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Artigo

CHEMICAL STUDY OF *Hortia superba* (Rutaceae) AND INVESTIGATION OF THE ANTIMYCOBACTERIAL ACTIVITY OF CRUDE EXTRACTS AND CONSTITUENTS ISOLATED FROM *Hortia* SPECIES

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In this paper, the chemical study of *Hortia superba* and antimycobacterial potential of *Hortia* species were investigated. Crude extracts and limonoids, alkaloids, dihydrocinnamic acid derivatives and coumarins isolated from *Hortia superba*, *Hortia oreadica* and *Hortia brasiliana* were evaluated against *Mycobacterium tuberculosis* H37Rv, *Mycobacterium kansasii* and *Mycobacterium avium*. The results obtained demonstrated an inhibitory effect of the dichloromethane extract of leaves of *H. oreadica* (MIC 31.25 μg mL^{-1}), indolequinazoline (15.62 μg mL^{-1}) and furoquinoline (31.25 μg mL^{-1}) alkaloids, and dihydrocinnamic acid derivatives (62.50 μg mL^{-1}), on the growth of *M. tuberculosis*. These results are promising in relation to the search for biologically active natural products and could be useful in the development of effective new drugs against mycobacteria.

Keywords: *Hortia* species; alkaloids; dihydrocinnamic acid derivatives; antimycobacterial potential.

INTRODUCTION

Natural products, or their direct derivatives, play a crucial role in the modern day chemotherapy of mycobacterial infections. There is currently a re-emerging interest in natural products able to provide novel structures for drug discovery, particularly those which are effective as antibacterial leads.[1]

The Rutaceae family is comprised of around 150 genera with more than 1500 terrestrial species, which are considered to be sources of alkaloids, coumarins, flavonoids and limonoids.[2] *Hortia* is a neotropical genus of Rutaceae that comprises 10 species,[3] distributed in South America from Panama to the state of São Paulo, Brazil, most of them occurring in the Amazonian region.[3] Previous studies have established a relationship between the compounds isolated from this genus and biological properties, including inhibition of the enzymes -glucosidase, α-amylase and lipase,[4] against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*[5] and antibacterial action against oral pathogens[6] and *Xylella fastidiosa*.[7] However, to date, no information on the antimycobacterial activity of this genus has been reported. The development of antimycobacterial agents from plant compounds attracts interest based on the premise that natural products may be less toxic than synthetic antimycobacterial agents. In this context, this paper describes preliminary investigations on three *Hortia* species employing crude extracts and ten isolated compounds of the classes of terpenoids (limonoids), alkaloids, dihydrocinnamic acid derivatives and coumarins. Their antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (ATCC 27294)*, Mycobacterium kansasii* (ATCC 12478) and *Mycobacterium avium* (ATCC 15769) was evaluated. A chemical study on the methanolic extract obtained from *Hortia superba* stems is also reported.

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EXPERIMENTAL

General experimental procedures

All organic solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were recorded in deuterated chloroform, with tetramethylsilane as the internal standard, at ambient temperature on a Bruker DRX 400 instrument operating at 400 and 100 MHz, for ^{1}H and ^{13}C respectively. A Shimadzu HPLC, model LC-6AD, equipped with a Shimadzu SPD-6AV UV detector (detection UV λ 217 and 254) and a Shodex Asahipak GS-310 2Ga column (460 x 25 mm, 10 μm particle size) was used for the analysis. For the column chromatography, Silica gel 60 (Acros Organics) and Sephadex LH 20 (Amersham Pharmacia Biotech AB) were used.

Plant material

Hortia brasiliana Vand. ex DC. was collected (May 2000) in Linhares, Espírito Santo state, Brazil. *Hortia oreadica* Groppo, Kallunki & Pirani was collected (Sep 2005) in the Adolpho Ducke Forest Reserve, Itacoatiara, Amazonas state, Brazil. *Hortia superba* Ducke was collected (Dec. 2001) along the road running from Manaus to Itacoatiara, at km 31, Amazonas State, Brazil. All plants were identified by Prof. Dr. José Rubens Pirani of the Department of Botany, University of São Paulo, and vouchers are deposited in the herbarium of the same department (Pirani 4672, Groppo Jr. 458 and Groppo Jr. 950, respectively).

Preparation of the crude extracts

The dried and powdered stem bark and stems of *H. superba*, leaves and stems of *H. brasiliana* and ground taproots, stems and leaves of *H. oreadica* were successively extracted using hexane (hex) (10 L), dichloromethane (DCM) (10 L) and methanol (MeOH) (10 L), at room temperature (3 times, at 3-day intervals, totaling 9 days). Evaporation of the solvents under reduced pressure produced

crude extracts (see Table 1), which were submitted to biological assays against *Mycobacterium tuberculosis*, *M. avium* and *M. kansasii*.

Table 1. Data on the crude extracts obtained from *Hortia* species

Plant species	Plant part extracted (mass in g)	Solvent	Mass of crude extract (in g)
H. oreadica	ground taproots (3330)	hexane	85.9
		dichloromethane	67.6
		methanol	244.0
	stems (2480)	hexane	8.2
		dichloromethane	8.4
		methanol	50.6
	leaves (3180)	hexane	93.0
		dichloromethane	161.0
		methanol	42.0
H. superba	stem bark (800)	methanol	0.5
	stems (2280)		18.1
H. brasiliana	leaves (655)	hexane	16.0
		dichloromethane	21.7
		methanol	25.9
	stems (938)	methanol	26.2

The crude extracts that provided a minimum inhibitory concentration (MIC) value of ≤ 250 μg mL^{-1} against at least one of the tested mycobacterium were selected for fractionation (see Table 2).

The isolation of compounds **1**, **2**, **3**, **4**, **6, 7** and **10** from *H. oreadica* and *H. brasiliana* have been described previously.[5,8]

Isolation of compounds from *H. superba*

The concentrated methanol extract (5.0 g) obtained from stems of *H. superba* was partitioned into hex (1.5 L) (concentrated 1.4 g), DCM (1.5 L) (concentrated 2.3 g) and hydroalcoholic (4.0 L) (concentrated 1.3 g) soluble fractions. The hexane fraction was subjected to column chromatography (CC) over silica gel (230-400 mesh, Φ x h= 6.5 x 63.0 cm) and elution with a gradient of hex-ethyl acetate (EtOAc)-MeOH afforded 9 fractions (9-1 to 9-9). Fraction 9-2 yielded compound **6** (17.8 mg). Fraction 9-3 (48.5 mg) was subjected to recycling high performance liquid chromatography (R-HPLC) (MeOH-DCM 8:2, flow rate 5.0 mL min^{-1}) to afford compounds **3** (6.4 mg) and **8** (7.5 mg) after recycling once. Fraction 9-4 yielded compound **3** (127.9 mg). Fraction 9-9 (31.9 mg) was rechromatographed on Sephadex LH 20 (Φ x h= 2.0 x 30.0 cm) using DCM-MeOH (1:9, 1.5 L) to give compounds **1** (1.9 mg) and **9** (1.5 mg). The dichloromethane fraction was subjected to CC over silica gel (230-400 mesh, Φ x h= 6.5 x 72.0 cm). Elution with a gradient of hex-acetone-MeOH yielded 25 new fractions. Based on the thin-layer chromatography (TLC) plate monitoring, all fractions were combined into 8 analytically distinct fractions (8-1 to 8-8). Fraction 8-3 (89.5 mg) was chromatographed on silica gel (Φ x h= 4.0 x 65.0 cm) and eluted with hex-EtOAc (6:4) to give compound **7** (4.1 mg). Fraction 8-4 (116.4 mg) was subjected to R-HPLC (MeOH, flow rate 5.0 mL min^{-1}) to afford compound **5** (7.4 mg) after recycling once. Fraction 8-7 (373.6 mg) was rechromatographed on silica gel (230-400 mesh, Φ x h= 1.5 x 23.0 cm) eluting with hex-EtOAc (6:4) affording 4 new fractions (8-7-1 to 8-7-4). Fraction 8-7-1 was purified by CC (Φ x h= 2.0 x 25.0 cm) on Sephadex LH 20 using MeOH (1 L) to afford compound **9** (7.1 mg).

Biological activity

The antimycobacterial activity of the crude extracts and compounds was assayed *in vitro.* The MIC values were determined in triplicate using the microdilution technique on a REMA, adapted from a procedure reported in the literature.[9] The microorganisms used were *M. tuberculosis* H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478) and M. *avium* (ATCC 15769). The crude extracts and compounds were dissolved in DMSO and serially diluted in Middlebrook 7H9 broth before inoculation. The concentrations used ranged from 10 to 2000 μg mL^{-1}. In order to determine the maximum concentration of

Table 2. Minimum inhibition concentration (μg mL^{-1}) of the crude extracts obtained from *Hortia* species and reference antibiotic

Microorganisms	Tested samples[a]									
	H. oreadica									
	HEGT	DEGT	MEGT	HES	DES	MES	HEL	DEL	MEL	RA[b]
M. tuberculosis	250	250	250	2000	1000	250	500	31.25	1000	0.03 (0.22 μM)
M. avium	1000	1000	2000	> 2000	2000	> 2000	2000	1000	> 2000	1.00 (7.30 μM)
M. kansasii	500	250	1000	> 2000	500	1000	500	250	500	1.00 (7.30 μM)
	H. brasiliana									
	HEL	DEL	MEL	MES						
M. tuberculosis	2000	2000	2000	500						
M. avium	> 2000	> 2000	> 2000	2000						
M. kansasii	> 2000	2000	> 2000	250						
	H. superba									
	MESB	MES								
M. tuberculosis	1000	250								
M. avium	> 2000	> 2000								
M. kansasii	2000	2000								

Tested samples[a]: **HEGT:** hexane extract of the ground taproots; **DEGT:** dichloromethane extract of the ground taproots; **MEGT:** methanol extract of the ground taproots; **HES:** hexane extract of the stem; **DES:** dichloromethane extract of the stem; **MES:** methanol extract of the stem; **HEL:** hexane extract of the leaves; **DEL:** dichloromethane extract of the leaves; **MEL:** methanol extract of the leaves; **MESB:** methanol extract of the stem bark. RA[b]: reference antibiotic (isoniazid).

DMSO in the samples that allowed the test microorganisms to reach normal growth, DMSO concentrations of up to 0.3% (v:v) were used. Isoniazid was used as the reference antibiotic drug.

RESULTS AND DISCUSSION

The screening of crude extracts and natural products for antimicrobial activity has previously shown that higher plants represent a potential source of new anti-infective agents,[10] as well as aiding the discovery of drugs obtained from natural products for primary lead compounds.[11] Given their high metabolite content and common pathways which can be manipulated, as well as their high diversity, plants represent a main source of natural products compared to other organisms such as marine sponges, algae, fungi and cyanobacteria.[12] Plant extracts containing terpenes, steroids, alkaloids, flavonoids, chalcones, coumarins, lignans, phenols, polyketides, alkanes, alkenes, alkynes, simple aromatics and peptides have been used in the treatment of different human diseases[13] around the globe, including tuberculosis.[12]

In this context, the crude extracts obtained from three *Hortia* species (see Table 2) were tested for their antimycobacterial activity against *M. tuberculosis*, *M. avium* and *M. kansasii.* The samples with MIC values ≤ 250 μg mL^{-1} against at least one mycobacterium were considered active, although for *M. tuberculosis* H37Ra other authors[14] have considered inactive the extracts of plant species distributed among 17 families from Turkey that could not prevent growth of this microorganism up to a concentration of 200 μg mL^{-1}. Thus, we focused our attention on five active extracts obtained from *H. oreadica* [hexane extract of the ground taproots (HEGT), dichloromethane extract of the ground taproots (DEGT), methanol extract of the ground taproots (MEGT), methanol extract of the stem (MES) and dichloromethane extract of the leaves (DEL)], one from *H. brasiliana* [methanol extract of the stem (MES)] and one from *H. superba* [methanol extract of the stem (MES)]. Extensive chromatographic separation led to the isolation of the following secondary metabolites, which have been previously reported in the literature: hortiolide D **(1)**,[8] guyanin **(2)**,[15] rutaecarpine **(3)**,[5] γ-fagarine **(4)**,[16] 4-methoxy-N-methyl-2-quinolone **(5)**,[17] 5,7-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran-6-propanoic acid **(6)**,[8] 5,6-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran-8-propanoic acid **(7)**,[8] 5-methoxyseselin **(8)**,[18] seselin **(9)**,[19] and bergaptene **(10)**[20] (see Figure 1).

In this paper we report the chemical study of *H. superba*, although all isolated compounds from this specie have been described in the literature.

The isolates were tested against selected microorganisms (see Table 3) and the results demonstrated the inhibitory effect of some natural products, in particular indolequinazoline **(3)** (MIC 15.62 μg mL^{-1}) and furoquinoline **(4)** (MIC 31.25 μg mL^{-1}) alkaloids and dihydrocinnamic acid derivatives **(6-7)** (MIC 62.50 μg mL^{-1}), on the growth of *M. tuberculosis*, but the activity against the other microorganisms was weak. The MIC values obtained are higher than that of isoniazid (0.03 μg mL^{-1} for *M. tuberculosis* and 1.00 μg mL^{-1} for *M. avium* and *M. kansasii*). However, these inhibitory concentrations were compared to the MIC of pyrazinamide (another first-line antimycobacterial drug), 20-100 μg mL^{-1}.[21] Rutaecarpine **(3)** was isolated from the most active crude extract (dichloromethane extract of the leaves of *H. oreadica* - MIC 31.25 μg mL^{-1}) and showed good bioactivity against *M. tuberculosis*, but this compound had no relevant activity against the other microorganisms.

Previous investigations[22] have revealed the antimycobacterial activity of the indole alkaloids ibogaine, voacangine, and texalin against *M. tuberculosis* H37Rv, *M. avium* and *M. kansasii*. The compound ibogaine was the most active (MIC 50 μg mL^{-1}) against *M. tuberculosis* and *M. kansasii,* suggesting that the indole skeleton might

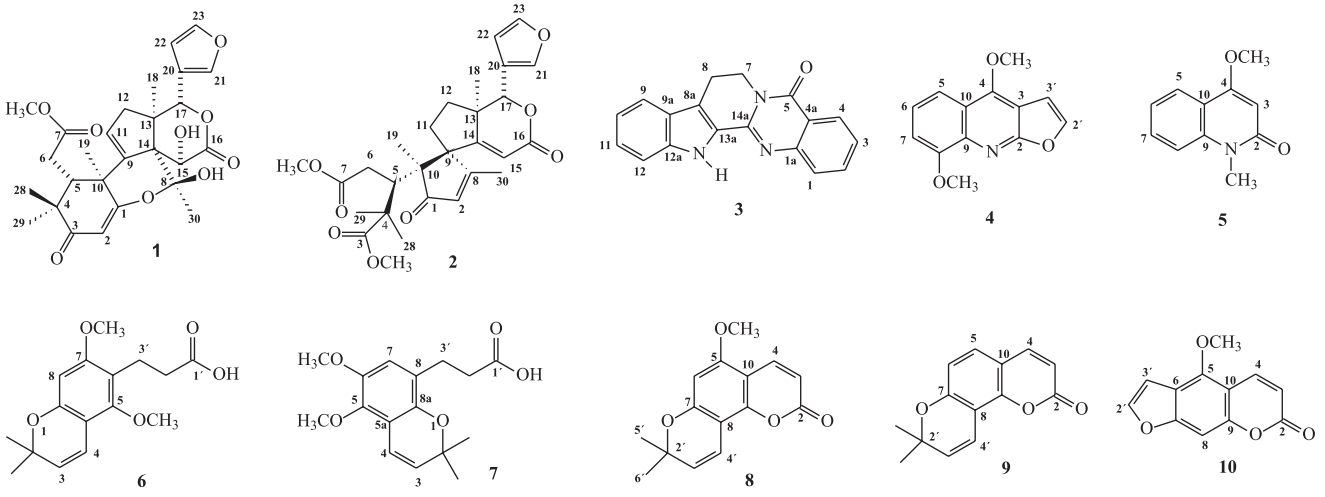

Figure 1. *Structure of compounds isolated from three species of Hortia*

Table 3. Minimum inhibition concentration (μg mL^{-1}) of compounds isolated from *Hortia* species

Microorganisms	1	2	3	4	5	6	7	8	9	10	RA[a]
M. tuberculosis	> 2000	> 2000	15.62 (54.0 μM)	31.25 (0.14 mM)	> 2000	62.50 (0.21 mM)	62.50 (0.21 mM)	500 (1.94 mM)	> 2000	250 (1.16 mM)	0.03 (0.22 μM)
M. avium	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000	1.00 (7.30 μM)
M. kansasii	> 2000	> 2000	2000 (6.97 mM)	2000 (8.73 mM)	2000 (10.6 mM)	1000 (3.42 mM)	2000 (6.84 mM)	2000 (7.75 mM)	> 2000	> 2000	1.00 (7.30 μM)

RA[a]: reference antibiotic (isoniazid).

be responsible for the observed activity, including in this study. In addition, authors have previously reported[23] the antimicrobial activity of a root extract of *Tabernaemontana elegans* Stapf., which was rich in alkaloids, and the alkaloidal fraction containing voacangine and dregamine as major components, against *Mycobacterium* species (MIC of around 128–256 μg mL^{-1}), which suggests that compounds in this class are potential candidates for antimicrobials.

With regard to compound **4**, a previous study[24] has shown that it was active against *M. tuberculosis* H37Rv (MIC 30 μg mL^{-1}), which is in agreement with the result obtained in this research, and the same study revealed that the compound dictamnine, a direct biosynthetic precursor to γ-fagarine and skimmianine, was also active against *M. tuberculosis* (MIC 31.25 μg mL^{-1}). According a report in the literature,[25] skimmianine, a quinoline-type alkaloid with an additional *O*-methyl group at position C7, showed an MIC of 25 μg mL^{-1} against *M. tuberculosis*. Thus, the quinoline skeleton of these alkaloids may play an important role in mediating the antimycobacterial activity.

Secondary metabolites of terpenoid origin are among the most promising classes of natural products with antimycobacterial activity.[26] In this context, studies have demonstrated the activity of terpenes, including monoterpenes, diterpenes, sesquiterpenes, triterpenes and steroids.[13,27] However, in our study the limonoids **1** and **2** did not display any antimycobacterial activity against the three microorganisms at the concentrations tested (MIC > 2000 μg mL^{-1}).

With regard to the structure-activity relationships of coumarins, it appears that the linear furo-cycle in **10** slightly increases the antimicrobial activity, notably against *M. tuberculosis*, when compared to pyrano-coumarins **8** and **9**. However, **10** exhibited a higher MIC value and the antimycobacterial activity was considered weak.

For the dihydrocinnamic acid derivatives, a review of the Rutaceae species revealed that the major source of these compounds with prenyl or pyrano substituents is various species of *Hortia*.[8] Consequently, we examined the activity of **6** and **7** and both showed good inhibitory potency against *M. tuberculosis* (MIC 62.50 μg mL^{-1}). Thus, the antimycobacterial activity of this compound class is being reported herein for the first time.

CONCLUSIONS

In this investigation we report the chemical study of *H. superba* and the antimycobacterial activity of some crude extracts and compounds isolated from three *Hortia* species, which may be related to the presence of alkaloids and dihydrocinnamic acid derivatives, or to more than one component, in the bioactive extracts. Further pharmacological and toxicity studies on the bioactive compounds **3**, **4**, **6** and **7** are required to confirm the biological action of these natural products. Therefore, this study provides an important basis for further investigations regarding the isolation of other compounds present in the active extracts of *Hortia* species, which will permit the establishment of a correlation between structure and antimycobacterial activity.

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