Antibacterial Activity of the Essential Oil from *Rosmarinus officinalis* and its Major Components against Oral Pathogens

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The essential oil of *Rosmarinus officinalis* L. (rosemary) was obtained by hydro-distillation and analysed by gas chromatography-mass spectrometry. Sixty-two constituents were identified, representing 98.06% of the total oil content. Oxygenated monoterpenes were the predominant components. The rosemary oil was characterized as having prominent (> 5%) contents of camphor (18.9%), verbenone (11.3%), α-pinene (9.6%), β-myrcene (8.6%), 1,8-cineole (8.0%), and β-caryophyllene (5.1%). The antimicrobial activity of the oil as well as of its major constituents was tested against the following microorganisms: *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus salivarius*, *Streptococcus sobrinus*, and *Enterococcus faecalis*, which are potentially responsible for the formation of dental caries in humans. The microdilution method was used for determination of the minimum inhibitory concentration (MIC) during evaluation of the antibacterial activity. The essential oil displayed low activity against the selected microorganisms. In the present study, the pure major compounds were more active than the essential oil. Among all the microorganisms tested, the pathogen *S. mitis* was the most susceptible and *E. faecalis* was the most resistant to the evaluated samples. This is the first report on antimicrobial activity of the major components of rosemary oil against oral pathogens.

Key words: Rosmarinus officinalis, Antibacterial Activity, Oral Pathogens

Introduction

Medicinal plants have been used in developing countries as alternative treatments to health problems. In many parts of Brazil there is a rich tradition of using herbal medicine in the treatment of various infectious diseases (Johann *et al.*, 2007; Oliveira *et al.*, 2007). Many studies have shown that aromatic plants traditionally used in folk medicine exert inhibitory effects on bacteria, fungi, and yeasts (Bozin *et al.*, 2007; Hammer *et al.*, 1999).

Dental caries and periodontal disease are associated with oral pathogens, and several plant derivatives have been evaluated with respect to their antimicrobial activities against some oral pathogens. Recent studies undertaken in our laboratory have demonstrated the great importance of natural products, both plant extracts and isolated compounds, as natural antibacterial agents against several oral pathogens (Ambrosio *et al.*, 2008; Cunha *et. al.*, 2007; Porto *et al.*, 2009).

The antimicrobial properties of essential oils have been known for many years, and oils from popular, commercially available aromatic plants, such as *Rosmarinus officinalis* L. (rosemary, Lamiaceae family), have been used extensively to treat bacterial and fungal infections (Bozin *et al.*, 2007; Daferera *et al.*, 2000).

R. officinalis is an edible evergreen shrub native to the Mediterranean area, which is widely used around the world for culinary and medicinal purposes (González-Trujano *et al.*, 2007; Leal *et al.*, 2003). Despite the several pharmacological applications of *R. officinalis*, studies on its antimicrobial properties against oral bacteria have been scarce (Bernardes *et al.*, 2010; Rasooli *et al.*, 2008).

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As part of our ongoing research on medicinal plants (Cunha *et al.*, 2003, 2008, 2010; Neto *et al.*, 2004), we report herein the *in vitro* antimicrobial activity of the essential oil obtained from the leaves of *R. officinalis*, as well as the antimicrobial activity of its major constituents, against some important oral pathogens. The essential oil was also characterized by gas chromatography-mass spectrometry (GC-MS) analyses, in order to identify its major components.

Material and Methods

Plant material

Rosmarinus officinalis 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 Dr. Milton Groppo. A voucher specimen (collector M. Groppo, number 1871, SPFR #11912) was deposited in the herbarium of the Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras, Universidade de São Paulo, Brazil.

Essential oil extraction

The essential oil was obtained from 40 g of fresh plant parts by hydrodistillation for 3 h using a Clevenger-type system. The extracted oil was dried with sodium sulfate. Oil samples were stored at –5 °C in sealed glass vials prior to use. The major components of the oil were purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA.

Gas chromatography-mass spectrometry (GC-MS) analysis

The identification of volatile constituents was performed using a Shimadzu QP-2010 gas chromatograph, equipped with a mass-selective detector and DB-5MS (30 m × 0.25 mm × 0.25 μm) capillary column. GC-MS analyses were performed by split injection, with the injector and column temperatures set at 240 and 60 °C, respectively, using a heating ramp of 3 °C min^{-1} and a final temperature of 240 °C for the column. The ion source and the transfer line temperatures were set at 250 °C. Helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. The GC-MS electron ionization system was set at 70 eV. Mass spectrum acquisition was performed in the mass range from 40 to 500 *m/z*. A sample of the essential oil was solubilized in ethyl acetate for the analyses. Retention indices (RI) were determined by co-injection of hydrocarbon standards. The oil components were identified by comparison with literature data (Adams, 2001) and the profiles from the Wiley 7 library, and by co-injection of authentic standards, if available.

Microorganisms

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

Antimicrobial assay

The antimicrobial assays were based on the methodology described by Palomino *et al.* (2002), while MIC (minimum inhibitory concentration) values (the lowest concentration of essential oil from *R. officinalis* or its major compounds capable of inhibiting the microorganism growth) were determined in triplicate using the broth microdilution method in 96-well microplates (NCCLS, 2006).

The samples were dissolved in DMSO (dimethyl sulfoxide) at 0.5 mg mL^{-1}, followed by dilution in tryptic soy broth; concentrations ranging from 2000 to 20 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 so that a cell concentration of $5 \cdot 10^5$ colony forming units (CFU) mL^{-1} was achieved. One inoculated well was included, so as to control the adequacy of the broth. In order to ensure medium sterility, one non-inoculated well containing no antimicrobial agent was also included. Chlorhexidine was used as positive control. In order to determine the MIC values for chlorhexidine, concentrations ranging from 5.90 to 0.01 μg mL^{-1} were employed. The microplates were sealed with a plastic film and incubated at 37 °C for 24 h. After that, resazurin (30 μL) in aqueous solution (0.02%) was added to the microplates.

Results

The result of the analysis of the chemical composition of the essential oil of *R. officinalis* by CG-MS are shown in Table I. From this oil, 62 constituents were identified, representing 98.06% of the total oil content. Oxygenated monoterpenes were the predominant components (48.23%). The rosemary oil was characterized as having prominent (> 5%) contents of camphor (18.9%), verbenone (11.3%), α-pinene (9.6%), β-myrcene (8.6%), 1,8-cineole (8.0%), and β-caryophyllene (5.1%).

The effects of the essential oil and the major compounds on the growth of the selected cariogenic bacteria are depicted in Table II.

Table I. Chemical composition of the essential oil of *R. officinalis*.

Peak no.	Compound[a]	Retention time [min]	RI[b]	Content (%)
1	Tricyclene	4.93	922	0.44
2	α-Thujene	4.98	924	0.07
3	α-Pinene	5.20	932	9.61
4	Camphene	5.63	949	2.81
5	2,4(10)-Thujadien	5.70	951	0.13
6	β-Pinene	6.38	977	2.54
7	3-Octanone	6.52	983	0.15
8	β-Myrcene	6.83	989	8.56
9	α-Phellandrene	7.20	1006	0.16
10	α-Terpinene	7.54	1016	0.38
11	*o*-Cymene	7.81	1023	2.59
12	Limonene	7.98	1028	2.52
13	1,8-Cineole	8.12	1032	7.97
14	γ-Terpinene	8.96	1055	1.03
15	*trans*-Sabinene hydrate	9.41	1068	0.10
16	Terpinolene	9.96	1083	0.87
17	β-Linalool	10.58	1100	2.51
18	Nonanal	10.73	1104	0.03
19	Chrysanthenone	11.38	1120	0.69
20	Camphor	12.59	1148	18.91
21	Isopulegol	12.89	1157	0.08
22	Pinocamphone	12.96	1158	0.11
23	Pinocarvone	13.04	1160	0.30
24	Borneol	13.51	1172	2.52
25	Iso-pinocamphone	13.62	1174	0.33
26	1-Terpinen-4-ol	13.83	1180	1.45
27	*p*-Cymene-8-ol	14.24	1190	4.08
28	Verbenone	15.13	1211	11.32
29	β-Citronellol	15.79	1226	0.10
30	*R*-(+)-Pulegone	16.23	1236	0.07
31	*cis*-Myrtanol	16.61	1245	0.11
32	*p*-Menth-1-en-3-one	16.89	1252	0.09
33	*n*-Decanol	17.77	1272	0.15
34	Bornyl acetate	18.21	1282	1.57
35	Thymol	18.58	1291	0.14
36	Carvacrol	18.88	1298	0.21
37	4-Allyl-guaiacol	21.05	1349	0.07
38	α-Ylangene	21.75	1365	0.16
39	α-Copaene	22.02	1371	0.06
40	1-Undecanol	22.09	1373	0.16
41	*cis*-Jasmone	22.78	1389	0.24
42	Methyl-eugenol	23.17	1398	0.14
43	β-Caryophyllene	23.90	1416	5.10

Table I (continued).

Peak no.	Compound[a]	Retention time [min]	RI[b]	Content (%)
44	Calarene	24.26	1425	0.03
45	Aromadendrene	24.59	1433	0.02
46	α-Humulene	25.32	1450	1.16
47	γ-Muurolene	26.13	1470	0.33
48	γ-Curcumene	26.29	1472	0.04
49	Zingiberene	26.95	1490	0.11
50	α-Muurolene	27.10	1494	0.10
51	β-Bisabolene	27.53	1504	0.34
52	γ-Cadinene	27.66	1508	0.19
53	δ-Cadinene	27.89	1514	0.45
54	β-Sesquiphellandrene	28.14	1520	0.13
55	Caryophyllene oxide	30.33	1576	1.15
56	Humulene epoxide II	31.39	1603	0.21
57	*Epi*-Cadinol	32.65	1637	0.11
58	Methyl jasmonate	32.74	1639	0.26
59	α-Cadinol	33.18	1651	0.72
60	α-Bisabolol	34.37	1683	1.92
61	Hexadecanol	41.30	1879	0.05
62	Ferruginol	54.46	2310	0.11
	Total of identified compounds			98.06
	Monoterpene hydrocarbons			31.69
	Oxygenated monoterpenes			48.23
	Aromatic oxygenated monoterpenes			5.36
	Sesquiterpenes			8.02
	Oxygenated sesquiterpenes			4.11
	Aliphatic compounds			0.65

[a] Compounds listed in order of elution from a DB-5MS column.
[b] RI, retention indices relative to C_9–C_{24} *n*-alkanes on a DB-5MS column.

Table II. Minimum inhibitory concentration values [μg mL^{-1} (mM)] against oral pathogens for the essential oil obtained from *R. officinalis* as well as for its major compounds.

Sample	Microorganism					
	E. faecalis	*S. salivarius*	*S. sanguinis*	*S. mitis*	*S. mutans*	*S. sobrinus*
Essential oil	> 2000	600	> 2000	> 2000	> 2000	500
Camphor	> 2000 (> 13.14)	400 (2.63)	400 (2.63)	300 (1.97)	1500 (9.85)	1500 (9.85)
Verbenone	> 2000 (> 13.31)	400 (2.62)	400 (2.62)	300 (6.57)	1000 (2.00)	1000 (6,57)
α-Pinene	> 2000 (> 14.68)	400 (2.94)	400 (2.94)	400 (2.94)	2000 (14.68)	1000 (7.34)
β-Myrcene	> 2000 (> 14.68)	400 (2.94)	1500 (11.01)	400 (2.94)	400 (2.94)	2000 (14.68)
1,8-Cineole	> 2000 (12.96)	400 (2.59)	400 (2.59)	300 (1.94)	1500 (9.72)	1500 (9.72)
β-Caryophyllene	> 2000 (> 9.79)	400 (1.96)	400 (1.96)	300 (1.47)	300 (1.47)	400 (1.96)
Chlorhexidine[a]	0.37 (0.00073)	0.09 (0.00018)	0.74 (0.0015)	0.37 (0.00073)	0.09 (0.00018)	0.09 (0.00018)

[a] Positive control.

Discussion

Several antibiotics such as ampicillin and chlorhexidine among others have been very effective in preventing dental caries (Chung *et al.*, 2006; Tsui *et al.*, 2008). However, various adverse effects such as tooth and restoration staining, increasing of calculus formation, diarrhea, and disarrangements of the oral and intestinal flora have been associated with the use of these chemicals (Chung *et al.*, 2006; More *et al.*, 2008). Recent studies have demonstrated the great importance of natural products, both plant extracts and isolated compounds, as natural antibacterial agents in oral care products (Ambrosio *et al.*, 2008; Cunha *et al.*, 2007; Porto *et al.*, 2009). Essential oils and their components have been recently investigated more thoroughly as promising agents for the prevention or treatment of dental plaque-related diseases (Botelho *et al.*, 2007; Rasooli *et al.*, 2008).

The essential oil obtained from rosemary displayed low antibacterial activity toward the selected microorganisms in the case of the undertaken protocol. The results obtained with the microorganism *S. mutans* were different from those reported by Rasooli *et al.* (2008). The different composition and concentrations of the components present in the rosemary oil are probably the causes that would explain this contrast. On the other hand, in the present study, the pure major compounds were more active than the essential oil. However, the MIC values for pure compounds were much lower than those obtained for chlorhexidine. Among all the microorganisms, the pathogen *S. mitis* was the most susceptible and *E. faecalis* was the most resistant to the evaluated samples.

Previous studies on the essential oil obtained from *R. officinalis* revealed that it displays antimicrobial activity (Bozin *et al.*, 2007; Santoyo *et al.*, 2005). Among the major compounds identified in our study, some were previously reported to have antimicrobial activity, including 1,8-cineole (Mazzanti *et al.*, 1998), verbenone (Koutsoudaki *et al.*, 2005), α-pinene (Soković and Van Griensven, 2006), camphor (Zuzarte *et al.*, 2009), myrcene (O'Bryan *et al.*, 2008), and β-caryophyllene (Ugur *et al.*, 2009). However, to the best of our knowledge, this is the first report on the antimicrobial activity of the major components of rosemary oil against several oral pathogens. In our study, the essential oil of *R. officinalis* has lower antibacterial activity compared to its major components. In other studies, it is suggested that minor components of the essential oil may contribute to an antagonistic effect on the antimicrobial activity (Botelho *et al.*, 2007). According to the literature, as typical lipophiles, this essential oil and its constituents pass through the cell wall and cytoplasmic membrane, disrupting the structure of the different layers of polysaccharides, fatty acids, and phospholipids in the cell, thereby leading to cell permeabilization. Cytotoxicity appears to include such membrane damage, but the mode of action depends largely on the chemical composition of the essential oil as well as on the bacterial strain (Bakkali *et al.*, 2008).

In conclusion, the obtained results have demonstrated that the essential oil from *R. officinalis* as well as its major compounds were not active against some important oral pathogens. Nevertheless, further studies with other essential oils are in progress to identify promising compounds that could be used as lead to develop natural anticaries agents.

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