BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Antibacterial activity of *Calendula officinalis* and *Echinacea purpurea* extracts against the causal agent of tomatoes' bacterial canker: *Clavibacter michiganensis* subsp. *michiganensis*

[Actividad antibacteriana de los extractos de *Calendula officinalis* y *Echinacea purpurea* contra el agente causal del cancro bacteriano del tomate: *Clavibacter michiganensis* subsp. Michiganensis]

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Section: **Biological activity**

Received: 25 September 2020
Accepted: 24 January 2021
Accepted corrected: 26 February 2021
Published: 30 September 2021

Citation:
Aksoy HM, Arslanoğlu SF, Edbeib MF, Kaya Y, Marakli S. Antibacterial activity of *Calendula officinalis* and *Echinacea purpurea* extracts against the causal agent of tomatoes' bacterial canker: *Clavibacter michiganensis* subsp. *michiganensis*. **Bol Latinoam Caribe Plant Med Aromat** 20 (5): 496 - 502 (2021). https://doi.org/10.37360/blacpma.21.20.5.36

Abstract: We aimed to investigate the effects of *Calendula officinalis* and *Echinacea purpurea* extracts in terms of growth parameters, antibacterial activity and phenolic profile in tomato infected by *Clavibacter michiganensis* subsp. michiganensis (*Cmm*T7). A significant difference was observed in *E. purpura* extract, indicating the highest effects on plant height (27.25 cm), fresh plant weight (28.45 cm), root length (24.42 cm), and root weight (6.74 g) (p<0.05). Moreover, *Calendula officinalis* and *Echinacea purpurea* extracts showed significant inhibitory activity against CmmT7 (p<0.05). Among phenolic compounds, the only chlorogenic acid amounts were varied in the tomato seedlings leaves with *C. officinalis* extract (K3) + *Cmm*T7, *E. purpurea* extract (E3) + *Cmm*T7 and *Cmm*T7 (p<0.01). Moreover, chlorogenic acid amount was approximately 9 times higher than in *Cmm*T7-treated leaves when compared to control. The results showed that application of the extracts of these plants had a significant influence on bacterial canker and growth parameters.

Keywords: Leaf extract; Plant pathogenic bacterium; Plant bioactive compounds; Medicinal plants; Tomato

Resumen: Nuestro objetivo fue investigar los efectos de los extractos de *Calendula officinalis* y *Echinacea purpurea* en términos de parámetros de crecimiento, actividad antibacteriana y perfil fenólico en tomate infectado por *Clavibacter michiganensis* subsp. michiganensis (*Cmm*T7). Se observó una diferencia significativa en el extracto de *E. purpura*, que indica los mayores efectos sobre la altura de la planta (27,25 cm), el peso de la planta fresca (28,45 cm), la longitud de la raíz (24,42 cm) y el peso de la raíz (6,74 g) (p<0,05). Además, los extractos de *Calendula officinalis* y *Echinacea purpurea* mostraron una actividad inhibidora significativa contra CmmT7 (p<0,05). Entre los compuestos fenólicos, las únicas cantidades de ácido clorogénico se variaron en las hojas de las plántulas de tomate con extracto de *C. officinalis* (K3) *Cmm*T7, extracto de *E. purpurea* (E3) *Cmm*T7 y *Cmm*T7 (p<0.01). Además, la cantidad de ácido clorogénico fue aproximadamente 9 veces mayor que en las hojas tratadas con CmmT7 en comparación con el control. Los resultados mostraron que la aplicación de los extractos de estas plantas tuvo una influencia significativa sobre el cancro bacteriano y los parámetros de crecimiento.

Palabras clave: Extracto de hoja; Bacteria fitopatógena; Compuestos bioactivos vegetales; Plantas medicinales; Tomate

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* is a Gram-positive bacterium, causing bacterial canker on *Solanum lycopersicum* and also huge financial losses in marketable tomato production zones (Gleason *et al*., 1993; Moustaine *et al*., 2019). Bacteria pass through a natural opening such as stoma before they move into the xylem (Gartemann *et al*., 2003; Ansar *et al.*, 2019). As a result of stunting and wilting, crop quality and loss may occur in plant and "bird's eye" on the fruit (Utkhede and Koch, 2004; Osdaghi *et al*., 2020). The solution of the tomato bacterial canker disease is based on the rotation of the crop, clean transportation practices, and even the usage of healthy seeds (Tireng Karut et al., 2019). In addition to these natural solutions, chemical pesticides have been applied but the disease has still been appeared and also caused several symptoms on plants. Therefore, the prevention of bacterial disease by applying chemical pesticides are restricted or prohibited in many countries (Valdés *et al*., 2017). For this reason, biological control as natural methods has been commonly used for plant protection. In these methods, naturally extracted substances obtained from plants are commonly used as a biological control against plant pathogens (Valdés *et al*., 2017; Bahaman *et al*., 2020).

C. officinalis L. (Asteraceae) has been one the best known traditional medicinal plants with stimulant and antispasmodic properties since ancient times (Latifian *et al*., 2018). The plants are used in folk medicine of different part of world, especially Europe. The plant contains different phenolic compounds including quinones, volatile oil, carotenoids, triterpenoids, flavonoids, coumarins etc. (Muley *et al*., 2009). *C. officinalis* was chosen for its properties to inhibit different anaerobic and facultative aerobic periodontal bacteria including *Porphyromonas gingivalis*, *Prevotella* spp., *Furobacterium nucleatum*, *Caphocytophaga gingivalis*, *Veilonella parvula*, *Eikenella corrodens*, *Peptostreptococcus micros* and *Actinomyces odontolyticus* (Lauk *et al*., 2003; Khare 2004; Ben-Erik and Michael, 2004; Gazim *et al*., 2008). Furthermore, *C. officinalis* extracts indicated anti-cancer effects *in vitro* studies on tumor cell lines (Jimenez-Medina *et al*., 2006). Another plant used in this study, *Echinacea purpurea* L., has been widely used to alleviate colds, rethroats and other upper respiratory infections. This plant consists of caffeic acid derivatives, alkamides, polysaccharides and glycoproteins. Especially caffeic acid derivatives possess many bioactive functions such as antioxidant, antidiabetic, antihyaluronidase, antiviral activities (Chiou *et al*., 2017).

In this study, phenolic profile of *C. officinalis* and *E. purpurea* was analysed. Moreover, antibacterial effects and also phenolic profiles of *C. officinalis* and *E. purpurea* extracts in tomato seedlings infected by *C. michiganensis* subsp. *michiganensis* (*Cmm*T7) were also investigated by using disc diffusion method and HPLC, respectively in addition to growth parameters.

MATERIALS AND METHODS

Plant material and preparation of extracts

C. officinalis and *E. purpurea* were collected from Field Crops Department's experimental area of Agricultural Faculty of Ondokuz Mayis University, Samsun, Turkey. The harvested samples were transferred in the clean plastic bags and numbered. For one week, the plants were air-dried in darkness followed by drying in an oven at 35ºC for 24 hours. The plant leaves were pulverized and 2 ml methanol (80%, v/v) was added to 200 mg plant powder for methanolic extraction. Then, samples were incubated with shaking overnight. The extracts were obtained by centrifuging at 10000 *x g* for 30 min. The supernatants were collected and kept at 4ºC until use.

Disc diffusion test

Antibacterial effects of plant extracts on *Cmm*T7 were evaluated by Kirby-Bauer disc diffusion method (Tortora *et al*., 2001). The bacterial isolate, *Cmm*T7, was provided by Ondokuz Mayis University, Agricultural Faculty, Plant Protection Department, Bacteriology Lab. (Samsun, Turkey). The bacterial isolate was incubated for 24-48 h at 26ºC on Nutrient Agar-NA (Difco). Then, the turbidity of the suspension was adjusted to an optical density at 600 nm (OD_{600}) of 0.7 (~1 × 10^8 CFU/mL). Samples from 100 µl of the inoculum media were added on NA plaques under aseptic conditions and spread using sterilized cotton swabs. Filter paper discs (Oxoid) soaked with 15 µl of each plant aqueous extract (1 g/ml) were placed on NA in Petri dishes, incubating at 26ºC for 24-48 h. The inhibition zone was measured in diameters using a stereomicroscope (Olympus SZ-61). The experiment was repeated three times.

Effect of the plant extracts and CmmT7 on plant growth

4-week-old tomato seedling cv. H 2274 was used for the determination of antibacterial effects of *C. officinalis* and *E. purpurea*. The concentration of

bacteria suspensions was 10^8 CFU/ml and the plant extracts were 20 g/l. Both bacteria and extracts were applied to tomato plants for one week. There were six treatments: (i) tomato seedlings with *C. officinalis* extract (K3)+*Cmm*T7, (ii) tomato seedlings with *E. purpurea* extract (E3)+*Cmm*T7, (iii) tomato seedlings with *C. officinalis* extract (K3), (iv) tomato seedlings with *E. purpurea* extract (E3), (v) tomato seedlings + *Cmm*T7, (vi) tomato seedlings with deionized sterile water (control). Only plant extract applications (iii – iv) were as follows: lateral roots of 4 weeks-old tomato seedlings were pruned with a scalpel and soaked in 50 ml of the extracts of *C. officinalis* and *E. purpurea* for 2 h at room temperature. Then, *Cmm*T7 was inoculated onto the same tomato seedlings by the cotyledon clipping method for i-ii applications. Moreover, *Cmm*T7 isolate was inoculated to the 4-week-old tomato plantlet for the treatment v by using the cotyledon clipping method (Xu *et al*., 2010). Cotyledons of tomato plantlets were obtained using deionized sterile water method for the control treatment (vi). After applications, tomato plantlets were planted into 250 ml pots including a soil mixture (1:1 soil:sand, w/w). The samples were then transferred in sterilised plastic saucers on greenhouse benches to prevent contamination. The pots were randomly placed in growing chambers at 24ºC, 16/8 day/night conditions and 70% relative humidity. Experiments were repeated three times for each bacterial strain and plant extract combinations. Plant heights, fresh plant weights, root lengths and root weights were analysed in addition to phenolic compounds.

Extraction of phenolic compounds

Tomato seedling leaves were analysed for the determination of phenolic compounds. Samples were quickly transferred into a liquid nitrogen tank to avoid spoilage and stored at -80ºC. Then the phenolic compounds were identified by HPLC analysis. The standard technique was performed with minor modifications (Yang *et al*., 2015). Every single sample (1 g) was mixed with 20 ml of 80% methanol-water mixture (v/v) used as an extraction liquid and vortexed for 10 min. Afterwards, the solutions of extracts were transferred for 1 h at 50ºC. After centrifugation at 12.000 rpm at 4ºC for 10 min, the supernatants were filtered through PTFE hydrophilic syringe filter (0.45 µm pore size, 13 mm diameter). Dried samples were resuspended in 1.0 mL HPLC grade methanol by vortexing and stored at 4ºC for further analysis.

Statistical analyses

Kolmogorov-Smirnov One Sample test results presented that all traits could be assumed normally distributed ($p<0.05$). Levene variance homogeneity test results showed that all traits had homoscedasticity ($p<0.05$). Then, One-Way ANOVA test was performed on all data, Duncan's multiple range test was performed to compare the means. Moreover, relations among traits were also investigated by using Pearson Correlation analyses with SPSS 20.0 program.

RESULTS AND DISCUSSION

Disc diffusion test

The inhibitory values (mm) of *C. officinalis* and *E. purpurea* extracts against *Cmm*T7 were indicated in Table No. 1. *C. officinalis* and *E. purpurea* extracts showed significant similar inhibitory activity against *Cmm*T7.

Table No. 1
Antibacterial activity of *C. officinalis* and *E. purpurea*

Treatments	***Cmm*T7**
	Mean diameter zone of inhibition (mm)* (Mean ± SE)
Control	0.0 ± 0.0**b**
C. officinalis (K3)	42.0 ± 1.76**a**
E. purpurea (E3)	47.5 ± 1.48**a**

Effect of the plant extracts and CmmT7 on plant growth

Tomato seedlings were treated with *C. officinalis*, *E. purpurea* extracts alone and also in combination with *Cmm*T7 in addition to deionized sterile water (control), showing similar effects on all plant growth parameters including root weights, root length, plant height and fresh plant weight (Figure No. 1). Moreover, a significant difference was also recorded between plant extracts - treated tomato seedlings and other treatments (K3 + *Cmm*T7, E3 + *Cmm*T7, and *Cmm*T7) ($p<0.05$). Among the treatments, extract of E3 showed the highest effects on plant height (27.25 cm), fresh plant weight (28.45 g), root length (24.42 cm), and root weight (6.74 g) ($p<0.05$) (Figure No. 1).

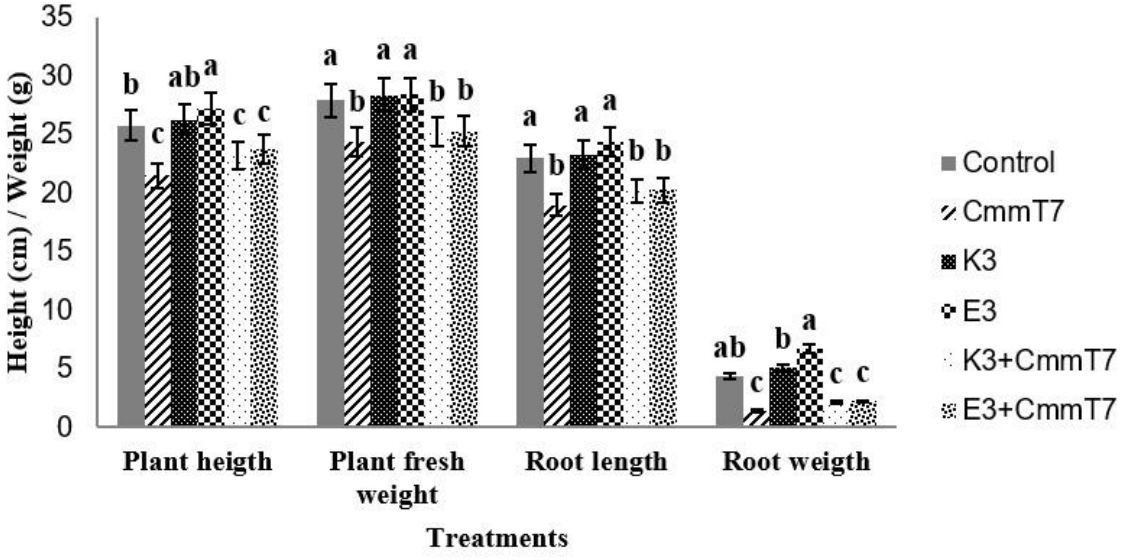

Figure No. 1
The antibacterial effects of *C. officinalis* and *E. purpurea* alone and in combination with *Cmm*T7 on growth of tomato (The values are expressed in mean ± standard deviation. Different letters above the bars are significant at $p<0.05$ as a result of Duncan's Multiple Range Test).

Phenolic contents

Phenolic compounds consisting of vanillic acids, tannic, catechin, rosmarinic, rutin, salicylic, caffeic, cinnamic, ferulic, gallic and oxalic were detected in trace amounts in all treatments (<0.01, <0.025, <0.01, <0.01, <0.04, <0.01, <0.05, <0.01, <0.05, <0.01 and <0.01 µg/100 g dry weight, DW). Only one phenolic compound, chlorogenic acid was significantly presented. The accumulation of chlorogenic acid (8.90 µg/100 g DW) was measured as the highest amount in leaves with treated *Cmm*T7, indicating 9 times higher than control plants. In addition, K3 + *Cmm*T7 and E3 + *Cmm*T7 – treated leaves, were significantly higher than KE and E3 extracts and even control (Figure No. 2).

In this study, both *in vivo* and *in vitro* experiments showed that the extracts of *C. officinalis* and *E. purpurea* had a positive effect on growth parameters and also antibacterial activity on *Cmm*T7. Supporting to our result, Goyal and Mathur (2011) also reported that there was an excellent inhibition response of *C. officinalis* extracts against *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* sp., coagulase-positive *Staphylococcus* sp., together with coagulase-negative *Staphylococcus* sp., *C. albicans* and *Candida parapsilosis*. In addition to antibacterial activity, antifungal activities of *C. officinalis* extracts have also been investigated (Gazim *et al*., 2008; Goyal & Mathur, 2011). One of them was carried out by Mehtab *et al*. (2017). They concluded the antifungal activity of the *C. officinalis* on *A. pori*, *A. niger* and Diplodia, resulting high amount of inhibition. The other experiment was performed by Efstratiou *et al*. (2012), revealing antifungal activity when compared to Standard antibiotic, Fluconazole. There are different antimicrobial activities response against different kind of microorganisms. This could be variations of microorganisms (Maeda *et al*., 2008).

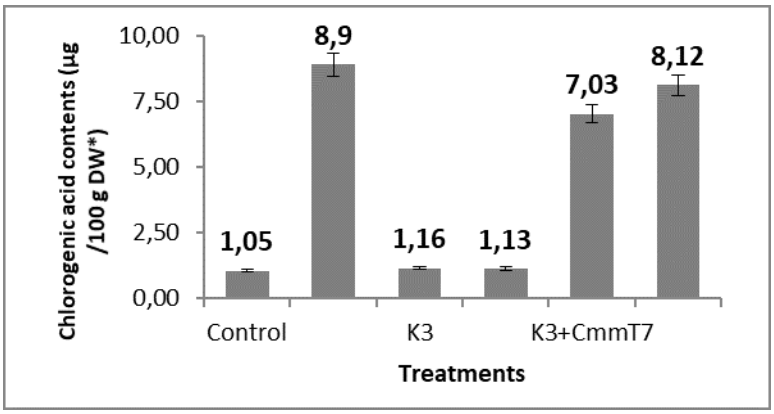

Figure No. 2
Total phenolic compounds contents in tomato seedlings leave (*: DW-dry weight. The values are expressed in mean ± standard deviation. Different letters above the bars are significant at $p<0.01$ as a result of Duncan's Multiple Range Test)

The antibacterial activity of *E. purpurea* also showed similar results with *C. officinalis*. Our findings were concordant with Oláh *et al.* (2017) who showed anti-inflammatory effects of *Echinacea purpurea* - derived alkylamides on atopic eczema. In addition, Tsai *et al.* (2012) also examined antioxidant and antimutagenic effects of *E. purpurea* flower extracts, reporting antioxidant and antimutagenic activities in freeze-dried extracts. Another study was also related to phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of *E. purpurea* and *E. angustifolia* extracts, showing different chemical content and biological effects (Aarland *et al*., 2017).

Phenolic compounds have been widely investigated in many studies. Lin *et al.* (2011) reported that cichoric acid was the main and caftaric acid was the second phenolic compounds in dried, *E. purpurea* materials. Moreover, García-Risco *et al.* (2017) examined Asteraceae (*Achillea millefolium* and *Calendula officinalis*) and Lamiaceae (*Melissa officinalis and Origanum majorana*) extracts in terms of the total content of phenolic compounds. They reported that Asteraceae extracts determined lower content of flavonoids and phenolics than Lamiaceae plant extracts. We identified caffeic, catechin, cinnamic, ferulic, gallic, oxalic, rosmarinic, rutin, salicylic, tannic and vanillic acids in all treatments but chlorogenic acid was found in *Cmm*T7-treated leaves (9-fold) when compared to control. There are many studies related to pathogenic bacteria and chlorogenic acid interactions (Baker *et al*., 2017). One of them was performed by López-Gresa *et al.* (2011). They supported our results and concluded that a rapid increase in the concentration of chlorogenic acid in tomato plants after infection with the bacterial pathogen *Pseudomonas syringae*. Moreover, Aksoy *et al.* (2017) also analysed the effect of *Pseudomonas putida* on tomato infected by *C. michiganensis* subsp. *michiganensis*. They revealed that *P. putida* - treated and *C. michiganensis* subsp. *michiganensis* - treated plants showed varying amounts in terms of chlorogenic acid, caffeic acid, catechin and rutin phenols. Moreover, they reported a direct relationship between the level of catechin and seedling survivability. Therefore, we could conclude that different phenols might play important roles in different bacterial diseases.

CONCLUSION

There is no effective pesticide for bacterial canker. Therefore, biological control is an important phenomenon to protect plants also commonly used as alternatives to pesticides. Our results contribute to valuable information related to the effects of phenolic compounds and antibacterial activity of *C. officinalis* and *E. purpurea* on *Cmm*T7 for the development of natural biopesticides. Further studies are necessary to evaluate which phenolic compounds are responsible for antimicrobial activity in bacterial canker in detail and also the antimicrobial effects of *C. officinalis* and *E. purpurea* in different bacterial diseases.

ACKNOWLEDGEMENTS

The authors would like to thank Project Management Office of Ondokuz Mayis University for financial support of the project (PYO.ZRT.1901.14.008)

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