Antimicrobial Activity of *Rosmarinus officinalis* against Oral Pathogens: Relevance of Carnosic Acid and Carnosol

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The *in vitro* inhibitory activity of crude EtOH/H_2O extracts from the leaves and stems of *Rosmarinus officinalis* L. was evaluated against the following microorganisms responsible for initiating dental caries: *Streptococcus mutans*, *S. salivarius*, *S. sobrinus*, *S. mitis*, *S. sanguinis*, and *Enterococcus faecalis*. Minimum inhibitory concentrations (*MIC*) were determined with the broth microdilution method. The bioassay-guided fractionation of the leaf extract, which displayed the higher antibacterial activity than the stem extract, led to the identification of carnosic acid (**2**) and carnosol (**3**) as the major compounds in the fraction displaying the highest activity, as identified by HPLC analysis. Rosmarinic acid (**1**), detected in another fraction, did not display any activity against the selected microorganisms. HPLC Analysis revealed the presence of low amounts of ursolic acid (**4**) and oleanolic acid (**5**) in the obtained fractions. The results suggest that the antimicrobial activity of the extract from the leaves of *R. officinalis* may be ascribed mainly to the action of **2** and **3**.

Introduction. – Dental caries is a common oral bacterial pathology caused by a biofilm consisting of microorganisms present on the tooth surface [1][2]. This disease has been associated with *Streptococcus* spp., mainly *Streptococcus mutans* and *S. sobrinus* [3][4]. Several antimicrobial substances, such as ampicillin, chlorhexidine, sanguinarine, metronidazole, and phenolic and quaternary ammonium antiseptics, have been very effective in preventing dental caries [3][5]. However, various adverse effects such as tooth and restoration staining, increase of calculus formation, diarrhea, and disarrangements of the oral and intestinal flora have been associated with the use of such compounds [3][6]. Thus, these drawbacks justify the search for new effective anticariogenic agents that could be employed in caries prevention.

The use of plant extracts and their constituents with known antimicrobial properties can be of great significance in therapeutic treatments. Within this context, the antimicrobial properties of plant extracts and isolated compounds have been investigated by a number of researchers worldwide [6][7]. Recent studies undertaken in our laboratory have demonstrated the great importance of natural products, both plant extracts and isolated compounds, as natural antimicrobial agents in oral care products [8][9].

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Rosmarinus officinalis L. (Lamiaceae) is an edible evergreen shrub native to the Mediterranean area, and it is widely used around the world for culinary and medicinal purposes [10–12]. Its main constituents are rosmarinic acid (**1**), carnosic acid (**2**), carnosol (**3**), ursolic acid (**4**), oleanolic acid (**5**), genkwanin, apigenin, and luteolin (*Fig. 1*) [13–15]. Despite several pharmacological applications of *R. officinalis*, studies on its antimicrobial properties against oral bacteria have been scarce [16].

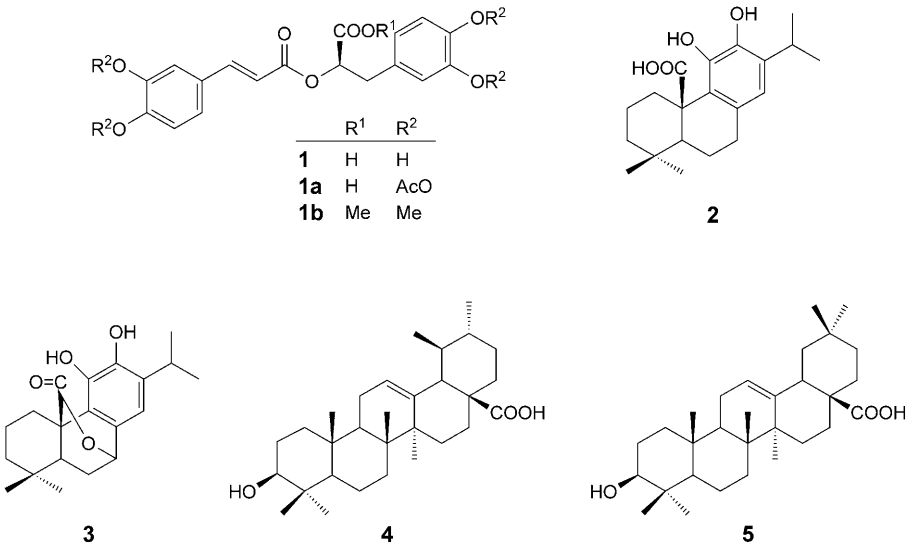

Fig. 1. *Chemical structures of rosmarinic acid* (**1**) *and its derivatives* (**1a** and **1b**), *carnosic acid* (**2**), *carnosol* (**3**), *ursolic acid* (**4**), *and oleanolic acid* (**5**)

As part of our ongoing research on medicinal plants [17–20], we report herein the *in vitro* antimicrobial activity of crude EtOH/H_2O extracts obtained from stems (HEROS) and leaves (HEROL) of *R. officinalis* against some important oral pathogens. Additionally, we have carried out a bioassay-guided fractionation and HPLC analysis of the HEROL, the more active extract, in order to identify the main compounds responsible of this biological activity.

Results and Discussion. – The effects of the extracts of *R. officinalis* (HEROS and HEROL) on the growth of the selected cariogenic bacteria are shown in the *Table*. The lowest *MIC* values were obtained for the EtOH/H_2O extract obtained from leaves (HEROL).

Among the seven fractions (*Frs. 1–7*) achieved by the fractionation of the HEROL, *Fr. 3* displayed the highest antibacterial activity against the selected bacteria (*Table*). HPLC analysis of this fraction (*Fig. 2*) showed that its major compounds are carnosic acid (**2**, t_R 32.16 min) and carnosol (**3**, t_R 27.41 min). Regarding their antimicrobial activity, both **2** and **3** displayed significant inhibitory effects against the selected oral pathogens (*Table*). They displayed better antibacterial activity than thymol, a commercially available antibacterial agent, which was used as positive control (*Table*).

Table. *Minimum Inhibitory Concentrations* (*MIC*) *of Extracts and Fractions of* R. officinalis, *Rosmarinic Acid* (**1**) *and Its Derivatives* (**1a** and **1b**), *Carnosic Acid* (**2**), *and Carnosol* (**3**) *against Oral Pathogens*

Sample	*MIC* [μg/ml]					
	E. faecalis	*S. salivarius*	*S. sanguinis*	*S. mitis*	*S. mutans*	*S. sobrinus*
HEROS[a])	>400	>400	350	400	350	>400
HEROL[a])	350	160	50	170	90	80
Fr. 1	>400	>400	>400	>400	>400	>400
Fr. 2	200	300	50	60	90	70
Fr. 3	70	12	50	12	50	10
Fr. 4	400	200	50	200	200	200
Fr. 5	>400	400	200	400	400	400
Fr. 6	>400	300	90	300	300	300
Fr. 7	>400	300	100	>400	>400	>400
1	>400	>400	>400	>400	>400	>400
1a	>400	400	>400	300	>400	>400
1b	>400	300	300	200	200	300
2	70	30	50	15	30	40
3	100	35	35	35	75	50
Chlorhexidine	0.37	0.09	0.74	0.37	0.09	0.09
Thymol	>400	400	>400	300	>400	300

[a]) HEROS and HEROL are crude EtOH/H_2O extracts from stems and leaves of *R. officinalis*, respectively.

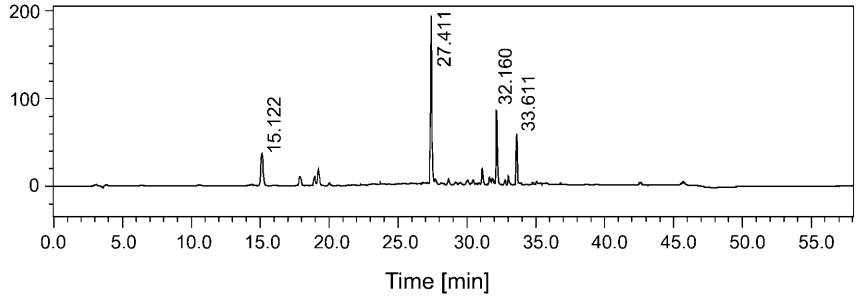

Fig. 2. *HPLC Profile obtained with* Method 1 *for* Fr. 3 *of the EtOH/H_2O extract of the leaves of* R. officinalis *showing the major compounds: carnosol* (**3**, t_R 27.41 min) *and carnosic acid* (**2**, t_R 32.16 min)

The literature reports sparse information about the antimicrobial activities of *R. officinalis*, and most of this work refers to its essential oil [21][22]. A recent *in vitro* antimicrobial evaluation of commercial rosemary extract formulations against some bacteria showed that *Gram*-positive bacteria were more sensitive to the formulations than *Gram*-negative ones, especially in the case of oil-soluble extracts containing carnosic acid as the major phenolic compound [23]. Moreover, a recent study also demonstrated the antimicrobial activities of the leaf extract of *R. officinalis* as well as of carnosic acid against bacteria and yeasts with dermatological relevance [24].

On the other hand, rosmarinic acid (**1**, t_R 7.47 min), which was detected as major compound in *Fr. 7*, did not display antimicrobial activity against the tested oral

pathogens. In addition, the acetyl and methyl ester derivatives, **1a** and **1b**, respectively, prepared from **1** were not able to improve its inhibitory activity.

The HPLC analysis also showed that both ursolic acid (**4**) and oleanolic acid (**5**) are present in small concentrations in *Frs. 2* and *3* of the HEROL. Concerning the antibacterial activity of these triterpene acids, previous studies undertaken in our laboratory revealed that both **4** and **5** possess moderate activity against oral pathogens [9].

The antibacterial activities of the two extracts and the isolated compounds against the selected oral pathogens were also compared to chlorhexidine, an antiseptic that possesses several detrimental side effects [3][6]. The high bactericidal activity of chlorhexidine is due to its ability to inhibit glycosydic and proteolytic activities. Moreover, it is able to reduce matrix metalloproteinase activities in a huge variety of oral bacteria [25][26].

Several phenolic compounds, such as thymol, have been reported for their inhibition of oral bacteria [27]. It was shown that the OH moiety attached to the aromatic ring is required for the high antibacterial activity of these type of compounds [27][28]. However, it was observed that rosmarinic acid, which is a polyphenolic compound, did not show antibacterial activity against the selected oral pathogens. In addition, it has been reported that some polyphenolic compounds present in tea extracts possess low anticariogenic activity against oral pathogens [29][30]. Thus, the occurrence of phenol groups may not be the only essential chemical characteristics for the antibacterial activity of the phenolic compounds.

In summary, we suggest that the antimicrobial activity against oral pathogens of *R. officinalis* leaf extract may be mainly attributed to the effects of carnosic acid (**2**) and carnosol (**3**). Therefore, with respect to an antimicrobial application, **2** and **3** should be considered as appropriate compounds for the quality control of *R. officinalis* leaf extract and its formulations.

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Experimental Part

Plant Material. Rosmarinus officinalis L. (Lamiaceae) was collected in the urban perimeter of Patrocínio city (18° 56′ 35″ S, 46° 59′ 31″ W, Minas Gerais, Brazil, in May 2007). The plant material was identified by *M. G.* A voucher specimen (collector *M. G.*, number 1871, SPFR 11912) was deposited with the Herbarium of the Departmento de Biologia, Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, (Herbarium SPFR).

Extraction and Fractionation. The aerial parts of *R. officinalis* were divided into stems and leaves, dried in a stove with circulating air (40°), and powdered by means of a blender. The obtained powders (130 g each) were exhaustively extracted with EtOH/H_2O 8:2 (*v*/*v*) by maceration at r.t., followed by filtration. The filtered extracts were concentrated under reduced pressure, affording the stem (HEROS, 2.6 g) and leaf (HEROL, 18.6 g) crude extracts. Because of its higher antimicrobial activity, the leaf EtOH/H_2O extract of *R. officinalis* (12.0 g) was chromatographed over 300 g silica gel *60* (SiO_2, 0.063–0.200 mm; *Merck*) by vacuum liquid chromatography [31] to afford seven fractions of 1000 ml each: *Fr. 1* (hexane), *Fr. 2* (hexane/AcOEt 75:25 (*v*/*v*)), *Fr. 3* (hexane/AcOEt 50:50 (*v*/*v*)), *Fr. 4* (AcOEt), *Fr. 5* (AcOEt/EtOH 75:25 (*v*/*v*)), *Fr. 6* (AcOEt/EtOH 50:50 (*v*/*v*)), and *Fr. 7* (EtOH). All the fractions obtained were analyzed by HPLC. A portion of the most active fraction (*Fr. 3*) was dissolved in MeOH/H_2O 1:1 (*v*/*v*) and chromatographed on a prep. RP-HPLC *Shimadzu Shim-pack ODS* column (250 × 20 mm; 5 μm), equipped with a pre-column of the same material using H_2O with 0.1% AcOH (*A*) and

MeOH (*B*) as eluents (isocratic step of 50% *B* during 5 min, followed by a linear gradient to 100% *B* in 25 min, and maintaining this composition for 10 min; the system was then re-equilibrated to the initial composition in 10 min) at a flow rate of 10 ml/min, affording compounds **2** and **3**. *Fr. 7* led to the isolation of **1**, under the same conditions as used for the analysis of *Fr. 3*. Moreover, *Fr. 2* provided compounds **4** and **5**.

HPLC Analysis. Two HPLC methods were used for the identification of the compounds in the rosemary fractions. The HPLC equipment employed in this work was a *Shimadzu LC-6AD* system equipped with a degasser *DGU-20A5*, a UV-DAD detector *SPD-M20A* series with a *CBM-20A* module, and a *Reodyne* manual injector. Separations of the micromolecules were carried out on a *Shimadzu Shim-pack ODS* column (250 × 4.60 mm; 5 μm) equipped with a pre-column of the same material. The MeOH used was HPLC grade (*J. T. Baker*), and ultrapure H_2O was obtained by passing redist. H_2O through a *Direct-Q UV3* system. The eluents utilized for the chromatographic analysis of **1**, **2**, and **3** (*Method 1*) were H_2O with 0.1% AcOH (*A*) and MeOH (*B*). An isocratic step of 50% *B* during 5 min was run, followed by a linear gradient to 100% *B* in 25 min, and maintaining this composition for 10 min; the system was then re-equilibrated to the initial composition in 15 min. The flow rate was 1.7 ml/min. The mobile phase for the chromatographic analysis of **4** and **5** (*Method 2*) was MeOH/H_2O with 0.1% AcOH 85 : 15. Additionally, the compounds were identified by comparison of their t_R and UV spectra with those of the corresponding standards. All standards were acquired from *Sigma-Aldrich* (St. Louis, MO, USA).

Preparation of Rosmarinic Acid Derivatives. Rosmarinic acid (1.0 g) was treated with excess Ac_2O in pyridine, to give the AcO derivative **1a** (0.73 g). In another preparation, rosmarinic acid (0.53 g) was treated with CH_2N_2 in Et_2O, to yield the Me ester derivative **1b** (0.54 g). The derivatives were purified by column chromatography (CC) on *Sephadex LH-20* (*Acros Organics*, New Jersey, USA).

Structure Identification. Structures of all the compounds were determined by spectroscopic methods. ^{1}H- and ^{13}C-NMR spectra (at 400 and 100 MHz, resp.) were recorded on a *Bruker DPX-400* spectrometer in (D_6)DMSO or $CDCl_3$ using Me_4Si as internal standard. HR-ESI-MS were recorded on a *Bruker Ultra-TOF* mass spectrometer.

Microorganisms. All the bacterial strains were acquired from the *American Type Culture Collection*. The following microorganisms were used in this study: *Enterococcus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *S. mitis* (ATCC 49456), *S. mutans* (ATCC 25275), *S. sobrinus* (ATCC 33478), and *S. sanguinis* (ATCC 10556).

Antimicrobial Assay. The *MIC* values (the lowest concentration of the extracts or pure compounds from *R. officinalis* capable of inhibiting microorganism growth) were determined in triplicate using the broth microdilution method in 96-well microplates [32]. The samples were dissolved in DMSO at 0.5 mg/ml, followed by dilution in tryptic soy broth; concentrations ranging from 400 to 1 μg/ml were achieved. The final DMSO content was 5% (*v*/*v*), and this soln. 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}. One inoculated well was included, to control the adequacy of the broth. To ensure medium sterility, one non-inoculated well containing no antimicrobial agent was also included. Chlorhexidine and thymol were used as positive controls. To determine the *MIC* values for chlorhexidine, concentrations ranging from 5.90 to 0.01 μg/ml were used. The microplates were sealed with plastic film and incubated at 37° for 24 h. After incubation, resazurin (30 μl) in aq. soln. (0.02%) was added to the microplates. This procedure was based on the methodology described by *Palomino et al.* [33].

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