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Evaluation of the *In vitro* leishmanicidal and *In vivo* acute oral toxicity of the *Caesalpinia echinata* L. extracts as source of natural products against leishmaniasis

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ABSTRACT

With the purpose of discovery leishmanicidal secondary metabolites from natural products, crude ethanolic extract (EE) from stems of the Caesalpinia echinata was assayed to verify its in vitro leishmanicidal activity. The EE showed in vitro growth inhibition activities of 90% against amastigote-like of Leishmania (Leishmania) amazonensis. The EE was then submitted to fractionation by Gel Permeation Chromatography (GPC) yielding fifteen fractions (F1 to F15). The same biological assay was performed for the fifteen fractions and the fractions F9 to F11 showed in vitro growth inhibition activities around 80%. The fractions F9 to F11 were pooled to produce an enriched fraction named EF. Evaluation of the acute toxicity of the EE and EF were carried out with Swiss-Webster mice, orally treated by a single oral dose of 300mg of the samples (EE and EF)/kg of body to verify changes in hematological and biochemical profiles and 5.0g of the samples (EE and EF)/kg body to verify the toxicity and safety in using EE and EF as therapeutic agents in the treatment of the leishmaniasis. After preliminary results, the LD_{50} concentration was estimated to be greater than 5.0g/kg body for both samples (EE and EF) by oral route. The EE and EF of the C. echinata were actives in vitro experiments and nontoxic for mice, moreover these experiments proved to be the first steps towards the development of leishmanicidal agents from C. echinata.

Key words: *Caesalpinia echinata;* Leishmaniasis; Leishmanicidal activity; Acute toxicity.

INTRODUCTION

Brazilwood or “Pau-brasil”(*Caesalpinia echinata* Lam. Family-Fabaceae) is a brazilian arboreal endemic species that can reach up to 30 meters long. It is typical in the dense forest occurring in the region stretching from Rio Grande do Norte to Rio de Janeiro.

Brazilwood was the first brazilian arboreal species to be exploited commercially on a large scale by the Portugueses after the discovery of the Brazil due to the presence of dyes [1-2]. Recently, studies about seedling development [3] phenology, pollination, and breeding system have provided knowlegde for its sustainable use and conservation [4-5]. The phytochemical investigations of the *C. echinata* is not well known affording few results on its pharmacological potential. Brazilin (**1**) and brazilein (**2**), figure 1, are the main dyes used for clothing and were identified in the stem from Brazilwood [6]. Brazilein and brazilin are related structurally to flavonoids. These dyes were identified as homoisoflavonoids (3-benzylidene-4-chromanones). Brazilein and brazilin have showed biological activities as cytotoxic agent against six cancer cell lines (HepG2, Hep3B, MDA-MB-231, MCF7, A549, Ca9-22) with IC_{50} values ranging from 8 to 34 µM [7]. Brazilein and ethanolic extract from *Caesalpinia sappan* inhibited the proliferation of T lymphocyte stimulated by Concanavalin A (Con A) and the proliferation of B lymphocyte stimulated by lipopolysaccharides (LPS) [8].

Anti-malarial, antibacterial, antioxidant, antiviral and cytotoxic activities have been reported for cassane diterpenes [9-14], a class of secundary metabolites that occur in species of the genus [15-20], (Fig. 1).

Other species of this genus have been evaluated *in vivo* for several biological effects, i. e. extracts from seeds of the *Caesalpinia bonduc* at a dose of 300mg/kg body have shown significant ability to prevent alterations due to stress [21] and the crude aqueous extract of the fruits from *Caesalpinia ferrea* Mart. (Leguminosae) showed anti-inflammatory effect in the carrageenan induced rat hind paw edema, by oral administration of 300mg/kg body [22]. Aqueous and ethanol extracts of *C. bonducella* seeds exhibited significant hypoglycemic and anti-hyperglycemic activities at 100 mg/kg body in normal and SZ-hyperglycemic rats, respectively [23].

Leishmaniasis are neglected diseases that affect millions of people around the world, mainly in low-incoming countries. The disease affects poorest people and is associated with all factors of poverty of these populations: malnutrition, a weak immune, poor housing and lack of resources [24]. Discovery of new drugs for this disease is necessary because the current therapeutical arsenal or is becoming little effective or are very toxic. Aiming at identifying new leishmanicidal compounds from natural sources, this study was designed to exploring the therapeutical potential of *C. echinata* as leishmanicidal agent based on the results of the growth inhibition activity (*in vitro*) and nontoxic effects (*in vivo*).

MATERIALS AND METHODS

Collection and authentication of plant material

Stems were collected at Zôo-Botânica Foundation, a savannah-like region in Belo Horizonte, Minas Gerais, Brazil (19°51'45.30"S; 44°0'38.49"W). A voucher specimen was identified and deposited at the Herbarium of the Belo Horizonte, Zôo-Botânica Foundation under code BHZB 6458.

Preparation of the crude ethanolic extract

Fresh stems were washed quickly in water; shade dried at room temperature (25±2 ºC) for 14 days and then manually cut in pieces. The plant material was powdered in a Wiley mill and subjected to extraction by maceration, using ethanol at room temperature for 7 days. The extraction was exhaustively repeated. The extract was reduced to dryness in a rotary evaporator at 40°C.

Fractionation of the crude extract

Gel Permeation Chromatography (GPC) was carried out using a system constituted by a glass column of 50 mm diameter and 250 mm length coupled in series to the other two columns of 50 mm diameter and 480 mm length. Columns were filled with Sephadex™ LH-20 (GE HealthCare) gel and using ethanol as mobile phase. The fractions were collected using an automatic SF-2120 (Advantec) collector. The crude ethanolic extract was dissolved in ethanol and injected in the GPC system. The solvent (ethanol) was pumped at 2 ml/min and collected in tubes of 22ml at 10 minutes per tube. Thin Layer Chromatography (TLC) analyses were obtained using silica gel G-60/F254 (0.25 mm, Merck). TLC were eluted with dichloromethane/methanol (95/5 v/v) or ethyl acetate/methanol/water (70/20/10 v/v) and spots were visualized at wavelengths of 254 and 366 nm and after spraying them with vanillin/sulfuric acid. It was obtained 240 fractions that were monitored by means of thin-layer chromatographic analyses (TLC). This experiment was repeated three times, using about six grams of the crude extract each time. By means of the comparison of the chemical profile by TLC, it was possible to obtain fifteen groups named F1 to F15.

Chromatographic analysis by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Analytical RP-HPLC analyses were developed in a Finnigan Surveyor HPLC system (Thermo Scientific) equipped with a Diode Array Detector (PDA). Analyses were carried out by a Waters Atlantis® dC18 column (3 µm, 2.10 mm x 150 mm) eluted with gradient of acetonitrile (0.1%HCOOH)/water (0.1%HCOOH) from 5% to 100% of

acetonitrile in 20 minutes, staying for 4 minutes at 100% then return to initial conditions in 6 minutes at flow rate of 0.310 mL/min.

Tests with amastigote-like of *Leishmania (Leishmania) amazonensis*

Extracellular promastigotes of *Leishmania (*Leishmania) *amazonensis* (IFLA/BR/196/PH-8), obtained from lesions of hamsters were cultured in Schneider medium pH 7.2 at 26°C. After seven days of cultivation, when growth of promastigotes reach stationary phase, the culture was centrifuged for 10 minutes at 1000 RCF, resuspended in Schneider medium pH 6.0 and incubated at 32°C, allowing the determination of amastigotes. An aliquot of 90 µL of an inoculum containing 1 x 10^8 parasites/ml and 10 µL of the samples (compound assayed to verify activity) at 200 µg/ml (water in 1% DMSO) were added to each well. The microplates were incubated at 32°C for 72 hours and cell viability were determined colorimetrically using 10 µL methyltiazoiltetrazolium bromide (5 mg/ml) at 570 nm. All samples were analyzed in triplicate and the results expressed as percentage of growth inhibition compared to control without sample. Amphotericin B (0.2 µg/ml) was used as the standard leishmanicidal drug. In addition, others controls such as untreated parasites and culture medium were used [25-27].

Acute toxicity tests in vivo

The experimental design for *in vivo* acute toxicity tests was elaborated using two levels of concentrations for each EE and EF samples. Low dose (300 mg/kg body) named LD and high dose (5.0 g/kg body) named HD, respectively. Each experiment was developed with twelve animals (six female and six male of Swiss-Webster especies). Control group with the same number of mice. The samples were prepared using EE and EF dissolved in soybean oil containing 5% ethanol (V/V) as vehicle.

Hematological parameters

Hematological parameters were performed by tests realized at LD level. Mice weighting approximately 24 g for female and 26 g for male were inoculated by gavage with a volume of 0.2 ml in a single dose of EE / EF at 300 mg/kg body. The control group received a single administration of 0.2 ml of soybean oil containing 5% of ethanol. After fourteen days, animals were euthanized using 5% ketamine hydrochloride and xylazine hydrochloride 2% in volume. Blood samples were collected in glass test tubes containing 10% EDTA anti-clotting. Hematological analyses were performed using an automatic hematological analyzer (Celm CC-530®). The analyzed hematological parameters were: atypical lymphocytes (%), band neutrophils (%), eosinophils (%), haematocrit (%), haemoglobin (g/dL), leukocytes (mm^3), lymphocytes (%), mean corpuscular volume (%), monocytes (%), platelets (mm^3) and ring cell (%). For biochemical analysis, blood was centrifuged at 1000 RCF for 10 minutes and biochemical profile was obtained from analysis of serum by alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), γ-glutamyltranspetidase (GGT), total protein and urea using Bioclin® commercials kits.

Histopathologic evaluation

Damage to organs was evaluated by experiments developed at HD level. Mice weighting approximately 23 g for female and 26 g for male were inoculated by gavage with a volume of 0.2 ml in a single dose of the EE / EF at a concentration of 5.0 g/kg body. The control group orally received a single administration of 0.2 ml of soybean oil containing 5% of ethanol. After administration, animals were observed within 30, 60, 120, 240 and 360 minutes. Later, the mice were observed every 24 hours for a period of 14 days, after onset of acute toxicity test. Clinical signs of autonomic effects such as salivation, diarrhea and urination and the central nerve effects including tremors and convulsions, changes in the level of activity, gait, posture and mortality were observed. From 24 hours up to 14 days after dosing, the weight of the animals was monitored daily. Clinical parameters were assessed using analogue scales ranging from unipolar 0 to 4 points (cyanosis, piloerection, writhing, ptosis, tremors, convulsions, ataxia), or ranging from bipolar 0-8 points, with 4 representing the value of normal (motor activity, respiration, corneal reflex, body tone, touch response, reflex headset) [28]. All animals were euthanized and necropsied after the 14th day. Macroscopic features of the lungs, kidneys, spleen, brain, liver and heart were observed. Vital organs were weighed, fixed 10% neutral buffered formalin and routinely processed by paraffin embedding technique to 4 µm hematoxylin-eosin stained sections for morphological assessment [29].

Ethical considerations

The maintenance and handling of animals during the experiments were conducted respecting the "Guide for the Care and Use of Laboratory Animals, National Research Council, Washington, DC, 1996, in support of the Ethics Committee on Animal Use - CEUA – FIOCRUZ, licence number LW-48/10.

Statistical analysis
Results were analyzed using matched paired *t*-test on GraphPad Prim 5 software programmer Verson 5.0, Inc 1994-2007 (www.graphpad.com) and were expressed as mean ± S.E.M. (standard error of the mean), *p* values of < 0.05 was considered statistically significant.

RESULTS

Gel Permeation Chromatography of the crude extract
By means of GPC was possible to obtain a mass distribution as shown at figure 2. This distribution was guided by thin layer chromatography (data not shown) according to similar or not similar chemical profile from each collected tube. According to mechanism of separation of GPC, low size and/or more polar molecules are more retained than big size and/or less polar molecules. Our experiments showed that the crude ethanolic extract presented size distribution constituted mainly of low size or more polar molecules, as presented in figure 2, after fractions F7 to F13.

***In vitro* leishmanicidal activity assay**
The crude ethanolic extract (EE) and the fractions F1 to F15 were assessed to verify *in vitro* leishmanicidal activity against amastigote-*like* of *Leishmania (Leishmania) amazonensis*. It was noticed that EE, F9, F10 and F11 showed growth inhibition around 80-90 % at 20μg/ml while the fractions F1 and F4 showed a mild growth stimulation (fig. 2). The fractions F9, F10 and F11 were grouped to produce a sample named Enriched Fractions (EF). This decision was based on the quantity of the material, results of the *in vitro* activity and the chemical profile. *In vivo* assays were performed with this fraction.

RP-HPLC analysis
The figure 3 presents the RP-HPLC analysis of EE and EF. As shown, both profiles were very complex, showing lot of compounds that could be solved using this methodology and the difference between crude (total components) and enriched fraction. According to the profile of the EE and EF on RP-HPLC chromatograms, it was possible to verify more polar characteristic in the case of the EE profile, whereas EF RP-HPLC chromatogram, the less polar constituents were majority.

Hematological analysis
Almost all hematological parameters were unaltered, except for the leukocytes level which was significantly increased ($P < 0.05$) at male EE group [(9267 ±1199)/mm^3] treated with 300 mg/kg compared with the control group [(6667 ± 1024)/mm^3], Table 1. The mechanism of the immune stimulation produced by EE is not clear.

Biochemical analysis
Table 2 shows the results of the biochemical analysis carried out to verify the capability of EE and EF in changing the normal biochemical profile. Serum creatinine decreased at female from EE [(0.45±0.03) mg/L] and EF [(0.34 ± 0.0) mg/L] compared with the control group [(0.56±0.03) mg/ml] and serum γ-glutamyltranspetidase level (GGT) was increased only in male from EF group [(15.19 ± 2.50) U/L] compared with the control group [(5.90 ± 2.63) U/L]. These differences were statistically significant at level ($p < 0.05$).

Effects of the EE and EF treatments on the weight of body
The body weights of the mice (control, EE and EF) treated by oral administration with a single dose at 5.0 g/kg are shown in figure 4. All groups (control, EE and EF) gained weight over the two weeks of observation. The EE group of male exhibited a more significant increased of the body weights after 14 days of treatment, compared to control group, while the EF was more significant for weight increase of the female group.

Evaluation of acute toxicity
Oral administration of EE and EF *of C. echinata* at a single dose of 5.0 g/kg body did not produced acute toxicity. There were no deaths during the entire observation period (14 days) after oral administration of single dose of EE and EF, which is considered the threshold dose for acute toxicological evaluation [30-31]. For this reason, there were no calculation of the lethal dose (LD_{50}) so it was estimated be greater than 5.0 g/kg by oral rote. At 24 h post-administration, no animal had cyanosis, piloerection, writhing, ptosis, tremors, convulsions and ataxia. Also there were no significant differences among groups for the parameters motor activity, respiration, corneal reflex, body tone, touch response and reflex headset.

Morphological analysis

The macroscopic analysis of the target organs of the treated animals by oral administration of EE and EF at a single dose of 5.0 g/kg body did not show changes in color and texture when compared with the control group. In addition, the microscopical analysis did not show histological alterations in all organs examined (data not shown).

The present study showed that the crude ethanolic extract (EE) and the enriched fraction (EF) of *C. echinata* had *in vitro* leishmanicidal activity at 20 μg/ml. Although Brazilwood has not been used by traditional medicine, EE and EF showed a pharmacological potential. The results indicated that *C. echinata* was nontoxic when administred acute and orally in mice. Mortality or adverse symptoms was not observed neither after the dose of the 300 mg/kg body nor 5.0 g/kg body, as expected. Indeed, animal behaviour such as appetite, water ingestion and morphological analyses suggested that EE and EF of *C. echinata* were nontoxic.

However, for animals treated with the lowest dose of the *C. echinata* the increase of leukocytes at male of EE group and the proportion of the other cells did not differ significantly between the groups. Those results were not clinically significants for the animal health as they are in the acceptable rate expected in this analysis for wild-type mice [32]. Administration of EF of *C. echinata* significantly increased γ-glutamyltranspetidase levels at male in serum, but neither others hepatobiliary enzyme nor total protein levels were affected by the treatments. Although creatinine levels have decreased at female from both EE and EF groups, urea levels were not altered. Histopathological examinations showed no evidence of necrosis, tumors, masses or foreign bodies in the liver, lungs, brain, spleen, heart or kidney analyzed, corroborating with these biochemical parameters, however, the animals were sacrificed fourteen days after administration of the sample and observations were not conduced for a long period of time, necessary to the development of the neoplasias, since this study was designed to assess the acute toxicity.

DISCUSSION

Secondary metabolites from natural sources such as plants, animals, algae, fungi, bacteria, have become a promising source for the discovery of new drugs against a myriad of diseases in which existing therapies are inefficient, very toxic or are not of interest to large pharmaceutical industries as in the cases of neglected diseases. The natural products offer chemical possibilities that contrast with the synthetic chemical, especially when we are talking about the production of complex molecules produced on a large scale.

By means of chromatographic techniques and bio monitoring of the obtained fractions, these bioactive compounds, from their crude extracts, can be isolated. In this study, Gel Permeation Chromatography (GPC) was the first chromatographic methodology employed to reduce the complexity of the crude extract. The mechanism of the separation provides us a wide range of different polarities for each fraction (fig 3) and a choice to perform a two-dimensional separation technique using, for example, reverse phase or normal phase chromatography as second dimension.

The biologic activity of the fractions is potentiated when crude extracts are fractionated due to high concentration of active component or by disruption of the antagonistic effect after separation. Synergism is also observed, mainly in situations where fractions have low or none activity but crude extracts are very potent. As shown in figure 2, the fractions F9 to F11 presented great quantity of mass and biologic activity. Thus the mechanism of the activity of the crude extract may be a reflection of the mechanism of the activity of the compounds in these fractions.

A good strategy for the development of a safety drug used in the treatment of neglected diseases would be those that could be applied in a single dose in high concentration, while avoiding the risk of an acute toxicity. As our proposal was identify biological activity and acute toxicity, the evaluation of the hematological parameters observed at low dose level (LD) could not be conclusive about deleterious effects to health, even that modifications on leukocytes level had been increased in the case of male EE group, decreasing in the creatinine level from female EE and EF and increasing in the γ-glutamyltranspetidase from female EF. It was observed a healthy body weight gain profile, showed us a healthy behavior, figure 4. The administration of high dose level (5.0 g/kg body) was safety but it was one dose experiments (acute toxicity). Experiments need to be prepared to learn if there are harmful effects against organs and/or development of tumors when these substances are used for long period at high level.

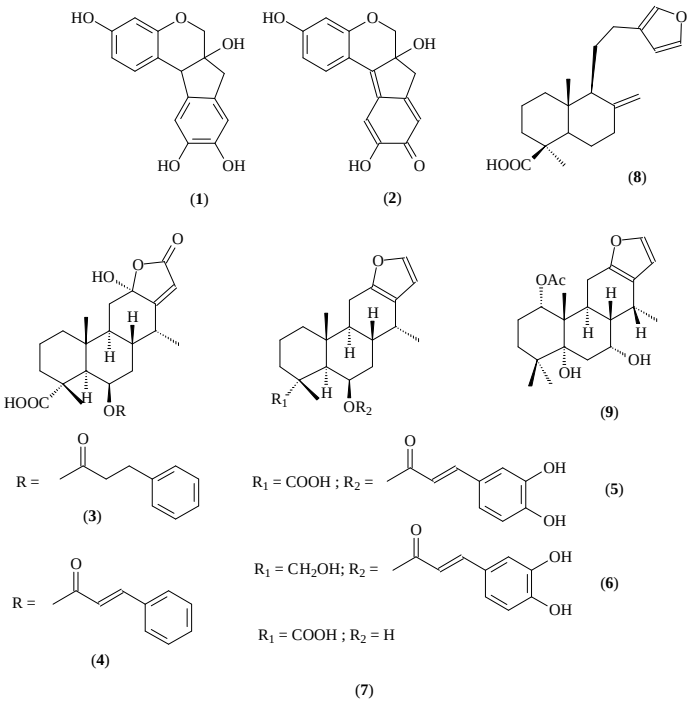

Figure 1 – Secondary metabolites isolated from *Caesalpinia* genus –Brazilin (1), Brazilein (2), derivatives of cassane terpenes (3-9).

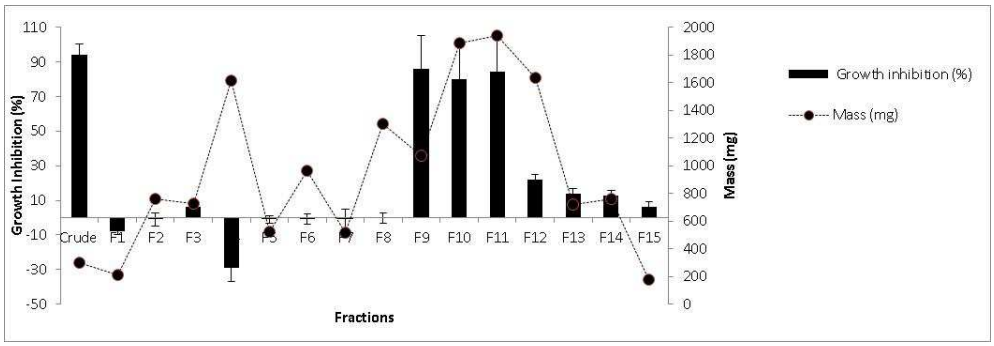

Figure 2 - Profile of the mass distribution and growth inhibition activity associated to each fraction obtained by GPC.

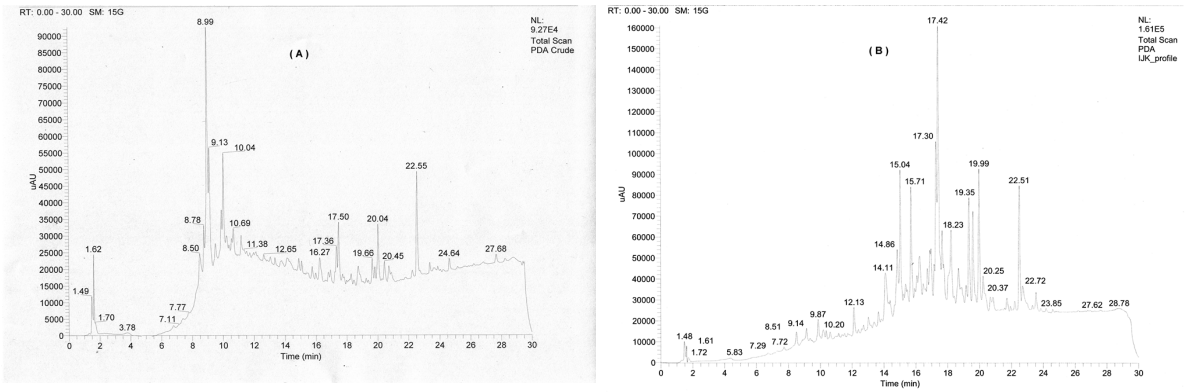

Figure 3 – RP-HPLC profile of the crude ethanolic extract (A) and enriched fractions from pooled of F9, F10 and F11 (B).

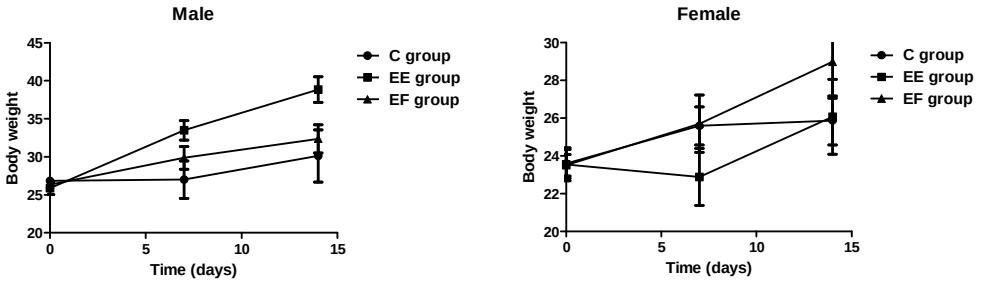

Figure 4. Body weight gain curves of male and female mice treated. C control group = mice orally treated with soybean oil containing 5% of ethanol; EE group: mice orally treated with single dose crude ethanol extract dissolved in soybean oil containing 5% of ethanol at a concentration of 5.0 g/kg and EF group: mice orally treated with single dose of active enriched fraction dissolved in soybean oil containing 5% of ethanol at a concentration of 5.0 g/kg. The values are expressed as mean.

Table - 1. Effect of single oral administration of 300 mg/kg body of the ethanol extract (EE) and enriched fraction (EF) of *Caesalpinia echinata* L. stems on hematological parameters in Swiss-Webster mice.

Parameter	C group		EE group		EF group		*P* values	*P* value
	Male	Female	Male	Female	Male	Female	Male	Female
Atypical lymphocytes (%)	2.33 ± 1.17	2.60 ± 0.84	0.33 ± 0.21	1.00 ± 0.51	2.16 ± 0.87	0.83 ± 0.47	(1) 0.1245 (2) 0.9115	(3) 0.1228 (4) 0.0878
Band neutrophils (%)	0.00	1.33 ± 0.61	0.50 ± 0.34	0.33 ± 0.21	0.83 ± 0.40	0.83 ± 0.48	(1) 1.0000 (2) 0.5413	(3) 0.1548 (4) 0.5350
Eosinophils (%)	0.67 ± 0.49	0.0	0.33 ± 0.21	0.33 ± 0.33	0.33 ± 0.21	0.16 ± 0.16	(1) 0.5490 (2) 0.5490	(3) 1.0000 (4) 0.5490
Haematocrit (%)	8.68 ± 0.68	8.26 ± 0.33	8.79 ± 0.31	8.65 ± 0.30	7.88 ± 0.22	7.66 ± 0.21	(1) 0.8946 (2) 0.2865	(3) 0.4070 (4) 0.1585
Haemoglobin (g/dL)	13.95 ± 0.80	15.03 ± 0.32	15.03 ± 0.35	15.43 ± 0.54	14.30 ± 0.27	13.90 ± 0.63	(1) 0.2436 (2) 0.6862	(3) 0.5364 (4) 0.1376
Leukocytes/mm^3	6667 ± 1024	10788 ± 1428	9267±1199	11217 ± 672	4233 ± 262	8533 ± 1277	(1) 0.1302 (2) 0.0441	(3) 0.7916 (4) 0.2664
Lymphocytes (%)	85.33 ± 6.57	84.50 ± 3.17	85.83 ± 1.92	88.50 ± 2.65	76.50 ± 4.58	82.33 ± 3.18	(1) 0.9432 (2) 0.2960	(3) 0.3562 (4) 0.6398
MCV (%)	40.33 ± 2.028	41.67± 1.085	38.00±0.7303	42.83±1.537	39.50 ± 1.176	43.33 ± 0.9888	(1) 0.3044 (2) 0.7296	(3) 0.5490 (4) 0.2828
Monocytes (%)	0.67 ± 0.21	1.00 ± 0.45	0.83 ± 0.65	0.17 ± 0.17	1.00 ± 0.51	1.17 ± 0.48	(1) 0.8133 (2) 0.5634	(3) 0.1114 (4) 0.8040
Platelets/mm^3	636000±15144	632333±50887	729000±66918	737200±77922	525000±93185	730000±48889	(1) 0.4437 (2) 0.3155	(3) 0.2742 (4) 0.1965
Ring cell (%)	1.83 ± 0.70	1.33 ± 0.61	0.50 ± 0.34	2.50 ± 0.43	3.33 ± 1.54	1.33 ± 0.88	(1) 0.1189 (2) 0.3969	(3) 0.1504 (4) 1.0000

Values are expressed as mean ±SEM.; MCV: mean corpuscular volume.; C: control group. EE: crude extract group. EF: enriched fraction group.; Student's t-test for the comparison of two means. (1) C male group vs. EE male group, (2) C male group vs. EF male group, (3) C female group vs. EE female group, (4) C female group vs. EF female group; p values ≤0.05 were considered significant.

Table - 2. Effect of single oral administration of 300 mg/kg body of the ethanol extract (EE) and enriched fraction (EF) of *Caesalpinia echinata* L. stems on biochemical parameters in Swiss-Webster mice.

Parameter	C group		EE group		EF group		*P* value	*P* value
	Male	Female	Male	Female	Male	Female	Male	Female
ALP (U/L)	41.58±13.56	25.81 ±2.20	42.46±8.55	29.87± 2.93	35.51±10.35	14.89 ±4.78	(1) 0.9574	(3) 0.3130
							(2) 0.7315	(4) 0.8554
ALT (U/L)	120.21±15.35	92.78±24.98	99.35 ± 6.17	80.98±18.42	89.66 ± 10.41	99.00±17.19	(1) 0.2728	(3) 0.7136
							(2) 0.1305	(4) 0.8427
AST (U/L)	244.31± 28.16	236.76 ± 30.00	214.06 ± 9.83	208.87 ± 46.15	204.11 ± 29.29	171.10 ± 17.43	(1) 0.3497	(3) 0.6144
							(2) 0.3607	(4) 0.0949
Creatinine (mg/L)	0.49 ± 0.03	0.56 ± 0.03	0.46 ± 0.02	0.45 ± 0.03	0.42 ± 0.02	0.34 ± 0.00	(1) 0.3514	(3) 0.0246*
							(2) 0.0738	(4) 0.0001*
GGT (U/L)	5.90 ± 2.63	6.40 ± 2.47	11.52 ± 1.35	7.74 ± 1.00	15.19 ± 2.50	8.59 ± 4.67	(1) 0.0863	(3) 0.6251
							(2) 0.0327*	(4) 0.6869
TP (g/L)	6.27 ± 0.11	6.83 ± 0.14	5.90 ± 0.14	6.63 ± 0.18	6.13 ± 0.08	6.32 ± 0.20	(1) 0.0716	(3) 0.4049
							(2) 0.3628	(4) 0.0612
Urea (mg/L)	67.44 ± 4.05	64.53 ± 2.20	65.64 ± 4.16	64.18 ± 5.73	57.11 ± 4.27	66.33 ± 2.42	(1) 0.7624	(3) 0.9559
							(2) 0.1094	(4) 0.5934

Values are expressed as mean ±SEM; ALP: alkaline phosphatase, ALT: alanine amino transferase, AST: aspartate amino transferase, γ-glutamyltranspetidase (GGT) and TP: total protein; C: control group. EE: crude extract group. EF: enriched fraction group. Student's t-test for the comparison of two means. (1) C male group vs. EE male group, (2) C male group vs. EF male group, (3) C female group vs. EE female group, (4) C female group vs. EF female group; p values ≤0.05 were considered significant.

CONCLUSION

The discovery of the new leishmanicidal agents is necessary because the arsenal of the therapeutic drugs are very toxic or the pathologic agents are becoming resistant. These preliminary studies were the first steps to show the pharmacological potential of the *C. echinata* and its application as nontoxic leishmanicidal agent.

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