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CROP PROTECTION

Activity of *Piperaceae* extracts and fractions in the control of *Phytomonas serpens*

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ABSTRACT: *Protozoa of the genus Phytomonas are harmful parasites to several agricultural crops of economic importance. Due to their recognized biological activity, crude extracts of Piper aduncum, P. crassinervium, P. hispidum, and P. amalago leaves, were tested using the microdilution plate technique to assess the antiparasitic potential against Phytomonas serpens. Results showed that the ethanolic crude extract of P. crassinervium and P. amalago presented the best inhibitory concentration for 50% of the cells (IC_{50}), 16.5 µg mL^{-1} in chloroform phase, and 18 µg mL^{-1} in aqueous phase, respectively, after 48 h treatment. Cytotoxicity analyses were performed using the colorimetric method of sulforhodamine-B in LLCMK$_2$ mammalian cells. The chloroform phase of P. crassinervium was subjected to the fractionation process, in which the ethyl acetate and dichloromethane fractions obtained better IC_{50} values. Scanning electron microscopy (SEM) images showed alterations in the cell membrane of the treated parasites. The data obtained indicate a potential antiparasitic effect of the Piper species analyzed against P. serpens, being considered promising candidates for formulations of bioproducts to control the parasite.*
Key words: *antiparasitic activity, medicinal plants, Piper, Trypanosomatid, Phytomonas.*

Atividade de extratos e frações de *Piperaceae* no controle de *Phytomonas serpens*

RESUMO: *Protozoários do gênero Phytomonas são parasitas prejudiciais a várias culturas agrícolas de importância econômica. Devido a sua atividade biológica reconhecida, extratos brutos de folhas de Piper aduncum, P. crassinervium, P. hispidum e P. amalago, foram testadas pela técnica de microdiluição em placa para avaliar o seu potencial antiparasitário contra Phytomonas serpens. Os resultados mostraram que o extrato bruto etanólico de P. crassinervium e P. amalago apresentaram as melhores concentrações inibitórias para 50% das células (IC_{50}), 16,5 µg mL^{-1} na fase clorofórmio e 18 µg mL^{-1} na fase aquosa, respectivamente, após 48 h de tratamento. Análises de citotoxicidade foram realizadas através do método colorimétrico da sulforodamina-B, em células de mamíferos LLCMK2. A fase clorofórmio de P. crassinervium foi submetida ao processo de fracionamento, no qual as frações acetato de etila e diclorometano obtiveram melhores valores de IC_{50}. Imagens de microscopia eletrônica de varredura (MEV) mostraram alterações na membrana celular dos parasitas tratados com fase aquosa de P. amalago. Os dados obtidos indicam potencial efeito antiparasitário das espécies de Piper analisadas contra P. serpens, sendo consideradas candidatas promissoras para formulações de bioprodutos para controle do parasito.*
Palavras-chave: *atividade antiparasitária, plantas medicinais, Piper, Tripanossomatídeo, Phytomonas.*

INTRODUCTION

Species of the *Trypanosomatidae* family are pathogenic protozoa that cause various diseases, affecting vertebrate animals, humans, and plants. In humans, these flagellates are etiological agents of Chagas disease (*Trypanosoma cruzi*) and Leishmaniasis (*Leishmania* spp.). In plants, *Phytomonas* is a genus of trypanosomatids, composed of several species recognized as significant pathogens, responsible for

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producing diseases in tropical agricultural crops of great economic interest (MASLOV et al., 2018).

Studies showed that some species of *Phytomonas* spp. are capable of causing lethal plant diseases, while others cause minor damage, infecting different plant tissues such as dairy ducts, phloem, seeds, flowers, and fruits. *Phytomonas* are an example of dixenic trypanosomatids, which affect both the invertebrate host and plants as a promastigote (CAMARGO et al., 1999). Transmitted by phytophages, these hemipterous insects are popularly known as bedbugs, and they are responsible for spreading the infection to more than 100 plant species (KAUFER et al., 2017). Among them, important crops such as tomatoes, coffee, cassava, cocoa trees, and palm trees that produce oil and coconut (PORCEL et al., 2014; FROLOV et al., 2019).

For a long time there were doubts about correlating the genus *Phytomonas*, as being phytopathogen or only endophytic to plant hosts, because its differentiation is complex and it does not produce easily detectable effects on plant growth or yield (ABREU FILHO et al., 2001). Since initial research on the pathogenicity of *Phytomonas* begun, two main species producing plant diseases have been identified: *Phytomonas staheli* that causes sudden wilt in coconut trees (*Cocos nucifera*) and slow wilt in oil palm (*Elaeis guineensis*). In these cases, leaf wilt leads to root rot. Additionally, *Phytomonas leptovasorum* causes phloem necrosis in Arabica and Liberica coffee (JASKOWSKA et al., 2015). The species *Phytomonas serpens* is isolated only in tomato fruit. The fruit infected by the insects *Phthia picta* and *Nezara viridula* present yellowish spots that result in nutritional loss and; consequently, lead to commercial impracticability of the product (JANKEVICIUS et al., 1989). *Phytomonas serpens* is also attributed to the production of auxin, a class of phytohormones that ends up causing interference with the plant metabolism, thus affecting plant development (IENNE et al., 2014). Despite the damage caused to crops, *P. serpens* is not considered a phytopathogen parasite, since the promastigote form of the parasite remains restricted to the place of infection (OLIVEIRA et al., 2017).

Despite being globally present, such as in African, European, and Asian countries, species of *Phytomonas* are considered endemic in South America, with a significant number of species in Brazil, which makes them a food security risk for many economies around the world. (DA SILVA et al., 2013). The most significant losses are registered mainly in developing countries, such as Colombia, Brazil, Ecuador and Costa Rica, which concentrate their export economies on tropical crops affected by *Phytomonas*. Besides, there are no adequate chemical control and treatment against these microorganisms, which requires the felling of the sick plant or the removal of infected plant material (JASKOWSKA et al., 2015).

In this sense, many studies point to plants as a potential source of new phytochemical substances, whose extracts can be used as antimicrobial agents (PEREIRA et al., 2018). The genus *Piper* has around 2000 species, and it is common in tropical and subtropical regions of the Atlantic Forest (PRANDO et al., 2014). Traditionally, these plants are used by the population of these regions in the treatment of influenza, cough, and rheumatism once they have ethnomedicinal properties. They are popularly known as pepper plants or false jaborandi (LAGO and KATO, 2007).

A complex of natural products is synthesized from the secondary metabolism of *Piper*, consisting in a mixture of bioactive organic compounds such as phenols, terpenes, esters, alcohols, among others (KUMAR et al., 2018). Several studies recognized these compounds for their biological activity as bactericides and fungicides (COSTA et al., 2016; PASCOLI et al., 2018; FERNANDEZ et al, 2019), antiprotozoans (GARCIA et al., 2013; VILLAMIZAR et al., 2017), antitumoral (LONGATO et al., 2011) and antivirals (BERTOL et al., 2012).

Thus, the objective of this study was to evaluate the antimicrobial activity of the crude extract and fractions of *Piper amalago, P. aduncum, P. hispidum*, and *P. crassinervium,* against *P. serpens*, aiming the use of natural compounds with antiparasitic action.

MATERIALS AND METHODS

Plant material

The plant specimens analyzed in this study were identified by Dr. Adriana Lenita Albiero from the State University of Maringá and deposited in its Herbarium. The leaves of (1) *Piper amalago* L. (HUEM 9885), (2) *Piper aduncum* L. (HUEM 9651) and (3) *Piper hispidum* Sw (HUEM 9137), were collected from Dr. Luiz Teixeira Mendes forest reserve in Maringá- Paraná, Brazil (coordinates: 23° 26'03.1 "S 5° 58'04.7). The leaves of the plant (4) *Piper crassinervium* H.B. & K. (HUEM 9884) were collected from Prof. Irenice Silva Medicinal Herbs Garden in the campus of the State University of Maringá (coordinates 23º 24´12.0" S, 51º 56´ 22.5" W).

Preparation of crude extract and fractionation

The fresh leaves were dried in air circulation greenhouse (*QuimisR*©, model Q-31, Diadema-SP,

Brazil) at 40 °C. Three days later, the material was crushed in a knife grinder (*Tecnal MarconiR*©, model TE048, Piracicaba, Brazil) and then powdered. Extracts of *Piper* species were obtained by the maceration method at room temperature with ethanol: water (9: 1, v/v), resulting in raw extracts. The extracts were filtered and evaporated under vacuum at 40 °C to obtain an aqueous extract. This freeze-dried aqueous extract was subjected to direct extraction with chloroform (without partition), generating a fraction soluble in chloroform, which was later removed generating the aqueous chloroform extract used in clinical tests (Figure 1).

The extracts were kept in a freezer at approximately -20 °C. The active chloroform extract of *P. crassinervium* was processed by vacuum adsorption column chromatography on silica gel 60 (70 to 230 mesh) and eluted with hexane, hexane: dichloromethane (50: 50; v/v), dichloromethane, ethyl acetate, and methanol, yielding 5 fractions.

Maintenance of Phytomonas serpens

Promastigote forms of *P. serpens* (15^T strain) were kept in Warren medium (Warren, 1960) pH 7.0, supplemented with 10% inactivated bovine fetal serum (SFB - Gibco Invitrogen Corporation, New York, USA) at 28 ºC.

Antipromastigote activity

Crude extracts and fractions of *Piper* species assessed were dissolved in dimethylsulfoxide (DMSO) and Phosphate-bufferid saline (PBS), obtaining a stock solution of 10,000 µg mL^{-1}. Subsequently, dilutions were performed in Warren medium to obtain final concentrations of 1000, 500, 100, 50, and 10 µg mL^{-1}. In these concentrations, protozoa with 48 h culture (initial inoculum of $1x10^6$ cells/mL) were added. The assays were performed in 24-well microplates, incubated at 28 ºC. After 48 h, growth was evaluated by counting the protozoa

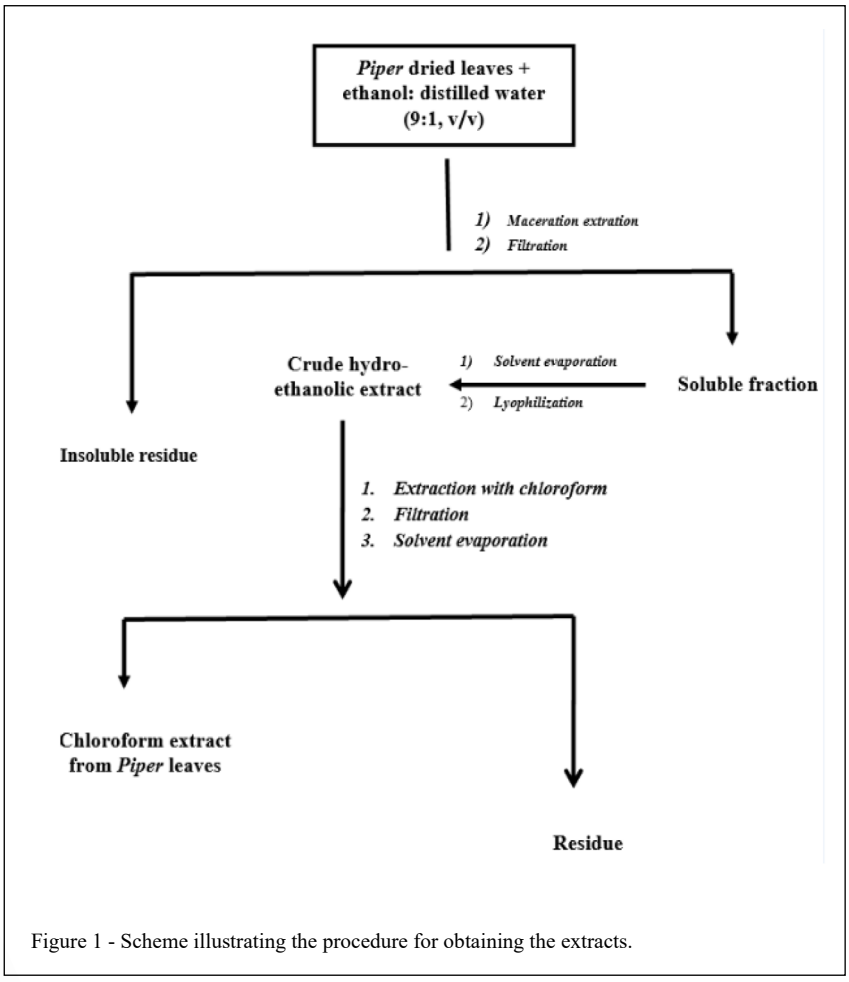

Figure 1 - Scheme illustrating the procedure for obtaining the extracts.

in the Neubauer chamber. Later, the percentage that inhibited 50% of protozoa growth was calculated (IC_{50}). Protozoa culture with no addition of extracts was used as a negative control.

Cytotoxicity assay

A suspension of 100 µL of $LLCMK_2$ cells (*Macaca mulatta* kidney epithelial cells) at a concentration of $2.5x10^5$ cels/mL in DMEM (Dulbecco Modified Eagle Medium-GibcoR®) was seeded in 96-well plates and incubated at 37 °C with a 5% CO_2 tension. After 24 h incubation, 100 µL of the various concentrations of crude extract solutions - *P. aduncum, P. crassinervium, P. hispidum, and P. amalag*o - were added and incubated for additional 96 h. Cell growth was evaluated by the colorimetric method of sulforodamine B, according to SKEHAN et al. (1990). The reading was performed in an ELISA reader (*Bio-Tek* FL-600 Microplate Fluorescence Reader) at an optical density of 530 nm and then the CC_{50} (cytotoxic concentration for 50% of cells) was calculated. The results were expressed as the percentage of growth inhibition with regards to the control.

Scanning electron microscopy (SEM)

Promastigotes (1×10^6 cells/mL) were treated with 18 µg mL^{-1} of *P. amalago* aqueous extract for 48 h at 28 ºC and then fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 h. Afterwards, the parasites were adhered to L-lysine coated coverslips and dehydrated in increasing concentrations of ethanol. The samples were dried at a critical CO_2 point, gold coated, and observed with a Shimadzu SS-550 scanning electron microscope (Japan) (GARCIA et al., 2013).

Statistical analysis

All tests were carried out in triplicate, and the data were analyzed through Analysis of Variance (ANOVA). Tukey's test was conducted, and a p-value of ≤0,05 was considered significant compared with the control group. The statistical analysis was performed with the program Graph-Pad Prism 4, USA.

RESULTS AND DISCUSSION

Among the tools that are most explored to biological control we have the use of plant extracts. Several studies have shown the wide biological activity of plant species that belong to the genus *Piper.* Recently FERNANDEZ et al. (2019) has reported promising results *in vitro* of dichloromethane fractions activity of *Piper corcovadensis* extract (Miq.) against *Mycobacterium tuberculosis*. PASCOLI et al. (2018) showed the antibacterial activity of crude extract and fractions of *Piper peltatum* and *Piper marginatum* against *Alicyclobacillus acidoterrestris.* The hexane-dichloromethane fraction of *P. peltatum* was the one that provided the best effect.

In our research, we first evaluated antiproliferative activity of hydroalcoholic crude extracts - chloroform phase (Figure 2), and aqueous phase (Figure 3) of *P. hispidum, P. aduncum, P. amalago* and *P. crassinervium* against promastigote forms of *P. serpens*, after 48 h incubation. The IC_{50} values obtained are shown in table 1. The chloroform phase of *P. crassinervium* demonstrated the best antiproliferative effect against *P. serpens* when compared with the chloroform phase of other *Piper* species. Among the aqueous phase extracts, *P. amalago* presented the lowest IC_{50} in *P. serpens.*

Similar results were presented by LOPES et al. (2008) in the assay with isolated compounds of *P. crassinervium* leaves that were evaluated against epimastigote forms of *T. cruzi*. The prenylated hydroquinone exhibited trypanocidal activity with IC_{50} of 6.10 µg mL^{-1}. Likewise, CARRARA et al. (2012) demonstrated the activity against promastigote forms of *L. amazonensis* from *P. amalago* leaf extraction. The extract showed significant antiproliferative activity against promastigotes, with an IC_{50} of 15 µg mL^{-1}.

An essential criterion in the research for active compounds with antiprotozoal activity is their toxicity on mammalian cells. For this purpose, the cytotoxicity of the extracts was evaluated on mammalian cells $LLCMK_2$. After 96 h treatment, the cytotoxic concentration for 50% of the cells (CC_{50}) was determined using the colorimetric method of sulforhodamine-B. CC_{50} values are exposed in Table 1. The CC_{50} obtained in the chloroform phase for all evaluated *Piper* species showed moderate toxicity, whereas the aqueous extracts showed low toxicity, with CC_{50} >850 µg mL^{-1}.

Extracts cytotoxicity of *Piper* species on $LLCMK_2$ mammalian cells was compared with the antiproliferative activity against promastigotes of *P. serp*ens, using the selectivity index (SI), the ratio between CC_{50} for $LLCMK_2$ cells and IC_{50} for protozoa. According to table 1, the aqueous fraction of *P. amalago* extract presented the best SI among the *Piper* species analyzed, which means that it was 47.5 times more toxic to promastigote forms of *P. serpens* than $LLCMK_2$ cells.

Therefore, the best selectivity indexes were presented by *P. amalago* in aqueous phase and by *P. crassinervium* in chloroform phase. Most

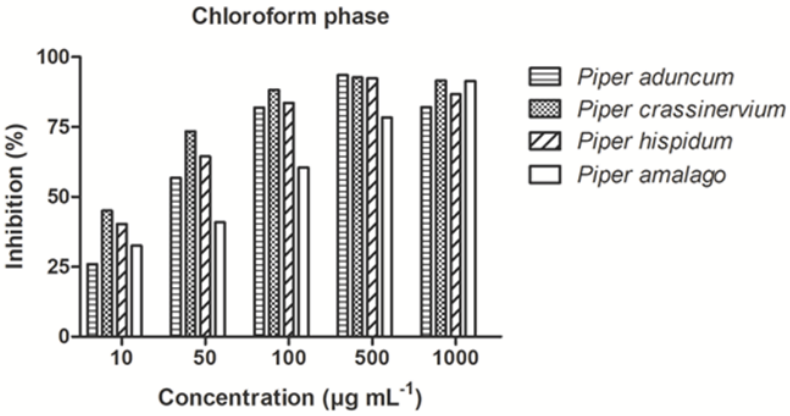

Figure 2 - Effect of *Piper* species extracts, in chloroform phase, on the proliferation of promastigote forms of *P. serpens*.

extracts were more toxic to the parasite than to mammalian cells.

In our results, the chloroform phase of *P. crassinervium* and the aqueous phase of *P. amalago* were selected for additional tests once they presented a more significant antiproliferative effect and a better selectivity index. The chloroform phase of *P. crassinervium* was submitted to the fractioning process. According to CARGNIN et al. (2013) and BAPELA et al. (2017) the compounds that present significant antiprotozoal activity are among those capable of being extracted by apolar solvents.

Figure 4 shows the antiproliferative activity of the five fractions obtained from *P. crassinervium* (Hexane fraction, Hexan-Dichloromethane fraction (1:1), Dichloromethane fraction, Ethyl acetate fraction, and Methanol fraction). The most active fractions were dichloromethane and ethyl acetate, both with an IC_{50} <10 µg mL^{-1}. The methanol fraction showed IC_{50} of 13 µg mL^{-1} and the hexane, and hexane dichloromethane fractions presented IC_{50} of 84 and 47 µg mL^{-1}, respectively. DMSO at the

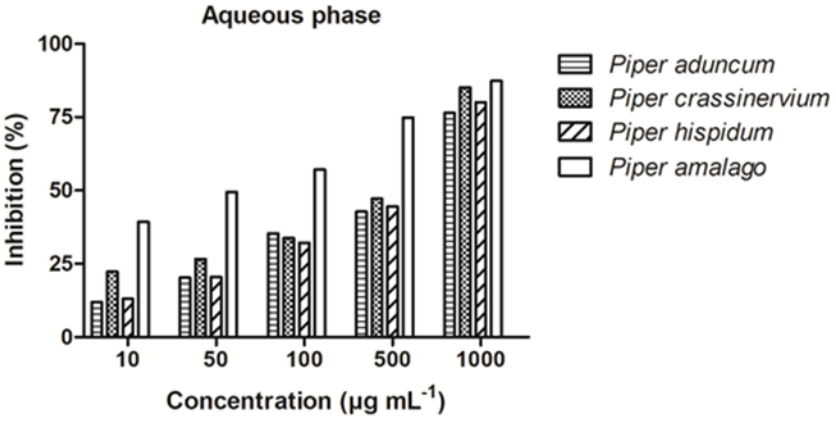

Figure 3 - Effect of *Piper* species extracts, in aqueous phase, on the proliferation of promastigote forms of *P. serpens*.

Table 1 - IC_{50} values for promastigotes of *Phytomonas serpens*; cytotoxic effects (CC_{50}) for $LLCMK_2$ cells and their respective selectivity indexes (SI).

Crude Extracts	Promastigotes IC_{50} (µg mL^{-1})	Cytotoxicity CC_{50} (µg mL^{-1})	SI
Piper crassinervium			
Chloroform phase	16.5 ± 0.7	210.0 ± 14.0	12.7
Aqueous phase	557.5 ± 3.5	>1000 ± 0	>1.8
Piper hispidum			
Chloroform phase	22.0 ± 0	87.5 ± 5.0	4.0
Aqueous phase	535.0 ± 21.2	>1000 ± 0	>1.9
Piper aduncum			
Chloroform phase	45.0 ± 2.8	39.0 ± 22.6	0.9
Aqueous phase	585.0 ± 7.0	>1000 ± 0	>1.7
Piper amalago			
Chloroform phase	55.0 ± 4.5	275.0 ± 49.9	5.0
Aqueous phase	18.0 ± 11.1	855.0 ± 145.0	47.5

maximum concentration used (1%) did not interfere with parasite growth.

RODRIGUES-SILVA et al. (2009) reported the *in vitro* activity of hydroalcoholic extract and fractions of *Piper ovatum,* against *Leishmania amazonensis*. A progressive increase in the antileishmanial effect was observed in the course of the fractionation. The dichloromethane and ethyl acetate fractions exhibited the best action against protozoa with IC_{50} values of 2,1 µg mL^{-1} for promastigotes and 24 µg mL^{-1} for amastigotes. These data corroborate the results of this study, since the same fractions showed higher antiprotozoal activity against *P. serpens*.

The action of the aqueous phase extracted from *P. amalago* was analyzed using scanning electron microscopy. The photomicrographs (Figure 5) reveal the differences in the morphology of promastigote cells treated with the extract when compared with the control (untreated cells). The control cells presented typical morphology of *P. serpens,* with elongated cell body, smooth cell

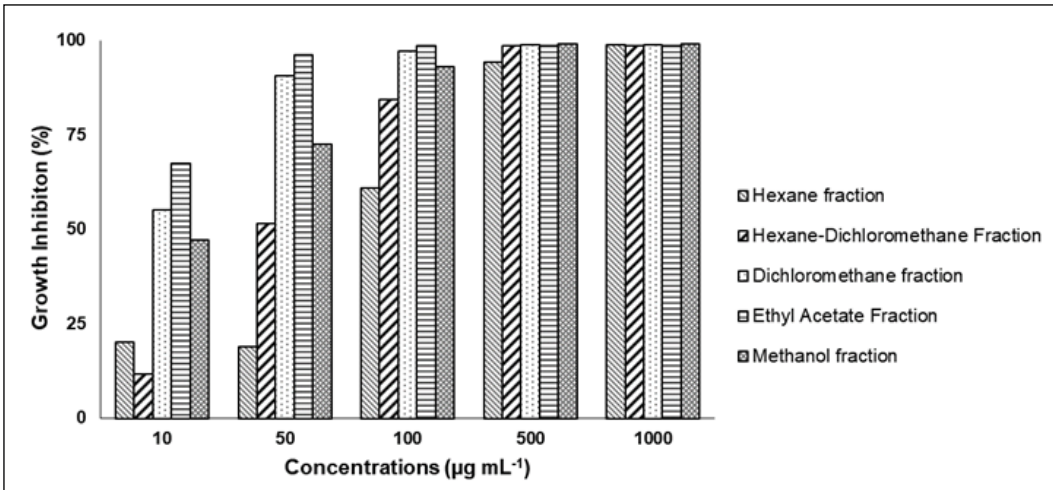

Figure 4 - Effect of the fractions from the extract, chloroform phase of *P. crassinervium*, on the proliferation of promastigote forms of *P. serpens*.

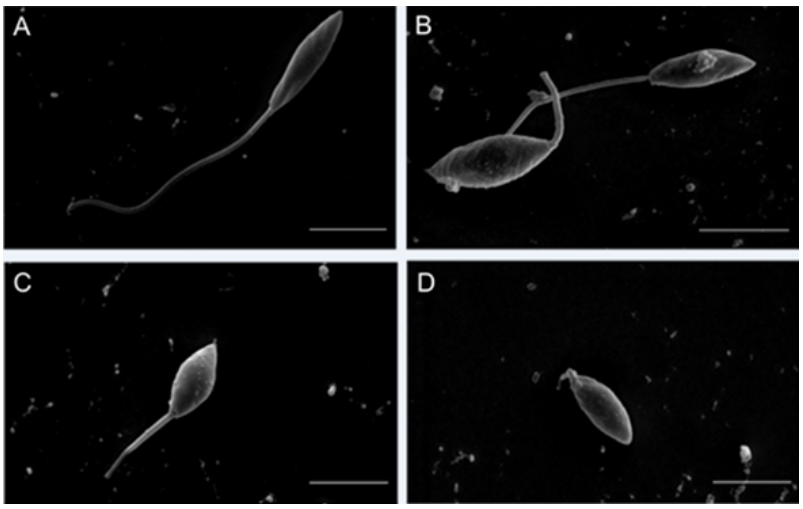

Figure 5 - Scanning electron microscopy of *P. serpens* promastigote cells, after 48 h treatment with the extract, aqueous phase of *P. amalago*: A) Control promastigote cells; B, C and D) promastigote cells treated with 18 µg mL^{-1} (IC_{50}). Bar = 5 µm.

surface, and preserved terminal flagellum (Figure 5A). On the contrary, when promastigotes were treated with their respective IC_{50} values (Table 1), there were some changes in their structural integrity. The aqueous phase of *P. amalago*, induced changes in the cell membrane, detected by deformities on the cell surface (Figure 5B), with a reduction in volume and rounding of the cell body (Figure 5C). Besides, it caused shortening and/or loss of the flagellum (Figure 5D).

There are currently no control records for *P. serpens*, and significant research efforts have been made to achieve formulations that can be used in bioproducts for parasite management. According to SILVA et al. (2019), the essential oil of *Varronia curassavica* (Cordiaceae) has an antiprotozoal activity against *P. serpens* by causing changes in the permeability of the cytoplasmic membrane of promastigote cells, reaching up to 75% reduction in cellular proliferation of parasites exposed to the essential oil.

MEDINA et al. (2015) investigated the toxicity of alkaloids produced by tomato plants, called tomatine and tomatidine, in inhibiting the growth of *P. serpens*. Results indicated that the tomatine is capable of disrupting the structure of the plasma cell membrane, causing the death of the parasite. However, tomatidine only interferes with growth due to the inhibition of sterol synthesis. Similar results have been described by EVANGELISTA et al. (2018).

CONCLUSION

Ethanolic crude extracts and *Piperaceae* fractions were effective against *P. serpens*, mainly because they showed higher selectivity to parasites than to $LLCMK_2$ mammalian cells. Our results showed through the inhibitory concentrations obtained after *P. crassinervium* fractionation, an improvement in the antiprotozoal activity of dichloromethane and ethyl acetate fractions. Moreover, the damage detected by scanning electron microscopy confirmed the effect of treatment with the crude extract *of P. amalago,* which was able to generate evident morphological changes in promastigote cells of *P. serpens*.

Later studies are essential to determine the inhibiton in pathways caused by the extracts since the evaluation carried out in this study points out that extracts of the different *Piper* species can be considered promising sources for the bio-guided isolation of new active compounds, indicating biotechnological potential in the development of chemical controls.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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