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АНТИБАКТЕРИАЛНА АКТИВНОСТ НА РАСТИТЕЛНИ ЕКСТРАКТИ ОТ *CHELIDONIUM MAJUS* СРЕЩУ ПРИЧИНИТЕЛИТЕ НА БАКТЕРИЙНО СТРУПЯСВАНЕ ПО ДОМАТИ – *XANTHOMONAS VESICATORIA* AND *XANTHOMONAS GARDNERI*

ANTIBACTERIAL ACTIVITY OF PLANT EXTRACTS FROM *CHELIDONIUM MAJUS* AGAINST *XANTHOMONAS VESICATORIA* AND *XANTHOMONAS GARDNERI* CAUSING BACTERIAL SPOT OF TOMATO

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Abstract

Chelidonium majus is an important medical plant used in traditional and folk medicine throughout the world but also a common weed in nature and around human habitats. *C. majus* extracts exhibit antiviral, antitumour, antimicrobial and anti-inflammatory effects. The present study is focused on the investigation of extracts from *C. majus* against *Xanthomonas vesicatoria* and *Xanthomonas gardneri* causing bacterial spot of tomato in Bulgaria as an alternative means of control. The obtained extracts possessed antibacterial activity against the pathogens. One of the fractions showed the best potential for control of bacterial spot of tomato.

Key words: *Chelidonium majus*, *Xanthomonas vesicatoria, Xanthomonas gardneri,* bacterial spot, tomato.

INTRODUCTION

Bacterial spot of tomato caused by *Xanthomonas vesicatoria* and *Xanthomonas gardneri* is an economically important disease which causes yield losses yearly. Control is mainly based on extensive use of copper-based chemicals. However, recent studies showed that most of the Bulgarian strains are resistant to copper in concentration of 0.1% and only a small percent are strongly sensitive to copper in concentration of 0.2% (Kizheva et al., 2013).

Different plant substances provide promising perspectives for alternative control of phytopathogens (Stangarlin et al., 1999; Schwan-Estrada et al., 2005) and plant extracts are being investigated recently for antimicrobial activities. Celandine (*Chelidonium majus*) is a common weed rich in secondary metabolites like coptisine, chelidonine, sanguinarine, chelerythrine, etc. (Wichtl, 2004). It has been used in traditional and folk medicine and *C. majus* extracts exhibit antiviral and antimicrobial effects. However, the plant has been tested mainly against clinically significant pathogens (Ćirić et al., 2008; HMPC, 2012).

In the present research we demonstrate the antibacterial activity of extracts from *C. majus* against *X. vesicatoria* and *X. gardneri* causing bacterial spot of tomato as an alternative 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 six *X. vesicatoria* strains (39t, 31t, 53t, 55t, 58t, 74t) and three *X. gardneri* strains (66t, 73t, 80t) isolated from tomato from the collection of Prof. DSci N. Bogatzevska, ISSAPP "N. Pushkarov" originating from the regions of Sofia, Kostinbrod, Plovdiv, Radnevo, and Topolovgrad (Bulgaria).

Extractions: Methanol and 96% ethanol/H_2O were used as solvents. Extractions with methanol were prepared from 100g freezed plant parts 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.

Fresh aerial parts (50 g) and roots (15 g) were soaked in 96% ethanol/H_2O (1:1) (until fully covered) 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 fraction.

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 28ºC for 48 h. The antibacterial activity was determined by measuring the inhibition zones in millimeter (diameter) on the 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 controls. Water solution of DMSO was used as negative control. The experiments were performed in triplicate and the standard deviation for the fraction activities 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.

Seedling treatment tests: Seedling treatment tests were carried out with artificially infected tomato seedlings from the sensitive cultivars Pink heart and Neven by vacuum infiltration (Bogatzevska, 1988) in two variants five repeats each. Treatments were carried out with 4% colored liquid fraction (Solution 1), 50% ethanol/water extract from fresh aerial parts (Solution 2), and 20% clear liquid fraction (Solution 3).

Variant 1. Healthy seedlings were infiltrated with bacterial suspension of a 48h culture $1x10^6$ cfu/ml of each pathogen under vacuum (1 atm.) for 2x1 min (Bogatzevska and Vitanov, 1989). The seedlings were leaved to dry leaves for 24h and treated with the extract under vacuum at the same conditions.

Variant 2. Healthy seedlings were treated with the extract under vacuum and after 24h infiltrated with bacterial suspension at the same conditions.

Controls: healthy seedlings inoculated with sterile distilled water, healthy seedlings treated with the fractions but not inoculated with pathogen and healthy seedlings artificially inoculated but not treated.

Seedlings were kept in laboratory, in sterile water, at room temperature, at indirect sunlight. Observations were made on the 3, 5, 8, and 12 day.

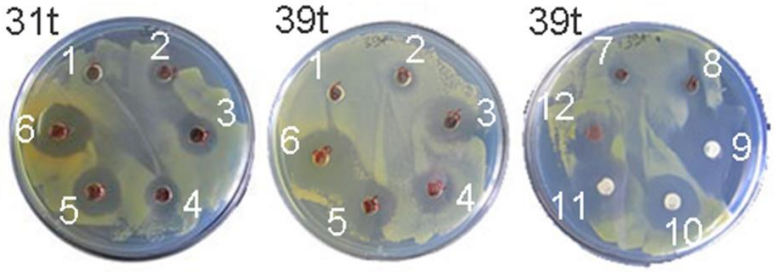
RESULTS AND DISCUSSION

Methanol extracts 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).

Таблица 1. Получени екстракти и фракции
Table 1. Tested extracts or fractions

Означение/ Letter of substance	***Описание на субстанцията/ Description of substance***
B	Clear liquid fraction of methanol extract from freezed plants
D	Colored liquid fraction of methanol extract from freezed 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

Extract B had no effect on the development of the tested strains. Extracts D, F, G and H exhibited different antibacterial activity (fig. 1, tabl. 2). Extracts G and H gave weak activities against the tested tomato isolates. Fraction F gave large variations in its activity against the tested strains from *X. vesicatoria* and *X. gardneri* ranging from lack of effect to very good effect (>15mm inhibition zone). Fraction D as 2% solution exhibited low to satisfactory effect *in vitro* and good to very good effect as 5-10% solutions. Differences between the two pathogens *X. vesicatoria* and *X. gardneri* were not observed (tabl. 2).



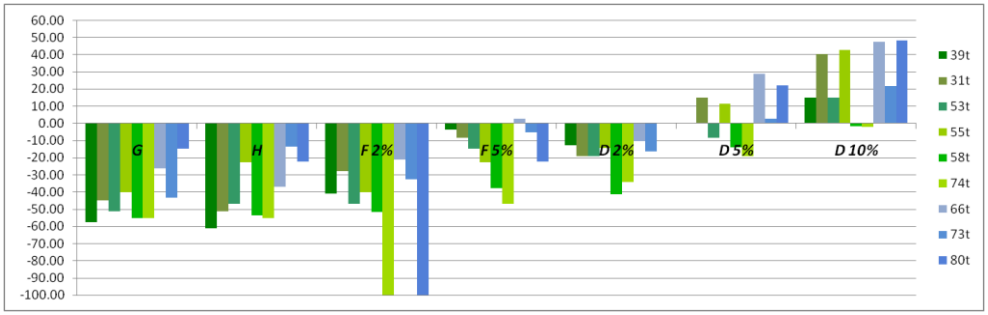
Фиг. 1. *Антибактериална активност на екстракти/фракции от C. majus срещу X. vesicatoria, изолирани от домати*
Fig. 1. *Antibacterial activity of extracts/fractions from C. majus against X. vesicatoria isolated from tomato*
1 – B; 2 – F 2%; 3 – F 5%; 4 – D 2%; 5 – D 5%; 6 – D 10%; 7 – G; 8 – H; 9 – gentamycin; 10 – kanamycin; 11 – erythromycin; 12 – tetracycline

Таблица 2. Антибактериална активност на екстракти/фракции от *C. majus* срещу *X. vesicatoria* и *X. gardneri* от домати (инхибиторни зони в мм±стандартно отклонение)
Table 2. Antibacterial activity of extracts/fractions from *C. majus* against *X. vesicatoria* and *X. gardneri* isolated from tomato (mean values of the inhibition zones in mm±standard error)

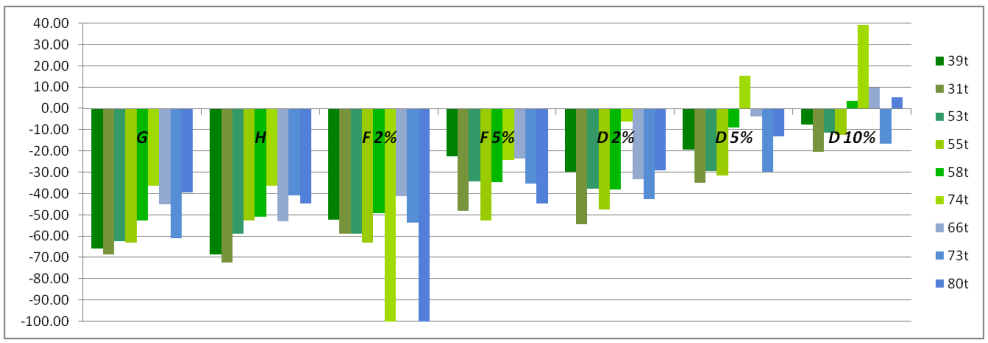
Щам Strain	***G***	***H***	***F 2%***	***F 5%***	***D 2%***	***D 5%***	***D 10%***
39t	7,67±1,15	7,00±0,00	10,67±1,53	17,33±0,58	15,67±0,58	18,00±1,00	20,67±1,53
31t	8,67±1,53	7,67±1,15	11,33±0,58	14,33±0,58	12,67±1,53	18,00±0,00	22,00±1,00
53t	7,67±1,15	8,33±1,15	8,33±1,15	13,33±0,58	12,67±1,53	14,33±2,52	18,00±0,00
55t	7,00±0,00	9,00±1,73	7,00±0,00	9,00±3,46	10,00±3,00	13,00±0,00	16,67±2,89
58t	8,67±1,53	9,00±0,00	9,33±1,53	12,00±0,00	11,33±1,53	16,67±2,31	19,00±1,73
74t	7,00±0,00	7,00±0,00	0,00	8,33±1,15	10,33±1,15	12,67±0,58	15,33±1,15
66t	9,33±2,08	8,00±1,73	10,00±1,00	13,00±2,65	11,33±1,53	16,33±0,58	18,67±2,08
73t	7,00±0,00	10,67±1,15	8,33±2,31	11,67±1,53	10,33±0,58	12,67±2,08	15,00±2,00
80t	7,67±1,15	7,00±0,00	0,00	7,00±0,00	9,00±0,00	11,00±0,00	13,33±1,53

The extracts activity was evaluated compared to four antibiotic controls (fig. 2 and tabl. 3). Extracts with the lowest activities (G, H, and F 2%) gave worst results against all four tested antibiotics. Activities of F 5% and D 2% were average 17,74% and 18,63% lower than the activity of tetracycline, respectively. D as 5% and 10% solution exhibited better results than tetracycline. D 10% gave commensurate result to erytromycin and kanamycin and only 15% lower activity compared to gentamycin.

a



b



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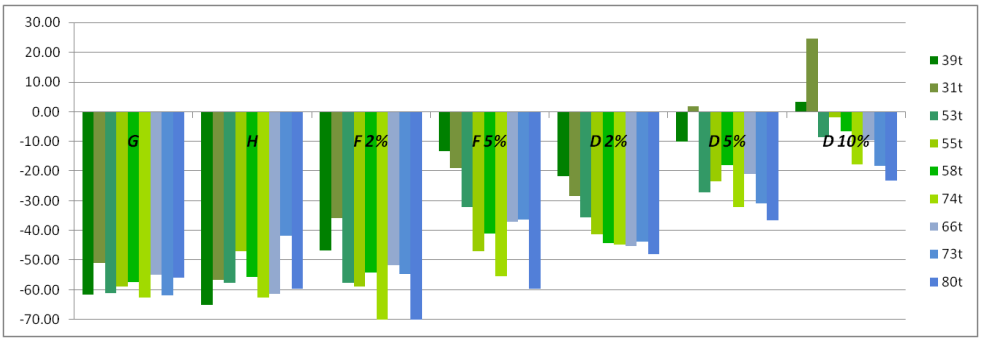
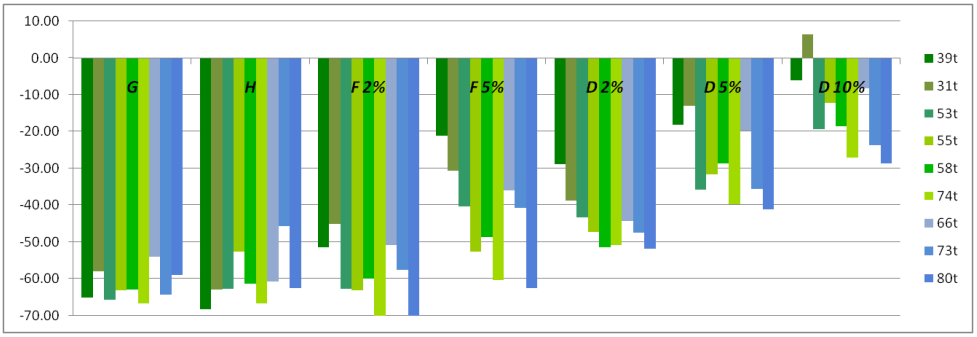


d



Фиг. 2. *Антимикробиален индекс (%) на тестваните екстракти/фракции спрямо контроли антибиотици:* ***a*** *– tetracycline,* ***b*** *– erythromycin,* ***c*** *– kanamycin,* ***d*** *– gentamycin*

Fig. 2*. Antimicrobial index (%) of the tested extracts compared to the antibiotic control: a - tetracycline, b - erythromycin, c - kanamycin, d - gentamycin*

Таблица 3. Средни активности на екстрактите/фракциите от *C. majus* спрямо средните активности на антибиотици (в %)
Table 3. Average activities of extracts/fractions from *C. majus* compared to average antibiotics activities (in %)

Антибиотик Antibiotic	***G***	***H***	***F 2%***	***F 5%***	***D 2%***	***D 5%***	***D 10%***
Tetracyclin	-43,11	-40,35	-51,16	-17,74	-18,63	4,30	25,14
Erytromycin	-54,95	-53,14	-64,16	-35,54	-35,36	-17,34	-1,14
Kanamycin	-58,31	-56,36	-62,14	-37,88	-39,17	-21,93	-6,44
Gentamycin	-62,11	-60,38	-65,66	-43,66	-44,88	-29,23	-15,26

Extract D, G and B were used for the seedlings treatment tests (Solutions 1, 2 and 3). Solution 2 expressed phytotoxic effect on the 2nd day after the treatments and was discarded from the following observations.

The two variants of the seedlings test allowed us to determine the potential of the solutions for preventive or post-infection treatment effects. The plants which were treated after inoculation with the pathogen died around the 12 day after infection. The plants which were treated with Solutions 1 and 3 prior to inoculation with pathogen exhibited single dried leaves or single small leaf spots until the 8th day after infection.

After the 8th day the plants developed without further disorders. The inoculated with pathogens non-treated control plants died or showed many spots, extensive chlorosis and leaf dieback.

Best antibacterial effect was observed for the colored liquid fraction of methanol extract from freezed plants of *C. majus*. This fraction also showed a good potential for prevention of bacterial spot disease and is suitable for further examinations of both seedlings and older plants. Less expectedly, a good potential for disease prevention exhibited also the clear liquid fraction of the methanol extract. This fraction had not antibacterial activity but was also able to prevent disease in tomato seedlings from both tested cultivars. At the present stage of the study it cannot be specified if the preventive effect is due to creation of a physical barrier to the pathogens or induction of resistance.

CONCLUSIONS

The obtained methanol and water-ethanol extracts from *C. majus* possessed antibacterial activity against the causal agents of bacterial spot of tomato - *X. vesicatoria* and *X. gardneri*. Best potential for control of bacterial spot of tomato showed the colored liquid fraction of methanol extract which exhibited both best activity in the *in vitro* assays and good protection of the treated tomato plants against the pathogens.

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