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Research Paper

Volatile compounds of Lamiaceae exhibit a synergistic antibacterial activity with streptomycin

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Abstract

Bacterial infections cause thousands of deaths in the world every year. In most cases, infections are more serious because the patient is already weakened, and often, the bacteria are already resistant to the antibiotics used. Counterparting this negative scenario, the interest in medicinal plants as an alternative to the synthetic antimicrobial drugs is blossoming worldwide. In the present work, we identified the volatile compounds of ethanol extracts of *Melissa officinalis*, *Mentha sp*., *Ocimum basilicum*, *Plectranthus barbatus*, and *Rosmarinus officinalis* by gas chromatography/mass spectrometry (GC/MS). Also was evaluated antimicrobial activity of ethanol extracts against 6 bacteria of clinical interest, and was tested the interaction of these extracts with a commercial antibiotic streptomycin. Phytol was a compound identified in all extracts by GC/MS, being majoritary component in *Plectranthus barbatus* and *Rosmarinus officinalis*. The Gram-positive bacteria were more sensitive to ethanol extracts, and *Plectranthus barbatus* and *Rosmarinus officinalis* were the most active extracts. Ethanol extracts exhibited a synergetic effect with streptomycin. These results encourage additional studies, in order to evaluate the possibilities of using ethanol extracts of Lamiaceae family as natural source for antibacterial activity.

Key words: Lamiaceae, antibacterial, ethanol extracts, volatile compounds, streptomycin.

Introduction

The increasing incidence of bacterial infections from clinical and non-clinical isolates resistant to most of the antimicrobial drugs currently available has severely threatened the therapeutic choices and became a global health problem in the last decade. Additionally, the emergence of infections caused by multidrug-resistant (MDR) microorganisms turned the prognostic of the treatment even worse (Bereket *et al.*, 2012; Kumarasamy *et al.*, 2010). Despite of the substantial advances of the biomedical sciences and public health polices in the past century, the world has witnessed an expansion of the emerging and re-emerging infectious diseases (Yang *et al.*, 2012). Parallel to that, the number of antimicrobial drugs launched to the market by the pharmaceutical companies in the last decade decreased drastically. Counterparting this negative scenario, the interest in medicinal plants as an alternative to the synthetic antimicrobial drugs is blossoming worldwide (Tavares *et al.*, 2008).

Among the many families of plants investigated, the Lamiaceae family deserves some special interest. This family has a cosmopolitan distribution, including about 300 genera and 7500 species. In Brazil, there are 26 genera with approximately 350 species. Many of these aromatic species are used as to prepare condiments or teas, such as lemon balm (*Melissa officinalis*), mint (*Mentha sp*.), basil (*Ocimum basilicum*), brazilian-boldo (*Plectranthus barbatus*), and rosemary (*Rosmarinus officinalis*) (Souza

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and Lorenzi, 2005). It is well-known that many spices especially those belonging to the Lamiaceae family possess a wide range of biological and pharmacological activities. Some of them are specifically related to the essential oils like antimicrobial, spasmolytic, carminative, hepatoprotective, antiviral, and anticarcinogenic activities (Bozin *et al.*, 2006). However, to our knowledge, there are few reports in the literature about the antimicrobial activity of the ethanol extracts of lemon balm, mint, basil, brazilian-boldo, and rosemary. In the present work, we identified the volatile compounds presents in the ethanol extracts of *Melissa officinalis*, *Mentha sp*., *Ocimum basilicum*, *Plectranthus barbatus*, and *Rosmarinus officinalis* by gas chromatography/mass spectrometry (GC/MS). The antimicrobial activity of each extract was also evaluated against 6 bacteria of clinical interest. Furthermore, the interaction of these extracts with a commercial antibiotic streptomycin was investigated.

Materials and Methods

Plant material and extraction

The Lamiaceae species were collected in Carmópolis de Minas, Minas Gerais, Brazil, in April 2011. The voucher specimens were deposited at the Instituto de Ciências Biológicas Herbarium, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil (*Melissa officinalis* BHCB 147242, *Mentha sp*. BHCB 147244, *Ocimum basilicum* BHCB 147240, *Plectranthus barbatus* BHCB 147241, and *Rosmarinus officinalis* BHCB 147245).

The fresh plant material was extracted by cold maceration in ethanol P.A (Vetec, Brazil) for a period of 10 days at room temperature. After it was filtrate and concentrated in a rotary evaporator at 40 °C under reduced pressure and lyophilized to yield ethanol extract (*Melissa officinalis* 2.31 g, *Mentha sp*. 10.37 g, *Ocimum basilicum* 2.78 g, *Plectranthus barbatus* 3.17 g, and *Rosmarinus officinalis* 6.30 g).

GC/MS analysis of ethanol extracts

The ethanol extracts profile were analyzed with a Shimadzu model QP5050A instrument equipped with DB-5 column (30 m x 0.25 mm, 0.25 μm df). The oven program had an initial temperature of the column was 80 °C for 1 min, then a 7 °C/min ramp to 300 °C and held for 5 min; injector temperature was kept at 250 °C (split 1:20) and detector temperature was at 260 °C. The carrier gas helium (He) at linear flow-rate of 39.3 mL/min (115.4 kPa). For each analysis 1 μL of the sample was injected in GC. The scan range was from 50 to 500 *m/z* at a scan rate of 0.50 scan/s. Solvent delay was 2.5 min. The compounds were identified by mass spectral database search (NIST) followed by matching of MS data and expressed in relative percentage of each compound, calculated by internal normalization of the chromatographic peak area. All volatiles showing mass spectra with match factors ≥ 90% were put on a "positive list" of tentatively identified metabolites.

Culture and maintenance of the bacteria isolates

Six bacteria strains, which included Gram-positive *Enterococcus faecalis* ATCC 19433, *Staphylococcus aureus* ATCC 29213, *Streptococcus mutans* ATCC 25175 and Gram-negative *Escherichia coli* O157:H7ATCC 43895, *Klebsiella pneumoniae* ATCC 4252 and *Pseudomonas aeruginosa* ATCC 27853, were used in the biological assays. All bacteria strains were stored at -80 °C.

Suspensions from the cultures of the bacteria were prepared in accordance with the guidelines in the CLSI M7-A6 document (2006) to obtain a final suitable inoculum of 1.5 x 10^6 UFC/mL.

Determination of the minimal inhibitory concentrations

The Minimal Inhibitory Concentration (MIC) values were obtained for broth microdilution testing performed in accordance with the guidelines in the CLSI M7-A6 document (2006).

Ethanol extracts dried were dissolved in sterile dimethylsulfoxide (DMSO) (Synth, Brazil) in final concentration of 2%. Later, serial dilutions were made with MH, maintaining a constant volume of 1000 μL in each tube. In this way, the samples were tested at eight concentrations that varied from 2000-15.62 μg/mL. An inoculum of 125 μL of cell culture was added to 25 μL of each concentration of extract in Mueller-Hinton broth (MH) in 96-well plates. After inoculation of bacteria, plates were incubated at 37 °C for 24 h. Streptomycin (Sigma-Aldrich, U.S.A.) was included as positive control, being the stock solution prepared in water. Mueller Hinton broth (MH) medium (Himedia, India) without samples or solvents was used as a control for growth and sterility. DMSO at 2% (v/v) was used as control for toxicity.

The MIC was assessed based on the lowest concentration of sample required to inhibit microbial growth (detected as the lack of visible turbidity) and by spectrophotometry by measuring the absorbance at 490 nm (Powder Wave XS2, Biotec, U.S.A.). The experiments were performed in triplicate and repeated three times.

Determination of minimal bactericidal concentrations

For assays to determine the minimum bactericidal concentration (MBC), aliquots of 25 μL were removed from wells without visible turbidity and placed on Mueller Hinton agar (Himedia, India) by a spread-plate method (Tortora *et al.*, 2005). After incubation at 37 °C for 24 h, colonies were counted. The concentration of sample that resulted in a growth 0.1% of initial inoculum (1.5 x 10^6 UFC/mL) was determined as the MBC (Torres *et al.*, 2010). All assays were performed in triplicate and repeated at least once.

Checkerboard assay

The synergistic combinations were investigated in the preliminary checkerboard method (Lee *et al.*, 2012) performed using ethanol extract and streptomycin, with adaptations. Samples of ethanol extract of the species studied were serially diluted in DMSO 2% v/v in concentrations ranging from 15.62 to 2000 μg/mL and streptomycin was used in concentrations ranging from 0.93 to 120 μg/mL. Subsequently, solutions of the same concentration were combined in a 1:1 ratio to evaluate the antimicrobial effect resulting from the interaction of ethanol extract and streptomycin. The MIC was defined as the lowest concentration of drug alone or in combination that inhibited the visible growth. The fractional inhibitory concentration (FIC) of streptomycin was calculated as the MIC of streptomycin in the presence of ethanol extract divided by the MIC of streptomycin alone. The FIC of ethanol extract was calculated in the same fashion. The FIC index (FICI) was calculated by adding both FICs. With this method, the synergistic activities were defined in a range of FICI ≤ 0.5, the additive or indifferent effects are defined in a range 0.5 < FICI < 2.0, and the antagonistic effects are defined by FICI > 2.0 (He and Chen, 2006; White *et al.*, 1996). All experiments were independently repeated three times.

Results and Discussion

Ethanol extracts obtained from five species of Lamiaceae family were tested against six pathogenic bacteria, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mutans, Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) are present in Table 1. It was considered that for the extracts with MIC values less than 100 μg/mL, the antimicrobial activity was good; from 100 to 500 μg/mL, the antimicrobial activity was moderate; from 500 to 1000 μg/mL the antimicrobial activity was weak, and over 1000 μg/mL the extract was considered inactive (Holetz *et al.*, 2002).

Ethanol extracts of *Plectranthus barbatus* and *Rosmarinus officinalis* showed moderate activity. Ethanol extract of *Plectranthus barbatus* had MIC value of 250 μg/mL against *Escherichia coli* and *Pseudomonas aeruginosa*, being showed moderate activity. Ethanol extract of *Rosmarinus officinalis* presented moderate activity, inhibited the growth *Staphylococcus aureus* and *E. coli,* at a concentration of 250 μg/mL, and *E. faecalis and S. mutans,* at a concentration of 500 μg/mL. Ethanol extracts of *Melissa officinalis, Mentha sp* and *Ocimum basilicum* showed moderate activity against *Streptococcus mutans* with MIC values of 250, 500 and 250 μg/mL, respectively. These extracts showed weak activity or were inactive against other bacteria tested.

The Gram-positive bacteria were more sensitive to ethanol extracts. *S. mutans* causes dental caries (Tahmourespour *et al.*, 2011), and was the more sensitive bacteria to ethanol extracts tested, being this an important result.

Ethanol extracts of *P. barbatus* and *R. officinalis* presented minimal bactericidal concentration (MBC) for *S. aureus*, with values 2000 and 250 μg/mL, respectively. Extract of *P. barbatus* also showed bactericidal effect against *P. aeruginosa,* and extract of *R. officinalis* against *E. faecalis* and *S. mutans.*

To explore the possibility of developing more powerful combination therapies of streptomycin with ethanol extracts, the checkerboard micro-titer test was performed with combined samples. Table 2 shows the occurrence of the synergistic effect of streptomycin with ethanol extracts of *M. officinalis, Mentha sp., O. basilicum* and *R. officinalis* against *S. mutans*; *P. barbatus* and *R. officinalis* against *E. coli*; *P. barbatus* against *P. aeruginosa,* and *R. officinalis* against *E. faecalis.* When streptomycin was combined with ethanol extracts, it was observed that the total amount of dispensed streptomycin can be diminished. This is an important observation, because ototoxicity and nephrotoxi-

Table 1 - Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) of ethanol extracts from five species of Lamiaceae family against six bacteria of clinical interest.

Extracts	*Melissa officinalis*		*Mentha* sp		*Ocimum basilicum*		*Plectranthus barbatus*		*Rosmarinus officinalis*		Streptomycin	
Bacteria	MIC μg/mL	MBC μg/mL	MIC μg/mL	MBC μg/mL	MIC μg/mL	MBC μg/mL	MIC μg/mL	MBC μg/mL	MIC μg/mL	MBC μg/mL	MIC μg/mL	MBC μg/mL
Enterococcus faecalis	2000	> 2000	1000	> 2000	1000	> 2000	1000	> 2000	500	2000	15	30
Staphylococcus aureus	> 2000	ND	> 2000	ND	> 2000	ND	1000	2000	250	250	7.5	15
Streptococcus mutans	250	> 2000	500	> 2000	250	> 2000	1000	> 2000	500	2000	15	30
Escherichia coli	> 2000	ND	> 2000	ND	> 2000	ND	250	> 2000	250	> 2000	15	30
Klebsiella pneumoniae	> 2000	ND	> 2000	ND	> 2000	ND	> 2000	ND	2000	> 2000	7.5	15
Pseudomonas aeruginosa	> 2000	ND	> 2000	ND	> 2000	ND	250	2000	> 2000	ND	15	30

ND: Non determined.

Table 2 - Fractional inhibiting concentration (FIC) and FIC index (FICI) of ethanol extracts from species of Lamiaceae family against bacteria of clinical interest.

Bacteria	Samples	FIC	FICI	Effect
Enterococcus faecalis	1. *Rosmarinus officinalis*	0.25	0.5	S
	2. Streptomycin	0.25		
Staphylococcus aureus	1. *Rosmarinus officinalis*	0.5	1.0	I
	2. Streptomycin	0.5		
Streptococcus mutans	1. *Melissa officinalis*	0.25	0.5	S
	2. Streptomycin	0.25		
Streptococcus mutans	1. *Mentha sp*	0.25	0.5	S
	2. Streptomycin	0.25		
Streptococcus mutans	1. *Ocimum basilicum*	0.25	0.5	S
	2. Streptomycin	0.25		
Streptococcus mutans	1. *Rosmarinus officinalis*	0.25	0.5	S
	2. Streptomycin	0.25		
Escherichia coli	1. *Plectranthus barbatus*	0.25	0.5	S
	2. Streptomycin	0.25		
Escherichia coli	1. *Rosmarinus officinalis*	0.25	0.5	S
	2. Streptomycin	0.25		
Pseudomonas aeruginosa	1. *Plectranthus barbatus*	0.25	0.5	S
	2. Streptomycin	0.25		

FICI ≤ 0.5: synergistic effect (S); 0.5 < FICI < 2.0: additive or indifferent effect (I); FICI > 2.0: Antagonistic effect (A).

city are related to both the cumulative dose and the peak serum concentration of aminoglycosides (Arbex *et al.*, 2010).

The results of the checkerboard assay also can be represented graphically by plotting the FIC values on a graph known as an isobologram. If the two agents have additive antimicrobial activity, the line connecting the *x*- and *y*-intercepts and the intervening points (the isobologram) will be straight. If the two agents have synergistic antimicrobial activity, the FIC values of each agent will be lower, and the isobologram will be concave. For factors that are antagonistic in combination, the isobologram will be convex (Singh *et al.*, 2000).

The isobolograms of ethanol extracts with streptomycin were showed in Figure 1. The isobolograms confirm the results found in the FIC index. Combination of streptomycin with *R. officinalis* produced the additive or indifferent interactions against *S. aureus* (Figure 1b). In contrast, the combined action of streptomycin with *M. officinalis*, *Mentha* sp., *O. basilicum* and *R. officinalis* against *S. mutans*; *P. barbatus* and *R. officinalis* against *E. coli*; *P. barbatus* against *P. aeruginosa,* and *R. officinalis* against *E. faecalis* were synergistic as reflected by the concavity of the isobolograms (Figure 1a, c, d, e, f, g, h and i).

The ethanol extracts from five species of Lamiaceae family were investigated by gas chromatography/mass spectrometry (GC/MS) in order to identify the majoritary compounds. It was possible to identify 13 dominant components (Figure 2) and their percentages are shown in Table 3. Phytol was identified in all ethanol extracts, being this majoritary compound present in *P. barbatus* and *R. officinalis*. Phytol showed antibacterial activity, inhibiting the growth of *Staphylococcus aureus* (Yoshihiro *et al.*, 2005), corroborating with the results obtained for *P. barbatus* and *R. officinalis* against *S. aureus*.

Other compounds identified in the five species of Lamiaceae family also presented antimicrobial activity. Camphor and verbenone showed activity against *Staphylococcus aureus* and *Escherichia coli* (Santoyo *et al.*, 2005). Camphor and verbenone are present in P. barbatus and *R. officinalis* confirming the antibacterial activity against these microorganisms. Cadinene, heptadecane, 2-decen-1-ol and 2, 6-dimethyl heptane also demonstrated activity against several microorganisms (Galvão *et al.*, 2012; Ozdemir *et al.*, 2004; Samy *et al.*, 2006; Vukovic *et al.*, 2007), explaining the activity of *M. officinalis*, *Mentha* sp. and *O. basilicum* against *S. mutans*. The fact of several compounds identified in ethanol extracts presented antimicrobial activity suggests that this activity of plant extracts is related with possible synergistic interactions between their components (Dorman and Deans, 2000).

Conclusion

Phytol was a compound identified in all extracts by GC/MS, being majoritary component in *Plectranthus barbatus* and *Rosmarinus officinalis.* The Gram-positive bacteria were more sensitive to ethanol extracts, and *Plectranthus barbatus* and *Rosmarinus officinalis* were the

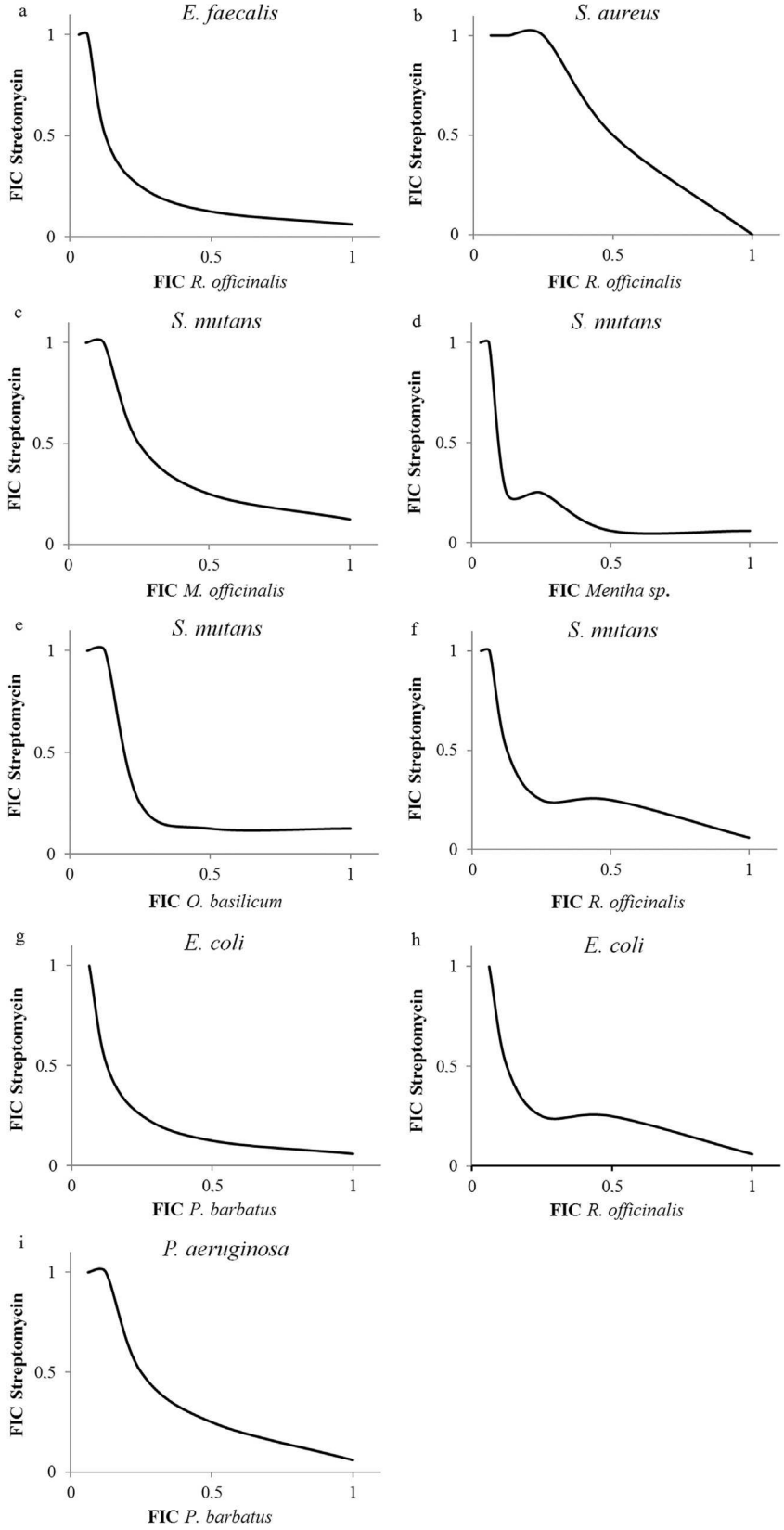

Figure 1 - Isobolograms showing interactions between streptomycin and ethanol extracts from species of Lamiaceae family. Data are the fractional inhibitory concentrations (FICs) of the two samples in combination. *y*-axis, streptomycin; *x*-axis, ethanol extracts from species of Lamiaceae family. Straight lines, result expected if the factors had additive interactions; concave and convex lines, synergistic and antagonistic interactions, respectively.

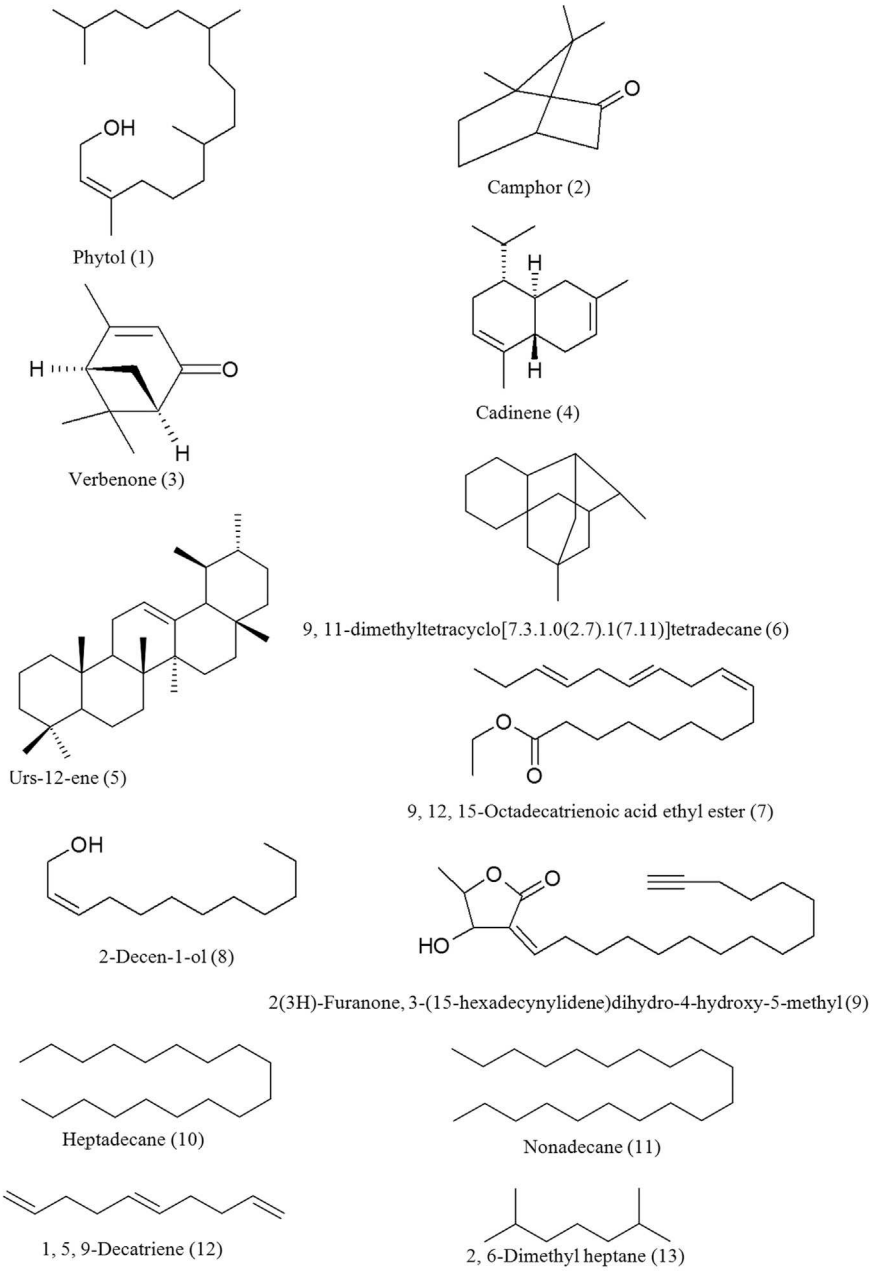

Figure 2 - Structures of majoritary compounds 1-13 identified by gas chromatography/mass spectrometry obtained from ethanol extracts of five species of Lamiaceae family.

Table 3 - Majoritary compounds of ethanol extracts from species of Lamiaceae family.

Species	Majoritary compounds (%)*
Melissa officinalis	nonadecane (25.13%); heptadecane (18.32%); 2, 6-dimethyl heptane (17.81%); phytol (13.84%)
Mentha sp.	2-decen-1-ol (23.55%); 1, 5, 9-decatriene (13.50%); phytol (12.55%); 2(3H)-furanone 3-(15-hexadecynylidene)dihydro-4-hydroxy-5-methyl (11.94%); cadinene (8.5%)
Ocimum basilicum	9, 12, 15-octadecatrienoic acid ethyl ester (26.19%); phytol (18.36%); cadinene (10.92%)
Plectranthus barbatus	phytol (35.18%); verbenone (12.89%); camphor (10.55%)
Rosmarinus officinalis	phytol (40.32%); urs-12-ene (19.57%); verbenone (12.76%); camphor (12.11%); 9, 11-dimethyltetracyclo[7.3.1.0(2.7).1(7.11)]tetradecane (11.38%)

*Concentration in percentage, calculated relative to the normalized areas of the peaks.

most active extracts. Ethanol extracts exhibited a synergetic effect with streptomycin. These results encourage additional studies, in order to evaluate the possibilities of using ethanol extracts of Lamiaceae family as natural source for antibacterial activity.

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Conflict of interest

The authors do not have any conflicts of interest.

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