Extraction of bioactive alkaloids from *Melocactus zehntneri* using supercritical fluid

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ABSTRACT

The purpose of this study was to extract alkaloids from *Melocactus zehntneri* aerial parts using supercritical fluid extraction (SFE) and to evaluate their cytotoxic action. SFE process was compared to different classical low pressure solvent extraction processes in terms of global yield, extract profile and biological activity. Two specimens of *M. zehntneri* were evaluated: young and adult aerial parts. The extracts were analyzed by HPLC-UV/DAD as well as they were evaluated against HMVII and *Trichomonas vaginalis*. The results indicated a qualitative difference in chromatographic profile of the extracts. Finally, the results point out the potential of the extract obtained from aerial parts of young specimens (Y-RM) through SFE at 300 bar/55°C against *T. vaginalis* and HMVII, presenting relevant information for development of new studies about cytotoxic.

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1. Introduction

*Melocactus zehntneri* (Cactaceae), popularly named as coroa-de-frade, is a medicinal cactus native from a Brazilian xeric biome, Caatinga [1]. Besides, chemotaxonomy reinforces *M. zehntneri* as specie pharmacologically interesting since isoquinoline and indole alkaloids, both bioactive classes present in several drugs, have been identified from a wide range of species by including plants from Cactaceae family [2]. In Brazilian Northeast, this specie plant has been pointed out by a quantitative ethnopharmacologic study as one of the most important species regarding therapeutic approaching [3]. It has been traditionally used in Brazilian folk medicine as herbal medicine for treatment of helminthiasis, hemorrhoid, bronchitis, cough, physical weakness and intestinal disorders [1–3]. The importance of *M. zehntneri* has been scientifically supported by antimicrobial (against *Staphylococcus epidermidis*) and antiparasitic (against *Trichomonas vaginalis*) properties of this specie [4,5]. Among these, particular attention has been given to the last.

Trichomoniasis is the most common non-viral sexually transmitted disease in the world affecting 276 million each year [6]. This target is important since the pathology is related to many health complications affecting women worldwide including pregnancy outcomes, infertility, cervical cancer and increasing human immunodeficiency virus transmission (HIV) rate [7]. Besides, there are just 5-nitroimidazole drugs as the current chemotherapy, being metronidazole used as the first choice [8]. It is a health problem since metronidazole-resistant strains have been reported including in vitro and clinical isolates that showed increasing therapy failure rates to high doses of metronidazole [7]. For this reason, new therapeutic researches from natural products bioactive against *Trichomonas vaginalis* have been encouraged to find out a new alter-
native drug. Recent studies demonstrated that aqueous extracts from roots and aerial parts from *M. zehntneri* obtained by percolation showed a potential bioactivity against the parasite [5]. However, no progress was found to obtain the fraction or the compounds responsible for this action.

Among the several factors which determine the effectiveness of a medicinal herb, the extraction technique is the key factor to obtain bioactive compounds with success. In the last years, the application of supercritical fluid extraction (SFE) has been widely studied for obtaining alkaloids from plants [9,10]. In comparison to traditional extraction methods, SFE has been widely used for offering the possibility of easy change in operating conditions allowing the selective extraction of compounds. Besides, SFE has several advantages such as environmental safety, selectivity and capability to extract thermally labile compounds under mild temperatures conditions. In addition, the solvating power of supercritical fluid is directly related to pressure, temperature of the process; these, in turn, enable to selective extraction on the matrix of the analyze dissolving the soluble compounds [11]. The use of supercritical CO$_2$ (SC–CO$_2$) to extract alkaloids such as caffeine from coffee beans and tea led to numerous patents [12]. Some classes of alkaloids such as pyrrolizidine, quinoline, isoquinoline, pyridine [9–13], indole [14–10] and phenanthrene [15] were extracted with supercritical CO$_2$ with or without modifiers. These studies demonstrate the technical and economic feasibility of SFE process to obtain bioactive compounds like alkaloids with efficiency when compared with conventional processes.

In order to obtain an specific bioactive compound or an active fraction, several factors could influence the extraction processes and must be considered, for example, solvent polarity, sample preparation, solvent/plant ratio, extraction time, age of the plant and fingerprint of the extracts. Thereby, the aim of this study was to extract alkaloids from *M. zehntneri* aerial parts using supercritical CO$_2$. Two kind of raw material were evaluated: specimens with inflorescence (Adult Raw Material: A-RM); and the specimens without inflorescence (Young Raw Material: Y-RM). The global yield and the profile of the SFE extracts were compared with those obtained by conventional techniques, including maceration and ultrasound-assisted extraction. In addition, the extracts were assayed against Human Malignant Melanoma cell line – HMVII (a vaginal epithelial tumoral lineage), and *Trichomonas vaginalis* (an eucariotic amitochondriated protest and the etiologic agent of trichomoniasis).

2. Material and methods

2.1. Plant material

*Melocactus zehntneri* was collected in July 2014 at Currais Novos, in Rio Grande do Norte, Brazil. The species was identified at Herbarium of Federal University do Rio Grande do Norte (UFRN) with the voucher specimen number 19860. In this study, the aerial part from cactus, a succulent, globular and thorny organ was used. The aerial parts which presented an inflorescence were named adult (A-RM), while the specimens without this structure were named young (Y-RM). Aerial parts (1.55 kg and 1.75 kg from Y-RM and A-RM, respectively) were lyophilized (Liptop, model L202, Brazil) and the dried material was fragmented using a knife mill (Tecnal, model TEF-631/2-Piracicaba, Brazil). The processed aerial parts were classified according to size of the particles using an agitator (Bertel, model NOVO 110/220-São Paulo, Brazil) with standard sieves of the Tyler series. The raw material used for the extractions was composed of particles with mesh sizes of 24 and 32. The global yield for a given operating condition was calculated as the ratio of the total mass of extract by the initial mass (dried raw material) of *M. zehntneri*.

2.2. Extraction methods

2.2.1. Supercritical fluid extraction (SFE)

The supercritical fluid extraction was performed with aerial parts from adults and young specimens in parallel experiments. The extraction was carried out in an extraction cell of the SFE system (Model SFE-100 System, Thar Technologies Inc). The system included a stainless steel extraction cell (100 mL capacity), cooler, CO$_2$ pump, co-solvent pump, flash stainless steel extraction collector (250 mL capacity) and heater. It was used a $2^2$ factorial design experiment, considering the pressure (100 and 300 bar) and temperature (35 and 55 °C) as the variables of the process. The experimental errors were measured by triplicate assays at the central point (250 bar/45 °C). The experiments (Y-RM and A-RM samples) were carried out using 3 g/min of total solvent flow rate, $4 \times 10^{-3}$ kg of raw material, and the methodology described by Pereira et al. [14].

2.2.2. Maceration

This extraction was performed using $0.5 \times 10^{-3}$ kg of raw material composed by aerial parts from adults and young specimens (1:1, w/w) submitted to maceration in ethanol-water (95:5, v/v) during 24, 48 and 72 h at 1:10 (w/v) of raw material/solvent ratio, according to studies of Cujic et al. [16]. The system was stirred in each 8 h. After the extraction, the solvent was evaporated using a vacuum evaporator (Buchi, model R-3, Switzerland), and stored at 4 °C for the analysis.

2.2.3. Ultrasound-assisted extraction (UAE)

This extraction strategy was performed using $0.5 \times 10^{-3}$ kg of raw material composed by aerial parts from adults and young specimens (1:1, w/w). UAE was performed in an ultrasound probe device (Model VCX 750 W, Vibra-cell). The samples were processed in an ice bath since ultrasound waves provided a slight increase in the temperature (i.e., 45 ± 5 °C). The experiments were carried out using the optimized conditions obtained in previous analysis: amplitude level at 27% of equipment power (750 W), during 45 min using 1:10 (w/v) of raw material/solvent ratio. Ethanol-water (95-5, v/v) was used as solvent based on studies of Cujic et al. [16] and Kaiser et al. [17].

In order to be clear about the samples used in our work, additional information about these is detailed here. Preliminary studies, performed with the extracts obtained by maceration and UAE processes using individual samples (only Y-RM and only A-RM), showed that the extracts of Y-RM and A-RM obtained by these conventional methods presented the same qualitative chromatographic profile and few quantitative differences were observed taking into account the absorbance of the peaks. Thus, in order to reduce the number of experiments and analysis, we decided to perform the study of classical extraction methods considering a representative sample (Y-RM + A-RM). On the other hand, only SFE process got show in the preliminary analysis the difference between these two raw materials: Y-RM and A-RM. Then, these samples were analyzed independently in the SFE process.

2.3. Analysis of the extracts

2.3.1. Thin layer chromatography (TLC)

The extracts were submitted to analytical thin layer chromatography (TLC) on chromatoplate of silica gel (60GF254) using chloroform:methanol (2:8 – v/v) as eluent system. The samples were observed at 365 nm and also by using Dragendorff reactive, specific for alkaloids.
2.3.2. HPLC-UV/DAD analyzes

The extracts obtained were analyzed by HPLC (Hitachi/Cromaster model) coupled to DAD detector, with UV at 254 and 279 nm. The wavelengths 254 and 279 nm were selected to analyze the data since literature indicates that the Cactaceae alkaloids are mostly derived from phenylalanine and tryptophan belonging to indole and isoquinoline alkaloids which present these wavelengths as the maximum absorption [18,19].

Data were processed at the EZChrom Elite software and the samples (20 µL) were injected on a Thermo Scientific C18 column (250 × 4.6 mm, 5 µm) with the oven temperature at 30 °C. The mobile phase was used in gradient (A: 0.5% formic acid in water and B: acetonitrile) with a flow rate 2.0 mL/min: 0–120 min [0% to 100% B]; 120–130 min [100% B]. All of extracts were prepared and analyzed at 7 mg/mL.

2.4. Biological assays

The cytotoxicity was performed in all extracts (SFE, maceration, UAE), and the analysis was carried out according to the following methodologies.

2.4.1. Culture of Trichomonas vaginalis

The T. vaginalis isolate 30236 (from the American Type Culture Collection, ATCC, USA) was used in this study. Trichomonads were cultured in vitro in tryptophan-yeast extract maltose (TYM) medium, pH 6.0, supplemented with 10% (v/v) heat-inactivated serum, and incubated at 37 °C [20]. Organisms in the logarithmic phase of growth and exhibiting more than 95% viability and normal morphology were harvested, centrifuged and suspended on new TYM medium for testing the extracts. All experiments were performed in triplicate, and with at least three independent cultures (n = 3).

2.4.2. Anti-T. vaginalis activity assay

The cytotoxicity of extracts against T. vaginalis was determined in vitro. The extracts were dissolved in DMSO (Dimethyl sulfoxide, 2.5%). For the assay, 96-microtiter plates were used where 50 µL TYM medium were added to all wells and a twofold serial dilutions were carried out by adding 50 µL of the sample stock solution (8.0 mg/mL) into the first well. Afterwards, 150 µL of the parasite suspension (1.3 × 10⁵ trophozoites/mL) was added in each well resulting in a final density of 1.0 × 10⁵ trophozoites/mL. Two controls were carried out considering the negative control with parasites only (100% trophozoites viability), and the positive control with 100 µM metronidazole (no viable trophozoites) (Sigma-Aldrich, St. Louis, MO, USA). The microplates were incubated for 24 h at 37 °C, 5% CO₂. The number of viable trophozoites was assessed by counting parasites using exclusion by trypan blue dye (0.2%) and hemocytometer. Results were expressed as the percentage of living organisms compared to untreated parasites, considering motility and normal morphology. Results were obtained from three independent experiments (n = 3).

2.4.3. Cytotoxicity against HMVII cells

The HMVII cell line, representative of vaginal epithelial cells (human vaginal malignant melanoma, from Health Protection Agency Culture Collections, UK), was used to evaluate the cytotoxicity of bioactive samples. HMVII lineage was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C, 5% CO₂. For the assay, 1.5 × 10⁴ cells/well were seeded in 96-well microtiter plate during 24 h. After this period, the medium was replaced by fresh medium containing or not (control condition) the bioactive samples. A solution containing 0.2% Triton X-100 was added as a positive control. The plates were incubated for 24 h, and then, after one wash with PBS (phosphate buffered saline), a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added and kept in the wells for 1 h. The plates were washed twice with PBS, and the insoluble purple formazan was dissolved in DMSO. The amount of reduced MTT was measured at 570 nm. The experiment was performed in triplicate.

2.4.4. Hemolytic assay

The hemolytic assay was performed according to literature with some modifications [21]. Fresh human blood was collected from healthy voluntary donors. The Research Ethical Committee of Federal University of Rio Grande do Sul approved documents, procedures and project under authorization CAAE 47423415.5.0000.5347. Blood samples were centrifuged at 2000 rpm for 5 min. The erythrocyte fraction was washed three times with PBS (pH 7.0) and re-suspended to give a 1.0% suspension. Samples under investigation were dissolved using DMSO and purified water to achieve a final concentration of 1.0 mg/mL. Using microtubes, the 1.0% erythrocyte suspension was mixed with the samples solution and/or water to obtain a final concentration of approximately 0.7% of erythrocytes. The microtubes were incubated at 37 °C under horizontal agitation for 1 h and then centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. The experiment was performed in triplicate, and the percentage of hemolysis was calculated in comparison to 100% hemolysis attributed to the hemolytic action of the positive control 0.1% triton X-100.

3. Results and discussion

The extraction efficiency is influenced by several aspects and the optimization of the extraction conditions could be useful to investigate the influence of different factors on the extraction of natural products. In this sense the comparison between extracts afforded by three extraction techniques was performed in terms of global yield, chromatographic profile and the cytotoxic potential of M. zehntneri.

3.1. Extraction methods

Fig. 1 shows the global yield obtained for SFE process at different conditions of temperature and pressure.

According to Fig. 1, the higher global yield was obtained at 300 bar/35 °C (0.68%), using A-RM, followed by extracts obtained at 35 °C using Y-RM (0.52%). Considering the Y-RM, the pressure had no significant effect on the global yield, and only the temperature presented effect on this material. On the other hand, for the A-RM, both the temperature as the pressure affected the global yield.

The effect of temperature on supercritical extraction is many times antagonistic. The increase in temperature causes a decrease in the density and thereby a decrease the solubility of the compounds in supercritical fluids; on the other hand, the increase in temperature also causes an increase in the vapor pressure of the solute, and this increases the solubility of the solutes in supercritical fluid. According to the results, for Y-RM, at a constant pressure (at 100 bar, for instance), it is observed that increasing the temperature (from 35 to 55 °C), the density decreased (from 712.81 to 325.07 kg/m³), and also the global yield decreased (0.52–0.46%). It indicates that the effect of the solvent density was superior to the effect of the solute vapor pressure for this raw material. The same behavior was observed at 300 bar. For the A-RM, this effect is observed at higher pressure. At 100 bar increasing the temperature (35–55 °C), the density decreased, however, the global yields were similar (0.41 ± 0.11 and 0.42 ± 0.10%), however at 300 bar, the same range of temperature (from 35 to 55 °C) decreased the solvent density (929.11–850.22 kg/m³), and also global yield decreased
Considering the conventional methods, two techniques were used to compare the results: maceration and UAE. The highest extraction efficiency was observed by maceration with a global yield of $14 \pm 1\%$ using 72 h of procedure. For the experiments using 24 and 48 h, no significant differences were observed ($11 \pm 2\%$ and $12 \pm 1\%$, respectively). The global yield for UAE was $13 \pm 1\%$, similar to maceration yield, in other words, ultrasonic waves had no extra effect in this raw material. These results were superior to that observed when supercritical fluid extraction was used (Fig. 1). However, it is known that the global yield is only the first step in the evaluation. Therefore, it is important to proceed the analysis of the chemical profile of the extracts to define the best method to be used.

### 3.2. Extract analysis

#### 3.2.1. TLC analysis

TLC analysis performed in all extracts indicated the presence of alkaloids only in the extracts obtained at 300 bar/55°C in both raw material (Y-RM and A-RM) since orange spots were revealed with Dragendorff reactive. In Y-RM sample one spot at Rf 0.05 while in A-RM two spots (Rf 0.33 and 0.75) were observed.

For TLC analysis one information must be highlight. Polysaccharides are well known to have nucleophilic and encapsulating properties. Its presence on the extracts could promote reduction of the alkaloids detection and/or the encapsulation of these compounds (alkaloids are electrophiles agents), resulting in false negative result on TLC chromatoplates analysis [22]. In other words, even if there were alkaloids in the extracts obtained by conventional processes, they could had formed aggregates with the polysaccharides or could be encapsulated them.

Therefore, it was important to know if this aggregates were formed, and for that was performed one analysis to evaluate the presence or not of polysaccharides. Tanaka et al. [23] described that the solution containing alcohol undergoing temperature changes (4–25°C) in cycles are applied to precipitation of polysaccharides. In order to evaluate the presence of polysaccharides in maceration extracts, tests using the methodology of Tanaka et al. [23] were carried out, and precipitates were observed in the maceration extracts. Then, a new analysis of TLC was performed consider-
ing two extracts: one using the first sample (with all compounds including the precipitates), and another sample removing the polysaccharides (as indicated by Tanaka et al. [23]). The results indicated that after spraying Dragendorff reagent in the chromatoplate, the sample free of polysaccharide provided intense spot and better resolution in the plates. This result suggests that the polysaccharides can hide the presence of alkaloid and provide false negative results.

Although SFE had presented low global yields, this technique proved to be quite selective for alkaloid extraction. These characteristics can be attributed to the high selectivity of SC-CO$_2$ to alkaloids, the low solubility of polysaccharides in SC-CO$_2$ and properties acquired by the solvent under the supercritical conditions.

3.2.2. **HPLC-UV/DAD analysis**

In order to carry out a deep comparison between the SFE extracts in different matrices, analyses of the different extracts by HPLC-UV/DAD were performed. The chromatographic profiles of the extracts from Y-RM and A-RM obtained by SFE are presented in Figs. 2 and 3.

The results demonstrate that the compounds with the retention time ($R_t$) at 67, 91, 105 and 116 min could be classified in the main alkaloid classes found in Cactaceae [18,19] since their UV spectrum are comparable to the isoquinoline and indole alkaloids chromophores. Comparing the chromatograms of the Y-RM, it is observed that the operating conditions at 100 bar and 200 bar/45 °C (Fig. 2c–e, Y-RM) were more efficient for the extraction of compounds corresponding to the retention time ($R_t$) at 105 and 116 min, respectively. While the operating condition at 300 bar was more efficient to extract compounds corresponding to $R_t = 67$ and 91 min (Fig. 2a, b, Y-RM). For the compound corresponding to $R_t = 70$ min, it was observed a larger peak area in samples obtained at 100 bar/35 °C and 200 bar/45 °C (Fig. 2e and c, Y-RM, respectively).

Analyzing the chromatographic profiles of samples from A-RM it is observed that the compound corresponding to $R_t = 67$ min was more efficiently extracted by the process at 300 bar/55 °C (Fig. 2a, A-RM). The compound corresponding to $R_t = 70$ min was obtained in all conditions, except at 100 bar/35 °C (Fig. 2e, A-RM). All conditions extracted compounds identified in the $R_t = 67, 91, 105$ and 116 min.

In the chromatograms obtained at 279 nm (Fig. 3), it is observed that the compound with $R_t = 91$ min was presented in all extracts being purest in the extract obtained at 300 bar/35 and 55 °C for Y-RM and 100 bar/35 °C for A-RM, however considering the results presented in Fig. 1, the extract obtained at 100 bar/35 °C presented higher deviation due to the problem of hygroscopicity of the raw material, making difficult the work with this raw material.

![Fig. 3. Chromatographic profiles from young (Y-RM) and adult (A-RM) specimens obtained by SFE at (a) 300 bar/55 °C, (b) 300 bar/35 °C, (c) 200 bar/45 °C, (d) 100 bar/55 °C and (e) 100 bar/35 °C and visualized at 279 nm.](image)

![Fig. 4. Chromatographic profiles of extracts obtained by (a) SFE 300 bar/55 °C, Y-RM, (b) UAE (27%, 45 min and 1:10, w/v) and (c) maceration extracts (72 h, 1:10, w/v), using HPLC-UV/DAD at 254 nm and 279 nm.](image)
Evaluating the effect of temperature and pressure, for Y-RM, it is observed that at a constant pressure, the temperature increase promoted a decrease in the efficiency in the extraction of compound with \( R_t = 70 \text{ min} \). On the other hand, at constant temperature, the pressure increase improved the extraction of the compound with \( R_t = 91 \text{ min} \). For A-RM, it is observed that at constant pressure, the temperature increase promoted an increase in the efficiency in the extraction of compound with \( R_t = 67 \text{ min} \). On the other hand, at constant temperature, the pressure increase reduced the extraction of the compound \( R_t = 91 \text{ min} \).

In general, it is observed high amount of the compound identified by \( R_t = 91 \text{ min} \) followed by that with \( R_t = 67 \text{ min} \) in both raw materials (Y-RM and A-RM). Moreover, in extracts obtained at high pressures the peaks presented higher intensity. Thus, it was chosen the chromatogram of the extract obtained at 300 bar/55 °C (from Y-RM) to be used in the comparison with the chromatograms of the extracts obtained by conventional extraction.

The samples obtained from the aerial parts of *M. zehntneri* extracted by maceration (72 h), SFE (300 bar/55 °C, Y-RM) and UAE (27%, 45 min and 1:10, w/v) were analyzed by HPLC-UV/DAD at 254 nm and 279 nm. Up to now, it has not been reported in the literature analysis by HPLC/DAD for *M. zehntneri*. Fig. 4 shows the chromatograms of the different extracts.

According to Fig. 4, the extracts obtained by maceration and UAE techniques showed more intense peak of compounds with \( R_t = 67 \text{ min} \), while SFE extract contains mainly the compound with \( R_t = 91 \text{ min} \) in higher amount, this is possibly attributed to higher solubility of this into SC-CO\(_2\). Furthermore, when the chromatographic profiles of all extracts are compared, it is observed that the condition at 300 bar/55 °C offered a sample with a lower number of peaks as well as alkaloid spots in TLC analysis, suggesting that this sample presents preferentially alkaloids.

The results suggest that despite the low yield the SFE was the most selective technique for extraction of the compound eluted at \( R_t = 91 \text{ min} \). An important observation is that the purity of the SFE extract could reduce the number of steps for purification, showing the feasibility of the SFE technique. However, the selection of the best process depends on the application, in this case it is related to the extract activity.

In order to try to clarify the chemical identity of the natural products present in the samples, literature data were evaluated. According to Sangster and Stuart [24], phenylalanine derivatives have in their molecular structure an aromatic ring which may or may not be conjugated with the carbonyl or olefin functions. When the non-conjugated type has a maximum absorbance at 260–280 nm, the absorption intensity depends on the presence of auxochrome substituents (OH and OCH\(_2\)) on the aromatic ring. In contrast, when these derivatives have aromatic ring conjugated, it is observed bathochromic shifts and an increase in absorption intensity in the spectrum. The ultraviolet spectra of the isoquinoline alkaloids are characterized by having one or more aromatic rings with unconjugated maximum of absorbance near 285 nm. When the aromatic rings are linked by the oxygen atom, differences are not observed in the maximum of absorbance. The same does not apply to the spectra of aromatic rings unconjugated, which indicates that the benzene rings are significantly decoupled. In addition, the aromatic rings isoquinolinic dehydrogenated have very complex spectra in 3 main regions 238 nm, 279 nm and 313–327 nm [24]. UV spectra of the indole alkaloid group have two characteristic absorbance maximums, the first band near 226 nm and a second between 280 and 292 nm [24]. However, the number and types of substituent on the aromatic ring can result in strong bathochromic shifts to longer wavelengths and this shift is even more pronounced when the carbonyl group is in a ring of 8 members [25].

Based on this information as well as the possibility of bathochromic shift, it is suggested that the spectrum corresponding to the peak at 105 min could be a phenylalanine derivative. In addition, the spectra corresponding to the peaks at 70 min, 91 min and 116 min are suggestive of indole derivatives. Finally, the spectrum corresponding to the peak at 67 min is suggestive of isoquinolinic derivative.

### 3.3. Cytotoxic potential

In this study all extracts were tested against *T. vaginalis* and the results are presented in Fig. 5.

According to Fig. 5, it is clear that the extract obtained by SFE at 300 bar/55 °C from Y-RM was able to impair the trophozoites viability at 1 mg/mL. The bioactive samples were also investigated for minimal inhibitory concentration (MIC) through a twofold serial dilution (from 1 mg/mL to 0.0078 mg/mL), however statistic differences in comparison to control just were observed at 1 mg/mL in both samples. Indeed, these results pointed out SFE extracts obtained at 300 bar/55 °C from Y-RM was moderately active against *T. vaginalis* trophozoites since it is in milligram order. Previous studies regarding natural products with anti-*T. vaginalis* potential were organized in a review [26] and it pointed out the heterogeneity of the bioactive extracts. Some plant extracts are active at μg/mL range while other ones were comparable with our data from *M. zehntneri*, at mg/mL order of magnitude.

However, the results opened another perspective for investigation of the biological potential. In addition to being a therapeutic target, *T. vaginalis* is considered to be a cellular model since this parasite does not present mitochondria, but possesses an unusual anaerobic membrane bounded organelle, named hydrogenosome, capable of producing H\(_2\) [27]. The absence of mitochondria in *T. vaginalis* makes it a suitable biochemical model for the study of cell death-related mechanisms since the mitochondrion is considered to be the “death signal integrator”, essential for triggering programmed cell death (PCD) by caspase-dependent and independent pathways [28]. In this sense, the bioactive samples against *T. vaginalis* were additionally assayed against erythrocytes (without nucleous and amitochondriated cell) and against HMVII (an epithelial and tumoral eukaryotic and mitochondriated cell line). The hemolytic test showed that the sample obtained by SFE at 300 bar/55 °C from Y-RM was not able to cause hemolysis since significant differences were not observed in comparison to control, then it could be hypothesized that the plasmatic membrane was not disrupted. Otherwise, this sample was cytotoxic against HMVII as showed in Fig. 6.

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**Fig. 5.** Anti-*T. vaginalis* activity of extractions fractions of *M. zehntneri*. Vehicle: DMSO 0.6%. (*) Represents statistical significance *p* < 0.005 by t-Student test compared with untreated control.
Overall the results point out that the sample obtained by SFE at 300 bar/55 °C from Y-RM was cytotoxic against both eukaryotic cells and that this cytotoxicity was not due to a physical damage like membrane disruption. Therefore, some mechanism of action could be involved at biochemical level since the absence of mitochondria, a key cellular organelle in cell death pathways, did not protect the cell from the samples toxicity. On the other hand, it can be suggested that the cytotoxicity of the bioactive sample against the tumoral cell line opened an important range of further studies on additional cell lines resistant to drugs which use mitochondrial pathways to signalize the cell death, like proapoptotic drugs. Finally, it is important to highlight that alkaloids are secondary metabolites historically recognized as cytotoxic agents so phytochemical results are in agreement with biological activity in our study.

3.3.1. Global discussion: final evaluation of the results

Considering the TLC analysis, HPLC-UV/DAD analysis, and cytotoxic assays, we could hypothesize that the compound responsible for the activity is that with Rf = 91 min. However, this compound should be isolated and tested against biological targets to assess and confirm the cytotoxic activity. This hypothesis is based on the presence of alkaloids in TLC plates just in samples obtained at 300 bar/55 °C for young and adults specimens, followed by activity found in the extract obtained at 300 bar/55 °C, for Y-RM. By chromatograms analysis, it was observed that the compounds with Rf = 67 and 91 min where present in all SFE extracts, in different concentrations. However, the extract obtained at 300 bar/55 °C was characterized by existence of few peaks, it enable us to suggest that this condition was selective for the compound with Rf = 91 min. For some medicinal plants the pharmacological action depends on the combined effect of multitudinous constituents, which may interact in a positive or negative manner [29]. In this way, the cytotoxic could be inhibits by constituents interaction such as other alkaloids in the extract [30].

4. Conclusions

The different results for extraction techniques suggest that the technique and properties of the solvent were the main factors determining the metabolite profile in the extract. Despite the low yield the SFE, this process was the most selective to extract alkaloid which absorbs at 254 nm and 279 nm. Besides, the SFE extracts were free of polysaccharides. This important feature, which reduces the number of steps for purification, associated with the CO2 SP properties show that SFE is the most sustainable technique. Finally, the results point out the potential of the extract obtained from aerial parts of young specimens (Y-RM) through SFE at 300 bar/55 °C against T. vaginalis and HMVII, presenting relevant information for development of new studies about cytotoxic potential.

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References


Fig. 6. Cytotoxic effect of the bioactive extract from M. zehntneri against HMVII cells. Vehicle: DMSO 0.6%.

against T. vaginalis and HMVII, presenting relevant information for development of new studies about cytotoxic potential.


