



Chemical Composition and Antibacterial Activity of the Essential Oil of *Vitex agnus-castus* L. (Lamiaceae)

REGIANE GONÇALVES¹, VANESSA F.S. AYRES¹, CARLOS E. CARVALHO¹, MARIA G.M. SOUZA², ANDERSON C. GUIMARÃES¹, GEONE M. CORRÊA¹, CARLOS H.G. MARTINS², RENATA TAKEARA¹, ELIANE O. SILVA³ and ANTÔNIO E.M. CROTTI⁴

¹Instituto de Ciências Exatas e Tecnológicas, Universidade Federal do Amazonas, Rua Nossa Senhora do Rosário, 3863, Tiradentes, 69103-128 Itacotiara, AM, Brazil

²Núcleo de Pesquisas em Ciências Exatas e Tecnológicas, Universidade de Franca, Av. Dr. Armando Salles de Oliveira, 201, Parque Universitário, 14404-600 Franca, SP, Brazil

³Departamento de Química Orgânica, Instituto de Química, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Ondina, 40170-115 Salvador, BA, Brazil

⁴Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, Monte Alegre, 14040-901 Ribeirão Preto, SP, Brazil

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ABSTRACT

Abnormal multiplication of oral bacteria causes dental caries and dental plaque. These diseases continue to be major public health concerns worldwide, mainly in developing countries. In this study, the chemical composition and antimicrobial activity of the essential oil of *Vitex agnus-castus* leaves (VAC-EO) collected in the North of Brazil against a representative panel of cariogenic bacteria were investigated. The antimicrobial activity of VAC-EO was evaluated in terms of its minimum inhibitory concentration (MIC) values by using the broth microdilution method in 96-well microplates. The chemical constituents of VAC-EO were identified by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). VAC-EO displayed some activity against all the investigated oral pathogens; MIC values ranged from 15.6 to 200 µg/mL. VAC-EO had promising activity against *Streptococcus mutans* (MIC= 15.6 µg/mL), *Lactobacillus casei* (MIC= 15.6 µg/mL), and *Streptococcus mitis* (MIC= 31.2 µg/mL). The compounds 1,8-cineole (23.8%), (*E*)-β-farnesene (14.6%), (*E*)-caryophyllene (12.5%), sabinene (11.4%), and α-terpinyl acetate (7.7%) were the major chemical constituents of VAC-EO. VAC-EO displays antimicrobial activity against cariogenic bacteria. The efficacy of VAC-EO against *S. mutans* is noteworthy and should be further investigated.

Key words: *Streptococcus mutans*, 1-8-cineole, cariogenic bacteria, oral pathogens.

INTRODUCTION

Acidogenic and aciduric bacteria that can form colonies in an organized biofilm cause dental plaque

(Crevelin et al. 2015). Brushing and flossing are the most effective strategies to remove microorganisms installed on the tooth surface and hence prevent caries and gum diseases. Controlling the formation of biofilm and completely removing the biofilm through mechanical means is complicated, and most of the

Correspondence to: Antônio Eduardo Miller Crotti
E-mail: millercrotti@ffclrp.usp.br

population fails to ensure proper oral care (Melo et al. 2015). Some products containing antimicrobial agents have been used as a complementary measure to help to decrease the formation of biofilm on the tooth surface (Crevelin et al. 2015). Chlorhexidine has been widely applied for this purpose because it displays anticariogenic properties. However, its regular use in oral care products often leads to several side effects and contributes to the emergence of microbial resistance (Aguilar et al. 2013a).

Vitex agnus-castus L. (Lamiaceae) is a small tree or shrub native to European, Mediterranean, and Central Asian countries. In Brazil, European immigrants introduced *V. agnus-castus* into the Amazon region (Zoghbi et al. 1999). This plant has well-known biological potential. Its diuretic, antiseptic, digestive, antifungal, anti-anxiety, aphrodisiac, anti-estrus, emmenagogue, antispasmodic, aperitif, and analgesic effects have been applied in folk medicine. *V. agnus-castus* has also been employed to treat mastopathies, to inhibit dopamine D2 and opioid receptors, and to inhibit the synthesis of prolactin, among other uses (Zahid et al. 2016). Moreover, this species is one of the most traditional and effective medicinal plants to be used in the relief of uterine cramps and in the regulation of menstruation (Gurmeet and Chhabra 2011, Mari et al. 2012). The essential oil (EO) extracted from the seeds of *V. agnus-castus* presents antimicrobial activity against *Candida* species that cause nosocomial infections (Asdadi et al. 2015), whereas the EO extracted from the leaves of this same plant species acts against some important plant pathogens such as *Agrobacterium tumefaciens*, *Erwinia carotovora* var. *carotovora*, *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Fusarium solani* (Badawy and Abdelgaleil 2014). The EO extracted from the fruits and leaves of *V. agnus-castus* exerts activity against *Salmonella typhimurium* and *Micrococcus flavus* (Stojković et al. 2011). However, the antimicrobial

activity of the EO of *V. agnus-castus* against oral pathogens has not been investigated yet.

As part of our ongoing project on the antimicrobial activity of essential oils (Aguilar et al. 2013a, Alves et al. 2015, Bardaji et al. 2015, Crevelin et al. 2015, Melo et al. 2015), we have investigated the chemical constituents and the antimicrobial activity of the EO of *Vitex agnus-castus* grown in the North of Brazil against a representative panel of cariogenic bacteria.

MATERIALS AND METHODS

PLANT MATERIAL

Vitex agnus-castus L. (Lamiaceae) was collected near the city of Itacotiara (58°26'54.3"S 3°8'28.8"W), state of Amazonas, Brazil in June 2009 and identified by Prof. Ari de Freitas Hidalgo (Universidade Federal do Amazonas, Brazil). A voucher specimen (HUAM 8268) was deposited at the Herbarium of Universidade Federal do Amazonas, Manaus, Brazil (Herbarium UFAM).

EXTRACTION OF THE ESSENTIAL OIL

Fresh *V. agnus-castus* leaves (1,500 g) were submitted to hydrodistillation in a Clevenger-type apparatus for 3 h. Before the extraction, the plant material was divided into three samples of 500 g each, and 500 mL of distilled water had been added to each sample. The essential oil of *V. agnus-castus* (VAC–EO) was manually collected, and traces of water were removed by addition of anhydrous sodium sulfate. After filtration, VAC–EO was stored at 4 °C, in the dark, until analysis. The yield (w/w) of VAC–EO was calculated from the weight of the fresh leaves and expressed as the average of triplicate analysis.

GAS CHROMATOGRAPHY (GC) AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSES

The chemical constituents of VAC–EO were identified using previously published methodology

(Tonuci et al. 2012, Aguiar et al. 2013b, Baldin et al. 2015, Melo et al. 2015, Wakabayashi et al. 2015, Vieira et al. 2017). To this end, ethyl ether was used as solvent, and the samples were analyzed on a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with FID and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30 m x 0.25-mm i.d.; 0.25 μ m film thickness) was employed. The temperature of the column was programmed to rise from 60 to 240 °C at 3 °C/min and was then held at 240 °C for 5 min. Helium (99.999%) at a flow rate of 1.0 mL/min was used as carrier gas. The injected sample volume was 0.1 μ L (split ratio of 1:10), and the temperatures of the injector and the detector were 240 and 280 °C, respectively. The relative concentrations of the chemical components of VAC–EO were obtained by normalization of the peak areas (%). The relative areas were the average of triplicate GC-FID analyses.

A Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler was used during the GC-MS analyses. The Rtx-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30 m length x 0.25 mm i.d. x 0.25 μ m film thickness) was employed for the analyses. The electron ionization mode was used at 70 eV. The carrier gas was helium (99.999%) at a flow rate of 1.0 mL/min, and the injection volume was 0.1 μ L (split ratio of 1:10). The temperatures of the injector and the ion source were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC–FID. The mass spectra were taken with a scan interval of 0.5 s, from 40 to 600 Da. The chemical components of VAC–EO were identified on the basis of their arithmetic retention indices on an Rtx-5MS capillary column under the same operating conditions used for GC–FID, relative to a homologous series of *n*-alkanes (C₈-C₂₀) (Van Den Dool and Kratz 1963). The

structures were computer-matched with the Wiley 7, NIST 08, and FFNSC 1.2 spectral libraries, and their fragmentation patterns were compared with literature data (Adams 2007).

BACTERIAL STRAINS AND ANTIMICROBIAL ASSAYS

The *in vitro* antimicrobial activity of VAC–EO was determined by minimum inhibitory concentration (MIC) assays based on the broth microdilution method (CLSI 2009). *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Lactobacillus casei* (ATCC 11578), and *Enterococcus faecalis* (ATCC 4082) were the standard strains used in the assay. Initially, the bacteria were transferred to blood agar (Difco Labs, Detroit, Mich, USA), and individual 24-h colonies were suspended in 10.0 mL of tryptic soy broth (Difco). A spectrophotometer (Femto, São Paulo, Brazil) at a wavelength (λ) of 625 nm was used to standardize the suspensions of each microorganism, to match the transmittance of 81, equivalent to 0.5 in the McFarland scale (1.5 x 10⁸ CFU/mL). Dilution of the standardized suspension generated the final concentration of 5 x 10⁵ CFU/mL. VAC–EO was dissolved in DMSO (Merck, Darmstadt, Germany) at 16.0 mg/mL. Concentrations ranging from 4000 to 3.9 μ g/mL were achieved after dilution of VAC–EO in tryptic soy broth (Difco). After the dilutions, the concentrations of DMSO lay between 4% and 0.0039% (v/v). Negative controls, three inoculated wells containing DMSO at concentrations ranging from 4% to 1%, and one non-inoculated well free of antimicrobial agent were included. One inoculated well helped to test whether the broth was adequate for microorganisms to grow. The positive control was chlorhexidine dihydrochloride (CHD) (Sigma-Aldrich, St. Louis) at concentrations ranging from 5.9 to 0.115 μ g/mL, diluted in tryptic soy broth

(Difco). The microplates (96-well) were sealed with parafilm and incubated at 37 °C for 24 h. After that, 30 mL of an aqueous solution of 0.02% resazurin (Sigma-Aldrich, St. Louis, MO, USA) was added into each microplate well to indicate the viability of the microorganism (Palomino et al. 2002). The lowest concentration of the sample that inhibited the growth of microorganism (MIC value) was determined as the lowest concentration of VAC-EO that was able to prevent the color of the resazurin solution from changing (Sarker et al. 2007). All the assays were conducted in triplicate.

RESULTS

CHEMICAL CONSTITUENTS OF THE ESSENTIAL OIL OF VITEX AGNUS-CASTUS (VAC-EO)

Vitex agnus-castus L. (Lamiaceae) leaves furnished a yellowish essential oil (VAC-EO) in 0.09 ± 0.01 % yield (w/w). Table I lists the 29 compounds identified in this oil. GC-FID and GC-MS analyses revealed that monoterpenes (62.4%) were the main constituents of VAC-EO, whereas 1,8-cineole (**2**, 23.8%), (*E*)- β -farnesene (**5**, 14.6%), (*E*)-caryophyllene (**4**, 12.5%), sabinene (**1**, 11.4%), and α -terpinyl acetate (**3**, 7.7%) were the major constituents (Figure 1).

ANTIMICROBIAL ACTIVITY OF VAC-EO

The *in vitro* antibacterial activity (MIC values, see Table II) of VAC-EO was evaluated against

a representative panel of cariogenic bacteria and compared the results with those obtained with chlorhexidine dihydrochloride (CHD, positive control). According to the literature, MIC values lower than 100 $\mu\text{g/mL}$, between 100 and 500 $\mu\text{g/mL}$, and between 500 and 1000 $\mu\text{g/mL}$ correspond to promising, moderate, and weak activities, respectively, whereas MIC values greater than 1000 $\mu\text{g/mL}$ denote inactivity (Gibbons 2004, Rios and Recio 2005, Saleem et al. 2010). On the basis of these criteria, VAC-EO displayed moderate activity against *Streptococcus salivarius*, *Streptococcus sanguinis* and *Enterococcus faecalis* (MIC= 200 $\mu\text{g/mL}$), and *Streptococcus sobrinus* (MIC= 125 $\mu\text{g/mL}$). On the other hand, VAC-EO had promising activity against *Streptococcus mutans* (MIC= 15.6 $\mu\text{g/mL}$), *Lactobacillus casei* (MIC= 15.6 $\mu\text{g/mL}$), and *Streptococcus mitis* (MIC= 31.25 $\mu\text{g/mL}$).

DISCUSSION

The chemical composition of the essential oil extracted from *Vitex agnus-castus* collected in Egypt (Badawy and Abdelgaleil 2014), Turkey (Sarikurkcu et al. 2009, Duymuş et al. 2014), Morocco (Asdadi et al. 2015), Serbia (Stojković et al. 2011) and Brazil (Zoghbi et al. 1999) has been investigated previously. Although the content and composition of an essential oil may vary greatly among specimens collected from different places and in distinct seasons (Baser and Buchbauer

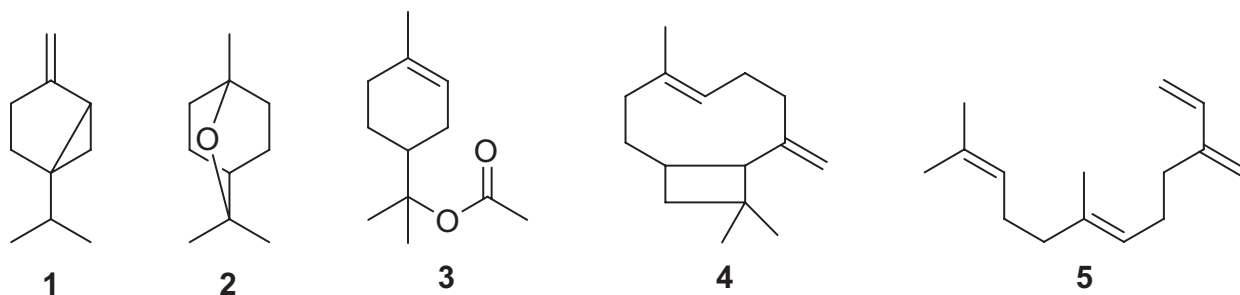


Figure 1 - Chemical structures of the monoterpenes sabinene (**1**), 1,8-cineole (**2**), α -terpinyl acetate (**3**), (*E*)-caryophyllene (**4**), and (*E*)- β -farnesene (**5**).

TABLE I
Chemical constituents of the essential oil of *Vitex agnus-castus* (VAC-EO).

Compound	RI _{exp} ¹	RI _{lit} ²	RA % ³	Identification ⁴
α -Thujene	924	931	0.2	RL MS
α -pinene	931	939	5.9	RL MS
Sabinene	971	976	11.4	RL MS
β -pinene	977	980	0.9	RL MS
myrcene	987	991	2.1	RL MS
1-phellandrene	1006	1139	0.6	RL MS
α -terpinene	1016	1018	0.3	RL MS
<i>p</i> -cymene	1023	1026	0.2	RL MS
limonene	1028	1031	2.8	RL MS
1,8-cineole	1031	1033	23.8	RL MS
(<i>E</i>)- <i>b</i> -ocimene	1043	1050	0.6	RL MS
γ -terpinene	1056	1062	0.7	RL MS
α -terpinolene	1084	1088	0.3	RL MS
Linalool	1100	1098	0.2	RL MS
4-terpineol	1179	1177	2.0	RL MS
α -terpineol	1194	1189	2.1	RL MS
α -terpinyl acetate	1345	1352	7.7	RL MS
Citronellyl acetate	1349	1354	0.6	RL MS
α -gurjunene	1402	1409	0.2	RL MS
(<i>E</i>)-caryophyllene	1415	1418	12.5	RL MS
(<i>E</i>)- β -farnesene	1452	1458	14.6	RL MS
Alloaromadendrene	1454	1461	0.6	RL MS
Bicyclogermacrene	1490	1494	6.6	RL MS
Sphatulenol	1571	1576	0.4	RL MS
Caryophyllene oxide	1575	1581	0.4	RL MS
Viridiflorol	1588	1590	0.2	RL MS
α -cadinol	1650	1653	0.2	RL MS
Hydrocarbon monoterpenes			26.0	
Oxygenated monoterpenes			36.4	
Hydrocarbon sesquiterpenes			34.5	
Oxygenated sesquiterpenes			1.2	
Total			98.1	

¹RI_{exp}: Retention index determined relative to *n*-alkanes (C₈-C₂₀) on the Rtx-5MS column; ²R_{lit}: retention index from ref. (Adams 2007); ³RA: relative area (peak area relative to the total peak area in the GC-FID chromatogram), average of three replicates; ⁴RL: comparison of RI_{exp} with the literature; MS: comparison of the mass spectra with those of the Wiley 7, NIST 08, and FFNSC 12 libraries.

TABLE II
Minimum inhibitory concentration (MIC) values ($\mu\text{g/mL}$)
of the essential oil of *Vitex agnus castus* (VAC–EO) against
selected cariogenic bacteria.

Microorganisms	VAC-EO	Chlorhexidine ¹
<i>Streptococcus mutans</i> (ATCC 25175)	15.6	1.8
<i>Streptococcus mitis</i> (ATCC 49456)	31.25	14.7
<i>Streptococcus sanguinis</i> (ATCC 10556)	200	7.4
<i>Streptococcus sobrinus</i> (ATCC 33478)	125	1.8
<i>Streptococcus salivarius</i> (ATCC 25975)	200	7.4
<i>Lactobacillus casei</i> (ATCC 11578)	15.6	3.7
<i>Enterococcus faecalis</i> (ATCC 4082)	200	14.7

¹Chlorhexidine dihydrochloride (positive control).

2010), few constituents have been identified in the essential oils of *Vitex agnus-castus* other than the monoterpenes 1,8-cineole, sabinene, α -pinene, and α -terpinyl acetate and the sesquiterpenes (*E*)- or (*Z*)- β -farnesene and (*E*)-caryophyllene. In the present study, we have detected 1,8-cineole, sabinene, α -terpinyl acetate, (*E*)-caryophyllene, and (*Z*)- β -farnesene as the major constituents of VAC–EO. The chemical composition of VAC–EO is very similar to the chemical composition of the essential oil extracted from a *V. agnus-castus* specimen collected in the North of Brazil (Zoghbi et al. 1999). However, the content of (*E*)-caryophyllene is considerably higher in VAC–EO.

Some reports have explained that the lipophilicity of the chemical constituents of essential oils is associated with their antimicrobial activity. The main lipophilic components of these oils are monoterpenes and sesquiterpenes (Vieira et al. 2017). Lipophilicity allows the components of essential oils to diffuse across the cell membranes easily and then affect the metabolic pathways or organelles of microorganisms. Essential oils can also inhibit the syntheses of DNA, RNA, proteins, and polysaccharides in bacterial cells (Abad et al.

2012). The correlation between the lipophilicity of the constituents of an essential oil and the antimicrobial activity of the oil has motivated us to investigate the antibacterial activity of some essential oils against cariogenic bacteria (Aguiar et al. 2013a, Bardaji et al. 2015, Crevelin et al. 2015). In this study, it was found that VAC–EO displays promising activity against *Streptococcus mutans* (MIC= 15.6 $\mu\text{g/mL}$), *Lactobacillus casei* (MIC= 15.6 $\mu\text{g/mL}$), and *Streptococcus mitis* (MIC= 31.25 $\mu\text{g/mL}$). This is remarkable because *S. mutans* is one of the primary causative agents of dental caries, and its inhibition by natural compounds is unusual (Saleem et al. 2010).

The essential oils from different parts of *V. agnus-castus* have been reported to display antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* (Görler et al. 1985, Ekundayo et al. 1990). Previous studies have associated the antibacterial activity of the essential oils of *Vitex* sp. to the content of 1,8-cineole because the activity of the essential oil increases with increasing content of 1,8-cineole (Nyiligira et al. 2004). Once inside microbial cell, 1,8-cineole might interact with enzymes and proteins in the membrane to reverse the flow of protons, which would affect cellular activity (Stojković et al. 2011). However, 1,8-cineole alone does not present significant activity (MIC > 1000 $\mu\text{g/mL}$, data not shown). In summary, the antimicrobial activity of VAC–EO may be correlated to other major components or even to minor chemicals that might underlie or even increase the activity of the major chemical constituents of this essential oil in a synergistic mechanism. However, although the antimicrobial activity of VAC–EO may be related to the lipophilicity of the monoterpenes thereof, the exact mechanism through which VAC–EO exerts its antimicrobial action is not clear and should be further investigated.

CONCLUSIONS

The findings presented here highlight that the essential oil of *Vitex agnus-castus* leaves (VAC–EO) displays interesting and promising antimicrobial activity against some important cariogenic bacteria, including *Streptococcus mutans*. In this sense, VAC–EO might be used as a promising component of new oral care products because *S. mutans* is one of the main causative agents of oral disorders, such as dental caries. Further studies to identify the active chemical constituents of VAC–EO and to determine its antimicrobial mechanism are underway.

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