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ORIGINAL PAPER

Control of *Agrobacterium tumefaciens* with Essential Oils Compared to Antagonistic *Agrobacterium radiobacter* Strain K84

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ABSTRACT

Crown gall is one of the most hazardous diseases for nurseries of stone fruit plants. Biological control of crown gall caused by *Agrobacterium tumefaciens* using *Agrobacterium radiobacter* K84 is more comparatively effective in disease control compared to chemical means. In the present work, the distinguished components of essential oils of caraway (*Carum carvi* L.) were carvone (79.38157%) and limonene (18.78265%), and those for thyme (*Thymus vulgaris* L.) were identified as thymol (14.79336%), 1,8-Cineol (14.45795%), borneol (13.22024%), β-caryophyllene (10.86938%) and bornyl acetate (5.08278%). The inhibition zone produced by the antagonist *A. radiobacter* against *A. tumefaciens* 27AS_ Pp4 was 11.8 mm in diameter, whereas it was 10.2 and 11.0 mm in caraway and thyme essential oils, respectively. The minimal inhibitory concentration (MIC) of essential oils against *A. tumefaciens* 27AS_Pp4 was 12.8 µl/ml of both caraway and thyme as compared with the minimal bactericidal concentration (MBC) being 25.6 and 12.8 µl/ml for caraway and thyme, respectively. When *A. tumefaciens* 27AS Pp4 was treated with caraway and thyme, the crystal violet uptake was increased. No disease syndromes were observed on apricot (*Prunus armeniaca* L.) seedlings inoculated with the pathogen *A. tumefaciens* 27AS_Pp4 and the antagonist *A. radiobacter*. Moreover, a significant decrease in the number of galls was observed in caraway and thyme essential oil treatments compared to the positive control. The fresh and dry weight of galls per plant confirmed the effectiveness of caraway and thyme treatments. The treatment of caraway essential oil showed a significant decrease in the fresh weight of shoots and roots compared to both positive and negative controls and *A. radiobacter* treatments. No significant differences in the fresh weight of shoots and roots in thyme oil treatment compared to negative control and *A. radiobacter*. Determination of the dry weight of shoots and roots comparatively confirmed these results. On the other hand, the essential oil of caraway showed a significant decrease in plant length compared to thyme, *A. radiobacter*, and positive and negative control treatments. In the case of thyme oil, the length of the plant has increased significantly compared to the negative control, with no significant difference between thyme oil and *A. radiobacter* treatments. These results suggest that the essential oils of caraway and thyme have a reasonable potential for controlling crown gall disease. Consideration should be given to the potential effect of the essential oil of caraway on plant growth. Further work with different horticulture seedling nurseries is needed.

Keywords: Apricot, *Prunus armeniaca*, Crown gall, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter* strain K84, Essential oils, Caraway, *Carum carvi*, Thyme, *Thymus vulgaris*.

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INTRODUCTION

Agrobacterium tumefaciens (= *Rhizobium radiobacter*) is a soil-borne bacterium that causes a neoplastic disease (crown gall), principally in the most dicotyledonous plants. It is most dangerous for the nursery production of many plants and is responsible for extensive economic losses in seedling production of fruit trees, roses, and grapevines in many countries (Young *et al*., 2001 and Pulawska, 2010).

Chemicals and antibiotics give partial control of crown gall and may be phytotoxic (Canfield *et al*., 1992). On the other hand, biological control of crown gall with *Rhizobium rhizogenes* strain K84 (= *A. radiobacter* K84 = *A. rhizogenes* K84) is more effective in controlling the disease than chemical control (Moore, 1977; Slater *et al*., 2009; and Velázquez *et al*., 2010). The use of strain K84 is considered a good example of effective microbial biological control (Schroth and Hancock, 1981). Some genetic factors limit the application of strain K84 as a bioagent against crown gall disease, such as (i) some *A. tumefaciens* strains (octopine type) are resistant to agrocin 84, (ii) some pathogenic agrocin84-sensitive agrobacteria mutate to an agrocin-resistant phenotype, (iii)

pAgK84 can be transferred from strain K84 into virulent agrobacteria, where it can become an agrocin producer and resistant to strain K84, and (iv) oncogenic agrobacteria may provide strain K84 with a Ti plasmid, allowing it to develop into virulent strains that produce agrocin while also being resistant to it (Otten *et al*., 2008). In other studies, bacterial and actinomycetes isolates were tested for antagonistic activity against *Agrobacterium tumefaciens* or their ability to control crown gall, but none were developed further, despite the fact that some were effective (Oskay *et al*., 2004; Tawfik *et al*., 2005; and Abd El-Rahman *et al*. 2019).

Plant essential oils demonstrate antibacterial activity against plant pathogenic bacteria and have been used to control bacterial diseases in plants (Koul *et al*., 2008; Oboo *et al*., 2014; and Bouaichi *et al*., 2015). The plant essential oils have a toxic and inhibitory effect on organisms due to their antimicrobial components (Isman, 2000 and Nazzaro *et al*., 2013). The composition of essential oils can be separated into two groups of components: the first has terpenes and terpenoids, and the second has aromatic and aliphatic compounds (Faleiro, 2011). Many factors, such as the environment, growing region, and cultivation practices, can affect the isolation, yield, and chemical composition of the essential oils (Hudaib and Aburjai, 2007).

The essential oils, as well as their distinguished components, are responsible for antimicrobial activity (Vyas, 2012). Iacobellis *et al*. (2005) found that essential oils of *Cuminum cyminum* and *Carum carvi* had antibacterial activity against Gram-positive and Gram-negative bacterial species, with the activity being particularly strong against the genera *Clavibacter*, *Curtobacterium*, *Rhodococcus*, *Erwinia*, *Xanthomonas*, *Ralstonia*, and *Agrobacterium*. Thyme oil is widely used for inhibiting gram-negative pathogens (Vyas, 2012). The mechanisms of action of the essential oil include the degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm coagulation, damaging the membrane proteins, increasing membrane permeability, and decreasing ATP synthesis (Nazzaro *et al*., 2013 and Yang *et al*., 2015).

This work aims to study the main components of local caraway and thyme essential oils. Determination of the minimal inhibitory concentration (MIC), the minimal bactericidal concentration (MBC), and study of the antibacterial mechanism of caraway and thyme essential oils against *A. tumefaciens*, the causal agent of crown gall disease. Evaluation of the antibacterial activity of local caraway and thyme essential oils against *A. tumefaciens in vitro* compared to *A. radiobacter* K84, as well as efficacy in suppressing gall formation on apricot seedlings by *A. tumefaciens* in pots.

MATERIALS AND METHODS

The source of bacterial isolates and antibiotics:

Two isolates of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* K84 were obtained from the collection of the Bacterial Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. Virulent *A. tumefaciens* 27AS_Pp4 isolate was previously recovered from peach by Barakat *et al.* (2009). To check virulence, the pathogenicity test was repeated using tomato seedlings. The 16S rDNA analysis was used to confirm the identity. 16S rRNA gene sequence was amplified using forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5-'GGTTACCTTGTTACGACTT-3' (Eden *et al*., 1991). PCR product was sequenced with the reverse primer. For the significant alignments, the obtained sequences were compared to those available in the GeneBank network services of the National Center for Biotechnology Information (NCBI) at http://blast.ncbi.nlm.nih.gov/Blast.cgi. MEGA 11 was used to construct the phylogenetic tree (Tamura *et al*., 2021) using the Neighbor-Joining method (Saitou and Nei, 1987). The partial 16S rRNA gene sequence of the *A. tumefaciens* 27AS_Pp4 isolate was submitted to GenBank of the NCBI to receive the accession number. The *A. radiobacter* K84 isolate was previously recovered from the biological prevention product, GALLTROL (AgBioChem, Inc., Orinda, California, U.S.A.). The antibiotic cephalexin is manufactured by SmithKline Beecham in Giza, Egypt for GlaxoSmithKline S.A.E., Cairo, Egypt. The antibiotic ciprofloxacin is produced by Organo for pharmaceutical and chemical industries (Organo Pharma) in Obour City, Egypt.

Plant materials and extraction of essential oils:

Local caraway (*Carum carvi* L.) seeds and thyme (*Thymus vulgaris* L.) herbs were obtained from the Medicinal and Aromatic Plants Research Institute, ARC, Egypt. Essential oils of both caraway seeds and thyme herbs were obtained through hydro-distillation using a

Clevenger type apparatus according to Guenther (1961). The essential oils were dried with anhydrous sodium sulphate and subjected to gas chromatography analysis.

Gas chromatography analysis:

The essential oil samples were analyzed using a Ds Chrom 6200 Gas Chromatograph apparatus with a capillary column BPX-5, 5 phenyl (equiv.) polysillphenylene-siloxane 30 x 0.25 mm ID x 0.25 film in the laboratory of the Medicinal and Aromatic Plants Research Department, Horticulture Research Institute (ARC). The temperature program varied in the range of 70–200°C, at a rate of 10°C/min. The flow rates for gases were 1 ml/min for nitrogen, 30 ml/min for hydrogen, and 330 ml/min for air. The temperatures of the detector and injector were 300 and 250°C, respectively. The identification of the compounds was done by matching their retention times with those of authentic samples injected under the same conditions (Hamouda, 2013).

Preparation of bacterial suspensions:

A. tumefaciens 27AS_Pp4 was inoculated in Petri dishes containing King's B agar medium (20g proteose peptone; 1.5g $MgSO_4$; 1.5g K_2HPO_4; 10 ml glycerol; 20g agar; distilled water to 1000 ml; pH was adjusted to 7.2), and the *A. radiobacter* K84 isolate was inoculated in Petri dishes containing YEM agar media (1g yeast extract; 10g mannitol; 0.5g K_2HPO_4; 0.2g $MgSO_4$; 0.1g NaCl; 20g agar; distilled water to 1000 ml; pH was adjusted to 7.2). Inoculated Petri dishes were incubated for 48 hours at a temperature of 28 ± 1 °C. Bacterial growth was harvested and the cell suspension was adjusted to 10^8 CFU/ml for each isolate (Matthysse, 2006 and Song *et al*., 2014). The bacterial cells were harvested and diluted in phosphate-buffered saline (1.44g Na_2HPO4; 0.24g KH_2PO4; 8g NaCl; 0.2 g KCl; distilled water to 1000 ml and final pH 7.4) until the optical density (at = 600 nm) was between 0.07 and 0.1 using a UV/Visible spectrophotometer (MODEL: 2000 UV-UNICO INSTRUMENTS CO., LTD, USA). This is approximately equivalent to 1×10^8 CFU/ml (Daly *et al*., 2017).

Antibacterial activity of essential oils:

The antibacterial activity of caraway and thyme essential oils against *A. tumefaciens* was tested by using a filter paper disc diffusion technique. Suspension of *A. tumefaciens* 27AS_Pp4 (1 ml of 10^8 CFU/ml per flask containing 250 ml medium) was inoculated in King's B agar medium containing 20g agar/l and poured into Petri dishes (20 ml medium/dish). Whatman standard filter paper discs, No.3 (6 mm), were autoclaved for 20 min at 121°C before drying overnight. The sterile paper disc was impregnated with 10 µl of undiluted essential oil. Disc impregnated with 10 µl suspension (10^8 CFU/ml) of *A. radiobacter* K84 was used as a control treatment. A disc impregnated with 10 µl sterilized distilled water was used as a negative control. Disc impregnated with 10 µl of cephalexin (10 µg) and Disc impregnated with 10 µl of ciprofloxacin (30 µg) were used as positive control treatments (Boruga *et al*., 2014). The paper disc was placed on the surface of the inoculated Petri dish. Five replications for each treatment were used. Closed Petri dishes were left for 1h at room temperatures for the diffusion of essential oils across the medium and then sealed with parafilm. All Petri dishes were incubated at 28°C for 48 hours. The diameter of the inhibition zone (mm) was determined (Vasinauskiene *et al*., 2006).

Determination of MIC and MBC:

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined using the serial dilution technique (Remmal *et al.*, 1993; Bouaichi *et al*. 2015; and Radaelli *et al*. 2016). Yeast peptone glucose (YPG) broth medium (5g yeast extract; 5g peptone; 10g glucose; distilled water to 1000 ml; pH 7.2) was used in all tests. Dimethyl sulfoxide (5 ml) was added to each essential oil (20.48 ml) and the volume was completed to 50 ml with sterile YPG (containing 1% Tween 80) to provide a filtered stock solution containing approximately 409.6 µl/ml of oil. Serial dilutions of each essential oil with YPG (containing 1% Tween 80) were prepared under aseptic conditions to obtain oil concentrations ranging from 409.6 to 0.8 µl/ml. One millimeter of essential oil concentration was added to YPG medium (3 ml) tubes inoculated with *A. tumefaciens* 27AS_Pp4. The final volume of each tube was 4 ml containing 2.5 X 10^5 CFU/ml of *A. tumefaciens* 27AS_Pp4. Final essential oil concentrations were 102.4, 51.2, 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4 and 0.2 µl/ml. Tubes of YPG medium at the same composition without *A. tumefaciens* 27AS_Pp4 were used as a negative control. Tubes of YPG medium at the same composition except essential oil were inoculated with *A. tumefaciens* 27AS_Pp4 and were used as a positive control. The tubes were incubated at 28 ± 1°C for 24 hours. After that, 50 µl of resazurin indicator at a concentration of 0.5 mg/ml in sterile water was added to each tube. After 2h of incubation at 28°C, the MIC was determined. MBC was determined by

spreading 100 µl of each tube of the concentration greater than or equal to the MIC on the YPG agar medium. After spreading, the incubation was conducted at 28 ± 1°C for 48h. The trial was replicated three times. The MBC/MIC ratio was used to determine the nature of the antibacterial effect of essential oils; when the ratio was less than 4, the essential oil was considered bactericidal, and when the ratio was greater than 4, the essential oil was considered bacteriostatic.

Antibacterial mechanism:

Crystal violet assay:

The crystal violet assay was used to determine the change in membrane permeability. *A. tumefaciens* 27AS_Pp4 was harvested and washed twice with phosphate buffer saline (pH7.4) after incubation in MG medium (5.0g mannitol; 1.0g L-glutamic acid; 250 mg K_2HPO_4; 100 mg NaCl; 100 mg $MgSO_4.7H_2O$; 5g tryptone; 2.5g yeast extract; 20.0g agar, distilled water to 1000 ml, final pH 7.0 and biotin at 1µg/1000 ml after autoclaving) at 30 ± 1°C for 24 hours. Biotin was prepared in distilled water at 1 mg/100 ml, filter sterilized, stored at -20°C, and 100 µl of biotin stock was added to 1000 ml of MG medium before use. The pellets were resuspended in a sterile 0.9% NaCl solution and mixed with the tested essential oil dissolved in 10% dimethyl sulfoxide at 1/2 MIC, MIC, and 2 MIC concentrations for 4 hours. The cells were harvested at 10,000 rpm for 5 min before being treated with 10 µg/ml crystal violet in the dark for 15 minutes. NaCl solution (0.9%) with 10% dimethyl sulfoxide was used as a negative control. Ethylenediaminetetraacetic acid [EDTA (0.25 M)], was used as a positive control. Three replications for each treatment were used. The absorbance of the supernatant was evaluated after centrifugation by measuring the OD590 nm with a UV/Visible spectrophotometer. The crystal violet solution was assumed to have a 100% absorbance. Crystal violet uptake was calculated using the formula: % of takeup= 100-[(OD of the sample)/(OD of the crystal violet solution) X 100] (Sparks *et al*., 2014; Khan *et al*., 2017; and Hsouna *et al*., 2019).

Pot experiments:

Apricot seedlings (cv. Baladi, 2 months old) in pots (20 cm diam.) were wounded in the crown region (Barakat *et al*., 2011) before soil infestation (100 ml/pot) with *A. tumefaciens* 27AS_Pp4 suspension (10^8 CFU/ml). To test the ability of caraway and thyme essential oils to control crown gall disease, one treatment of each essential oil at dose 26.6 µl/ml was used (100 ml/pot), immediately after soil infestation with *A. tumefaciens* 27AS_Pp4 suspension. Instead of oils, 100 ml suspension of *A. radiobacter* (10^8 CFU/ml) and 100 ml of sterile water were used as a comparison and positive control treatments, respectively. Apricot seedlings wounded and only treated with 200 ml/pot sterilized water, were used as a negative control. Five replicates (with five plants per replicate) were used for each treatment. The disease progress and plant growth parameters were recorded after 45 days from *A. tumefaciens* 27AS_Pp4 inoculation. The disease progress was scored as a number and weight (g) of galls/plant. Growth parameters were also determined as the weight of shoots/plant (g), the weight of root/plant (g), and the length of the plant.

Statistical analysis:

A completely randomized design was used in all experiments. The collected data were analyzed using one-way ANOVA as outlined by Gomez and Gomez, 1984. For performing the mentioned statistical analysis, SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) statistical packages were used. Duncan's multiple range test (Duncan, 1955) was used to compare the means at probability (*P*) level 0.05.

RESULTS

Check out the *A. tumefaciens* Pp4 virulence, 16S rRNA analysis, and GenBank (NCBI) accession number:

After repeating the pathogenicity test on tomato seedlings, the *A. tumefaciens* Pp4 isolate produced typical symptoms of crown gall disease. The obtained partial 16S rRNA gene sequence of the *A. tumefaciens* Pp4 isolate compared to the sequences in the NCBI database showed 91.34% similarity with *A. tumefaciens* strain IAM 12048 and *A. tumefaciens* strain NCPPB2437. The partial 16S rRNA gene sequence of the *A. tumefaciens* Pp4 isolate has been deposited in GenBank (NCBI) under the accession number OK559740. The phylogenetic tree constructed using neighbor-joining method illustrated in (Fig. 1).

Chemical compositions of essential oils:

Data in Table (1) and Figures (2 and 3) showed that caraway and thyme essential oils have different components with different ratios. The essential oil of caraway contained carvone and limonene in high concentrations, while other components were less than one percent. Caraway oil has carvone (79.38157%), limonene (18.78265%), perilla alcohol (0.43729%), trance carveol (0.37461%), carveol (0.36041%), β-

caryophyllene (0.32851%), trance dihydrocarvone (0.16595%) and other components (0.16901%). While the essential oil of thyme contained thymol, 1,8-Cineol, borneol, β-caryophyllene, and bornylacetate with comparatively high content, while other components were less than five percent. Thyme oil has thymol (14.79336%), 1,8-Cineol (14.45795%), borneol (13.22024%), β-caryophyllene (10.86938%), bornyl acetate (5.08278%), camphor (4.77196%), α-terpinen (4.70931%), geraniol (4.18600%), carvacrol (4.09322%), linalool (2.67510%), myrcene (1.27368%) and other components (19.86702%).

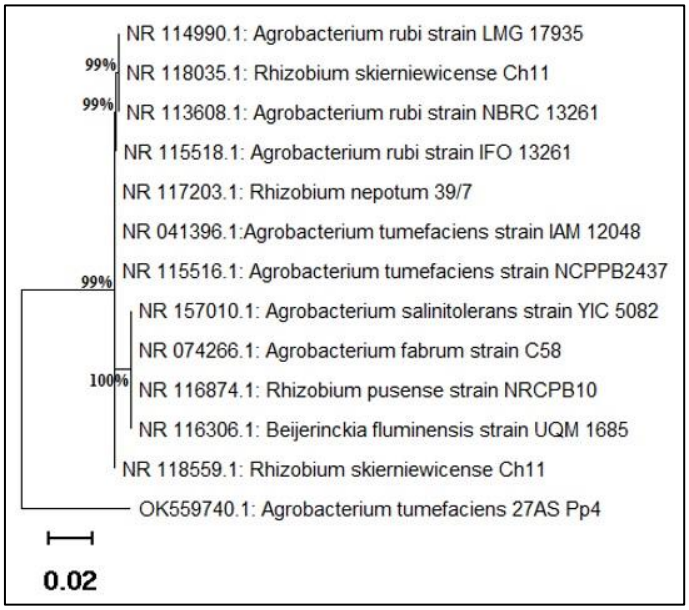

Fig. (1): Neighbor-joining phylogenetic tree based on the partial 16S rRNA sequences showing the relationship between *A. tumefaciens* 27AS_Pp4 and closely related isolates in GenBank of the NCBI using the Kimura 2-parameter model in MEGA 11. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 repetitions) are shown next to the branches.

Table (1): Chemical composition of caraway (*Carum carvi*) and thyme (*Thymus vulgaris*) essential oils.

Component	*Carum carvi*		*Thymus vulgaris*	
	Area (%) of total	PK NO. in Fig. 2	Area (%) of total	PK NO. in Fig. 3
Borneol	-	-	13.22024	11
Bornyl acetate	-	-	5.08278	14
Camphor	-	-	4.77196	10
Carvacrol	-	-	4.09322	17
Carveol	0.36041	6	-	-
Carvone	79.38157	4	-	-
1,8-Cineol	-	-	14.45795	7
Geraniol	-	-	4.18600	12
Limonene	18.78265	1	-	-
Linalool	-	-	2.67510	8
Myrcene	-	-	1.27368	3
Thymol	-	-	14.79336	15
Perilla alcohol	0.43729	5	-	-
Trance carveol	0.37461	3	-	-
Trance dihydrocarvone	0.16595	2	-	-
α-terpinen	-	-	4.70931	4
β-caryophyllene	0.32851	8	10.86938	18
Others	0.16901	7	19.86702	1, 2, 5, 6, 9, 13, 16, 19

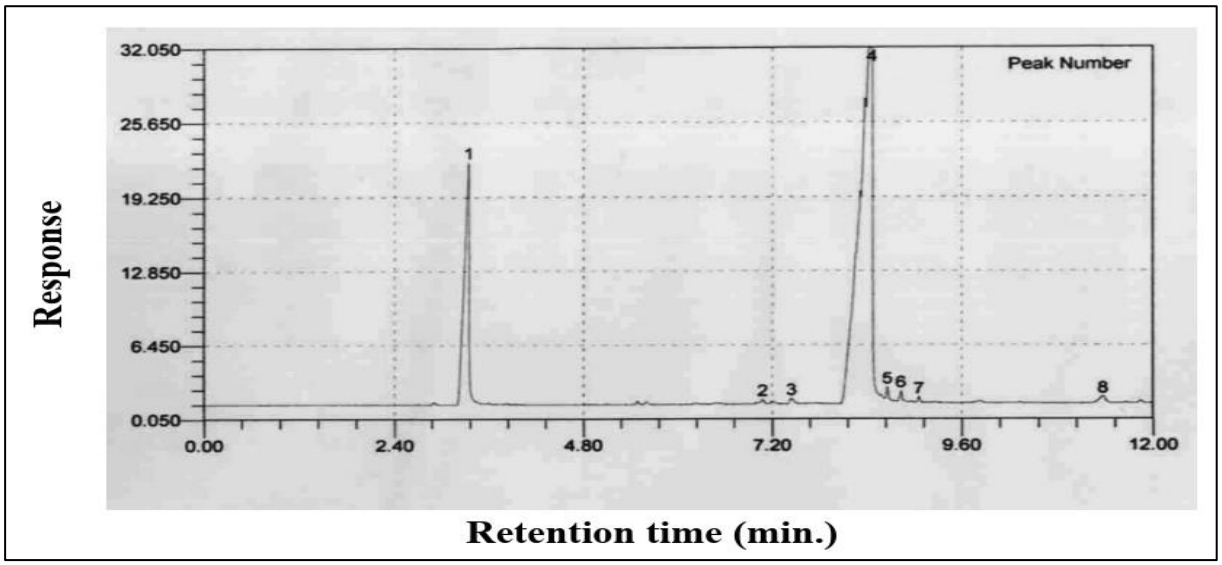

Fig. (2): GC chromatogram of essential oil of local caraway (*Carum carvi*) seeds. 1= Limonene; 2= Trance dihydrocarvone; 3= Trance carveol; 4= Carvone; 5= Perilla alcohol; 6= Carveol; 7= Others; 8= β-caryophyllene.

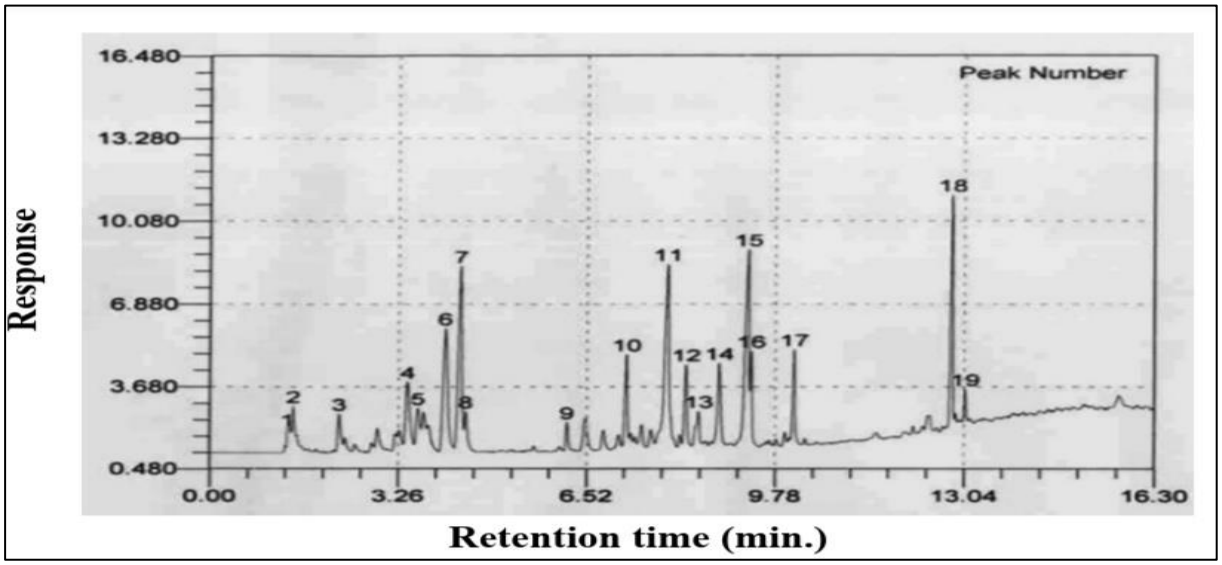

Fig. (3): GC chromatogram of essential oil of local thyme (*Thymus vulgaris*) herb. 3= Myrcene; 4 = α-terpinen; 7= 1,8-Cineol; 8= Linalool; 10= Camphor; 11= Borneol; 12= Geraniol; 14= Bornyl acetate; 15 =Perilla alcohol; 17= Carvacrol; 18= β-caryophyllene; 1, 2, 5, 6, 9, 13, 16, 19= Others.

Antibacterial activity of essential oils compared to chosen antibiotics and *A. radiobacter*:

Essential oils of caraway and thyme in addition to the antagonist *A. radiobacter* showed antibacterial activity against *A. tumefaciens* 27AS_Pp4 *in vitro*, as shown in Table (2). *A. radiobacter* showed an inhibition zone of 11.8 mm in diameter, while essential oils of caraway and thyme showed comparatively similar inhibition zones of 10.2 and 11.0 mm in diameter, respectively. Cephalexin and ciprofloxacin, on the other hand, showed inhibition zones of 0.0 and 27.2 mm in diameter, respectively (Figure, 4).

Table (2): Antibacterial activity of caraway and thyme essential oils compared to *A. radiobacter* K84 against *A. tumefaciens* 27AS_Pp4 *in vitro*.

Treatment	Inhibition zone (mm)*
Caraway	10.2^{c}
Thyme	11.0^{bc}
A. radiobacter	11.8^{b}
Cephalexin*	0.0^{d}
Ciprofloxacin*	27.2^{a}
Negative control	0.0^{d}

* Antibiotics; means of five replicates; the means in the table that share the same letter are not significantly different using Duncan's Multiple Range Test ($p \leq 0.05$).

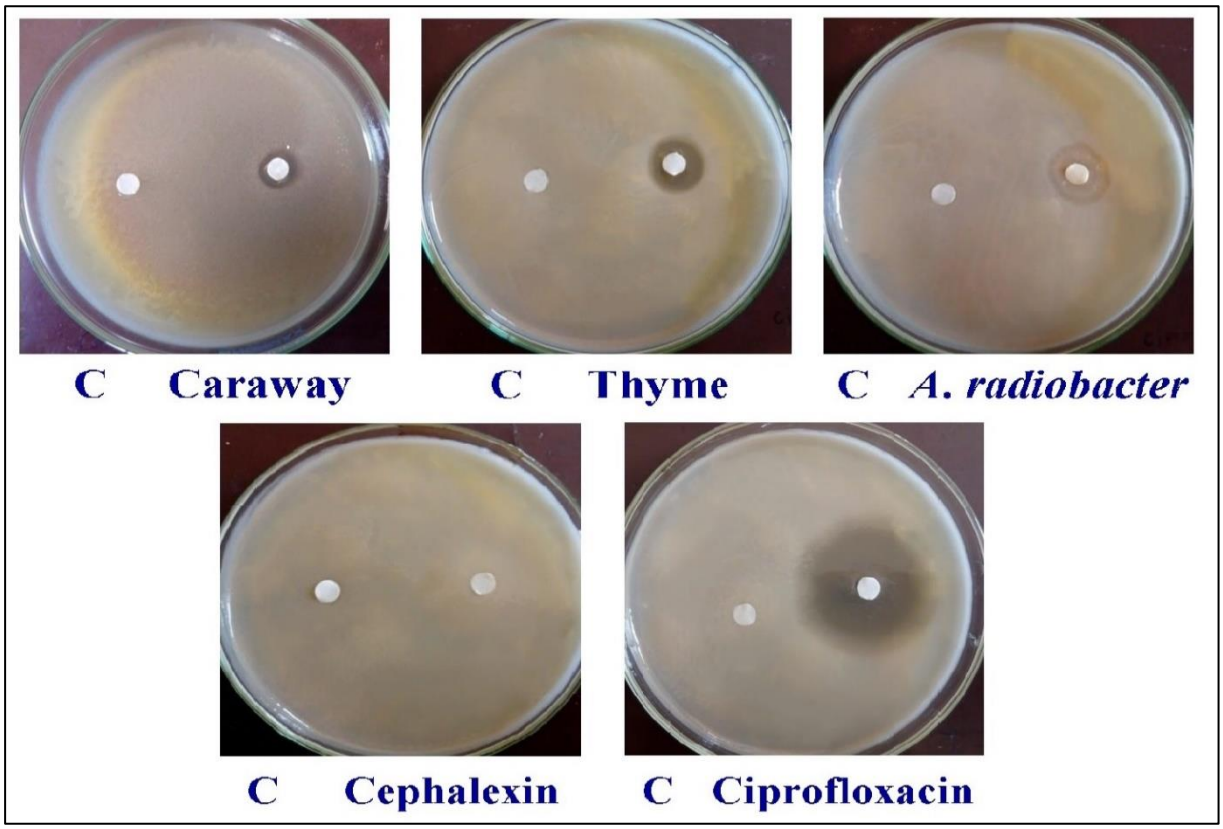

Fig. (4): *In vitro* antagonistic activity of caraway and thyme essential oils compared to *A. radiobacter* and antibiotics against *A. tumefaciens* 27AS_Pp4. C= 10 µl sterilized distilled water; caraway and thyme= 10 µl of undiluted essential oil; *A. radiobacter* K84= 10 µl suspension (10^8 CFU/ml); cephalexin= 10 µl (10 µg); ciprofloxacin= 10 µl (30 µg).

MIC and MBC of caraway and thyme essential oils against *A. tumefaciens* 27AS_Pp4:

The MICs and MBCs for essential oils of caraway and thyme were determined against *A. tumefaciens* 27AS_Pp4 (Table, 3). The MIC value is the lowest essential oil concentration when there is no color change of resazurin from blue to pink and was 12.8 µl/ml for both caraway and thyme essential oils (Figure 5). Whereas the MBC value is the lowest concentration when there is no growth (99.9% or more of the original inoculum was killed) after sub-culturing of the concentrations greater than or equal to the MIC on YPGA medium, and they were 25.6 and 12.8 µl/ml for caraway and thyme, respectively. The MBC/MIC ratios were 2 and 1 for caraway and thyme essential oils, respectively. The antibacterial effect of both caraway and thyme essential oils was a bactericidal effect against *A. tumefaciens* 27AS_Pp4.

Crystal violet assay

In the absence of essential oils, *A. tumefaciens* 27AS Pp4 uptake of crystal violet was 8.87 %. At 12.8 µl/ml (MIC) of caraway and thyme treatment, crystal violet uptake increased significantly to 39.08 and 51.06 %, respectively (Figure 6). The uptake of crystal violet in the presence of 0.25 M EDTA (positive control) was 43.93%.

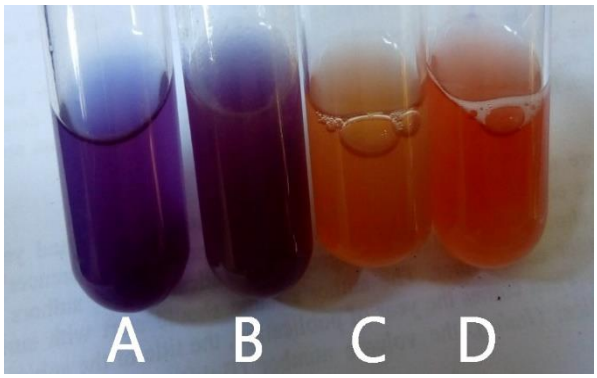

Fig. (5): MIC value (12.8 µl/ml) of thyme essential oil against *A. tumefaciens* 27AS_Pp4 using resazurin reaction test. (A) control (-) = YPG medium without *A. tumefaciens* 27AS_Pp4; (B) thyme 12.8 µl/ml = YPG medium with *A. tumefaciens* 27AS_Pp4 and thyme 12.8 µl/ml; (C) thyme 6.4 µl/ml = YPG medium with *A. tumefaciens* 27AS_Pp4 and thyme 6.4 µl/ml; (D) control (+) = YPG medium with only *A. tumefaciens* 27AS_Pp4.

Table (3): The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of caraway and thyme essential oils against *A. tumefaciens* 27AS_Pp4.

Essential oil	Essential oil concentration (µl/ml) *	Resazurin reaction**	MIC (µl/ml)	Growth on YPGA medium***	MBC (µl/ml)	MBC/MIC ratio	Nature of the antibacterial effect
Caraway	102.4	-	12.8	-	25.6	2	Bactericidal
	51.2	-		-			
	25.6	-		-			
	12.8	-		+			
	6.4	+		+			
	3.2	+		+			
	1.6	+		+			
	0.8	+		+			
	0.4	+		+			
	0.2	+		+			
Thyme	102.4	-	12.8	-	12.8	1	Bactericidal
	51.2	-		-			
	25.6	-		-			
	12.8	-		-			
	6.4	+		+			
	3.2	+		+			
	1.6	+		+			
	0.8	+		+			
	0.4	+		+			
	0.2	+		+			
Control (-)	0.00	-	ND	-	ND	ND	ND
Control (+)	0.00	+	ND	+	ND	ND	ND

* Final essential oil concentration; **color change from blue to pink (+) and no color change (-);***After sub-culturing from essential oil concentrations; bacterial cells growing (+) no growth (-); ND= not determined.

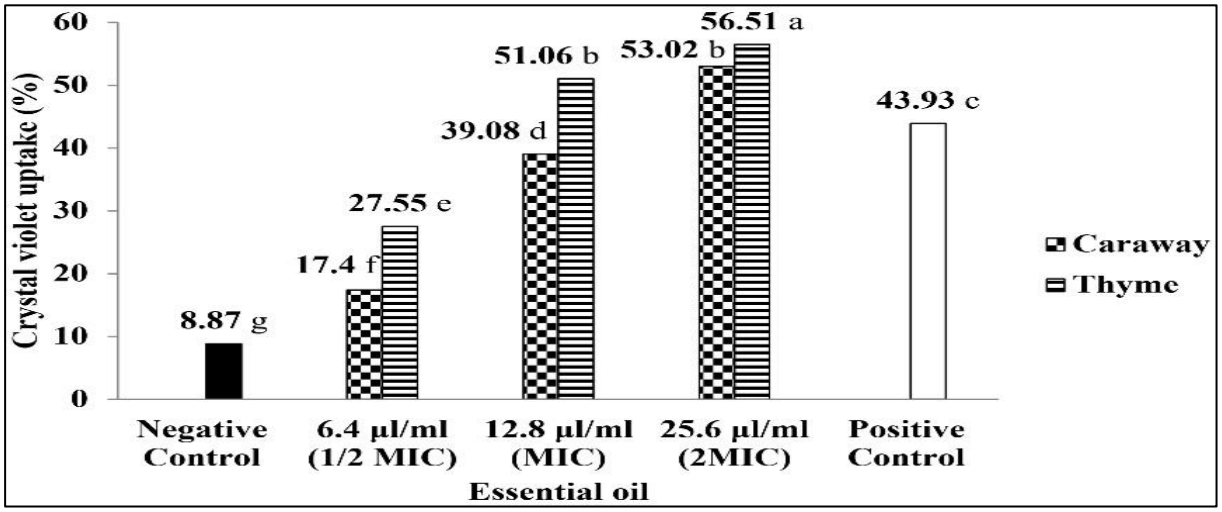

Fig. (6): Crystal violet uptake by *A. tumefaciens* 27AS_Pp4 treated with caraway and thyme essential oils [6.4 (1/2 MIC), 12.8 (MIC), and 25.6 (2MIC) µl/ml]. positive control = Ethylenediaminetetraacetic acid [EDTA (0.25 M)]; negative control = NaCl solution (0.9%) with 10% dimethyl sulfoxide; means of three replicates; the means in the figure that share the same letter are not significantly different using Duncan's Multiple Range Test ($p \leq 0.05$).

Control of crown gall disease on apricot seedlings in pots:

No disease symptoms were observed on apricot seedlings inoculated with the pathogen *A. tumefaciens* 27AS_Pp4 and the antagonist *A. radiobacter* (Table, 4). A significant decrease in the number and weight of galls was observed in caraway and thyme essential oil treatments compared to the positive control. The average number of galls was 1.4 and 0.4 gall per plant in caraway and thyme essential oil treatments, respectively, compared to 3.8 gall per plant in the positive control. The fresh and dry weight of galls per plant confirmed the effectiveness of caraway and thyme treatments. The fresh weights of galls per plant were 0.26 and 0.07g in caraway and thyme essential oil treatments, respectively, compared to 0.52g in positive control. While the dry weights of galls per plant were 0.12 and 0.03g in caraway and thyme essential oil treatments, respectively, compared with 0.25g in positive control.

Treatments of caraway essential oil showed a significant decrease in the fresh weight of shoots and roots compared to the positive control, negative control, and *A. radiobacter*. In the case of thyme oil treatment, there were insignificant differences in the fresh weight of shoots and roots compared to negative control and *A. radiobacter* (Table 4). Measuring dry weight comparatively confirmed these results. The fresh weight of shoots per plant was 2.44 and 2.79g in caraway and thyme essential oil treatments, respectively, compared to 5.05g in positive control and 3.02g in negative control treatments. While the fresh weight of shoots per plant was 3.27g in *A. radiobacter* treatment. The fresh weights of roots per plant were 0.92 and 1.33g in caraway and thyme essential oil treatments, respectively, compared to 2.27g in positive control and 1.32g in negative control treatments. While the fresh weight of roots per plant was 1.39g in *A. radiobacter* treatment.

Treatments of caraway essential oil showed a significant decrease in plant length compared to thyme, *A. radiobacter*, positive control, and negative control (Table 4). In case of thyme oil treatment, the length of the plant was increased significantly compared to the negative control, and an insignificant difference in length of plants between thyme oil and *A. radiobacter* treatments was obtained. The average lengths of the plants were 20.4, 25.2, 26.4, 28.4, and 23.5cm in caraway, thyme, *A. radiobacter*, positive control, and negative control treatments, respectively.

Table (4): Efficacy of caraway and thyme oils compared to *A. radiobacter* K84 in suppression of gall formation on apricot seedlings by *A. tumefaciens* 27AS_Pp4 in pots.

Treatment	Average number of galls/plant	Average weight of galls/plant (g)		Average weight of shoots/plant (g)		Average weight of roots/plant (g)		Average length of plant (cm)
		Fresh	Dry	Fresh	Dry	Fresh	Dry	
Caraway	1.4^{b}	0.26^{b}	0.12^{b}	2.44^{c}	1.17^{b}	0.92^{c}	0.55^{c}	20.4^{d}
Thyme	0.4^{c}	0.07^{c}	0.03^{c}	2.79^{bc}	1.26^{b}	1.33^{b}	0.80^{b}	25.2^{b}
A. radiobacter	0.0^{c}	0.0^{c}	0.0^{c}	3.27^{b}	1.41^{b}	1.39^{b}	0.79^{b}	26.4^{b}
Positive control	3.8^{a}	0.52^{a}	0.25^{a}	5.05^{a}	2.19^{a}	2.27^{a}	1.31^{a}	28.4^{a}
Negative control	0.0^{c}	0.00^{c}	0.0^{c}	3.02^{b}	1.32^{b}	1.32^{b}	0.78^{b}	23.5^{c}

Means of five replicates; the means in the same column that share the same letter are not significantly different using Duncan's Multiple Range ($p \leq 0.05$).

DISCUSSION

Many factors, such as the environment, location, and farming practices, can affect the isolation, yield, and chemical composition of the essential oils (Hudaib and Aburjai, 2007). The main components of *Carum carvi* (caraway) essential oil (hydrodistillation from fruits) were reported to be carvone 23.3%, limonene18.2%, germacrene D 16.2%, and trans-dihydrocarvone 14.0% (Iacobellis *et al*., 2005) and in Romanian *Thymus vulgaris* (thyme), by steam distillation from the aerial part of the plant, were thymol 47.59%, γ-terpinene 30.90% and p-cymene 8.41% (Boruga *et al*., 2014). In the present work, the essential oil of local seeds of caraway contained carvone (79.38157%), limonene (18.78265%), perilla alcohol (0.43729%), trance carveol (0.37461%), carveol (0.36041%), β-caryophyllene (0.32851%), trance dihydrocarvone (0.16595%) and other components (0.16901%). While the essential oil of local thyme herbs contained thymol (14.79336%), 1,8-Cineol (14.45795%), borneol (13.22024%), β-caryophyllene (10.86938%), bornyl acetate (5.08278%), camphor (4.77196%), α-terpinen (4.70931%), geraniol (4.18600%), carvacrol (4.09322%), linalool

(2.67510), myrcene (1.27368%) and other components (19.86702%). Differences in quantities and varieties of essential oils reported herein and those reported by others worldwide can be attributed to differences in climate, environment, location, farming practices, plant variety and/or cultivar, and plant organs used in the extraction of either seeds or foliage (Iacobellis *et al*., 2005 and Hudaib and Aburjai, 2007). The methods of determination of essential oils may be a cause of such differences (Ferhat *et al*., 2007).

The essential oils as well as their distinguished components are responsible for antimicrobial activity (Vyas, 2012 and Iacobellis *et al*., 2005). Among five tested essential oils (lavender, sage, lemon balm, clove, and thyme oil (BioZell) against five important phytopathogenic bacteria (*Erwinia amylovora*, Xanthomonas *arboricola* pv. *corylina*, *Xanthomonas arboricola* pv. *juglandis*, *Pseudomonas syringae* pv. *syringae* and *Agrobacterium tumefaciens*), the largest inhibition zone (17.6 mm) was observed by BioZell on the growth of *A. tumefaciens* (Mikiciński *et al*., 2012). The antibacterial activity of 13 plant essential oils against the two phytopathogenic bacteria, *A. tumefaciens* and *Erwinia carotovora* subsp. *carotovora*, revealed that cinnamon, clove, chenopodium, caraway, rosemary, and thyme were the most effective oils, with greater inhibitory activity against the two tested bacteria. Furthermore, caraway and thyme oils demonstrated similar activity (El-Zemity *et al*., 2008). *A. rhizogenes* strains (K84 and K1026) were effective against nopaline-producing strains *in vitro* (Rhouma *et al*., 2008). In the present work, *A. radiobacter* showed an inhibition zone of 11.8 mm in diameter against *A. tumefaciens* 27AS_Pp4. While essential oils of caraway and thyme showed inhibition zones of 10.2 and 11.0 mm in diameter, respectively.

The minimal inhibitory quantities (MIQs) of caraway essential oil ranged from 170.2 to 7280µg (The MIQ was calculated using the average densities of *C. cyminum* and *C. carvi* essential oils, which were considered 0.92 and 0.91 g/ml, respectively) against many species of the following bacterial genera: *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Rhodococcus*, *Erwinia*, *Xanthomonas*, *Ralstonia*, and *Agrobacterium* (Iacobellis *et al*., 2005). The MICs of thyme essential oil against several bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Mycobacterium smegmatis*, ranged from 75 to 1100 µg/ml (Liu *et al*., 2017). Caraway essential oil had MIC values ranging from 100 to 300 ppm and MBC values ranging from 200 to 400 ppm against ten pathogenic bacteria (Begum *et al*., 2008). The MIC values of the essential oil of thyme were 5 and 1 µl/ml and the MBC values were 5 and 1µl/ml against *P. carotovorum* and *R. solanacearum*, respectively (Nezhad *et al*., 2012). In the present work, the MIC value of essential oils of caraway and thyme was 12.8 µl/ml for both essential oils against *A. tumefaciens* 27AS_Pp4. While the MBC values were 25.6 and 12.8 µl/ml for caraway and thyme, respectively.

The main components of caraway essential oil are carvone and limonene, while thymol and carvacrol are important components of thyme essential oil. The integrity and permeability of the cell wall and cell membrane could be destroyed by limonene, potentially leading to cell death. Protein, nucleic acid, and AKPase leakage, as well as the results of PI fluorescence staining, could help to confirm this conclusion (Han *et al*., 2021). Carvacrol and thymol disrupt bacterial membrane integrity, increase membrane permeability, and induce protons and potassium leakage, eventually leading to loss of membrane potential (Xu *et al*., 2008). In the absence of carvacrol, *E. coli* uptake of crystal violet was 4.8 %, which increased by 26 and 42 % at 200 and 450 µg/ml (MIC) of carvacrol treatment, respectively (Khan *et al*., 2017). The essential oil of *Mentha piperita* L. increased the uptake of crystal violet by *A. tumefaciens* from 23% to 94% (Hsouna *et al*., 2019). In the present work, *A. tumefaciens* 27AS Pp4 uptake of crystal violet was 8.87 % in the absence of essential oils but increased to 39.08 and 51.06 % with 12.8 µl/ml (MIC) of caraway and thyme treatments, respectively.

At a concentration of 200 mg/ml, peppermint essential oil completely inhibited the formation of tumours on tomato plants inoculated with the pathogenic strain *A. tumefaciens* (Hsouna *et al*., 2019). In Egypt, Mahmoud *et al*. (2004) tested the efficiency of some natural oils, including caraway and thyme, in inhibiting the growth of *A. tumefaciens in vitro* and controlling the crown gall disease of apricot seedlings in the greenhouse. *A. radiobacter* K84 completely inhibited crown gall disease on plants infected with *A. tumefaciens*. When a mixture of pathogenic *A. tumefaciens* isolates was used for inoculation, none of the plants were affected by crown gall following treatment with K84 (Khmel *et al*., 1998). In the present work, no

disease symptoms were observed on apricot seedlings inoculated with the pathogen *A. tumefaciens* 27AS_Pp4 and the antagonist *A. radiobacter*. Significant decreases in the number and weight of galls were observed in caraway and thyme essential oil treatments compared to the positive control. Treatments of caraway essential oil showed a significant decrease in the fresh weight of shoots and roots compared to the positive control, negative control, and *A. radiobacter*. In the case of thyme oil treatment, there were non-significant differences in the fresh weight of shoots and roots in thyme oil treatment compared to negative control and *A. radiobacter*. The dry weight of the shoots and roots partly confirmed these results. Treatments of caraway essential oil showed a significant decrease in the length of the plant compared with thyme, *A. radiobacter*, positive control, and negative control. In case of thyme oil treatment, the length of the plant was increased significantly compared to the negative control, and an insignificant difference in length of plants was obtained between thyme oil and *A. radiobacter* treatments. At different concentrations (0 to 20 ml/l), essential oils of Eucalyptus, Camphor, and Lemongrass were used in experiments on seed germination and seedling growth of *Parthenium hysterophorus*, and all essential oils reduced seed germination and seedling length (Paudel and Gupta, 2009). The essential oil of *Carum carvi* seeds inhibited the germination and seedling growth of wheat, maize, flax and canary grass in a dose-dependent manner. Canary grass and wheat germination and radicle growth were the most sensitive (Marichali *et al*., 2014). The results of the present work suggest that the essential oils of caraway and thyme have a reasonable potential for controlling crown gall disease. Consideration should be given to the potential effect of essential oil of caraway on plant growth. Further work with different horticulture seedlings nurseries is needed along with different diseases.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest.

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