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ORIGINAL ARTICLE

Hyptis pectinata essential oil: chemical composition and anti-*Streptococcus mutans* activity

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OBJECTIVES: The aim of the present study was to evaluate the anti-*Streptococcus mutans* activity of *Hyptis pectinata* essential oil, and present its promising potential against oral diseases.

MATERIALS AND METHODS: The essential oil of *H. pectinata* was obtained by hydrodistillation from dried leaves and analyzed by GC/MS. The effectiveness of this essential oil regarding the antimicrobial activity against several *S. mutans* strains was investigated by the agar diffusion and microdilution methods, and chlorohexidine was used as a standard control.

RESULTS: The *H. pectinata* essential oil exhibited considerable inhibitory effect against either all the clinical isolates obtained from patients' saliva or the ATCC strains tested, with minimum inhibitory and bactericidal concentrations of 200 μg ml^{-1}. The study also compared the efficiency of the emulsifying agents Tween 20, Tween 80, dimethyl sulfoxide and propylene glycol in *H. pectinata* essential oil when tested against *S. mutans*. The data obtained confirmed the better inhibitory effect of the oil when using all tested diluents, although Tween 80 seemed to be more suitable for emulsification.

CONCLUSION: According to our results, *H. pectinata* essential oil can be considered a promising alternative to chlorhexidine for the control of oral bacteria-related diseases and hygiene.

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Keywords: *Hyptis pectinata*; essential oil; *Streptococcus mutans*; Tween; DMSO; antimicrobial activity

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Introduction

The most common type of oral disease, dental caries, may significantly impact a person's overall health, and it seems to occur when the normal balance between the microorganisms and the host is disturbed in some way. Dental caries is a multifactorial infection disease, usually associated with increased numbers of *Streptococcus mutans* at the site of the disease. Estimation of the salivary levels of this organism may be useful for assessing caries risk in patients and for monitoring their response to preventive measures (Hardie, 1992).

Streptococcus mutans is an important component of the biofilms on teeth (dental plaque) associated with many forms of dental caries. This bacterium rapidly metabolizes dietary carbohydrates, resulting in the formation of acid end products that can contribute to the demineralization of tooth enamel during caries development (McNeill and Hamilton, 2004).

Numerous long-term studies, including some with *S. mutans*, have demonstrated the effectiveness of mouth rinses containing antimicrobial active ingredients, such as chlorhexidine, and essential oil, in preventing and controlling both supragingival plaque and gingivitis, when used adjunctively to mechanical oral hygiene regimens (Fine *et al*, 2000). Antimicrobial mouth rinses have a variety of therapeutic and cosmetic clinical uses, which are primarily dependent upon the ability of the products to decrease the quantity and pathogenicity of the oral microbiota (Fine *et al*, 2005).

Essential oils are odorous, volatile products of plant secondary metabolism, found in many leaves and stems. They have been formulated into several over-the-counter oral hygiene products, and the efficacy of mouth rinses containing essential oil has been reported since the 1890s, and, concerning oral microorganisms, they proved to be beneficial and safe on daily oral health routines (Bispo *et al*, 2001; Alviano *et al*, 2005). On the other hand, chlorhexidine is one of the most widely used biocides in antiseptic products, in both hand

washing and oral products, particularly effective against gingivitis and periodontitis. However, there are side effects of chlorhexidine treatment such as an objectionable taste, tooth discoloration, and desquamation and soreness of the oral mucosa. Recent reports suggested that chlorhexidine was ineffective against dental caries in clinical trials, and it has been implicated as the potential cause of the selection and persistence of bacteria with low level antibiotic resistance (Filoche *et al*, 2005).

Hyptis pectinata (L.) Poit (Lamiaceae), popularly known in Brazil as 'sambacaitá', or 'canudinho', is a herbaceous plant with aromatic leaves. In folk medicine, this plant is recommended for several conditions, among them rhinopharyngitis, nasal congestion, certain skin diseases (Malan *et al*, 1988), gastric disorders and fever, and bacterial infections (Rojas *et al*, 1992; Bispo *et al*, 2001). *Hyptis pectinata* has also been extensively used as mouth rinses by the population in the state of Sergipe, Brazil.

Although some investigations of the various activities of *H. pectinata* have been made, no references were found concerning the essential oil antibacterial activity against *S. mutans*. As the development of new therapies for the treatment of oral diseases is of great relevance, the purpose of the present work was to determine the chemical composition and evaluate the antimicrobial activity of the essential oil of *H. pectinata*, cultivated in Sergipe State, Brazil, against clinical isolates and reference strains of *S. mutans*. Additionally, when performing these tests, the study also compared the efficiency of some emulsifying agents for the adequate dispersion of this essential oil on *in vitro* antimicrobial tests.

Materials and methods

Plant material

The culture of *H. pectinata* was established at the Experimental Farm from Federal University of Sergipe (UFS), Sergipe, Brazil. A voucher specimen was deposited at the UFS herbarium (registry number 7454). Leaves of *H. pectinata* were dried in an oven (40°C) with air renewal and circulation until complete dehydration and coarsely ground into powder.

Essential oil extraction and analysis

The essential oil was obtained by hydrodistillation (3 h) of *H. pectinata* leaves in a Clevenger-type apparatus, until the condensing oil could no more be seen. The essential oil was separated from the aqueous solution (hydrolate), dried with anhydrous Na_2SO_4 (yield 0.5% v/w), transferred into an amber glass flask and kept at a temperature of −10°C until used (Guenther, 1972). Oil sample analysis was performed on a Shimadzu QP5050A gas chromatograph (SHIMADZU, São Paulo, Brazil) interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column DB5 (30 cm × 0.25 mm i.d., composed of 5% phenylmethylpolysiloxane), connected to an ion trap detector operating in Electron Impact mode at 70 eV; He as carrier gas, flow rate of 1 ml min^{-1}; split mode, ratio of 1:5; injection volume of 0.5 μl (in CH_2Cl_2); injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 80°C (isothermal for 2 min), with an increase of 3°C min^{-1}, to 180°C, then 10°C min^{-1} to 300°C, ending with a 10-min isothermal at 300°C. The calculation of the retention indexes was made through co-injection with an n-alkane series (Baydar *et al*, 2004). Identification of the oil constituents was made based on the retention indexes (McNeill and Hamilton, 2004), and by comparison of mass spectra with computer search using NIST21 and NIST107 libraries. The concentrations of the compounds were calculated from the GC peak areas and they were arranged in order of GC elution (Adams, 1995).

Streptococcus mutans *isolation from saliva*

The saliva samples were obtained from 10 dentate volunteers (males and females, age range 20–35 years), with a catalogued high standard of oral hygiene and gingival health. Volunteers with fixed or removable appliances, or dental prosthesis, were excluded, as well as those with any medical or pharmacological history that could compromise the conducting of the study (Pitten and Kramer, 1999). The unstimulated saliva samples (2 ml) were collected at the odontological clinic of the University Hospital/UFS, and taken to the laboratory in a sterile vial maintained in ice, where they were processed immediately.

As a representative microorganism of the oral microbiota, *S. mutans* was used in this study, mainly because of its involvement in caries diseases. For the *S. mutans* isolation from saliva, the samples were streaked on the selective medium Mitis salivarius agar (Difco, Detroit, MI, USA) added with bacitracin (0.2 U ml^{-1}) and 20% sucrose, and incubated at 37°C for 72 h under microaerophilic conditions (Gold *et al*, 1973). Typical *S. mutans* colonies obtained in the selective media were then cultivated on a Columbia blood agar base (Merck, Darmstadt, Germany), with the addition of 5% desfibrinated sheep blood under the same conditions, and after growth they were stocked in freezer at −80°C. Nine clinical isolates of *S. mutans* were obtained and two ATCC (10449 and 27175) strains were also included in the experiments.

Antibacterial activity of essential oil against S. mutans*: diffusion method*

Bacterial cells growing in Mitis salivarius medium were centrifuged at 1500 *g* 4°C^{-1} 15 min^{-1} and washed twice in phosphate-buffered saline (pH 7.2). An aliquot (100 μl) of a 10^5 cells ml^{-1} suspension was streaked on brain heart infusion (BHI; Difco) agar plates and, after 10 min, 20 μl of pure and diluted essential oil in different dispersion solvents (as shown in Table 1), were dropped on the medium surface. Likewise, 20 μl of each different solvent were also tested to confirm their lack of antibacterial activity, while chlorhexidine was used as a positive control (Table 2). The plates were refrigerated for 2 h in order to delay bacterial growth and facilitate diffusion of the substances, and then incubated at 37°C

Table 1 Chemical composition of *Hyptis pectinata* essential oil

Peak no.	*RT (min)*	*Compound*	*%*	*RRI exp.*[a]	*RRI lit.*[b]
1	4.050	α-Pinene	1.39	937	939
2	4.700	1-Octen-3-ol	2.23	975	978
3	4.817	β-Pinene	6.95	982	980
4	5.867	Limonene	2.06	1030	1031
5	7.550	Linalool	1.54	1099	1098
6	16.375	α-Copaene	2.40	1381	1376
7	16.867	β-Elemene	2.15	1396	1391
8	17.800	β-Caryophyllene	28.34	1425	1418
9	18.900	α-Caryophyllene	1.50	1459	1454
10	19.758	Germacrene-D	3.07	1486	1480
11	20.050	Guaiene	3.76	1495	1490
12	20.258	Bicyclogermacrene	1.49	1502	1494
13	20.525	β-Bisabolenel	1.17	1510	1509
14	20.758	γ-Cadinene	1.75	1518	1513
15	21.025	Trans-Calamene	2.40	1527	1532
16	21.958	Epi-Longipanool	1.07	1558	1561
17	22.708	Globulol	1.24	1583	1583
18	22.892	Caryophyllene oxide	28.00	1589	1581
19	23.683	Not detected	1.17	1618	–
20	23.833	Cubenol	1.66	1621	1614
21	25.767	Not detected	4.68	1687	–

[a]IRR exp. (Relative retention index calculated applying Van den Dool H, Kratz PD (1963). J Chrom 11: 463).
[b]Comparative relative retention time originally data reported by Adams (1995).

Table 2 Preparations used in the assay

Solutions	*Description*
Control	
+	Chlorhexidine 0.12% oral solution (Periogard, Colgate-Palmolive®, São Paulo, Brazil)
–	Canola oil solution diluted 1:1 in Tween 80, Tween 20, DMSO and glycol propylene (1% in distilled water)
Test	Essential oil of *Hyptis pectinata*, pure and diluted 1:1 in: Tween 80, Tween 20, DMSO and propylene glycol (1% in distilled water)
	Hydrolate of *H. pectinata*

for 48 h under microaerophilic conditions, after which the diameter (mm) of inhibition zones was measured (Hili *et al*, 1997).

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

Inhibition of *S. mutans* growth was determined using a microdilution assay in sterile 96-well microtiter plate (Filoche *et al*, 2005). The *H. pectinata* essential oil was tested at concentrations ranging from 0.1 to 10 mg ml^{-1} (diluted in Tween 80). Each well contained 100 μl twofold serially diluted essential oil, 100 μl double strength BHI growth medium, and 20 μl of an overnight culture of *S. mutans*, representing, approximately, 5×10^6 cells ml^{-1}. The controls comprised pure growth medium and inoculated growth medium without test agent. The results were based on *S. mutans* visual growth, which were confirmed spectrophotometrically (at 600 nm), after 24 and 48 h using a microtiter plate reader (Awareness Technology Inc., Palm City, FL, USA). The plate was shaken for 30 s prior to each reading.

The MIC was defined as the minimum concentration of the test agent limiting turbidity to <0.06 OD at 600 nm, which represented complete growth inhibition or, at least, growth inhibition $>80\%$, compared to the growth of the untreated cultures. To assess whether the action of essential oil was bacteriostatic or bactericidal (MBC) to *S. mutans*, 20 μl of the bacterial culture resuspended in 200 μl of sterile saline was taken from concentrations equal to or higher than MIC. This volume was streaked on BHI agar plates and incubated at 37°C for 48 h. No growth at concentrations higher than or equal to the MIC indicated bactericidal action. Control tests were run simultaneously by adding solvents, without the essential oil. All experiments were made in duplicate and average values were calculated.

Results

Essential oil analysis

In the present study the composition and relative percentages of the essential oil of *H. pectinata* cultivated in Sergipe State, in the northeast region of Brazil, was determined (Table 1). Twenty major constituents were identified with high contents of caryophyllene (28.34%), caryophyllene oxide (28%) and β-pinenes (6.95%), respectively.

Susceptibility of S. mutans *strains to* H. pectinata *essential oil and the effects of diluents*

Nine strains of *S. mutans* were isolated from the saliva of the 10 volunteers, after growth in the selective medium, Mitis salivarium. Tests for *in vitro* studies for the antimicrobial activity of essential oil of *H. pectinata* against these strains, as well as the two ATCC reference strains (Table 3), have shown that the essential oil, pure or diluted in emulsifying agents Tween 20, Tween 80, DMSO (dimethyl sulfoxide) and propylene glycol (1:1), inhibited all strains tested to different extents. The profile of inhibition was similar to that observed for the positive control, chlorhexidine. The mean values for the inhibition zones diameter produced varied from 10 to 18.5 mm. No inhibition zone was observed when canola oil or hydrolate of *H. pectinata* (negative controls) were tested.

MIC and MBC of H. pectinata *essential oil controlling the growth of* S. mutans *strains*

The MIC was 200 μg ml^{-1} for all clinical strains tested. This value was also the same obtained for the MBC.

Discussion

It has been demonstrated that mouth rinses with essential oils can be a beneficial, safe component of daily oral health routines (Claffey, 2003), but the antimicrobial activity and selective toxicity of these preparations, as well as their chemical composition, are poorly known.

Table 3 Inhibition halo diameter of *Streptococcus mutans* induced by *Hyptis pectinata* essential oil pure and diluted in different diluents (DMSO, Tween 20 and Tween 80 and propylene glycol 1%) in drop tests

		Tested strains (halo formation in mm)										
		ATCC		*Clinical strains*								
Diluents	*Oil dilution*	*10449*	*25175*	*S 1*	*S 2*	*S 3*	*S 4*	*S 5*	*S 6*	*S 7*	*S 8*	*S 9*
–	–	12	13	12	10	14	12	15	14	11	12	13
DSMO	1:1	11	14	11	14	14	15	14	15	12	11	15
Tween 20	1:1	16	16	12	14	16	17	14	18	12	16	15
Tween 80	1:1	16	15	14	17	16	14	12	18	18	14	20
Propylene glycol	1:1	12	14	13	12	12	14	15	15	11	12	12
Controls												
Hydrolate of *H. pectinata*		–	–	–	–	–	–	–	–	–	–	–
Canole oil		–	–	–	–	–	–	–	–	–	–	–
Chlorhexidine		13	15	13	15	13	14	13	15	16	15	16

The main constituents in the essential oil of *H. pectinata* cultivated in Sergipe State, used in this study, were characterized by high concentrations of β-caryophyllene, caryophyllene oxide, and β-pinene. In contrast to the present findings, studies on the essential oil from *H. pectinata*-cultivated plants originating from western Africa showed cymene and thymol as the major compounds (more than 60%), while caryophyllene, caryophyllene oxide and β-pinene were present only as trace constituents (Malan *et al*, 1988). This finding strongly suggests that the high content of sesquiterpens in the sample analyzed by our group is probably due to environmental conditions, as both climate and soil are very different, supporting the existence of diverse chemotypes of these species. Indeed, it is well known that the chemical composition of essential oils depends on climatic, seasonal and geographic conditions, as well as the harvest period and the distillation technique (Perry *et al*, 1999).

Essential oils rich in phenolic compounds (or terpenes), such as caryophyllene and pinenes, which were detected in high amounts in our samples, are widely reported to possess high levels of antimicrobial activity (Baydar *et al*, 2004). In addition, their antibacterial activity depends on the type, composition and concentration of the essential oil, the type and concentration of the target microorganism and the processing and storage conditions (Hammer *et al*, 1999; Marino *et al*, 2001; Baydar *et al*, 2004). Tellez *et al* (2000) studied the composition and biologic activities of essential oil from *Callicarpa americana* and demonstrated that constituents such as sesquiterpenes, caryophyllene, and caryophyllene oxide proved to have good antifungal activity (Cheng *et al*, 2005). The inherent activity of the oil can be expected to be related to the chemical configuration of the components, the proportions in which they are present, and the interactions between them (Burt, 2004). Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components mixed (Gill *et al*, 2002; Burt, 2004), which suggests that the minor components are critical to the activity, and may have a synergistic effect or enhancing influence.

Kalemba and Kunicka (2003) showed that the susceptibility of a given microorganism to essential oils depends, first, on the properties of the essential oil and the microorganism itself. Factors that may contribute to divergent results include differences between the types and numbers of bacterial isolates tested in each study, and the methods used, including the criteria for determining MICs. Recent isolates may exhibit an increased resistance to antimicrobial compounds, which possibly derives from their interaction with host cells.

Clinical studies were undertaken to test the essential oil bioactivity of an exotic plant *Lippia mulflore*. It was proved that its oil was highly effective against some ATCC reference strains such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermides*, *Escherichia coli* and *Klebsiella pneumoniae,* as well as against some oral microbiota isolates (Kalemba and Kunicka, 2003).

In the present study, we used multiple isolates of *S. mutans* from human saliva and ATCC control strains. By using the microdilution method, the essential oil of *H. pectinata* showed good efficacy against all the *S. mutans* strains tested, and the results of the MICs found with the clinical strains were similar to those of the reference strains (200 μg ml^{-1}). This concentration was also bactericidal against all strains of *S. mutans* selected for this study independently of their origin.

An emulsifier, or solvent, is generally used to dissolve the essential oil, or to stabilize it in water-based culture media. However, a number of researchers found its use doubtful (Burt, 2004). In this sense, the efficiency of several emulsifying agents was tested. Generally, the use of the emulsifier, tested in the dilution of 1:1 resulted in a better inhibitory effect of the essential oil against all tested strains when compared with the concentrated oil. Therefore, the emulsification of the *H. pectinata* essential oil may facilitate its dispersion in the agar diffusion method, assuring better results. Our results contrasted with those described by other authors who found that the use of solvents and detergents decreased the antibacterial effects of the essential oil (Burt, 2004).

The effectiveness of alternative methods for controlling bacterial growth is extremely important nowadays.

Species are developing drug resistance and there is resurgence in the use of natural alternative therapies instead of synthetic pharmaceuticals, often with many side effects. Oral hygiene has a direct effect on oral health and is based on mouth rinse as a corrective treatment and on reduction and/or elimination of bacterial accumulations on the teeth surface and between teeth by daily tooth brushing and frequent dental cleaning or prophylaxes (Loesche and Grossman, 2001). In Brazil, ethnopharmacological studies have shown the use of several plants, including *H. pectinata*, as mouth washes in popular medicine for treating oral infections (MF Arrigoni-Blank, unpublished data).

The results of this preliminary study, focused on the essential oil of *H. pectinata*, tend to reinforce the use of this plant as an anti-infective agent in folk medicine (Pereda-Miranda *et al*, 1993). The use of this plant extract by inhabitants of financially unprivileged regions could represent an efficient and unrestricted alternative to prevent and control oral infections in underdeveloped areas. Such findings should be especially relevant for local inhabitants of 'caatinga' in the northeast region of Brazil, where this plant is abundant. As this essential oil is not toxic for mammalian cells (MF Arrigoni-Blank, unpublished data), its use in rinse or gel preparations produced by pharmaceutical industries certainly has a clinical application in the treatment of oral diseases. However, additional tests, including experimental models and pharmacological applicability, are required before considering the essential oil of *H pectinata* a real promising compound.

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