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Par

Francisco Humberto XAVIER-JÚNIOR

Titre de la thèse

Systèmes dispersés pour l'administration orale du paclitaxel à base de microémulsion et de nanocapsules mucoadhésives contenant de l'huile de copaïba

Directeurs de thèse :

Christine VAUTHIER, Directeur de Recherche CNRS, (Université Paris-Sud)

Eryvaldo Sócrates TABOSA DO EGITO, Professeur (UFRN)

Composition du jury :

Gilles PONCHEL, Professeur (Université Paris-Sud)

Patrick SAULNIER, PU-PH (Université d'Angers)

Sylvie CRAUSTE-MANCIET, MCU-PH HDR (Université de Bordeaux)

« C'est le temps que tu as perdu pour ta rose qui fait ta rose si importante »

Antoine de Saint-Exupéry

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ABSTRACT

The oral route encourages a progressive interest in anticancer drug administration, currently, mainly administered by parenteral route. However, that route is limited by problems related to the physicochemical properties of drugs, physiological factors and dosage forms that reduce the overall bioavailability of the drug. To overcome limitations, systems based on lipids and polymeric nanoparticles have been used. Therefore, the aim of this project was to develop dispersed systems for oral route containing copaiba oil in their internal phase as vehicle for an anticancer drug, paclitaxel. Researches were developed in two directions aiming to formulate suitable microemulsions and nanocapsules. In the first part of the project, copaiba oil was analyzed; a method of paclitaxel dosage in this oil was developed and validated. Then formulation of the copaiba oil/water microemulsion was assessed taking into account solubility parameter of both oil components and surfactants. This led to the obtaining of stable microemulsion with the copaiba essential oil having remarkably high volume fraction of dispersed phase (19.6%) at low surfactant concentration (13.7%). It was demonstrated that paclitaxel could be incorporated in the microemulsion without disturbing the characteristics of the system. The second system developed in this work consisted mucoadhesive nanocapsules encapsulating copaiba oil. The formulation was based on an experimental design approach which one was also used during encapsulation of the paclitaxel. The development of this system included its labeling with a fluorescent probe and incorporating radiolabeled paclitaxel. Stability of the nanocapsules in simulated gastrointestinal medium was investigated and mucoadhesion on gut mucosa was evaluated. This work has proposed two formulations of paclitaxel in nanosystems which are ready for an evaluation for their capacity to deliver this anticancer drug by the oral route.

KEYWORDS: Oral route, copaiba oil, paclitaxel, microemulsion, nanocapsules, hydrophilic-lipophilic balance, chitosan, mucoadhesion.

RÉSUMÉ

La voie orale suscite un intérêt pour l'administration des médicaments anticancéreux, qui sont encore administrés essentiellement par voie parentérale. Cette voie est limitée par les problèmes liés aux propriétés physico-chimiques des principes actifs, aux facteurs physiologiques qui limitent fortement leur biodisponibilité orale. Pour surmonter certaines limitations, l'utilisation de systèmes à base de lipides et de nanoparticules polymères peuvent se montrer très performants. L'objectif de ce travail a consisté à développer des systèmes dispersés pour la voie orale contenant dans leur phase interne de l'huile de copaïba servant de véhicules à des médicaments anticancéreux comme le paclitaxel. Ce travail de recherche a été mené selon deux grands axes: l'un orienté vers les systèmes de type microémulsion et l'autre vers les nanocapsules. Dans la première partie du travail, l'huile de copaïba a été analysée et une méthode de dosage du paclitaxel dans l'huile de copaïba a été développée et validée. Dans la suite du travail, des microémulsions d'huile de copaïba/eau ont été formulée suivant une approche basée sur les paramètres de solubilité des composés de l'huile et des tensioactifs. Ce travail a permis l'obtention de microémulsion contenant des fractions volumiques importantes de l'huile essentielle de copaïba (19.6%) tout en maintenant les concentrations en tensioactifs faible (13.7%). Du paclitaxel a pu être incorporé dans les microémulsions sans perturber notablement les caractéristiques du system. Le deuxième système développé dans ce travail a été des nanocapsules mucoadhésives contenant de l'huile de copaïba. La formulation a été réalisée en mettant en œuvre un plan d'expérience à 2 niveaux avec trois facteurs. Des nanocapsules incorporant du paclitaxel et marquée par une sonde fluorescente et du paclitaxel radiomarqué ont également été développées. La stabilité de ces nanocapsules a été étudiée dans des milieux gastrique et intestinaux simulés. Leur mucoadhésion a été évaluée sur des fragments de muqueuse intestinale prélevés chez le rat. Les résultats de ces travaux ont conduit au développement de deux formulations de paclitaxel dans des nanosystèmes originaux qui pourront par la suite être évalués pour en étudier leur capacité à délivrer l'agent anticancéreux par voie orale.

MOTS CLES : voie orale, huile de copaïba, paclitaxel, microémulsion, nanocapsules, équilibre hydrophile-lipophile, chitosane, mucoadhésion.

RESUMO

A via oral suscita um interesse crescente para a administração de medicamentos anticancerígenos, os quais, na atualidade, ainda são administrados essencialmente pela via parenteral. No entanto, essa via é limitada por problemas relacionados às propriedades físico-químicas do fármaco, fatores fisiológicos e as formas farmacêuticas que reduzem a biodisponibilidade oral do medicamento. Para superar essas limitações, sistemas lipídicos e poliméricos nanoparticulados têm sido utilizados. O objetivo deste trabalho foi desenvolver sistemas dispersos para via oral, contendo na fase interna óleo de copaíba servindo de veículos para fármacos anticancerígenos, como paclitaxel. Os estudos foram desenvolvidos em dois sentidos com o objetivo de formular adequadas microemulsões e nanocápsulas mucoadesivas. Na primeira parte do projeto, o óleo de copaíba foi analisado e um método de dosagem de paclitaxel neste óleo foi desenvolvido e validado. Posteriormente, microemulsão de óleo de copaíba em água foi desenvolvida em função dos cálculos dos parâmetros de solubilidade entre os componentes do óleo de copaíba e os surfactantes. Tal processo levou à obtenção de microemulsão estável com o óleo essencial de copaíba cotendo elevada fração volumétrica da fase dispersa (19,6%) e uma baixa concentração de surfactante (13,7%). O Paclitaxel foi incorporado na microemulsão sem causar perturbação nas características do sistema. O segundo sistema desenvolvido neste trabalho consistiu de nanocápsulas mucoadesivas para encapsulação do óleo de copaíba. A formulação foi baseada na abordagem de planejamento experimental, o qual também foi usado durante a encapsulação do paclitaxel. O desenvolvimento deste sistema ainda incluiu a marcação com uma sonda fluorescente e incorporação de paclitaxel radioativo. A estabilidade das nanocápsulas foi investigada em meio gastrointestinal simulado. A mucoadesão foi avaliada em mucosa intestinal de ratos. Os resultados deste trabalho conduziram ao desenvolvimento de duas formulações de paclitaxel em nanosistemas originais que estão prontos para avaliação da sua capacidade de entregar de fármaco anticancerígeno pela via oral.

PALAVRAS-CHAVES: Via oral, óleo de copaíba, paclitaxel, microemulsão, nanocápsulas, equilíbrio hidrófilo-lipofílico, quitosana, mucoadesão.

TABLE DES MATIERES

ABBREVIATIONS / ABREVIATIONS

%CI	Creaming index
ANOVA	Analysis of variance
BSTFA	N,O-Bis(trimethylsilyl) trifluoroacetamide
CEO	Copaiba essential oil
Ch	Chitosan
Cop	Copaiba oil
CRO	Copaiba resin oil
CS	Clinical strain
DL	Drug loading
DLS	Dynamic light scattering
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethyl sulfoxide
EE	Entrapment efficiency
Eh	Hydrogen bonding forces
ELISA	Enzyme-linked immunosorbent assay
Fd	London dispersion forces
Fp	Keesom dipolar force
GC-FID	Gas chromatography–flame ionization detector
GC-MS	Gas chromatography–mass spectrometry
HLB	Hydrophilic-lipophilic balance
HLB_0	Hydrophilic-lipophilic balance requis
HPLC	High-performance liquid chromatography
IBCA	Isobutyl cyanoacrylate
IPP	Isopentenyl pyrophosphate
LOD	Limit of detection
LOQ	Limit of quantification
ME	Microemulsion

MECop	Copaiba oil-loaded microemulsion
MECop Ptx	Paclitaxel into copaiba oil-loaded microemulsion
MIC	Minimum inhibitory concentration
MWCO	Molecular weight cut off
NC	Nanocapsule
NCC	Copaiba oil-loaded chitosan-poly (isobutyl cyanocrylate) core-shell nanocapsules
NCC [H^3]-Ptx	Radioactive paclitaxel encapsulated into copaiba oil-loaded chitosan-poly (isobutyl cyanocrylate) core-shell nanocapsules
NCC Ptx	Paclitaxel encapsulated into copaiba oil-loaded chitosan-poly (isobutyl cyanocrylate) core-shell nanocapsules
NCCdry Ptx	Paclitaxel encapsulated into copaiba oil-loaded chitosan-poly (isobutyl cyanocrylate) core-shell nanocapsules after drying process
NCCfluo Ptx	Polyfluor® 570 labeled in paclitaxel encapsulated into copaiba oil-loaded chitosan-poly (isobutyl cyanocrylate) core-shell nanocapsules
O/W	Oil-in-water
OD	Optical density
PdI	Polydispersity index
PIT	Phase inversion technique
Ptx	Paclitaxel
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TTC	Tetrazolium chloride
W/O	Water-in-oil

Introduction Générale

Le cancer est une des maladies les plus importantes dans le monde aussi bien par l'augmentation de son incidence, sa prévalence et de sa mortalité. Il s'agit d'un problème de santé prioritaire qui est motivé par le fait que le cancer est une cause de décès majeure dans la plupart des pays dans monde (Fang *et al.*, 2011; Siegel *et al.*, 2014). Le paclitaxel est l'un des plus puissants agents anti-cancéreux actuellement utilisé pour le traitement des cancers du poumon et du sein, de la leucémie aiguë, ainsi que des cancers de l'ovaire avancé, du cerveau et des carcinomes du col de l'utérus (Forastiere, 1994; Rowinsky *et al.*, 1995; Weaver, 2014). C'est un pseudoalkaloide qui présente une structure diterpénoïde. Cette molécule qui a été initialement isolée de *Taxus brevifolia* (Fang *et al.*, 2005) fonctionne en favorisant la stabilisation des microtubules inhibant ainsi la prolifération cellulaire et finalement l'induction de l'apoptose (Schiff *et al.*, 1979; Hamel *et al.*, 1981; Horwitz, 1992). Comme de nombreux agents anti-cancéreux, le paclitaxel est administrés par voie intraveineuse, car il présente une faible biodisponibilité orale (Weingart *et al.*, 2008). Cependant, l'administration par la voie intraveineuse présente de nombreux inconvénients comme l'extravasation du médicament ou du sang au site d'injection, l'infection de cathéter, et la thrombose qui peuvent être évités par l'administration du médicament par voie orale (Roger *et al.*, 2011). L'administration des traitements de chimiothérapie par voie orale présente plusieurs intérêts majeurs outre celui d'améliorer le confort du patient au moment de l'administration. Elle permet d'améliorer l'observance du traitement par sa simplicité de mise en œuvre, de diminuer le coût du traitement et d'améliorer la sécurité du traitement pour le patient (Borner *et al.*, 2001; Batlle *et al.*, 2004).

Malgré ces avantages potentiels, l'administration orale du paclitaxel est limitée par les propriétés physico-chimiques du médicament qui sont inappropriées pour permettre une

bonne biodisponibilité par cette voie d'administration (solubilité, lipophilie, pKa). Elle est également limitée par des facteurs physiologiques (temps de transit intestinal, pH gastro-intestinal, mécanismes d'absorption, métabolisme rapide dans les entérocytes de l'épithélium digestif) et à d'autres aspects liés aux formes galéniques (faible perméabilité, instabilité). L'ensemble de ces facteurs ne permettent pas d'obtenir une biodisponibilité satisfaisante de la molécule par voie orale (Prabhu *et al.*, 2005; Ensign *et al.*, 2012). Il existe plusieurs facteurs qui contribuent à l'efficacité et à l'utilité des préparations pharmaceutiques en tant que supports pour la délivrance orale de molécules insolubles dans l'eau. Il s'agit notamment de la capacité des médicaments à se dissoudre, de la vitesse du transit intestinal et de l'absorption des médicaments par la muqueuse intestinale après administration orale (Liu *et al.*, 2011). Donc, l'amélioration de la biodisponibilité orale de tels médicaments permettrait d'améliorer encore l'efficacité thérapeutique des molécules et la compliance du patient (Singh *et al.*, 2009).

Au cours des dernières années, divers formes galéniques ont été proposés en vue d'améliorer la délivrance orale de molécules difficiles à administrer par cette voie d'administration. Certaines font appel à des nanomédecines comme des nanoparticules, des nanocapsules, des micelles, des microémulsions, des liposomes, des matériaux nanoporeux et des multicouches polymères. Parmi eux, les systèmes à base de lipides et les nanoparticules polymères sont apparus utiles pour surmonter certaines limitations. Par exemple, au cours de la dernière décennie, il a été montré que les formulations à base de lipides présentent un meilleur potentiel pour solubiliser des molécules difficilement solubles dans des milieux aqueux et notamment les molécules liposolubles. Simultanément, ils améliorent la stabilité et protègent les molécules contre une dégradation. L'amélioration de la solubilité et de la stabilité des molécules actives s'accompagne généralement d'une augmentation de leur biodisponibilité orale (Shah *et*

al., 1994; Constantinides, 1995; Zhao *et al.*, 2005; Poullain-Termeau *et al.*, 2008; Kalepu *et al.*, 2013). Les nanocapsules biodégradables sont des systèmes vésiculaires dans lesquels un principe actif est confiné dans une cavité constituée d'un cœur liquide, elles ont été proposées pour contrôler la libération de principes actifs et le cibler vers les sites d'absorption. Quelque soit le système retenu, son utilisation est de délivrer de manière contrôlée et la plus efficace possible le principe actif. Pour cela, le système doit être conçu de manière à intégrer les différentes fonctionnalités qui lui permettront au final d'améliorer la biodisponibilité de la molécule pharmacologiquement active transportée (Li *et al.*, 2001; Tong *et al*., 2008; Mora-Huertas *et al.*, 2010; Perrier *et al.*, 2010; Roger *et al.*, 2010a; Groo *et al.*, 2013).

Les huiles végétales entrent dans la composition de nombreux systèmes d'administration orale de principes actifs (Lee *et al.*, 1995; Dantas *et al.*, 2010; Attaphong *et al.*, 2012). Certaines huiles végétales ont des propriétés pharmacologiques utilisées en médecine traditionnelle. Par exemple, l'huile de Copaïba, *Copaifera langsdorffi*, produite au Brésil est largement utilisés en médecine traditionnelle pour le traitement de maladies inflammatoires, microbiologiques et cancéreuse (Gomes *et al.*, 2007; Mendonça *et al.*,2009a; Comelli-Júnior *et al.*, 2010; Souza *et al.*, 2011). Son activité pharmacologique est liée à sa composition en composés diterpéniques et sesquiterpéniques (Veiga-Junior *et al.*, 2002; Gomes *et al.*, 2008). Il pourrait être proposé que de telles huiles deviennent un constituant majeur d'un système d'administration de médicament anticancéreux et pourrait ainsi éventuellement potentialiser l'activité biologique d'un principe actif associé au vecteur.

L'objectif de notre travail de thèse a été de développer des systèmes d'administration des médicaments anticancéreux pour la voie orale à base d'huile végétale thérapeutique.

L'huile thérapeutique sélectionnée pour ce travail a été l'huile de copaïba et le principe actif anticancéreux retenu a été le paclitaxel. Les travaux ont été menés en trois parties. La première partie a été consacrée au développement de méthodes destinées à analyser et doser l'huile de copaïba (chapitre I) et à doser le paclitaxel dans l'huile de copaïba pour en déterminer sa solubilité dans l'huile de copaïba et son coefficient de partage (chapitre II).

La deuxième partie du travail qui regroupe les chapitres III à VI, a été consacrée à des travaux de formulation du paclitaxel dans des microémulsions et d'en évaluer la capacité à promouvoir l'attachement du paclitaxel sur du tissu intestinal *ex-vivo*. Ainsi, le chapitre III de la thèse propose une revue de l'état de l'art du développement de micro-émulsion avec des huiles naturelles. Le chapitre IV présente les résultats d'un travail initial qui avait pour objectif de mettre en place de l'huile de copaïba dans des systèmes émulsionnés. Il est intitulé « Prospective study for the development of emulsion systems containing natural oil products». Le chapitre V présente la formulation et la caractérisation d'une microémulsion préparée avec de l'huile de copaïba. La stratégie de formulation s'est appuyée sur l'utilisation des paramètres de solubilité destinée à optimiser la miscibilité des parties lipophiles des tensioactifs dans les constituants majeurs de l'huile de copaïba. L'incorporation du paclitaxel dans la microémulsion et l'évaluation de la mucoadhésion est décrite dans le chapitre VI.

La troisième partie de nos travaux consignée dans les chapitres VII à VIII a été consacrée à l'étude de formulations de nanocapsules polymères. Le chapitre VII présente l'optimisation de l'encapsulation d'huile de copaïba dans les nanocapsules recouvertes de chitosane en utilisant une approche basée sur la mise en œuvre d'un plan

d'expérience factoriel 2^3. Le chapitre VIII s'intéresse aux études de mucoadhésivité du paclitaxel encapsulé dans des nanocapsules recouvertes de chitosane.

Enfin, une discussion générale permet de mettre en parallèle et de discuter l'ensemble de tous les résultats obtenus dans le cadre de ce projet, puis de dégager les avantages et les inconvénients de la stratégie consistant à utiliser des systèmes de délivrance nanotechnologiques pour l'administration orale de médicaments anticancéreux à base de l'huile de copaïba.

Références

ATTAPHONG, C., DO, L. & SABATINI, D. A. 2012. Vegetable oil-based microemulsions using carboxylate-based extended surfactants and their potential as an alternative renewable biofuel. *Fuel*, 94, 1, 606-613.

BATLLE, J. F., ARRANZ, E. E., CASTRO CARPEÑO, J., SÁEZ, E. C., AUÑÓN, P. Z., SÁNCHEZ, A. R. & BARÓN, M. G. 2004. Oral chemotherapy: potential benefits and limitations. *Clin Transl Oncol*, 6, 6, 335-340.

BORNER, M., SCHEITHAUER, W., TWELVES, C., MAROUN, J. & WILKE, H. 2001. Answering patients' needs: oral alternatives to intravenous therapy. *Oncologist*, 6 Suppl 4, 12-6.

COMELLI-JÚNIOR, E., SKINOVSKI, J., SIGWALT, M. F., BRANCO, A. B., LUZ, S. R. & BAULÉ, C. P. 2010. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaíba *(Copaifera Iangsdorfii)* oil. *Acta Cir Bras*, 25, 362-367.

CONSTANTINIDES, P. P. 1995. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm Res*, 12, 11, 1561-72.

DANTAS, T. N. C., SILVA, H. S. R. C., NETO, A. A. D., MARCUCCI, M. C. & MACIEL, M. A. M. 2010. Development of a new propolis microemulsion system for topical applications. *Rev bras farmacogn*, 20, 3, 368-375.

ENSIGN, L. M., CONE, R. & HANES, J. 2012. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Adv Drug Deliv Rev*, 64, 6, 557-70.

FANG, J., NAKAMURA, H. & MAEDA, H. 2011. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev*, 63, 3, 136-51.

FANG, W. S. & LIANG, X. T. 2005. Recent progress in structure activity relationship and mechanistic studies of taxol analogues. *Mini Rev Med Chem*, 5, 1, 1-12.

FORASTIERE, A. A. 1994. Current and future trials of Taxol (paclitaxel) in head and neck cancer. *Ann Oncol*, 5 Suppl 6, S51-4.

GOMES N, M., REZENDE C, M., FONTES, S. P., HOVELL, A. M., LANDGRAF, R. G., MATHEUS, M. E., PINTO A, C. & FERNANDES, P. D. 2008. Antineoplasic activity of Copaifera multijuga oil and fractions against ascitic and solid Ehrlich tumor. *J Ethnopharmacol*, 119, 1, 179-84.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-492.

GROO, A. C., SAULNIER, P., GIMEL, J. C., GRAVIER, J., AILHAS, C., BENOIT, J. P. & LAGARCE, F. 2013. Fate of paclitaxel lipid nanocapsules in intestinal mucus in view of their oral delivery. *Int J Nanomedicine*, 8, 1178-2013 (Electronic), 4291-302.

HAMEL, E., CAMPO, A. A., LOWE, M. C. & LIN, C. M. 1981. Interactions of taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J Biol Chem*, 256, 22, 11887-94.

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci*, 13, 4, 134-6.

KALEPU, S., MANTHINA, M. & PADAVALA, V. 2013. Oral lipid-based drug delivery systems – an overview. *Acta Pharm Sin B*, 3, 6, 361-372.

LEE, M. J., LEE, M. H. & SHIM, C. K. 1995. Inverse targeting of drugs to reticuloendothelial system-rich organs by lipid microemulsion emulsified with poloxamer-338. *Inter J Pharm*, 113, 2, 175-187.

LI, Y., PEI, Y., ZHANG, X., GU, Z., ZHOU, Z., YUAN, W., ZHOU, J., ZHU, J. & GAO, X. 2001. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. *J Control Release*, 71, 2, 203-11.

LIU, Y., WANG, P., SUN, C., ZHAO, J., DU, Y., SHI, F. & FENG, N. 2011. Bioadhesion and enhanced bioavailability by wheat germ agglutinin-grafted lipid nanoparticles for oral delivery of poorly water-soluble drug bufalin. *Int J Pharm*, 419, 1-2, 260-5.

MENDONÇA, D. E. & ONOFRE, S. B. 2009a. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev Bras Farmacogn*, 19, 577-581.

MORA-HUERTAS, C. E., FESSI, H. & ELAISSARI, A. 2010. Polymer-based nanocapsules for drug delivery. *Int J Pharm*, 385, 1-2, 113-42.

PERRIER, T., SAULNIER, P. & BENOIT, J. P. 2010. Methods for the functionalisation of nanoparticles: new insights and perspectives. *Chemistry*, 16, 38, 11516-29.

POULLAIN-TERMEAU, S., CRAUSTE-MANCIET, S., BROSSARD, D., MUHAMED, S., NICOLAOS, G., FARINOTTI, R., BARTHELEMY, C., ROBERT, H. & ODOU, P. 2008. Effect of oil-in-water submicron emulsion surface charge on oral absorption of a poorly water-soluble drug in rats. *Drug Deliv*, 15, 8, 503-14.

PRABHU, S., ORTEGA, M. & MA, C. 2005. Novel lipid-based formulations enhancing the in vitro dissolution and permeability characteristics of a poorly water-soluble model drug, piroxicam. *Int J Pharm*, 301, 1–2, 209-216.

ROGER, E., LAGARCE, F. & BENOIT, J. P. 2011. Development and characterization of a novel lipid nanocapsule formulation of Sn38 for oral administration. *Eur J Pharm Biopharm*, 79, 1, 181-8.

ROGER, E., LAGARCE, F., GARCION, E. & BENOIT, J. P. 2010a. Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery. *Nanomedicine (Lond)*, 5, 2, 287-306.

ROWINSKY, E. K. & DONEHOWER, R. C. 1995. Paclitaxel (taxol). *N Engl J Med*, 332, 15, 1004-14.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SHAH, N. H., CARVAJAL, M. T., PATEL, C. I., INFELD, M. H. & MALICK, A. W. 1994. Self-emulsifying drug-delivery systems (SEDDS) with polyglycolyzed glycerides for improving in-vitro dissolution and oral absorption of lipophilic drugs. *Int J Pharm*, 106, 1, 15-23.

SIEGEL, R., MA, J., ZOU, Z. & JEMAL, A. 2014. Cancer statistics, 2014. *CA Cancer J Clin*, 64, 1, 9-29.

SINGH, A. K., CHAURASIYA, A., AWASTHI, A., MISHRA, G., ASATI, D., KHAR, R. K. & MUKHERJEE, R. 2009. Oral bioavailability enhancement of exemestane from self-microemulsifying drug delivery system (SMEDDS). *AAPS PharmSciTech*, 10, 3, 906-16.

SOUZA, A. B., MARTINS, C. H., SOUZA, M. G., FURTADO, N. A., HELENO, V. C., SOUSA, J. P., ROCHA, E. M., BASTOS, J. K., CUNHA, W. R., VENEZIANI, R. C. & AMBROSIO, S. R. 2011. Antimicrobial activity of terpenoids from *Copaifera langsdorffii Desf.* against cariogenic bacteria. *Phytother Res*, 25, 2, 215-20.

TONG, R. & CHENG, J. 2008. Paclitaxel-initiated, controlled polymerization of lactide for the formulation of polymeric nanoparticulate delivery vehicles. *Angew Chem Int Ed Engl*, 47, 26, 4830-4.

VEIGA-JUNIOR, V. F. & PINTO, A. C. 2002. O gênero copaifera L. *Quim Nova*, 25, 273-286.

WEAVER, B. A. 2014. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*, 25, 18, 2677-81.

WEINGART, S. N., BROWN, E., BACH, P. B., ENG, K., JOHNSON, S. A., KUZEL, T. M., LANGBAUM, T. S., LEEDY, R. D., MULLER, R. J., NEWCOMER, L. N., O'BRIEN, S., REINKE, D., RUBINO, M., SALTZ, L. & WALTERS, R. S. 2008. NCCN Task force report: Oral chemotherapy. *J Natl Compr Canc Netw*, 6 Suppl 3, S1-14.

ZHAO, X. L., CHEN, D. W., GAO, P., DING, P. T. & LI, K. X. 2005. Synthesis of ibuprofen eugenol ester and its microemulsion formulation for parenteral delivery. *Chem Pharm Bull* 53, 10, 1246-1250.

Section I

Développement des méthodes

Chapter I

Development of gas-chromatography method for the analysis of copaiba oil.

Le travail présenté dans ce premier chapitre avait pour objectif de développer une méthode rapide, simple et précise pour la quantification des huiles copaïba. Les méthodes a été développées et validées en utilisant une technique chromatographie en phase gazeuse pour être appliquée à l'analyse de l'huile résine et de l'huile essentielle de copaïba. L'huile essentielle de copaïba a été efficacement extraite par la méthode de l'hydrodistillation à partir de l'huile de résine de copaïba. La réaction de dérivatisation de l'huile résine a été effectuée et confirmée par technique de chromatographie sur couche mince qui a permis l'identification des composés diterpéniques. Les analyses en chromatographie en phase gazeuse couplée à la spectrométrie de masse ont été mises au point et en œuvre pour déterminer la composition des huiles copaïba et identifier la position des pics de ces composants. Les principaux composés identifiés dans l'huile essentielle de copaïba ont été le β-bisabolène (23,6%), le β-caryophyllène (21,7%) et l'α-bergamotène (20,5%). Les principaux composant identifiés dans l'huile résine de copaïba méthylée ont été l'acide copalique (15,6%), le β-bisabolène (12,3%), le β-caryophyllène (7,9%), l'α-bergamotène (7,1%) et le l'acide Labd-8 (20) -ène-15,18-dioïque (6,7%). Une bonne corrélation entre les chromatographies en phase gazeuse en utilisant les détecteurs à ionisation de flamme et de spectrométrie de masse ont été obtenus au cours de la transposition de la méthode d'analyse. La méthode a démontré une haute performance pour les paramètres de validation pris en compte incluant la sensibilité, la spécificité, la linéarité, la précision, l'exactitude et les limites de détection et de quantification pour les analyses du β-caryophyllène, α- humulène et l'oxyde de caryophyllène dans les huiles de copaïba. Ce travail a été adapté à la quantification fiable dans le contrôle de la qualité de l'huile de copaïba et peut également être utilisé pour mesurer l'huile de copaïba lorsqu'il est chargé dans les formulations pharmaceutiques ou cosmétiques.

Mots-clés: Huile de copaïba, composition chimique, chromatographie en phase gazeuse à haute résolution, validation, β-caryophyllène, α-humulène, oxyde de caryophyllene

DEVELOPMENT OF GAS-CHROMATOGRAPHY METHOD FOR THE ANALYSIS OF COPAIBA OIL.

Xavier-Junior, F.H.[1, 2], Maciuk, A.[3], Morais, A.R.V. [1, 2], Alencar, E.N.[1], Rehder, V. L. G.[4], Egito, E.S.T.[1], Vauthier, C.[2]*,

[1] Universidade Federal do Rio Grande do Norte, Centro de Ciências da Saúde, Departamento de Farmácia, Laboratório de Sistemas Dispersos (LaSiD). Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

[3] Université Paris Sud, Laboratoire de Pharmacognosie - UMR CNRS 8076 BioCIS - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

[4] Universidade Estadual de Campinas (UNICAMP) – Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas. Rua Alexandre Cazelatto, 999, Vila Betel, Paulínia – SP.

*Corresponding author: Christine Vauthier

Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Christine.vauthier@u-psud.fr

ABSTRACT

A rapid, simple and precise method for the quantification of copaiba oils (*Copaifera langsdorffii)* have been developed and validated using gas chromatography analyses. Copaiba essential oil was efficiently extracted by hydrodistillation method from the copaiba resin oil. Oil derivatization was performed and confirmed by Thin Layer Chromatography technique which allowed the identification of diterpenes compounds. Gas chromatography coupled to mass spectrometry analyses was effectively developed to determine the composition of the copaiba oils. The main compounds identified in the copaiba essential oil were β-Bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene (20.5%). On the other hand, from the methylated copaiba resin oil were copalic acid (15.6%), β-Bisabolene (12.3%), β-caryophyllene (7.9%), α-bergamotene (7.1%) and Labd-8(20)-ene-15,18-dioic acid (6.7%) were found. A good correlation between the gas chromatography interfaced with flame ionization and mass spectrometry detectors were obtained favoring the transposition of the methodology analyses. The method showed a high performance concerning sensitivity, specificity, linearity, precision, accuracy, limits of detection and quantification parameters for the β- caryophyllene, α- humulene and caryophyllene oxide analyses in the copaiba oils. This work should be suitable to the reliable quantification in the quality control of copaiba oil and can also be used to copaiba oil quantification when loaded in pharmaceutical or cosmetic formulations.

Keywords: Copaiba oil, *Copaifera langsdorffii*, Chemical composition, high resolution gas chromatography, Validation, β- caryophyllene, α- humulene, caryophyllene oxide

1.0. INTRODUCTION

The significant use and development of pharmaceuticals originated from synthetic and natural sources have taken place along with the analytical methods responsible for determining, identifying and quantifying those products (Aturki *et al.*, 2014). Several methods are responsible for analyses of drugs, impurities, intermediates, degradation products, mixtures of compounds, phytoextracts etc (Maggio *et al.*, 2014). Among these methods, such as, potentiometric, spectroscopic and microbiological, the chromatographic ones stand out due to the useful property of separation and its powerful performance for the analysis of complex products such as natural oils, which can be further enhanced interestingly along with other possible features.

Concerning the use for pharmaceutical application, the most known chromatographic methods include high performance liquid chromatography, liquid chromatography coupled to mass spectrometer and gas chromatography coupled to different detectors, besides the traditional techniques such as thin layer chromatography, especially concerning screening in complex mixtures. As in any chromatographic method, the methods might allow to access separation, identification and quantification. Accordingly, gas chromatography is well used in several fields of science (Skoog *et al.*, 2007; Attimarad *et al.*, 2011; Marriott *et al.*, 2012).

Gas chromatography technique is one of the most efficient concerning separations of complex mixtures such as natural products in which compounds can be volatilized. Natural products have been widely studied regarding development of novel pharmaceutical compounds due to their pharmacological activities and their renewable character. The active compounds obtained from vegetable sources are usually complex mixtures of plant's secondary compounds known by their protective activity against

predators. However, on the human body, these compounds are pharmacologically active, and they are likely to have evolved in order to interact with cell membranes and interact with specific target proteins (Stone & Williams, 1992; Saleem *et al.*, 2010).

Copaiba oil is extracted from trees of the genus *Copaifera*. The trees from this genus are distributed amongst South America, Central America and Africa. However, the greater number of species is located in South America, specifically, in Brazil (Veiga Junior & Pinto, 2002). The oil extracted from the trunks of the trees has been used in folk medicine since ancient times. A complex mixture of diterpenes and sesquiterpenes comprises the oil (Gramosa & Silveira, 2005; Sousa, J. P. B. *et al.*, 2011; Gelmini *et al.*, 2013; Alencar, É. N. *et al.*, 2015). Besides the pharmacological activities that these compounds provide to the copaiba oil, they can be useful to identify and quantify copaiba oil species in pharmaceutical systems. Amongst the currently studied pharmacological activities of copaiba oil, its antibacterial, antifungal, anti-inflammatory, healing, anti-Leishmania and anti-cancer activities have attracted the attention of many researchers (Vieira *et al.*, 2009; Deus *et al.*, 2011; Santos *et al.*, 2011; Santos *et al.*, 2013; Sousa *et al.*, 2013).

Over the last years, biological studies on copaiba oil justified its vast use in folk medicine and its importance for the development of new natural products (Xavier-Júnior *et al.*, 2012b). Therefore, the development of a validated analytical method to accurately quantify these compounds became mandatory. A method validation is performed in order to standardize the process and the use of the instrumentation aiming to minimize random error and ensure that the method may be trusted and be used in different location. Moreover, the development and the determination of many experimental parameters such as, accuracy, linearity, sensitivity, selectivity, precision

and the knowledge of the limits of quantification and detection is necessary to ensure that the method is validated (González *et al.*, 2014; Mujawar *et al.*, 2014; Nikolaou *et al.*, 2015). Therefore, the aim of this work was to develop a method of validation for copaiba oil by gas chromatography using the β-caryophyllene, α- humulene and caryophyllene oxide as standards. In addition, this work established a correlation between gas chromatography interfaced with flame ionization and mass spectrometry analyses promoting an exhaustive study on the chemical composition of copaiba essential and resin oil constituents.

2.0 MATERIALS AND METHODS

2.1. Materials

Copaiba resin oil (*Copaifera langsdorffii*) was obtained from Flores & Ervas (Piracicaba, SP, Brazil). N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), diazomethane, β-caryophyllene, α- humulene and caryophyllene oxide were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Hexane and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA, EUA). Ultrapure water was obtained from a Millipore purification system (Milli-Q® plus, Millipore, St Quentin en Yvelines, France). All chemicals were of reagent grade and used as received.

2.2. Copaiba essential oil extraction

Copaiba essential oil was produced by the hydrodistillation method. 400 mL of copaiba resin oil with four times the volume of ultrapure water were placed in the Clevenger-

type apparatus for 3 h to essential oil extraction. Posteriorly, the essential oil extracted was dried with sodium sulphate, filtered through 0.22 μm cellulose membrane (Merck Millipore, Billerica, MA, USA) and stored in borosilicate glass vial at −20 °C until further use.

2.3. Copaiba resin oil derivatization

Copaiba resin oil was submitted to a derivatization reaction before gas chromatography analyses. A methylation reaction was achieved by diluting 20-30 mg of copaiba resin oil with 2 mL of ethyl acetate. This mixture was placed in an ice bath and 2 mL of diazomethane was slowly added. After reaction, the solvent was completely evaporated under air flow. Silylation derivatization was performed using BSTFA. Five mg of copaiba resin oil was diluted in 0.5 mL of ethyl acetate and an excess of silylating reagent was added. This solution was heated at 60 °C for 30 minutes. For both derivatization reactions, the blank reagent was prepared and the volume was adjusted to 1.5 mL in ethyl acetate prior gas chromatography analysis.

2.4. Copaiba oil analysis

2.4.1. Thin Layer Chromatography

Samples were spotted on pre-coated thin layer chromatography plates (silica gel 60 F254, 10 x 20cm, 0.25mm layer thickness, Merck Millipore, SP, Brazil) in order to indentify the oil profiles and to confirm the derivatization of copaiba resin oil before gas chromatography analyses. Samples were diluted in ethyl acetate and applied in the plate.

Mobile phase consisted of hexane/ethyl acetate (9:1) solution. After elution the samples were analyzed in ultraviolet-visible at 254 nm, following anisaldehyde solution application and drying at 105 °C for 5 min. Retention factor (Rf) was calculated by the ratio of migration distance of substance present in copaiba oils and the migration distance of solvent front.

2.4.2. Gas-Chromatography Mass Spectrometry

Identification of copaiba resin and essential oil constituent were performed by gas chromatography coupled to mass spectrometry (GC-MS) using Hewlett-Packard 6890 gas chromatograph with HP-5975 mass selective detector. The column used was a HP-5MS cross-linked fused silica capillary column (30 m× 0.25 mm × 0.25 µm). (Agilent J&W, Santa Clara, CA, USA). Chromatographic parameters to copaiba resin and essential oils analysis are described in Table 1. The injected volume for all samples was 1µL. The split ratio was 1:25 and the electron ionization system was set at 70 eV. Helium was the carrier gas at a flow rate of 1 $mL.min^{-1}$. Data acquisition and integration were carried out using the MSD ChemStation software. Copaiba oils components were identified by comparing their mass fragmentation with both the National Institute of Standards and Technology (NIST) mass spectral library data and the published data elsewhere. β- caryophyllene, α- humulene and caryophyllene oxide injected as standards were identified by comparison of its retention time and mass spectrum with the ones found on the NIST library.

Table 1- Chromatographic parameters to copaiba resin and essential oil analysis by GC-MS

Parameters	Copaiba Resin oil	Copaiba essential oil
Initial oven temperature:	110 °C	60 °C
Initial rate:	5 °C.min^{-1}	3 °C.min^{-1}
Final temperature:	280 °C (final hold time of 26.0 min at 300°C)	240 °C (final hold time of 7.0 min at 250 °C)
Injector temperature	250 °C	220 °C
Detector temperature	300 °C	250 °C

2.4.3. Gas chromatography – Flame Ionization Detector

The quantification of volatile constituents were performed using a PR2100 Gas-Chromatography (Alpha MOS, Toulouse, France) interfaced with a Flame Ionization Detector (GC-FID). A fused silica capillary column (25 m × 0.32 mm i.d., 0.5 µm) film thickness coated with cross-linked 5% Phenyl Polysilphenylene-siloxane (SGE Analytical Science Pty Ltd, Victoria, Australia) was used. The work temperatures for the copaiba oils were as follows: oven temperature started at 90 °C, isothermal, then heating 2 °C.min^{-1} to 150 °C, and after, isothermally heating 20 °C.min^{-1} to 300 °C. The injector temperature was 250 °C and the detector temperature was 300 °C. The volume injected for all samples was 1 µL. The split ratio was 1:80. The nitrogen was the carrier gas at a flow rate of 1 mL.min^{-1}. Data acquisition and integration were carried out using Winilab 3 software. β -caryophyllene, α- humulene and caryophyllene oxide were

selected as the standard for the quantification of the main components presented in the copaiba oils.

2.5. Method validation for β- caryophyllene, α- humulene and caryophyllene oxide

The validation procedures for β- caryophyllene, α- humulene and caryophyllene oxide were performed following the international conference on harmonization (ICH) (Validation of analytical procedures: Text and Methodology, ICH-Q2 (R1), 2005) and the Food and Drug Administration (*Food and Drug Administration, Guidance for Industry. Bioanalytical Method Validation* 2001) guidelines. The validation procedures followed the good manufacturing practices. All equipments and volumetric glassware were evaluated and calibrated before analysis. The balance (Sartorius MSA-224S-000-DU Cubis Analytical Balance, Elk Grove, USA) was calibrated to minimal measures of 0.1mg. Specificity, selectivity, linearity range, accuracy, precision, detection and quantification limits were evaluated using the GC-FID.

2.5.1. Preparation of stock solutions

Three individual stock solutions of β- caryophyllene, α- humulene and caryophyllene oxide were prepared in ethyl acetate at 1 $mg.mL^{-1}$, placed in an amber vial hermetically sealed and kep at -20 °C until use. These stock solutions were diluted to obtain the concentrations required for preparation of standard working solutions. For all substances, working solutions ranged from 40 to 160 $\mu g.mL^{-1}$ (40, 70, 100, 130 and 160 $\mu g.mL^{-1}$) were prepared in 1 mL of ethyl acetate and used for the validation analyzes.

2.5.2. Specificity and selectivity

The specificity/selectivity of the analytical method was confirmed by the analyses of solutions containing 100% of the normal working concentration of β- caryophyllene, α-humulene and caryophyllene oxide. The ability to separate all the compounds (related substances, degradation products and excipients) from standard samples was analyzed.

2.5.3. Linearity

Standard calibration curves of individual stock solutions of β-caryophyllene, α-humulene and caryophyllene oxide were prepared a concentration ranged from 40 to 160 $\mu g.mL^{-1}$ (40, 70, 100, 130 and 160 $\mu g.mL^{-1}$). Peak area ratios of the standards were individually plotted against the analyte concentrations. Standard calibration curves of the compounds were developed by calculation of the regression line using the least squares method. Linearity curves were performed on 3 different days.

2.5.4. Determination of the limit of detection and quantification

The Limit of Detection (LOD) was determined based on the ratio between the standard deviation of the response and the slope estimated from the calibration curve of the standards multiplied by 3.3. The Limit of Quantitation (LOQ) was determined as the lowest amount of analyte that was reproducibly quantified. This parameter was calculated by the ratio of the standard deviation of the response and the slope of the calibration curve of the standards multiplied by 10.

2.5.5. Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80, 100 and 120 %) of bulk samples of β- caryophyllene, α-humulene and caryophyllene oxide along with the linearity range taken in triplicate. Then percentage recovery values were determined by the absolute percentage deviation at each concentration of the standard solutions.

2.5.6. Precision

Precision was estimated by: intra-day (repeatability) and inter-day precision. Intra-day precision was investigated by injecting triplicate samples of β- caryophyllene, α-humulene and caryophyllene oxide solutions of three different concentrations (40, 100 and 160 $\mu g.mL^{-1}$). Inter-day precision was assessed by injecting the same three samples over three consecutive days. Inter- and intra-day precisions were expressed as the relative standard deviation (RSD).

2.6. Determination of the β- caryophyllene, α- humulene and caryophyllene oxide in copaiba oils by GC-FID

Stock solutions (10 $mg.mL^{-1}$) of copaiba essential and resin oils were prepared in triplicate with ethyl acetate. These stock solutions were injected in GC-FID in the same conditions of the validation studies, as described above. The dosages of β-caryophyllene, α- humulene and caryophyllene oxide were performed based in the standard calibration curves of individual compound.

2.7. Statistical analyses

All the experiments were conducted in triplicates. All values are expressed as their mean and standard deviation. Means of two groups were compared using non-paired Student's t-tests. When comparing multiple groups, one way analysis of variance (ANOVA) was applied with the Tukey multiple comparison procedure. The statistical data were considered significant at $p < 0.05$

3.0 RESULTS AND DISCUSSION

Copaiba essential oil was obtained by hydrodistillation from the copaiba resin oil using Clevenger apparatus to separate the colorless volatile fraction of the viscous residue. The yield of this extraction was calculated according to the ratio (w/w) of the volatile oil obtained and the resin oil material used initially for extraction. Thus, the copaiba essential oil yield was of 11 ± 0.8 %. Gelmini et al obtained a yield of 22.5 % for *C. langsdorffii* using steam distillation as the extraction method (Gelmini *et al.*, 2013). Although the yield has been lower in this work, this method was considered of high performance since the amount of essential oil extracted was elevated (44 mL) and there were minimal loss of volatile substances due the operation in a closed circuit with cohobation of the water. In addition, there was no step using chemical solvents and the extraction time was considered ideal to prevent degradation of chemical compounds.

Derivatization reactions were performed in order to identify the diterpenoic acids and derivative components presented in copaiba resin oil but normally no detectable in gas chromatography. This method modifies an analyte's functionality in order to enable chromatographic separations (Orata, 2012). The reaction of diazomethane (CH_2N_2) with

a carboxylic acid formed instantly methyl esters compounds. The derivative shows lower polarity, relative to the parent substance due the replacement of the hydrogen by an alkyl group (Figure 1A). Silylation is the introduction of a silyl group into a molecule, usually in substitution for active hydrogen. The reaction is a nucleophilic attack upon the silicon atom of the silyl donor. Replacement of the active hydrogen by a silyl group reduces the polarity of the compound (Figure 1B).

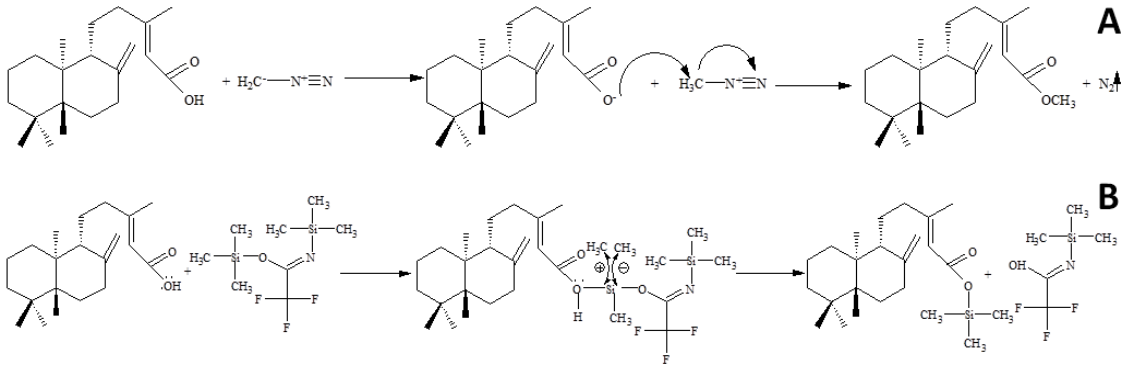

Figure 1: Derivatization reactions of carboxylic acid (i.e. copalic acid). The Figure A and B show the methylation and silylation reactions, respectively.

Thin layer chromatography was used to identify the profile of copaiba oils and to confirm the derivatization reactions (Figure 2). It was observed that after derivatization, two initial groups formed by a large compound mixtures at retention factor (Rf) of 0.1 and 0.47, respectively, from the copaiba resin oil became the eluted in four compounds at Rf of 0.25, 0.37, 0.59 and 0.71, respectively, for the methylation and three compounds at Rf of 0.25, 0.59 and 0.71, respectively, for the silylation reaction. These results showed indications that the derivatization reactions were successful performed due to the conversion of polar compounds (as diterpenic acids) in derivative ones with lower polarity, which can be observed after the elution of the mobile phase.

Derivatization reactions did not change the Rf profile of the compounds present in the copaiba essential oil on the plate in the thin layer chromatography analysis (data not shown).

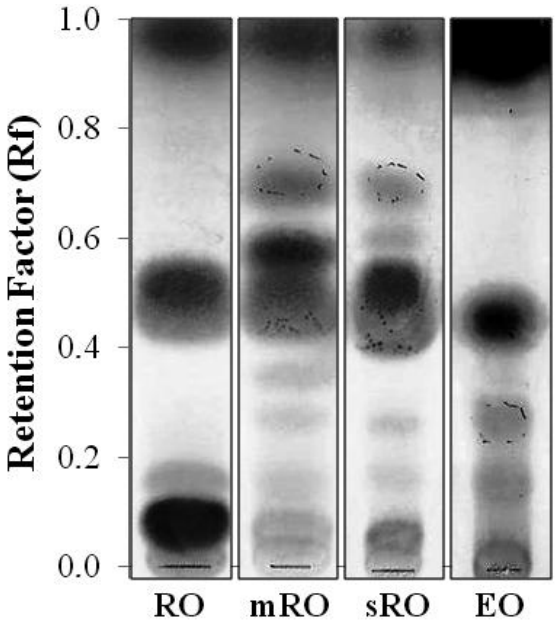

Figure 2- Thin layer chromatography profile of copaiba oils. Where RO is the copaiba resin oil, mRO is the copaiba resin oil after methylation, sRO copaiba resin oil after silylation and EO is the copaiba essential oil.

In order to analyze the compounds presented in the copaiba resin and essential oils, the GC-MS method was developed. Figure 3 shows the chromatographic profile of copaiba oils. The chromatogram showed a series of peaks indicating a good separation between the compounds during elution. It can be observed that the derivatization reaction preserved the sesquiterpenes compounds profile. However it was possible to identify a large amount of compounds which were eluted at high intensity in typical region of

identification of the diterpenes (Alencar, É. N. *et al.*, 2015). After derivatization by silylation and methylation reactions, same chromatographic profiles were also obtained.

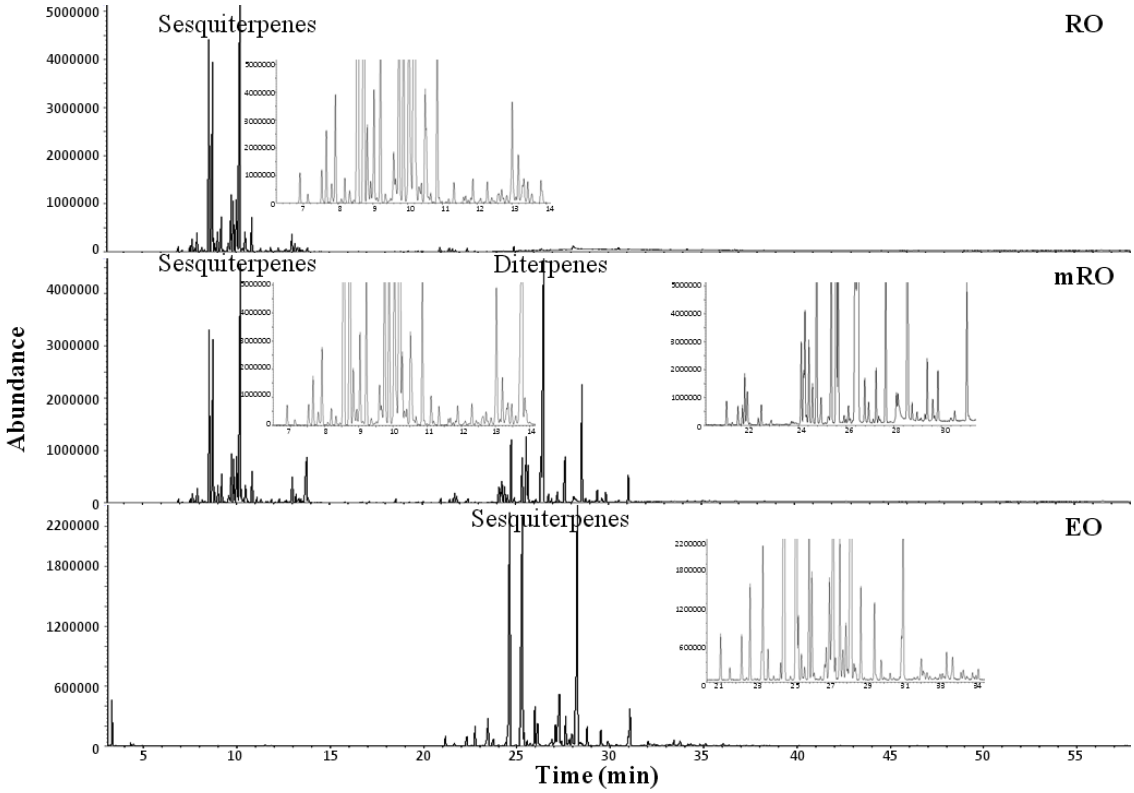

Figure 3- Gas chromatography profile of copaiba oils. Where RO is the copaiba resin oil, mRO is the copaiba resin oil after methylation and EO is the copaiba essential oil.

The residue fraction obtained after extraction of the essential oil possessed a lower amount of sesquiterpenes (less that 40 ± 3%), indicating that sesquiterpenes compounds were extracted by hydrodistillation and concentrated in the essential oil (Figure 4). In the same way, it was observed an increase in the concentration of diterpenes compounds in the methylated residue fraction (Figure 4 B).

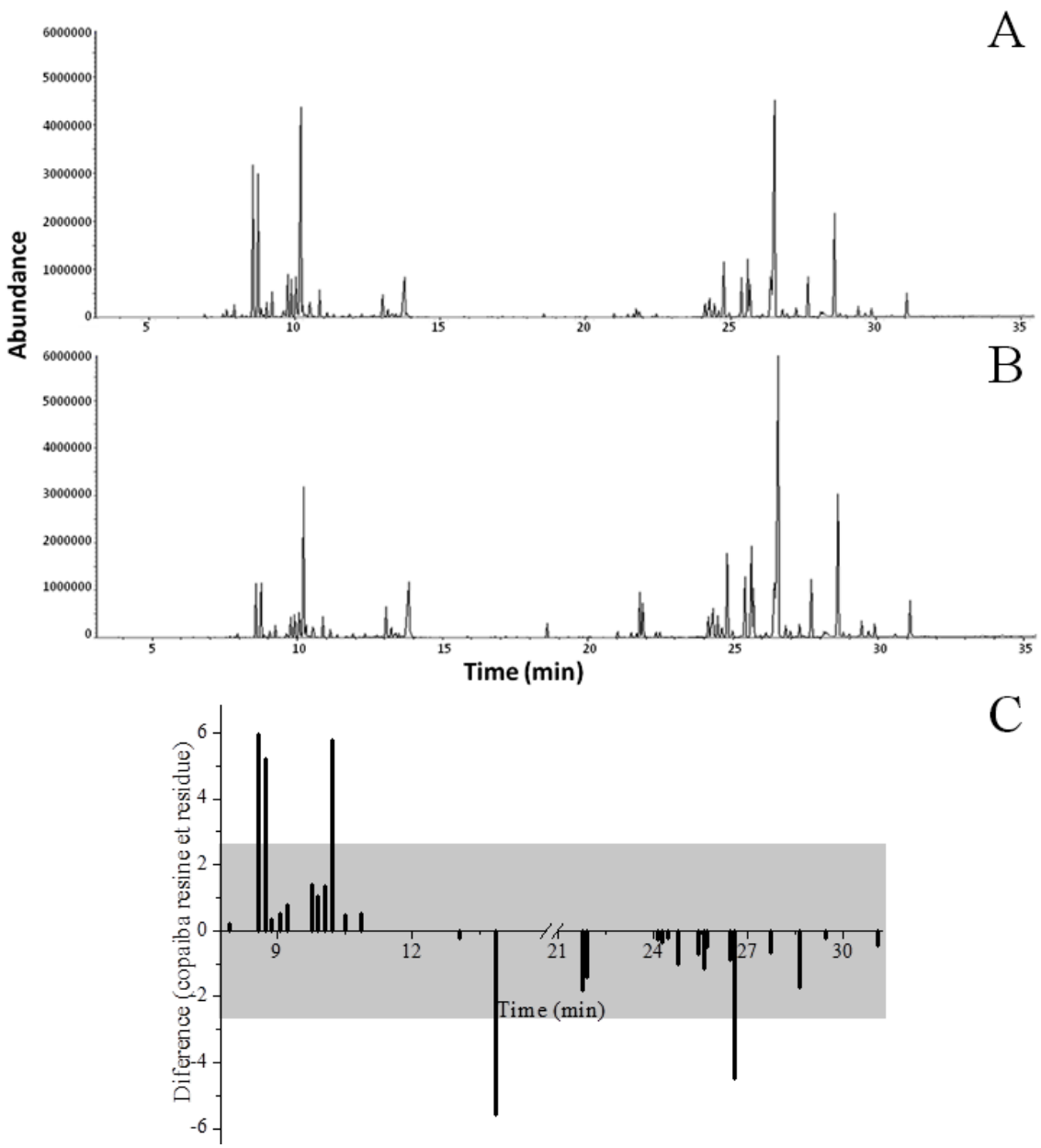

Figure 4- Difference between the methylated copaiba resin oil (A) before start the hydrodistillation process and methylated residue fractions (B) obtained after extraction of the copaiba essential oil (C). The gray color represents the precision of the method to determination of copaiba oil compounds

The parameters of the chromatographic copaiba essential oil analysis were different in comparison with to the ones of copaiba resin oil. The low rate of heating of the copaiba essential oil was performed, in order to better separate the compounds and to preserve the chemical structure of possible unstable molecules from their degradation. Therefore, the retention times of the sesquiterpenes compounds were different, but the chromatographic profile of the essential oil possessed the same sequence of the elution of the fraction from the copaiba resin oil. The chemical composition of copaiba essential and resin oils were obtained by GC-MS (Table 2). All chromatograms showed small peaks but only components giving a peak area greater than 0.1 % of all peak areas detected on the chromatograms were further considered in this work. The assays were able to detected 38 components from the non-derivatized copaiba resin oil, corresponding to 98.1 % of all compounds presented in the oil of which 95.9 % were sesquiterpenes. After the methylation reaction, it was possible to identify 52 compounds in the copaiba oil, in which 44.8 % of the relative area corresponded to sesquiterpenes and 48.2 % to diterpenes, totaling 93 % of detected compounds. In the copaiba essential oil, only sesquiterpenes were detected, corresponding to 97.5 % of all peak areas on the chromatograms.

Table 2- GC–MS analyses of *C. langsdorffii*. Peak identification, retention time (RT, min) and relative area percentage of RO: Copaiba resin oil, mRO: Methylated copaiba resin oil, EO: Copaiba essential oil.

Compounds	MW	RT (min)	RO Area (%)	mROArea (%)	RT (min)	EO Area (%)
δ-Elemene	204	6.90	0.4	0.2	21.17	0.6
α-Cubebene	204	7.13	0.1	0.1	21.67	0.2
(+)-Cyclosativene	204	7.53	0.5	0.2	22.31	0.7
α-Copaene	204	7.66	1.0	0.4	22.77	1.4
trans-α-Bergamotene	204	7.81	0.3	0.1	23.39	0.3
(-)-β-Elemene	204	7.92	1.5	0.6	23.46	2.0
δ-Selinene	204	8.19	0.4	0.1	23.74	0.5
(Z,β)-Farnesene	204	8.33	0.2	-	-	-
β-Caryophyllene	204	8.57	18.7	7.9	24.63	21.7
α-Bergamotene	204	8.75	16.0	7.1	25.29	20.5
α-Guaiene	204	8.84	1.2	0.5	25.38	0.9
Aromadendrene	204	8.95	0.3	0.1	25.54	0.4
α-Humulene	204	9.04	2.9	1.3	25.96	2.9
β-Farnesene	204	9.23	1.6	0.8	26.11	1.7
(+)-Valencene	204	-	-	-	26.81	0.3
τ-Muurolene	204	9.60	0.8	0.4	26.89	0.5
β-Cubebene	204	9.75	4.8	2.2	27.06	1.7
β-Selinene	204	9.88	4.4	2.0	27.27	6.1
α-Selinene	204	-	-	-	27.62	2.3

τ-Gurjunene	204	-	-	-	27.79	0.5
β-Chamigrene	204	10.04	5.7	2.7	27.95	0.9
β-Bisabolene	204	10.21	25.2	12.3	28.24	23.6
γ-Cadinene	204	10.40	0.3	0.1	-	-
δ-Cadinene	204	10.51	2.4	1.2	28.76	1.4
α-Bisabolene	204	10.86	2.7	1.4	-	-
ni	204	11.34	0.3	0.2	29.50	1.2
Caryophyllene oxide	220	11.89	0.4	0.2	31.04	4.1
ni	220	12.30	0.3	0.2	-	-
ni	220	12.72	0.2	0.1	-	-
ni	220	12.86	0.1	-	-	-
ni	204	13.03	1.8	1.4	33.40	0.4
Aromadendrane <dehydro>	206	13.21	0.7	0.4	-	-
ni	204	-	-	-	33.72	0.5
α-Cadinol	222	13.31	0.2	0.2	-	-
α-Bisabolol	222	13.84	0.5	0.4	35.11	0.2
Hexadecanoic methyl ester	270	18.58	-	0.2	-	-
Kaur-16-ene	272	20.99	0.4	0.3	-	-
ni	272	21.45	0.3	-	-	-
ni	286	21.66	0.3	0.2	-	-
Linoleic acid methyl ester	294	21.75	-	0.5	-	-
ni	296	21.82	0.2	-	-	-
ni	286	22.45	0.3	0.2	-	-
ni	286	24.22	-	0.5	-	-
ni	320	24.28	-	1.9	-	-

ni	320	24.44	-	0.8	-	-
ni	320	24.77	-	3.3	-	-
ni	286	24.95	0.5	0.3	-	-
Kaur-16-en-18-oic acid methyl ester	318	25.38	-	2.4	-	-
Methyl copalate	318	25.59	-	3.5	-	-
Kauran-19-oic acid methyl ester	318	25.67	-	1.9	-	-
ni	318	26.38	-	3.8	-	-
Copalic acid methyl ester	330	26.51	-	15.6	-	-
ni	332	26.79	-	0.4	-	-
ni	318	26.94	-	0.2	-	-
ni	332	27.26	-	0.5	-	-
ni	330	27.66	-	2.3	-	-
ni	336	28.11	-	0.4	-	-
Labd-8(20)-ene-15,18-dioic acid methyl ester	364	28.58	-	6.7	-	-
ni	364	28.76	-	0.2	-	-
ni	362	29.40	-	0.6	-	-
ni	376	30.55	0.2	0.1	-	-
ni	376	31.06	-	1.4	-	-
Total of Sesquiterpenes			95.9	44.8		97.5
Total of Diterpenes			2.2	48.2		0.0
Total detected			98.1	93.0		97.5

MW = molecular weight; ni = not identified; - absent

The major compounds identified in the copaiba essential oil were β-caryophyllene (21.7 %), α-bergamotene (20.5 %) and β-Bisabolene (23.6 %). From the copaiba resin oil, the major compounds were β-caryophyllene (18.7 %), α-bergamotene (16 %) and β-Bisabolene (25.2 %). In the methylated copaiba resin oil were β-caryophyllene (7.9 %), α-bergamotene (7.1 %), β-Bisabolene (12.3 %), copalic acid methyl ester (15.6 %) and Labd-8(20)-ene-15,18-dioic acid methyl ester (6.7 %) were the major compounds. Gramosa *et al.* reported that the major component in the copaiba essential oil was β-caryophyllene (53%) (Gramosa & Silveira, 2005). Soares *et al.* also detected the high level of β-caryophyllene (42.3%) in the volatile fraction rich in sesquiterpenes. On the other hand, the nonvolatile fraction consisted of a higher amount of copalic acid content (49.9%) (Soares *et al.*, 2013). Other studies reveal the presence of large amounts of α-bergamotene and copalic acid about 48 and 22 %, respectively for the copaiba essential oil and for the copaiba resin oil (Gelmini *et al.*, 2013).

It is noteworthy this amount variation of the main compound found in the copaiba essential oil and the copaiba resin oil reported by different authors. However, differences between the major compounds of the natural oils from different studies in the literature are well recognized, since it is well known that the chemical composition of natural oils is not constant. The variation may be attributed to several factors, which influence their composition, such as: the geographical origin of the plants, the environmental factors such as light, temperature, soil composition and season, the period and harvest time, as well as the plant organ, age and stage in the vegetative cycle (Raileanu *et al.*, 2013).

To perform the validation studies, the same analytical profile of the copaiba essential oil and the copaiba resin oil was developed using GC-FID. The goal was to find the

correlation between the two methods of analysis for further development of the dosages of the components presented in copaiba oil samples. Although mass spectrometers are sometimes considered the most powerful detectors for chromatographic methods, the flame ionization is the most used detector in gas chromatography because it has adequate sensitivity, large linear response range and low noise for most needed analysis, becoming, therefore, less expensive than mass spectrometers (Skoog *et al.*, 2007; Marriott *et al.*, 2012; Yuan *et al.*, 2015). Figure 5 presented the difference between all peaks areas detected on the chromatograms analyzed by GC-MS and GC-FID of copaiba resin oil (A) and copaiba essential (B) oil, respectively. There were no major changes in the areas of the compounds analyzed by both methods (maximum variation of 4%). The differences between the two methods were not statistically significant (p>0.05). In addition, the main compounds detected by GC-MS were also identified by the GC-FID in the same retention time, indicating correspondence between both methods.

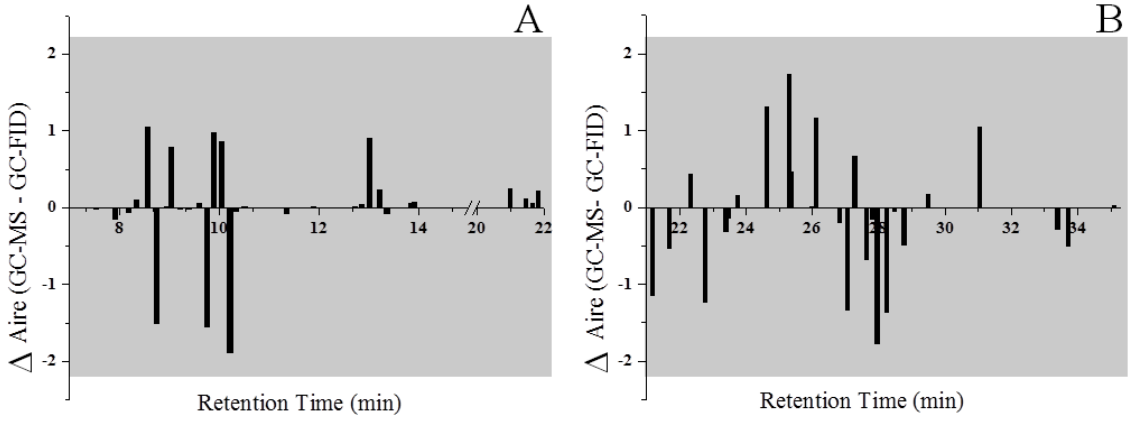

Figure 5- The main difference between all peaks areas detected on the chromatograms analyzed by GC-MS and GC-FID of copaiba resin oil (A) and copaiba essential (B) oil, respectively. The results were calculated based on the percentage area (%) difference

between the compounds. The gray color represents the precision of the method to determination of copaiba oil compounds

GC-FID methods of copaiba oils were initially performed, aiming to increase the resolution of the peaks and to reduce the analysis time between the samples. This process did not alter the sequence and neither the area percentage of the eluted compounds in the copaiba oil. This small adjustment in the methodology presented only a slightly change in the retention time. However, the analysis quality rest unchanged. Therefore, in order to quantify the compounds presented in the copaiba oil, the validation procedure was performed using β- caryophyllene, α- humulene and caryophyllene oxide as reference substances. These compounds, which represent an important group of sesquiterpenes, were selected due to their presence in all copaiba oil samples previously analyzed.

The studied standards showed good resolution peaks, indicating high specificity and selectivity (Figure 6). This method was specific for the standards with no interference of the peaks at the retention time. The purity of the peaks was confirmed by mass fragmentation in the GC-MS. The retention times measured by GC-MS analyses for β-caryophyllene, α- humulene and caryophyllene oxide were 13.15, 14.87 and 21.52 minutes, respectively. The examination of the chromatogram in Figure 6A revealed the presence of impurities that was eluted after β- caryophyllene (peak at 14.9 minutes). However, this fact was already expected due the quality of the sample. This small peak can be α- humulene, which has the same retention time. Therefore, the proposed method was considered adequate for β- caryophyllene, α- humulene and caryophyllene oxide

assay because the peak of the standards were well separated from each other compounds and no peaks interfered with the observed analyte peaks.

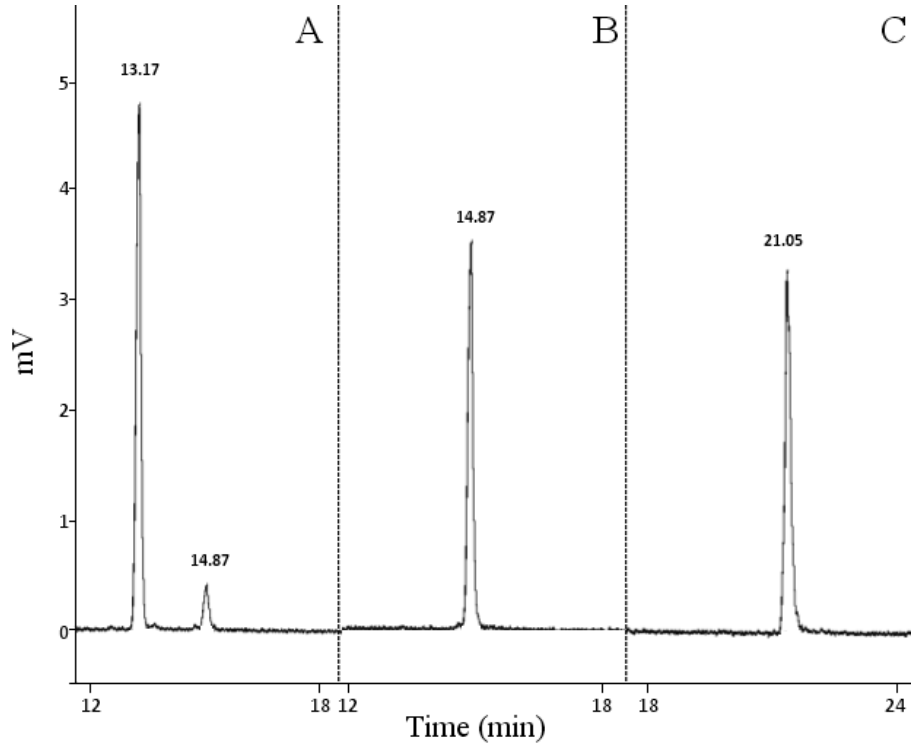

Figure 6- Representative GC-FID chromatograms of the β- caryophyllene (A), α-humulene (B) and caryophyllene oxide (C) standards at concentration of 160, 130 and 130 µg.mL^{-1}, respectively.

A linear range equation was judged to produce the best fit of the concentration / response relationship. Linearity of the analytical procedure was evaluated by plotting detector response (peak area) against analyzed concentration. Calibration plots were constructed after analysis of β- caryophyllene, α- humulene and caryophyllene oxide solutions at concentrations of 40, 70, 100, 130 and 160 µg.mL^{-1}. Each level was injected

in triplicate and the goodness to fit for concentrations were consistently greater than 0.99 during the course of the validation and study period. The regression equation was showed in the Table 3. Correlation coefficients for the method were 0.999, 0.997 and 0.998 for β- caryophyllene, α- humulene and caryophyllene oxide, respectively. The intercept was very small and the correlation coefficient closes to the unity for all standards. All these results indicated that the method was linear over the range of 40–160 $\mu g.mL^{-1}$.

Table 3- Validation parameters of β- caryophyllene, α- humulene and caryophyllene oxide

Parameters	**β-Caryophyllene**	***α*-Humulene**	**Caryophyllene oxide**
Retention Time (min)	13.15 ± 0.02	14.87 ± 0.02	21.52 ± 0.04
Linearity			
a (slope)	0.204 ± 0.007	0.202 ± 0.001	0.227 ± 0.005
b (intercept)	-1.332 ± 0.395	- 0.5044 ± 0.255	- 2.354 ± 0.038
Correlation coefficient(R^2)	0.999	0.997	0.998
Detection limit ($\mu g.mL^{-1}$)	6.38	4.16	0.55
Quantitation limit ($\mu g.mL^{-1}$)	19.36	12.62	1.67
Accuracy (%RSD)	3.21	3.46	2.24

LOD was identified as the lowest concentration of an analyte that the assay can reliably differentiate from the background noise (Validation of analytical procedures: Text and Methodology, ICH-Q2 (R1), 2005). β- caryophyllene, α- humulene and caryophyllene

oxide showed a LOD of 6.38, 4.16 and 0.55 $\mu g.mL^{-1}$, respectively (Table 3). LOQ was identified as the lowest concentration of an analyte in a sample that could be determined with acceptable precision and accuracy under the stated experimental conditions for this method (*Food and Drug Administration, Guidance for Industry. Bioanalytical Method Validation* 2001)). LOQ for β- caryophyllene, α- humulene and caryophyllene oxide was 19.36, 12.62 and 1.67 $\mu g.mL^{-1}$, respectively (Table 3).

The accuracy of the assay was defined as the absolute value of the ratio between the calculated mean values of the quality control samples and their normal values. Standards accuracy was determined on the range of 80–120% of the analytical working concentration by calculating recovery. The accuracy of the β- caryophyllene, α- humulene and caryophyllene oxide ranged from 98.81 to 102.51 %, 99.87 to 103.66 %, and 98.61 to 101.11 %, respectively. These results values showed a RSD lower than 3.21, 3.46 and 2.24 % for β- caryophyllene, α- humulene and caryophyllene oxide, respectively (Table 3). Thus, it can be inferred that the method demonstrated a good correlation between the theoretical and the practical values, satisfying the drug quality research requirements.

Precision was estimated by the intra-day (repeatability) and the inter-day precision. Intra-day precision was investigated by injecting triplicate samples of β- caryophyllene, α- humulene and caryophyllene oxide solutions at three different concentrations (40, 100 and 160 $\mu g.mL^{-1}$). Inter-day precision was determined by evaluating the repeatability of the analytical procedure, if re-produced in the same laboratory, but under the analysis carried out on another day. The results obtained for the inter- and intra-day precision studies are presented in Table 4. Based on these results, these

methods were considered satisfactory, presenting lower random errors (p <0.05) and representing a true measure of the obtained analytical results proximity.

Table 4- Intra and inter-day variations of β- caryophyllene, α- humulene and caryophyllene oxide

Spiked Concentration ($\mu g.mL^{-1}$)	**Measured Concentration**								
	β- caryophyllene			**α- humulene**			**Caryophyllene oxide**		
	Mean ($\mu g.mL^{-1}$)	**SD**	**RSD (%)**	**Mean ($\mu g.mL^{-1}$)**	**SD**	**RSD (%)**	**Mean ($\mu g.mL^{-1}$)**	**SD**	**RSD (%)**
Intra-day variation									
40	41.4	1.0	2.3	40.3	1.2	3.0	41.1	1.7	4.2
100	99.4	2.4	2.4	103.5	2.0	1.9	100.5	3.9	3.9
160	162.3	3.2	2.0	161.3	1.9	1.2	161.1	2.5	1.5
Inter-day variation									
40	40.8	1.2	2.9	41.5	1.8	4.3	41.5	1.8	4.3
100	98.8	4.3	4.4	105.5	2.5	2.4	105.5	2.0	1.9
160	159.1	4.9	3.1	162.1	2.1	1.3	162.1	1.9	1.2

Values are for n= 3 observations; S.D., standard deviation and R.S.D., relative standard deviation.

Finally, this validated analytical method was used to determine the amount of the compounds in the copaiba resin oil and copaiba essential oil by GC-FID. The stock solution at 10 $mg.mL^{-1}$ of copaiba essential oil contained 1982 ± 13, 279 ± 25 and 24 ± 0.9 $\mu g.mL^{-1}$ of β- caryophyllene, α- humulene and caryophyllene oxide, respectively; In

contrast, the copaiba resin oil showed standards dosages of 808 ± 25, 97 ± 6 and 16 ± 0.6 $\mu g.mL^{-1}$ for β- caryophyllene, α- humulene and caryophyllene oxide, respectively. As chromatograms always show the same ratio between the peaks, these ratios can be used to determine the concentration of other components from the copaiba oil samples. The results of the ratio among β- caryophyllene and others compounds identified in the copaiba oils were shown in Figure 7.

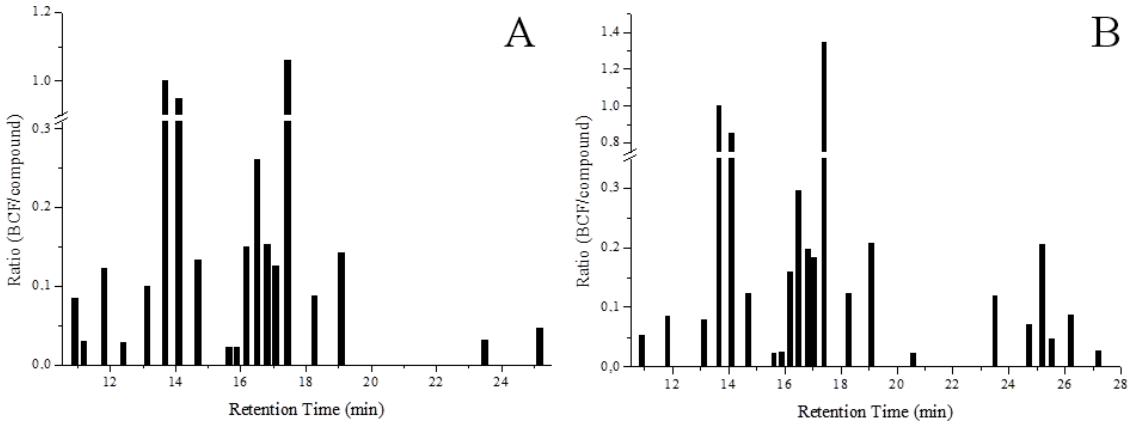

Figure 7- Ratio among the peaks areas of β- caryophyllene (BCF) and the others peak area compounds detected on the chromatograms from the copaiba essential oil (A) and the copaiba resin oil (B).

4.0 CONCLUSION

Compounds presented in the copaiba oils were effectively identified by GC-MS analysis. Furthermore, the derivatization of the copaiba resin oil was performed promoting the identification of diterpenes compounds. A good correlation between the GC-FID and GC-MS analysis were obtained, favoring the transposition of the

methodology analysis. A rapid, simple, accurate and precise GC-FID method for the quantitation of β- caryophyllene, α- humulene and caryophyllene oxide in copaiba oil samples was developed and validated. Acceptable values were obtained for the following validation parameters, such as: Linearity, LOD, LOQ, precision and accuracy. The methods described in this work were successfully used for quantifying the β-caryophyllene, α- humulene and caryophyllene oxide in the copaiba oils samples. This work should be suitable to the reliable quantification for quality control of different copaiba oil species and can also be used for copaiba oil quantitation when loaded in pharmaceutical or cosmetic formulations.

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REFERENCES

ALENCAR, É. N., XAVIER-JÚNIOR, F. H., MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G. & EGITO, E. S. T. 2015. Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions. *J Nanosci Nanotechnol*, 15, 1, 880-888.

ATTIMARAD, M., MUEEN AHMED, K. K., ALDHUBAIB, B. E. & HARSHA, S. 2011. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. *Pharm Methods*, 2, 2, 71-75.

ATURKI, Z., ROCCO, A., ROCCHI, S. & FANALI, S. 2014. Current applications of miniaturized chromatographic and electrophoretic techniques in drug analysis. *J Pharm Biomed Anal*, 101, 0, 194-220.

DEUS, R. J. A., ALVES, C. N. & ARRUDA, M. S. P. 2011. Avaliação do efeito antifúngico do óleo resina e do óleo essencial de copaíba (Copaifera multijuga Hayne). *Rev Bras Plantas Med*, 13, 01-07.

Food and Drug Administration, Guidance for Industry. Bioanalytical Method Validation 2001.

GELMINI, F., BERETTA, G., ANSELMI, C., CENTINI, M., MAGNI, P., RUSCICA, M., CAVALCHINI, A. & MAFFEI FACINO, R. 2013. GC-MS profiling of the phytochemical constituents of the oleoresin from Copaifera langsdorffii Desf. and a preliminary in vivo evaluation of its antipsoriatic effect. *Int J Pharm*, 440, 2, 170-8.

GONZÁLEZ, O., BLANCO, M. E., IRIARTE, G., BARTOLOMÉ, L., MAGUREGUI, M. I. & ALONSO, R. M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *J Chromatogr A*, 1353, 0, 10-27.

GRAMOSA, N. V. & SILVEIRA, E. R. 2005. Volatile constituents of *Copaifera langsdorffii* from the Brazilian northeast. *J Essent Oil Res*, 17, 2, 3.

MAGGIO, R. M., CALVO, N. L., VIGNADUZZO, S. E. & KAUFMAN, T. S. 2014. Pharmaceutical impurities and degradation products: Uses and applications of NMR techniques. *J Pharm Biomed Anal*, 101, 0, 102-122.

MARRIOTT, P. J., CHIN, S.-T., MAIKHUNTHOD, B., SCHMARR, H.-G. & BIERI, S. 2012. Multidimensional gas chromatography. *Trends Analyt Chem*, 34, 0, 1-21.

MUJAWAR, S., UTTURE, S. C., FONSECA, E., MATARRITA, J. & BANERJEE, K. 2014. Validation of a GC–MS method for the estimation of dithiocarbamate fungicide residues and safety evaluation of mancozeb in fruits and vegetables. *Food Chem*, 150, 0, 175-181.

NIKOLAOU, P., PAPOUTSIS, I., DONA, A., SPILIOPOULOU, C. & ATHANASELIS, S. 2015. Development and validation of a GC/MS method for the simultaneous determination of levetiracetam and lamotrigine in whole blood. *J Pharm Biomed Anal*, 102, 0, 25-32.

ORATA, F. Derivatization Reactions and Reagents for Gas Chromatography Analysis, Advanced Gas Chromatography. In: MOHD, D. M. A. (Ed.). Progress in Agricultural, Biomedical and Industrial Applications: InTech, 2012.

RAILEANU, M., TODAN, L., VOICESCU, M., CIUCULESCU, C. & MAGANU, M. 2013. A way for improving the stability of the essential oils in an environmental friendly formulation. *Mater Sci Eng C Mater Biol Appl*, 33, 6, 3281-8.

SALEEM, M., NAZIR, M., ALI, M. S., HUSSAIN, H., LEE, Y. S., RIAZ, N. & JABBAR, A. 2010. Antimicrobial natural products: an update on future antibiotic drug candidates. *Nat Prod Rep*, 27, 2, 238-54.

SANTOS, A. O., COSTA, M. A., UEDA-NAKAMURA, T., DIAS-FILHO, B. P., DA VEIGA-JÚNIOR, V. F., DE SOUZA LIMA, M. M. & NAKAMURA, C. V. 2011. Leishmania amazonensis: Effects of oral treatment with copaiba oil in mice. *Exp Parasitol*, 129, 2, 145-151.

SANTOS, E. C. G., DONNICI, C. L., CAMARGOS, E. R., AUGUSTO DE REZENDE, A., ANDRADE, E. H., SOARES, L. A., FARIAS LDE, M., ROQUE DE CARVALHO, M. A. & ALMEIDA, M. 2013. Effects of Copaifera duckei Dwyer oleoresin on the cell wall and cell division of Bacillus cereus. *J Med Microbiol*, 62, Pt 7, 1032-7.

SKOOG, D. A., HOLLER, F. J. & CROUCH, S. R. Instrumental Analysis. Int. India: Cengage learning, 2007.

SOARES, D. C., PORTELLA, N. A., RAMOS, M. F., SIANI, A. C. & SARAIVA, E. M. 2013. Trans- beta -Caryophyllene: An Effective Antileishmanial Compound Found in Commercial Copaiba Oil (Copaifera spp.). *Evid Based Complement Alternat Med*, 2013, 761323.

SOUSA, A. A. V. O., LIMA, F. D., MACHADO, A. A., VERA, M. O., FERNANDES, L. M. P., LIMA, R. R. & MAIA, C. D. S. F. 2013. Tissular and motor analysis of neuroprotective and anti-inflammatory/INS; effects of copaiba oil (Copaifera reticulata Ducke) after focal cortical ischemia. *J Neurol Sci* 333, Supplement 1, 0, e247.

SOUSA, J. P. B., BRANCALION, A. P. S., SOUZA, A. B., TURATTI, I. C. C., AMBRÓSIO, S. R., FURTADO, N. A. J. C., LOPES, N. P. & BASTOS, J. K. 2011. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal*, 54, 4, 653-659.

STONE, M. J. & WILLIAMS, D. H. 1992. On the evolution of functional secondary metabolites (natural products). *Mol Microbiol*, 6, 1, 29-34.

Validation of analytical procedures: Text and Methodology, ICH-Q2 (R1). International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, 2005, Geneva.

VEIGA JUNIOR, V. F. & PINTO, A. C. 2002. O gênero *copaifera* L. *Quim Nova*, 25, 273-286.

VIEIRA, R. C., BOMBARDIERE, E., DE OLIVEIRA, J. J., DE SOUZA LINO JÚNIOR, R., BRITO, L. A. B. & JUNQUEIRA-KIPNIS, A. P. 2009. Topical Copaíba oil treatment of surgical wound alters the repair and the cell migration in the presence of a foreign body in BALB/c mice. *Vet Immunol Immunopathol*, 128, 1–3, 319.

XAVIER-JÚNIOR, F. H., SILVA, K. G. H., FARIAS, I. E. G., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., OLIVEIRA, A. G. & EGITO, E. S. T. 2012b. Prospective study for the development of emulsion systems containing natural oil products. *J Drug Del Sci Tech*, 22, 4, 273-373.

YUAN, C., CHEN, D. & WANG, S. 2015. Drug confirmation by mass spectrometry: Identification criteria and complicating factors. *Clin Chim Acta*, 438, 0, 119-125.

Chapter II

HPLC method for the dosage of paclitaxel in copaiba oil. Development, validation, application to the determination of the solubility and partition coefficients

Le deuxième chapitre de ce mémoire de thèse présente une méthode très sensible, simple, rapide, innovante et économique a été développée et validée pour la quantification de paclitaxel dans l'huile de copaïba utilisant la chromatographie en phase liquide à haute performance couplée à une détection par absorption dans l'ultraviolet. La séparation chromatographique a été réalisée par sur une colonne de type Uptisphere Strategy 100A en phase inverse C-18 (150 mm x 3 µm x 3 mm). La phase mobile était constituée d'acétonitrile et d'eau (50:50). Le débit était de 0,4 $mL.min^{-1}$ et la détection a été effectuée à 228 nm. Aucun pic d'interférence a été observés lors de l'élution de paclitaxel sur un temps d'analyse totale de 15 min. La courbe d'étalonnage du paclitaxel dans un milieu contenant 10 $\mu g.mL^{-1}$ d'huiles résine ou d'huile essentielles de copaïba était linéaire sur la gamme de concentration de 50 à 2000 $ng.mL^{-1}$ avec un coefficient de détermination de 0,999 et des résidus de régression faibles avec une dispersion homoscédastique. Les limites de quantification et de détection étaient basse à 21,03 et 6,31 $ng.mL^{-1}$, respectivement. L'exactitude et la précision des déterminations étaient inférieures ou égales à 0,77 et 0,65%, respectivement. La méthode développée a été appliquée avec succès pour l'évaluation de la solubilité du paclitaxel dans des échantillons de l'huile de copaïba par des études de coefficient de partage. Le paclitaxel a montré une caractéristique lipophile (log P> 1) et une plus grande solubilité dans les huiles résine et essentielle de copaïba par rapport à l'eau. En conclusion, la méthode a montré une sensibilité, linéarité, précision, exactitude et spécificité nécessaires pour le succès de la quantification du paclitaxel dans des échantillons d'huile de copaïba. Ces méthodes sont adaptées pour une application aux analyses quantitatives de paclitaxel incorporé dans les systèmes d'administration de médicaments contenant de l'huile de copaïba .

Mots-clés: paclitaxel, huile de copaïba, validation, chromatographie en phase liquide à haute performance, solubilité, coefficient de partage

HPLC METHOD FOR THE DOSAGE OF PACLITAXEL IN COPAIBA OIL. DEVELOPMENT, VALIDATION, APPLICATION TO THE DETERMINATION OF THE SOLUBILITY AND PARTITION COEFFICIENTS.

Xavier-Junior, F. H. [1,2], Gueutin, C.[2], Morais, A. R. V. [1,2], Alencar, E. N.[1], Egito, E. S. T. [1], Vauthier, C.[2] *

[1] Universidade Federal do Rio Grande do Norte, Centro de Ciências da Saúde, Departamento de Farmácia, Laboratório de Sistemas Dispersos (LaSiD). Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Corresponding author

Christine Vauthier, Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Christine.vauthier@u-psud.fr

ABSTRACT

A highly sensitive, simple, rapid, innovative and economic method has been developed and validated for the quantification of paclitaxel in copaiba oil using High-Performance Liquid Chromatography (HPLC) with UV detection. Chromatographic separation was performed by Uptisphere Strategy 100A reversed-phase C-18 (150 mm x 3 µm x 3 mm) column. The mobile phase was constituted of acetonitrile and water (50:50). The flow rate was 0.4 $mL.min^{-1}$ and the detection was performed at 228 nm. No interfering peaks were observed during the paclitaxel elution at the total run time of 15 min. Standard curves of paclitaxel containing 10 mg of copaiba resin oil was linear over the concentration range from 50 to 2000 $ng.mL^{-1}$ with a determination coefficient of 0.999 and lower regression residues with a homoscedastic dispersion. The lower quantification and detection limits were 21.03 and 6.31 $ng.mL^{-1}$, respectively. The accuracy and precision determinations were less or equal to 0.77 and 0.65 %, respectively. The method developed was successfully applied to the evaluation of paclitaxel in copaiba oil samples by solubility and partition coefficient studies. Paclitaxel showed a lipophilic characteristic (logP>1) and a higher solubility in copaiba resin oil and copaiba essential oil. In conclusion, the method showed sensitivity, linearity, precision, accuracy and specificity necessary for successfully quantification of the paclitaxel in copaiba oil samples. This analytical method can be, therefore, also applied to quantify paclitaxel in drug delivery systems in which copaiba oil is associated, such as lipid and polymer systems.

Keywords: Paclitaxel, Copaiba Oil, Validation, HPLC, Solubility, Partition Coefficient

1.0 INTRODUCTION

Paclitaxel ($C_{47}H_{51}NO_{14}$) is a pseudoalkaloid with a diterpenoid structure having unique tri- or tetracyclic 20 carbon skeletons and a molecular weight of 853 Da (Fang & Liang, 2005). Originally, it was isolated in early 1960s from the bark of Pacific Yew (*Taxus brevifolia*; family Taxaceae) (Wani *et al.*, 1971). Paclitaxel has been the most effective antitumor agent developed in the past three decades (Kim *et al.*, 2005). However, the production of paclitaxel in large-scale has been limited due to the low abundance and slow growth of Taxus trees, combined to a low concentration of the drug in the trees (Frense, 2007). Nowadays, the paclitaxel has been produced by semi-synthesis reaction from 10-deacetylbaccatin III, a renewable precursor found in the needles of the European yew tree (*Taxus baccata*) (Gueritte-Voegelein *et al.*, 1994) or through of the suspension culture of Taxus cells (Kajani *et al.*, 2012), as promising alternative less expensive, less difficult and nondestructive methods to the Taxus species.

Paclitaxel is an antineoplastic agent used effectively to treatment of a variety of cancers including refractory ovarian, breast, small and non-small cell lung, head and neck carcinoma and AIDS-related Kaposi's sarcoma (Wani *et al.*, 1971; Rowinsky *et al.*, 1990; Rowinsky *et al.*, 1992; Huizing *et al.*, 1995; Singla *et al.*, 2002). Paclitaxel has a unique mechanism of action. It disrupts the dynamic equilibrium within the microtubule system promoting the hyper-stabilization of the cellular microtubules, forming an incomplete metaphase plate of chromosomes and an abnormal organization of spindle microtubules (Horwitz, 1992; Rao *et al.*, 1994). Therefore, it blocks cells in the late G2 phase and M phase of the cell cycle, thereby, inhibiting cell replication and promoting the cellular apoptosis (Schiff *et al.*, 1979; Panchagnula, 1998).

The diterpenoid pseudoalkaloid drug paclitaxel is insoluble in aqueous media and show a poor permeability across biological membranes that complicates its application in therapeutic formulations (Singla *et al.*, 2002). Furthermore, paclitaxel is a substrate for P-glycoprotein, a membrane transporter that serves as a drug efflux pump and can alter paclitaxel pharmacokinetics and sensitivity to tumor cells (Guo *et al.*, 2003). To overcome this mechanism of cancer drug resistance, the use of additional substances in the formulation is required. In this sense, copaiba oil is a natural oil, rich in caryophyllene compounds, that can be used to increase the uptake of paclitaxel by inhibition of the P-glycoprotein efflux transporters (Legault & Pichette, 2007). The copaiba oil consists of a mixture of sesquiterpenes and diterpenes hydrocarbons (Sousa, J. P. *et al.*, 2011; Alencar, E. N. *et al.*, 2015; Xavier-Junior, Chapter I, 2015a). This oil has been traditionally used in folk medicine due its therapeutic properties as anticancer, anti-inflammatory, antioxidant, antimicrobial, antitetanus, and antiseptic among others (Gomes, N. M. *et al.*, 2007; Santos, A. O. *et al.*, 2008; Leandro *et al.*, 2012).

In order to associate the anticancer activity of paclitaxel and copaiba oil in a single nanomedicine formulation, a method capable to dose paclitaxel in a medium that contained copaiba resin oil and copaiba essential oils were of grand interest. Such a method would be also needed to determine the solubility of paclitaxel in the copaiba oil and to evaluate its partition coefficient, which would be good predictor characteristics for the success of a nanomedicine formulation containing it. In fact, the majority of methods used to produce nanomedicines involve dispersions of an organic phase into an aqueous phase.

Over the course of the current analysis, the complex components mixture from copaiba oil can hamper the quantification of paclitaxel when associated with these formulations.

Accordingly, the need for an accurate and precise analysis method of paclitaxel in copaiba oil is mandatory for quality control and drug development. Several High-Performance Liquid Chromatography (HPLC) methods for the separation and determination of paclitaxel from biological samples were reported in the literature (Song & Au, 1995; Supko *et al.*, 1999). However, there are only few studies that have reported the analysis of this drug in highly complex matrices such as vegetable oils. Most of the proposed methods displayed high sensitivity including HPLC- MS and immunoassays, but their high cost prevents their application on a routine analytical use (Wang *et al.*, 2003). Moreover, HPLC- UV, a simple and efficient method, can be a suitable analytical tool to perform paclitaxel analyzes at a low cost with high reproducibility.

Thus, the aim of the present work was to develop and validated a simple, fast, specific and sensitive HPLC-UV method for the quantification of paclitaxel associate with copaiba oil. The method was investigated to be applied on the determination of the solubility of paclitaxel in the copaiba oils and to evaluate its partition coefficient.

2.0 MATERIALS AND METHODS

2.1 Materials

Copaiba oil (*Copaifera langsdorffii*) was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Paclitaxel was obtained from CHEMOS GmbH (Regenstauf, Germany). Methanol, ethanol, n-octanol and acetonitrile were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q® plus, Millipore, St Quentin en Yvelines, France).

2.2. Copaiba essential oil extraction

Copaiba essential oil was obtained from 400 mL of copaiba resin oil by hydro-distillation using a Clevenger-type apparatus for 3 h. The extract was dried with sodium sulphate, filtered and stored at −20°C.

2.3 Chromatographic equipment and conditions

A Waters HPLC system equipped with a Waters 515 pump, a Waters 717 plus autosampler, and a Waters 486- Tunable Absorbance detector (Waters Corp., Milford, MA) were used. Chromatographic separations were achieved using a Uptisphere Strategy 100A reversed-phase C-18 (150 mm x 3 µm x 3 mm) column and a Uptisphere Strategy C18-2 (10 mm x 3 µm x 4 mm) guard column (Interchim SA, Montluçon, France). The detection wavelength was set at 228 nm, which was the maximum absorbance level observed for paclitaxel. The mobile phase consisting of acetonitrile: water (50:50) was pumped through the column at a flow rate of 0.4 $mL.min^{-1}$ at 30°C. 25 µL samples were introduced onto the HPLC system every 15 min. The mobile phase and the samples were filtered through a 0.20 µm hydrophilic nylon membrane filter (Merck Millipore, Billerica, MA, EUA) prior to use. Chromatographic data were monitored and analyzed using Azur software (Datalys, France)

2.4. Validation methods

The validation of the chromatographic method was performed according to the ICH validation guidelines (Validation of analytical procedures: Text and Methodology, ICH-

Q2 (R1), 2005) for specificity/selectivity, linearity, precision, accuracy, and limit of detection (LOD) and limit of quantification (LOQ). Validation tests followed the good manufacturing practices and all equipments and volumetric glassware were evaluated and calibrated before analysis. Three individual stock solutions of paclitaxel at 1 $mg.mL^{-1}$ were prepared in ethanol, placed into a hermetically sealed amber vial and stored at -20 °C until use. The analytical standards at concentrations ranging from 50 to 2000 $ng.mL^{-1}$ (50, 200, 500, 800, 1100, 1400, 1700 and 2000 $ng.mL^{-1}$) were prepared in the mobile phase and used for the validation analyzes.

2.4.1 Specificity/selectivity

The specificity/selectivity of the analytical method was confirmed by analyzes of the paclitaxel analytical standard solutions at concentration of 200 $ng.mL^{-1}$ prepared with either 1 mL of mobile phase, or 1 mL of mobile phase with 10 mg of copaiba resin or copaiba essential oils. These analyses were repeated six times. The ability to separate all compounds demonstrating the lack of chromatographic interference from standard samples was analyzed.

2.4.2. Linearity

Paclitaxel stock solution was diluted to give a series of sub-stock solutions with concentrations ranging from 50 to 2000 $ng.mL^{-1}$ (50, 200, 500, 800, 1100, 1400, 1700 and 2000 $ng.mL^{-1}$). Sub-stock solutions were prepared by appropriate dilution of paclitaxel stock solutions to a final volume of 1 mL of the mobile phase containing 10

mg of copaiba resin oil. Calibration curves were obtained by least-squares linear regression, weighted by the reciprocal of the concentration, using the peak area of the drug. Linearity curves were performed on 3 different days.

2.4.3. Detection and quantification limits

The LOD was defined as the lowest concentration of paclitaxel resulting in a peak area greater or equal to three times from the background noise ($S/N \geq 3$). The LOQ was determined based on the standard deviation of the response and the slope with peak area greater or equal to ten times from the background noise ($S/N \geq 10$). LOD and LOQ were estimated from the calibration curve of the paclitaxel concentrations ranging from 50 to 2000 $ng.mL^{-1}$ in 1mL of mobile phase containing 10 mg of copaiba resin oil.

2.4.4. Accuracy

The accuracy of the HPLC method was demonstrated by the percentage of deviation. Accuracy was determined by six replicates of paclitaxel concentrations (ranged from 50 to 2000 $ng.mL^{-1}$) in 1 mL of mobile phase with 10 mg of copaiba resin oil. The concentrations found were obtained by refitting peak response ratios from paclitaxel solutions of concentrations added into a derived regression equation. The found and added concentrations were, then, used to determine the absolute percentage of deviation at each paclitaxel concentration containing copaiba resin oil.

2.4.5. Precision

The precision of the method was assessed by analyzing the intra- and inter-day variability of paclitaxel samples. The intra-day precision was determined by quantification of paclitaxel samples at three distinct concentrations (800, 1400 and 2000 $ng.mL^{-1}$) in 1 mL of mobile phase with 10 mg of copaiba resin oil. The samples were injected six times and prepared within a day. The inter-day precision was assessed separately from the obtained peak areas by injecting the same three drug concentrations in distinct days. Posteriorly, concentrations were calculated by refitting peak response ratios into a derived regression equation from the calibration curve and the relative standard deviation (%RSD) were calculated for the intra- and inter-day precision.

2.5. Solubility evaluation of paclitaxel

Paclitaxel solubility was determined in milli-Q® water, copaiba essential oil and copaiba resin oil by adding a surplus of drug substance in a glass vial with 1 mL solvent. The vials were sealed and shaken at room temperature for 24 h to assure saturation. After equilibration, samples were removed and centrifuged at 15,000 rpm for 15 min (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) to remove insoluble crystals of paclitaxel. The supernatants were filtered through 0.20 µm nylon filter (Merck Millipore, Billerica, MA, EUA). The filtrates were diluted as required with the mobile phase and stirred in an ultrasound bath (Elma Elmasonic S10H, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) for 5 minutes. The resulting solutions were analyzed by the HPLC method as previously described. Paclitaxel

concentrations in the samples were obtained using the linear regression equation from the calibration curve.

2.6. Partition coefficient of paclitaxel

The partition coefficient of paclitaxel between n-octanol/water, copaiba resin oil/water and copaiba essential oil/water were determined at 25 °C. One mL of each lipophilic substance and 1 mL of milli-Q® water were placed separately in glass stoppered flasks. These flasks were previously shaken by magnetic stirrer (C-MAG HS 7 IKA, Staufen, Germany) for 24 hours at 25 ± 1°C. Thus, 1 mg of the drug was added, following by magnetic stirrer for another 24 hours at 25 ± 1°C. To promote the separation of the aqueous and oily phases, the samples were centrifuged for 5 min at 3,000 rpm (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany). Posteriorly, the aqueous (Milli-Q® water) and oily (n-octanol, copaiba essential oil and copaiba resin oil) phases were carefully separated and centrifuged at 15,000 rpm for 15 min to precipitate insoluble crystals of paclitaxel. The supernatant from each separated systems (n-octanol/water, copaiba resin oil/ water and copaiba essential oil/ water) were filtered through a 0.20 µm nylon filter and diluted if required in 1 mL of the mobile phase using ultrasound bath for 5 min. The resulting solutions were analyzed by the HPLC method as previously described. The partition coefficient of the paclitaxel was taken as the logarithm ratio between the drug concentration (w/v) solubilized in each lipophilic and hydrophilic phases (logP).

2.7 Statistical analyzes

All the experiments were conducted in triplicates. All values are expressed as their mean ± standard deviation (SD). Means of two groups were compared using non-paired Student's t-tests. When comparing multiple groups, one way analyzes of variance (ANOVA) was applied. The statistical data were considered significant at $p < 0.05$

3.0 RESULTS AND DISCUSSION

The development and validation of a HPLC-UV method for the quantification of paclitaxel in natural oils from *Copaifera langsdorffii* was performed. Chromatographic conditions were developed to achieve a routine analysis for paclitaxel in copaiba oils, with high reproducibility and sensibility, combined to a simple, rapid and economic way. The copaiba essential oil, obtained from the copaiba resin oil by the hydro-distillation method with yield of 11 ± 0.8%, was transparent. Previous studies showed that copaiba essential oil is constituted by a mixture of sesquiterpenes compounds, which the major identified compounds were β-caryophyllene (21.7 %), α-bergamotene (20.5 %) and β-bisabolene (23.6 %). However, the copaiba resin oil showed 64 different compounds, among them, 47.7 % of diterpenes, especially copalic acid (15.6 %) and labd-8(20)-ene-15,18-dioic acid (6.7 %), besides sesquiterpene compounds as β-caryophyllene (7.9 %), α-bergamotene (7.1 %) and β-bisabolene (12.3 %) (Xavier-Junior, Chapter I, 2015a).

The complex mixtures of copaiba oil compounds make difficult the identification and quantification of lipophilic molecules when incorporated into this oil because of the presence of innumerable potential interfering compounds. Therefore, the development

of an effective methodology able to identify the paclitaxel when incorporated in copaiba oil samples was required.

The specificity/selectivity of the analytical method was confirmed by analyzing solutions containing 200 $ng.mL^{-1}$ of the working drug and known amount of copaiba oils (Figure 1). The chromatograms showed a good resolution and separation of paclitaxel from other peaks attributed to compounds presented in the copaiba oil. No statistically significant difference was observed between the chromatogram areas of samples containing copaiba resin oil or copaiba essential oils in comparison with the chromatogram obtained only with the mobile phase. Peak purity values for paclitaxel in the chromatograms were in the range from 0.996 to 1.0. These results indicated that the peaks were homogenous with a retention time at 9.7 ± 0.2 minutes and showed a significant ability to separate the paclitaxel from the complex mixture of copaiba oil.

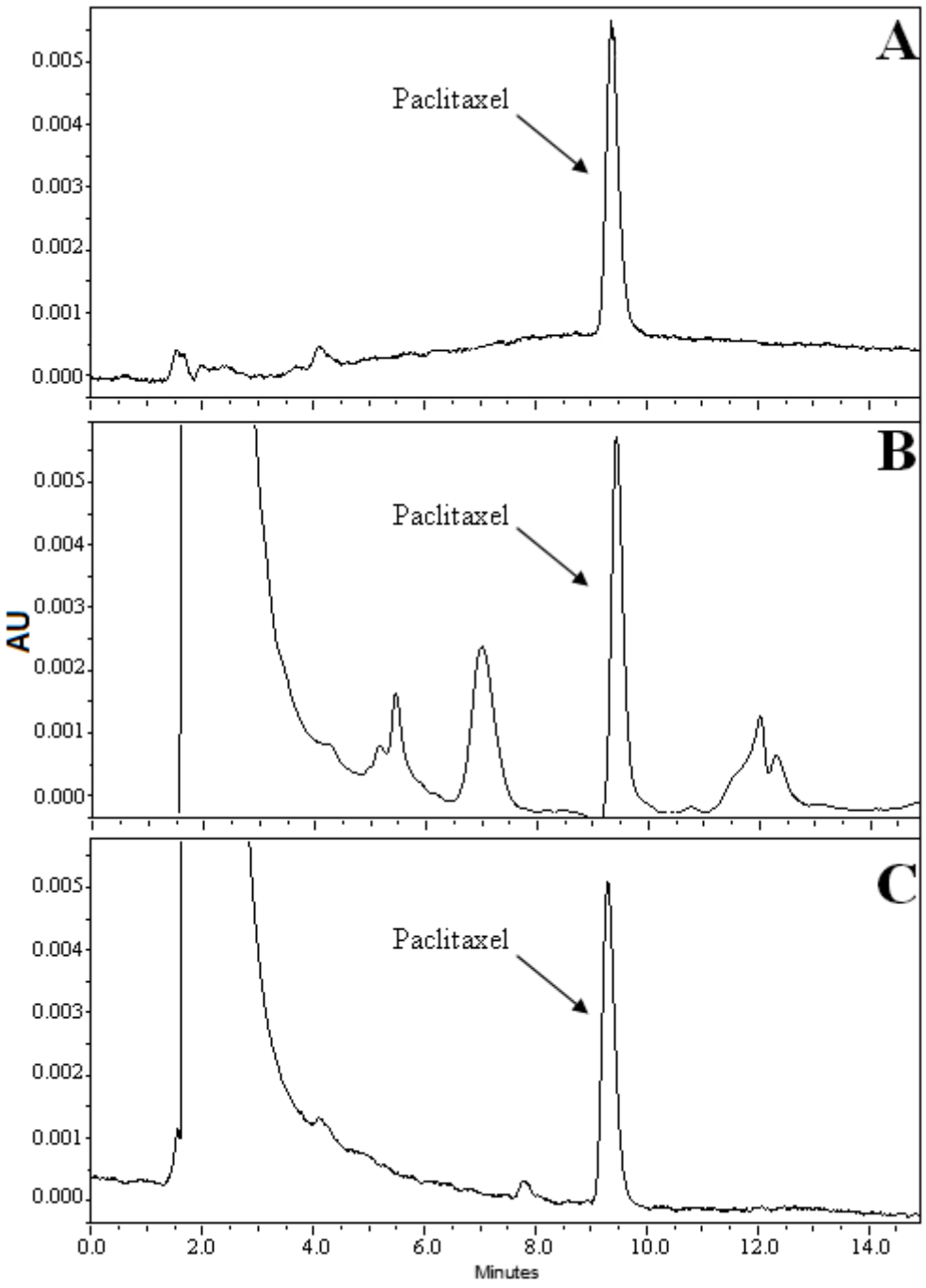

Figure 1- Chromatograms showing the elution peak of paclitaxel spiked at the concentration of 200 $ng.mL^{-1}$ in the mobile phase only (A), in the mobile phase containing 10 mg of copaiba oil resin (B) and in the mobile phase containing 10 $mg.mL^{-1}$ of copaiba essential oil (C).

The calibration curve of the HPLC-UV assay demonstrated that the method was linear (r^2 = 0.999) in the range from 50 to 2000 ng.mL^{-1}. The standard deviation of the curve of the analyzed drug was not significant. The straight-line equation was y = 0.198x (± 0.0003) + 4.401 (±0.395) (Where, x is the concentration of paclitaxel (ng.mL^{-1}) and y the peak area (mAu s) (Figure 2A). Through the ANOVA analyzes, it was possible to perform the significance test for linear regression in order to determine if the regression model was suitable to generate a linear relation between the response variable y and some of the regression variables x. Thus, as $F_{calculated=}$ 706741> $F_{tabulated}$ = 5.99 for α=0.05 for three measures from the calibration curve containing 10 mg of copaiba resin oil, the proposed model was considered adequate to describe the linear regression. In addition, the Figure 2B showed homoscedasticity of the residues, indicating a higher normal distribution of values found around the regression line of the model.

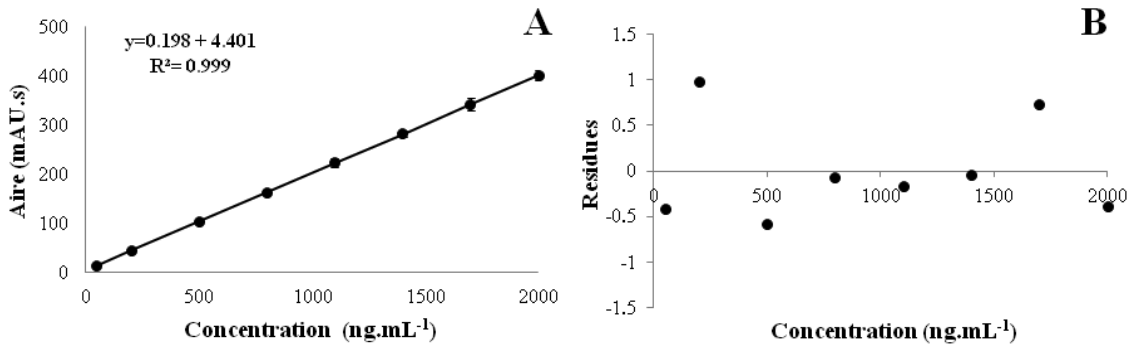

Figure 2- Calibration curve (A) and residual plots (B) of paclitaxel in the mobile phase containing 10 mg.mL^{-1} of copaiba resin oil analyzed by HPLC-UV.

The LOQ was determined based on the standard deviation values of the response and the slope produced from the calibration curve data. The LOQ to produce the requisite precision and accuracy for this method was 21.03 ng.mL^{-1}, under the stated experimental conditions. The LOD was determined based on the signal-to-noise ratio using an analytical response of three times the background noise. Thus, the LOD of

paclitaxel was 6.31 ng.mL^{-1}. The sensitivity of this method was comparable with most other HPLC–UV methods developed for paclitaxel dosages using complex compound mixtures (Wang *et al.*, 2003; Kim *et al.*, 2005; Mohammadi *et al.*, 2009; Choudhury *et al.*, 2014).

Accuracy was determined by quantifying six samples of paclitaxel at all concentrations of the calibration curve (range from 50 to 2000 ng.mL^{-1}) in copaiba resin oil (Table 1). The recoveries ranged from 97.1 to 102.7 % and the RSD were less than 0.77 %. These results satisfied the drug quality research requirements.

Table 1- Accuracy of paclitaxel assay in mobile phase containing 10 mg.mL^{-1} of copaiba resin oil

Concentration (ng.mL^{-1})	**Concentration found (ng.mL^{-1})***	**SD**	**Accuracy (%)**	**RSD (%)**
50	48.6	0.4	97.1	0.77
200	205.4	0.9	102.7	0.46
500	498.5	1.3	99.7	0.27
800	805.5	3.9	100.7	0.48
1100	1117.7	7.8	101.6	0.70
1400	1392.3	4.2	99.4	0.30
1700	1731.8	12.4	101.9	0.72
2000	2009.3	7.8	100.5	0.39

*n=6, SD= standard deviation, RSD= relative standard deviation

Precision was estimated by the intra-day (repeatability) and the inter-day precision. Intra-day precision was investigated by the quantitation of triplicate samples of paclitaxel solutions at three different concentrations (800, 1400 and 2000 $ng.mL^{-1}$). Inter-day precision was assessed by quantifying the same three samples over three consecutive days (Table 2). RSD for intra- and inter-day were less or equal to 0.65 %, which indicates that the method possessed low values of random errors. In other words, the method does not suffer significant changes between analyzes.

Table 2- Intra-day and inter-day precision analyzes of paclitaxel.

Parameters	**Concentration ($ng.mL^{-1}$)**	**Concentration found ($ng.mL^{-1}$)***	**SD**	**RSD (%)**
Intra-day precision	800	802.9	3.9	0.49
	1400	1390.0	3.7	0.27
	2000	2009.1	9.6	0.48
Inter-day precision	800	796.5	3.1	0.39
	1400	1401.3	8.1	0.57
	2000	2003.6	13.1	0.65

*n=3, SD= standard deviation, RSD= relative standard deviation

To investigate the suitability of this analytical method, the solubility and partition coefficient studies of paclitaxel were performed. Paclitaxel solubility was determined in water and in both copaiba essential oil and copaiba resin oil. The paclitaxel solubility in

water was lower than 0.4 µg.mL^{-1}, which is in agreement to the data of the literature (Konno *et al.*, 2003; Zhao *et al.*, 2010). Paclitaxel solubility in copaiba essential oil and copaiba resin oil were 52.9 (± 8.7) and 797.2 (± 79.1) µg.mL^{-1}, respectively, which were respectively 132 and 2,000 times higher compared to the solubility in water. This considerable increase of solubility may be explained by the lipophilic character of the drug molecule.

The evaluation of the partition coefficient is an important step prior studies of the physicochemical characteristics of drugs for the development of liquid formulations (Silva *et al.*, 2006). In fact, the distribution of the compound in the multi-phase system is directly related to its partitioning behavior (Balbach & Korn, 2004). Consistently with the results of solubility determined earlier in the present work and with the data of the literature (Lee *et al.*, 2006; Surapaneni *et al.*, 2012; Zabaleta *et al.*, 2012), the partition of paclitaxel in n-octanol/water showed a logP of 3.89 (Table 3). When the lipophilic phase was copaiba essential oil and copaiba resin oil, the paclitaxel partition coefficients were 3.21 and 2.63, respectively (Table 3). These quite high values of paclitaxel partition coefficients explain the high solubility of paclitaxel in the oils and its extremely low solubility in water. Moreover, the high oil solubility and partition coefficient (lipophilicity) of the paclitaxel are favorable to achieve a higher drug entrapment in the internal phase of drug delivery systems. Therefore, copaiba oil can be selected as the oil phase for the development of drug delivery systems in which the combination of the anticancer activity of the oil and paclitaxel is desired in a single formulation.

Table 3- Partition coefficients of paclitaxel in, n-octanol, copaiba essential oil and copaiba resin oil.

Samples *	Compounds	Concentration ($\mu g.mL^{-1}$)	SD	P value (Oil/Water)	LogP
1	**n-Octanol**	1207.5	24.3	7803.4	3.89
	Water	0.2	0.1		
2	**Copaiba resin oil**	645.8	37.9	1634.6	3.21
	Water	0.4	0.1		
3	**Copaiba essential oil**	162.4	14.3	423.3	2.63
	Water	0.4	0.1		

*n=3, SD= standard deviation,

4.0 CONCLUSION

A rapid, simple, specific and sensitive HPLC method for the quantification of paclitaxel in samples containing copaiba oils was developed and validated. This method was highly sensitive for paclitaxel separation from a mixture of natural compounds found in copaiba oil and its essential oil extract. Calibration curves were reproducible. No significant differences were shown between the slope and the y-intercept and the residues were homoscedastic. This method showed a low LOD and LOQ levels, besides small coefficient of variation at 1% for accuracy and precision, satisfying the drug quality research requirements. It is also noteworthy that the method can be applied in all lab equipped with a basic HPLC system due to the simplicity of the material used while the cost of the analysis will remain low. The method was successful applied to the

evaluation of concentrations of paclitaxel in various samples. Solubility characteristics of paclitaxel in copaiba oil and corresponding partition coefficients deduced from those experiments are encouraging to achieve the development of a drug delivery system that will combine the oil and paclitaxel in a single nanomedicine with the aim to potentiate their anticancer activity and enhanced efficacy of existing treatments.

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REFERENCES

ALENCAR, E. N., XAVIER-JUNIOR, F. H., MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G. & EGITO, E. S. T. 2015. Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. *J Nanosci Nanotechnol*, 15, 880-888.

BALBACH, S. & KORN, C. 2004. Pharmaceutical evaluation of early development candidates "the 100 mg-approach". *Int J Pharm*, 275, 1-2, 1-12.

CHOUDHURY, H., GORAIN, B., KARMAKAR, S. & PAL, T. K. 2014. Development and validation of RP-HPLC method: scope of application in the determination of oil solubility of paclitaxel. *J Chromatogr Sci*, 52, 1, 68-74.

FANG, W. S. & LIANG, X. T. 2005. Recent progress in structure activity relationship and mechanistic studies of taxol analogues. *Mini Rev Med Chem*, 5, 1, 1-12.

FRENSE, D. 2007. Taxanes: perspectives for biotechnological production. *Appl Microbiol Biotechnol*, 73, 6, 1233-40.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-92.

GUERITTE-VOEGELEIN, F., GUENARD, D., DUBOIS, J., WAHL, A. & POTIER, P. 1994. [Chemical and biological studies on Taxol (Paclitaxel) and Taxotere (Docetaxel), new antineoplastic agents]. *J Pharm Belg*, 49, 3, 193-205.

GUO, P., MA, J., LI, S. & GALLO, J. M. 2003. Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 798, 1, 79-86.

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci*, 13, 4, 134-6.

HUIZING, M. T., MISSER, V. H., PIETERS, R. C., TEN BOKKEL HUININK, W. W., VEENHOF, C. H., VERMORKEN, J. B., PINEDO, H. M. & BEIJNEN, J. H. 1995. Taxanes: a new class of antitumor agents. *Cancer Invest*, 13, 4, 381-404.

KAJANI, A. A., MOGHIM, S. & MOFID, M. R. 2012. Optimization of the basal medium for improving production and secretion of taxanes from suspension cell culture of Taxus baccata L. *Daru*, 20, 1, 54.

KIM, S. C., YU, J., LEE, J. W., PARK, E. S. & CHI, S. C. 2005. Sensitive HPLC method for quantitation of paclitaxel (Genexol in biological samples with application to preclinical pharmacokinetics and biodistribution. *J Pharm Biomed Anal*, 39, 1-2, 170-6.

KONNO, T., WATANABE, J. & ISHIHARA, K. 2003. Enhanced solubility of paclitaxel using water-soluble and biocompatible 2-methacryloyloxyethyl phosphorylcholine polymers. *J Biomed Mater Res A*, 65, 2, 209-14.

LEANDRO, L. M., VARGAS F, S., BARBOSA, P. C., NEVES, J. K., SILVA, J. A. & VEIGA-JUNIOR, V. F. 2012. Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins. *Molecules*, 17, 4, 3866-89.

LEE, H., SOO, P. L., LIU, J., BUTLER, M. & ALLEN, C. Polymeric Micelles for Formulation of Anti-Cancer Drugs. In: AMIJI, M. M. (Ed.). Nanotechnology for Cancer Therapy: CRC Press, 2006. p.329.

LEGAULT, J. & PICHETTE, A. 2007. Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. *J Pharm Pharmacol*, 59, 12, 1643-7.

MOHAMMADI, A., ESMAEILI, F., DINARVAND, R., ATYABI, F. & WALKER, R. B. 2009. Development and validation of a stability-indicating method for the quantitation of Paclitaxel in pharmaceutical dosage forms. *J Chromatogr Sci*, 47, 7, 599-604.

PANCHAGNULA, R. 1998. Pharmaceutical aspects of paclitaxel. *Inter J Pharm*, 172, 1–2, 1-15.

RAO, S., KRAUSS, N. E., HEERDING, J. M., SWINDELL, C. S., RINGEL, I., ORR, G. A. & HORWITZ, S. B. 1994. 3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin. *J Biol Chem*, 269, 5, 3132-4.

ROWINSKY, E. K., CAZENAVE, L. A. & DONEHOWER, R. C. 1990. Taxol: a novel investigational antimicrotubule agent. *J Natl Cancer Inst*, 82, 15, 1247-59.

ROWINSKY, E. K., ONETTO, N., CANETTA, R. M. & ARBUCK, S. G. 1992. Taxol: the first of the taxanes, an important new class of antitumor agents. *Semin Oncol*, 19, 6, 646-62.

SANTOS, A. O., UEDA-NAKAMURA, T., DIAS FILHO, B. P., VEIGA JUNIOR, V. F., PINTO, A. C. & NAKAMURA, C. V. 2008. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the Copaifera genus. *Mem Inst Oswaldo Cruz*, 103, 3, 277-81.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SILVA, K. G. H., XAVIER-JUNIOR, F. H., FARIAS, I. E. G., CALDAS-NETO, J. A., SILVA, A. K. A. S., NAKASHIMA-JUNIOR, T., ARAUJO, I. B., OLIVERIA, A. G., MEDEIROS, A. C. & EGITO, E. S. T. 2006. A new insight about pharmaceutical dosage forms for benzathine penicillin G. *J Basic Appl Pharm Sci*, 27, 1, 21-26.

SINGLA, A. K., GARG, A. & AGGARWAL, D. 2002. Paclitaxel and its formulations. *Int J Pharm*, 235, 1-2, 179-92.

SONG, D. & AU, J. L. 1995. Isocratic high-performance liquid chromatographic assay of taxol in biological fluids and tissues using automated column switching. *J Chromatogr B Biomed Appl*, 663, 2, 337-44.

SOUSA, J. P., BRANCALION, A. P., SOUZA, A. B., TURATTI, I. C., AMBROSIO, S. R., FURTADO, N. A., LOPES, N. P. & BASTOS, J. K. 2011. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal*, 54, 4, 653-9.

SUPKO, J. G., NAIR, R. V., SEIDEN, M. V. & LU, H. 1999. Adaptation of solid phase extraction to an automated column switching method for online sample cleanup as the basis of a facile and sensitive high-performance liquid chromatographic assay for paclitaxel in human plasma. *J Pharm Biomed Anal*, 21, 5, 1025-36.

SURAPANENI, M. S., DAS, S. K. & DAS, N. G. 2012. Designing Paclitaxel Drug Delivery Systems Aimed at Improved Patient Outcomes: Current Status and Challenges. *ISRN Pharmacology*, 2012, 623139.

Validation of analytical procedures: Text and Methodology, ICH-Q2 (R1). International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, 2005, Geneva.

WANG, L. Z., HO, P. C., LEE, H. S., VADDI, H. K., CHAN, Y. W. & YUNG, C. S. 2003. Quantitation of paclitaxel in micro-sample rat plasma by a sensitive reversed-phase HPLC assay. *J Pharm Biomed Anal*, 31, 2, 283-9.

WANI, M. C., TAYLOR, H. L., WALL, M. E., COGGON, P. & MCPHAIL, A. T. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc*, 93, 9, 2325-7.

XAVIER-JUNIOR, F. H., MACIUK, A., MORAIS, A. R. V., ALENCAR, E. N., REHDER, V. L. G., EGITO, E. S. T., VAUTHIER, C. Chapter I, 2015a. Development of gas-chromatography method for the analysis of copaiba oil. *Chapter I- Article in preparation*

ZABALETA, V., PONCHEL, G., SALMAN, H., AGUEROS, M., VAUTHIER, C. & IRACHE, J. M. 2012. Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: permeability and pharmacokinetic study. *Eur J Pharm Biopharm*, 81, 3, 514-23.

ZHAO, D., ZHAO, X., ZU, Y., LI, J., ZHANG, Y., JIANG, R. & ZHANG, Z. 2010. Preparation, characterization, and in vitro targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. *Int J Nanomedicine*, 5, 669-77.

Section II

Les systèmes d'administration de médicaments à base de lipides

Chapter III

Prospective study for the development of emulsion systems containing natural oil products

Le troisième chapitre de cette thèse présente un article intitulé « Prospective study for the development of emulsion systems containing natural oil products ». Il a été publié dans le « Journal of Drug Delivery Science and Technology» le 19 Janvier 2012. L'étude consignée dans cet article marque le début des travaux sur les systèmes dispersés de l'huile de copaïba par notre groupe de recherche. Le choix de l'huile de copaiba avait été motivé par plusieurs raisons. Les huiles extraites des plantes ont un certain nombre d'avantages par rapport aux huiles minérales. Elles sont moins toxiques, biodégradables et renouvelables. Au cours de ces dernières années, les huiles végétales sont devenues plus attrayantes en raison de leurs avantages économiques et de leur renouvellement. En particulier, l'huile de copaïba, présente un riche mélange de composés de diterpènes et sesquiterpènes, largement utilisé dans la médecine populaire pour leurs propriétés anti-inflammatoires, anti-infectieuses et anticancéreuses. L'objective de notre étude menée sur l'huile de copaiba a été le développement et la caractérisation de systèmes émulsionnés avec cette l'huile. La première étape de notre travail a été de déterminer l'équilibre hydrophile-lipophile requis (HLB) de l'huile de copaïba sur une base expérimetale . Ensuite, des diagrammes de phases pseudo-ternaires ont été réalisés pour vérifier la formation de systèmes lipidiques différentes en changeant les concentrations des composants. Enfin, une étude de la stabilité et les méthodes de production des systèmes d'intérêt ont été réalisées. Les échantillons ont été préparés en utilisant différents mélanges binaires de tensioactifs obtenir une série de valeurs de HLB compris entre 4,5 et 16,5. Le HLB requis de l'huile de copaïba a été determiné pour une valeur de 14,8. L'émulsion obtenue a été montré une très bonne stabilité sur une période d'une année. . Les diagrammes de phases pseudo-ternaires ont été utiles pour décrire les proportions des mélanges des composants idéales pour la formation de différents systèmes disperses. Ces résultats indiquent que l'émulsion basée

de l'huile de copaïba pourrait être un moyen prometteur pour l'administration de médicaments.

Mots-clés: Émulsion, huile de copaïba, équilibre hydrophile-lipophile, diagramme de phase pseudo-ternaire, stabilité.

Prospective study for the development of emulsion systems containing natural oil products

F.H. Xavier-Júnior[1], K.G.H. Silva[2], I.E.G. Farias[2], A.R.V. Morais[2], E.N. Alencar[2], I.B. Araújo[1, 2], A.G. Oliveira[3], E.S.T. Egito[1, 2]*

[1]Universidade Federal do Rio Grande do Norte (UFRN), Centro de Ciências da Saúde (CCS), Programa de Pós-Graduação em Ciências da Saúde (PPGCSa), Laboratório de Sistemas Dispersos (LaSiD),
Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN, Brazil
[2]UFRN, Departamento de Farmácia, LaSiD, Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN, Brazil
[3]UNESP, Faculdade de Ciências Farmacêuticas, Grupo de Sistemas Biomiméticos, Fármacos,
Rodovia Araraquara-Jau, km 01, 14801-902, Araraquara-SP, Brazil
*Correspondence: socrates@ufrnet.br or socrates@pq.cnpq.br

Copaiba oil, when used as anti-inflammatory and anti-infective agent, presents two major problems: i) low absorption and bioavailability and ii) an unpleasant taste. Once an appropriate emulsion was achieved, the samples were prepared using different surfactant binary mixtures to obtain a range of HLB values between 4.5 and 16.5. The HLB value of copaiba oil was 14.8; this system has been stable for more than one year, and the pseudo-ternary diagrams were useful to describe the component proportions. These results indicated that the copaiba oil emulsion might be a promising vehicle for topical delivery of drugs and active cosmetic ingredients.

Key words: Emulsion – Copaiba oil – Hydrophilic-lipophilic balance – Pseudo-ternary phase diagram – Stability analysis.

Oils extracted from plant species have a number of potential advantages compared to their mineral counterparts. They are less toxic, biodegradable, and renewable, while petroleum chemicals are finite [1]. Vegetable oils, which have become more attractive recently because of their economic benefits and renewability, are used as components in many manufactured products [2]. Essential oils are used primarily as natural preservatives, flavorings, and fragrances in cosmetic products [3]. The oils are also used in the flavor and cosmetic industries [4] and as diesel-like fuels [5].

Copaifera langsdorffii Desf. (*Leguminosae*), popularly known as "copaiba", is a large tree that grows abundantly in the Brazilian States of Amazonas, Pará, and Ceará [6]. Phytochemical studies of *Copaifera langsdorffii* oleo resin revealed the presence of essential oils (8 % sesquiterpens: β-caryophyllene, caryophyllene oxide, β-elemane, α-cis-bergamotene, ar-curcumene, and α-trans-bergamotene) and a mixture of diterpenes (70 % kaurenoic and polyalthic acids) [6]. However, significant differences in chemical composition were found among the species [7]. The benefits attributed to copaiba oil in popular medicine are its anti-inflammatory, antitumor, and anti-tetanus properties. Moreover, copaiba oil has been used as a urinary anti-septic, and also in the treatment of bronchitis, syphilis, skin diseases, ulcers, and wounds [8].

Emulsions are mixtures of two (or more) immiscible liquids dispersed one within the other in droplet form. They are thermodynamically unstable dispersions with droplets of a diameter ranging from 0.5 to 100 μm [9]. In general, emulsions are used as delivery systems for water insoluble drugs and have the potential for sustained release and drug-targeting delivery by binding ligands for various cell surface receptors [10].

The hydrophile-lipophile balance (HLB), first described by Griffin [11], has been used to describe the simultaneous attraction of surfactants or surfactant blends and their ability to form stable emulsions, whose value is close to that required of the oil phase [12]. On the other hand, pseudo-ternary phase diagrams provide the boundaries of the different structures as a function of the component composition. Because of the presence of more than three components, one of the axes is frequently a fixed ratio of the other two (usually the mixture of the surfactant and co-surfactant), while the other axes represent the oil and water components [13]. The border regions can be classified as emulsion, phase separation, and microemulsion, among others. The pseudo-ternary phase diagram is constructed to determine the composition of polar, non-polar, and surfactant phases that will yield an emulsion [14].

The aim of this study was the development and characterization of copaiba oil emulsion systems. First, the critical hydrophilic-lipophilic balance (HLBc) of copaiba oil was determined. Then, a pseudo-ternary phase diagram study was developed. In addition, a study of the stability and the production methods for such systems was carried out.

I. MATERIALS AND METHODS

1. Materials

Copaiba oil was from Flores & Ervas (Piracicaba, SP, Brazil), Span 80 (sorbitan 80 monooleate) was purchased from Sigma Aldrich Inc. (St Louis, MO, United States), and Tween 20 (polyoxyethylene 20 sorbitan monolaurate) was from Vetec (Rio de Janeiro, RJ, Brazil). Distilled water was used throughout the experiments. All chemicals were of pharmaceutical grade and were used as received without further purification.

2. Methods

2.1. Hydrophilic-lipophilic balance design

The final HLB value of each system varied according to the individual percentage of each surfactant. A series of 13 emulsions with HLB values ranging from 4.5 to 16.5 (variation among total HLB values comprising one unit) was first prepared by blending the emulsifiers at different ratios, according to *Equation 1* [11]:

$$HLB = (W_{Tw} HLB_{Tw} + W_{Sp} HLB_{Sp})/(W_{Tw} + W_{Sp}) \quad \textbf{Eq. 1}$$

where W_{Tw} is the amount (weight) of Tween 20 used, W_{Sp} the amount

(weight) of Span 80 used at the "optimum ratio", HLB_{Tw} and HLB_{Sp} are the assigned HLB values for Tween 20 and Span 80, respectively, and HLB is the "final HLB" value of the system for the type of emulsion being studied.

A second step involved emulsion preparation using smaller ratio intervals between the two most stable emulsions from the first step.

2.2. Emulsion preparation

The oil-in-water emulsion-based formulation containing 5 % (w/w) copaiba oil, 93 % (w/w) water, and 2 % (w/w) surfactant blends was prepared using the phase inversion technique (PIT) [15]. The required amount of Span 80 was dispersed in the oil phase. Tween 20 was then dispersed in the aqueous phase. Both phases were heated separately at 70 °C. Final emulsions were obtained after homogenization of oil and aqueous phases using an Ultra-Turrax T 25 homogenizer (IKA, Staufen, Germany) at 13,000 rpm for 10 min.

2.3. Emulsion characterization

2.3.1. Morphological analysis

Morphological examination of the emulsions was performed using an optical microscope (Olympus, Center Valley, PA, United States) following blue staining with a 2 % (w/w) methylene blue solution. The preparation was observed under 4×, 10×, and 40× lenses [16].

2.3.2. Macroscopic aspect and creaming analysis

The color of the emulsions, as well as their stability variation (presence of creaming, coalescence, or phase separation), was determined visually. The creaming stability of emulsions, assessed by transferring 10 mL of emulsion into a test tube and storing it at ambient temperature (approximately 25 ± 2 °C), was observed for one year. During this time, the volume of emulsion creamed at the top was recorded and the creaming percentage calculated as follows (*Equation 2*) [17]:

$$\%CI = (Cl/Ct) \times 100 \qquad \textbf{Eq. 2}$$

where %CI is creaming index, Cl is the height of the cream layer, and Ct is the total height of the emulsion tube.

2.3.3. pH Evaluation

Emulsion pH was measured by using a pre-calibrated pH meter PG-2000 (Gehaka, São Paulo, SP, Brazil) at 25 ± 2 °C.

2.3.4. Conductivity analysis

The electrical conductivity of the samples was measured using a DM-32 conductivity meter (Digicrom Analytical, Campo Grande, SP, Brazil) with a cell constant of 0.11 cm^{-1}. The measurements were performed at 25 ± 2 °C.

2.3.5. Droplet size analysis

For the series of formulations made in the second step, the droplet size of the emulsion was determined by laser diffraction. The formulation (0.1 mL) was freshly dispersed in 50 mL of water in a volumetric flask and gently mixed by inverting the flask. A measurement was taken instantaneously after the dilution process using a Mastersizer 2000 (Malvern Instruments, Worcestershire, United Kingdom). Light scattering was monitored at 25 °C at a 90° angle.

2.3.6. Rheological measurements

For the series of formulations made in the second step, sample rheology was evaluated using a Haake Rheostress 600 rheometer (Thermo Electron, Takkebijsters, BL, Germany) fitted with a stainless steel cone/plate measuring 35 mm in diameter, screw angle of 2°, and a 105 μm hole. The jacketed sample cup was connected to a circulating water bath operating at 25 °C. A sample volume of 1 mL was used. The measurements were performed in triplicate. Steady flow measurements were carried out using a single sample for the whole curve in the range of shear stresses corresponding to shear rates from 0 to 200/s for 300 s, and the rheological parameters, such as shear stress, shear rate, and apparent viscosity, were obtained from the software. The apparent viscosity (ηapp) was determined at a shear rate of 60 s^{-1}.

2.4. Stability studies

2.4.1. Micro-emultocrit technique (short-term stability)

The micro-emultocrit technique was used in this study to evaluate the creaming index [16]. The technique was performed by filling 75 % of a heparin-free capillary tube with each formulation and placing it in a micro-centrifuge (Fanen, São Paulo, SP, Brazil) at 11 500 g for 10 min.

2.4.2. Stability under centrifugation

Centrifugation tubes filled with 10 mL of emulsion underwent a centrifugal acceleration of 1000, 2500, and 3500 g for 15 min at 25 °C. To avoid changes induced by possible heating, the temperature, which should not exceed 30 °C, was measured in one tube at the end of the experiment.

2.4.3. Freeze/thaw cycles

Hermetically-sealed test tubes filled with the emulsion were stored vertically for 16 h in a freezer at -21 °C and then for 8 h at room temperature (25 ± 2 °C). The emulsion was observed and any change was recorded. This cycle was repeated six times [18].

2.4.4. Intrinsic stability

For the series of formulations made in the second step, test tubes were filled with 10 mL of the emulsion and then hermetically sealed. They were then stored vertically at room temperature (25 ± 2 °C). Weekly macroscopic aspects were evaluated for one year. Any change such as phase separation or creaming rate was recorded [18].

2.4.5. Stability under storage (long-term stability)

For the series of formulations made in the second step, test tubes were filled as described in section 2.4.4. The tubes were stored vertically in a hot air oven at 45 ± 2, 25 ± 2 and 4 ± 2 °C, following the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Weekly macroscopic aspects were evaluated for one year.

2.5. Construction of pseudo-ternary phase diagrams

In order to determine the range of component concentration for the existing emulsion range, pseudo-ternary phase diagrams were constructed using the water titration method at ambient temperature and three different stirring processes [19, 20]. One phase diagram was built for each production method. Tween 20 and Span 80 were mixed at a weight ratio of the copaiba oil HLBc value to obtain the Smix. Copaiba oil and Smix were then mixed at a weight ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, respectively. This concentration variation allowed the creation of 90 formulations. The mixtures of oil, surfactant, and co-surfactant at a certain weight ratio were diluted with water dropwise, using three stirring processes: i) vortex stirring, ii) magnetic stirring, and iii) sonic probe followed by ultrasonic bath cleaner. After equilibrium, the mixtures and their microscopic appearance were assessed visually, using cross-polarized light microscopy (Olympus BX 41, Shinjuku-ku, TOY, Japan). The first method, mechanical stirring, was generated by using Vortex (IKA, Staufen, Germany). The second method involved using a magnetic stirrer (IKA, Staufen, Germany). In the last method, sonic probe, a sonicator (Heat Systems, Farmingdale, NY, United States) was used, followed by the use of an ultrasonic bath cleaner (Unique, Indaiatuba, SP, Brazil). All the stirring processes, performed in duplicate, were carried out for 2 min for each sample. All the samples were then analyzed overnight.

The pseudo-ternary phase diagrams were determined to classify the systems as microemulsions, crude emulsions, nanoemulsions, gels, or phase separation. No attempt was made to distinguish between oil-in-water, water-in-oil, or bi-continuous microemulsions and/or emulsions. Gels were selected for clear and highly viscous mixtures that did not show a change in meniscus after being tilted to an angle of 90°.

II. RESULTS

The first HLB interval of the emulsions was evaluated macroscopically and microscopically for 60 days. The more stable preparations pointed to the HLB interval that was used to construct the second step of formulations. Then, for the second set of preparations, stability studies were conducted to determine the most stable formulation over the time. Finally, the HLBc of the copaiba oil was defined.

Microscopic images showed that copaiba oil emulsions stabilized with the Tween 20 and Span 80 admixtures had individual droplets, which were homogeneously distributed with no sign of flocculation (*Figure 1*). However, formulations with low HLB values (11.5) showed wide variations in droplet size, suggesting coalescence.

Despite the wide range of HLB values (4.5 to 16.5), the PIT produced stable emulsions until the eighth day of analysis, with milky and white aspects and a lower percentage of cream (*Table I*).

Macroscopic aspect showed that the formulations with HLB values of 16.5, 15.5, 14.5, and 12.5 maintained constant characteristics over the two months of the study. These formulations showed lower creaming index values during this period. Inversely, emulsions with lower HLB values showed phase separation or higher creaming percentage.

Table I shows that all formulations presented cream during the 60-day period. However, HLB formulations of 16.5, 15.5, 14.5, and 13.5 can be considered the most stable ones, since the creaming phenomenon was not as marked as that of the others.

Because the previously described studies were unable to find a narrow HLB interval, which could express the most stable emulsions, auxiliary tests were used to obtain a more reliable HLB interval for copaiba oil. The results of pH analysis (*Table II*) show significant pH variation in the formulations, but the HLB samples of 15.5 and 14.5 values had a lower standard deviation of pH values, which remained constant throughout analysis.

The systematic variation of the electrical conductivity profile in the systems (*Table II*) demonstrates that all emulsions underwent significant variations in conductivity values over time, suggesting the onset of the instability characteristic of emulsion systems. The emulsions with the lowest HLB values showed low conductivity values due to the instability process. This finding corroborates the pH results, creaming rate at 25 °C, and macroscopic aspects. Emulsions with an HLB range between 15.5 and 14.5 (F2.0 and F3.0) showed the best conductivity values, since the standard deviation during analysis was the lowest, showing better stability throughout storage.

The full set of results indicated that systems with HLB values between 15.5 and 14.5 were the more stable ones. Therefore, in order to obtain the most reliable HLBc value of copaiba oil, the second set of emulsion formulations was performed within this HLB range.

From the second set of formulations, the emulsions stored at 25 °C demonstrated good characteristics, such as macroscopic appearance, white and milky aspect, and low cream values. Over the two months, the creaming index showed that the emulsions presented about 4 % cream. The standard deviation (SD) in each sample represents the variation in cream percentage throughout the study, suggesting instability during the process. The formulations with HLB values between 15.1 and 14.6 showed smaller SD variations in the creaming index.

The pH values (around 5.1) were smaller from samples F2.4 (HLB 15.1) to F2.7 (HLB 14.8). Conductivity analysis shows that the conductivity values of samples F2.3, F2.5, and F2.7 did not oscillate significantly, presenting a mean of 41 μS/cm, characteristic of an emulsion with an external aqueous phase [21, 22].

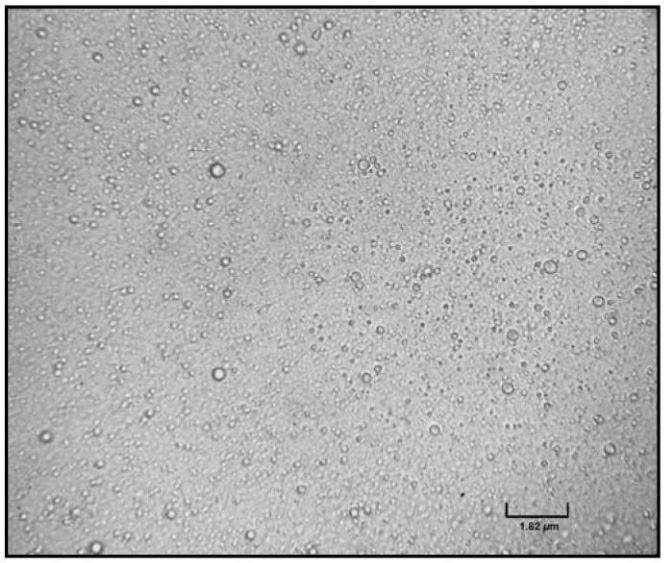

Figure 1 - Microscopic droplets of the emulsified systems with HLB value of 14.8 (40 x 20 μm).

Table I - Macroscopic aspect and creaming index of the emulsion formulations based on copaiba oil stored at 25 °C for 60 days (first step). D: day; regular: milky aspect; italics: yellowish aspect and coalescence; bold: phase separation.

Formulations	HLB value	D1	D3	D5	D8	D15	D30	D60
F1.0	16.5	0.00	0.00	1.43	1.43	1.45	1.45	1.52
F2.0	15.5	0.00	0.00	0.00	1.43	1.45	1.45	1.52
F3.0	14.5	0.00	0.00	1.45	1.47	1.47	1.47	1.47
F4.0	13.5	0.00	0.00	1.39	2.86	2.94	2.94	3.03
F5.0	12.5	0.00	0.00	1.43	1.47	2.94	2.94	3.17
F6.0	11.5	0.00	0.00	1.56	3.33	3.39	3.39	10.71
F7.0	10.5	0.00	0.00	2.86	2.99	3.03	3.03	*3.17*
F8.0	9.5	0.00	2.82	2.82	2.94	4.41	4.41	*4.55*
F9.0	8.5	1.43	2.86	2.86	*2.99*	*4.31*	*4.41*	*4.76*
F10.0	7.5	4.29	5.80	7.14	*7.46*	*7.58*	*10.61*	*10.77*
F11.0	6.5	5.71	7.14	8.82	*10.29*	*10.45*	**11.76**	**14.06**
F12.0	5.5	7.04	8.57	10.29	*10.29*	*11.76*	**14.49**	**12.31**
F13.0	4.5	7.04	8.45	8.82	*11.76*	**11.94**	**16.42**	**18.75**

Table II - pH and conductivity of copaiba oil emulsions with HLB between 16.5 and 4.5.

Formulations	HLB value	pH	Conductivity
F1.0	16.5	5.13 ± 0.30	35.2 ± 2.65
F2.0	15.5	4.95 ± 0.23	33.9 ± 1.82
F3.0	14.5	4.89 ± 0.23	36.5 ± 2.00
F4.0	13.5	4.88 ± 0.23	38.4 ± 2.37
F5.0	12.5	5.00 ± 0.25	41.1 ± 2.28
F6.0	11.5	4.94 ± 0.21	42.3 ± 2.04
F7.0	10.5	4.88 ± 0.26	46.5 ± 2.49
F8.0	9.5	4.93 ± 0.27	47.9 ± 1.58
F9.0	8.5	4.87 ± 0.21	48.2 ± 2.30
F10.0	7.5	4.82 ± 0.24	46.3 ± 2.17
F11.0	6.5	4.89 ± 0.19	44.3 ± 10.30
F12.0	5.5	4.83 ± 0.22	36.8 ± 3.68
F13.0	4.5	4.92 ± 0.33	37.3 ± 8.25

With regard to the viscosity, as revealed by the flow curves, the emulsion systems showed a nonlinear relationship between shear stress and shear rate, characteristic of a non-Newtonian flux material (*Figure 2*). In this case, viscosity can be termed "apparent viscosity" and is dependent on the shear rate, pressure, temperature, and time

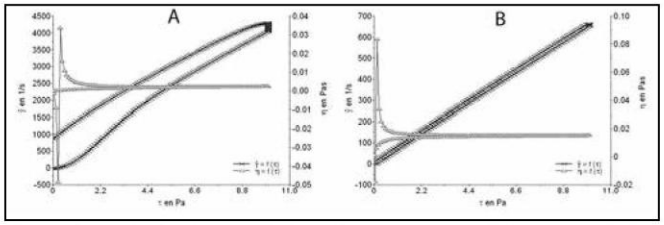

Figure 2 - Rheological analysis of formulation 2.7 (HLB of 14.8) (A) and copaiba oil (B).

Table III - Droplet diameter and apparent viscosity value of copaiba oil emulsions.

Formu-lations	HLB value	D(4.3) (μm)	D(0.5) (μm)	D(0.9) (μm)	Span factor	Viscosity (mPa.s)
F2.0	15.5	0.77	0.56	1.30	1.80	2.269
F2.1	15.4	1.18	0.82	2.23	2.35	2.306
F2.2	15.3	1.36	0.78	2.62	2.97	2.323
F2.3	15.2	0.97	0.49	1.77	3.07	2.252
F2.4	15.1	1.20	0.66	2.28	3.03	2.276
F2.5	15.0	0.99	0.60	2.00	2.85	2.282
F2.6	14.9	1.05	0.70	2.13	2.63	2.290
F2.7	14.8	0.80	0.62	1.25	1.50	2.238
F2.8	14.7	1.06	0.70	2.04	2.50	2.274
F2.9	14.6	1.56	0.68	2.16	2.73	2.251
F3.0	14.5	0.89	0.46	1.84	3.45	2.252

[23] (*Table III*). Furthermore, the rheological analysis showed that the systems behaved like a pseudoplastic and power-law model, which can be used to describe the flow characteristics of the emulsion. Owing to the external character of the emulsion, the viscosity found here was very near that of water, which is completely different from the pure copaiba oil, which exhibited Newtonian behavior and had a viscosity of 15.53 mPa.

Mean emulsion droplet diameters are shown in *Table III*. Low Span factor rates, low polydispersity, and a narrow size distribution define a good system [24]. The sample with an HLB value of 14.8 (F2.7) showed the lowest polydispersity rate, with 90 % of the droplets smaller than 1.25 µm, median droplet size of 0.62 μm, and mean diameter of 0.80 μm.

Based on all previously experimental results, the HLB value of copaiba oil was found to be 14.8. A deep stability study of this formulation was carried out to confirm such assumption.

In the present study, emulsion stability was evaluated through the creaming phenomena, which is the measure of the amount of cream phase separated on the top layer of the o/w emulsion. All copaiba oil emulsions with an HLB value of 14.8 (F2.7) were stable at 25 °C throughout the 1-year study period.

According to the creaming rate, the emulsions stored at 25 °C contained 4 % cream, on average. However, among the emulsions stored at 45 °C, the samples with an HLB value of 14.8 were the most resistant to temperature, maintaining complete stability up to 60 days of analysis.

The micro-emultocrit showed a creaming index of 2 % for the preparation with an HLB value of 14.8. Centrifuge tests showed that all emulsions from the second set of formulations had good physical stability. In order to observe the presence of cream or emulsion phase separation when subjected to gravitational stress, the resistance to centrifugation test was performed.

The study's freeze-thaw cycle assesses the stability of emulsion systems under different storage conditions in regular time intervals. However, the F2.7 (HLB value of 14.8) submitted to a freeze-thaw cycle was stable until the 6th cycle. Later, the instability led to phase separation.

The Tween 20:Span 80 ratio of the surfactant mixture was previously established at 85:15(w/w), which generates an HLB value of 14.8. The phase diagrams related to the HLBc of copaiba oil were investigated based on a water/Tween 20/Span 80/quaternary system of copaiba oil (*Figures 3 to 5*). In this study, all the phase diagrams were based on the proposed model of colloidal-system formation designed by Winsor. In fact, the mixtures of several compounds with different physicochemical properties generate four types of monophasic Winsor IV systems.

III. DISCUSSION

Throughout the study, the emulsions stored at 25 °C showed increasing layers of cream, leading to phase separation, because the increase in temperature accelerates the onset of instability. The kinetic energy of the system likely increases, as does the motility of water molecules, which, in most cases, is the solvent responsible for chemically-unstable reactions [25].

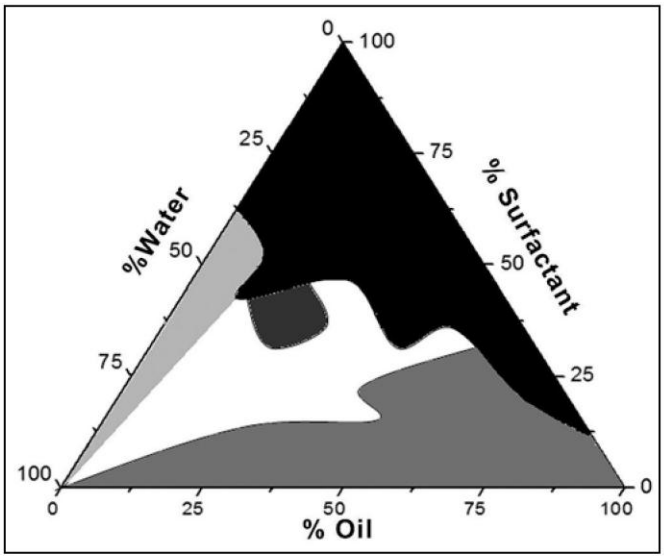

Figure 3 - Phase diagram of copaiba oil produced from vortex stirring at the required HLBc value. Black: microemulsion; dark grey: gel (emulsion or microemulsion?); medium grey: phase separation; light grey: nanoemulsion; white: emulsion.

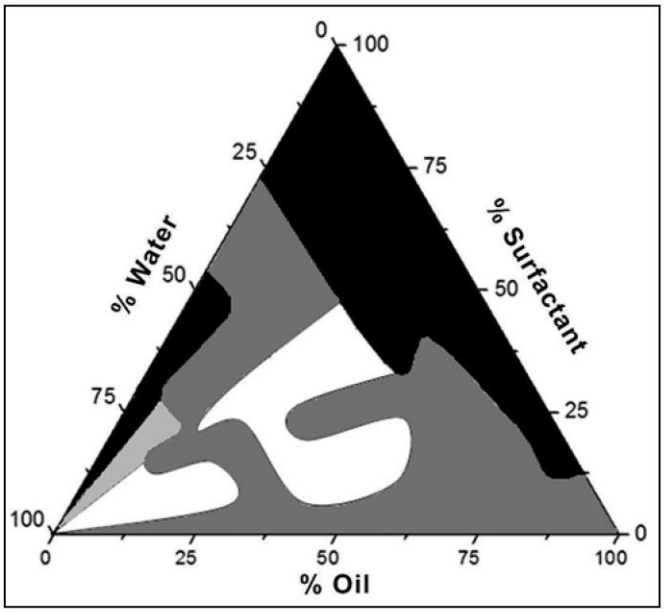

Figure 4 - Phase diagram of copaiba oil produced from magnetic stirring at the required HLBc value. Black: microemulsion; medium grey: phase separation; light grey: nanoemulsion; white: emulsion.

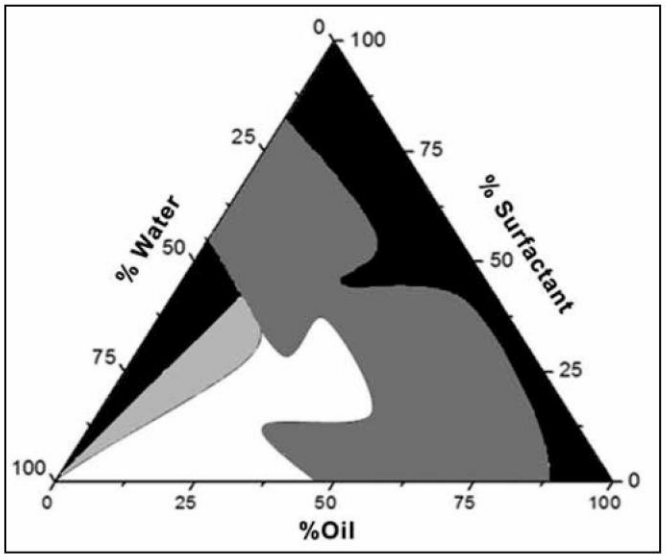

Figure 5 - Phase diagram of copaiba oil produced from sonic probe stirring at the required HLBc value. Black: microemulsion; medium grey: phase separation; light grey: nanoemulsion; white: emulsion.

Creaming is an important parameter to evaluate the stability of emulsion systems against different HLB values, since the occurrence of surface creaming is a precursor of coalescence, allowing phase separation and total loss of stability [9, 26].

Among the parameters, the pH value is a simple tool that suggests the onset of instability, since a significant decrease in this parameter indicates possible surfactant chain degradation that will cause emulsion phase instability between the internal and external phase of the emulsion [27]. Formulations with the lowest HLB had the highest pH oscillation due to emulsion instability.

Conductivity is one of the parameters used to study emulsion stability as a function of time because this method is sensitive to minimal changes in the structure of the emulsified system. The electric current is conducted by the ions present in the external phase of o/w emulsions, while the w/o emulsion behaves as an electrical insulator [9, 26, 28, 29].

Viscosity is an important measurement, as Stokes' law states that the rate of phase separation between two immiscible liquids is directly dependent on the droplet square radius and inversely proportional to system viscosity. Therefore, this measurement is useful in determining appropriate consistency or fluidity as well as indicating the product performance over time. The Stokes' law equation states that the velocity at which a droplet will rise or fall in a liquid varies with the square of its diameter. Therefore, sedimentation velocity of the droplets in a liquid medium arises from the difference in droplet density and in the liquid medium. According to the Stokes' law equation, the smaller the diameters of oil droplets present in the emulsion, the lower their settling velocity, providing more stability to the emulsion [30].

In this study, the instability likely occurs because the non-ionic surfactants are sensitive to temperature changes. On the other hand, the formulation stored at 4 °C was more stable for over one year, due to the low temperature and consequent low collision energy among the droplets.

The mechanical stress to determine whether there is a tendency toward sedimentation or flotation of phases, inducing phase separation. The micro-emultocrit technique is a useful tool to efficiently and rapidly determine the critical HLB of emulsion systems, and reveal possible instabilities not detectable after preparation [16]. Moreover, this creaming index could be explained by the high gravitational force applied to the system. Centrifugation resistance of an emulsion depends on the difference in density between the oily and aqueous phases and also the interfacial film resistance. With similar formulations exhibiting small density differences, stability under centrifugation reflects the strength of the interfacial film. It was observed that for the sample with an HLB of 14.8 no phase separation occurred when the samples were submitted to high gravitational forces.

The global results from the stability study confirm that the copaiba oil presents an HLBc value of 14.8 for o/w emulsion. This data is of utmost importance for the further studies concerning the pseudo-ternary phase diagrams, which will indicate the boundaries of copaiba oil, surfactant, and water that produce emulsified systems.

The progressive change in the composition of a mixture of immiscible solvents can produce significant variations in the thermodynamic properties of its compounds [31]. The pseudo-ternary phase diagram is an efficient scanning method to obtain different formulations such as emulsion and other systems that can be used for effective therapy [32, 33]. These systems are of great interest to the pharmaceutical industry, not only for their extensive use worldwide, but also for their easy preparation, low-cost drug delivery system, and improved bioavailability [32].

Colloidal system formation was observed at room temperature using three different stirring methods. In vortex shaking an internal cyclone is formed that, irrespective of system viscosity, provides not only good homogenization, but also offers more advantages, such as the use of a small amount of sample at room temperature (*Figure 3*). The magnetic stirring process also allows the use of a small amount of sample at room temperature. However, this process is not suitable for larger amounts of sample or for high viscosity (*Figure 4*). The main effect of ultrasound is the acoustical effect, where the collapse between micro-bubbles increases until they implode, producing both high pressure and temperatures during the final collapse stages (*Figure 5*).

No significant differences in phase diagram regions were found in the microemulsion system for the three production processes. This likely occurs due to the spontaneous formation of such systems, in which the potential energy involved in the production process does not determine the structural formation. There was no significant difference between the numbers of emulsion regions (30 %) produced by the three methods, but there were differences between the percentages of the formulation components that formed the emulsion areas. This same trend was observed for the formation of other colloidal systems, compared to the composition of the formed regions, but the values of the area showed no significant variations. The sonic probe method generates a larger nanoemulsion region (10 %) than the others, probably due to the high energy input involved in the production process that forms small droplets [34]. Other studies have found similar results.

Formariz *et al*. (2007) found, besides other colloidal structures, a microemulsion region of 40 % using the ultra-sound stirring process and cholesterol as oil phase. Additionally, Podlogar *et al*. (2004) used the magnetic stirring process and isopropyl myristate as lipophilic phase and found an emulsion region of approximately 30 %. However, according to the literature, there is a wide difference among chemical composition, concentration of surfactants, and the stirring process for production of phase diagrams.

Future studies will be developed to assay the influence of the addition of pharmaceutical excipients in the formulation in order to enhance the pharmacological and cosmetic properties of Copaiba oil and to evaluate the influence on the HLBc of the emulsions. Furthermore, other studies are needed to evaluate the anti-inflammatory, antimicrobial activity, and toxicological effects on human cells of the colloidal system based on Copaiba oil.

The results of this study show that it is possible to obtain oil-in-water emulsions of copaiba oil stabilized by a surfactant admixture of Tween 20/Span 80 with an HLB value of 14.8. A rheological study of the system revealed a pseudoplastic shear-thinning fluid behavior with thixotropic characteristic. The emulsion with that HLB value

remained stable for over a year, withstanding physicochemical stress tests as well.

Pseudo-ternary phase diagrams make it easier to determine the concentration range of the triphasic component system. Furthermore, the various dispersed systems may have a different apparent viscosity over a particular region. The formation of colloidal structures also depends on the production method, and the behavior of pseudo-ternary phase diagrams is dependent on the energy supplied to the system. In fact, the stirring process induces the formation of different structures, according to the stirring intensity, which improves the degree of inter-component interaction. It can be inferred, however, that the microemulsion regions produced in the phase diagrams were not dependent on the level of energy supplied to the system, probably due to the thermodynamic stability of such systems [13]. Moreover, due to the inherent instability of these systems, each stirring process showed considerable percentage variations in the different regions. It was also possible to conclude that the copaiba oil emulsion system investigated here may be a promising vehicle for drugs and/or active cosmetics.

This work demonstrates the feasibility of production of emulsion systems using natural oil products. As all the mandatory steps on the physicochemical characterization of both the oil and the emulsion system are described in detail, this work could serve as a simple manual for production of emulsion systems from natural products.

REFERENCES

1. Dossat V., Combes D., Marty A. - Lipase-catalysed transesterification of high oleic sunflower oil. - Enzym. Microb. Technol., **30**, 90-94, 2002.
2. Aluyor E.O., Ozigagu C.E., Oboh O.I., Aluyor P. - Chromatographic analysis of vegetable oils: a review. - Sci. Res. Essays, **4**, 191-197, 2009.
3. Orafidiya L.O., Oladimeji F.A. - Determination of the required HLB values of some essential oils. - Int. J. Pharm., **237**, 241-249, 2002.
4. Veiga Jr V.F., Pinto A.C. - O gênero Copaifera L. - Quim. Nov., **25**, 273-286, 2002.
5. Monti H., Tiliacos N., Faure R. - Two diterpenoids from copaiba oil. - Phytochem., **42**, 1653-1656, 1996.
6. Paiva L.A.F., Gurgel L.A., Sousa E.T., Silveira E.R., Silva R.M., Santos F.A., Rao V.S.N. - Protective effect of Copaifera langsdorffii oleo-resin against acetic acid-induced colitis in rats. - J. Ethnopharmacol., **93**, 51-56, 2004.
7. Santos O., Ueda-Nakamura T., Filho P.D.B. - Antimicrobial activity of copaiba oils obtained from different species of Copaifera in Brazil. - Planta. Med., **73**, 860-860, 2007.
8. Gomes N.M., Rezende C.M., Fontes S.P., Matheus M.E., Fernandes P.D. - Antinociceptive activity of Amazonian Copaiba oils. - J. Ethnopharmacol., **109**, 486-492, 2007.
9. Lieberman H.A. Ed. - Pharmaceutical Dosage Forms-Disperse Systems. - M. Dekker, New York, 1988.
10. Cui G.H., Wang L., Davis P.J., Kara M., Liu H. - Preparation and physical characterization of a novel marine oil emulsion as a potential new formulation vehicle for lipid soluble drugs. - Int. J. Pharm., **325**, 180-185, 2006.
11. Griffin W.C. - Classification of surface-active agents by "HLB". - J. Soc. Cosmet. Chem., 311-326, 1949.
12. Kantaria S., Rees G.D., Lawrence M.J. - Formulation of electrically conducting microemulsion-based organogels. - Int. J. Pharm., **250**, 65-83, 2003.
13. Santos P., Watkinson A.C., Hadgraft J., Lane M.E. - Application of microemulsions in dermal and transdermal drug delivery. - Skin Pharmacol. Physiol., **21**, 246-259, 2008.
14. Tenjarla S. - Microemulsions: An overview and pharmaceutical applications. - Crit. Rev. Ther. Drug Carr. Syst., **16**, 461-521, 1999.
15. Yu W., Egito E.S.T., Barratt G., Fessi H., Devissaguet J.P., Puisieux F. - A novel-approach to the preparation of injectable emulsions by a spontaneous emulsification process. - Int. J. Pharm., **89**, 139-146, 1993.
16. Macedo J.P., Fernandes L.L., Formiga F.R., Reis M.F., Junior T.N., Soares L.A., Egito E.S.T. - Micro-emultocrit technique: a valuable tool for determination of critical HLB value of emulsions. - Aaps. Pharmscitech., **7**, 21, 2006.
17. Onunkwo G.C., Adikwu M.U. - Stability of veegum/mucuna gum emulsions. - STP Pharm. Sci., **7**, 320-325, 1997.
18. Roland I., Piel G., Delattre L., Evrard B. - Systematic characterization of oil-in-water emulsions for formulation design. - Int. J. Pharm., **263**, 85-94, 2003.
19. Tai C.Y., Lee M.H., Wu Y.C. - Control of zirconia particle size by using two-emulsion precipitation technique. - Chem. Eng. Sci., **56**, 2389-2398, 2001.
20. Yan C., Resau J.H., Hewetson J., West M., Rill W.L., Kende M. - Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique. - J. Control. Release, **32**, 231-241, 1994.
21. Boonme P., Krauel K., Graf A., Rades T., Junyaprasert V.B. - Characterization of microemulsion structures in the pseudoternary phase diagram of isopropyl palmitate/water/Brij 97: 1-butanol. - AAPS Pharmscitech., **7**, 2006.
22. Hathout R.M., Woodman T.J., Mansour S., Mortada N.D., Geneidi A.S., Guy R.H. - Microemulsion formulations for the transdermal delivery of testosterone. - Eur. J. Pharm. Sci., **40**, 188-196, 2010.
23. Atkinson H.V. - Modelling the semisolid processing of metallic alloys. - Prog. Mater. Sci., **50**, 341-412, 2005.
24. Mondal N., Samanta A., Pal T.K., Ghosal S.K. - Effect of different formulation variables on some particle characteristics of poly (DL-lactide-co-glycolide) nanoparticles. - Yakugaku Zasshi, **128**, 595-601, 2008.
25. Saito H., Shinoda K. - The stability of W/O type emulsions as a function of temperature and of the hydrophilic chain length of the emulsifier. - J. Colloid Interface Sci., **32**, 647-651, 1970.
26. Le Hir A. Ed. - Pharmacie galénique, bonnes pratiques de fabrication des médicaments. - Masson, Paris, 2001.
27. van Ruth S.M., Roozen J.P., Posthumus M.A., Jansen F.J. - Volatile composition of sunflower oil-in-water emulsions during initial lipid oxidation: influence of pH. - J. Agric. Food Chem., **47**, 4365-4369, 1999.
28. Latreille B., Paquin P. - Evaluation of emulsion stability by centrifugation with conductivity measurements. - J. Food Sci., **55**, 1666-1668, 1990.
29. Masmoudi H. - The evaluation of cosmetic and pharmaceutical emulsions aging process using classical techniques and a new method: FTIR. - Int. J. Pharm., **289**, 117-131, 2005.
30. Robins M.M. - Emulsions - creaming phenomena. - Curr. Opin. Colloid Interface Sci., **5**, 265-272, 2000.
31. Oliveira A.G., Scarpa M.V., Correa M.A., Cera L.F.R., Formariz T.P. - Microemulsões: estrutura e aplicações como sistema de liberação de fármacos. - Quim. Nov., **27**, 131-138, 2004.
32. Shafiq-Un-Nabi S., Shakeel F., Talegaonkar S., Ali J., Baboota S., Ahuja A., Khar R.K., Ali M. - Formulation development and optimization using nanoemulsion technique: A technical note. - AAPS Pharmscitech., **8**, 2007.
33. Oliveira A.G., Scarpa M.V. - Microemulsão I: Fundamentos teóricos da formação do sistema microemulsionado. - Pharm. Bras., **1**, 73-79, 2001.
34. Leong T.S.H., Wooster T.J., Kentish S.E., Ashokkumar M. - Minimising oil droplet size using ultrasonic emulsification. - Ultrason. Sonochem., **16**, 721-727, 2009.

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MANUSCRIPT

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Chapter IV

Microemulsion-based drug delivery systems containing natural oils

Le chapitre IV de ce manuscrit consiste en une revue de l'état de l'art de la production de microémulsions avec des huiles naturelles utilisés comme systemes pour la délivrance de principes actifs. Les produits naturels sont des mélanges très complexes contenants des composés de nature chimique différente. Certains ont des activités physiologiques ou thérapeutiques qui peuvent agir seules ou en synergie. Pour cette raison, plusieurs de ces huiles naturelles sont utilisées dans l'industrie pharmaceutique, agronomique, alimentaire, sanitaire et cosmétique. Aujourd'hui, leur intérêt est tourné vers leur immense potentiel pour prévenir et traiter de nombreuses maladies humaines. La formulation en microémulsions est apparue particulièrement appropriée pour améliorer les propriétés pharmaceutiques et biopharmaceutiques de ces huiles. Les microémulsions sont des dispersions thermodynamiquement stable, transparentes et isotropes constitué d'un mélange d'huile et d'eau stabilisé par un film interfacial de tensioactifs, typiquement en combinaison avec un co-tensioactif. Ces dispersions peuvent protéger des composés labiles d'une dégradation prématurée, contrôler la libération d'une molécule active, augmenter la solubilité d'un composé actif et par conséquent d'améliorer la biodisponibilité d'un médicament faiblement biodisponible. L'objectif de ce travail de revue bibliographique a été d'examiner les divers avantages des produits naturels formulés dans les systèmes de microémulsion à être utilisés comme des systèmes de livraison de composés bioactifs.

Mots-clés: microémulsion, produit naturel, huile naturel, huiles végétales, système de livraison, effet synergique

MICROEMULSION-BASED DRUG DELIVERY SYSTEMS CONTAINING NATURAL OILS

Xavier-Junior, F.H.[1,2], Vauthier, C.[2], Morais, A.R.V.[1], Alencar, E.N.[1], Egito, E.S.T.[1]*

[1] Universidade Federal do Rio Grande do Norte, Centro de Ciências da Saúde, Departamento de Farmácia, Laboratório de Sistemas Dispersos (LaSiD). Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Corresponding author

Christine Vauthier, Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Christine.vauthier@u-psud.fr

ABSTRACT

Natural products are extremely complex mixtures containing compounds of different chemical nature. Some have physiological or therapeutical activities that may act either alone or in synergy. So many of them are used in the pharmaceutical, agronomic, food, sanitary and cosmetic industries. Today, their interest is growing toward their immense potential to prevent and treat numerous human diseases. Formulation in microemulsions appeared suitable to improve pharmaceutical and biopharmaceutical properties. Microemulsions are thermodynamically stable, transparent and isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactants, typically in combination with a cosurfactant. They can protect labile compounds from premature degradation, control drug release, increase drug solubility hence enhance the bioavailability of a poorly bioavailable drug. This paper was aimed to review the various advantages of natural products formulated in microemulsion systems to be used as delivery systems for bioactive compounds. The state of the art of parameters involved in the microemulsion formation is summarized as well.

Keywords: Microemulsion, natural product, natural oil, plant oils, delivery systems, synergic effect

INTRODUCTION

Microemulsion (ME) has attracted much interest for several years in terms of their delivery and target potentials (Tenjarla, 1999; Gupta & Moulik, 2008; Muzaffar *et al.*, 2013). Microemulsions (MEs) are thermodynamically stable-phase transition systems, transparent, optically isotropic, which possess low surface tension and small droplet size (Danielsson, Ingvar & Lindman, Björn, 1981; Hegde *et al.*, 2013). These systems are formed by two immiscible liquids (water and oil) mixed to form a single phase stabilized by an interfacial film of alternating surfactant and cosurfactant molecules (Singh *et al.*, 2011; Lawrence & Rees, 2012).

It is well established today that ME can appear in at least three major microstructures: swollen micellar (oil-in-water, O/W), reverse micellar (water-in-oil, W/O) and bicontinuous structures (Ghosh & Murthy, 2006; Lakshmi *et al.*, 2013). These structures can be formed depending on the concentrations, nature, and arrangements of the molecules present in the formulation (Mcclements, 2012). The structure of micelles containing solubilized oil molecules may be spheroids (e.g., micelles or reverse micelles), cylinder-like structure (such as rod-micelles or reverse micelles), plane-like structure (e.g., lamellar structures) or sponge-like structures (e.g., bicontinuous) (Jonsson *et al.*, 1998).

MEs have many advantages as drug delivery systems, including improved appearance, high stability, easiness of preparation and small droplet size, resulting in large surface area from which the active substance can partition and be absorbed or permeate through membranes (Tenjarla, 1999; Lawrence & Rees, 2012; Ritika *et al.*, 2012; Lakshmi *et al.*, 2013). Also, these systems possess the ability to enhance the bioavailability of poorly soluble drugs by maintaining them in molecular dispersion, consequently

allowing for controlled or sustained release of the active agent (Schmalfub *et al.*, 1997; Tenjarla, 1999). They form spontaneously (zero energy input). Therefore, they are ease of manufacture (no process dependent) and scale-up (Al-Adham *et al.*, 2000; Vandamme, 2002; Ghosh & Murthy, 2006). These special properties of the ME offer a high potential for numerous practical applications, including enhanced oil recovery, pharmaceutical and cosmetic formulations, edible coatings for food, and others industrial applications (Singh *et al.*, 2011).

Recently, natural products MEs have been of increasing interest to researchers and have shown great potential in industrial applications. ME utility lies from the fact that they can incorporate a large amount of active natural products in the continuous or disperse phase which are otherwise difficult to formulate (Gupta *et al.*, 2006; Biruss *et al.*, 2007; Safaei-Ghomi & Ahd, 2010; Lawrence & Rees, 2012). Natural products are extremely complex mixtures containing compounds of various chemical natures which are widely exploited by industries around the world. Chemical constituents of natural oils act either alone or in synergy with other compounds giving a global therapeutic activity when incorporated in formulations (Guenther, 1972; Cowan, 1999; Bakkali *et al.*, 2008; Vigan, 2010; Bilia *et al.*, 2014).

Natural products and/or their volatile constituents are used widely to prevent and treat human disease. The possible role and mode of action of these natural products is discussed with regard to the prevention and treatment of very severe disease including cancer, Alzheimer's and cardiovascular diseases, as well as their bioactivity as spasmolythic, revulsive, anti-inflammatory, analgesic and acaricide, antibacterial, antiviral, antipsoriatic, antioxidants and antidiabetic agents (Buchbauer, 2004; Ali *et al.*,

2008; Adorjan & Buchbauer, 2010; Astani *et al.*, 2010; Chaiyana *et al.*, 2010; Safaei-Ghomi & Ahd, 2010; Bassole & Juliani, 2012).

Due to the therapeutic advantages and the complex composition of the natural products, various formulation approaches including carrier technology as ME offer an intelligent approach for the in vivo delivery of several molecules. The present review is divided into two sections. The first section covers the basic concepts of the physicochemical formulation of ME systems, main aspects and energy responsible for their formations. The second section describes the natural products uses and reviews their recent applications in ME systems.

BACKGROUND

The term "microemulsion" was first introduced in 1943 by Hoar and Schulman. It is characterized as a self-assembled homogeneous isotropic system, with thermodynamic stability and it contains extremely high oil/water interfacial areas, offering ultra-low interfacial tension (less than 0.1 mN/m) and viscosity. This system is comprised of two immiscible liquids, such as oil and water, which are stabilized by surfactants and co-surfactants (Hoar, T. P. & Schulman, J. H. , 1943). The dispersed phase occurred as very small droplets (10 - 100 nm), therefore which hardly scattered light in the visible wavelength domain explaining their tendency to appear as transparent. ME usage may result in high drug absorption and permeation, and hence, strong possibility of drug delivery.

The molecular structures of the surfactants consist of an apolar tail region and a polar head group. The surfactants decrease the surface tension of the oil-water interface and

change the entropy of the system. However, it is required a relatively large amount of surfactant in order to stabilize the large interfacial area of ME (Jha, S. K. *et al.*, 2011). The low interfacial tension compensates the dispersion entropy, hence the system is thermodynamically stable (Tenjarla, 1999) and form spontaneously under a specific set of composition and environmental conditions (Rao & Mcclements, 2011).

When dispersed in water or in non-aqueous solvents, the surfactants self-associate into a variety of equilibrium phases. Once the surfactants is an amphiphilic molecule (posses within their structure a part that has an affinity for oil and a part that has affinity for water), during the mixing, the surfactants molecules migrate to the oil-water interface to form a film. The monolayer of surfactant in the interface can exert a two-dimensional surface pressure due to the expansion of the film until the pressure at both sides of the interface becomes constant. After the surfactants occupy the entire interface, the addition of more surfactant will result in micelle formation (Chen *et al.*, 1986; Tenjarla, 1999). Depending on the structure of the surfactants present (greater affinity for water or for oil), it will be determined which type of the system will be formed, once a surfactant more soluble in water than in oil will influence the direction of the ME oil in water (o/ w ME), vice-versa for a more soluble surfactant in the oil than in water (w /o ME) or bi-continuous ME. Various structures may be formed when the surfactants are combined with water, oil or both, such as spherical micelle, reverse micelle, rod shaped micelle, hexagonal phase, lamellar phase and reverse hexagonal phase (Figure 1) (Lawrence & Rees, 2012). In addition, the required concentration of surfactants in the systems will depend of its structure, since a lower concentration of surfactant that strongly favors orientation at the oil/water interface is required in comparison with a surfactant that partitions strongly into either the oil or water phase (Bagwe *et al.*, 2001). Concerning the concentration and the several geometry from the surfactants structures,

it is reasonable that the surfactants film in ME may have different shapes (Soderman & Nydén, 1999). It is possible to verify the formation of elongated, rod-like micelles and W/O spherical droplets at low water content. Whereas, at high water concentration, the most frequent form observed is O/W droplets. Additionally, bicontinuous structures may be formed in ME with similar contents of waters and oil (Podlogar *et al.*, 2005).

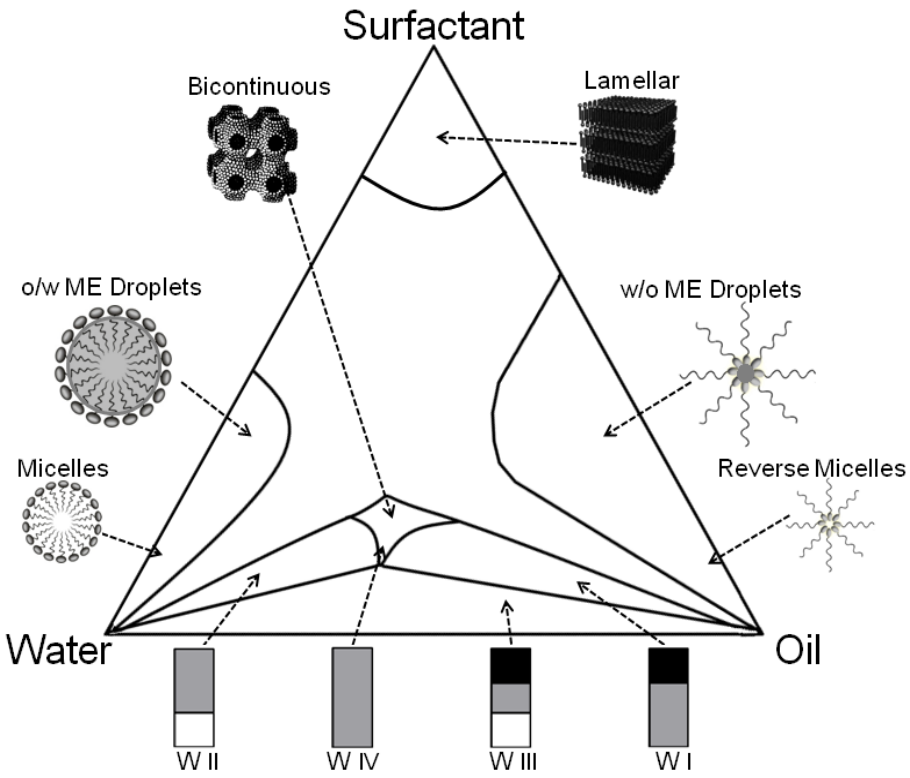

Figure 1: A predictive pseudo-ternary phase diagram with the existence fields of different systems: conventional micelles, reverse micelles, W/O ME, O/W ME and bi-continuous ME and of the Winsor systems (white is the water-excess region, black is the oil-excess region and gray is the microemulsion) (adapted from (Lawrence & Rees, 2012)

At low surfactant concentration, there are four types of ME phases that exist in equilibrium; these phases are commonly referred as Winsor phases (Figure 1) (Winsor, 1954). They are, Winsor I: With two phases, a lower O/W ME phase in equilibrium with the upper excess oil. Winsor II: with two phases, an upper (W/O) ME phase in equilibrium with the water excess. Winsor III: With three phases, a middle ME phase (O/W plus W/O, called bi-continuous) in equilibrium with the upper oil excess and lower excess water. Winsor IV: In single phase, where both oil and water are completely dispersed in the surfactant ME phase. Inter-conversion among the above-mentioned phases can be achieved by adjusting proportions of the constituents.

Attempts have been made to rationalize surfactant behavior in ME formation. These approaches are fairly empirical but can be a useful guide to surfactant selection. In this context, the hydrophilic –lipophilic balance (HLB), the critical packing parameter and the solubility parameters approach were proposed in order support surfactant selection for ME application. The HLB takes into account the relative contribution of hydrophilic and hydrophobic fragments of the surfactant molecule. The W/O ME are formed through the surfactants high dispersion rates in oil, beyond the limit to form reverse micelles (Griffin, W. C., 1949b; Bagwe *et al.*, 2001). Surfactants used to produce this type of ME have a HLB ranging from 3 to 8. On the other hand, to produce W/O ME, the oil is dispersed using surfactants having HLB ranging from 8 to 18. The formation of bicontinuous ME (HLB ≈ 10) is explained by various models, such as the Scriven model, the Random-lattice model, the Cubic random-cell model and the disordered open-connected model (outside the scope of this review) (Tenjarla, 1999).

In contrast, the critical packing parameter relates the ability of surfactants to form particular aggregates to the geometry of the molecule itself. This parameter measures

the preferred geometry adopted by the surfactant, and consequently, it is predictive of the type of aggregate that is likely to form (Lawrence & Rees, 2012). The geometric position of the surfactant at the interface can be another factor influencing the ME structure (Ho *et al.*, 1996). The size and shape of the ME are mainly governed by the curvature free energy of the interface between water ad oil and are determined by the bending elastic constant and curvature of the surfactant film. The elasticity of the film depends on the type of surfactant and on the thermodynamic conditions, but also on the presence of additives like alcohols, electrolytes, block copolymers, and polyelectrolytes. Co-surfactant such as short chain alcohols can improve the film's flexibility (Komesvarakul *et al.*, 2006).

The solubility parameter theory is based on the premise that when the solubility parameters of two chemical compounds are equal, the compounds are infinitely soluble (Hildebrand, 1916; Hildebrand & Scott, 1950; Burke, 1984). The intermolecular forces that cause chemical species to dissolve are the same forces that prevent those materials from boiling away until a specific temperature is reached (Vaughan, 1985). Hansen *et al.* included molecules interacting by dipolar and hydrogen bonding forces (as well as dispersion forces) on this theory, by making the assumption that the solubility parameter could be represented by an additive function of three components (Hansen, C. M., 1967). In this theory, for complete miscibility, two liquids need each of these parameters to be similar. The solubility parameter offers a far more comprehensive system than the HLB system concept. The HLB does not take onto account the chemical match between the surfactant and the phase components of the ME. In other words, it did not take into account the miscibility properties of the surfactants with solvents composing each phase of the ME However, solubility parameter has the disadvantage of being very complex with several alternative expressions available (Vaughan, 1993).

ME droplets have a larger effective interaction volume for the type O/W than W/O, which is due to a strong repulsive term introduced by the presence of an electrical double layer at the surface of the O/W droplet when the ionic surfactants are used. However, when a non-surfactant is used to stabilize O/W ME, the predominant repulsive factor might be attributed to steric interactions, although the polar head groups produce hydration shell. Additionally, the preparation process of W/O ME is easier then O/W ME, since its interfacial tension tends to be lower due to the easier surfactant arrangement at an interface with high curvature, given that the surfactant tails extend outwards into a continuous oil phase, which is entropically more favorable as the hydrocarbon tails have more directional freedom (Lawrence & Rees, 2012).

THEORY OF MICROEMULSION FORMATION

The formation and as well as stability of ME can be affected by various factors such as nature of surfactant, molecular weight of surfactant, alcohol chain length, temperature etc. The reduction of the interfacial free energy to a very low value is of prime importance in the ME formation. Accordingly, the ME formation has been explained by the following three approaches: interfacial or mixed film theory, solubilization theory and thermodynamic theory (Paul & Moulik, 1997; Mehta & Kaur, 2011; Lawrence & Rees, 2012).

Interfacial or mixed film theory

Postulated by Bowcott & Schulman in 1955, this theory describes that the interfacial film is considered to be a duplex in nature (region bounded by water on one side and oil on the other), with an inner and an outer interfacial tension acting independently. (Bowcott & Schulman, 1955). Such a specialized liquid has been based on the assumption that interactions in the interface and reducing the original O/W interfacial tension to zero are capable to form a ME spontaneously. Nevertheless, the ME formation is not ensure by zero interfacial tension although the interfacial tension is generally extremely low, but it depends on the kind of molecular interactions in the liquid interface.

Based on it, Robbins *et al.* developed the theory of MEs phase behavior which discuss that the changes on the direction and extent of curvature are due to the interactions in a mixed film, which can estimate the type and size of the ME droplets (Robbins, 1976). Furthermore, the differential tendency of water to swell the heads and oil to swell the tails of the surfactants impose the ideal kind and degree of curvature of the surfactant film molecules included in the interface to ME formation.

Solubilization theory

Since the 70's it is possible to explain that ME are swollen micelles in which either the water is solubilized in reverse micelles, or the oil is solubilized in normal micelles (Shinoda & Friberg, 1975). A model of this theory was presented by Adamson et al reporting that the W/O emulsion is formed because of the balance achieved in the Laplace and osmotic pressure and that the electrical double layer system with internal

aqueous phase is partially responsible for the interfacial energy, which presented positive free energy, contradicting the concept of negative interfacial tension (Adamson, 1969).

Thermodynamic theory

Concerning the thermodynamic theory, it is important to consider that the free energy must be negative to form thermodynamically stable MEs and the ME formation depends on the reduction of the surface tension of the oil – water interface by the surfactants and the change in entropy of the system (Ruckenstein & Krishnan, 1980). Schulman explained that the formation of a thermodynamically stable ME occurs with a very low interfacial tension of the order of 10^{-4} to 10^{-5} dynes/cm. Moreover, the interfacial charge is responsible for controlling the phase continuity, once the thermodynamic approach accounts for the free energy of the electric double layer along with the van der Waals and the electrical double layer interaction potentials among the droplets (Hoar, T. P. & Schulman, J. H. , 1943; Mehta & Kaur, 2011). However, a significant favorable entropic change should be accompanied by large reductions in surface tension in order to achieve a negative free energy of formation, resulting in spontaneous microemulsification and in a thermodynamically stable system. This entropic change arises from monomer-micelle surfactant exchange, surfactant diffusion in the interfacial layer and the mixing of one phase in the other in the form of large numbers of small droplets (Lawrence & Rees, 2012).

METHOD OF PREPARATION

Although MEs may form spontaneously, external factors can be used to overcome kinetic barriers hence reducing time to obtain the formation of this system. Some factors that can accelerate and facilitate the formation of the ME system can be the order of component addition, the application of a mechanical agitation, the use of ultrasounds or of heat for instance. Therefore, in order to accelerate their formation on a kinetic standpoint, two different methods were proposed to accelerate their formation. These include a phase inversion and a phase titration methods. The change in spontaneous curvature of the surfactant is used by the phase inversion method. The phase inversion may occur in response to temperature or upon dilution of excess of dispersed system inducing drastic physical changes as changes in particle size that can affect drug release both *in vivo* and *in vitro* (Jha, S. K. *et al.*, 2011). The concept of phase inversion temperature (PIT) was introduced by Shinoda and Arai showing the importance of temperature on surfactant properties (particularly nonionic surfactants) (Shinoda & Arai, 1964; Shinoda & Arai, 1967). During the PIT the interfacial properties of the system are balanced and the very small droplet sizes are produced. The nature of the emulsified oils as well as the HLB and concentration of surfactants are important parameters for the PIT (Venkatesh *et al.*, 2014). Additionally, changing the water volume fraction can induce a transition in the spontaneous radius of curvature (Jha, S. K. *et al.*, 2011).

The phase titration method uses the spontaneous diffusion of surfactant or solvent molecules into the continuous phase due to ultra low interfacial tension. The use of diagrams is a useful tool to understand the complex series of interactions that can occur when different components are mixed together. Pseudoternary phase diagram is often

constructed when there are 4 components in the formulation, wherein one corner is the mix of surfactants and the others are the oil and the water. To construct the phase diagram all the components of formulation are mixed in proportions varying from 0 to 100%. Subsequently, each system was characterized and demarcated the phase boundaries formed (Shinoda & Lindman, 1987; Hegde *et al.*, 2013; Muzaffar *et al.*, 2013; Venkatesh *et al.*, 2014).

USES AND APPLICATIONS OF MICROEMULSION SYSTEMS

MEs have been used in a variety of chemical and industrial processes, such as in enhanced oil recovery, as fuels, as coatings and textile finishing, as lubricants, cutting oils and corrosion inhibitors, in detergency, cosmetics, agrochemicals, food, biotechnology, environmental remediation and detoxification, in analytical applications, microporous media synthesis, in pharmaceuticals and as liquid membranes (Paul & Moulik, 2001; Kartsev *et al.*, 2009).

Pharmaceutical preparations such as liquid crystals, micelles and emulsion forming systems have been studied by several authors as a method to solubilize drugs, once the solubilization using cosolvents was the conventional approach. However, the use of cosolvents cannot be employed for parenteral administration for several drugs, furthermore, other disadvantages such as precipitation of the drug on dilution, severe pain at injection site and hemolysis are related to the use of cosolvents (Date & Nagarsenker, 2008). Nevertheless, the instability of emulsions and low solubilization capacity of micelles are disadvantageous. ME is a better proposition over other compartmentalized systems due to their thermodynamic stability, minimum energy

necessary for formation, easiness of preparation, long- term shelf life, low viscosity, surfactant-provoked permeability and reduction of various diffusion barriers by acting as penetration enhancer, protecting against enzymatic degradation, improving drug stability and solubilization capacity which allow a large amount of drug to be incorporated. However, MEs show the disadvantage of having a high concentration of surfactants, which can be toxic to cells depending on their nature (Rozman *et al.*, 2009; Saha *et al.*, 2012).

These structures have been investigated as drug delivery systems for the purpose of drug targeting and controlled release. They improve the bioavailability of poorly soluble drugs due to the capacity of solubilizing both lipophilic or hydrophilic drugs, and partitioning them between the dispersed and the continuous phases, or even administering them together in the same preparation (Nazar *et al.*, 2009). Another important factor is their small droplet size which results in large surface area from which the drug can partition hence it improves problems linked with the dissolution of drugs. A that can be better absorbed or permeate through biological membranes (Gupta & Moulik, 2008). Accordingly, the enhancement of the bioavailability of the drug can reduce the dose required to provide the same pharmacological action and hence reduce associated side-effects associated (Paul & Moulik, 2001). In addition to these advantages, concerning the parenteral delivery systems, the ME improves the drug residence in the blood circulation and reduces the drug irritation (Ren *et al.*, 2012). Furthermore, MEs cause minimum immune reactions or fat embolism in contrast to emulsions.

MEs have been used to sustain or control drug release for percutaneous, peroral, topical, transdermal, ocular and parenteral administration, enhancing absorption of drugs,

modulating the kinetics of the drug release and decreasing the toxicity (Paul & Moulik, 2001; Singh *et al.*, 2011). However, the uses of MEs may be limited due to the maintenance of thermodynamic stability in the temperature range between 0° and 40 °C, constant pressure during storage, low solubilizing capacity for high molecular weight drugs, toxicity and possible incompatibility of surfactants and cosurfactants, requirement at high concentrations for formulations (Paul & Moulik, 2001).

NATURAL PRODUCTS

Mother nature has been a source of medicinal agents for thousands of years. Human started to use plant as medicine since 60,000 years ago, approximately, and today 65% of the world's population relies on plant for their primary health care (Cowan, 1999). Various medicinal plants have been used for years in daily life to treat disease all over the world (Bown, 2003; Nair *et al.*, 2005). Oldest forms of healthcare include the use of leaves, flowers, stem, berries and root of herbs because of their therapeutic or medicinal value (Joseph *et al.*, 2013). Today, it's estimated that there are 250,000 to 500,000 plant species identified so far, about 35,000 are used worldwide for medicinal purposes (Borris, 1996; Moerman, 1996).

Natural medicine is based on the premise that plants contain substances that can promote health and alleviate illness, usually with minimal toxic side effects (Chahlia, 2009; Balakumar *et al.*, 2011; Rajan *et al.*, 2011; Bilia *et al.*, 2014). Focus on plant research especially medicinal plants used in traditional systems has increased all over the world (Dahanukar & Kulkarni, 2000; Biswas & Mukherjee, 2003). Ethnobotanical information has contributed to health care worldwide through the isolation of bioactive

compounds for direct use in medicines (Soejarto *et al.*, 2012; Ntie-Kang *et al.*, 2013; Ezuruike & Prieto, 2014). Indeed, plant extracts represent excellent renewable resources for human applications.

The plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). The most important of these biologically active constituents are alkaloids, flavonoids, tannins and phenolic compounds (Sukumaran *et al.*, 2011). These secondary metabolites in the plants have a prominent function of protection as antibacterials, antivirals, anti-fungals, insecticides and also against herbivores by reducing their appetite for such plants (Cowan, 1999). It is believed that most of the 100,000 known secondary metabolites are involved in plant chemical defense systems, however, only 12,000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1978; Wink, 1999).

Some metabolites are also involved in defense mechanisms against abiotic stress (e.g., UV-B exposure) and are important in the interaction of plants with other organisms (e.g., attraction of pollinators) (Schafer & Wink, 2009; Bassole & Juliani, 2012). Some, such as terpenoids, give plants their odors and flavor (e.g., the capsaicin from chili peppers); others (quinones and tannins) are responsible for plant pigment; and more some of the herbs and spices used by humans to season food (Cowan, 1999). In human, these natural compounds are predominantly used as antioxidant, antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic, and they even have vasodilatory and neuroprotective properties (Martino *et al.*, 2009; Rasooli, 2011; Leandro *et al.*, 2012).

Regarding natural oils studies, the composition of most seed oils are made up of a wide range of fatty acids with six dominating fatty acids: palmitic, stearic, oleic, linoleic,

linolenic and lauric acids. Such fatty acids include those with chain lengths between 8 and 24 carbon atoms, containing varying numbers of double bonds, conjugated systems or functional groups such as acetylenic bond (triple bond), epoxy group (oxygen containing) and hydroxy group (Carlsson, 2009). The advantage about plant oil uses is the excellent renewable sources for industrial usage and further they are structurally similar to the long-chained hydrocarbons derived from petroleum (Wagner *et al.*, 2001).

Essential oil is one of the main classes of secondary metabolites. These oils are volatile, natural, highly enriched in isoprene structure characterized by a strong odor (Cowan, 1999; Bakkali *et al.*, 2008). Essential oils are liquid at room temperature, though a few of them are solid or resinous, limpid, rarely colored, hydrophobic, generally they have a lower density than that of water, they are soluble in lipids and in organic solvents (Balz, 1999; Bakkali *et al.*, 2008). They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. Essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and Tropical countries where they represent an important part of the traditional pharmacopoeia (Bakkali *et al.*, 2008). It is known that the percentage of the components of essentials oils varies amongst species, plants parts, age and stage in the vegetative cycle, as well as according to environmental factors such as light, temperature, soil composition and season, the geographical origin of the plants and the period of harvest (Angioni *et al.*, 2006; Raileanu *et al.*, 2013).

Complex mixtures of volatile compounds come from two groups of distinct biosynthetic pathways. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight (Croteau,

1987; Croteau *et al.*, 2000; Betts, 2001; Pichersky *et al.*, 2006). Terpenes are made from combinations of several isoprene precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The mevalonate route operates in the cytoplasm and mitochondria, and the deoxyxylulose pathway in the plastids (Figure 2). After isoprene precursor's formation, the terpenes biosynthesis consists of repetitive addition of IPPs and DMAPPs molecules and modification by terpene specific synthetases to form the terpene skeleton (Geranyl pyrophosphate). Finally, secondary enzymatic modification of the skeleton occurs to attribute functional properties to the different terpenes (Pichersky *et al.*, 2006; Bakkali *et al.*, 2008). A monoterpene have a general chemical structure of $C_{10}H_{16}$ and they occur as diterpenes, triterpenes, and tetraterpenes (C 20, C 30 and C 40), as well as hemiterpenes (C 5) and sesquiterpenes (C 15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Mcgarvey & Croteau, 1995; Cowan, 1999). These compounds are largely synthesized from acetate units, and despite sharing their origins with fatty acids, they differ of these because they contain extensive branching and are cyclized. Aromatic compounds occur less frequently than terpenes and they are generated from phenylpropane (C6-C3) derivatives (Cowan, 1999; Vigan, 2010).

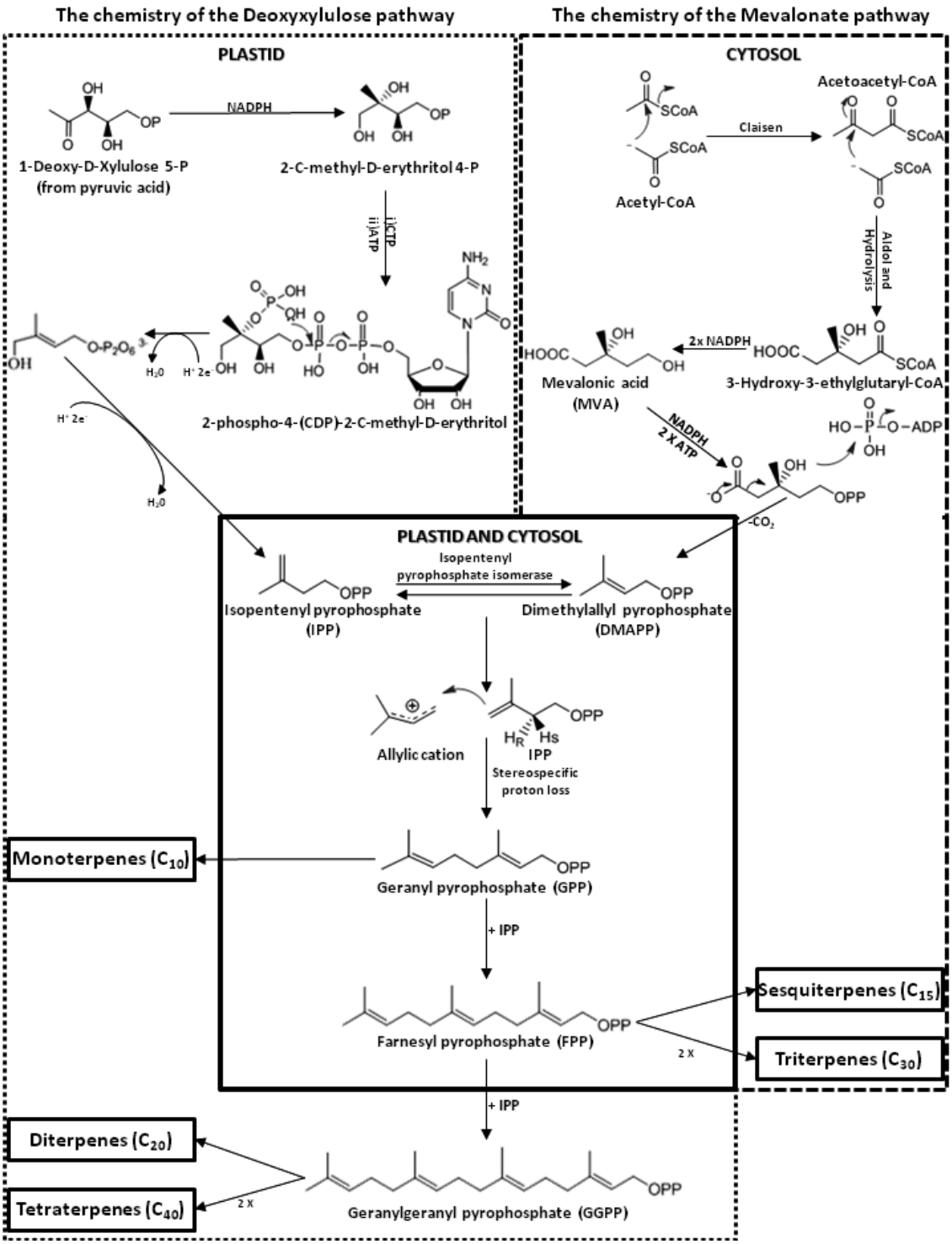

Figure 2: The terpenes biosynthesis by deoxyxylulose and mevalonate pathways

Regarding their biological properties, it has to be kept in mind that essential oils are complex mixtures of numerous molecules that have different mechanisms of action (Guenther, 1972; Koul *et al.*, 2008).It is likely that several components of the essential oils play a role in cell penetration and distribution, due the lipophilic or hydrophilic attraction and fixation on cell walls and membranes (Cal, 2006; Bakkali *et al.*, 2008). In general, the terpenes compounds including limonene (Clarys *et al.*, 1998; Lim *et al.*, 2006), menthol, terpineol, menthone, pulegone, carvone (Jain *et al.*, 2002), thymol, carvacrol, trans- anethole, linalool (Kararli *et al.*, 1995), 1,8-cineole (Williams & Barry, 1991), geraniol (Arellano *et al.*, 1996; Hanif *et al.*, 1998) show a low systemic toxicity and skin irritancy in addition to having good penetration enhancing abilities (Cornwell *et al.*, 1996). This feature is very important because the distribution of the oil in the cell determines the different types of produced radical reactions, depending on their compartmentalization in the cell (Hansen *et al.*, 2006).

Studies also showed that antioxidant activity may be attributed primarily to the high content of phenolic components of the essential oil (Kahkonen *et al.*, 1999; Dorman & Deans, 2000). In general, these compounds can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affecting ionic Ca^{++} cycling and other ionic channels, reducing the pH gradient, collapsing of the proton pump and depletion of the ATP pool (Richter & Schlegel, 1993; Sikkema *et al.*, 1994; Vercesi *et al.*, 1997; Turina *et al.*, 2006; Pasqua *et al.*, 2007). They may change the fluidity of membranes, which might become abnormally permeable, resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure, which may explain their pharmacological and possible toxic effects (Armstrong, 2006; Lorenzi *et al.*, 2009; Devi *et al.*, 2010). Others studies indicated that plant extracts anti-mutagenic properties may be due to inhibition of

penetration of the mutagens into the cells, inactivation of the mutagens by direct scavenging, inhibition of metabolic conversion by P450 of promutagens into mutagens, or activation of enzymatic detoxification (Bakkali *et al.*, 2008). The major anti-mutagenic compounds related are tannic acid, apigenine (Kuo *et al.*, 1992), α-bisabolol (Gomes-Carneiro *et al.*, 2005), thuyone, 1,8-cineole, camphor, limonene (Vukovic-Gacic *et al.*, 2006), (-)-Menthol, (-)-α-pinene, (+)-α-pinene, β-ionone, α- terpinene, α-terpineol, citronellal, and others (Gomes-Carneiro *et al.*, 1998; Oliveira *et al.*, 1999).

Essential oils possess antibacterial, anti-fungal (Safaei-Ghomi & Ahd, 2010; Bassole & Juliani, 2012) and antiviral (Astani *et al.*, 2010) properties. It may be used for prevention and treatment of cancer (Bhalla *et al.*, 2013; Bayala *et al.*, 2014), cardiovascular diseases including atherosclerosis and thrombosis (Lahlou *et al.*, 2005; Santos, M. R. *et al.*, 2007). They might also be used as analgesic (Sousa, 2011), sedative (Buchbauer, 2004), anti-inflammatory (Hajhashemi *et al.*, 2003; Silva *et al.*, 2003), spasmolytic (Magalhaes *et al.*, 2004), local anesthetic, antipyretic activities (Chakraborty *et al.*, 2010), food preservative (Tiwari *et al.*, 2009) and their fragrance can be used in cosmetic applications (Buchbauer, 2004; Adorjan & Buchbauer, 2010).

Several techniques can be used to extract essential oils from different parts of the aromatic plant, including solvent extraction, expression under pressure, enfleurage, and distillation extractions, but hydro or steam distillation are the most commonly used method (Figure 3) (Stashenko *et al.*, 1999; Bassole & Juliani, 2012). The steam distillation separation process based on the difference in composition between a liquid mixture and the vapor formed from it. The mechanical process is used exclusively for citrus fruit: their essential oils are contained in microvesicles located in the peel and may be extracted by pressure or friction. Dry distillation, without addition of water

vapour, is used for wood, bark and roots (Vigan, 2010). For perfume uses, extractions with lipophilic solvents and sometimes with supercritical carbon dioxide are desired. Thus, the chemical profile of the essential oil products differs not only in the number of molecules but also in the stereochemical types of molecules extracted, according to the type of extraction, thereby, the type of extraction is chosen according to the purpose of the use (Bakkali *et al.*, 2008). Their composition can also change after extraction. Depending on the storage conditions, they can quickly become oxidized, and this oxidation is responsible in some cases for variation on the pharmacological activities (Karlberg & Dooms-Goossens, 1997). To monitor these phenomena, most of the commercialized plant extracts are chemotyped by gas chromatography and mass spectrometry analysis (Stashenko *et al.*, 1999). Analytical monographs have been published in the pharmacopoeia from different country to ensure good quality of essential oils (Smith *et al.*, 2005).

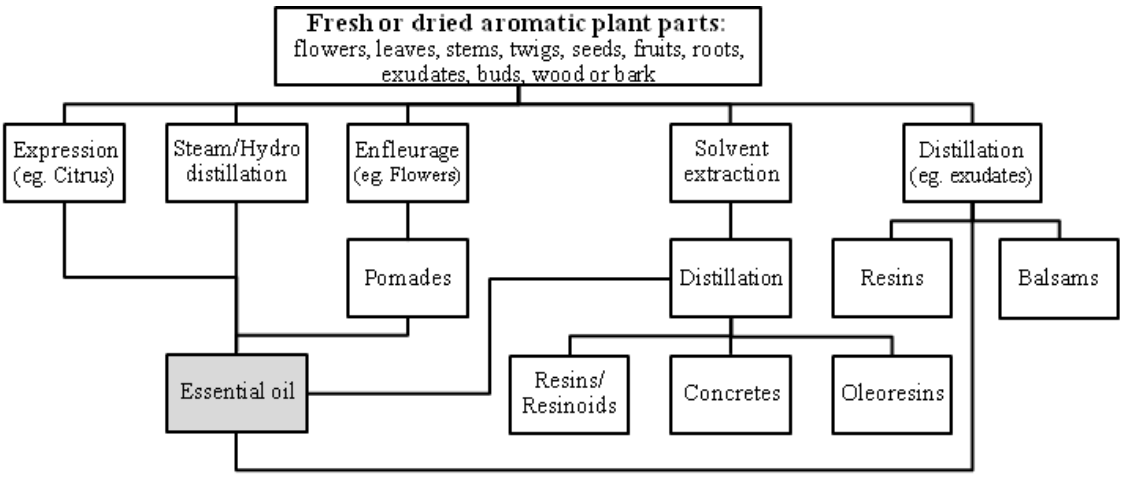

Figure 3- Main extraction process to essential oils from fresh or dried aromatic plant parts

Among these secondary metabolites, it is estimated that over 3,000 essential oils are known, of which about 300 are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Van De Braak & Leijten, 1999; Bakkali *et al.*, 2008). Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure one or another organ dysfunction or systemic disorder (Hajhashemi *et al.*, 2003; Silva *et al.*, 2003). The new attraction for natural products as essential oils is important due some of them constitute being an effective or complements alternatives to synthetic compounds used in chemical industry, without showing the same secondary effects and protecting the ecological equilibrium (Carson & Riley, 2003; Bakkali *et al.*, 2008).

In some cases, the bioactivities of essential oil are closely related with the activity of the main components of the oils (Juliani *et al.*, 2002). However, some studies have demonstrated that whole essential oil usually have higher activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity, though antagonistic and additive effects have also been observed (Figure 4) (Hammer *et al.*, 1999; Dorman & Deans, 2000; Santana-Rios *et al.*, 2001; Gill *et al.*, 2002; Yoon *et al.*, 2011). An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less expressive when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects (Burt, 2004; Bassole & Juliani, 2012). Thus, the uses of these combinations are strategies to potential synergic effects. In that sense, for ME application, it is more relevant to study whole oil rather than some of its components because the concept of synergism appears to be more

meaningful, and the addition effect of the disperse system and the natural product can be expected.

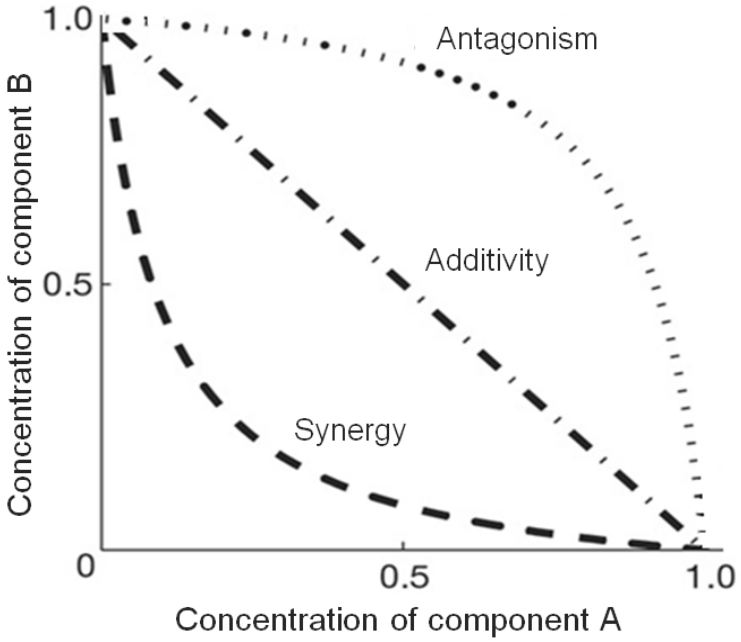

Figure 4: Schema of synergistic, antagonistic and additive effects between components in essential oil (adapted from Chait et al (Chait *et al.*, 2007)).

MICROEMULSION BASED ON NATURAL PRODUCTS

Development of ME including natural products have been of increasing interest to researchers and have shown great potential in industrial applications (Guenther, 1972; Cowan, 1999; Bakkali *et al.*, 2008; Vigan, 2010; Bilia *et al.*, 2014). The association of the intrinsic advantages from ME may be able to act in synergy with the natural compounds (Gupta *et al.*, 2006; Biruss *et al.*, 2007; Lawrence & Rees, 2012). For the purposes of this review, recent developments will for the most part constitute an

evaluation of the literature in the area of ME with natural products. Table 1 show the main works of ME containing natural oils and its respective therapeutic application.

Table 1- Microemulsion containing natural oils

Oil	Source	Data about therapeutic applications	Reference
Babchi oil	*Psoralea corylifolia*	Psoriasis	(Ali *et al.*, 2008)
Cassia oil	*Cinnamomum cassia*	Antifungal activity against *Geotrichum citri-aurantii*	(Xu *et al.*, 2012)
Cinnamon oil		Fungicide	(Wang *et al.*, 2014)
Citrus oil			(Fanun, 2010b)
Clove oil			(Gupta *et al.*, 2006)
Clove oil	*Syzygium aromaticum*	Leishmaniasis	(Gupta *et al.*, 2005)
Coconut oil			(Rukmini *et al.*, 2012)
Copaiba oil	*Copaifera Langsdorffii*		(Xavier-Junior, Chapter V, 2015)
Corn oil			(Gupta *et al.*, 2006)
Cottonseed oil			(Gupta *et al.*, 2006)
Davana oil	*Artemisia pallens*	Topical delivery	(Salunkhe *et al.*, 2013)
Eucalyptus oil	*Eucalyptus Spp*	Absorption promoter to transdermal drug	(Majhi & Moulik, 1999; Maghraby, 2008)
Lemon grass	*Cymbopogon citratus*	Alzheimer's	Chaiyanaetal., 2010
Lemon oil			(Rao & Mcclements, 2011)
Linseed oil			(Mitra *et al.*, 1994)

Lizard tail	*Houttuynia Cordra*		(Yi *et al.*, 2010)
Makrut lime	*Citrus hystrix*	Alzheimer's	(Chaiyana *et al.*, 2010)
Neem oil	*Azadirachta indica*	Acaricidal activity against *Sarcoptes scabiei* var. cuniculi larvae	(Xu *et al.*, 2010)
Orange oil		Absorption promoter to transdermal drug	(Yotsawimonwat *et al.*, 2006)
Orange oil			(Gupta *et al.*, 2006)
Palm oil			(Mitra *et al.*, 1994)
Peppermint oil	*Mentha piperita L.*		(Fanun, 2010a)
Peppermint oil			(Gupta *et al.*, 2006)
Plai oil	*Zingiber cassumunar*	Alzheimer's	(Okonogi & Chaiyana, 2012)
Ricebran oil			(Mitra *et al.*, 1994)
Saffola oil			(Mitra *et al.*, 1994)
Sesame oil			(Mitra *et al.*, 1994)
Soyabean oil			(Mitra *et al.*, 1994)
Tea tree oil	*Melaleuca Alternifolia*	Psoriasis	(Khokhra & Diwan, 2011)

Numerous studies have considered the development of MEs incorporating natural products. Recently, the O/W ME including a high volume fraction of a natural oil (copaiba oil) (19.6%) has been developed (Xavier-Junior, Chapter V, 2015). The formulation approach was based on the chemical match between components of the oil and the lipophilic part of surfactants according to Hansen approach. This ME showed a reduced concentration of surfactant and high values of the oil/surfactant ratio (1.43).

ME for oral route was obtained using copaiba essential oil at final composition of 19.6 %, Pluronic F-68® 0.15 %, Brij O10 ® 13.55 %, and milli-Q ® water 66.7% (w/w). This system showed an incorporation of 3.8 $mg.mL^{-1}$ of β-caryophyllene from copaiba oil.

Gupta et al studied the phase diagram of a new pseudoternary system consisting of clove oil (*Syzygium aromaticum*)/ Tween® 20/water at ratio of 5/30/65. Several compositions from the single-phase region were selected and their stability toward time, temperature, and electrolytes were examined (Gupta *et al.*, 2005). Others authors have observed in detail the development in the phase behavior of Eucalyptus oil/ Tween 20/ Butanol/ Water and Eucalyptus oil/ Tween® 20/ Cinnamic Alcohol/ Water systems. Triangular and tetrahedral representations have been considered to understand the topological nature of the multicomponent mixtures between the compound mixtures (Majhi & Moulik, 1999).

Yotsawimonwat et al investigate the formation of ME considering orange oil in ME. Pseudoternary phase diagrams of orange oil, ethyloleate or a 1:1 mixture (w/w) of orange oil and ethyloleate as oil components, and 6:4 (w/w) mixture of polyoxyethylene 20 sorbitan monooleate and sorbitan monolaurate as surfactant components and water or propylene glycol as hydrophilic components were investigated. The authors observed a smaller ME region on the phase diagram when orange oil was used as a substitute for ethyloleate. In addition, the dimension of solution-type ME areas in the phase diagrams was likely to depend on the miscibility of components and larger ME areas were found when ethyloleate was used instead of orange oil and propylene glycol was used instead of water. Moreover, orange oil incorporation as a penetration enhancer into a topical ME affects its physical characteristics; this, in turn, may lead to instability of the ME and/or can influence the release patterns of drugs (Yotsawimonwat *et al.*, 2006).

Water/propylene glycol/sucrose laurate/ethoxylated mono-di-glyceride/citrus oil ME systems were formulated. In this system, the free energy of solubilization decreased with water content in the water-in-oil ME region and increased in the oil-in-water region. Futhermore, the free energy of solubilization decreased with increasing ethoxylated mono-di-glyceride content in the mixed surfactants. The authors also observed that the hydrodynamic diameter of the diluted ME decreases with the increase in temperature (Fanun, 2010b).

Microemulsification of vegetable oils (ricebran, saffola, soyabean, sesame, palm and linseed) with water using aerosol-OT and cinnamic alcohol as mixed amphiphiles have been studied. The biological formed MEs covered on the average approximately 27% of single phase area in the triangular phase diagram. Saffola oil ME at a reasonable water/aerosol mole ratio presented a moderate increase in conductance with temperature. Amongst the studied systems (sesame, saffola and ricebran), the viscosity of the first two decreased with the rate of shear whereas the ricebran's viscosity increased. When cinnamic alcohol was used as the oil, the trend of viscosity was similar to that of sesame and saffola (Mitra *et al.*, 1994). Another study developed a topical davana oil (*Artemisia pallens)* ME formulation and the same results were observed. The authors found that with the increase of Tween® 80 concentration, the solubilization capacity of davana oil into the ME system was also increased leading to enhancement in ME region. However, transcutol P decreasing interfacial free energy and reducing surface tension promoting the homogenized droplets formation. Optimized formulation was prepared using *Artemisia pallens* based oil (15% w/v), tween 80 (15% w/v), transcutol P (5% w/v), and water (65% w/v) (Salunkhe *et al.*, 2013).

Gupta *et al.* studied microemulsification of various combinations of water with corn oil, cottonseed oil, clove oil, orange oil, and peppermint oil using several non-ionic surfactants (Tween® 20, Brij® 30, and Brij® 92) and cosurfactants (ethanol and isopropanol). Both ternary (oil/surfactant/water) and pseudoternary (oil/surfactant + co-surfactant/water) phase diagrams were constructed. The ternary systems produced larger ME forming zones than pseudoternary systems. Interestingly, the peppermint oil/ isopropyl alcohol /water and 1:1 (v/v) peppermint oil + isopropyl myristate / isopropyl alcohol /water combinations were used to form the proportion of single-phase of the majority ME. All systems showed excellent stability within 1 year and they withstood to temperature variations (Gupta *et al.*, 2006). Fanum *et al.* also developed a peppermint oil-containing ME. The author observed O/W ME formation with droplets of up to 12 nm diameter. The solubilization capacity of water in the oil is dependent on the surfactants and ethanol/oil mixing ratios (w/w). In addition, a progressive transformation of the W/O to bicontinuous and inversion to O/W ME occurred upon dilution with water (Figure 5). The diffusion coefficients of the surfactants at the interface increased while increasing the water volume fraction (Fanun, 2010a).

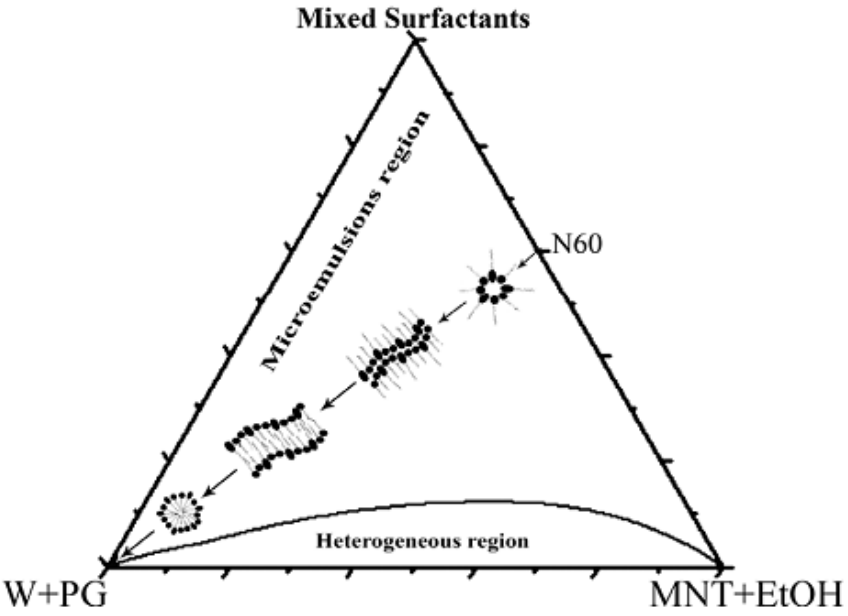

Figure 5- Schematic presentation (not for scale) of the structural transitions along the N60 dilution line in the pseudo-ternary phase diagram. Pseudoternary phase behavior of water (W) / propylene glycol (PG)/ sucrose laurate (L1695) / ethoxylated mono-di-glyceride (EMDG)/ peppermint oil (MNT)/ethanol (EtOH) system at 25 ˚C. The mixing ratios (w/w) were mixed surfactants (L1695/EMDG) =1/1, W/PG=2/1 and MNT/EtOH=1/1 (Fanun, 2010a).

A stable coconut oil-containing ME was prepared based on the HLB concept from a mixture of three surfactants using a ternary mixture of non-ionic surfactants. The W/O ME was successfully formulated with a surfactant blend composed of 16.6% of Tween® 20, 15.0% of Span® 20, and 68.4% of Span® 80. Transparent ME could only be formed when the ratio of water and surfactants was at least 1:4.5 and the ratio of water/surfactants and coconut oil was kept bellow 1:3.5. These ME remained stable during storage for up to 2 months, even after centrifugation, but they were not stable

when subjected to heating at 70 ºC or higher (Rukmini *et al.*, 2012). Rao *et al.* established conditions to fabricate stable ME from a nonionic surfactant (Tween® 80) and flavor oil (lemon oil). The stable lemon oil-containing ME was produced only by heating the colloidal dispersion containing high surfactant-to-oil ratio. The authors suggested that there was a kinetic barrier at ambient temperature that prevented the system from reaching its most kinetically or thermodynamically stable state. In addition, the application of heating appeared to be much more effective than the application of mechanical energy at overcoming this kinetic barrier. In this study when higher temperatures (from 62 to 90°C) were applied at high surfactant concentration (20 % Tween® 80 and 10% of lemon oil) the system was not transparent by turbidity analyses, while upon cooling back to ambient temperature, the turbidity of the system decreased and remained low at ambient temperature (Rao & Mcclements, 2011).

Xu *et al.* developed a food-grade water-dilutable ME containing cassia oil (*Cinnamomum cassia)* as oil, ethanol as cosurfactant, Tween® 20 as surfactant and water. Antifungal activity *in vitro* and *in vivo* against *Geotrichum citri-aurantii* was assessed. According to the authors, the phase diagram confirmed the feasibility of formulating such a ME including cassia oil. The ME was composed from cassia oil/ethanol/Tween 20 at a weight ratio of 1:3:6 (w/w/w) for each of these ingredients respectively. The average droplet size was 6.3 nm. The *in vitro* antifungal experiments showed that the ME inhibited fungal growth on solid medium and prevented arthroconidium germination in liquid medium. Cassia oil had a stronger activity when encapsulated in the ME. The *in vivo* antifungal experiments indicated that the water-dilutable ME was effective in preventing postharvest diseases of citrus fruits caused by *G. citri-aurantii* (Xu *et al.*, 2012).Yifei *et al.* also developed the ME as a potential alternative to chemical fungicides, but their system was composed of cinnamon

essential oil. The ME reduced significantly the decay incidence by 18.7% of postharvest gray mold of pears (*Pyrus pyrifolia*) in comparison to that non-ME after 4 days storage at 20 °C. In the vapor phase, the cinnamon ME with the lowest concentration had the best control for decay incidence and lesion diameter. The authors concluded that ME may be an alternative way to control the gray mold of pears without a negative influence on its qualities (Wang *et al.*, 2014).

Preparation of neem (*Azadirachta indica*) oil containing ME was investigated as well as the acaricidal activity in vitro. In this systems, the mixture of Tween® 80 and the sodium dodecyl benzene sulfonate (4:1) w/w was used as surfactants; the mixture of surfactants and hexyl alcohol (cosurfactant) (4:1) w/w was used as emulsifiers. The ME was composed of a mixture of neem oil, emulsifiers and water (1:3.5:5.5) w/w. The ME formed after stirring at 800 rpm for 15 min at 40 °C. It showed globular and uniform droplets, and have a viscosity of 9.96 mPa s at 25 °C. The ME was still clear after 6 months at room temperature. The lethal time was used to evaluate the acaricidal activity in vitro using *Sarcoptes scabiei* var. cuniculi larvae. The ME containing 10% neem oil showed a median lethal time value (LC50) was 81.75 min. against *Sarcoptes scabiei* var. cuniculi larvae. These results acknowledged an effective anti parasite activity for the neem oil containing ME (Wang *et al.*, 2014).

Natural product/oil containing ME has been developed to topical treat of the psoriasis. Ali *et al.* have investigated and evaluated a ME gel-based system of babchi oil (*Psoralea corylifolia*) for the treatments of psoriasis. Babchi oil is used because its chief constituent psoralen, this action inhibits DNA synthesis and causes decrease in cell proliferation. Thus, the authors suggested that a ME gel could be a potential vehicle for improved topical delivery of psoralen hence it could be a potential vehicle to improved

topical delivery of babchi oil in psoriasis lesions (Ali *et al.*, 2008). The ME was prepared by titration of the aqueous phase into the mixture from oil and surfactant. It consisted of 1.67% v/v of babchi oil, 8.33% v/v of oleic acid, 55% v/v of Tween® 80/ Transcutol-P (S/Co ratio 1:1) and 35% v/v of distilled water. The ME gel was a potential vehicle for improved topical delivery of psoralen and showed a potential vehicles for improved topical delivery of babchi oil. A ME formulated with tea tree oil, another natural oil containing active molecules against psoriasis including terpinin-4-ol was proposed by Khokhra *et al.* The ME was formulated with 5% tea tree oil, different concentrations of polysorbate 80 as surfactant and isopropyl Myristate and isopropyl alcohol as cosurfactants . The tea tree oil-containing ME showed droplets of spherical shape with a size ranging between 84 and 115 nm. These MEs showed a low viscosity . The maximum terpinen-4-ol compound content observed was 1.68 µg/mg of ME. The release profile of terpinen-4-ol from ME depicted that there was a total of 14.5% release through the excised skin from Wistar rats using Franz-type diffusion cells after 24 hours and this ME showed no signs of erythema and skin irritation (Khokhra & Diwan, 2011).

Essential oils of three edible Thai plants, *Cymbopogon citratus* (Gramineae), *Citrus hystrix* (Rutaceae) and *Zingiber cassumunar* (Zingiberaceae) were comparatively tested for acetylcholinesterase and butyrylcholinesterase inhibitory activities in order to for enhancing the acetylcholine levels in Alzheimer's patients. Among the three oils, the *C. citratus* oil exhibited the highest cholinesterase inhibitory activity. Brij® 97, Triton X®-114, Tween® 20 and Tween® 85 were employed as surfactant whereas ethanol and hexanol were used as cosurfactants in the formulation of the ME. Formulating the *C. citratus* oil containing ME, results revealed that the type and concentration of surfactant and co-surfactant exhibited different characteristics than influence in the formation of a large ME region in the pseudoternary phase diagram. Tween 20® was used in

combination with ethanol rather than hexanol, to be more suitable as co-surfactant to ME formation. The mixture Tween® 20/ethanol was hardly influenced by both the pH and the ionic strength of the aqueous phase regarding the formation of the ME. The inhibitory activities of the ME based on water/*C. citratus* oil/Tween® 20/ethanol was significantly greater when compared with the native oil (Chaiyana *et al.*, 2010). The acetylcholinesterase and butyrylcholinesterase inhibitory activities of *Zingiber cassumunar* oil was enhanced by 20 to 25 times respectively while formulating in a ME compared with the activity reported for the non-formulated native oil (Okonogi & Chaiyana, 2012). Chaiyana et al interestingly developed a system with the alkaloidal extract from *Tabernaemontana divaricata* loaded in the *Zingiber cassumunar* oil (Plai oil) containing ME. This system was composed for Plai oil, Triton X®-114, ethanol and water with the oil:surfactant ratios of 1:5 and 2:5. A reverse micellar phase, W/O MEs, liquid crystallines systems and coarse emulsions structures were formed along the aqueous dilution line at both oil:surfactant ratios. Formulations with the oil:surfactant ratio of 1:5 containing 0.1 µg/mL extract from *T.divaricata* showed a significantly higher acetylcholinesterase inhibition than those with the oil:surfactant ratio of 2:5. The ME system containing oil:surfactant ratio of 1:5 significantly increased the transdermal delivery of the *T.divaricata* extract within 24 h (Chaiyana *et al.*, 2013).

Some authors have also studied MEs base formulations for solubilization of volatile oils. Yi *et al.* studied the solubilization of volatile oil from *Houttuynia Cordra* in O/W ME. The ME was developed by titration method, but the varieties and amount of surfactant and co-surfactants had effects on solubilization for volatile oil. The ME was composed by medium-chain triglycerides as oil phase, polyoxyethylene castor oil EL-35 as surfactant and propylene glycol as cosurfactant (at ratio of 2). This system was

capable to solubilize the volatile oil from *Houttuynia Cordra* and this system has a broad range of therapeutic activities (Yi *et al.*, 2010.

A number of reports detail ME formulations designed to potentiate topical or transdermal permeability of drugs. Maghraby has analyzed the effects of cosurfactants on the transdermal delivery of hydrocortisone from eucalyptus oil containing ME. Pseudoternary phase diagrams were constructed in the presence and absence of cosurfactants. ME formulations containing 20% eucalyptus oil, 20% water and 60% of either Tween® 80 or 1:1 surfactant/cosurfactant mixture were compared. On most cases during this study, the incorporation of cosurfactants expanded the ME zone. The cosurfactant free ME was viscous and showed pseudo-plastic flow. In contrast, the ME prepared with a cosurfactant was less viscous and showed a Newtonian flow. In this study, the hydrocortisone was used as model drug. The drug loading and release rate were increased in the presence of cosurfactants (ethanol being the most efficient among the tested cosurfactants) with the release depending on the viscosity, affecting the phase behavior and the transdermal delivery potential of ME (Maghraby, 2008).

CONCLUSION

Natural products have a promising potential in maintaining and promoting health, as well as preventing and potentially treating some diseases. The natural products are extremely complex mixtures of different functional-group classes. Drug delivery systems of natural products, as ME, represent a promising strategy for overcoming the limitations of these products, as low solubility, biodisponibility and efficacy, degradation of the active components in the presence of air, light, moisture and

temperatures. Several studies have been developed on the last years concerning the formulation of new ME systems containing natural products, such as extracts and oils, those studies are discovering that these systems are promising and are also an innovative approach that has potential applications in medicinal and health research, which can result in decrease of the dose, long-term safety increasing, absorption and biodisponibility enhancing, despite reducing systemic side effects. MEs have been shown to be able to protect labile drug, control drug release, improving water solubility of hydrophobic ingredients, enhance the efficacy and reduce patient variability. Furthermore, these systems can promote the retention of ingredients in the internal phase, mask the taste and reduce toxic side effects.

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REFERENCES

ADAMSON, A. W. 1969. A model for micellar emulsions. *J Colloid Interface Sci*, 29, 2, 261-7.

ADORJAN, B. & BUCHBAUER, G. 2010. Biological properties of essential oils: an updated review. *Flavour Fragr J*, 25, 6, 407-426.

AL-ADHAM, I. S. I., KHALIL, E., AL-HMOUD, N. D., KIERANS, M. & COLLIER, P. J. 2000. Microemulsions are membrane-active, antimicrobial, self-preserving systems. *J Appl Microbiol* 89, 32-39.

ALI, J., AKHTAR, N., SULTANA, Y., BABOOTA, S. & AHUJA, A. 2008. Antipsoriatic microemulsion gel formulations for topical drug delivery of babchi oil *(Psoralea corylifolia). Methods Find Exp Clin Pharmacol,* 30, 4, 277-85.

ANGIONI, A., BARRA, A., CORONEO, V., DESSI, S. & CABRAS, P. 2006. Chemical composition, seasonal variability, and antifungal activity of *Lavandula stoechas L. ssp.* stoechas essential oils from stem/leaves and flowers. *J Agric Food Chem,* 54, 12, 4364-70.

ARELLANO, A., SANTOYO, S., MARTIN, C. & YGARTUA, P. 1996. Enhancing effect of terpenes on the in vitro percutaneous absorption of diclofenac sodium. *Inter J Pharm,* 130, 1, 141-145.

ARMSTRONG, J. S. 2006. Mitochondrial membrane permeabilization: the sine qua non for cell death. *Bioessays,* 28, 3, 253-60.

ASTANI, A., REICHLING, J. & SCHNITZLER, P. 2010. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytother Res,* 24, 5, 673-9.

BAGWE, R. P., KANICKY, J. F., PALLA, B. J., PATANJALI, P. K. & SHAH, D. O. 2001. Improved drug delivery using microemulsions: rationale, recent progress, and new horizons. *Crit Rev Ther Drug Carrier Syst,* 18, 1, 77-140.

BAKKALI, F., AVERBECK, S., AVERBECK, D. & IDAOMAR, M. 2008. Biological effects of essential oils-a review. *Food Chem Toxicol,* 46, 2, 446-75.

BALAKUMAR, S., RAJAN, S., THIRUNALASUNDARI, T. & JEEVA, S. 2011. Antifungal activity of *Ocimum sanctum Linn.* (Lamiaceae) on clinically isolated dermatophytic fungi. *Asian Pac J Trop Med,* 4, 8, 654-7.

BALZ, R. The healing power of essential oils. Twin Lakes, WI, USA: Lotus Press, 1999.

BASSOLE, I. H. & JULIANI, H. R. 2012. Essential oils in combination and their antimicrobial properties. *Molecules,* 17, 4, 3989-4006.

BAYALA, B., BASSOLE, I. H., SCIFO, R., GNOULA, C., MOREL, L., LOBACCARO, J. M. & SIMPORE, J. 2014. Anticancer activity of essential oils and their chemical components - a review. *Am J Cancer Res,* 4, 6, 591-607.

BETTS, T. J. 2001. Chemical characterisation of the different types of volatile oil constituents by various solute retention ratios with the use of conventional and novel commercial gas chromatographic stationary phases. *J Chromatogr A*, 936, 1-2, 33-46.

BHALLA, Y., GUPTA, V. K. & JAITAK, V. 2013. Anticancer activity of essential oils: a review. *J Sci Food Agric*, 93, 15, 3643-53.

BILIA, A. R., GUCCIONE, C., ISACCHI, B., RIGHESCHI, C., FIRENZUOLI, F. & BERGONZI, M. C. 2014. Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. *Evid Based Complement Alternat Med*, 2014, 651593.

BIRUSS, B., KAHLIG, H. & VALENTA, C. 2007. Evaluation of an eucalyptus oil containing topical drug delivery system for selected steroid hormones. *Int J Pharm*, 328, 2, 142-51.

BISWAS, T. K. & MUKHERJEE, B. 2003. Plant medicines of Indian origin for wound healing activity: a review. *Int J Low Extrem Wounds*, 2, 1, 25-39.

BORRIS, R. P. 1996. Natural products research: perspectives from a major pharmaceutical company. *J Ethnopharmacol*, 51, 1-3, 29-38.

BOWCOTT, J. E. & SCHULMAN, J. H. 1955. Emulsions. *Z Elektrochem*, 59, 283-290.

BOWN, D. Royal horticultural society encyclopedia of herbs and their uses. Dorling Kindersley, 2003.

BUCHBAUER, G. 2004. On the biological properties of fragrance compounds and essential oils. *Wien Med Wochenschr*, 154, 21-22, 539-47.

BURKE, J. Solubility Parameters: Theory and Application. Craig Jensen Ed, 1984.

BURT, S. 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. *Int J Food Microbiol*, 94, 3, 223-53.

CAL, K. 2006. Skin penetration of terpenes from essential oils and topical vehicles. *Planta Med*, 72, 4, 311-6.

CARLSSON, A. S. 2009. Plant oils as feedstock alternatives to petroleum - A short survey of potential oil crop platforms. *Biochim*, 91, 6, 665-70.

CARSON, C. F. & RILEY, T. V. 2003. Non-antibiotic therapies for infectious diseases. *Commun Dis Intell Q Rep*, 27 Suppl, S143-6.

CHAHLIA, N. 2009. Effect of *capparis decidua* on hypolipidemic activity in rats. *J Med plant res*, 3, 6, 481-484.

CHAIT, R., CRANEY, A. & KISHONY, R. 2007. Antibiotic interactions that select against resistance. *Nature*, 446, 7136, 668-671.

CHAIYANA, W., RADES, T. & OKONOGI, S. 2013. Characterization and in vitro permeation study of microemulsions and liquid crystalline systems containing the anticholinesterase alkaloidal extract from *Tabernaemontana divaricata*. *Int J Pharm*, 452, 1-2, 201-10.

CHAIYANA, W., SAEIO, K., HENNINK, W. E. & OKONOGI, S. 2010. Characterization of potent anticholinesterase plant oil based microemulsion. *Int J Pharm*, 401, 1-2, 32-40.

CHAKRABORTY, A., DEVI, B. R., SANJEBAM, R., KHUMBONG, S. & THOKCHOM, I. S. 2010. Preliminary studies on local anesthetic and antipyretic activities of Spilanthes acmella Murr. in experimental animal models. *Indian J Pharmacol*, 42, 5, 277-9.

CHEN, S. J., EVANS, D. F., NINHAM, B. W., MITCHELL, D. J., BLUM, F. D. & PICKUP, S. 1986. Curvature as a determinant of microstructure and microemulsions. *J Phys Chem*, 90, 5, 842-847.

CLARYS, P., ALEWAETERS, K., JADOUL, A., BAREL, A., MANADAS, R. O. & PRÉAT, V. 1998. In vitro percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *Eur J Pharm Biopharm*, 46, 3, 279-283.

CORNWELL, P. A., BARRY, B. W., BOUWSTRA, J. A. & GOORIS, G. S. 1996. Modes of action of terpene penetration enhancers in human skin; Differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies. *Inter J Pharm*, 127, 1, 9-26.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev*, 12, 4, 564-582.

CROTEAU, R. 1987. Biosynthesis and catabolism of monoterpenoids. *Chem Rev*, 87, 929-954

CROTEAU, R., KUTCHAN, T. M. & LEWIS, N. G. Natural products (secondary metabolites). American Society of Plant Physiologists., 2000.

DAHANUKAR, S. A. & KULKARNI, R. A. 2000. Pharmacology of medicinal plants and natural products. *Indian J Pharmacol*, 32, 81-118.

DANIELSSON, I. & LINDMAN, B. 1981. The definition of microemulsion. *Colloids Surf*, 3, 4, 391-392.

DATE, A. A. & NAGARSENKER, M. S. 2008. Parenteral microemulsions: An overview. *Int J Pharm* 355, 1–2, 19-30.

DEVI, K. P., NISHA, S. A., SAKTHIVEL, R. & PANDIAN, S. K. 2010. Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *J Ethnopharmacol*, 130, 1, 107-15.

DORMAN, H. J. & DEANS, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol*, 88, 2, 308-16.

EZURUIKE, U. F. & PRIETO, J. M. 2014. The use of plants in the traditional management of diabetes in Nigeria: Pharmacological and toxicological considerations. *J Ethnopharmacol*, 155, 2, 857-924.

FANUN, M. 2010a. Formulation and characterization of microemulsions based on mixed nonionic surfactants and peppermint oil. *J Colloid Interface Sci*, 343, 2, 496-503.

_____. 2010b. Properties of microemulsions with mixed nonionic surfactants and citrus oil. *Colloids Surf A Physicochem Eng Asp*, 369, 1–3, 246-252.

GEISSMAN, T. A. Flavonoid compounds, tannins, lignins and related compounds. New York, N.Y: Elsevier, 1963.

GHOSH, P. K. & MURTHY, R. S. R. 2006. Microemulsions: A potential drug delivery system. *Curr Drug Deliv*, 3, 167-180.

GILL, A. O., DELAQUIS, P., RUSSO, P. & HOLLEY, R. A. 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *Int J Food Microbiol*, 73, 1, 83-92.

GOMES-CARNEIRO, M. R., DIAS, D. M., OLIVEIRA, A. C. & PAUMGARTTEN, F. J. 2005. Evaluation of mutagenic and antimutagenic activities of alpha-bisabolol in the Salmonella/microsome assay. *Mutat Res*, 585, 1-2, 105-12.

GOMES-CARNEIRO, M. R., FELZENSZWALB, I. & PAUMGARTTEN, F. J. 1998. Mutagenicity testing (+/-)-camphor, 1,8-cineole, citral, citronellal, (-)-menthol and terpineol with the Salmonella/microsome assay. *Mutat Res*, 416, 1-2, 129-36.

GRIFFIN, W. C. 1949b. Classification of Surface Active Agents by HLB. *J Soc Cosmet Chem* 1, 311-326.

GUENTHER, E. The essential oils. Malabar, Fla, USA: Krieger Publishing Company, 1972.

GUPTA, S. & MOULIK, S. P. 2008. Biocompatible microemulsions and their prospective uses in drug delivery. *J Pharm Sci*, 97, 1, 22-45.

GUPTA, S., MOULIK, S. P., LALA, S., BASU, M. K., SANYAL, S. K. & DATTA, S. 2005. Designing and testing of an effective oil-in-water microemulsion drug delivery system for *in vivo* application. *Drug Deliv*, 12, 5, 267-73.

GUPTA, S., SANYAL, S. K., DATTA, S. & MOULIK, S. P. 2006. Preparation of prospective plant oil derived micro-emulsion vehicles for drug delivery. *Indian J Biochem Biophys*, 43, 4, 254-7.

HAJHASHEMI, V., GHANNADI, A. & SHARIF, B. 2003. Anti-inflammatory and analgesic properties of the leaf extracts and essential oil of Lavandula angustifolia Mill. *J Ethnopharmacol*, 89, 1, 67-71.

HAMMER, K. A., CARSON, C. F. & RILEY, T. V. 1999. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol*, 86, 6, 985-90.

HANIF, R. M., QINENG, P. & ZHAN, G. 1998. Penetration enhancing effect of tetrahydrogeraniol on the percutaneous absorption of 5-fluorouracil from gels in excised rat skin. *J Control Release*, 55, 2-3, 297-302.

HANSEN, C. M. 1967. The Three Dimensional Solubility Parameter. *J Paint Technol* 39

HANSEN, J. M., GO, Y. M. & JONES, D. P. 2006. Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu Rev Pharmacol Toxicol*, 46, 215-34.

HEGDE, R. R., VERMA, A. & GHOSH, A. 2013. Microemulsion: new insights into the ocular drug delivery. *ISRN Pharm*, 2013, 826798.

HILDEBRAND, J. H. 1916. Solubility. *J Am Chem Soc*, 38, 1442 - 1473

HILDEBRAND, J. H. & SCOTT, R. L. The Solubility of Non-electrolytes. New York: 1950.

HO, H.-O., HSIAO, C.-C. & SHEU, M.-T. 1996. Preparation of microemulsions using polyglycerol fatty acid esters as surfactant for the delivery of protein drugs. *Int J Pharm*, 85, 2, 138-143.

HOAR, T. P. & SCHULMAN, J. H. 1943. Transparent water-in-oil dispersions: the oleopathic hydro-micelle. *Nature*, 152, 102-104.

JAIN, A. K., THOMAS, N. S. & PANCHAGNULA, R. 2002. Transdermal drug delivery of imipramine hydrochloride. I. Effect of terpenes. *J Control Release*, 79, 1-3, 93-101.

JHA, S. K., DEY, S. & KARKI, R. 2011. Microemulsions- Potential Carrier for Improved Drug Delivery. *Asian J Biomed Pharm Sci*, 1, 1, 5-9.

JONSSON, B., LINDMAN, B., HOLMBERG, K. & KRONBERG, B. Surfactants & Polymers in Aqueous Solutions. Chichester, U.K.: John Wiley & Sons, 1998.

JOSEPH, B., GEORGE, J. & MORAN, J. 2013. Pharmacology and Traditional Uses of *Mimosa pudica*. *Int J Pharm Sci Drug Res*, 5, 2, 41-44.

JULIANI, H. R., JR., BIURRUN, F., KOROCH, A. R., OLIVA, M. M., DEMO, M. S., TRIPPI, V. S. & ZYGADLO, J. A. 2002. Chemical constituents and antimicrobial activity of the essential oil of *Lantana xenica*. *Planta Med*, 68, 8, 762-4.

KAHKONEN, M. P., HOPIA, A. I., VUORELA, H. J., RAUHA, J. P., PIHLAJA, K., KUJALA, T. S. & HEINONEN, M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem*, 47, 10, 3954-62.

KARARLI, T. T., KIRCHHOFF, C. F. & PENZOTTI JR, S. C. 1995. Enhancement of transdermal transport of azidothymidine (AZT) with novel terpene and terpene-like enhancers: In vivo-in vitro correlations. *J Control Release*, 34, 1, 43-51.

KARLBERG, A. T. & DOOMS-GOOSSENS, A. 1997. Contact allergy to oxidized d-limonene among dermatitis patients. *Contact Dermatitis*, 36, 4, 201-6.

KARTSEV, V. N., SHTYKOV, S. N., BOGOMOLOVA, I. V. & RYZHOV, I. P. 2009. Thermodynamic stability of microemulsion based on sodium dodecyl sulfate. *J Mol Liq*, 145, 3, 173-176.

KHOKHRA, S. & DIWAN, A. 2011. Microemulsion based transdermal drug delivery of tea tree oil. *Int J Drug Development Res*, 3, 1, 191-198.

KOMESVARAKUL, N., SANDERS, M. D., SZEKERES, E., ACOSTA, E. J., FALLER, J. F., MENTLIK, T., FISHER, L. B., NICOLL, G., SABATINI, D. A. & SCAMEHORN, J. F. 2006. Microemulsions of triglyceride-based oils: The effect of co-oil and salinity on phase diagrams. *J Cosmet Sci*, 57, 4, 309-325.

KOUL, O., WALIA, S. & DHALIWAL, G. S. 2008. Essential oils as green pesticides: Potential and constraints. *Biopestic Int*, 4, 1, 63-84.

KUO, M. L., LEE, K. C. & LIN, J. K. 1992. Genotoxicities of nitropyrenes and their modulation by apigenin, tannic acid, ellagic acid and indole-3-carbinol in the Salmonella and CHO systems. *Mutat Res*, 270, 2, 87-95.

LAHLOU, S., MAGALHAES, P. J., SIQUEIRA, R. J., FIGUEIREDO, A. F., INTERAMINENSE, L. F., MAIA, J. G. & SOUSA, P. J. 2005. Cardiovascular effects of the essential oil of Aniba canelilla bark in normotensive rats. *J Cardiovasc Pharmacol*, 46, 4, 412-21.

LAKSHMI, J., KUMAR, B. A. & GUPTA, S. 2013. Investigation of microemulsion as a potential carrier for advanced transdermal delivery: An overview. *Int J Pharm Sci Rev Res* 20, 2, 51-59.

LAWRENCE, M. J. & REES, G. D. 2012. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 64, 175-193.

LEANDRO, L. M., VARGAS F, S., BARBOSA, P. C., NEVES, J. K., SILVA, J. A. & VEIGA-JUNIOR, V. F. 2012. Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins. *Molecules*, 17, 4, 3866-89.

LIM, P. F., LIU, X. Y., KANG, L., HO, P. C., CHAN, Y. W. & CHAN, S. Y. 2006. Limonene GP1/PG organogel as a vehicle in transdermal delivery of haloperidol. *Int J Pharm*, 311, 1-2, 157-64.

LORENZI, V., MUSELLI, A., BERNARDINI, A. F., BERTI, L., PAGES, J. M., AMARAL, L. & BOLLA, J. M. 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. *Antimicrob Agents Chemother*, 53, 5, 2209-11.

MAGALHAES, P. J., LAHLOU, S. & LEAL-CARDOSO, J. H. 2004. Antispasmodic effects of the essential oil of Croton nepetaefolius on guinea-pig ileum: a myogenic activity. *Fundam Clin Pharmacol*, 18, 5, 539-46.

MAGHRABY, G. M. 2008. Transdermal delivery of hydrocortisone from eucalyptus oil microemulsion: effects of cosurfactants. *Int J Pharm*, 355, 1-2, 285-92.

MAJHI, P. R. & MOULIK, S. P. 1999. Physicochemical studies on biological macro- and microemulsions vi : Mixing behaviors of eucalyptus oil, water and polyoxyethylene sorbitan monolaurate (tween 20) assisted by n-butanol or cinnamic alcohol. *J Dispers Sci Technol*, 20, 5, 1407-1427.

MARTINO, L., FEO, V., FRATIANNI, F. & NAZZARO, F. 2009. Chemistry, antioxidant, antibacterial and antifungal activities of volatile oils and their components. *Nat Prod Commun*, 4, 12, 1741-50.

MCCLEMENTS, D. J. 2012. Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, 8, 1719-1729

MCGARVEY, D. J. & CROTEAU, R. 1995. Terpenoid metabolism. *Plant Cell*, 7, 7, 1015-26.

MEHTA, S. K. & KAUR, G. Microemulsions: Thermodynamic and Dynamic Properties. InTech, 2011.

MITRA, N., MUKHOPADHYAY, L., BHATTACHARYA, P. K. & MOULIK, S. P. 1994. Biological microemulsions: Part IV-Phase behaviour and dynamics of microemulsions prepared with vegetable oils mixed with aerosol-OT, cinnamic alcohol and water. *Indian J Biochem Biophys*, 31, 2, 115-20.

MOERMAN, D. E. 1996. An analysis of the food plants and drug plants of native North America. *J Ethnopharmacol*, 52, 1, 1-22.

MUZAFFAR, F., SINGH, U. K. & CHAUHAN, L. 2013. Review on microemulsion as futuristic drug delivery. *Int J Pharm Pharm Sci*, 5, 39-53

NAIR, R., KALARIYA, T. & SUMITRA, C. 2005. Antibacterial activity of some selected Indian Medicinal Flora Turk. *J Bot*, 29, 41-47.

NAZAR, M. F., KHAN, A. M. & SHAH, S. S. 2009. Microemulsion System with Improved Loading of Piroxicam: A Study of Microstructure. *AAPS PharmSciTech*, 10, 4, 1286-1294.

NTIE-KANG, F., LIFONGO, L., MBAZE, L., EKWELLE, N., OWONO OWONO, L., MEGNASSAN, E., JUDSON, P., SIPPL, W. & EFANGE, S. M. 2013. Cameroonian medicinal plants: a bioactivity versus ethnobotanical survey and chemotaxonomic classification. *BMC Complement Altern Med*, 13, 1, 147.

OKONOGI, S. & CHAIYANA, W. 2012. Enhancement of anti-cholinesterase activity of *Zingiber cassumunar* essential oil using a microemulsion technique. *Drug Discov Ther*, 6, 5, 249-55.

OLIVEIRA, A. C., FIDALGO-NETO, A. A. & PAUMGARTTEN, F. J. 1999. In vitro inhibition of liver monooxygenases by beta-ionone, 1,8-cineole, (-)-menthol and terpineol. *Toxicology*, 135, 1, 33-41.

PASQUA, R., BETTS, G., HOSKINS, N., EDWARDS, M., ERCOLINI, D. & MAURIELLO, G. 2007. Membrane toxicity of antimicrobial compounds from essential oils. *J Agric Food Chem*, 55, 12, 4863-70.

PAUL, B. K. & MOULIK, S. P. 1997. Microemulsion: An overview. *J Dispers Sci Technol*, 18, 4, 301-367.

_____. 2001. Uses and applications of microemulsions. *Soft Condens Matter*, 80, 8, 990-1001.

PICHERSKY, E., NOEL, J. P. & DUDAREVA, N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science*, 311, 5762, 808-11.

PODLOGAR, F., BESTER ROGAC, M. & GASPERLIN, M. 2005. The effect of internal structure of selected water–Tween 40®–Imwitor 308®–IPM microemulsions on ketoprofene release. *Inter J Pharm*, 302, 1–2, 68-77.

RAILEANU, M., TODAN, L., VOICESCU, M., CIUCULESCU, C. & MAGANU, M. 2013. A way for improving the stability of the essential oils in an environmental friendly formulation. *Mater Sci Eng C Mater Biol Appl*, 33, 6, 3281-8.

RAJAN, S., THIRUNALASUNDARI, T. & JEEVA, S. 2011. Anti-enteric bacterial activity and phytochemical analysis of the seed kernel extract of *Mangifera indica Linnaeus* against *Shigella dysenteriae (Shiga, corrig.) Castellani and Chalmers*. *Asian Pac J Trop Med*, 4, 4, 294-300.

RAO, J. & MCCLEMENTS, D. J. 2011. Formation of flavor oil microemulsions, nanoemulsions and emulsions: influence of composition and preparation method. *J Agric Food Chem*, 59, 9, 5026-35.

RASOOLI, I. Bioactive compounds in phytomedicine InTech 2011.

REN, X., SVIRSKIS, D., ALANY, R. G., ZARGAR-SHOSHTARI, S. & WU, Z. 2012. In-situ phase transition from microemulsion to liquid crystal with the potential of prolonged parenteral drug delivery. *Inter J Pharm*, 431, 1–2, 130-137.

RICHTER, C. & SCHLEGEL, J. 1993. Mitochondrial calcium release induced by prooxidants. *Toxicol Lett*, 67, 1-3, 119-27.

RITIKA, A., HARIKUMAR, S. L. & AGGARWAL, G. 2012. Microemulsion system in role of expedient vehicle for dermal application. *J Drug Deliv Ther*, 2, 4, 23-28.

ROBBINS, M. L. Theory of the phase behaviour of microemulsions. Oil recovery symposium of the society of petroleum engineers of AIME. Oklahoma, 5839, 22-24.

ROZMAN, B., ZVONAR, A., FALSON, F. & GASPERLIN, M. 2009. Temperature-Sensitive Microemulsion Gel: An Effective Topical Delivery System for Simultaneous Delivery of Vitamins C and E. *AAPS PharmSciTech*, 10, 1, 54-61.

RUCKENSTEIN, E. & KRISHNAN, R. 1980. Effect of electrolytes and mixtures of surfactants on the oil-water interfacial tension and their role in formation of microemulsions. *J Colloid Interface Sci*, 76, 1, 201-211.

RUKMINI, A., RAHARJO, S., HASTUTI, P. & SUPRIYADI, S. 2012. Formulation and stability of water-in-virgin coconut oil microemulsion using ternary food grade nonionic surfactants. *Inter Food Res J*, 19, 1, 259-264.

SAFAEI-GHOMI, J. & AHD, A. A. 2010. Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*. *Pharmacogn Mag*, 6, 23, 172-5.

SAHA, R., RAKSHIT, S., MITRA, R. K. & PAL, S. K. 2012. Microstructure, Morphology, and Ultrafast Dynamics of a Novel Edible Microemulsion. *Langmuir*, 28, 22, 8309-8317.

SALUNKHE, S. S., THORAT, J. D., MALI, S. S., HAJARE, A. A. & BHATIA, N. M. 2013. Formulation, development and evaluation of *Artemisia pallens* (davana) oil based topical microemulsion. *World J Pharm Pharm Sci*, 2, 6, 5725-5736.

SANTANA-RIOS, G., ORNER, G. A., AMANTANA, A., PROVOST, C., WU, S. Y. & DASHWOOD, R. H. 2001. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. *Mutat Res*, 495, 1-2, 61-74.

SANTOS, M. R., CARVALHO, A. A., MEDEIROS, I. A., ALVES, P. B., MARCHIORO, M. & ANTONIOLLI, A. R. 2007. Cardiovascular effects of Hyptis fruticosa essential oil in rats. *Fitoterapia*, 78, 3, 186-91.

SCHAFER, H. & WINK, M. 2009. Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. *Biotechnol J*, 4, 12, 1684-703.

SCHMALFUB, U., NEUBERT, R. & WOHLRAB, W. 1997. Modification of drug penetration into human skin using microemulsions. *J Control Release*, 46, 3, 279-285.

SCHULTES, R. E. The kingdom of plants. New York, N.Y.: McGraw-Hill Book Co., 1978.

SHINODA, K. & ARAI, H. 1964. The Correlation between Phase Inversion Temperature in Emulsion and Cloud Point in Solution of Nonionic Emulsifier. *J Phys Chem*, 68, 12, 3485-3490.

SHINODA, K. & ARAI, H. 1967. The effect of phase volume on the phase inversion temperature of emulsions stabilized with nonionic surfactants. *J Colloid Interface Sci*, 25, 3, 429-431.

SHINODA, K. & FRIBERG, S. 1975. Microemulsions: Colloidal aspects. *Adv Colloid Interface Sci*, 4, 4, 281-300.

SHINODA, K. & LINDMAN, B. 1987. Organized surfactant systems: microemulsions. *Langmuir*, 3, 2, 135-149.

SIKKEMA, J., BONT, J. A. & POOLMAN, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem*, 269, 11, 8022-8.

SILVA, J., ABEBE, W., SOUSA, S. M., DUARTE, V. G., MACHADO, M. I. & MATOS, F. J. 2003. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. *J Ethnopharmacol*, 89, 2-3, 277-83.

SINGH, V., BUSHETTII, S. S., APPALA, R. S., RIZWAN, A., MAMTA, S. & BISHT, A. 2011. Microemulsions as Promising Delivery Systems: A Review. *Ind J Pharm Edu Res* 45, 4, 392-401.

SMITH, R. L., COHEN, S. M., DOULL, J., FERON, V. J., GOODMAN, J. I., MARNETT, L. J., PORTOGHESE, P. S., WADDELL, W. J., WAGNER, B. M., HALL, R. L., HIGLEY, N. A., LUCAS-GAVIN, C. & ADAMS, T. B. 2005. A procedure for the safety evaluation of natural flavor complexes used as ingredients in food: essential oils. *Food Chem Toxicol*, 43, 3, 345-63.

SODERMAN, O. & NYDÉN, M. 1999. NMR in microemulsions. NMR translational diffusion studies of a model microemulsion. *Colloids Surf A Physicochem Eng Asp*, 158, 1–2, 273-280.

SOEJARTO, D. D., GYLLENHAAL, C., KADUSHIN, M. R., SOUTHAVONG, B., SYDARA, K., BOUAMANIVONG, S., XAIVEU, M., ZHANG, H. J., FRANZBLAU, S. G., TAN, G. T., PEZZUTO, J. M., RILEY, M. C., ELKINGTON, B. G. & WALLER, D. P. 2012. An ethnobotanical survey of medicinal plants of Laos toward the discovery of bioactive compounds as potential candidates for pharmaceutical development. *Pharm Biol*, 50, 1, 42-60.

SOUSA, D. P. 2011. Analgesic-like activity of essential oils constituents. *Molecules*, 16, 3, 2233-52.

STASHENKO, E. E., CERVANTES, M., COMBARIZA, Y., FUENTES, H. & MARTÍNEZ, J. R. 1999. HRGC/FID and HRGC/MSD analysis of the secondary metabolites obtained by different extraction methods from *Lepechinia schiedeana*, and *in vitro* evaluation of its antioxidant activity. *J High Resolut Chromatogr*, 22, 6, 343-349.

SUKUMARAN, S., KIRUBA, S., MAHESH, M., NISHA, S. R., MILLER, P. Z., BEN, C. P. & JEEVA, S. 2011. Phytochemical constituents and antibacterial efficacy of the flowers of *Peltophorum pterocarpum (DC.) Baker ex Heyne*. *Asian Pac J Trop Med*, 4, 9, 735-8.

TENJARLA, S. 1999. Microemulsions: An overview and pharmaceutical applications. *Crit Rev Ther Drug Carrier Syst*, 16, 5, 461-521.

TIWARI, B. K., VALDRAMIDIS, V. P., O'DONNELL, C. P., MUTHUKUMARAPPAN, K., BOURKE, P. & CULLEN, P. J. 2009. Application of natural antimicrobials for food preservation. *J Agric Food Chem*, 57, 14, 5987-6000.

TURINA, A. V., NOLAN, M. V., ZYGADLO, J. A. & PERILLO, M. A. 2006. Natural terpenes: self-assembly and membrane partitioning. *Biophys Chem*, 122, 2, 101-13.

VAN DE BRAAK, S. A. A. J. & LEIJTEN, G. C. J. J. Essential oils and oleoresins: A survey in the netherlands and other major markets in the european union. Netherlands: Rotterdam, 1999.

VANDAMME, T. F. 2002. Microemulsions as ocular drug delivery systems: recent developments and future challenges. *Prog Retin Eye Res*, 21, 1, 15-34.

VAUGHAN, C. D. 1985. Using Solubility Parameters in Cosmetic Formulations. *J Soc Cosmet Chem*, 36, 319 - 333.

_____. 1993. Solubility Parameters for Characterising New Raw Materials. *Cosmet Toiletries* 108, 57-62.

VENKATESH, D. N., KUMAR, S. S., KIRAN, H. C., MEGALA, M., MONISHA, C., MYTHILI, L. & SPOORTHY, G. 2014. Microemulsion: An update. *Inter J Pharm Sci*, 4, 6, 802-811.

VERCESI, A. E., KOWALTOWSKI, A. J., GRIJALBA, M. T., MEINICKE, A. R. & CASTILHO, R. F. 1997. The role of reactive oxygen species in mitochondrial permeability transition. *Biosci Rep*, 17, 1, 43-52.

VIGAN, M. 2010. Essential oils: renewal of interest and toxicity. *Eur J Dermatol*, 20, 6, 685-92.

VUKOVIC-GACIC, B., NIKCEVIC, S., BERIC-BJEDOV, T., KNEZEVIC-VUKCEVIC, J. & SIMIC, D. 2006. Antimutagenic effect of essential oil of sage (Salvia officinalis L.) and its monoterpenes against UV-induced mutations in Escherichia coli and Saccharomyces cerevisiae. *Food Chem Toxicol*, 44, 10, 1730-8.

WAGNER, H., LUTHER, R. & MANG, T. 2001. Lubricant base fluids based on renewable raw materials: Their catalytic manufacture and modification. *Appl Catal A Gen*, 221, 1–2, 429-442.

WANG, Y., ZHAO, R., YU, L., ZHANG, Y., HE, Y. & YAO, J. 2014. Evaluation of cinnamon essential oil microemulsion and its vapor phase for controlling postharvest gray mold of pears (*Pyrus pyrifolia). J Sci Food Agric*, 94, 5, 1000-4.

WILLIAMS, A. C. & BARRY, B. W. 1991. Terpenes and the lipid-protein-partitioning theory of skin penetration enhancement. *Pharm Res*, 8, 1, 17-24.

WINK, M. Functions of plant secondary metabolites and their exploitation in biotechnology. UK: Sheffield Academic Press, 1999.

WINSOR, P. A. Solvent Properties of Amphiphilic Compounds. London: Butterworth, 1954.

XAVIER-JUNIOR, F. H., HUANG, N., VACHON, J. J., REHDER, V. L. G., MACIUK, A., EGITO, E.S.T., VAUTHIER, C. Chapter V, 2015. Match of solubility parameters between oil and surfactants as a rational approach for the formulation of O/W microemulsion with high dispersed volume of copaiba oil and low surfactant content. *Article in preparation*

XU, J., FAN, Q. J., YIN, Z. Q., LI, X. T., DU, Y. H., JIA, R. Y., WANG, K. Y., LV, C., YE, G., GENG, Y., SU, G., ZHAO, L., HU, T. X., SHI, F., ZHANG, L., WU, C. L., TAO, C., ZHANG, Y. X. & SHI, D. X. 2010. The preparation of neem oil microemulsion (*Azadirachta indica*) and the comparison of acaricidal time between neem oil microemulsion and other formulations *in vitro*. *Vet Parasitol*, 169, 3-4, 399-403.

XU, S. X., LI, Y. C., LIU, X., MAO, L. J., ZHANG, H. & ZHENG, X. D. 2012. In vitro and in vivo antifungal activity of a water-dilutable cassia oil microemulsion against Geotrichum citri-aurantii. *J Sci Food Agric*, 92, 13, 2668-71.

YI, H., GAO, J. & YANG, H. 2010. Solubilization of O/W microemulsion for volatile oil from *Houttuynia Cordra*. *Zhongguo Zhong Yao Za Zhi*, 35, 1, 49-52.

YOON, J. I., BAJPAI, V. K. & KANG, S. C. 2011. Synergistic effect of nisin and cone essential oil of *Metasequoia glyptostroboides Miki ex Hu* against *Listeria monocytogenes* in milk samples. *Food Chem Toxicol*, 49, 1, 109-14.

YOTSAWIMONWAT, S., OKONOKI, S., KRAUEL, K., SIRITHUNYALUG, J., SIRITHUNYALUG, B. & RADES, T. 2006. Characterisation of microemulsions containing orange oil with water and propylene glycol as hydrophilic components. *Pharmazie*, 61, 11, 920-6.

Chapter V

Match of solubility parameters between oil and surfactants as a rational approach for the formulation of O/W microemulsion with high dispersed volume of copaiba oil and low surfactant content

Le but du travail présenté dans le chapitre V était de développer une microémulsion huile dans eau comprenant une fraction de haut volume d'une huile naturelle (huile de copaïba) tandis que la concentration en tensioactif sera maintenu à un niveau faible. L'approche de formulation a été basée sur l'appariement chimique entre les composants de l'huile et la partie lipophile de tensioactifs en appliquant une approche basée sur l'analyse des paramètres de solubilité des différents composés développé par Hansen. Les tensioactifs montrant le meilleur appariement de leurs paramètres de solubilité avec ceux des principaux composantes de l'huile de copaïba ont été sélectionnés pour préparer des mélanges dans des différentes proportions pour être ajustées au HLB requis de l'huile puis ces mélanges ont été utilisés pour étudier deleur effect sur la formation de microémulsions. La concentration minimale du mélange de tensioactif nécessaire pour obtenir une microémulsion a ensuite été recherchée par titrage d'un mélange contenant 75% de l'eau et 25% de l'huile de copaïba à 25 °C. Les microémulsions ont été obtenues à la composition finale de l'huile essentielle à 19,6%, le Pluronic F-68® 0,15%, Brij-O10® 13,55% et eau milli-Q® 66,7%. Les microémulsions sont apparus isotrope par l'observation en lumière polarisée. Ils ont été caractérisés par une taille de gouttelettes de 42 ± 0,5 nm avec une distribution unimodale. La microémulsion a permis de disperser 94% des composés trouvés dans l'huiles essentielles de copaïba, ce-qui correspondant à une concentration de 3,8 $mg.mL^{-1}$ de β-caryophyllène. L'approche basée sur l'analyse des paramètres de solubilité des composants de l'huile et de la fraction lipophile des tensioactifs a été utile pour préparer des microémulsions caractérisés par des valeurs élevées du rapport huile / tensioactif (1,43) et la de fraction volumique d'huile (19,6%) par rapport aux microémulsions décrites dans la littérature. Comme la réduction de la concentration de l'agent tensioactif et l'augmentation de la fraction volumique de la phase dispersée est une préoccupation générale lors de la

formulation de microémulsions pour l'administration de médicaments, l'approche proposée dans ce travail est apparue intéressante. Elle a permis de nouveaux progrès vers le développement de microémulsions qui pourront être proposées pour la formulation de médicaments anticancéreux qui ont une mauvaise biodisponibilité raison d'une faible solubilité aqueuse destinés à la voie orale.

Mots-clés: paramètres de solubilité, microémulsion, équilibre hydrophile-lipophile, huile de copaïba, voie orale.

MATCH OF SOLUBILITY PARAMETERS BETWEEN OIL AND SURFACTANTS AS A RATIONAL APPROACH FOR THE FORMULATION OF O/W MICROEMULSION WITH HIGH DISPERSED VOLUME OF COPAIBA OIL AND LOW SURFACTANT CONTENT.

Xavier-Junior, F.H.[1,2], Huang, N.[2], Vachon, J. J. [2], Rehder, V. L. G.[3], Maciuk, A.[4], Egito, E.S.T.[1], Vauthier, C.[2]*

[1] Universidade Federal do Rio Grande do Norte, Centro de Ciências da Saúde, Departamento de Farmácia, Laboratório de Sistemas Dispersos (LaSiD). Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

[3] Universidade Estadual de Campinas (UNICAMP) – Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas. Rua Alexandre Cazelatto, 999, Vila Betel, Paulínia – SP.

[4] Université Paris Sud, Laboratoire de Pharmacognosie - UMR CNRS 8076 BioCIS - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

***Corresponding author: Christine Vauthier**

Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

christine.vauthier@u-psud.fr

ABSTRACT

The aim of this work was to develop an O/W microemulsion including a high volume fraction of a natural oil (copaiba oil) while the concentration in surfactant will be kept at a low level. The formulation approach was based on the chemical match between components of the oil and the lipophilic part of surfactants according to Hansen approach. Surfactants showing the best match on their solubility parameters with that of the main complements of copaiba oil were selected to prepare blends at different proportions and to investigate the formation of microemulsions. The minimum concentration of the blend of surfactant required to obtain a microemulsion was then searched by titration of a mixture containing 75% water and 25 % copaiba oil at 25°C. Microemulsions were obtained with essential oil at final composition of 19.6 %, Pluronic F-68® 0.15 %, Brij O10 ® 13.55 %, and milli-Q ® water 66.7% (w/w). The microemulsions appeared isotropic by observation under polarized light by optical microscopy. They were characterized by a droplet size of 42 ± 0.5 nm with unimodal distribution. The microemulsion showed an incorporation of 94% of the mother copaiba essential oil compounds, corresponding at concentration of 3.8 $mg.mL^{-1}$ of β-caryophyllene. The approach based on the analysis of solubility parameters of oil components and lipophilic fraction from surfactant was useful to prepare microemulsions characterized by high values of the oil/surfactant ratio (1.43) and oil volume fraction (19.6 %) as compared with microemulsions of the literature. As reducing the concentration of surfactant and increasing volume fraction of the disperse phase is a general concern while formulating microemulsions for drug delivery, the approach proposed in this work appeared interesting to further progress toward the development of suitable microemulsion as formulation for oral delivery of anticancer drugs that have a poor biodisponibility because of a low aqueous solubility.

Keywords: Microemulsion, copaiba oil, solubility parameters, oral route.

1 INTRODUCTION

Microemulsions are colloidal system which have a great potential in pharmacy to improve delivery of drugs (Lawrence & Rees, 2012; Muzaffar *et al.*, 2013). These systems are isotropic, thermodynamically stable and single-phase liquid solution formed by mixing oil, water, and surfactants (Hoar, T. P. & Schulman, J. H. , 1943; Danielsson, Ingvar & Lindman, Björn, 1981; Mcclements, 2012). Microemulsions can increase the solubility of poorly water-soluble compounds and improve their penetration through biological membranes, thereby enhancing oral bioavailability (Araya *et al.*, 2005; Gibaud & Attivi, 2012).

Copaiba oil (*Copaifera langsdorffii*) has been utilized in folk medicine. Phytochemical properties of diterpenes and sesquiterpenes hydrocarbons are attributed various therapeutic effects including anti-inflammatory, antitumoral, antimicrobial, antitetanus, antiblenorrhagea, antileishmania and expectorant activities (Gomes, Niele Matos *et al.*, 2007; Gomes N *et al.*, 2008; Mendonça & Onofre, 2009b; Comelli Júnior *et al.*, 2010; Souza, Martins, Souza, Furtado, Heleno, De Sousa, *et al.*, 2011). However, the lipophilic nature of copaiba oil renders its use difficult in the folk medicine therapy due the low solubility, absorption and bioavailability by oral route. Microemulsion systems containing vegetable oils are a suitable alternative to enhance therapeutic effects, improving pharmacological activities and reduce toxicity of active compounds (Lee *et al.*, 1995; Dantas, T. N. C. *et al.*, 2010; Attaphong *et al.*, 2012).

Models for predicting solubility of substances in solvent mixtures have an important application in drug formulation (Barton, 1983; Thimmasetty *et al.*, 2009). For microemulsion development, solubility study were also found as an essential approach to optimize energy of mixing oil compounds in surfactant blends which range from non-

polar to highly polar substances. Solubility parameters are an intrinsic physicochemical property of a substance expresses as its square root of the cohesive energy density. The cohesive energy density itself is defined as the ratio of the vaporization energy to the molar volume at the same temperature (Hildebrand *et al.*, 1970). In 1967, Hansen suggested the splitting of the "global" Hildebrand solubility parameter into three parts derived from different types of cohesive forces including disperse, polar and hydrogen bond forces (Hansen, C.M. , 1967; Hildebrand *et al.*, 1970). Formulating microemulsions, choice of solvents and surfactants are often based on empirical data rather than on a rational approach. However, an initial estimate based on oil- surfactant/ co-surfactant solubility calculations could help optimizing the formulation of microemulsion minimizing experimental expenditure.

The aim of the present study was to develop and characterize a copaiba oil/ water microemulsion with a high volume fraction of the oil and a low concentration of surfactant from a rational approach based on the use of solubility parameters. Microemulsions are interesting systems because their intrinsic thermodynamic stabilities and small size which can promote the effective delivery of large amounts of copaiba oil. However, in general, microemulsions require the incorporation of a large amount of surfactants for stabilizing droplets, which can often cause toxicity (He *et al.*, 2010; Sapra *et al.*, 2013). It was then postulated that a rational approach of the formulation taking into account solubility parameters of the different components entering the composition of the microemulsion could improve the performance reducing the amount of surfactant while allowing the incorporation of a still interesting volume fraction of the dispersed phase. Such an approach was applied with success to the formulation of W/O microemulsions but to our knowledge it was not used to formulate O/W microemulsions containing natural oil so far.

2 MATERIALS AND METHODS

2.1 Materials

Copaiba oil was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Polyoxyethylene (10) oleyl ether (Brij® O10), Polyethylene glycol sorbitan monolaurate (Tween® 20), Polyoxyethylene-polyoxypropylene block copolymer (Pluronic® F-68), and β-caryophyllene were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q plus, Millipore, St Quentin en Yvelines, France). Ethyl acetate and Ethanol were purchased from Fisher Scientific (Illkirch, France). All chemicals reagent grade were used as received.

2.2. Copaiba essential oil extraction

Copaiba essential oil was obtained from 400 mL of copaiba resin oil by hydrodistillation using a Clevenger-type apparatus for 3 h. The extract was dried with sodium sulphate, filtered and stored at -20 °C. Aliquots (10-20 mg) of the oil were dissolved in ethyl acetate (1 mL) and analyzed by a validated gas chromatography method (Xavier-Junior, Chapter I, 2015b).

2.3. Gas chromatography – Flame Ionization Detector and mass spectrometry analyses

Analysis of the composition of copaiba oil was performed by gas chromatography-flame ionization detector and mass spectrometry as described by Xavier-Junior *et al.* (Xavier-Junior, Chapter I, 2015b). Briefly, the essential compounds showed in copaiba oil-loaded microemulsion and copaiba oil alone were determined. The apparatus used was a PR2100 gas chromatography (Alpha MOS, Toulouse, France) interfaced with a Flame Ionization Detector (GC-FID) and Hewlett-Packard 6890 gas chromatography (Agilent Technologies, Santa Clara, CA, EUA) with HP-5975 mass selective detector (GC-MS). A fused silica capillary column (25 m × 0.32 mm i.d., 0.5 µm) film thickness coated with cross-linked 5% phenyl polysilphenylene-siloxane (SGE Analytical Science Pty Ltd, Victoria, Australia) was used as the separation capillary. The injector and detector temperatures were set at 250 and 300 °C, respectively. The start of column heating was set at 90 °C, with heating ramp of 2 C.min^{-1} to 150 °C, then isothermally heating 20 °C min^{-1} to 300 °C. Helium was used as carrier gas at 1 mL.min^{-1}. The GC-MS electron ionization system was set at 70 eV. The volume injected for all samples was 1 µL. The oil components were identified by comparing their mass fragmentation with data from the electronic library from the Wiley 6, aro_cnrs, F&F_Lib_Argeville, MainLib and Aromes libraries and published data elsewhere. The β-caryophyllene was selected as the standard for the studies of copaiba oil quantification.

2.4 Solubility Parameters: method of calculation

The group contribution method was used to calculate the solubility parameters of the main compounds composing copaiba oil and surfactants knowing their molecular structure. In this study, the theoretical determination of solubility parameters were provided to main compounds presented in the copaiba oils and the lipophilic chain to the main surfactants used for oral route based in the partial chemical group contribution in the Table 1. The Hansen approach, which is one of the most common methods by which each solubility parameter contribution can be estimated was used using the equations (1 and 2) , while chemical group contribution were taken from tables given in the literature (Van Krevelen & Hoftyzer, 1976).

Table 1- Chemical group contributions to the dispersion partial solubility parameter using the group contribution method of van Krevelen and Hoftyzer (Van Krevelen & Hoftyzer, 1976)

Chemical group	**V (cm^3mol^{-1})**	**F_{di} ($cal^{1/2}\,cm^{3/2}\,mol^{-1}$)**	**F_{pi} ($cal^{1/2}\,cm^{3/2}mol^{-1}$)**	**E_{hi} ($calmol^{-1}$)**
$-CH_2$	16.1	132.0	0.0	0.0
$-CH_3$	33.5	205.0	0.0	0.0
-CH=	13.5	98.0	0.0	0.0
-C	-19.2	-34.2	0.0	0.0
-CH	-1.0	39.0	0.0	0.0
$-C(CH_3)_2$	49.0	308.0	0.0	0.0
-COO-	18.0	191.0	240.0	1675.0
-O-	3.8	49.0	196.0	1467.0
-COOH	20.5	259.2	205.4	4889.9

Where: $F_{di,}$ F_{pi} and E_{hi} are the molar attraction constants due to dispersion, polar and hydrogen bonding energy of the partial group, respectively, and V is the group contribution to molar volume.

$$\delta_{d=}\frac{\sum F_{di}}{V} \quad \delta_{p=}\frac{\sqrt{\sum F_{pi}^2}}{V} \quad \delta_{h=}\sqrt{\frac{\sum E_{hi}}{V}} \qquad (Eq1)$$

$$\delta_t^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \qquad (Eq2)$$

Where: δ_d, δ_p, and δ_h are the dispersive, polar and hydrogen bonding solubility parameter components, respectively.

2.5 Calculation of the HLB_0 of copaiba oil and of surfactants

The required Hydrophilic-Lipophilic Balance (HLB_0) of copaiba oils main compounds were determined following equations (3):

$$HLB_0 = \frac{20}{1+K/\left[\delta_d^2 + 0.25\,\delta_p^2 + 0.25\,\delta_h^2\right]_L} \qquad (Eq3)$$

Where: the K value can assume the value of 39 to be applied to the formulation of an O/W emulsion (Beerbower & Hill, 1971).

HLB of non-ionic surfactants were calculated from the molecular weight ratio between their hydrophilic moieties and entire molecule (equation 4). The determination of the

HLB of surfactant blends and HLB_0 total of the copaiba oils were calculated according the equation 5 (Griffin, W. C., 1949a; Griffin, 1954).

$$HLB_{value}=20.\ \frac{\text{Mw hydrophilic potion}}{\text{Mw entire molecule}} \qquad \text{(Eq4)}$$

$$HLB_{blend}=\frac{W_A HLB_A + W_B HLB_B}{W_A + W_B} \qquad \text{(Eq5)}$$

Where W_A and W_B are the amount (weight) of the first and second surfactants/compound used, respectively, the HLB_A and HLB_B are the assigned HLB values for surfactants/compound A and B, respectively.

The best surfactant blends were selected based in the closer solubility parameters among the surfactants and copaiba oils compounds. This approach was expected to provide stability of the disperse system at lower levels of surfactant(s) at the same HLB_0 from copaiba oils. Tween 20®, Brij O10® and PluronicF-68® were used to produce microemulsion loaded copaiba oil. Surfactant blends were Tween 20®:Brij $O10^{®}_{(w/w)}$ (4.2:95.8 and 13.4:86.6 ratios) and Pluronic F-68®: Brij $O10^{®}_{(w/w)}$ (1.1:98.9 and 3.4:96.6 ratios).

2.6 Preparation of the microemulsion

Copaiba oils and milli-Q water were weighed at 25:75 and 15:85 $_{(w/w)}$ ratios of 1 g per batch. Thereafter, surfactant either pure or in blends Tween 20®:Brij $O10^{®}_{(w/w)}$ or

PluronicF-68®: Brij O10®$_{(w/w)}$ were sequentially added to the copaiba oil/milli-Q® water mixtures. After each addition of 50 mg of surfactant, the system was sonicated (Misonix XL 2020 sonicator, Farmingdale, NY, U.S.A) at 40 % amplitude for 60 seconds followed by an ultrasonic bath for 10 minutes (Elma Elmasonic S10H, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) and the turbidity was monitored out on the UV-VIS-Fibre Optics Spectrometer AVS-S2000 with DH-2000 deuterium Halogen light source and AvaSoft software package (Avantes, Apeldoorn, Netherlands). The addition of surfactant was pursued until a clear and transparent system was obtained.

2.7 Characterization of the microemulsion

2.7.1 Transmittance measurements

Temperature-scanning transmittance measurements were used to obtain information about potential changes in the microstructure of the samples during heating. Temperature versus transmittance scans were then performed on the samples using a UV-VIS-Fibre Optics Spectrometer. The line emission spectra were observed between 400 and 800 nm wavelength and 650 nm to analysis. The samples were heated from 25 to 45 °C a rate of 2.5 °C.min^{-1}.

2.7.2 Polarized light microscopy

In order to determine optical isotropy, copaiba oil/ water microemulsion was examined under polarized light microscopy, with a Nikon E600 Eclipse direct microscope

(Champigny/Marne, France). The microscope was equipped with a long focus objective (LWD 40 x 0.55; 0-2mm) and a Nikon Coolpix 950 camera was used to record the images with a resolution of 1600 x 1200 pixels.

2.7.3 pH analysis

The pH values of the microemulsions loaded copaiba oils were measured by a pH meter (model HI 8417, Hanna Instruments Inc., Woonsocket, USA), at 20 ± 2 °C.

2.7.4 Size measurement

The hydrodynamic mean diameter and the size distribution of the microemulsion were determined by dynamic light scattering, using a He-Ne laser (wavelength of 633 nm) and a detector angle of 90° in a Malvern Zetasizer (NANO ZS90, Malvern Instruments Limited, UK). For size distribution measurements, a dispersion of diluted microemulsion in milli-Q® water (1:100) was analyzed at 25 °C in a polystyrene cell. Cumulates analysis provides the characterization of a sample through the mean value (z-average) for the droplet size and polydispersity index (PdI).

2.7.5 Morphology of the microemulsion

Droplets morphology of selected copaiba oil/water-microemulsion was observed by transmission electron microscopy (TEM) using an electron microscope JEOL 1400 (JEOL Ltd, Tokyo, Japan), equipped with a high resolution CCD Gatan digital camera

(SC1000 Orius, France) and operated at 80kV. One drop of the diluted microemulsion in milli-Q® water (1:100) was placed on a carbon coated copper grid. One drop of 2% phosphotungstic acid was added to it. The superfluous marker on sample was wiped off by filter paper. Finally, the sample was air dried and observed in TEM at ambient temperature.

2.7.6 *Rheological behavior*

Rheological properties of the microemulsion were determined using a rotational rheometer AR-G2 (TA instruments, New Castle, USA). Measurements were performed with an aluminum cone/plate geometry with a diameter of 40 mm, an angle of 1° and a truncation gap of 28 µm. Samples were maintained at 37ºC using a Peltier plate. Analyses were carried out by gradually increasing the shear rate from 10^{-1} to 10^{3} s^{-1}, after 5 minutes of equilibrium time. Measurements were performed in triplicate.

2.8 Determination of copaiba oil content in the microemulsion

The copaiba oil content in the microemulsion was determined as follows. Briefly, 1 mL of the microemulsion loaded copaiba essential oil was centrifugated at speed of 8500 x g for 15 min (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) to eliminate the titanium residues that may have been released from the ultrasound tip. The supernatant was used to determination of the drug content in the microemulsion formulation. Thus, 20 µL were solubilized in 1 mL of ethyl acetate to extraction using ultrasonic bath (Elma Elmasonic S10H, Elma Hans Schmidbauer GmbH & Co. KG,

Singen, Germany) for 15 minutes. The solution was filtered using a 0.1 µm teflon filter (Merck Millipore, Billerica, MA, EUA). GC-FID method was used to measure the content of copaiba oil, in particular, β-Caryophyllene according the previous validation studies as described above (Xavier-Junior, Chapter I, 2015b).

2.9 Statistical analysis

All the experiments were conducted in triplicates. Means of two groups were compared using non-paired Student's t-tests. All values are expressed as their mean ± S.D. When comparing multiple groups, one way analysis of variance (ANOVA) was applied with the Tukey multiple comparison procedure. The statistical data were considered significant at $p < 0.05$

3. RESULTS AND DISCUSSION

3.1 Copaiba oil characterization

Figure 1 shows chromatograms given from the analysis of the resin and essential oil from *Copaifera langsdorffii* (Alencar, É. N. *et al.*, 2015; Xavier-Junior, Chapter I, 2015b). In agreement with previous work, the assays allowed to identify 20 components in the copaiba resin oil, which 10 were sesquiterpenes and the other half were diterpenes compounds. Copaiba essential oil showed 15 sesquiterpenes compounds. In this work, components with concentrations greater than 6.7 % were further considered as they appeared as the main components of the resin and essential oil which composition are

given in Table 2. Partial solubility parameters were calculated for these compounds as they were the main components composing copaiba oil.

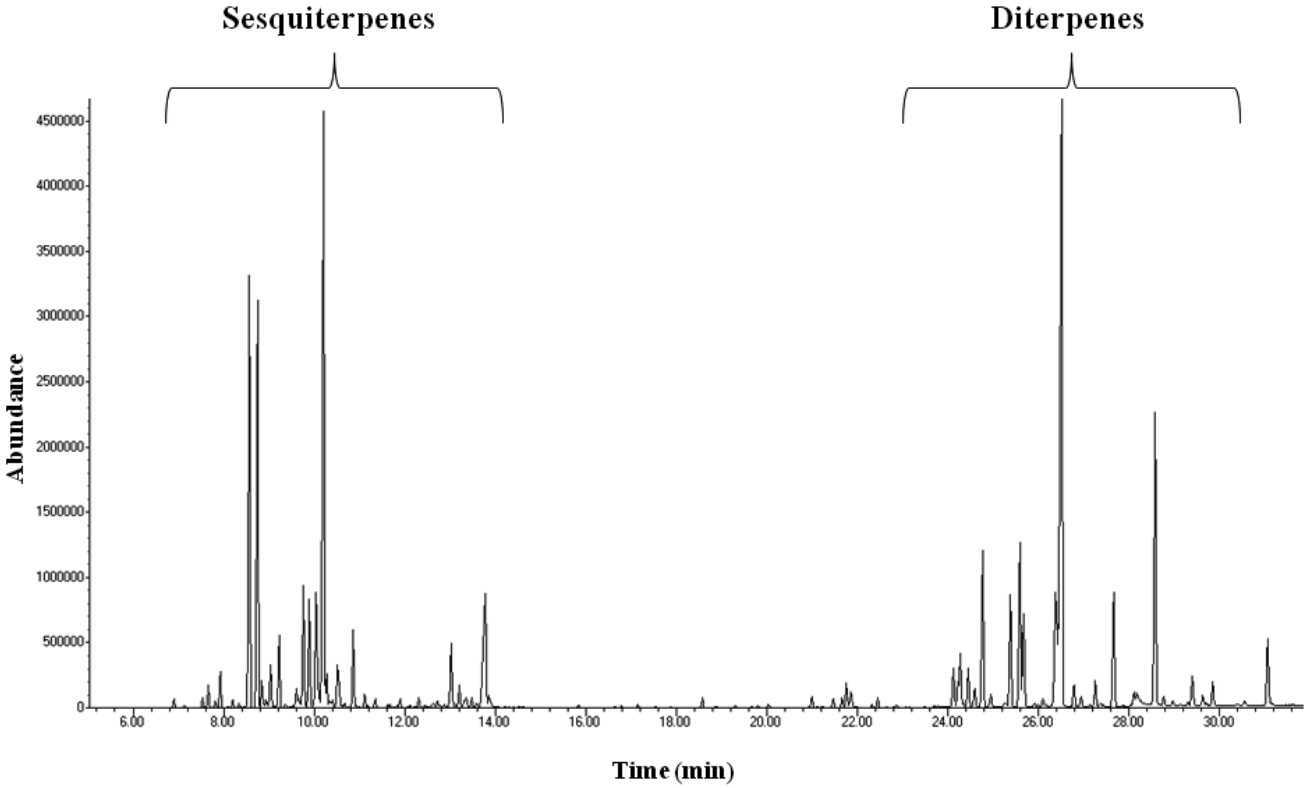

Figure 1- The copaiba resin oil chromatogram, with a typical sesquiterpenes and diterpenes compounds region. * Diterpenes compounds were obtained after methylation derivatization reaction.

Table 2 – Percentage of major compounds from Copaiba (*Copaifera langsdorffii*) resin and essential oils

Chemical Compound*	RT (min)	Resin oil (%)	Essential oil (%)
β-Caryophyllene	8.57	7.9	21.7
α-Bergamotene	8.75	7.1	20.5
β-Bisabolene	10.21	12.3	23.6
Copalic acid	26.51	15.6	-
Labd-8(20)-ene-15,18-dioic acid	28.58	6.7	-
Compounds concentration (<6.7 %)		43.3	32.0
Not detected compounds (%)		7.1	2.2
Total identified (%)		93.0	97.5

* compounds which composition were above 6.7 % of total components. RT (min): Retention time; (-) No detected.

3.2 Calculation of solubility parameters of oil components and of lipophilic parts of surfactants

Solubility parameters can be used to predict interactions between molecules and their miscibility and solubility (Long *et al.*, 2006). This parameter can be calculated from the contribution of cohesive energy of different chemical groups composing the chemical structure of a component (Hansen, C.M. , 1967). Regarding miscibility and solubility of

chemicals, a general principle based on like dissolves in like can be used to identify pairs of compounds that are miscible or appropriate solvents to dissolve a define molecule. Basis of this approach which is widely used in formulation is to match solubility parameters shown by each component (Greenhalgh *et al.*, 1999; Verheyen *et al.*, 2001). Formulating O/W-microemulsions including copaiba oil as the dispersed phase, the aim of our work was to incorporate a high volume fraction of copaiba oil using a minimum amount of surfactant. Success in formulation of inverse microemulsions (W/O) with high volume ratio (up to 40%) of the dispersed phase and stabilized with only few percent of surfactant (5%) were obtained following a rational choice of the nature of the surfactants based on their miscibility predicted from their solubility parameters . While successful to formulate W/O microemulsion but not yet applied to the formulation of O/W-microemulsion to our knowledge, the approach was applied to the formulation of O/W-microemulsions in the present work. Thus, Figure 2 showed the chemical structure of surfactants and main components found in copaiba resin and essential oils. The molecules were shown highlighting their lipophilic and hydrophilic parts respectively. The composition in chemical groups of the lipophilic part of each molecule was summarized in Table 3 where results of the calculation of partial (δ_p, δ_d, δ_h) and total (δ_t) solubility parameters of the main copaiba oils components and of the lipophilic part of the surfactants were reported.

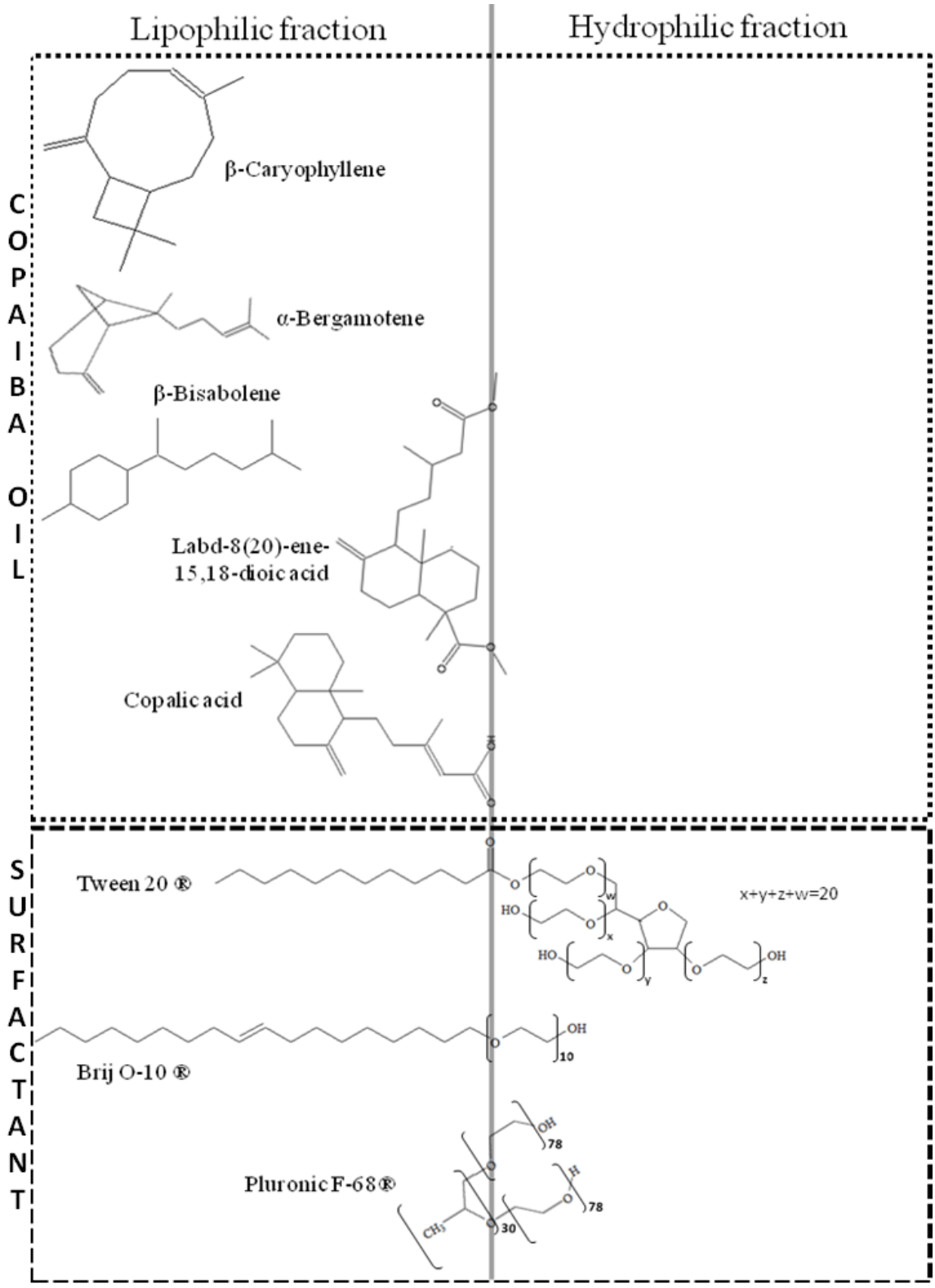

Figure 2- Chemical structure of the main compounds found in copaiba resin and essential oils showing their lipophilic and hydrophilic parts.

Table 3- Solubility parameters ($cal^{1/2}cm^{-3/2}$) and required Hydrophilic-Lipophilic Balance (HLB_0) of the main compounds from copaiba resin and essential oils and lipophilic chains of surfactants

Compound ($cal^{1/2}/cm^{3/2}$)	**Chemical contribution**	δ_d	δ_p	δ_h	δ_t	HLB_0	**HLB**
α-Bergamotene	$6(-CH_2) + 1(-CH_3) + 1(-CH=) + 2(-C) + 2(-CH) + 1\ (C(CH_3)_2)$	9.3	0.0	0.0	9.3	13.8	
β-Caryophyllene	$6(-CH_2) + 3(-CH_3) + 1(-CH=) + 3(-C) + 2(-CH)$	9.8	0.0	0.0	9.8	14.2	
β-Bisabolene	$7(-CH_2) + 2(-CH_3) + 3(-CH=) + 1\ (-C(CH_3)_2)$	7.2	0.0	0.0	7.2	11.4	
Copalic acid	$8(-CH_2) + 2(-CH_3) + 1(-CH=) + 3(-C) + 2(-CH) + 1\ C(CH_3)_2 + 1(-COOH)$	9.6	0.9	4.7	10.7	14.3	
Labd-8(20)-ene-15,18-dioic acid	$9(-CH_2) + 3(-CH_3) + 2(-C) + 3(-CH) + 2(-COO-)$	9.3	1.4	3.7	10.1	14.0	
Tween 20®	$10(-CH_2) + 1(-CH_3) + 1(-COO-)$	8.1	1.1	2.8	8.6		17.0
Brij-O10®	$15(-CH_2) + 1(-CH_3) + 2(-C=) + 1\ (-O-)$	8.1	0.3	1.1	8.2		12.9
Pluronic- F68®	$30(-CH_2) + 30(-CH_3) + 30(-CH) + 30(-O-)$	8.1	0.7	3.7	8.9		29.0*

* Result from the literature (Prakash, 2010)

Solubility parameters of the lipophilic parts of the surfactants shown in the Table 3 were quite well match with values calculated for the main components of copaiba oil. Therefore, the similar calculated total and partial solubility parameters between lipophilic chains of the surfactants and copaiba oil compounds were closely related. Total solubility parameters were calculated according to the percentage contribution of main compounds in the complex mixture from copaiba oils. Copaiba essential and resin oils showed the calculated solubility parameters of 8.7 and 9.4 $cal^{1/2}/cm^{3/2}$, respectively. A low calculated values of solubility parameters were obtained, due these samples presented a rich mixture of sesquiterpenes and diterpenes hydrocarbons. The HLB_0 of the main compounds presents in the copaiba oils were calculated based in the equation 3. All compounds blended in the copaiba essential and resin oil showed a HLB_0 of 13.1 and 13.5, respectively as calculated from equation 5. The HLB_0 calculated through solubility parameters approach was very close to HLB_0 experimental found to copaiba resin oil (14.8) (Xavier-Júnior *et al.*, 2012a).

The solubility parameters of the lipophilic chains of each surfactant used to oral application were calculated. Tween 20®, Brij O10® and Pluronic F-68® showed the total solubility parameter calculated of 8.6, 8.2 and 8.9 $cal^{1/2}/cm^{3/2}$, respectively. These surfactants showed the best chemical match with the oil component comparing each partial solubility parameters. Indeed, the chemical similarity determined between the lipophilic tail of the surfactant and the terpenes hydrocarbon group on the copaiba oil was taken as the key factor for the formation of optimized microemulsions optimizing the miscibility of the components. The HLB of the Tween 20® and Brij O10® were calculated based in the equation 4, being quite consistent with the results provided by the supplier. The HLB of the Pluronic F-68® was provided from the literature, due the Griffin's equation is applied to surfactant which HLB less than 20. To produce the

microemulsion, the surfactants were mixed together at a composition where the HLB of the surfactant blend where identical then the required HLB of copaiba oil. The corresponding composition of the surfactants blends was calculated from equation 5 using the HLB of the each surfactant and the HLB_0 value found for copaiba oils. Thus, the selects blends of surfactant were composed of Tween 20®:Brij O10® $_{(w/w)}$ (4.2:95.8) and PluronicF-68®: Brij O10®$_{(w/w)}$ (1.1:98.9) to copaiba essential oil; and Tween 20®:Brij O10®$_{(w/w)}$ (13.4:86.6) and PluronicF-68®: Brij O10® $_{(w/w)}$ (3.4:96.6) to copaiba resin oil. Solubility parameters and HLB_0 determinations were assumed to show the best compromise to stabilize droplets of copaiba oil in the internal phase of the O/W-microemulsions.

3.3 Development of microemulsions

A high volume fraction of copaiba oil and a low concentration of surfactant were determined as the goal of this work. Therefore, the copaiba oils and milli-Q® water were placed in test tube at 25:75$_{(w/w)}$ or 15:85 $_{(w/w)}$ ratios corresponding as starting compositions. These mixtures were then titrated with surfactant blends and with pure surfactants, sonicated and analyzed. The maximal concentration of surfactant added in the system corresponded to 13.7 % (i.e. oil/surfactant ratio 1.43) as above such a quite high concentration of surfactant it was considered that the formulations will be out of the range of the acceptable specifications defined for this work. Figure 3 showed regions (in grey) where microemulsions with copaiba essential and resin oils were formed for different concentrations of surfactants. The copaiba resin oil was not able to form stable microemulsion at high volume fraction of the oil and with a low concentration of surfactant. The presence of high molecular weight acid resinous

compounds in the complex mixture of the raw copaiba oil may interfere with surfactants to form a stable interface allowing the obtaining of microemulsion with a reasonable concentration of surfactant. In contrast, the copaiba essential oil formed microemulsions over a large range of surfactant concentrations and considering the two blends of surfactant selected above. Microemulsions could form with concentrations of surfactant as low as 13.7% while the content in oil in the microemulsion remained high 19.6 %. Main compounds found in the essential oils were purified from heavy resinous components. The approach applied to select surfactants based on the chemical pairing between surfactants and oil component led to formulations which compositions complied with our specifications. It can be assumed that the purity of the sesquiterpenes in the essential oil contributed to the success of the method of chemical paring based on the match of the solubility parameters between the oil components and surfactants.

Composition of the starting system (Oil/water Ratio)	**Copaiba resin oil/surfactant Ratio**							
	1.5	**1.3**	**1.0**	**0.7**	**0.6**	**0.5**	**0.4**	**Surfactant blend**
15:85								Brij O10
15:85								Tween 20: Brij O10 (13.4:86.6)
25:75								Tween 20: Brij O10 (13.4:86.6)
15:85								Tween 20
15:85								Pluronic F-68: Brij O10 (3.4:96.6)
25:75								Pluronic F-68: Brij O10 (3.4:96.6)
15:85								Pluronic F-68

Composition of the starting system (Oil/water Ratio)	**Copaiba essential oil/surfactant Ratio**							
	1.5	**1.3**	**1.0**	**0.7**	**0.6**	**0.5**	**0.4**	**Surfactant blend**
15:85								Brij O10
15:85								Tween 20: Brij O10 (4.2:95.8)
25:75								Tween 20: Brij O10 (4.2:95.8)
15:85								Tween 20
15:85								Pluronic F-68: Brij O10 (1.1:98.9)
25:75								Pluronic F-68: Brij O10 (1.1:98.9)
15:85								Pluronic F-68

Figure 3- Formations of microemulsion systems with copaiba resin and essential oil at different surfactants blends ratios and copaiba oil/water ratios. Grey indicated the obtaining of transparent system.

Figure 4 show a macroscopic view of the microemulsions formulated with copaiba essential oil and blends of either Pluronic F-68 ®: Brij O10 ® and Tween 20 ®: Brij O10 ®. They appeared homogeneous and showed the characteristic tyndall effect of colloidal dispersions. They were stable and isotropic when observed under polarized light microscopy. The true microemulsion are isotropic materials, i.e, the sample does not rotate the plane of light polarization, because different crystallographic axes of the material have constant refractive indices; therefore, the light ray propagates with the same speed in all directions (Boonme *et al.*, 2006b; Polizelli *et al.*, 2006).

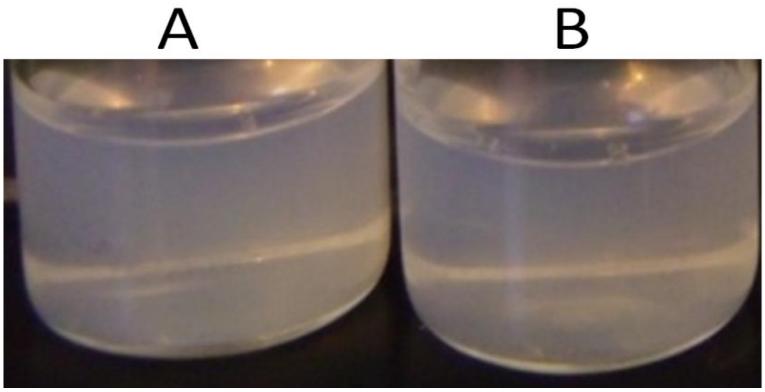

Figure 4: Macroscopic aspect of microemulsions formulated with copaiba essential oil and a blend of Pluronic F-68 ®: Brij O10 ® at 1.1:98.9 ratio (A) and with a blend of Tween 20 ®: Brij O10 ® at 4.2:95.8 ratio (B). The oil content and surfactant content of the both microemulsions were 19.6 and 13.7 %, respectively.

At 25°C, the light transmittance of the two microemulsions was 72 and 55 % for the microemulsion Pluronic F-68 ®: Brij O10 ® (1.1:98.9) and Tween 20 ®: Brij O10 ® (4.2:95.8), respectively consistently with their tyndall effect. By heating the microemulsions up to 45 °C, they became totally transparent above 40 °C (Figure 5). The systems remained within the microemulsion domain by increasing the temperature.

The fact the microemulsions became more transparent can be explained by a decrease in size of the microemulsion droplets thanks to an improvement of the structure of the interface where the lipophilic moiety of the surfactant interact with the components of the oil at the oil droplet surface.

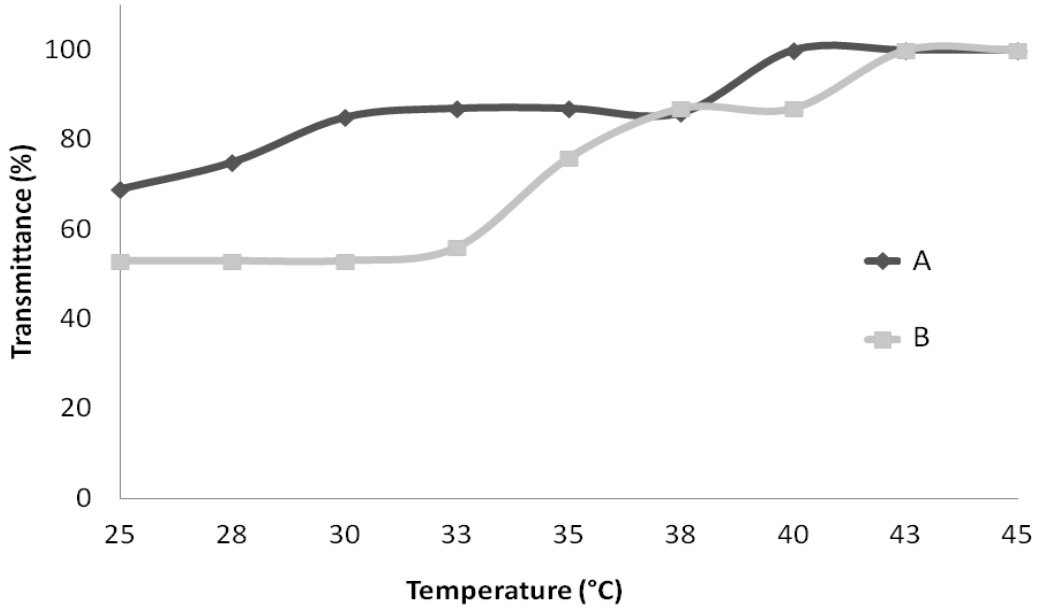

Figure 5: Temperature effect on the light transmittance of the microemulsion composed of copaiba essential oil 19.6 %, Pluronic F-68 ®: Brij O10 ® mixtures at 1.1:98.9 ratio 13.7 %, 66.7% of water (A), and copaiba essential oil 19.6 %; Tween 20 ®: Brij O10 ® mixtures at 4.2:95.8 ratio 13.7%, Water of 66.7 % (B).

The results of droplet size measurement, PdI and pH of the formulations were summarized in the Table 5. Microemulsion formulated with Pluronic F-68®: Brij O10® mixtures showed lower mean diameter and PdI than the formulation with Tween 20 ®: Brij O10 ®. The size distribution with a PdI of less than 0.25 indicates a narrow size distribution of the microemulsion and consequently a homogenous monomodal

distribution. After one week, the size of the microemulsion formulated with the Tween 20 ®: Brij O10 ® was increased.

Table 4- The droplet size, PdI and pH of the microemulsions formulated with copaiba essential oil and a blend of Pluronic F-68 ®: Brij O10 ® at 1.1:98.9 ratio (A) and with a blend of Tween 20 ®: Brij O10 ® at 4.2:95.8 ratio (B). The oil content and surfactant content of the both microemulsions were 19.6 and 13.7 %, respectively.

Microemulsion	Characteristic	Size (nm)	PdI	pH
A	Translucent or tyndall effect, homogeneous and isotropic	42 ± 0.5	0.13 ± 0.01	6.5 ± 0.4
B	Translucent or tyndall effect, homogeneous and isotropic	95 ± 10	0.31 ± 0.03	6.0 ± 0.5

TEM analysis was performed in order to determine the microstructure of microemulsion loaded copaiba essential oil formulated with Pluronic F-68 ®: Brij O10 ® (Figure 6). The microemulsion showed a spherical shape and uniform droplet size with droplets size about 45 nm that confirm the similar size obtained by dynamic light scattering analysis. This results corroborates with others studies than identified disperses and spherical micro-droplets using TEM analysis (Poullain-Termeau *et al.*, 2008; Hu *et al.*, 2011; Tian *et al.*, 2012).

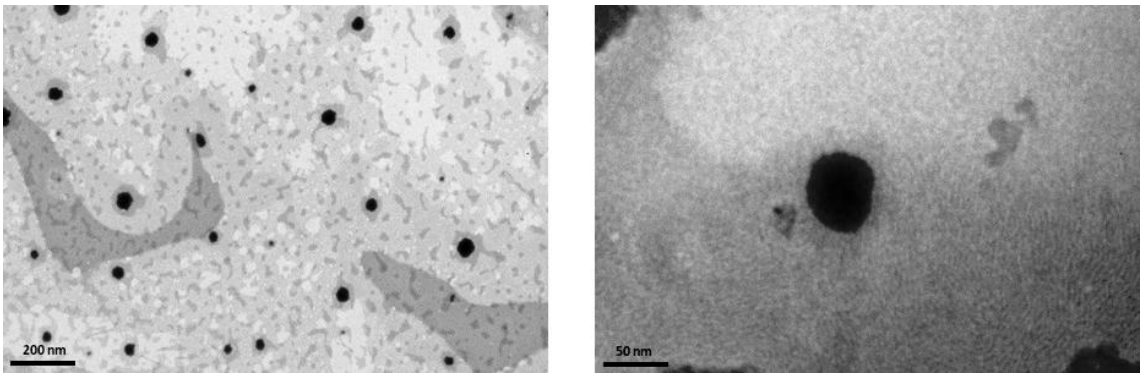

Figure 6-TEM images of the microemulsion loaded copaiba essential oil formulated with Pluronic F-68 ®: Brij O10 ® (scale bar= 200 and 50 nm, respectively)

Rheology behavior is a fundamental approach to provide useful information about the microemulsion structure and stability (Formariz *et al.*, 2010; Pal, 2011). The Figure 7 show the rheology behavior of the copaiba essential oil containing microemulsion formulated with Pluronic F-68 ®: Brij O10 ® at 1.1:98.9 ratio (A) and with Tween 20 ®: Brij O10® at 4.2:95.8 ratio (B) surfactants blends. Both formulations (A) and (B) were shear-thinning. Formulation (A) was shear-thinning from 1 to about 500 s^{-1}, and seems to reach the second Newtonian plateau above 500 s^{-1}. In contrast, formulation (B) was shear-thinning on the whole studied shear rate range. Between 0.1 and 10 s^{-1}, both formulations exhibited similar viscosity values. Above 10 s^{-1}, formulations (B) had higher viscosities values than formulation (A). Thus, the relatively low viscosity values may indicate that the microemulsion was composed of individual spherical droplets and non-anisometric aggregates (Moulik & Paul, 1998; Djordjevic *et al.*, 2004; Acharya & Hartley, 2012), confirming the spherical droplet obtained by TEM analysis.

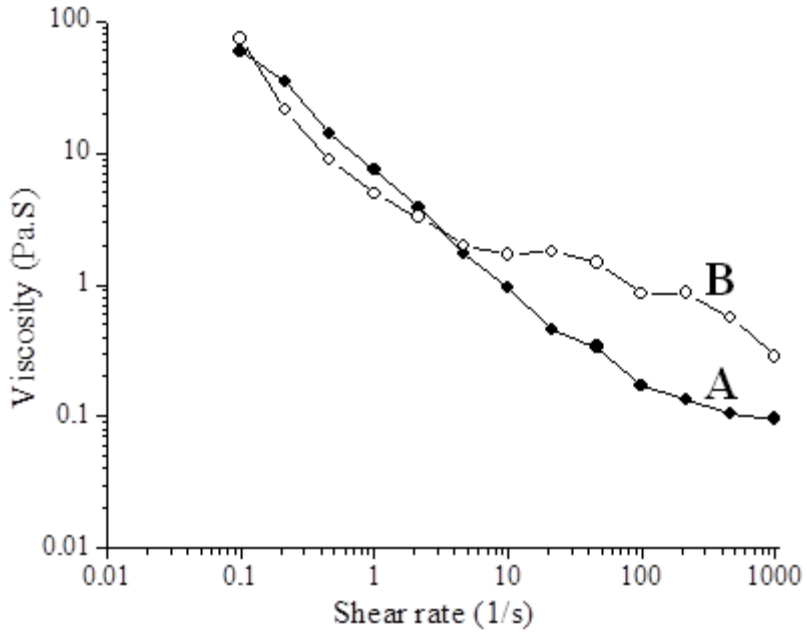

Figure 7- Flow curves of microemulsions formulated with copaiba essential oil and with a blend of Pluronic F-68 ®: Brij O10 ® at 1.1:98.9 ratio (A) and with a blend of Tween 20 ®: Brij O10 ® at 4.2:95.8 ratio (B). The oil content and surfactant content of both microemulsions were 19.6 and 13.7 %, respectively.

Figure 8 showed the comparison of gas chromatograms obtained after analysis of the copaiba essential oil and the microemulsion. The data indicated that there was no significant difference in the composition and compound concentration between the copaiba oil included in the microemulsion and copaiba essential oil itself (p>0.05). The precision of the method to quantify the copaiba oil was 2.4%, the differences in the area of the peaks were less than 2 %, indicating no significant differences between the copaiba essential oil and microemulsion dosages. It was also possible to determine that 94% of the compounds found in copaiba essential oil were also present in the microemulsion; the others 6% represented minor compounds not identified. The small

loss of compounds in the copaiba essential oil and the microemulsion was the same, indicating that the loss was due to the production process but not to the oil volatilization. The copaiba oil has a rich mixture of components which gives numerous therapeutic activities. One of the most important, whether in relation to its high concentration or interesting biological activities, is the β-caryophyllene. This sesquiterpenes showed synergistic effect in the anticancer therapy, including the membrane permeabilization, block of P-glycoprotein in drug-resistant cancer cells and lipid peroxidation activities increasing the anticancer activity of the drug. From the quantitative analysis of the chromatograms, it could be determined that the copaiba essential oil-loaded microemulsion which contain the highest volume fraction for the lower concentration in surfactant (13.7 % of Pluronic F-68 ®: Brij O10 ® blend) included a concentration of 3.8 $mg.mL^{-1}$ of β-caryophyllene.

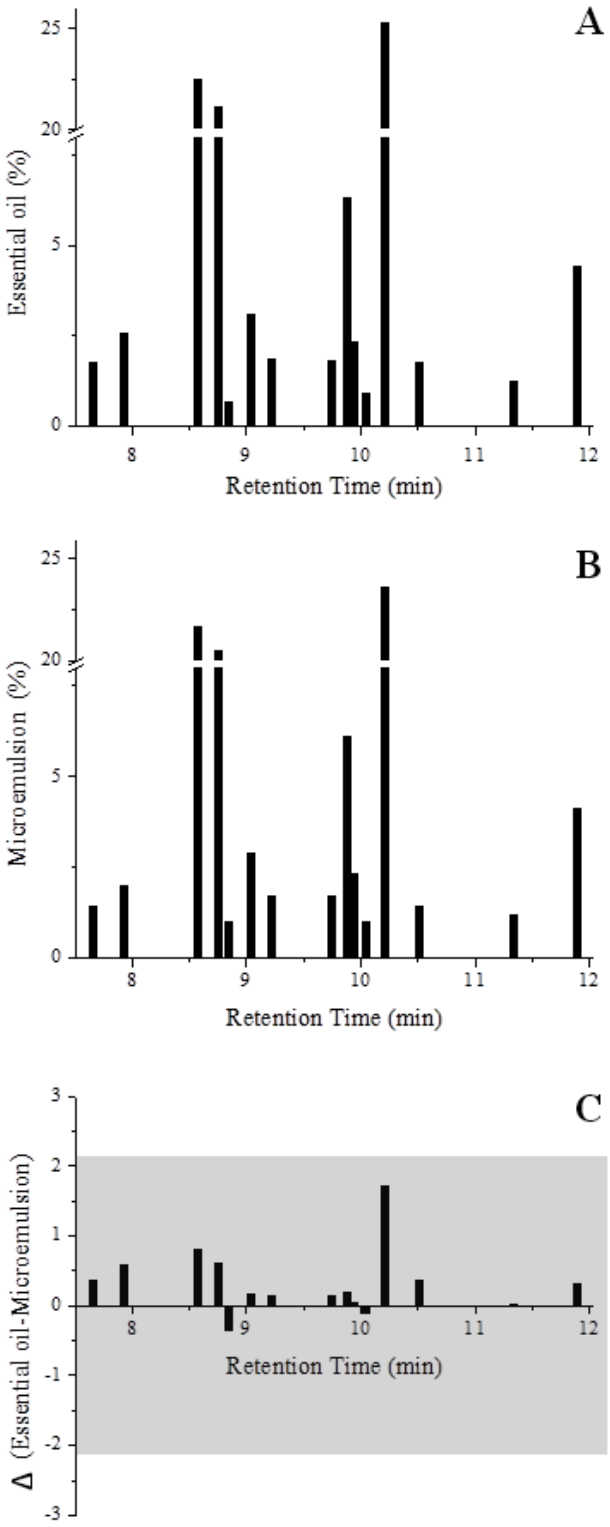

Figure 8- Comparison of chromatograms obtained by gas chromatography analysis of copaiba essential oil (A) and microemulsion prepared with copaiba essential oil (B) by GC-FID analysis. C is the percentage difference between A and B. The gray color represents the precision of the method to determination of copaiba oil compounds

The use de microemulsion has been growing in recent years. To develop such systems, infinity of different ratios, components and methods can be combined to formation of the O/W-microemulsion. In this work, it was possible to obtain a microemulsion containing a high volume fraction of natural oil and a low surfactant concentration compared with previous microemulsion developed in the literature (Figure 9). The major systems produced showed lower oil concentration dispersed in the microemulsion, and, in general, this oil is only used as promoter of the drug solubility (Figure 9A). Associated this fact, the high concentration of surfactant is utilized to stabilizer the dispersion of the droplets in the aqueous medium. Thus, a large part of the work produced in the literature presenting oil / surfactant ratio of less than 1, corresponding at large amount of surfactant required to stabilize the oil droplets (Figure 9B). In the present work, microemulsion with a oil/surfactant ratio above 1 could be formulated using a rational approach for the selection of surfactants which presented high chemical compatibility with the oil based on the analysis of solubility parameters of the different components. The microemulsions formulated in this work have an increased amount of natural therapeutic oil compared to the O/W-microemulsion described in the literature. Additionally, their surfactant contents was reduced which is very interesting because high concentrations of surfactants in microemulsions limits their *in vivo* use being generally responsible for toxicity.

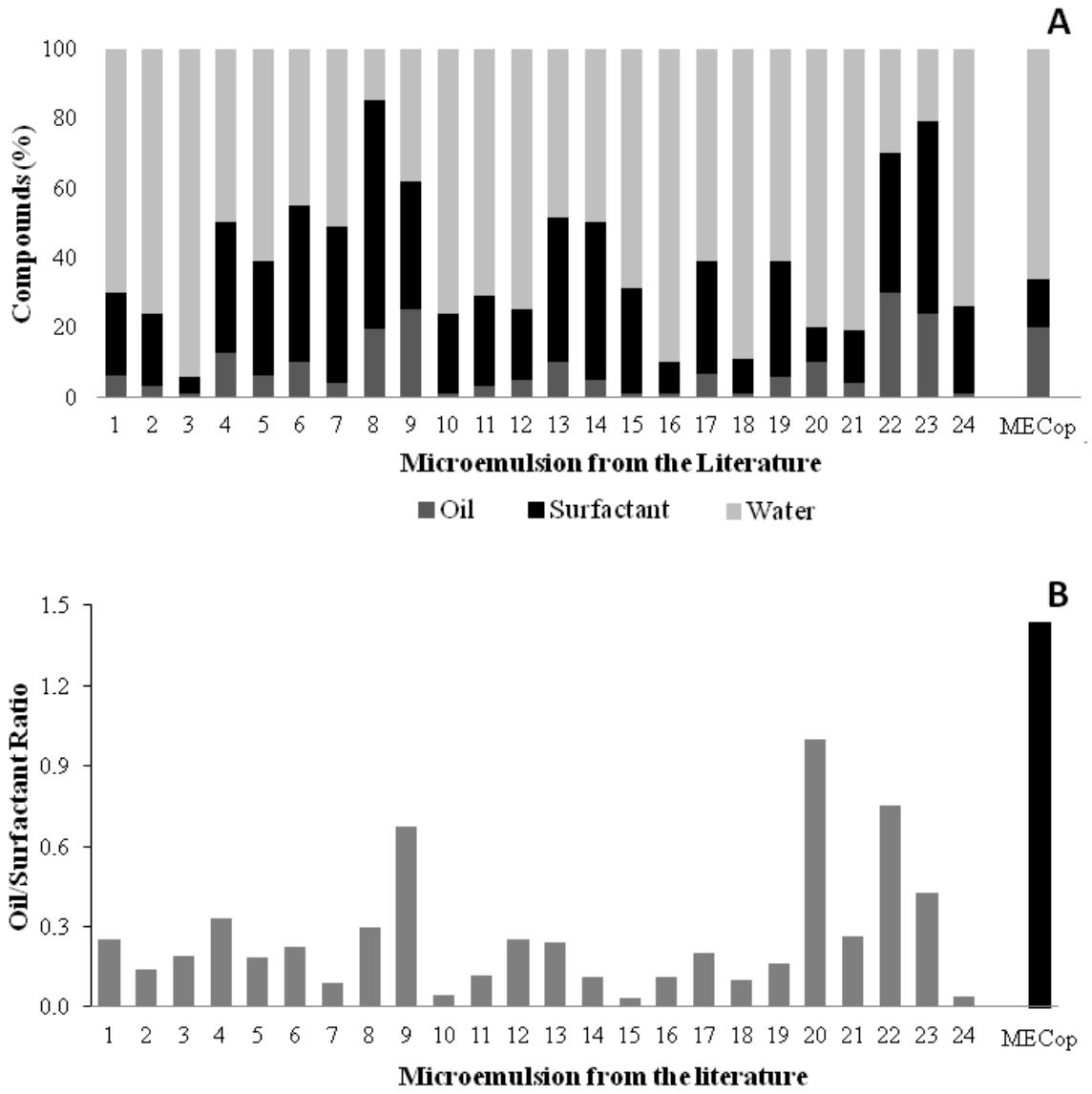

Figure 9- Comparison among the microemulsion containing copaiba oil obtained by solubility parameter approach (MECop) with other different microemulsion from the literature. A- represent the ratio of the aqueous phase/surfactant+cosurfactant/Oil phase composing the microemulsion, where medium gray, light gray and black colors represent the oil phase, the surfactant (and/or cosurfactant) and aqueous phase contributions (%) to microemulsion composition, respectively. B- show the ratio between the oil and surfactant (and/or cosurfactant) compounds, gray columns showed the data from the literature and the dark column denoted MECop showed the corresponding composition of the microemulsion produced in the present work ussing

Pluronic F-68 ®: Brij O10 ® at 1.1:98.9 ratio. 1 (Ghosh *et al.*, 2013), 2 (Teixeira *et al.*, 2007), 3 (Hamed *et al.*, 2012), 4 (Zhang *et al.*, 2008), 5 (Djordjevic *et al.*, 2004), 6 (Boonme *et al.*, 2006b), 7 (Surjyanarayan *et al.*, 2009), 8 (Borhade *et al.*, 2008), 9 (Hu *et al.*, 2011), 10 (Dantas, T. N. C. *et al.*, 2010), 11 (Yi *et al.*, 2012), 12 (Spernath *et al.*, 2002), 13 (Rao & Mcclements, 2011), 14 (Jha, S.K. *et al.*, 2011), 15 (Polizelli *et al.*, 2006), 16 (Tian *et al.*, 2012), 17 (Biresh & Shiv, 2011), 18 (Mrestani *et al.*, 2010), 19 (Solanki *et al.*, 2012), 20 (Pestana *et al.*, 2008b), 21 (Gao *et al.*, 1998), 22 (Acharya *et al.*, 2001), 23 (Agatonovic-Kustrin *et al.*, 2003), 24 (Zeng *et al.*, 2010).

4. CONCLUSION

The determination of the solubility parameter approach and HLB required were used to select surfactants blends which showed lipophilic portions of good chemical compatibility with the majority copaiba oil compounds. The use of mixtures of these surfactants allowed the microemulsions formulation with high volume fraction and low surfactant concentrations using the essential oil which is the purified fraction. This work valid the application of this predictive approach to development for directs O/W microemulsions formulations. The composition of the oil recovered in the microemulsion was identical to the starting oil. The pharmacological properties of the copaiba oil should be preserved in the microemulsion and may be used in synergy with other active ingredients incorporated in the microemulsions to anticancer activity for oral route.

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REFERENCES

ACHARYA, A., SANYAL, S. K. & MOULIK, S. P. 2001. Physicochemical investigations on microemulsification of eucalyptol and water in presence of polyoxyethylene (4) lauryl ether (Brij-30) and ethanol. *Int J Pharm*, 229, 1-2, 213-26.

ACHARYA, D. P. & HARTLEY, P. G. 2012. Progress in microemulsion characterization. *Curr Opin Colloid Interface Sci*, 17, 5, 274-280.

AGATONOVIC-KUSTRIN, S., GLASS, B. D., WISCH, M. H. & ALANY, R. G. 2003. Prediction of a stable microemulsion formulation for the oral delivery of a combination of antitubercular drugs using ANN methodology. *Pharm Res*, 20, 11, 1760-5.

ALENCAR, É. N., XAVIER-JÚNIOR, F. H., MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G. & EGITO, E. S. T. 2015. Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions. *J Nanosci Nanotechnol*, 15, 1, 880-888.

ARAYA, H., TOMITA, M. & HAYASHI, M. 2005. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. *Int J Pharm*, 305, 1-2, 61-74.

ATTAPHONG, C., DO, L. & SABATINI, D. A. 2012. Vegetable oil-based microemulsions using carboxylate-based extended surfactants and their potential as an alternative renewable biofuel. *Fuel*, 94, 1, 606-613.

BARTON, A. F. M. Handbook of solubility parameters and other cohesive parameters. New York: 1983.

BEERBOWER, A. & HILL, M. W. Detergents and emulsifiers. Allured Publishing Co, 1971.

BIRESH, K. S. & SHIV, S. H. 2011. Microemulsion drug delivery system for oral bioavailability enhancement of glipizide. *J Adv Pharm Technol Res*, 1, 4, 195-200.

BOONME, P., KRAUEL, K., GRAF, A., RADES, T. & JUNYAPRASERT, V. B. 2006b. Characterization of microemulsion structures in the pseudoternary phase diagram of isopropyl palmitate/water/Brij 97:1-butanol. *AAPS PharmSciTech*, 7, 2, E45.

BORHADE, V., NAIR, H. & HEGDE, D. 2008. Design and evaluation of self-microemulsifying drug delivery system (SMEDDS) of tacrolimus. *AAPS PharmSciTech*, 9, 1, 13-21.

COMELLI JÚNIOR, E., SKINOVSKI, J., SIGWALT, M. F., BRANCO, A. B., LUZ, S. R. & BAULÉ, C. D. P. 2010. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaíba (Copaifera Iangsdorfii) oil. *Acta Cir Bras*, 25, 362-367.

DANIELSSON, I. & LINDMAN, B. 1981. The definition of microemulsion. *Colloids Surf*, 3, 4, 391-392.

DANTAS, T. N. C., SILVA, H. S. R. C., DANTAS-NETO, A. A., MARCUCCI, M. C. & MACIEL, M. A. M. 2010. Development of a new propolis microemulsion system for topical applications. . *Rev bras farmacogn*, 20, 3, 368-375.

DJORDJEVIC, L., PRIMORAC, M., STUPAR, M. & KRAJISNIK, D. 2004. Characterization of caprylocaproyl macrogolglycerides based microemulsion drug delivery vehicles for an amphiphilic drug. *Int J Pharm*, 271, 1-2, 11-9.

FORMARIZ, T. P., CHIAVACCI, L. A., SCARPA, M. V., SILVA-JÚNIOR, A. A., EGITO, E. S. T., TERRUGI, C. H. B., FRANZINI, C. M., SARMENTO, V. H. V. & OLIVEIRA, A. G. 2010. Structure and viscoelastic behavior of pharmaceutical biocompatible anionic microemulsions containing the antitumoral drug compound doxorubicin. *Colloids Surf B Biointerfaces*, 77, 1, 47-53.

GAO, Z.-G., CHOI, H.-G., SHIN, H.-J., PARK, K.-M., LIM, S.-J., HWANG, K.-J. & KIM, C.-K. 1998. Physicochemical characterization and evaluation of a microemulsion system for oral delivery of cyclosporin A. *Int J Pharm*, 61, 1, 75-86.

GHOSH, V., SARANYA, S., MUKHERJEE, A. & CHANDRASEKARAN, N. 2013. Antibacterial microemulsion prevents sepsis and triggers healing of wound in wistar rats. *Colloids Surf B Biointerfaces*, 105, 152-157.

GIBAUD, S. & ATTIVI, D. 2012. Microemulsions for oral administration and their therapeutic applications. *Expert Opin Drug Deliv*, 9, 8, 937-51.

GOMES N, M., REZENDE C, M., FONTES, S. P., HOVELL, A. M., LANDGRAF, R. G., MATHEUS, M. E., PINTO A, C. & FERNANDES, P. D. 2008. Antineoplasic activity of Copaifera multijuga oil and fractions against ascitic and solid Ehrlich tumor. *J Ethnopharmacol*, 119, 1, 179-84.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-492.

GREENHALGH, D. J., WILLIAMS, A. C., TIMMINS, P. & YORK, P. 1999. Solubility parameters as predictors of miscibility in solid dispersions. *J Pharm Sci*, 88, 11, 1182-90.

GRIFFIN, W. C. 1949a. Classification of Surface-Active Agents by 'HLB'. *J Soc Cosmet Chem*, 1, 5, 311-26.

_____. 1954. Calculation of HLB Values of Non-Ionic Surfactants. *J Soc Cosmet Chem*, 5, 4, 249-56.

HAMED, S. F., SADEK, Z. & EDRIS, A. 2012. Antioxidant and antimicrobial activities of clove bud essential oil and eugenol nanoparticles in alcohol-free microemulsion. *J Oleo Sci*, 61, 11, 641-8.

HANSEN, C. M. 1967. The three dimensional solubility parameter - key to paint component affinities ii. - dyes, emulsifiers, mutual solubility and compatibility, and pigments. *J Paint Technol*, 39, 511, 505-510.

HE, C. X., GAO, J.-Q. & GAO, J. Q. 2010. Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs. *Expert Opin Drug Deliv*, 7, 4, 445-60.

HILDEBRAND, J. H., PRAUSNITZ, J. M. & SCOTT, R. L. Regular and related solutions. New York: Van Nostrand Reinhold, 1970.

HOAR, T. P. & SCHULMAN, J. H. 1943. Transparent water-in-oil dispersions: the oleopathic hydro-micelle. *Nature*, 152, 102-104.

HU, L., WU, H., NIU, F., YAN, C., YANG, X. & JIA, Y. 2011. Design of fenofibrate microemulsion for improved bioavailability. *Int J Pharm*, 420, 2, 251-5.

JHA, S. K., KARKI, R., VENKATESH, D. P. & GEETHALAKSHAMI, A. 2011. Formulation development and characterization of microemulsion drug delivery systems containing antiulcer drug. *Int J Drug Dev Res*, 3, 4, 336-343.

LAWRENCE, M. J. & REES, G. D. 2012. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 64, 175-193.

LEE, M. J., LEE, M. H. & SHIM, C. K. 1995. Inverse targeting of drugs to reticuloendothelial system-rich organs by lipid microemulsion emulsified with poloxamer-338. *Inter J Pharm*, 113, 2, 175-187.

LONG, C., ZHANG, L. & QIAN, Y. 2006. Preparation and Crystal Modification of Ibuprofen-Loaded Solid Lipid Microparticles. *Chin J Chem Eng*, 14, 4, 518-525.

MCCLEMENTS, D. J. 2012. Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, 8, 1719-1729

MENDONÇA, D. E. & ONOFRE, S. B. 2009b. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev. Bras. Farmacogn*, 19, 577-581.

MOULIK, S. P. & PAUL, B. K. 1998. Structure, dynamics and transport properties of microemulsions. *Adv Colloid Interface Sci*, 78, 2, 99-195.

MRESTANI, Y., BEHBOOD, L., HARTL, A. & NEUBERT, R. H. 2010. Microemulsion and mixed micelle for oral administration as new drug formulations for highly hydrophilic drugs. *Eur J Pharm Biopharm*, 74, 2, 219-22.

MUZAFFAR, F., SINGH, U. K. & CHAUHAN, L. 2013. Review on microemulsion as futuristic drug delivery. *Int J Pharm Pharm Sci*, 5, 39-53
PAL, R. 2011. Rheology of simple and multiple emulsions. *Curr Opin Colloid Interface Sci*, 16, 1, 41-60.

PESTANA, K. C., FORMARIZ, T. P., FRANZINI, C. M., SARMENTO, V. H., CHIAVACCI, L. A., SCARPA, M. V., EGITO, E. S. & OLIVEIRA, A. G. 2008b. Oil-in-water lecithin-based microemulsions as a potential delivery system for amphotericin B. *Colloids Surf B Biointerfaces*, 66, 2, 253-9.

POLIZELLI, M. A., TELIS, V. R. N., AMARAL, L. Q. & FEITOSA, E. 2006. Formation and characterization of soy bean oil/surfactant/water microemulsions. *Colloids Surf A*, 281, 1–3, 230-236.

POULLAIN-TERMEAU, S., CRAUSTE-MANCIET, S., BROSSARD, D., MUHAMED, S., NICOLAOS, G., FARINOTTI, R., BARTHELEMY, C., ROBERT, H. & ODOU, P. 2008. Effect of oil-in-water submicron emulsion surface charge on oral absorption of a poorly water-soluble drug in rats. *Drug Deliv*, 15, 8, 503-14.

RAO, J. & MCCLEMENTS, D. J. 2011. Formation of flavor oil microemulsions, nanoemulsions and emulsions: influence of composition and preparation method. *J Agric Food Chem*, 59, 9, 5026-35.

SAPRA, B., BHANDARI, S., SOOD, J., JINDAL, M., TIWARY, A. & TIWARY, A. 2013. A critical appraisal of microemulsions for drug delivery: part I. *Ther Deliv*, 4, 12, 1547-64.

SOLANKI, S. S., SARKAR, B. & DHANWANI, R. K. 2012. Microemulsion drug delivery system: for bioavailability enhancement of ampelopsin. *ISRN Pharm*, 2012, 108164.

SOUZA, A. B., MARTINS, C. H., SOUZA, M. G., FURTADO, N. A., HELENO, V. C., DE SOUSA, J. P., ROCHA, E. M., BASTOS, J. K., CUNHA, W. R., VENEZIANI, R. C. & AMBROSIO, S. R. 2011. Antimicrobial activity of terpenoids from Copaifera langsdorffii Desf. against cariogenic bacteria. *Phytother Res*, 25, 2, 215-20.

SPERNATH, A., YAGHMUR, A., ASERIN, A., HOFFMAN, R. E. & GARTI, N. 2002. Food-grade microemulsions based on nonionic emulsifiers: media to enhance lycopene solubilization. *J Agric Food Chem*, 50, 23, 6917-22.

SURJYANARAYAN, M., SNIGDHA, S. M. & NAAZNEEN, S. 2009. Design and development of Saquinavir microemulsion for the oral bioavailabili ty enhancement. *Int J PharmTech Res*, 1, 1442-48.

TEIXEIRA, P. C., LEITE, G. M., DOMINGUES, R. J., SILVA, J., GIBBS, P. A. & FERREIRA, J. P. 2007. Antimicrobial effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. *Int J Food Microbiol*, 118, 1, 15-9.

THIMMASETTY, J., SUBRAHMANYAM, C. V. S., VISHWANATH, B. A. & SATHESH BABU, P. R. 2009. Solubility parameter estimation of celecoxib by current methods. *Asian J Res Chem*, 2, 2

TIAN, Q., REN, F., XU, Z., XIE, Y. & ZHANG, S. 2012. Preparation of high solubilizable microemulsion of naproxen and its solubilization mechanism. *Int J Pharm*, 426, 1-2, 202-10.

VERHEYEN, S., AUGUSTIJNS, P., KINGET, R. & VAN DEN MOOTER, G. 2001. Determination of partial solubility parameters of five benzodiazepines in individual solvents. *Int J Pharm*, 228, 1-2, 199-207.

XAVIER-JUNIOR, F. H., MACIUK, A., MORAIS, A. R. V., ALENCAR, E. N., REHDER, V. L. G., EGITO, E. S. T., VAUTHIER, C. Chapter I, 2015b. Development of gas-chromatography method for the analysis of copaiba oil. *Article in preparation*

XAVIER-JÚNIOR, F. H., SILVA, K. G. H., FARIAS, I. E. G., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., OLIVEIRA, A. G. & EGITO, E. S. T. 2012a. Prospective study for the development of emulsion systems containing natural oil products. *J Drug Del Sci Tech*, 22, 4, 367-372.

YI, C., ZHONG, H., TONG, S., CAO, X., FIREMPONG, C. K., LIU, H., FU, M., YANG, Y., FENG, Y., ZHANG, H., XU, X. & YU, J. 2012. Enhanced oral bioavailability of a sterol-loaded microemulsion formulation of Flammulina velutipes, a potential antitumor drug. *Int J Nanomedicine*, 7, 5067-78.

ZENG, Z., ZHOU, G., WANG, X., HUANG, E. Z., ZHAN, X., LIU, J., WANG, S., WANG, A., LI, H., PEI, X. & XIE, T. 2010. Preparation, characterization and relative bioavailability of oral elemene o/w microemulsion. *Int J Nanomedicine*, 5, 567-72.

ZHANG, H., SHEN, Y., BAO, Y., HE, Y., FENG, F. & ZHENG, X. 2008. Characterization and synergistic antimicrobial activities of food-grade dilution-stable microemulsions against Bacillus subtilis. *Food Res Int*, 41, 5, 495-499.

Chapter VI

Paclitaxel-loaded copaiba oil in water microemulsion as oral drug delivery systems: preparation and evaluation of mucoadhesion.

Le but de ce travail était de préparer une microémulsion chargée en paclitaxel et d'en évaluer le potentiel comme les systèmes de délivrance de ce principe actif anticancéreux pour son administration orale . Les formulations de microémulsion ont été élaborés sur la base des études précédentes en utilisant les approches des paramètres de solubilité pour produire une microémulsion avec un volume élevé d'huile dispersé pour une faible concentration des tensioactifs. Le paclitaxel a été incorporé dans l'huile copaïba / eau microémulsion par sonication à 25 °C. L'évaluation de l'efficacité d'encapsulation et le taux de la charge de médicament ont été évaluées en utilisant la méthode HPLC validée au cours d'un travail précédent (Chapitre 2). La composition de la microémulsion retenue est donnée dans le rapport huile essentielle de copaïba/ Pluronic F-68®/ Brij-O10 ® / eau milli-Q® de 19,6: 0,15: 13,55: 66,7, respectivement. Le systeme d'apparence homogène et transparent, présentait des caractéristiques isotropes. La taille des gouttelettes était de 51 ± 1,2 nm, avec une distribution unimodale alors que la structure était inchangée par rapport à des taux de dilution de 0,4 à 50 %. La microémulsion ont été rhéofluidifiantes. L'incorporation maximale du paclitaxel dans le système était de 0,37 $mg.mL^{-1}$ correspondant à 1,89 mg de paclitaxel par g de l'huile de copaïba, ce qui est équivalent une augmentation de 36 fois par rapport à la solubilité du paclitaxel dans l'huile essentiel de copaïba. Une microémulsion radiomarqué a été préparée en utilisant du [3H] -paclitaxel marqué au tritium en vue d'une étude visant à déterminer des propriétés de mucoadhésion. L'incorporation du paclitaxel radiomarqué dans la microémulsion n'a pas modifié les caractéristiques physico-chimiques de la microémulsion. La microémulsion a été marqué avec une radioactivité spécifique de 232 $kBq.mL^{-1}$ de microémulsion telle que déterminée par scintillation liquide. Le test de mucoadhésion *ex-vivo* a été réalisée sur de la muqueuse intestinale de rat fraichement prélevée et montée dans des chambres d'Ussing. L'association maximale du paclitaxel

retrouvé sur la muqueuse a été de 4,4 ± 0,7% de la quantité initiale apportée par la microémulsion radioactive, ce qui correspondant à une quantité de 9,25 µg de paclitaxel qui se fixe par cm² de muqueuse intestinale.

Mots-clés: Microémulsion, paclitaxel, huile de copaïba, mucoadhésion, administration de médicaments par voie orale.

PACLITAXEL-LOADED COPAIBA OIL IN WATER MICROEMULSION AS ORAL DRUG DELIVERY SYSTEMS: PREPARATION AND EVALUATION OF MUCOADHESION.

Xavier-Junior, F.H.[1,2], Huang N.[2], Morais, A.R.V. [1,2], Alencar, E.N.[1], Gueutin, C. [1], Chacun, H.[2], Egito, E.S.T.[1], Vauthier, C.[2]*

[1] UFRN, DFAR, Laboratório de Sistemas Dispersos (LaSiD), Natal – RN, Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

*Corresponding author: Christine Vauthier

Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

christine.vauthier@u-psud.fr

ABSTRACT

The aim of this work was to prepare and evaluate paclitaxel-loaded copaiba oil in water microemulsion as delivery systems for oral administration of this anticancer drug. The microemulsion formulations were developed based on previous studies using solubility parameters approach to produce a microemulsion with a high oil volume and using a low concentration in surfactant. Paclitaxel was incorporated in the copaiba oil/water microemulsion by sonication at 25 °C. Evaluation of the encapsulation efficiency and drug loading was assessed using a validated HPLC method. A microemulsion having a composition of copaiba essential oil, Pluronic F-68®, Brij O10 ® and milli-Q ® water at 19.6: 0.15: 13:55: 66.7(w/w) ratios, respectively was, homogeneous, transparent and showed isotropic characteristics. The droplet size was 51 ± 1.2 nm with unimodal distribution and the structure was unchanged from dilution ratios ranging from 0.4 to 50%. The systems were shear-thinning. The maximum incorporation of the paclitaxel in the system was 0.37 $mg.mL^{-1}$ corresponding to 1.89 mg of paclitaxel per g of copaiba oil. Radiolabeled microemulsion was developed using [3H]-paclitaxel to mucoadhesion evaluation. The microemulsion showed the similar characteristics of the nonradioactive system with specific radioactivity of 232 kBq per mL of microemulsion as determined by liquid scintillation. Ex-vivo mucoadhesion test was performed in excised rat intestinal mucosa mounted in Ussing Chambers. The maximum association was 4.4 ± 0.7 % of the initial amount of [3H]-paclitaxel-loaded microemulsion, corresponding at 92.5 mg of paclitaxel by m^2 of the intestinal mucosa.

Keywords: Microemulsion, paclitaxel, copaiba oil, solubility parameters, mucoadhesion, oral drug delivery.

1. INTRODUCTION

Nanomedicine has become one of the most promising pathways for developing effective targeted therapies with particular impact in oncology (Duncan, 2004; Engineering., 2004; Kateb *et al.*, 2011). In recent years, several efforts have been made in order to develop anticancer drug delivery systems for oral intake. Paclitaxel has been used as an anticancer agent due to its inhibitory effect of cellular growth by stabilizing the microtubule assembly, causing the death of the cell by disrupting the normal tubule dynamics required for cell division and vital interphase process (Schiff *et al.*, 1979; Hamel, Del Campo, *et al.*, 1981; Horwitz, 1992). This drug is particularly active against primary epithelial ovarian carcinoma, breast cancer, colon, head, non-small cell lung cancer, and AIDS related Kaposi's sarcoma (Forastiere, 1994; Rowinsky & Donehower, 1995). However, it has a poor oral bioavailability partly due to its poor solubility in aqueous medium and to its high metabolization in the gastrointestinal epithelial cells (Adams *et al.*, 1993; Singla *et al.*, 2002; Kasim *et al.*, 2004).

In order to overcome this problem, the reformulation of the paclitaxel in systems that can enhance its solubility and permeability and being able to decrease its adverse effects, has been the goal of the new drug-based anticancer therapy (Kawasaki & Player, 2005). Amongst various drug delivery systems, microemulsions are considered as ideal alternatives for the oral delivery of low water solubility compounds (Constantinides, 1995; Kawakami, Yoshikawa, Hayashi, *et al.*, 2002; Kawakami, Yoshikawa, Moroto, *et al.*, 2002; Takahashi *et al.*, 2002; Araya *et al.*, 2005). Microemulsions are clear, thermodynamically stable, isotropic liquid mixtures of oil, water and surfactant, normally used in combination with a co-surfactant. Microemulsions are also characterized by a low viscosity and ultralow interfacial

tension. They may form a number of different structures (oil-in-water (O/W), water-in-oil (W/O) droplets and bicontinuous) (Bhargava *et al.*, 1987). For microemulsions occurring as dispersions of droplets dispersed in a continuous phase, droplet size generally ranged between 20 and 200 nm (Hoar, T. P. & Schulman, J.H., 1943; Schulman *et al.*, 1959; Danielsson, I. & Lindman, B., 1981; Lawrence & Rees, 2000; Talegaonkar *et al.*, 2008). The major advantages of these systems include high potential of drug solubilization, thermodynamic stability, protection against degradation, improved dissolution of lipophilic molecules and surfactant-induced permeability enhancement (Shah *et al.*, 1994; Constantinides, 1995; Zhao *et al.*, 2005). It is noteworthy that microemulsion formulated with oils extracted from vegetal has increasing interest in technological applications over the last two decades (Lee *et al.*, 1995; Dantas, T. N. C. *et al.*, 2010; Attaphong *et al.*, 2012; Xavier-Junior, Chapter IV, 2015)

In a previous work, microemulsion composed of a high content of natural oil dispersed in an aqueous phase was formulated with a low concentration of surfactant from the application of a rational approach considering the miscibility of the oil components with surfactants based on the match of their solubility parameters (Xavier-Junior, Chapter V, 2015). The microemulsion incorporated an important amount of copaiba oil (*Copaifera langsdorffii)* (94 %), an oil that contains sesquiterpenes and diterpenes having interesting therapeutic activity which include among others antitumor, anti-inflammatory, antimicrobial, antileishmanial, expectorant, diuretic, antinociceptive activities (Veiga-Junior & Pinto, 2002; Gomes, Niele Matos *et al.*, 2007; Gomes N *et al.*, 2008; Mendonça & Onofre, 2009b; Comelli Júnior *et al.*, 2010; Souza, Martins, Souza, Furtado, Heleno, De Sousa, *et al.*, 2011). For instance, the composition of the essential oil is β-Bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene

(20.5%) The aim of the present study was to formulate a microemulsion incorporating paclitaxel and copaiba oil in a single formulation designed for oral administration. The rational behind this work was to design a new anticancer drug formulation combining the activity of paclitaxel with that of compounds founds in copaiba oil. Incorporation of paclitaxel in the copaiba oil containing microemulsion was developed and the physicochemical characteristics were determined. The amount of paclitaxel-loaded microemulsion was determined by a validated high-performance liquid chromatography (HPLC) method. As the microemulsion was designed for oral administration, the retention of [3H]-paclitaxel at the level of rat intestinal mucosa mounted in Ussing chamber was evaluated as an indicator of the mucoadhesion promoter.

2 MATERIALS AND METHODS

2.1 Materials

Copaiba oil (*Copaifera langsdorffii)* was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Paclitaxel was obtained from CHEMOS GmbH (Regenstauf, Germany). [3H]-paclitaxel (3 $Ci.mmol^{-1}$) was acquired from Isobio (Fleurus, Belgium). Hionic-Fluor® and Ultima-Gold® (Packard, Rungis, France) were used as scintillating cocktails for radioactive analyses. Soluene-350® used to dissolve biological samples was obtained from Perkin-Elmer (Courtaboeuf, France). Polyoxyethylene (10) oleyl ether (Brij O10®) and polyoxyethylene-polyoxypropylene block copolymer (Pluronic F-68®) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Ethanol, acetonitrile and sodium sulphate were purchased from Fisher Scientific (Illkirch, France). Ultrapure

water was obtained from a Millipore purification system (Milli-Q plus, Millipore, St Quentin en Yvelines, France).

2.2. Copaiba essential oil extraction

Hydrodistillation extraction using a Clevenger-type apparatus was performed in order to obtain the copaiba essential oil from 400 mL of copaiba resin oil. The system was operated by 3 h. The extract obtained was dried with sodium sulphate, filtered and stored at −20°C.

2.3 Preparation of the microemulsion

Surfactants were selected to obtain optimal miscibility of the lipophilic moiety in the oil phase using an approach based on the match of their solubility parameters. O/W-microemulsion was the prepared by a titration method as described by Xavier Junior *et al.* (Xavier-Junior, Chapter V, 2015). The microemulsion was prepared with copaiba essential oil: Pluronic F-68®: Brij O10®: milli Q® water at a weight ratio of 19.6: 0.15: 13.55: 66.7, respectively. The compounds were weighted to total volume of 1 g and sonicated (Misonix XL 2020 sonicator, Farmingdale, NY, U.S.A) at 40 % amplitude for 60 seconds followed by an ultrasonic bath for 10 minutes (Elma Elmasonic S10H, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany). 1 mg of paclitaxel was added in the dispersion, thus resonicated at 20 % amplitude for 40 seconds. The system was filtrated using a 0.22 µm teflon filter (Merck Millipore, Billerica, MA, EUA) to remove

the excess of paclitaxel which remained undissolved in the microemulsion and was not incorporated in the dispersion.

For mucoadhesion studies, the microemulsion were labeled with [3H]-paclitaxel. The microemulsions were prepared following the protocol described above with minor changes. [3H]-paclitaxel initially solubilized in ethanol was dried in nitrogen gas atmosphere under constant pressure to eliminate the organic phase. Thus, to produce radiolabeled microemulsion, 1g of microemulsion was added in this bottle with cold paclitaxel (1 mg), in order to obtain a final radioactivity of 370 kBq of [3H]-paclitaxel per mL of microemulsion.

2.4 Characterization of paclitaxel-loaded microemulsion

2.4.1 Transmittance measurements

Temperature-scan and transmittance measurements were used to obtain information about potential changes in the microstructure of the samples during heating. The transmittance of the samples was monitored using a UV-VIS-Fibre Optics Spectrometer AVS-S2000 with DH-2000 deuterium Halogen light source and AvaSoft software package (Avantes, Apeldoorn, Netherlands) while the temperature of the sample varied from 25 to 45°C at an increment rate of 2.5 °C.min^{-1}. The fiber optics spectrometer was working in the wavelength range between 400 and 800 nm. The wavelength of 650 nm was retained to monitor the transmittance of the sample.

2.4.2 Polarized light microscopy

Paclitaxel-loaded microemulsion was examined by polarized light microscopy using a Nikon E600 Eclipse direct microscope (Champigny/Marne, France). The microscope was equipped with a long focus objective (LWD 40 x 0.55; 0-2mm) and a Nikon Coolpix 950 camera was used to record the images with a resolution of 1600 x 1200 pixels.

2.4.3 Size measurement

The hydrodynamic mean diameter and the size distribution of the microemulsion were determined by dynamic light scattering, using a He-Ne laser (wavelength of 633 nm) and a detector angle of 90° in a Malvern Zetasizer, NANO ZS90 (Malvern Instruments Limited, UK). For size distribution measurements, the dilution curve at concentrations ranging from 0.2 to 50% of microemulsion in milli-Q® water was developed and analyzed in a polystyrene cell at 25 °C. Cumulates analysis provides the characterization of a sample through the mean value (z-average) for the droplets size, and a width parameter known as polydispersity index (PdI).

2.4.4 Morphology study

Transmission electron microscopy (TEM) images were obtained on a JEOL 1400 microscope (JEOL Ltd, Tokyo, Japan), equipped with a high resolution CCD Gatan digital camera (SC1000 Orius, France) and operated at 80kV as the acceleration voltage. For TEM analyses, one drop of the diluted sample (1:100) was placed on a carbon-

formvar coated copper grid and then a drop of 1% phosphotungstic acid covered on the microemulsion. The superfluous of phosphotungstic acid on the sample was wiped off with a filter paper.

2.4.5 Rheological behavior

Rheological properties of paclitaxel-loaded and unloaded microemulsion were determined using a rotational rheometer AR-G2 (TA instruments, New Castle, USA) . Measurements were performed with an aluminum cone/plate geometry with a diameter of 40 mm, an angle of 1° and a truncation gap of 28 µm. Samples were maintained at 37ºC using a Peltier plate. Analyses were carried out by gradually increasing the shear rate from 10^{-1} to 10^{3} s^{-1}, after 5 minutes of equilibrium time. Measurements were performed in triplicate.

2.5 Maximum incorporation of paclitaxel-loaded microemulsion

Maximum incorporation of paclitaxel-loaded microemulsion was determined by a centrifugation method. Briefly, 1 mL of sample was centrifuged at 8500 × g (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) for 15 minutes to remove the drug excess. The supernatant was recovered and carefully filtered using a 0.22 µm membrane. Thus, the filtrate was diluted in the mobile phase or scintillating cocktail and it was placed in ultrasound bath for 15 minutes. The quantitative analysis of the paclitaxel-loaded microemulsion was performed using HPLC and liquid scintillation methods.

A previous HPLC method was developed and validated to quantification of paclitaxel in copaiba oil (Xavier-Junior, Chapter II, 2015). The chromatographic system used was a Waters series, equipped with a Waters 515 pump, a Waters 717 plus autosampler and a Waters 486- Tunable Absorbance detector (Waters Corp., Milford, MA). The separation of paclitaxel was carried out at 30 °C using a Uptisphere Strategy 100A reversed-phase C-18 (150 mm x 3 µm x 3 mm) column and a Uptisphere Strategy C18-2 guard column (10 mm x 3 µm x 4 mm) (Interchim SA, Montluçon, France). The mobile phase, pumped at 0.4 $mL.min^{-1}$, was acetonitrile: water (50:50) and effluent was monitored with UV detection at 228 nm. 25 µL samples were introduced onto the HPLC system every 15 min. Chromatographic data were monitored and analyzed using Azur software (Datalys, France). Under these conditions, the paclitaxel was eluted at 9.7 minutes and microemulsion adjuvants were not able to change the specificity of drug identification. The calibration curves were designed over the range from 50 to 2000 $ng.mL^{-1}$ (r^2=0.999), the accuracy of the method was less or equal to 0.77 %, and relative standard deviation for intra- and inter-day precision were less or equal to 0.65 %. The limit of quantification and detection were calculated to be 21.03 and 6.31 $ng.mL^{-1}$, respectively.

Radiolabeled [3H]-paclitaxel loaded microemulsion was preformatted in liquid scintillation counter (Model LS 6000 TA, Beckman, France). The samples were mixed with 10 mL of a scintillating cocktail and analyzed. For tissue digestion, 2 mL of Soluene-350® at 65 °C was used overnight. Posteriorly, 10 mL of scintillating cocktail was added to measure the [3H]-paclitaxel radioactivity.

2.6 Evaluation of mucoadhesion

The studies were performed according to the recommendations of the ethical committee of the French Ministry of Higher Education and Research, project 2003-055 regarding the care and use of animals for experimental procedures. Male Wistar rats (200–250 g) (Charles River, Paris) were used for the mucoadhesion *ex vivo* assays. Animals were euthanized with an overdose of pentobarbital by intraperitoneal injection. Jejunum from fresh small intestine of sacrificed rats were excised, rinsed with chilled physiological saline solution (0.9 %) and cut into segments of 2–3 cm length. After visual examination of the tissue, sections containing Peyer's Patches were discarded.

Jejunum portions were mounted in Ussing chambers (the intestinal surface tested was 1 cm^2) and bathed with ringer buffer at pH 7.4. The system was maintained at constant temperature (37 °C) and continuously oxygenated with O_2 /CO_2 (95 % / 5 %). After equilibration at the temperature of the experiment for 30 min, the transport buffer was removed and 50 µL of radiolabeled microemulsion was added to the donor compartment. Each compartment of the Ussing chamber was filled with 3 mL of ringer buffer. The experiment was performed over a period of 2 hours to insure the attachment equilibrium. Over incubation time, microemulsion dispersion was removed. Tissue was rinsed three times with 2 mL of ringer buffer, to eliminate non-attached microemulsion. Subsequently, the tissue with attached microemulsion was digested overnight in 2 mL of Soluene-350® at 65°C. Then, 10 mL of scintillating liquid were added and finally samples were analyzed by liquid scintillation to determine the amount of [3H]-paclitaxel which associated with the mucosa. Each sample was tested in three different rats in duplicate.

2.7 Statistical analysis

Means of two groups were compared using non-paired Student's t-tests. All values are expressed as their mean ± S.D. When comparing multiple groups, one way analysis of variance (ANOVA) was applied with the Tukey multiple comparison procedure. The statistical data were considered significant at $p < 0.05$

3. RESULTS AND DISCUSSION

The aim of the work was to produce paclitaxel-loaded copaiba essential oil/water-microemulsion and to evaluate their mucoadhesive properties on intestinal fragments. The system was developed based on previous studies which were based on the match of solubility parameter approach to select suitable surfactants to formulate a microemulsion incorporating copaiba oil (Xavier-Junior, Chapter V, 2015). A microemulsion with a high volume fraction of copaiba essential oil ad a relatively low content of surfactant could be formulated. The essential oil from *Copaifera langsdorffii* provided a transparent oil rich in sesquiterpenes hydrocarbons compounds, including β-Bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene (20.5%) as main compounds (Xavier-Junior, Chapter I, 2015b). These sesquiterpenes present interesting therapeutic properties, highlighting the increase the drug anticancer activity, as paclitaxel, facilitating the passage of drug through the membrane and thus potentiates its therapeutic activity (Legault & Pichette, 2007).

Copaiba essential oil: Pluronic F-68®: Brij O10®: Milli-Q water were blended at 19.6: 0.15: 13.55: 66.7 ratios to prepare the microemulsion. Paclitaxel was added to the prepared microemulsion. The paclitaxel-loaded microemulsion appeared homogeneous

and transparent (Figure 1). The formulation prepared several times displayed the same characteristics acknowledging the reproducibility and stability of the microemulsion. The paclitaxel-loaded microemulsion appeared isotropic by analysis through polarized light microscopy indicating that it was constituted by a dispersion of droplets in a continuous phase (Danielsson, I. & Lindman, B., 1981; Djordjevic *et al.*, 2004).

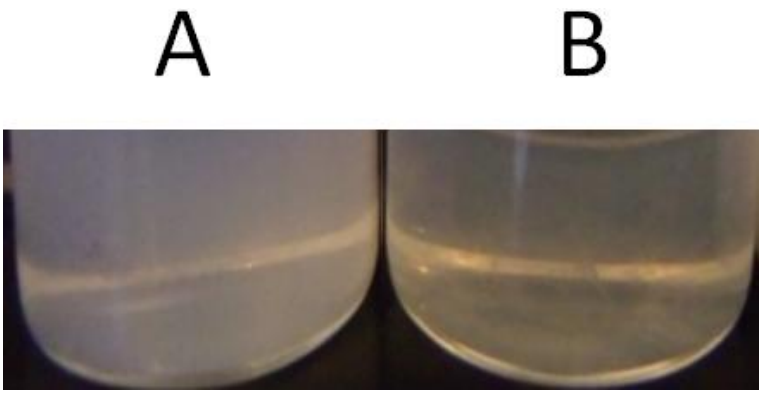

Figure 1: Macroscopic aspect of the paclitaxel unloaded (A) and loaded (B) microemulsions

Paclitaxel-loaded microemulsion showed the maximum transmittance at temperatures ranging from 20 to 45 °C A significant difference in the variation of transmittance with the temperature was observed comparing the paclitaxel-loaded microemulsion and the corresponding non loaded microemulsion. In microemulsion apparently the paclitaxel was placed in the interface, reducing the surface tension and enhance the formation of more stable system.

To evaluate the diameter of the microemulsion droplets by DLS, the microemulsion needed to be diluted. As shown on Figure 2, Diluting the microemulsion provided a curve with a constant diameter of the droplets ($p>0.05$) at the highest volume fraction of

the dispersed phase ranging from 1 to 50% At volume fractions below 0.4%, the diameter of the droplets increased dramatically indicating a loss of physical integrity of microemulsion (Li *et al.*, 2005; Borhade *et al.*, 2008) ($p>0.05$). After 24 hours, the diluted microemulsions did not show any phase separation, modification of droplet size and drug precipitation. This effect was related by Mohsin *et al.*, where was presented the schematic diagram of the possible phases formed on dilution, the excess of the water can promotes the changes in the interface systems and formation of bicontinuous system (Mohsin *et al.*, 2009). For further analysis, the dilution of the mother microemulsion dispersion at 1% was used as it permitted to keep the initial characteristics of the droplets.

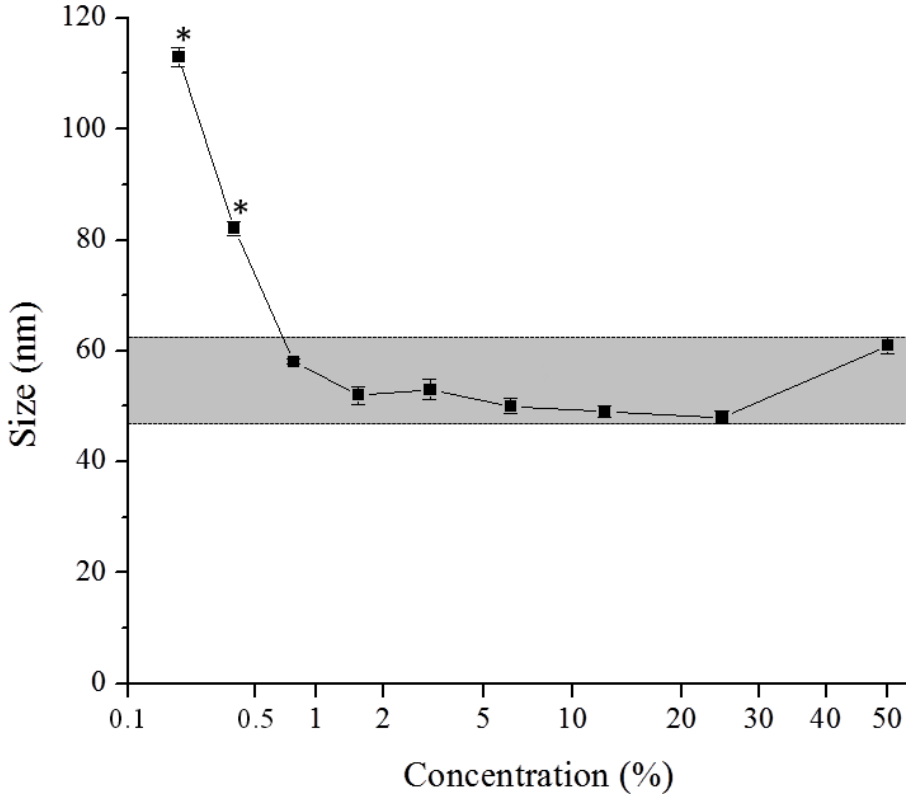

Figure 2- Dilution effect in the paclitaxel-loaded microemulsion analyzed by DLS at concentration ranged from 0.1 to 50 %. *= $p<0.05$

Results of droplet size, PdI, pH, maximum incorporation of the paclitaxel-loaded and unloaded microemulsions were summarized in Table 1. Incorporation of paclitaxel in the microemulsion increased slightly the diameter of the microemulsion while the PDI remained low indicating a monomodal and a homogeneous distribution of the size of the droplets in both cases (Figure 3A). The microemulsions were composed of copaiba oil droplets of very small size hence provided with a large interfacial surface area, drug diffusion and kept the drug in solution throughout its passage through the gastrointestinal tract (Pouton, 2000; Nicolaos *et al.*, 2003; Constantinides & Wasan, 2007).

Table 1- The droplet size, PDI, pH, maximum incorporation of the paclitaxel-loaded microemulsion (MECop Ptx) in comparison with the system without the drug (MECop)

Samples	Characteristic	Size (nm)	PdI	pH	Incorporation ($mg.mL^{-1}$)
MECop	Translucent or tyndall effect, homogeneous and isotropic	42 ± 0.5	0.13 ± 0.01	6.5 ± 0.4	
MECop Ptx	Translucent , homogeneous and isotropic	51 ± 1.2	0.21 ± 0.03	6.1 ± 0.3	0.37 ± 0.03

The pH of paclitaxel-loaded microemulsion was of 6.1 ± 0.3 that was suitable for the application of the microemulsion as a delivery system for the oral administration of drugs. The slight pH difference between paclitaxel-loaded and unloaded microemulsion was not statistically significant. Paclitaxel-loaded microemulsion was also characterized

by TEM (Figure 3B). The microstructure analyses showed a spherical shape and uniform droplet size distribution consistently with the results of size measurement performed by DLS.

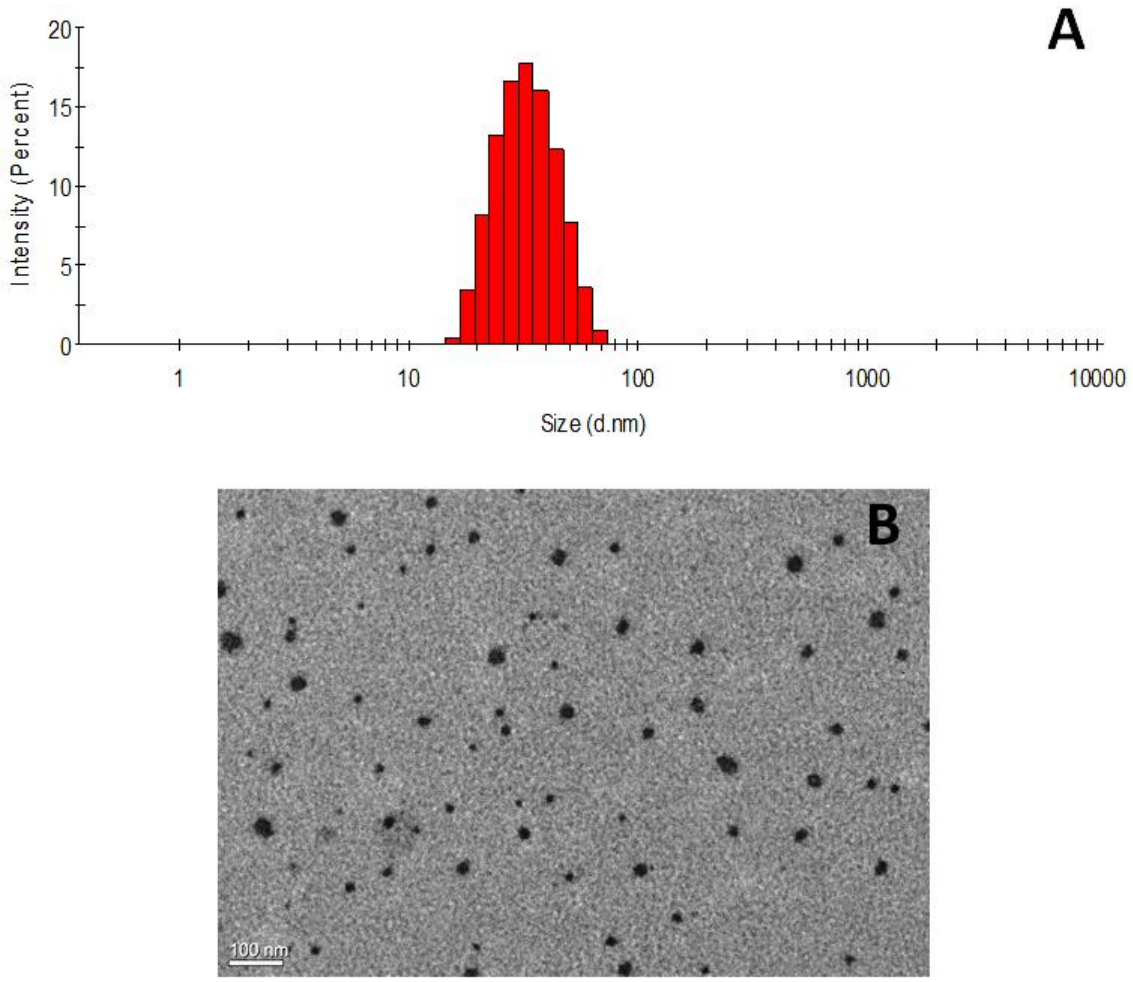

Figure 3- Size and morphological characteristics of the paclitaxel-loaded microemulsion. Typical droplet size distribution obtained from measurements performed by DLS (A) and aspect of the microemulsion observed by TEM. Scale Bar = 100 nm.(B)

The maximum incorporation of paclitaxel in the microemulsion was 0.37 mg of paclitaxel per mL of microemulsion. This corresponded to an incorporation of 1.89 mg of paclitaxel per g of copaiba oil. This incorporation rate corresponds to 37% of the initial amount of the drug added in the microemulsion. The high association of the paclitaxel into microemulsion system can be related at its high partition coefficient in copaiba oils (Xavier-Junior, Chapter II, 2015). Compared with other formulations reported in the literature that were considering lipid formulations, microemulsions or self emulsifying systems, the present formulation incorporated much higher amount of paclitaxel while at the same time, concentrations in surfactant and oil were considerably reduced (Nornoo *et al.*, 2008; Wang *et al.*, 2011; Li *et al.*, 2012).

Radiolabeled microemulsion was effectively developed with [3H]-paclitaxel. This system showed the similar characteristics with the nonradioactive microemulsion. The maximum radioactivity of the [3H]-paclitaxel was 232 kBq per mL of microemulsion.

Rheology of microemulsion can provide useful information about their structure. Regarding the rheology, in microemulsion, formation of liquid crystalline stage coincides with formation of non-spherical aggregates (cylindrical or lamellar aggregates), which obstructs the flow in the dispersion medium (Ambade *et al.*, 2008). The flow curves presented in the Figure 4 revealed that the microemulsions were shear-thinning. The microemulsion containing copaiba oil was shear-thinning from 1 to about 500 s^{-1}, and seemed to reach the second Newtonian plateau above 500 s-1. These results indicate that the systems developed were isotropic and contained spherical dispersed droplets which offer a low resistance to flow besides the fact that they exhibited low viscosity characteristics to microemulsions (Ambade *et al.*, 2008). The rheological characteristic property of microemulsions is interesting in order to can facilitate

microemulsion preparation but is also to achieve drug administration suitable for oral delivery. In addition, the more ordered system in the dispersion may be responsible for prolonging or modifying the drug release profile (Lapasin *et al.*, 2001; Krishnan *et al.*, 2002; Pestana *et al.*, 2008a).

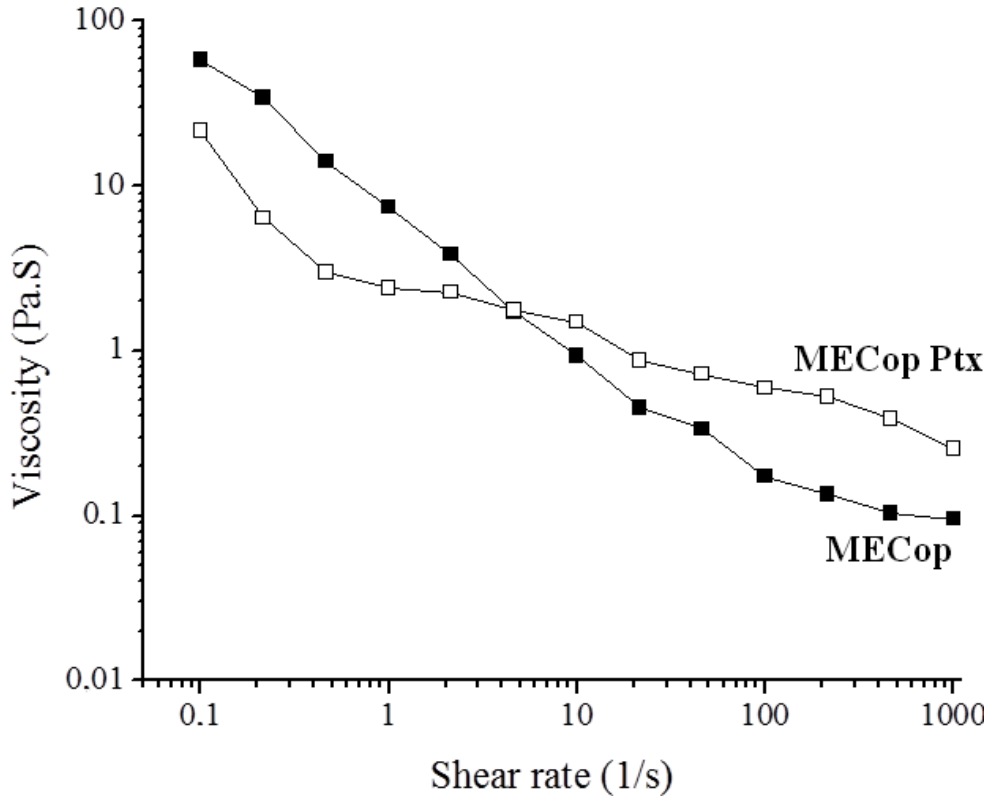

Figure 4- Rheological behavior of the paclitaxel -loaded (MECop Ptx) (open squares) and unloaded microemulsion (MECop) (full squares).

The use of mucoadhesive molecules can be an important strategy to retain the preparation at the action site and to direct the drug to a specific site or tissue, decreasing the drug administration frequency and increasing the patient compliance to the therapy (Huang *et al.*, 2000; Woodley, 2001). The mechanisms of mucoadhesion involve the contact and the consolidation stages of the ligation between the system and the mucus.

This mechanism can be affected by different factors as the molecular weight, flexibility, cross-linking density, hydrogen bonding capacity, hydration, charge and concentration of the molecules in the formulation and the mucus (Boddupalli *et al.*, 2010). The mucus contains glycoproteins, lipids, inorganic salts and 95% water, which mucin is the most important glycoprotein of mucus and is responsible for its structure. The mucoadhesiveness of the paclitaxel-loaded microemulsion containing copaiba oil was evaluated directly on rat intestinal mucosa mounted in Ussing Chambers using [3H]-paclitaxel-loaded microemulsion. Thus, only the radioactivity associated with paclitaxel incorporated in the microemulsion could be evaluated by this method. The percentage of the association of the radioactivity of paclitaxel with the rat intestine mucosa was 4.4 ± 0.7 % of the initial dose introduced in the Ussing Chamber. Based on these results, mucoadhesion 1 mL of the paclitaxel-loaded microemulsion when in administration for oral route will need $4x10^{-3}$ m² of the intestinal area to total formulation association. The mucoadhesion of the paclitaxel-loaded microemulsion was calculated at 92.5 mg of paclitaxel per m² of the intestinal mucosa. In the clinic, to obtained an ideal anticancer therapy to adult patients receiving chemotherapy, it is required a total paclitaxel amount of 313.3 mg to achieve the commonly dose prescription (175 mg/m² of body surface area) (Van Den Bongard *et al.*, 2004; Sacco *et al.*, 2010). Taking into account the large surface area of the human intestine (about 30 m² (Helander & Fandriks, 2014)), this formulation when applied to human can associate itself with the mucus at total drug association area of 3.4 m². This association is important to oral drug delivery because it could be related to increase bioavailability of the drug. For the microemulsion developed this interaction can be related to the mucoadhesive effect of poly(oxyethylene) substances presents in the Pluronic F-68® and Brij O10® used to

produce the microemulsion developed in this work (Tiwari, Goldman, Sause, *et al.*, 1999; Tiwari, Goldman, Town, *et al.*, 1999; Singh & Ahuja, 2002).

4. CONCLUSION

Paclitaxel, a widely used anticancer agent could be incorporated in an O/W microemulsion characterized by a low amount of surfactant and a large volume fraction of dispersed copaiba essential oil without disturbing too much the physicochemical characteristic of the original microemulsion. The concentration of paclitaxel solubilized in the microemulsion was considerable regarding the solubility of this drug in water. The system showed a good fluidity that is important for its production and its use as a drug delivery system for oral administration. Paclitaxel contained in the microemulsion associated well with rat intestinal mucosa in experiments designed to evaluate the *ex-vivo* mucoadhesion. The paclitaxel-loaded microemulsion proposed in this work showed promising properties to be further developed as a drug delivery system for oral administration of the anticancer molecule, however more studies are required in order to ensure the drug therapeutic dose for oral route. It is assumed that synergetic anticancer effect could be obtained with this system which contained copaiba essential oil including various components showing an activity against cancer.

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REFERENCES

ADAMS, J. D., FLORA, K. P., GOLDSPIEL, B. R., WILSON, J. W., ARBUCK, S. G. & FINLEY, R. 1993. Taxol: a history of pharmaceutical development and current pharmaceutical concerns. *J Natl Cancer Inst Monogr*, 15, 141-7.

AMBADE, K. W., JADHAV, S. L., GAMBHIRE, M. N., KURMI, S. D., KADAM, V. J. & JADHAV, K. R. 2008. Formulation and evaluation of flurbiprofen microemulsion. *Curr Drug Deliv*, 5, 1, 32-41.

ARAYA, H., TOMITA, M. & HAYASHI, M. 2005. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. *Int J Pharm*, 305, 1-2, 61-74.

ATTAPHONG, C., DO, L. & SABATINI, D. A. 2012. Vegetable oil-based microemulsions using carboxylate-based extended surfactants and their potential as an alternative renewable biofuel. *Fuel*, 94, 1, 606-613.

BHARGAVA, H. N., NARUKAR, A. & LIEB, L. M. 1987. Using microemulsions for drug delivery. *Pharm Technol*, 11, 46 - 54.

BODDUPALLI, B. M., MOHAMMED, Z. N., NATH, R. A. & BANJI, D. 2010. Mucoadhesive drug delivery system: An overview. *J Adv Pharm Technol Res*, 1, 4, 381-7.

BORHADE, V., NAIR, H. & HEGDE, D. 2008. Design and evaluation of self-microemulsifying drug delivery system (SMEDDS) of tacrolimus. *AAPS PharmSciTech*, 9, 1, 13-21.

COMELLI JÚNIOR, E., SKINOVSKI, J., SIGWALT, M. F., BRANCO, A. B., LUZ, S. R. & BAULÉ, C. D. P. 2010. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaíba (Copaifera Iangsdorfii) oil. *Acta Cir Bras*, 25, 362-367.

CONSTANTINIDES, P. P. 1995. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm Res*, 12, 11, 1561-72.

CONSTANTINIDES, P. P. & WASAN, K. M. 2007. Lipid formulation strategies for enhancing intestinal transport and absorption of P-glycoprotein (P-gp) substrate drugs: in vitro/in vivo case studies. *J Pharm Sci*, 96, 2, 235-48.

DANIELSSON, I. & LINDMAN, B. 1981. The definition of a microemulsion. *Colloids Surf*, 3, 391-392.

DANTAS, T. N. C., SILVA, H. S. R. C., NETO, A. A. D., MARCUCCI, M. C. & MACIEL, M. A. M. 2010. Development of a new propolis microemulsion system for topical applications. *Rev bras farmacogn*, 20, 3, 368-375.

DJORDJEVIC, L., PRIMORAC, M., STUPAR, M. & KRAJISNIK, D. 2004. Characterization of caprylocaproyl macrogolglycerides based microemulsion drug delivery vehicles for an amphiphilic drug. *Int J Pharm*, 271, 1-2, 11-9.

DUNCAN, R. 2004. Nanomedicines in action. *Pharm J*, 273, 485-488.

ENGINEERING., R. S. R. A. O. Nanoscience and nanotechnologies: Opportunities and uncertainties. London, UK: Royal Society, 2004.

FORASTIERE, A. A. 1994. Current and future trials of Taxol (paclitaxel) in head and neck cancer. *Ann Oncol*, 5 Suppl 6, S51-4.

GOMES N, M., REZENDE C, M., FONTES, S. P., HOVELL, A. M., LANDGRAF, R. G., MATHEUS, M. E., PINTO A, C. & FERNANDES, P. D. 2008. Antineoplasic activity of Copaifera multijuga oil and fractions against ascitic and solid Ehrlich tumor. *J Ethnopharmacol*, 119, 1, 179-84.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-492.

HAMEL, E., DEL CAMPO, A. A., LOWE, M. C. & LIN, C. M. 1981. Interactions of taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J Biol Chem*, 256, 22, 11887-94.

HELANDER, H. F. & FANDRIKS, L. 2014. Surface area of the digestive tract - revisited. *Scand J Gastroenterol*, 49, 6, 681-9.

HOAR, T. P. & SCHULMAN, J. H. 1943. Transparent water-in-oil dispersions: The oleopathic hydro-micelle. *Nature* 152, 102-103.

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci*, 13, 4, 134-6.

HUANG, Y., LEOBANDUNG, W., FOSS, A. & PEPPAS, N. A. 2000. Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces. *J Control Release*, 65, 1-2, 63-71.

KASIM, N. A., WHITEHOUSE, M., RAMACHANDRAN, C., BERMEJO, M., LENNERNAS, H., HUSSAIN, A. S., JUNGINGER, H. E., STAVCHANSKY, S. A., MIDHA, K. K., SHAH, V. P. & AMIDON, G. L. 2004. Molecular properties of WHO essential drugs and provisional biopharmaceutical classification. *Mol Pharm*, 1, 1, 85-96.

KATEB, B., CHIU, K., BLACK, K. L., YAMAMOTO, V., KHALSA, B., LJUBIMOVA, J. Y., DING, H., PATIL, R., PORTILLA-ARIAS, J. A., MODO, M., MOORE, D. F., FARAHANI, K., OKUN, M. S., PRAKASH, N., NEMAN, J., AHDOOT, D., GRUNDFEST, W., NIKZAD, S. & HEISS, J. D. 2011. Nanoplatforms for constructing new approaches to cancer treatment, imaging, and drug delivery: what should be the policy? *Neuroimage*, 54 Suppl 1, S106-24.

KAWAKAMI, K., YOSHIKAWA, T., HAYASHI, T., NISHIHARA, Y. & MASUDA, K. 2002. Microemulsion formulation for enhanced absorption of poorly soluble drugs. II. In vivo study. *J Control Release*, 81, 1-2, 75-82.

KAWAKAMI, K., YOSHIKAWA, T., MOROTO, Y., KANAOKA, E., TAKAHASHI, K., NISHIHARA, Y. & MASUDA, K. 2002. Microemulsion formulation for enhanced absorption of poorly soluble drugs. I. Prescription design. *J Control Release*, 81, 1-2, 65-74.

KAWASAKI, E. S. & PLAYER, A. 2005. Nanotechnology, nanomedicine, and the development of new, effective therapies for cancer. *Nanomedicine*, 1, 2, 101-9.

KRISHNAN, K., CHAPMAN, B., BATES, F. S., LODGE, T. P., ALMDAL, K. & BURGHARDT, W. R. 2002. Effects of shear flow on a polymeric bicontinuous microemulsion: Equilibrium and steady state behavior. *J Rheol*, 46, 2, 529-554.

LAPASIN, R., GRASSI, M. & COCEANI, N. 2001. Effects of polymer addition on the rheology of o/w microemulsions. *Rheol Acta*, 40, 2, 185-192.

LAWRENCE, M. J. & REES, G. D. 2000. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 45, 1, 89-121.

LEE, M. J., LEE, M. H. & SHIM, C. K. 1995. Inverse targeting of drugs to reticuloendothelial system-rich organs by lipid microemulsion emulsified with poloxamer-338. *Inter J Pharm*, 113, 2, 175-187.

LEGAULT, J. & PICHETTE, A. 2007. Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. *J Pharm Pharmacol*, 59, 12, 1643-7.

LI, G., FAN, Y., LI, X., WANG, X., LI, Y., LIU, Y. & LI, M. 2012. In vitro and in vivo evaluation of a simple microemulsion formulation for propofol. *Int J Pharm*, 425, 1-2, 53-61.

LI, P., GHOSH, A., WAGNER, R. F., KRILL, S., JOSHI, Y. M. & SERAJUDDIN, A. T. 2005. Effect of combined use of nonionic surfactant on formation of oil-in-water microemulsions. *Int J Pharm*, 288, 0378-5173 (Print), 27-34.

MENDONÇA, D. E. & ONOFRE, S. B. 2009b. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev. Bras. Farmacogn*, 19, 577-581.

MOHSIN, K., LONG, M. A. & POUTON, C. W. 2009. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: precipitation of drug after dispersion of formulations in aqueous solution. *J Pharm Sci*, 98, 10, 3582-95.

NICOLAOS, G., CRAUSTE-MANCIET, S., FARINOTTI, R. & BROSSARD, D. 2003. Improvement of cefpodoxime proxetil oral absorption in rats by an oil-in-water submicron emulsion. *Int J Pharm*, 263, 1-2, 165-71.

NORNOO, A. O., OSBORNE, D. W. & CHOW, D. S. 2008. Cremophor-free intravenous microemulsions for paclitaxel I: formulation, cytotoxicity and hemolysis. *Int J Pharm*, 349, 1-2, 108-16.

PESTANA, K. C., FORMARIZ, T. P., FRANZINI, C. M., SARMENTO, V. H., CHIAVACCI, L. A., SCARPA, M. V., EGITO, E. S. & OLIVEIRA, A. G. 2008a. Oil-in-water lecithin-based microemulsions as a potential delivery system for amphotericin B. *Colloids Surf B*, 66, 2, 253-9.

POUTON, C. W. 2000. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. *Eur J Pharm Sci*, 11 Suppl 2, S93-8.

ROWINSKY, E. K. & DONEHOWER, R. C. 1995. Paclitaxel (taxol). *N Engl J Med*, 332, 15, 1004-14.

SACCO, J. J., BOTTEN, J., MACBETH, F., BAGUST, A. & CLARK, P. 2010. The average body surface area of adult cancer patients in the UK: a multicentre retrospective study. *PLoS One*, 5, 1, e8933.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SCHULMAN, J. H., STOECKENIUS, W. & PRINCE, L. M. 1959. Mechanism of formation and structure of micro emulsions by electron microscopy. *J Phys Chem* 63, 1677-1680.

SHAH, N. H., CARVAJAL, M. T., PATEL, C. I., INFELD, M. H. & MALICK, A. W. 1994. Self-emulsifying drug-delivery systems (SEDDS) with polyglycolyzed glycerides for improving in-vitro dissolution and oral absorption of lipophilic drugs. *Int J Pharm*, 106, 1, 15-23.

SINGH, B. & AHUJA, N. 2002. Development of controlled-release buccoadhesive hydrophilic matrices of diltiazem hydrochloride: optimization of bioadhesion, dissolution, and diffusion parameters. *Drug Dev Ind Pharm*, 28, 4, 431-42.

SINGLA, A. K., GARG, A. & AGGARWAL, D. 2002. Paclitaxel and its formulations. *Int J Pharm*, 235, 1-2, 179-92.

SOUZA, A. B., MARTINS, C. H., SOUZA, M. G., FURTADO, N. A., HELENO, V. C., DE SOUSA, J. P., ROCHA, E. M., BASTOS, J. K., CUNHA, W. R., VENEZIANI, R. C. & AMBROSIO, S. R. 2011. Antimicrobial activity of terpenoids from Copaifera langsdorffii Desf. against cariogenic bacteria. *Phytother Res*, 25, 2, 215-20.

TAKAHASHI, Y., KONDO, H., YASUDA, T., WATANABE, T., KOBAYASHI, S. & YOKOHAMA, S. 2002. Common solubilizers to estimate the Caco-2 transport of poorly water-soluble drugs. *Int J Pharm*, 246, 1-2, 85-94.

TALEGAONKAR, S., AZEEM, A., AHMAD, F. J., KHAR, R. K., PATHAN, S. A. & KHAN, Z. I. 2008. Microemulsions: a novel approach to enhanced drug delivery. *Recent Pat Drug Deliv Formul*, 2, 3, 238-57.

TIWARI, D., GOLDMAN, D., SAUSE, R. & MADAN, P. L. 1999. Evaluation of polyoxyethylene homopolymers for buccal bioadhesive drug delivery device formulations. *AAPS PharmSci*, 1, 3, E13.

TIWARI, D., GOLDMAN, D., TOWN, C., SAUSE, R. & MADAN, P. L. 1999. In vitro-in vivo evaluation of a controlled release buccal bioadhesive device for oral drug delivery. *Pharm Res*, 16, 11, 1775-80.

VAN DEN BONGARD, H. J., KEMPER, E. M., VAN TELLINGEN, O., ROSING, H., MATHOT, R. A., SCHELLENS, J. H. & BEIJNEN, J. H. 2004. Development and validation of a method to determine the unbound paclitaxel fraction in human plasma. *Anal Biochem*, 324, 1, 11-5.

VEIGA-JUNIOR, V. F. & PINTO, A. C. 2002. O gênero copaifera L. *Quim Nova*, 25, 273-286.

WANG, Y., WU, K. C., ZHAO, B. X., ZHAO, X., WANG, X., CHEN, S., NIE, S. F., PAN, W. S., ZHANG, X. & ZHANG, Q. 2011. A novel paclitaxel microemulsion containing a reduced amount of Cremophor EL: pharmacokinetics, biodistribution, and in vivo antitumor efficacy and safety. *J Biomed Biotechnol*, 2011, 854872.

WOODLEY, J. 2001. Bioadhesion: new possibilities for drug administration? *Clin Pharmacokinet*, 40, 2, 77-84.

XAVIER-JUNIOR, F. H., GUEUTIN, C., MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T., VAUTHIER, C. Chapter II, 2015. HPLC method for the dosage of paclitaxel in copaiba oil. Development, validation, application to the determination of the solubility and partition coefficients. *Article in preparation*

XAVIER-JUNIOR, F. H., HUANG, N., VACHON, J. J., REHDER, V. L. G., MACIUK, A., EGITO, E.S.T., VAUTHIER, C. Chapter V, 2015. Match of solubility parameters between oil and surfactants as a rational approach for the formulation of O/W microemulsion with high dispersed volume of copaiba oil and low surfactant content. *Article in preparation*

XAVIER-JUNIOR, F. H., MACIUK, A., MORAIS, A. R. V., ALENCAR, E. N., REHDER, V. L. G., EGITO, E. S. T., VAUTHIER, C. Chapter I, 2015b. Development of gas-chromatography method for the analysis of copaiba oil. *Article in preparation*
XAVIER-JUNIOR, F. H., VAUTHIER, C., MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T. Chapter IV, 2015. Microemulsion-based drug delivery systems containing natural oils. *Article in preparation*

ZHAO, X. L., CHEN, D. W., GAO, P., DING, P. T. & LI, K. X. 2005. Synthesis of ibuprofen eugenol ester and its microemulsion formulation for parenteral delivery. *Chem Pharm Bull* 53, 10, 1246-1250.

Section III

Les systèmes d'administration de médicaments à base des polymères

Chapter VII

Experimental design approach applied to the development of chitosan coated poly(isobutylcyanocrylate) nanocapsules encapsulating copaiba oil

Le but du travail présenté dans ce chapitre était le développement, la caractérisation et l'optimisation de nanocapsules polymères mucoadhésives composées d'un cœur d'huile de copaïba. L'enveloppe des nanocapsules est composée de poly(cyanoacrylate d'isobutyle) recouverte de chitosane pour lui conférer des propriétés mucoadhésives. La conception de ce vecteur destiné à l'administration d'agents anticancéreux par voie orale a été abordée par une démarche faisant appel à un plan d'expérience. Les nanocapsules ont été obtenus par le développent d'une méthode originale de polymérisation interfaciale du cyanoacrylate d'isobutyle en utilisant du chitosane comme agent de stabilisation des nanocapsules et d'ajustement des propriétés de surface. Des études préliminaires ont été réalisées en utilisant différentes masses moléculaires de chitosane, différentes caractéristiques de l'huile de copaïba et en modifiant la composition de la phase organique utilisée pour préparer les nanocapsules par la méthode de polymérisation interfaciale. Ensuite, les caractéristiques de taille et potentiel zêta des nanocapsules ont été optimisées par un plan d'expérience à 2 niveaux avec trois facteurs (le pH, la température et la concentration du chitosane dans le milieu de polymériation) et des points centraux afin d'identifier les conditions de synthèse produisant des nanocapsules stables des plus petites dimensions et présentent un potentiel zêta le plus élevé de valeur positive. Les échantillons ont été observés par microscopie électronique à transmission. L'huile encapsulée dans les nanocapsules a été analysé par une méthode validée en chromatographie en phase gazeuse en utilisant le β-caryophyllène comme référence pour la caractérisation de l'huile de copaïba. Finalement, l'efficacité d'encapsulation de l'huile a été déterminée. Les résultats ont montré que la taille des nanocapsules variait de 300 à 1200 nm en fonction des différents facteurs étudiés. Des valeurs de charge de surface positive ont été obtenues dans tous les cas, témoignant de la présence du chitosane à la surface des nanoparticles.

Les plus petites nanocapsules stables qui ont été obtenues ont un diamètre de 473 nm et un potentiel zêta de + 34 mV. L'efficacité d'encapsulation de l'huile de copaïba a été de 75,8%, ce qui correspond à une teneur en β-caryophyllène de 55,5 µg.mg^{-1} de nanocapsules. La composition de l'huile encapsulée dans les nanocapsules est identique à celle de l'huile initiale indiquant que le procédé d'encapsulation préserve la qualité des caractéristiques de l'huile de copaïba.

Mots-clés: Nanocapsules, huile de copaïba, chitosane, plans d'expériences, poly(cyanoacrylate d'isobutyle), polymérisation interfaciale

EXPERIMENTAL DESIGN APPROACH APPLIED TO THE DEVELOPMENT OF CHITOSAN COATED POLY(ISOBUTYLCYANOCRYLATE) NANOCAPSULES ENCAPSULATING COPAIBA OIL

Xavier-Junior, F.H.[1, 2], Egito, E.S.T.[1], Morais, A.R.V. [1, 2], Alencar, E.N.[1], Maciuk, A.[3], Vauthier, C.[2]*,

[1] UFRN, DFAR, Laboratório de Sistemas Dispersos (LaSiD), Natal – RN, Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

[3] Université Paris Sud, Laboratoire de Pharmacognosie - UMR CNRS 8076 BioCIS - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Corresponding author

Christine Vauthier, Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Christine.vauthier@u-psud.fr

ABSTRACT

The aim of this work was to develop, characterize and optimize the natural copaiba oil-loaded chitosan decorated poly(isobutylcyanocrylate) nanocapsules. These systems were obtained by developing an original method of interfacial polymerization of isobutyl cyanoacrylate using chitosan as a stabilizer for the nanocapsules. A preliminary study investigated the influence of the molecular weight of chitosan, the characteristics of the copaiba oil and of the solvent phase. This showed that nanocapsules could only be produced with copaiba resin oil, while the size varied from 300 to 1200nm. Nanocapsule size and zeta potential were then optimized by two-level three-variable full-factorial experimental design. By transmission electron microscopy, samples showed spherical objects. The composition of the oil entrapped in the nanocapsules as analyzed by a validated method of gas chromatography using β- caryophyllene with reference to copaiba oil characterization revealed that the oil encapsulated was of the same composition then the initial oil. Nanocapsules with positive zeta potential were obtained consistently with the expected distribution of chitosan on the nanocapsule surface. Optimal nanocapsules showed a diameter of 473 nm, a zeta potential of +34 mV and an encapsulation efficiency of the oil of 74 % including 55.5 µg of β-caryophyllene/mg of nanocapsules. The designed nanocapsules show valuable characteristics to be further development as oral carrier for anticancer molecules including paclitaxel to develop a synergistic effect between oil component and the chemo-therapeutic agent.

Keywords: nanocapsules, copaiba oil, chitosan, poly(isobutylcyanoacrylate), interfacial polymerization

1 INTRODUCTION

Nanoparticles composed of mucoadhesive polymers are promising systems for oral drug delivery applications (Ponchel & Irache, 1998; Petit *et al.*, 2012). Generally, nanoparticles are defined as solid colloidal particles that include both nanospheres and nanocapsules (Guterres *et al.*, 2007). The nanocapsules are vesicular systems in which the drug is confined in a liquid/solid cavity surrounded by a polymeric membrane. The upper size limit is ∼1000 nm in diameter (Fessi *et al.*, 1989; Quintanar-Guerrero *et al.*, 1998).

In general, nanoparticulate systems show promise as drug carrier due to their capacity to modulate drug biodistribution to release the drug in a controlled manner, increase intracellular uptake and improve the stability of active substances (Cruz *et al.*, 2006; Pinto Reis *et al.*, 2006a; Leite *et al.*, 2007; Anton *et al.*, 2008). Nanoparticles made of biodegradable polymers, including poly(isobutylcyanocrylate), may provide an alternative solution for oral delivery of drugs across the gastrointestinal barrier thanks to their extremely small size. Their surface may be turned to increase mucoadhesion (Bravo-Osuna, Vauthier, *et al.*, 2007; Bravo-Osuna *et al.*, 2008). For instance, chitosan has been a widely used polysaccharide in formulation of mucoadhesive drug delivery systems (Thanou *et al.*, 2001; Chen *et al.*, 2014). This polysaccharide is biocompatible and nontoxic. Its inherent mucoadhesive properties come from its chemical structure including amino groups able to promote electrostatic interactions with sialic acid groups of the mucus (Gåserod *et al.*, 1998; Illum *et al.*, 2001). In addition, the positive charges of chitosan are also believed to be essential to increase permeability of the intestinal epithelium thanks to its capacity to disturb calcium concentration balance near the tight junction (Bernkop-Schnurch *et al.*, 2006; Bravo-Osuna, Millotti, *et al.*, 2007). Although

widely used to improve mucoadhesion of nanospheres, this polysaccharide was not yet used to improve mucoadhesion of polymeric nanocapsules which are interesting drug delivery systems for delivery of lipophilic drugs.

The copaiba oil-resin (*Copaifera langsdorffii*) is an oily plant extract which is used in folk medicine in its *in-natura* form (Sousa, J. P. *et al.*, 2011). Phytochemical studies on oil-resin reveal that it contains a complex mixture of diterpenes and sesquiterpenes hydrocarbons (Veiga Junior *et al.*, 2007; Alencar, E. N. *et al.*, 2015), giving this oil many interesting therapeutic activities. For instance, these include anti-inflammatory, antitumor, anti-tetanus, antimicrobial, antileishmania activities among others (Gomes, N. M. *et al.*, 2007; Santos, A. O. *et al.*, 2008; Leandro *et al.*, 2012). Although used for years in folk medicine, it is believed that pharmacological activities of this oil may be increased developing appropriate formulations.

Thus, the aim of this work was to develop an original oral formulation of copaiba oil by encapsulating the oil in mucoadhesive polymer nanocapsules. These systems were chosen because they appeared the more appropriate formulations for the delivery of oil, while their small size is desirable to promote mucoadhesion on the gut mucosae. The polymer composing the nanocapsule envelope was a critical choice and poly(isobutylcyanocrylate) was selected because of its capacity to formulate nanocapsule that resist well to the gastric medium and promote release in the intestinal medium (Aboubakar *et al.*, 2000). Although the development of mucoadhesive oil containing nanocapsule of poly(isobutylcyanocrylate) was not described before, this development was based on the use of an experimental design approach that was never applied so far while developing new formulations of oil containing nanocapsules prepared by interfacial polymerization of isobutylcyanocrylate. It is noteworthy that

experimental design approach was not so much applied in development of nanocapsules while such an approach could be helpful tool to optimize formulations limiting the number of experiments to perform (Patel *et al.*, 2013).

2 MATERIALS AND METHODS

2. 1. Materials

Copaiba resin oil was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Isobutyl cyanoacrylate was provided by ORAPI engineered solutions worldwide (Vaulx-en-Velin, France). Water soluble chitosan Mw 20,000 g/mol was purchased from Amicogen (Jinju, South Gyeongsang, South Korea). Ethanol, acetone, 2-propanol, sodium hydroxide, ethyl acetate, nitric acid were provided by Fisher Scientific (Pittsburgh, PA, EUA). Diazomethane and β-caryophyllene were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q plus, Millipore, St Quentin en Yvelines, France). All chemicals were reagent grade and used as received.

2.2 Copaiba essential oil extraction

Copaiba essential oil was obtained from 400 mL of copaiba resin oil by hydrodistillation using a Clevenger-type apparatus for 3 h. The extracted essential oil was dried with sodium sulphate, filtered, stored in a refrigerator and protected from light until use.

2.3 Method of preparation of the nanocapsules

Copaiba oil-loaded chitosan-decorated poly(isobutylcyanocrylate) nanocapsules were elaborated by the method of interfacial polymerization (Couvreur *et al.*, 1979; Fallouh *et al.*, 1986) that was adapted because of the use of chitosan. Thus, 0.25 mL of copaiba oil and 0.032mL of isobutylcyanoacrylate were solubilized in 6.25 mL of ethanol to produce the organic phase. This phase was slowly injected dropwise in 12.5 mL of chitosan solution (0.3, 0.6 or 0.9 %) (Polymerization medium) prepared at various pH (3, 6 or 9) and homogenized for 10 min at 1250 rpm (at 5, 25 or 45 °C) (Fisher-Bioblock Scientific AM 3001K, Illkirch, France). The obtained colloidal dispersion was concentrated by rotary evaporator for 20 min at 35°C / 43mBar (BÜCHI Rotavapor R-125, Heating Bath B-491, Vacuum pump V-700, recirculating Chiller F-108, Flawil, Switzerland) to eliminate ethanol. Posteriorly, the formed nanocapsules were filtered through a 5 µm minisart NML membrane (Sartorius GmbH, Goettingen, Germany). The obtained nanocapsules dispersion were purified by dialysis (Spectra/Por Biotech membranes, cellulose ester, 100,000 g/mol molecular weight cut off (MWCO), Rancho Dominquez, CA, USA) against ultrapure water three times for 60 min and once overnight to remove non associated chitosan. After dialysis, the nanocapsules were stored at +4°C for 24 hours before characterization.

2.4 Experimental design and nanocapsules optimization process

In the present study, a 2^3 full-factorial experimental design with center points leading to 11 experimental randomized runs was used to optimize formulation and process parameters for the preparation of copaiba oil-loaded chitosan decorated-

poly(isobutylcyanocrylate) nanocapsules. For the optimization of the preparation of the nanocapsules, three independent variables including, the pH of the polymerization media (x_1) (3, 6 and 9), the temperature of production (x_2) (5, 25, 45 °C) and the concentration of chitosan 20 kDa (x_3) (0.3, 0.6, 0.9 %) were selected. Each variable was set at a low, middle and high level. The size and zeta potential of nanocapsules were chosen as the dependent output response variables. The effects of the studied variables were graphically and statistically interpreted using the Statistic software (Version 7.0, StatSoft Inc., USA) to validate the statistical design. Response surface plots were generated to visualize the simultaneous effect of each variable on each response parameter.

2.5 Characterization of the nanocapsules.

Size measurement

Hydrodynamic mean diameter and size distribution of the nanocapsules were determined at 25°C by quasi-elastic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Orsay, France). The scattered angle was fixed at 90°. The samples were diluted 1:100 before analysis. Each measurement was done in triplicate, and the average effective diameter and polydispersity were recorded.

Determination of the zeta potential

Zeta potential of the nanocapsules was deduced from the electrophoretic mobility by Laser Doppler Electrophoresis (Zetasizer Nano ZS90 (Malvern Instruments Ltd, Orsay,

France). Nanocapsules suspensions were diluted (1:100) with NaCl at 1 mmol/L. Values are presented as mean from three replicate samples.

Morphology of nanocapsules

Transmission electron microscopy (TEM) analysis of copaiba oil-loaded chitosan-decorated poly(isobutylcyanocrylate) nanocapsules was performed using a JEOL 1400 apparatus (JEOL Ltd, Tokyo, Japan), and Gatan CCD digital camera (Orius SC1000) high-resolution to investigate the morphology of formed samples. Nanocapsules were directly observed at 60kV after staining with phosphotungstic acid 2% (pH 7.4) for 30 seconds.

2.6 Analysis of the encapsulated copaiba oil

Copaiba oil composition was analyzed by gas chromatography- Flame Ionization Detector. PR2100 gas chromatography (Alpha MOS, Toulouse, France) equipped with 5% Phenyl Polysilphenylene-siloxane (SGE Analytical Science Pty Ltd, Victoria, Australia) non polar fused silica capillary column (25 m × 0.32 mm i.d., 0.5 µm) film thickness coated with cross-linked was used. This method was previously validated for the analysis of the composition of copaiba oil (Xavier-Junior, Chapter I, 2015b). Samples were diluted with dichloromethane and 1.0 µL was injected in the chromatograph. The operating conditions to the samples were: oven temperature program from 90 °C (2 °C min^{-1}) to 150 °C, after isothermally heating 20 °C.min^{-1} to 300 °C, kept for 5 min at the final temperature. Split injection was 20 mL.min^{-1}, carrier

gas helium, flow rate 1 mL.min^{-1}, temperature of injector and detector fixed at 250 °C and 300 °C, respectively. Composition of the major compounds present in the copaiba oil encapsulated in the nanocapsules was analyzed and compared with that of the initial oil taken prior to encapsulation.

2.7 Determination of encapsulation efficiency, encapsulation rate and concentration in the nanocapsule dispersion

For the determination of the encapsulation efficiency, the encapsulationn rate and the concentration of copaiba oil in the nanocapsules, samples were prepared as explained bellow prior to their analysis by gas chromatography .

Copaiba oil-loaded chitosan-decorated poly(isobutylcyanocrylate) nanocapsules were recovered by an ultrafiltration method. The nanocapsules were centrifugated in a Microcon centrifugal filter unit (Ultracel YM-100, regenerated cellulose, Merck Millipore**,** Billerica, MA, USA) at a speed of 10,000 rpm for 20 min (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) to remove the dispersion phase. Copaiba oil-loaded chitosan-decorated poly(isobutylcyanocrylate) nanocapsules were separated in the different fractions. Nanocapsules dispersion, nanocapsules retained on the membrane and dispersion phase (i.e. the ultrafiltrate). These fractions were then ressupensed in 1 mL of dichloromethane, sonicated for 1 hour and filtered through a 0.22 μm millipore filter. β-Caryophyllene on trapped in copaiba oil containing nanocapsules was analyzed by gas chromatography, as previously described. The encapsulation efficiency was calculated as follows (Equation 1):

$$EE\ (\%) = \frac{(\text{total amount of copaiba oil used - amount of copaiba oil unloaded})}{\text{total amount of copaiba oil used}} \times 100 \quad \text{(Eq1)}$$

The nanocapsule concentration in the dispersion was evaluated by gravimetry. 1g of the purified nanocapsule dispersion was freeze-dried and the dry residue was weighted to deduce the percentage of nanocapsules contained in 1g of the dispersion. The encapsulation rate was determined by the ratio between weights of the β-caryophyllene on trapped in copaiba oil loaded on the nanocapsules and the total weight of the nanocapsule analyzed by gas chromatography.

2.8 Statistical analysis

The results of these experiments were compared using analysis of variance (ANOVA), which was able to determine if the variables and the interactions between variables were significant. Regression model, t-tests and F-test with a 95% confidence level ($p<0.05$) were performed. To statistical analysis were used the Graph Pad Prism (Version 5.0, La Jolla, CA, USA) and Statistic software (Version 7.0, StatSoft Inc., USA).

3 RESULTS AND DISCUSSION

Copaiba oil nanocapsules were obtained from a new method of interfacial polymerization of poly (isobutylcyanocrylate) performed with chitosan and in absence

of surface active compounds. Preliminary studies were performed in order to identify the best substances to nanocapsule production. In this context, chitosan of different molecular weight (20, 70 and 250 kDa) were investigated. Copaiba resin and essential oils were also used to produce nanocapsules. In addition, selection of an ideal solvent to solubilize the compounds of the organic phase was achieved based on the use of ethanol, 2-propanol and acetone. Nanocapsules obtained were characterized by measurement of the particle size and zeta potential (Figure 1).

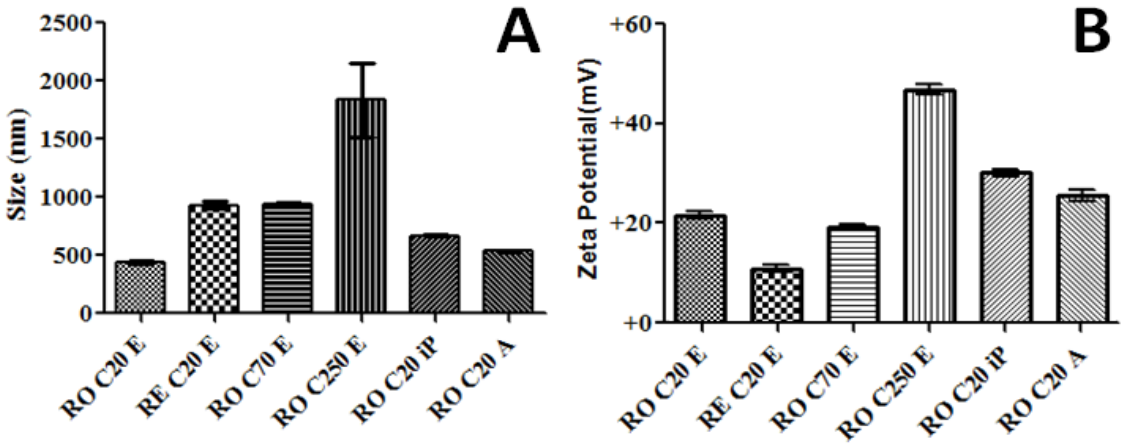

Figure 1- Particle size (A) and zeta potential (B) of copaiba oil nanocapsules with chitosan coated in the surface. Wherein RO and RE corresponds to nanocapsules produced with copaiba resin and essential oils, respectively; C20, C70 and C250 referred to the three different chitosan molecular weight of 20, 70 and 250 kDa; and E, iP and A indicated the type of organic solvent used, ethanol, 2-propanol and acetone respectively, used to nanocapsules production.

As can be seen in the Figure 1 A, the mean diameters of the nanocapsules ranged from 440 ± 8 nm for copaiba resin oil and 932 ± 28 nm for copaiba essential oil. The size increase observed in these nanocapsules probability are associated to their concentration process, since the ethanol and water evaporation at reduced pressures may promote diffusion (drag) of essential oil molecules from nanocapsule. Thus, the oil can promote a positive pressure into the capsule, and this fact may be associated to expansion and size increase of the system. Nanocapsules prepared with higher molecular weight chitosan presented a significant increase of the size, compared to those prepared with smaller molecular weight chitosan (Figure 1A). Saremi *et al.* suggested that this may be due to the higher viscosity of polymeric droplets of higher molecular weight chitosan (Saremi *et al.*, 2013). Also worth noting the morphological differences observed in the nanocapsules by TEM (Figure 2). Nanocapsules obtained with 20 and 70 kDa chitosan were spherical and uniform suggesting that the oily core was surrounded by an envelope typically characteristic of these systems. However, the nanocapsule obtained with 250 kDa chitosan appeared as elliptical structure. Formation of such elliptical structure may be possible due to different spatial conformations related to the viscosity of the reaction medium (Pastoriza-Santos & Liz-Marzán, 2009). However because of the size of the particle it should not completely discarded that the nanocapsule shape was modified during drying on the grid due to the softness of its cavity filled by the oil. Although it will be necessary to confirm the shape of these nanocapsules and these objects are far too large regarding the aim of the study, these images clearly showed that large objects encapsulating copaiba oil in a polymer membrane can be produced.

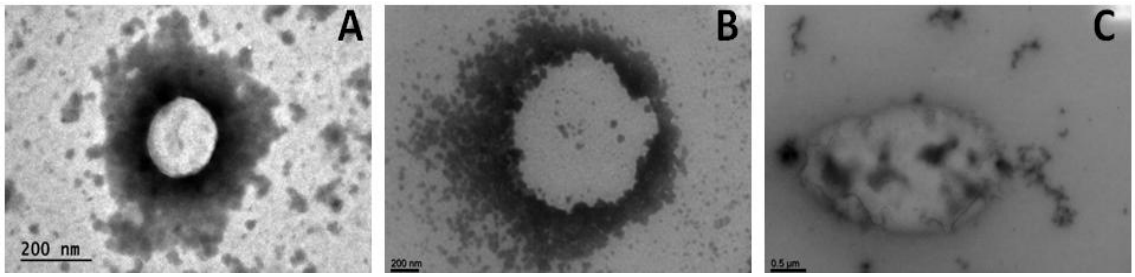

Figure 2- Transmission electron microphotographs of the nanocapsules at different molecular weight chitosan. Copaiba oil-loaded poly(isobutylcyanocrylate) nanocapsules coated with Chitosan 20 (A), 70 (B) and 250 kDa (C). Scale bar A and B 200nm; and C 0.5µm

Organic solvents play an important role in nanocapsules development. Solvents are responsible for the dissolution of the oil and/or drug, and rapid nanoparticle formation as a process due to differences in surface tension, wherein aqueous phase pulls more strongly on the surrounding liquid than one with a low surface tension. Consequently, turbulences and thermal inequalities at the interface of both liquids cause violent spreading of the solvent flow away from regions of low surface tension and the polymer that formed by rapid polymerization tends to aggregate on the oil surface and forms the nanocapsule envelope (Quintanar-Guerrero *et al.*, 1998). Copaiba resin oil-loaded in nanocapsules coated with chitosan formulated with ethanol, 2-propanol and acetone as solvent phase presented average particle size of 440 ± 8, 669 ± 8 and 536 ± 7 nm, respectively (p<0.05) (Figure 1A).

Concerning the electrophoretic mobility results, all nanocapsules presented positive values of zeta potential indicating the presence of chitosan on the nanocapsule surface.

The charges on the nanocapsule envelope were statistically different depending on conditions of preparation. Zeta potential was also an important index to judge for the stability of nanoparticle dispersions. Nanocapsules with high absolute value of zeta potential can be stabilized by repulsive electrostatic forces preventing aggregation of the particles in the dispersion (Zhang & Feng, 2006). Zeta potential of formulations containing copaiba essential oil was much lower although still positive (less than +12 mV) (Figure 1B).

Based on results obtained from this series of preparation, optimization of the procedure of preparation of chitosan-coated nanocapsules containing copaiba oil was pursue using chitosan 20kDa, ethanol and copaiba resin oil. The optimization was carried out following a 2^3 full-factorial experimental design approach with center points. The pH of the polymerization media (x_1), the temperature of production (x_2) and the concentration of chitosan 20 kDa (x_3) were selected as independent variables for nanocapsules optimization. The pH of the polymerization medium was considered in regard with the mechanism of polymerization of isobutylcyanoacrylate which is highly sensitive to pH and in regard with the solubility properties of chitosan which depends on hydroxyl and hydrogen ion concentrations. The amount of chitosan added in the medium was also considered important as this component was assumed to insure stability of the formed nanocapsule and was expected to confer mucoadhesive proprieties to the nanocapsules. Temperature was included as design variable because it may obviously influence interactions between components during the formation of the nanocapsules. Thus, chitosan 20 kDa in the different concentrations, temperature and the pH of the polymerization media were studied in order to perform the optimization of the preparation of the nanocapsule according to the experimental design presented in the

Table 1. Optimization was aimed to obtain small and stable nanocapsules which parameters were evaluated by measuring the size and zeta potential of each preparation.

Table 1: Variables and levels chosen to define the experimental region and their corresponding coded values for nanocapsule production

	Independent Variable	***Level***		
i	**x_i**	**-1**	**0**	**+1**
1	**pH**	3	6	9
2	**Temperature (°C)**	5	25	45
3	**Chitosan 20kDa concentration (%)**	0.3	0.6	0.9
	Dependent Variable (y_i)	***Desired Response***		
1	**Mean globule size (nm)**	Minimize		
2	**Zeta potential (mV)**	Maximize		

The mean particle hydrodynamic diameter was strongly influenced by the variables selected for the study emphasizing their relevancy. The size value varied from 200 to 1200 nm. Standardized effects of the independent variables and their interactions on the dependent variable were investigated by preparing a Pareto chart (Figure 3). The length of each bar in the chart indicated the standardized effect of the corresponding variable on the response. Negative values in the response of the standardized effects indicated unfavorable or antagonistic effect on the nanocapsule development, while positive coefficients of the response of the standardized effects showed a favorable or synergistic effect.

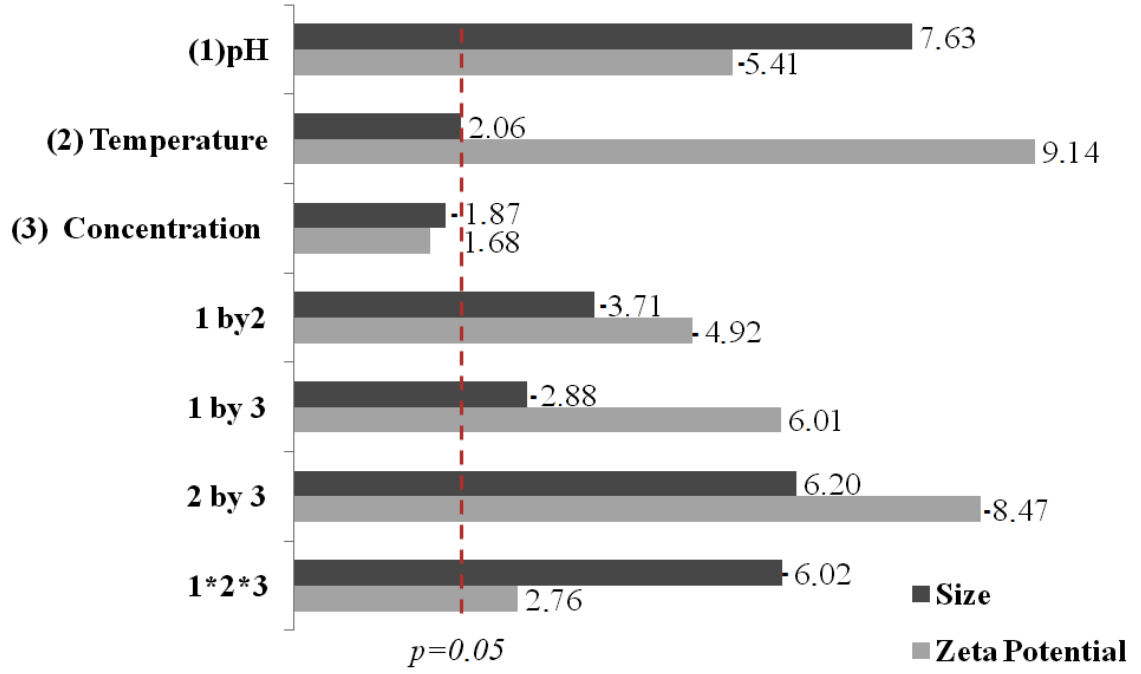

Figure 3: The Pareto Chart of standardized effects to size and zeta potential dependent variables (p<0.05)

According to the Pareto's chart, an increase of the pH of the polymer medium had a statistically positive effect in the increase of nanocapsule droplet size. Additionally, the interaction of this variable with the other studied variables contributed to modify the nanocapsule size. This modification of size may be due to the hydration of free-amino groups of chitosan in acid solution (pH of 3.0). With a pKa value of 6.3, chitosan is a polycation when dissolved in acid solution and its amino groups are protonated giving free $-NH_3^+$ sites (Ravikumara & Madhusudhan, 2011). The increase in particle size at pH 9 might be due to a lower degree of protonation of the amino groups of chitosan (Zhao *et al.*, 2002). At low pH, the reaction rate is too slow to allow the formation of small particles, corroborating the findings found by McCarron et al (Mccarron *et al.*, 1999). Although the polymerization was slow down which gave possibility of

coalescence of the emulsion droplets before polymerization of the isobutylcyanoacrylate required to form the nanocapsule envelope. While at high pH, a too fast and uncontrolled polymerization may produce polymer aggregates.

The increase of the temperature contributed for a particle size increment. The concentration of chitosan used when analyzed alone did not show significant effect on the size of the nanocapsule produced. However, the high concentration of chitosan in association with temperature variable showed a positive significant effect. In the acid solution at high concentration of chitosan, the increase of the temperature from 5 to 45 °C provoked the increase of the nanocapsule size from 250 to 1070 nm, while in basic polymerization medium the temperature variation did not show significant size changes (size of 930 nm). Opposite effect in the nanocapsules size, was observed between the concentration of chitosan and the pH of the medium. The concentration of chitosan, the temperature and the pH of the polymerization medium are parameters that have an influence on the viscosity of the polymerization medium. In turn, the viscosity of the polymerization medium may be an important factor that can promote the ideal interaction among the compounds in nanocapsule production. An increase of the temperature decreases the viscosity of the polymerization medium, due to the increase of the thermal motion of the polymer with temperature (Desbrieres, 2002; El-Hefian *et al.*, 2010). The viscosity of the polymerization medium also decreased while reducing the concentration in chitosan which is a macromolecule. It is also decreased when the pH reached acid values due to the screening effect of anionic groups of chitosan in solution (Wang *et al.*, 1994; Martínez-Ruvalcaba *et al.*, 2004).

The significance of independent variables and their interactions were tested by analysis of variance. An alpha-level of 0.05 was used to determine the statistical significance

between all analyses. The model's goodness of fit was checked by the coefficient of determination (R^2). The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions (fluctuation) (Cochran & Cox, 1957). These experiments were determined statistically significant with linear relationship of R^2=0.96, indicating that 96% of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (Adj R^2 = 0.94) was also very important to confirm a high significance of the model. These ensured a satisfactory adjustment of the polynomial model to the experimental data (Liu *et al.*, 2004). For this study, the adjusted R^2 was very close to the experimental R^2 value.

By applying multiple regression analysis on the design matrix and analyzing the responses given in the experiments, the first-order polynomial equation given in equation 2 in the coded form was established to size droplets:

$$Y_1 = 834 + 214x_1 + 58x_2 - 104x_1x_2 - 81x_1x_3 + 174x_2x_3 - 169x_1x_2x_3 \quad \text{(Eq2)}$$

Where Y_1 was the predicted droplets size (nm), x_1, x_2 and x_3 are the coded terms for three independent test variables including the pH, the temperatures and the concentration of the chitosan, respectively.

According to the regression model's ANOVA, it was possible to observe that the linear model was significant ($p<0.05$). This was evidenced from the Fisher's *F* –test which provided a *F*-value of the model (F_{model} = 23.5) much greater than the tabulated *F*-

value (F_{Tab}= 2.4) at the 5% level, indicating that the computed Fisher's variance ratio at this level was large enough to justify a very high degree of adequacy of the linear model and also to indicate that treatment combinations are highly significant (Liu *et al.*, 2004; Sen & Swaminathan, 2004). Since the rapport F_{model}/F_{tab} was about 10, the Fisher's *F*-test was concluded with 95% certainty that the regression model explained a significant amount of the variation in the dependent variable.

The normal (percentage) probability plot of the residuals was an important diagnostic tool to detect and explain the systematic departures from the assumptions that errors were normally distributed and were independent of each other and that the error variances are homogeneous (Liu *et al.*, 2004). In this study, a plot of normal probability of the residuals indicated almost no serious violation of the assumptions underlying the analyses (F_{model} = 3.5). This value was found to be lower than the tabulated *F*- value (F_{Tab}= 4.26) at the 5% level, indicating that the experiment exhibited predictive results (residues model *F*-calculated/ tabulated *F*- value < 1). This satisfactory normal distribution confirmed the normality assumptions previously made and the independence of the residuals.

Aiming the straight forward examination of the experimental variables on the responses, the three-dimensional response surfaces were used. The Figure 4 (A) shows a three-dimensional diagram of calculated size response surface relating both pH and temperature to copaiba oil nanocapsule size. It can be observed that the decrease in pH and temperature reduced the size considerably. The linear nature of the response surface demonstrated that there were considerable interactions between each of the independent variables and the nanocapsules sizes.

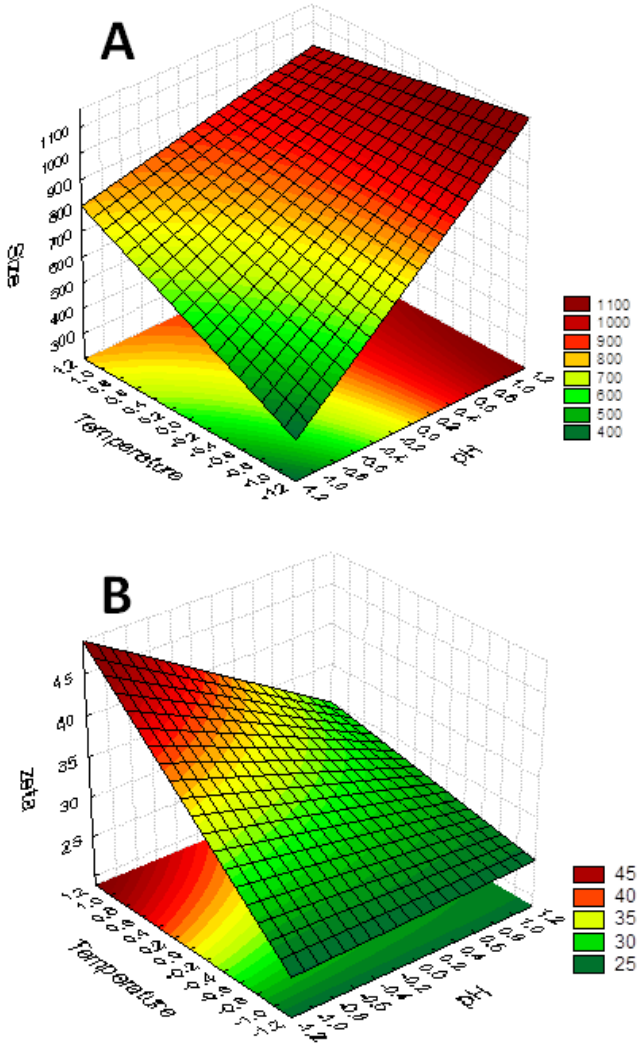

Figure 4: 3D Response surface for size droplets (A) and zeta potential (B) variable dependency with temperature and pH independents variables to production of nanocapsules.

Regarding the zeta potential, the copaiba oil nanocapsules showed values ranging from + 15 to + 55 mV. These positive values might be explained by the exposure of chitosan's positive charges on the nanocapsule surface. Otherwise, poly(isobutylcyanocrylate) nanocapsules obtained by the same method but without

chitosan generally show negative values (Aboubakar, Puisieux, Couvreur, Deyme, *et al.*, 1999; Cournarie *et al.*, 2004).

In the zeta potential analysis, the standardized effects of the independent variables and their interactions on the dependent variable were also investigated by preparing a Pareto chart. The increasing temperature and the decreasing pH enhanced positively surface charge more significantly than the chitosan concentration variable (Figure 3). Possibly, because the polymer revealed itself to be more soluble and protonated, it exposed the carbohydrate free amino groups easily, being responsible for the increasingly positive zeta potential values. The concentration of the chitosan in polymerization medium when analyzed individually showed no significant difference in the amount of surface charges in the nanocapsules. However, the interaction of chitosan concentration with the other studied variables showed significant changes in the nanocapsules charges.

These experiments presented the linearity regression of R^2=0.94, therefore this model explains 94% of the variability in the response. The value of the adjusted determination coefficient (Adj R^2 = 0.92) presented a high significance of the model. The *F* test applied in the mathematical model shows the significative (Regression model *F* calculated/ *F* tabulated > 10) and predictive results (residues model *F* calculated/ tabulated *F* value < 1) of this experiments. In addition, the full first-order response surface was plotted for analysis of the optimal zeta potential on the nanocapsules (Figure 4 B). High positive values can be achieved by maintaining a low pH and increasing the temperature of the polymerization medium. The high temperature promotes particle size increase, however the opposite does not change the charge of the zeta potential sufficiently to cause destabilization of the system.

The result of the optimization study following a 2^3 full-factorial experimental design approach allowed to identify the optimal conditions for the nanocapsules preparation that have both a small particle size and positive zeta potential as initially researched. The optimal characteristics of the nanocapsule preparation were pH 3, temperature 4 °C and with a concentration of chitosan of 0.9 % (w/v). The samples prepared under these conditions showed a droplet size of 473 ± 1 nm, the size distribution was uni-modal with a polydispersion index of 0.20 ± 0.02, and a zeta potential of +34.8 ± 0.2 mV.

TEM analysis showed that the optimal nanocapsules prepared by interfacial polymerization were spherical and not aggregated (Figure 5 A). The diameter of the nanocapsules observed by microscopy agreed well with particle sizes determination by dynamic light scattering. In addition, the nanocapsules had an oily core surrounded by an envelope typically characteristic of these systems. Also in Figure 5 B, it can be observed the dark details in the nanocapsule surface around the oil core and poly(isobutylcyanocrylate) envelope decorated with chitosan, confirming effective coating of this polymer on the system developed.

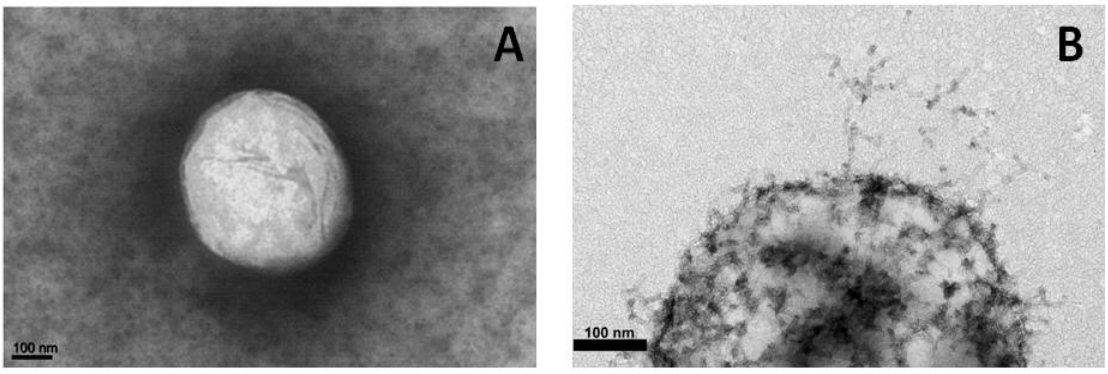

Figure 5: Transmission electron microscopy analysis of the optimal formulation of copaiba oil- loaded chitosan- poly (isobutylcyanocrylate) nanocapsules stained with phosphotungstic acid (2%) at 60 kV (Imagif). Scale bar of 100nm.

The gas chromatography analysis was performed to characterize the copaiba oil recovered from the nanocapsule and compared it with the chromatogram obtained from the oil before nanoencapsulation (Figure 6). The Figure 6C gives the difference in the area of the peaks between the 2 samples. All components found in the original oil were also found in the nanocapsules. The differences in concentrations of the components present in copaiba oil were less that 2% which is within the range of precision of the gas chromatography method used for the determination. In addition, these results indicated that there was no modification of the oil during the preparation of the nanocapsules. The method preserved well the oil which was encapsulated under its native form.

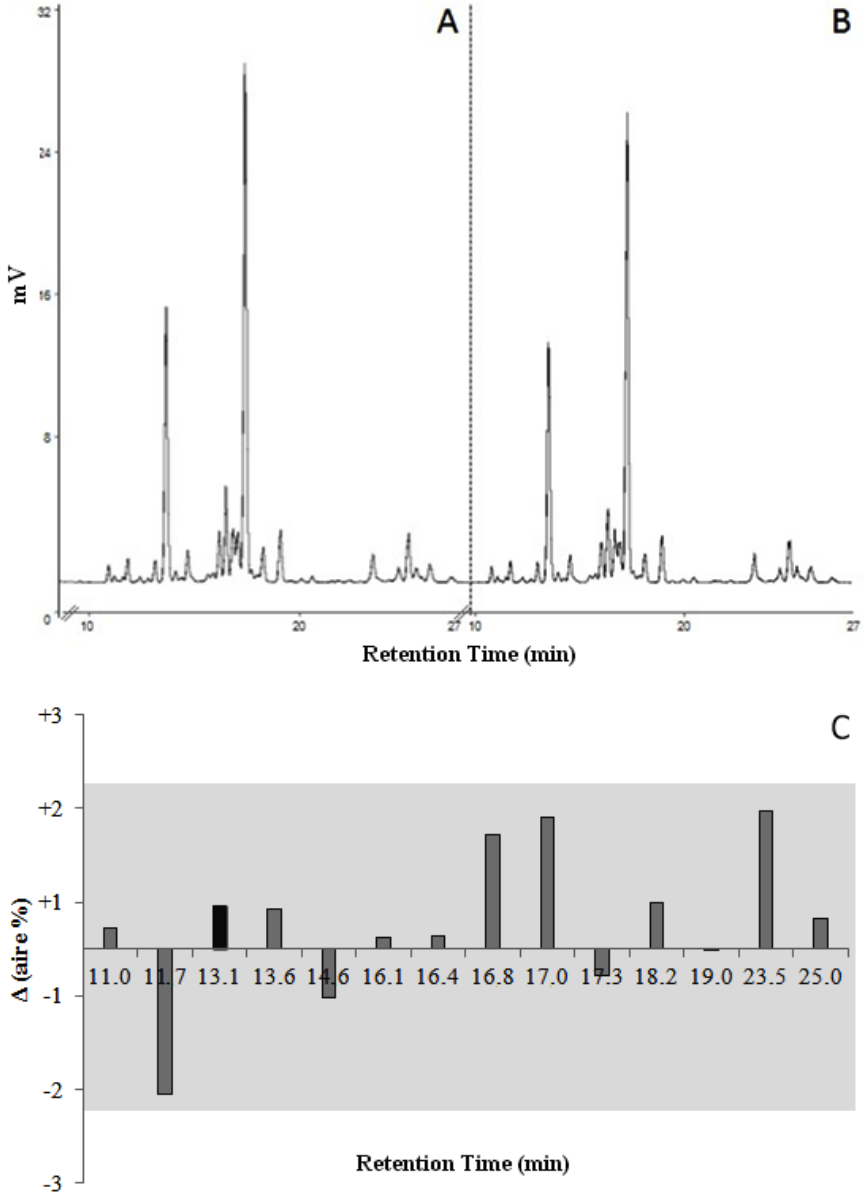

Figure 6: Major compounds encapsulated in copaiba resin oil-nanocapsules coated with chitosan. Figures A and B represent the copaiba resin oil chromatograms before and after recovery from the nanocapsule. Figure C gives the difference in the area of the peaks between the copaiba resin oil in native form and the oil encapsulated in the nanocapsules. The gray color represents the precision of the method to determination of copaiba oil compounds

The encapsulation efficiency of copaiba oil was high at 75.8 ± 3 %. It could be calculated that the nanocapsule dispersion contained 2.5 mg of β-caryophyllene encapsulated in the nanocapsules per mL of the dispersion while the β-caryophyllene loading efficiency was 55.5 µg /mg of nanocapsules. This high encapsulation indicated that over the course of the preparation, copaiba oil immediately diffused in the internal phase of nanocapsules, and was then encapsulated by the polymer coat after addition of the organic phase into the polymerization medium. These high values are an important parameter that was used to evaluate nanocarriers and may improve therapeutic effects and lower the dose of drug required (Zhao *et al.*, 2013).

β-Caryophyllene presented in copaiba resin oil-nanocapsules coated with chitosan show a major potential impact in therapeutic application on human health. Klauke *et al.* suggested an average daily β-caryophyllene intake in the range of 10- 200 mg, which corresponds to human daily dose of 0.16- 3.3 mg/kg of the β-caryophyllene for a 60 kg human (Klauke *et al.*, 2014). Thanks to potential mucoadhesive of these nanocapsules and the high concentration of β-caryophyllene encapsulated, it can be expected to achieve a therapeutic dose even smaller and more efficient than currently suggested (0.18- 3.6 g of nanocapsules). In addition, studies carried out by Legault *et al.* suggest the β-caryophyllene may accumulate in the membranes of cancer cells and increase membrane permeability of bioactive compounds (Legault & Pichette, 2007). Thus, it is expected that the β-caryophyllene presented in copaiba resin oil-nanocapsules coated with chitosan may be capable to increase intracellular accumulation of lipophilic anticancer drugs by the oral route and consequently enhance potential anticancer activity of another anticancer drug associated with the nanocapsules thanks to a synergetic effect between the oil and a co-encapsulated drug in the nanocapsules.

4 CONCLUSION

Poly(isobutylcyanocrylate) nanocapsules incorporating copaiba oil could be prepared by the method of interfacial polymerization while surfactant was omitted and replaced by chitosan. As assumed, chitosan associated with the nanocapsules conferring positives charges to the nanocapsule surface. Experimental design approach was useful for the optimization of the formulation to provide nanocapsules of small diameter and high positive zeta potential. This approach also highlighted the role of three important variables that may vary during the preparation of the nanocapsules and that greatly influenced the nanocapsule characteristics. Copaiba oil was encapsulated at high encapsulation efficiency and the composition of the encapsulated oil was identical to that of the native oil. These nanocapsules are expected to be mucoadhesive and suitable to serve as carrier system for lipophilic anticancer drugs by the oral route with possible synergistic effect between the oil and the drug.

ACKNOWLEDGEMENTS

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REFERENCES

ABOUBAKAR, M., COUVREUR, P., PINTO-ALPHANDARY, H., GOURITIN, B., LACOUR, B., FARINOTTI, R., PUISIEUX, F. & VAUTHIER, C. 2000. Insulin-loaded

nanocapsules for oral administration: In vitro and in vivo investigation. *Drug Dev Res*, 49, 2, 109-117.

ABOUBAKAR, M., PUISIEUX, F., COUVREUR, P., DEYME, M. & VAUTHIER, C. 1999. Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization. *J Biomed Mater Res*, 47, 4, 568-76.

ALENCAR, E. N., XAVIER-JUNIOR, F. H., MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G. & EGITO, E. S. T. 2015. Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. *J Nanosci Nanotechnol*, 15, 880-888.

ANTON, N., BENOIT, J. P. & SAULNIER, P. 2008. Design and production of nanoparticles formulated from nano-emulsion templates - A review. *J Control Release*, 128, 3, 185-199.

BERNKOP-SCHNURCH, A., HEINRICH, A. & GREIMEL, A. 2006. Development of a novel method for the preparation of submicron particles based on thiolated chitosan. *Eur J Pharm Biopharm*, 63, 2, 166-72.

BRAVO-OSUNA, I., MILLOTTI, G., VAUTHIER, C. & PONCHEL, G. 2007. In vitro evaluation of calcium binding capacity of chitosan and thiolated chitosan poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Int J Pharm*, 338, 1-2, 284-90.

BRAVO-OSUNA, I., VAUTHIER, C., CHACUN, H. & PONCHEL, G. 2008. Specific permeability modulation of intestinal paracellular pathway by chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles. *Eur J Pharm Biopharm*, 69, 2, 436-44.

BRAVO-OSUNA, I., VAUTHIER, C., FARABOLLINI, A., PALMIERI, G. F. & PONCHEL, G. 2007. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomater*, 28, 13, 2233-43.

CHEN, C. K., WANG, Q., JONES, C. H., YU, Y., ZHANG, H., LAW, W. C., LAI, C. K., ZENG, Q., PRASAD, P. N., PFEIFER, B. A. & CHENG, C. 2014. Synthesis of pH-responsive chitosan nanocapsules for the controlled delivery of doxorubicin. *Langmuir*, 30, 14, 4111-9.

COCHRAN, W. G. & COX, G. M. E. D. Experimental designs. New York: Wiley, 1957. 346-354.

COURNARIE, F., CHERON, M., BESNARD, M. & VAUTHIER, C. 2004. Evidence for restrictive parameters in formulation of insulin-loaded nanocapsules. *Eur J Pharm Biopharm*, 57, 2, 171-9.

COUVREUR, P., KANTE, B., ROLAND, M., GUIOT, P., BAUDUIN, P. & SPEISER, P. 1979. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J Pharm Pharmacol*, 31, 5, 331-2.

CRUZ, L., SCHAFFAZICK, S. R., DALLA COSTA, T., SOARES, L. U., MEZZALIRA, G., DA SILVEIRA, N. P., SCHAPOVAL, E. E., POHLMANN, A. R. & GUTERRES, S. S. 2006. Physico-chemical characterization and in vivo evaluation of indomethacin ethyl ester-loaded nanocapsules by PCS, TEM, SAXS, interfacial alkaline hydrolysis and antiedematogenic activity. *J Nanosci Nanotechnol*, 6, 9-10, 3154-62.

DESBRIERES, J. 2002. Viscosity of semiflexible chitosan solutions: influence of concentration, temperature, and role of intermolecular interactions. *Biomacromolecules*, 3, 2, 342-9.

EL-HEFIAN, E. A., ELGANNOUDI, E. S., MAINAL, A. & YAHAYA, A. H. 2010. Characterization of chitosan in acetic acid: Rheological and thermal studies. *Turk J Chem*, 34, 1, 47-56.

FALLOUH, N. A., ROBLOTTREUPEL, L., FESSI, H., DEVISSAGUET, J. P. & PUISIEUX, F. 1986. Development of a New Process for the Manufacture of Polyisobutylcyanoacrylate Nanocapsules. *Int J Pharm*, 28, 2-3, 125-132.

FESSI, H., PUISIEUX, F., DEVISSAGUET, J. P., AMMOURY, N. & BENITA, S. 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. *Int J Pharm*, 55

GÅSEROD, O., JOLLIFFEB, I. G., HAMPSONB, F. C., DETTMARB, P. W. & SKJÅK-BRÆKA, G. 1998. The enhancement of the bioadhesive properties of calcium alginate gel beads by coating with chitosan. *Int J Pharm*, 175, 2, 237–246.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-92.

GUTERRES, S. S., ALVES, M. P. & POHLMANN, A. R. 2007. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. *Drug Target Insights*, 2, 147-57.

ILLUM, L., JABBAL-GILL, I., HINCHCLIFFE, M., FISHER, A. N. & DAVIS, S. S. 2001. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev*, 51, 1-3, 81-96.

KLAUKE, A. L., RACZ, I., PRADIER, B., MARKERT, A., ZIMMER, A. M., GERTSCH, J. & ZIMMER, A. 2014. The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. *Eur Neuropsychopharmacol*, 24, 4, 608-20.

LEANDRO, L. M., VARGAS F, S., BARBOSA, P. C., NEVES, J. K., SILVA, J. A. & VEIGA-JUNIOR, V. F. 2012. Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins. *Molecules*, 17, 4, 3866-89.

LEGAULT, J. & PICHETTE, A. 2007. Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. *J Pharm Pharmacol*, 59, 12, 1643-7.

LEITE, E. A., GRABE-GUIMARAES, A., GUIMARAES, H. N., MACHADO-COELHO, G. L., BARRATT, G. & MOSQUEIRA, V. C. 2007. Cardiotoxicity reduction induced by halofantrine entrapped in nanocapsule devices. *Life Sci*, 80, 14, 1327-34.

LIU, H. L., LAN, Y. W. & CHENG, Y. C. 2004. Optimal production of sulphuric acid by Thiobacillus thiooxidans using response surface methodology. *Process Biochem* 39, 12, 1953-1961.

MARTÍNEZ-RUVALCABA, A., CHORNET, E. & RODRIGUE, D. 2004. Dynamic rheological properties of concentrated chitosan solutions. *J Text Stud*, 35, 53-74.

MCCARRON, P. A., WOOLFSON, A. D. & KEATING, S. M. 1999. Response surface methodology as a predictive tool for determining the effects of preparation conditions on the physicochemical properties of poly(isobutylcyanoacrylate) nanoparticles. *Int J Pharm*, 193, 1, 37-47.

PASTORIZA-SANTOS, I. & LIZ-MARZÁN, L. M. 2009. N,N-Dimethylformamide as a Reaction Medium for Metal Nanoparticle Synthesis. *Adv Funct Mater*, 19, 5, 679-688.

PATEL, B. K., PARIKH, R. H. & ABOTI, P. S. 2013. Development of oral sustained release rifampicin loaded chitosan nanoparticles by design of experiment. *J Drug Deliv*, 2013, 370938.

PETIT, B., BOUCHEMAL, K., VAUTHIER, C., DJABOUROV, M. & PONCHEL, G. 2012. The counterbalanced effect of size and surface properties of chitosan-coated

poly(isobutylcyanoacrylate) nanoparticles on mucoadhesion due to pluronic F68 addition. *Pharm Res*, 29, 4, 943-52.

PINTO REIS, C., NEUFELD, R. J., RIBEIRO, A. J. & VEIGA, F. 2006a. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomed*, 2, 1, 8-21.

PONCHEL, G. & IRACHE, J. 1998. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv Drug Deliv Rev*, 34, 2-3, 191-219.

QUINTANAR-GUERRERO, D., ALLEMANN, E., FESSI, H. & DOELKER, E. 1998. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. *Drug Dev Ind Pharm*, 24, 12, 1113-28.

RAVIKUMARA, N. R. & MADHUSUDHAN, B. 2011. Chitosan nanoparticles for tamoxifen delivery and cytotoxicity to MCF-7 and Vero cells. *Pure Appl Chem*, 83, 11, 2027-2040.

SANTOS, A. O., UEDA-NAKAMURA, T., DIAS FILHO, B. P., VEIGA JUNIOR, V. F., PINTO, A. C. & NAKAMURA, C. V. 2008. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the Copaifera genus. *Mem Inst Oswaldo Cruz*, 103, 3, 277-81.

SAREMI, S., DINARVAND, R., KEBRIAEEZADEH, A., OSTAD, S. N. & ATYABI, F. 2013. Enhanced oral delivery of docetaxel using thiolated chitosan nanoparticles: preparation, in vitro and in vivo studies. *Biomed Res Int*, 2013, 150478.

SEN, R. & SWAMINATHAN, I. 2004. Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. *Biochem Eng J*, 21, 2, 141-148.

SOUSA, J. P., BRANCALION, A. P., SOUZA, A. B., TURATTI, I. C., AMBROSIO, S. R., FURTADO, N. A., LOPES, N. P. & BASTOS, J. K. 2011. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal*, 54, 4, 653-9.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001. Oral drug absorption enhancement by chitosan and its derivatives. *Adv Drug Deliv Rev*, 52, 2, 117-26.

VEIGA JUNIOR, V. F., ROSAS, E. C., CARVALHO, M. V., HENRIQUES, M. G. & PINTO, A. C. 2007. Chemical composition and anti-inflammatory activity of copaiba

oils from Copaifera cearensis Huber ex Ducke, Copaifera reticulata Ducke and Copaifera multijuga Hayne--a comparative study. *J Ethnopharmacol*, 112, 2, 248-54.

WANG, W., WANG, L. & QIN, W. 1994. Dilute solution behavior of chitosan in different acid solvents *Chin J Polym Sci*, 12, 1

XAVIER-JUNIOR, F. H., MACIUK, A., MORAIS, A. R. V., ALENCAR, E. N., REHDER, V. L. G., EGITO, E. S. T., VAUTHIER, C. Chapter I, 2015b. Development of gas-chromatography method for the analysis of copaiba oil. *Article in preparation*

ZHANG, Z. & FENG, S.-S. 2006. The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)–tocopheryl polyethylene glycol succinate nanoparticles. *Biomater*, 27, 21, 4025-4033.

ZHAO, K. S., ASAMI, K. & LEI, J. P. 2002. Dielectric analysis of chitosan microsphere suspensions: study on its ion adsorption. *Colloid Polym Sci*, 280, 11, 1038-1044.

ZHAO, Y. Q., WANG, L. P., MA, C., ZHAO, K., LIU, Y. & FENG, N. P. 2013. Preparation and characterization of tetrandrine-phospholipid complex loaded lipid nanocapsules as potential oral carriers. *Int J Nanomedicine*, 8, 4169-4181.

Chapter VIII

Preparation of paclitaxel-loaded chitosan- poly (isobutylcyanoacrylate) core-shell nanocapsules and evaluation of their mucoadhesion by in vitro methods

Le dernier chapitre de cette thèse présente les résultats de l'étude visant à évaluer la mucoadhésion du paclitaxel encapsulé dans les nanocapsules d'huile de copaïba développées précédemment (Chapitre VII). Ces nanocapsules de poly(cyanoacrylate d'isobutyle) enrobées de chitosane ont été développées pour l'administration de médicaments par voie orale. La formulation de nanocapsules de paclitaxel a été optimisée en faisant varier la concentration de l'huile de copaïba, du cyanoacrylate d'isobutyle utilisé comme monomère et du paclitaxel. La rhodamine B et [3H]-paclitaxel radioactif ont également été ajoutés à la formulation en vue de pouvoir suivre les nanocapsules et le paclitaxel au cours d'expériences menées *in vitro* et *ex-vivo* visant à évaluer les propriétés mucoadhésives des formulations. Toutes les nanocapsules produites ont été caractérisées par la taille des particules, le potentiel zêta et des observations en microscopie électronique à transmission. L'efficacité d'encapsulation et l'adhésion aux tissus muqueux du paclitaxel a été déterminée par HPLC en utilisant une méthode qui a déjà validée au début de nos travaux de thèse (Chapitre I) et par mesure de radioactivité en scintillation liquide. Une étude de stabilité des nanocapsules dans les fluides gastro-intestinaux simulés a été réalisée et une étude de stabilité au stockage après application d'un processus de séchage a été effectuée. *In vitro*, les propriétés mucoadhésives ont été explorées par la mise en œuvre d'un test d'agrégation *in-vitro* avec la mucine. Ces études ont été complétées par des travaux visant à evaluer la mucoadhésion du principe actif apporté sous forme de nanocapsules sur une muqueuse intestinale de rat fraîchement excisée sur un modèle d'étude *ex vivo* en chambre d'Ussing. Les nanocapsules chargées en paclitaxel ont montré un diamètre hydrodynamique moyen de 470 nm, un indice de polydispersité faible et une forme sphérique. L'efficacité d'encapsulation et le taux de charge en paclitaxel était de 74 ± 1% et 1,70 ± 0,02%, respectivement, ce qui correspond à une teneur 16.8 ± 0.27 µg du

paclitaxel par mg de nanocapsules. Après séchage, les nanocapsules peuvent être redispersées sans changement de leur caractéristiques de taille ni de structure. Les dispersions de nanocapsules sont apparues stables en milieu gastrique simulé sur une période de 120 minutes et après six mois de conservation à 4 °C dans l'eau milli-Q®. Le potentiel zêta était +37 mV. Cette valeur nettement positive indique la présence de chitosane sur la surface des nanocapsules. Les nanocapsules ont montré des propriétés mucoadhésives intéressantes avec les mucines. Les études menées avec le modèle d'étude de la mucoadhésion *ex-vivo* confirme le potentiel mucoadhésif des nanocapsules. Elles ont permis d'associées 3.4 g de [3H] -paclitaxel encapsulé par m² de muqueuse intestinale de rat démontrant leur potentiel à transporter le principe actif et à le fixer au plus proche des sites d'absorption.

Mots-clés: Mucoadhésion; nanocapsules; paclitaxel; livraison orale; poly (cyanoacrylate d'isobutyle); chitosane; huile de copaïba

PREPARATION OF PACLITAXEL-LOADED CHITOSAN- POLY (ISOBUTYLCYANOACRYLATE) CORE-SHELL NANOCAPSULES AND EVALUATION OF THEIR MUCOADHESION BY IN VITRO METHODS

Xavier-Junior, F.H.[1,2], Gueutin, C. [1], Chacun, H.[2], Egito, E.S.T.[1], Vauthier, C.[2]*

[1] UFRN, DFAR, Laboratório de Sistemas Dispersos (LaSiD), Natal – RN, Brazil.

[2] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 chatenay-Malabry Cedex – France.

***Corresponding author: Christine Vauthier**

Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 chatenay-Malabry Cedex – France.

christine.vauthier@u-psud.fr

ABSTRACT

The aim of this work was to study mucoadhesive property of paclitaxel encapsulated into copaiba oil containing-poly(isobutylcyanoacrylate) nanocapsules coated with chitosan designed for oral drug delivery. Samples were produced by interfacial polymerization. Formulations of paclitaxel containing nanocapsules were optimized by varying copaiba oil, isobutylcyanoacrylate and paclitaxel concentrations. Rhodamine B and radioactive [3H]-paclitaxel were also added in the formulation. All produced nanocapsules were characterized by particle size, zeta potential and transmission electron microscopy. Encapsulation efficiency of paclitaxel and paclitaxel adhering to mucosal tissue were determined by HPLC using a method that was previously validated and liquid scintillation analyses. Simulated gastrointestinal fluids, drying process and storage stability studies were performed. Mucoadhesion tests were performed by i*n-vitro* aggregation test with mucin and *ex-vivo* in Ussing Chamber using freshly excised rat intestinal mucosa. Nanocapsule-loaded paclitaxel showed a mean hydrodynamic diameter of 470 nm, a low polydispersity index and a spherical form. The encapsulation efficiency and drug loading of paclitaxel were 74 ± 1% and 1.70 ± 0.02%, respectively. After drying, nanocapsules could be redispersed with no change of the nanocapsule structure. Dispersions of nanocapsules were stable in simulated gastric medium for 120 min and after six months at 4°C. Potential zeta was + 37 mV due to the presence of chitosan on the nanocapsule surface. The nanocapsules showed interesting mucoadhesive properties with mucins. They could promote association of 9 % of the amount of [3H]-paclitaxel encapsulated with the intestinal mucosa of the rat.

Keywords: Mucoadhesion; Nanocapsules; Paclitaxel; Oral Delivery; Poly(isobutylcyanoacrylate); Chitosan; Copaiba Oil

1. INTRODUCTION

Cancer remains a major cause of death in most countries in the world, and its incidence increases over the years (Fang *et al.*, 2011). Recently, many works have been focused on the development of oral anticancer drugs to improve the ease of treatments for patients (Roger *et al.*, 2010b; Mazzaferro *et al.*, 2013a; b). Paclitaxel ($C_{47}H_{51}NO_{14}$) is a pseudoalkaloid anticancer drug with a diterpenoid structure, extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) (Wani *et al.*, 1971; Fang & Liang, 2005). This drug's mechanism of action is based on the inhibitory effect of cellular growth by hyperstabilizing the cellular microtubules. Hence inhibiting cell replication in the late G2 mitotic phase of the cell cycle which in turn leads to apoptosis (Schiff *et al.*, 1979; Horwitz, 1992). Paclitaxel has a powerful antitumor ability against a wide spectrum of cancers, such as breast and lung cancers, acute leukemia, advanced ovarian, head and neck carcinomas (Horwitz, 1992; Rowinsky & Donehower, 1995; Allen, 2002; Rivkin *et al.*, 2010). Theoretically, oral administration of paclitaxel as many other drugs are a preferable choice compared to other routes due to various advantages: higher convenience for patient hence, better compliance to treatment, lower cost and higher safety. Unfortunately, paclitaxel that is insoluble in aqueous based medium and metabolized over absorption by epithelial cells of the gut mucosa shows a limited oral bioavailability (<10%), which complicates its oral administration (Malingre *et al.*, 2001; Peltier *et al.*, 2006).

Nowadays, strategies based on the use of nanoparticles are proposed to overcome these limitations. Indeed, it was shown that association of drugs with nanoparticles may be efficient to increase bioavailability of many drugs including paclitaxel by protecting the drug against degradation and eventually enhancing the permeability across the intestinal

epithelium (Ponchel & Irache, 1998; Brigger *et al.*, 2002; Bae *et al.*, 2007; Rivkin *et al.*, 2010; Zabaleta *et al.*, 2013). Additionally, these systems can reduce toxicity controlling the biodistribution from the blood compartment and once in tissue, it can enhance delivery of the drug to resistant cancer cells over expressing the P-glycoprotein (Verdiere *et al.*, 1994; Sparreboom *et al.*, 1997; Verdiere *et al.*, 1997; Varma *et al.*, 2003; Jabr-Milane *et al.*, 2008).

A large part of published works reported the delivery of drugs including paclitaxel after association with nanospheres. Drawbacks of these systems are their generally low payload which makes the part of the drug composing the nanoparticles only few percent of the composition of the carrier. Nanocapsules are vesicles appear more suitable systems to achieve high payload especially when the drug is soluble in the component filling out the nanocapsule cavity and have favorable partition coefficient to remain in this medium during the nanocapsule preparation (Quintanar-Guerrero *et al.*, 1998; Buzea *et al.*, 2007).

In a previous work, we have designed new poly(isobutylcyanoacrylate) nanocapsules decorated with chitosan and filled with natural oil having different interesting biological activities including anticancer properties(Xavier-Junior, Chapter VII, 2015). They can be used as “passive tumor targeting” due to accumulation in certain solid tumors by the enhanced permeability and retention effect (Maeda *et al.*, 2000; Arias *et al.*, 2001; Danhier *et al.*, 2010). These systems are attractive to enhance drug delivery by the oral route as suggested from previous work carried on insulin delivery (Damge *et al.*, 1988). They were stable in gastric environment while they appeared to be rapidly translocated in the blood from the intestine despite observations of relative *in vitro* instability in simulated intestinal medium (Aboubakar *et al.*, 2000; Pinto-Alphandary *et al.*, 2003).

The new nanocapsule exhibiting chitosan on their surface are expected to demonstrate mucoadhesive properties which are assumed to further potentialize oral administration of the drug. Paclitaxel would be a suitable drug to incorporate in these nanocapsules as it was recently demonstrated that it is well soluble in copaiba oil, the component included in the cavity of the chitosan-decorated poly(isobutylcyanoacrylate) nanocapsules (Xavier-Junior, Chapter II, 2015).

The aim of the present work was to investigate the encapsulation of paclitaxel in the copaiba oil nanocapsules decorated with chitosan and to evaluate their potential to interact with the gut mucosa of rats thanks to the presence of chitosan on their surface. Optimization of the paclitaxel-loaded nanocapsules was achieved using a statistical interaction approach considering three variable parameters including the concentration of copaiba oil, the concentration in isobutylcyanoacrylate and the concentration of paclitaxel used to prepare the nanocapsules by interfacial polymerization of isobutylcyanoacrylate. Stabilitty of paclitaxel-loaded nanocapsules were then evaluated in simulated gastrointestinal fluids, under different storage conditions and after drying. Mucoadhesive properties were evaluated based on an aggregation test with mucins and on the evaluation of their retention at the level of rat intestinal mucosa mounted in Ussing Chamber.

2 MATERIALS AND METHODS

2.1 Materials

Isobutylcyanoacrylate was provided by ORAPI engineered solutions worldwide (Vaulx-en-Velin, France). Copaiba oil was purchased from Flores & Ervas (Piracicaba, SP,

Brazil). Chitosan 20,000 Da was purchased from Amicogen (Jinju, South Gyeongsang, South Korea). PolyFluor® 570: methacryloxyethyl thiocarbamoyl rhodamine B (N-[9-(2-carboxy-x-methacryloxy-ethylthiocarbamoylphenyl)-6-diethylamino-3H-xanthen-3-ylidene]-N-ethyl-ethanaminium chloride) was provide from Biovalley Polyscience (Marne-la-Vallée, France). Paclitaxel was obtained from CHEMOS GmbH (Regenstauf, Germany). [3H]-paclitaxel (3 Ci/mmol) was purchased from Isobio (Fleurus, Belgium). Hionic-Fluor® and Ultima-Gold® (Packard, Rungis, France) were used as scintillating cocktails for radioactive analyses. Soluene-350® used to dissolve biological samples was obtained from Perkinelmer (Courtaboeuf, France). Pancreatin, pepsine, sodium chloride, hydrochloric acid, sodium hydroxide, monobasic potassium phosphate and mucin from porcine stomach were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ethanol, Acetonitrile and nitric acid were provided by Fisher Scientific (Illkirch, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q plus, Millipore, St Quentin en Yvelines, France). All chemicals were reagent grade and used as received.

2.2 Preparation of nanocapsules

Nanocapsules were prepared by interfacial polymerization as described by Al Khouri Fallouh et al (Al Khouri Fallouh *et al.*, 1986) following the modification introduced by Xavier-Junior *et al.* (Xavier-Junior, Chapter VII, 2015). Briefly, an organic phase composed of ethanol (6.25 mL), copaiba oil (0.250, 0.350 and 0.450 mL), isobutylcyanoacrylate (0.032, 0.037 and 0.042 mL) and paclitaxel (2, 6 and 10 mg) were introduced dropwise in an 5 °C aqueous medium containing 12.5 mL of chitosan (0.9 %) which pH was adjusted at 3. Stirring rate was fixed at 1,250 rpm during addition

and over the next 10 minutes. Ethanol was then evaporated using rotary evaporator at 35 °C for 20 min at 43 mBa (BÜCHI Rotavapor R-125, Heating Bath B-491, Vacuum pump V-700, recirculating Chiller F-108, Flawil, Switzerland). The obtained nanocapsule dispersions were filtered (5µm nylon membrane filter, Merck Millipore, Billerica, MA, EUA). Then, they were purified and concentrated to 2 mL in Amicon Ultra centrifugal filter, 100 kDa molecular weight cut off (Merck Millipore, Billerica, MA, USA). This system was placed in centrifuge (Eppendorf centrifuge 5804 R, Rotor S-4-72, Hamburg, Germany) under 4,000 g at 20 °C against Milli-Q® water three times for 20 min. Optimization of the paclitaxel encapsulation was achieved using statistical interaction approach between the concentrations of copaiba oil, isobutylcyanoacrylate and paclitaxel variables. The dependent variables analyzed were particle size and zeta potential.

For mucoadhesion and stability studies, the nanocapsules were labeled with PolyFluor® 570: methacryloxyethyl thiocarbamoyl rhodamine B, and with [3H]-paclitaxel. The nanocapsules were prepared following the protocol described above with minor changes. Initially, 0.25 µL of PolyFluor® 570 (1 $mg.mL^{-1}$ in ethanol) was added in the organic phase, immediately in this phase were added copaiba oil and isobutylcyanoacrylate for fluorescent nanocapsules preparation For radiolabeled nanocapsules containing paclitaxel, 4.2 kBq of [3H]-paclitaxel per mL of final nanocapsules suspension were dissolved in organic phase before polymerization. For some experiments non chitosan-coated nanocapsules were also needed. These nanocapsules were prepared with Pluronic F-68® according previous work (Al Khouri Fallouh *et al.*, 1986; Gallardo *et al.*, 1993; Aboubakar, Puisieux, Couvreur & Vauthier, 1999).

2.3 Characterization of nanocapsules

The hydrodynamic diameter and the size distribution of the nanocapsules were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Worcestershire, UK) at 25°C. The scattered angle was fixed at 90 °. Samples were diluted at 1:100 with Milli-Q® water before analysis. Results were expressed as the mean hydrodynamic diameter, the standard deviation of the size distribution and the polydispersity index (PdI). Zeta potential of the nanocapsules was measured by Laser Doppler Electrophoresis using Zetasizer Nano ZS90 (Malvern Instruments Ltd, Worcestershire, UK). To maintain a constant ionic strength, samples were diluted (1:100) in saline solution (NaCl) at 1 mM. All results corresponded to the average of three determinations.

Transmission electron microscopy (TEM) was used to investigate the nanocapsule morphology. Observations were performed using a JEOL 1400 transmission electron microscope (JEOL Ltd, Tokyo, Japan) coupled with a Gatan CCD high-resolution digital camera (Orius SC1000). One drop of the diluted nanocapsule in Milli-Q® water (1:100) was placed on a formvar-carbon coated copper grid for 5 minutes. Thereafter, unfixed nanocapsules were removed by filter paper and one drop of 2% phosphotungstic acid (pH 7.4) was added to it for 30 seconds. The superfluous marker on sample was wiped off by filter paper. Finally, the grid was air dried prior to its introduction in the electron microscope.

Fluorescence microscopy studies were conducted to test the stability of the nanocapsules in simulated gastrointestinal fluids. They were performed using a fluorescent microscope (Leitz Diaplan, Wild Leitz GmBH, Wetzlar, Germany) equipped with a filter N 2.1 adapted to PolyFluor® 570: methacryloxyethyl thiocarbamoyl

rhodamine B (excitation 515–561 nm, emission cutoff 580 nm). The images were captured using QED capture version 2.0.24. A control experiment was performed by diluting the nanocapsules in Milli-Q® water (1:50) instead of using simulated gastrointestinal fluids. The same parameters observations were maintained for all samples.

2.4 Determination of paclitaxel

2.4.1 HPLC analysis

The amount of paclitaxel associated with the nanocapsules was quantified by high-performance liquid chromatography (HPLC). The method was developed and validated in a previous work (Xavier-Junior, Chapter II, 2015). The chromatographic system used was a Waters 515 pump, a Waters 717 plus autosampler and a Waters 486- Tunable Absorbance detector (Waters Corp., Milford, MA). Chromatographic separations were achieved using a Uptisphere Strategy 100A reversed-phase C-18 (150 mm x 3 µm x 3 mm) column and a Uptisphere Strategy C18-2 guard column (10 mm x 3 µm x 4 mm) (Interchim SA, France). The mobile phase, pumped at 0.4 $mL.min^{-1}$, was acetonitrile: water (50:50) at 30 °C monitored with UV-detection at 228 nm. The mobile phase and the samples were filtered through a 0.20 µm hydrophilic nylon membrane filter (Merck Millipore, Billerica, MA, EUA) prior to use. Under these conditions, the run time was 15 min and the paclitaxel was eluted at retention time of 9.7 minutes. Chromatographic data were monitored and analyzed using UV Winilab3 software (Perkin Elmer, Shelton, USA). The method was validated demonstrating that it was linear ($r^2 = 0.999$) within the range of concentration comprised between 50 to 2000 $ng.mL^{-1}$, recovery ranged from

97.1 to 101.9 % and relative standard deviation for intra- and inter-day precision were less or equal to 0.65 %. The specificity was tested in presence of the nanocapsules adjuvant and demonstrated that these factors did not alter the paclitaxel assay. The limit of quantification and limit of detection were 21.03 and 6.31 $ng.mL^{-1}$, respectively.

2.4.2 Radioactivity analysis

Radiolabeled [3H]-paclitaxel loaded copaiba oil- poly(isobutylcyanoacrylate) nanocapsules coated with chitosan was determined by liquid scintillation counter (Model LS 6000 TA, Beckman, France). Samples (40 µL) were vortexed for 1 minute with 10 mL of a scintillating cocktail and analyzed. For analysis in the rat intestinal tissue, 1 cm^2 from excised tissue was digested in 2 mL of Soluene-350® at 65 °C overnight. Then, 10 mL of scintillating cocktail was added into the bottle, vortexed for 1 minute and measured the [3H]-paclitaxel radioactivity.

2.5 Drug loading and encapsulation efficiency

Free drug was determined in the clear supernatant obtained after separation of nanocapsules from aqueous dispersion medium by ultrafiltration-centrifugation technique (Microcon centrifugal filter, Ultracel YM-100, regenerated cellulose, Merck Millipore**,** Billerica, MA, USA). Nanocapsules were centrifugated at 10,000 rpm for 20 min (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) over the ultrafiltration unit. Drug loading (DL) and encapsulation efficiency (EE) were expressed as percentages and deduced from equations 1 and 2, respectively:

$$\textbf{DL (\%)} = \frac{\text{Weight of nanocapsules loaded paclitaxel}}{\text{Total weight of nanocapsules}} \text{ X } 100\% \qquad \textbf{(Eq1)}$$

$$\textbf{EE (\%)} = \frac{\text{Experimental drug loading}}{\text{Theorical drug loading}} \text{ X } 100\% \qquad \textbf{(Eq2)}$$

2.6 Stability of paclitaxel loaded nanocapsules

Paclitaxel loaded copaiba oil- poly(isobutylcyanoacrylate) nanocapsules coated with chitosan dispersion stability was evaluated for over a period of 6 months in terms of size and zeta potential while storage was achieved at 4 °C and 25 °C. Stability of the nanocapsules was investigated after incubation in simulated gastrointestinal fluids, according to the conditions described in United States Pharmacopoeia XXXIV (Convention, 2011). Simulated gastric fluid medium was composed by 0.2 % of sodium chloride, 8 % of hydrochloric acid (1M) and 0.32 % (w/v) of pepsin with a pH of 1.2. Simulated intestinal fluid medium was formed by 0.62 % monobasic potassium phosphate solution, 7.7 % of sodium hydroxide with pancreatin 1 % (w/v) (pH 6.8). For this study, nanocapsules labeled with PolyFluor® 570 were added in the simulated fluid at dilution of 1:50 and incubated at 37 °C for various time over of total 120 minutes. At defined times, the samples were collected and analyzed by DLS for size measurement. Integrity of the nanocapsules was appreciated by fluorescence microscopy observations as previously described. Stability studies were also investigated after drying the nanocapsules dispersion in Eppendorf Vacufuge® 5301 vacuum centrifuge (Eppendorf

AG, Hamburg, Germany) at ambient temperature. The dried nanocapsules were analyzed by DLS and TEM after rehydration and redispersion in the equivalent amount to recovered initial volume of Milli-Q® water.

2.7 Evaluation of mucoadhesion

2.7.1 Aggregation of nanocapsules in presence of mucin

Mucin from porcine stomach was prepared using Milli-Q® water for 2 h at room temperature (20 °C) to obtain dispersion at 1 % (w/v). Dispersions of nanocapsules were prepared in Milli-Q® water at different concentrations: 1.5; 2.0; 2.5; 3.0; 3.5 and 4.0 $mg.mL^{-1}$. Then, 75 µL of the nanocapsules dispersion were placed in the wells of a 96- microwell plate with polystyrene conical bottom. The absorbance initial (A_0) of each suspension was evaluated at 450 nm using a microplate reader (Multiskan Anscent, Labsystems SA, Cergy-Pontoise, France). 30 µL of mucin suspension (0.25 $mg.mL^{-1}$) was added and the plate was incubated for 1 h at 37 C. To quantify the aggregation, the absorbance A_{1h} was evaluated after centrifugation (240 g) for 5 min at 25 °C (Eppendorf centrifuge 5804 R, Rotor A- 4- 81 with MTP/Flex carrier, Hamburg, Germany). Each experiment was repeated three times and the difference between A_0 and A_{1h} (A_0 - A_{1h}) was plotted as a function of the dilution performed for each nanocapsule dispersion.

2.7.2 Mucoadhesion assay on rat intestinal mucosa

Animal experiments were carried out according to the recommendations of the ethics committee of the French Ministry of Higher Education and Research, project 2003-055 regarding the care and use of animals for experimental procedures. Male Wistar rats (200–250 g) (Charles River, Paris) were used for the mucoadhesion *ex vivo* assays. Rats were allowed free access to water and food, and housed under controlled environmental conditions (constant temperature, humidity, and a 12 h dark-light cycle). Animals were euthanized with an overdose of pentobarbital by intraperitoneal injection. Fresh small intestine (jejunum) portion was excised, rinsed with physiological saline (NaCl 0.9 %) and cut into small segments of 2-3 cm length. After visual examination of the tissue, sections containing Peyer's patches were discarded.

Intestinal portions were mounted in Ussing chambers with a delimited intestinal mucosa surface area (1 cm^2). The systems were maintained in ringer buffer at 37 °C, continuously oxygenated with O_2 /CO_2 95%/ 5%. After removing the transport buffer, 0.1 mL of radiolabeled nanocapsules in ringer buffer (pH 7.5) were applied to the mucosal surface. Each compartment of the Ussing chamber was filled with 3 mL of ringer solution. The experiment was performed over a period of 2 hours to insure the attachment equilibrium. After incubation for 2 hours, the nanocapsule dispersion was removed. Tissue was rinsed three times with 3 mL of ringer buffer, to eliminate non-attached nanocapsules while it was still mounted in the Ussing Chamber. Subsequently, the mucosa with the attached nanocapsules was recovered and let to dissolve in 2 mL of Soluene-350® at 65 °C overnight. Then, 10 mL of scintillating liquid were added and finally samples were analyzed by liquid scintillation to determine the amount of [3H]-paclitaxel which associated with the mucosa. Results were expressed as the amount of

attached nanocapsules per apparent surface (g/m^2) and by the number of attached nanocapsules in the tissue, as defined by the equation 3:

$$N = \frac{m_T\ 6}{\rho\ S\ d} \qquad \textbf{(Eq3)}$$

where N is the number of attached nanocapsules, m_T the mass of attached nanocapsules (g), ρ the nanocapsules density oil estimated (0.99 g/cm^3), d the nanocapsules diameter (cm) and S the nanocapsules surface: $S= 4\pi\ (d/2)^2$. Each sample was tested in three different rats in duplicate.

2.8 Statistical analysis

All experiments were conducted in triplicates. All values were expressed as their mean ± standard deviation (SD). Means of two groups were compared using non-paired Student's t-test. When comparing multiple groups, one way analysis of variance (ANOVA) was applied with the Tukey multiple comparison procedure. The analyses were performed using the Graph Pad Prism (Version 5.0, La Jolla, CA, USA) and Statistic software (Version 7.0, StatSoft Inc., USA). The statistical data were considered significant at $p < 0.05$

3.0 RESULTS AND DISCUSSION

3.1 Optimization of the preparation of paclitaxel loaded nanocapsules

Paclitaxel loaded copaiba oil- poly(isobutylcyanoacrylate) nanocapsules coated with chitosan were produced by the new method of interfacial polymerization of isobutylcyanoacrylate carried out in the presence of chitosan while surfactant was absent in the polymerization medium (Xavier-Junior, Chapter VII, 2015). This method was used to obtain chitosan-coated nanocapsules encapsulating paclitaxel assuming that they will show mucoadhesive properties.

Optimization of the incorporation of paclitaxel was studied considering the influence of the isobutylcyanoacrylate, copaiba oil and paclitaxel concentrations on the size and zeta potential of the produced nanocapsules (Figure 1). It was considered that nanocapsules with smaller sizes promote mucoadhesion (Ponchel *et al.*, 1994; Ponchel & Irache, 1998; Bertholon *et al.*, 2006). Furthermore, after reaching the blood, small nanocapsules can undergo capillary distribution and uniform perfusion.

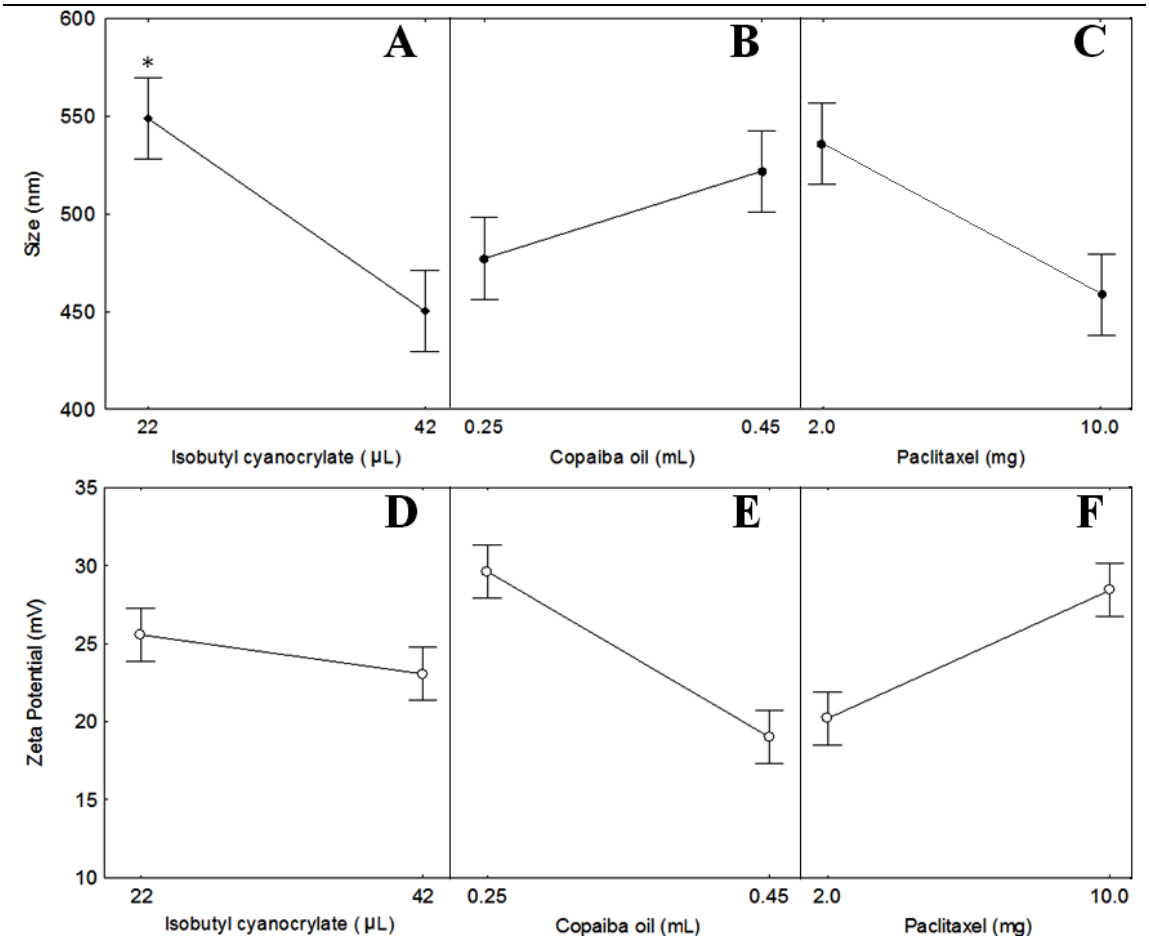

Figure 1- Size (top line) and zeta potential (bass line) influence in paclitaxel loaded nanocapsules formed at low and high concentration of isobutylcyanoacrylate (A and D), copaiba oil (B and E) and paclitaxel (C and F). * Broad distribution size

An opposite and significant effect was observed in the size when increasing concentrations in isobutylcyanoacrylate and paclitaxel in the polymerization medium ($p < 0.05$). Considering the concentration of monomer, a decrease of the diameter of the nanocapsules of 100 nm was observed by increasing the amount of isobutylcyanoacrylate introduced in the polymerization medium from 0.022 to 0.042 mL (Figure 1A). The nanocapsule dispersion obtained with concentration in isobutylcyanoacrylate of 0.022 mL were unstable showing aggregates while size

distribution was broad. This can be associated with the formation of a low polymer concentration, possibly insufficient to effectively cover the available surface of the copaiba oil droplets which form during dispersion of the organic phase in the polymerization medium (Douglas *et al.*, 1985). By increasing the concentration of drug from 2 to 10 $mg.mL^{-1}$ in the organic phase, the diameter of the nanocapsule which formed was decreased (Figure 1C). Analyzing the chemical structure of paclitaxel, a slight amphiphilicity can be highlighted (Figure 2). It may be enough to promote formation of emulsion droplets of lower size then those obtained in the absence of paclitaxel during dispersion of the organic phase in the polymerization medium hence of the nanocapsules. A slight increase in the nanocapsule size was observed when increased the volume of copaiba oil loaded in the systems from 0.25 to 0.45 mL, however, this difference was not statistically significant ($p > 0.05$) (Figure 1B).

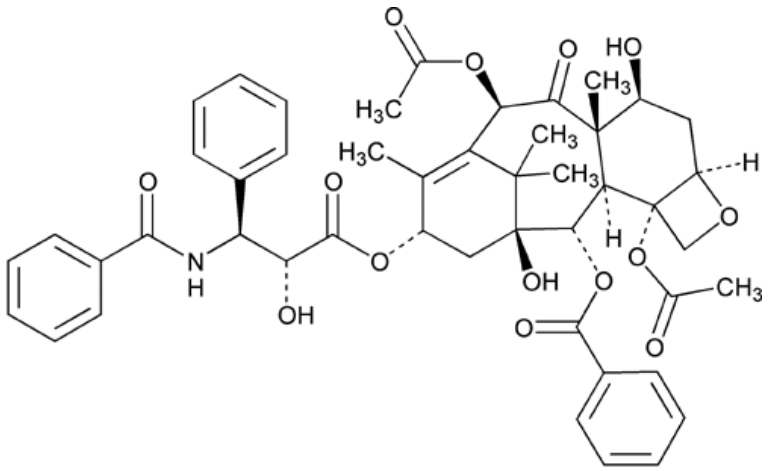

Figure 2: Chemical structure of the paclitaxel

Positive zeta potential values were observed with all nanocapsules suggesting that chitosan was covering the nanocapsules surface as expected. The rather strong positive zeta potential may be explained by the presence of quaternary ammonium groups in

chitosan (Bathool *et al.*, 2012) at the almost neutral pH of measurement. The positive zeta potential of the nanocapsules was expected to promote mucoadhesion thanks to electrostatic interactions with the mucus surface, which is negatively charged at physiological pH (Bernkop-Schnurch, 2005). Higher value of zeta potential (positive or negative), are also highly favorable to obtain stable dispersions of nanocapsules as electrostatic repulsion forces between particles having the same electric charge prevent aggregation of the dispersion (Feng & Huang, 2001). No significant difference ($p > 0.05$) was observed between nanocapsules prepared with two concentrations in isobutylcyanoacrylate (Figure 1D). Zeta potential was much influenced by the amount of copaiba oil introduced in the polymerization so did modification of the concentration of paclitaxel but in the opposite way (Figure 1 E and F). To explain these effect either small amounts of copaiba oil and paclitaxel could adsorbed on the nanocapsule surface or they influenced the coverage of the nanocapsule surface by the chitosan molecules leading to masking or exhibiting the positive charge of the polysaccharide (Harivardhan Reddy & Murthy, 2003; Mohammadpour Dounighi *et al.*, 2012).

In order to obtain nanocapsules of small size, but at the same time large amounts of chitosan coated on the surface, it can be deduced that optimized paclitaxel loaded nanocapsules were produced using 0.032 mL of isobutylcyanoacrylate, 0.25 mL of copaiba oil and 6 mg of paclitaxel.

3.2 Characteristics of the optimized paclitaxel loaded nanocapsules

The mean particle diameter of these nanocapsules was found to be 486 ± 3 nm with a narrow PdI of 0.17 (Figure 3). Zeta potential of those nanocapsules was frankly positive

(+ 37.1 ± 0.3 mV), indicating presence of chitosan on the nanocapsule surface. Preparation of unloaded nanocapsules performed in same conditions but omitting paclitaxel provided with nanocapsule which characteristic did not differ significantly ($p > 0.05$) from that of the loaded nanocapsules to size, zeta potential and morphology analyses.

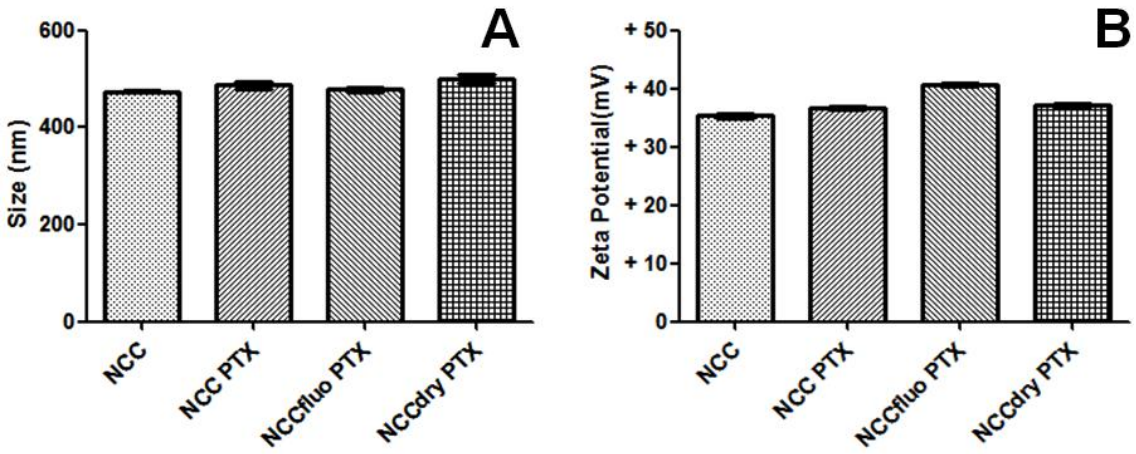

Figure 3: Size (A) and zeta potential (B) of different nanocapsules. NCC: Copaiba oil-loaded chitosan-poly (isobutylcyanocrylate) core-shell nanocapsules; NCC PTX: Paclitaxel into NCC; NCCfluo Paclitaxel: PolyFluor® 570 labeled NCC PTX; NCCdry PTX: NCC PTX after drying process.

TEM images showed the spherical shape and surface morphology of the paclitaxel unloaded and loaded nanocapsules (Figure 4 A and B, respectively). The nanocapsules appeared well separated on all preparations. As confirmed by TEM examination, nanocapsules suggesting that they are formed by an oily cavity (copaiba oil where paclitaxel was dissolved) surrounded by a polymer membrane. The actual diameter

observed by microscopy around 475 nm which was consistent with values determined by DLS.

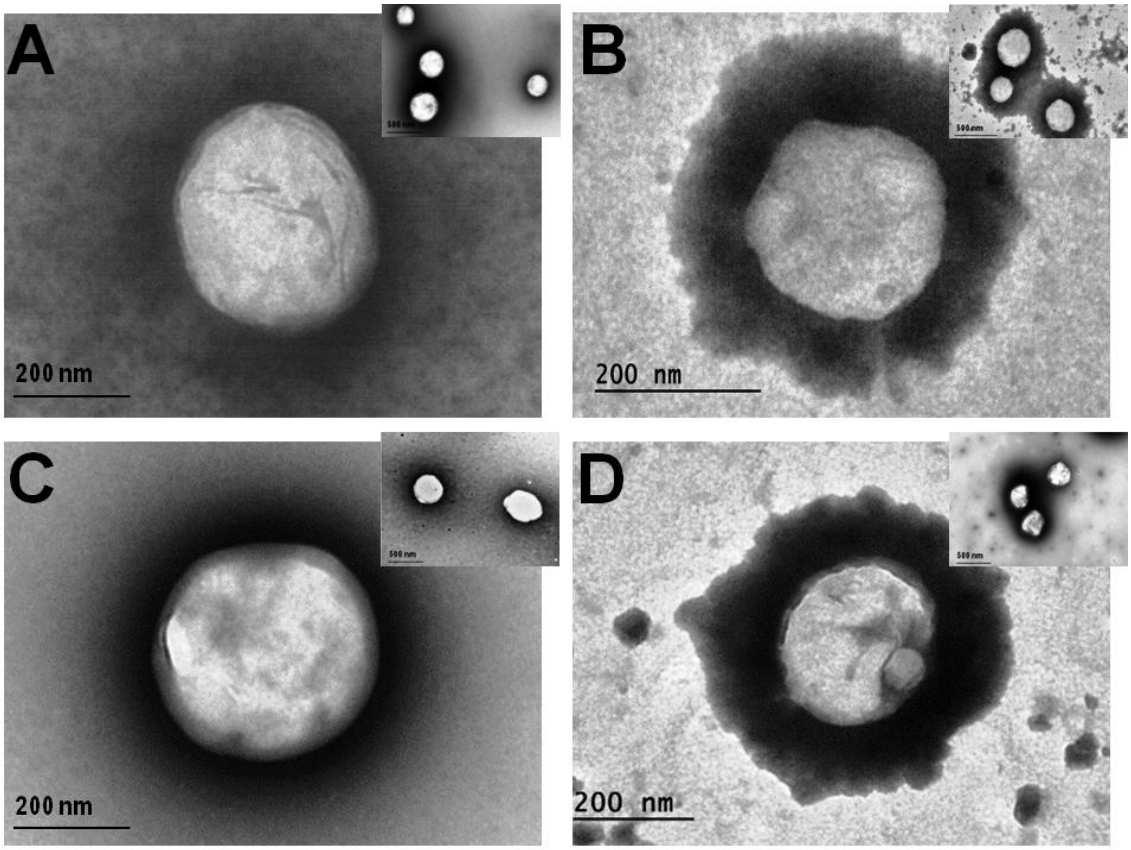

Figure 4- TEM of copaiba oil-loaded chitosan-poly (isobutylcyanocrylate) core-shell nanocapsules (NCC) (A); Paclitaxel into NCC (NCC PTX) (B); NCC PTX after drying process (C); PolyFluor® 570 labeled NCC PTX (D). Scale bar of 200 nm and 500 nm to isolated and grouped nanocapsules, respectively.

Concerning the maximum EE and DL of paclitaxel loaded in nanocapsules, the results showed values of 74.5 ± 1.2% and 1.7 ± 0.02% (w/w), respectively. The drug concentration loaded in the formulation was 16.8 ± 0.3 µg of paclitaxel per mg of

nanocapsules, corresponding at 746 ± 12 µg.mL^{-1} of nanocapsules dispersion. This corresponding to an excellent association of the drug with the nanosystem compared with previously described systems. The DL showed by these nanocapsules was also relevant with a therapeutic dose when it is compared with existing injectable therapies. For instance, in the Ambraxane®, paclitaxel which is formulated in a nanomedicine is administrated at concentration of the dispersions of 5 mg.mL^{-1}. Previous studies, reported 70% of paclitaxel EE into PEGylated poly (lactide-co-glycolide) nanoparticles by nanoprecipitation method (Danhier *et al.*, 2009). Huang et al showed DL of 0.18 and 0.56 % in paclitaxel-loaded poly (butylcyanoacrylate) nanoparticle obtained by in emulsion and microemulsion polymerization methods (Huang *et al.*, 2007). To explain the high DL obtained in the nanocapsules, it is believed that the good solubility of the paclitaxel in copaiba oil combined with its high partition coefficient in favor to copaiba oil had contributed favorably to the success of the encapsulation method developed in the present work (Xavier-Junior, Chapter II, 2015).

Biopharmaceutical studies require the labeling of particles in order to localize them *in vivo* and/or *in vitro* during assays carried out under various experimental conditions (Bravo-Osuna, Ponchel, *et al.*, 2007; Vauthier & Bouchemal, 2009). Fluorescent labeled nanocapsules were synthesized by incorporation of methacryloxyethyl thiocarbamoyl rhodamine B (Polyfluor® 570), a fluorescent co-monomer, which reacts with isobutylcyanoacrylate during the formation of the nanocapsules. Fluorescent labeled nanocapsules did not show significant difference in size and morphology compared with the non-labeled nanocapsules. They only differed by a slight increase of zeta potential (Figure 3 and 4D). The nanocapsules appeared clearly fluorescent under observation by fluorescent microscope.

Radiolabeled nanocapsules were effectively developed with [3H]-paclitaxel. This dispersion showed a radioactive activity of 3.14 ± 0.07 kbq.mL^{-1}. The encapsulation efficiency was similar to that of the nanocapsules prepared with the non-radioactive drug, indicating that the use of radiolabeled drug did not modify characteristics of the nanocapsules. The non chitosan-coated nanocapsules prepared with Pluronic F-68® were characterized by a size of 230 ± 3.2 nm, a PdI of 0.18, a zeta potential of -21.1 mV ±0.06. These characteristics were consistent with nanocapsules synthesized in previous works.

3.3 Stability of the nanocapsules upon storage

The storage of nanocapsules was investigated as dispersion at 4 and 25 °C and after elimination of the water to obtain a dried powder. Upon storage under the form of a dispersion particle size and zeta potential were evaluated over a period of 6 months. Results presented in Figure 5 showed that nanocapsule dispersion stored at 4 °C maintained the initial properties and no aggregation were observed ($p > 0.05$). The nanocapsule dispersion stored at 25 °C remained stable over 3 months with only slight variation of their mean diameter and zeta potential by 5 and 7 %, respectively. After 6 months, the sample presented a statistic significant increase of the size by 19 % while the zeta potential decreased by 20 % ($p < 0.05$). These effects were probably caused by a fusion growth process which is more pronounced at elevated temperatures. These results were consistent with Lemoine *et al.* and Coffin *et al.*, where minor changes in the in nanoparticles size were observed when stored at 4 or 5 °C in contrast with higher temperatures (25 or 37 °C) (Coffin & Mcginity, 1992; Lemoine *et al.*, 1996).

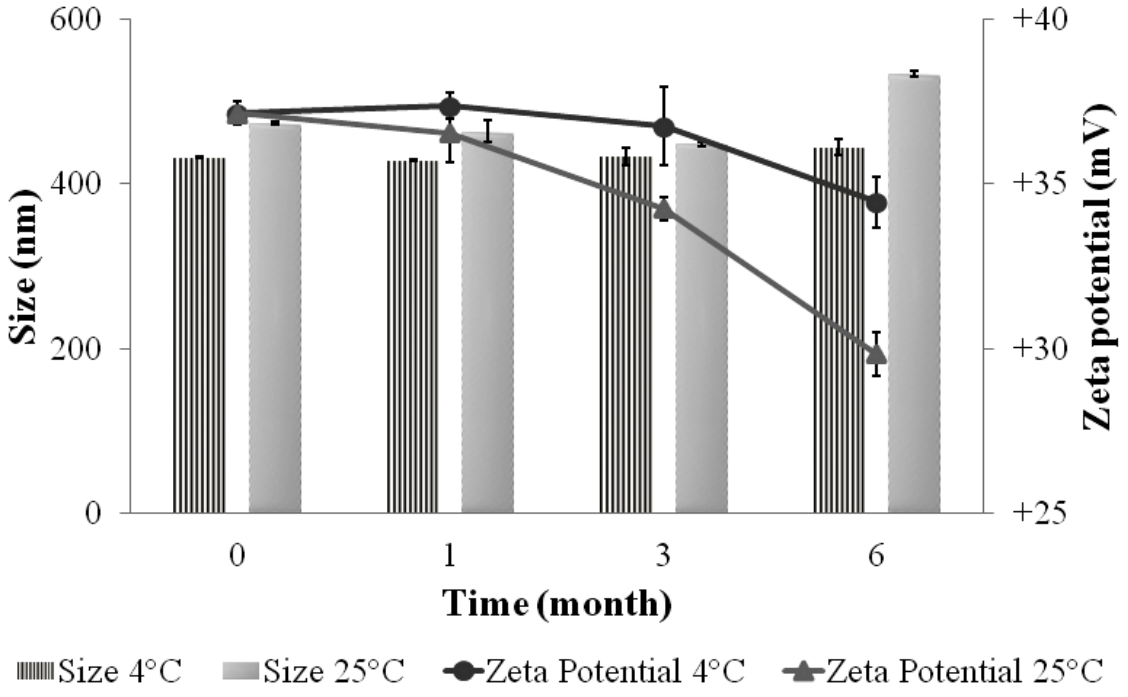

Figure 5- Size and zeta potential of paclitaxel into copaiba oil-loaded chitosan-poly(isobutylcyanocrylate) core-shell nanocapsules stability stored at 4 °C and 25° C over a period of 6 months.

Storage of formulations under a dried form is generally preferable. However, one major problem with nanoparticles is that they often aggregate during the drying process and the recovery of dispersion having the same size characteristics than the parent dispersion is the main difficulty. Besides irreversible aggregation, nanocapsules can be destroyed by the drying process (Abdelwahed, Degobert & Fessi, 2006; Abdelwahed, Degobert, Stainmesse, *et al.*, 2006; Tewa-Tagne *et al.*, 2007). Here the nanocapsules were dried and characterized by DLS and TEM after redispersion to reconstitute the parent dispersion. Particle size (513.5 ± 5.4 nm with polydispersion index of 0.19), zeta potential (+ 37.2 ± 0.2 mV) and morphology of the nanocapsules appeared similar to the initial dispersion after drying and reconstitution in water (Figure 3 and 4 C). Comparing

the size and polydispersity index of the two nanocapsule dispersions the difference was not statistically significant ($p > 0.05$). The nanostructure as observed by TEM was also well preserved demonstrating that the nanocapsule can be dried without losing their physicochemical characteristics and morphology.

3.4 Stability of nanocapsules in simulated gastrointestinal fluids

The size and the fluorescent image of the labeled nanocapsules were followed over a period of 120 minutes of incubation in simulated gastric and intestinal media (Figure 6 and 7). In the reconstituted gastric medium, no important increase of the size occurred after 120 minutes of incubation ($p>0.05$). By fluorescent microscopy, the fluorescence remains confined within the nanocapsules for at least 1 hour while released of fluorescence clearly started after 120 minutes of incubation in the gastric medium. This indicated that the nanocapsules were quite stable in the gastric environment while they start to leak after 120 minutes in this medium. Although the nanocapsules became leaky, no significant aggregation was observed. At strong acid pH, chitosan having a pKa of 6.3 is highly protonated hence positively charged which can explain that the nanocapsules remained well dispersed (Meng *et al.*, 2010).

During the first 30 min of incubation in the intestinal medium, the nanocapsules dispersion showed a dramatic increase of the size of the nanocapsule corresponding to five times more and PdI of 0.64, while observations by fluorescent microscopy revealed a marked fluorescent background. Aggregation of the fluorescent nanocapsules, only shown at 120 minutes, suggesting that this system were leaky and already started to degrade with time as well as the size measured by DLS over the time of the experiment.

In the intestinal medium, chitosan protonation is low hence the charge. Nanocapsules may tend to aggregate because the electrostatic forces contributing to the colloidal stability may be diminished. The enzymatic environment is also greatly favorable to the loss of stability of the poly(isobutylcyanoacrylate) nanocapsules. Esterase's can degrade the polymer causing leakage as well as swelling of the polymer envelope (Lenaerts *et al.*, 1984; Scherer *et al.*, 1994). The present observations, we were very consistent with those reported by Aboubakar *et al.* on insulin nanocapsules (Aboubakar *et al.*, 2000).

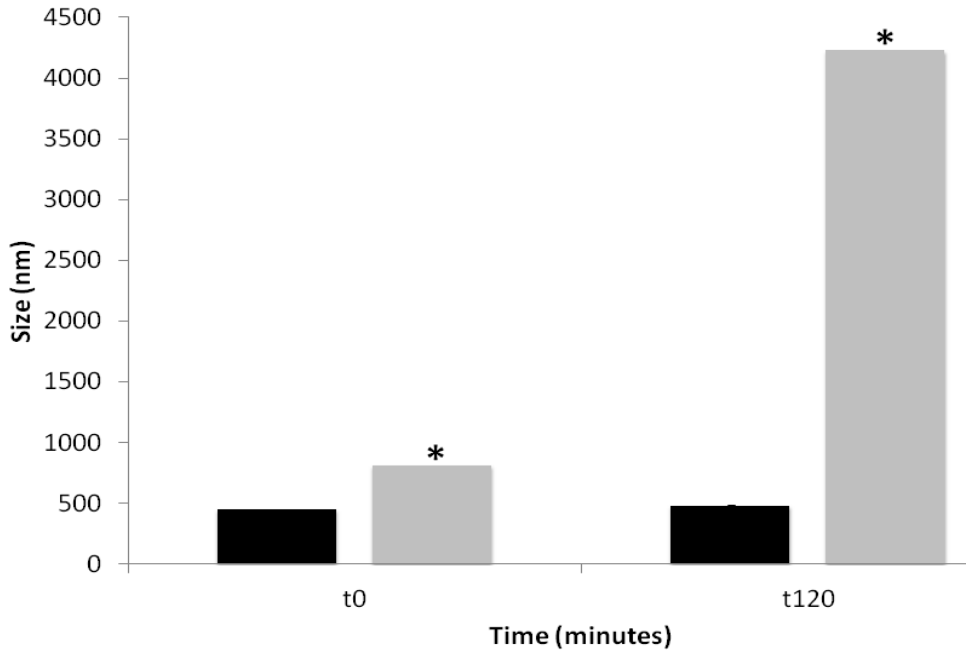

Figure 6- The size of the polyFluor® 570 labeled nanocapsules in different media simulating the pH environment in the gastrointestinal tract after two hours of incubation. Dark bars= Simulated gastric fluid, light bar= Simulated intestinal fluid and *=$p<0.05$

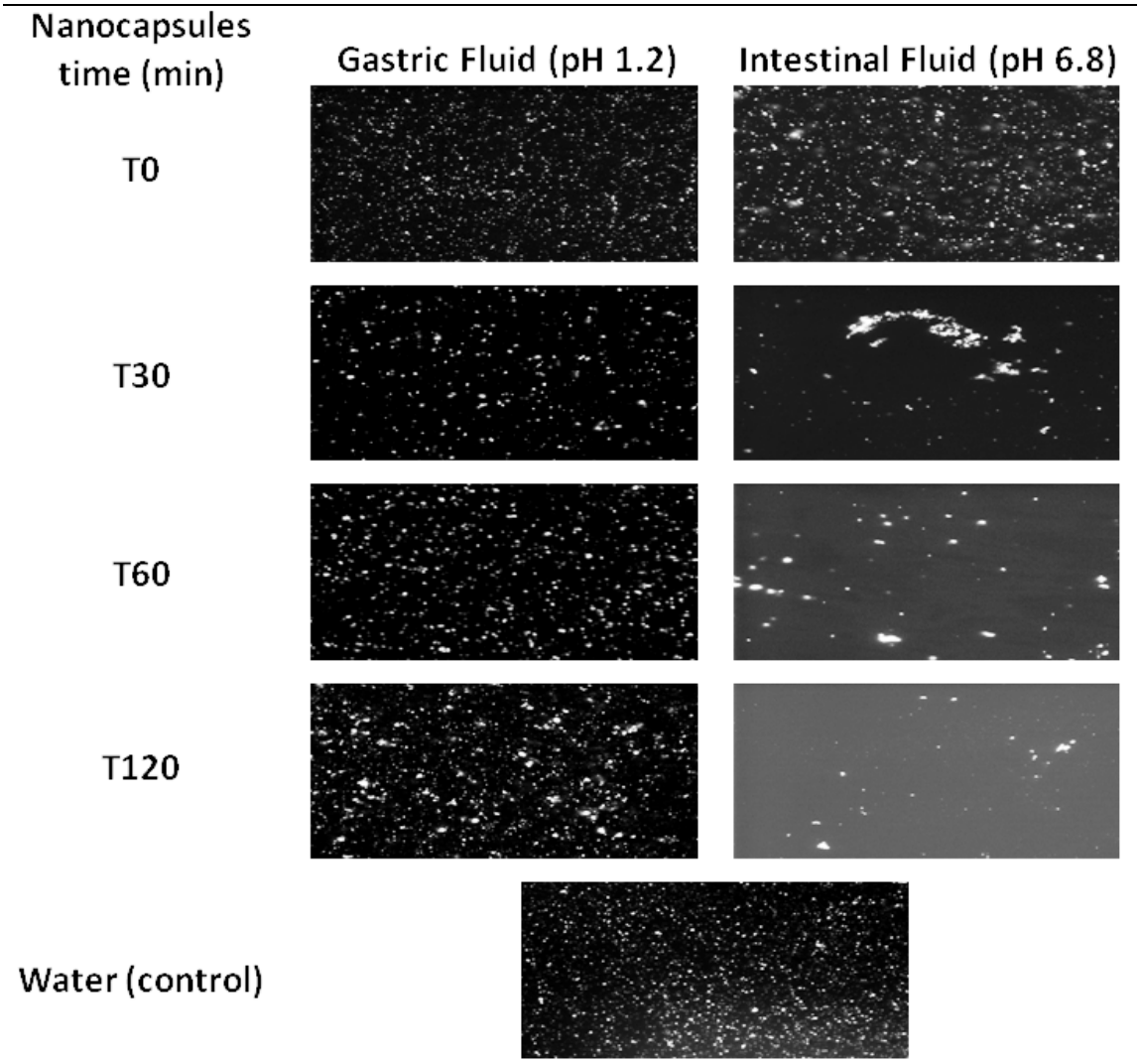

Figure 7- Polyfluor® 570 labeled nanocapsules stability in reconstituted gastric and intestinal fluids by 120 minutes.

3.5 Mucoadhesion studies

The potential use of small mucoadhesive polymer particle formulations lies in possible prolongation of the residence time near absorption sites of the drug either through non-specific (van der Waals and/or hydrophobic interactions) or specific interactions between components of the particles and the mucus. In general, increases of the

bioavailability of the loaded drug were then obtained (Ponchel & Irache, 1998; Hagerstrom *et al.*, 2003; Moghaddam *et al.*, 2009a). Mucus which is composed of highly hydrated glycoprotein called mucins cover mucosa forming a continuous adherent blanket on the surface of the epithelium. While adhering to the mucosa, nanocapsules would have to interact with mucins. There are several methods that are suitable to investigate mucoadhesion of nanocapsules on the gut mucosa (Neves *et al.*, 2011; Laffleur & Bernkop-Schnurch, 2013). For the present study, the method evaluating aggregation of nanocapsules in presence of mucin was selected as a convenient *in-vitro* test. Adhesion of nanocapsules was also evaluated directly on rat intestinal mucosa. Results from the aggregation of mucin are presented on Figure 8 considering unloaded and loaded-paclitaxel nanocapsules coated with chitosan. There were compared with results obtained with nanocapsules composed of poly(isobutylcyanoacrylate), copaiba oil but which were not coated with chitosan. These nanocapsules were stabilized by using Pluronic including PEG chains that are also know to display mucoadhesive properties (Kim *et al.*, 2007; Jones *et al.*, 2009; Gratieri *et al.*, 2010).

Different profiles of mucin-nanocapsule aggregation were obtained comparing the different types of nanocapsules. At concentrations bellow 2.5 $mg.mL^{-1}$ the loaded and unloaded paclitaxel nanocapsules coated with chitosan showed a marked difference while above this concentration, the two types of nanocapsules showed a maximum of aggregation in the presence of mucins which was acknowledged by the plateau observed on the curves. Both types of nanocapsules were coated with chitosan. The occurrence of the plateau indicated occurrence of a saturation phenomenon. This was consistent considering that interactions between the nanocapsules and the mucins consisted of chemical bonds between the positively charged amino groups of chitosan and the

negatively charged sialic acid residues of mucus glycoproteins at concentrations above 2.5 mg.mL^{-1} in nanocapsules (Rossi *et al.*, 2000). As can be observed in Figure 8, the presence of Pluronic F-68® on the nanocapsule surface did not provoke aggregation of the mucin over the range of concentrations studied. In contrast with the nanocapsules coated with chitosan, the nanocapsules coated with Pluronic did not interacted with mucins. These results showing higher interactions of chitosan-coated nanocapsules with mucins compared with Pluronic-coated nanocapsules suggested that the addition of chitosan on the nanocapsule surface should promote their mucoadhesion.

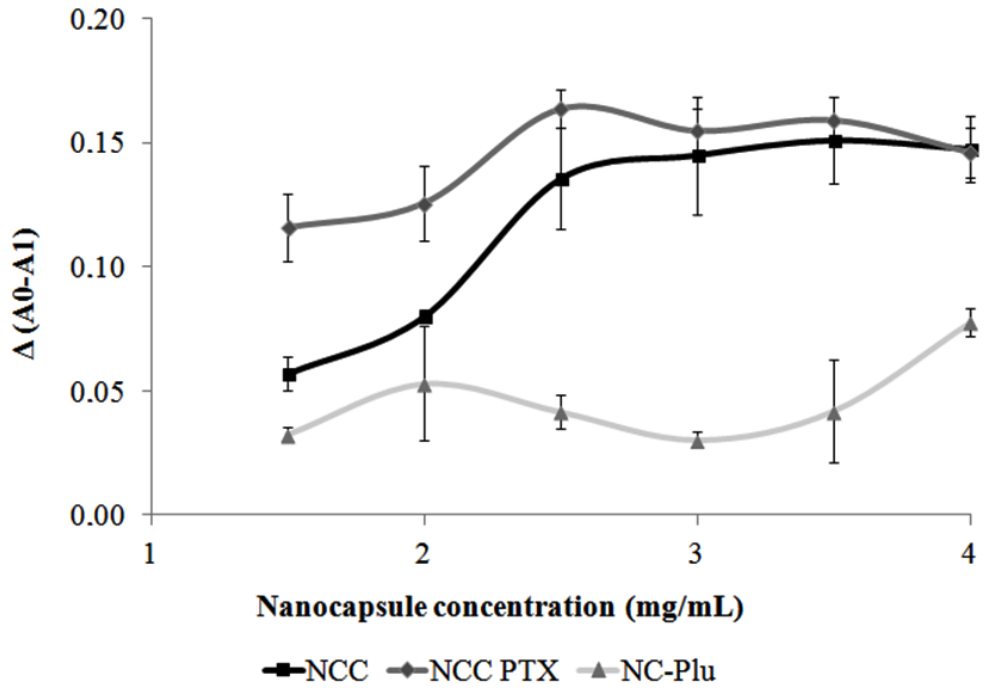

Figure 8- *in-vitro* mucin aggregation test to evaluate mucoadhesive properties of the nanocapsules. NCC: Copaiba oil-loaded chitosan-poly (isobutylcyanocrylate) core-shell nanocapsules; NCC PTX: Paclitaxel into NCC; NC-Plu: Copaiba oil-loaded Pluronic-poly (isobutylcyanocrylate) nanocapsules.

Mucoadhesion of the nanocapsules was also evaluated on freshly excised rat intestinal mucosa while the tissue was mounted in Ussing Chambers. The nanocapsules were loaded with [3H]-paclitaxel. Thus, only mucoadhesion of the radiolabeled nanocapsules could be evaluated by this method. The nanocapsule concentration used was the same as in the plateau observed in the mucin aggregation test described above. After incubation of the nanocapsules with the mucosa in the Ussing chambers, 9 ± 1.1% of the initial amount of the radioactivity introduced with the nanocapsules was associated with the mucosa after two hours. This corresponded to a deposition and strong attachment of 6.2 $x10^9$ nanocapsules per square centimeter of the intestinal mucosa or to 3.4 g of nanocapsules per square meter. In the literature, Bravo-Osuna *et al.* have observed that the presence of chitosan on the poly(isobutylcyanoacrylate) nanoparticle surface increased dramatically the mucoadhesive behavior of the nanoparticles thanks to the formation of hydrogen and ionic bonds between the positively charged amino groups of the polysaccharide and the negatively charged sialic acid residues of mucin glycoproteins (Bravo-Osuna, Vauthier, *et al.*, 2007). The authors have also reported a relation between the amount of attached chitosan 20 kDa-coated nanoparticles per square meter of intestinal mucosa of rat at concentration of 2 $mg.mL^{-1}$ corresponding at nanoparticles adhesive interaction about of 1.5 g/m^2. Thus, comparing the studies developed in this work with Bravo-Osuna *et al.*, it was observed an increase by 2-folds in the nanocapsules adhesive interaction with the mucosa. Results from the studies devolopped in the present work also agreed well with those reported by Moghaddam *et al.* who have found approximately the same amount of nanoparticles attached on the intestinal mucosa of rat while investigating the mucoadhesion of chitosan 20kDa-pHEMA nanoparticles (Moghaddam *et al.*, 2009b).

In the clinic, paclitaxel dose is commonly prescribed at 175 mg/m² and administered intravenously over a period of 3-24 hours (Van Den Bongard *et al.*, 2004). Sacco *et al.* observed the overall mean body surface area of 1.79 m^2 (95% CI 1.78–1.80) to patients receiving chemotherapy for head and neck, ovarian, lung, upper GI/pancreas, breast or colorectal cancers (Sacco *et al.*, 2010). Thus, to obtain an ideal anticancer therapy, a dose of 313.3 mg of paclitaxel is required. Paclitaxel loaded copaiba oil-poly(isobutylcyanoacrylate) nanocapsules coated with chitosan developed in this work show a important impact as new anticancer therapy by oral route for paclitaxel. Taking into account the ideal therapy, is required 18.6 g of nanocapsules containing paclitaxel. Although the amount administered of nanocapsules appears to be high, other factors such as the synergic effect of copaiba oil in the cancer therapy and character of the modified release have not been considered in order to reduce the traditional dose.

4 CONCLUSION

New poly(isobutylcyanoacrylate) based nanocapsules were synthesized by interfacial polymerization having a surface coated with chitosan. Paclitaxel could be incorporated in the nanocapsules which cavity was filled out with copaiba oil while chitosan conferred interesting mucoadhesive properties to the new formulation. Properties of the nanocapsules agreed well with those expected for a formulation designed to enhance oral bioavailability of the associated drug. Taking together, results from the present work are encouraging to pursue the development of the chitosan-coated nanocapsules for oral delivery of paclitaxel as new treatment for cancer with possible synergetic anticancer effect with therapeutically active components found in copaiba oil.

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REFERENCES

ABDELWAHED, W., DEGOBERT, G. & FESSI, H. 2006. Freeze-drying of nanocapsules: impact of annealing on the drying process. *Int J Pharm*, 324, 1, 74-82.

ABDELWAHED, W., DEGOBERT, G., STAINMESSE, S. & FESSI, H. 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv Drug Deliv Rev*, 58, 15, 1688-713.

ABOUBAKAR, M., COUVREUR, P., PINTO-ALPHANDARY, H., GOURITIN, B., LACOUR, B., FARINOTTI, R., PUISIEUX, F. & VAUTHIER, C. 2000. Insulin-loaded nanocapsules for oral administration: In vitro and in vivo investigation. *Drug Dev Res*, 49, 2, 109-117.

ABOUBAKAR, M., PUISIEUX, F., COUVREUR, P. & VAUTHIER, C. 1999. Physico-chemical characterization of insulin-loaded poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization. *Int J Pharm*, 183, 1, 63-6.

AL KHOURI FALLOUH, N., ROBLOT-TREUPEL, L., FESSI, H., DEVISSAGUET, J. P. & PUISIEUX, F. 1986. Development of a new process for the manufacture of polyisobutylcyanoacrylate nanocapsules. *Inter J Pharm*, 28, 2–3, 125-132.

ALLEN, T. M. 2002. Ligand-targeted therapeutics in anticancer therapy. *Nat Rev Cancer*, 2, 10, 750-63.

ARIAS, J. L., GALLARDO, V., GOMEZ-LOPERA, S. A., PLAZA, R. C. & DELGADO, A. V. 2001. Synthesis and characterization of poly(ethyl-2-cyanoacrylate) nanoparticles with a magnetic core. *J Control Release*, 77, 3, 309-21.

BAE, K. H., LEE, Y. & PARK, T. G. 2007. Oil-encapsulating PEO-PPO-PEO/PEG shell cross-linked nanocapsules for target-specific delivery of paclitaxel. *Biomacromolecules*, 8, 2, 650-6.

BATHOOL, A., VISHAKANTE, G. D., KHAN, M. S. & SHIVAKUMAR, H. G. 2012. Development and characterization of atorvastatin calcium loaded chitosan nanoparticles for sustain drug delivery *Adv Mat Lett*, 3, 6, 466-470.

BERNKOP-SCHNURCH, A. 2005. Mucoadhesive systems in oral drug delivery. *Drug Discov Today Technol*, 2, 1, 83-7.

BERTHOLON, I., PONCHEL, G., LABARRE, D., COUVREUR, P. & VAUTHIER, C. 2006. Bioadhesive properties of poly(alkylcyanoacrylate) nanoparticles coated with polysaccharide. *J Nanosci Nanotechnol*, 6, 9-10, 3102-9.

BRAVO-OSUNA, I., PONCHEL, G. & VAUTHIER, C. 2007. Tuning of shell and core characteristics of chitosan-decorated acrylic nanoparticles. *Eur J Pharm Sci*, 30, 2, 143-54.

BRAVO-OSUNA, I., VAUTHIER, C., FARABOLLINI, A., PALMIERI, G. F. & PONCHEL, G. 2007. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomater*, 28, 13, 2233-43.

BRIGGER, I., DUBERNET, C. & COUVREUR, P. 2002. Nanoparticles in cancer therapy and diagnosis. *Adv Drug Deliv Rev*, 54, 5, 631-51.

BUZEA, C., PACHECO, II & ROBBIE, K. 2007. Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases*, 2, 4, MR17-71.

COFFIN, M. D. & MCGINITY, J. W. 1992. Biodegradable pseudolatexes: the chemical stability of poly(D,L-lactide) and poly(epsilon-caprolactone) nanoparticles in aqueous media. *Pharm Res*, 9, 2, 200-5.

CONVENTION, U. S. P. U.S. Pharmacopeia National Formulary United States Pharmacopeial: Pck Sup edition, 2011.

DAMGE, C., MICHEL, C., APRAHAMIAN, M. & COUVREUR, P. 1988. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. *Diabetes*, 37, 2, 246-51.

DANHIER, F., LECOUTURIER, N., VROMAN, B., JEROME, C., MARCHAND-BRYNAERT, J., FERON, O. & PREAT, V. 2009. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in vitro and in vivo evaluation. *J Control Release,* 133, 1, 11-7.

DANHIER, F., UCAKAR, B., MAGOTTEAUX, N., BREWSTER, M. E. & PREAT, V. 2010. Active and passive tumor targeting of a novel poorly soluble cyclin dependent kinase inhibitor, JNJ-7706621. *Int J Pharm,* 392, 1-2, 20-8.

DOUGLAS, S. J., ILLUM, L. & DAVIS, S. S. 1985. Particle size and size distribution of poly(butyl 2-cyanoacrylate) nanoparticles. II. Influence of stabilizers. *J Colloid Interface Sci,* 103, 1, 154-163.

FANG, J., NAKAMURA, H. & MAEDA, H. 2011. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev,* 63, 3, 136-51.

FANG, W. S. & LIANG, X. T. 2005. Recent progress in structure activity relationship and mechanistic studies of taxol analogues. *Mini Rev Med Chem,* 5, 1, 1-12.

FENG, S. & HUANG, G. 2001. Effects of emulsifiers on the controlled release of paclitaxel (Taxol) from nanospheres of biodegradable polymers. *J Control Release,* 71, 1, 53-69.

GALLARDO, M., COUARRAZE, G., DENIZOT, B., TREUPEL, L., COUVREUR, P. & PUISIEUX, F. 1993. Study of the mechanisms of formation of nanoparticles and nanocapsules of polyisobutyl-2-cyanoacrylate. *Inter J Pharm,* 100, 1–3, 55-64.

GRATIERI, T., GELFUSO, G. M., ROCHA, E. M., SARMENTO, V. H., DE FREITAS, O. & LOPEZ, R. F. 2010. A poloxamer/chitosan in situ forming gel with prolonged retention time for ocular delivery. *Eur J Pharm Biopharm,* 75, 2, 186-93.

HAGERSTROM, H., EDSMAN, K. & STROMME, M. 2003. Low-frequency dielectric spectroscopy as a tool for studying the compatibility between pharmaceutical gels and mucous tissue. *J Pharm Sci,* 92, 9, 1869-81.

HARIVARDHAN REDDY, L. & MURTHY, R. S. R. 2003. Polymerization of n-butyl cyanoacrylate in presence of surfactant: study of influence of polymerization factors on particle properties, drug loading and evaluation of its drug release kinetics. *Ars Pharmaceutica,* 44, 4, 351-369

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci,* 13, 4, 134-6.

HUANG, C. Y., CHEN, C. M. & LEE, Y. D. 2007. Synthesis of high loading and encapsulation efficient paclitaxel-loaded poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion. *Int J Pharm*, 338, 1-2, 267-75.

JABR-MILANE, L. S., VAN VLERKEN, L. E., YADAV, S. & AMIJI, M. M. 2008. Multi-functional nanocarriers to overcome tumor drug resistance. *Cancer Treat Rev*, 34, 7, 592-602.

JONES, D. S., BRUSCHI, M. L., FREITAS, O., GREMIAO, M. P., LARA, E. H. & ANDREWS, G. P. 2009. Rheological, mechanical and mucoadhesive properties of thermoresponsive, bioadhesive binary mixtures composed of poloxamer 407 and carbopol 974P designed as platforms for implantable drug delivery systems for use in the oral cavity. *Int J Pharm*, 372, 1-2, 49-58.

KIM, T. H., AHN, J. S., CHOI, H. K., CHOI, Y. J. & CHO, C. S. 2007. A novel mucoadhesive polymer film composed of carbopol, poloxamer and hydroxypropylmethylcellulose. *Arch Pharm Res*, 30, 3, 381-6.

LAFFLEUR, F. & BERNKOP-SCHNURCH, A. 2013. Strategies for improving mucosal drug delivery. *Nanomedicine (Lond)*, 8, 12, 2061-75.

LEMOINE, D., FRANCOIS, C., KEDZIEREWICZ, F., PREAT, V., HOFFMAN, M. & MAINCENT, P. 1996. Stability study of nanoparticles of poly(epsilon-caprolactone), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide). *Biomater*, 17, 22, 2191-7.

LENAERTS, V., COUVREUR, P., CHRISTIAENS-LEYH, D., JOIRIS, E., ROLAND, M., ROLLMAN, B. & SPEISER, P. 1984. Degradation of poly (isobutyl cyanoacrylate) nanoparticles. *Biomater*, 5, 2, 65-8.

MAEDA, H., WU, J., SAWA, T., MATSUMURA, Y. & HORI, K. 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release*, 65, 1-2, 271-84.

MALINGRE, M. M., BEIJNEN, J. H. & SCHELLENS, J. H. 2001. Oral delivery of taxanes. *Invest New Drugs*, 19, 2, 155-62.

MAZZAFERRO, S., BOUCHEMAL, K. & PONCHEL, G. 2013a. Oral delivery of anticancer drugs I: general considerations. *Drug Discov Today*, 18, 1-2, 25-34.

_____. 2013b. Oral delivery of anticancer drugs III: formulation using drug delivery systems. *Drug Discov Today*, 18, 1-2, 99-104.

MENG, X., TIAN, F., YANG, J., HE, C. N., XING, N. & LI, F. 2010. Chitosan and alginate polyelectrolyte complex membranes and their properties for wound dressing application. *J Mater Sci Mater Med*, 21, 5, 1751-9.

MOGHADDAM, F. A., ATYABI, F. & DINARVAND, R. 2009a. Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. *Nanomed*, 5, 2, 208-15.

_____. 2009b. Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. *Nanomedicine*, 5, 2, 208-15.

MOHAMMADPOUR DOUNIGHI, N., ESKANDARI, R., AVADI, M., ZOLFAGHARIAN, H., MIR MOHAMMAD SADEGHI, A. & REZAYAT, M. 2012. Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system. *J Venom Anim Toxins Incl Trop Dis*, 18, 44-52.

NEVES, J., BAHIA, M. F., AMIJI, M. M. & SARMENTO, B. 2011. Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale. *Expert Opin Drug Deliv*, 8, 8, 1085-104.

PELTIER, S., OGER, J. M., LAGARCE, F., COUET, W. & BENOIT, J. P. 2006. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules. *Pharm Res*, 23, 6, 1243-50.

PINTO-ALPHANDARY, H., ABOUBAKAR, M., JAILLARD, D., COUVREUR, P. & VAUTHIER, C. 2003. Visualization of insulin-loaded nanocapsules: in vitro and in vivo studies after oral administration to rats. *Pharm Res*, 20, 7, 1071-84.

PONCHEL, G., DURRER, C., IRACHE, J. M. & DUCHÊNE, D. Study of the interactions between nanoparticles and intestinal mucosa. In: OTTEWILL, R. H. & RENNIE, A. R. (Ed.). Trends in Colloid and Interface Science VIII: Steinkopff, v.97, 1994. cap. 60, p.275-280.

PONCHEL, G. & IRACHE, J. 1998. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv Drug Deliv Rev*, 34, 2-3, 191-219.

QUINTANAR-GUERRERO, D., ALLEMANN, E., FESSI, H. & DOELKER, E. 1998. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. *Drug Dev Ind Pharm*, 24, 12, 1113-28.

RIVKIN, I., COHEN, K., KOFFLER, J., MELIKHOV, D., PEER, D. & MARGALIT, R. 2010. Paclitaxel-clusters coated with hyaluronan as selective tumor-targeted nanovectors. *Biomater*, 31, 27, 7106-14.

ROGER, E., LAGARCE, F., GARCION, E. & BENOIT, J. P. 2010b. Reciprocal competition between lipid nanocapsules and P-gp for paclitaxel transport across Caco-2 cells. *Eur J Pharm Sci*, 40, 5, 422-9.

ROSSI, S., FERRARI, F., BONFERONI, M. C. & CARAMELLA, C. 2000. Characterization of chitosan hydrochloride-mucin interaction by means of viscosimetric and turbidimetric measurements. *Eur J Pharm Sci*, 10, 4, 251-7.

ROWINSKY, E. K. & DONEHOWER, R. C. 1995. Paclitaxel (taxol). *N Engl J Med*, 332, 15, 1004-14.

SACCO, J. J., BOTTEN, J., MACBETH, F., BAGUST, A. & CLARK, P. 2010. The average body surface area of adult cancer patients in the UK: a multicentre retrospective study. *PLoS One*, 5, 1, e8933.

SCHERER, D., ROBINSON, J. R. & KREUTER, J. 1994. Influence of enzymes on the stability of polybutylcyanoacrylate nanoparticles. *Int J Pharm*, 101, 1–2, 165-168.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SPARREBOOM, A., VAN ASPEREN, J., MAYER, U., SCHINKEL, A. H., SMIT, J. W., MEIJER, D. K., BORST, P., NOOIJEN, W. J., BEIJNEN, J. H. & VAN TELLINGEN, O. 1997. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A*, 94, 5, 2031-5.

TEWA-TAGNE, P., BRIANCON, S. & FESSI, H. 2007. Preparation of redispersible dry nanocapsules by means of spray-drying: development and characterisation. *Eur J Pharm Sci*, 30, 2, 124-35.

VAN DEN BONGARD, H. J., KEMPER, E. M., VAN TELLINGEN, O., ROSING, H., MATHOT, R. A., SCHELLENS, J. H. & BEIJNEN, J. H. 2004. Development and validation of a method to determine the unbound paclitaxel fraction in human plasma. *Anal Biochem*, 324, 1, 11-5.

VARMA, M. V., ASHOKRAJ, Y., DEY, C. S. & PANCHAGNULA, R. 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol Res*, 48, 4, 347-59.

VAUTHIER, C. & BOUCHEMAL, K. 2009. Methods for the preparation and manufacture of polymeric nanoparticles. *Pharm Res*, 26, 5, 1025-58.

VERDIERE, A. C., DUBERNET, C., NEMATI, F., POUPON, M. F., PUISIEUX, F. & COUVREUR, P. 1994. Uptake of doxorubicin from loaded nanoparticles in multidrug-resistant leukemic murine cells. *Cancer Chemother Pharmacol*, 33, 6, 504-8.

VERDIERE, A. C., DUBERNET, C., NEMATI, F., SOMA, E., APPEL, M., FERTE, J., BERNARD, S., PUISIEUX, F. & COUVREUR, P. 1997. Reversion of multidrug resistance with polyalkylcyanoacrylate nanoparticles: towards a mechanism of action. *Br J Cancer*, 76, 2, 198-205.

WANI, M. C., TAYLOR, H. L., WALL, M. E., COGGON, P. & MCPHAIL, A. T. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc*, 93, 9, 2325-7.

XAVIER-JUNIOR, F. H., EGITO, E. S. T., MORAIS, A. R. V., ALENCAR, E. N., MACIUK, A., VAUTHIER, C. Chapter VII, 2015. Experimental design approach applied to the development of chitosan coated poly(isobutylcyanocrylate) nanocapsules encapsulating copaiba oil. *Article in preparation*

XAVIER-JUNIOR, F. H., GUEUTIN, C., MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T., VAUTHIER, C. Chapter II, 2015. HPLC method for the dosage of paclitaxel in copaiba oil. Development, validation, application to the determination of the solubility and partition coefficients. *Article in preparation*

ZABALETA, V., CALLEJA, P., ESPUELAS, S., CORRALES, L., PIO, R., AGUEROS, M. & IRACHE, J. M. 2013. Mucopenetrating nanoparticles: vehicles for the oral administration of paclitaxel. *Ann Pharm Fr*, 71, 2, 109-18.

Discussion Générale

La voie orale est certainement la voie préférée des patients pour l'administration de médicaments, en effet c'est la meilleur voie du point de vue du leur confort, en termes d'observance, de simplicité, de coûts du traitement et également de sécurité d'emploi pour le patient (Borner *et al.*, 2001; Batlle *et al.*, 2004). Toutefois, elle est limitée pour certains principes actifs dont les propriétés physico-chimiques, les facteurs physiologiques et d'autres aspects liés aux formes galéniques conduisent à une dissolution et une absorption réduite, ce qui entraîne une faible biodisponibilité orale (Prabhu *et al.*, 2005; Ensign *et al.*, 2012). Il est donc nécessaire que la formulation permette d'obtenir un profil pharmacocinétique pertinent, caractérisé par une biodisponibilité suffisante, ce qui améliorerait encore l'efficacité thérapeutique et la compliance du patient (Singh *et al.*, 2009). Au cours du temps, les nanomédecines sont apparus comme des outils de formulation qui peuvent aider à surmonter ces difficultés (Duncan, 2004; Engineering., 2004; Kateb *et al.*, 2011).

Au cours des dernières années, de nombreaux efforts ont été réalisés afin de développer des systèmes d'administration de médicaments anticancéreux pour la voie orale. Ces efforts de reformulations fournissent également la possibilité d'améliorer l'efficacité de la thérapie à base de médicaments anti-cancéreux (Kawasaki & Player, 2005). Les nouveaux systèmes d'administration de médicaments par voie orale s'avèrent très utiles car ils permettront d'augmenter l'observance des patients, d'améliorer la biodisponibilité, et ainsi de fournir de meilleurs effets thérapeutiques tout en augmentant la qualité de vie des patients (Verma & Garg, 2001; Roger *et al.*, 2010b; Gibaud & Attivi, 2012; Mazzaferro *et al.*, 2013a; b). Les systèmes d'administration modernes empruntés aux nanomédecines sont également capables d'améliorer la stabilité des molécules actives fragiles ou rapidement dégradées dans le milieu gastro-intestinale, réduire la toxicité non-spécifique et améliorer la perméabilité à travers

l'épithélium intestinal tout en diminuant la dose de médicaments délivrés aux cellules cancéreuses (Sparreboom *et al.*, 1997; Varma *et al.*, 2003; Jabr-Milane *et al.*, 2008; Muthu *et al.*, 2009).

De cette façon, l'encapsulation des molécules anticancéreuses dans des vecteurs nanothérapeutiques s'est avérée être une solution prometteuse pour le traitement du cancer. Dans cette thèse, le paclitaxel a été utilisé comme molécule anticancéreuse pour plusieurs raisons. C'est en premier lieu l'un des plus puissants agents anti-cancéreux utilisé pour le traitement des cancers et il fait partie à la classe biopharmaceutique IV (peu soluble et faiblement perméable). Ces raisons justifient de rechercher des formulations originales capables d'améliorer les caractéristiques physico-chimiques du paclitaxel pour augmenter sa biodisponibilité par voie orale (Forastiere, 1994; Rowinsky & Donehower, 1995; Weaver, 2014). Il est intéressant de noter que cette molécule est d'origine végétale extrait du *Taxus brevifolia* (Fang & Liang, 2005). C'est un pseudo-alcaloïde qui présente une structure diterpénoïde. Son mode d'action est defavoriser la stabilisation des microtubules dans le cytoplasme des cellules inhibant ainsi la prolifération cellulaire et induisant finalement l'apoptose (Schiff *et al.*, 1979; Hamel, Campo, *et al.*, 1981; Horwitz, 1992).

Cette molécule anticancéreuse étant d'origine naturelle, l'encapsulation dans des nanosystèmes contenant des huiles d'origine végétale peut être éventuellement intéressant pour augmenter le taux d'encapsulation, la stabilité et favoriser un effet thérapeutique synergique. (Lee *et al.*, 1995; Dantas, T. N. C. *et al.*, 2010; Attaphong *et al.*, 2012). De plus, les huiles extraites des plantes ont un certain nombre d'avantages potentiels en comparaison aux huiles minérales, elles présentent une faible toxicité, sont biodégradables et renouvelables. (Dossat *et al.*, 2002; Aluyor *et al.*, 2009). Dans ce

contexte, l'huile végétale de copaïba (*Copaifera langsdorffi)* semble être une bonne candidate. Cette huile formée par une complexe mélange de composés diterpéniques et sesquiterpéniques permet de lutter contre maladies inflammatoires, microbiologiques et cancéreuse (Veiga-Junior & Pinto, 2002; Gomes, Niele Matos *et al.*, 2007; Gomes N *et al.*, 2008; Mendonça & Onofre, 2009a; Comelli-Júnior *et al.*, 2010; Souza, Martins, Souza, Furtado, Heleno, Sousa, *et al.*, 2011).

Cette thèse avait pour de but développer des systèmes miniaturisés pour améliorer la délivrance de médicaments anticancéreux, le paclitaxel, par voie orale en utilisant l'huile végétale de copaïba qui présente elle-même des propriétés thérapeutique. Dans ce contexte, deux systèmes différents ont été proposés. L'un de nature lipidique et l'autre polymére. Les travaux expérimentaux initiaux sur l'équilibre hydrophile-lipophile et le développement d'émulsions d'huile de copaïba ont été réalisés au Laboratorio de Sistemas Dispersos (LASID) à l'Universidade Federal do Rio Grande (UFRN) à Natal, au Brésil, sous la Direction du Professeur Dr. Sócrates Egito. La deuxième partie de cette thèse concernant le développement des formulations orales à base de microémulsions et de nanocapsules de l'huile de copaïba pour l'encapsulation du paclitaxel ont été élaborées au sein de l'Institut Galien Paris-Sud (UMR 8612), dans l'Université Paris-Sud, à Châtenay Malabry, en France, sous la Direction du Dr Christine Vauthier. Ce travail pu être effectué grâce à une collaboration bilatérale développées dans le cadre d'un projet financé par la « Coordination de perfectionnement du personnel de l'enseignement supérieur du Ministère de l'Education brésilien-CAPES/MEC», le « Comité Français d'Évaluation de la Coopération Universitaire et Scientifique avec le Brésil -COFECUB » et la mise en place d'un accord de co-tutelle de thèse entre les universités brésilienne et française.

Afin d'associer les activités anticancéreuses de l'huile de copaïba et d'un principe actif utilisé couramment en cancérologie dans une formulation de la nanomédecine unique, le développement de méthodes destinées à analyser et doser l'huile de copaïba et le paclitaxel solubilisé dans cette huile ont été développées. La validation de la méthode a été réalisée afin de normaliser le processus et l'utilisation de l'instrumentation visant à minimiser l'erreur aléatoire et s'assurer que la méthode peut être digne de confiance. La détermination de nombreux paramètres expérimentaux ont été nécessaire pour garantir que la méthode est validée (González *et al.*, 2014; Mujawar *et al.*, 2014; Nikolaou *et al.*, 2015). La validation de la méthode a été réalisée par la détermination de la précision, linéarité, sensibilité, sélectivité, et des limites de détection et quantification.

Les critères qui ont été retenus pour le choix des méthodes d'analyses et de dosage du paclitaxel et de l'huile de copaïba étaient basés sur la rapidité de l'analyse, la simplicité de mise en œuvre, la précision, la sensibilité et la performance économique de la mesure. Une analyse par chromatographie gazeuse a été proposée pour l'huile de copaïba. Plusieurs composants ont été identifiés et la méthode a été développée pour permettre leur quantification à l'aide d'un détecteur à ionisation de flamme. Les principaux composés identifiés dans l'huile essentielle de copaïba ont été le β-bisabolène (23,6%), le β-caryophyllène (21,7%) et l'α-bergamotène (20,5%). Les composés identifiés dans l'huile résine de copaïba sont l'acide copalic (15,6%), le β-bisabolène (12,3%), le β-caryophyllène (7,9%), l'α-bergamotène (7,1%) et l'acide Labd-8(20)-ene-15,18-dioïque (6,7%). En utilisant un détecteur à ionisation de flamme, la méthode est linéaire pour une gamme de concentration en 0,99 du 40 à 160 $\mu g.mL^{-1}$. L'analyse quantitative peut être réalisée sur un temps d'analyse de 13,15, 14,87 et 21,52 pour le β- caryophyllène, l'α- humulène et l'oxyde caryophyllene, respectivement. La limite de détection a été déterminée à 6,38, 4,16 et 0,55 $\mu g.mL^{-1}$ et la limite de

quantification à été établie à 19,36, 12,62 et 1,67 µg.mL^{-1} pour le β- caryophyllène, l'α-humulène et l'oxyde caryophyllene, respectivement. La précision et l'exactitude de la méthode ont été inférieures à 4,4 et 3,5 %, respectivement.

Le mélange de composants complexes de l'huile de copaïba peut entraver la quantification du paclitaxel lorsqu'il est associé à ses formulations. En conséquence, le besoin d'une méthode d'analyse exacte et précise de paclitaxel dans l'huile de copaïba est obligatoire pour le contrôle de qualité et le développement de médicaments. Par conséquent, l'HPLC UV a été un outil analytique approprié pour effectuer des analyses de paclitaxel à faible coût avec une haute reproductibilité et une méthode simple et efficace. Le dosage du paclitaxel a pu être réalisé par une méthode HPLC développées sur un temps d'analyse de 9,7 min. La courbe d'étalonnage obtenue est linéaire (r^2=0.9998) pour une gamme de concentration du 50 à 2000 ng.mL^{-1} et les résidus de régression présentait une dispersion homoscédastique. Les limites de détection et de quantification ont été établies à 21.03 et 6.31 ng.mL^{-1}, respectivement. Les déterminations de l'exactitude et la précision ont été inférieures ou égales à 0.77 et 0.65 %, respectivement. La méthode a été appliquée à la déterminer de la solubilité du paclitaxel dans l'huile de copaïba et des coefficients de partage du paclitaxel entre différents milieux lipophiles et l'eau. Ainsi, la solubilité du paclitaxel dans l'huile de copaïba résine est intéressante 0,8 mg.mL^{-1} puisqu'elle est apparue 2000 fois supérieure à celle de l'eau. Les coefficients de partage déterminé pour l'huile résine et essentielle sont de 3,2 et 2,6 respectivement. La bonne solubilité du paclitaxel dans l'huile de copaïba est un paramètre favorable en vue de l'association de ce principe actif à des systèmes de délivrance de médicaments issus des nanomédecines.

La détermination de l'équilibre hydrophile-lipophile (HLB) requis pour l'huile de copaïba pour être compatible avec un mélange d'agents tensio-actifs capable de

stabiliser les émulsions du type huile dans l'eau a été développé. Le système HLB est le rapport (ou balance) entre les portions hydrophiles de l'agent tensioactif non- ionique à la partie lipophile. Des valeurs HLB des agents stabilisants affectent la formation et stabilité des systèmes lipidiques dévelopés (Griffin, W.C, 1949). Donc, différents systèmes lipidiques développés. La valeur du HLB requis pour l'huile de copaïba a été déterminé expérimentalement en préparat différents systèmes lipidiques avec des mélanges de tensio-actifs de HLB différents et en étudiant les diagrammes de phase pseudo-ternaires des mélanges de tensioactifs / co-tensioactifs / huile de copaïba / eau. Les systèmes dispersés répondant au cahier des charge fixé ont été obtenus dans la région optimale de HLB du tensio-actif de 14,8, donnant un HLB requis pour l'huile de copaïba de 14.8. Il est intéressant de noter que la dispersion de l'huile de copaïba dans les systèmes émulsionnés était stable pendant plus d'un an, et différents systèmes dispersés (microémulsions, nanoémulsions…) ont été produites en utilisant des diagrammes de phases.

En utilisant les informations obtenues sur l'HLB de l'huile de copaïba, un système lipidique plus stable avec une taille de gouttelettes plus petites pouvant transporter une plus grande quantité d'huile et de medicament ont été développés. Pour se faire, nous nous sommes intéressés à la formulation de microémulsions. D'après a litérature, ces systèmes ont un fort pouvoir de solubilisation des principes actives lipophiles. De plus, ils agissent comme des promoteurs d'absorption pour les molécules de classe IV comme le paclitaxel. Les microémulsions peuvent aussi moduler le profil de libération des principes actifs encapsulés ainsi que promouvoir l'absorption des formulations administrées par voie orale par le système lymphatique évitant ainsi l'effet du premier passage hépatique (Schmalfub *et al.*, 1997; Tenjarla, 1999; Singh *et al.*, 2011; Lawrence & Rees, 2012; Mcclements, 2012; Ritika *et al.*, 2012; Lakshmi *et al.*, 2013). L'approche

suivie pour la formulation des microémulsions a été basée sur une analyse des paramètres de solubilité des composés dans le but de privilégier un choix basé sur leur miscibilité, a priori favorable pour favoriser la stabilité des interfaces huile/eau créées lors de la préparation des microémulsions. Cette démarche a permis de formuler des microémulsions contenant des fractions volumiques importantes d'huile essentielle de copaïba (19,6%) tout en maintenant la concentration en tensioactifs faible (13.7 %). Le paclitaxel a pu être incorporé dans les microémulsions avec une efficacité d'encapsulation de 37% donnant une concentration en paclitaxel de 0,37 $mg.mL^{-1}$ de microémulsion. Cette incorporation ne perturbe pas notablement le diamètre des gouttelettes de la microémulsion ni la structure. Une étude de mucoadhésion a montré que la microémulsion contenant le paclitaxel permet de concentrer ce principe actif sur la muqueuse intestinale de rat.

Dans cette thèse nous avons également chercher à développer une formulation de nanocapsules polyméres mucoadhésives pour le transport simultané de l'huile de copaïba et du paclitaxel par voie orale. Les nanocapsules ont été choisi car elles présentent un noyau huileux enveloppé dans une coque de polymère et que ceses systèmes se sont déjà montrés prometteurs pour permettre d'améliorer la biodisponibilité de molécules actives administrées par la voie orale (Cruz *et al.*, 2006; Pinto Reis *et al.*, 2006b; Leite *et al.*, 2007; Anton *et al.*, 2008). L'objectif du travail a été de développer des nanocapsules contenant de l'huile de copaïba et du paclitaxel avec des propriétés mucoadhésives grâce à l'utilisation de chitosane. Des nanocapsules ayant les propriétés recherchées ont été obtenus par le développent d'une méthode originale de polymérisation interfaciale du cyanoacrylate d'isobutyle en présence de chitosane comme agent assurant la stabilité colloïdale de la dispersion et devant promouvoir la mucoadhésion des formulations. L'obtention de nanocapsules de petite dimension et de

potentiel zêta positif de valeur absolue élevée a été optimisez par un plan d'expérience à 2 niveaux en cosidérant trois variables indépendantes (pH, température et concentration du chitosane dans le milieu). Les systèmes développés et optimisés ont montré un diamètre de 473 nm, un potentiel zêta de +34 mV et une efficacité d'encapsulation de l'huile de copaïba de 75,8% soit une association de 55,5 µg de β- caryophyllène / mg de nanocapsules. Le procédé d'encapsulation n'a pas modifié la composition de l'huile qui est demeurée inchangée après encapsulation par rapport à l'huile de départ. Les valeurs du potentiel zêta positives sont en conformité avec la distribution attendue chitosane sur la surface des nanocapsules (Thanou *et al.*, 2001; Mao *et al.*, 2004). Le paclitaxel incorporé n'a pas modifiée la taille, la morphologie et le potentiel zêta des nanocapsules. Le rendement d'encapsulation du paclitaxel était 75%, ce qui correspond à 17 $\mu g.mg^{-1}$ de nanocapsules. Cette formulation était stable en milieu gastrique reconstitué pendant 120 minutes et au bout de six mois à 4 °C en suspension dans l'eau. Les études de mucoadhésion ont permis de montrer que 9% de la quantité de paclitaxel apporté sous forme de nanocapsules avaient adhéré à une surface de 1 cm^2 de muqueuse intestinale après 2 heures d'incubation en chambre de Ussing ce qui correspond à une quantité de 3.4 g de nanocapsules par m^2 de la muqueuse intestinale. Ces systèmes ont montré une bonne corrélation avec d'autres nanoparticules mucoadhésives développés dans la littérature (Moghaddam *et al.*, 2009b) et l'augmentation de la mucoadhesion en 2 fois par rapport autres études en utilisant nanoparticules recouverte avec chitosan 20 kDa (Bravo-Osuna, Vauthier, *et al.*, 2007).

Les deux systèmes développés dans cette thèse ont présenté une efficacité de transport du paclitaxel associé avec l'huile de copaïba par voie orale. Cependant, la quantité de paclitaxel dans les nanocapsules a été deux fois plus que les microémulsions et ceux-là ont été stable à séchage. Les nanocapsules ont aussi été systèmes plus performant pour

le transport du paclitaxel, car ils ont présenté une plus haute mucoadhésion correspondant à une plus grande association du anticancéreux dans les nanocapsules avec la muqueuse intestinale chez le rat.

Les résultats de ces travaux ont conduit au développement de formulations de paclitaxel contenant l'huile naturel de copaïba dans des nanosystèmes originaux qui pourront par la suite être évalués pour en étudier leur capacité à délivrer l'agent anticancéreux par voie orale. La biodisponibilité du paclitaxel administré par voie orale à l'aide de ces formulations sera à évaluer de même que l'effet synergique possible de l'huile de copaïba qui a été utilisé comme composant des formulations et elle présente des propriétés pharmacologiques anticancéreuses.

Références

ALUYOR, E. O., OZIGAGU, C. E., OBOH, O. I. & ALUYOR, P. 2009. Chromatographic analysis of vegetable oils: A review. *Sci Res Essays*, 4, 4, 191-197.

ANTON, N., BENOIT, J. P. & SAULNIER, P. 2008. Design and production of nanoparticles formulated from nano-emulsion templates - A review. *J Control Release*, 128, 3, 185-199.

ATTAPHONG, C., DO, L. & SABATINI, D. A. 2012. Vegetable oil-based microemulsions using carboxylate-based extended surfactants and their potential as an alternative renewable biofuel. *Fuel*, 94, 1, 606-613.

BATLLE, J. F., ARRANZ, E. E., CASTRO CARPEÑO, J., SÁEZ, E. C., AUÑÓN, P. Z., SÁNCHEZ, A. R. & BARÓN, M. G. 2004. Oral chemotherapy: potential benefits and limitations. *Clin Transl Oncol*, 6, 6, 335-340.

BORNER, M., SCHEITHAUER, W., TWELVES, C., MAROUN, J. & WILKE, H. 2001. Answering patients' needs: oral alternatives to intravenous therapy. *Oncologist*, 6 Suppl 4, 12-6.

BRAVO-OSUNA, I., VAUTHIER, C., FARABOLLINI, A., PALMIERI, G. F. & PONCHEL, G. 2007. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomater*, 28, 13, 2233-43.

COMELLI-JÚNIOR, E., SKINOVSKI, J., SIGWALT, M. F., BRANCO, A. B., LUZ, S. R. & BAULÉ, C. P. 2010. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaíba *(Copaifera Iangsdorfii)* oil. *Acta Cir Bras*, 25, 362-367.

CRUZ, L., SCHAFFAZICK, S. R., DALLA COSTA, T., SOARES, L. U., MEZZALIRA, G., DA SILVEIRA, N. P., SCHAPOVAL, E. E., POHLMANN, A. R. & GUTERRES, S. S. 2006. Physico-chemical characterization and in vivo evaluation of indomethacin ethyl ester-loaded nanocapsules by PCS, TEM, SAXS, interfacial alkaline hydrolysis and antiedematogenic activity. *J Nanosci Nanotechnol*, 6, 9-10, 3154-62.

DANTAS, T. N. C., SILVA, H. S. R. C., NETO, A. A. D., MARCUCCI, M. C. & MACIEL, M. A. M. 2010. Development of a new propolis microemulsion system for topical applications. *Rev bras farmacogn*, 20, 3, 368-375.

DOSSAT, V., COMBES, D. & MARTY, A. 2002. Lipase-catalysed transesterification of high oleic sunflower oil. *Enzyme Microb Technol*, 30, 1, 90-94.

DUNCAN, R. 2004. Nanomedicines in action. *Pharm J*, 273, 485-488.

ENGINEERING., R. S. R. A. O. Nanoscience and nanotechnologies: Opportunities and uncertainties. London, UK: Royal Society, 2004.

ENSIGN, L. M., CONE, R. & HANES, J. 2012. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Adv Drug Deliv Rev*, 64, 6, 557-70.

FANG, W. S. & LIANG, X. T. 2005. Recent progress in structure activity relationship and mechanistic studies of taxol analogues. *Mini Rev Med Chem*, 5, 1, 1-12.

FORASTIERE, A. A. 1994. Current and future trials of Taxol (paclitaxel) in head and neck cancer. *Ann Oncol*, 5 Suppl 6, S51-4.

GIBAUD, S. & ATTIVI, D. 2012. Microemulsions for oral administration and their therapeutic applications. *Expert Opin Drug Deliv*, 9, 8, 937-51.

GOMES N, M., REZENDE C, M., FONTES, S. P., HOVELL, A. M., LANDGRAF, R. G., MATHEUS, M. E., PINTO A, C. & FERNANDES, P. D. 2008. Antineoplasic

activity of Copaifera multijuga oil and fractions against ascitic and solid Ehrlich tumor. *J Ethnopharmacol*, 119, 1, 179-84.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-492.

GONZÁLEZ, O., BLANCO, M. E., IRIARTE, G., BARTOLOMÉ, L., MAGUREGUI, M. I. & ALONSO, R. M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *J Chromatogr A*, 1353, 0, 10-27.

GRIFFIN, W. C. 1949. Classification of surface-active Agents by "HLB". *J Soc Cosmet Chem*, 311-326.

HAMEL, E., CAMPO, A. A., LOWE, M. C. & LIN, C. M. 1981. Interactions of taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J Biol Chem*, 256, 22, 11887-94.

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci*, 13, 4, 134-6.

JABR-MILANE, L. S., VAN VLERKEN, L. E., YADAV, S. & AMIJI, M. M. 2008. Multi-functional nanocarriers to overcome tumor drug resistance. *Cancer Treat Rev*, 34, 7, 592-602.

KATEB, B., CHIU, K., BLACK, K. L., YAMAMOTO, V., KHALSA, B., LJUBIMOVA, J. Y., DING, H., PATIL, R., PORTILLA-ARIAS, J. A., MODO, M., MOORE, D. F., FARAHANI, K., OKUN, M. S., PRAKASH, N., NEMAN, J., AHDOOT, D., GRUNDFEST, W., NIKZAD, S. & HEISS, J. D. 2011. Nanoplatforms for constructing new approaches to cancer treatment, imaging, and drug delivery: what should be the policy? *Neuroimage*, 54 Suppl 1, S106-24.

KAWASAKI, E. S. & PLAYER, A. 2005. Nanotechnology, nanomedicine, and the development of new, effective therapies for cancer. *Nanomedicine*, 1, 2, 101-9.

LAKSHMI, J., KUMAR, B. A. & GUPTA, S. 2013. Investigation of microemulsion as a potential carrier for advanced transdermal delivery: An overview. *Int J Pharm Sci Rev Res* 20, 2, 51-59.

LAWRENCE, M. J. & REES, G. D. 2012. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 64, 175-193.

LEE, M. J., LEE, M. H. & SHIM, C. K. 1995. Inverse targeting of drugs to reticuloendothelial system-rich organs by lipid microemulsion emulsified with poloxamer-338. *Inter J Pharm*, 113, 2, 175-187.

LEITE, E. A., GRABE-GUIMARAES, A., GUIMARAES, H. N., MACHADO-COELHO, G. L., BARRATT, G. & MOSQUEIRA, V. C. 2007. Cardiotoxicity reduction induced by halofantrine entrapped in nanocapsule devices. *Life Sci*, 80, 14, 1327-34.

MAO, S., SHUAI, X., UNGER, F., SIMON, M., BI, D. & KISSEL, T. 2004. The depolymerization of chitosan: effects on physicochemical and biological properties. *Int J Pharm*, 281, 1-2, 45-54.

MAZZAFERRO, S., BOUCHEMAL, K. & PONCHEL, G. 2013a. Oral delivery of anticancer drugs I: general considerations. *Drug Discov Today*, 18, 1-2, 25-34.

_____. 2013b. Oral delivery of anticancer drugs III: formulation using drug delivery systems. *Drug Discov Today*, 18, 1-2, 99-104.

MCCLEMENTS, D. J. 2012. Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, 8, 1719-1729

MENDONÇA, D. E. & ONOFRE, S. B. 2009a. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev Bras Farmacogn*, 19, 577-581.

MOGHADDAM, F. A., ATYABI, F. & DINARVAND, R. 2009b. Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. *Nanomedicine*, 5, 2, 208-15.

MUJAWAR, S., UTTURE, S. C., FONSECA, E., MATARRITA, J. & BANERJEE, K. 2014. Validation of a GC–MS method for the estimation of dithiocarbamate fungicide residues and safety evaluation of mancozeb in fruits and vegetables. *Food Chem*, 150, 0, 175-181.

MUTHU, M. S., RAJESH, C. V., MISHRA, A. & SINGH, S. 2009. Stimulus-responsive targeted nanomicelles for effective cancer therapy. *Nanomedicine (Lond)*, 4, 6, 657-67.

NIKOLAOU, P., PAPOUTSIS, I., DONA, A., SPILIOPOULOU, C. & ATHANASELIS, S. 2015. Development and validation of a GC/MS method for the simultaneous determination of levetiracetam and lamotrigine in whole blood. *J Pharm Biomed Anal*, 102, 0, 25-32.

PINTO REIS, C., NEUFELD, R. J., RIBEIRO, A. J. & VEIGA, F. 2006b. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomedicine*, 2, 1, 8-21.

PRABHU, S., ORTEGA, M. & MA, C. 2005. Novel lipid-based formulations enhancing the in vitro dissolution and permeability characteristics of a poorly water-soluble model drug, piroxicam. *Int J Pharm*, 301, 1–2, 209-216.

RITIKA, A., HARIKUMAR, S. L. & AGGARWAL, G. 2012. Microemulsion system in role of expedient vehicle for dermal application. *J Drug Deliv Ther*, 2, 4, 23-28.

ROGER, E., LAGARCE, F., GARCION, E. & BENOIT, J. P. 2010b. Reciprocal competition between lipid nanocapsules and P-gp for paclitaxel transport across Caco-2 cells. *Eur J Pharm Sci*, 40, 5, 422-9.

ROWINSKY, E. K. & DONEHOWER, R. C. 1995. Paclitaxel (taxol). *N Engl J Med*, 332, 15, 1004-14.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SCHMALFUB, U., NEUBERT, R. & WOHLRAB, W. 1997. Modification of drug penetration into human skin using microemulsions. *J Control Release*, 46, 3, 279-285.

SINGH, A. K., CHAURASIYA, A., AWASTHI, A., MISHRA, G., ASATI, D., KHAR, R. K. & MUKHERJEE, R. 2009. Oral bioavailability enhancement of exemestane from self-microemulsifying drug delivery system (SMEDDS). *AAPS PharmSciTech*, 10, 3, 906-16.

SINGH, V., BUSHETTII, S. S., APPALA, R. S., RIZWAN, A., MAMTA, S. & BISHT, A. 2011. Microemulsions as Promising Delivery Systems: A Review. *Ind J Pharm Edu Res* 45, 4, 392-401.

SOUZA, A. B., MARTINS, C. H., SOUZA, M. G., FURTADO, N. A., HELENO, V. C., SOUSA, J. P., ROCHA, E. M., BASTOS, J. K., CUNHA, W. R., VENEZIANI, R. C. & AMBROSIO, S. R. 2011. Antimicrobial activity of terpenoids from *Copaifera langsdorffii Desf.* against cariogenic bacteria. *Phytother Res*, 25, 2, 215-20.

SPARREBOOM, A., VAN ASPEREN, J., MAYER, U., SCHINKEL, A. H., SMIT, J. W., MEIJER, D. K., BORST, P., NOOIJEN, W. J., BEIJNEN, J. H. & VAN TELLINGEN, O. 1997. Limited oral bioavailability and active epithelial excretion of

paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A*, 94, 5, 2031-5.

TENJARLA, S. 1999. Microemulsions: An overview and pharmaceutical applications. *Crit Rev Ther Drug Carrier Syst*, 16, 5, 461-521.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001. Oral drug absorption enhancement by chitosan and its derivatives. *Adv Drug Deliv Rev*, 52, 2, 117-26.

VARMA, M. V., ASHOKRAJ, Y., DEY, C. S. & PANCHAGNULA, R. 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol Res*, 48, 4, 347-59.

VEIGA-JUNIOR, V. F. & PINTO, A. C. 2002. O gênero copaifera L. *Quim Nova*, 25, 273-286.

VERMA, R. K. & GARG, S. 2001. Current status of drug delivery technologies and future directions. *Pharm Technol*, 251 - 14.

WEAVER, B. A. 2014. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*, 25, 18, 2677-81.

Conclusion et Perspective

Les travaux présentés dans cette thèse, s'inscrivent dans le cadre de recherches menées sur le développement d'un nouveau système galénique oral pour la libération d'agents anticancéreux tel le paclitaxel, en utilisant de l'huile végétale de copaïba. L'objectif de ces travaux était donc de mettre au point une forme galénique optimale pour l'administration par voie orale pour le traitement du cancer utilisant des huiles végétales de copaïba. Le but recherché par la nouvelle formulation galénique est d'améliorer et de permettre la réduction des effets indésirables qui compliquent le suivi du traitement anticancéreux. Les travaux expérimentaux ont été réalisés grâce à un accord de coopération international établi entre le Laboratorio de Sistemas Dispersos (LASID) à l'Universidade Federal do Rio Grande (UFRN) à Natal, au Brésil et l'Institut Galien Paris-Sud (UMR 8612), dans l'Université Paris-Sud, à Châtenay Malabry, en France et à la mise en place d'un accord de cotutelle pour la préparation de la thèse.

Dans cette thèse, des méthodes analytiques ont été développés et validées pour réaliser l'analyse qualitative et quatitative de l'huile de copaïba d'une part et pour doser le paclitaxel dans des milieux contenant de l'huile de copaiba d'autre part. Les méthodes développées chromatographies gazeuses et par l'HPLC ont montré une haute performance sur les paramètres de la validation. La méthode HPLC a été appliquée pour déterminer la solubilité et les coefficient de partage du paclitaxel dans l'huile de copaïba puis pour doser le paclitaxel dans les formulations développées avec l'huile de copaiba. .

La formulation des émulsions et microémulsions huile dans eau contenant de l'huile de copaiba a été basée sur l'utilisation d'approches rationelles pour le choix des tensio-actifs. Dans le cas des émulsions, le HLB requis de l'huile a été recherché avec différents mélanges de tensio-actifs puis la formulation optimale a été identifiée en étudiant les diagrammes de phase pseudoternaires. Pour aborder la formulation de

micromulsions incorporant une phase dispersées d'huile importante à faible concentration de tensio-actif une autre stratégie a été adoptée. Elle a été basée sur la recherche de tensio-actifs pour lesquels la partie lipophile présentaient la meilleure miscibilité avec les composés de l'huile de copaiba sur la base de l'analyse des paramètres de solubilité. Cette démarche a permis de proposer une formulation de microémulsion montrant des performances très au dessus des microémulsions de la litérature en terme de quantité d'huile dispersées et une concentration en tensio-actif qui reste en dessous de celle des microémulsions de la litérature. Du paclitaxel a été incorporé à la microémulsion sans changer de manière importante les propriétés et en permettant d'augmenter de manière importante la quantité solubilisée par unité de volume comparée à la solubilité en milieu aqueux qui présente une limite pour l'administration de ce principe actif. Enfin, au cours de ce travail, il a été montré que le paclitaxel incorporé dans la microémulsion pouvait être capturé par la muqueuse intestinale de rat.

Au cours de cette thèse, nous nous sommes aussi intéréssés à la formulation de nanocapsules mucoadhésives. Des nanocapsules originales incorporant de l'huile de copaiba et formées d'une enveloppe de poly(cyanoacrylate d'isobutyle) recouverte de chitosane ont été développées et optimisées par l'application d'un plan d'expérience. Nous avons montré qu'il était possible d'encapsuler du paclitaxel dans le cœur d'huile de copaïba de ces nanocapsules obtenues avec un rendement de fabrication satisfaisant et une teneur élevée en paclitaxel. Ces nanocapsules chargées en paclitaxel sont apparues stables dans les milieux gastro-intestinaux et dans les conditions de conservation étudiées. Ces nanocapsules ont été marquées avec une sonde fluorescente et du [3H]-paclitaxel radiomarqué permettant de démontrer leur intérêt pour leur

capacité à associer le paclitaxel à la muqueuse intestinale de rat grâce à leur propriété mucoadhésive.

Les travaux réalisés au cours de cette thèse ont apporté deux formulations de paclitaxel mucoadhésives de nature différentes incorporant de l'huile de copaiba, une huile naturelle utilisée en médecine traditionnelle pour ses propriétés anticancéreuses. Les études menée sur l'évaluation de la capacité de ces systèmes à promouvoir une association du paclitaxel avec la muqueuse digestive ont montré un transfert de concentrations intéressantes de cette molécule au tissu intestinal de rat. Cependant, les nanocapsules ont été plus efficace par rapport à les microémulsions en ce qui concerne à la mucoadhésion dans la muqueuse intestinale chez le rat.

De nombreuses perspectives s'ouvrent à l'issue de ce travail et peuvent être proposées pour la suite. En effet, plusieurs études complémentaires permettraient de conforter et d’affiner certaines hypothèses et d’approfondir certains aspects qui n'ont pas encore pu être explorés. Ainsi, les travaux sur ces formulations méritent d'être poursuivis en vue d'en évaluer la capacité à améliorer la biodisponibilité du paclitaxel administré par voie orale dans un traitement aux anticancéreux et à en comprendre le mécanisme d'action. Il sera également intéressant d'entreprendre des travaux pour étudier l'hypothèse d'une synergie d'action issue de l'association du paclitaxel avec l'huile de copaiba. La synergie pourrait intervenir à différents niveaux. L'huile de copaiba pourrait jouer un rôle de promoteur d'absorption au niveau de la muqueuse intestinale permettant ainsi d'augmenter significativement la biodisponibilité orale du paclitaxel. Des études complémentaires utilisant le modèle des chambres d’Ussing pourraient permettre d'élucider cet effet avant d'étudier l'impact de la présence de l'huile de copaiba dans les formulations sur la biodisponibilité du paclitaxel administré par voie orale chez

l'animal. L'huile de copaiba contenant des composants anti-cancéreux, celle du paclitaxel pourrait être potentialisée par celle des composants de l'huile. Pour ces études, nous pourrions envisager des travaux menés sur des lignées cellulaires en culture et destinés à évaluer l'activité anticancéreuses des formulations. Ces travaux pourront ensuite être complétés par une étude de l'efficacité d'un traitement appliqué à un modèle de tumeur développé chez l'animal. Dans le cas où une synergie pourra être mise en évidence, une nouvelle étude pourrait avoir pour objectif de réduire la dose de paclitaxel administrée. Une étude similaire pourrait aussi être proposée dans le cas où les formulations modifient fortement la pharmacocinétique et la biodistribution en favorisant une distribution dans le tissue tumoral.

En fonction des résultats, les études toxicologiques aiguës et chroniques seront à entreprendre rapidement. En supplément d'études de toxicologies devant répondre à un cahier des charges bien précis, des études complémentaires pourront être réalisées en évaluant la présence ou la réduction des effets secondaires décrits pour le paclitaxel lorsqu'il sera administré sous la forme d'une microémulsion d'huile de copaiba ou des nanocapsules formulées au cours de ce travail. Les formulations étant destinées à l'administration par voie orale, une étude de la toxicité de ces formulations sur le tissu gastro-intestinale paraît également pertinente à proposée en vue de vérifier que ces formulations n'entraîne pas de modification des fonctionnalités physiologiques des tissus digestifs.

Enfin, d'autres principes actifs lipophiles pourraient être associées aux nanocapsules d'huile de copaiba ou aux microémulsions en vue d'en améliorer leur potentiel pour en développer des médicaments efficaces par voie orale.

Références

ABDELWAHED, W., DEGOBERT, G. & FESSI, H. 2006. Freeze-drying of nanocapsules: impact of annealing on the drying process. *Int J Pharm*, 324, 1, 74-82.

ABDELWAHED, W., DEGOBERT, G., STAINMESSE, S. & FESSI, H. 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv Drug Deliv Rev*, 58, 15, 1688-713.

ABOUBAKAR, M., COUVREUR, P., PINTO-ALPHANDARY, H., GOURITIN, B., LACOUR, B., FARINOTTI, R., PUISIEUX, F. & VAUTHIER, C. 2000. Insulin-loaded nanocapsules for oral administration: In vitro and in vivo investigation. *Drug Dev Res*, 49, 2, 109-117.

ABOUBAKAR, M., PUISIEUX, F., COUVREUR, P., DEYME, M. & VAUTHIER, C. 1999. Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization. *J Biomed Mater Res*, 47, 4, 568-76.

ABOUBAKAR, M., PUISIEUX, F., COUVREUR, P. & VAUTHIER, C. 1999. Physico-chemical characterization of insulin-loaded poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization. *Int J Pharm*, 183, 1, 63-6.

ACHARYA, A., SANYAL, S. K. & MOULIK, S. P. 2001. Physicochemical investigations on microemulsification of eucalyptol and water in presence of polyoxyethylene (4) lauryl ether (Brij-30) and ethanol. *Int J Pharm*, 229, 1-2, 213-26.

ACHARYA, D. P. & HARTLEY, P. G. 2012. Progress in microemulsion characterization. *Curr Opin Colloid Interface Sci*, 17, 5, 274-280.

ADAMS, J. D., FLORA, K. P., GOLDSPIEL, B. R., WILSON, J. W., ARBUCK, S. G. & FINLEY, R. 1993. Taxol: a history of pharmaceutical development and current pharmaceutical concerns. *J Natl Cancer Inst Monogr*, 15, 141-7.

ADAMSON, A. W. 1969. A model for micellar emulsions. *J Colloid Interface Sci*, 29, 2, 261-7.

ADORJAN, B. & BUCHBAUER, G. 2010. Biological properties of essential oils: an updated review. *Flavour Fragr J*, 25, 6, 407-426.

AGATONOVIC-KUSTRIN, S., GLASS, B. D., WISCH, M. H. & ALANY, R. G. 2003. Prediction of a stable microemulsion formulation for the oral delivery of a combination of antitubercular drugs using ANN methodology. *Pharm Res*, 20, 11, 1760-5.

AL-ADHAM, I. S. I., KHALIL, E., AL-HMOUD, N. D., KIERANS, M. & COLLIER, P. J. 2000. Microemulsions are membrane-active, antimicrobial, self-preserving systems. *J Appl Microbiol* 89, 32-39.

AL KHOURI FALLOUH, N., ROBLOT-TREUPEL, L., FESSI, H., DEVISSAGUET, J. P. & PUISIEUX, F. 1986. Development of a new process for the manufacture of polyisobutylcyanoacrylate nanocapsules. *Inter J Pharm*, 28, 2–3, 125-132.

ALENCAR, E. N., XAVIER-JUNIOR, F. H., MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G. & EGITO, E. S. T. 2015. Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. *J Nanosci Nanotechnol*, 15, 880-888.

ALI, J., AKHTAR, N., SULTANA, Y., BABOOTA, S. & AHUJA, A. 2008. Antipsoriatic microemulsion gel formulations for topical drug delivery of babchi oil *(Psoralea corylifolia). Methods Find Exp Clin Pharmacol*, 30, 4, 277-85.

ALLEN, T. M. 2002. Ligand-targeted therapeutics in anticancer therapy. *Nat Rev Cancer*, 2, 10, 750-63.

ALUYOR, E. O., OZIGAGU, C. E., OBOH, O. I. & ALUYOR, P. 2009. Chromatographic analysis of vegetable oils: A review. *Sci Res Essays*, 4, 4, 191-197.

AMBADE, K. W., JADHAV, S. L., GAMBHIRE, M. N., KURMI, S. D., KADAM, V. J. & JADHAV, K. R. 2008. Formulation and evaluation of flurbiprofen microemulsion. *Curr Drug Deliv*, 5, 1, 32-41.

ANGIONI, A., BARRA, A., CORONEO, V., DESSI, S. & CABRAS, P. 2006. Chemical composition, seasonal variability, and antifungal activity of *Lavandula stoechas L. ssp.* stoechas essential oils from stem/leaves and flowers. *J Agric Food Chem*, 54, 12, 4364-70.

ANTON, N., BENOIT, J. P. & SAULNIER, P. 2008. Design and production of nanoparticles formulated from nano-emulsion templates - A review. *J Control Release*, 128, 3, 185-199.

ARAYA, H., TOMITA, M. & HAYASHI, M. 2005. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. *Int J Pharm*, 305, 1-2, 61-74.

ARELLANO, A., SANTOYO, S., MARTIN, C. & YGARTUA, P. 1996. Enhancing effect of terpenes on the in vitro percutaneous absorption of diclofenac sodium. *Inter J Pharm*, 130, 1, 141-145.

ARIAS, J. L., GALLARDO, V., GOMEZ-LOPERA, S. A., PLAZA, R. C. & DELGADO, A. V. 2001. Synthesis and characterization of poly(ethyl-2-cyanoacrylate) nanoparticles with a magnetic core. *J Control Release*, 77, 3, 309-21.

ARMSTRONG, J. S. 2006. Mitochondrial membrane permeabilization: the sine qua non for cell death. *Bioessays*, 28, 3, 253-60.

ASTANI, A., REICHLING, J. & SCHNITZLER, P. 2010. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytother Res*, 24, 5, 673-9.

ATKINSON, H. V. 2005. Modelling the semisolid processing of metallic alloys. *Prog Mater Sci*, 50, 3, 341-412.

ATTAPHONG, C., DO, L. & SABATINI, D. A. 2012. Vegetable oil-based microemulsions using carboxylate-based extended surfactants and their potential as an alternative renewable biofuel. *Fuel*, 94, 1, 606-613.

ATTIMARAD, M., MUEEN AHMED, K. K., ALDHUBAIB, B. E. & HARSHA, S. 2011. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. *Pharm Methods*, 2, 2, 71-75.

ATURKI, Z., ROCCO, A., ROCCHI, S. & FANALI, S. 2014. Current applications of miniaturized chromatographic and electrophoretic techniques in drug analysis. *J Pharm Biomed Anal*, 101, 0, 194-220.

BAE, K. H., LEE, Y. & PARK, T. G. 2007. Oil-encapsulating PEO-PPO-PEO/PEG shell cross-linked nanocapsules for target-specific delivery of paclitaxel. *Biomacromolecules*, 8, 2, 650-6.

BAGWE, R. P., KANICKY, J. F., PALLA, B. J., PATANJALI, P. K. & SHAH, D. O. 2001. Improved drug delivery using microemulsions: rationale, recent progress, and new horizons. *Crit Rev Ther Drug Carrier Syst*, 18, 1, 77-140.

BAKKALI, F., AVERBECK, S., AVERBECK, D. & IDAOMAR, M. 2008. Biological effects of essential oils-a review. *Food Chem Toxicol*, 46, 2, 446-75.

BALAKUMAR, S., RAJAN, S., THIRUNALASUNDARI, T. & JEEVA, S. 2011. Antifungal activity of *Ocimum sanctum Linn.* (Lamiaceae) on clinically isolated dermatophytic fungi. *Asian Pac J Trop Med*, 4, 8, 654-7.

BALBACH, S. & KORN, C. 2004. Pharmaceutical evaluation of early development candidates "the 100 mg-approach". *Int J Pharm*, 275, 1-2, 1-12.

BALZ, R. The healing power of essential oils. Twin Lakes, WI, USA: Lotus Press, 1999.

BARTON, A. F. M. Handbook of solubility parameters and other cohesive parameters. New York: 1983.

BASSOLE, I. H. & JULIANI, H. R. 2012. Essential oils in combination and their antimicrobial properties. *Molecules*, 17, 4, 3989-4006.

BATHOOL, A., VISHAKANTE, G. D., KHAN, M. S. & SHIVAKUMAR, H. G. 2012. Development and characterization of atorvastatin calcium loaded chitosan nanoparticles for sustain drug delivery *Adv Mat Lett*, 3, 6, 466-470.

BATLLE, J. F., ARRANZ, E. E., CASTRO CARPEÑO, J., SÁEZ, E. C., AUÑÓN, P. Z., SÁNCHEZ, A. R. & BARÓN, M. G. 2004. Oral chemotherapy: potential benefits and limitations. *Clin Transl Oncol*, 6, 6, 335-340.

BAYALA, B., BASSOLE, I. H., SCIFO, R., GNOULA, C., MOREL, L., LOBACCARO, J. M. & SIMPORE, J. 2014. Anticancer activity of essential oils and their chemical components - a review. *Am J Cancer Res*, 4, 6, 591-607.

BEERBOWER, A. & HILL, M. W. Detergents and emulsifiers. Allured Publishing Co, 1971.

BERNKOP-SCHNURCH, A. 2005. Mucoadhesive systems in oral drug delivery. *Drug Discov Today Technol*, 2, 1, 83-7.

BERNKOP-SCHNURCH, A., HEINRICH, A. & GREIMEL, A. 2006. Development of a novel method for the preparation of submicron particles based on thiolated chitosan. *Eur J Pharm Biopharm*, 63, 2, 166-72.

BERTHOLON, I., PONCHEL, G., LABARRE, D., COUVREUR, P. & VAUTHIER, C. 2006. Bioadhesive properties of poly(alkylcyanoacrylate) nanoparticles coated with polysaccharide. *J Nanosci Nanotechnol*, 6, 9-10, 3102-9.

BETTS, T. J. 2001. Chemical characterisation of the different types of volatile oil constituents by various solute retention ratios with the use of conventional and novel commercial gas chromatographic stationary phases. *J Chromatogr A*, 936, 1-2, 33-46.

BHALLA, Y., GUPTA, V. K. & JAITAK, V. 2013. Anticancer activity of essential oils: a review. *J Sci Food Agric*, 93, 15, 3643-53.

BHARGAVA, H. N., NARUKAR, A. & LIEB, L. M. 1987. Using microemulsions for drug delivery. *Pharm Technol*, 11, 46 - 54.

BILIA, A. R., GUCCIONE, C., ISACCHI, B., RIGHESCHI, C., FIRENZUOLI, F. & BERGONZI, M. C. 2014. Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. *Evid Based Complement Alternat Med*, 2014, 651593.

BIRESH, K. S. & SHIV, S. H. 2011. Microemulsion drug delivery system for oral bioavailability enhancement of glipizide. *J Adv Pharm Technol Res*, 1, 4, 195-200.

BIRUSS, B., KAHLIG, H. & VALENTA, C. 2007. Evaluation of an eucalyptus oil containing topical drug delivery system for selected steroid hormones. *Int J Pharm*, 328, 2, 142-51.

BISWAS, T. K. & MUKHERJEE, B. 2003. Plant medicines of Indian origin for wound healing activity: a review. *Int J Low Extrem Wounds*, 2, 1, 25-39.

BODDUPALLI, B. M., MOHAMMED, Z. N., NATH, R. A. & BANJI, D. 2010. Mucoadhesive drug delivery system: An overview. *J Adv Pharm Technol Res*, 1, 4, 381-7.

BOONME, P., KRAUEL, K., GRAF, A., RADES, T. & JUNYAPRASERT, V. B. 2006a. Characterization of microemulsion structures in the pseudoternary phase diagram of isopropyl palmitate/water/Brij 97 : 1-butanol. *Aaps Pharmscitech*, 7, 2

BORHADE, V., NAIR, H. & HEGDE, D. 2008. Design and evaluation of self-microemulsifying drug delivery system (SMEDDS) of tacrolimus. *AAPS PharmSciTech*, 9, 1, 13-21.

BORNER, M., SCHEITHAUER, W., TWELVES, C., MAROUN, J. & WILKE, H. 2001. Answering patients' needs: oral alternatives to intravenous therapy. *Oncologist*, 6 Suppl 4, 12-6.

BORRIS, R. P. 1996. Natural products research: perspectives from a major pharmaceutical company. *J Ethnopharmacol*, 51, 1-3, 29-38.

BOWCOTT, J. E. & SCHULMAN, J. H. 1955. Emulsions. *Z Elektrochem*, 59, 283-290.

BOWN, D. Royal horticultural society encyclopedia of herbs and their uses. Dorling Kindersley, 2003.

BRAVO-OSUNA, I., MILLOTTI, G., VAUTHIER, C. & PONCHEL, G. 2007. In vitro evaluation of calcium binding capacity of chitosan and thiolated chitosan poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Int J Pharm*, 338, 1-2, 284-90.

BRAVO-OSUNA, I., PONCHEL, G. & VAUTHIER, C. 2007. Tuning of shell and core characteristics of chitosan-decorated acrylic nanoparticles. *Eur J Pharm Sci*, 30, 2, 143-54.

BRAVO-OSUNA, I., VAUTHIER, C., CHACUN, H. & PONCHEL, G. 2008. Specific permeability modulation of intestinal paracellular pathway by chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles. *Eur J Pharm Biopharm*, 69, 2, 436-44.

BRAVO-OSUNA, I., VAUTHIER, C., FARABOLLINI, A., PALMIERI, G. F. & PONCHEL, G. 2007. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomater*, 28, 13, 2233-43.

BRIGGER, I., DUBERNET, C. & COUVREUR, P. 2002. Nanoparticles in cancer therapy and diagnosis. *Adv Drug Deliv Rev*, 54, 5, 631-51.

BUCHBAUER, G. 2004. On the biological properties of fragrance compounds and essential oils. *Wien Med Wochenschr*, 154, 21-22, 539-47.

BURKE, J. Solubility Parameters: Theory and Application. Craig Jensen Ed, 1984.

BURT, S. 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. *Int J Food Microbiol*, 94, 3, 223-53.

BUZEA, C., PACHECO, II & ROBBIE, K. 2007. Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases*, 2, 4, MR17-71.

CAL, K. 2006. Skin penetration of terpenes from essential oils and topical vehicles. *Planta Med*, 72, 4, 311-6.

CARLSSON, A. S. 2009. Plant oils as feedstock alternatives to petroleum - A short survey of potential oil crop platforms. *Biochim*, 91, 6, 665-70.

CARSON, C. F. & RILEY, T. V. 2003. Non-antibiotic therapies for infectious diseases. *Commun Dis Intell Q Rep*, 27 Suppl, S143-6.

CHAHLIA, N. 2009. Effect of *capparis decidua* on hypolipidemic activity in rats. *J Med plant res*, 3, 6, 481-484.

CHAIT, R., CRANEY, A. & KISHONY, R. 2007. Antibiotic interactions that select against resistance. *Nature*, 446, 7136, 668-671.

CHAIYANA, W., RADES, T. & OKONOGI, S. 2013. Characterization and in vitro permeation study of microemulsions and liquid crystalline systems containing the anticholinesterase alkaloidal extract from *Tabernaemontana divaricata*. *Int J Pharm*, 452, 1-2, 201-10.

CHAIYANA, W., SAEIO, K., HENNINK, W. E. & OKONOGI, S. 2010. Characterization of potent anticholinesterase plant oil based microemulsion. *Int J Pharm*, 401, 1-2, 32-40.

CHAKRABORTY, A., DEVI, B. R., SANJEBAM, R., KHUMBONG, S. & THOKCHOM, I. S. 2010. Preliminary studies on local anesthetic and antipyretic activities of Spilanthes acmella Murr. in experimental animal models. *Indian J Pharmacol*, 42, 5, 277-9.

CHEN, C. K., WANG, Q., JONES, C. H., YU, Y., ZHANG, H., LAW, W. C., LAI, C. K., ZENG, Q., PRASAD, P. N., PFEIFER, B. A. & CHENG, C. 2014. Synthesis of pH-responsive chitosan nanocapsules for the controlled delivery of doxorubicin. *Langmuir*, 30, 14, 4111-9.

CHEN, S. J., EVANS, D. F., NINHAM, B. W., MITCHELL, D. J., BLUM, F. D. & PICKUP, S. 1986. Curvature as a determinant of microstructure and microemulsions. *J Phys Chem*, 90, 5, 842-847.

CHOUDHURY, H., GORAIN, B., KARMAKAR, S. & PAL, T. K. 2014. Development and validation of RP-HPLC method: scope of application in the determination of oil solubility of paclitaxel. *J Chromatogr Sci*, 52, 1, 68-74.

CLARYS, P., ALEWAETERS, K., JADOUL, A., BAREL, A., MANADAS, R. O. & PRÉAT, V. 1998. In vitro percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *Eur J Pharm Biopharm*, 46, 3, 279-283.

COCHRAN, W. G. & COX, G. M. E. D. Experimental designs. New York: Wiley, 1957. 346-354.

COFFIN, M. D. & MCGINITY, J. W. 1992. Biodegradable pseudolatexes: the chemical stability of poly(D,L-lactide) and poly(epsilon-caprolactone) nanoparticles in aqueous media. *Pharm Res*, 9, 2, 200-5.

COMELLI-JÚNIOR, E., SKINOVSKI, J., SIGWALT, M. F., BRANCO, A. B., LUZ, S. R. & BAULÉ, C. P. 2010. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaíba *(Copaifera Iangsdorfii)* oil. *Acta Cir Bras*, 25, 362-367.

CONSTANTINIDES, P. P. 1995. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm Res*, 12, 11, 1561-72.

CONSTANTINIDES, P. P. & WASAN, K. M. 2007. Lipid formulation strategies for enhancing intestinal transport and absorption of P-glycoprotein (P-gp) substrate drugs: in vitro/in vivo case studies. *J Pharm Sci*, 96, 2, 235-48.

CONVENTION, U. S. P. U.S. Pharmacopeia National Formulary United States Pharmacopeial: Pck Sup edition, 2011.

CORNWELL, P. A., BARRY, B. W., BOUWSTRA, J. A. & GOORIS, G. S. 1996. Modes of action of terpene penetration enhancers in human skin; Differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies. *Inter J Pharm*, 127, 1, 9-26.

COURNARIE, F., CHERON, M., BESNARD, M. & VAUTHIER, C. 2004. Evidence for restrictive parameters in formulation of insulin-loaded nanocapsules. *Eur J Pharm Biopharm*, 57, 2, 171-9.

COUVREUR, P., KANTE, B., ROLAND, M., GUIOT, P., BAUDUIN, P. & SPEISER, P. 1979. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J Pharm Pharmacol*, 31, 5, 331-2.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev*, 12, 4, 564-582.

CROTEAU, R. 1987. Biosynthesis and catabolism of monoterpenoids. *Chem Rev*, 87, 929-954

CROTEAU, R., KUTCHAN, T. M. & LEWIS, N. G. Natural products (secondary metabolites). American Society of Plant Physiologists., 2000.

CRUZ, L., SCHAFFAZICK, S. R., DALLA COSTA, T., SOARES, L. U., MEZZALIRA, G., DA SILVEIRA, N. P., SCHAPOVAL, E. E., POHLMANN, A. R. & GUTERRES, S. S. 2006. Physico-chemical characterization and in vivo evaluation of indomethacin ethyl ester-loaded nanocapsules by PCS, TEM, SAXS, interfacial alkaline hydrolysis and antiedematogenic activity. *J Nanosci Nanotechnol*, 6, 9-10, 3154-62.

CUI, G. H., WANG, L., DAVIS, P. J., KARA, M. & LIU, H. 2006. Preparation and physical characterization of a novel marine oil emulsion as a potential new formulation vehicle for lipid soluble drugs. *Int J Pharm*, 325, 1-2, 180-185.

DAHANUKAR, S. A. & KULKARNI, R. A. 2000. Pharmacology of medicinal plants and natural products. *Indian J Pharmacol*, 32, 81-118.

DAMGE, C., MICHEL, C., APRAHAMIAN, M. & COUVREUR, P. 1988. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. *Diabetes*, 37, 2, 246-51.

DANHIER, F., LECOUTURIER, N., VROMAN, B., JEROME, C., MARCHAND-BRYNAERT, J., FERON, O. & PREAT, V. 2009. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in vitro and in vivo evaluation. *J Control Release*, 133, 1, 11-7.

DANHIER, F., UCAKAR, B., MAGOTTEAUX, N., BREWSTER, M. E. & PREAT, V. 2010. Active and passive tumor targeting of a novel poorly soluble cyclin dependent kinase inhibitor, JNJ-7706621. *Int J Pharm*, 392, 1-2, 20-8.

DANIELSSON, I. & LINDMAN, B. 1981. The definition of microemulsion. *Colloids Surf*, 3, 4, 391-392.

DANTAS, T. N. C., SILVA, H. S. R. C., NETO, A. A. D., MARCUCCI, M. C. & MACIEL, M. A. M. 2010. Development of a new propolis microemulsion system for topical applications. *Rev bras farmacogn*, 20, 3, 368-375.

DATE, A. A. & NAGARSENKER, M. S. 2008. Parenteral microemulsions: An overview. *Int J Pharm* 355, 1–2, 19-30.

DESBRIERES, J. 2002. Viscosity of semiflexible chitosan solutions: influence of concentration, temperature, and role of intermolecular interactions. *Biomacromolecules*, 3, 2, 342-9.

DEUS, R. J. A., ALVES, C. N. & ARRUDA, M. S. P. 2011. Avaliação do efeito antifúngico do óleo resina e do óleo essencial de copaíba (Copaifera multijuga Hayne). *Rev Bras Plantas Med*, 13, 01-07.

DEVI, K. P., NISHA, S. A., SAKTHIVEL, R. & PANDIAN, S. K. 2010. Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *J Ethnopharmacol*, 130, 1, 107-15.

DJORDJEVIC, L., PRIMORAC, M., STUPAR, M. & KRAJISNIK, D. 2004. Characterization of caprylocaproyl macrogolglycerides based microemulsion drug delivery vehicles for an amphiphilic drug. *Int J Pharm*, 271, 1-2, 11-9.

DORMAN, H. J. & DEANS, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol*, 88, 2, 308-16.

DOSSAT, V., COMBES, D. & MARTY, A. 2002. Lipase-catalysed transesterification of high oleic sunflower oil. *Enzyme Microb Technol*, 30, 1, 90-94.

DOUGLAS, S. J., ILLUM, L. & DAVIS, S. S. 1985. Particle size and size distribution of poly(butyl 2-cyanoacrylate) nanoparticles. II. Influence of stabilizers. *J Colloid Interface Sci*, 103, 1, 154-163.

DUNCAN, R. 2004. Nanomedicines in action. *Pharm J*, 273, 485-488.

EL-HEFIAN, E. A., ELGANNOUDI, E. S., MAINAL, A. & YAHAYA, A. H. 2010. Characterization of chitosan in acetic acid: Rheological and thermal studies. *Turk J Chem*, 34, 1, 47-56.

ENGINEERING., R. S. R. A. O. Nanoscience and nanotechnologies: Opportunities and uncertainties. London, UK: Royal Society, 2004.

ENSIGN, L. M., CONE, R. & HANES, J. 2012. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Adv Drug Deliv Rev*, 64, 6, 557-70.

EZURUIKE, U. F. & PRIETO, J. M. 2014. The use of plants in the traditional management of diabetes in Nigeria: Pharmacological and toxicological considerations. *J Ethnopharmacol*, 155, 2, 857-924.

FALLOUH, N. A., ROBLOTTREUPEL, L., FESSI, H., DEVISSAGUET, J. P. & PUISIEUX, F. 1986. Development of a New Process for the Manufacture of Polyisobutylcyanoacrylate Nanocapsules. *Int J Pharm*, 28, 2-3, 125-132.

FANG, J., NAKAMURA, H. & MAEDA, H. 2011. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev*, 63, 3, 136-51.

FANG, W. S. & LIANG, X. T. 2005. Recent progress in structure activity relationship and mechanistic studies of taxol analogues. *Mini Rev Med Chem*, 5, 1, 1-12.

FANUN, M. 2010a. Formulation and characterization of microemulsions based on mixed nonionic surfactants and peppermint oil. *J Colloid Interface Sci*, 343, 2, 496-503.

_____. 2010b. Properties of microemulsions with mixed nonionic surfactants and citrus oil. *Colloids Surf A Physicochem Eng Asp*, 369, 1–3, 246-252.

FENG, S. & HUANG, G. 2001. Effects of emulsifiers on the controlled release of paclitaxel (Taxol) from nanospheres of biodegradable polymers. *J Control Release*, 71, 1, 53-69.

FESSI, H., PUISIEUX, F., DEVISSAGUET, J. P., AMMOURY, N. & BENITA, S. 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. *Int J Pharm*, 55

Food and Drug Administration, Guidance for Industry. Bioanalytical Method Validation 2001.

FORASTIERE, A. A. 1994. Current and future trials of Taxol (paclitaxel) in head and neck cancer. *Ann Oncol*, 5 Suppl 6, S51-4.

FORMARIZ, T. P., CHIAVACCI, L. A., SCARPA, M. V., SILVA-JÚNIOR, A. A., EGITO, E. S. T., TERRUGI, C. H. B., FRANZINI, C. M., SARMENTO, V. H. V. & OLIVEIRA, A. G. 2010. Structure and viscoelastic behavior of pharmaceutical biocompatible anionic microemulsions containing the antitumoral drug compound doxorubicin. *Colloids Surf B Biointerfaces*, 77, 1, 47-53.

FRENSE, D. 2007. Taxanes: perspectives for biotechnological production. *Appl Microbiol Biotechnol*, 73, 6, 1233-40.

GALLARDO, M., COUARRAZE, G., DENIZOT, B., TREUPEL, L., COUVREUR, P. & PUISIEUX, F. 1993. Study of the mechanisms of formation of nanoparticles and nanocapsules of polyisobutyl-2-cyanoacrylate. *Inter J Pharm*, 100, 1–3, 55-64.

GAO, Z.-G., CHOI, H.-G., SHIN, H.-J., PARK, K.-M., LIM, S.-J., HWANG, K.-J. & KIM, C.-K. 1998. Physicochemical characterization and evaluation of a microemulsion system for oral delivery of cyclosporin A. *Int J Pharm*, 61, 1, 75-86.

GÅSEROD, O., JOLLIFFEB, I. G., HAMPSONB, F. C., DETTMARB, P. W. & SKJÅK-BRÆKA, G. 1998. The enhancement of the bioadhesive properties of calcium alginate gel beads by coating with chitosan. *Int J Pharm*, 175, 2, 237–246.

GEISSMAN, T. A. Flavonoid compounds, tannins, lignins and related compounds. New York, N.Y: Elsevier, 1963.

GELMINI, F., BERETTA, G., ANSELMI, C., CENTINI, M., MAGNI, P., RUSCICA, M., CAVALCHINI, A. & MAFFEI FACINO, R. 2013. GC-MS profiling of the phytochemical constituents of the oleoresin from Copaifera langsdorffii Desf. and a preliminary in vivo evaluation of its antipsoriatic effect. *Int J Pharm*, 440, 2, 170-8.

GHOSH, P. K. & MURTHY, R. S. R. 2006. Microemulsions: A potential drug delivery system. *Curr Drug Deliv*, 3, 167-180.

GHOSH, V., SARANYA, S., MUKHERJEE, A. & CHANDRASEKARAN, N. 2013. Antibacterial microemulsion prevents sepsis and triggers healing of wound in wistar rats. *Colloids Surf B Biointerfaces*, 105, 152-157.

GIBAUD, S. & ATTIVI, D. 2012. Microemulsions for oral administration and their therapeutic applications. *Expert Opin Drug Deliv*, 9, 8, 937-51.

GILL, A. O., DELAQUIS, P., RUSSO, P. & HOLLEY, R. A. 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *Int J Food Microbiol*, 73, 1, 83-92.

GOMES-CARNEIRO, M. R., DIAS, D. M., OLIVEIRA, A. C. & PAUMGARTTEN, F. J. 2005. Evaluation of mutagenic and antimutagenic activities of alpha-bisabolol in the Salmonella/microsome assay. *Mutat Res*, 585, 1-2, 105-12.

GOMES-CARNEIRO, M. R., FELZENSZWALB, I. & PAUMGARTTEN, F. J. 1998. Mutagenicity testing (+/-)-camphor, 1,8-cineole, citral, citronellal, (-)-menthol and terpineol with the Salmonella/microsome assay. *Mutat Res*, 416, 1-2, 129-36.

GOMES N, M., REZENDE C, M., FONTES, S. P., HOVELL, A. M., LANDGRAF, R. G., MATHEUS, M. E., PINTO A, C. & FERNANDES, P. D. 2008. Antineoplasic activity of Copaifera multijuga oil and fractions against ascitic and solid Ehrlich tumor. *J Ethnopharmacol*, 119, 1, 179-84.

GOMES, N. M., REZENDE, C. M., FONTES, S. P., MATHEUS, M. E. & FERNANDES, P. D. 2007. Antinociceptive activity of Amazonian Copaiba oils. *J Ethnopharmacol*, 109, 3, 486-92.

GONZÁLEZ, O., BLANCO, M. E., IRIARTE, G., BARTOLOMÉ, L., MAGUREGUI, M. I. & ALONSO, R. M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *J Chromatogr A*, 1353, 0, 10-27.

GRAMOSA, N. V. & SILVEIRA, E. R. 2005. Volatile constituents of *Copaifera langsdorffii* from the Brazilian northeast. *J Essent Oil Res*, 17, 2, 3.

GRATIERI, T., GELFUSO, G. M., ROCHA, E. M., SARMENTO, V. H., DE FREITAS, O. & LOPEZ, R. F. 2010. A poloxamer/chitosan in situ forming gel with prolonged retention time for ocular delivery. *Eur J Pharm Biopharm*, 75, 2, 186-93.

GREENHALGH, D. J., WILLIAMS, A. C., TIMMINS, P. & YORK, P. 1999. Solubility parameters as predictors of miscibility in solid dispersions. *J Pharm Sci*, 88, 11, 1182-90.

GRIFFIN, W. C. 1949. Classification of surface-active Agents by "HLB". *J Soc Cosmet Chem*, 311-326.

_____. 1954. Calculation of HLB Values of Non-Ionic Surfactants. *J Soc Cosmet Chem*, 5, 4, 249-56.

GROO, A. C., SAULNIER, P., GIMEL, J. C., GRAVIER, J., AILHAS, C., BENOIT, J. P. & LAGARCE, F. 2013. Fate of paclitaxel lipid nanocapsules in intestinal mucus in view of their oral delivery. *Int J Nanomedicine*, 8, 1178-2013 (Electronic), 4291-302.

GUENTHER, E. The essential oils. Malabar, Fla, USA: Krieger Publishing Company, 1972.

GUERITTE-VOEGELEIN, F., GUENARD, D., DUBOIS, J., WAHL, A. & POTIER, P. 1994. [Chemical and biological studies on Taxol (Paclitaxel) and Taxotere (Docetaxel), new antineoplastic agents]. *J Pharm Belg*, 49, 3, 193-205.

GUO, P., MA, J., LI, S. & GALLO, J. M. 2003. Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 798, 1, 79-86.

GUPTA, S. & MOULIK, S. P. 2008. Biocompatible microemulsions and their prospective uses in drug delivery. *J Pharm Sci*, 97, 1, 22-45.

GUPTA, S., MOULIK, S. P., LALA, S., BASU, M. K., SANYAL, S. K. & DATTA, S. 2005. Designing and testing of an effective oil-in-water microemulsion drug delivery system for *in vivo* application. *Drug Deliv*, 12, 5, 267-73.

GUPTA, S., SANYAL, S. K., DATTA, S. & MOULIK, S. P. 2006. Preparation of prospective plant oil derived micro-emulsion vehicles for drug delivery. *Indian J Biochem Biophys*, 43, 4, 254-7.

GUTERRES, S. S., ALVES, M. P. & POHLMANN, A. R. 2007. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. *Drug Target Insights*, 2, 147-57.

HAGERSTROM, H., EDSMAN, K. & STROMME, M. 2003. Low-frequency dielectric spectroscopy as a tool for studying the compatibility between pharmaceutical gels and mucous tissue. *J Pharm Sci*, 92, 9, 1869-81.

HAJHASHEMI, V., GHANNADI, A. & SHARIF, B. 2003. Anti-inflammatory and analgesic properties of the leaf extracts and essential oil of Lavandula angustifolia Mill. *J Ethnopharmacol*, 89, 1, 67-71.

HAMED, S. F., SADEK, Z. & EDRIS, A. 2012. Antioxidant and antimicrobial activities of clove bud essential oil and eugenol nanoparticles in alcohol-free microemulsion. *J Oleo Sci*, 61, 11, 641-8.

HAMEL, E., CAMPO, A. A., LOWE, M. C. & LIN, C. M. 1981. Interactions of taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J Biol Chem*, 256, 22, 11887-94.

HAMMER, K. A., CARSON, C. F. & RILEY, T. V. 1999. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol*, 86, 6, 985-90.

HANIF, R. M., QINENG, P. & ZHAN, G. 1998. Penetration enhancing effect of tetrahydrogeraniol on the percutaneous absorption of 5-fluorouracil from gels in excised rat skin. *J Control Release*, 55, 2-3, 297-302.

HANSEN, C. M. 1967. The three dimensional solubility parameter - key to paint component affinities ii. - dyes, emulsifiers, mutual solubility and compatibility, and pigments. *J Paint Technol*, 39, 511, 505-510.

HANSEN, J. M., GO, Y. M. & JONES, D. P. 2006. Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu Rev Pharmacol Toxicol*, 46, 215-34.

HARIVARDHAN REDDY, L. & MURTHY, R. S. R. 2003. Polymerization of n-butyl cyanoacrylate in presence of surfactant: study of influence of polymerization factors on particle properties, drug loading and evaluation of its drug release kinetics. *Ars Pharmaceutica*, 44, 4, 351-369

HATHOUT, R. M., WOODMAN, T. J., MANSOUR, S., MORTADA, N. D., GENEIDI, A. S. & GUY, R. H. 2010. Microemulsion formulations for the transdermal delivery of testosterone. *Eur J Pharm Sci*, 40, 3, 188-196.

HE, C. X., GAO, J.-Q. & GAO, J. Q. 2010. Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs. *Expert Opin Drug Deliv*, 7, 4, 445-60.

HEGDE, R. R., VERMA, A. & GHOSH, A. 2013. Microemulsion: new insights into the ocular drug delivery. *ISRN Pharm*, 2013, 826798.

HELANDER, H. F. & FANDRIKS, L. 2014. Surface area of the digestive tract - revisited. *Scand J Gastroenterol*, 49, 6, 681-9.

HILDEBRAND, J. H. 1916. Solubility. *J Am Chem Soc*, 38, 1442 - 1473

HILDEBRAND, J. H., PRAUSNITZ, J. M. & SCOTT, R. L. Regular and related solutions. New York: Van Nostrand Reinhold, 1970.

HILDEBRAND, J. H. & SCOTT, R. L. The Solubility of Non-electrolytes. New York: 1950.

HO, H.-O., HSIAO, C.-C. & SHEU, M.-T. 1996. Preparation of microemulsions using polyglycerol fatty acid esters as surfactant for the delivery of protein drugs. *Int J Pharm*, 85, 2, 138-143.

HOAR, T. P. & SCHULMAN, J. H. 1943. Transparent water-in-oil dispersions: the oleopathic hydro-micelle. *Nature*, 152, 102-104.

HORWITZ, S. B. 1992. Mechanism of action of taxol. *Trends Pharmacol Sci*, 13, 4, 134-6.

HU, L., WU, H., NIU, F., YAN, C., YANG, X. & JIA, Y. 2011. Design of fenofibrate microemulsion for improved bioavailability. *Int J Pharm*, 420, 2, 251-5.

HUANG, C. Y., CHEN, C. M. & LEE, Y. D. 2007. Synthesis of high loading and encapsulation efficient paclitaxel-loaded poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion. *Int J Pharm*, 338, 1-2, 267-75.

HUANG, Y., LEOBANDUNG, W., FOSS, A. & PEPPAS, N. A. 2000. Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces. *J Control Release*, 65, 1-2, 63-71.

HUIZING, M. T., MISSER, V. H., PIETERS, R. C., TEN BOKKEL HUININK, W. W., VEENHOF, C. H., VERMORKEN, J. B., PINEDO, H. M. & BEIJNEN, J. H. 1995. Taxanes: a new class of antitumor agents. *Cancer Invest*, 13, 4, 381-404.

ILLUM, L., JABBAL-GILL, I., HINCHCLIFFE, M., FISHER, A. N. & DAVIS, S. S. 2001. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev*, 51, 1-3, 81-96.

JABR-MILANE, L. S., VAN VLERKEN, L. E., YADAV, S. & AMIJI, M. M. 2008. Multi-functional nanocarriers to overcome tumor drug resistance. *Cancer Treat Rev*, 34, 7, 592-602.

JAIN, A. K., THOMAS, N. S. & PANCHAGNULA, R. 2002. Transdermal drug delivery of imipramine hydrochloride. I. Effect of terpenes. *J Control Release*, 79, 1-3, 93-101.

JHA, S. K., DEY, S. & KARKI, R. 2011. Microemulsions- Potential Carrier for Improved Drug Delivery. *Asian J Biomed Pharm Sci*, 1, 1, 5-9.

JHA, S. K., KARKI, R., VENKATESH, D. P. & GEETHALAKSHAMI, A. 2011. Formulation development and characterization of microemulsion drug delivery systems containing antiulcer drug. *Int J Drug Dev Res*, 3, 4, 336-343.

JONES, D. S., BRUSCHI, M. L., FREITAS, O., GREMIAO, M. P., LARA, E. H. & ANDREWS, G. P. 2009. Rheological, mechanical and mucoadhesive properties of thermoresponsive, bioadhesive binary mixtures composed of poloxamer 407 and carbopol 974P designed as platforms for implantable drug delivery systems for use in the oral cavity. *Int J Pharm*, 372, 1-2, 49-58.

JONSSON, B., LINDMAN, B., HOLMBERG, K. & KRONBERG, B. Surfactants & Polymers in Aqueous Solutions. Chichester, U.K.: John Wiley & Sons, 1998.

JOSEPH, B., GEORGE, J. & MORAN, J. 2013. Pharmacology and Traditional Uses of *Mimosa pudica*. *Int J Pharm Sci Drug Res*, 5, 2, 41-44.

JULIANI, H. R., JR., BIURRUN, F., KOROCH, A. R., OLIVA, M. M., DEMO, M. S., TRIPPI, V. S. & ZYGADLO, J. A. 2002. Chemical constituents and antimicrobial activity of the essential oil of *Lantana xenica*. *Planta Med*, 68, 8, 762-4.

KAHKONEN, M. P., HOPIA, A. I., VUORELA, H. J., RAUHA, J. P., PIHLAJA, K., KUJALA, T. S. & HEINONEN, M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem*, 47, 10, 3954-62.

KAJANI, A. A., MOGHIM, S. & MOFID, M. R. 2012. Optimization of the basal medium for improving production and secretion of taxanes from suspension cell culture of Taxus baccata L. *Daru*, 20, 1, 54.

KALEPU, S., MANTHINA, M. & PADAVALA, V. 2013. Oral lipid-based drug delivery systems – an overview. *Acta Pharm Sin B*, 3, 6, 361-372.

KANTARIA, S., REES, G. D. & LAWRENCE, M. J. 2003. Formulation of electrically conducting microemulsion-based organogels. *Int J Pharm*, 250, 1, 65-83.

KARARLI, T. T., KIRCHHOFF, C. F. & PENZOTTI JR, S. C. 1995. Enhancement of transdermal transport of azidothymidine (AZT) with novel terpene and terpene-like enhancers: In vivo-in vitro correlations. *J Control Release*, 34, 1, 43-51.

KARLBERG, A. T. & DOOMS-GOOSSENS, A. 1997. Contact allergy to oxidized d-limonene among dermatitis patients. *Contact Dermatitis*, 36, 4, 201-6.

KARTSEV, V. N., SHTYKOV, S. N., BOGOMOLOVA, I. V. & RYZHOV, I. P. 2009. Thermodynamic stability of microemulsion based on sodium dodecyl sulfate. *J Mol Liq*, 145, 3, 173-176.

KASIM, N. A., WHITEHOUSE, M., RAMACHANDRAN, C., BERMEJO, M., LENNERNAS, H., HUSSAIN, A. S., JUNGINGER, H. E., STAVCHANSKY, S. A., MIDHA, K. K., SHAH, V. P. & AMIDON, G. L. 2004. Molecular properties of WHO essential drugs and provisional biopharmaceutical classification. *Mol Pharm*, 1, 1, 85-96.

KATEB, B., CHIU, K., BLACK, K. L., YAMAMOTO, V., KHALSA, B., LJUBIMOVA, J. Y., DING, H., PATIL, R., PORTILLA-ARIAS, J. A., MODO, M., MOORE, D. F., FARAHANI, K., OKUN, M. S., PRAKASH, N., NEMAN, J., AHDOOT, D., GRUNDFEST, W., NIKZAD, S. & HEISS, J. D. 2011. Nanoplatforms for constructing new approaches to cancer treatment, imaging, and drug delivery: what should be the policy? *Neuroimage*, 54 Suppl 1, S106-24.

KAWAKAMI, K., YOSHIKAWA, T., HAYASHI, T., NISHIHARA, Y. & MASUDA, K. 2002. Microemulsion formulation for enhanced absorption of poorly soluble drugs. II. In vivo study. *J Control Release*, 81, 1-2, 75-82.

KAWASAKI, E. S. & PLAYER, A. 2005. Nanotechnology, nanomedicine, and the development of new, effective therapies for cancer. *Nanomedicine*, 1, 2, 101-9.

KHOKHRA, S. & DIWAN, A. 2011. Microemulsion based transdermal drug delivery of tea tree oil. *Int J Drug Development Res*, 3, 1, 191-198.

KIM, S. C., YU, J., LEE, J. W., PARK, E. S. & CHI, S. C. 2005. Sensitive HPLC method for quantitation of paclitaxel (Genexol in biological samples with application to preclinical pharmacokinetics and biodistribution. *J Pharm Biomed Anal*, 39, 1-2, 170-6.

KIM, T. H., AHN, J. S., CHOI, H. K., CHOI, Y. J. & CHO, C. S. 2007. A novel mucoadhesive polymer film composed of carbopol, poloxamer and hydroxypropylmethylcellulose. *Arch Pharm Res*, 30, 3, 381-6.

KLAUKE, A. L., RACZ, I., PRADIER, B., MARKERT, A., ZIMMER, A. M., GERTSCH, J. & ZIMMER, A. 2014. The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. *Eur Neuropsychopharmacol*, 24, 4, 608-20.

KOMESVARAKUL, N., SANDERS, M. D., SZEKERES, E., ACOSTA, E. J., FALLER, J. F., MENTLIK, T., FISHER, L. B., NICOLL, G., SABATINI, D. A. & SCAMEHORN, J. F. 2006. Microemulsions of triglyceride-based oils: The effect of co-oil and salinity on phase diagrams. *J Cosmet Sci*, 57, 4, 309-325.

KONNO, T., WATANABE, J. & ISHIHARA, K. 2003. Enhanced solubility of paclitaxel using water-soluble and biocompatible 2-methacryloyloxyethyl phosphorylcholine polymers. *J Biomed Mater Res A*, 65, 2, 209-14.

KOUL, O., WALIA, S. & DHALIWAL, G. S. 2008. Essential oils as green pesticides: Potential and constraints. *Biopestic Int*, 4, 1, 63-84.

KRISHNAN, K., CHAPMAN, B., BATES, F. S., LODGE, T. P., ALMDAL, K. & BURGHARDT, W. R. 2002. Effects of shear flow on a polymeric bicontinuous microemulsion: Equilibrium and steady state behavior. *J Rheol*, 46, 2, 529-554.

KUO, M. L., LEE, K. C. & LIN, J. K. 1992. Genotoxicities of nitropyrenes and their modulation by apigenin, tannic acid, ellagic acid and indole-3-carbinol in the Salmonella and CHO systems. *Mutat Res*, 270, 2, 87-95.

LAFFLEUR, F. & BERNKOP-SCHNURCH, A. 2013. Strategies for improving mucosal drug delivery. *Nanomedicine (Lond)*, 8, 12, 2061-75.

LAHLOU, S., MAGALHAES, P. J., SIQUEIRA, R. J., FIGUEIREDO, A. F., INTERAMINENSE, L. F., MAIA, J. G. & SOUSA, P. J. 2005. Cardiovascular effects of the essential oil of Aniba canelilla bark in normotensive rats. *J Cardiovasc Pharmacol*, 46, 4, 412-21.

LAKSHMI, J., KUMAR, B. A. & GUPTA, S. 2013. Investigation of microemulsion as a potential carrier for advanced transdermal delivery: An overview. *Int J Pharm Sci Rev Res* 20, 2, 51-59.

LAPASIN, R., GRASSI, M. & COCEANI, N. 2001. Effects of polymer addition on the rheology of o/w microemulsions. *Rheol Acta*, 40, 2, 185-192.

LATREILLE, B. & PAQUIN, P. 1990. Evaluation of emulsion stability by centrifugation with conductivity measurements. *J Food Sci*, 55, 6, 1666-1668.

LAWRENCE, M. J. & REES, G. D. 2000. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 45, 1, 89-121.

_____. 2012. Microemulsion-based media as novel drug delivery systems. *Adv Drug Deliv Rev*, 64, 175-193.

LE HIR, A. Pharmacie galénique - bonnes pratiques de fabrication des médicaments. 8ª. Paris: Masson, 2001. 402.

LEANDRO, L. M., VARGAS F, S., BARBOSA, P. C., NEVES, J. K., SILVA, J. A. & VEIGA-JUNIOR, V. F. 2012. Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins. *Molecules*, 17, 4, 3866-89.

LEE, H., SOO, P. L., LIU, J., BUTLER, M. & ALLEN, C. Polymeric Micelles for Formulation of Anti-Cancer Drugs. In: AMIJI, M. M. (Ed.). Nanotechnology for Cancer Therapy: CRC Press, 2006. p.329.

LEE, M. J., LEE, M. H. & SHIM, C. K. 1995. Inverse targeting of drugs to reticuloendothelial system-rich organs by lipid microemulsion emulsified with poloxamer-338. *Inter J Pharm*, 113, 2, 175-187.

LEGAULT, J. & PICHETTE, A. 2007. Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. *J Pharm Pharmacol*, 59, 12, 1643-7.

LEITE, E. A., GRABE-GUIMARAES, A., GUIMARAES, H. N., MACHADO-COELHO, G. L., BARRATT, G. & MOSQUEIRA, V. C. 2007. Cardiotoxicity

reduction induced by halofantrine entrapped in nanocapsule devices. *Life Sci*, 80, 14, 1327-34.

LEMOINE, D., FRANCOIS, C., KEDZIEREWICZ, F., PREAT, V., HOFFMAN, M. & MAINCENT, P. 1996. Stability study of nanoparticles of poly(epsilon-caprolactone), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide). *Biomater*, 17, 22, 2191-7.

LENAERTS, V., COUVREUR, P., CHRISTIAENS-LEYH, D., JOIRIS, E., ROLAND, M., ROLLMAN, B. & SPEISER, P. 1984. Degradation of poly (isobutyl cyanoacrylate) nanoparticles. *Biomater*, 5, 2, 65-8.

LEONG, T. S. H., WOOSTER, T. J., KENTISH, S. E. & ASHOKKUMAR, M. 2009. Minimising oil droplet size using ultrasonic emulsification. *Ultrason Sonochem*, 16, 6, 721-727.

LI, G., FAN, Y., LI, X., WANG, X., LI, Y., LIU, Y. & LI, M. 2012. In vitro and in vivo evaluation of a simple microemulsion formulation for propofol. *Int J Pharm*, 425, 1-2, 53-61.

LI, P., GHOSH, A., WAGNER, R. F., KRILL, S., JOSHI, Y. M. & SERAJUDDIN, A. T. 2005. Effect of combined use of nonionic surfactant on formation of oil-in-water microemulsions. *Int J Pharm*, 288, 0378-5173 (Print), 27-34.

LI, Y., PEI, Y., ZHANG, X., GU, Z., ZHOU, Z., YUAN, W., ZHOU, J., ZHU, J. & GAO, X. 2001. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. *J Control Release*, 71, 2, 203-11.

LIEBERMAN, H., RIEGER, M. & BANKER, G. Pharmaceutical dosage forms-disperse systems. In: (Ed.). New York: M. Dekker, 1988. p.49-90; 210-12; 85-366

LIM, P. F., LIU, X. Y., KANG, L., HO, P. C., CHAN, Y. W. & CHAN, S. Y. 2006. Limonene GP1/PG organogel as a vehicle in transdermal delivery of haloperidol. *Int J Pharm*, 311, 1-2, 157-64.

LIU, H. L., LAN, Y. W. & CHENG, Y. C. 2004. Optimal production of sulphuric acid by Thiobacillus thiooxidans using response surface methodology. *Process Biochem* 39, 12, 1953-1961.

LIU, Y., WANG, P., SUN, C., ZHAO, J., DU, Y., SHI, F. & FENG, N. 2011. Bioadhesion and enhanced bioavailability by wheat germ agglutinin-grafted lipid nanoparticles for oral delivery of poorly water-soluble drug bufalin. *Int J Pharm*, 419, 1-2, 260-5.

LONG, C., ZHANG, L. & QIAN, Y. 2006. Preparation and Crystal Modification of Ibuprofen-Loaded Solid Lipid Microparticles. *Chin J Chem Eng*, 14, 4, 518-525.

LORENZI, V., MUSELLI, A., BERNARDINI, A. F., BERTI, L., PAGES, J. M., AMARAL, L. & BOLLA, J. M. 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. *Antimicrob Agents Chemother*, 53, 5, 2209-11.

MACEDO, J. P., FERNANDES, L. L., FORMIGA, F. R., REIS, M. F., JUNIOR, T. N., SOARES, L. A. & EGITO, E. S. T. 2006. Micro-emultocrit technique: a valuable tool for determination of critical HLB value of emulsions. *Aaps Pharmscitech*, 7, 1, 21.

MAEDA, H., WU, J., SAWA, T., MATSUMURA, Y. & HORI, K. 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release*, 65, 1-2, 271-84.

MAGALHAES, P. J., LAHLOU, S. & LEAL-CARDOSO, J. H. 2004. Antispasmodic effects of the essential oil of Croton nepetaefolius on guinea-pig ileum: a myogenic activity. *Fundam Clin Pharmacol*, 18, 5, 539-46.

MAGGIO, R. M., CALVO, N. L., VIGNADUZZO, S. E. & KAUFMAN, T. S. 2014. Pharmaceutical impurities and degradation products: Uses and applications of NMR techniques. *J Pharm Biomed Anal*, 101, 0, 102-122.

MAGHRABY, G. M. 2008. Transdermal delivery of hydrocortisone from eucalyptus oil microemulsion: effects of cosurfactants. *Int J Pharm*, 355, 1-2, 285-92.

MAJHI, P. R. & MOULIK, S. P. 1999. Physicochemical studies on biological macro- and microemulsions vi : Mixing behaviors of eucalyptus oil, water and polyoxyethylene sorbitan monolaurate (tween 20) assisted by n-butanol or cinnamic alcohol. *J Dispers Sci Technol*, 20, 5, 1407-1427.

MALINGRE, M. M., BEIJNEN, J. H. & SCHELLENS, J. H. 2001. Oral delivery of taxanes. *Invest New Drugs*, 19, 2, 155-62.

MAO, S., SHUAI, X., UNGER, F., SIMON, M., BI, D. & KISSEL, T. 2004. The depolymerization of chitosan: effects on physicochemical and biological properties. *Int J Pharm*, 281, 1-2, 45-54.

MARRIOTT, P. J., CHIN, S.-T., MAIKHUNTHOD, B., SCHMARR, H.-G. & BIERI, S. 2012. Multidimensional gas chromatography. *Trends Analyt Chem*, 34, 0, 1-21.

MARTÍNEZ-RUVALCABA, A., CHORNET, E. & RODRIGUE, D. 2004. Dynamic rheological properties of concentrated chitosan solutions. *J Text Stud*, 35, 53-74.

MARTINO, L., FEO, V., FRATIANNI, F. & NAZZARO, F. 2009. Chemistry, antioxidant, antibacterial and antifungal activities of volatile oils and their components. *Nat Prod Commun*, 4, 12, 1741-50.

MASMOUDI, H. 2005. The evaluation of cosmetic and pharmaceutical emulsions aging process using classical techniques and a new method: FTIR. *Int J Pharm*, 289, 1-2, 117-131.

MAZZAFERRO, S., BOUCHEMAL, K. & PONCHEL, G. 2013a. Oral delivery of anticancer drugs I: general considerations. *Drug Discov Today*, 18, 1-2, 25-34.

_____. 2013b. Oral delivery of anticancer drugs III: formulation using drug delivery systems. *Drug Discov Today*, 18, 1-2, 99-104.

MCCARRON, P. A., WOOLFSON, A. D. & KEATING, S. M. 1999. Response surface methodology as a predictive tool for determining the effects of preparation conditions on the physicochemical properties of poly(isobutylcyanoacrylate) nanoparticles. *Int J Pharm*, 193, 1, 37-47.

MCCLEMENTS, D. J. 2012. Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, 8, 1719-1729
MCGARVEY, D. J. & CROTEAU, R. 1995. Terpenoid metabolism. *Plant Cell*, 7, 7, 1015-26.

MEHTA, S. K. & KAUR, G. Microemulsions: Thermodynamic and Dynamic Properties. InTech, 2011.

MENDONÇA, D. E. & ONOFRE, S. B. 2009a. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev Bras Farmacogn*, 19, 577-581.

_____. 2009b. Atividade antimicrobiana do óleo-resina produzido pela copaiba - Copaifera multijuga Hayne (Leguminosae). *Rev. Bras. Farmacogn*, 19, 577-581.

MENG, X., TIAN, F., YANG, J., HE, C. N., XING, N. & LI, F. 2010. Chitosan and alginate polyelectrolyte complex membranes and their properties for wound dressing application. *J Mater Sci Mater Med*, 21, 5, 1751-9.

MITRA, N., MUKHOPADHYAY, L., BHATTACHARYA, P. K. & MOULIK, S. P. 1994. Biological microemulsions: Part IV-Phase behaviour and dynamics of microemulsions prepared with vegetable oils mixed with aerosol-OT, cinnamic alcohol and water. *Indian J Biochem Biophys*, 31, 2, 115-20.

MOERMAN, D. E. 1996. An analysis of the food plants and drug plants of native North America. *J Ethnopharmacol*, 52, 1, 1-22.

MOGHADDAM, F. A., ATYABI, F. & DINARVAND, R. 2009a. Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. *Nanomed*, 5, 2, 208-15.

_____. 2009b. Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. *Nanomedicine*, 5, 2, 208-15.

MOHAMMADI, A., ESMAEILI, F., DINARVAND, R., ATYABI, F. & WALKER, R. B. 2009. Development and validation of a stability-indicating method for the quantitation of Paclitaxel in pharmaceutical dosage forms. *J Chromatogr Sci*, 47, 7, 599-604.

MOHAMMADPOUR DOUNIGHI, N., ESKANDARI, R., AVADI, M., ZOLFAGHARIAN, H., MIR MOHAMMAD SADEGHI, A. & REZAYAT, M. 2012. Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system. *J Venom Anim Toxins Incl Trop Dis*, 18, 44-52.

MOHSIN, K., LONG, M. A. & POUTON, C. W. 2009. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: precipitation of drug after dispersion of formulations in aqueous solution. *J Pharm Sci*, 98, 10, 3582-95.

MONDAL, N., SAMANTA, A., PAL, T. K. & GHOSAL, S. K. 2008. Effect of different formulation variables on some particle characteristics of poly (DL-lactide-co-glycolide) nanoparticles. *Yakugaku Zasshi*, 128, 4, 595-601.

MONTI, H., TILIACOS, N. & FAURE, R. 1996. Two diterpenoids from copaiba oil. *Phytochemistry*, 42, 6, 1653-1656.

MORA-HUERTAS, C. E., FESSI, H. & ELAISSARI, A. 2010. Polymer-based nanocapsules for drug delivery. *Int J Pharm*, 385, 1-2, 113-42.

MOULIK, S. P. & PAUL, B. K. 1998. Structure, dynamics and transport properties of microemulsions. *Adv Colloid Interface Sci*, 78, 2, 99-195.

MRESTANI, Y., BEHBOOD, L., HARTL, A. & NEUBERT, R. H. 2010. Microemulsion and mixed micelle for oral administration as new drug formulations for highly hydrophilic drugs. *Eur J Pharm Biopharm*, 74, 2, 219-22.

MUJAWAR, S., UTTURE, S. C., FONSECA, E., MATARRITA, J. & BANERJEE, K. 2014. Validation of a GC–MS method for the estimation of dithiocarbamate fungicide residues and safety evaluation of mancozeb in fruits and vegetables. *Food Chem*, 150, 0, 175-181.

MUTHU, M. S., RAJESH, C. V., MISHRA, A. & SINGH, S. 2009. Stimulus-responsive targeted nanomicelles for effective cancer therapy. *Nanomedicine (Lond)*, 4, 6, 657-67.

MUZAFFAR, F., SINGH, U. K. & CHAUHAN, L. 2013. Review on microemulsion as futuristic drug delivery. *Int J Pharm Pharm Sci*, 5, 39-53
NAIR, R., KALARIYA, T. & SUMITRA, C. 2005. Antibacterial activity of some selected Indian Medicinal Flora Turk. *J Bot*, 29, 41-47.

NAZAR, M. F., KHAN, A. M. & SHAH, S. S. 2009. Microemulsion System with Improved Loading of Piroxicam: A Study of Microstructure. *AAPS PharmSciTech*, 10, 4, 1286-1294.

NEVES, J., BAHIA, M. F., AMIJI, M. M. & SARMENTO, B. 2011. Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale. *Expert Opin Drug Deliv*, 8, 8, 1085-104.

NICOLAOS, G., CRAUSTE-MANCIET, S., FARINOTTI, R. & BROSSARD, D. 2003. Improvement of cefpodoxime proxetil oral absorption in rats by an oil-in-water submicron emulsion. *Int J Pharm*, 263, 1-2, 165-71.

NIKOLAOU, P., PAPOUTSIS, I., DONA, A., SPILIOPOULOU, C. & ATHANASELIS, S. 2015. Development and validation of a GC/MS method for the simultaneous determination of levetiracetam and lamotrigine in whole blood. *J Pharm Biomed Anal*, 102, 0, 25-32.

NORNOO, A. O., OSBORNE, D. W. & CHOW, D. S. 2008. Cremophor-free intravenous microemulsions for paclitaxel I: formulation, cytotoxicity and hemolysis. *Int J Pharm*, 349, 1-2, 108-16.

NTIE-KANG, F., LIFONGO, L., MBAZE, L., EKWELLE, N., OWONO OWONO, L., MEGNASSAN, E., JUDSON, P., SIPPL, W. & EFANGE, S. M. 2013. Cameroonian medicinal plants: a bioactivity versus ethnobotanical survey and chemotaxonomic classification. *BMC Complement Altern Med*, 13, 1, 147.

OKONOGI, S. & CHAIYANA, W. 2012. Enhancement of anti-cholinesterase activity of *Zingiber cassumunar* essential oil using a microemulsion technique. *Drug Discov Ther*, 6, 5, 249-55.

OLIVEIRA, A. C., FIDALGO-NETO, A. A. & PAUMGARTTEN, F. J. 1999. In vitro inhibition of liver monooxygenases by beta-ionone, 1,8-cineole, (-)-menthol and terpineol. *Toxicology*, 135, 1, 33-41.

OLIVEIRA, A. G. & SCARPA, M. V. 2001. Microemulsão I: fundamentos teóricos da formação do sistema microemulsionado. *Pharma Bras*, 1, 8, 73-79.

OLIVEIRA, A. G., SCARPA, M. V., CORREA, M. A., CERA, L. F. R. & FORMARIZ, T. P. 2004. Microemulsões: estrutura e aplicações como sistema de liberação de fármacos. *Quim Nova*, 27, 131-138.

ONUNKWO, G. C. & ADIKWU, M. U. 1997. Stability of veegum/mucuna gum emulsions. *Stp Pharma Sciences*, 7, 4, 320-325.

ORAFIDIYA, L. O. & OLADIMEJI, F. A. 2002. Determination of the required HLB values of some essential oils. *Int J Pharm*, 237, 1-2, 241-9.

ORATA, F. Derivatization Reactions and Reagents for Gas Chromatography Analysis, Advanced Gas Chromatography. In: MOHD, D. M. A. (Ed.). Progress in Agricultural, Biomedical and Industrial Applications: InTech, 2012.

PAIVA, L. A. F., GURGEL, L. A., CAMPOS, A. R., SILVEIRA, E. R. & RAO, V. S. N. 2004. Attenuation of ischemia/reperfusion-induced intestinal injury by oleo-resin from Copaifera langsdorffii in rats. *Life Sci*, 75, 16, 1979-1987.

PAIVA, L. A. F., GURGEL, L. A., SOUSA, E. T., SILVEIRA, E. R., SILVA, R. M., SANTOS, F. A. & RAO, V. S. N. 2004. Protective effect of Copaifera langsdorffii oleo-resin against acetic acid-induced colitis in rats. *J Ethnopharmacol*, 93, 1, 51-56.

PAL, R. 2011. Rheology of simple and multiple emulsions. *Curr Opin Colloid Interface Sci*, 16, 1, 41-60.

PANCHAGNULA, R. 1998. Pharmaceutical aspects of paclitaxel. *Inter J Pharm*, 172, 1–2, 1-15.

PASQUA, R., BETTS, G., HOSKINS, N., EDWARDS, M., ERCOLINI, D. & MAURIELLO, G. 2007. Membrane toxicity of antimicrobial compounds from essential oils. *J Agric Food Chem*, 55, 12, 4863-70.

PASTORIZA-SANTOS, I. & LIZ-MARZÁN, L. M. 2009. N,N-Dimethylformamide as a Reaction Medium for Metal Nanoparticle Synthesis. *Adv Funct Mater*, 19, 5, 679-688.

PATEL, B. K., PARIKH, R. H. & ABOTI, P. S. 2013. Development of oral sustained release rifampicin loaded chitosan nanoparticles by design of experiment. *J Drug Deliv*, 2013, 370938.

PAUL, B. K. & MOULIK, S. P. 1997. Microemulsion: An overview. *J Dispers Sci Technol*, 18, 4, 301-367.

_____. 2001. Uses and applications of microemulsions. *Soft Condens Matter*, 80, 8, 990-1001.

PELTIER, S., OGER, J. M., LAGARCE, F., COUET, W. & BENOIT, J. P. 2006. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules. *Pharm Res*, 23, 6, 1243-50.

PERRIER, T., SAULNIER, P. & BENOIT, J. P. 2010. Methods for the functionalisation of nanoparticles: new insights and perspectives. *Chemistry*, 16, 38, 11516-29.

PESTANA, K. C., FORMARIZ, T. P., FRANZINI, C. M., SARMENTO, V. H., CHIAVACCI, L. A., SCARPA, M. V., EGITO, E. S. & OLIVEIRA, A. G. 2008a. Oil-in-water lecithin-based microemulsions as a potential delivery system for amphotericin B. *Colloids Surf B*, 66, 2, 253-9.

_____. 2008b. Oil-in-water lecithin-based microemulsions as a potential delivery system for amphotericin B. *Colloids Surf B Biointerfaces*, 66, 2, 253-9.

PETIT, B., BOUCHEMAL, K., VAUTHIER, C., DJABOUROV, M. & PONCHEL, G. 2012. The counterbalanced effect of size and surface properties of chitosan-coated poly(isobutylcyanoacrylate) nanoparticles on mucoadhesion due to pluronic F68 addition. *Pharm Res*, 29, 4, 943-52.

PICHERSKY, E., NOEL, J. P. & DUDAREVA, N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science*, 311, 5762, 808-11.

PINTO-ALPHANDARY, H., ABOUBAKAR, M., JAILLARD, D., COUVREUR, P. & VAUTHIER, C. 2003. Visualization of insulin-loaded nanocapsules: in vitro and in vivo studies after oral administration to rats. *Pharm Res*, 20, 7, 1071-84.

PINTO REIS, C., NEUFELD, R. J., RIBEIRO, A. J. & VEIGA, F. 2006a. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomed*, 2, 1, 8-21.

PODLOGAR, F., BESTER ROGAC, M. & GASPERLIN, M. 2005. The effect of internal structure of selected water–Tween 40®–Imwitor 308®–IPM microemulsions on ketoprofene release. *Inter J Pharm*, 302, 1–2, 68-77.

POLIZELLI, M. A., TELIS, V. R. N., AMARAL, L. Q. & FEITOSA, E. 2006. Formation and characterization of soy bean oil/surfactant/water microemulsions. *Colloids Surf A*, 281, 1–3, 230-236.

PONCHEL, G., DURRER, C., IRACHE, J. M. & DUCHÊNE, D. Study of the interactions between nanoparticles and intestinal mucosa. In: OTTEWILL, R. H. & RENNIE, A. R. (Ed.). Trends in Colloid and Interface Science VIII: Steinkopff, v.97, 1994. cap. 60, p.275-280.

PONCHEL, G. & IRACHE, J. 1998. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv Drug Deliv Rev*, 34, 2-3, 191-219.

POULLAIN-TERMEAU, S., CRAUSTE-MANCIET, S., BROSSARD, D., MUHAMED, S., NICOLAOS, G., FARINOTTI, R., BARTHELEMY, C., ROBERT, H. & ODOU, P. 2008. Effect of oil-in-water submicron emulsion surface charge on oral absorption of a poorly water-soluble drug in rats. *Drug Deliv*, 15, 8, 503-14.

POUTON, C. W. 2000. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. *Eur J Pharm Sci*, 11 Suppl 2, S93-8.

PRABHU, S., ORTEGA, M. & MA, C. 2005. Novel lipid-based formulations enhancing the in vitro dissolution and permeability characteristics of a poorly water-soluble model drug, piroxicam. *Int J Pharm*, 301, 1–2, 209-216.

QUINTANAR-GUERRERO, D., ALLEMANN, E., FESSI, H. & DOELKER, E. 1998. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. *Drug Dev Ind Pharm*, 24, 12, 1113-28.

RAILEANU, M., TODAN, L., VOICESCU, M., CIUCULESCU, C. & MAGANU, M. 2013. A way for improving the stability of the essential oils in an environmental friendly formulation. *Mater Sci Eng C Mater Biol Appl*, 33, 6, 3281-8.

RAJAN, S., THIRUNALASUNDARI, T. & JEEVA, S. 2011. Anti-enteric bacterial activity and phytochemical analysis of the seed kernel extract of *Mangifera indica Linnaeus* against *Shigella dysenteriae (Shiga, corrig.) Castellani and Chalmers*. *Asian Pac J Trop Med*, 4, 4, 294-300.

RAO, J. & MCCLEMENTS, D. J. 2011. Formation of flavor oil microemulsions, nanoemulsions and emulsions: influence of composition and preparation method. *J Agric Food Chem*, 59, 9, 5026-35.

RAO, S., KRAUSS, N. E., HEERDING, J. M., SWINDELL, C. S., RINGEL, I., ORR, G. A. & HORWITZ, S. B. 1994. 3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin. *J Biol Chem*, 269, 5, 3132-4.

RASOOLI, I. Bioactive compounds in phytomedicine InTech 2011.

RAVIKUMARA, N. R. & MADHUSUDHAN, B. 2011. Chitosan nanoparticles for tamoxifen delivery and cytotoxicity to MCF-7 and Vero cells. *Pure Appl Chem*, 83, 11, 2027-2040.

REN, X., SVIRSKIS, D., ALANY, R. G., ZARGAR-SHOSHTARI, S. & WU, Z. 2012. In-situ phase transition from microemulsion to liquid crystal with the potential of prolonged parenteral drug delivery. *Inter J Pharm*, 431, 1–2, 130-137.

RICHTER, C. & SCHLEGEL, J. 1993. Mitochondrial calcium release induced by prooxidants. *Toxicol Lett*, 67, 1-3, 119-27.

RITIKA, A., HARIKUMAR, S. L. & AGGARWAL, G. 2012. Microemulsion system in role of expedient vehicle for dermal application. *J Drug Deliv Ther*, 2, 4, 23-28.

RIVKIN, I., COHEN, K., KOFFLER, J., MELIKHOV, D., PEER, D. & MARGALIT, R. 2010. Paclitaxel-clusters coated with hyaluronan as selective tumor-targeted nanovectors. *Biomater*, 31, 27, 7106-14.

ROBBINS, M. L. Theory of the phase behaviour of microemulsions. Oil recovery symposium of the society of petroleum engineers of AIME. Oklahoma, 5839, 22-24.

ROBINS, M. M. 2000. Emulsions -- creaming phenomena. *Curr Opin Colloid Interface Sci*, 5, 5-6, 265-272.

ROGER, E., LAGARCE, F. & BENOIT, J. P. 2011. Development and characterization of a novel lipid nanocapsule formulation of Sn38 for oral administration. *Eur J Pharm Biopharm*, 79, 1, 181-8.

ROGER, E., LAGARCE, F., GARCION, E. & BENOIT, J. P. 2010a. Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery. *Nanomedicine (Lond)*, 5, 2, 287-306.

_____. 2010b. Reciprocal competition between lipid nanocapsules and P-gp for paclitaxel transport across Caco-2 cells. *Eur J Pharm Sci*, 40, 5, 422-9.

ROLAND, I., PIEL, G., DELATTRE, L. & EVRARD, B. 2003. Systematic characterization of oil-in-water emulsions for formulation design. *Int J Pharm*, 263, 1-2, 85-94.

ROSSI, S., FERRARI, F., BONFERONI, M. C. & CARAMELLA, C. 2000. Characterization of chitosan hydrochloride-mucin interaction by means of viscosimetric and turbidimetric measurements. *Eur J Pharm Sci*, 10, 4, 251-7.

ROWINSKY, E. K., CAZENAVE, L. A. & DONEHOWER, R. C. 1990. Taxol: a novel investigational antimicrotubule agent. *J Natl Cancer Inst*, 82, 15, 1247-59.

ROWINSKY, E. K. & DONEHOWER, R. C. 1995. Paclitaxel (taxol). *N Engl J Med*, 332, 15, 1004-14.

ROWINSKY, E. K., ONETTO, N., CANETTA, R. M. & ARBUCK, S. G. 1992. Taxol: the first of the taxanes, an important new class of antitumor agents. *Semin Oncol*, 19, 6, 646-62.

ROZMAN, B., ZVONAR, A., FALSON, F. & GASPERLIN, M. 2009. Temperature-Sensitive Microemulsion Gel: An Effective Topical Delivery System for Simultaneous Delivery of Vitamins C and E. *AAPS PharmSciTech*, 10, 1, 54-61.

RUCKENSTEIN, E. & KRISHNAN, R. 1980. Effect of electrolytes and mixtures of surfactants on the oil-water interfacial tension and their role in formation of microemulsions. *J Colloid Interface Sci*, 76, 1, 201-211.

RUKMINI, A., RAHARJO, S., HASTUTI, P. & SUPRIYADI, S. 2012. Formulation and stability of water-in-virgin coconut oil microemulsion using ternary food grade nonionic surfactants. *Inter Food Res J*, 19, 1, 259-264.

SACCO, J. J., BOTTEN, J., MACBETH, F., BAGUST, A. & CLARK, P. 2010. The average body surface area of adult cancer patients in the UK: a multicentre retrospective study. *PLoS One*, 5, 1, e8933.

SAFAEI-GHOMI, J. & AHD, A. A. 2010. Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*. *Pharmacogn Mag*, 6, 23, 172-5.

SAHA, R., RAKSHIT, S., MITRA, R. K. & PAL, S. K. 2012. Microstructure, Morphology, and Ultrafast Dynamics of a Novel Edible Microemulsion. *Langmuir*, 28, 22, 8309-8317.

SAITO, H. & SHINODA, K. 1970. The stability of W/O type emulsions as a function of temperature and of the hydrophilic chain length of the emulsifier. *J Colloid Interface Sci*, 32, 4, 647-651.

SALEEM, M., NAZIR, M., ALI, M. S., HUSSAIN, H., LEE, Y. S., RIAZ, N. & JABBAR, A. 2010. Antimicrobial natural products: an update on future antibiotic drug candidates. *Nat Prod Rep*, 27, 2, 238-54.

SALUNKHE, S. S., THORAT, J. D., MALI, S. S., HAJARE, A. A. & BHATIA, N. M. 2013. Formulation, development and evaluation of *Artemisia pallens* (davana) oil based topical microemulsion. *World J Pharm Pharm Sci*, 2, 6, 5725-5736.

SANTANA-RIOS, G., ORNER, G. A., AMANTANA, A., PROVOST, C., WU, S. Y. & DASHWOOD, R. H. 2001. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. *Mutat Res*, 495, 1-2, 61-74.

SANTOS, A. O., COSTA, M. A., UEDA-NAKAMURA, T., DIAS-FILHO, B. P., DA VEIGA-JÚNIOR, V. F., DE SOUZA LIMA, M. M. & NAKAMURA, C. V. 2011. Leishmania amazonensis: Effects of oral treatment with copaiba oil in mice. *Exp Parasitol*, 129, 2, 145-151.

SANTOS, A. O., UEDA-NAKAMURA, T., DIAS FILHO, B. P., VEIGA JUNIOR, V. F., PINTO, A. C. & NAKAMURA, C. V. 2008. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the Copaifera genus. *Mem Inst Oswaldo Cruz*, 103, 3, 277-81.

SANTOS, E. C. G., DONNICI, C. L., CAMARGOS, E. R., AUGUSTO DE REZENDE, A., ANDRADE, E. H., SOARES, L. A., FARIAS LDE, M., ROQUE DE CARVALHO, M. A. & ALMEIDA, M. 2013. Effects of Copaifera duckei Dwyer oleoresin on the cell wall and cell division of Bacillus cereus. *J Med Microbiol*, 62, Pt 7, 1032-7.

SANTOS, M. R., CARVALHO, A. A., MEDEIROS, I. A., ALVES, P. B., MARCHIORO, M. & ANTONIOLLI, A. R. 2007. Cardiovascular effects of Hyptis fruticosa essential oil in rats. *Fitoterapia*, 78, 3, 186-91.

SANTOS, O., UEDA-NAKAMURA, T. & FILHO, P. D. B. 2007. Antimicrobial activity of copaiba oils obtained from different species of Copaifera in Brazil. *Planta Med*, 73, 9, 860-860.

SANTOS, P., WATKINSON, A. C., HADGRAFT, J. & LANE, M. E. 2008. Application of microemulsions in dermal and transdermal drug delivery. *Skin Pharmacol Physiol*, 21, 5, 246-259.

SAPRA, B., BHANDARI, S., SOOD, J., JINDAL, M., TIWARY, A. & TIWARY, A. 2013. A critical appraisal of microemulsions for drug delivery: part I. *Ther Deliv*, 4, 12, 1547-64.

SAREMI, S., DINARVAND, R., KEBRIAEEZADEH, A., OSTAD, S. N. & ATYABI, F. 2013. Enhanced oral delivery of docetaxel using thiolated chitosan nanoparticles: preparation, in vitro and in vivo studies. *Biomed Res Int*, 2013, 150478.

SCHAFER, H. & WINK, M. 2009. Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. *Biotechnol J*, 4, 12, 1684-703.

SCHERER, D., ROBINSON, J. R. & KREUTER, J. 1994. Influence of enzymes on the stability of polybutylcyanoacrylate nanoparticles. *Int J Pharm*, 101, 1–2, 165-168.

SCHIFF, P. B., FANT, J. & HORWITZ, S. B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277, 5698, 665-7.

SCHMALFUB, U., NEUBERT, R. & WOHLRAB, W. 1997. Modification of drug penetration into human skin using microemulsions. *J Control Release*, 46, 3, 279-285.

SCHULMAN, J. H., STOECKENIUS, W. & PRINCE, L. M. 1959. Mechanism of formation and structure of micro emulsions by electron microscopy. *J Phys Chem* 63, 1677-1680.

SCHULTES, R. E. The kingdom of plants. New York, N.Y.: McGraw-Hill Book Co., 1978.

SEN, R. & SWAMINATHAN, I. 2004. Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. *Biochem Eng J*, 21, 2, 141-148.

SHAFIQ-UN-NABI, S., SHAKEEL, F., TALEGAONKAR, S., ALI, J., BABOOTA, S., AHUJA, A., KHAR, R. K. & ALI, M. 2007. Formulation development and optimization using nanoemulsion technique: A technical note. *Aaps Pharmscitech*, 8, 2

SHAH, N. H., CARVAJAL, M. T., PATEL, C. I., INFELD, M. H. & MALICK, A. W. 1994. Self-emulsifying drug-delivery systems (SEDDS) with polyglycolyzed glycerides for improving in-vitro dissolution and oral absorption of lipophilic drugs. *Int J Pharm*, 106, 1, 15-23.

SHINODA, K. & ARAI, H. 1964. The Correlation between Phase Inversion Temperature in Emulsion and Cloud Point in Solution of Nonionic Emulsifier. *J Phys Chem*, 68, 12, 3485-3490.

SHINODA, K. & ARAI, H. 1967. The effect of phase volume on the phase inversion temperature of emulsions stabilized with nonionic surfactants. *J Colloid Interface Sci*, 25, 3, 429-431.

SHINODA, K. & FRIBERG, S. 1975. Microemulsions: Colloidal aspects. *Adv Colloid Interface Sci*, 4, 4, 281-300.

SHINODA, K. & LINDMAN, B. 1987. Organized surfactant systems: microemulsions. *Langmuir*, 3, 2, 135-149.

SIEGEL, R., MA, J., ZOU, Z. & JEMAL, A. 2014. Cancer statistics, 2014. *CA Cancer J Clin*, 64, 1, 9-29.

SIKKEMA, J., BONT, J. A. & POOLMAN, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem*, 269, 11, 8022-8.

SILVA, J., ABEBE, W., SOUSA, S. M., DUARTE, V. G., MACHADO, M. I. & MATOS, F. J. 2003. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. *J Ethnopharmacol*, 89, 2-3, 277-83.

SILVA, K. G. H., XAVIER-JUNIOR, F. H., FARIAS, I. E. G., CALDAS-NETO, J. A., SILVA, A. K. A. S., NAKASHIMA-JUNIOR, T., ARAUJO, I. B., OLIVERIA, A. G., MEDEIROS, A. C. & EGITO, E. S. T. 2006. A new insight about pharmaceutical dosage forms for benzathine penicillin G. *J Basic Appl Pharm Sci*, 27, 1, 21-26.

SINGH, A. K., CHAURASIYA, A., AWASTHI, A., MISHRA, G., ASATI, D., KHAR, R. K. & MUKHERJEE, R. 2009. Oral bioavailability enhancement of exemestane from self-microemulsifying drug delivery system (SMEDDS). *AAPS PharmSciTech*, 10, 3, 906-16.

SINGH, B. & AHUJA, N. 2002. Development of controlled-release buccoadhesive hydrophilic matrices of diltiazem hydrochloride: optimization of bioadhesion, dissolution, and diffusion parameters. *Drug Dev Ind Pharm*, 28, 4, 431-42.

SINGH, V., BUSHETTII, S. S., APPALA, R. S., RIZWAN, A., MAMTA, S. & BISHT, A. 2011. Microemulsions as Promising Delivery Systems: A Review. *Ind J Pharm Edu Res* 45, 4, 392-401.

SINGLA, A. K., GARG, A. & AGGARWAL, D. 2002. Paclitaxel and its formulations. *Int J Pharm*, 235, 1-2, 179-92.

SKOOG, D. A., HOLLER, F. J. & CROUCH, S. R. Instrumental Analysis. Int. India: Cengage learning, 2007.

SMITH, R. L., COHEN, S. M., DOULL, J., FERON, V. J., GOODMAN, J. I., MARNETT, L. J., PORTOGHESE, P. S., WADDELL, W. J., WAGNER, B. M., HALL, R. L., HIGLEY, N. A., LUCAS-GAVIN, C. & ADAMS, T. B. 2005. A procedure for the safety evaluation of natural flavor complexes used as ingredients in food: essential oils. *Food Chem Toxicol*, 43, 3, 345-63.

SOARES, D. C., PORTELLA, N. A., RAMOS, M. F., SIANI, A. C. & SARAIVA, E. M. 2013. Trans- beta -Caryophyllene: An Effective Antileishmanial Compound Found in Commercial Copaiba Oil (Copaifera spp.). *Evid Based Complement Alternat Med*, 2013, 761323.

SODERMAN, O. & NYDÉN, M. 1999. NMR in microemulsions. NMR translational diffusion studies of a model microemulsion. *Colloids Surf A Physicochem Eng Asp*, 158, 1–2, 273-280.

SOEJARTO, D. D., GYLLENHAAL, C., KADUSHIN, M. R., SOUTHAVONG, B., SYDARA, K., BOUAMANIVONG, S., XAIVEU, M., ZHANG, H. J., FRANZBLAU, S. G., TAN, G. T., PEZZUTO, J. M., RILEY, M. C., ELKINGTON, B. G. & WALLER, D. P. 2012. An ethnobotanical survey of medicinal plants of Laos toward the discovery of bioactive compounds as potential candidates for pharmaceutical development. *Pharm Biol*, 50, 1, 42-60.

SOLANKI, S. S., SARKAR, B. & DHANWANI, R. K. 2012. Microemulsion drug delivery system: for bioavailability enhancement of ampelopsin. *ISRN Pharm*, 2012, 108164.

SONG, D. & AU, J. L. 1995. Isocratic high-performance liquid chromatographic assay of taxol in biological fluids and tissues using automated column switching. *J Chromatogr B Biomed Appl*, 663, 2, 337-44.

SOUSA, A. A. V. O., LIMA, F. D., MACHADO, A. A., VERA, M. O., FERNANDES, L. M. P., LIMA, R. R. & MAIA, C. D. S. F. 2013. Tissular and motor analysis of neuroprotective and anti-inflammatory/INS; effects of copaiba oil (Copaifera reticulata Ducke) after focal cortical ischemia. *J Neurol Sci* 333, Supplement 1, 0, e247.

SOUSA, D. P. 2011. Analgesic-like activity of essential oils constituents. *Molecules*, 16, 3, 2233-52.

SOUSA, J. P., BRANCALION, A. P., SOUZA, A. B., TURATTI, I. C., AMBROSIO, S. R., FURTADO, N. A., LOPES, N. P. & BASTOS, J. K. 2011. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal*, 54, 4, 653-9.

SOUSA, J. P. B., BRANCALION, A. P. S., SOUZA, A. B., TURATTI, I. C. C., AMBRÓSIO, S. R., FURTADO, N. A. J. C., LOPES, N. P. & BASTOS, J. K. 2011. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal*, 54, 4, 653-659.

SOUZA, A. B., MARTINS, C. H., SOUZA, M. G., FURTADO, N. A., HELENO, V. C., DE SOUSA, J. P., ROCHA, E. M., BASTOS, J. K., CUNHA, W. R., VENEZIANI, R. C. & AMBROSIO, S. R. 2011. Antimicrobial activity of terpenoids from Copaifera langsdorffii Desf. against cariogenic bacteria. *Phytother Res*, 25, 2, 215-20.

SPARREBOOM, A., VAN ASPEREN, J., MAYER, U., SCHINKEL, A. H., SMIT, J. W., MEIJER, D. K., BORST, P., NOOIJEN, W. J., BEIJNEN, J. H. & VAN TELLINGEN, O. 1997. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A*, 94, 5, 2031-5.

SPERNATH, A., YAGHMUR, A., ASERIN, A., HOFFMAN, R. E. & GARTI, N. 2002. Food-grade microemulsions based on nonionic emulsifiers: media to enhance lycopene solubilization. *J Agric Food Chem*, 50, 23, 6917-22.

STASHENKO, E. E., CERVANTES, M., COMBARIZA, Y., FUENTES, H. & MARTÍNEZ, J. R. 1999. HRGC/FID and HRGC/MSD analysis of the secondary metabolites obtained by different extraction methods from *Lepechinia schiedeana*, and *in vitro* evaluation of its antioxidant activity. *J High Resolut Chromatogr*, 22, 6, 343-349.

STONE, M. J. & WILLIAMS, D. H. 1992. On the evolution of functional secondary metabolites (natural products). *Mol Microbiol*, 6, 1, 29-34.

SUKUMARAN, S., KIRUBA, S., MAHESH, M., NISHA, S. R., MILLER, P. Z., BEN, C. P. & JEEVA, S. 2011. Phytochemical constituents and antibacterial efficacy of the flowers of *Peltophorum pterocarpum (DC.) Baker ex Heyne*. *Asian Pac J Trop Med*, 4, 9, 735-8.

SUPKO, J. G., NAIR, R. V., SEIDEN, M. V. & LU, H. 1999. Adaptation of solid phase extraction to an automated column switching method for online sample cleanup as the basis of a facile and sensitive high-performance liquid chromatographic assay for paclitaxel in human plasma. *J Pharm Biomed Anal*, 21, 5, 1025-36.

SURAPANENI, M. S., DAS, S. K. & DAS, N. G. 2012. Designing Paclitaxel Drug Delivery Systems Aimed at Improved Patient Outcomes: Current Status and Challenges. *ISRN Pharmacology*, 2012, 623139.

SURJYANARAYAN, M., SNIGDHA, S. M. & NAAZNEEN, S. 2009. Design and development of Saquinavir microemulsion for the oral bioavailabili ty enhancement. *Int J PharmTech Res*, 1, 1442-48.

TAI, C. Y., LEE, M.-H. & WU, Y.-C. 2001. Control of zirconia particle size by using two-emulsion precipitation technique. *Chem Eng Sci*, 56, 7, 2389-2398.

TAKAHASHI, Y., KONDO, H., YASUDA, T., WATANABE, T., KOBAYASHI, S. & YOKOHAMA, S. 2002. Common solubilizers to estimate the Caco-2 transport of poorly water-soluble drugs. *Int J Pharm*, 246, 1-2, 85-94.

TALEGAONKAR, S., AZEEM, A., AHMAD, F. J., KHAR, R. K., PATHAN, S. A. & KHAN, Z. I. 2008. Microemulsions: a novel approach to enhanced drug delivery. *Recent Pat Drug Deliv Formul*, 2, 3, 238-57.

TEIXEIRA, P. C., LEITE, G. M., DOMINGUES, R. J., SILVA, J., GIBBS, P. A. & FERREIRA, J. P. 2007. Antimicrobial effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. *Int J Food Microbiol*, 118, 1, 15-9.

TENJARLA, S. 1999. Microemulsions: An overview and pharmaceutical applications. *Crit Rev Ther Drug Carrier Syst*, 16, 5, 461-521.

TEWA-TAGNE, P., BRIANCON, S. & FESSI, H. 2007. Preparation of redispersible dry nanocapsules by means of spray-drying: development and characterisation. *Eur J Pharm Sci*, 30, 2, 124-35.

THANOU, M., VERHOEF, J. C. & JUNGINGER, H. E. 2001. Oral drug absorption enhancement by chitosan and its derivatives. *Adv Drug Deliv Rev*, 52, 2, 117-26.

THIMMASETTY, J., SUBRAHMANYAM, C. V. S., VISHWANATH, B. A. & SATHESH BABU, P. R. 2009. Solubility parameter estimation of celecoxib by current methods. *Asian J Res Chem*, 2, 2

TIAN, Q., REN, F., XU, Z., XIE, Y. & ZHANG, S. 2012. Preparation of high solubilizable microemulsion of naproxen and its solubilization mechanism. *Int J Pharm*, 426, 1-2, 202-10.

TIWARI, B. K., VALDRAMIDIS, V. P., O'DONNELL, C. P., MUTHUKUMARAPPAN, K., BOURKE, P. & CULLEN, P. J. 2009. Application of natural antimicrobials for food preservation. *J Agric Food Chem*, 57, 14, 5987-6000.

TIWARI, D., GOLDMAN, D., SAUSE, R. & MADAN, P. L. 1999. Evaluation of polyoxyethylene homopolymers for buccal bioadhesive drug delivery device formulations. *AAPS PharmSci*, 1, 3, E13.

TIWARI, D., GOLDMAN, D., TOWN, C., SAUSE, R. & MADAN, P. L. 1999. In vitro-in vivo evaluation of a controlled release buccal bioadhesive device for oral drug delivery. *Pharm Res*, 16, 11, 1775-80.

TONG, R. & CHENG, J. 2008. Paclitaxel-initiated, controlled polymerization of lactide for the formulation of polymeric nanoparticulate delivery vehicles. *Angew Chem Int Ed Engl*, 47, 26, 4830-4.

TURINA, A. V., NOLAN, M. V., ZYGADLO, J. A. & PERILLO, M. A. 2006. Natural terpenes: self-assembly and membrane partitioning. *Biophys Chem*, 122, 2, 101-13.

Validation of analytical procedures: Text and Methodology, ICH-Q2 (R1). International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, 2005, Geneva.

VAN DE BRAAK, S. A. A. J. & LEIJTEN, G. C. J. J. Essential oils and oleoresins: A survey in the netherlands and other major markets in the european union. Netherlands: Rotterdam, 1999.

VAN DEN BONGARD, H. J., KEMPER, E. M., VAN TELLINGEN, O., ROSING, H., MATHOT, R. A., SCHELLENS, J. H. & BEIJNEN, J. H. 2004. Development and validation of a method to determine the unbound paclitaxel fraction in human plasma. *Anal Biochem*, 324, 1, 11-5.

VAN RUTH, S. M., ROOZEN, J. P., POSTHUMUS, M. A. & JANSEN, F. J. 1999. Volatile composition of sunflower oil-in-water emulsions during initial lipid oxidation: influence of pH. *J Agric Food Chem*, 47, 10, 4365-9.

VANDAMME, T. F. 2002. Microemulsions as ocular drug delivery systems: recent developments and future challenges. *Prog Retin Eye Res*, 21, 1, 15-34.

VARMA, M. V., ASHOKRAJ, Y., DEY, C. S. & PANCHAGNULA, R. 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol Res*, 48, 4, 347-59.

VAUGHAN, C. D. 1985. Using Solubility Parameters in Cosmetic Formulations. *J Soc Cosmet Chem*, 36, 319 - 333.

_____. 1993. Solubility Parameters for Characterising New Raw Materials. *Cosmet Toiletries* 108, 57-62.

VAUTHIER, C. & BOUCHEMAL, K. 2009. Methods for the preparation and manufacture of polymeric nanoparticles. *Pharm Res*, 26, 5, 1025-58.

VEIGA-JUNIOR, V. F. & PINTO, A. C. 2002. O gênero copaifera L. *Quim Nova*, 25, 273-286.

VEIGA JUNIOR, V. F., ROSAS, E. C., CARVALHO, M. V., HENRIQUES, M. G. & PINTO, A. C. 2007. Chemical composition and anti-inflammatory activity of copaiba oils from Copaifera cearensis Huber ex Ducke, Copaifera reticulata Ducke and Copaifera multijuga Hayne--a comparative study. *J Ethnopharmacol*, 112, 2, 248-54.

VENKATESH, D. N., KUMAR, S. S., KIRAN, H. C., MEGALA, M., MONISHA, C., MYTHILI, L. & SPOORTHY, G. 2014. Microemulsion: An update. *Inter J Pharm Sci*, 4, 6, 802-811.

VERCESI, A. E., KOWALTOWSKI, A. J., GRIJALBA, M. T., MEINICKE, A. R. & CASTILHO, R. F. 1997. The role of reactive oxygen species in mitochondrial permeability transition. *Biosci Rep*, 17, 1, 43-52.

VERDIERE, A. C., DUBERNET, C., NEMATI, F., POUPON, M. F., PUISIEUX, F. & COUVREUR, P. 1994. Uptake of doxorubicin from loaded nanoparticles in multidrug-resistant leukemic murine cells. *Cancer Chemother Pharmacol*, 33, 6, 504-8.

VERDIERE, A. C., DUBERNET, C., NEMATI, F., SOMA, E., APPEL, M., FERTE, J., BERNARD, S., PUISIEUX, F. & COUVREUR, P. 1997. Reversion of multidrug resistance with polyalkylcyanoacrylate nanoparticles: towards a mechanism of action. *Br J Cancer*, 76, 2, 198-205.

VERHEYEN, S., AUGUSTIJNS, P., KINGET, R. & VAN DEN MOOTER, G. 2001. Determination of partial solubility parameters of five benzodiazepines in individual solvents. *Int J Pharm*, 228, 1-2, 199-207.

VERMA, R. K. & GARG, S. 2001. Current status of drug delivery technologies and future directions. *Pharm Technol*, 251 - 14.

VIEIRA, R. C., BOMBARDIERE, E., DE OLIVEIRA, J. J., DE SOUZA LINO JÚNIOR, R., BRITO, L. A. B. & JUNQUEIRA-KIPNIS, A. P. 2009. Topical Copaíba oil treatment of surgical wound alters the repair and the cell migration in the presence of a foreign body in BALB/c mice. *Vet Immunol Immunopathol*, 128, 1–3, 319.

VIGAN, M. 2010. Essential oils: renewal of interest and toxicity. *Eur J Dermatol*, 20, 6, 685-92.

VUKOVIC-GACIC, B., NIKCEVIC, S., BERIC-BJEDOV, T., KNEZEVIC-VUKCEVIC, J. & SIMIC, D. 2006. Antimutagenic effect of essential oil of sage (Salvia officinalis L.) and its monoterpenes against UV-induced mutations in Escherichia coli and Saccharomyces cerevisiae. *Food Chem Toxicol*, 44, 10, 1730-8.

WAGNER, H., LUTHER, R. & MANG, T. 2001. Lubricant base fluids based on renewable raw materials: Their catalytic manufacture and modification. *Appl Catal A Gen*, 221, 1–2, 429-442.

WANG, L. Z., HO, P. C., LEE, H. S., VADDI, H. K., CHAN, Y. W. & YUNG, C. S. 2003. Quantitation of paclitaxel in micro-sample rat plasma by a sensitive reversed-phase HPLC assay. *J Pharm Biomed Anal*, 31, 2, 283-9.

WANG, W., WANG, L. & QIN, W. 1994. Dilute solution behavior of chitosan in different acid solvents *Chin J Polym Sci*, 12, 1

WANG, Y., WU, K. C., ZHAO, B. X., ZHAO, X., WANG, X., CHEN, S., NIE, S. F., PAN, W. S., ZHANG, X. & ZHANG, Q. 2011. A novel paclitaxel microemulsion containing a reduced amount of Cremophor EL: pharmacokinetics, biodistribution, and in vivo antitumor efficacy and safety. *J Biomed Biotechnol*, 2011, 854872.

WANG, Y., ZHAO, R., YU, L., ZHANG, Y., HE, Y. & YAO, J. 2014. Evaluation of cinnamon essential oil microemulsion and its vapor phase for controlling postharvest gray mold of pears (*Pyrus pyrifolia)*. *J Sci Food Agric*, 94, 5, 1000-4.

WANI, M. C., TAYLOR, H. L., WALL, M. E., COGGON, P. & MCPHAIL, A. T. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc*, 93, 9, 2325-7.

WEAVER, B. A. 2014. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*, 25, 18, 2677-81.

WEINGART, S. N., BROWN, E., BACH, P. B., ENG, K., JOHNSON, S. A., KUZEL, T. M., LANGBAUM, T. S., LEEDY, R. D., MULLER, R. J., NEWCOMER, L. N., O'BRIEN, S., REINKE, D., RUBINO, M., SALTZ, L. & WALTERS, R. S. 2008. NCCN Task force report: Oral chemotherapy. *J Natl Compr Canc Netw*, 6 Suppl 3, S1-14.

WILLIAMS, A. C. & BARRY, B. W. 1991. Terpenes and the lipid-protein-partitioning theory of skin penetration enhancement. *Pharm Res*, 8, 1, 17-24.

WINK, M. Functions of plant secondary metabolites and their exploitation in biotechnology. UK: Sheffield Academic Press, 1999.

WINSOR, P. A. Solvent Properties of Amphiphilic Compounds. London: Butterworth, 1954.

WOODLEY, J. 2001. Bioadhesion: new possibilities for drug administration? *Clin Pharmacokinet*, 40, 2, 77-84.

XAVIER-JUNIOR, F. H., EGITO, E. S. T., MORAIS, A. R. V., ALENCAR, E. N., MACIUK, A., VAUTHIER, C. Chapter VII, 2015. Experimental design approach applied to the development of chitosan coated poly(isobutylcyanocrylate) nanocapsules encapsulating copaiba oil. *Article in preparation*

XAVIER-JUNIOR, F. H., GUEUTIN, C., MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T., VAUTHIER, C. Chapter II, 2015. HPLC method for the dosage of paclitaxel in copaiba oil. Development, validation, application to the determination of the solubility and partition coefficients. *Article in preparation*

XAVIER-JUNIOR, F. H., HUANG, N., VACHON, J. J., REHDER, V. L. G., MACIUK, A., EGITO, E.S.T., VAUTHIER, C. Chapter V, 2015. Match of solubility parameters between oil and surfactants as a rational approach for the formulation of O/W microemulsion with high dispersed volume of copaiba oil and low surfactant content. *Article in preparation*

XAVIER-JUNIOR, F. H., MACIUK, A., MORAIS, A. R. V., ALENCAR, E. N., REHDER, V. L. G., EGITO, E. S. T., VAUTHIER, C. Chapter I, 2015a. Development of gas-chromatography method for the analysis of copaiba oil. *Chapter I- Article in preparation*

XAVIER-JÚNIOR, F. H., SILVA, K. G. H., FARIAS, I. E. G., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., OLIVEIRA, A. G. & EGITO, E. S. T. 2012a.

Prospective study for the development of emulsion systems containing natural oil products. *J Drug Del Sci Tech*, 22, 4, 367-372.

XAVIER-JÚNIOR, F. H., SILVA, K. G. H., FARIAS, I. E. G., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., OLIVEIRA, A. G. & EGITO, E. S. T. 2012b. Prospective study for the development of emulsion systems containing natural oil products. *J Drug Del Sci Tech*, 22, 4, 273-373.

XAVIER-JUNIOR, F. H., VAUTHIER, C., MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T. Chapter IV, 2015. Microemulsion-based drug delivery systems containing natural oils. *Article in preparation*

XU, J., FAN, Q. J., YIN, Z. Q., LI, X. T., DU, Y. H., JIA, R. Y., WANG, K. Y., LV, C., YE, G., GENG, Y., SU, G., ZHAO, L., HU, T. X., SHI, F., ZHANG, L., WU, C. L., TAO, C., ZHANG, Y. X. & SHI, D. X. 2010. The preparation of neem oil microemulsion (*Azadirachta indica*) and the comparison of acaricidal time between neem oil microemulsion and other formulations *in vitro*. *Vet Parasitol*, 169, 3-4, 399-403.

XU, S. X., LI, Y. C., LIU, X., MAO, L. J., ZHANG, H. & ZHENG, X. D. 2012. In vitro and in vivo antifungal activity of a water-dilutable cassia oil microemulsion against Geotrichum citri-aurantii. *J Sci Food Agric*, 92, 13, 2668-71.

YAN, C., RESAU, J. H., HEWETSON, J., WEST, M., RILL, W. L. & KENDE, M. 1994. Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique. *J Control Release*, 32, 3, 231-241.

YI, C., ZHONG, H., TONG, S., CAO, X., FIREMPONG, C. K., LIU, H., FU, M., YANG, Y., FENG, Y., ZHANG, H., XU, X. & YU, J. 2012. Enhanced oral bioavailability of a sterol-loaded microemulsion formulation of Flammulina velutipes, a potential antitumor drug. *Int J Nanomedicine*, 7, 5067-78.

YI, H., GAO, J. & YANG, H. 2010. Solubilization of O/W microemulsion for volatile oil from *Houttuynia Cordra*. *Zhongguo Zhong Yao Za Zhi*, 35, 1, 49-52.

YOON, J. I., BAJPAI, V. K. & KANG, S. C. 2011. Synergistic effect of nisin and cone essential oil of *Metasequoia glyptostroboides Miki ex Hu* against *Listeria monocytogenes* in milk samples. *Food Chem Toxicol*, 49, 1, 109-14.

YOTSAWIMONWAT, S., OKONOKI, S., KRAUEL, K., SIRITHUNYALUG, J., SIRITHUNYALUG, B. & RADES, T. 2006. Characterisation of microemulsions containing orange oil with water and propylene glycol as hydrophilic components. *Pharmazie*, 61, 11, 920-6.

YU, W., EGITO, E. S. T., BARRATT, G., FESSI, H., DEVISSAGUET, J. P. & PUISIEUX, F. 1993. A novel-approach to the preparation of injectable emulsions by a spontaneous emulsification process. *Int J Pharm*, 89, 2, 139-146.

YUAN, C., CHEN, D. & WANG, S. 2015. Drug confirmation by mass spectrometry: Identification criteria and complicating factors. *Clin Chim Acta*, 438, 0, 119-125.

ZABALETA, V., CALLEJA, P., ESPUELAS, S., CORRALES, L., PIO, R., AGUEROS, M. & IRACHE, J. M. 2013. Mucopenetrating nanoparticles: vehicles for the oral administration of paclitaxel. *Ann Pharm Fr*, 71, 2, 109-18.

ZABALETA, V., PONCHEL, G., SALMAN, H., AGUEROS, M., VAUTHIER, C. & IRACHE, J. M. 2012. Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: permeability and pharmacokinetic study. *Eur J Pharm Biopharm*, 81, 3, 514-23.

ZENG, Z., ZHOU, G., WANG, X., HUANG, E. Z., ZHAN, X., LIU, J., WANG, S., WANG, A., LI, H., PEI, X. & XIE, T. 2010. Preparation, characterization and relative bioavailability of oral elemene o/w microemulsion. *Int J Nanomedicine*, 5, 567-72.

ZHANG, H., SHEN, Y., BAO, Y., HE, Y., FENG, F. & ZHENG, X. 2008. Characterization and synergistic antimicrobial activities of food-grade dilution-stable microemulsions against Bacillus subtilis. *Food Res Int*, 41, 5, 495-499.

ZHANG, Z. & FENG, S.-S. 2006. The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)–tocopheryl polyethylene glycol succinate nanoparticles. *Biomater*, 27, 21, 4025-4033.

ZHAO, D., ZHAO, X., ZU, Y., LI, J., ZHANG, Y., JIANG, R. & ZHANG, Z. 2010. Preparation, characterization, and in vitro targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. *Int J Nanomedicine*, 5, 669-77.

ZHAO, K. S., ASAMI, K. & LEI, J. P. 2002. Dielectric analysis of chitosan microsphere suspensions: study on its ion adsorption. *Colloid Polym Sci*, 280, 11, 1038-1044.

ZHAO, X. L., CHEN, D. W., GAO, P., DING, P. T. & LI, K. X. 2005. Synthesis of ibuprofen eugenol ester and its microemulsion formulation for parenteral delivery. *Chem Pharm Bull* 53, 10, 1246-1250.

ZHAO, Y. Q., WANG, L. P., MA, C., ZHAO, K., LIU, Y. & FENG, N. P. 2013. Preparation and characterization of tetrandrine-phospholipid complex loaded lipid nanocapsules as potential oral carriers. *Int J Nanomedicine*, 8, 4169-4181.

Annexe

Article

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Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions

Éverton N. Alencar[1], Francisco H. Xavier-Júnior[2,3], Andreza R. V. Morais[2], Teresa R. F. Dantas[2], Nednaldo Dantas-Santos[2], Lourena M. Verissimo[2], Vera L. G. Rehder[4], Guilherme M. Chaves[1], Anselmo G. Oliveira[5], and E. Sócrates T. Egito[1,2,*]

[1] *Programa de Pós-Graduação em Ciências Farmacêuticas (PPGCF), Centro de Ciências da Saúde (CCS), Universidade Federal do Rio Grande do Norte (UFRN), Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil*
[2] *UFRN, CCS, Faculdade de Farmácia, Laboratório de Sistemas Dispersos (LaSiD), Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil*
[3] *UFRN, Rede Nordeste de Biotecnologia (RENORBIO), Programa de Pós-Graduação, 59000-000, Natal-RN-Brazil*
[4] *Centro Pluridisciplinar de Pesquisas Químicas, Universidade Estadual de Campinas (UNICAMP), Biológicas e Agrícolas, Rua Alexandre Cazelatto, 999, Vila Betel, Paulínia, SP, Brazil*
[5] *Faculdade de Ciências Farmacêuticas (UNESP), Departamento de Fármacos e Medicamentos, Rodovia Araraquara, Jaú Km 01, 14801-902 Araraquara, SP, Brazil*

The aim of this work was to investigate the antimicrobial activity of nanostructured emulsions based on copaiba (*Copaifera langsdorffii*) resin-oil, copaiba essential oil, and bullfrog (*Rana catesbeiana* Shaw) oil against fungi and bacteria related to skin diseases. Firstly, the essential oil was extracted from copaiba resin-oil and these oils, along with bullfrog oil, were characterized by gas chromatography combined with mass spectrometry (GC-MS). Secondly, nanostructured emulsion systems were produced and characterized. The antimicrobial susceptibility assay was performed, followed by the Minimum Inhibitory Concentration (MIC) determination, the bioautography assay, and the antibiofilm determination. Strains of the genera *Staphylococcus*, *Pseudomonas*, and *Candida* were used. The CG-MS analysis was able to identify the components of copaiba resin-oil, copaiba essential oil, and bullfrog oil. The MIC assay in association with the bioautography revealed that some esters of palmitic and oleic acids—α-curcumene, α-himachalene, isothujol, and α-fenchene—probably inhibited some strains. The nanostructured emulsions based on copaiba resin-oil and essential oil improved the antimicrobial activity of the pure oils, especially against *Staphylococcus* and *Candida,* resistant to azoles. The bullfrog oil nanostructured emulsion showed a lower antimicrobial effect when compared to the copaiba samples. However, bullfrog oil-based nanostructured emulsion showed a significant antibiofilm activity ($p < 0.05$). Given the significant antimicrobial and antibiofilm activities of the evaluated oils, it may be concluded that nanostructured emulsions based on copaiba and bullfrog oils are promising candidates for the treatment of infections and also may be used to incorporate other antimicrobial drugs.

Keywords: Nanostructured Emulsion, Copaiba (*Copaifera langsdorffii*) Oil, Bullfrog (*Rana catesbeiana*) Oil, Antimicrobial Activity.

1. INTRODUCTION

Natural oils have been used in popular medicine as antimicrobial agents for treatment of various infections.[1] Nowadays, due to the substantial number of drugs resistant to microorganisms, these oils and other natural products have become scientifically recognized, encouraging the introduction of new products originated from animal and vegetable sources in the market.[2] Among these products, bullfrog (*Rana catesbeiana* Shaw) and copaiba (*Copaifera langsdorffii*) oils are widely used in popular medicine.

Copaiba oil is extracted from trees known as Copaibeiras (*Copaifera* spp.), which are distributed in South America

*Author to whom correspondence should be addressed.

BHI agar for 24 h and Sabouraud dextrose agar for 48 h,

2.2.3. Antimicrobial Susceptibility Assay

and Africa.[3] The copaiba resin-oil is traditionally used in popular medicine due to its anti-inflammatory and antimicrobial activities, among other pharmacological and cosmetic properties already elucidated in the literature.[3–6] Phytochemical studies of resin-oil reveal that it contains about 28 diterpenes and 72 sesquiterpenes hydrocarbons.[7] The essential oil extracted from the copaiba resin-oil concentrates the majority of the sesquiterpenes, which are associated with the pharmacological activity of this compound. Therefore, the copaiba essential oil has become a highly demanded product in the pharmaceutical field.[8]

The bullfrog oil is biotechnologically extracted from bullfrog adipose tissues, which are usually discarded in the frog farms in the food industry. In this way, it is possible to reuse an animal residue to provide an active oil to treat diseases.[9] Because it belongs to the muscular tissue and skin, the bullfrog oil is rich in polyunsaturated fatty acids such as the omega group, which are pharmacologically relevant for their medicinal use.[10] Additionally, other chemical components from bullfrogs, such as peptides from their stomach, have been studied due to their antimicrobial activity.[11]

Because the skin acts as a physical barrier of the human organism, it is constantly exposed to several pathogenic microorganisms through the contact with the external environment. Therefore, this organ may be susceptible to infections. Among the microorganisms that are involved with such infections, gram-positive bacteria of the genus *Staphylococcus*, gram-negative bacteria such as *Pseudomonas aeruginosa*, and yeasts belonging to the genus *Candida* are prevalently found.[12, 13] It is well known that many antimicrobial agents are no longer effective against several microorganisms that may have become resistant to antimicrobial agents. Nevertheless, these microorganisms are still sensitive to natural products. On the other hand, the use of oils *in natura* leads to low adherence to treatment due to its unpleasant organoleptic characteristics. Natural oils may also not provide desirable pharmacokinetic properties and permeability. Therefore, the delivery systems, such as nanostructured emulsions, are viable systems to improve these characteristics. Additionally, such systems can help the understanding of the mechanism of action of the substances present in these natural oils, a subject barely discussed on the literature.

Nanostructured emulsion systems containing natural oils are suitable alternatives against infectious pathogens because of the synergic effect induced by the antimicrobial activity of the renewable source of oils and the advantages conferred to the nanostructured emulsions.[14] The development of these systems is economically viable and aims to improve the bioavailability, to enhance or modify the release of the oil's active components, and to improve their organoleptic characteristics.[15] Those emulsified systems may be defined as homogeneous milky systems formed by two phases that are immiscible, in which one phase is dispersed within the other in the form of droplets stabilized by surfactant molecules.[16]

The aim of this work was to determine the antimicrobial activity of nanostructured emulsions based on bullfrog and copaiba (resin and essential) oils. Therefore, first, a chemical characterization of the oils used to develop the nanostructured emulsions was performed to identify their antimicrobial compounds. Moreover, the antimicrobial activity of the nanostructured emulsion systems produced through different methods was evaluated.

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Chemicals

The copaiba (*Copaifera langsdorffii*) resin-oil was purchased from Flores and Ervas (Piracicaba, SP, Brazil). Bullfrog (*Rana catesbeiana* Shaw) oil was a gift from Asmarana Natural Products (Natal, Brazil). Span 80®, methylene blue dye, glucose, and tetrazolium chloride were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Tween 20®, DMSO, hexane, ethyl acetate, ethanol, and violet crystal were purchased from Vetec Quimica Fina LTDA (Rio de Janeiro, RJ, Brazil). The copaiba essential oil was obtained by hydrodistillation of copaiba resin-oil using a Clevenger apparatus for 4 h at 100 °C. Brain Heart Infusion (BHI) broth, Sabouraud dextrose agar, Mueller-Hinton agar, and Mueller-Hinton broth came from Himedia (Mumbai, MU, India). Yeast Extract-Peptide-Dextrose (YPD) broth was a gift from MMML (Medical and Molecular Mycology Laboratory), UFRN (Natal, Brazil). All other chemicals were of at least analytical grade.

2.1.2. Microorganisms

The bacteria strains used in this study were *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, and two clinical strains of each species. The yeasts used during the experiments were *Candida albicans* ATCC 90027, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 2001, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 13803, and also a clinical strain of each aforementioned tested species. Yeasts clinical strains isolated from patients were collected according to two different protocols approved by the local Research Ethics Committee from the Onofre Lopes University Hospital, Natal, Rio Grande do Norte, Brazil under the numbers: 595/11þ (vaginal isolates) and 494/10 (skin and mucosal isolates). All the fungal isolates belong to The Laboratório de Micologia Médica e Molecular culture collection, UFRN, Rio Grande do Norte, Brazil. The bacteria were maintained in Nutrient broth (Mumbai, MU, India) medium, and the yeasts were maintained in Sabouraud Dextrose broth medium (Mumbai, MU, India) containing 20% glycerol, frozen at −80 °C until the moment of the experiment. All stored bacteria and yeasts were cultured in

BHI agar for 24 h and Sabouraud dextrose agar for 48 h, both at 37 °C respectively, before the tests.

2.2. Methods

2.2.1. Chemical Characterization

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the oils was performed on a Hewlett-Packard 6890 gas chromatograph interfaced to a HP-5975 mass selective detector. The column used was a HP-5MS cross-linked fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Before the experiments, the bullfrog oil and copaiba resin-oil were methylated using diazomethane aiming to perform the methyl ester's identification of acid compounds. The work temperatures for the copaiba essential oil were as follows: oven temperature started at 60 °C, isothermal, then heating 3 °C/min to 240 °C, and isothermally for 7 minutes at 250 °C. The injector temperature was 220 °C. The following are the conditions for both copaiba resin-oil and bullfrog oil: oven temperature started at 110 °C, isothermal, then, heating 5 °C/min to 280 °C, and isothermally for 26 minutes at 300 °C. The injector temperature was 250 °C. The volume injected for all samples was 1 μL. The split ratio was 1:25 and the ionization voltage 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. The separated components were identified by matching them with the National Institute of Standards and Technology (NIST) mass spectral library data and by comparing their Kovat's indices with those of authentic components and with published data. The quantitative determination was carried out by peak area integration.

2.2.2. Nanostructured Emulsion Preparation and Characterization

The three different nanostructure emulsions were prepared containing copaiba resin-oil, copaiba essential oil, and bullfrog oil according to previous studies.[15] The system was produced using the following composition: oil at 5% (w/w), water at 93% (w/w), Tween 20® at 1.56% (w/w), and Span 80® at 0.44% (w/w). Phase inversion technique (PIT) was used to produce the nanostructured emulsion.[17] The amount of Span® 80 was dispersed in the oil (phase 1) while Tween® 20 was dispersed in the water (phase 2). The phases were heated separately to 70° C and then phase 1 was emulsified into phase 2 by an Ultra-Turrax® T25 (IKA, Staufen, Germany) homogenization for 10 minutes at 13,000 rpm.

Additionally, physicochemical analyses were carried out. pH was measured using a PG-2000 pHmeter (GEHAKA, Morumbi, SP, Brazil) and electrical conductivity measurement was performed using a DM-32 conductivity (Digicrom Analytical, Campo Grande, SP, Brazil). Droplet size, polydispersity, and zeta potential analysis were evaluated by Dynamic Light Scattering (DLS) using a ZetaPlus (Holtsville, NY, USA). All analyses were performed at 25 ± 2 °C.

2.2.3. Antimicrobial Susceptibility Assay

Bacterial and fungal sensitivity of all strains described above was initially performed using a susceptibility test with Muller-Hinton agar for bacteria and Mueller-Hinton agar + 2% glucose and 0.5 μg/mL methylene blue dye for yeasts.[18, 19] The inocula were prepared in NaCl 0.9% (w/v) solution and adjusted to the 0.5 McFarland standard. Subsequently, 10 μL of the copaiba resin-oil, copaiba essential oil, bullfrog oil, and the three nanostructured emulsions containing each of these oils were added to the wells. The oils were dispersed in DMSO (1% (w/w)) for all tests. Chloramphenicol (1.5 mg/mL) and ketoconazole (2.5 mg/mL) were used as synthetic antimicrobial controls for bacteria and yeasts, respectively. Tween 20® at 1.56% (w/w), Span 80® at 0.44% (w/w), and DMSO 1% (w/w) were also tested individually. Assays were carried out in triplicate, three times ($n = 9$).

2.2.4. Broth Microdilution Assay

The inocula of microorganisms in NaCl 0.9% (w/v) solution were adjusted to the 0.5 McFarland standard. They were then used to prepare further dilutions in Mueller-Hinton Broth, containing 0.5 to 2.5×10^3 CFU/mL. A broth microdilution assay with serial dilutions of the samples was performed with sterile Muller-Hinton Broth in a 96-well microplate from 0.0001 to 248.7 mg/mL.[20] Subsequently, 100 μL of each microorganism cell suspension was then added to each well. The plates containing bacteria were incubated for 24 hours at 35 °C while the fungi were incubated for 48 hours at 37 °C in an orbital shaker (TE–420 Tecnal, Piracicaba, SP, Brazil) at 150 rpm for Minimal Inhibitory Concentration (MIC) determination. Assays were carried out in triplicate.

2.2.5. Bioautography Assay

The Thin Layer Chromatography (TLC) was developed with copaiba-oil/methanol and bullfrog-oil/methanol (2:8) and the hexane/ethyl acetate (9:1) solution as the eluent system. The TLC plates were revealed with vanillin-sulphuric acid to observe the chromatographic profile. Subsequently, the TLC plates were reproduced and loaded into culture plates prepared with Mueller-Hinton agar and the bacterial inoculum adjusted to the McFarland 0.5 standard. For the fungi strains, Mueller-Hinton agar plus 2% (w/w) glucose and 0.5 μg/mL methylene blue dye were used. The inhibition halos were observed using tetrazolium chloride (TTC) and the Retention Factors (*Rf*s) were calculated after an incubation period of 24 and 48 hours for bacteria and fungi, respectively, at 35 °C. The silica was removed at the *Rf* for extraction of the chemical retained compounds. The extraction was performed with ethyl acetate in an ultrasound bath for 20 minutes and filtration. Moreover, the samples were analyzed in GC-MS. Assays were carried out in triplicate.

2.2.6. Antibiofilm Assay

Cell suspensions were prepared according to the microdilution assay. The nanostructured emulsions and the respective oils (99:1 (w/w) in DMSO) were added (12.5% (v/v)) separately in sterile 96-well microliter plates containing sterile Mueller-Hinton Broth and microorganism suspensions, incubated at 35 °C for 24 h and 48 h for bacteria and yeasts, respectively, in an orbital shaker at 150 rpm. Then, the supernatants were removed and the wells were washed with sterile water to remove non-adherent cells. The biofilms were stained with crystal violet for 20 minutes. Subsequently, 200 μL of absolute ethanol was added and the optical density (OD) was measured with an ELISA microplate reader (BioTek, μQuant) at 570 nm.[21] Ketoconazole and chloramphenicol were used as control antimicrobial agents according to the antimicrobial screening. Assays were carried out in triplicate.

2.2.7. Statistical Analysis

The results are presented as the mean ± S.D. Statistical significance between 3 groups was performed by Analysis of Variance (ANOVA) followed by Tukey's post-test. Student's t-test was used between 2 unpaired groups. p values less than 0.05 ($p < 0.05$) were considered significant.

3. RESULTS AND DISCUSSION

3.1. Chemical Characterization

Concerning the samples' characterization, the GC-MS was a useful tool to identify the chemical compounds of the studied oils. The technique showed a required resolution and peak separation, allowing analyzing the compounds individually in comparison with the spectra library and the retention times found in the literature. Prior to the CG-MS analysis, the extraction of copaiba essential oil showed a 10% (v/v) yield, which was superior to that reported by Gramosa and Silveira, 2005.[22] The CG-MS analyses showed that terpenes have lower retention time in copaiba resin-oil analysis when compared to the same compounds in the copaiba essential oil (Figs. 1(A) and (B)). These characteristic terpene compound peaks can be observed in two other studies that analyzed the chemical composition of copaiba oils.[3, 23]

The analysis of copaiba resin-oil chromatograms suggests that β-bisabolene was the major identified compound (Table I). Other components, such as β-caryophyllene and α-bermagotene, were also present at high amounts. The residue fraction obtained after extraction of the essential oil showed a lower amount of sesquiterpenes, indicating that these compounds were extracted by hydrodistillation and were concentrated in the essential oil (data not shown).

The bullfrog oil GC-MS analyses showed separated peaks in which methyl oleate was the ester presented as the most abundant compound. The oleic acid esters were the predominant compounds, followed by esters of palmitic and linoleic acid (Fig. 1(C)). Thus, these three

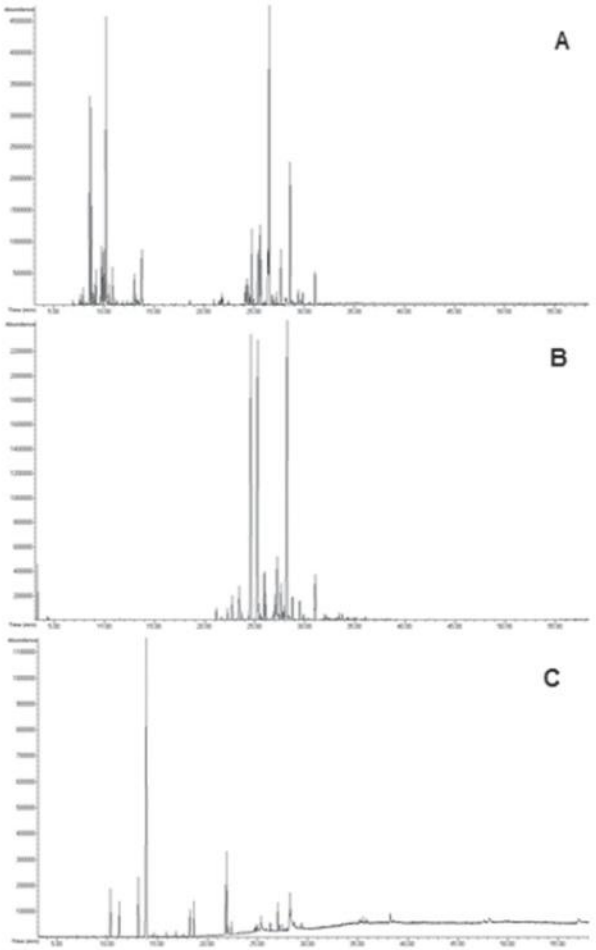

Figure 1. Chromatogram of natural oils. (A) Copaiba resin-oil; (B) copaiba essential oil; (C) bullfrog oil.

compounds were the predominant monounsaturated, saturated, and polyunsaturated fatty acids, respectively.

These results are also in accordance with those observed in the literature concerning the composition of bullfrog oil,[9, 24] which reported different methodologies for bullfrog oil extraction and also indicated the predominance of oleic, linoleic, and palmitic fatty acids (Table II).

3.2. Nanostructured Emulsion Characterization

The systems were macroscopically presented as milky and fluid dispersions. Concerning their organoleptic characteristics, a change in their natural oils inherent odor was observed after the nanostructured emulsion process of these oils. Furthermore, the formulations were not creamy on top. The conductivity (from 187.51 μS to 226.20 μS) confirmed the external aqueous phase of the systems. The DLS analysis showed a nanosized droplet size for all nanostructured emulsions of about 200 nm, as well as a low polydispersity. Additionally, pH values were low, ranging from 3.22 to 3.48, probably due to the presence of

Table I. Percentage of terpenes from copaiba (*Copaifera langsdorffii*) oils.

Chemical composition	RT (min)	CO (%)	RT (min)	CEO (%)
δ-elemene	6.90	0.37	21.17	0.63
(+)-cyclosativene	7.52	0.45	22.31	0.67
α-copaene	7.66	0.93	22.76	1.39
β-elemene	7.92	1.44	23.46	2.41
β-caryophyllene	8.57	18.30	24.62	21.68
α-bergamotene	8.74	15.61	25.29	20.53
α-guaiene	8.83	1.17	25.38	0.88
β-farnesene	9.03	1.61	26.11	1.56
α-caryophyllene	9.22	2.80	25.95	2.85
τ-muurolene	9.60	0.82	26.88	0.53
β-cubebene	9.75	4.83	27.06	1.72
β-selinene	9.88	4.34	27.27	6.16
α-selinene	–	–	27.62	2.31
τ-gurjenene	–	–	27.78	0.89
β-chamigrene	10.04	5.63	–	–
β-bisabolene	10.21	24.76	28.24	23.67
β-sesquiphellandrene	10.55	2.44	–	–
α-curcumene	12.29	0.35	–	–
α-cedrene	12.85	0.53	–	–
β-cedrene	–	–	28.76	1.40
α-himachalene	13.01	1.74	33.39	0.46
Kaurene	20.98	0.39	–	–

Notes: RT (min): Retention time; CO: Copaiba resin-oil; CEO: Copaiba essential oil; (–) Not detected.

Table II. Percentage of fatty acid esters from bullfrog (*Rana catesbeiana* Shaw) oil.

Chemical composition	RT (min)	BO (%)
Methyl palmitoleate	18.25	2.23
Methyl palmitate	18.65	5.87
Methyl linoleate	21.84	4.79
Methyl oleate	21.94	9.26
Glyceryl monooleate	28.22	7.59

Notes: RT (min): Retention time; BO: Bullfrog oil.

fatty acids from both oils. The zeta potential was distinct for the three systems, probably because this property is influenced by the chemical composition of the nanostructured emulsion components, especially the oils (Table III). The bullfrog oil nanostructured emulsion showed the lowest zeta potential (-11.86 ± 1.99), which could predict low stability. However, all the nanostructured samples remained quite stable over three months at 25 °C.

Table III. Physicochemical characterization of the nanostructured emulsions.

	pH	Conductivity (μS)	Droplet size (nm)	Polydispersity	Zeta potential
COE	3.40	187.51	200 ± 0	0.24 ± 0.01	−34.37 ± 2.50
CEOE	3.48	226.20	280 ± 1	0.14 ± 0.02	−27.08 ± 0.89
BOE	3.22	220.30	260 ± 0	0.27 ± 0.01	−11.86 ± 1.99

Notes: COE: Copaiba resin-oil nanostructured emulsion; CEOE: Copaiba essential oil nanostructured emulsion; BOE: Bullfrog oil nanostructured emulsion.

3.3. Antimicrobial Susceptibility Assay

The antimicrobial screening performed by the agar diffusion method was performed to investigate whether the tested strains were sensitive to the oils used to produce the nanostructured emulsion systems. The inhibition halos showed that some strains were sensitive to copaiba oils. The resin-oil and the essential oil exhibited an inhibition halo of 6.6 mm and close to 9.0 mm, respectively, against *S. aureus* (Table IV). This result corroborates the findings of other studies with copaiba oil from other species against *S. aureus*, including the copaiba oil extracted from *Copaifera multijuga*, which showed inhibition halos of 7.0 mm against *S. aureus*.[25]

On the other hand, the bullfrog oil showed no significant antibacterial or antifungal activity against all tested strains. However, it is important to emphasize that studies based solely on susceptibility tests using agar diffusion are not conclusive. In some occasions the antimicrobial compounds are not able to migrate through the agar or are present at low concentrations. Therefore, further microbiological assays such as broth microdilution and bioautography would be required to confirm these results.[20]

The microbiological behavior of the resin-oil and that of the essential oil revealed in this work were significantly different, probably because of their different chemical compositions. In fact, the essential oil showed an activity of about 50% more efficient than the resin-oil against *S. epidermidis* ATCC 12228 and was more effective than the resin-oil for most of the tested strains. This indicates that the highest concentration of sesquiterpenes, such as β-caryophyllene and α-himachalene, provides greater antibacterial and antifungal activity to the essential oil (Table IV).[26]

Several works found in the literature concerning the antimicrobial activity of *C. langsdorffii* oil by agar diffusion technique show a different profile of response. In fact, the copaiba oil used in this work presented an inhibition halo that was about 50% lower for some reference strains. This could be explained by the variability of the methodology or by the variability of the chemical composition of the oil in plants of the same species. Therefore, the

Table IV. Microbial sensitivity (inhibition halos (mm)) of copaiba resin-oil and copaiba essential oil against susceptible strains.

Microorganisms	CO	CEO	Ref.
S. aureus ATCC 29213	6.66 ± 1.32	9.88 ± 2.14	*27.78 ± 2.04
S. epidermidis ATCC 12228	5.44 ± 0.72	14.00 ± 2.39	*35.11 ± 3.68
S. epidermidis CS1	–	11.89 ± 1.96	*27.89 ± 3.14
S. epidermidis CS2	–	13.11 ± 3.14	*24.44 ± 6.34
C. glabrata ATCC 2001	6.33 ± 2.06	12.89 ± 6.03	**28.33 ± 2.50
C. glabrata 15V3C (CS)	–	9.88 ± 0.78	**28.11 ± 2.08
C. krusei ATCC 6258	–	13.67 ± 3.42	**24.67 ± 2.87
C. krusei LMM54 (CS)	–	13.78 ± 3.23	**24.22 ± 4.35

Notes: (–) Exhibited no inhibition halo; CO: Copaiba resin-oil; CEO: Copaiba essential oil; Ref: Antimicrobial synthetic reference; *Chloramphenicol; **Ketoconazole.

evaluation of the chemical composition of the oils is a crucial step in the development of nanostructured emulsions using natural oil products.[27]

Concerning the antifungal activity, it must be emphasized that copaiba essential oil has potential activity against reference strains of *C. krusei* (12.89 ± 6.03) and *C. glabrata* (13.67 ± 3.42), which are known for possible resistance to azole antifungals widely used for the treatment of superficial infections. On the other hand, strains of *C. albicans*, *C. tropicalis*, and C. *parapsilosis* had full growth and were not sensitive to the essential oil. Therefore, each strain should be tested with the essential oil independently of the genus or species. However, these Candida species are considered less able to develop resistance to synthetic antifungal drugs currently in use.[28]

It is important to note that both the DMSO (used to solubilize the oils) and the surfactants (Tween 20® and Span 80®) present no activity when separately used. This finding is relevant to prove that the surfactants would not directly influence the antimicrobial activity of the nanostructured emulsion systems.

3.4. Broth Microdilution Assay

Following the evaluation of the sensitivity of the strains to the oils, a broth microdilution assay was performed with the nanostructured emulsion systems containing copaiba resin-oil, copaiba essential oil, and bullfrog oil. The copaiba oil-based nanostructured emulsions presented lower MIC values when compared to the pure oil samples, specifically for *C. glabrata* and *C. krusei* (Table V). It is important to highlight that, although with only 5% of oil, the nanostructured emulsion systems containing the copaiba resin-oil and the copaiba essential oil showed activity equivalent to or better than that of the pure oil alone (Table V). Such improvement of activity could be due to the nanostructured emulsion system, in which the oil was dispersed as droplets and which may improve the activity of the compounds, resulting in a better activity of natural oils.[15,29] This was again demonstrated by the significant decrease ($p < 0.05$) in the MIC for *C. glabrata* ATCC 2001 using the emulsion based on the copaiba resin-oil rather than the oil itself.

However, a likely paradoxical effect phenomenon for the clinical strain of *C. glabrata*, which presented inhibition only at low concentrations, but cells restarted to grow with higher concentrations of the oil, might have occurred (results not shown). Despite the fact that Chamilos et al. (2007), testing the effect of a new group of antifungal agents, the echinocandins,[30] have described that only *Candida* species other than *C. glabrata* presented paradoxical growth effect, we cannot rule out the possibility that this phenomenon has happened for this species in the presence of the oil. Nevertheless, additional studies are mandatory to confirm this finding.

Since the nanostructured emulsion based on bullfrog oil and the pure oil showed no activity in the susceptibility assay, the MIC was determined only against the ATCC strains in order to confirm the preliminary results. As expected, both the bullfrog oil nanostructured emulsion and the pure oil exhibited MIC values $> 228.5 \pm 0.0$ mg/mL for all tested strains. However, because the oils presented different composition, the ineffective activity found for the bullfrog oil compared to the copaiba oils could be due to the choice of the strains used in this study. Concerning its active compounds, other strains not discussed in this study might show sensitivity to the bullfrog oil chemical compounds. It is important to note that the action of natural products may be either species specific or even strain specific. It is well described in the literature that susceptibility to antifungal drugs may vary within different strains belonging to the same species.[27] Therefore, a microbiological activity assay with additional strains would reveal the efficacy of such product and the fatty acids found in its composition, as claimed by the literature.[31]

3.5. Bioautography

The bioautography of copaiba resin-oil revealed two different chromatographic zones that caused inhibition on the microorganisms' growth. The first chromatographic band inhibited *P. aeruginosa* ATCC 27853 (*Rf* 0.2) and the second one inhibited the growth of *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 (*Rf* 0.15). The GC-MS analysis showed traces of α-curcumene, α-himachalene,

Table V. Microdilution assay results (MIC) of copaiba oils and nanostructured emulsions (mg/mL).

Microorganisms	CO	COE	CEO	CEOE
S. aureus ATCC 29213	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$55.4 \pm 0.0^{*}$	$> 249.3 \pm 0.0^{*}$
S. epidermidis ATCC 12228	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	221.7 ± 0.0	$> 249.3 \pm 0.0$
S. epidermidis CS1	$0.0009 \pm 0.0^{**}$	$0.0455 \pm 0.022^{**}$	221.7 ± 0.0	$> 249.3 \pm 0.0$
S. epidermidis CS2	$> 234.0 \pm 0.0$	$> 249.750 \pm 0.0$	221.7 ± 0.0	$> 249.3 \pm 0.0$
C. glabrata ATCC 2001	$> 234.0 \pm 0.0^{*}$	$0.9717 \pm 0.00^{***}$	$0.1083 \pm 0.076^{***}$	$15.6 \pm 0.0^{***}$
C. glabrata 15V3C (CS)	0.0009 ± 0.0	0.0001 ± 0.0	$0.1083 \pm 0.038^{***}$	$0.9736 \pm 0.0^{***}$
C. krusei ATCC 6258	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$34.7 \pm 0.0^{*}$	$15.6 \pm 0.0^{*}$
C. krusei LMM54 (CS)	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$34.7 \pm 0.0^{*}$	$3.9 \pm 0.0^{*}$

Notes: CO: Copaiba resin-oil; COE: Copaiba resin-oil nanostructured emulsion; CEO: Copaiba essential oil; CEOE: Copaiba essential oil nanostructured emulsion; MIC = Minimum Inhibitory Concentration. $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$, when comparing the emulsions with the oils themselves.

Isothujol, and α-fenchene in the first band, while the second fraction showed α-himachalene (1.46%) and traces of α-curcumene in its composition (results not shown). Pharmacological and toxicological data involving these isolated terpenes are not well described yet in the literature. Therefore, their mechanism of action of such substances was not elucidated yet. However, there are some registers about derivatives and isomers of himachalene, especially β-himachalene, as being a potential antifungal and insecticidal agent in the food poisoning.[32,33]

Additionally, other terpenes from the copaiba oil have been reported as active compounds by several studies in which the antimicrobial activity of β-caryophyllene, caryophyllene oxide, and copalic acid, usually present in large quantities in the copaiba resin-oils, was investigated.[34] Thus, it may be suggested that the terpenes, identified by the GC-MS after the bioautography method, were probably responsible for the antimicrobial activity of the copaiba oil. However, further studies are required in order to investigate their activity individually.

The bioautography of bullfrog oil followed by CG-MS analysis revealed the presence of esters of oleic (48.6%) and palmitic (0.9%) acids in the zone related to the inhibition growth of *P. aeruginosa* ATCC 27853 (*Rf* 0.6). As it can be seen, the oleic acid was predominant in this oil (results not shown). Issacs et al. (1995) demonstrated that oleic acid and monoglycerides are responsible for the antimicrobial activity in breast milk.[31] Therefore, these fatty acids present in large amounts in the bullfrog oil may be the compound responsible for its antimicrobial activity. It is important to note that although the bioautography results did not corroborate with the antimicrobial screening, it provides the possibility of testing concentrated compounds through chromatographic bands, which provides better sensitivity to the method and highlights the effective antimicrobial activity of the bullfrog oil.

3.6. Antibiofilm Activity

The reduction in biofilm formation is an important tool to corroborate the antimicrobial activity because phenotypic changes may occur with microorganisms, making them more invasive than planktonic cells. In most cases, biofilm formation confers greater resistance to antimicrobial molecules and the immune defense of the host.[35]

Both copaiba resin-oil and copaiba essential oil were able to provide effective inhibition on the biofilm formation (Fig. 2). The nanostructured emulsions were able to inhibit the biofilm formation of all ATCC strains tested. Additionally, considering most of the strains, the oils themselves and their nanostructured emulsions presented the same profile of inhibition with no significant difference ($p > 0.05$). However, for *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 strains, a significant decrease ($p < 0.05$) in the biofilm formation was observed for the copaiba essential oil nanostructured emulsion when compared to the pure oil. In contrast, *C. krusei* CS improved

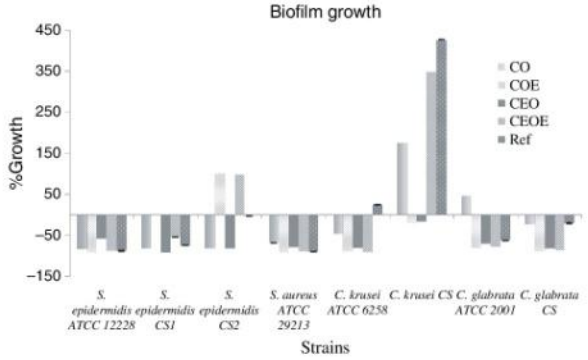

Figure 2. Percentage of biofilm growth in the presence of copaiba samples. CO: Copaiba resin-oil; COE: Copaiba resin-oil nanostructured emulsion; CEO: Copaiba essential oil; CEOE: Copaiba essential oil nanostructured emulsion; Ref.: Chloramphenicol (bacteria) and Ketoconazole (fungi).

the biofilm formation when cells were grown in the presence of this system and ketoconazole (Fig. 2). However, the copaiba essential oil nanostructured emulsion showed a better antibiofilm activity than ketoconazole for C. *krusei* ATCC 6258, clearly demonstrating that the improvement of biofilm formation may vary within different *C. krusei* strains. Concerning *C. glabrata* ATCC 2001 and CS, the nanostructured emulsions were more effective by inhibiting biofilm formation than the oils themselves (Fig. 2).

The biofilm inhibition induced by natural oils has been already studied for different bacteria and fungi. As discussed by Carvalho and Fonseca (2007),[36] terpene compounds may be useful to inhibit the biofilm formation by acting in both the cellular membrane structure and the cell surface hydrophobicity. Interestingly, in spite of the parenteral lipid emulsion induced *Candida* biofilm formation on medical catheters,[37] the nanostructured emulsions based on copaiba oil containing the terpene compounds were able to inhibit it (Fig. 2).

The analysis of the biofilm formation for bullfrog oil nanostructured emulsion showed a significant inhibitory activity against most of the yeast strains when compared to the pure oil (Fig. 3). It is important to highlight that although bullfrog oil nanostructured emulsion did not present positive results for *Pseudomonas aeruginosa* ATCC 90027 in the microbial sensitivity assay and in the broth microdilution assay, a significant inhibition of the biofilm formation was observed, suggesting that the bullfrog oil may act on the cells that form the biofilm, impairing either adhesion initial steps or exopolymeric matrix secretion, but not in planktonic cells of *P. aeruginosa*.

Additionally, bullfrog oil nanostructured emulsion inhibited the biofilm formation of most of the tested *Candida* species. Thus, this system could be used to treat fungal infections triggered by the biofilm formation caused by yeasts of the genus *Candida*, which are responsible for about 80% of fungal infections in the hospital environment.[38]

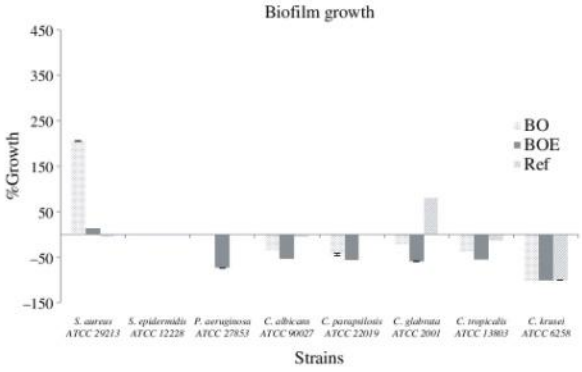

Figure 3. Percentage of biofilm growth in the presence of bullfrog samples. BO: Bullfrog oil; BOE: Bullfrog oil nanostructured emulsion; Ref.: Chloramphenicol (bacteria) and Ketoconazole (fungi).

It is important to highlight that the biofilm formation is related to the severity of the infection. Therefore, its inhibition is a mandatory step to evaluate whether a product has a good antimicrobial activity, even if the concentration used to inhibit biofilm formation is below the MIC. Moreover, the nanostructured emulsion system here evaluated could be a therapeutic choice to treat patients without the use of synthetic antibacterial or antifungal agents.

4. CONCLUSION

Copaiba essential oil assembles the majority of sesquiterpenes, identified as main antimicrobial compounds, presented in the copaiba resin-oil while bullfrog oil contains a pool of omega fatty acids. Moreover, copaiba essential oil exhibited better antimicrobial activity than the similar resin-oil, especially against *Staphylococcus* and *Candida* species. Bullfrog oil and its nanostructured emulsion, on the other hand, showed no significant antimicrobial sensitivity against the tested strains in some experiments. However, the oleic acid, which was identified as the main antimicrobial compound in this product, exhibited antimicrobial activity against *P. aeruginosa*.

The nanostructured emulsions, even containing only 5% (w/w) of oil in its formulation, showed their importance in preserving and enhancing the antimicrobial activity of copaiba oils. Moreover, the nanostructured emulsion systems were able to improve the antimicrobial activities of the original oils, especially the bullfrog oil nanostructured emulsion, which demonstrated a poor antimicrobial activity. Additionally, this system showed a significant result concerning the biofilm inhibition against *Pseudomonas aeruginosa,* an important multidrug resistant pathogen responsible for hospital infections in immunocompromised patients.

Therefore, given the relevant antimicrobial and antibiofilm activities found for the copaiba essential oil and the bullfrog oil nanostructured emulsions against pathogenic species of bacteria and fungi involved with cutaneous infections, it may be concluded that these formulations are suitable alternatives to develop new medicines for future use in the treatment of infections triggered by several microorganisms.

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References and Notes

1. M. Saleem, M. Nazir, M. S. Ali, H. Hussain, Y. S. Lee, N. Riaz, and A. Jabbar, *Nat. Prod. Rep.* 27, 238 (**2010**).
2. P. Cos, A. J. Vlietinck, D. V. Berghe, and L. Maes, *J. Ethnopharmacol.* 106, 290 (**2006**).
3. V. F. V. Junior and A. C. Pinto, *Quim. Nova* 25, 273 (**2002**).
4. A. O. Santos, T. Ueda-Nakamura, B. P. D. Filho, V. F. V. Junior, A. C. Pinto, and C. V. Nakamura, *Memórias do Instituto Oswaldo Cruz.* 103, 277 (**2008**).
5. J. C. T. Carvalho, V. Cascon, L. S. Possebon, M. S. S. Morimoto, L. G. V. Cardoso, M. A. C. Kaplan, and B. Gilbert, *Phytotherapy Research* 19, 946 (**2005**).
6. S. R. M. Lima, V. F. V. Junior, H. B. Christo, A. C. Pinto, and P. D. Fernandes, *Phytotherapy Research* 17, 1048 (**2003**).
7. V. F. V. Junior, L. Zunino, M. L. Patitucci, A. C. Pinto, and J. B. Calixto, *J. Pharm. Pharmacol.* 58, 1405 (**2006**).
8. J. P. B. Sousa, A. P. S. Brancalion, A. B. Souza, I. C. C. Turatti, S. R. Ambrósio, N. A. J. C. Furtado, N. P. Lopes, and J. K. Bastos, *J. Pharm. Biomed. Anal.* 54, 653 (**2011**).
9. V. S. Lopes, T. N. C. Dantas, A. F. Cunha, E. F. Moura, and M. A. M. Maciel, *Rev. Ser. Cienc. Vida.* 30, 85 (**2010**).
10. A. A. Gonçalves and M. C. M. Otta, *Rev. Bras. Eng. Pesca.* 3 (**2008**).
11. I. Minn, H. S. Kim, and S. C. Kim, *Biochim. Biophys. Acta* 1, 31 (**1998**).
12. M. Rosenthal, D. Goldberg, A. Aiello, E. Larson, and B. Foxman, *Infect. Genet. Evol.* 11, 839 (**2011**).
13. A. L. Dawson, R. P. Dellavalle, and D. M. Elston, *Dermatol. Clin.* 30, 141 (**2012**).
14. A. Yorgancioglu and E. E. Bayramoglu, *Ind. Crop. Prod.* 44, 378 (**2013**).
15. F. H. Xavier-Júnior, K. G. H. Silva, I. E. G. Farias, A. R. V. Morais, E. N. Alencar, I. B. Araújo, A. G. Oliveira, and E. S. T. Egito, *J. Drug Del. Sci. Tech.* 22, 367 (**2012**).
16. J. Bibette, F. L. Calderon, and P. Poulin, *Rep. Prog. Phys.* 62, 969 (**1999**).
17. W. Yu, G. Barratt, E. S. T. Egito, H. Fessi, J. P. Devissaguet, and F. Puisieux, *Int. J. Pharm.* 89, 139 (**1993**).
18. CLSI, Performance Standards for Antimicrobial Disk Susceptibility Tests—CLSI Document M02-A10, Wayne, PA (**2009**).
19. CLSI, Method for Antifungal Disk Difusion Susceptibility Testing of Yeasts—CLSI Document M44-A2, Wayne, PA (**2009**).
20. E. A. Ostrosky, B. R. Freitas, M. K. Mizumoto, M. E. L. Lima, T. M. Kaneko, and S. O. Nishikawa, *Rev. Bras. Farmacogn.* 18, 301 (**2008**).
21. N. Wakimoto, J. Nishi, J. Sheikh, J. P. Nataro, J. Sarantuya, M. Iwashita, K. Manago, K. Tokuda, M. Yoshinaga, and Y. Kawano, *Am. J. Trop. Med. Hyg.* 71, 687 (**2004**).
22. N. V. Gramosa and E. R. Silveira, *J. Essent. Oil Res.* 17, 130 (**2005**).
23. A. G. B. Júnior, E. C. B. Junior, V. F. V. Junior, A. C. Pinto, S. F. Carvalhaes, and M. A. M. Maciel, *Quim. Nova.* 28, 719 (**2005**).

24. E. Méndez, J. Sanhueza, S. Nieto, H. Speisky, and A. Valenzuela, *J. Am. Oil Chem. Soc.* 75, 79 **(1998)**.
25. D. E. Mendonça and S. B. Onofre, *Rev. Bras. Farmacogn.* 19, 577 **(2009)**.
26. A. B. Souza, C. H. Martins, M. G. Souza, N. A. Furtado, V. C. Heleno, J. P. de Sousa, E. M. Rocha, J. K. Bastos, W. R. Cunha, R. C. Veneziani, and S. R. Ambrosio, *Phytother Res.* 25, 215 **(2011)**.
27. F. A. Pieri, V. O. Silva, C. F. Souza, J. C. M. Costa, L. F. Santos, and M. A. S. Moreira, *Arq. Bras. Med. Vet. Zoo.* 64, 241 **(2012)**.
28. Y.-L. Yang, H.-T. Chen, C.-C. Lin, W.-L. Chu, and H.-J. Lo, *Diagnostic Microbiology and Infectious Disease* 76, 182 **(2013)**.
29. M. Ferreira, R. Santiago, T. Souza, E. S. T. Egito, E. Oliveira, and L. Soares, *AAPS PharmSciTech.* 11, 1383 **(2010)**.
30. G. Chamilos, R. E. Lewis, N. Albert, and D. P. Kontoyiannis, *Antimicrob. Agents Chemother.* 51, 2257 **(2007)**.
31. C. E. Isaacs, R. E. Litov, and H. Thormar, *J. Nutr. Biochem.* 6, 362 **(1995)**.
32. D. Singh and S. K. Agarwal, *J. Chem. Ecol.* 14, 1145 **(1988)**.
33. M. Daoubi, R. Hernandez-Galan, A. Benharref, and I. G. Collado, *J. Agric Food Chem.* 53, 6673 **(2005)**.
34. L. M. Leandro, F. S. Vargas, P. C. S. Barbosa, J. K. O. Neves, J. A. Silva, and V. F. Veiga-Junior, *Molecules* 17, 3866 **(2012)**.
35. C. R. Arciola, D. Campoccia, P. Speziale, L. Montanaro, and J. W. Costerton, *Biomaterials* 33, 5967 **(2012)**.
36. C. C. de Carvalho and M. M. da Fonseca, *FEMS Microbiol Ecol.* 61, 406 **(2007)**.
37. K. Swindell, A. A. Lattif, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum, *J. Infect Dis.* 200, 473 **(2009)**.
38. A. L. Colombo and T. Guimarães, *Rev. Soc. Bras. Med. Trop.* 36, 599 **(2003)**.

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Curriculum Vitae

Francisco Humberto XAVIER JUNIOR

Universidade Federal do Rio Grande do Norte, CCS, Departamento de Farmácia. Laboratório de Sistemas Dispersos (LASID). R. Gustavo Cordeiro de Farias s/n. Petrópolis. CEP: 59010780, - Natal RN – Brasil

Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 chatenay-Malabry Cedex – France.

Mail: ffhxjunior@yahoo.com.br

Né le 01 novembre 1986 à Conceição/PB – Brasil

Nationalité brésillienne

Formation

2013 - ce jour Doctorante en biopharmacie et technologie pharmaceutique
UMR CNRS 8612 Physico-chimie, Pharmacotechnie et Biopharmacie, Faculté de Pharmacie, Université Paris-Sud 11. Châtenay-Malabry - France.

2011 - ce jour Doctorante en biotechnologie
Programa de Pós-Graduação em Biotecnologia (Renorbio).
Universidade Federal do Rio Grande do Norte, Laboratório de Sistemas Dispersos, à Natal, Brésil

2011 - ce jour Pharmacien clinique dans l'étude de phase III de vaccins pour les maladies infectieuses
Centro de Estudos e Pesquisas em Molestias Infecciosas LTDA, sponsor Sanofi Pasteur AS

2011-2011 Maître de conférences en pharmacognosie, contrat de courte durée
Universidade Federal do Rio Grande do Norte, Laboratório de Sistemas Dispersos, à Natal, Brésil

2008 - 2011 Master en Sciences de la Santé.
Programa de Pós-Graduação em Ciências da Saúde,
Universidade Federal do Rio Grande do Norte, Laboratório de Sistemas Dispersos, à Natal, Brésil

2004 - 2008 Licence en pharmacie.
Universidade Federal do Rio Grande do Norte

Compétences

Expertise scientifique :	Maîtrise des stratégies de devellopment des vector pour l'amélioration du passage des barrières biologiques par des médicaments, characterization physico-chimique des systèmes lipidiques et polimeriques, contrôle de la qualité physico-chimique et microbiologique des médicaments, encapsulation d'agents anticancéreux et de traceurs fluorescents, maitrise des moyens de solubilisation de principes actifs, domaine de l'extraction, l'isolement et la caractérisation des produits naturels, maitrise du plan d'expériences pour l'optimisation des processus, essais cliniques de phase III.
Expertise technique:	Chromatographie en phase liquide à haute performance (HPLC), chromatographie en phase gazeuse (GC-MS, GC-FID), Rheologie, mesures du diamètre par diffusion quasi-élastique de la lumière et du potentiel zêta, Microscopie électronique à transmission, radioactivité, infrarouge, l'analyse thermique, spectrophotométrie, lyophilisation, manipulation en culture cellulaire, culture de microrganismes, études *in-vivo* chez les rats, mucoadhésion et étude du passage de principes actifs à travers de la Chambre d'Ussing.
Savoir-faire organisationnel :	Encadrement de stagiaires, services administratifs et organisationnels, recherche des collaborations scientifiques, organisation de congrès scientifiques, communication orale et écrite.
Langues :	Portugais (langue maternelle); Français, Anglais et Espagnol (courants)
Connaissances informatiques :	Microsoft Office, Windows, Statistic, GraphPad, Origin, Endnote, Prezi, Lightroom, Photoshop

Production scientifique

Articles

1. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., VERISSIMO, L. M., REHDER, V. L. G., CHAVES, G. M., OLIVEIRA, A. G., EGITO, E. S. T. Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions. Journal of Nanoscience and Nanotechnology, v.15, p.880 - 888, 2015.

2. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., FARIAS, I. E. G., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., OLIVEIRA, A. G., EGITO, E. S. T. Prospective study for the development of emulsion systems containing natural oil products. Journal of Drug Delivery

Science and Technology. , v.22, p.367-372, 2012.

3. SILVA, K. G. H., **XAVIER-JUNIOR, F. H.** , FARIAS, I. E. G., SILVA, A. K. A., SOUZA, L. C. A., CALDAS NETO, J. A., SANTIAGO, R. R., ALEXANDRINO-JUNIOR, F., NAGASHIMA JUNIOR, T., SORES, L. A. L., MAGALHÃES, N. S. S., EGITO, E. S. T. Stationary cuvette: a new approach to obtaining analytical curves by UV-VIS spectrophotometry. Phytochemical Analysis on line. , v.20, p.265 - 271, 2009.

4. SILVA, K. G. H., **XAVIER-JUNIOR, F. H.** , FARIAS, I. E. G., CALDAS NETO, J. A., SILVA, A. K. A., NAGASHIMA JUNIOR, T., MEDEIROS, A. C., DIMESTEIN, EGITO, E. S. T. A new insight about pharmaceutical dosage forms for benzathine penicillin G. Revista de Ciências Farmacêuticas Básica e Aplicada. , v.27, p.21 - 26, 2006.

Communications Scientifiques avec résumé étendu publiés

1. FARIA, M. A. S., ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., MACHADO, L. A., RUTCKEVISKI, R., DANTAS, T. R. F., OLIVEIRA, C. M., DANTAS-SANTOS, N., CHAVES, G. M., EGITO, E. S. T. Emulsions based on copaiba essential oil: a source of treatment for infectious diseases In: XXI International Conference on Bioencapsulation, 2013, Berlim.

2. FARIA, M. A. S., ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., MACHADO, L. A., RUTCKEVISKI, R., DANTAS, T. R. F., OLIVEIRA, C. M., DANTAS-SANTOS, N., CHAVES, G. M., EGITO, E. S. T. Evaluation of antibiofilm activity of cobaiba essential oil emulsion In: XXI International Conference on Bioencapsulation, 2013, Berlim.

3. SILVA-FILHO, M. A., MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MACHADO, L. A., OLIVEIRA, C. M., DANTAS, T. R. F., SILVEIRA, W. L. L., DANTAS-SANTOS, N., EGITO, E. S. T. Evaluation of the antifungal activity of microemulsions containing Amphotericin B after the lyophilization process In: XXI International Conference on Bioencapsulation, 2013, Berlim.

4. SILVA-FILHO, M. A., MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., RUTCKEVISKI, R., OLIVEIRA, C. M., DANTAS, T. R. F., DANTAS-SANTOS, N., SILVA-JUNIOR, A. A., EGITO, E. S. T. Preliminary design of freeze-dried microemulsion containing Amphotericin B In: XXI International Conference on Bioencapsulation, 2013, Berlim.

5. SILVA-FILHO, M. A., RUTCKEVISKI, R., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., MACHADO, L. A., OLIVEIRA, C. M., DANTAS, T. R. F., DANTAS-SANTOS, N., EGITO, E. S. T. Quality control of bullfrog (Rana catesbeiana Shaw) oil: a contribuition for the extraction and chemical identification In: XXI International Conference on Bioencapsulation, 2013, Berlim.

6. SILVA, K. G. H., MARTINS, A. S., **XAVIER-JUNIOR, F. H.** , EGITO, E. S. T. Copaiba oil microemulsion containing ketoconazole: Assessment of the rate of encapsulation In: XVIII International Conference on Bioencapsulation, 2010, Lisboa.

7. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., ALENCAR, E. N., MORAIS, A. R. V., ARAÚJO, I. B., EGITO, E. S. T. Phase diagrams of copaiba oil: comparative study on their production process In: XVIII International Conference on Bioencapsulation, 2010, Porto.

8. SILVA, K. G. H., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., EGITO, E. S. T. Copaiba oil microemulsions: A new system for future use in inflammatory diseases In: XVIIth International Conference on Bioencapsulation, 2009, Groningen, Netherlands.

Communications Scientifiques avec résumés publiés

1. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MACHADO, L. A., RUTCKEVISKI, R., OLIVEIRA, C. M., DANTAS, T. R. F., BARRATT, G., EGITO, E. S. T. Preparation of freeze-dried emulsion system based on copaíba oil by design of experiment In: XIV Journée de l'école de doctorale, 2014, Paris.

2. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MACHADO, L. A., DANTAS, T. R. F., OLIVEIRA, C. M., DANTAS-SANTOS, N., VERISSIMO, L. M., EGITO, E. S. T. Analyses of the glass transition temperature of microemulsion containing glucose and lactose by differential scanning calorimetry In: 3rd Conference on Innovation in Drug Delivery - Advances in Local Drug Delivery, 2013, Pisa.

3. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., MACHADO, L. A., DANTAS, T. R. F., OLIVEIRA, C. M., DANTAS-SANTOS, N., CHAVES, G. M., VERISSIMO, L. M., EGITO, E. S. T. Copaiba oil-resin (Copaifera langsdorfii) emulsion as potent biofilm inhibitors In: 3rd Conference on Innovation in Drug Delivery - Advances in Local Drug Delivery, 2013, Pisa.

4. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., MACHADO, L. A., RUTCKEVISKI, R., DANTAS-SANTOS, N., EGITO, E. S. T., VAUTHIER, C. Emulsions based on natural oils as antimicrobial agents In: XIIIèmes Journées de I'École Doctorale - Innovaton Thérapeutique, 2013, Kremlin-Bicêtre.

5. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., DANTAS, T. R. F., OLIVEIRA, C. M., NASCIMENTO, A. E. G., RUTCKEVISKI, R., DANTAS-SANTOS, N., EGITO, E. S. T. Influence of cryoprotectants on the surface tension of microemulsions In: 3° Encontro Brasileiro para Inovação Terapêutica, 2013, Jaboatão dos Guararapes.

6. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MACHADO, L. A., OLIVEIRA, C. M., DANTAS, T. R. F., DANTAS-SANTOS, N., EGITO, E. S. T. Optimization of lyophilization process of microemulsion containing lactose and glucose by experiment design In: 3° Encontro Brasileiro para Inovação Terapêutica, 2013, Jaboatão dos Guararapes.

7. DANTAS, T. R. F., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., DANTAS-SANTOS, N., EGITO, E. S. T. A influência do potencial zeta na estabilidade de sistemas dispersos contendo ativos naturais In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

8. SILVA, K. G. H., ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS-SANTOS, N., CHAVES, G. M., EGITO, E. S. T.Antibiofilm Activity of Bullfrog (Rana Castebeiana Shaw) Oil Emulsion In: Groupe Thematique de Recherche sur la Vectorisation - GTRV, 2012, Paris.

9. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS, A. B., PAULA, P. R., CHAVES, G. M., DANTAS-SANTOS, N., EGITO, E. S. T.Anti-Candida activity of Copaiba Oils: A comparative study between volatile and resin oils In: IV Simpósio Nacional de Produtos Naturais, 2012, João Pessoa.

10. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., EGITO, E. S. T.Atividade anti-Staphylococcus do óleo essencial e resina de Copaíba (Copaifera langsdorffii): estudo comparativo In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

11. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., OLIVEIRA, C. M.,

DANTAS-SANTOS, N., EGITO, E. S. T.Caracterização de sistemas microemulsionados de óleo de copaíba a partir de estudos termoanalíticos em calorimetria exploratória diferencial In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

12. OLIVEIRA, C. M., MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., DANTAS-SANTOS, N., EGITO, E. S. T.Centrifugação analítica como método para determinação da estabilidade de sistemas emulsionados In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

13. SILVA, K. G. H., ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS-SANTOS, N., CHAVES, G. M., EGITO, E. S. T.Copaiba Oil (Copaifera langsdorffii) Emulsions: An Alternative to Treat Cutaneous Infections In: Groupe Thematique de Recherche sur la Vectorisation - GTRV, 2012, Paris.

14. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., PAULA, P. R., DANTAS-SANTOS, N., EGITO, E. S. T. opaiba oil emulsions: characterization of pseudoternary phase diagram by analytical centrifugation In: IV Simpósio Nacional de Produtos Naturais, 2012, João Pessoa.

15. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., OLIVEIRA, C. M., DANTAS-SANTOS, N., EGITO, E. S. T. Crioprotetores e o seu mecanismo de ação em sistemas coloidais liofilizados In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

16. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., PAULA, P. R., OLIVEIRA, C. M., DANTAS-SANTOS, N., EGITO, E. S. T. Critical HLB of Rana catesbeiana oil emulsions: Preliminary study In: IV Simpósio Nacional de Produtos Naturais, 2012, João Pessoa.

17. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS, T. R. F., DANTAS-SANTOS, N., EGITO, E. S. T. Desenvolvimento de sistemas emulsionados sem tensoativos contendo Óleo de Copaíba: estudo In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

18. **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., PAULA, P. R., OLIVEIRA, C. M., DANTAS-SANTOS, N., EGITO, E. S. T. Development of emulsified systems based on Bullfrog (Rana catesbeiana) oil In: IV Simpósio Nacional de Produtos Naturais, 2012, João Pessoa.

19. OLIVEIRA, C. M., MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., DANTAS-SANTOS, N., EGITO, E. S. T. Efeito da cristalização em soluções de crioprotetores para liofilização de sistemas coloidais In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

20. DANTAS-SANTOS, N., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., MACHADO, L. A., EGITO, E. S. T. Estudo das propriedades terapêuticas da rã-touro (Rana catesbeiana Shaw) In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

21. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., DANTAS-SANTOS, N., REHDER, V. L. G., EGITO, E. S. T. Extraction, emulsion development and CG-MS characterization of copaiba essential oil (Copaifera langdorffii). In: Groupe Thematique de Recherche sur la Vectorisation - GTRV, 2012, Paris.

22. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., SOUZA, E. S.,

PAULA, P. R., DANTAS, A. B., OLIVEIRA, C. M., DANTAS-SANTOS, N., REHDER, V. L. G., CHAVES, G. M., EGITO, E. S. T. Gas chromatography-mass spectrometryanalysis of antimicrobial compounds of bullfrog (Rana catesbeiana shaw) oil In: XXI Congresso Latinoamericano de Microbiologia (XXI ALAM), 2012, Santos

23. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., PAULA, P. R., EGITO, E. S. T. Identification of antimicrobial compounds of Copaiba oil by Bioautography: a preliminary study In: AAPS Anual Meeting and Exposition, 2012, Chicago.

24. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., OLIVEIRA, C. M., DANTAS-SANTOS, N., EGITO, E. S. T. Influence of Multiple Lyophilization Factors on the Microemulsion Droplet Size: An Experimental Design In: Groupe Thematique de Recherche sur la Vectorisation - GTRV, 2012, Paris.

25. GOES, P. A., **XAVIER-JUNIOR, F. H.** , ALVES, M.S.C.F Integralidade na Promoção da Saúde do Idoso: Relato de Experiência In: World Nutrition Rio2012, 2012, Rio de Janeiro.

26. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., PAULA, P. R., SILVEIRA, W. L. L., OLIVEIRA, A. G., EGITO, E. S. T. Lyophilization of microemulsions: Influence of different cryoprotectants in their droplet size In: AAPS Anual Meeting and Exposition, 2012, Chicago.

27. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., DANTAS, A. B., PAULA, P. R., DANTAS-SANTOS, N., CHAVES, G. M., EGITO, E. S. T. Natural oils as potent inhibitors of biofilm formation In: IV Simpósio Nacional de Produtos Naturais, 2012, João Pessoa.

28. DANTAS-SANTOS, N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., MACHADO, L. A., EGITO, E. S. T. Obtenção de sistemas coloidais contendo Óleo de Copaíba, Tween 20, Phosal 50 e água In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

29. MACHADO, L. A., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., DANTAS-SANTOS, N., EGITO, E. S. T. Perfil de prescrição de antimicrobianos no Hospital Universitário Dr. Mário Araújo de Bagé In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

30. GOES, P. A., **XAVIER-JUNIOR, F. H. .** Práticas educativas como promoção de saúde-Relato de experiencia em um grupo de idosos In: Simpósio Norteriograndense de Geriatria e Gerontologia, 2011, Natal.

31. DANTAS, T. R. F., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., DANTAS-SANTOS, N., EGITO, E. S. T. Recentes avanços do desenvolvimento de sistemas de liberação contendo produtos naturais para tratamento de doenças infecciosas In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

32. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., OLIVEIRA, C. M., DANTAS-SANTOS, N., EGITO, E. S. T. Study of Microemulsion Glass Trasition Temperature Containing Maltose and Mannitol by Differential Scanning Calorimetry In: Groupe Thematique de Recherche sur la Vectorisation - GTRV, 2012, Paris.

33. MACHADO, L. A., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., DANTAS-SANTOS, N., EGITO, E. S. T. Uso de ativos naturais no tratamento de asma: revisão da literatura In: XIV Congresso Científico da UnP e XIII Mostra de Extensão da UnP, 2012, Natal.

34. SILVA, G. H. F., MELO, M. C. B., **XAVIER-JUNIOR, F. H. .** Análise do indice peso/idade em crianças beneficiadas do Programa Bolsa Familia In: 11° Congresso Nacional de Nutrição Baseada em Evidencia, 2011, Fortaleza.

35. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., SILVA, K. G. H., PAULA, P. R., EGITO, E. S. T. Antimicrobial Activity Evaluation of Copaiba Oil Microemulsion System: A Preliminary Study In: II internacional Symposium of Pharmaceutical Sciences, 2011, Natal.

36. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., SILVA, K. G. H., PAULA, P. R., EGITO, E. S. T. Aplicação da Análise Térmica no Controle de Qualidade de Óleos Vegetais In: XIII Congresso Científico e a XII Mostra de Extensão, 2011, Natal.

37. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., SILVA, K. G. H., BARBOSA, L. M. Q., ARAÚJO, I. B., EGITO, E. S. T. Comparação de metodologias para determinação da atividade antifungica do oleo de copaiba In: I Workshop Brasileiro de Tecnologia Farmacêutica e Inovação, 2011, Aracaju.

38. FERREIRA, L. F., SILVEIRA, W. L. L., **XAVIER-JUNIOR, F. H.** , DAMASCENO, B. P. G. L., EGITO, E. S. T. Development and Validation of an Analytical Method for Quantification of Simvastatin in Microemulsion by High Performance Liquid Chromatography In: II International Symposium of Pharmaceutical Sciences, 2011, Natal.

39. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., SILVA, K. G. H., PAULA, P. R., EGITO, E. S. T. Emulsões Sem Tensoativos: Recentes Avanços e Aplicabilidades In: XIII Congresso Científico e a XII Mostra de Extensão, 2011, Natal.

40. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., SILVA, K. G. H., ARAÚJO, I. B., EGITO, E. S. T. Estudo de parâmentros físico-químicos do cloranfenicol e seu doseamento em microemulsões In: I Workshop Brasileiro de Tecnologia Farmacêutica e Inovação, 2011, Aracaju.

41. SILVA, G. H. F., MELO, M. C. B., **XAVIER-JUNIOR, F. H. .** Indices antropométricos e o acompanhamento nutricional de crianças beneficadas do Programa Bolsa Familia In: 11° Congresso Nacional de Nutrição Baseada em Evidencia, 2011, Fortaleza.

42. **XAVIER-JUNIOR, F. H.** Inserção sócio-cultural utilizando o método científico In: XVII semana de ciência, tecnologia e cultura, 2011, Natal.

43. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., SILVA, K. G. H., PAULA, P. R., EGITO, E. S. T. Métodos de Análise Antimicrobiana de Óleos Vegetais In: XIII Congresso Científico e a XII Mostra de Extensão, 2011, Natal.

44. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., SILVA, K. G. H., PAULA, P. R., EGITO, E. S. T. O Processo de Liofilização nas Indústrias: Revisão Bibliografica In: XIII Congresso Científico e a XII Mostra de Extensão, 2011, Natal.

45. SILVA, G. H. F., MELO, M. C. B., **XAVIER-JUNIOR, F. H. .** Programa Bolsa Familia: Negligencia ao acompanhamento das medidas antropométricas de crianças beneficiadas In: 11° Congresso Nacional de Nutrição Baseada em Evidencia, 2011, Fortaleza.

46. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., ALENCAR, E. N., MORAIS, A. R. V., SANTOS, E. C. G., EGITO, E. S. T. Controle de qualidade microbiológico e estudo da atividade antibacteriana de sistemas microemulsionados e do óleo de copaíba contra

staphylococcus epidermidis In: 62ª Reunião Anual da Sociedade Brasileira para o Progresso da Ciência, 2010, Natal.

47. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., MORAIS, A. R. V., ALENCAR, E. N., SANTOS, E. C. G., EGITO, E. S. T. Estudo da concentração inibitória mínima do óleo de copaíba em cepas bacterianas do gênero staphylococcus In: 62ª Reunião Anual da Sociedade Brasileira para o Progresso da Ciência, 2010, Natal.

48. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., SANTOS, E. C. G., MORAIS, A. R. V., ALENCAR, E. N., ALEXANDRINO-JUNIOR, F., ARAÚJO, I. B., EGITO, E. S. T.Evaluation of antibacterial activity of copaiba oil colloidal systems In: 70th FIP World Congress of Pharmacy/Pharmaceutical Sciences, 2010, Lisboa.

49. **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., SILVA, K. G. H., ARAÚJO, I. B., EGITO, E. S. T. Incorporação de cloranfenicol em microemulsão de óleo de copaíba In: XXI Simpósio de Plantas Medicinais do Brasil, 2010, João Pessoa.

50. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., ALEXANDRINO-JUNIOR, F., SILVA, A. K. A., ALENCAR, E. N., MORAIS, A. R. V., ARAÚJO, I. B., EGITO, E. S. T. Introducing the scientific method in the high school classroom: Pharmaceutical Sciences as a background In: 70th FIP World Congress of Pharmacy/Pharmaceutical Sciences, 2010, Lisboa.

51. **XAVIER-JUNIOR, F. H.** , MORAIS, A. R. V., ALENCAR, E. N., SILVA, K. G. H., ARAÚJO, I. B., EGITO, E. S. T. O ensino de praticas em saúde por meio de instrumentos pedagógicos In: II Congresso Multiprofissional da Saúde, 2010, Natal.

52. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., SILVA, K. G. H., SANTOS, E. C. G., ARAÚJO, I. B., EGITO, E. S. T. Óleo de Copaíba como antifúngico natural: estudo pela técnica de difusão em ágar In: XXI Simpósio de Plantas Medicinais do Brasil, 2010, João Pessoa.

53. SILVA, K. G. H., **XAVIER-JUNIOR, F. H.** , FARIAS, I. E. G., SANTIAGO, R. R., ALEXANDRINO-JUNIOR, F., EGITO, E. S. T. A new tool to obtaining analytical curves by UV-Vis spectrophotometry In: 21 st International Symposium on Pharmaceutical and Biomedical Analysis, 2009, Orlando, USA.

54. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., SILVA, K. G. H., SANTOS, E. C. G., EGITO, E. S. T., MORAIS, A. R. V. Avaliação da atividade antimicrobiana do óleo de copaíba In: I Simpósio Nacional em Ciências Farmacêuticas Básicas e Aplicadas : Fronteiras do Conhecimento e Políticas de Inovação, 2009, Natal.

55. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., ALENCAR, E. N., MORAIS, A. R. V., ARAÚJO, I. B., EGITO, E. S. T. Avaliação do Perfil dos Alunos de Escolas Públicas Participantes dos Cursos de Férias de Farmácia In: XI Congresso Científico: Educação, Inclusão E Sustentabilidade: Grandes Desafios Da Ciência, 2009, Natal.

56. ALENCAR, E. N., **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., MORAIS, A. R. V., EGITO, E. S. T. Controle de Qualidade do Óleo de Copaíba: Estudo de Pré-Formulação para o Desenvolvimento de Sistemas Emulsionados In: XX Congresso de Iniciação Científica da UFRN - CIC2009, 2009, Natal.

57. MORAIS, A. R. V., **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., ALENCAR, E. N., EGITO, E. S. T. Desenvolvimento de Diagramas de Fases Pseudoternário de Óleo de Copaíba/Tween 20/ Phospholipon 100H e Água In: XX Congresso de Iniciação Científica da UFRN - CIC2009, 2009, Natal.

58. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., FARIAS, I. E. G., ALENCAR, E. N., MORAIS, A. R. V., ARAÚJO, I. B., NAGASHIMA JUNIOR, T., EGITO, E. S. T. Development of Based Microemulsion System of Copaiba Oil In: 7th International Congress of Pharmaceutical Sciences CIFARP, 2009, Ribeirão Preto.

59. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., EGITO, E. S. T. Diagrama de Fases de Óleo de Copaíba: Estudo Comparativo para a Produção de Microemulsão In: XI Congresso Científico: Educação, Inclusão E Sustentabilidade: Grandes Desafios Da Ciência, 2009, Natal.

60. **XAVIER-JUNIOR, F. H.** , ALENCAR, E. N., MORAIS, A. R. V., SILVA, K. G. H., ARAÚJO, I. B., EGITO, E. S. T. Diagramas de Fases Pseudoternário do Óleo de Copaíba: Estudo de Regiões de Emulsões In: XI Congresso Científico: Educação, Inclusão E Sustentabilidade: Grandes Desafios Da Ciência, 2009, Natal.

61. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., EGITO, E. S. T. Freeze-Dried Microemulsion Copaiba Oil: Preliminary Studies In: 7th International Congress of Pharmaceutical Sciences CIFARP, 2009, Ribeirão Preto.

62. MARTINS, A. S., **XAVIER-JUNIOR, F. H.** , GUARDA, L. T. A., LOUSADO, E. S. Judicialização do direito a saúde: uma abordagem crítica In: XI Congresso Científico e X Mostra de Extensão da Universidade, 2009, Natal.

63. **XAVIER-JUNIOR, F. H.** , SILVA, K. G. H., MORAIS, A. R. V., ALENCAR, E. N., ARAÚJO, I. B., EGITO, E. S. T. Perfil dos Professores Participantes dos Cursos de Férias de Farmácia In: XI Congresso Científico: Educação, Inclusão E Sustentabilidade: Grandes Desafios Da Ciência, 2009, Natal.

Résumé

Des systèmes dispersés pour la voie orale contenant dans leur phase interne de l'huile de copaïba servant de véhicules pour le paclitaxel ont été développées. Des microémulsions et des nanocapsules bioadhésives ont été formulées selon deux approches originales, l'une basée sur l'appariement chimique des composés de la microémulsion, et l'autre et sur la mise en œuvre d'un plan d'expérience. Deux méthodes d'analyses originales ont été développées et validées destinées à l'analyse de la composition de l'huile de copaiba et au dosage du paclitaxel dans les formulations contenant de l'huile de copaiba.

MOTS CLES : voie orale, huile de copaïba, paclitaxel, microémulsion, nanocapsules, équilibre hydrophile-lipophile, chitosane, mucoadhésion.

LABORATOIRE DE RATTACHEMENT :
Institut Galien Paris Sud, UMR CNRS 8612
Equipe 6 : Amélioration du passage des barrières par les molécules biologiquement actives
5, Rue Jean Baptiste Clément
92296 Châtenay-Malabry, France

PÔLE : PHARMACOTECHNIE ET PHYSICO-CHIMIE PHARMACEUTIQUE

UNIVERSITÉ PARIS-SUD 11
UFR «FACULTÉ DE PHARMACIE DE CHATENAY-MALABRY »
5, rue Jean Baptiste Clément
92296 CHÂTENAY-MALABRY Cedex