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Leishmania amazonensis and *Leishmania chagasi: In vitro* leishmanicide activity of *Virola surinamensis* (rol.) warb

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Leishmania amazonensis and *Leishmania chagasi: in vitro* leishmanicide activity of *Virola surinamensis* (Rol.) Warb.

ABSTRACT

Virola species have been used in traditional medicine as healing in skin infections. From *V. surinanensis* oil were isolated several sesquiterpene as the nerolidol which showed activity against species of *Leishmania*. The current study aimed to evaluate the leishmanicide activity and toxicity of extracts, fractions and surinamesin obtained from leaves of *Virola surinamensis*. Hexane, Ethyl Acetate, and Methanol extracts were obtained from powder of dry leaves of *V. surinamensis.* The hexane and ethyl acetate extracts were fractionated by silica gel column chromatography and increasingly polar gradient. The viability of *L. chagasi* and *L. amazonensis* promastigotes was assessed by tetrazolium salt assay (MTT). Peritoneal macrophages were exposed to *L. amazonensis* promastigotes. The treatment was performed with the extracts for 24 hours. Then, the coverslips were stained and the infection index was determined. Cytotoxicity was determined in macrophage cells by peritoneum viability assay (MTT). The selectivity index was calculated as the product of cytotoxic concentration 50% and inhibitory concentration 50%. The hexane extract showed leishmanicide activity in promastigotes. The ethyl acetate, methanol extracts and fractions (C1 – C6), were inactive against promastigote form of *L. chagasi* and *L. amzazonensis*. None extract showed effect on *L. amazonensis* amastigotes. All samples tested showed low cytotoxicity (CC_{50}> 500 µg/mL). The selectivity index of the hexane extract was greater than 5. The hexane extract of *V. surinamensis* was active against *L. chagasi and L. amazonensis* promastigotes. The extract fractionation did not increase significantly its antipromastigote activity. The surinamensin is probably not responsible for the activity. The extracts were inactive against amastigotes of *L. amazonensis*.

Keywords: Leishmanicide; *Virola surinamensis*; Cytotoxic

1. Introduction

Due to its urbanization, the Leishmaniasis becomes increasingly important (Desjeux 2004; Shimabukuro et al., 2011). Over 90% of visceral leishmaniasis cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan. The majority of cutaneous Leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic (WHO, 2016).

The pharmacological treatment for leishmaniasis include pentavalent antimony, pentamidine, and amphotericin B (Khraiwesh et al., 2016). However, there was a significant increase of drug therapeutic failure. Moreover, the drugs have considerable degree of toxicity to the host (Mitropoulos et al., 2010).

Plant-derived extracts have been active in experimental leishmaniasis and have presented less toxicity (Fournet et al., 1996, Pontin et al., 2008, Ezatpour et al., 2015). Secondary metabolites as hydroquinones, naphthoquinones, terpenoids, flavonoids, alkaloids, and lignans have demonstrated antileishmanial activity and low degree of toxicity (Fournet and Muñoz 2002; Queiroz et al., 2016).

Neolignans surinamensin (Gottlieb, 1978; Fig. 1A), virolin (Oliveira and Sampaio, 1978; Fig. 1A) and grandisin (Carvalho et al., 2010; Fig. 1B) were isolated from *Virola surinamensis* (Rol.) (Myristicaceae). Surinamensin was active *in vitro* against *L. donovani* promastigotes at 50 μM. The neolignan presented no selective toxicity when tested against *L. donovani* amastigotes in the mouse peritoneal macrophage (Barata et al., 2000).

Figure 1

This study evaluated the antileishmanial activity of different extracts, fractions, and surinamensin from leaves of *V. surinamensis*. Also, assessed the cytotoxicity in peritoneal macrophages.

2. Methods

2.1. Plant material and obtaining and fraction of extracts

Plants were collected in Moju, State of Pará, Brazil (S02°10' 52.2''; W 048° 47' 43.9''), and identified by EMBRAPA botanists. The voucher specimen (180980) was deposited in the Herbário IAN EMBRAPA. Plants were dried at room temperature for seven days and the material was powdered. The extracts were prepared by extraction with hexane, ethyl acetate, and methanol successively, followed by concentration in a rotary evaporator (Fisatom). The extracts were later fractionated in chromatographic column (CC) with silica gel (Macherey-Nagel) as stationary phase and solvents at increasing polarities as movel phase (hexane, dichloromethane, ethyl acetate and methanol). The fractions were grouped based on chromatographic profile. The subfractions were submitted to new division into chromatographic column. Nuclear magnetic resonance was used to identificate the isolated compounds.

Surinamesin: ^{1}H NMR spectral data (300 MHz, $CDCl_3$): δ 6.61 (2H, *s*), 4.62 (1H, *d*, *J*= 8.0 Hz), 4.14 (*m*), 1.19 (3H, *d*, *J*= 6.0 Hz); 6.9 (*m*), 6.37 (1H, *d*, *J*=16 Hz); 6.24 (*m*), 1.87 (*d*, *J*=5.5Hz), 3.82 (3H, *s*), 3.86 (3H, *s*), 3.90 (3H, *s*); ^{13}C NMR spectral data (75 MHz, $CDCl_3$): δ 137.7 (C-1), 104.3 (C-2), 153.2 (C-3), 135.6 (C-4), 153.2 (C-5), 104.3 (C-6), 83.9 (C-7), 78.6 (C-8), 17.1 (C-9), 133.5 (C-1'), 109.2 (C-2'), 150.7 (C-3'), 146.6

(C-4'), 118.7 (C-5'), 118.9 (C-6'), 130.4 (C-7'), 124.9 (C-8'), 18.3 (C-9'), 55.7 (3'-OMe), 56.1 (3-OCH_3 and 5-OCH_3), 60.8 (4-OCH_3).

2.2. Antipromastigote activity assay

The leishmaniasis strain (*Leishmania (L.) amazonensis* MHOM/BR/2009/M26361 and MCAN/BR/2011/M27289 *Leishmania (L.) chagasi*) were obtained from Instituto Evandro Chagas, Ananindeua, Brazil. *L.amazonensis* and *L. chagasi* promastigotes were obtained after primary isolation on NNN blood slopes. Then, the strains were sub-cultured and adapted to RPMI (Roswell Park Memorial Institute 1640) medium (Sigma-Aldrich®, USA). The promastigotes were cultivated at 26ºC in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL, Mota et al., 2015). Culture of promastigote forms in logarithm phase was adjusted to 4×10^6 parasites/100 µL. The susceptibility test was performed in 96-well plates. The extracts were tested in triplicate in a concentration gradient (200 to 3.125 µg/mL). The negative control contained only parasites and the incubation medium, and the positive control was made with amphotericin B (Cristália®, 25–0.3906 µg/mL). After 24h of incubation at 26ºC, it was addicted 10 µL of the tetrazolium salt (Sigma-Aldrich) to each well (5 mg/mL), and the parasites were quantified in enzyme-linked immunosorbent-assay plate reader (Biotek model ELX 808). The IC_{50} was determined by linear regression (Graph Pad Prism version 5.04). The results were classified as: IC_{50} ≤100 µg/mL was considered active, IC_{50} between 101 and 200 µg/mL was considered moderate active, and IC_{50}≥ 200 µg/mL were considered to be inactive (Mota et al. 2015).

2.3. Antiamastigote activity assay

Macrophages obtained from the peritoneum of Balb/c mice (with concentration adjusted to 4 $\times 10^5$ cells/50 µL) grew in RPMI-1640 medium (Sigma Aldrich®, USA), supplemented with 5 % of fetal calf serum (Gibco, Grand Island, NY, USA), kept in a 5% CO_2 atmosphere at 37°C. The cells were added to circular cover slips (4 x 10^5 cells /50 µL RPMI-1640 supplemented). After, *L. amazonensis* promastigotes were added (4 $\times$ 10^6 parasites). Treatment with the samples was performed with concentrations of 500, 250 and 125 µg/mL/24h. The coverslips were removed and stained with Giemsa (Dinâmica®). Then, the infection rate of macrophages was determined.

2.4. Viability assay and selective index

Cell viability was determined by the MTT [3- (4,5- dimethyltrazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MOSMAN, 1983). Macrophages obtained from the peritoneum of Balb/c mice (4 $\times$ 10^5 cells/0.1 mL) grew in RPMI-1640 medium (Sigma Aldrich®, USA), supplemented with 5% of fetal calf serum, kept in a 5% CO_2 atmosphere at 37°C. The cells were treated with extracts, fractions or surinamesin in different concentrations (between 500 and 25 µg/mL). After 24h of incubation, the MTT (Sigma-Aldrich®) was added (5.0 mg/mL). The plate was incubated at 37°C in an atmosphere of 5 % CO_2 for 4h. Dimethyl sulfoxide was added to each well to solubilize the formazan crystals. The optical density was determined at 490 nm (Biotek ELX 808 microplate reader). The cell viability was expressed as the control absorbance percentage of the untreated cells after subtracting the appropriate background. The cytotoxic concentration (CC_{50}) was determined by linear regression. Samples with

CC_{50} >200 μg/mL were considered as low cytotoxicity. Selectivity index (SI) for the antipromastigote activity was calculated based on the rate between CC_{50} and IC_{50} for *in vitro* activity against *L.amazonensis* and *L. chagasi* (REIMÃO, 2009).

All procedures were in accordance with the ethical principles of Animal Experimentation. The study was approved by the Ethics Committee (CEUA/IEC/SVS/MS) under report number 0022/2011.

2.5. Statistical Analysis

All tests were performed in triplicate and the statistical analysis was performed using ANOVA followed by the Tukey test for multiple comparisons. Calculations were performed by means of program GraphPad Prism 6.0 ®. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Phytochemical studies

The hexane extract obtained from leaves of *V. surinamensis* was subjected to fractionation. Then, fractions 4, 5, and 17 were obtained (yield 8.78%) (24.6%) with adequate mass and low chemical complexity. From fractions 4 and 5 were obtained the sub-fractions C1 and C2. Fraction 17 was fractionated again in order to give a mixture of virolin and surinamensin (C3, Fig. 2). Surinamensin was isolated in sufficient quantity to carry out biological studies.

The ethyl acetate extract obtained from the leaves was subjected to fractionation and the surinamensin was isolated after successive fractioning. The methanol extract was not submitted to fractionation (Figure 2).

Figure 2

3.2 Antileishmanial activity and selectivity index

Strains of *L. amazonensis* and *L. chagasi* were used to evaluate the antipromastigote activity. However, for antiamastigote activity only *L. amazonensis* was used. Only extracts were subjected to tests to evaluate antiamastigote activity.

The hexane extract inhibited the growth of *L. chagasi* (IC_{50}= 86.40 µg/mL) and *L. amazonensis* promastigotes (IC_{50} = 79.7 $\pm$ 1.3 µg/mL). However, its fractionation reduced the antipromastigote activity in *L. amazonensis* (IC_{50} >200 µg/mL). Treo-surinamensin was inactive against promastigotes (Table 1).

Table 1

Antipromastigote activity of extract of *Virola surinamensis*, fractions and surinamesin.

Samples	*L. infantun chagasi*		*L (L) amazonensis*	
	IC_{50}[d] (µg/mL)	Result interpretation	IC_{50} (µg/mL)	Result interpretation
HEVS[a]	86.40	Active	79.7 $\pm$ 1.3	Active
EAEVS[b]	>200	Inactive	>200	Inactive
MEVS[c]	141,4	Moderate	>200	Inactive
C1	-	-	>200	Inactive
C2	-	-	>200	Inactive
C3	-	-	>200	Inactive
C4	-	-	>200	Inactive
C5	-	-	>200	Inactive
C6	-	-	>200	Inactive
C7- surinamesin	-	-	>200	Inactive
Amphotericin	0.062	active	0.169	Active

HEVS= hexane extract;
EAEVS= ethyl acetate extract;
MEVS= methanol extract; C3 and C7* - surinamesin;
IC50 = 50% inhibitory concentration.

The ethyl acetate extract and its fractions were inactive in promastigote form of *L. amazonensis* (IC_{50} >200 µg/mL) whereas, the methanol extract showed antileishmanial activity against *L. chagasi* promastigotes at the highest concentration used in this study with CI_{50} >141,4 µg/mL, considered moderately active. In *L. amazonensis*, this extract was inactive (IC_{50} >200 µg/mL, Table 1).

The HEVS, EAEVS, MEVS in all concentrations (Figures 3D, 3E and 3F) did not cause reductions in the number of amastigotes present in infected cells compared to the negative control (NC, Fig. 3A). The destruction of murine macrophages was observed, and probably caused by intracellular forms of *L. amazonensis*. Observing a high number of parasites around the cell infected with 100% destruction of the infected macrophages treated with the extracts of *V. surinamensis* in the concentrations used in this study.

Figure 3

When not infected macrophages were treated with Extracts, fractions and surinamesin, it was found that these substances were not toxic to peritoneal macrophages of mice in the concentrations used in this study (CC_{50} > 500 µg/mL, Table 2). The relation between antipromastigote activity and cytotoxicity showed high selectivity of the hexane extract in both species: *L. chagasi* (SI= >5.8, Table 2), and *L. amazonensis* (SI= >6.2, Table 2).

Table 2
Cytotoxicity in macrophage and selectivity index of the extracts of *V. surinamensis* and surinamensin.

Samples	CC_{50}[a] (µg/mL)	SI[b]- *L chagasi* promastigote	SI[b]–*L.amazonensis* promastigote
HEVS[a]	>500	>5.8	> 6.2
EAEVS	>500	>2.0	-
MEVS	>500	> 3.5	-
C1	>200	-	-
C2	>200	-	-
C3 and C7*	>500	-	-
C4	>500	-	-
C5	>500	-	-
C6	>500	-	-
Amphotericin	>100	>1613	>2943

CC_{50}[a] = Cytotoxic concentration 50%.
SI[b] = Selectivity index calculated in relation to the promastigote forms.
HEVS= Hexane extract.
EAEVS= Ethyl acetate extract.
MEVS= Methanolic extract.
Subfractions: C1, C2, C4, C5, C6; C3 and C7* - surinamesin.

4. Discussion

The hexane extract of *V. surinamensis* should contain long chain hydrocarbons mixture, glycerides esters, steroids and aryl propanoides (Saraiva, 2012). However, the major constituents are neolignans. Probably the antipromastigote activity must be related to a minority constituent of this extract. The secondary metabolites involved in antipromastigote activity have nonpolar character, probably not present in the other extracts. This may explain the lack of activity of other extracts.

Neolignans and lignans correspond to 50% of Myristicaceae family constituents (Lopes et al., 2004; Morais, 2008). These metabolites have been assigned as antifungal (Zacchino et al., 1997), antimalarial and Chagas's disease activities (Lopes et al., 2004). The leishmanicide activity of different Virola species has been related to the presence of neolignans (Fernandes et al., 1993). However, another metabolite seems to be involved in the activity. Another possibility is the synergism between neolignans and other

metabolites. The antipromastigote activity of hexane extract may result from the synergistic effect. The fractionation reduces biological activity.

This paper reports the antipromastigote effect of hexane extract. The hexane extract of *V. surinamensis* presented high activity against promastigote of *L. chagasi and L. amazonensis*. The selectivity index was higher than 5.0. The extract fractionation did not increase significantly its antipromastigote activity. The surinamensin is probably not responsible for the activity. The ethyl acetate and methanol extracts were no active in *L. chagasi* and *L. amazonensis* promastigotes. The extracts, fractions and surinamesine were inactives in *L. amazonensis* amastigotes.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Title of Figures / Subtitles

Fig. 1. Chemical structures of substances isolated from *V. surinamensis*.

Legend: Surinamensin R= OCH_3, Virolin R= H ; Grandisin.

Fig. 2. Flowchart fractionation of hexane extracts and ethyl acetate.

Fig. 3. Antiamastigote assay of *V. surinamensis* against *L. amazonensis*.

Negative Control (NC, uninfected macrophages) (a), Macrophages Infected with *L. amazonensis* amastigotes (b), infected macrophages treated with Amphotericin B (100

ug / ml) (c), infected macrophages treated with HEVs (500 ug / mL) *V. surinamensis* (d), infected macrophages treated with EAEVS (500 ug /mL) (e), and infected macrophages treated with MEVS (500 ug / mL) *V. surinamensis* (f). Red arrows Indicate cell destruction (macrophages) and black arrows Indicate the presence of amastigotes around the destroyed cell. All images in a 100x magnification.

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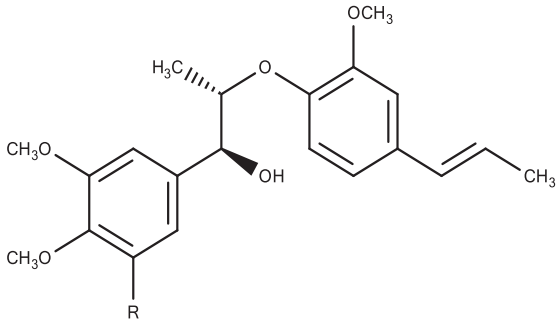
Figure 1

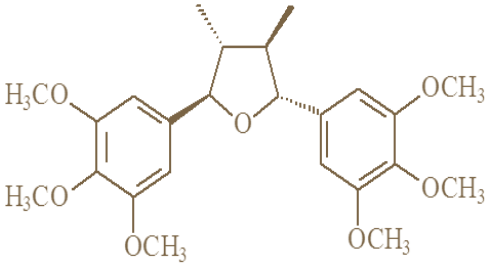
A)



Virolin: R= H

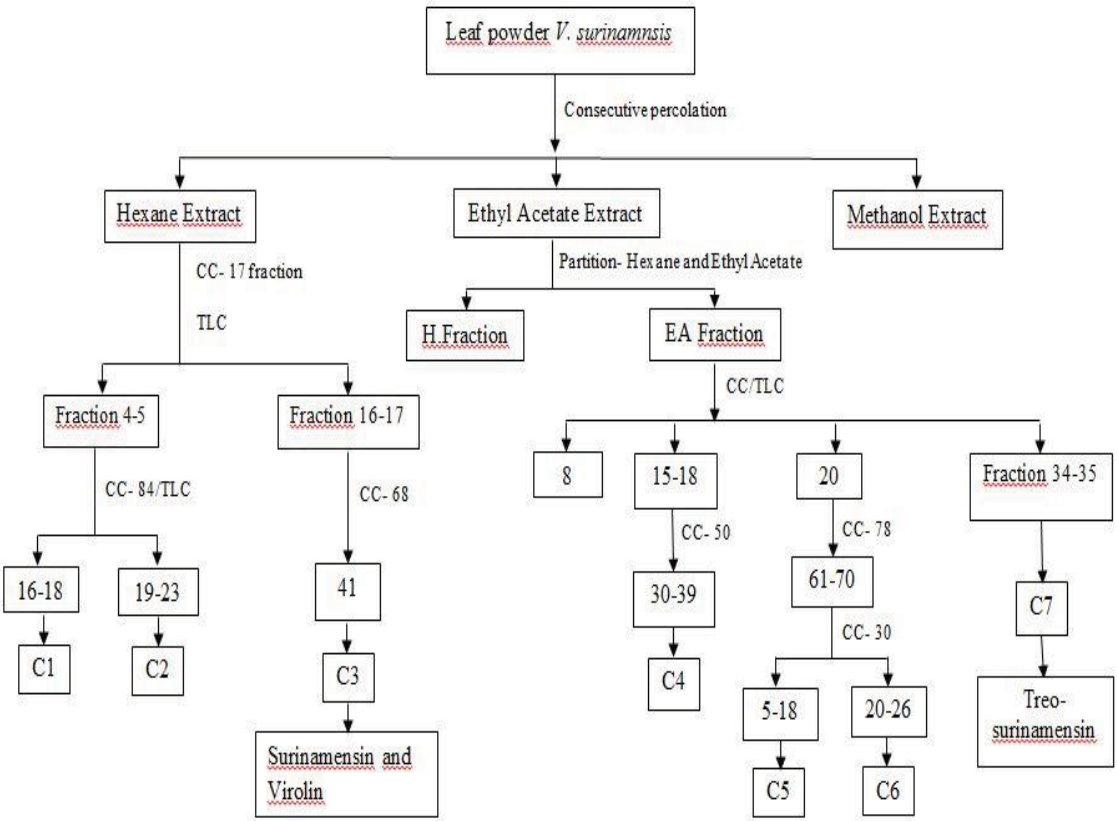
Surinamensin: R= OCH3

B)



Grandisin

Figure 2


Leaf powder V. surinamnsis
Consecutive percolation
Hexane Extract
Ethyl Acetate Extract
Methanol Extract
CC- 17 fraction
TLC
Partition- Hexane and Ethyl Acetate
H.Fraction
EA Fraction
CC/TLC
Fraction 4-5
Fraction 16-17
8
15-18
20
Fraction 34-35
CC- 84/TLC
CC- 68
CC- 50
CC- 78
16-18
19-23
41
30-39
61-70
C7
CC- 30
C1
C2
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C4
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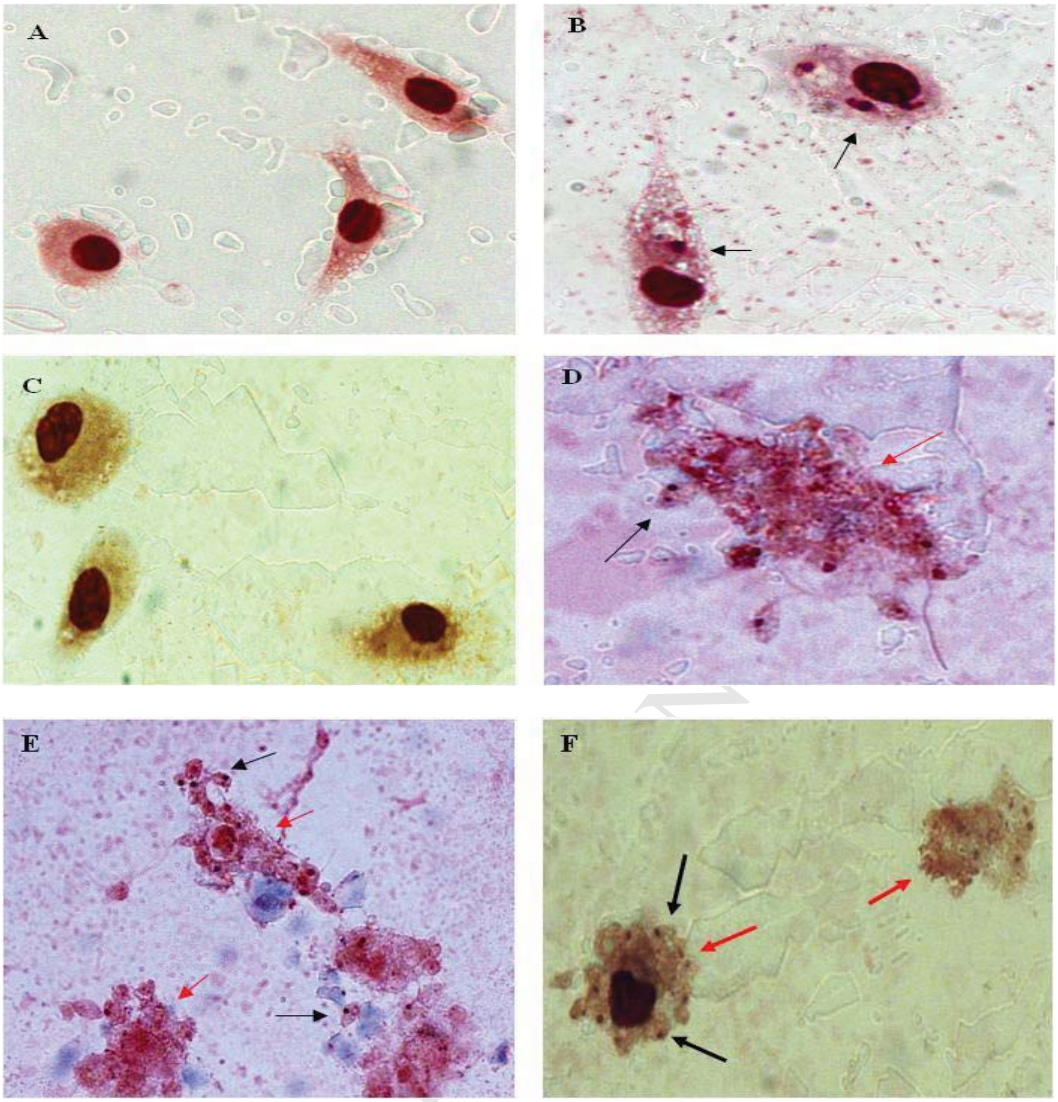
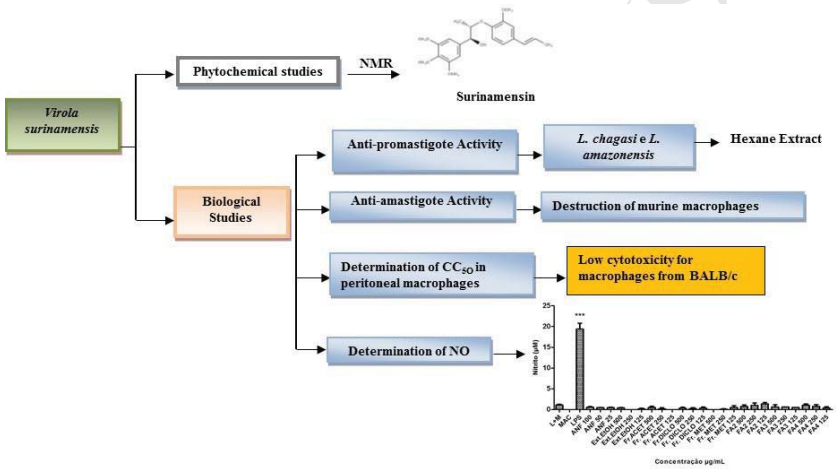
20-26
Treo-surinamensin
Surinamensin and Virolin
C5
C6

Figura 3



GRAPHICAL ABSTRACT



HIGHLIGTS

- The *Virola surinamensis* activity of the samples was evaluated.
- Extract fractionation did not increase the antipromastigote activity significantly.
- The surinamesin is not problably responsible for leishmanicide activity.