Antibacterial and leishmanicidal effects of the hexane extract of *Mitracarpus frigidus* (Rubiaceae) aerial parts

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ABSTRACT

In the present study it was investigated the antibacterial and leishmanicidal activities of the hexane extract and fractions of *Mitracarpus frigidus* aerial parts. The biological assays were evaluated against six strains of bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Escherichia coli* and *Enterobacter cloacae*), promastigote forms of four species of *Leishmania* (*L. major*, *L. braziliensis*, *L. chagasi* and *L. amazonensis*) and amastigote forms of *L. major*. Cytotoxicity against mammalian cells and phytochemical analysis of the major groups of phytoconstituents were also reported. The samples showed significant antimicrobial activity, mainly for *P. aeruginosa*, *S. dysenteriae* and *S. typhimurium* with MIC < 100 µg/mL. A pronounced antileishmanial potential was also verified for promastigote and amastigote forms, being the samples more specific for the intracellular stage. Despite the samples showed some toxicity against mammalian cells, they were more selective for the intracellular parasite than to host cells. The most active samples showed the presence of steroids, terpenes and flavonoids.

KEYWORDS: *Mitracarpus frigidus*, *Leishmania major*, antibacterial, terpenes, flavonoids.

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RESUMO

No presente estudo foram investigadas as atividades antimicrobiana e leishmanicida do extrato em hexano e frações das partes aéreas de *Mitracarpus frigidus*. Os ensaios biológicos foram avaliados contra seis cepas de bactérias (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Escherichia coli* e *Enterobacter cloacae*), formas promastigotas de quatro espécies de *Leishmania* (*L. major*, *L. braziliensis*, *L. chagasi* e *L. amazonensis*) e formas amastigotas de *L. major*. Avaliação da citotoxicidade contra células de mamíferos e triagem fitoquímica também foram estudados. As amostras apresentaram significativa atividade antimicrobiana, principalmente para *P. aeruginosa*, *S. dysenteriae* e *S. typhimurium* com CIM < 100 μg/mL. Um pronunciado potencial leishmanicida foi também verificado para as formas promastigotas e amastigotas, sendo que as amostras apresentaram especificidade para o estágio intracelular. Apesar destas amostras apresentaram certa toxidez contra células de mamíferos, estas foram mais seletivas para o parasita intracelular do que para as células hospedeiras. Esteroides, terpenos e flavonoides foram identificados nas amostras mais ativas.

PALAVRAS-CHAVE: *Mitracarpus frigidus*, *Leishmania*, antibacteriano, terpenos, flavonoides.

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INTRODUCTION

In recent years, the frequency of microbial resistance and its association with serious infectious diseases have increased gradually (Aguilar, 2009). Many micro-organisms have developed resistance against both the well-established conventional and the last generation antibiotics, causing serious public health problems and economic loss (Castro *et al.*, 2002; Aguilar, 2009). Not less important, leishmaniasis is a disease that causes serious public health problems in most tropical countries (Santos *et al*., 2008).

The use of medicinal plants for the treatment of many diseases is associated with folk medicine in different parts of the world (Araújo & Leon, 2001). Different cultures of the most distinguished countries, developed or not, know and use the therapeutic potential of plants in the treatment of diseases. These practices accompany men since prehistoric times and have evolved with them over the years (Coutinho *et al*., 2004).

The genus *Mitracarpus* is native to Brazil and is found mainly in tropical and subtropical countries. Some species of *Mitracarpus* have ethnopharmacological use reported, as *M. scaber* Zucc that is widely used in West African traditional medicine for headache, toothache, amenorrhea, dyspepsia, liver diseases, venereal diseases and leprosy. The juice of the plant is applied topically to dermatological diseases (Dalzier, 1936; Kerharo & Adam, 1974). There are no reports on the traditional uses of *M. frigidus* (Willd. ex Roem. & Schult.) K. Shum, an annual shrub commonly found in South America, including Brazil. Previous studies developed with the extracts obtained from this plant revealed the presence of flavonoids, tannins, alkaloids, terpenes and quinones, and presented antimicrobial, leishmanicidal, cytotoxic, laxative and anti-inflammatory activities (Fabri *et al.,* 2009; Fabri *et al.,* 2012a; Fabri *et al.*, 2013). Recently, kaempferol, kaempferol-O-rutenoside, rutin, ursolic acid and psychorubrin were identified in this plant (Fabri *et al.,* 2009; Fabri *et al*., 2012b; Fabri *et al*., 2013).

Due to the importance of microbial and leishmaniasis diseases, the aim of this study was to evaluate the antibacterial and leishmanicidal potential and the *in vitro* citotoxicity of the hexane extract and fractions of *M. frigidus* aerial parts. Furthermore, it was investigated their main groups of phytoconstituents.

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MATERIAL AND METHODS

Plant material

The aerial parts of *Mitracarpus frigidus* (Reem Willd. ex Schult.) K. Schum. were collected in Juiz de Fora, Minas Gerais, Brazil, in May 2009. The voucher specimen was deposited in the Herbarium Leopoldo Krieger (CESJ 46,076) of the Federal University of Juiz de Fora.

Fractionation of the hexane extract

The hexane extract (MFH) (16 g) obtained from the methanolic extract of *M. frigidus* aerial parts (Fabri *et al.*, 2009) was fractionated using column chromatography on silica gel (Merck, 70-230 mesh ASTM) size 74 x 4 cm, with an increasing gradient elution (hexane, dichloromethane and MeOH). The fractions obtained were analyzed by thin layer chromatography (TLC) and grouped according to their chromatographic profile in 9 fractions (MFH1 to MFH9). These fractions were concentrated at reduced pressure using a rotary evaporator, weighed and kept refrigerated until the completion of the biological tests.

The hexane extract and fractions were analyzed by thin layer chromatography (TLC) on silica gel 60 F254 (Merck) to identify the chemical constituents. The mobile phases were: I) hexane: EtOAc, 70:30, *v/v* for MFH1-MFH4; II) hexane: EtOAc, 30:70, v/v for hexane extract and MFH5-MFH9. The plates were visualized under UV light at 254 and 365 nm and revealed with sulfuric acid and vanillin, as universal revealer; 10% KOH (Borntrager Reagent) for the identification of anthraquinones and coumarins; Dragendorff Reactive for alkaloids; NP/PEG for flavonoids; Liebermann-Burchard Reagent for steroids and triterpenes and 0.1% $FeCl_3$ for phenolic compounds and tannins.

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Antimicrobial assay

Microbial strains

The samples were evaluated against a panel of microorganisms, *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia co*li (ATCC 10536), *Shigella dysenteriae* (ATCC 13313), *Salmonella enterica* sorovar *typhimurium* (ATCC 13311) and *Enterobacter cloacae* (ATCC 10699). These strains were cultured overnight at 37ºC in Mueller Hinton agar (MHA).

Serial dilution assay for determination of the minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of each sample was determined by using broth microdilution techniques as described by NCCLS (2002). MIC values were determined in Mueller Hinton broth (MHB). Bacteria were cultured overnight at 37ºC for 24 h in Mueller Hinton Agar (MHA). Samples stock solutions were two-fold diluted from 5000 to 2.5 µg/mL (final volume = 80 µL) and a final DMSO concentration ≤ 1%. Then, 100 µL of MHB were added onto microplates. Finally, 20 µL of 10^6 CFU/mL (according to McFarland turbidity standards) of standardized bacterial suspensions were inoculated onto microplates and the test was performed in a volume of 200 µL. Plates were incubated at 37ºC for 24 h. The same tests were performed simultaneously for growth control (MHB + bacteria) and sterility control (MHB + plant samples). Chloramphenicol (500 to 0.24 µg/mL) was used as positive control. The MIC values were calculated as the highest dilution showing complete inhibition of tested strain. The analyses were performed in duplicate.

Quantitative evaluation of antimicrobial activity

The antimicrobial activity of plant extracts may be expressed in different ways based on technique used. The agar diffusion method is commonly used as a preliminary test in the screening of plants for antimicrobial activity. While the micro-dilution method yields MIC values, the minimum concentration at which inhibition is observed (µg/mL). In this study other ways of expressing antimicrobial efficiency as comparative numerical values are used. Beside results being recorded in terms of MIC (µg/mL), percent activity values which demonstrate the total antimicrobial potency of particular extracts, and microbial susceptibility

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index (MSI), which is used to compare the relative susceptibility among the microbial strains were employed (Bonjar, 2004):

Percent activity (%) = (100 x No. of susceptible strains to a specific extract) / (Total nº of tested microbial strains). The percent activity demonstrates the total antimicrobial potency of particular extracts. It shows the number of microbial found susceptible to one particular extract.

Microbial susceptible index (MSI) = (100 x No. of extracts effective against each microbial strain) / (No. of total samples). MSI is used to compare the relative susceptibility among the microbial strains. MSI values ranges from '0' (resistant to all samples) to '100' (susceptible to all samples).

Antileishmanial assay

Parasites

Four *Leishmania* species for *in vitro* screening were used: *L. amazonensis* (IFLA/Br/67/PH8), *L. major* (MRHO/SU/59/P), *L. braziliensis* (MHOM/Br/75/M2903) and *L. chagasi* (MHOM/Br/74/PP75). Promastigotes of *L. amazonensis* were cultured in Warren's medium (brain heart infusion – BHI – enriched by hemin and folic acid), promastigotes of *L. major* and *L. braziliensis* were maintained in BHI medium, and promastigotes of *L. chagasi* were maintained in Medium 199, both supplemented with 10% fetal bovine serum at 24 °C.

Promastigote forms

The antileishmanial activity was performed according to a previously described method (Braga *et al*., 2007). Briefly, promastigotes from a logarithmic phase culture were suspended to yield 2×10^6 cells/mL (*L. amazonensis*) and 3×10^6 cells/mL (*L. chagasi, L. braziliensis* and *L. major*) after Neubauer chamber counting. The test was performed in 96-well microtiter plates maintained at 24°C. The analyses were performed in triplicate. The parasites were exposed to increasing concentrations of the samples (nonserial six dilutions: from 100 to 0.39 µg/mL) for 72 h at 24°C. Controls with 0.5% DMSO were also performed.

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The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a three day incubation period. Amphotericin B was used as reference drug.

Amastigote forms

Concerning the amastigotes *in vitro* model, inflammatory macrophages were obtained from BALB/c mice previously inoculated intraperitoneal with 2 mL of 3% thioglycollate medium (Morais-Teixeira *et al*., 2008). Briefly, peritoneal macrophages were plated at 2×10^6 cells/mL on coverslips (13 mm diameter) previously arranged in a 24-well plate in RPMI 1640 medium supplemented with 10% inactivated FBS and allowed to adhere for 24 h at 37°C in 5% CO_2. Adherent macrophages were infected with *L. major* (MRHO/SU/59/P) promastigotes in the stationary growth phase using a ratio of 1:5 at 33°C for 3 h. Non-internalized promastigotes were eliminated and solutions of tested compounds were added (nonserial five dilutions: from 50 to 1.2 µg/mL) and maintained at 33°C in 5% CO_2 for 72 h. Slides were fixed and stained with Giemsa for parasite counting (optical microscopy, 1000 x magnification). The parasite burden was evaluated by counting the intracellular parasite, uninfected and infected macrophages (at minimum 100 cells infected) in treated and untreated cultures and the survival index was obtained by multiplying the percentage of infected macrophages by the mean number of amastigote forms per infected cell (Santos *et al*., 2008). Control with 0.5% DMSO was also performed. The analyses were performed in triplicate.

Cytotoxicity against mammalian cells

Mouse peritoneal macrophages were obtained and cultured as described before. Briefly, the macrophages were used for cytotoxicity assay in a concentration of $2x10^6$ cells/mL in 96-well culture plates in RPMI 1640 medium supplemented with 10% inactivated FBS, at 37 °C and 5% CO_2 atmosphere. After 24 h, the adherent macrophages were incubated with the extracts in a serial dilution (with concentration ranging from 150 to 15 µg/mL), in duplicate at each concentration for 72 h at 37 °C and 5% CO_2 atmosphere. The viability of the macrophages was determined with the MTT assay using a multiwall scanning spectrophotometer (Multiskan EX microplate reader), as described above, and was confirmed by comparing the morphology with the control group via light microscopy (Carmo *et al*.,

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2011). Dose response curves were plotted (values expressed as percentage of control optical density) and CC_{50} values (50% inhibitory concentration) were obtained. The analyses were performed in triplicate.

Statistical Analysis

For the antileishmanial activity and cytotoxicity on macrophages, the IC_{50} and CC_{50} values, respectively, were carried out at 5% significance level ($p < 0.05$, CI 95%), using a nonlinear regression curve (GraFit Version 5 software). For *Leishmania* amastigote assays, the statistical analysis was performed with the software GraphPad Prism 4.

RESULTS AND DISCUSSION

Phytochemical analysis

The fractionation of the hexane extract yielded nine fractions (MFH1 - MFH9) which were weighed and analysed by TLC (Table 1).

Table 1– Yield (% *w/w*) and phytochemical screening of hexane extract (MFH) and fractions of *Mitracarpus frigidus*

Samples	Yield (% w/w)[a]	Phytocompounds[b]							
		St	Tr	Cm	An	Ph	Al	Fl	Ta
MFH	-	+	+	+	-	+	+	+	-
MFH1	4.7	-	+	-	-	-	-	-	-
MFH2	1.1	-	+	-	-	-	-	-	-
MFH3	2.7	-	+	-	-	-	-	-	-
MFH4	1.4	-	+	-	-	-	-	-	-
MFH5	2.2	-	+	-	-	-	-	-	-
MFH6	63.3	+	+	+	-	+	-	+	-
MFH7	10.9	+	+	-	-	+	-	+	-
MFH8	1.1	+	+	-	-	+	-	+	-
MFH9	3.4	+	+	-	-	+	+	+	-

[a] Yield in relation to the hexane extract (MFH)

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The TLC analysis identified the presence of triterpenoids in all samples tested, and steroids and flavonoids in the most polar samples (MFH6 - MFH9). Alkaloids and coumarins were found only in MFH6 and MFH9, respectively.

Previous study with MFH not detected the presence of triterpenes and coumarins (Fabri *et al.*, 2009). It occurred for, in our work, TLC and specific reagents were utilized instead of colorimetric assays in test tubes.

Antibacterial activity

The antibacterial activity was concentrated in MFH7 and MFH8 with MIC ≤ 125 µg/mL. *S. thyphimurium*, *P. aeruginosa*, *S. dysentereae* and *E. coli* were the most susceptible microorganisms. Importantly, for *S. dysenteriae*, the MIC value found to MFH8 was lower than that observed to the positive control (chloramphenicol) (Table 2).

The percent activity values and MSI were calculated for the samples that presented MIC < 1000 µg/mL. The percentage of antibacterial activity was greater for MFH8 with 100% activity, followed by MFH and MFH7 with 83%. The MSI revealed that *S. dysenteriae*, *S. typhimurium* and *E. cloacae* strains were susceptible to 83% of the samples tested, followed by *P. aeruginosa* (67%).

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Table 2 – Antibacterial activity of the hexane extract (MFH) and fractions of the *Mitracarpus frigidus.*

Samples	Minimum inhibitory concentration – MIC (µg/mL)[a]					
	Sa	*Pa*	*Sd*	*St*	*Ec*	*Ecl*
MFH	250	>1000	250	500	500	250
MFH1	1000	500	500	500	1000	1000
MFH2	1000	1000	1000	1000	1000	1000
MFH3	>1000	>1000	1000	1000	>1000	1000
MFH4	>1000	>1000	>1000	>1000	>1000	>1000
MFH5	>1000	>1000	>1000	>1000	>1000	500
MFH6	1000	1000	1000	1000	1000	1000
MFH7	1000	500	250	125	125	500
MFH8	250	62.5	31.3	31.3	500	500
MFH9	1000	500	500	500	1000	500
Chl[b]	25	>100	50	3.12	1.56	3.12

[a]*Sa - Staphylococcus aureus*; *Pa - Pseudomonas aeruginosa*; *Sd - Shigella dysenteriae*; *St - Salmonella typhimurium*; *Ec - Escherichia coli*; *Ecl - Enterobacter cloacae*
[b]Chl – Chloramphenicol

Previous studies had shown that substances with antibacterial activity from plants appear to be more active against Gram-positive than Gram-negative organisms (Herrera *et al*., 1996). The reason for this difference in sensitivity can be attributed to the morphological characteristics of these organisms. Gram-negative bacteria have an outer phospholipid layer with structural components of lipopolysaccharides. For this reason, Gram-positive should be more susceptible to the action of substances because these have only an outer layer of peptidoglycan as effective barrier (Wilkens *et al*., 2002).

The broad spectrum of activity against Gram-negative bacteria found for the fractions can be attributed to the presence of the lipophilic substances, triterpenes and steroids, which may be related to lipophilic components of plant extracts. These components increase permeability and loss of cellular components, and a change variety of enzyme systems, including those involved in the production of cellular energy and synthesis of structural components, inactivating or destroying genetic material (Bagamboula *et al*., 2004). Flavonoids are a broad class of phenolics that are known to possess antimicrobial activity,

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essentially by enzyme inhibition of DNA gyrase (Cushnie & Lamb, 2005). These results are extremely important as Gram-negative bacteria such as *S. thyphimurium* and *P. aeruginosa* are responsible for a high incidence of infections. The most common infections in Intensive Care Unit (ICU) are those caused by Gram-negative such as *P. aeruginosa*, especially ventilator-associated pneumonia. At ICU of large hospitals, resistance to *P. aeruginosa* is 20 to 75% for beta-lactamic and aminoglycosides antibiotics and this is accompanied by cross-resistance (Arruda, 1998). *Salmonella* infections cause diarrhea, fever, abdominal pain, and people usually recover without treatment. However, some people like the elderly, children and those with compromised immune systems, the diarrhea may be so severe that the patient must be hospitalized. In these patients, the infection can spread from the intestine into the bloodstream and then to other body sites and can lead to death (Hohmann, 2001). *S. dysenteriae*, a gram-negative bacterium, is a significant cause of gastroenteritis in both developing and industrialized countries (Boumghar-Bourtchai *et al*., 2008). People infected with *Shigella* develop diarrhoea, fever and stomach cramps starting a day or two after they are exposed to the bacterium. It is typically associated with mild self-limiting infection (DeLappe *et al*., 2003).

Antileishmanial activity and cytotoxicity

Protozoa parasites of the *genus Leishmania* cause visceral, cutaneous, and mucosal diseases in humans, which are collectively referred to as leishmaniasis. These diseases affect more than 12 million people worldwide and are responsible for high rates of mortality in tropical and subtropical countries. The drugs of choice for the treatment of leishmaniasis are pentavalent antimonials, but toxic side effects, limited efficacy to control parasite proliferation and drug resistance are frequently encountered (Santos *et al*., 2008; Cruz *et al*., 2009). Considering the side effects and the resistance that pathogenic protozoan parasites develop against these drugs, more attention should be given to the extracts and biologically active compounds isolated from plant species (Mendonça-Filho *et al*., 2004).

Table 3 shows the antileishmanial activity of MFH and fractions against the promastigotes forms of four *Leishmania* species: *L. amazonensis*, *L. braziliensis, L. major* and *L. chagasi*. MFH, MFH1 and MFH6 to MFH9 showed the best antiproliferative activities against *Leishmania* species. MFH and MFH9 showed the highest activity against the four species. In general, *L. major* promastigotes were the most sensitive parasite to the samples tested.

Table 3 – Effects of hexane extract (MFH) and fractions of the *Mitracarpus frigidus* against *Leishmania* promastigote and amastigote forms and murine macrophages.

Samples	Antileishmanial activity - IC$_{50}$ (µg/mL)[a]					Peritoneal macrophages	SE[d]	SS[e]
	Promastigote				Amastigote			
	La	Lb	Lm	Lc	Lm	CC$_{50}$ (µg/mL)[b]		
MFH	58.5 ± 0.10	30.2 ± 1.24	23.9 ± 1.1	27.5 ± 0.44	6.6 ± 1.1	18.2 (13.9 – 27.8)	2.8	3.6
MFH1	44.1 ± 2.00	43.7 ± 1.88	> 100	34.5 – 19.5	-	90.3 (55.5 -111.0)	-	-
MFH2	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH3	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH4	> 100	> 100	> 100	65.1 ± 0.08	-	> 100	-	-
MFH5	> 100	> 100	> 100	> 100	-	> 100	-	-
MFH6	> 100	47.4 ± 2.4	18.6 ± 3.03	32.7 ± 1.17	5.7 ± 2.0	29.4 (27.8 – 55.5)	5.2	3.3
MFH7	> 100	> 100	17.8 ± 0.3	36.7 ± 0.8	1.3 ± 0.2	20.3 (13.9 – 27.8)	15.6	13.7
MFH8	> 100	48.7 ± 0.85	28.6 ± 2.25	55.3 ± 0.3	2.6 ± 0.5	46.5 (27.8 – 55.5)	17.9	11.0
MFH9	60.6 ± 0.88	24.7 ± 0.66	16.6 ± 1.57	38.4 ± 0.43	3.7 ± 0.9	17.1 (13.9 – 27.8)	4.6	4.5
Amp B[c]	0.2 ± 0.009	0.1 ±0.02	0.3 ± 0.07	0.1 ± 0.004	0.3 ± 0.02	> 100	-	-

[a]La – *Leishmania amazonensis*; Lb – *L. braziliensis*; Lm – *L. major*; Lc – *L. chagasi*. Mean of triplicate assays ± SE
[b] 95% confidence limits in parenthesis
[c] Amphotericin B
[d]SE (Selectivity) - CC$_{50}$ of macrophages / IC$_{50}$ of amastigotes of *L. major*
[e]SP (Specificity) - IC$_{50}$ promastigotes of *L. major* / IC$_{50}$ amastigotes of *L. major*

As *L. major* was the most sensitive strain for MFH and MFH6 to MFH9, *L. major*-infected peritoneal macrophage model was assayed in order to investigate if they were also active against the intracellular stage of the parasite, the amastigote forms (Table 3 and 4). All

tested fractions showed a significant effect against the amastigote forms of *L. major*. MFH7 and MFH8 showed the best activity against intracellular amastigotes, with an IC_{50} value of 1.3 and 2.6 µg/mL, respectively. Despite these samples presented some toxicity against mammalian cells, MFH7 and MFH8 were 15.6 and 17.9 times more selective for the intracellular parasite than to the host cells, respectively (Table 3). According to Muylder *et al.* (2011), values > 2 was the cut-off chosen to define a compound as more active against the intracellular amastigote stage; while a specificity value < 0.4 indicated a compound more active against promastigotes; compounds with specificity values between 0.4 and 2 were considered active against both stages. So, MFH7 and MFH8 were considered more active against amastigotes, the relevant parasite stage in clinical disease.

This is the first report of amastigote activity for *Mitracarpus frigidus* against *L. major*. It is important to point out that this form is related to the Old World cutaneous leishmaniasis (previously known as zoonotic or rural zoonotic cutaneous leishmaniasis) and can be found in several countries including the Eastern Mediterranean Region and Asia (WHO, 2010).

Table 4 presents more information about the anti-amastigote activity of MFH and MFH6 to MFH9. When the parasites were treated with those fractions, a significant dose-dependent decrease of intracellular amastigote form was observed by values of the survival index.

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Table 4 – Effect of hexane extract (MFH) and active fractions on *Leishmania major* interiorized in peritoneal macrophage cells after 72 hours of treatment.

Samples		Survival Index for Amastigotes (µg/mL) (% Inhibition)			
		1.2	2.5	5.0	10.0
Control	257.3 ± 18.5	-	-	-	-
MFH	-	228.6 ± 1.7 (11)	232.8 ± 2.6 (15)	141.3 ± 1.0 (49)	112.8 ± 1.3 (59)
MFH6	-	198.3 ± 1.5 (23)	200.7 ± 0.3 (22)	171.7 ± 6.1 (33)	153.3 ± 1.8 (40)
MFH7	-	168.1 ± 1.5 (35)	143.2 ± 11.7 (44)	14.6 ± 0 (94)	9.0 ± 0.1 (97)
MFH8	-	170.7 ± 1.4 (33)	155.9 ± 0.8 (39)	24.2 ± 0.1 (91)	18.4 ± 1.6 (93)
MFH9	-	205. 3 ± 2.3 (26)	188.4 ± 0.7 (32)	151.7 ± 2.8 (41)	139.6 ± 6.9 (46)

[a]Survival Index – number of amastigotes per cells x % infected cells
[b]Data are presented as mean ± standard deviation

The leishmanicidal activity of MFH against promastigote forms of *L. amazonensis* and *L. chagasi* was similar that found by Fabri *et al.* (2009). Already the cytotoxicity of MFH was significantly different studies, whereas in our study, we detected a CC_{50} value of 18.2 µg/mL and Fabri *et al.* (2009), the extract not showed toxicity. This fact probably occurred because works show different methods for this activity.

Phytoconstituents isolated from plants have shown potent antileishmanial activity, including terpenes, steroids and phenolic compounds like flavonoids (Sen & Chatterjee, 2011). Terpenes and steroids increased the production of NO in *Leishmania* infected macrophages, along with directly targeting the parasite as evidenced by mitochondrial swelling and alterations in the organization of nuclear and kinetoplast chromatin (do Socorro *et al*., 2003). Furthermore, these compounds showed antileishmanial activity via targeting of DNA topoisomerases I and II, and preventing DNA cleavage, ultimately inducing apoptosis in parasites (Chowdhury *et al*., 2003). Flavonoids have been shown to inhibit the synthesis of parasite DNA via inhibition of topoisomerase II mediated linearization of kDNA minicircles,

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culminating in arresting of cell cycle progression (Mittra *et al*., 2000). Additionally, these compounds can chelate iron, which translates into a decreased availability of the iron dependent ribonucleotide reductase, a rate limiting enzyme for DNA synthesis (Sen *et al*., 2008). Extracts rich in flavonoids exhibited antileishmanial activity by increasing generation of reactive nitrogen intermediates that was further enhanced by the addition of IFN-γ (Gomes *et al*., 2010).

CONCLUSION

The results were encouraging, as *M. frigidus* showed significant antimicrobial and leishmanicidal activities. It is suggested that *M. frigidus* could be of use as a source of natural antimicrobial and leishmanicidal component for pharmaceutical industry. Active compounds are being isolated and elucidated for chemical characterization.

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