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Antibacterial Activity of Plant Extracts against Bacterial Pathogens of Tomato

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Abstract

Tomato plants of all ages are susceptible to *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas* sp. and *Pseudomonas syringae* pv. *tomato.* Control is based mainly on copper-containing chemicalsand is often unsatisfactory. The object of this study was to test the effect of extracts from different, common for Bulgaria, medicinal and weed plants against referent strains*in vitro*.

Fresh plant aerial parts were oven-dried or freezed before extraction. Methanol and *n*-hexane were used as solvents. Extractions were prepared in Soxhlet extractor at 80°C/4h. Methanol and hexane were recovered in a vacuum evaporator. The fractions were diluted in water (%, w/v) with dimethylsulfoxide as dilution agent for some extracts. The *in vitro* test was completed by the agar diffusion method in triplicate with 50µl of each substance. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone. A total of 25 plants and 47 methanol and *n*-hexane extracts were tested against the pathogenic bacteria of tomato. Extracts from seven plants have potential to be used against *C. michiganensis* subsp. *michiganensis.* Methanol extract from *C. majus* has the biggest potential for control of *Xanthomonas* of tomato and also has some effect against *C. michiganensis* subsp. *michiganensis. H. spectabile* has the potential to control all four bacteria but higher concentrations need to be tested. Methanol extract from *Chaenomeles* sp. has a good potential for control of all pathogens in concentrations of 5%.

Key words: plant extracts, tomato, antibacterial activity, *Clavibacter michiganensis, Xanthomonas, Pseudomonas syringae* pv. *tomato*

Резюме

Доматените растения са чувствителни към патогените от род *Xanthomonas, Clavibacter michiganensis* subsp. *michiganensis* и *Pseudomonas syringae* pv. *tomato* във всички фази от своето развитие. Контролът се основава главно на мед-съдържащи препарати и често е незадоволителен. Целта на настоящето изследване е да се тестват екстракти от различни, нативни за България, медицински и плевелни видове растения срещу референтни щамове на патогените *in vitro*.

Свежите растителни части са изсушени или замразени преди екстракция. Екстракциите са извършени в екстрактор на Soxhlet при 80°C за 4h с метанол или *n*-хексан. Екстрактите са концентрирани във вакуум изпарител. Фракциите са тествани като водни разтвори (%, w/v) с диметилсулфоксид като агент за някои от тях, в количества по 50 µl по метода дифузия в агар, трикратно. Антибактериалната активност е оценена чрез измерване на диаметъра на инхибиторните зони. Проучен е антимикробният ефект на общо 47 метанолови и *n*-хексановиекстракта от 25 растения срещу патогените по домати. Екстрактите от седем растителни вида имат потенциал да бъдат използвани за контрол на *C. michiganensis* subsp. *michiganensis.* Метаноловият екстракт от *C. majus* се отличава с най-голям потенциал за контрол на бактерии от род *Xanthomonas* по домати като показва ефект и срещу *C. michiganensis* subsp. *michiganensis. H. spectabile* притежава активност срещу всички тествани патогени, но е нужно тестването на екстракта в по-високи концентрации за постигане на по-добри резултати. Метаноловият екстракт от *Chaenomeles* sp. е с добър потенциал за контрол на патогените в концентрация 5%.

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Introduction

Bacterial diseases of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas vesicatoria, Xanthomonas gardneri*, and *Pseudomonas syringae* pv. *tomato* have become a significant factor in tomato production all over the world, causing great crop losses in greenhouses and fields every year. Tomato plants of all ages are susceptible to bacterial canker, bacterial spot, and bacterial speck. The pathogens can be present in low levels on asymptomatic plants, multiplying rapidly in favorable weather conditions.

Control of bacterial diseases of tomato is difficult and with unsatisfactory effect. Measures include mainly the use of pathogen-free planting material, cultural practices, and general sanitation measures (Gleason *et al.,* 1993; Obradovic *et al.,* 2004). Copper-based chemicals are extensively used. However, they only reduce epiphytic populations of *C. michiganensis* subsp. *michiganensis* (Gleason *et al.,* 1993) and recent studies have shown that most of the Bulgarian *Xanthomonas* strains are resistant to copper in a concentration of 0.1% and only a small percent are strongly sensitive to copper in a concentration of 0.2% (Kizheva *et al.,* 2013).

Yearly crop losses and the limitations of known measures for control require elaboration of alternative techniques, especially for use in organic farming. The use of natural products derived from plants does not affect the environment and provides an economical and efficient alternative for disease control. Effects of plant extracts and essential oils against pathogens have been extensively studied recently. Plant species can be rich in secondary metabolites some of which exhibit antimicrobial properties against various microorganisms, insects, and herbivores. Nevertheless, only a small percent of the plant species on the earth have been investigated (Cowan, 1999; Stangarlin *et al*., 1999; Schwan-Estrada and Stangarlin, 2005).

Some essential oils provide promising results *in vitro*. Oils from cinnamon, basil, fenchel, thyme, oregano, dictamnus, and marjoram were effective against C. *michiganensis* subsp. *michiganensis* (Daferera *et al.,* 2003; Tanovic *et al*., 2007; Tobias *et al*., 2007) and essential oil of *Origanum minutiflorum* - against *X. vesicatoria* (Altundag and Aslim, 2011), but oils often greatly decrease germination ability (Tobias *et al*., 2007). Indian clove essential oil is not toxic but it provides only 53.0% control of *X. vesicatoria* (Lucas *et al*., 2012). Plant extracts provide a more promising field of research but they are still weakly investigated. Extracts from *Rauvolfia tetraphylla* and *Physalis minima* were effective against *X. vesicatoria* (Shariff *et al.,* 2006) and *Carya illinoensis* in Mexico was effective against *C. michiganensis* subsp. *michiganensis* (Castillo *et al*., 2011). Some extracts from Moroccan plants possessed activity against *P. syringae* pv. *tomato* (Elkhalfi *et al.,* 2013). Crude extracts from *Allium sativum* and *Ficus carica* fruits showed antibacterial effects against *X. vesicatoria, P. syringae* pv. *tomato* and *C. michiganensis subsp. michiganensis,* but the extracts were non-durable (Balestra *et al*., 2009).

The object of this study was to test the effect of different extracts from plants growing on the territory of Bulgaria against phytopathogenic bacteria of tomato.

Material and Methods

Plant material

Fresh plant aerial parts were collected from 24 plant species from the region of Sofiysko pole (Sofia Valley), Bulgaria (Table 1). Fruits from *Chaenomeles* sp. were collected from the fields of the Research Institute of Mountain Stockbreeding and Agriculture, Troyan. Plant materials were oven-dried (22-60°C) to absolute dry weightor freezed at -10°C before extraction.

Bacterial strains

Test bacteria were strains from the collection of Prof. DSci N. Bogatzevska, ISSAPP "N. Pushkarov" originating from tomatoes from Bulgaria: *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato*, *X. vesicatoria, and X. gardneri.*

Extractions

Two solvents with different polarity were used: methanol and *n*-hexane. Extractions were prepared in Soxhlet extractor at 80°C for 4 hours. Methanol was recovered at 55°C, 300 mbar. The firstfraction (clear liquid) was collected at 70°C, 72 mbar. A second, colored fraction (for ACHMI, CHQMA, POROL, and SALHI) and a third, colored fraction were obtained in the vacuum flask based on their solubility in distilled water and 96% ethanol. *N*-hexane was recovered at 40°C, 325 mbar until a single solid fraction was obtained. The *n*-hexane extracts and fractions from methanol extracts 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 a diluting agent for the

Table 1. Plant species tested for antibacterial activity

Plant name	Family	Bayer Code	Common name	Solvents
Achillea clypeolata Smith	*Asteraceae*	ACHCP	yellow yarrow	methanol *n*-hexane
Achillea millefolium L.	*Asteraceae*	ACHMI	common yarrow, milfoil, thousand-leaf	methanol *n*-hexane
Ambrosia atemisiifolia L.	*Asteraceae*	AMBEL	annual/common ragweed, hogweed	methanol *n*-hexane
Artemisia absinthium L.	*Asteraceae*	ARTAB	absinthium, wormwood	methanol *n*-hexane
Chaenomeles sp.	*Rosaceae*	1CNMG	Japanese quince	methanol
Chelidonium majus L.	*Papaveraceae*	CHQMA	greater celandine, tetterwort, nipplewort	methanol *n*-hexane
Clematis vitalba L.	*Ranunculaceae*	CLVVT	old man‘s beard	methanol *n*-hexane
Conium maculatum L.	*Apiaceae*	COIMA	devil‘s bread/porridge, poison hemlock/parsley, spotted corobane/hemlock	methanol *n*-hexane
Consolida regalis Gray, 1821	*Ranunculaceae*	CNSRE	Branching/field/ forking larkspur	methanol
Datura stramonium L.	*Solanaceae*	DATSL	Jimson weed, datura, purple thorn apple	methanol *n*-hexane
Echium vulgare L.	*Boraginaceae*	EHIVU	viper‘s bugloss, blue thistle, blueweed	methanol *n*-hexane
Equisetum arvense L.	*Equisetaceae*	EQUAR	common/field horsetail, marestails, toad pipe	methanol *n*-hexane
Forsithia viridissima Lindley	*Oleaceae*	FOSVI	chinese gold bell, green-stem forsythia	methanol *n*-hexane
Hedera helix L.	*Araliaceae*	HEEHE	common ivy	methanol *n*-hexane
Hylotelephium spectabile (Boreau) Ohba	*Crassulaceae*	SEDSL	showy/butterfly stonecrop, ice plant	methanol
Hypericum perforatum L.	*Hypericaceae*	HYPPE	St John‘s wort, goatweed	methanol *n*-hexane
Iva xanthifolia (Nutt.)	*Asteracea*	IVAXA	burweed marsh elder, false ragweed, giant sumpweed	methanol *n*-hexane
Melilotus albus Medicus	*Fabaceae*	MEUAL	honey/white sweet-clover	methanol *n*-hexane
Melilotus officinalis (L.) Pall.	*Fabaceae*	MEUOF	yellow/ribbed/common melilot, yellow sweetclover	methanol *n*-hexane

Plantago major L.	*Plantaginaceae*	PLAMA	rat's-tail/ large plantain, ripple-seed	methanol
Portulaca oleracea L.	*Portulacaceae*	POROL	common purslane, fatweed	methanol
Ribes nigrum L.	*Grossulariaceae*	RIBNI	black currant	methanol
Salvia hispanica L.	*Lamiaceae*	SALHI	chia	methanol
Tagetes patula var. nana L.	*Asteraceae*	TAGPA	marigold	methanol
Tanacetum vulgare L.	*Asteraceae*	CHYVU	common tansy	methanol *n*-hexane

n-hexane extracts and some of the colored fractions from the methanol extracts.

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 (d=5mm) were filled with 50µl of each substance and left for 2 h prior to incubation. Incubation was held at 28ºC for 48 h.The antimicrobial activity was assessed by measuring the diameter of the inhibition zone after 24 and 48 hours.The antibacterial assay was performed in triplicate.

Results

The clear liquid fractions did not show any antibacterial effect. Water solutions (2% and 5%) of twenty extracts (methanol and *n*-hexane) from 16 plant species did not possess antibacterial activity either (Table 2).

Satisfactory results (11-16 mm inhibition zones) were observed from 5% extracts from ACHMI, ARTAB, CHQMA, 1CNMG, CLVVT, DATSL, SEDSL, HYPPE, IVAXA, RIBNI, and SALHI and from 2% extracts from CHQMA, HYPPE, IVAXA, and MEUOF. The greatest number of plant extracts showed activity against *C. michiganensis* subsp. *michiganensis* – 20 methanol and *n*-hexane extracts from 17 plants. Both methanol and *n*-hexaneextracts from EHIVU and MEUOF had antibacterial properties, though unsatisfactory (≤ 10mminhibition zones) at the tested solutions. Methanol extracts from HYPPE and1CNMG, and *n*-hexane extract from IVAXA gave satisfactory results against this pathogen (Table 2).

P. syringae pv. *tomato* seemed to be the least susceptible towards the tested extracts as only four extracts revealed activity - 1CNMG, SEDSL, POROL, and RIBNI. Seven plant species possessed antibacterial activity against the causal agents of bacterial spot of tomato – *X. vesicatoria* and *X. gardneri*. Good results were observed from extracts from CHQMA and 1CNMG against these two pathogens. SALHI also gave satisfactory results but only against *X. gardneri.*

Extracts from four plants were active against three of the tomato pathogens and only 1CNMG and SEDSL showed effect against all four pathogenic bacteria. However, most of the inhibitory zones formed by SEDSL were not large enough at the tested concentrations.

Discussion

The lack of adequate products for control of plant pathogenic bacteria and the increasing resistance to copper-based chemicals (Gleason *et al.*, 1993; Kizheva *et al.*, 2013) have raised the need to seek new alternatives and environmentally friendly means of plant protection. Plants as sources of secondary metabolites with certain biological activities have been investigated but mainly in the aspect of human health and medicine. Knowledge of plant activities in the aspect of plant protection is still highly insufficient and needs to be enriched.

The plants used in this study are common for Bulgaria species (with the exception of *S. hispanica*), which can easily be found in nature or grown without the need of special facilities. The plants have been purposefully selected to meet this requirement so that the collection of plant biomass is economically advantageous and eventual future commercial production is possible. Studies including screening for antibacterial properties like this one are essential for the next steps of analysis of

Table 2. Antibacterial activity of plant extracts against bacterial pathogens of tomato

Plant species	Extract	Pathogen			
		C. michiganensis subsp. *michiganensis*	*P. syringae* pv. *tomato*	*X. vesicatoria*	*X. gardneri*
ACHCP	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	7	7
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
ACHMI	2% (w/v)met fr-2	8+1 pg*	0	8	7
	5% (w/v) met fr-2	11	0	0	0
	2% (w/v)met fr-3	0	0	0	0
	5% (w/v) met fr-3	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
AMBEL	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	9 pg	0	0	0
ARTAB	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	7	0	0	0
	5% (w/v) hex	11	0	0	0
1CNMG	5% (w/v)met	12	14	12	13
CHQMA	5% (w/v)met fr-2	0	0	13	15
	10% (w/v) met fr-2	0	0	16	18
	2% (w/v)met fr-3	7+2 pg	0	7	11
	5% (w/v) met fr-3	10+2 pg	0	12	16
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
CLVVT	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	8	0	0	0
	5% (w/v) hex	11	0	0	0
COIMA	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	8+1 pg	0	0	0
CNSRE	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
DATSL	2% (w/v)met	8	0	0	0
	5% (w/v) met	11	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
DATSL (seed)	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0

EHIVU	2% (w/v)met	0	0	0	0
	5% (w/v) met	8+1 pg	0	0	0
	2% (w/v) hex	7 pg	0	0	0
	5% (w/v) hex	8	0	0	0
EQUAR	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
FOSVI	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
HEEHE	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	7+2 pg	0	0	0
SEDSL	2% (w/v)met	7	9 pg	7 pg	9 pg
	5% (w/v) met	9	12	9 pg	9
HYPPE	2% (w/v)met	13	0	0	0
	5% (w/v) met	15	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	0	0
IVAXA	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	11	0	0	0
	5% (w/v) hex	13	0	0	0
MEUAL	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	0	0	9 pg	0
MEUOF	2% (w/v)met	11+8 pg	0	0	0
	5% (w/v) met	9	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	9	0	0	0
PLAMA	2% (w/v)met	0	0	0	0
	5% (w/v) met	7 pg	0	0	9
POROL	2% (w/v)met fr-2	0	0	0	0
	5% (w/v) met fr-2	0	0	0	9 pg
	2% (w/v) met fr-3	0	0	0	0
	5% (w/v) met fr-3	7 pg	9 pg	0	0
RIBNI	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	11	9	9
SALHI	2% (w/v)met fr-2	0	0	0	0
	5% (w/v) met fr-2	0	0	0	11
	2% (w/v)met fr-3	0	0	0	0
	5% (w/v) met fr-3	0	0	0	11
TAGPA	2% (w/v)met	0	0	0	0
	5% (w/v) met	7	0	0	0

CHYVU	2% (w/v)met	0	0	0	0
	5% (w/v) met	0	0	0	0
	2% (w/v) hex	0	0	0	0
	5% (w/v) hex	7 pg	0	8	8

*average value;
pg – poor growth of the bacterial strain alone or next to the sterile inhibitory zone;
met – methanol extract; met fr-2 – second fraction from the methanol extract; met fr-3 – third fraction from the methanol extract; hex – *n*-hexane extract

plant active substances, optimizations of extract preparation and working concentrations, *in vivo* testing on crops, and application optimization.

Even though the potential of plants for the purposes of crop protection is still weakly investigated, most of the studies of antibacterial activities concern essential oils or extracts from herbaceous plants (Daferera *et al.,* 2003; Tanovic *et al.,* 2007; Tobias *et al*., 2007; Elkhalfi *et al*., 2013). The plants selected in this study expand the group of potential donors of substances with antibacterial properties by including weed and fruit species. The unpretentious fruit shrubs like black currant and Japanese quince open new opportunities for application of these human health friendly plants.

Conclusion

A total of 25 plants and 47 methanol and *n*-hexane extracts were tested against the economically important pathogenic bacteria of tomato *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato*, *X. vesicatoria,* and *X. gardneri.* Extracts from seven plants have the potential to be used against *C. michiganensis* subsp. *michiganensis.* Methanol extract from *C. majus* has the biggest potential for control of *Xanthomonas* of tomato and also has some effect against *C. michiganensis* subsp. *michiganensis. H. spectabile* has the potential for control of the bacteria but higher concentrations need to be tested. Methanol extracts from *Chaenomeles* sp. has good potential for control of all pathogens in concentrations of 5%.

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