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Synergistic antibacterial interaction between Melissa officinalis extracts and antibiotics

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ABSTRACT

Synergism between water, ethanol and ethyl acetate extract of *Melissa officinalis* and five commonly used antibiotics (streptomycin, chloramphenicol, tetracycline, amoxicillin, rifamycine) were investigated by disc diffusion method in relation to antibiotic-susceptible and antibiotic resistant human-pathogenic bacteria. The total phenol content in the extracts were determined by Folin-Ciocalteau`s method while the flavonoid concentration by aluminium chloride method. The extracts, at least with one antibiotic, showed synergistic interaction. Depending on the species of bacteria, the zones of inhibition in extract/antibiotic plates were in the range of 0.5 – 11.5 mm wider than in controls. The certain extract/antibiotic combinations exhibited significant results against antibiotic resistant bacteria (the inhibition zones were 7-11mm wider than in controls). The ethanol and ethyl acetate extracts showed better synergistic capacity than water extract. The least susceptible bacteria to extract/antibiotic combinations were *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. The highest amount of total phenols was measured in water extract while the highest amount of flavonoids had ethyl acetate extract.

Keywords: Herb-drug interaction, *Melissa officinalis*, plant extracts, total phenol content, flavonoid concentration.

INTRODUCTION

Healing potential of plants has been known for thousands of years. Medicinal properties of plants can be attributed to biologically active substances. Plants produce a whole series of different compounds which are not of particular significance for primary metabolism, but represent an adaptive ability of a plant to adverse abiotic and biotic environmental conditions. They can have a remarkable effect to other plants, microorganisms and animals from their immediate or wider environment. All these organic compounds are defined as biologically active substances, and generally represent secondary metabolites, given the fact that they occur as an intermediate or end products of secondary plant metabolism. These secondary metabolites, apart from determining unique plant traits, such as: colour and scent of flowers and fruit, characteristic flavour of spices, vegetables, they also complete the functioning of plant organism, showing both biological and pharmacological activity of a plant.

Melissa officinalis L., member of Lamiaceae family, is one of the well known aromatic medicinal plant species. This plant with white or pale pink flowers is commonly referred to as Lemon Balm because of its lemon-like flavor and fragrance. It often takes as a tea after a meal for its ability to reduce indigestion and gas. Lemon balm tea was also traditionally used in Europe as a mild sedative and to treat headache, migraine, nervous tension and insomnia, as well as to treat cold, fever and cough. Based on research, *M. officinalis* extracts have strong antimicrobial properties against viral, bacterial and fungal infections (Iauk *et al*., 2003; Ertürk, 2006; Nolkemper *et al*., 2006). Applied externally as a cream, lemon balm extract significantly decreases healing time of herpes mouth sores (recurring herpes labialis), prevents their spread and reduces the symptoms of itching (Koytchev *et al*., 1999). The aqueous extract of *M. officinalis* also has potent anti-HIV-1 activity (Yamasaki *et al*., 1998). Furthermore, *M. officinalis* is interested due to its demonstrated antioxidant (Hohmann *et al*., 1999; Canadanović-Brunet *et al*., 2008), anti-inflammatory (Drozd and Anuszewska, 2003), sedative (Soulimani *et al*., 1991), hypolipidemic (Bolkent *et al*., 2005) and antiulcerogenic properties (Khayyal *et al*., 2001).The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In recent years there have been many studies about the beneficial role of bioactive plant extracts and pure isolated compounds in increasing the *in vitro* efficacy of commonly used antibiotics against variety of microorganisms (Betoni *et al*., 2006; Esimone *et al*., 2006; Sibanda and Okoh, 2007; Stefanovic *et al*., 2009a; Stefanovic *et al*., 2009b; Stefanovic *et al*., 2011; Stefanovic *et al*., 2012). The ability of plant extracts to act synergistically with antibiotics could be a new approach to solve the problem of bacterial resistance and less susceptible bacteria. Considering that extracts of lemon balm show broad-spectrum antimicrobial activity, the aim of this study was to research *in vitro* the possible existence of synergy between water, ethanol and ethyl acetate extract of *Melissa officinalis* and commonly used antibiotics (streptomycin, chloramphenicol, tetracycline, amoxicillin, rifamycine). The second aim was to measure total phenol content and concentration of flavonoids as one of bioactive secondary metabolites responsible for antibacterial activity.

MATERIALS AND METHODS

General

The following reagents were used: Folin-Ciocalteu phenol reagent and aluminium chloride hexahydrate (from Fluka Chemie AG, Buchs, Switzerland), gallic acid and rutin hydrate (from Sigma Chemicals Co., St Louis, MO, USA). All solvents (ethanol, ethyl acetate, methanol) and sodium hydrogen carbonate were purchased from Zorka pharma, Sabac, Serbia, except dimethylsulfoxide (DMSO) was from Merck, Germany. Nutrient media: Mueller-Hinton broth and Mueller-Hinton agar were purchased from Liofilchem, Italy. Standard discs of antibiotics (amoxicillin, chloramphenicol, streptomycin, tetracycline, rifamycine) were from Torlak, Belgrade, Serbia.

Plant material

Melissa officinalis (leaves) was obtained commercially. Dried, ground leaves (50g for each extract) were extracted with water, ethanol and ethyl acetate by maceration. After being concentrated, stock solutions of extracts (80mg/ml) were obtained by dissolving a certain amount of crude extract in solvent (5% DMSO). The yields of the extracts were 11,6g for water extract, 5g for ethanol extract and 5,3g for ethyl acetate extract.

Microorganisms

The bacteria used in the antibacterial tests were: Gram-positive (*Bacillus subtilis* PMFKg-B2, *Staphylococcus aureus* PMFKg-B30) and Gram-negative (*Enterobacter cloacae* PMFKg-B22, *Escherichia coli* PMFKg-B32, *Klebsiella pneumoniae* PMFKg-B26, *Pseudomonas aeruginosa* PMFKg-B41, *Proteus mirabilis* PMFKg-B29). All strains were clinical isolates, a generous gift from the Institute of Public Health, Kragujevac and stored in microbiological collection at the Laboratory of Microbiology (Faculty of Science, University of Kragujevac).

Inoculum preparation

Bacterial suspension were prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspent into 5ml of sterile 0,85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 Mc Farland standard. When adjusted to the turbidity of a 0.5 Mc Farland standard, a suspension of bacteria contains about 10^8 colony forming units (CFU)/ml. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve 10^6CFU/ml.

Antibacterial screening test

Before the combination assays between plant extracts and antibiotics were evaluated, the minimum inhibitory concentrations (MIC) of water, ethanol and ethyl acetate extract were determined by tube dilution method (NCCLS, 1997). Two-fold serial dilutions of the extracts were prepared in Mueller Hinton broth. The tested concentration range was from 40 mg/ml to 1.25 mg/ml. The synergistic interactions were evaluated by standard diffusion method using commercial antibiogram tablets. One mililiter of solutions of plant extracts were incorporated into 9 ml of molten Mueller Hinton agar in order to obtain the final concentration of 1/4 MIC and poured into petri dishes. One-fourth the MIC was considered as the sub-inhibitory concentration of plant extracts. Petri dishes, controls and with plant extracts, were inoculated with bacterial suspensions using sterile swab, and, finally, standard discs of antibiotics (amoxicillin 25µg; chloramphenicol 30µg; streptomycin 30µg; tetracycline 30µg; rifamycine 5µg) were set up. The diameters (mm) of the each inhibition zone were recorded after incubation at 37ºC/24h. The synergistic effect was evaluated by comparing the size of inhibition zone in plates containing plant extract and in control plates without plant extract. Each test included negative control (5% DMSO) and control of sterility.

All tests were performed in triplicate and the results are expressed as mean ± standard deviation. Antibiotic resistance

profile of test strains were determined by antibiogram test using standard discs of tested antibiotics.

Total phenol content

Total phenol content in the plant extracts was measured using spectrophotometric method (Singleton *et al.*, 1999). The methanol solution of the extract in concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanol solution of the extract, 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water and 2 ml of 7.5% $NaHCO_3$. The blank was prepared containing 0.5 ml of methanol, 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of 7.5% of $NaHCO_3$. The samples were incubated in the thermostat at 45 ˚C for 45 min. The absorbance was measured using spectrophotometer at λ_{max}= 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained.

The same procedure was repeated for the standard solution of gallic acid and a calibration curve was created. Based on the measured absorbance, the concentration of phenolics was read from the calibration curve; then the content of phenolics in the extracts was expressed in terms of gallic acid equivalent, (GAE) per gram of extract.

Flavonoid concentration

The content of flavonoids in the plant extracts was determined using spectrophotometric method (Quettier-Deleu *et al*., 2000). The sample contained 1 ml of methanol solution of the extract in concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was measured using spectrophotometer at λ_{max}= 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and a calibration curve was created. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration curve; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (RUE) per gram extract.

RESULTS

Synergistic, antibacterial activity

The results of antibacterial activity of tested *M. officinalis* extracts and results of joint activity between extracts and antibiotics against human-pathogenic bacteria are presented in Table 1. The solvent control (5% DMSO) did not inhibit growth of the tested bacteria. Antibiotic resistance profile of test strains is presented in Table 2. In general, the water extract showed antibacterial activity with MIC values in the range of 5 mg/ml – 20 mg/ml, the ethanol extract in the range of 5 mg/ml – 40 mg/ml and MICs for the ethyl acetate extract were 10 mg/ml for all of the tested bacteria. Combinations of antibiotics with water, ethanol and ethyl acetate extract were tested for possible synergistic interactions. Synergistic interaction between two agents means that their joint effect is stronger than the sum of effects of the individual agents. In general, the zones of inhibition in antibiotic/plant extract plates were in the range of 0.5 – 11.5 mm wider than the zones of inhibition in the control plates depending on the species of bacteria. Enlargement of inhibition zone over 5 mm was considered significantly. The better synergistic capacity showed chloramphenicol, tetracycline and amoxicillin than rifamycin and streptomycin. Combining effects of chloramphenicol with tested extracts showed the inhibition zones 7-8 mm wider than controls in relation to *E. cloacae*. *P. aeruginosa* was resistant to tetracycline and chloramphenicol, but even that tetracycline/ethanol extract and tetracycline/ethyl acetate extract combinations showed synergistic activity (the inhibition zones were 11mm and 10.5 mm wider than controls, respectively) as well as chloramphenicol/ethyl acetate extract combinations (the inhibition zone was 9.5 mm wider than control). All bacterial strains showed reduced sensitivity or resistance to amoxicillin. Significant enlargement of inhibitory zones showed amoxicillin with water and ethanol extract against *E. cloacae*, amoxicillin/water extract against *S. aureus* and amoxicillin/ethanol extract and amoxicillin/ethyl acetate extract against *K. pneumoniae*. The least susceptible bacteria to extract/antibiotic combinations were *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. In all cases, rifamycin and streptomycin showed

Table. 1: Synergism between *Melissa officinalis* L. extracts and antibiotics.

Species	MIC (mg/ml)			Streptomycine + 1/4 MIC (mm)			Chloramphenicol + 1/4 MIC (mm)		
	W	E	Et	W	E	Et	W	E	Et
Bacillus subtilis	5	5	10	27 ± 0.00*	29.5 ± 0.71	28.5 ± 0.71	28 ± 0.00	34.5 ± 0.71	32 ± 1.41
Enterobacter cloacae	10	5	10	30.5 ± 0.71	32.5 ± 0.71	29.5 ± 0.71	39 ± 1.41	39 ± 1.41	40 ± 1.41
Klebsiella pneumoniae	5	5	10	28.5 ± 0.71	29.5 ± 0.71	28.5 ± 0.71	29.5 ± 0.71	33.5 ± 0.71	30.5 ± 0.71
Staphylococcus aureus	10	10	10	29.5 ± 0.71	30 ± 1.41	29 ± 1.41	33.5 ± 0.71	34.5 ± 0.71	34.5 ± 0.71
Pseudomonas aeruginosa	20	40	10	24.5 ± 0.71	28 ± 1.41	27 ± 1.41	9	14.5 ± 0.71	18.5 ± 0.71
Proteus mirabilis	20	40	10	17.5 ± 0.71	20.5 ± 0.71	19,5 ± 0.71	9	13.5 ± 0.71	9
Escherichia coli	20	40	10	23.5 ± 0.71	17.5 ± 0.71	20.5 ± 0.71	9	9	9

Table. 1: Synergism between *Melissa officinalis* L. extracts and antibiotics (*continued*).

Species	Tetracycline + 1/4MIC (mm)			Amoxicillin + 1/4 MIC (mm)			Rifamycin + 1/4 MIC (mm)		
	W	E	Et	W	E	Et	W	E	Et
Bacillus subtilis	30±1.41	36±0.00	34.5±0.71	20.5±0.7	25.5±0.71	23.5±0.71	20.5±0.71	21.5±0.71	21.5±0.71
Enterobacter cloacae	25.5±0.71	29.5±0.71	32±1.41	27±1.41	26.5±0.71	21.5±0.71	17±0.00	22.5±0.71	21.5±2.12
Klebsiella pneumoniae	30±0.00	28.5±0.71	35±1.41	19.5±0.7	29.5±0.71	27±1.41	22.5±0.71	24.5±0.71	23.5±0.71
Staphylococcus aureus	21.5±0.71	23.5±0.71	27±1.41	27±1.41	20.5±0.71	17±0.00	16.5±0.71	20.5±0.71	20.5±0.71
Pseudomonas aeruginosa	14.5±0.71	20±1.41	19.5±0.71	9	9	9	13±0.00	9	9
Proteus mirabilis	9	15.5±0.71	9	9	9	9	9	13±0.00	9
Escherichia coli	9	9	9	9	9	9	13.5±0.71	12.5±0.71	9

diameter of the zone of inhibition including the diameter of disc; "9"= diameter of the antibiotic disc (equivalent to "no effect"); W-water extract, E-ethanol extract, Et-ethyl acetate extract.

Table. 2: Antibiotic resistance profile of tested bacterial strains.

Species	Streptomycine (mm)	Chloramphenicol (mm)	Tetracycline (mm)	Amoxicillin (mm)	Rifamycin (mm)
Bacillus subtilis	27.5 ± 0.71* (S)**	28.5 ± 0.71 (S)	29.5±0.71 (S)	19.5±0.71 (R)	19.5±0.71 (R)
Enterobacter cloacae	26 ± 1.41 (S)	32 ± 2.82 (S)	25±0.00 (I)	18±1.41 (R)	17±0.00 (R)
Klebsiella pneumoniae	29 ± 1.41 (S)	29 ± 0.00 (S)	30.5±0.71 (S)	19.5±0.71 (R)	19.5±0.71 (R)
Staphylococcus aureus	25 ± 0.00 (I)	30 ± 0.00 (S)	21±1.41 (R)	15.5±0.71 (R)	17.5±0.71 (R)
Pseudomonas aeruginosa	25 ± 0.00 (I)	9 (R)	9 (R)	9 (R)	9 (R)
Proteus mirabilis	19 ± 1.41 (R)	9 (R)	9 (R)	9 (R)	9 (R)
Escherichia coli	21 ± 1.41 (R)	9 (R)	9 (R)	9 (R)	9 (R)

diameter of the zone of inhibition including the diameter of disc; ** S - susceptible; I - moderate susceptible; R- resistant (according to the manufacturer's guide-Institute of Virology and Immunology, Torlak, Belgrade); "9"- diameter of the antibiotic disc (equivalent to "no effect").

indifferent effect, except streptomycin/ethanol extract combination against *Enterobacter cloacae*.

Total phenol and flavonoid content

Since phenols and flavonoids significantly contribute to the overall antibacterial activity, it was reasonable to determine their total amount in the tested extracts. The total phenol and flavonoid content is shown in Table 3. The highest amount of total phenolics was measured in water extract, 319.11 mg of GAE/g of extract and flavonoids in ethyl acetate extract, 190.77 mg of RUE/g of extract.

Table. 3: Total phenol content and flavonoid concentration in *Melissa officinalis* L. Extracts.

Type of extract	Total phenol content (mgGAE/g of extract)	Flavonoid concentration (mgRUE/g of extract)
Water extract	319.11 ± 1.52	31.05 ± 0.55
Ethanol extract	85.65 ± 0.65	136.85 ± 1.15
Ethyl acetate extract	24.55 ± 0.84	190.77 ± 1.01

DISSCUSION

The extracts of *M. officinalis*, among themselves, showed equable antibacterial activity while the tested bacteria showed sensitivity at different concentrations. The concentrations were ranged from 5 mg/ml to 40 mg/ml. *Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis* showed low sensitivity to ethanol extract. Weaker activity of lemon balm to mentioned Gram-negative bacterium was also noted in the work of Canadanović-Brunet *et al.,* 2008. Antimicrobial activity of different *Melissa officinalis* extracts was also tested by other scientists who showed different level of antimicrobial activity with their research (Iauk *et al*., 2003; Ertürk, 2006; Uzun *et al*., 2004). The water, ethanol and ethyl acetate extracts of *M. officinalis* enhance the activities of amoxicillin, streptomycine, tetracycline and chloramphenicol. Synergistic activity with antibiotics were demonstrated even that the sub-inhibitory concentration of extracts were used. The observed enhancement of antibiotic activity could be explained by the presence of biologically active compounds in these extracts.

The extracts of *M. officinalis* have been known to contain a number of antimicrobial compounds. Phytochemical screening of this plant has shown the presence of flavonoids (isoquercitrin, rhamnocitrin, luteolin, apigenin-7-O-glucoside) (Patora and Klimek, 2002; Herodez *et al*., 2003), caffeic acid, rosmarinic acid, vanillic acid, *p*-coumaric acid (Canadanović-Brunet *et al*., 2008; Herodez *et al*., 2003), phenolic substances and tannin (Hohmann *et al*., 1999). In this work high concentration of total phenolics was measured in water extract. The high total phenol content was, also, determined by Dastmalchi *et al*., 2008, the water-ethanol extract had 268.9 mgGAE/g of extract and by Trendafilova *et al*., 2010, water extract had 1126.5 mg/L GAE/g of extract. The significant concentrations of flavonoids were determined in ethanol and ethyl acetate extract. These bioactive phytocompounds inhibit the growth of bacteria and their joint activity with antibiotics lead to an enhanced antibacterial effect and continuation of the useful life time of antibiotics. The mechanism governing the joint action of plant extract compounds and antibiotics is still unknown. Some authors suggest that phytocompounds disturb cell wall or increase permeability of the cytoplasmic membrane and thereby facilitate the influx of antibiotics, produce efflux pump inhibitors or inhibit penicillin-binding proteins (Sibanda and Okoh, 2007; Zhao *et al*., 2001; Shiota *et al*., 2004).

CONCLUSIONS

This work confirms the antibacterial activity of *M. officinalis* extract and shows their potential use as agents which enhance antibiotic activity. Future works of ours will be isolation and identification of active compounds responsible for these interesting activities and closer investigation of a mechanism of action between phytochemicals and antibiotics.

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