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EVALUATION OF ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *TIBOUCHINA CANDOLLEANA* (MELASTOMATACEAE), ISOLATED COMPOUNDS AND SEMI-SYNTHETIC DERIVATIVES AGAINST ENDODONTIC BACTERIA

Fernanda M. dos Santos, Maria Gorete de Souza, Antônio E. Miller Crotti, Carlos H. G. Martins, Sérgio R. Ambrósio, Rodrigo C. S. Veneziani, Márcio L. Andrade e Silva, Wilson R. Cunha*

Núcleo de Pesquisa em Ciências Exatas e Tecnológicas da Universidade de Franca, Franca, SP, Brasil.

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ABSTRACT

This work describes the phytochemical study of the extracts from aerial parts of *Tibouchina candolleana* as well as the evaluation of the antimicrobial activity of extracts, isolated compounds, and semi-synthetic derivatives of ursolic acid against endodontic bacteria. HRGC analysis of the *n*-hexane extract of *T. candolleana* allowed identification of β-amyrin, α-amyrin, and β-sitosterol as major constituents. The triterpenes ursolic acid and oleanolic acid were isolated from the methylene chloride extract and identified. In addition, the flavonoids luteolin and genistein were isolated from the ethanol extract and identified. The antimicrobial activity was investigated via determination of the minimum inhibitory concentration (MIC) using the broth microdilution method. Amongst the isolated compounds, ursolic acid was the most effective against the selected endodontic bacteria. As for the semi-synthetic ursolic acid derivatives, only the methyl ester derivative potentiated the activity against *Bacteroides fragilis*.

Key words: *Tibouchina candolleana*, ursolic acid, antimicrobial activity

INTRODUCTION

The periodontal disease is described as a set of inflammatory and infectious processes that attack the periodontal tissues. The inflammatory process is triggered mainly by Gram negative anaerobic bacteria. Adjuvant drug therapies have played a key role in assisting in cases involving development or persistence of infection during or after prescription of appropriate endodontic therapy (3).

Chemical agents have been employed, in order to promote bacteria eradication. Nevertheless, the different susceptibilities exhibited by oral pathogens, as well as the toxicity and allergenicity displayed by these chemicals, have made treatment very difficult (13,20,21,29). This scenario illustrates the need for discovering new compounds that can be used as complement to instrumental procedures.

Plant species are an excellent source for the discovery of new antimicrobial drugs, mainly considering that the molecular

***Corresponding Author. Mailing address:** Núcleo de Pesquisa em Ciências Exatas e Tecnológicas da Universidade de Franca, Av. Dr. Armando Salles de Oliveira 201, Parque Universitário, 14404-600, Franca, SP, Brazil.; Tel.: +55-16-37118878.; E-mail: wrcunha@unifran.br

diversity of natural products is much higher than that derived from chemical syntheses. This fact has stimulated investigation of the antimicrobial activity of different plant extracts, in order to promote the development of new pharmaceuticals that can control many diseases such as oral diseases. In this context, our research group has recently concentrated efforts on the assessment of the antimicrobial properties of natural products from plants, aiming to detect new compounds that display antibacterial activity against oral pathogens and that can be employed as adjuvant therapy in dentistry (4,6,9,26,27).

The genus *Tibouchina*, belonging to the Melastomataceae, is mainly distributed in tropical and subtropical regions of the Americas and includes about 350 species (25). In Brazil, *Tibouchina* species are known as "quaresmeira" and are used as ornamental plants because of their dark purple flowers. Previous studies on *Tibouchina* species have evidenced the presence of tannins, flavonoids, and benzoquinones (5,14,30,34-36). Crude plant extracts from *Tibouchina* and their isolated compounds have been reported to exhibit antioxidant and antifungal activities (16,22,23). However, to the best of our knowledge, there is no previous report on *Tibouchina candolleana*. This has led us to investigate the phytochemical and the antimicrobial activity of the aerial parts of this plant.

MATERIALS AND METHODS

Plant Material

T. candolleana (Mart. ex DC.) Cogn. was collected in Peixoto (Minas Gerais, Brazil), near Usina Mascaranhas de Moraes, in September, 2003. The plant was identified by Dr. Angela Borges Martins, Instituto de Biologia, UNICAMP, Brazil. A voucher specimen (UEC 142048) has been deposited in the Herbarium of the same Institute.

Extraction and Fractionation

The aerial parts of *T. candolleana* were air-dried at 40°C and ground. The powdered material (1.0 kg) was exhaustively extracted by maceration at room temperature using *n*-hexane, methylene chloride, and ethanol, in this sequence, to afford the crude extracts (10.0 g, 15.5 g, and 44.3 g, respectively).

An aliquot of the *n*-hexane extract (500 mg) was filtered over a Celite/Norit 3:1 w/w mixture (60 g) and eluted with ethyl acetate. An aliquot (10 mg) of the resulting fraction was resuspended in chloroform (3 mL) and percolated through a sep-Kap column (Alltech, silica gel 200 mg, 3 mL). The column was eluted with chloroform (10 mL) and evaporated to dryness at room temperature, followed by HRGC analysis.

A sample of the methylene chloride extract (10.0 g) was chromatographed over silica gel 60 (300 g, 0.063-0.200 mm, Merck) using different solvents and vacuum liquid chromatography, which furnished five fractions of 1000 mL each (F1: *n*-hexane; F2: methylene chloride; F3: methylene chloride/AcOEt 50:50 v/v; F4: AcOEt; F5: EtOH). Fraction F3 (4.53 g) exhibited the highest antimicrobial activity, so part of this fraction (1.50 g) was filtered through a mixture of a Celite/Norit 3:1 w/w mixture (60 g) and eluted with AcOEt, which afforded a mixture containing ursolic acid and oleanolic acid 730 mg). These compounds were separated by HPLC, yielding ursolic acid (1) and oleanolic acid (2) as white amorphous solids (R_t: 20.67 min and R_t: 21.52 min, respectively) (15).

A sample of the ethanolic extract (13.0 g) was chromatographed over silica gel 60 (300 g, 0.063-0.200 mm, Merck) by vacuum liquid chromatography using different solvents, giving six fractions of 1000 mL each (Fr1: *n*-hexane/AcOEt 75:25 v/v ; Fr2: *n*-hexane/AcOEt 50:50 v/v; Fr3: AcOEt ; Fr4: AcOEt/EtOH 75:25 v/v ; Fr5: AcOEt/EtOH 50:50 v/v; Fr6: EtOH). Fraction Fr-3 (300 mg) was chromatographed over Sephadex LH-20 using methanol as eluent, followed by semi-preparative reverse-phase HPLC purification using methanol/H_2O (75:25 v/v) as mobile phase, to give rise to luteolin (3) (28 mg) and genistein (4) (15 mg).

Preparation of C-3 ester derivatives of ursolic acid

In order to obtain some triterpene acid derivatives, ursolic

acid (20 mg) was treated with excess acetic anhydride in pyridine (11), to furnish the 3-acetoxyl derivative (15 mg) (1a). Ursolic acid (50 mg) was also treated with appropriate long-chain fatty acids (44 mg lauric acid or 62.6 mg stearic acid), *N*,*N*'-dicyclohexylcarbodiimide (DCC, 113.5 mg), and 4-dimethylamino-pyridine (DMAP, 67 mg) in 1,2-dichloroethane. The reaction mixture was stirred at room temperature for 24 h, which was followed by filtration and evaporation under reduced pressure (18). The residue was purified by silica gel column chromatography with *n*-hexane, to afford the corresponding fatty acid esters 1b (35 mg) and 1c (38 mg).

Preparation of the C-28 methyl ester derivative of ursolic acid

Ursolic acid (20 mg) was treated with CH_2N_2 in Et_2O (11), yielding the respective C-28 methyl ester derivative 1d (18 mg), which was purified by column chromatography on silica gel 60 (0.063-0.200 mm, Merck, Darmstadt, Germany).

Structural Identification

The structural elucidation of the isolated compounds was performed on the basis of MS as well as ^{1}H and ^{13}C NMR spectroscopic and comparison with published data. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Brüker DPX-400 spectrometer. Samples were dissolved in DMSO-d_6 or $CDCl_3$ using TMS as internal standard. High-resolution ESI-MS spectra were registered on a Bruker Ultro-TOF mass spectrometer.

Gas chromatography analysis

A portion (50 mg) of the *T. stenocarpa n*-hexane extract was cleaned up and submitted to HRGC analysis according to a previously reported methodology (8). HRGC analysis was performed on a Hewlett-Packard model 5980 series II gas chromatograph equipped with a split injector (split ratio 1:60) operating at 260°C and a flame ionization detector operating at 330°C. The injected volume was 2 μL. HP-50 (cross-linked 50% phenyl-methyl-silicone, 30 m x 0.25 m x 0.25 μm) and HP-1 (cross-linked methyl-silicone, 30 m x 0.25 m x 0.25 μm) capillary columns were employed. Hydrogen was used as the carrier gas at an average linear velocity of 44 $cm.s^{-1}$. Data were processed on a Hewlett-Packard model 3395 integrator.

Determination of the antimicrobial activity

The minimum inhibitory concentration (MIC) values of the samples were determined in triplicate by using the broth microdilution method in 96-well microplates (7). The tested strains were obtained from the American Type Culture Collection (ATCC). The following microorganisms were employed: *Bacteroides fragilis* (ATCC 25285), *Actinomyces naeslundii* (ATCC 19039), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella nigrescens* (ATCC 33563), *Fusobacterium nucleatum* (ATCC 49256), *Bacteriodes thetaiotaomicrom* (ATCC 29741), and *Peptostreptococcus anaerobius* (ATCC 27337)

The samples were dissolved in DMSO at 1.0 mg mL^{-1}, followed by dilution in schaedler broth (DIFCO, MO, USA) supplemented with hemin (5.0 μg mL^{-1}) and vitamin K1 (10 μg mL^{-1}); concentrations ranging from 400.0 to 0.25 μg mL^{-1} were achieved. The final DMSO content was 5% (v/v), and this solution was used as negative control. The inoculum was adjusted for each organism, to yield a cell concentration of 5×10^5 colony forming units (CFU) mL^{-1}, according to previously standardization by the Clinical Laboratory Standards Institute (7). One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well, free of antimicrobial agent, was also included, to ensure medium sterility. Chlorhexidine dihydrochloride was utilized as positive control. The microplates (96-wells) were sealed with plastic film. The time necessary for growth was 96 hours for anaerobes incubated at 37 °C under appropriate gaseous conditions [anaerobic work station (Don Whitley Scientific, Bradford, UK), in an atmosphere of 5–10% H_2, 10%

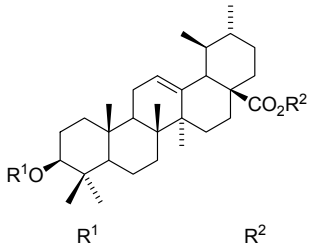
CO_2, and 80–85% N_2]. Resazurin (30 μL) in aqueous solution (0.02%) was then added to the microplates, to indicate microorganism viability (28). The MIC value was determined as the lowest concentration of the compound capable of inhibiting microorganism growth.

RESULTS AND DISCUSSION

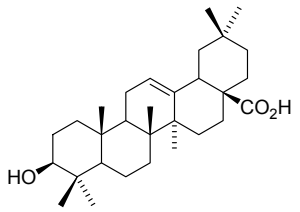
Studies on *Tibouchina* species are scarce in the literature. This is the first time that *T. candolleana* has been investigated. Figure 1 brings the chemical structures of the compounds identified in *T. candollena* extracts. The *n*-hexane extract, analyzed by HRGC, allowed identification of the compounds *β*-sitosterol (5), *β*-amirina (6), and *α*-amirina (7).

The NMR-^{1}H and ^{13}C data of fraction F-2, obtained from fractionation of the methylene chloride extract, revealed the presence of a mixture of ursolic and oleanoic acids (12). This fraction was further chromatographed by HPLC, which enabled isolation of ursolic acid (1) and oleanolic acid (2) (Figure 1). Both acids consist of triterpenoids that are widely distributed in the plant kingdom, and they have been frequently isolated from other species belonging to Melastomataceae family as mutually isomeric mixtures (10,11,31). However, this is the first time that ursolic acid and oleanolic acid have been reported in this genus. These triterpene acids display several biological activities (17).

In addition, fractionation of the ethanolic extract allowed isolation and identification of the flavonoids luteolin (3) (33) and genistein (4) (24) (Fig. 1).



	R^1	R^2
1	H	H
1a	$COCH_3$	H
1b	$CO(CH_2)_{10}CH_3$	H
1c	$CO(CH_2)_{16}CH_3$	H
1d	H	CH_3



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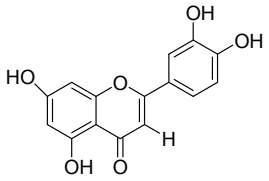

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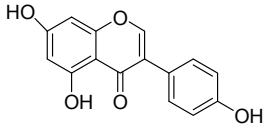

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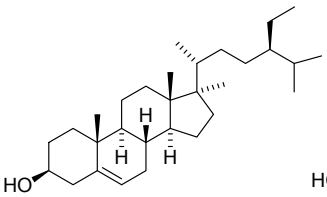

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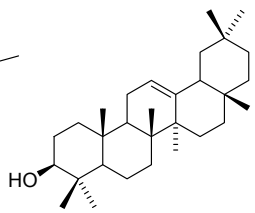

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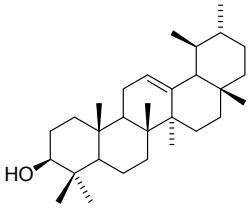

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Figure 1. Chemical structures of compounds present in *Tibouchina candolleana* extracts and of semi-synthetic derivatives.

Regarding the effects of the *T. candollena* extracts on the growth of the selected endodontic bacteria, all crude extracts exhibited low antimicrobial activity, as shown in Table 1.

Amongst the compounds evaluated in the present work, the mixture of ursolic and oleanolic acids (1+2) was the most active against *Bacteroides fragilis,* with a MIC value of 20 μg mL^{-1}. The mixture was more active than pure ursolic acid (MIC = 80 μg mL^{-1}), indicating a possible synergistic effect. The mixture 1+2 was also effective against *Actinomyces naeslundii* and *Porphyromonas gingivalis*, with MIC values of 20 μg mL^{-1} and 40 μg mL^{-1}, respectively.

Ursolic acid (1) was the most effective against most of the tested bacteria, with MIC values of 20 μg mL^{-1} for *Actinomyces naeslundii* and *Porphyromonas gingivalis*, 80 μg mL^{-1} for *Bacteroides fragilis*, and 90 μg mL^{-1} for *Prevotella nigrescens*. Oleanolic acid (2) also afforded significant results for the strains *Actinomyces naeslundii* and *Porphyromonas gingivalis*, with MIC values of 20 μg mL^{-1} and 40 μg mL^{-1}, respectively.

The flavonoids genistein (3) and luteolin (4) were not satisfactorily active against the investigated bacteria tested, since MIC values higher than or equal to 400 μg mL^{-1} were achieved.

Table 1. Minimum inhibitory concentration values (μg mL^{-1}) obtained for the crude extracts of *Tibouchina candolleana*, isolated compounds, and semi-synthetic derivatives.

	Microorganisms						
Samples	***Bacteroides fragilis***	***Actinomyces naeslundii***	***Porphyromonas gingivalis***	***Prevotella nigrescens***	***Fusobacterium nucleatum***	***Bacteroides thetaiotaomicron***	***Peptostreptococcus anaerobius***
[a]Hex	>400	>400	>400	>400	>400	>400	>400
[b]MC	>400	>400	>400	>400	>400	>400	>400
[c]EtOH	>400	400	>400	>400	>400	>400	>400
1	80	20	20	90	>400	>400	200
2	>400	20	40	200	>400	>400	400
1+2	20	20	40	300	400	>400	400
3	>400	400	400	400	>400	>400	>400
4	>40	>400	>400	>400	>400	>400	400
1a	>400	>400	>400	>400	>400	>400	400
1b	>400	>400	>400	>400	400	>400	400
1c	>400	>400	>400	>400	400	>400	400
1d	>400	200	>400	300	400	400	400
[d]Positive Control	14.8	7.4	3.7	3.7	14.8	29.0	7.4

[a]*n*-hexane extract; [b] methylene chloride extract; [c]ethanolic extract
[d]Positive control: chlorhexidine

According to several authors (3,32), drugs derived from natural products can serve not only as new drugs themselves, but also as lead compounds for chemical modifications that could furnish derivatives with better activity and pharmacokinetic properties, new mechanisms of action, and fewer adverse side effects. In an effort to obtain more active compounds, semi-synthetic derivatives of ursolic acid (1a – 1d) were prepared (Figure 1). Mallavadhani and co-workers (19) have demonstrated that lipophilicity is an important parameter in the development of biological agents. The authors stated that molecules with carbon chains above C-10 are fairly lipophilic and consequently good candidates for pharmacological assessment. In view of these observations, two lipophilic 3-*O*-fatty acid ester chain derivative of ursolic acid (1b and 1c) were synthesized herein. The *3β*-dodecanoate (1b) and the *3β*-octadecanoate (1c) derivatives of ursolic acid did not promote lower MIC values against any of the studied microorganisms. The difference in outcomes may be related to the types of

bacteria used in the several literature works, and the mechanisms involved in the activity are probably different.

The presence of the 3-acetoxy group in derivative 1a did not enhance the bactericide activity as compared to ursolic acid, while the C-28 methyl ester derivative (1d) had improved the activity against *Bacteroides fragilis* only, with a MIC value of 20 μg mL^{-1}. Thus, comparison between the MIC values of ursolic acid and those of its semi-synthetic derivatives suggests that the COOH and OH groups attached to carbons 3 and 17, respectively, are important for the activity against these bacteria.

In summary, our study indicates promising results for the antimicrobial activity of ursolic acid (1) and oleanolic acid (2) against some endodontic bacteria. It has been reported that these compounds are not toxic (1,18), which makes them particularly interesting for future developments of novel antimicrobial agents against endodontic bacteria.

It is also important to emphasize that in view of the promising results obtained for these compounds, other biological studies to elucidate the mechanism of antimicrobial action as well as the structure-activity relationship are necessary. In this context, additional antimicrobial studies to detail the kinetics, synergistic activities, and mode of action of such compounds must also be conducted, so that better understanding of their characteristics and potential can be gained.

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