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RESEARCH PAPER

Antibacterial Action of Extracts of *Clinopodium vulgare* L. Curative Plant

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ABSTRACT

Clinopodium vulgare *L. is one of the curative plants used in Bulgarian folk medicine, mainly during wars for the purposes of healing wounds. The antibacterial activity was studied based on its phytochemical properties. By colony forming unit (CFU)/ml values obtained in different intervals after inoculation of 5% extracts of* Clinopodium vulgare *L. in ethanol and propylene glycol, it has been proved that the plant showed a very strong action on bacteria. The effects of this action are on gram-positive and gram-negative microorganisms and also on isolated microorganisms at laboratory conditions from significant urocultures with multiple resistance. These results are very important as a basis for searching possibilities for utilizing the antibacterial properties of this plant pharmaceutically.*

INTRODUCTION

Clinopodium vulgare L. is one of the curative plants applied in the folk medicine of Bulgaria. The plant has been used mostly during wars for the purposes of healing wounds. Bulgarian literature since 1920 has been investigated, but no data have been found about its phytochemical properties or about the action of the plant. Authors from Poland and Moldova (1,2) prove the contents of some biologically active substances in the plant (saponins), but they do not give any data about their exact structure. They also claimed that they contain some aglicon from the group of the triterpens; after hydrolysis, the sugars are galactosa, arabinosa, and ramnosa, and there are tripentenoids, phytol, phytosterenes, and ursolic acid. Also, some higher fatty acids were isolated (3). Obreshkova et al. (4,5) proved for the first time that it contained the following substances: flavonoids (hesperidin, hiperozid, ruthin, querzetin, luteolin, apigenin, akacetin), phenol carbonic acids (coffeinic, rosmarinic, *P*-kumarinic, ferrulic); oleanolitic acid, ursolic acid, and fluor were isolated from the triterpen mixture.

Until now, no preparation known in medicine or cosmetics has been prepared on the basis of the extract of this plant. Considering the above-mentioned compound, the antibacterial action of 5% ethanol and 5% propylene

glycol extracts of *Clinopodium vulgare* L. have been studied.

MATERIALS AND METHODS

Herb

The perennial grass-plant *Clinopodium vulgare* L. is one of the branches of the Clinopodium family Lamiaceae (Labiatae). In the German botanical literature resources, it is called Calmintha; in the English, it is called Savory. The plant grows in dry, sandy soils in rare woods and among bushes at altitudes not over 1700 m in Europe and Asia.

Extract Production

To produce the extract of *Clinopodium vulgare* L., 5% ethanol extract is obtained from the total superterrestrial part of the plant by triple-stage extraction with ethanol 96% in the ratio herb/extragent 1:3. Standardizing is done after identification of flavonoids and tannic products and quantitative determination of flavonoids toward quercetin. The content of flavonoids in the extract was 12% to 20% as determined by density measurement using the outer standard method. The relative density D_n^{20} was 0.807:0.840. The factor of rotation N_d^{20} was 10.330–10.416. Residual ash measured 6.00–7.80%.

The 5% propylene glycol extract was obtained by means of double-stage extraction with propylene glycol in the ratio herb/extragent 1:2 utilizing the total superterrestrial part of the plant. Standardizing was done by means of identification of flavonoids and tannic products. The content of flavonoids in the extract was 8% to 10% as defined by means of density measurement of flavonoids toward quercetin. The relative density D_n^{20} was 1.010–1.120. The factor of rotation N_d^{20} was 1.370–1.450. The acidic value in milligrams per 1 g of the product was 5.10–40.0.

Bacterial Strains

The experiments used the standard strains *Staphylococcus aureus* 209 P and *Klebsiella pneumoniae* 52145 (Pasteur Institute) from the Bulgarian Type Culture Collection (BTCC, Sofia) and recent clinical isolates from significant urocultures, identification of which was carried out by conventional microbiological methods, with the isolates showing polyresistance toward antibiotics (Table 1). The culture media was lactose broth (LB), meat-peptone agar (MPA), Mueller-Hinton broth (MHB), and Mueller-Hinton agar (MHA). The nutritive properties of the media were proved according to the USP (6).

Antibacterial Agents

The experiments used ampicillin, chloramphenicol, and tetracycline 250-mg capsules, carbenicillin, and cefamandol fl. 1.0 g (Antibiotic Co., Razgrad, Bulgaria); gentamicin amp. 40 mg and Nelidix tab. 500 mg (Sofarma, Bulgaria); rifampicin 150-mg capsules (Ciba Geigy,

Table 1

Resistance to Antibiotics of Bacterial Strains Isolated from Urocultures

	Bacterial Strains[a]				
Antibiotics	*E. cloacae* 213	*K. pneumoniae* 277	*P. mirabilis* 249	*K. Oxytoca* 202	*E. coli* 1
Ampicillin	R	R	R	R	R
Carbenicillin	R	R	R	R	R
Cefamandol	R	R	R	R	R
Cefotaxime	S	S	S	S	S
Cloramphenicol	R	R	R	I	R
Gentamycin	R	R	R	R	R
Nelidix	R	R	R	R	R
Rifampicin	R	R	R	S	R
Streptomycin	R	R	R	R	I
Tetracyclin	R	R	R	R	R

[a] R, resistance (MIC $> 10^3$ mg/L for all antibiotics); S, sensitivity; I, intermediate.

Switzerland); cefotaxime fl. 500 mg (LEK Slovenia); and streptomycin fl. 500 mg (Medexport V/O Moscow).

Testing of Antimicrobial Activity

All procedures were carried out in a sterile laminar airflow box. For preparation of inoculum, to harvest the bacterial cultures, and to wash membrane filters, a pyrogen-free saline test solution (TS) containing 9 g sodium chloride in water to make 1000 ml (6) was chosen; it did not have antibacterial properties under test conditions. Bacterial suspensions were prepared from 24 hr agar cultures in saline TS and adjusted to the 0.5 McFarland turbidity standard, and 9 ml of 5% extract of *Clinopodium vulgare* L. were inoculated with 1 ml of bacterial suspension containing about 10^4 cells/ml. The plate count method was carried out according to the USP (6).

Membrane filtration was carried out according to the USP (6) with the 47-mm Sterifil apparatus as a filter holder and 0.22-μm filters and a hydrophobic edge GSEP 047 AO Millipore. The whole sample contaminated with bacteria was filtered, and after washing the membrane three times by filtering about 100 ml of sterile saline TS, membranes were immediately inoculated in MHA and incubated at 37°C for 24 hr.

To measure the influence of herb extracts on bacterial cells, at different time intervals following the contamination the number of colony forming units per ml (CFU/ml) was determined. The presented numbers of CFU/ml in the figures are the logarithms of the mean values of three determinations by the plate count method. For the validation of the ability of the plate count method to demonstrate the reduction of the count of viable microorganisms, the membrane filtration method was used. The results were expressed as logarithms of reduction calculated according to the following formula: log (number of microorganisms before exposition) − log (number of microorganisms after exposure).

Determination of the Minimal Inhibitory Concentrations

The disk method on MHA and serial dilutions in MHB were used to determine the minimal inhibitory concentrations (MICs). The determination of the resistance (R) and MIC values was based on National Committee for Clinical Laboratory Standards (NCCLS) criteria (7,8).

RESULTS

The effect of the extracts on test microorganisms was evaluated using the value CFU/ml, which was determined in different intervals after inoculation. Both tested extracts (5% propylene glycol and 5% ethanol) of the herb showed strong activity on bacteria even in the first hour after their inoculation with the standard strains *Staphylococcus aureus* and *Klebsiella pneumoniae* (Figs. 1, 2, respectively). The growth and development of the test microorganisms only in the 5% ethanol and in the 5% propylene glycol, measured by the log of CFU/ml, does not differ from the growth and development in ster-

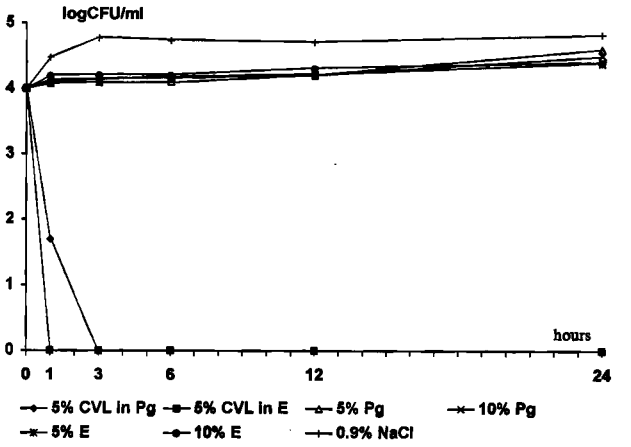

Figure 1. Influence of 5% propylene glycol (PG) and 5% ethanol (E) extracts of *Clinopodium vulgare* L. (CVL) on *Staphylococcus aureus* 209 P at different intervals after inoculation with 10^4 microorganisms/ml.

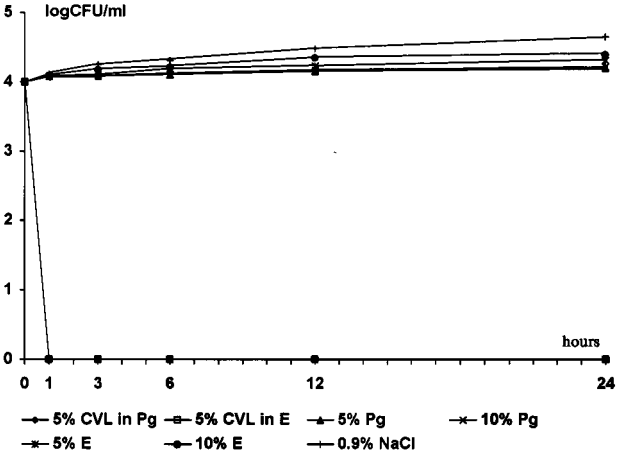

Figure 2. Influence of 5% propylene glycol (PG) and 5% ethanol (E) extracts of *Clinopodium vulgare* L. (CVL) on *Klebsiella pneumoniae* 52145 in different intervals after inoculation with 10^4 microorganisms/ml.

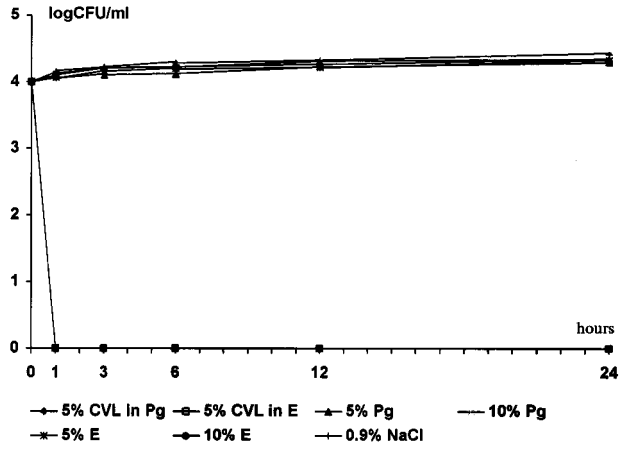

Figure 3. Influence of 5% propylene glycol (PG) and 5% ethanol (E) extracts of *Clinopodium vulgare* L. (CVL) on a polyresistant strain of *Klebsiella oxytoca* 202 at different intervals after inoculation with 10^4 microorganisms/ml.

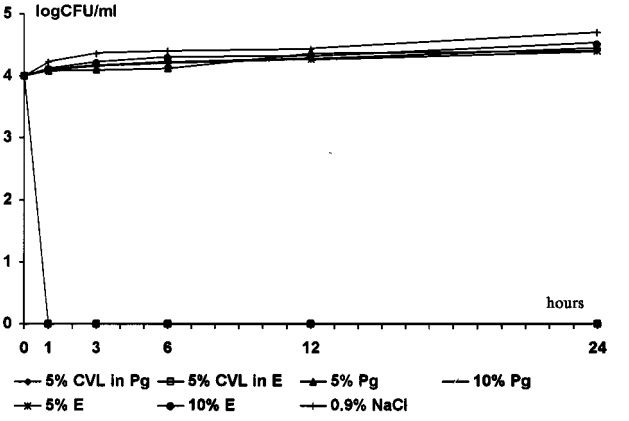

Figure 5. Influence of 5% propylene glycol (PG) and 5% ethanol (E) extracts of *Clinopodium vulgare* L. (CVL) on a polyresistant strain of *Enterobacter cloacae* 213 at different intervals after inoculation with 10^4 microorganisms/ml.

ile saline TS; this fact makes us conclude that the antimicrobial action was due to the presence of the plant extract. Its action on the recent laboratory isolates *Enterobacter cloacae* 213, *Escherichia coli* 1, *Klebsiella pneumoniae* 277, *Klebsiella oxytoca* 202, and *Proteus mirabilis* 249, which are resistant to antibiotics (Table 1), was also similar (Figs. 3, 4, and 5).

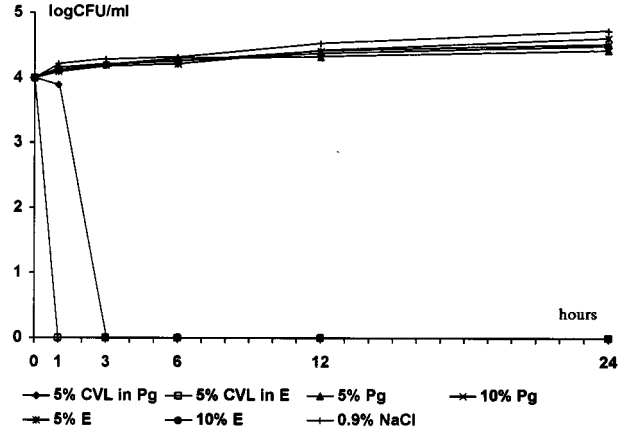

Figure 4. Influence of 5% propylene glycol (PG) and 5% ethanol (E) extracts of *Clinopodium vulgare* L. (CVL) on a polyresistant strain of *Serratia marcescens* 206 at different intervals after inoculation with 10^4 microorganisms/ml.

The results obtained with membrane filtration are presented in Table 2; they also prove a very strong antibacterial effect of the 5% extracts of *Clinopodium vulgare* L. against chosen test microorganisms.

DISCUSSION

The results presented in this report proved a very strong bactericidal effect of the 5% extracts of *Clinopodium vulgare* L. in vitro toward chosen standard strains. These extracts also showed effects on the widely found gram-negative microorganisms that were polyresistant to antibiotics (9,10) and which are of great importance for people in respect to their health and pathology (11,12), especially in causing urine infections. They also have ecological significance and influence on nosocomial infections (13,14).

We chose for our experiments the time-killing curve method despite the fact that the NCCLS has suggested other techniques (15) and despite the evolvement of rapid automated technologies for this purpose over a decade (16). For the purposes of accuracy and reproducibility, the method we used was validated with membrane filtration. The validation of the results proves its accuracy and ability to demonstrate the bactericidal action of the 5% extracts of *Clinopodium vulgare* L.

The possibility of utilizing the antibacterial properties of this plant is a very important feature, taking into consideration its strong activity on resistant strains, as well

Table 2

Antibacterial Effect of Both 5% Extracts of the Herb Tested by Membrane Filtration

	Antibacterial Activity Against[a]				
Herb Extracts	*S. aureus* P 209	*K. pneumoniae* 52145	*E. cloacae* 313	*P. mirabilis* 249	*E. coli* 1
5% Ethanol extract	3.0	3.0	3.0	3.0	3.0
5% Propylene glycol extract	3.0	3.0	3.0	3.0	3.0
Saline TS	−0.22	−0.9	−0.11	−0.8	−0.12
5% Ethanol	−0.18	−0.15	−0.16	−0.1	−0.19
5% Propylene glycol	−0.28	−0.35	−0.21	−0.25	−0.27

[a] As logarithms of reduction after 3 hr at 24°C.

as the rising bacterial resistance to already existing antibacterial agents such as antibiotics (17–19), disinfectants (20–23), and preservatives (24,25). This obviously is one of the reasons for the tendency for the spread of curative plants in contemporary medicine, in most cases preferably instead of synthetic substances (26–29), especially antimicrobials (30–34). The use of natural substances, particularly plants, to control diseases is a centuries-old practice that has led to the discovery of more than half of all ''modern'' pharmaceuticals. Yet, only a small amount of the world's resources of curative plants is being applied in practice, probably due to the small amount of curative plants with chemical properties that have been determined and studied, despite of advancements in this field. The role of natural products will continue as long as there are unexplored sources of novel natural products.

In addition, based on the results obtained, we will try to add raw material for pharmaceutical and cosmetic purposes.

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