CIÊNCIAS BOTÂNICAS:

Evolução e diversidade de plantas

Vanessa da Fontoura Custódio Monteiro
Pedro Henrique Abreu Moura
(Organizadores)

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APRESENTAÇÃO

Com grande extensão territorial e diversidade de domínios morfoclimáticos, o Brasil possui a flora mais rica do mundo. Esta obra *"Ciências botânicas: evolução e diversidade de plantas"* é um pequeno compilado de pesquisas desenvolvidas em várias regiões do país, contribuindo com o avanço científico.

O primeiro capítulo é dedicado às algas, que também são estudadas em Botânica Criptogâmica. O capítulo traz resultados de um levantamento de algas marinhas bentônicas dos estados do Piauí e Maranhão, sendo encontrados representantes de algas pardas (Phaeophyta), algas vermelhas (Rhodophyta) e algas verdes (Chlorophyta).

Nos segundo e terceiro capítulos, as briófitas ganham destaque. A riqueza de espécies de musgos encontrados no estado do Mato Grosso é apresentada, contribuindo com a ampliação do conhecimento sobre a diversidade e ecologia de plantas avasculares no estado.

E claro, as samambaias também são abordadas nesta obra, mais especificamente no capítulo 4, onde os autores trazem respostas morfoecológicas de *Tectaria incisa* Cav. (Tectariaceae) em Floresta Atlântica no estado do Rio de Janeiro.

A diversidade de Angiospermas é retratada nos capítulos subsequentes. O capítulo 5 é referente à flora do Amapá, com foco na família Vitaceae. No capítulo 6, é apresentado a importância ecológica, econômica e social de *Parkia platycephala* Benth. (Fabaceae) no Cerrado. O capítulo 7 traz resultados de uma pesquisa sobre a atividade biológica de *Hesperozygis ringens* (Benth.) Epling (Lamiaceae), uma planta endêmica da região Sul do Brasil.

Já os capítulos 8 e 9 estão voltados especificamente para orquídeas, trazendo resultados de pesquisas sobre o desenvolvimento da semente e do protocormo de *Cleistes libonii* (Rchb.f.) Schltr. e de análises cienciométricas sobre pesquisas de micropropagação *in vitro* de *Cattleya walkeriana* Gardner.

E para encerrar este livro, os autores do último capitulo investigam as concepções de estudantes de licenciatura em Ciências Biológicas sobre a célula, propondo estratégias para a construção de um conceito científico de célula por meio da investigação, da experimentação e da modelagem.

Desejamos a cada autor que contribuiu com esta obra os nossos agradecimentos. Aos leitores, desejamos uma leitura proveitosa e muito amor pelas Ciências Botânicas.

Vanessa da Fontoura Custódio Monteiro
Pedro Henrique Abreu Moura

SUMÁRIO

CAPÍTULO 7

INVESTIGATION OF BIOLOGICAL ACTIVITIES OF *Hesperozygis ringens*

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ABSTRACT: *Hesperozygis ringens* (Lamiaceae) is an aromatic plant, native to the South of Brazil, popularly called “espanta pulga”. Although there are some proven pharmacological activities for the species, their knowledge is very modest. Thus, the objective of this work was to evaluate the antitrypanosomal, nematicidal, and antibacterial activities of extracts of *H. ringens* obtained by

supercritical fluid extraction (SFE–CO_2) and ultrasonic assisted extraction with ethanol (UAE–EtOH). Both extracts, at concentrations 2500 μg mL^{-1}; 1250 μg mL^{-1}; 625 μg mL^{-1} (SFE–CO_2) and 250 μg mL^{-1}; 62.5 μg mL^{-1}; 31.25 μg mL^{-1} (UAE–EtOH) eliminated 100% of *Trypanosoma evansi* at of the time evaluated in the experiment (9h). Both the SFE–CO_2 extract and UAE–EtOH extract in 10 and 80 mg mL^{-1} and in their dilutions, respectively, eliminated 80% J_2 larvae of *Javanic Meloidogyne*. The UAE-EtOH extract did not show antimicrobial activity against the tested microrganisms. The SFE-CO_2 extract showed an inhibition halo in the diffusion disk assay against the *Pseudomoas aeruginosa* bacteria and the fungus *Candida albincans*, with lowest inhibiting concentrationof 1.46 mg mL–1 in the microdilution assay. Considering that *H. ringens* showed action against bacteria, nematodes, protozoa and can be considered a possible alternative for the development of new drugs.
KEYWORDS: Medicinal plant, Extracts, Secondary metabolites, Biological activity.

INVESTIGAÇÃO DE ATIVIDADES BIOLÓGICAS DE *Hesperozygis ringens*

RESUMO: *Hesperozygis ringens* (Lamiaceae) é uma planta aromatica, endêmica da região Sul do Brasil, popularmemte chamada de “espanta pulga”. Embora existam algumas propriedades farmacológicas comprovadas para espécie, ainda é pouco estudada. Dessa forma, o objetivo desse trabalho foi avaliar a atividade antitripanosoma, nematicida e antibacteriana dos extratos de *H. ringens* obtidos por extração com fluido supercrítico (SFE–CO_2) e extração assistida por ultrassom com etanol (UAE–EtOH). Ambos extratos na concentração 2500 μg mL^{-1}; 1250 μg mL^{-1}; 625 μg mL^{-1} (SFE–CO_2) and 250 μg mL^{-1}; 62.5 μg mL^{-1}; 31.25 μg mL^{-1} (UAE–EtOH) eliminaram 100% de *Trypanosoma evansi* no tempo avaliado no experimento (9h). Os dois extratos SFE–CO_2 e UAE–EtOH respectivamente, 10 e 80 mg mL^{-1} e diluições eliminaram 80% de larvas J_2 de *Meloidogyne Javanica.* O extato UAE-EtOH não apresentu atividade antibacteriana frente aos microrganismos testados. O extrato SFE-CO_2 mostrou halo de inibição no teste de disco-difusão frente a bacteria *Pseudomoas aeruginosa* e ao fungo *Candida albincans*, sendo a concentração inibitória mínima de 1.46 $mgmL^{-1}$. Considerando que *H. ringens* mostrou atividade frente a bactérias, nematóides e protozoários pode ser considerada uma aternativa para o desevolvimento de novo fármacos.
PALAVRAS-CHAVE: Plantas medicinais, Extratos, Metabólitos secundários, Atividades biológicas.

1 | INTRODUCTION

Hesperozygis ringens is a plant native to south of Brazil, belongs to the Lamiaceae family and is popularly known as “espanta pulga” (freedom translation - scare flea). The essential oil of this species presents anesthetic action (Silva et al. 2014), allelopathic (Von Poser et al. 1996, Pinheiro et al. 2016), antiparasitic (Bandeira Junior et al. 2017), larvicide (Ribeiro et al. 2010; Silva et al. 2014) and antimicrobial (Sutili et al. 2015; Bandeira Junior et al. 2017). Due to the benefits that plant species have to offer, and due to growing problems such as: resistance, environmental pollution and toxicity of conventional medicines, compounds of plant origin can become an alternative (Wang et al. 2017).

Most reports in the literature on *H. ringens* involve the characterization of essential oil (Pinheiro et al. 2016; Rosa et al. 2019). Thus, in order to expand the potential of the plant, in previous studies we investigated ultrasonic assisted extraction with ethanol as solvent (UAE–EtOH), detecting rosmarinic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, ρ-coumaric acid, vanillic acid, resveratrol, luteolin, quercetin, apigenin and kaempferol. Beside, studied the supercritical fluid extraction with carbon dioxide (SFE–CO2) detecting pulegone, limonene, linalool, ρ-mentone, neo-menthol, transcariophylene and β-Sitosterol as major compounds (Dolwitsch et al., 2020). Considering that *H. ringens* presents a series of promising activities against bacteria and parasites which may contribute to the development of new drugs. This work aims to evaluate the nematicidal and trypanocidal activity of different extracts obtained by SFE–CO_2 and UAE–EtOH in order to obtain a more detailed knowledge about the species, aiming its safe use.

2 | MATERIALS AND METHODS

2.1 Samples

Leaves of *Hesperozygis ringens* were collected in the municipality of Santa Maria, RS, Brazil, district of Santo Antão (S29º 37' 18.6" W053º 52' 26.0"), in March 2017. A voucher was deposited in the Herbarium of the Botanical Garden of the Federal University of Santa Maria under registration number 17543.

2.2 Preparation and extraction

The leaves were dried in oven with air circulation at 40°C until constant weight. The samples were subsequently ground and subjected to extraction with supercritical CO_2 and ultrasound probe using ethanol as solvent, as described in Dolwistch et al. (2020).

2.3 Trypanocidal Activity

The culture medium for *T. evansi* was adapted (Baltz et al. 1985) as previously published by Baldissera et al. (2013). The trypomastigotes were acquired from the infection of one rat (R1) with a T. evansi isolate. Five days post-infection R1 showed high parasitemia $7.5x10^6$ trypanosomes μL^{-1}) and it was anesthetized with isoflurane for blood collection by cardiac puncture stored in EDTA tubes. For blood separation, each 200 µL was diluted in complete culture medium (200 µL), stored in microcentrifuge tubes and centrifuged during 10 min at 400G. The supernatant was removed and resuspended in culture medium and the number of parasites was counted in a Neubauer chamber. *Method of culture*: The culture medium for *T. evansi* was adapted from Baltz et al. (1985) using minimal essential medium (MEM) without glutamine (0.376 g), glutamine (0.016 g), sodium bicarbonate (0.088 g), glucose (0.04 g), sodium-free HEPES (0.238 g), solution 100× non-essential amino acid (200 µL), penicillin (1596 U/mL) and streptomycin (100 µg/mL). The ingredients were

dissolved and homogenized in 30 mL of water with pH adjusted to 7.1 using NaOH. Then, it was filled up with ultrapure water to a volume of 42 mL of medium and the osmolarity was checked (0.30). The culture medium was then sterilized by filtration (at 0.22 µm) and stored in a refrigerator. In the days of testing, 10 mL were separated in falcon tubes, adding 1 µL/mL of 50 mM hypoxanthine (dissolved in NaOH, 0.1 M) and 2 µL/mL of 1.2 mM 2-mercaptoethanol. Subsequently, the complete culture medium was equilibrated for 2 h (37 °C with 5% CO_2). The components used for the preparation of the culture medium (except the antibiotics) were purchased from Sigma. *In vitro bioassays*: The culture medium with the parasites was distributed in microtiter plates (270 µL/pool), followed by the addition of 5 µL of extract SFE-CO_2 at concentrations of 2500 µg mL^{-1}, 1250 µg mL^{-1} and 625 µg mL^{-1} and UAE–EtOH extract at 250 µg mL^{-1}, 62,5 µg mL^{-1} , 15,62 µg mL^{-1} , diluted in DMSO. Two negative controls (DMSO and culture medium) were also carried in same volume. The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 9 h after the onset of the experiment in Neubauer chambers. The microtiter plates were placed in a 5% CO_2 incubator at 37 ºC. *Statistical analysis* - All experiments were conducted in triplicate. Data are expressed as the Mean±SEM.

2.4 Nematicidal activity

The solvents of the extracts were evaporated and the residue was resuspended in sterile distilled water and tweem 80 for the SFE–CO_2 extract and sterile distilled water for the UAE–EtOH extract. Serial dilution was performed from 10 mg mL^{-1} (SFE–CO_2) and 80 mg mL^{-1} (UAE–EtOH) concentrations to obtain 1: 2, 1: 4, 1: 6, 1: 8 fractions in both extracts. *Obtainment and preparation of the inoculum of Meloidogyne javanica*: The nematode inoculum used in the experiments was obtained from tomato roots kept in greenhouse. The eggs were extracted by the technique of Hussey and Berker (1973), modified by Boneti and Ferraz (1981). Resulting suspension was calibrated using the microscope and Peters chamber. After the extraction of the eggs, an outbreak chamber was assembled to obtain the juveniles in stage two (J2) to be used in the tests. *Action of extracts on J_2 larvae of Meloidogyne javanica:* In Elisa plates, previously sterilized, 100 µl of each extract and 100 µl of suspension containing 30 juveniles J2 of *Meloidogyne javanica* were added. The control treatment was performed in the same way, using distilled water. These plates were incubated at 26 °C for up to 48 h. At the end of the incubation period, the numbers of live nematodes 24 and 48 h after exposure to the extracts were evaluated. Analysis of the number of J2 was quantified by Microscope (Olímpicus CX41) with coupled chamber (20x). *Statistical analysis*: Data were assessed by One-Way ANOVA followed by post-hoc Tukey's test using GraphPad Prism 5.01 statistical software (GraphPad Software, Inc, CA, USA). Differences were considered significant at $p<0.05$. Data are expressed as the Mean±SEM.

2.5 Antimicrobial activity

The following tests were performed based on the methodologies described by dos Santos Alves et al. (2016) and Jobim et al. (2014).

Microorganisms: In this study, bacterial and fungical standard strains (American Type Culture Collection - ATCC) and clinical isolates of *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 6538), *Acinetobacter baumannii* (ATCC 19606), *Aeromonas caviae* (Clinical isolate - IC) *Candida albicans* (ATCC 24433) and *Pseudomonas aeruginosa* (PA01) were used.

Preparation of inoculums: The inoculum sizes were standardized according to the Clinical Laboratory Standards Institute guidelines (CLSI). Isolated colonies were grown for 18-24 h in Mueller Hinton Agar (Himedia) and the suspension was prepared in a saline solution (NaCl 0.85%) with density adjusted to 0.5 on the McFarland scale (1.5×10^8 CFU/mL).

Kirby-Bauer method: The Kirby-Bauer or disc-diffusion method was performed as a preliminary screening test to evaluate antimicrobial susceptibility (Bauer et al. 1966). In order to do so, sterile discs of paper with size of 6 mm were impregnated with 15 µL of phenolic and volatile extracts in the concentration of 120 and 93.86 mg/mL, respectively. The discs were placed in Müller-Hinton Broth Agar (Himedia) whose surface was previously inoculated with the microorganisms by a sterile swab, and then incubated at 37 °C for 24 h. At the same time, a control was performed with dimethylsulfoxide (DMSO, Vetec Química Fina Ltda.). All tests were performed in triplicate. After the incubation, we only checked whether or not there was formation of inhibition halos. For the extract (volatile) that showed microbial inhibition, the minimum inhibitory concentration assay was performed.

Determination of minimum inhibitory concentration (MIC): The antibacterial and antifungal activity of the extracts against *Aeromonas caviae* and *Candida albicans* was evaluated using the broth microdilution method. The assay was carried out in 96-well microtiter plates using Mueller Hinton Broth (Himedia). The extract (volatile) was diluted in DMSO, giving rise to serial concentrations that ranged from 93.86 to 1.46 mg/mL. The inoculum was mixed in each dilution of the extract. The plates were incubated at 37 °C and the minimal inhibitory concentration (MIC) was recorded after 24 h of incubation. The MICs were defined as the lowest concentration of extract that inhibits visible bacterial growth. This test was performed in triplicate. 2,3,5-triphenyltetrazolium chloride was used as an indicator of microbial growth. All experiments were conducted in triplicate

3 I RESULTS AND DISCUSSION

3.1 Trypanocidal Activity

The SFE–CO_2 extract (Fig 1A) in concentrations of 2500 µg mL^{-1} and 1250 µg mL^{-1},

eliminated 100% of the trypomastigotes after 1 hour of exposure. At the concentration 625 µg mL^{-1}, there was a reduction in the number of trypomastigotes in 1 h, with death after 3h. Believed that the compounds found in the SFE–CO_2 extract are responsible for reducing the number of trypomastigotes in vitro, since monoterpenes and sesquiterpenes from other plants have already shown trypanocidal effect in vitro against *T. cruzi*, *T. brucei* and *T. evansi.* (Baldissera et al. 2017). For the UAE–EtOH extract (Fig. 1B) at 250 µg mL^{-1} there were no live trypomastigotes after 1 hour. At the concentrations of 62.5 µg mL^{-1} and 15.62 µg mL^{-1}, the death of trypanosomes occurred in 3 and 6 hours, respectively. These results are related to the chemical composition of the extract, because, some authors demonstrate that phenolic compounds and flavonoids have anti-Trypanosoma activity (dos Santos Alves et al. 2016; Amisigo et al. 2019).

There was a reduction in the death of the parasites with the decrease of the concentrations; however, at the end of the experiment time (9h), all the concentrations presented trypanocidal action. Considering the anti-trypanosome activity the studies suggest promising antiparasitic activities, indicating a possible therapeutic alternative in the treatment of trypanosomiasis. These results are relevant because there are few drugs for the treatment of *Trypanosoma evansi*, which are often associated with cases of bacterial resistance and hepatic and renal damage (Borges et al. 2012; Baldissera et al. 2014; dos Santos Alves et al. 2016).

No studies were found on the anti-Trypanosoma activity of *H. ringens*, but our results are in agreement with other existing works. Baldiserra et al. (2014) evaluated aqueous, methanolic and ethanolic extracts of *Achyrocline satureioides*, which trypanocidal effect after 1 hour of treatment at concentrations of 500 and 1000 µg mL^{-1} and after 9 hours at concentrations of 50 and 100 µg mL^{-1}. Gressler et al. (2015) observed that curcumin at 100 µg mL^{-1} concentration killed all parasites within 1 hour of treatment. In relation to the study of dos Santos Alves et al. (2016), our results showed superior activity, since the dichloromethane, ethyl acetate and butanolic extracts of *Equisetum hyemale* at concentration 25000 µg mL^{-1}, eliminated *trypanosoma evansi* only after 9 hours of treatment.

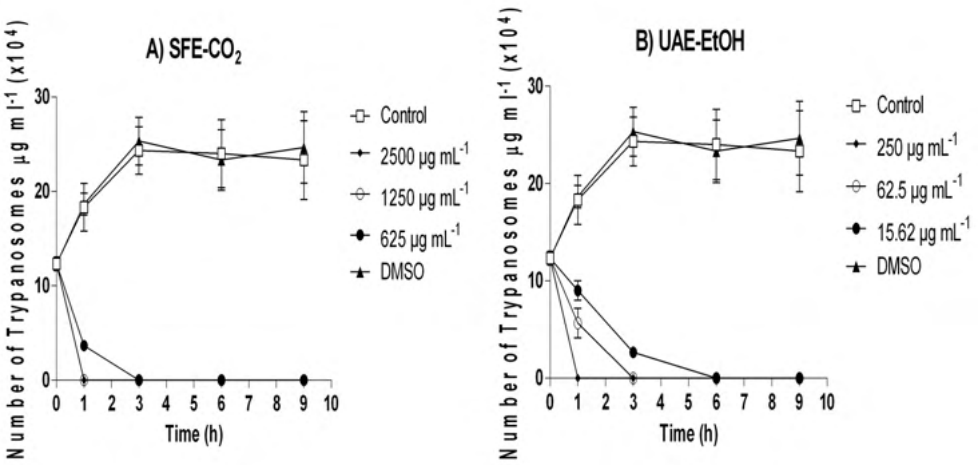

Figure 1. In vitro, trypanocida activity of extracts SFE-CO_2 (A) and UAE-EtOH (B) of *H. ringens* against Trypanosoma evansi when compared control (untreated) and DMSO.

3.2 Nematicidal activity

Figure 2 (A and B) shows the relationship between the action of both extracts of H. ringens at different concentrations and the number of live *Meloidogyne javanica* J_2 larvae compared to the control. According to the data obtained, 80% of J_2 larvae were killed for the SFE–CO_2 and UAE–EtOH extracts at concentrations 10 and 80 mg mL^{-1} and in their dilutions, respectively.

The SFE-CO_2 extract had nematicidal activity at a lower concentration than the UAE–EtOH extract, which was already expected, once that, according to Dolwitsch et al. (2020) this extract is composed mainly of terpenes, among them pulegone which has antiparasitic action reported (Ribeiro et al. 2010; Bandeira-Junior et al. 2017).

Other authors corroborate the concentrations tested for this assay, Lopes at al. (2005) found that aqueous basil extract (*O. basilicum*) at 100 mg mL^{-1} showed action against *Meloidogyne incognita*. For Neves et al. (2008) the papaya seed extract (*Carica papaya*) at 100 mg mL^{-1} caused the death of 100% of *M. javanica* juveniles. According to Kong et al. (2006) evaluated the essential oils obtained from 88 different medicinal plants at a concentration of 10 mg mL^{-1} on *Bursaphelenchus xylophilus* nematoid. The authors observed that essential oils of cinnamon (*Cinnamomum zeylanicum*), citronella (*Cymbopogon nardus*), clove (*Eugenia caryophyllata Thumberg*), coriander (*Coriandrum sativum*), lemongrass (*C. citratus*), oregano (*Origanum vulgare*), black pepper (*Pimenta officinalis*), clove (*Satureja hortensis*) and thyme (*Thymus vulgaris*) caused 100% lethality to the nematode after 24 hours of exposure.

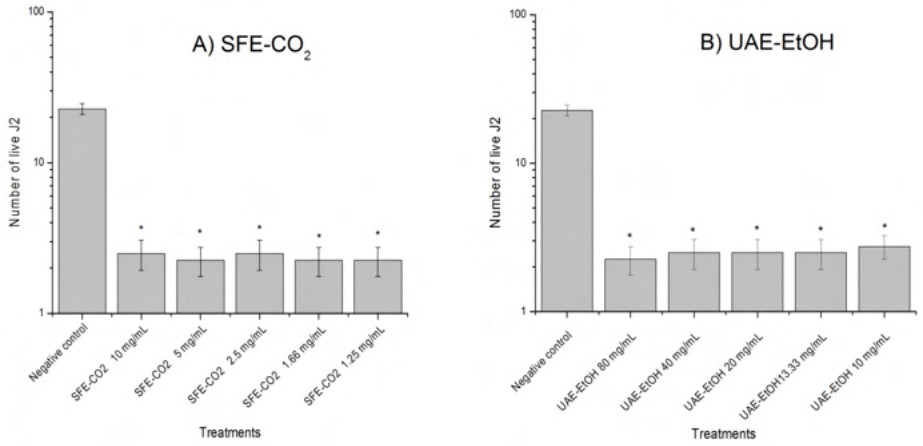

Figure 2. Number of juveniles of second stage (J2) of Meloidogyne alive after action of the extracts SFE-CO2 (A) and UAE-EtOH (B) in different concentrations of H. ringens compared to the negative control.

3.3 Antimicrobial activity

The UAE–EtOH extract did not show activity for microorganisms, in the concentration of 120 mg/mL. The volatile extract in the concentration of 93.86 mg/mL showed activity against the Gram negative bacteria *Aeromonas caviae* and against the fungus *Candida albincans*. The MICs of 1.46 mg/mL were found for *Aeromonas caviae* and against the fungus *Candida albincans*.

It is likely that the negative result the UAE–EtOH extract may be related to the antagonism between the constituents of the extract (Aneja 2012).

There are only few studies in the literature on the antimicrobial activity of *H. ringens.* (Sutili et al. 2015). verified the antibacterial activity of the essential oil of *H. ringens* against *Aeromonas hydrophila*. The MIC found in the study ranged from 800 to 3.200 µg/mL. Although the authors used *Clevenger* extraction and tested another bacterial species, the results support what was found in this study. Gomes et al. (2012) found antifungal activity of *H. ringens* front of other fungi, among them: *Fusarium solani*, *Fusarium oxysporum lycopersisi* and *Guignardia citricarpa*. As for other species of the genus *Hesperozygis*, Martini et al. (2011) demonstrated that the essential oil of *H. myrtoides* was active against *Lactobacillus casei*, *Staphylococcus aureus*, *Escherichia coli*, *Candida Albincans* and *Candida glabrata*. Rosa et al. (2019) investigated the antibacterial activity of *H. ringens* by determining the minimum inhibitory concentration (MIC) of hexane and ethanolic extracts obtained by soxhlet against *Aeromonas hydrophila*, *Raoultella ornithinolytica*, and *Citrobacter freundii* from infected fish, being that only the hexane extract had activity against the tested microorganisms. Although the aforementioned studies involve different microorganisms,

they corroborate with what was found in this work regarding the antibacterial and antifungal potential of the species. Through these results, it was verified that the volatile extract of *H. ringens* presented antibacterial and antifungal activity against microorganisms not yet reported for the species, which have important clinical relevance.

The antimicrobial properties of the SFE-CO_2 extract of *H. ringens* are ratified by the compounds that were found, since pulegone (Pellegrini et al. 2017), menthone (Desam et al. 2019), limonene (Ambrosio, 2016) are recognized by these activities.

Analysing the antimicrobial activity suggesting that the plant can contribute to the development of therapeutic alternatives in the treatment of bacterial infections.

4 I CONCLUSION

Both extracts presented action against *T. evansi* and second stage larvae (J_2) of *M. javanica*, thus, this extracts has the potential a new therapeutic option in the treatment of trypanosomiasis and nematodes.

The extract obtained by SFE–CO_2 presented antimicrobial activity against *Aeromonas caviae* and *Candida albicans*, so it may be an alternative for the development of new antimicrobial agents.

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ÍNDICE REMISSIVO

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