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Biological activities and chemical composition of crude extracts from *Chresta exsucca*

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Crude extracts of Chresta exsucca *were investigated for their* in vitro *trypanocidal, leishmanicidal, antibacterial and antifungal activities. Trypomastigote forms of* Trypanosoma cruzi, *amastigote-like forms of* Leishmania amazonensis *and twenty strains of microorganisms including Gram-positive and Gram-negative bacteria and yeasts were utilized in the bioassays. The best results were found for the leishmanicidal activity. The chemical composition of hexanic and ethanolic extracts of this species was determinate using chromatographic techniques as HRGC and HPLC-ESI-MS, respectively. Steroids, triterpenes and flavonoids were identified.*

Uniterms
- *Chresta exsucca*
- Asteraceae
- Biological activities
- HRGC
- HPLC-ESI-MS

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INTRODUCTION

The use and research of natural products have increased in the last years because of the biological activities of their constituents, including the studies performed with plants species for treatment of parasitic and microbial diseases (Kayser *et al*., 2003a; Newman *et al*., 2003; Truiti *et al*., 2003; Oumzil *et al*., 2002).

In South America, the Chagas disease is a public health problem and represents the first cause of cardiac lesions in young and economically productive adults (Moncayo, 2003). Nearly 20 million people are infected by *Trypanosoma cruzi* while 90 million are at risk in endemic areas (WHO, 1991, 2000). The transmission of the disease occurs mainly by the vector (Triatomine), blood transfusion and congenital routes (Coura, Castro, 2002). There is no satisfactory chemotherapy for the disease in any of its phases. The scarcity of serological control for blood donors in endemic areas and the restricted alternative of chemoprophylaxis using gentian violet, have caused a need to the development of new drugs against this disease (Araya *et al*., 2003; WHO, 2000).

Other problem that occurs in South America is the leishmaniases that comprise a group of cutaneous, mucocutaneous and visceral diseases caused by haemoflagellate protozoan parasites that survive and multiply in macrophages in the mammalian host and are transmitted by phlebotomine sandflies (WHO, 2000; Barata *et al*., 2000; WHO, 1990). The incidence of this disease has increased in the last years and therapy available mainly include antimonials, pentamidine and amphotericin B, which are toxic, difficult to apply in the field and not always effective (Peters, 1999; Thakur *et al*., 1999).

In addition, the development of resistant strains of pathogenic bacteria to antibiotics currently in use in the

therapeutic is a problem of continuing concern to public health (Karaman *et al*., 2003).

Asteraceae family comprises many species with antimicrobial and antiprozoal activities, such as, for example, *Lychnophora staavioides* (Takeara *et al*., 2003) and *Podolepsis hieracioides* (Kayser *et al*., 2003b). The genus *Chresta*, belonging to this family, comprises 12 endemic species from Brazilian Central Plateau (Robinson, 1999). *Chresta exsucca* is perenial herbs adapted to the arid conditions of shrubby "cerrado" and is known principally from the state of Goiás (MacLeish, 1985). Here we report for the first time the biological activity of crude extracts from *C. exsucca* against two protozoan parasites (*Trypanosoma cruzi* trypomastigotes and *Leishmania amazonensis* amastigotes) and several strains of bacteria and fungi. The chemical composition of bioactive hexanic and ethanolic extracts was also determined by chromatographic analysis (HRGC and LC-ESI-MS).

MATERIALS AND METHODS

Plant material

Chresta exsucca was collected in Alto do Paraíso (GO-Brazil) in October 1998 and identified by Dr. João Semir (Instituto de Biologia, Unicamp, Campinas, SP-Brasil). A voucher specimen is deposited in the Herbarium of FFCLRP/USP (SPFR6873).

Preparation of plant extracts

Plant material (2100 g) was dried, pulverized and stored in dark bags to protect them from humidity and light. The powdered material was extracted by maceration with *n*-hexane, dichloromethane and ethanol at room temperature. The solvents used in each extraction were evaporated under reduced pressure and different extracts were obtained. These extracts were evaluated against *Trypanosoma cruzi* trypomastigote forms, *Leishmania (L.) amazonensis* amastigote forms, Gram-positive and Gram-negative bacteria strains and yeasts. In addition, the chemical composition of hexanic and ethanolic extracts was determined by gas chromatography and HPLC-ESI-MS, using standard, respectively.

Clean up procedure

An aliquot (10 mg) of the hexane extract was resuspended in analytical grade chloroform (3 mL) and percolated through a sep-kap column (Alltech, silica-gel 200 mg, 3 mL). The column was eluted with hexane (10 mL) and chloroform (10 mL). Fractions were collected separately and evaporated to dryness at room temperature. Chloroform phase was analyzed by HRGC in duplicate. The ethanolic extract (10 mg) was resuspended in methanol-water (7:3) (3 mL) and percolated through a sep-kap column (Alltech, C-18, 200 mg, 3 mL). The column was eluted with analytical grade methanol (10 mL) and this phase was analyzed by HPLC-ESI-MS.

Gas chromatographic analysis

Chloroform phase was analyzed by HRGC on a Hewlett-Packard model 5890 Series II Gas Chromatograph with a split injector (split ratio 1:60) at 260 °C and a flame ionization detector at 330 °C. The injected volume was 2 mL. Hydrogen was employed as carrier gas at an average linear velocity of 44 cm/s (HP-50) and 42 cm/s (HP-1). The HP-50 (cross-linked 50% phenyl-methyl-silicone, 30 m x 0.25 mm x 0.25 mm) and HP-1 (cross-linked methyl-silicone, 30 m x 0.25 mm x 0.25 mm) capillary columns were employed. For HP-50 the column temperature was 280 °C (isotherm) and for HP-1 the column temperature program was 250 °C held for 12 min, increased at 6 °C/min to 280 °C, and held this temperature for 30 min. Data were processed on a Hewlett-Packard model 3395 injector. The standards used in the gas chromatography are listed in Table III.

HPLC-ESI-MS analysis

Ethanolic extract was analyzed by HPLC system consisting of LC10AD solvent pumps, SLC10A system controller, 7125 Rheodyne injector (Shimadzu, Japan), Shim –Pack ODC-18 analytical column (4,6 x 250 mm) with a RP-18 guard column (LiChrospher® 100,5 mm, 4 x 4 mm, Merck, Germany). An UV detector (SPD10A, Shimadzu, Japan) set at 280 nm was used. The mobile phase conditions were as follows: 15% MeOH at 0 min, 75% at 40 min, held constant until 45 min, 15% at 60 min. The next sample was injected after a further 10 min. The flow-rate was 1 mL min^{-1}, at room temperature, and the injection volume was 20 mL. The ESI-MS spectra were acquired in a Quattro LC quadrupole mass spectrometer fitted with a electrospray interface operating in the negative ion mode (Micromass, United Kingdom). The source and desolvation temperatures were 70 °C and 100 °C, respectively. Cone voltage was 30 V. The parent ion and the retention time were compared with previously isolated standards.

In vitro trypanocidal activity

For the trypanocidal activity blood of Swiss albino mice infected with *T. cruzi* (Y strain) was used. It was collected by cardiac puncture at the peak of parasitemia and was diluted to contain 10^6 trypomastigotes/mL. Stock solutions (crude extracts) were prepared in 2.5% of dimethyl sulfoxide (DMSO) and were added to blood samples to provide a final concentration of 4000 mg/mL. After incubation for 24 hours at 4 °C, the number of parasites was determined according to Brener (1962). In the tests, gentian violet (250 mg/mL) was used as positive control and DMSO 2.5% as negative control. All experiments were performed in triplicate.

In vitro leishmanicidal activity

Axenic *L. (L.) amazonensis* (strain designation MPRO/BR/72/M 1841) amastigotes were serially cultivated at 33 °C in modified UM-54 mediun (Pral *et al*., 2003) and were used at the beginning of the stationary phase. Washed parasites were resuspended in RPMI-1640 medium supplemented with 4% fetal calf serum, pH 5.0 and incubated at 33 °C for 24 hours with crude extracts (1000 mg/mL) dissolved previously in RPMI-1640. As controls, parasite suspensions were incubated in RPMI alone or RPMI containing 0.1% DMSO. Amastigote viability was assessed colorimetrically by reduction of a tetrazolium salt (MTT) as described by Mosmann (1983). Amphotericin B (20 mg/mL) was used as the positive control and DMSO/RPMI-1640 (1:99) negative control. The experiments were carried out in triplicate.

Antimicrobial activity

The effect of crude extracts was evaluated against twenty strains of microorganisms, including Gram-positive and Gram-negative bacteria and yeasts. The following microorganisms were used: *Escherichia coli* – ATCC 10538; *Pseudomonas aeruginosa* – ATCC 27853; *P. aeruginosa* – 290 D (field strain); *Micrococcus luteus* – ATCC 9341; *Staphylococcus aureus* – ATCC 25923 and 6538; *S. aureus* 7+ penicilinase producer; *S. aureus* 8- penicilinase non-producer; *Staphylococcus epidermidis* 6epi and epiC (field strain); *Candida albicans* – ATCC 1023; *C. albicans* cas and *Candida tropicalis* ct (field strains), cultivated for 24 hours at 37 °C in Mueller Hinton broth (Difco) – MHb; *Enterococcus faecalis* – ATCC 10541; *Streptococcus mutans* – ATCC 25175; *S. mutans* (strains 11.1; 9.1; fab3; 11.2) and *Streptococcus sobrinus* 180.3 (field strains), incubated for 24 hours at 37 °C in Brain Heart Infusion (Difco) – BHI. The standard and field strains (oral cavity) were collected from "Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto – SP, Brasil". Sensitivity tests were performed by a modified agar-well diffusion method (well technique in double layer) according to Okeke *et al*. (2001), Cole (1994), Grove, Randall (1955). The Mueller Hinton Medium (Difco) - MH agar plates, containing an inoculum size of 10^6 cfu/mL of *Escherichia*, *Pseudomonas*, *Micrococcus*, *Staphylococcus* and *Candida* strains or 10^6 cfu/mL of *Enterococcus* and *Streptococcus* strains on Brain Heart Infusion Agar (Difco) – BHIa plates, were used. The inoculum size of each test strain was standardized according to National Committee for Clinical Laboratory Standard (NCCLS, 1993). Subsequently, aliquots of 20 mL of each test-drug solution were applied into 5.0 mm diameter wells. Crude extract solutions were prepared at 5000 mg/mL in propyleneglycol/sterile water (5:95). After incubation at 37 °C for 24 hours, the inhibition zone corresponding to the halo (H) formed from well edge to the beginning of the region of microbial growth was measured in millimeters (mm). In the tests, bacitracine (0.2 UI/mL) and ketoconazole (100 mg/mL) were used as positive controls and propyleneglycol/sterile water (5:95) served as negative control. The experiments were performed in duplicate for each strains analyzed.

RESULTS AND DISCUSSION

The results of bioactivity of crude extracts from *C. exsucca* for activity against *T. cruzi* trypomastigotes and *L. amazonensis* amastigote-like stages are showed in Table I. When examined against *T. cruzi* trypomastigotes, hexanic extract from *C. exsucca* (total plant) given at 4000 mg/mL did not interfere appreciably with parasite viability, whereas dichloromethanic and ethanolic extracts reduced parasite viability to 46.7 and 49.2%, respectively. In these assays mice infected blood containing 2.5% of DMSO were used as negative control and did not interfere with *T. cruzi* tryposmastigotes viability, while the gentian violet at 250 mg/mL were used as positive control and showed 100% of activity.

On the other hand, all the extracts evaluated interfere markedly with viability of *L. amazonensis* amastigotes. Hexanic, dichlorometanic and ethanolic extracts were clearly leishmanicidal and parasite viability were reduced to 1.8, 4.7 and 0.2%, respectively. Amphotericin B (20 mg/mL), here used as a positive control, reduced amastigote viability to 3.7%. Suspensions of *L.*

TABLE I - *In vitro* activity of crude extracts from *Chresta exsucca* against *Trypanosoma cruzi* trypomastigotes and axenic *Leishmania amazonensis* amastigotes

Extracts	Antiprotozoal activity[a]	
	% viability of *T. cruzi* trypomastigotes	% viability of *L. amazonensis* amastigotes
Hexane	89.06 ± 0.64	1.83 ± 0.01
Dichloromethane	46.71 ± 1.67	4.72 ± 0.04
Ethanol	49.15 ± 1.46	0.28 ± 0.02
Amphotericin B (20 mg/mL)[b]	-	3.70 ± 0.05
DMSO/RPMI-1640 (1:99)[c]	-	100.00 ± 0.02
Gentian violet (250 mg/mL) [d]	0.00 ± 0.00	-
DMSO (2.5%)[e]	100.00 ± 0.00	-

[a] = Results expressed as% viability (± standard deviation) of the *T. cruzi* trypomastigotes (extracts evaluated at 4000 mg/mL) and *L. amazonensis* amastigotes (extracts evaluated at 1000 mg/mL); [b] = positive control of the anti-leishmanial assay; [c] = negative control of the antileishmanial assay; [d] = positive control of the trypanocidal assay; [e] = mice infected blood containing 2.5% of DMSO was used as negative control of the trypanocidal assay; - = not done.

amazonensis amastigotes incubated in medium alone or in medium containing 0.1% DMSO remained viable during the 24 h incubation period (% amastigote viability=100.0%), a result that excludes spontaneous parasite damage during the bioassay.

The extracts from *C. exsucca* were also screened for antibacterial and antifungal activity and the results are showed in Table II. While ethanolic extract was inactive (at 5000 mg/mL) against all eighteen strains of microorganisms tested, dichloromethanic extract was effective against only strains the *Streptococcus* (three strains of *S. mutans* and one strain of *S. sobrinus*) and hexanic extract inhibited the growth of five strains including *S. aureus*, *S. mutans*, *S. sobrinus*, *E. coli* and *P. aeruginosa*. As indicated in Table II, the extracts of *C. exsucca* assayed were ineffective against *Candida* strains tested. Bacitracine at 0.2 UI/mL (against bacteria strains) and ketoconazole at 100 mg/mL (against fungi strains), here were used as positive controls, inhibited the growth of all microorganism assayed. Media containing propyleneglycol/ sterile water (5:95) were used as negative control for which no inhibitory effect was observed.

The biological activities of crude extracts from *C. exsucca* are being evaluated for the first time and the results obtained indicate the potential of this plant as a source of bioactive compounds.

TABLE II - Antimicrobial activity of crude extracts from *Chresta exsucca*

Microorganisms	A	B	C	Bacitracine (0.2 UI/mL)[a]	Ketoconazole (100 mg/mL)[a]
	H	H	H	H	H
S. aureus (ATCC 6538)[b]	-	-	-	23	-
S. aureus penicilinase + (7+)[c]	12	-	-	25	-
S. aureus penicilinase – (8-)[c]	-	-	-	25	-
S. epidermidis (6ep)[c]	-	-	-	31	-
M. luteus (ATCC 9341)[b]	-	-	-	25	-
E. faecalis (ATCC 10541)[b]	-	-	-	28	-
S. mutans (ATCC 25175)[b]	9	8	-	24	-
S. mutans (fab3)[c]	-	-	-	22	-
S. mutans (9.1)[c]	-	10	-	22	-
S. mutans (11.1)[c]	8	8	-	22	-
S. mutans (11.2)[c]	-	-	-	22	-
S. sobrinus (180,3)[c]	-	7	-	23	-
E. coli (ATCC 10538)[b]	7	-	-	32	-
P. aeruginosa (ATCC 27853)[b]	11	-	-	22	-
P. aeruginosa (290D)[c]	-	-	-	22	-
C. albicans (cas)[c]	-	-	-	-	30
C. albicans (ATCC 1023)[b]	-	-	-	-	30
C. tropicalis (ct)[c]	-	-	-	-	15

H = halo of inhibition (mm); - = absence of inhibition of microbial growth at 5000 mg/mL; A = hexane extract; B = dichloromethane extract; C = ethanol extract; [a]: positive control; [b] = standard strains; [c] = field strains (oral cavity).

TABLE III - Triterpenes and sterols identified by HRGC (HP-50 colunm) in hexanic extract of *Chresta exsucca*

Standards	RR[a]	Hexanic extract
		% area
Campesterol	1238	-
Stigmasterol	1308	0.095
β-sitosterol	1453	-
D^7-stigmastenol	1513	-
Spinasterol	1683	-
Taraxerone	1628	-
Epitaraxerol	1656	-
Taraxerol	1698	-
β-Amyrin	1768	0.165
α-Amyrin	1992	1.335
Lupeol	2052	1.080
β-Friedelanol	2491	-
Friedelin	2724	-
Pseudotaraxasterol	2479	-
Taraxasterol	2570	-
11-oxours-12-ene	3160	-
11-oxoolean-12-ene	3586	0.240
Taraxerol acetate	1872	-
β-amyrin acetate	1930	9.365
α-amyrin acetate	2154	29.640
Lupeol acetate	2234	30.335
Bauerenyl acetate	2508	-
11a,12a-oxidetaraxeryl acetate	2810	-
β-friedelanol acetate	2871	-
α-amyrinonil acetate	3653	8.615
β-amyrinonil acetate	4126	1.935

RR[a] = relative retention to cholesterol (internal standard); -: compound not detected

Seeking to verify the chemical composition of the bioactive crude extracts from *C. exsucca*, analysis employing the chromatographic techniques HRGC and HPLC-ESI-MS was proceeded.

Gas chromatography has been the traditional technique for the analysis of terpenoids. Improved technology in GC fused-silica capillary columns has enabled the use of much shorter (<10 m) columns. Several studies on the characterization of wood extractives show that capillary GC is by far the most convenient and comprehensive technique available for separating the individual components in wood extractives (Vasconcelos *et al*., 2000; Fernandez *et al*., 2001). To identify the triterpenes and sterols from hexanic extract of *C. exsucca*, 26 authentic standards were used. These standards were obtained from several plant species, which were studied in our laboratory. In Table III are summarized the HRGC results of the sterols and triterpenes extracted and identified. These structures were unequivocally confirmed by co-injection of authentic standards and identified by relative retention value.

HPLC-ESI-MS represents the combination of a high-resolution separation system with a powerful detection/characterization technique, which has been successfully applied to the determination of compounds present in material from a variety of natural product source. A specific application of HPLC-ESI-MS natural product mixture analysis is the procedure known as dereplication. This process is rapid, precise and efficient besides has become one of the key processes for maintaining samples from natural source (Strege, 1999). Lin *et al*. (2000) utilized the HPLC-ESI-MS technique to detect the presence flavonoids in red clover.

TABLE IV - Flavonoids identified by HPLC-ESI-MS in ethanolic extract of *Chresta exsucca*

Standards	Rt (min)[a]	$[M-H]^-$ (*m/z*)[b]	Present flavonoids
Apigenin	16.86	269	+
Luteolin	16.22	285	+
Genkwanin	18.80	283	+
Crysoeriol	16.99	299	+
Velutin	18.74	313	-
Kaempferol	16.05	285	+
Tiliroside	15.53	579	-
Luteolin 3'-O-β-D-glucopyranoside	15.04	447	+
Luteolin 4'-O-β-D-glucopyranoside	13.51	447	+
Luteolin 7-O-β-D-glucopyranoside	9.17	447	+
Vicenin-2	11.37	593	+

Rt[a] = retention time values; [b] $[M-H]^-$ = molecular íon; + = presente flavonoids; - = compound not detected

In order to verify the correct identification of the present flavonoids in the ethanolic extract of *C. exsucca*, 11 standards, all obtained from *C. scapigera* (Schinor, 2004), were used. Standard solutions of flavonoids were chromatographed to determine their retention times and MS data for comparison with the chromatograms of the plant extract. The results are showed in the Table IV.

Thus, the chemical composition of hexanic and ethanolic extract from *C. exsucca* could be determined without the need of isolation and purification processes of compounds through classical phytochemistry, which involves methods that are both costly and time-consuming. Steroids, triterpenes and flavonoids were identified in crude extracts of *C. exsucca*. Some of these compounds showed to be active against microorganisms (protozoal, bacteria and fungi) as reported previously by the literature (Salvador *et al*., 2004; Schinor *et al*., 2004; Taleb-Contini *et al*., 2003; Pena *et al*., 2001). However, further biological studies should be performed, including *in vitro* and *in vivo* investigations, as well as to evaluation of the toxicity, looking toward a clinical employment of these bioactive natural products.

CONCLUSION

Crude extracts of *C. exsucca* showed trypanocidal, leishmanicidal and antibacterial *in vitro* activities. The chemical composition of hexanic and ethanolic extracts was determined using HRGC and HPLC-ESI-MS chromatographic techniques. Steroids, triterpenes and flavonoids were identified.

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RESUMO

Atividades biológicas e composição química dos extratos brutos de *Chresta exsucca*

As atividades tripanocida, leishmanicida, antibacteriana e antifungica dos extratos brutos de Chresta exsucca *foram investigadas. Formas tripomastigotas do* Trypanosoma cruzi, *formas amastigotas de* Leishmania amazonensis *e vinte cepas de microrganismos, incluindo bactérias Gram-positivas, Gram-negativas e leveduras, foram utilizadas nos ensaios biológicos. Os melhores resultados foram obtidos para a atividade leishmanicida. A composição química dos extratos hexânicos e etanólicos dessa espécie foi determinada empregando-se técnicas cromatográficas como HRGC e HPLC-ESI-MS, respectivamente. Esteróides, triterpenos e flavonóides foram identificados.*

UNITERMOS: Chresta exsucca. *Asteraceae. Atividades biológicas. HRGC. HPLC-ESI-MS.*

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