ANTIBACTERIAL ACTIVITY OF EXTRACTS FROM *CHELIDONIUM MAJUS* AGAINST *XANTHOMONAS EUVESICATORIA* CAUSING BACTERIAL SPOT OF PEPPER

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ABSTRACT

Bacterial spot of pepper caused by *Xanthomonas euvesicatoria* is an economically important disease which causes yield losses yearly. Control is difficult and is based mainly on copper-based chemicals. However, recent studies showed that copper in concentrations that are most used in practice give contradictory results. The aim of this study was to test the effect of different plant extracts of the common weed *Chelidonium majus* against the causal agent of bacterial spot *X. euvesicatoria* as an alternative mean of control. Methanol, ethanol/water and n-hexane extracts from fresh and freezed plants were obtained and tested. Two of the fractions of methanol extract from freezed aerial parts and ethanol/water extract from roots of *C. majus* possess best antibacterial activity against *X. euvesicatoria* and are suitable for further experiments for control of bacterial spot of pepper.

***Key words**: Chelidonium majus, Xanthomonas euvesicatoria, bacterial spot, phytopathogen*

INTRODUCTION

Bacterial spot of pepper caused by *Xanthomonas euvesicatoria* is an economically important disease which causes yield losses yearly. Control is difficult and is based mainly on copper-based chemicals which are extensively used. However, recent studies showed that copper in concentrations that are most used in practice give contradictory results. Copper (0,1-0,2%) has practically no effect on tomato population of *X. euvesicatoria* in Bulgaria (Vancheva et al., 2014) although pepper strains tend to be more susceptible. Pepper strains from USA are resistant (Garton J. E. 2009).

Natural substances derived from plants provide promising perspectives for alternative control of phytopathogens (Stangarlin et al., 1999; Schwan-Estrada et al., 2005) and are suitable for bioproduction. Different plants extracts are intensively being investigated recently for antimicrobial activities against pathogens but still only a small number of plant species have been investigated.

Celandine (*Chelidonium majus*) is a common weed in nature and around human habitats rich in secondary metabolites. The alkaloid fraction which contains coptisine, chelidonine, sanguinarine, chelerythrine, etc. (Wichtl, 2004) is generally assumed to contribute to the antimicrobial activity of plant extracts (Hahn and Nahrstedt, 1991; HMPC, 2012).

The aim of this study was to test the effect of different plant extracts of *C. majus* against the causal agent of bacterial spot *X. euvesicatoria* as possible mean of control.

MATHERIALS AND METHODS

Plant material: Fresh plant aerial parts and roots were collected during the flowering stage from the region of Sofia field, Bulgaria. Plant materials were used fresh or freezed at -10 °C before extraction.

Bacterial strains: Test bacteria were seven *X. euvesicatoria* strains from the collection of Prof. DSci N. Bogatzevska, ISSAPP ”N. Pushkarov” originating from Bulgaria and Macedonia.

Extractions: Three solvents with different polarity were used: methanol, 96% ethanol/H_2O, and *n*-hexane. Extractions with methanol and *n*-hexane were prepared in Soxhlet extractor at 80 °C for 4 hours. Methanol extracts were concentrated in vacuum evaporator at 55 °C, 300 mbar. After

the evaporation of the solvent a clear liquid fraction was collected at 70 °C, 72 mbar. A second colored liquid fraction and a third soft fraction were separated in the vacuum flask.

N-hexane extracts were concentrated in vacuum evaporator at 40 °C, 325 mbar until a single solid fraction was obtained.

Fresh aerial parts and roots were soaked in 96% ethanol/H_2O (1:1) for 48h in dark bottles at room temperature and filtrated.

The extracts and fractions were stored at 16 °C in air tight brown bottles.

The fractions were diluted in water (%, v/v, w/v) 18 h before the assay. Dimethylsulfoxide (DMSO) was used as dilution agent for the soft and the solid fractions.

Antibacterial assay: The *in vitro* test for antibacterial activity was completed by the agar diffusion method on Nutrient agar with 0.2% glucose. Bacterial suspensions of 100 µl, $1.5x10^7$ cfu/ml were used for inoculums. The wells were filled with 50µl of each substance and left for 2 h prior to incubation. Incubation was held at 28 ºC for 48 h. The antibacterial activity was determined by measuring the inhibition zones in millimeter (diameter) on the 24^{th} and 48^{th} hour. Standard antibiotic discs tetracyclin (30 µg/disk), gentamycin (30 µg/disk), kanamycin (30 µg/disk) and erytromycin (15 µg/disk) were used as control. The experiments were performed in triplicate and the standard deviation was calculated.

The antimicrobial activity was assessed by measuring the diameter of the inhibition zone. The antimicrobial index (AI) was calculated to evaluate the efficacy of the tested extracts and fractions compared to the control antibiotic and expressed in percent:

$$AI(\%) = \left(\frac{E}{A} - 1\right) x\ 100$$

where A is the average inhibitory zone (mm) of the antibiotic and E is the average inhibitory zone (mm) of the tested extract.

RESULTS AND DISCUSSION

Methanol extracts from fresh and freezed plants were separated in three fractions after the evaporation of the solvent and were tested individually. Ethanol/water extracts were tested as crude extracts (tabl. 1).

Tabl. 1. Tested extracts or fractions

Letter of tested substance	***Description of tested substance***
A	Clear liquid fraction of methanol extract from fresh plants
B	Clear liquid fraction of methanol extract from freezed plants
C	Colored liquid fraction of methanol extract from fresh plants
D	Colored liquid fraction of methanol extract from freezed plants
E	Soft fraction of methanol extract from fresh plants
F	Soft fraction of methanol extract from freezed plants
G	Ethanol/water extract from fresh aerial parts
H	Ethanol/water extract from fresh roots
K	N-hexane solid extract

Extracts A, B and K had no effect on the development of the tested *X. euvesicatoria* strains. Extracts C-H exhibited different antibacterial activity (fig. 1, tabl. 2).

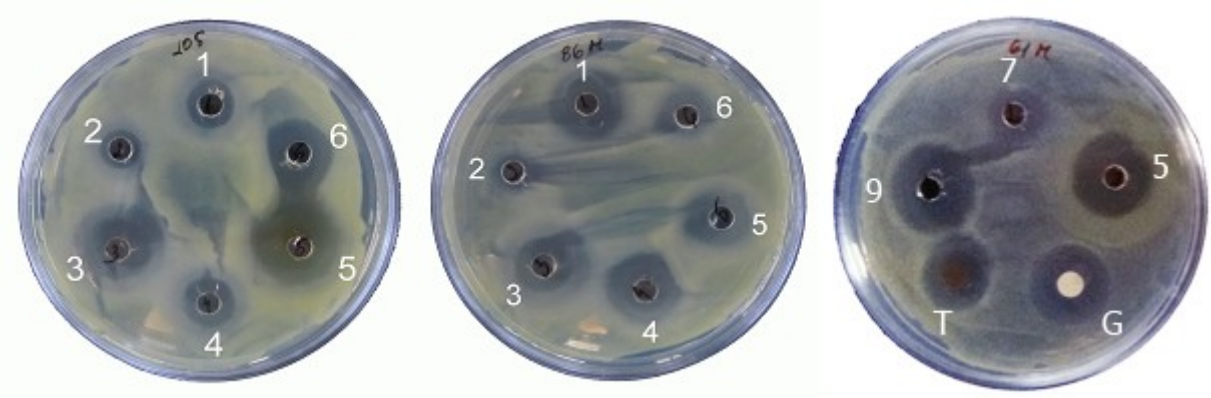

Fig. 1. Antibacterial activity of extracts from *C. majus*
1-5%E, 2-2%E, 3-5%F, 4-2%F, 5-10%D, 6-5%D, 7-G, 8-50%G, 9-H, 10-50%H,
T-tetracycline, G- gentamycin

Tabl. 2. Antibacterial activity of extracts from *C. majus* (mean values of the inhibition zones in mm±standard error)

Strain	E-5%	E-2%	F-5%	F-2%	D-10%	D-5%	G	H
5б	11,00±1	10,00±1,73	14,33±0,58	11,00±0	16,33±0,58	13,67±0,58	12,67±0,58	16,00±1
10б	11,33±0,58	10,33±1,53	15,33±0,58	12,33±0,58	17,00±0	14,00±1	13,00±0	16,33±1,15
71б	10,00±0	9,33±1,53	14,00±1	10,00±0	18,33±1,15	13,00±0	12,67±1,15	18,00±1,73
73б	10,67±0,58	9,67±0,58	14,33±2,52	13,33±2,52	17,33±0,58	11,67±1,53	13,67±0,58	15,67±1,15
61M	12,00±2	9,00±1	13,67±0,58	10,00±0	18,33±1,15	13,67±1,15	11,67±2,31	18,33±1,15
73M	11,67±1,53	7,67±0,58	12,67±0,58	10,67±0,58	17,67±1,15	13,00±1	13,33±2,31	16,00±1
86M	14,00±3	11,33±2,08	13,00±1	12,33±2,31	16,33±0,58	13,33±1,15	13,33±1,53	16,33±1,15

Based on the preliminary results extract C had significantly lower activity compared to D. 2% extract E had low effect on the tested strains as all average zones were below 12 mm. The 5% dilution of E (from fresh plants) had better activity which was commensurate to the values achieved by the 2% extract F (from freezed plants). Good antibacterial activity was achieved by extracts F-5%, D-5% and G. Largest inhibition zones were measured for extracts D-10% and H.

Compared to the extracts from the fresh plants, the extracts from the freezed plants exhibited much better activity. This refers both for the colored liquid fraction and the soft fraction. This could be explained by the cell disruption caused by the freeze-defreeze and the easier migration of the active secondary metabolites in the solvent for the time of the extraction. Differences between the Bulgarian and Macedonian strains were not observed.

The extracts gave satisfactory or good activity which was evaluated compared to four antibiotic controls (fig. 2). 5% extract F had activity close to that of tetracycline. D-10% showed activity commensurate to that of erythromycin and better than tetracycline and kanamycin. Ethanol/water extract from roots (H) exhibited better activity than tetracycline, commensurate to kanamycin and erythromycin. All extracts formed smaller zones compared to gentamycin. Extracts E and G both gave values smaller than the tested antibiotics.

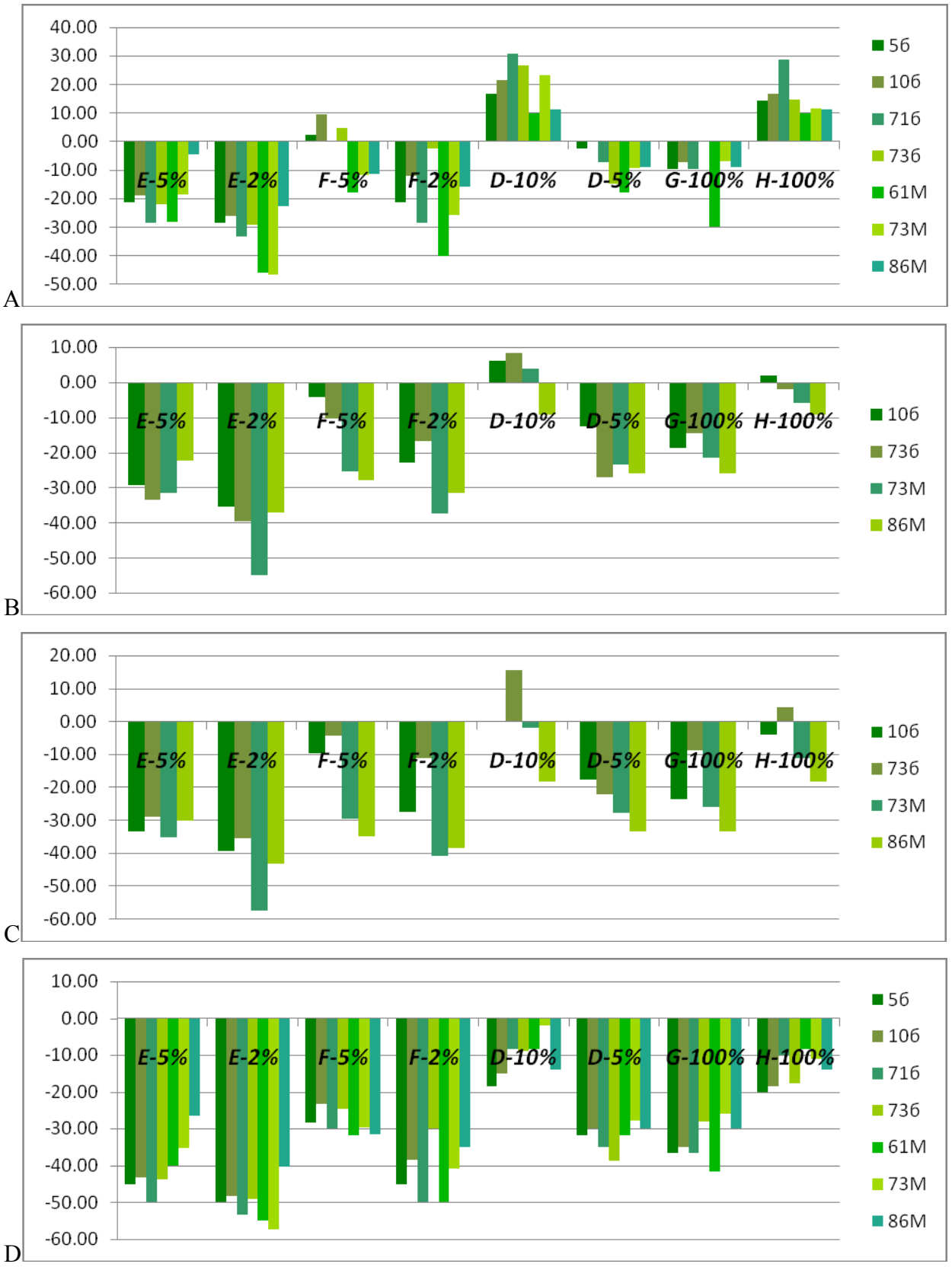

Fig. 2. Antimicrobial index (%) of the tested extracts compared to the antibiotic control: A-tetracycline, B-kanamycin, C-erythromycin, D-gentamycin

C. majus has been tested against several bacterial species but mainly clinically significant pathogens (Ćirić et al., 2008; HMPC, 2012). The plant accumulates several quantitatively significant alkaloids of the benzylisoquinoline type in the aerial parts and the roots and only smaller amounts of acids, flavonoids and carotenoids (Wichtl, 2004; HMPC, 2012). The overall content of alkaloids in the aerial parts is up to 1%, and in the roots - up to 3% (Wichtl, 2004). Since only the extracts obtained by polar solvents (methanol, ethanol/water) possessed antibacterial activity against *X. euvicatoria*, we can generally assume that this activity is due to the alkaloid fractions. The characteristic orange-yellow latex also contains proteolytic enzymes (Wichtl, 2004) but the temperatures for extraction used in the present study eliminate enzyme activity.

According to some data in literature the alkaloid content varies strongly depending on origin and time of harvest and seems to be especially low during the flowering period. If this is the case, the obtained extracts would give the minimum possible inhibitory activity and give even higher results if the plants are collected in the pre-flowering or post-flowering periods.

A comparative study of the antibacterial activity of extracts from plants collected in different periods of vegetation and an analysis of the alkaloid content in the obtained extracts would give more profound answer to these questions.

CONCLUSION

Two of the fractions of methanol extract from freezed aerial parts and ethanol/water extract from roots of *C. majus* possess best antibacterial activity against *X. euvesicatoria* and are suitable for further experiments for control of bacterial spot of pepper.

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