EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS AND ISOLATED CONSTITUENTS FROM *CHRESTA SCAPIGERA*

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ABSTRACT

Crude extracts and eight isolated compounds from *Chresta scapigera* were evaluated for antibacterial and antifungal activities by the agar-well diffusion method. Twenty strains, including Gram-positive and Gram-negative bacteria and yeasts were used in the bioassay. Hexane extracts presented the best results while ethanol extracts did not indicate inhibition of the microbial growth. Amongst the evaluated compounds β-amyrin acetate, tiliroside and luteolin showed the strongest antimicrobial effect.

Key words: *Chresta scapigera*, flavonoids, triterpenes, antimicrobial activity

INTRODUCTION

The emergence of human pathogenic microorganisms that are resistant to major classes of antibiotics has increased in recent years, due to the indiscriminate use of antimicrobial drugs (9). This has caused many clinical problems in the treatment of infectious diseases and the antibiotics commonly used are sometimes associated with adverse effects on the host, which include hypersensitivity, allergic reaction and immunosuppression (12). Therefore, research for development of new antimicrobial agents is an urgent need.

Plants are known to produce some chemicals, which are naturally toxic to bacteria and fungi (2). Traditionally, the dried herbs are used either as boiled in water to make a tea or as an infusion to treat systemic bacterial and fungal infections, as well applied directly on the skin or nails in a plaster form to treat local infections (20).

Some species of Asteraceae family present antimicrobial action, such as *Arnica montana* (8), *Carthamus lanatus* (17), *Moquinia kingii* (16). The genus *Chresta*, belonging to this family, comprises 12 endemic species of "Cerrado Brasileiro" (15) and there has been no reports about biological activities of plants belonging to this genus.

Thus, the aim of this investigation was to evaluate the *in vitro* antimicrobial activity of crude extracts (root, stem, leaves and inflorescence) and eight isolated compounds (kaempferol, apigenin, luteolin, tiliroside, crysoeriol, β-amyrin acetate, lupeol and 11α,12α-oxidetaraxeryl acetate) from *Chresta scapigera* against twenty strains of microorganisms, including Gram-positive and Gram-negative bacteria and yeasts.

MATERIALS AND METHODS

Plant material

The plant material of *Chresta scapigera* was collected in Furnas (MG-Brazil) in July 1998, and identified by Dr. João Semir (Instituto de Biologia, Unicamp, Campinas, SP-Brasil). A voucher specimen is deposited in the Herbarium of Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto/Universidade de São Paulo (register number SPFR 6874).

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Extraction and fractionation

Roots (188.0 g), stem (252.0 g), leaves (313.0 g) and inflorescences (99.0 g) were separated, dried, powdered 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, respectively, at room temperature. The *n*-hexanic extract of roots (9.521 g) was chromatographed on a column of silica gel 60 (0.063-0.200 mm, Merck), eluted with hexane, ethyl acetate, methanol and mixtures of these solvents in increasing polarity. This extract furnished 18 fractions and the fractions 3 and 4 were purified by preparative thin layer chromatography (PTLC) yielding compounds **(I)** (16 mg) and **(II)** (10 mg). The *n*-hexanic extract of inflorescences (2.594 g) was chromatographed using the same conditions and furnished 14 fractions. Preparative TLC was used to purify the fraction 4, which yielded compound **(III)** (30 mg). The ethanolic crude inflorescence extract (2.6 g) was partitioned between dichloromethane and methanol. The hidroalcoholic phase (1.3 g) was submitted to filtration on sephadex LH-20 and the fractions 6 and 7 were purified by preparative high performance liquid chromatography (HPLC) in reverse phase (column ODS Shimadzu 5 μm, 20 x 250 mm, eluent: methanol:water in gradient, flow 9 mL/min, UV detection: 280 nm) resulting in compounds **(IV)** (22 mg), **(V)** (12 mg) and **(VI)** (10 mg). While the dichloromethanic phase (391 mg) was chormatographed on a silica gel column and was eluted with hexane, ethyl acetate, methanol and mixtures of these solvents in increasing polarity. The fraction 3 was purified by preparative TLC resulting in compound **(VII)** (3 mg). The ethanolic crude extract of stem was chromatographed on polyvinylpyrrolidone by vacuum liquid chromatography eluted with $CHCl_3$, methanol and mixtures of these solvents in increasing polarity. Filtration on sephadex LH-20 was used to purify the fraction 1, which yielded compounds **(V)** (2 mg), **(VII)** (3 mg) and **(VIII)** (3 mg).

The structures of all compounds were determined by ^{1}H-NMR and ^{13}C-NMR spectra, which were measured in $CDCl_3$ (for triterpenes) and DMSO-d_6 (for flavonoids) using tetramethylsilane as the internal standard by a BRUCKER DRX instrument (400MHz for ^{1}H and 100MHz for ^{13}C).

Antimicrobial activity

Crude extracts and isolated compounds were tested for antimicrobial activity 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); *Kocuria rhizophila* - ATCC 9341; *Staphylococcus aureus* - ATCC 25923 and 6538; *S. aureus* 7+ penicilinase producer; *S. aureus* 8- penicilinase non-producer; *Staphylococcus epidermidis* 6ep and epiC (field strain); *Candida albicans* - ATCC 1023; *C. albicans* cas and *Candida tropicalis* ct (field strains), cultivated for 24 hours at 37ºC 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 37ºC in Brain Heart Infusion (Difco) - BHI. The standard and field strains (oral cavity) were provided by 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.* (14), Cole (3), Grove and Randall, (7). The Mueller Hinton Medium (Difco) - MH agar plates, containing an inoculum size of 10^6 cfu/mL of *Escherichia*, *Pseudomonas*, *Kocuria*, *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 tested strain was standardized according to Clinical and Laboratory Standard Institute (13). Subsequently, aliquots of 20 μl of each test-sample solution were applied into 5.0 μm diameter wells. Solutions were prepared in propyleneglycol/ sterile water (5:95) at 5000 mg/ml for the crude extracts and 2500 mg/ml for pure compounds in the screening. After incubation at 37ºC 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 μg/ml) were used as positive experimental controls for bacteria and fungi strains, respectively, and propyleneglycol/sterile water (5:95) as negative experimental control.

The minimal inhibitory concentration (MIC) was determined for each isolated compound using the agar diffusion method to give a concentration between 25 and 2500 mg/ml (5,6,14,18). In MIC determination, media containing sterile water and propyleneglycol (5:95) was used as negative control in which any inhibitory effect could be observed.

The tests were performed in duplicates for each microorganism evaluated and the final results were presented as the arithmetic average.

RESULTS

The chemical structures of all isolated compounds were determined by ^{1}H-NMR and ^{13}C-NMR spectra and β-amyrin acetate **(I)**, 11*a*,12α-oxidetaraxeryl acetate **(II)**, lupeol **(III)**, tiliroside **(IV)**, apigenin **(V)**, kaempferol **(VI)**, crysoeriol **(VII)**, luteolin **(VIII)** were identified by a comparison with the literature values (10,19).

The data for antibacterial and antifungal activities for the crude extracts and the isolated compounds of *C. scapigera* are show in Table 1 and Table 2, respectively. The antimicrobial activity assay was performed for crude extracts and isolated

Table 1. Antimicrobial activity of the crude extracts from *Chresta scapigera* using diffusion method.

Microorganisms	A	B	C	D	E	F	G	H	Bacitracine (0.2 UI/ml)[a]	Ketoconazole (100 mg/ml)[a]
	h	h	h	h	H	h	h	h	h	H
Staphylococcus aureus (ATCC 6538)[b]	8	12	8	8	—	—	—	—	23	—
S. aureus penicilinase + (7+)[c]	12	14	5	3	—	—	—	—	25	—
S. aureus penicilinase – (8-)[c]	—	10	—	7	—	—	—	—	25	—
Staphylococcus epidermidis (6ep)[c]	—	—	—	—	—	—	—	—	31	—
S. epidermidis (epiC)[c]	—	—	—	—	—	—	—	—	31	—
Kocuria rhizophila (ATCC 9341)[b]	—	10	—	12	—	—	—	—	25	—
Enterococcus faecalis (ATCC 10541)[b]	—	—	—	—	—	—	—	—	28	—
Streptococcus mutans (ATCC 25175)[b]	9	—	8	—	—	—	8	—	24	—
S. mutans (fab3)[c]	—	—	—	—	—	—	—	—	22	—
S. mutans (9.1)[c]	9	—	8	—	9	10	10	10	22	—
S. mutans (11.1)[c]	10	11	9	10	—	—	7	—	22	—
S. mutans (11.2)[c]	—	12	—	8	—	—	—	—	22	—
Streptococcus sobrinus (180,3)[c]	—	—	—	—	—	—	—	—	23	—
Escherichia coli (ATCC 10538)[b]	7	11	8	10	—	—	—	—	32	—
Pseudomonas aeruginosa (ATCC 27853)[b]	11	15	9	15	—	—	—	—	22	—
P. aeruginosa (290D)[c]	—	—	—	—	—	—	—	—	22	—
Candida albicans (cas)[c]	—	—	—	—	—	—	—	—	—	30
C. albicans (ATCC 1023)[b]	—	—	—	—	—	—	—	—	—	30
Candida tropicalis (ct)[c]	—	—	—	—	—	—	—	—	—	15

h: halo of inhibition in mm; —: not active at 5000 µg/ml; A: hexane extract of inflorescence; B: hexane extract of stem; C: hexane extract of leaves; D: hexane extract of root; E: dichloromethane extract of inflorescence; F: dichloromethane extract of stem; G: dichloromethane extract of leaves; H: dichloromethane extract of root; a: positive control; b: standard strain, c: field strain (oral cavity).

compounds against 14 strains of Gram-positive bacteria, 3 strains of Gram-negative bacteria and 3 strains of yeast. Among the crude extracts evaluated, the hexanic extracts were active and inhibited the growth of *S. aureus, K. rhizophila, S. mutans, E. coli* and *P. aeruginosa*. As indicated in Table 2, the bioactive compounds were mainly flavonoids and triterpenoids.

The bacitracine (0.2UI/mL) here used as a positive experimental control against all bacteria strains assayed, produced halo of inhibition (H) of 22-32 mm, while ketoconazole (100 µg/mL) served as the positive experimental control for all fungi strains assayed with halo of inhibition of 15-30 mm (Table 1). The media containing propyleneglycol/RPMI-1640 (5:95) was used as negative control for which no inhibitory effect could be observed.

DISCUSSION

As shown in Table 1, all the hexanic extracts obtained from *C. scapigera* inhibited the growth of the bacterial strains and yeast, including *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 10538), while the ethanolic extracts did not interfere in the microbial growth at 5000 µg/ml. The dichloromethanic extracts were weakly

active against *S. mutans* (strain 9.1). The screening conducted with compounds isolated from *C. scapigera* revealed that the antimicrobial activity was associated mainly with Gram-positive bacteria and yeasts (Table 2). Among the evaluated flavonoids, luteolin, which has a hydroxyl group at the 3' position, showed antimicrobial activity on *S. mutans*, *S. aureus* and *C. tropicalis* strains, whereas apigenin had very little effect. Crysoeriol, a 3'-methoxy-flavone, did not affect the growth of the evaluated microorganisms. Thus, a hydroxyl group at the 3' position can be responsible for the antimicrobial activity. This is in agreement with results presented by Yamamoto and Ogawa (19). Among flavonoids evaluated kaempferol was the most active, being active against great number of microorganisms at 500 µg/ml, while tiliroside, an kaempferol derivative with a acil-glicoside portion, presented activity mainly at 2500 µg/ml (Table 2).

Flavonoids are known to be synthesized by plants in response to microbial infection. Hence, it should not be surprising that they have been found to be effective antimicrobial substances against a wide array of microorganisms, when tested *in vitro*. Their activity is probably due to their ability to react with extracellular and soluble proteins and to complex with bacterial cell walls (4).

Table 2. Antimicrobial minimum inhibitory concentration activity of the isolated compounds from *Chresta scapigera*.

Microorganisms	MIC (µg/ml)							
	I	II	III	IV	V	VI	VII	VIII
Staphylococcus aureus (ATCC 6538)[a]	500	500	500	2500	100	2500	500	—
S. aureus (ATCC 25923)[a]	500	500	500	500	100	—	100	—
S. aureus penicilinase + (7+)[b]	500	—	—	2500	500	—	500	—
S. aureus penicilinase – (8-)[b]	2500	—	—	2500	—	—	—	—
Staphylococcus epidermidis (6ep)[b]	500	—	—	500	500	—	—	—
S. epidermidis (epiC)[b]	500	—	—	2500	500	—	—	—
Kocuria rhizophila (ATCC 9341)[a]	—	—	—	—	—	—	—	—
Enterococcus faecalis (ATCC 10541)[a]	2500	—	—	—	500	—	—	—
Streptococcus mutans (ATCC 25175)[a]	2500	—	—	2500	—	—	500	—
S. mutans (fab3)[b]	—	—	—	—	—	—	—	—
S. mutans (9.1)[b]	2500	—	—	2500	500	—	—	—
S. mutans (11.1)[b]	2500	—	—	2500	500	—	—	—
S. mutans (11.2)[b]	2500	—	—	2500	500	—	—	—
Streptococcus sobrinus (180,3)[b]	2500	—	—	2500	500	—	500	—
Escherichia coli (ATCC 10538)[a]	—	—	—	—	2500	—	2500	—
Pseudomonas aeruginosa (ATCC 27853)[a]	—	—	—	—	—	—	—	—
P. aeruginosa (290D)[b]	—	—	—	—	—	—	—	—
Candida albicans (cas)[b]	2500	—	—	2500	—	—	—	—
C. albicans (ATCC 1023)[a]	2500	—	—	2500	—	2500	—	—
Candida tropicalis (ct)[b]	2500	2500	2500	2500	—	2500	—	—

MIC: Minimum Inhibitory Concentration in µg/ml; —: without inhibitory activity; [a]: standard strain;[b]: field strain (oral cavity).

There are some reports showing that polar extracts inhibited the growth of both Gram-positive and Gram-negative bacteria (9,11). However in the present work, the ethanolic extracts of *C. scapigera* were inactive at 5000 µg/ml against all tested microorganisms. This can be explained by the chemical composition of the evaluated extracts, since a variation in concentrations of the active compounds present in these extracts can occur as well as antagonism effects. Despite this, the compounds (**IV**), (**V**), (**VI**) and (**VII**) were isolated of ethanolic extracts and were active against some of the tested microorganisms.

The obtained results for the hexanic extracts indicate the presence of antimicrobial constituents in these extracts. Bioactive compounds, such as (**I**), (**II**) and (**III**), were isolated from hexanic extract. Among the triterpenes, compound (**I**) was the most active on Gram-positive bacteria and yeast strains at concentrations (2500 and 500 mg/ml).

Our data showed that the response in terms of susceptibility to tested drugs varied among the strains. The differences in susceptibility may be explained by differences in cell wall composition and/or genetic content of plasmids that can be easily transferred among bacterial strains (9).

In conclusion, our results demonstrated that hexanic and dichloromethanic crude extracts and flavonoids and triterpenoids isolated from *C. scapigera* present antimicrobial activity. However, further studies about the safety and toxicity of the extracts and isolated compounds are needed, in order to evaluate possible clinical application in therapy of infectious diseases.

RESUMO

Avaliação da atividade antimicrobiana dos extratos brutos e dos constituintes de *Chresta scapigera*

Os extratos brutos e oito constituintes isolados de *Chresta scapigera* foram avaliados para as atividades antibacteriana e antifúngica, utilizando o método de difusão em ágar. Vinte cepas indicadoras, incluindo bactérias (Gram-positivas e Gram-negativas) e leveduras, foram utilizadas no bioensaio. Os melhores resultados foram obtidos para os extratos hexânicos,

enquanto os extratos etanólicos não inibiram o crescimento microbiano. Acetato de β-amirina, tilirosídeo e luteolina foram os mais eficazes dentre os constituintes avaliados.

Palavras-chave: *Chresta scapigera*, flavonóides, triterpenos, atividade antimicrobiana

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