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Full Length Research Paper

In vitro antibacterial efficacy of *Clinopodium vulgare* L. extracts and their synergistic interaction with antibiotics

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***In vitro* antibacterial activity of ethanol, ethyl acetate and acetone extract from *Clinopodium vulgare* and their synergistic interaction with gentamicin and cephalexin were researched. Antibacterial efficacy of the extracts was defined by determining minimum inhibitory concentrations (MIC) using microdilution method. The values were in the range from 0.625 to >20 mg/ml. The most sensitive bacteria were gram-positive bacteria: *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*. Herb-drug interactions tested by checkerboard method and expressed as fractional inhibitory concentration (FIC) index showed indifferent and synergistic effects. Synergism was observed in relation to *B. subtilis* and *Klebsiella pneumoniae*. In the presence of sub-inhibitory concentrations (1/4 and 1/8 MIC) of the extracts, the activity of gentamicin and cephalexin was increased up to 16-fold. The highest amount of phenols and flavonoids had ethyl acetate extract while the lowest amount was measured for acetone extract.**

Key words: *Clinopodium vulgare*, antibacterial activity, plant extracts, herb-drug interaction, total phenol, flavonoid content.

INTRODUCTION

Clinopodium vulgare L. (*Satureja vulgaris* L.), a member of family Lamiaceae is an erect up to 45 cm tall perennial herbaceous plant. The oval leaves are supported by a short stem and are toothed at the edges. The flowers with tubular lipped corollas of a pinkish colour are arranged on the stem in several crowded bristly rings. It grows well in dry grassy places along banks and hedgerows and open woodland, widely distributed in Europe, Western Asia and North Africa. It is also found in Canada and has been introduced to the United States (Grieve, 1995). In traditional medicine, *C. vulgare* is not much used, although it has value as a heart tonic, an expectorant and a diuretic (Saric, 1989). In external use it is considered as an antiseptic for wounds and injuries (Opalchenova and Obreshkova, 1999). In addition, the leaves are traditionally used as spice (Seidemann, 2005). Based on the researches, aqueous extract of *C. vulgare* showed strong antitumour activity when tested *in vitro* on A2058 (human metastatic melanoma), HEp-2 (epidermoid carcinoma larynx, human) and L5178Y (mouse lymphoma) cell lines (Dzhambazov et al., 2002). Water and 80% acetone extract showed good antioxidant activity (Kratchanova et al., 2010) while only ethanol extract was tested for antibacterial activity (Opalchenova and Obreshkova, 1999). The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial properties of medicinal plants. Moreover, the increasing use of plant extracts in food, cosmetic and pharmaceutical industries suggests that in order to determine the active compounds a systematic study of medicinal plants is very important (Hammer et al., 1999; Nostro et al., 2000).

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

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commonly used antibiotics against variety of microorganisms (Betoni et al., 2006; Esimone et al., 2006; Nostro et al., 2006; Nagoshi et al., 2006; Horiuchi et al., 2007; Stefanovic et al., 2009a, b). Synergistic interaction between two agents in which one agent enhances the action of the other and together act more effectively than a single agent alone could be a new approach to solve the problem of bacterial resistance and less susceptible bacteria. Whereas the chemical components and antibacterial property of *C. vulgare* are insufficiently investigated; the aim of this work was to evaluate the antibacterial activity of ethanol, ethyl acetate and acetone extract of this plant and their possible synergistic interaction with commonly used antibiotics (gentamicin and cephalexin). The second aim was to determine a total phenol and flavonoid content in the extracts.

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, acetone, methanol) and sodium hydrogen carbonate were purchased from Zorka pharma, Sabac, Serbia, except dimethylsulfoxide (DMSO) was from Merck, Germany. Resazurin powder was obtained from Alfa Aesar, Germany. Nutrient media: Mueller-Hinton broth and Mueller-Hinton agar were purchased from Liofilchem, Italy. Antibiotics (gentamicin and cephalexin) were from Panfarma, Beograd, Serbia.

Plant material

Aerial parts of *C. vulgare* were collected from the region of Trgoviste (Serbia) during the summer of 2009. Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac. The voucher specimen (200991) of the plant is deposited in the Herbarium of the Faculty of Science, University of Kragujevac.

Extraction

Dried, ground plant material was extracted by direct maceration with ethanol, ethyl acetate and acetone. Briefly, 30 g of plant material was soaked with 150 ml of solvent for 24 h at room temperature, after that the resulting extract was filtered through filter paper (Whatman no.1). The residue from the filtration was extracted again, twice, using the same procedure. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40°C. The percentage of crude extracts yield was calculated using the formula:

$$\%\text{yield} = \frac{W_{\text{crude extract}}}{W_{\text{dried plant}}} \times 100$$

$W_{\text{crude extract}}$ = weight of crude extract

$W_{\text{dried plant}}$ = weight of dried plant material

Stock solutions of crude extracts were obtained by dissolving in DMSO and then diluted into Mueller-Hinton broth to achieve a concentration of 10% DMSO. Solutions of antibiotics were obtained by dissolving in a Mueller-Hinton broth.

Test microorganisms

The following bacteria were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and clinical isolate of *S. aureus* (PMFKg-B30), *Bacillus subtilis* (PMFKg-B2), *Enterococcus faecalis* (PMFKg-B22), *Klebsiella pneumoniae* (PMFKg-B26), *E. coli* (PMFKg-B32), *Pseudomonas aeruginosa* (PMFKg-B28) and *Proteus mirabilis* (PMFKg-B29). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Bacteria are 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 5 ml of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 Mc Farland standard (0.5 ml 1.17% w/v $BaCl_2 \times 2H_2O$ + 99.5 ml 1% w/v H_2SO_4) (Andrews, 2001). When adjusted to the turbidity of a 0.5 Mc Farland standard, a suspension of bacteria contain about 10^8 colony forming units (CFU)/ml. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve 10^6 CFU/ml.

Antibacterial assay

Antibacterial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using microdilution plate method with resazurin (Sarker et al., 2007). Briefly, the 96-well microplate was prepared by dispensing 100 µl of Mueller-Hinton broth into each well. A 100 µl from the stock solution of tested extract (concentration of 40 mg/ml) was added into the first row of the plate. Then, two-fold, serial dilutions were performed by transferring 100 µl of solution from one row to another using a multichannel pipette. The obtained concentration range was from 20 to 0.156 mg/ml. 10 µl of each 10^6 CFU/ml bacterial suspension was added to wells. Finally, 10 µl of resazurin solution was added. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of tested compound that prevented resazurin color change from blue to pink. Cephalexin was used as positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Moreover, because of the two-fold serial dilution assay, the final concentration of DMSO used in the experiment was 5% and lower. Each test included growth control and sterility control.

All tests were performed in duplicate and MICs were constant. Minimum bactericidal concentration was determined by plating 10 µl of samples from wells where no indicator color change was recorded on Mueller-Hinton agar. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum bactericidal concentration.

MIC index

The MIC index (MBC/MIC) was calculated for each extract and positive control drug to determine whether an extract has bactericidal (MBC/MIC≤4) or bacteriostatic (>4 MBC/MIC<32) effect on growth of bacteria (Cutler et al., 1994).

Combination assay

The synergistic interactions were evaluated by checkerboard method (Satish et al., 2005). Briefly, a series of twofold dilutions of gentamicin and cephalexin concentration from MIC to 1/32 MIC and a series of twofold dilutions of ethanol, acetone and ethyl acetate extract concentration from MIC to 1/32 MIC were prepared. 36 different combinations were tested for one bacterium. 100 µl of Mueller-Hinton broth were added into 36 wells of a 96-well microplate. 50 µl of each dilutions of extract were added horizontally into six rows and 50 µl of each dilutions of antibiotic were added vertically into six columns. The final volume was 200 µl.

Because the final volume is four times as great as the volume of each antimicrobial, the antimicrobial concentrations used in the stock solutions are four times greater than the desired final concentrations. Each well contained unique combination of plant extract/antibiotic concentration. The plate was inoculated with 10 µl of the bacterial suspension prepared as those used for MIC assay. Finally, 10 µl of resazurin solution was added. The plate was incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agents in combination at which visible bacterial growth was inhibited. Each test included growth control, solvent control and sterility control.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index using the following formula:

$$\text{FIC index} = \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}} + \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

Interpretation of the FIC index (FICI) was as follows: FICI ≤ 0.5 synergy; FICI > 0.5 to 4 indifference and FICI > 4 antagonism (Satish et al., 2005; White et al., 1996). The action of antimicrobial agents is considered to be:

i) Synergistic if their joint effect is stronger than the sum of effects of the individual agents.
ii) Indifferent if their joint effect is equal to the effect of either individual agent.
iii) Antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent.

If the MIC of any agent alone occurred at the highest concentration tested, the FIC index was considered not determinable and synergy could not be assessed. Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC index was expressed as the average of the FIC index values.

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 (mg/ml) from the calibration curve; then the content of phenolics in the extracts was expressed in terms of gallic acid equivalent, GAE (mg of GAE/g of extract).

Total flavonoid content

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 (mg of RUE/g of extract).

RESULTS AND DISCUSSION

Antibacterial activity

The results of antibacterial activity of ethanol, ethyl acetate and acetone extract from *C. vulgare* against 10 species of gram-positive and negative pathogenic bacteria are presented in (Table 1). The ethanol extract gave the highest yield of 15.6%, followed by ethyl acetate yield of 5.04% and acetone extract yield of 3.6% in extraction procedures. The organic solvent extracts showed selective antibacterial properties and the activity depended both on the species of bacteria and on the type and concentration of extract. In an earlier study, 5% ethanol extract of *C. vulgare*, based on colony forming unit (CFU)/ml values showed a very strong action on bacteria (Opalchenova and Obreshkova, 1999). In this study, the better activity showed ethyl acetate and acetone extract than ethanol extract. The MIC values of *C. vulgare* ethyl acetate extract were in the range from 0.625 to 10 mg/ml for acetone extract 0.625 to 20 mg/ml and for ethanol extract 1.25 to >20 mg/ml. The MBC values of tested extracts were in the range from 2.5 to >20 mg/ml. According to the MIC index, in 53.33% the extracts showed bactericidal activity against tested bacteria. The observed antibacterial activity is attributed to the presence of different bioactive compounds (phenols, flavonoids, tannins, coumarins, alkaloids) which have an impact on growth and metabolism of microorganisms (Cowan, 1999). Since phenols and flavonoids significantly contribute to the overall antibacterial activity, it was reasonable to determine their total amount in

Table 1. Antibacterial activity of ethanol, ethyl acetate and acetone extract from *Clinopodium vulgare.*

Species	Ethanol extract			Ethyl acetate extract			Acetone extract			Cephalexin		
	MIC[a]	MBC[a]	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC[b]	MBC[b]	MIC index
E. coli ATCC 25922	20	> 20	n.d.	10	10	1	10	10	1	6.25	6.25	1
S. aureus ATCC 25923	2.5	10	4	2.5	2.5	1	0.625	2.5	4	6.25	6.25	1
P. aeruginosa ATCC 27853	20	20	1	10	> 20	n.d.	10	10	1	> 1000	> 1000	n.d.
B. subtilis	1.25	10	8	0.625	2.5	4	1.25	5	4	12.5	12.5	1
S. aureus	> 20	> 20	n.d.	10	20	2	10	10	1	1.56	1.56	1
E. faecalis	> 20	> 20	n.d.	10	10	1	10	20	2	3.125	6.25	2
E. coli	> 20	> 20	n.d.	10	> 20	n.d.	20	> 20	n.d.	1.56	1.56	1
K. pneumoniae	> 20	> 20	n.d.	20	> 20	n.d.	20	> 20	n.d.	500	1000	2
P. aeruginosa	> 20	> 20	n.d.	10	> 20	n.d.	10	> 20	n.d.	> 1000	> 1000	n.d.
P. mirabilis	20	20	1	10	10	1	10	10	1	> 1000	> 1000	n.d.

[a]Values are expressed in mg/ml; [b] values are expressed in µg/ml. n.d: not determined.

Table 2. Total phenol content and total flavonoid content of ethanol, ethyl acetate and acetone extract from *Clinopodium vulgare.*

Plant extract	Total phenol content[a] (mg of GAE/g)	Total flavonoid content[b] (mg of RU/g)
Ethanol extract	36.37 ± 0.13	104.99 ± 0.96
Ethyl acetate extract	55.07 ± 0.09	167.96 ± 0.87
Acetone extract	24.71 ± 0.21	82.56 ± 0.79

[a]Total phenolics are expressed as gallic acid equivalent/gram of extract (GAE/g); [b]total flavonoids are expressed as rutin equivalent/gram of extract (RU/g). Values represent mean ± S.D.

the tested extracts. The total phenol and flavonoid content is shown in (Table 2).

The ethyl acetate extract of *C. vulgare* had the highest phenolic content with 55.07 mg of GAE/g of extract while the lowest content was measured for the acetone extract with 24.71 mg of GAE/g of extract. Total flavonoid content was ranged between 82.56 mg of RUE/g of extract for acetone extract to 167.96 mg of RUE/g of extract for ethyl acetate extract. The activity of ethyl acetate extract was in relation with total phenol and flavonoid content. The most sensitive bacteria towards ethanol, ethyl acetate and acetone extract of *C. vulgare* were gram-positive bacteria: *S. aureus* ATCC 25923 and *B. subtilis*. The reason for higher sensitivity of the gram-positive bacteria than gram-negative bacteria could be ascribed to their differences in cell membrane constituents and their arrangement. The gram-positive bacteria contain an outer peptidoglycan layer which is an ineffective permeability barrier (Scherrer and Gerhardt, 1971). The resistance of gram-negative bacteria towards antibacterial substances may be due to outer membrane carrying the structural lipopolysaccharide components which makes it impermeable to lipophilic solutes and porins constitute a selective barrier to the hydrophilic

Table 3. Fractional inhibitory concentration (FIC) indices for the combinations between the extracts of *Clinopodium vulgare* and antibiotics.

Species	Antibiotic	MIC (µg/ml)	Ethanol extract	Ethyl acetate extract	Acetone extract
E. coli ATCC 25922	Gentamicin	0.39	1.56 (I)	1.56 (I)	1.35 (I)
	Cephalexin	6.25	1.45 (I)	1.33 (I)	1.33 (I)
S. aureus ATCC 25923	Gentamicin	0.19	1.24 (I)	1.12 (I)	1.24 (I)
	Cephalexin	6.25	1.34 (I)	1.19 (I)	1.195 (I)
P. aeruginosa ATCC 27853	Gentamicin	0.098	1.187 (I)	1.137 (I)	1.187 (I)
	Cephalexin	>1000	n.d.	n.d.	n.d.
B. subtilis	Gentamicin	3.125	0.44 (S)	0.44 (S)	0.395 (S)
	Cephalexin	12.5	0.44 (S)	0.44 (S)	1.29 (I)
S. aureus	Gentamicin	0.39	n.d.	1.24 (I)	1.184 (I)
	Cephalexin	1.56	n.d.	1.23 (I)	1.07 (I)
E. faecalis	Gentamicin	0.39	n.d.	1.34 (I)	1.30 (I)
	Cephalexin	3.125	n.d.	1.24 (I)	1.24 (I)
E. coli	Gentamicin	1.56	n.d.	1.67 (I)	1.33 (I)
	Cephalexin	1.56	n.d.	1.67 (I)	1.67 (I)
K. pneumoniae	Gentamicin	6.25	n.d.	1.33 (I)	1.33 (I)
	Cephalexin	500	n.d.	1.67 (I)	0.5 (S)
P. aeruginosa	Gentamicin	>1000	n.d.	n.d.	n.d.
	Cephalexin	>1000	n.d.	n.d.	n.d.
P. mirabilis	Gentamicin	>1000	n.d.	n.d.	n.d.
	Cephalexin	>1000	n.d.	n.d.	n.d.

I: Indifference; S: synergism and n.d: not determined.

solutes (Nikaido and Vaara, 1985).

Combining effects of extracts and antibiotics

It has been known that one of the effective approaches to overcome bacterial resistance is restoration of antibiotic activity through the synergistic action of antibacterial materials from natural and synthesized agents (Zhao et al., 2001; Shiota et al., 2004). In this work, possible joint activity of *C. vulgare* extracts and two antibiotics, gentamicin and cephalexin was evaluated. The experiment was done against pathogenic bacteria which are often a cause of human infection and with whom a problem of drug resistance in clinical settings is connected. The results of checkerboard method expressed as FIC index are presented in (Table 3). For most of all tested bacteria, interactions were indifferent. The synergy was observed in relation to two tested bacteria. *B. subtilis* was affected synergistically by the combinations of all three extracts and gentamicin and the combinations of cephalexin with ethanol and ethyl acetate extract. With *K. pneumoniae*, resistant to cephalexin, the combination of acetone extract and cephalexin demonstrated synergy. It was found that the presence of sub-inhibitory concentrations (1/4, 1/8 MIC) of the extracts modulated the activity of gentamicin and cephalexin by reducing the concentration of antibiotic needed to inhibit the growth of bacteria. The concentration of antibiotics was decreased up to 16-fold.

Synergistic interactions are a result of a combined effect of active compounds from extracts and antibiotics. It seems that both active compounds from extracts and antibiotics directly or indirectly attach the same site on bacterial cell. 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 (Zhao et

al., 2001; Shiota et al., 2004; Sibanda and Okoh, 2007). However, the understanding of mechanism of synergy is fundamental to development of pharmacological agents against bacterial infection.

Conclusion

The results of this work indicate the potential antibacterial efficacy of *C. vulgare* ethanol, ethyl acetate and acetone extract against tested pathogenic bacteria. The significant amount of phenols and flavonoids contribute in total biological activity of this plant. The detection of synergy between the extracts and antibiotics demonstrates the potential of this plant as a source of antibiotic resistance modiflying compounds. The results were especially interesting in relation to tested resistant strain of *K. pneumoniae*, a causer of nosocomial infection.

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