Brazilian Journal of Microbiology (2009) 40: 535-540
ISSN 1517-8382

DETERMINATION OF THE ANTIBACTERIAL ACTIVITY OF CRUDE EXTRACTS AND COMPOUNDS ISOLATED FROM *HORTIA OREADICA* (RUTACEAE) AGAINST ORAL PATHOGENS

Vanessa Gisele Pasqualotto Severino[1]; Maria Fátima das Graças Fernandes da Silva[1]; Rodrigo Lucarini[2]; Lilian Bueno Montanari[2]; Wilson Roberto Cunha[2]; Adriana Helena Chicharo Vinholis[2]; Carlos Henrique Gomes Martins[2*]

[1]Centro de Ciências Exatas e de Tecnologia, Departamento de Química, Universidade Federal de São Carlos, São Carlos, SP, Brasil; [2]Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, Franca, SP, Brasil

Submitted: July 28, 2008; Returned to authors for corrections: October 27, 2008; Approved: May 03, 2009.

ABSTRACT

Extracts from *Hortia oreadica* afforded four dihydrocinnamic acid derivatives, isolated from the *n*-hexane extract, as well as limonoid guyanin and the furoquinoline alkaloid dictamnine, both isolated from the dichloromethane extract. The extracts and the isolated compounds were tested against some oral pathogens, so as to investigate their antibacterial activity. The results showed that the *n*-hexane extract and the compound dictamnine are the most active against the selected microorganisms

Key words: *Hortia oreadica*; Rutaceae; *a*ntibacterial activity; MIC; oral pathogens

The environmental characteristics of the oral cavity, such as high humidity, relatively constant temperature (34 to 36°C), pH close to neutral, and nutrient availability, allows the establishment of a highly complex microbiota composed of around 500 groups that inhabit the several areas of the mouth and cause periodontal diseases and caries.

Because pathogenic microorganisms can develop resistance against antibiotics, attention has been paid to extracts and biologically active compounds isolated from plant species (5). Antimicrobials of plant origin are efficient in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (7).

The genus *Hortia* belongs to the Rutaceae family and includes 12 species distributed throughout Brazil. The Rutaceae family comprises many species that present biological properties, being considered a large source of alkaloids, coumarins, flavonoids, and limonoids (19). Recent biological studies on *Hortia* species have demonstrated biological activities against the enzymes Adenine Phosphoribosyltransferase, from *Leishmania tarentolae*, and Glyceraldehyde-3-Phosphate Dehydrogenase, from *Trypanosoma* cruzi, *Plasmodium falciparum*, *Trypanosoma brucei rhodisiense*, and cancer cell lines COR-L23, C-32, and

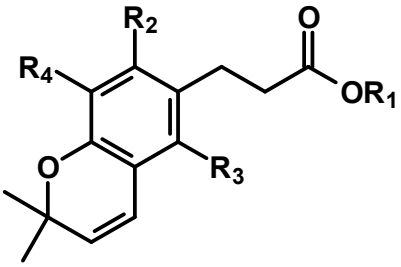
***Corresponding Author. Mailing address:** Universidade de Franca, Núcleo de Pesquisas em Ciências Exatas e Tecnológicas, SP, Franca, Brasil.; Tel: (+5516) 3711-8756 Fax: (+5516) 3711-8874.; E-mail: martinsc@unifran.br

MCF7 (2). However, little is known about the potential of secondary metabolites from *Hortia* against oral pathogens. The aim of the current study was twofold: to determine the *in* vitro antibacterial activity of crude extracts and compounds isolated from *Hortia oreadica* against oral pathogens, as well as discuss some aspects related with structure-activity.

Roots of *H. oreadica* were collected from the Forest Reserve Adolpho Ducke, Itacoatiara, Amazonas state, Brazil. Identification was carried out by Prof. Dr. José Rubens Pirani from the Botany Department of the São Paulo University, and vouchers were deposited in the Herbarium of the same department. The roots were dried in an oven (Fanem model 002CB) at 40º C and pulverized in a mill (Tecnal®, model TE 650), for preparation of a fine powder. The powdered roots (2.5 kg) were extracted with *n*-hexane and dichloromethane, respectively, at room temperature. After filtration, each extract was evaporated in a rotary evaporator (Büchi model R-114), affording the *n*-hexane extract - HE (0.739 kg) and the dichloromethane extract - DE (0.961 kg), which were stored at 4°C until use. The solvents were purchased from Merck (KgaA, Darmstadt, Germany).

Afterwards, part of the *n*-hexane extract (0.130 kg) was chromatographed on a silica gel 230-400 Mesh ASTM column with *n*-hexane/ethyl acetate (9/1v/v) as the mobile phase, affording dihydrocinnamic acid derivatives (compounds **I-IV**, Figure 1), which were identified by comparison with literature data (3, 15, 18).

Part of the dichloromethane extract (0.20 kg) was chromatographed on a silica gel 230-400 Mesh ASTM column with the mobile phases *n*-hexane/ethyl acetate and methanol. Compounds were separated and purified by Recycling High-Performance Liquid Chromatography (Shimadzu, model SCL-10A); the column was a Shodex Asahipak - model GS-310 2G (45.0 x 2.5 cm, 5 µm particle size) with elution in the isocratic mode: methanol/ dichloromethane (50:50 v/v), flow rate = 3.0 mL.min^{-1}. Detection (Shimadzu SCL-10A) was monitored at λ = 217 and 254 nm, and compounds **V** and **VI** (Figure 1) were identified by comparison with literature data (2, 8).



dihydrocinnamic acid derivatives

Substitutions on R positions were as follows:

I	$R_1 = H$	$R_2 = OCH_3$	$R_3 = OCH_3$	$R_4 = H$
II	$R_1 = CH_3$	$R_2 = H$	$R_3 = OCH_3$	$R_4 = H$
III	$R_1 = CH_3$	$R_2 = OCH_3$	$R_3 = OCH_3$	$R_4 = H$
IV	$R_1 = CH_3$	$R_2 = H$	$R_3 = OCH_3$	$R_4 = OCH_3$

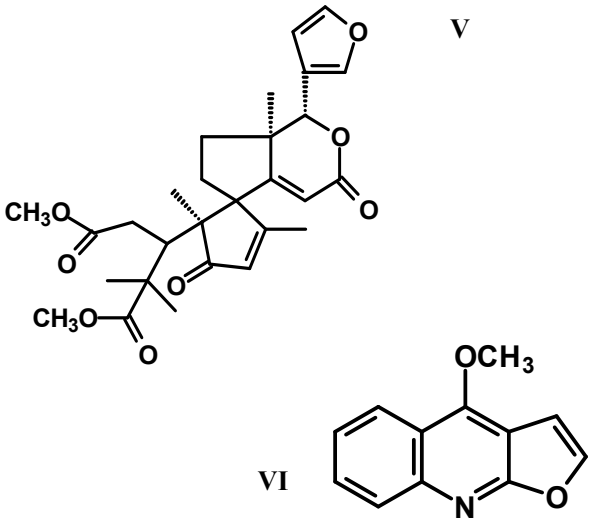


Figure 1. The chemical structures of the compounds isolated from *H. oreadica* evaluated for antibacterial activity against oral pathogens.

The following microorganisms were used in the evaluation of the antibacterial activity of the extracts and isolated compounds: *Enterococcus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *Streptococcus mitis* (ATCC 49456), *Streptococcus mutans* (ATCC 25275), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus sanguinis* (ATCC 10556), and *Lactobacillus casei* (ATCC 11578). All the strains were acquired from the American Type Culture Collection.

The Minimum Inhibitory Concentration (MIC) values of the compounds and crude extracts were determined in triplicate by using the broth microdilution method (9) in 96-well microplates (TPP®, EUA). The samples were dissolved in DMSO (1 $mg.mL^{-1}$), and then diluted in tryptic soy broth, to achieve concentrations ranging from 0.4 to 0.02 $mg.mL^{-1}$. The final DMSO concentration was 5 % (v/v), and this solution was used as negative control. The inoculum was adjusted to each organism, to yield a cell concentration of 10^8 Colony Forming Units ($CFU.mL^{-1}$). One well was used as control for microorganism growth in the medium, and one uninoculated well, free of antimicrobial agent, was used to ensure sterility of the medium. Chlorhexidine dihydrochloride (Sigma, Poole, Dorset, UK) was used as positive control. Two-fold serial dilutions were made in tryptic soy broth, so that concentrations ranging from 0.0059 to 0.00001 $mg.mL^{-1}$ would be achieved. The microplates were incubated at 37°C for 24 hours, and 30 μL resazurin (Sigma) in aqueous solution (0.01 %) was added, to indicate microorganism viability (10). The MIC values were determined as the lowest concentration of the compounds and extracts capable of inhibiting microorganism growth.

Results concerning the antibacterial activity of the extracts and isolated compounds are shown in Table 1. The chemical structures of the evaluated compounds are displayed in Figure 1. The similar MIC values for compounds **I-IV** are related to their chemical structures, which show differences only with respect to the positions of a methoxyl group in the aromatic ring or in the acid or ester functions in the side chain. The *n*-hexane extract displayed activity at lower concentrations than the corresponding isolated compounds, which may be related to the presence of lipophilic compounds, mainly long chain fatty acids, which are the major constituents of this extract. Lipophilicity is known to be closely related to permeation through a lipidic coating of bacteria (16). These compounds are often identified in natural apolar extracts that exhibit antimicrobial properties (6, 11). However, it is likely that the effects of these compounds on oral pathogens are potentialized by synergistic/additive effects of other minor chemical constituents present in the *n*-hexane extract. Compounds **V** and **VI** isolated from the dichloromethane extract displayed activity against the oral pathogens at lower concentrations than the corresponding dichloromethane extract. The results reveal that limonoid guyanin (**V**) exhibits some level of inhibition against the microorganisms *S. sobrinus, S. mutans, S. mitis,* and *L. casei.* This class of substances is well known for presenting many biological roles such as insecticide, citotoxic, anti-inflammatory, and antimalarial activities (17). In addition, the presence of two ester groups in the structure, as well as the furan ring, may contribute to the inhibitory activity of this limonoid. The furoquinoline alkaloid dictamnine (**VI**), a very common compound in the family Rutaceae, showed interesting MIC values against the microorganisms *S. sanguinis*, *S. mutans,* and *L. casei.* Dictamnine has been reported to be a phototoxic and photomutagenic compound (12, 14). It participates in the severe skin phototoxicity of the plant (13), and this photobiological activity has been shown to be connected with the reactive furan double bond (12).

Therefore, the result obtained in our assays suggests that the presence of the furan ring may also contribute to the observed inhibitory activity against the microorganisms.

It is important to emphasize that the reports displaying the antimicrobial activity of natural products against oral pathogens are scarce (4), and a consensus on the acceptable level of inhibition for natural products compared with standard antibiotics has not been reached. Thus, Aligianis *et al*. (1) proposed a classification for plant material based on the MIC results, considering MIC values up to 0.5 $mg.mL^{-1}$as strong inhibition , MIC values between 0.6 and 1.5 $mg.ml^{-1}$ as moderate inhibition, and MIC values above 1.6 $mg.mL^{-1}$ as weak inhibition .

In summary, the present study has shown the growth inhibitory activity of extracts and compounds isolated from *H. oreadica* against oral pathogens. These compounds could be useful for the further development of new agents that could be used to reduce both dental caries and plaque formation.

Table 1. Minimum Inhibitory Concentration (MIC) values ($mg.mL^{-1}$) of extracts and isolated compounds from *H.oreadica* roots against oral pathogens.

Microorganisms	Compounds and Extracts								
	I	**II**	**III**	**IV**	**V**	**VI**	**DE**	**HE**	**Chlorhexidine**
S. salivarius	0.3	0.3	0.3	0.3	*	*	0.3	0.2	0.0001
S. sanguinis	*	*	*	*	*	0.4	*	0.2	0.0004
S. sobrinus	*	*	*	*	0.3	*	*	0.3	0.0001
S. mutans	*	*	*	*	0.4	0.4	*	0.3	0.0001
S. mitis	*	*	*	*	0.3	*	*	0.3	0.0001
E. faecalis	*	*	*	*	*	*	*	*	0.0004
L. casei	*	*	*	*	0.3	0.1	*	0.4	0.0001

Compounds I to IV refer to the four dihydrocinnamic acid derivatives isolated from the crude *n*-hexane extract of *Hortia oreadica*; compounds V and VI refer to the guyanin and dictamnine isolated from the crude dichloromethane extract of *H. oreadica*; DE: dichloromethane extract of *H. oreadica*; HE: *n*-hexane extract of *H. oreadica*; Chlorhexidine: control positive. *Inactive in the evaluated concentrations

ACKNOWLEDGEMENTS

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) for financial support.

RESUMO

Determinação da Atividade Antibacteriana de Extratos Brutos e Substâncias Isoladas de *Hortia oreadica* (Rutaceae) frente à Patógenos Bucais

Extratos brutos de *Hortia oreadica*, forneceram quatro derivados do ácido diidrocinâmico, que foram isolados do extrato *n*-hexânico, bem como as substâncias guianina e dictamina, isoladas do extrato em diclorometano. Os extratos brutos e as substâncias isoladas foram avaliados frente a alguns patógenos bucais com o objetivo de investigar a atividade antibacteriana. Os resultados demonstraram que o extrato bruto n-hexânico e a substância dictamina foram os mais ativos frente ao conjunto de microrganismos avaliados.

Palavras-chave: *Hortia oreadica*; Rutaceae; atividade antibacteriana; CIM; patógenos bucais

REFERENCES

1. Aligianis, N.; Kalpoutzakis, E.; Mitaku, S.; Chinou, I.B. (2001). Composition and antimicrobial activity of the essential oil of two *Origanum* species. *J. Agric. Food Chem*., 49: 4168-4170.
2. Braga, P.A.C. (2005). *Estudo fitoquímico de espécies de Hortia (Rutaceae), importância quimiossitemática e atividades biológicas dos constituintes isolados*. São Carlos, Brasil, 269p. (D.Sc. Thesis. Departamento de Química. UFSCar).
3. Correa, D.B.; Gottlieb, O.R.; Padua, A.P. (1975). Chemistry of brazilian Rutaceae. I. dihydrocinnamic acids from *Hortia badinii*. *Phytochemistry*, 14: 2059-2060.
4. Cunha, L.C.S.; Silva, M.L.A.; Niege, A.J.; Cardoso Furtado, N.A.J.C.; Vinhólis, A.H.C.; Martins, C.H.G.; da Silva Filho, A.A.; Cunha, W.R. (2007). Antibacterial activity of triterpene acids and semi-synthetic derivatives against oral phatogens. *Z. Naturforsch. C.,* 62: 668-672.
5. Essawi, T.; Srour, M. (2000). Screening of some palestinian medicinal plants for antibacterial activity. *J. Ethnopharmacol.*, 70: 343-349.
6. Gyles, R.K.; Marquis, R.E.; Phan, T.N. (2002). Antimicrobial actions of glycerol fatty-acid esters for oral streptococci. In: IADR/AADR/CADR 80th General Session, San Diego, CA, p.1486.
7. Iwu, M.W.; Duncan, A.R.; Okunji, C.O. (1999). New antimicrobials of plant origin. *In*: Janick, J. (ed.) *Perspectives on new crops and new uses*. ASHS Press Alexandria, VA, USA, p. 457-462.
8. Jacobs, H.; Ramdayal, F. (1986). Guyanin, a novel tetranortriterpenoid. Structure elucidation by 2-D NMR spectroscopy. *Tetrahedron Lett.*, 27: 1453-1456.
9. NCCLS. (2003). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Sixth Edition. NCCLS document M7-A6. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania, USA.
10. Palomino, J.C.; Martin, A.; Camacho, M.; Guerra, H.; Swings, J.; Portaels, S. (2002). Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chem*., 46: 2720-2722.
11. Petschow, B.W.; Batema, R.P.; Ford, L.L. (1996). Susceptibility of *Helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free-fatty acids. *Antimicrob. Agents Chemother*., 40: 302-306.
12. Pfyffer, G.E.; Panfil, I.; Towers, G.H.N. (1982). Monofunctional covalent photobinding of dictamnine, a furoquinoline alkaloid, to DNA as a target *in vitro*. *Photochem. Photobiol.*, 35: 63–68.
13. Schempp, C.M.; Sonntag, M.; Scho¨pf, E.; Simon, J.C. (1996). Dermatitis bullosa striata pratensis durch *Dictamnus albus* L. (Brennender Busch). *Hautarzt*, 47: 708–710.
14. Schimmer, O.; Kühne, I. (1991). Furoquinoline alkaloids as photosensitizers in *Chlamydomonas reinhardtii*. *Mutat. Res*., 249: 105–110.
15. Suarez, L.E.C.; Menichini, F.; Monache, F.D. (2002). Tetranortriterpenoids and dihydrocinnamic acid derivatives from *Hortia colombiana*. *J. Braz. Chem. Soc*., 13: 339-344.
16. Tokuyama, R.; Takahashi, Y.; Tomita, Y.; Tsobouchi, M.; Yoshida, T.; Iwasaki, N.; Kado, N.; Okezaki, E.; Nagata, O. (2001). Structure-activity relationship (SAR) studies on oxazolidinone antibacterial agents. 2. Relationship between lipophilicity and antibacterial activity in 5-thiocarbonyl oxazolidinones. *Chem. Pharm. Bull*., 49: 353-360.
17. Vasanth, S.; Kundu, A.B. (1991). Biological activities of limonoids. *J. Sci. Ind. Res.*, 50: 884-896.
18. Vieira, P.C.; Alvarenga, M.A.; Gottlieb, O.R.; McDougall, M.N.V.; Reis, F.A.M. (1980). Estructural confirmation of dihydrocinnamic acids

from *Adiscanthus fusciflorus* by ^{13}C NMR. *Phytochemistry*, 19: 472-473.

19. Waterman, P.G.; Grundon, M.F. (1983). *In Chemistry and Chemical Taxonomy of Rutales*. Academic Press: New York.