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Artigo

Evaluation of the leishmanicide action of ethanol extracts of *Crotalaria retusa* L. (Fabaceae)

Louisianny Guerra da Rocha,[1] Cícero Flávio Soares Aragão,*[,2] Maria Iracema Bezerra Loiola,[3] Renata Aracyl Bezerril,[1] Nara Ruth Freitas Paiva,[2] Cecília Maria Carvalho Xavier de Holanda,[1] Maria Edileuza Felinto de Brito[4]

[1]Departamento de Microbiologia e Parasitologia, Universidade Federal do Rio Grande do Norte, Lagoa Nova, s/n, 59072-970 Natal-RN, Brazil,
[2]Laboratório de Controle de Qualidade de Medicamentos, Universidade Federal do Rio Grande do Norte, Av, General Cordeiro de Faria s/n, Petrópolis, 59010-180 Natal-RN, Brazil,
[3]Departamento de Botânica, Ecologia e Zoologia, Universidade Federal do Rio Grande do Norte, Lagoa Nova, s/n, 59072-970 Natal-RN, Brazil,
[4]Centro de Pesquisa Aggeu Magalhães, Fiocruz, Av. Professor Moraes Rego, s/n, Campus UFPE, 50670-420, Recife-PE, Brazil

RESUMO: "Avaliação da ação leishmanicida do extrato etanólico da *Crotalaria retusa* L. (Fabaceae)". O presente trabalho se propõe avaliar a atividade citotóxica do extrato etanólico bruto e da fração dos alcalóides totais (FAT) da planta *Crotalaria retusa* para células promastigotas metacíclicas de *Leishmania chagasi*. O estudo da cinética de extração por ultra-som para os alcalóides totais da *Crotalaria retusa*, tornou possível a otimização dos parâmetros de extração. Foi avaliada a ação leishmanicida da FAT da planta em estudo, a qual não mostrou atividade citotóxica em altas concentrações. Foi observado uma potente ação leishmanicida para os extratos etanólicos (10 e 30%) após a concentração de 5,6 mg/mL de *Crotalaria retusa* e do etanol presente na solução extrativa (10 e 30%) nas concentrações de 70 e 210 x 10^{-4} %, respectivamente. Estes resultados sugerem a citotoxicidade do extrato etanólico da *Crotalaria retusa* de 10 a 30% para células de *Leishmania chagasi*, associada possivelmente à concentração do etanol presente no extrato.

Unitermos: *Crotalaria retusa*, Fabaceae, ação leishmanicida.

ABSTRACT: The purpose of the present work is to conduct an evaluation of the cytotoxicity of ethanol extracts and the total alkaloid fraction (TAF) from *Crotalaria retusa* for procyclic promastigotes cells of *Leishmania chagasi*. The kinetic study of extraction assisted by ultrasound of the total alkaloids present in *Crotalaria retusa* made it possible the optimization of the extraction parameters. It was evaluated the leishmanicide action of the TAF which did not show toxic activity for cells of the parasite in high concentrations. It was observed a powerful leishmanicide action of the ethanol extracts (10 and 30%) after the concentration of 5.6 mg/mL of *Crotalaria retusa*, and the ethanol present in the extractive solution (10 and 30%) in the concentration from 70 and 210 x 10^{-4} %, respectively. These results suggest that the cytotoxicity of the ethanol extract of *Crotalaria retusa* at 10 and 30% for cells of *Leishmania chagasi*, can be associated only to the concentration of the alcohol present in the extract.

Keywords: *Crotalaria retusa*, Fabaceae, leishmanicide action.

INTRODUCTION

Leishmaniasis are diseases with wide geographical distribution around the world with higher prevalence in underdeveloped countries, caused by the parasite of the genus *Leishmania* spp. which are transmitted to humans through the bite of the phlebotomine insect (Ahua et al., 2007).

Major epidemiological factors contribute to its distribution, particularly deforestation, the parasite's resistance to drugs, ecological and climatic changes (Ostfeld et al., 2005).

It is widely known that these diseases are neglected, because they affect thousands of people but no satisfactory adequate drugs treatment has been developed (Trouiller et al., 2001). The research for new, more effective and less toxic drugs is needed for the treatment of these pathologies as well as an improvement in the patients' quality of life (Bharate et al., 2005).

The plants are still important in the discovery of new drugs as providers of the drug or semi-synthetical

* *E-mail: ciceroaragao@ufrnet.br, Tel. +55-84-3215-4351*

or synthetical drugs based in secondary compounds from plants (Amaral et al., 2006; Barbosa-Filho et al., 2007, 2008; Quintans-Júnior et al., 2008).

According to Rocha et al. (2005), plants of the Fabaceae family, showed leishmanicide effect for different species of *Leishmania* spp., such as, ethanol extracts of *Crotalaria barbata* R. Grah, methanolic extracts of *Desmodium gangetiurm* L. and saponine fraction of *Periandra mediterranea* Taub.

The pyrrolizidine alkaloids constitute a major class of phytotoxins found in 560 species of plants, with this compound being found with higher frequency in the representatives of the families Asteraceae, Boraginaceae, Fabaceae and Orchidaceae. The monocrotaline (Figure 1) is one of the most abundant alkaloids found in the species *Crotalaria retusa* L (Nobre et al., 2004, 2005).

Literature data reveal the toxicity of *Crotalaria retusa* for horses, pigs, chicken and geese, through hepatic changes characterized by hepatic fibrosis and megalocitosis. This toxic effect derives from the high concentration of alkaloids, mainly monocrotaline type present in the plant (Curran et al., 1996; Hooper & Scanlan, 1977; Alfonso et al., 1993).

The technique of extraction by ultrasound has been proved highly effective in the preparation of plants' extracts for therapeutical purposes. In this technique the relation mass plant drug/volume of solvent and extraction time is smaller when compared to other extraction methodologies (Celeghini et al., 2007)

The ultrasound is the form of acoustic energy, whose frequency goes beyond the human hearing limit (20 kHz), and it can be generated by a transductor which converts electrical energy into mechanical (Costa, 2004). This high frequency, shown in Figure 2, produces a vibratory effect in the plant cell, to the point of rupture, and thus releasing all its content, implying the release of active essence and the wearing out of the plant raw material, characterizing an exhaustive extraction method.

The purpose of the present work is to conduct an evaluation of the cytotoxicity of ethanol extracts and the alkaloid fraction from *Crotalaria retusa* for procyclic promastigotes cells of *Leishmania chagasi.*

MATERIAL AND METHODS

Collection and identification of the botanical material

For this study were used the leaves of *Crotalaria retusa* collected in an anthropogenic area in the outskirts of the Parque Estadual das Dunas, (coordinates 05°50'30"S and 35°11'42"W), in the district of Natal, Rio Grande do Norte/Brazil. L. Flowered and fructified branches were also gathered for the identification of the species. Samples of the plant are registered in the Herbarium of UFRN of the Universidade Federal do Rio Grande do Norte under the number 5439.

The identification of the species was achieved through comparative morphological studies, using specialized bibliography (Brito et al., 2006) and herborized material belonging to the collection of the UFRN herbarium.

Preparation of the extract by ultrasound

Using the leaves of the studied species, the extractions by ultrasound were conducted in an ultrasound of the UNIQUE, USC 1400 model.

Kinetic study

For the kinetic studies of the extraction assisted by ultrasound it was used 1 g of the plant, which was submitted to sonication using 10 ml of hydroalcoholic solvent (70:30 and 20:80), CR30 and CR80, respectively, in the times 1, 2, 3, 4, 5 and 10 minutes.

After the preparation of the extracts for the different types of solvents, the molar absorbtivity was determined in the ultraviolet region in the wavelengths of 205, 250, 270 and 330 nm.

The kinetic calculations were conducted with the help of the software Origin 5.0.

Preparation of the extracts for the evaluation of the leishmanicide activity

An extract was prepared by ultrasound for the study of the leishmanicide activity.. Using 1 g of the leaf which was submitted to sonication using 10 ml of different solvent systems: 1) water:ethanol 90:10 (CR10) and 2) water:ethanol 70:30 (CR30) in a 10 minute extraction time.

The CR30 extract was submitted to extraction for alkaloids where was obtained the total alkaloid fraction (TAF).

Extraction of the total alkaloid fraction (TAF)

The CR 30 extract was treated with a chlorydric acid solution at 3% under agitation, then filtrated by celite providing a residue and an acid solution which was submitted to several extractions with chloroform. The aqueous phase was transformed to pH = 9 with ammonium hydroxide and then extracted with chloroform until negative reaction with the Dragendorff reagent. The chloroform phase was washed with water, dried with $MgSO_4$ anhydrous, filtrated and had its solvent evaporated under pressure reduced to 55 ^oC. It was thus obtained the fraction of the total tertiary alkaloids (TAF) (2.8 mg) (Cortes et al., 1995).

Leishmania chagasi cultivation

The strains of *Leishmania chagasi* (MHOM/BR/2000/merivaldo2) (Paranhos-Silva et., 2003) promastigotes were cultivated (*in vitro*) in biphasic medium NNN/LIT supplemented with 20% of bovine foetal serum (SFB) in a proportion of 3:2 mL, incubated in a BOD camera in a 26 °C temperature.

Antileishmanial assay (in vitro) against *Leishmania chagasi*

Ethanol extracts of Crotalaria retusa L. CR10 and CR30

Cultures of procyclic promastigotes cells of the parasite immersed in the medium LIT + 20% SFB, were incubated for a 24 hour period in BOD camera at 26 °C with CR10 and CR30 samples in the concentrations of 0.4; 0.8; 1.6; 2.4; 3.2; 5.6; 7.2 and 9.6 mg/mL. The solutions at 10% of ethanol were also incubated with final concentrations equal to the analysed extracts, such as, 5, 10, 20, 30, 40, 70, 90 and 120 x 10^{-4} % and for the solutions at 30% of ethanol in the concentrations of 15, 30, 60, 90, 120, 210, 270 and 360 x 10^{-4} %. Afterwards were removed of the parasite suspension and quantified the viable cells in a Neubauer chamber, through the optical microscope in the 40x objective.

Alkaloid fraction of Crotalaria retusa

For the assays with the TAF of the plant, it was prepared a stock solution containing 7,0 mg/mL, which was kept in a refrigerator until the moment of the analysis. In essay tubes containing medium of culture LIT + 20% SFB and promastigotes cells of *Leishmania chagasi*, were added the following concentrations of the TAF: 35, 70, 107, 175, 245, 350, 420, 490, 560, 630 and 700 µg/mL and submitted to a 24 hour incubation period in BOD camera at 26 °C. The cytotoxic effect was evaluated through the count of viable cells in Neubauer camera using optical microscopy in the 40X objective.

Statistical treatment

The experiment was conducted in duplicate for each concentration and evaluated through the correlation analysis ($p < 0.0001$; $\alpha = 0.05$).

RESULTS AND DISCUSSION

Kinetics extraction by ultrasound

Ultrasound waves with frequencies above 20 kHz are generated by a transductor which converts electrical energy into mechanical. These waves travel in the liquid medium, originating variations of pressure responsible for the cavitation, that is, the creation and implosion of gas microbubbles in the center of a liquid. This high frequency forms shock waves in the moment of the bubble implosion (Barboza, 1992), and also produces a vibratory effect in the plant cell, capable of

Table 1. Effect of the ethanol extracts at 10% from *Crotalaria retusa* in growth of promastigotes *Leishmania chagasi.*

Ethanol extracts 10% (mg/mL)	% inhibition growth
0.4	*ni*
0.8	*ni*
1.6	*ni*
2.4	*ni*
3.2	1.3
5.6	55.3
7.2	77.7
9.6	100.0

ni = No inhibition.

Table 2. Effect of the ethanol solution at 10% in growth of promastigotes *Leishmania chagasi.*

Ethanol concentration (%)	% inhibition growth
5 x 10^{-4}	*ni*
10 x 10^{-4}	*ni*
20 x 10^{-4}	*ni*
30 x 10^{-4}	*ni*
40 x 10^{-4}	1.3
70 x 10^{-4}	55.3
90 x 10^{-4}	77.7
120 x 10^{-4}	100.0

ni = No inhibition.

Table 3. Effect of the ethanol extracts at 30% from *Crotalaria retusa* in growth of promastigotes *Leishmania chagasi.*

Ethanol extracts 30% (mg/mL)	% inhibition growth
0.4	*ni*
0.8	*ni*
1.6	*ni*
2.4	*ni*
3.2	*ni*
5.6	51.3
7.2	60.5
9.6	100.0

ni = No inhibition.

Table 4. Effect of the ethanol solution at 30% in growth of promastigotes *Leishmania chagasi.*

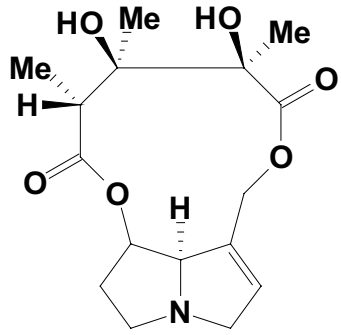
Ethanol concentration (%)	% inhibition growth
15 x 10^{-4}	*ni*
30 x 10^{-4}	*ni*
60 x 10^{-4}	*ni*
90 x 10^{-4}	*ni*
120 x 10^{-4}	*ni*
210 x 10^{-4}	51.3
270 x 10^{-4}	60.5
360 x 10^{-4}	100.0

ni = No inhibition.

Table 5. Effect of the alkaloid fraction (TAF) from *Crotalaria retusa* in growth of promastigotes *Leishmania chagasi.*

TAF (µg/mL)	% inhibition growth
35	*ni*
70	*ni*
107	*ni*
175	*ni*
245	*ni*
350	*ni*
420	*ni*
490	*ni*
560	*ni*
630	*ni*
700	*ni*

ni = No inhibition.



Monocrotaline

Figure 1. Chemical structure of the monocrotaline, one of the main alkaloids of *Crotalaria retusa*.

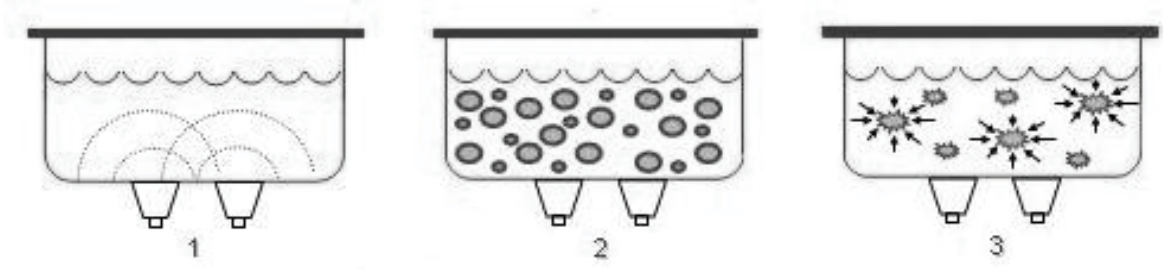


Figure 2. Ultrasound scheme. 1) acoustic energy in the ultrasound region; 2) plant cells before the ultrasound radiation; 3) Plant cell suffering the effect of cavitation which leads to its rupture increasing the efficiency of extraction and solubilization in the solvent.

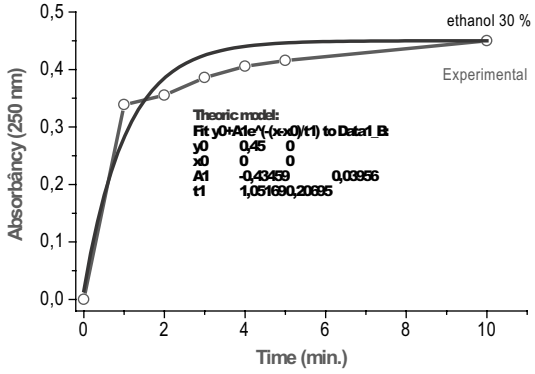


Figura 3. Kinetics extraction by ultrasound from *Crotalaria retusa* (experimental) and theoretical model by exponential (first order) with hydroalcoholic solvent 70:30 (H_2O:EtOH).

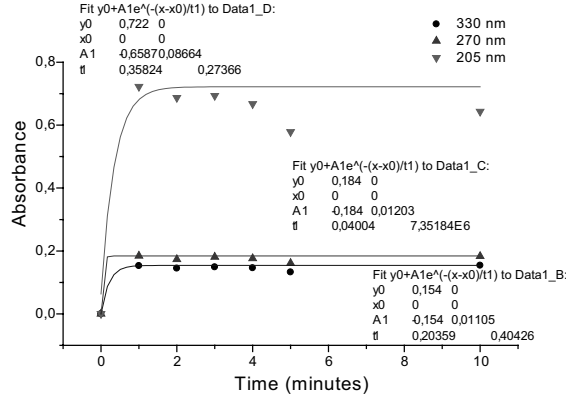


Figura 4. Kinetics extraction by ultrasound from *Crotalaria retusa* (experimental) and theoretical model by exponential (first order) with hydroalcoholic solvent 20:80 (H_2O:EtOH).

taking it into rupture and, with this, the release of its content.

The Figures 3 and 4 show the influence of the parameter time and the type of solvent, in the extraction of pyrrolizidinic alkaloids present in *Crotalaria retusa* monitoring the variation, through the analysis of the extract by spectrophotometry in different wavelengths.

The kinetic parameters for the extraction of the alkaloids present in this species, Figure 3, showed an increase according to the equation y = 0,45 - 0,43459

$e^{(-(x-0)/1,05169)}$, of the absorbtivity with the increase in the extraction time, and also, the absorbtivity was higher in the extracts using the solvent system 70:30 in the time of 10 minutes.

Figure 3 presented the following equations: y = 0,722 - 0,6587 $e^{(-(x-0)/0,35824)}$; y = 0,184 - 0,184 $e^{(-(x-0)/0,04004)}$ and y = 0,154 - 0,154 $e^{(-(x-0)/0,20359)}$ for the wavelengths of 205, 270 and 330 nm, respectively.

In the variation of the parameter time, Figures 3 and 4, it was also observed the rise of absorbtivity, calculated as pyrrolizidinic alkaloids present in *Crotalaria retusa w*ith the increase of the extraction time by ultrasound, being longer for the hydroalcoholic solvent 70:30 (H_2O:EtOH) in the λ = 254 nm and also for the hydroalcoholic solvent 20:80 (H_2O:EtOH) in the λ = 205 nm.

In these two systems the λ = 254 nm is more adequate since in a wavelength of 205 nm the analysis are very close to the "forbidden region" of the ultraviolet spectra which is the one that can suffer influences of other chemical compounds that present the transitions $n \rightarrow \pi^*$ characteristic of benzene nuclei.

The characterization and optimization of the kinetics parameters provided reliable data to the research and production laboratories, providing a reduction in the costs and extraction time.

The kinetic study of extraction assisted by ultrasound of the alkaloids present in *Crotalaria retusa* made it possible the optimization of the extraction parameters. Besides providing a reduction in the costs and extraction time, it generated products which were evaluated as to their possible leishmanicide activity.

Evaluation of the leishmanicide activity

The Leishmaniasis is a parasitic disease with wide distribution around the world affecting thousands of people. Many researches were conducted to assess the effect of the use of plants or of several of their metabolites for the prevention and treatment of this protozoosis (Bezerra et al., 2006; Nakamura et al., 2006; Paula-Junior et al., 2006; Ahua et al., 2007; Moreira et al., 2007; Rodríguez et al., 2008).

Studies were conducted with ethanol extracts of plants of the family Apocynaceae, Araliaceae, Asteraceae, Euphorbiaceae, Fabaceae, Gentianaceae, among others, showing cytotoxic action for the different species of the gender *Leishmania* spp (Rocha et al., 2005).

Nobre et al. (2005) observed in their studies acute intoxication in sheep by the use of *Crotalaria retusa* after ingestion of seeds in high quantity. They attributed this effect to the monocrotaline alkaloid present in the plant. Other authors report toxic effect of the plant in several animal species (Alfonso et al., 1993).

Frankenburg et al. (1998) used several solvents, such as glycerol, glycol polypropylene, ethanol and glucose, in the pharmaceutical formulations with amphotericin B in the treatment of the lesions of leishmaniasis. After the use of these formulations during 03 weeks of infection, it was observed that the ethanol in the concentrations between 5 - 25% showed a better therapeutical efficiency.

In this experiment for the evaluation of the leishmanicide activity of the plant *Crotalaria retusa*, were used ethanol extracts at 10 and 30%, total alkaloid fraction (TAF) of the plant and the promastigote form of *Leishmania chagasi*.

The leishmanicide effect of the ethanol extracts of the plant in study, as well as the effect of the extractive solution, is represented in the Tables 1, 2, 3 e 4.

Additionally it was evaluated the leishmanicide action of the TAF of the *Crotalaria retusa* in study which did not show toxic activity for cells of the parasite in high concentrations (Table 5). During for the preparation TAF the alcohol is eliminated alongside the acid aqueous fraction, alkaloid fraction (TAF) is alcohol free. Leishmanicide activity seen in the tables above is due to the ethanol and not to the alkaloids (TAF) of this plant in the experimental conditions employed.

After the evaluation of the effect of the extract in study through the correlation analysis, it was observed a powerful leishmanicide action of the ethanol extracts (10 and 30%) after the concentration of 5.6 mg/mL of *Crotalaria retusa* and the ethanol present in the extractive solution (10 and 30%) in the concentration from 70 and 210 x 10^{-4} %, respectively.

CONCLUSION

The results obtained in this work suggest that the cytotoxicity of the ethanol extract of *Crotalaria retusa* at 10 and 30% for cells of *Leishmania chagasi*, can be associated only to the concentration of the alcohol present in the extract and not to the concentration of the plant in study.

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