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Antimicrobial activity evaluation of *Hortia oreadica Groppo,* Kallunki & Pirani essentials oils (leaves and flowers) and crude ethanol extract and fractions of leaves

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Research Article

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ABSTRACT

Hortia oreadica (Rutaceae) known as “para-tudo, quina, quina do campo” ("for- everything, quinine, quinine of the field") is used in traditional medicine locally to treat stomach pain and fevers. The aim of this study was evaluate the antimicrobial activity of essential oils (leaves and flowers) and the crude ethanol extract and fractions of leaves. The samples were collected in Pirenópolis, Goiás, Brazil. The crude ethanol extract was obtained by maceration of the leaves powdered. The essential oils were obtained by hydro distillation using a Clevenger type apparatus and analyzed by GC/MS. The antimicrobial activity in vitro was performed by broth micro dilution method. The crude ethanol extract showed good antimicrobial activity against *Candida krusei* (MIC=31.25 µg/ml) and *Candida tropicalis* (MIC=15.62 µg/ml) and moderate activity against *Staphylococcus epidermidis* ATCC 12229 (MIC=250 µg/ml). The hexane fraction had moderate activity against *S. epidermidis* ATCC 12229 (MIC=250 µg/ml), *Pseudomonas aeruginosa* SPM 1 (MIC=250 µg/ml) and *Salmonella spp* ATCC 194430 (MIC=250 µg/ml), the oils of leaves and flowers showed weak antimicrobial activity or were inactive (MIC>500 µg/ml). This work represents the first study of antimicrobial activity of essential oils of leaves and flowers of *H. oreadica*.

INTRODUCTION

Hortia oreadica Groppo, Kallunki & Pirani is a shrub with about 1 m tall [1], popularly known as “para-tudo", "quina", "quina do campo” and its bitter bark is traditionally used to treat stomach pain and fever, also as a substitute for quinine alkaloid extracted from Cinchona (Rubiaceae) [2]. The dictamnine isolated from this plant is a quinoline derivative which may be responsible for its quinine like antimalarial action [3-5].

Phytochemical studies of *Hortia oreadica* led to the identification from dichloromethane extract of taproots limonoids [3] and dihydrocinnamic acid derivatives [4]. Severino et al.[5] verified antimicrobial activity of the hexane extract of *H. oreadica* roots and the dictamnine alkaloid isolated of this extract against oral pathogens *Enterococcus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *S. mitis* (ATCC 49456), *S. mutans* (ATCC 25275), *S. sobrinus* (ATCC 33478), *S. sanguinis* (ATCC 10556), and *Lactobacillus casei* (ATCC 11578). Severino, Monteiro, Silva, Lucarini and Martins [6] verified antimicrobial activity of the dichloromethane extract of leaves of *H. oreadica* (MIC 31.25 µg /ml), indolequinazoline (15.62 µg /ml) and furoquinoline (31.25 µg /ml) alkaloids, and dihydrocinnamic acid derivatives (62.50 µg/ml) against *M. tuberculosis.*

This study aimed to evaluate the antimicrobial activity of essential oils from leaves and flowers and from crude ethanol extract and fractions of leaves against Gram-positive bacteria, Gram negative bacteria and fungi.

MATERIAL AND METHODS

Plant material

The leaves and flowers of *H. oeradica* was collected in Pirenópolis, Goiás (15° 48'15" S, 48° 52' 48" W, 1295 m) and received botanic identification by Dr. Heleno Dias Ferreira, of the Institute of Biological Sciences, Federal University of Goiás (UFG). A voucher specimen has been deposited at the Herbarium of Federal University of Goiás, Brazil, Conservation Unit PRPPG, under code number UFG-47798.

Preparation of the crude ethanol extract and fractions of the leaves

H. oreadica leaves were dried by forced air at 35°C and then powdered by knife mill TE-625 (Tecnal Ltda, Piracicaba, SP, Brazil). The powder was extracted with ethanol P.A. 1:3 (w/v) at room temperature three times and evaporated under reduce pressure, on a rotatory evaporator at 40°C to obtain the crude extract.

The crude ethanol extract was solubilized with MeOH: H_2O solution (7:3 v/v) and then extracted successively with hexane, ethyl acetate, and butanol; these fractions were also evaporated under reduced pressure and the aqueous fractions were lyophilized [7].

Essential oils

For the essential oils, healthy leaves and flowers were collected of 10 different individuals of *H. oreadica* in November. Fresh plant material was powdered separately and submitted to hydro distillation in a Clevenger-type apparatus for two hours. At the end of each distillation the oils were collected, dried with anhydrous Na_2SO_4, measured, and transferred to glass flasks and kept at a temperature of -18°C.

Antimicrobial activity

To evaluate the antimicrobial activity of essential oils from the leaves and flowers and from crude ethanol extract and fractions of leaves was used the method micro dilution test in broth as recommended by the Clinical and Laboratory Standards Institute [8,9]. Experiments were carried out in duplicate.

Microorganisms used in the assays were standard strains from the American Type Culture Collection (ATCC) and clinical isolates provided by the Bacteriology and Mycology Laboratory of the Tropical Pathology and Public Health Institute of Federal University of Goiás (UFG). The following strains were used: Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *S. epidermidis* ATCC 12229, *Micrococcus luteus* ATCC 9341, *Micrococcus roseus* ATCC 1740, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633; Gram-negative bacteria: *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* (clinical isolated) HMA; FTA 502, *Serratia marcescens* ATCC 14756, *Pseudomonas aeruginosa* (clinical isolated) SPM 1, *P. aeruginosa* ATCC 90227, *Salmonella spp* ATCC 194430, *Salmonella enterica subsp. enterica serovar Typhi* ATCC 10749, *Klebisiella pneumoniae* ATCC 700603; fungi: *Candida krusei* (ATCC 34135), *Candida tropicalis* (ATCC 28707).

The bacteria were incubated in Casoy broth at 35 ºC for 24 h, transferred to an inclined Casoy agar, and incubated at 35 ºC for an additional 24 h to reactivate the cultures. Fungi were cultivated in Sabourad dextrose agar plates and incubated at room temperature for 24 h (*Candida spp*.)

The culture medium used in the antifungal activity test was RPMI 1640 and the medium for the antibacterial activity test was Müller Hinton broth (MH). Samples (crude ethanol extract and fractions) were solubilized in 10% dimethyl sulfoxide (DMSO) and essential oils in 10% DMSO and 0.02% Tween 80® and then diluted in MH broth (antibacterial activity) to obtain a concentration of 2000 µg/ml or RPMI (antifungal activity) to obtain the concentration of 1000 µg/ml.

For antibacterial tests were used sterile microplates with 96-well U bottom. 100 µL of MH were added to all wells from columns 2 to 12 and 200 µL of each sample were added to wells A through G in column 1. A multichannel pipette was used to obtain serial dilutions up to column 11, where the final 100 µL were discarded, for concentrations ranging from 2000 µg/ml to 1.95 µg/ml. 200 µL of MH broth containing 10% DMSO and 0.02% Tween 80® were added to line H as a negative control, and pure MH broth was added to column 12 to control for microbial growth. For the positive control for Gram-positive and Gram-negative bacteria, serial dilutions were made from the following concentrations: vancomycin (32 µg/ml) and gentamicin (128 µg/ml).

Bacterial inoculants were prepared in sterile saline solution (NaCl 0.85%) to obtain a turbidity equivalent to half of the McFarland 1.0 scale (79.4 to 83.2% transmittance, measured with a spectrophotometer at 625 nm). These inoculants were diluted 1:10 in saline to obtain a cell concentration of 107 CFU/ml. Five µL of the inoculants were placed in the wells, for a final concentration of 5 × 105 CFU/ml. No microorganisms were added to wells in line G, which were used as controls of the sterility of the medium and the samples tested. After bacterial inoculation, microplates were sealed and incubated at 35°C ± 2°C for 18 to 24 h. After the incubation period, 20 µL of 0.5% triphenyl tetrazolium chloride (TTC) were added to all wells, and the microplates

were reincubated for approximately half an hour. The appearance of a reddish color was considered proof of bacterial growth.

For the antifungal assays, we added 100 µL of RPMI to all microplate wells in columns 2 to 12 and 200 µL of each sample to wells A through G in column 1. Using a multichannel pipette, serial dilutions were performed up to column 11, where 100 µL were discarded. Fungal inoculants were prepared in a sterile saline solution (NaCl 0.85%) to obtain a turbidity equivalent to half of the McFarland 1.0 scale (83.2 to 85% transmittance, measured with a spectrophotometer at 530 nm). Two dilutions (first 1:50, then 1:20) were prepared in RPMI to obtain cellular concentrations between 1 and 5 × 105 CFU/ml. The final dilution of the inoculants in the wells ranged from 0.50 to 0.25 × 103 CFU/ml. 100 µL of each inoculate were added to each well to obtain sample concentrations between 1000 µg/ml and 0.97 µg/ml. No microorganisms were added to wells in line G, since they were used as controls of the sterility of the medium and of the samples tested. After inoculation, microplates were covered and incubated at room temperature for 48 h. The occurrence of fungal growth was checked visually. For the positive control serial dilutions were made from 500 µg/ml of itraconazole (Sigma).

The MIC was defined as the lowest concentration of the sample (in µg/ml) fully capable of inhibiting bacterial or fungal growth. The classification proposed by Holetz et al. [10] was used to interpret the results of the tests of antimicrobial activity. Using this classification, MIC values below 100 µg/ml indicate good inhibitory activity; values between 100 and 500 µg/ml, moderate inhibitory activity; values between 500 and 1000 µg/ml, weak inhibitory activity; and values above 1000 µg/ml characterize an inactive substance.

RESULTS

Essential oils

The yields of essential oil were 0.09% for the flowers and 0.50% for the leaves.

Antimicrobial activity

The crude ethanol extract of *H. oreadica* leaves had good antifungal activity against *C. krusei* and *C. tropicalis* and moderate antibacterial activity against *S. epidermidis* ATCC 12229 and weak activity against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *M. luteus* ATCC 9341, *M. roseus* ATCC 1740, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *P. aeruginosa* SPM 1, *P. aeruginosa* ATCC 90227, *Salmonella spp* ATCC 194430, *S. enterica subsp. enterica serovar Typhi* ATCC 10749 and *S. enterica*. The hexane fraction had moderate activity against *S. epidermides* ATCC 12229, *P. aeruginosa* SPM 1 and *Salmonella spp* ATCC 194430 and weak activity against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *M. luteus* ATCC 9341, *M. roseus* ATCC 1740, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 90227. The ethyl acetate fraction had weak activity against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 12229, *M. luteus* ATCC 9341, *M. roseus* ATCC 1740, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *P. aeruginosa* SPM 1, *P. aeruginosa* ATCC 90227, *Salmonella spp* ATCC 194430, *S. enterica subsp. enterica serovar Typhi* ATCC 10749 (**Table 1**).

Table 1. Antimicrobial activity of the essential oil from the leaves and flowers and crude ethanol extract and hexane, ethyl acetate, butanol and aqueous fractions of leaves of *H. oreadica*.

Bacteria	Minimum inhibitory concentration (µg/ml)							Vancomycin	Gentamicin	Itraconazole
	Essentials oils		Crude ethanol extract	Fractions						
	Leaves	Flowers		Hexane	Ethyl acetate	Butanol	Aqueous			
Gram-positive										
S. aureus ATCC 25923	500	2000	500	500	1000	2000	2000	1	-	-
S. aureus ATCC 29213	>2000	>2000	2000	2000	2000	>2000	1000	-	-	-
S. epidermidis ATCC 12228	2000	2000	500	500	1000	2000	2000	-	-	-
S. epidermidis ATCC 12229	500	1000	250	250	1000	2000	1000	1	-	-
M. luteus ATCC 9341	2000	2000	1000	500	500	2000	1000	0,25	-	-
M. roseus ATCC 1740	2000	>2000	1000	500	500	2000	1000	0,5	-	-
B. cereus ATCC 14579	1000	>2000	1000	500	1000	2000	1000	2	-	-
B. subtilis ATCC 6633	2000	>2000	1000	500	500	2000	1000	-	-	-
Gram-negative										
E. coli ATCC 8739	2000	>2000	2000	2000	2000	2000	2000	-	8	-

E. aerogenes ATCC 13048	2000	>2000	2000	2000	2000	2000	2000	-	0,125	-
E. cloacae (isolado clínico) HMA; FTA 502	2000	>2000	2000	2000	2000	2000	2000	-	4	-
S. marcenscens ATCC 14756	2000	>2000	2000	2000	2000	2000	2000	-	-	-
P. aeruginosa (isolado clínico) SPM 1	1000	2000	500	250	1000	2000	1000	-	4	-
P. aeruginosa ATCC 90227	1000	2000	1000	1000	1000	2000	1000	-	-	-
Salmonella spp ATCC 194430	1000	2000	500	250	1000	2000	1000	-	2	-
S. enterica subsp. enterica serovar Typhi ATCC 10749	500	1000	500	500	1000	2000	1000	-	-	-
K. pneumoniae ATCC 700603	1000	2000	2000	2000	2000	2000	2000	-	-	-
Fungi										
C. krusei ATCC 34135	>1000	>1000	31,25	1000	>1000	1000	1000	-	-	0,48
C. tropicalis ATCC 28707	>1000	>1000	15,62	1000	>1000	1000	1000	-	-	31,25

- Not tested

The aqueous fraction had weak activity against *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12229, *M. luteus* ATCC 9341, *M. roseus* ATCC 1740, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *P. aeruginosa* SPM 1*, P. aeruginosa* ATCC 90227, *Salmonella spp* ATCC 194430, *S. enterica subsp. enterica serovar Typhi* ATCC 10749. The butanol fraction had no antimicrobial activity (**Table 1**).

The essential oil of the leaves had weak activity against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12229, *B. cereus* ATCC 14579, *P. aeruginosa* SPM 1, *P. aeruginosa* ATCC 90227, *Salmonella spp* ATCC 194430, *S. enterica subsp. E. typhi* ATCC 10749 and *K. pneumoniae* ATCC 700603. The essential oil of flowers had weak activity against *S. epidermidis* ATCC 12229 and *Salmonella enterica subsp. enterica serovar Typhi* ATCC 10749 (**Table 1**).

DISCUSSION

The crude ethanol extract of *H. oreadica* had good antifungal activity against *C. krusei* and *C. tropicalis* and moderate antibacterial activity against *S. epidermidis* ATCC 12229. The hexane fraction had better antibacterial activity than the crude ethanol extract for *M. luteus* ATCC 9341, *M. roseus* ATCC1740. *B cereus* ATCC 14579 e *B. subtilis* ATCC 6633, *P. aeruginosa* (isolado clinico) SPM 1 e *Salmonella sp* ATCC 194430. The antifungal activity of ethanol extract is probably due to the synergistic action between the constituents, in view of the absence of such activity in the fractions. The essential oils from leaves and flowers and the ethyl acetate, butanol and aqueous fractions showed weak antimicrobial activity or were inactive (MIC>500 µg/ml). In other research, Severino et al. [5] detected antimicrobial activity of the hexane extract of *H. oreadica* roots against oral pathogens. Other species from Rutaceae family also showed antimicrobial activity: *Hortia brasiliana* (against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (MIC of 256 µg/ml) [11]; *Citrus medica Linn*. (against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*) [12] and *C. limon* against almost half of Candida strains analyzed, including *C. albicans* and *C. parapsilosis* [13]. Haddouchi et al. [14] found antifungal activity of essential oils of *Ruta angustifolia* and *Ruta graveolens* against *C. albicans*, *Fusarium oxysporum*, *Alternaria alternaria*, *Aspergillus flavus* and of *Ruta. chalepensis var. bracteosa* and *Ruta tuberculata* against *Cladosporium herbarum*, *F. oxysporum*, *A. flavus*.

CONCLUSIONS

In conclusion, it was verified good antifungal activity of ethanol extract and moderate antibacterial activity of ethanol extract and hexane fraction of the leaves of *H. oeradica* against bacteria implicated in skin and diarrheal infections. These results support the claims of the traditional therapies using *H. oeradica* leaves to treat several diseases. This work represents the first study of the antimicrobial activity of the essential oils from leaves and flowers of *H. oreadica* collected in Pirenópolis, Goiás. The knowledge gained from this study should be useful for further exploitation and application of the resource.

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