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LEAVES OF *Licania rigida* Benth AND *Turnera subulata* HAVE AN ANTICOAGULANT ACTIVITY BY THROMBIN INHIBITION

JEFFERSON ROMÁRYO DUARTE DA LUZ

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Rio Grande do Norte, como parte dos requisitos para a obtenção do título de Mestre em Ciências da Saúde.

Orientadora: Profa. Dra. Maria das Graças Almeida
Co-orientador: Prof. Dr. Hugo Alexandre de Oliveira Rocha

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DEDICATÓRIA

Dedico este trabalho

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Paulo Reglus Neves Freire

(Pedagogia da Autonomia - 1996)

RESUMO

O emprego de plantas medicinais para o tratamento, cura e prevenção de patologias é um hábito que acompanha a humanidade desde seus primórdios. Considerando que o Brasil apresenta uma grande biodiversidade para a produção de fitoterápicos, é de suma importância o estudo de plantas como fontes alternativas de tratamento, visando a busca de susbtâncias que possam auxiliar os fármacos atualmente prescritos na terapêutica clínica. Durante muitos anos, doenças cardiovasculares e doenças tromboembólicas tem sido as principais causas de morte por doenças no mundo, sendo responsáveis pela morte de cerca de 17,5 milhões de pessoas até o ano de 2012 (31% das mortes em todo o mundo). O sistema de coagulação está centralmente envolvido na formação do trombo venoso. Indicações atualmente definidas para anticoagulantes incluem a profilaxia e tratamento do tromboembolismo. Heparinas não fracionadas e heparinas de baixa massa molar são utilizadas como medicamentos anticoagulantes, no entanto, estes compostos são acompanhados de alguns efeitos secundários, tais como, trombocitopenia e um elevado risco de hemorragia. A efeito deste problema tem se gerado uma procura de novas substâncias, a fim de auxiliar a terapêutica anticoagulante. Neste contexto, este estudo teve como objetivo avaliar o potencial anticoagulante, efeitos tóxicos e hemorrágicos dos extratos foliares de *Licania rigida* Benth e *Turnera subulata*, espécies vegetais amplamente encontradas no semiárido nordestino. Os extratos foram obtidos a partir de etanol (50%), com posterior partição com solventes de polaridades crescentes, incluindo hexano e acetato de etila. A avaliação dos extratos frente ao sistema de coagulação mostrou uma atividade anticoagulante satisfatória pelo Tempo de Tromboplastina parcial Ativada e Tempo de Protrombina (100% de atividade), atividade Anti-Xa (~ 40% de inibição) e uma grande capacidade de inibibir diretamente da trombina (~ 80 a 100% de inibição) como principal mecanismo de ação. Além disso, observou-se que os extratos apresentam um baixo efeito hemorrágico, bem como, a ausência de toxicidade em modelos *in vitro* (Citotoxicidade por MTT) e *in vivo* (Toxicidade oral aguda). Este trabalho relata pela primeira vez o potencial anticoagulante de *Licania rigida* Benth e *Turnera subulata*.

ABSTRACT

The use of medicinal plants for the treatment, cure and prevention of pathologies is a habit that accompanies humanity since its beginnings. Considering that Brazil presents a great biodiversity for the production of phytotherapics, it is of paramount importance the study of plants as alternative sources of treatment, aiming at the search for substances that may help the drugs currently prescribed in clinical therapeutics. Over many years, cardiovascular disease and thromboembolic disorders have been the leading cause of death by disease in the world, being responsible for the death of approximately 17.5 million people by the year 2012 (31% of deaths worldwide). The coagulation system is centrally involved in the formation of venous thrombus. Currently defined indications for anticoagulants include prophylaxis and treatment of thromboembolism. Unfractionated heparins and low molecular heparins are used as anticoagulant drugs. However, these compounds are accompanied by several side effects such as thrombocytopenia and a high risk of systemic bleeding. The effect of this problem demanded the search for new substances in order to assist prolonged anticoagulant therapy. In this context, this study aimed to evaluate the anticoagulant potential, toxic and hemorrhagic effects from *Licania rigida* Benth and *Turnera subulata* leaves, species widely found in Northeast semiarid. The extracts were obtained from ethanol (50%) with subsequent partition with solvents of increasing polarities, including hexane and ethyl acetate.The crude extracts were obtained from ethanol (50%) and subsequent partition with increasingly polar solvents including hexane and ethyl acetate. The evaluation of the extracts against the coagulation system showed a satisfactory anticoagulant activity by Activated Partial Thromboplastin Time and Prothrombin Time (100% activity), Anti-Xa activity (~ 40% inhibition) and a large capacity to inhibit directly from Thrombin (~ 80 to 100% inhibition) as the main mechanism of action. In addition, the extracts were found to have a low hemorrhagic effect, as well as the absence of toxicity in in vitro models (MTT cytotoxicity) and in vivo (acute oral toxicity). This paper reports for the first time the anticoagulant potential of *Licania rigida* Benth and *Turnera subulata*.

LIST OF ABBREVIATIONS AND ACRONYMS

AFLR (Ethyl Acetate Fraction of *Licania rigida*)

AFTS (Ethyl Acetate Fraction of *Turnera subulata*)

ALT (Alanine Transaminase)

ANVISA (Brazilian National Health Surveillance Agency)

aPTT (activated Partial Tromboplastin Time)

AST (Aspartate Transaminase)

AT (Antithrombin)

ATIII (Antithrombin III)

AVK (Antagonist of Vitamin K)

$CaCl_2$ (Calcium Chloride)

Cell 3T3 (Mouse Fibroblast Cells)

Cell HEK-293 (Epithelial Embryonic Human Kidney Cells)

CELR (Crude Extract of *Licania rigida*)

CETS (Crude Extract of *Turnera subulata*)

CO_2 (Carbon Dioxide)

DMEM (Dulbecco´s Modified Eagle´s Medium)

DMSO (Dimethyl Sulfoxide)

FBS (Fetal Bovine Serum)

FIIa (Factor II Activated)

FXa (Factor X Activated)

FXIII (Factor XIII)

HCII (Heparin Cofator II)

HCT (Hematocrit)

HFLR (Hexane Fraction of *Licania rigida*)

HFTS (Hexane Fraction of *Turnera subulata*)

HGB (Hemoglobin)

IP (Intraperitoneal)

LMWH (Low Molecular Weight Heparin)

MCH (Mean Concentration Hemoglobin)

MCHC (Mean Corpuscular Hemoglobin Concentration)

MCV (Mean Corpuscular Volume)

MPV (Mean Platelet Volume)

MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide)

OECD (Organization for economic cooperation and development)

PAR-4 (Protease-Activated Receptor 4)

PCT (Procalcitonin)

PDW (Platelets distribution width)

PF4 (Platelet Factor 4)

PHC (Primary Health Care)

PLT (Platelets)

PT (Prothrombin Time)

RBC (Red Blood Cells)

RDW (Volume Change Mean Corpuscular)

RN (Rio Grande do Norte)

TF (Tissue Factor)

TFPI (Inhibitor of Tissue Factor)

UFH (Unfractionated Heparin)

V/V (Volume/Volume)

vWF (von Willebrand Factor)

WBC (Total Count of Leukocytes)

WHO (World Health Organization)

Y-GT (Gamma-Glutamyl Transferase)

LIST OF FIGURES

SUMMARY

1 INTRODUCTION

Over many years, cardiovascular disease and thromboembolic disorders has been the leading cause of death by disease in the world, being responsible for the death of approximately 17.5 million people by the year 2012 (31% of deaths worldwide). [1]

The pathogenesis of thrombosis involves three factors collectively known as the 'triad of Virchow ", postulated for more than 150 years ago by German pathologist Rudolf Virchow in 1856. These factors involve injury to the vascular wall, circulatory stasis and blood composition with imbalance in the pro and/or anticoagulant system. According to the current therapeutic knowledge, this triad is the basis for the assessment of risk factors for thromboembolism, the cause of which, most often is multifactorial (Figure 1). [2]

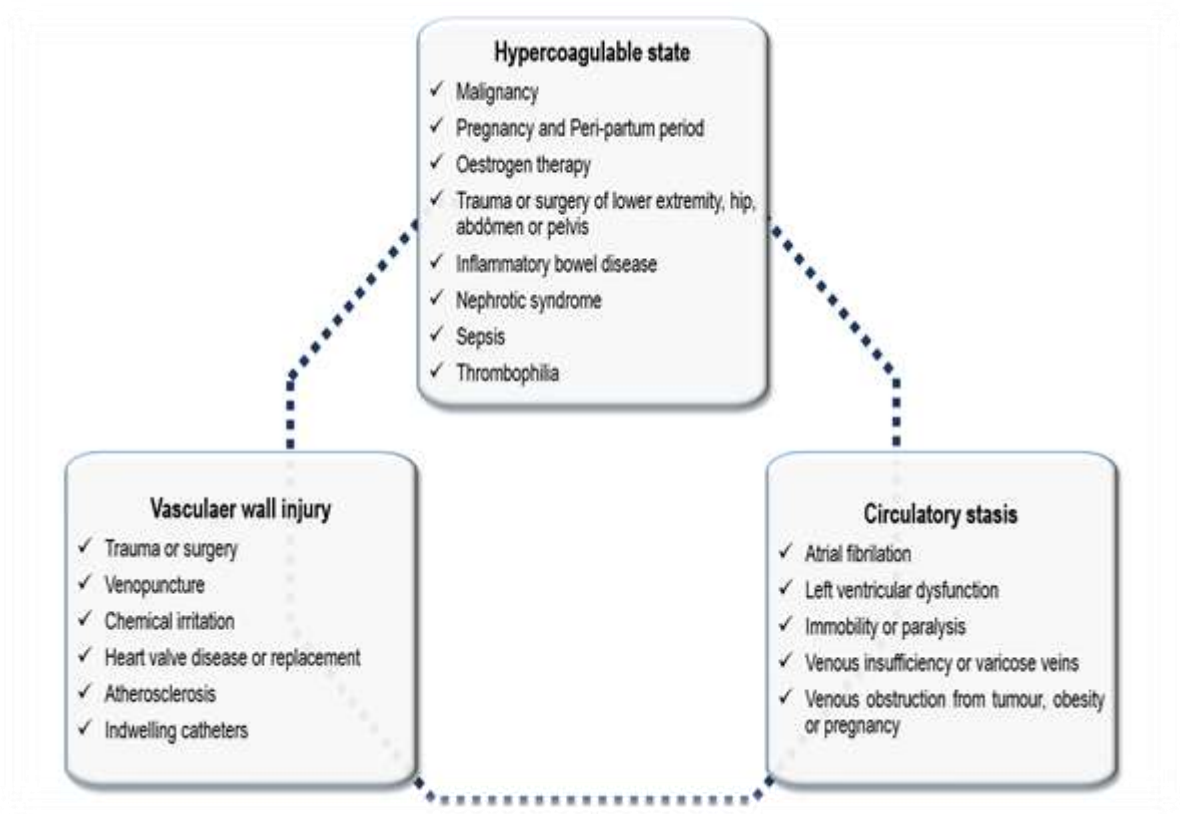

Figure 1 - Virchow's triad, risk factors for thromboembolism. Adapted from Haas et al.[3]

In order to elucidate the role of new anticoagulants in the prevention and treatment of thromboembolism, it is necessary to align some considerations about the hemostatic system, which is responsible for the normal balance between procoagulant and anticoagulant factors. [4]

Hemostasis is a protective physiologic response to vascular injury resulting in exposure of subendothelial layers of the vessel wall to the blood components. [5] It has the aim to stop the bleeding site of tissue injury where the repair of the lesion occurs quickly and fairly regulated manner. [6] Hemostasis of the organism depends on the existence of a precise balance between various physiological processes such as fibrinolysis and their natural inhibitors, inflammation, integrity of the monolayer of endothelial cells, coagulation and others. [7]

By way of elucidation of the functioning of the clotting mechanism has been proposed the "Cascade Model".[8] The "coagulation cascade" was initially described as two distinct classical pathways (the extrinsic and intrinsic), each with enzymatic chain reactions culminating in the formation of a stable fibrin clot. Recently, however, clotting has been described as a complex process whose model is based on the role of intervening cells, including presenting cells of the tissue factor (TF) and platelets. This system is divided into three interconnected stages: initiation; amplification and propagation (Figure 2). [9]

The initiation phase occurs in cells expressing TF. The association TF-FVIIa (Extrinsic tenase complex) accelerate the activation of the factors IX and X. The FXa, located on the cell surface is relatively protected from inactivation, but, when free, is rapidly inhibited by antithrombin (AT) and the pathway inhibitor of TF (TFPI). The FXa associates with FVa (Prothrombinase complex) and to Ca^{2+}, on the surface of the cells presenting TF, forming thrombin.

Once formed, thrombin can move from the cells presenting TF to the local platelets, initiating the amplification phase. The binding of thrombin to platelets releases and activates the FVIII that, in its inactive form, is linked to the von Willebrand Factor (vWF), besides activating the factors V and XI on the platelet surface.

The propagation phase occurs on activated platelets.The FIXa, generated in the initiation phase and by FXIa on the platelet surface, binds to FVIIIa forming the intrinsic tenase complex. As the FXa does not diffuse freely, it is necessary to produce it on the platelet surface by the FIXa/FVIIIa complex.

The FXa formed, is rapidly associated with FVa and Ca^{2+} on the surface of the plate. This prothrombinase complex catalyzes the conversion of prothrombin to thrombin, the essential protease of the coagulation system.

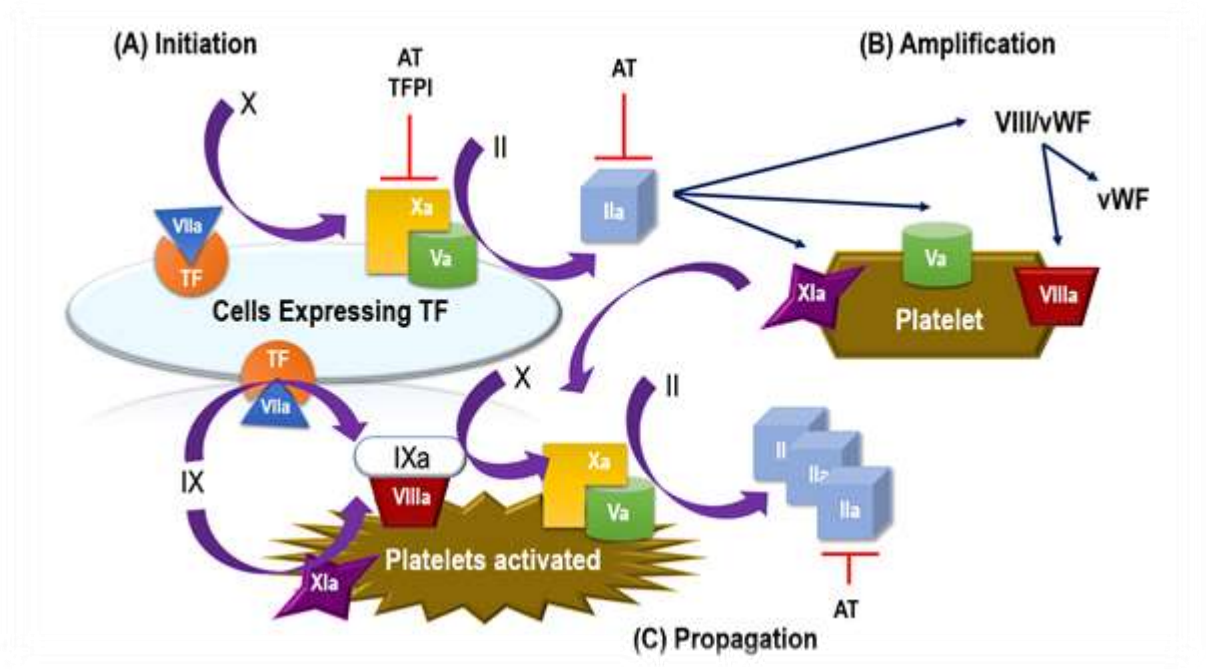

Figure 2 - Cellular model of blood coagulation. (A) Initiation phase. (B) Amplification phase. (C) Propagation phase. Adapted from Monroe *et al.*[9]

Although the ultimate aim of coagulation is the polymerization of fibrin, the crucial feature of the coagulation pathway is the generation of thrombin/factor IIa (FIIa). [10] Thrombin is a multifunctional enzyme that acts in the coagulation system that generates procoagulant reactions and anticoagulants, in addition to the conversion of soluble fibrinogen to an insoluble fibrin matrix, the thrombin is also responsible for clot stabilization by activation of factor XIII (FXIII), which favors the formation of cross-covalent bonds between fibrin molecules. [9,10]

Thrombin hinders the fibrinolytic action by activating the inhibitor of fibrinolysis mediated by thrombin, which removed lysine and arginine residues terminals of fibrin (binding sites of fibrinolytic enzymes), by binding to PAR-4 receptor that ensures a complete degranulation platelet and by the incorporation into the clot structure. This incorporation decreases the effectiveness of inactivation by antithrombin III (ATIII), which is allocated to the responsibility of inhibiting free coagulation factors (IIa, IXa, Xa and XIa), protecting the movement of liberated enzymes of the lesion site. [11,12]

The natural inhibitors of the clotting system plays essential functions in the maintenance of blood flow within the vessel. The paper highlighted that the FXa and especially thrombin play in the formation of blood clots, the main regulators of this system are involved in the inhibition of these two serine proteases, which are: the route of inhibiting the TF, the ATIII and heparin cofactor II (HCII). [13]

Disturbances in blood clotting can lead to disorders. The pharmacotherapy of thrombotic disorders arose in the first half of the twentieth century with the discovery of "Heparin" by McLean and his colleagues in 1916, being used, for the first time, in the treatment of thrombosis in 1936. Link, in 1939, synthesized dicumarol and a number of antagonistic substances of vitamin K (AVK). The more potent was called "Warfarin", whose chemical structure results from a modification of a coumarin, an isolated substance of *Melilotus* genus, firstly clinically used in 1955, acting as a cofactor to form gamma-glutamic acid, present in the factors II, VII, IX and X. [14,15]

As shown to significantly reduce the thromboembolic risk, heparins, leading the prophylaxis and acute and parenteral antithrombotic treatment, and AVK in oral and chronic treatment have been administered routinely for over 50 years. [16] However, both, AVKs as heparins (unfractionated Heparin - UFH and low molecular weight heparin - LMWH) are far from the ideal anticoagulant for presenting relevant contraindications. [17]

Heparins are natural products isolated from animal material, and as such provide a theoretical risk of contamination by pathogens such as viruses and prions, and can trigger cross-reactivity with some human proteins, particularly with the platelet factor 4 (PF4), resulting thrombocytopenic, and serious bleeding effects, requiring constant monitoring of laboratory clotting time. [18,19]

Within this context, the natural products have been investigated worldwide, as anticoagulant extracts. The substances of the plant kingdom, which has contributed several molecules in use in the prophylaxis and treatment of various diseases, have been among the most studied natural products under this approach.[20]

The use of plants in order to prevent, treat and cure disease is one of the oldest forms of medical practice known to humankind, such treatment is extremely important, especially for developing countries, where medicinal plants are widely used Primary Health Care (PHC). In these countries, they are used in raw form (not processed) as teas or decoctions, herbal (standardized and formulated plant extracts) and as a popular alternative to allopathic medicinal products. [21]

It is estimated that approximately 40% of the currently available drugs have been developed directly or indirectly from natural sources and subdivided: 25% of plants, 12% of micro-organisms and 3% of animals. [22]

In this context the Brazilian biodiversity stands out imposing form, it has the highest plant biodiversity in the world with about 60,000 species cataloged. [23] As scientific goal is to investigate the pharmacological properties of plant species in the ecosystem of the semiarid region, which has as unique biome, the caatinga, characterized by extreme heterogeneity of its vegetation type and composition, as well as lack of dedicated studies this ecosystem, to elucidate the true potential of its biodiversity. [24]

In this context, the popular use of leaves of oiticica (*Licania rigida* Benth) and chanana (*Turnera subulata*), species from the Brazilian semiarid region, which are used in the treatment of diabetes, inflammatory processes and diseases such as chronic degenerative characterized with the involvement of reactive oxygen species.

Therefore, the search for herbal medicines that may interfere with the hemostatic process, assisting the drugs currently used in the clinic, has been intensified since natural products have historically been the primary source of antithrombotic substances. [20]

Considering the problems created by the use of anticoagulants and the need for new substances that help the drugs currently used in the clinic, with fewer side effects, the objective of this study was to evaluate the anticoagulant potential, hemorrhagic and toxic effects of a crude extract and ethyl acetate fraction from *Licania rigida* Benth and *Turnera subulata* leaves.

2 BACKGROUND

Despite progress in diagnosis and treatment, thromboembolic diseases remain the cause of high morbidity and mortality globally.[1,25] Arterial thrombosis is the most common cause of acute myocardial infarction, strokes and ischemia, on the other hand, the complications of deep vein thrombosis include pulmonary embolism and post-thrombotic syndrome. [26] The coagulation system in interaction with platelet aggregation is centrally involved in the formation of arterial and venous thrombus. [27]

Indications currently defined for anticoagulants include the prophylaxis and treatment of venous thromboembolism, cardioembolic prevention in patients with cardiac arrhythmia or mechanical valve prostheses and secondary prevention in patients with acute coronary syndromes or undergoing percutaneous coronary intervention. [28]

Unfractionated heparins and low molecular heparins are used as anticoagulant drugs. However, these compounds are accompanied to several side effects such as thrombocytopenia and a high risk of systemic bleeding. [29,30] The effect of this problem generated demand for new substances in order to assist prolonged anticoagulant therapy. [31]

Therefore, the search for herbal medicines that may interfere with the hemostatic process, assisting the drugs currently used in the clinic, has been intensified since natural products have historically been the primary source of antithrombotic substances. [20]

Considering the problems created by the use of anticoagulants and the need for new herbal medicines that help the drugs currently used in the clinic, with fewer side effects, it is justifiable to evaluate the anticoagulant potential, toxic and hemorrhagic effects from *Licania rigida* Benth and *Turnera subulata* leaves, species widely found in semiarid Northeast.

3 OBJECTIVES

3.1 General objective

- Evaluate the effect of a crude extract and ethyl acetate fraction from *Licania rigida* Benth and *Turnera subulata* leaves, under coagulation, toxicity and hemorrhage.

3.2 Specific objectives

- Get crude extracts and ethyl acetate fractions from *Licania rigida* Benth and *Turnera subulata* leaves;
- Determine the chemical composition of extracts by High Performance Liquid Chromatography - HPLC;
- Evaluate the anticoagulant potential of the extracts studied by coagulation tests *in vitro* (Activated Partial Thromboplastin Time, Prothrombin Time, Anti-Xa and Anti-IIa assays)
- Elucidate the hemolytic activity of extracts under study;
- Determine the hemorrhagic ability of extracts of *Licania rigida* Benth and *Turnera subulata* by a modified model of topical scarification in the rat tail;
- Determine the acute toxicity of the plants under study extracts, using physiological, biochemical and hematological parameters related to liver and kidney functions.

4 MATERIAL AND METHODS

4.1 Collection and preparation of the crude extract and ethyl acetate fraction of *Licania rigida* Benth and *Turnera subulata* leaves

Leaves of *Licania rigida* Benth were collected in Florânia, Rio Grande do Norte, Brazil (SS-RN 6°14'07" S/36°78'21" O) and *Turnera subulata*, in Natal, Rio Grande do Norte, Brazil (SS-RN 5°52'17" S/35°10'45" O), both on November 2014. The plant material was identified by Dr. Jomar Gomes Jardim, and a voucher specimen was deposited at Herbarium of the Department of Botany and Zoology, Federal University of Rio Grande do Norte, Natal, RN, Brazil, under the reference number 8206/09 and 0674/08, respectively. The leaves were dried in a circulating air oven at 40 °C for 48 h and triturated mechanically. The extract from *L. rigid*a and *T. subulata* leaves were prepared by maceration in ethanol:water (50:50, v/v). After four days, the samples were filtered and later lyophilized. Thus, it was obtained the hidroethanolic extract (crude extract) from *L. rigida* (CELR) and *T. subulata* (CETS) leaves.

In order to characterize the active compounds from CELR and CETS, a portion of the extracts were resuspended in metanol and subjected to liquid-liquid partition with solvents of increasing polarity: n-hexane (3x300mL) and ethyl acetate (3x300mL). Two fractions were obtained of each crude extract (HFLR, AFLR, HFTS and AFTS), respectively. The amount of phenolic compound was measured in both fractions using Folin-Ciocalteau method, previously described [32] (data not show). Since phenolic compounds are absent in HFLR and HFTS, they were not used in the subsequent assays.

4.2 Chemical procedures (HPLC-DAD analysis)

Reverse phase chromatographic analyses were carried out on a Phenomenex C18 chromatography column (4.6×100 mm, particle size 2.6 µm, Torrance, CA, USA) using a HPLC (VARIAN ProStar HPLC system, Walnut Creek, CA, USA) equipped with a ProStar 240 quaternary pump, autosampler (ProStar 410) and a detector (mod. 355 PDA UV/V). We apply this methodology only for the *Turnera subulata* species, since the *Licania rigida* Benth species already possessed its elucidated chemical characterization, so CETS and AFTS (5 mg/mL) were dissolved in methanol, as

previously described [16]. The separation was conducted at room temperature with a flow rate of 1.3 mL/min. Mobile phase was water containing 0.1% formic (A phase) and acetonitrile (B phase) under following gradient conditions: 0-3 min, 5% B; 3-7 min, 5-20% B; 7-9 min, 20% B; 9-10 min, 2-23%, B; 10-15 min, 23% B; 15-19 min, 23-50% B; 19-20 min, 50-5% B. The injection volume was 9 µL. All the chromatography operations were carried out at room temperature and in triplicates. Chromatograms were recorded at 280 nm. All solutions were filtered through a 0.22 µm membrane (Millipore, Billerica, MA, USA). Chromatography peaks were identified by comparing its retention time with those of reference standards (gallic acid, chlorogenic acid, epigallocatechin, rutin, hiperin, quercetin, apigenin, kaempferol) and by DAD spectra (200 to 600 nm).

4.3 Activated partial thromboplastin time (aPTT) assay

The test was in accordance with the aPTT kit instructions (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CELR, AFLR, CETS and AFTS were dissolved in physiological saline at various concentrations (0.1, 1, 5, 10, 30, 50, and 100 µg/mL) and 10 µL of these solutions were incubated with 90 µL of plasma at 37 °C for 3 min. Then, 100 µL of bovine cephalin was added and incubated at 37 °C. After 3 min of incubation, 100 µL of pre-warmed 0.25 M $CaCl_2$ solution was added to the mixture and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

4.4 Prothrombin time (PT) assay

The test was in accordance with the PT kit instructions (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CELR, AFLR, CETS and AFTS were dissolved in physiological saline at various concentrations (5, 10, 15, 20, 30, 50, and 100 µg/mL) and 10 µL of these solutions were incubated with 90 µL of plasma at 37 °C for 3 min. Then, 200 µL of Soluplastin reagent was added and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

4.5 Assay for the anti-Xa activity

The anti-Xa activity assay was conducted in a 96-well microplate by using the Biophen Heparin Anti-Xa kit (HYPHEN Biomed, ref: 221010, Paris, France), according to the manufacturer's instructions. Briefly, 40 µL of antithrombin (AT) was incubated at 37 °C for 2 min in the presence of increasing concentrations of heparin, CELR, AFLR, CETS and AFTS diluted in buffer with a pH of 8.4 (0.05 M Tris, 0.175 M NaCl, 0.0075 M EDTA, containing 0.1% polyethylene glycol). Then, 40 µL of purified bovine Factor Xa (FXa) was added to each well, mixed, and incubated at 37 °C for 2 min. After, 40 µL of a chromogenic substrate for FXa was added and the mixture was incubated for 2 min at 37 °C. Following incubation, 80 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured at 405nm in Epoch Microplate Spectrophotometer (Epoch-Biotek, Winooski, VT, USA).

4.6 Thrombin Inhibition Assay

The thrombin Inhibition assay was conducted in a 96-well microplate by using the Biophen Heparin Anti-IIa kit (HYPHEN Biomed, ref: 221025, Paris, France), according to the manufacturer's instructions. Briefly, 50 µL of thrombin was incubated at 37 °C for 2 min in the presence of increasing concentrations of heparin, CELR, AFLR, CETS and AFTS, previously diluted in citrated fresh human plasma and again diluted in AT. After the addition of 50 µL of factor IIa (thrombin), purified bovine was homogenized in each well and incubated at 37 °C for 2 min. Then, 50 µL of chromogenic substrate for FIIa was added to homogenates and incubated at 37° C for 2 min. After incubation, 50 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured at 405 nm in Epoch Microplate Spectrophotometer (Epoch-Biotek, Winooski, VT, USA).

4.7 Residual hemorrhagic effects

The residual hemorrhagic effect of CELR, AFLR, CETS and AFTS fractions were analyzed by a modified model of topical scarification in the rat tail as described previously [33]. After anesthesia with ketamine and xylazine in a 1:1 (v/v) proportion, a scar was made with a surgical blade in the distal portion of the tail. Then, the tail was

then dipped vertically in physiological saline solution, dabbed with gauze and dipped again in fresh saline to observe bleeding. The tail was dipped in solutions containing CELR, AFLR, CETS, AFTS or heparin at concentration of 100 µg/mL for 2 min and then washed extensively with saline solution. The treated tail was immersed in new physiological saline solutions for 40 min and blood was quantified using the Drabkin assay. The results were expressed as the sum of the hemoglobin values of each tube minus the hemoglobin value present prior to exposure of the test substance (Figure 3).

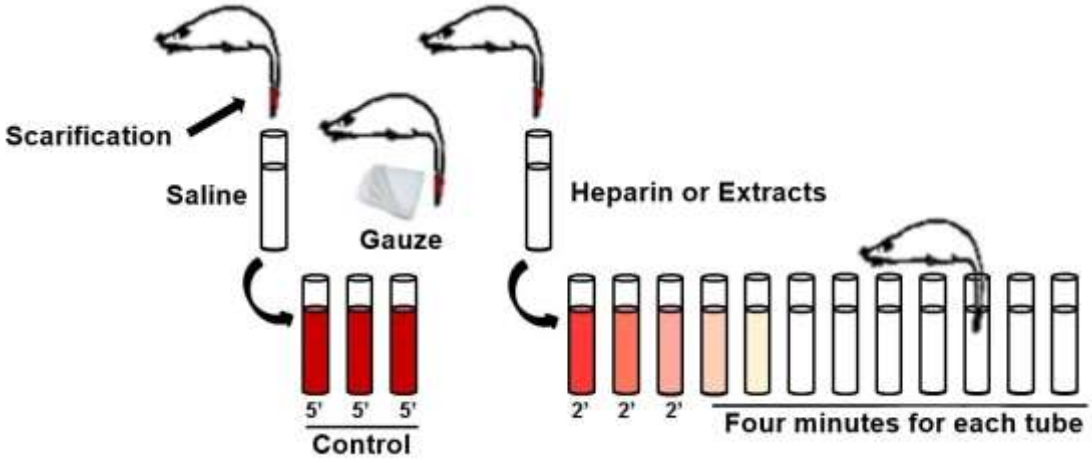

Figure 3 - Scheme scarification model rat tail to evaluate the hemorrhagic activity. Adapted from Araújo *et al*[34]

4.8 MTT assay (cytotoxicity)

The mouse fibroblast cells (3T3) and epithelial embryonic human kidney cells (HEK 293) were cultured under standard conditions in Dulbecco´s modified Eagle´s medium (DMEM), supplemented with fetal bovine serum (FBS) at a final concentration of 10%. Cells were maintained in cell culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2. Cells (1 x 10^4cells per well) were cultured for 24 h in 96-well microplates to promote adhesion. After 24 h, cells were treated with different concentrations of CELR, AFLR, CETS and AFTS (0.1, 1, 10, 100, and 1000 µg/mL) in triplicate and incubated at 37 °C for 24 h. After this period, 100 µL of MTT (5 mg/mL) dissolved in DMEM and was added to each well and the cells were incubated again. After 4 h, the culture medium was removed and then 100 µL of DMSO was added to each well. The reading was performed at 570 nm with a microplate reader (Epoch-Biotek, Winooski, VT, USA).

4.9 Hemolysis assay

The hemolysis assay was performed as described previously [35]. Briefly, 20 µL of CELR, AFLR, CETS and AFTS were diluted in a DMSO solution of 0.005 % (v/v) at concentrations of 50, 100, 250, and 500 µg/mL in triplicate and were added to 180 µL of a suspension of 1 % human erythrocytes. A solution of 0.05 % Triton 100X was used as a positive control test, to generate hemolysis. In one microplate, extracts and the positive control were incubated for 30 min at 37°C under constant agitation, centrifuged at 8,600 g for 10 min and the absorbance of the supernatant was measured at 540 nm with a microplate ELISA reader (Epoch-Biotek, Winooski, VT, USA).

4.10 Animals

Wistar rats (*Rattus norvegicus*), 250-300 g, three-months old, from both sexes, provided by the vivarium of the Health Sciences Center from Federal University of Rio Grande do Norte were used. The animals were maintained under standard environmental conditions and fed with food and water *ad libitum*. All the procedures involving rats were done in agreement with the recommendations of the Brazilian National Health Surveillance Agency (ANVISA) and the Organization for economic cooperation and development (OECD). The experimental protocols were approved by the Ethics Committee on Animal Use of Federal University of Rio Grande do Norte (protocol n°035/2015).

4.11 Acute Oral Toxicity

The acute oral toxicity test was performed according to experimental protocol of the Brazilian National Health Surveillance Agency nr. 90 (2004) [36] and OECD (2001) [37]. This evaluation used five groups, with gavage doses of 500 and 2000 mg/kg, compared to a control group that received only distilled water, not exceeding 1 mL/100 g of body weight. In the first 12 h, systematic behavioral observations were made (vocal tremor, piloerection, hyperactivity, tremors, abdominal cramps, diarrhea, and number of deaths). At the end of 14 days, the animals were euthanized with an overdose of sodium thiopental (100 mg/kg) by intraperitoneal (i.p) administration and then underwent laparotomy for blood collection by cardiac puncture and evisceration. Liver,

kidney, spleen, lung, heart, intestine, stomach, esophagus, and brain were removed for macroscopic and relative weight evaluations.

4.12 Biochemical and hematological parameters

The hematological parameters analyzed were total count of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean concentration hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), volume change mean corpuscular (RDW), the total count (WBC), differential leukocytes (lymphocytes, monocytes and granulocytes), procalcitonin (PCT), platelets (PLT), mean platelet volume (MPV) and platelets distribution width (PDW). Total protein, albumin, globulin, alanine transaminase enzymes (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (γ-GT), and bilirubin were evaluated as liver function markers. Creatinine and urea were determined for renal evaluation. Other parameters analyzed were total cholesterol, triglycerides, amylase, and glucose. Hematological tests were performed through ABX Micros 60 OT Equipment (ABX Diagnotics, France) and biochemical parameters were performed using LABTEST kits (Lagoa Santa, Brazil) through LABMAX PLENNO equipament (LABTEST, Lagoa Santa, Brazil).

4.13 Statistical analyses

Results were analyzed by a one-way ANOVA and Tukey's post hoc test. Values of $p < 0.05$ were considered indicative of statistical significance. The analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

5 ARTICLES PRODUCED

5.1 Article 1

The article "LEAVES OF *Licania rigida* BENTH HAS A POTENTIAL ANTICOAGULANT EFFECT BY THROMBIN INHIBITION" was submitted to the journal *Phytomedicine* which has an impact factor 2.937, Qualis B1 for CAPES, for area of Medicine II.

Leaves of *Licania rigida* Benth has a Potential Anticoagulant Effect by Thrombin Inhibition

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ABSTRACT:
Background: Cardiovascular diseases are the main causes of mortality and morbidity worldwide. Anticoagulants are used as a secondary prevention of thromboembolic events in patients with venous or arterial thrombosis and those with heart disease that may by predisposed to thrombus formation. The use of plants for medicinal purposes is already a global practice. Licania rigida Benth (Chrysobalanaceae Family) is a tree of large and evergreen foliage, found in semi-arid regions of Brazil.
Study design: In this study, we evaluated the toxicological risks *in vitro* and *in vivo*, as well as the *in vitro* anticoagulant potential of the crude extract (CELR) and ethyl acetate fraction (AFLR) of L. rigida leaves.
Methods: *In vitro* toxicity was assessed by an MTT assay in 3T3 and HEK-293 cells as also an acute oral toxicity test was performed in Wistar rats. The anticoagulant ability of the extracts was measured by the aPTT, PT, and inhibition of Xa and IIa factors tests.
Results: In relation to the toxic effects, no toxicity was observed after exposure to the extracts. The anticoagulant potential was demonstrated in the aPTT and PT tests where the two extracts have the ability to double the standard coagulation time at the concentration of 50 µg/mL. However, only the AFLR was capable of inhibiting the extrinsic pathway of coagulation by inhibiting 100% of the IIa factor at a concentration of 100 µg/mL, compared with that by the clinical anticoagulant, heparin.
Conclusion: CELR and AFLR demonstrated an anticoagulant ability by the blood coagulation without causing toxic damage to the body and can become a target for further studies to validate its potential as a phytomedication.

Key words: *Licania rigida* Benth; Coagulation; Toxicity.

Abbreviations: WHO, World Health Organization; CELR, Crude Extract of *Licania rigida* Benth; HFLR, Hexane Fraction of *Licania rigida* Benth; AFLR, Acetate Fraction of *Licania rigida* Benth; Hex, n-hexane; EtOAc, ethyl acetate; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; 3T3, mouse fibroblast cells; HEK-293, epithelial embryonic human kidney cells; DMEM, Dulbecco´s modified Eagle´s medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; i.p. intraperitoneal; ANVISA, Brazilizan National Health Surveillance Agency; OECD, Organization for economic cooperation and development; RBC, total count of red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean concentration hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, volume change mean corpuscular; WBC, total count leukocytes; L, Lymphocytes; M, Monocytes; G, Granulocytes; PCT, procalcitonin; PLT, platelets; MPV, mean platelet volume; PDW, platelets distribution width; Glic, glucose; Trig, triglycerides; Col, cholesterol; ALT, alanine transaminase enzymes; AST, aspartate transaminase; γ-GT, gamma-glutamyl transferase; TB, total bilirubin; BD, bilirubin direct; BI, bilirubin indirect; Cret, creatinine; TP, total protein; ALB, albumin; Glo, globulins; Ami, amylase; aPTT, activated partial thromboplastin time; PT, Prothrombin time; AT, antithrombin; TT, thrombin time.

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases are the leading cause of mortality and morbidity worldwide. One of the studied causes is the dysfunction of the heart valves (aortic, mitral, pulmonary, and tricuspid), which compromise their normal physiological behavior, preventing blood to flow properly through the heart chambers. The most common solution for this type of disease is the recovery or replacement of damaged valves (Rodriguez-Sanchez et al., 2015; WHO, 2016).

Artificial valves are fundamentally constituted by pyrolytic carbon in combination with metallic and polymeric components; these have a high durability and are viable in the long term for the patient (about 20 years). However, there is an increased risk of thromboembolic complications due to high shear stress, and anticoagulants are commonly prescribed for these patients (Sewell-loftin et al., 2011).

Anticoagulants are used as a secondary prevention of thromboembolic events in patients with venous or arterial thrombosis and those with heart disease that may by predisposed to thrombus formation (González-Barcénas et al., 2016). Despite its high efficiency, the drugs currently used are accompanied by a high risk of systemic bleeding (Zhu et al., 2015).

Compounds such as warfarin, dicumarol and heparin are used as anticoagulants in the treatment of thromboembolic complications associated with the use of artificial valves, however, at high risk of bleeding disorders that accompany these compounds, new substances are being studied in order to assist prolonged anticoagulant therapy (Brito et al., 2014).

Higher plants have the potential to be exploited in a variety of forms such as extracts for therapeutic purposes, as isolated molecules or with some structural modifications, or as

precursors for chemical synthesis. Thus, the use of plants with medicinal properties deserves attention in order to identify and characterize molecules, and develop new applications or methods of use (Atanasov et al., 2015). This explains the significant increase of medicinal products derived from medicinal plants, resulting from the structural diversity of secondary metabolites (Dutra et al., 2016).

Licania rigida Benth, popularly known as Oiticica, is a large tree, which belongs to Chrysobalanaceae family, order Rosales and superorder Rosiflorae, distributed in tropical and subtropical regions (Queiroga et al., 2013). The effects of *L. rigida* are associated with treating diseases related to oxidative stress, whereas phytochemical studies have shown the presence of various phenolic compounds, including tannins, flavonoids, triterpenoids, coumarins, and other antioxidants (Feitosa et al., 2012).

Considering the problems created by the use of anticoagulants and the need for new substances that help the drugs currently used in the clinic, with fewer side effects, the objective of this study was to evaluate the anticoagulant potential, toxic and hemorrhagic effects, of a crude extract and ethyl acetate fraction of *L. rigida* Benth leaves.

2. Material and Methods

2.1. Collection and preparation of the crude extract and ethyl acetate fraction of *Licania rigida* Benth leaves (CELR and AFLR, respectively)

Leaves of *L. rigida* Benth were collected in Florânia, Rio Grande do Norte, Brazil (SS-RN 6°14’07” S/36°78’21” O), on November 2014. The plant material was identified by Dr. Jomar Gomes Jardim, and a voucher specimen was deposited at Herbarium of the Department of Zoology, Ecology and Botany, Federal University of Rio Grande do Norte, Natal, RN, Brazil, under the reference number 8206/09. L. rigida Benth leaves were dried in a circulating air oven at 40°C for 48h and triturated mechanically. The extract from *L. rigida* leaves was prepared by maceration in ethanol:water (50:50, v/v), for four days, filtered and later lyophilized. It was obtained the hidroethanolic extract (crude extract) from *L. rigida* leaves (CELR).

In order to characterize the active compounds from CELR, a portion of the extract was resuspended in metanol and subjected to liquid-liquid partition with solvents of increasing polarity: n-hexane (Hex) (3x300mL) and ethyl acetate (EtOAc) (3x300mL). Two fractions were obtained (HFLR and AFLR), all fractions are dried under reduced pressure at 45ºC. Analysis of phenolic constituents of the extracts (data not shown), confirmed that the hexane fraction of *L. rigida*, it does not show a significant concentration of phenolic compounds, thus, the HFLR was removed for subsequent assays.

2.2.Cytotoxicity by MTT assay

Cytotoxicity was determined using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The mouse fibroblast cells (3T3) and epithelial embryonic human kidney cells (HEK 293) were cultured under standard conditions in Dulbecco´s modified Eagle´s medium (DMEM) (Invitrogen, San Diego, CA, USA), supplemented with fetal bovine serum (FBS) at a final concentration of 10%. Cells were maintained in cell culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 and were collected by treatment with trypsin. Cells (1 x 10^5 cells per well) were seeded in medium supplemented with FBS (10%) and cultured for 24 h in 96-well microplates to promote adhesion. After 24 h, cells were treated with different concentrations of CELR and AFLR (0.1, 1, 10, 100, and 1000 μg/mL) in triplicate and incubated at 37°C for 24 h. After this period, 100 μL of MTT (5 mg/mL) was added to each well and the cells were incubated again. After 4 h, the culture medium was removed and then 100 μL of DMSO was added to each well. The reading was performed at 570 nm with a microplate ELISA reader (Epoch-Biotek, Winooski, VT, USA).

2.3. Hemolysis assay

The hemolysis assay was performed as described by Wang et al. (2010). Briefly, 20 μL of CELR and AFLR were diluted in a DMSO solution of 0.005 % (v/v) at concentrations of 50, 100, 250, and 500 μg/mL in triplicate and were added to 180 μL of a suspension of 1 % human erythrocytes. A solution of 0.05 % Triton 100X was used as a positive control test, to generate hemolysis. In one microplate, extracts and the positive control were incubated for 30 min at 37°C under constant agitation, centrifuged at 8,600 g for 10 min and the absorbance of the supernatant was measured at 540 nm with a microplate ELISA reader (Epoch-Biotek, Winooski, VT, USA).

2.4. Animals

Wistar rats (*Rattus norvegicus*), 250-300g, three-months old, from both sexes, provided by the vivarium of the Health Sciences Center from Federal University of Rio Grande do Norte were used. The animals were maintained under standard environmental conditions and fed with food and water *ad libitum*. On the day of the experiment, the rats were placed in the experimental room for at least 1h to allow acclimatization prior use. At the end of the experimental, the rats were euthanized with an overdose of sodium thiopental (100 mg/kg) by intraperitoneal (i.p). All the procedures involving rats were done in agreement with the recommendations of the Brazilian National Health Surveillance Agency (ANVISA) and the

Organization for economic cooperation and development (OECD). The experimental protocols were approved by the Ethics Committee on Animal Use of Federal University of Rio Grande do Norte (protocol n°035/2015).

2.5. Acute Toxicity

The acute toxicity test was performed according to experimental protocol of the Brazilian National Health Surveillance Agency nr. 90 (2004) and OECD (2001). This evaluation used five groups, with gavage doses of 500 and 2000 mg/kg, compared to a control group that received only distilled water, not exceeding 1 mL/100 g of body weight. In the first 12 h, systematic behavioral observations were made (vocal tremor, piloerection, hyperactivity, tremors, abdominal cramps, diarrhea, and number of deaths). From the second day, the observations were made every 6 h and then at least twice a day. Moreover, water consumption and food intake were checked every 2 days and their weight was monitored every 7 days. At the end of 14 days, the animals were euthanized with an overdose of sodium thiopental (100 mg/kg) by intraperitoneal (i.p) and then underwent laparotomy for blood collection by cardiac puncture and evisceration. Liver, kidney, spleen, lung, heart, intestine, stomach, esophagus, and brain were removed for macroscopic and relative weight evaluations.

2.6. Biochemical and hematological parameters

The hematological parameters analyzed were total count of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean concentration hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), volume change mean corpuscular (RDW), the total count (WBC), differential leukocytes (lymphocytes, monocytes and granulocytes), procalcitonin (PCT), platelets (PLT), mean platelet volume (MPV) and platelets distribution width (PDW). Total protein, albumin, globulin, alanine transaminase enzymes (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (γ-GT), and bilirubin were evaluated as liver function markers. Creatinine and urea were determined for renal evaluation. Other parameters analyzed were total cholesterol, triglycerides, amylase, and glucose.

2.7. Residual hemorrhagic effects

The residual hemorrhagic effect of CELR and AFLR compounds were analyzed by a modified model of topical scarification in the rat tail (Cruz and Dietrich, 1967). After anesthesia with ketamine and xylazine in a 1:1 (v/v) proportion, a scar was made with a surgical blade in

the distal portion of the tail. Then, the tail was then dipped vertically in physiological saline solution, dabbed with gauze and dipped again in fresh saline to observe bleeding. The tail was dipped in solutions containing CELR, AFLR, or heparin at concentration of 100 µg/mL for 2 min and then washed extensively with saline solution. The treated tail was immersed in new physiological saline solutions for 40 min and blood was quantified using the Drabkin assay. The results were expressed as the sum of the hemoglobin values of each tube minus the hemoglobin value present prior to exposure of the test substance.

2.8. Activated partial thromboplastin time (aPTT) assay

The aPTT assay was performed according to the method of the kit aPTT test (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CELR, and AFLR were dissolved in physiological saline at various concentrations (0.1, 1, 5, 10, 30, 50, and 100 µg/mL) generating a volume of 10 µL, and incubated with 90 µL of plasma at 37°C for 3 min. Then, 100 µL of bovine cephalin was added and incubated at 37°C. After 3 min of incubation, 100 µL of pre-warmed 0.25 M $CaCl_2$ solution was added to the mixture and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

2.9. Prothrombin time (PT) assay

The PT assay was performed according to the method of the kit PT test (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CELR, and AFLR were dissolved in physiological saline at various concentrations (5, 10, 15, 20, 30, 50, and 100 µg/mL) generating a volume of 10 µL, and incubated with 90 µL of plasma at 37°C for 3 min. Then, 200 µL of Soluplastin reagent was added and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

2.10. Assay for the anti-Xa activity

The anti-Xa activity assay was conducted in a 96-well microplate by using the Biophen Heparin Anti-Xa kit (HYPHEN Biomed, ref: 221010, Paris, France), according to the manufacturer's instructions. Briefly, 40 µL of antithrombin (AT) was incubated at 37°C for 2 min in the presence of increasing concentrations of heparin, CELR, and AFLR diluted in buffer with a pH of 8.4 (0.05 M Tris, 0.175 M NaCl, 0.0075 M EDTA, containing 0.1% polyethylene glycol). Then, 40 µL of purified bovine Factor Xa (FXa) was added to each well, mixed, and incubated at 37°C for 2 min. Then, 40 µL of a chromogenic substrate for FXa was added and

the mixture was incubated for 2 min at 37°C. Following incubation, 80 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured against a corresponding blank.

2.11. Thrombin Inhibition Assay

The thrombin Inhibition assay was conducted in a 96-well microplate by using the Biophen Heparin Anti-IIa kit (HYPHEN Biomed, ref: 221025, Paris, France), according to the manufacturer's instructions. Briefly, 50 µL of thrombin was incubated at 37°C for 2 min in the presence of increasing concentrations of heparin, CELR, and AFLR, previously diluted in citrated fresh human plasma and again diluted in AT. After the addition of 50 µL of factor IIa (thrombin), purified bovine was homogenized in each well and incubated at 37°C for 2 min. Then, 50 µL of chromogenic substrate for FIIa was added to homogenates and incubated at 37°C for 2 min. After incubation, 50 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured at 405 nm.

2.12. Statistical analyses

Results were analyzed by a one-way ANOVA and Tukey's post hoc test. Values of $p < 0.05$ were considered indicative of statistical significance. The analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

3. Results and Discussion

3.1. Cytotoxicity by MTT assay

The assessment of the cytotoxicity of CELR and AFLR showed no toxicity to the mice fibroblast cells (3T3) (Figure 1A). No statistically significant differences in any of the tested concentrations were observed compared to the negative control (DMEM). However, with respect to human embryonic kidney cells (HEK-293), AFLR was able to maintain its integrity, as demonstrated by a subtle proliferation of these cells at the highest concentration tested (1000 µg/mL) (Figure 1B). The results show that CELR and AFLR extracts did not cause any toxic effect *in vitro* with respect to normal cells tested in this study. Kadan et al. (2016) tested several species of fractionated extracts of *Ocimum basilicum* against normal rat muscle cells using the MTT technique. They found that the methanol extract was safe at a concentration of 0.25 mg/mL, whereas with 0.5 mg/mL, the extract hexane already had a safe action, thus there was an absence of cytotoxicity using these concentrations.

In contrast, Miriam et al. (2016) measured methanol extracts of *Rhus Coriaria*, *Pistacia vera,* and *Pistacia khinjuk* and exposed endothelial cells of human umbilical cord to them for

24 h. The mortality rate was also measured by the MTT technique, which showed a toxic effect in a dose-dependent manner, using doses up to 100 μg/mL. The cell protection caused was approximately 60%, 20%, and 25%, respectively, compared to that in the control.

3.2. Anti-hemolytic effect

CELR and AFLR extracts tested in this study had a very low hemolytic effect, Figure 2 shows the effect of the extracts on the hemolysis of human erythrocytes using Triton 100X as positive control and saline as negative control, demonstrating their anti-hemolytic at doses of 50, 100, and 250 μg/mL.

CELR at a concentration of 500 μg/mL showed a 40 % rate of erythrocyte hemolysis, whereas AFLR at the same concentration showed no significant change in the hemolytic rate compared to that of the negative control.

Crude extract and fractions of *Maytenus royleanus* leaves have also been evaluated for their hemolytic activity. The ethyl acetate fraction had the lowest hemolysis rate, in a dose-dependent manner, where the point of 250 μg/mL gave the highest hemolytic rate (approximately 50 % erythrocyte hemolysis) compared to that in the positive control (distilled water) (Shabbir et al., 2013). The presence of flavonoids, coumarins, and tannins in the extracts of *M. royleanus* species had a higher polarity due to fractionation with ethyl acetate, suggesting greater protection to the cell lysis, whereas *L. rigida* extracts maintained a better level of protection by presenting more antioxidants (Feitosa et al., 2012).

3.3. Acute oral toxicity

With regard to behavioral effects, no episode described methodology was observed. Tables 1 and 2 show the levels of toxicity by the analysis of biochemical and hematological parameters of liver and kidney function. No significant differences were found in comparison to the control group. However, total cholesterol levels decreased on average by 60 % following treatment with the extracts, suggesting a possible hypolipidemic action, aiding its action in the treatment of cardiovascular diseases. The effects of plant compounds on hyperlipidemia has already been studied over the years, and most recently, it was shown that the *Cydonia oblonga* Miller leaves were able to regulate lipid metabolism in the blood of rats by lowering total cholesterol levels by up to 60% (Umar et al., 2015). Likewise, the aqueous extract obtained from *Cordia dichotoma* showed a decreased rate in total cholesterol levels by 41% in hyperlipidemic *Wistar* rats (El-Newary et al., 2016).

The weights of organs after euthanasia (Table 3) showed no significant differences compared to that in the control. Regarding the behavioral clinical parameters, such as water and food consumption, and body weight of the animals (data not shown), there were no signs of toxicity.

Evaluation of acute toxicity is a widely used methodology in the studies of plant compounds. This method is useful to identify the toxicity and minimize any ambiguity in the general population, who believe that natural products are devoid of toxic or adverse effects (Palhares et al., 2015). In addition to toxicity studies in animals, epidemiological and laboratory studies have shown that plant products can exert toxic effects by the production of secondary metabolites, leading to long vascular resistance in rodent embryo teratogenicity (Ouedraogo, 2012). The *Jatropha gossypifolia* L is an example of this aspect (therapeutic/toxic). It is rich in diterpene (jatrona) with various therapeutic and biological activities; however, toxicological tests have revealed its purgative and depressant effects on the respiratory and cardiovascular systems, as well as an important chronic toxicity (Mariz et al., 2008).

The hydroalcoholic extract of *Casearia sylvestris* leaves have been evaluated for acute oral toxicity tests in rats, using a single dose of 2000 mg/mL. No toxic effects on the parameters studied, biochemical and hematological parameters, body weight animals, consumption of water and food and relative weight of organs were found (Ameni et al., 2015). In addition, the ethanol extract of *Aralia elata* leaves in acute and subchronic tests showed no serious toxic effects at a concentration of 540 mg/kg (Li et al., 2015).

3.4 Evaluation of hemorrhagic activity

The prolonged use of unfractionated heparin is able to generate disturbances in hemostasis, leading to bleeding events (Brito et al., 2014); thus, the hemorrhagic residual effect of CELR and AFLR at a concentration of 100 µg/mL compared to that of clinical heparin (100 µg/ml) was investigated. CELR and AFLR present a reduced potential hemorrhagic effect compared to that by heparin, especially AFLR, which has practically no hemorrhagic effect (Figure 3).

Heparin has been used in clinical therapy for many years for its anticoagulant capacity; however, prolonged use may cause bleeding, followed by thrombocytopenia, osteoporosis, hypersensitivity, and necrosis of the skin. These same effects are seen with prolonged use of warfarin, a modified coumarin isolated from the genus *Melilotus* (Wang et al., 2016; Okishige et al., 2016).

3.5. APTT, PT, Anti-Xa and Anti-IIa assays

The anticoagulant activity of CELR and AFLR has been demonstrated experimentally by testing the aPTT and PT. Commercial heparin was tested as a standard positive control, where a significant anticoagulant aPTT activity of more than 240 seconds (negative control: 36.05 ± 0.03 s) and PT greater than 60 seconds (negative control: 16.65 ± 0.33 s) were expected. Figure 4 (A and B) shows the ability of CERL and AFLR in prolonging the normal clotting time, reaching the potential of the heparin anticoagulant at the highest concentrations tested (≥ 50 µg/mL). The results obtained are in accordance with certain plant extracts that possess anticoagulant activity (Félix-Silva et al., 2014; Nyansah et al., 2016) although these extracts were not tested in comparison to a standard drug.

Prolongation of the clotting time in the aPTT assay indicates the inhibition of the intrinsic coagulation pathway, whereas inhibition of the extrinsic pathway of blood coagulation was observed in the PT test. To evaluate the possible mechanism of anticoagulant action of CELR and AFLR, their effect was analyzed directly on the coagulation factors X and thrombin. Figure 4 (C and D) shows the results of percentage inhibition of CELR and AFLR on these factors, compared with that by commercial heparin used as positive control.

Both extracts were able to inhibit factor Xa on average 30-40 %, failing to reach the maximum inhibition, however, AFLR was able to achieve 100 % inhibition of thrombin at the highest concentration tested (100 µg/mL), whereas CELR, at 100 µg/mL, stabilized the inhibition of thrombin about 90 %. These results suggest that anticoagulant action of CELR and AFLR are primarily due to the inhibition of thrombin.

Plant extracts have been studied using anticoagulant tests, which are based on the ability to inhibit blood coagulation factors (Chaves et al., 2010). Among these substances, an oligomeric flavonoid extracted from *Alpinia platychilus* has shown anticoagulant activity in the thrombin time (TT) and PT tests, however, no action mechanism or potential bleeding hazards were suggested (Shen et al., 2015). Likewise, chloroform and methanol extracts of *Artemisia dracunculus* leaves are able to interfere with the coagulation cascade in the PT test (Duric et al., 2015).

4. Conclusions

The experimental results indicated that both (CELR and AFLR) extracts of *Licania rigida* Benth leaves have significant anticoagulant activity with low indication of bleeding, which is presented by anticoagulant drugs currently used in the clinic. Moreover, no considerable toxicological effect was found.

Therefore, the experimental results are promising, because both CELR and AFLR have shown potential as anticoagulants. However, more data is required to elucidate the mechanism of action of the extracts on blood coagulation, with the aim to found new substances in order to assist prolonged anticoagulant therapy.

Acknowledgements

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Conflict of interests

The authors have no conflict to declare.

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Table Legends

Table 1. Biochemical parameters of rats after 14 days of treatment with CELR and AFLR. Results are expressed as mean ± SD (n = 5); Control group treated with vehicle (distilled water). Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test.
* $p < 0.05$ compared with the control group.

Table 2. Hematological parameters of rats after 14 days of treatment with CELR and AFLR. Results are expressed as mean ± SD (n = 5); Control group treated with vehicle (distilled water). Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test and showed no significant statistical results. L = Lymphocytes, M = Monocytes, G = Granulocytes.

Table 3. Organs weight of the rats after 14 days of treatment with CELR and AFLR. Results are expressed as mean ± SD (n = 5); Control group treated with vehicle (distilled water). Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test and showed no significant statistical results. R= Right, L= Left.

Figure Legends

Fig. 1. Cytotoxicity effects of CELR and AFLR on mouse fibroblast cells (3T3) (A) and epithelial embryonic human kidney cells (HEK 293) (B), measured by MTT assays. Culture medium DMEM was used as a negative control of cytotoxicity. Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test.
* $p < 0.05$ compared with the control group.

Fig. 2. Hemolytic effects of CELR and AFLR. Saline solution was used as a negative control and Triton 100X used as a positive control of hemolysis. Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test.
* $p < 0.05$ compared with the positive control group.
$^{\#}$ $p < 0.05$ compared with the negative control group.

Fig. 3. Bleeding activity of CELR (100 µg/mL), AFLR (100 µg/mL) and porcine intestinal mucosa heparin (100 µg/mL) applied topically. The bleeding potency measured after 2 min following saline solution washing.

Fig. 4. Anticoagulant activity of CELR, AFLR, and Heparin by activated partial thromboplastin time (aPTT) assay (A), Prothrombin time (PT) assay (B), Inhibition of Xa Factor, (C) and Inhibition of IIa Factor (D).

Tables:

Table 1.

Biochemical parameters	Control	CELR 500 mg/Kg	CELR 2000 mg/Kg	AFLR 500 mg/Kg	AFLR 2000 mg/Kg
Glic (mg/dL)	122 ± 21.9	122 ± 20.1	125.6 ± 21.5	123 ± 19.2	129.4 ± 21.4
Trig (mg/dL)	53 ± 5.65	63 ± 3.89	68.6 ± 4.38	65 ± 2.34	68.7 ± 4.34
Col (mg/dL)	54.5 ± 6.06	22.5 ± 0.24*	22 ± 0.19*	21.5 ± 0.19*	22.5 ± 0.1*
ALT (U/L)	97 ± 4.24	86 ± 4.30	90 ± 4.20	89.1 ± 4.39	95 ± 5.29
AST (U/L)	250 ± 23.3	208 ± 25.3	209 ± 15.3	208.5 ± 24.8	210.3 ± 14.1
γ-GT (U/L)	12 ± 1.4	6 ± 1.5	0.66 ± 0.05	1 ± 0.23	1.5 ± 0.04
TB (mg/dL)	0.38 ± 0.01	0.190 ± 0.01	0.203 ± 0.01	0.210 ± 0.01	0.209 ± 0.02
BD (mg/dL)	0.10 ± 0.07	0.98 ± 0.05	0.106 ± 0.09	0.99 ± 0.05	0.143 ± 0.1
BI (mg/dL)	0.28 ± 0.01	0.12 ± 0.03	0.13 ± 0.09	0.11 ± 0.03	0.15 ± 0.08
Urea (mg/dL)	54.5 ± 7.7	44.5 ± 7.5	47.3 ± 7.04	45.9 ± 7.5	48.3 ± 7.75
Cret (mg/dL)	0.65 ± 0.07	0.45 ± 0.07	0.7 ± 0.06	0.50 ± 0.09	1 ± 0.07
TP (g/dL)	6.6 ± 0.1	6.62 ± 0.5	6.73 ± 0.60	6.01 ± 0.5	6.79 ± 0.6
ALB (g/dL)	3.1 ± 0.42	3.0 ± 0.41	2.6 ± 0.3	3.1 ± 0.41	3 ± 0.09
Glo (g/dL)	3.5 ± 0.42	3.9 ± 0.33	4.13 ± 0.55	3.9 ± 0.32	4.12 ± 0.54
Ami (U/L)	900 ± 3.53	801 ± 3.49	803 ± 2.09	802.5 ± 3.43	813.3 ± 2.07

Table 2.

Hematological parameters	Control	CELR 500 mg/kg	CELR 2000 mg/kg	AFLR 500 mg/kg	AFLR 2000 mg/kg
HGB (g/dL)	16 ± 0.28	18 ± 0.29	21.73 ± 7.5	17 ± 0.22	22.75 ± 7.9
HCT (%)	47.5 ± 0.98	44.3 ± 0.91	42.9 ± 0.2	44.9 ± 0.87	41.9 ± 0.21
RBC (10^6/mm^3)	8.49 ± 0.21	8.48 ± 0.24	9.02 ± 0.38	8.89 ± 0.12	9.25 ± 0.31
MCV (fm^3)	56.5 ± 3.5	55.5 ± 3.5	62.33 ± 9.7	59.5 ± 2.7	63.44 ± 9.8
MCH (pg)	18.8 ± 0.84	20.1 ± 0.81	25.6 ± 8.1	21.1 ± 0.41	26.6 ± 7.9
MCHC (g/dL)	33.35 ± 0.6	35.56 ± 1.2	41.56 ± 14.1	33.56 ± 1.1	40.35 ± 13.2
RDW (%)	13.05 ± 0.2	13.05 ± 0.2	15 ± 4.3	13.95 ± 0.9	14.1 ± 4.4
WBC (10^3/mm^3)	7.6 ± 0.84	7.5 ± 0.7	8 ± 0.8	7.6 ± 0.77	9 ± 0.81
L (%)	89.2 ± 1.76	81.2 ± 1.75	81.9 ± 7.79	85.8 ± 1.35	82.7 ± 7.87
M (%)	8.9 ± 1.41	8.97 ± 1.45	12.9 ± 1.95	8.1 ± 1.29	15.9 ± 1.87
G (%)	1.85 ± 0.35	2.95 ± 0.33	5.13 ± 0.06	2.85 ± 0.34	5.12 ± 0.09
PCT (ng/mL)	0.44 ± 0.01	0.89 ± 0.01	0.359 ± 0.01	0.66 ± 0.02	0.341 ± 0.01
PLT (10^3/mm^3)	865 ± 9.8	826 ± 9.9	813.3 ± 8.7	859 ± 8.9	823.3 ± 7.1
MPV (fL)	7.05 ± 0.91	7.09 ± 0.98	6.73 ± 0.11	6.09 ± 0.65	6.83 ± 0.01
PDW (fL)	9.05 ± 0.35	9.01 ± 0.6	8.43 ± 0.40	8.01 ± 0.31	8.93 ± 0.25

Table 3.

Organ	Control	CELR 500 mg/Kg	CELR 2000 mg/Kg	AFLR 500 mg/Kg	AFLR 2000 mg/Kg
Kidney (R)	0.33 ± 0.04	0.34 ± 0.04	0.33 ± 0.03	0.34 ± 0.04	0.34 ± 0.03
Kidney (L)	0.35 ± 0.06	0.33 ± 0.06	0.32 ± 0.09	0.38 ± 0.01	0.33 ± 0.01
Adrenal (R)	0.07 ± 0.001	0.08 ± 0.001	0.06 ± 0.001	0.05 ± 0.002	0.05 ± 0.001
Adrenal (L)	0.06 ± 0.001	0.08 ± 0.001	0.05 ± 0.001	0.09 ± 0.003	0.06 ± 0.003
Liver	3.41 ± 0.54	3.34 ± 0.51	2.86 ± 0.33	3.20 ± 0.23	2.89 ± 0.12
Spleen	0.244 ± 0.04	0.154 ± 0.01	0.177 ± 0.01	0.234 ± 0.06	0.189 ± 0.01
Heart	0.315 ± 0.02	0.314 ± 0.03	0.309 ± 0.01	0.307 ± 0.02	0.301 ± 0.01
Lung	0.52 ± 0.007	0.55 ± 0.006	0.511 ± 0.07	0.55 ± 0.008	0.450 ± 0.02
Stomach	0.95 ± 0.14	0.94 ± 0.17	1.66 ± 0.28	0.89 ± 0.18	1.36 ± 0.27

Figures:

Figure 1.

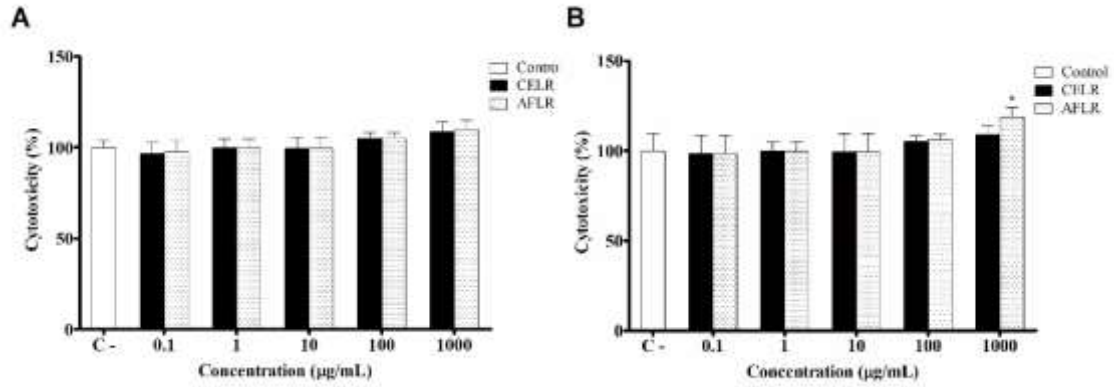


Figure 2.

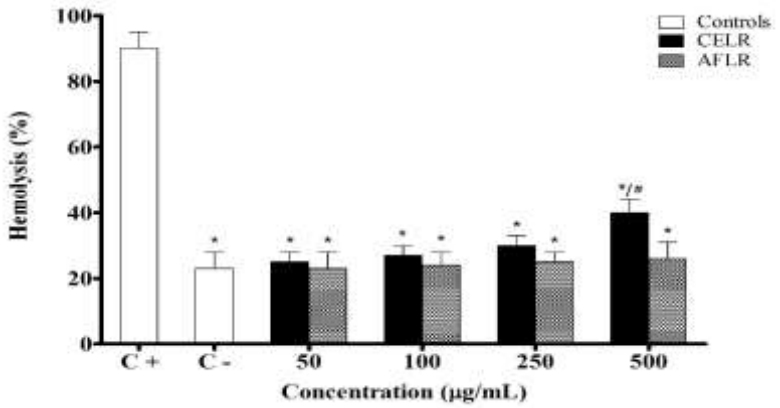


Figure 3.

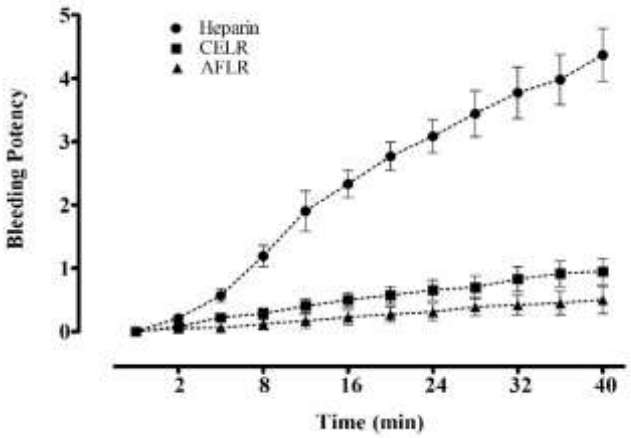
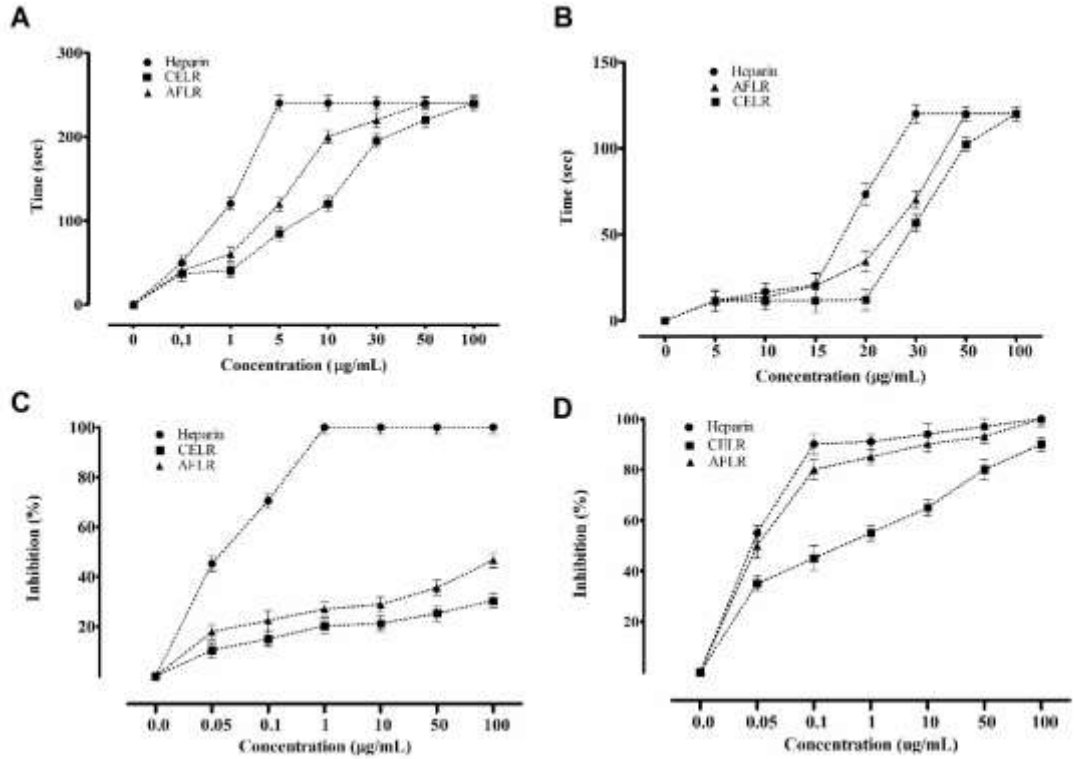


Figure 4.



5.2 Article 2

The article "EXTRACTS OBTAINED FROM LEAVES OF *Turnera subulata* INHIBITS BLOOD COAGULATION" was submitted to the *Journal of Pharmacy and Pharmacology* which has an impact factor 2.363, Qualis B1 for CAPES, for area of Medicine II.

Extracts Obtained from Leaves of *Turnera subulata* Inhibits Blood Coagulation

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Abstract

Objectives This study aimed to investigate the anticoagulant activity, toxicity and hemorragic effects of a crude extract and an ethyl acetate fraction from *Turnera subulata* leaves, a plant found in tropical and subtropical regions.
Methods The chemical composition of the samples was determined by HPLC-DAD. Toxicity *in vitro* was evaluated by MTT assay in normal cell lines (3T3 and HEK-293). Acute oral toxicity test was performed in *Wistar* rats (200-300g) in accordance to the OECD guidelines by evaluating hematological and biochemical parameters related to liver and kidney functions. The bleeding assay was performed by scarification test in rat tail. Activated partial thromboplastin time (aPTT), Prothrombin Time (PT) and inhibition of Xa and IIa factors were used to assess the anticoagulant potential.
Key findings Phytochemicals findings indicated the presence of compounds with similar RT and UV spectrum to flavonol -3-O-glycosilate and a flavone glycoside stood out as the majority class of chemicals, toxicity wasn´t reported by the experimental data with low hemorragic effects. Antihyperglycemic and hypolipidemic actions were indicated by the 50% reduction in glucose, triglycerides and total cholesterol levels in the acute toxicity model. Moreover, a strong anticoagulant activity was found in the tests, suggesting the direct thrombin inhibition as a main action mechanism.
Conclusions The leaves of *T. subulata* have active constituints with potential for therapeutic purposes and are promising for future studies aiming at the development of herbal formulations to assist the anticoagulant drugs used currently in therapy clinic.

Introduction

Despite progress in their diagnosis and treatment, thromboembolic diseases remain as causes of the high morbidity and mortality globally. [1-2] Arterial thrombosis is the most common cause of acute myocardial infarction, strokes and ischemia, on the other hand, the complications of deep vein thrombosis include pulmonary embolism and post-thrombotic syndrome. [3] The coagulation system in interaction with platelet aggregation is centrally involved in the formation of arterial and venous thrombus. [4]

Indications currently defined for anticoagulants include the prophylaxis and treatment of venous thromboembolism, cardioembolic prevention in patients with cardiac arrhythmia or mechanical valve protheses and secondary prevention in patients with acute coronary syndromes or undergoing percutaneous coronary intervention. [5] Unfractionated and low molecular heparins are used as anticoagulant drugs. However, these compounds are accompanied by several side effects such as thrombocytopenia and a high risk of systemic bleeding. [6-7] These side effects generated the need for new substances in order to assist prolonged anticoagulant therapy. [8]

Herbal extracts have shown a proven ability to inhibit blood coagulation cascade especially inhibiting the intrinsic and extrinsic pathways, including their. factors. [9-10] Some species of *Turnera* (family Passifloraceae) are widely distributed in tropical and subtropical regions. [11] They are some scientific evidence properties such as anti-inflammatory [12-13], antihyperglicemic [14], antifungal [15] and antioxidant [16]. Phytochemical studies have revealed that this genus contains various chemical constituents, including flavonols, alkaloids, tannins, cyanogenic glycosides, fatty acids, triterpenoids and phenolic compounds [17].

According to the literature, *Turnera* genus has been indicated as an interferent in the inflammatory process. Since the coagulation and inflammatory systems are closely linked, thrombin is the main link between these two pharmacological activities and considering the side effects of the currently used anticoagulants and the need for new substances with fewer side effects that would help the drugs currently used in the clinic, the objective of this study was to evaluate the anticoagulant potential, toxic and hemorrhagic effects of a crude extract and ethyl acetate fraction from *Turnera subulata* leaves.

Material and Methods

General

MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) and standard sugars were purchased from Sigma Chemical Company, St. Louis, MO, USA. Cell culture medium components (Dulbecco's Modified Eagle Medium-DMEM), trypsin and fetal calf serum (FCS) were obtained from Cultilab (Campinas, Brazil). L-glutamine, sodium bicarbonate, sodium pyruvate and phosphate buffered saline (PBS) were purchased from Invitrogen Corporation (Burlington, ON, USA). Dimethyl sulfoxide (DMSO), aPTT and PT kits were purchased from CLOT Bios Diagnostica (São Paulo, SP, Brazil). Biophen Heparin Anti-Xa and Anti-IIa kits were purchased from HYPHEN Biomed (Paris, France).

Collection and preparation of the crude extract and ethyl acetate fraction of *Turnera subulata* leaves (CETS and AFTS, respectively)

Leaves of *T. subulata* were collected in Natal, Rio Grande do Norte, Brazil (SS-RN 5°52'17" S/35°10'45" O), on November 2014. The plant material was identified by Dr. Jomar Gomes Jardim, and a voucher specimen was deposited at the Herbarium of the Department of Botany and Zoology, Federal University of Rio Grande do Norte, Natal, RN, Brazil, under the reference number 0674/08. *T. subulata* leaves were dried in a circulating air oven at 40 °C for 48 h and triturated mechanically. The extract from *T. subulata* leaves was prepared by maceration in ethanol:water (50:50, v/v). After four days, the sample was filtered and later lyophilized. Thus, the hidroethanolic extract (crude extract) from *T. subulata* leaves (CETS) was obtained.

In order to characterize the active compounds from CETS, a portion of the extract was resuspended in metanol and subjected to liquid-liquid partition with solvents of increasing polarity: n-hexane (3x300mL) and ethyl acetate (3x300mL). Two fractions were obtained (HFTS and AFTS, respectively). The amount of phenolic compound was measured in both fractions using Folin-Ciocalteau method, described previously [18] (data not show). Since these molecules were absent in HFTS, it was not used in the subsequent assays.

Chemical procedures (*HPLC-DAD analysis)*

Reverse phase chromatographic analyses were carried out on a Phenomenex C18 chromatography column (4.6×100 mm, particle size 2.6 µm, Torrance, CA, USA) using a HPLC (VARIAN ProStar HPLC system, Walnut Creek, CA, USA) equipped with a ProStar 240 quaternary pump, autosampler (ProStar 410) and a detector (mod. 355 PDA UV/V). CETS and AFTS (5 mg/mL) were dissolved in methanol, as described previously [16]. The separation

was conducted at room temperature with a flow rate of 1.3 mL/min. Mobile phase was water containing 0.1% formic (A phase) and acetonitrile (B phase) under following gradient conditions: 0-3 min, 5% B; 3-7 min, 5-20% B; 7-9 min, 20% B; 9-10 min, 2-23%, B; 10-15 min, 23% B; 15-19 min, 23-50% B; 19-20 min, 50-5% B. The injection volume was 9 µL. All the chromatography operations were carried out at ambient temperature and in triplicates. Chromatograms were recorded at 280 nm. All solutions were filtered through a 0.22 µm membrane (Millipore, Billerica, MA, USA). Chromatography peaks were identified by comparing its retention time with those of reference standards (gallic acid, chlorogenic acid, epigallocatechin, rutin, hiperin, quercetin, apigenin, kaempferol) and by DAD spectra (200 to 600 nm).

Activated partial thromboplastin time (aPTT) assay

The test was performed in accordance with the aPTT information kit (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CETS and AFTS were dissolved in saline at various concentrations (0.1, 1, 5, 10, 30, 50, and 100 µg/mL) and 10 µL of these solutions were incubated with 90 µL of plasma at 37 °C for 3 min. Then, 100 µL of bovine cephalin was added and incubated at 37 °C. After 3 min of incubation, 100 µL of pre-warmed 0.25 M $CaCl_2$ solution was added to the mixture and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

Prothrombin time (PT) assay

The test was performed in accordance with the PT kit information (CLOT Bios Diagnostica, São Paulo, SP, Brazil). Heparin, CETS and AFTS were dissolved in saline at various concentrations (5, 10, 15, 20, 30, 50, and 100 µg/mL) and 10 µL of these solutions were incubated with 90 µL of plasma at 37 °C for 3 min. Then, 200 µL of Soluplastin reagent was added and the clotting time was measured in triplicate using a Clot Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

Assay for the anti-Xa activity

The anti-Xa activity assay was conducted in a 96-well microplate, using the Biophen Heparin Anti-Xa kit (HYPHEN Biomed, ref: 221010, Paris, France), according to the manufacturer's instructions. Briefly, 40 µL of antithrombin (AT) was incubated at 37 °C for 2 min in the presence of increasing concentrations of heparin, CETS and AFTS diluted in buffer with a pH of 8.4 (0.05 M Tris, 0.175 M NaCl, 0.0075 M EDTA, containing 0.1% polyethylene glycol). Then, 40 µL of purified bovine Factor Xa (FXa) was added to each well, mixed, and incubated at 37 °C for 2 min. After, 40 µL of a chromogenic substrate for FXa was added and

the mixture was incubated for 2 min at 37 °C. Following incubation, 80 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured at 405nm in Epoch Microplate Spectrophotometer (Epoch-Biotek, Winooski, VT, USA).

Thrombin Inhibition Assay

The thrombin Inhibition assay was conducted in a 96-well microplate, using the Biophen Heparin Anti-IIa kit (HYPHEN Biomed, ref: 221025, Paris, France), according to the manufacturer's instructions. Briefly, 50 µL of thrombin was incubated at 37 °C for 2 min in the presence of increasing concentrations of heparin, CETS and AFTS, previously diluted in citrated fresh human plasma and again diluted in AT. After the addition of 50 µL of factor IIa (thrombin), purified bovine serum/albumin? was homogenized in each well and incubated at 37 °C for 2 min. Then, 50 µL of chromogenic substrate for FIIa was added to homogenates and incubated at 37° C for 2 min. After incubation, 50 µL of 30 % acetic acid was added to stop the reaction and the absorbance was measured at 405 nm in Epoch Microplate Spectrophotometer (Epoch-Biotek, Winooski, VT, USA).

Residual hemorrhagic effects

The residual hemorrhagic effect of CETS and AFTS compounds were analyzed by a modified model of topical scarification in the rat tail, as described previously [19]. After anesthesia with ketamine and xylazine in a 1:1 (v/v) proportion, a scar was made with a surgical blade in the distal portion of the tail. Then, the tail was then dipped vertically in physiological saline solution, dabbed with gauze and dipped again in fresh saline to observe bleeding. The tail was dipped in solutions containing CETS, AFTS, or heparin at concentration of 100 µg/mL for 2 min and then washed extensively with saline solution. The treated tail was immersed in new physiological saline solutions for 40 min and blood was quantified using the Drabkin assay. The results were expressed as the sum of the hemoglobin values of each tube minus the hemoglobin value present prior to exposure of the test substance.

MTT assay (cytotoxicity)

The mouse fibroblast cells (3T3) and epithelial embryonic human kidney cells (HEK 293) were cultured under standard conditions in Dulbecco´s modified Eagle´s medium (DMEM), supplemented with fetal bovine serum (FBS) at a final concentration of 10%. Cells were maintained in cell culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2. Cells (1 x 10^4cells per well) were cultured for 24 h in 96-well microplates to promote adhesion. After 24 h, cells were treated with different concentrations of CETS and AFTS (0.1, 1, 10, 100, and 1000 µg/mL) in triplicate and incubated at 37 °C for 24 h. After this period, 100 µL of MTT (5 mg/mL) dissolved in DMEM was added to each well and the cells were incubated again.

After 4 h, the culture medium was removed and then 100 µL of DMSO was added to each well. The reading was performed at 570 nm with a microplate reader (Epoch-Biotek, Winooski, VT, USA).

Animals

Wistar rats (*Rattus norvegicus*), 250-300g, three-months old, from both sexes, provided by the vivarium of the Health Sciences Center from Federal University of Rio Grande do Norte were used. The animals were maintained under standard environmental conditions and fed with food and water *ad libitum*. All the procedures involving rats were done in agreement with the recommendations of the Brazilian National Health Surveillance Agency (ANVISA) and the Organization for economic cooperation and development (OECD). The experimental protocols were approved by the Ethics Committee on Animal Use of Federal University of Rio Grande do Norte (protocol n°035/2015).

Acute Oral Toxicity

The acute oral toxicity test was performed according to experimental protocol of the Brazilian National Health Surveillance Agency nr. 90 (2004) [20] and OECD (2001) [21]. This evaluation used five groups, with gavage doses of 500 and 2000 mg/kg, compared to a control group that received only distilled water, not exceeding 1 mL/100 g of body weight. In the first 12 h, systematic behavioral observations were made (vocal tremor, piloerection, hyperactivity, tremors, abdominal cramps, diarrhea, and number of deaths). At the end of 14 days, the animals were euthanized with an overdose of sodium thiopental (100 mg/kg) by intraperitoneal (i.p) and then underwent laparotomy for blood collection by cardiac puncture and evisceration. Liver, kidney, spleen, lung, heart, intestine, stomach, esophagus, and brain were removed for macroscopic and relative weight evaluations.

Biochemical and hematological parameters

The hematological parameters analyzed were total count of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean concentration hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), volume change mean corpuscular (RDW), the total count (WBC) and differential leukocytes (lymphocytes, monocytes and granulocytes), procalcitonin (PCT), platelets (PLT), mean platelet volume (MPV) and platelets distribution width (PDW). Total protein, albumin, globulin, alanine aminotransferase enzymes (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (γ-GT), and bilirrubin were evaluated as liver function markers. Creatinine and urea were determined for renal evaluation. Other parameters analyzed were total cholesterol,

triglycerides, amylase, and glucose. Hematological tests were performed through ABX Micros 60 OT Equipment (ABX Diagnotics, France) and biochemical parameters by commercial kits.

Statistical analyses

Results were analyzed by a one-way ANOVA and Tukey's post hoc test. Values of $p < 0.05$ were considered indicative of statistical significance. The analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

Results

Chemical procedures (HPLC-DAD analysis)

HPLC analysis of CETS and AFTS revealed the presence of chromatographic peaks consistent with the pattern showed by the standards, such as rutin and apigenin (Figure 1). The groups were identified by comparison between UV spectra and retention times (RT) of the extracts and external standards (Table 1).

HPLC analysis of the extracts indicated the presence of compounds with a similar RT and UV spectrum to rutin (flavonol -3-O-glycosilate) with values (121,51 μg Eq/g CETS) and (262,36 μg Eq/g AFTS), and a compound with a similar UV spectrum of apigenin (flavone glycoside), with values of 80.57 μg Eq/g (CETS) and 252.48 μg Eq/g (AFTS).

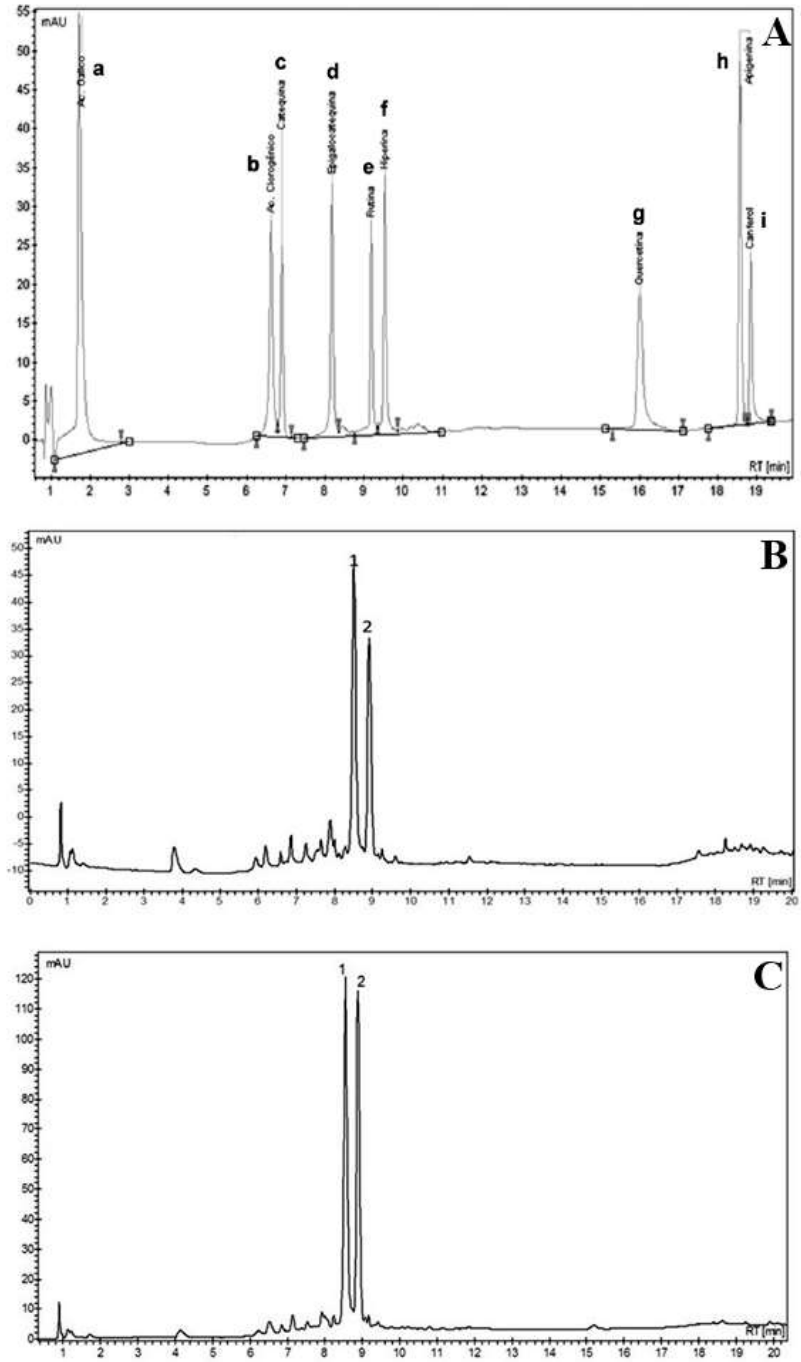

Figure 1 HPLC profile of phenolic compounds from *Turnera subulata* leaves, detected at 280nm. Profile (A): HPLC-UV chromatogram of standard phenolic compounds, (a) Gallic acid; (b) chlorogenic acid; (c) catechin; (d) (-)epigallocatechin gallate; (e) rutin; (f) hiperin; (g) quercetin; (h) apigenin; (i) kaempferol. Profile (B): Crude extract of *T. subulata* with two majoritariam peaks (1) related to flavonol-3-O-glycosilate like rutin (2) related to flavone glycoside like apigenin. Profile (C): Ethyl acetate fraction of *T. subulata* with two majoritariam peaks (1) related to flavonol-3-O-glycosilate like rutin (2) related to flavone glycoside like apigenin.

Table 1 Quantification parameters of nine phenolic compounds identified by comparison between UV spectra and retention times (RT) of the extracts and external standards based on the chromatographic method.

Compound	Retention Times (min)	Linear Range (μg/mL)	UV (nm)	R^2
Gallic acid	1.71	1.5-50	271	0.9945
Chlorogenic acid	6.47	1.5-50	326	0.9973
Catechin	7.19	1.5-50	278	0.9993
(-) Epigallocatechin gallate	8.18	1.5-50	275	0.9989
Rutin	9.20	1.81-100	275-354	0.9976
Hiperin	9.24	1.81-100	256-354	0.9995
Quercetin	16.03	1.5-50	255-371	0.9991
Apigenin	18.51	1.5-50	266-337	0.9989
Kaempferol	18.85	1.5-50	263-367	0.9984

Anticoagulant activity of *Turnera subulata*

The anticoagulant activity of CETS and AFTS has been demonstrated experimentally by testing the activated partial thromboplastin time and prothrombin time, as the factors X and II activated.

Heparin used in clinical was tested as a standard positive control, where a significant anticoagulant aPTT activity of more than 240 seconds (negative control: 36.05 ± 0.03 s) and PT greater than 60 seconds (negative control: 16.65 ± 0.33 s) were expected. In figure 2 (A), at 5 μg/mL, heparin, prolonged the clotting time for more than 240 s, detected by aPTT and in figure 2 (B), at 30 μg/mL, heparin prolonged the clotting time for more than 120 s, detected by PT. The same effects is achieved by CETS and AFTS at a higher concentration (100 μg/mL), demonstrating that both extracts are able to inhibit the clot formation via the intrinsic and extrinsic pathway.

Following, we examined whether heparin and extracts were able to inhibit directly the activity of Xa and IIa (thrombin) factors. As shown in figure 3 (A), heparin was able to hamper the activity of Xa factor in a dose dependent concentration, only 1 μg/mL was necessary to inhibit totally the factor. In figure 3 (B), heparin was able to completely hamper the activity of

thrombin at 100 μg/mL. Despite its lower activity, CETS and AFTS were capable of inhibiting the Xa factor activity in a concentration-dependent manner, reaching approximately 40 % of inhibition at about 100 μg/mL, as well as, the extracts were able to curb the thrombin activity, reaching nearly 80 % (AFTS) and 40 % (CETS) of inhibition at about 100 μg/mL.

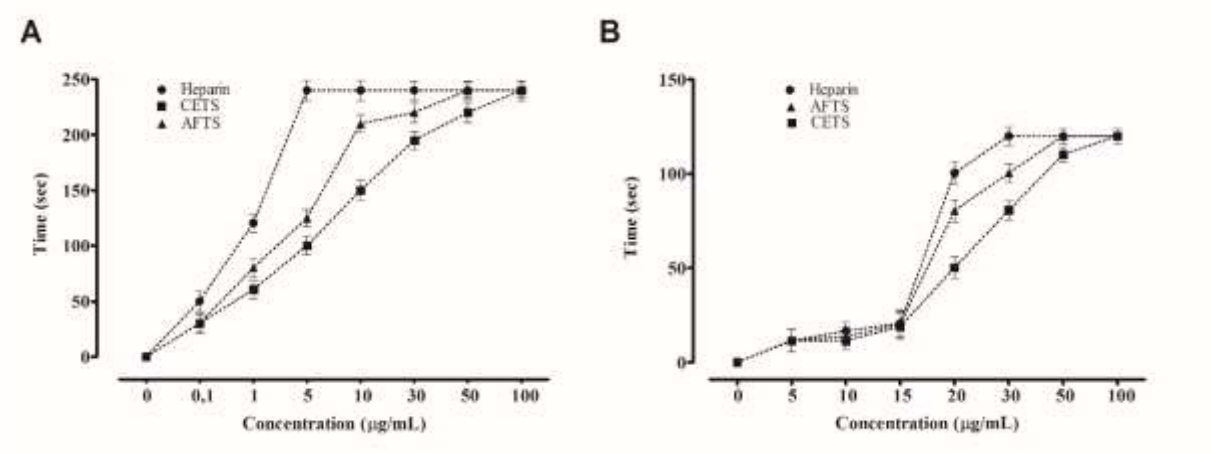

Figure 2 Anticoagulant activity of CETS, AFTS, and Heparin by activated partial thromboplastin time (aPTT) assay (A) and Prothrombin time (PT) assay (B).

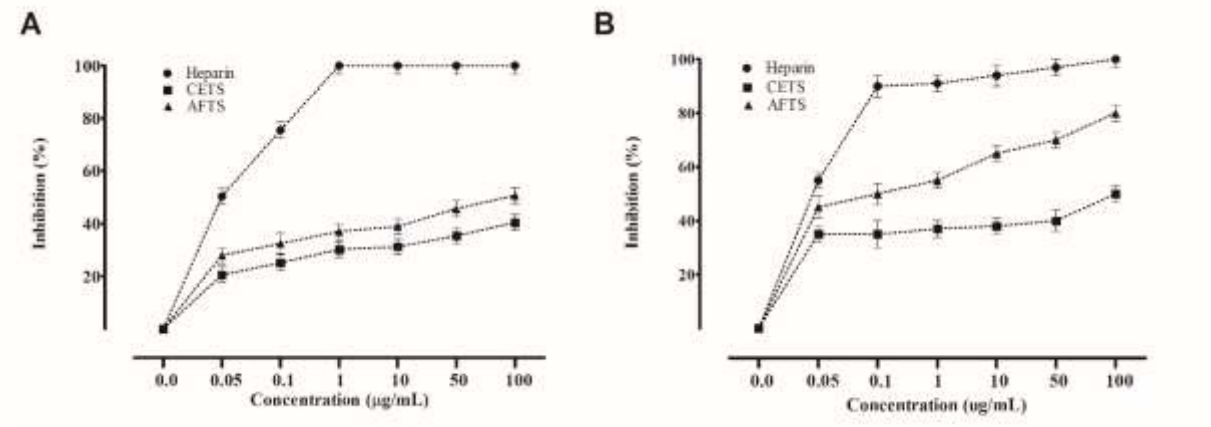

Figure 3 Anticoagulant activity of CETS, AFTS, and Heparin by inhibition of factor Xa activity (A) and inhibition of Thrombin activity (B).

Evaluation of hemorrhagic activity

The clinical use of heparin is limited by some inconvenient effects as thrombocytopenia and hemorrhagic complications due to its ability to interfere in the hemostatic balance. Thus, its of relevant importance to investigate the effects of CETS and AFTS on hemostasis. Figure 4 shows that heparin (100 μg/mL) possess a potent hemorrhagic effects, demonstrating a high level of residual bleeding through the scarification model by rat tail in a dosage of hemoglobin of treated animals.

Interestingly, CETS, despite, its anticoagulant potential, had a lower hemorrhagic action that commercial heparin at the concentration tested (100 μg/mL) on average 50% less side effect. Ultimately, AFTS, representing a fraction of the anticoagulant agent is most concentrated had an insignificant effect on bleeding (on average of 10%) when compared to clinical heparin at the same concentration tested, showed a negligible potential of bleeding.

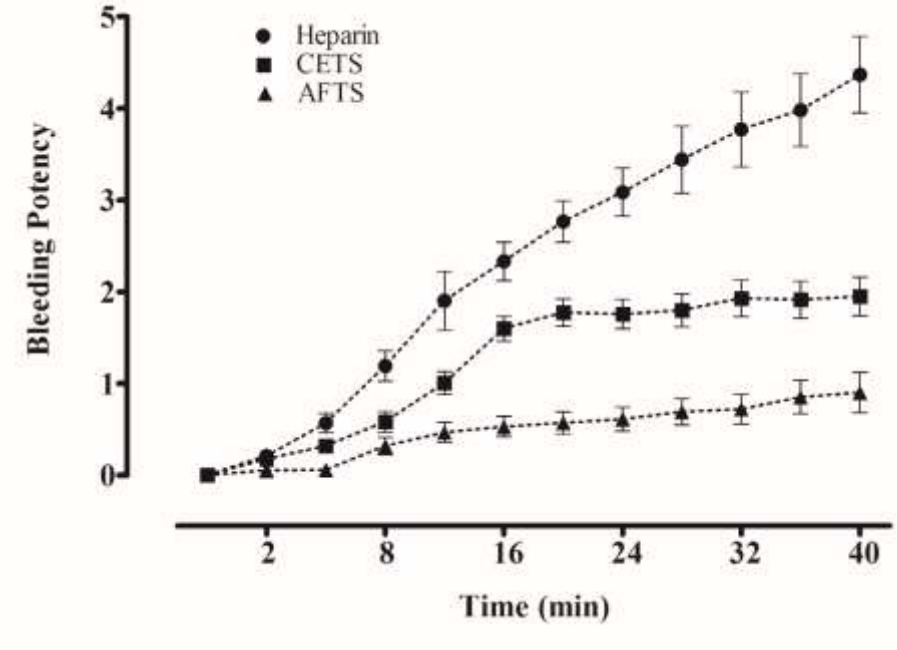

Figure 4 Bleeding activity of CETU (100 μg/mL), AFTU (100 μg/mL) and porcine intestinal mucosa heparin (100 μg/mL) applied topically. The bleeding potency measured after 2 min following saline solution washing.

MTT assay (cytotoxicity)

In order to verify the cytotoxic levels of the extracts studied, MTT assay was carried out with normal cell lines. The assessment of the cell viability of CETS and AFTS showed no toxicity to the mice fibroblast cells (3T3) (Figure 5A) and human embryonic kidney cells (HEK-293) (Figure 5B). No statistically significant differences in any of the tested concentrations were observed compared to the negative control (DMEM). The results show that CETS and AFTS extracts did not cause any toxic effect *in vitro* with respect to normal cells tested in this study.

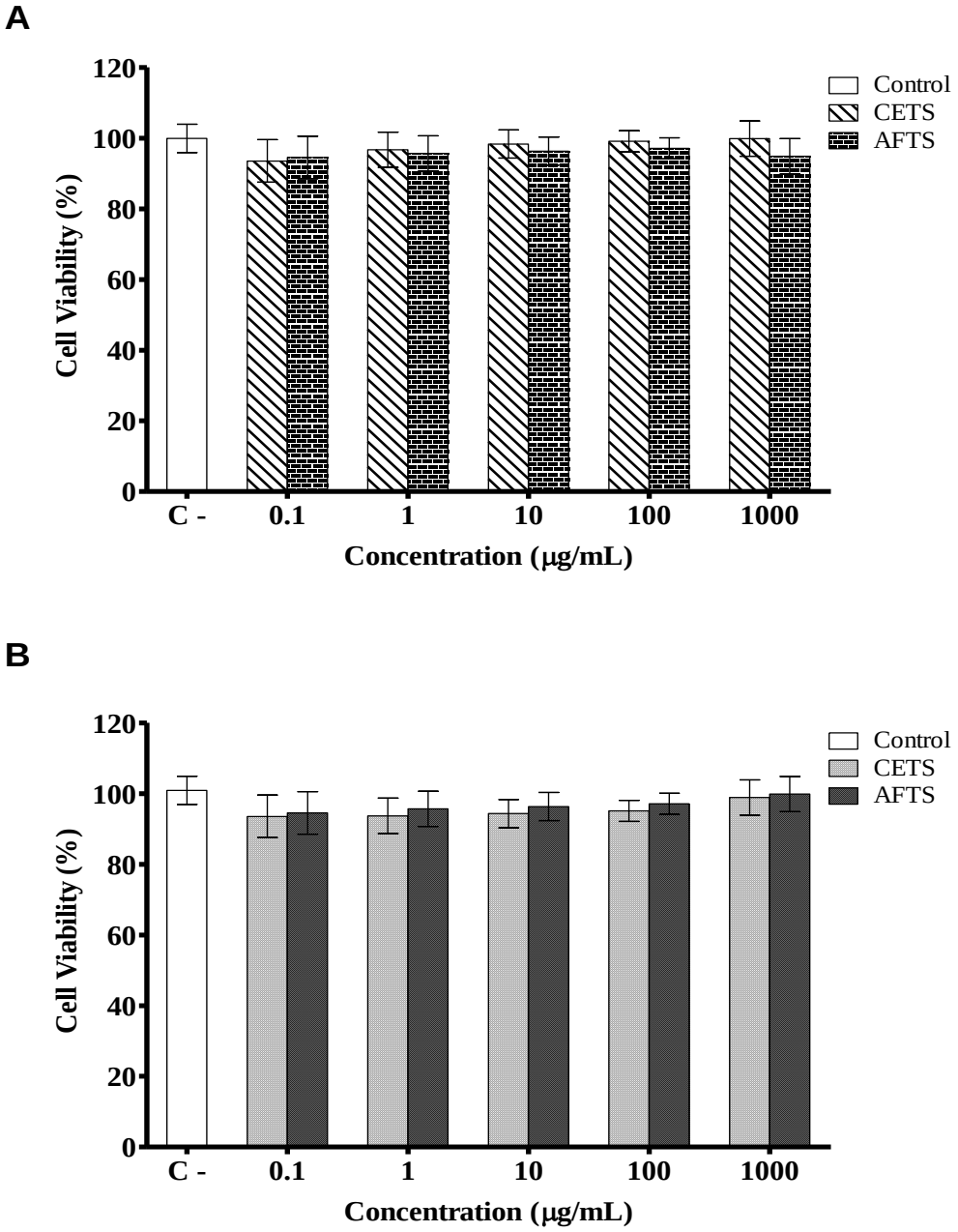

Figure 5 Cell viability (Cytotoxicity effects) of CETS and AFTS on mouse fibroblast cells (3T3) (A) and epithelial embryonic human kidney cells (HEK 293) (B), measured by MTT assays. Culture medium DMEM was used as a negative control of cytotoxicity. Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test.

Acute Toxicity

With regard to behavioral effects, no episode described in methods was observed. Table 2 show the analysis of biochemical parameters of animals (treated and control). Statistically significant data were found on three biochemical parameters analyzed when comparing the treated group and the control group with received distilled water. Glucose, triglycerides and total cholesterol levels decreased on average by 50% following treatment with the extracts.

Hematological parameters and the weights of organs after euthanasia showed no significant differences compared to that in the control. The tables relating to hematological parameters and relative weight of organs can be found in the supplementary material. Regarding the behavioral clinical parameters, such as water and food consumption, and body weight of the animals (data not shown), there were no signs of toxicity.

Table 2 Biochemical parameters of rats after 14 days of treatment with CETS and AFTS.
Results are expressed as mean ± SD (n = 5); Control group treated with vehicle (distilled water). Comparisons between groups were analyzed with an ANOVA and Tukey's post hoc test.
* $p < 0.05$ compared with the control group.

Biochemical parameters	Control 2000mg/kg	CETS 500 mg/Kg	CETS 2000 mg/Kg	AFTS 500 mg/Kg	AFTS 2000 mg/Kg
Glic (mg/dL)	122 ± 21.9	60 ± 20.1*	61.4 ± 21.6*	60.1 ± 19.2*	62 ± 21.4*
Trig (mg/dL)	53 ± 5.45	25 ± 3.89*	28.6 ± 4.38*	25 ± 2.32*	28.7 ± 4.34*
Col (mg/dL)	54.5 ± 6.05	24.5 ± 0.24*	22 ± 0.19*	25.5 ± 0.19*	21.5 ± 0.10*
ALT (U/L)	97 ± 4.24	86.1 ± 2.30	89 ± 2.20	89.9 ± 4.34	97 ± 5.29
AST (U/L)	250 ± 23.3	238 ± 25.3	249 ± 15.3	228.5 ± 24.8	240.3 ± 14.1
γ-GT (U/L)	12 ± 1.4	11 ± 1.5	10.66 ± 0.05	12 ± 0.23	11.5 ± 0.04
TB (mg/dL)	0.99 ± 0.01	0.90 ± 0.01	0.93 ± 0.01	0.90 ± 0.01	0.99 ± 0.02
BD (mg/dL)	0.99 ± 0.07	0.98 ± 0.05	0.97 ± 0.09	0.99 ± 0.05	0.110 ± 0.1
BI (mg/dL)	0.28 ± 0.01	0.13 ± 0.03	0.12 ± 0.09	0.15 ± 0.03	0.15 ± 0.08
Urea (mg/dL)	54.5 ± 7.7	54.5 ± 7.5	57.3 ± 7.04	55.9 ± 7.5	58.3 ± 7.75
Cret (mg/dL)	0.65 ± 0.07	0.65 ± 0.07	0.7 ± 0.03	0.59 ± 0.19	0.70 ± 0.07
TP (g/dL)	6.6 ± 0.1	6.2 ± 0.5	6.3 ± 0.64	6.1 ± 0.5	6.9 ± 0.6
ALB (g/dL)	3.1 ± 0.42	3.9 ± 0.42	2.9 ± 0.34	3.1 ± 0.42	3 ± 0.12
Glo (g/dL)	3.5 ± 0.42	3.8 ± 0.35	4.3 ± 0.54	3.1 ± 0.32	4.82 ± 0.54
Ami (U/L)	900 ± 3.53	900 ± 3.44	903 ± 2.09	902.5 ± 3.43	913.8 ± 2.07

Discussion

The genus *Turnera* (family Passifloraceae) has been well documented as presenting anti-inflammatory, antidiabetic and antiobesity properties in the literature[13, 17]; however, its anticoagulant activity have never been described.

Brito et al. [16] has showed high levels of phenolic compounds in *Turnera ulmifolia* Linn. var. *elegans* specie, including the presence of compounds with a similar RT and UV spectrum to flavonol -3-O-glycosilate like rutin and a flavone glycoside like apigenin at leaf hydroethanolic extract as majoritariam compounds, same founded in this paper. As expected, ethyl acetate fraction of *T. subulata* includes almost the same compounds when assessed by HPLC-UV method.

Plant extracts have been studied using anticoagulant tests, which are based on the ability to inhibit blood coagulation factors. [22] The family Passifloraceae has been studied for the treatment of cardiovascular diseases for many years, *Passiflora nitida* Kunth demonstrated a significant anticoagulant activity of the activated partial thromboplastin time test, suggesting an inhibitory effect of one or more factors of the intrinsic pathway of the coagulation cascade (factors VIII, IX, XI, XII). [23] The results of this work, showed that in addition to inhibiting the intrinsic pathway of blood coagulation cascade, CETS and AFTS, inhibited extrinsic pathway of the coagulation cascade by prothrombin time test, also suggesting that anticoagulant action of CETS and AFTS are more strongly due to the inhibition of thrombin (common pathway cascade). Experiments such as the inhibition of heparin cofactor II still need to be performed in order to uncover the actual mechanism of action of *Turnera subulata* over blood clotting inhibition.

Heparin has been used in clinical therapy for many years for its anticoagulant capacity, however its prolonged use may cause bleeding, followed by thrombocytopenia, osteoporosis, hypersensitivity, and necrosis of the skin. Effects such as bleeding, intestinal toxicity and others are seen with prolonged use of warfarin, a modified coumarin isolated from the genus Melilotus. [24-25] Considering this problems, the search for new substances that can help the anticoagulant drugs used in the clinic are now encouraged. However, unfortunately, bleeding assessment tests are not widespread in the scientific community regarding herbal plants.

With proven anticoagulant activity and having low hemorrhagic action, the efforts of this work were managed to find out if CETS and AFTS possessed some toxicological effects, therefore, toxicity *in vitro* and *in vivo* assays were performed. Cell lines of mice fibroblast and human embryonic kidney did not show toxicity signals, when compared with negative controls.

Avelino-Flores et al. [26] working with a methanol extract of *Turnera diffusa* specie, reported a low cytotoxicity in human normal fibroblast, without serious damage at 50μg/mL suggesting a safe action.

The evaluation of acute oral toxicity (*in vivo*) showed that the extracts have caused no toxic effects in biochemical and hematological parameters tested, especially in relation to hepatic and renal parameters. There are no data in the literature on the toxic potential of *Turnera subulata*, however, studies with species of the same genus corroborate the findings of the present article. Acute oral toxicity was related in *Turnera diffusa* Willd specie, that presented a low acute toxicity in an experiment using Sprague Dawley rats, showing no abnormal signs, behavioral changes, mortality or differences between control and treatment groups in relation to histological examination of kidney and liver, as weel as, biochemistry parameters, increasingly demonstrating the safe use of this genus. [27]

The results of the biochemical parameters evaluated in this paper (decreased values of glucose, triglycerides, and total cholesterol after the treatment with CETS and AFTS) suggest possible antihyperglycemic and hypolipidemic properties of these extracts, contributing to the use of CETS and AFTS as herbal medicines in the treatment of cardiovascular diseases. Plant species belonging to family Passifloraceae are described in the literature as antihyperglicemic, especially species of the Turnera genus [14,17,28].

Szewczyk and Zidorne [17] showed in a review of the *Turnera diffusa* antidiabetic actions the reduction in the hyperglycemic peaks at 15.9% in the experimental model using diabetic rabbits, even as, in alloxan-induced diabetic mice research model, observing a hypoglycemic activity of this kind. A methanolic extract of leaves of *Turnera ulmifolia* was evaluated for its potential activity on blood glucose levels in alloxan-induced diabetic and euglycemic rats, the results demonstrated an antihyperglycemic and euglycemic actions. [29]

The results of this paper are consistent with the pharmacological properties of inhibiting coagulation reported in the literature for flavonoids and flavones [29-30] suggestively present in phytochemical characterization performed in this study.

Conclusions

In phytochemical characterization of leaves of *Turnera subulata* the results indicated the presence of compounds with a similar RT and UV spectrum to flavonol -3-O-glycosilate and a flavone glycoside stood out as the majority class of chemicals, which can be attributed, in part, the biological effects studied. The anticoagulant activity of CETS and AFTS shows an

inhibition off all pathway of the blood coagulation cascade (intrinsic, extrinsic and common pathway), suggesting a direct thrombin inhibition as the main action mechanism, with low hemorrhagic effects. The extracts also showed low toxicity *in vitro* and *in vivo* experiments, showing a possible antihyperglycemic and hypolipidemic actions by biochemical analysis in the acute oral toxicity model in healthy *Wistar* rats. The leaves of *T. subulata* have active constituents with potential for therapeutic purposes and are promising for future studies aiming at the development of herbal formulations to assist the anticoagulant drugs used currently in clinical therapy.

Declarations

Conflict of interests

The authors have no conflict to declare.

Acknowledgements

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6 COMMENTS, CRITICAL AND CONCLUSIONS

The initial project entitled "Cytotoxicity in vitro and determination of trace minerals from plant extracts of medicinal species of the Northeastern semiarid region correlated to the antioxidant capacity and hemostasis" aimed to assess the cytotoxic activity and mineral content of extracts of *Turnera subulata*, *Spondias sp* and *Pseudobombax marginatum*, medicinal species of the Northeastern semiarid region correlated with antioxidant capacity and hemostatic profile. The participation of various minerals in cellular and/or toxic physiological processes is critical.

Micronutrients (e.g., zinc, copper, selenium) has a central role in maintaining the immune system and antioxidants affecting the micronutrient-gene interactions with anti- and pro-inflammatory functions. The proposal focused on the use of leaf extracts of endemic species of flora northeastern used in folk medicine for the treatment of diseases.

One of the main difficulties in the progress of the research was in relation to financial resources. The project was submitted to Universal notice MCTI/CNPq No. 14/2014 - Track B, but was not approved. In order to perform all the analyzes provided it was necessary to establish some partnerships. It was also required the collaboration of other research grants of the Multidisciplinary Laboratory (LabMult), particularly considering the reagents and overall material consumption. The Potiguar University (UnP) was also a major partner, contributing with reagents, kits and consumables.

The bad functioning of the Atomic Absorption Spectrophotometer from our laboratory, as well as the lack of funding for the purchase of multi-element lamps, were the great watershed in this research. We decided to abandon the analyzes of trace elements, such as the antioxidant analyzes, since our objective was to correlate the presence of minerals with this activity.

The focus of the research project then proceeded to evaluate the impact of our plant extracts in the hemostatic system in proposing to identify one or more extracts that possess anticoagulant activity with low bleeding effects and can be used as herbal medicines in aid anticoagulant drugs used currently in the clinic have a number of limitations to the prolonged use to cause significant side effects in the treatment of cardiovascular diseases with emphasis on venous thromboembolism.

Among the limitations identified, we emphasized the lack of financial resources for the purchase of specific kits for assessing the anticoagulant activity and the failure

of two of the study species in interfere with blood clotting system. *Spondias sp* and *Pseudobombax marginatum* did not inhibit the intrinsic and extrinsic pathways of the coagulation cascade by aPTT and PT tests, in a pilot study. Thus, our efforts were directed to *Turnera subulata* and other species studied by our research group, the *Licania rigida* Benth, which were already known ethnopharmacological as anti-inflammatory.

However, despite all the difficulties, the results were of great value to clarify the bioactive and pharmacological potential of the plant biodiversity that Brazil provides, and to assist the scientific community in the search for new substances that can assist in treatment of thromboembolic events, since together other cardiovascular diseases account for a mortality rate of 30% globally.

The execution of this work will allow the further development and expansion studies in a subject in which the student is interested since his undergraduate and runs through his professional work as Biologist. Throughout this process, we developed a lot of skills and competencies in relation to knowledge and implementation of necessary laboratory techniques for this research.

The extracts action research goals of *L. rigida* and *T. subulata* on hemostasis were achieved due to the commitment of the team involved in the project. As a future perspective, is carrying out work in order to formulate a standardized herbal medicine that can aid in the treatment of cardiovascular disease, and further clarify the mechanism of action of these extracts, which based on the results of this research, it is suggested that the extracts act on direct inhibition of thrombin and possibly inhibition of heparin cofactor II.

Concomitant with the activities of research, part of the data from this Project were presented in national and international scientific congresses. In addition, a scientific initiation student, who was present at all stages of development of this work, was guide. From the end of the work to date, the research has generated two articles: a submitted to *Phytomedicine* and the other to *Journal of Pharmacy and Pharmacolgy*, four abstracts presentations in national scientific congresses and two abstracts presentations in international scientific congresses, in addition to the insertion of the researcher in two research groups of Directory of Research Groups in Brazil's of National Council for Scientific and Technological Development - CNPq. This technical-scientific production generated by the research project is detailed below:

- Submitted Articles

- The article “LEAVES OF *Licania rigida* BENTH HAS A POTENTIAL ANTICOAGULANT EFFECT BY THROMBIN INHIBITION” was submitted to the journal *Phytomedicine* which has an impact factor 2.937, Qualis B1 for CAPES, for area of Medicine II.

- The article “EXTRACTS OBTAINED FROM LEAVES OF *Turnera subulata* INHIBITS BLOOD COAGULATION” was submitted to the *Journal of Pharmacy and Pharmacology* which has an impact factor 2.363, Qualis B1 for CAPES, for area of Medicine II.

- Academic Guidance of Scientific Initiation

- THAYSE EVELLYN SILVA DO NASCIMENTO (Scholarship) in the project entitled "Effect of extracts and fractions of leaves of *Turnera subulata* and *Licania rigid*a on hemostasis and cell death” – graduate in Pharmacy of UFRN, orientation period: 2014-2016.

- Abstracts published in National Events proceedings

- LUZ, J. R. D.; NASCIMENTO, T. E. S. ; CRUZ, A.K.M. ; REZENDE, A. A. ; LUCHESSI, A. D. ; URURAHY, M. A. G. ; ROCHA, H. A. O. ; ALMEIDA, M. G. Preliminary chemical composition, toxicity and anticoagulant activity of leaf extract of *Turnera ulmifolia* Linn. var. *elegans*. In: VIII Simpósio Ibero-Americano de Plantas Medicinais e o III Simpósio Ibero-Americano de Investigação em Câncer – SPM, 2016, Santa Catarina. Anais do VIII Simpósio Ibero-Americano de Plantas Medicinais e o III Simpósio Ibero-Americano de Investigação em Câncer, 2016.

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- Abstracts published in International Events proceedings

- LUZ, J. R. D.; NASCIMENTO, T. E. S. ; CRUZ, A.K.M. ; REZENDE, A. A. ; LUCHESSI, A. D. ; ROCHA, H. A. O. ; ALMEIDA, M. G. . Anticoagulant activity of a rich extract in flavonol-3-glycosylated of leaves of *Licania rigida* benth with low toxicity in vivo. In: XXIII Congress of International Union of Biochemistry and Molecular Biology - IUBMB and XLIV Annual Meeting of SBBq, 2015, Foz do Iguaçu. Anais do XXIII Congress of International Union of Biochemistry and Molecular Biology - IUBMB and XLIV Annual Meeting of SBBq, 2015.

- LUZ, J. R. D.; NASCIMENTO, T. E. S. ; CRUZ, A.K.M. ; REZENDE, A. A. ; URURAHY, M. A. G. ; LUCHESSI, A. D. ; ROCHA, H. A. O. ; ALMEIDA, M. G. . Low toxicity of a rich extract in flavonol-o-3-glycosylated of leaves of *Turnera subulata* with anticoagulant activity. In: 9th Congress of Toxicology in Developing Countries and XIX Congresso Brasileiro de Toxicologia, 2015, Natal. Anais do CTDC9 and XIX CBTox, 2015.

- Researcher insertion in research groups

- Research Group Bioanalysis (Certificate) – training year: 2000.
  Group Leaders: Profª Drª Tereza Maria Dantas de Medeiros and Profª Drª Valéria Soraya de Farias Sales, inserted in the Metabolic Diseases research line.

- Research Group Chronic Degenerative Diseases (Certificate) – training year: 2005. Group Leaders: Prof Dr José Brandão Neto and Profª Drª Maria das Graças Almeida, inserted in stress Oxidative and Antioxidants research line.

Finally, coming to the end of another training phase, the feeling of accomplishment is the fulfillment of this work and the path trodden with much effort and dedication throughout the phase of post-graduation.

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EXHIBITS

EXHIBITS 1

Universidade Federal do Rio Grande do Norte
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

PROTOCOLO N.º 035/2015

Professor/Pesquisador: *MARIA DAS GRAÇAS ALMEIDA*

Natal (RN), 07 de outubro 2015.

Certificamos que o projeto intitulado "**AVALIAÇÃO TOXICOLOGICA E HEMOSTÁTICA DE EXTRATOS VEGETAIS DE ESPÉCIES DO NORDESTE BRASILEIRO**", protocolo 035/2015, sob a responsabilidade de MARIA DAS GRAÇAS ALMEIDA, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei n.º 11.794, de 8 de outubro de 2008, do Decreto n.º 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado, *após esclarecimentos*, pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS da Universidade Federal do Rio Grande do Norte – CEUA/UFRN.

Vigência do Projeto	**AGOSTO 2018**
Número de Animais	**60**
Espécie/Linhagem	**Ratos Wistar**
Peso/Idade	**250g / 3 meses**
Sexo	**Machos e Fêmeas**
Origem	**Centro de Ciências da Saúde UFRN**

Informamos ainda que, segundo o Cap. 2, Art. 13 do Regimento, é função do professor/pesquisador responsável pelo projeto a elaboração de relatório de acompanhamento que deverá ser entregue tão logo a pesquisa for concluída.

Josy Carolina Covan Pontes
Coordenadora da CEUA