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THE INHIBITORY EFFECT OF SEVERAL ESSENTIAL OILS OF MEDICINAL PLANTS ON WILTING AND SOFT POTATO ROT FACTOR IN GREENHOUSE AND LABORATORY

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ABSTRACT

Potato is one of the plants that annually suffer from damages due to the soft rot and tuber disease in storage. Nowadays, utilization of herbal essential oils for acquisition of natural antibacterial agents has been of great interest to control plant-pathogens. In the present study, five medicinal herbs of *Thymus vulgaris L*, *Coriandrum sativum L*, *Cuminum cyminum L*, *Rosmarinus officinalis L* and *Eucalyptus Globules L* were extracted using distillation with water and their inhibitory effects on *Ralstonia solanacearum* bacteria (potato soft rot factor) and Pectobacterium carotovorum bacteria (potato wilting factor) examined through disc diffusion method. Tube dilution method was used to determine the minimum inhibitory concentration and minimum bactericidal concentration. The variance analysis was carried out on tests in the form of a completely randomized factorial design. Comparison of means was performed based on Duncan's multiple range test (MSTAT-C software). *Thymus vulgaris L.* essential oil was used in greenhouse stage, because it showed the highest level of inhibitory effect on both bacteria in the laboratory stage. The obtained results showed that *T. vulgaris L.* essential oil had the most antibacterial activity and effect compared with other essential oils by creating an inhibitory shaft diameter of 34.8 mm. For greenhouse experiments, *T. vulgaris L.* essential oil was used with a concentration of 0.5% by spray method and a concentration of 1% by pouring essential oil into the soil. According to the results, *T. vulgaris L.* reduced the occurrence of potato soft rot disease up to 41% and potato bacterial wilt up to 44%. Therefore, *T. vulgaris L.* essential oil can be utilized as an inhibitor to manage these two diseases in potato plant due to its appropriate antibacterial function.

Keywords: antibacterial effect, herbal essential oil, ralstonia solanacearum, pectobacterium carotovorum.

INTRODUCTION

Carotovora Pectobacterium is the causative agent of potato's soft rot and tuber disease in the cellar (Aazadvar *et al*., 2007; Hooker, 1981), so that about 24 to 38 percent of potato waste has been reported during the first three months of storage caused by this bacterium (Varns *et al*., 1985). The use of chemical compounds threatens human health in addition to environmental pollution. For this reason, utilizing natural compounds to control pests and plant diseases is one of the ways to reduce environmental hazards. Food safety is becoming more and more important with modern advances in human health and food production (WHO, 2002). It has been estimated that annually more than 30% of people in industrialized countries suffer from food contamination (WHO, 2002). Therefore, it is necessary to combine new methods with existing methods in order to reduce or eliminate factors of food-borne diseases (Leistner, 1978; Samavi *et al*., 2009). In this regard, communities make endeavors to move toward green usage and reduce the use of artificial additives with lower environmental impact (Tuley de Silva, 1995; Smid, 1999 & Gorris, 1998). It is recommended to use natural products as antibacterial compounds to reduce the risks to human health and economic losses caused by food-producing microbes (Conner Daferera *et al*., 1993) which is an appropriate solution to control pathogenic bacteria and increase the shelf life of food products (Shukla and Dwivedi, 2012). Plant essential oils appear to be an appropriate option as natural anti-bacterial additives, so that the anti-bacterial properties of many essential oils and their compounds are proven (Mari *et al*., 2003). Many herbal essences, especially medicinal plants and their constituent elements have been known for antibacterial activity on a wide range of microorganisms including gram-negative (Sivropoulou *et al*., 1996) and gram-positive (Kim *et al*., 1995) bacteria. It has usually been shown that gram-negative bacteria are more resistant to the antagonistic effects of essential oils than gram-positive bacteria, due to the presence of lipopolysaccharides[1] in the outer membrane of the bacterial cell; but this result has not always been correct (Burtet *et al*., 2004; Karapinar and Aktug, 1987). In the past, the antibacterial properties of essential oil of some plants have been tested on bacteria. In a particular study, it has been shown that essential oil of *Coriandrum sativum L* prevents the growth of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimuruim* bacteria (Broomand *et al*., 2008; Shukla and Dwivedi, 2012). In addition, the antibacterial properties of *T. vulgaris L* oil on Fire Blight bacterium (with bacteria agent of *Erwinia amylovora*) of grainy trees has been proven (Hasanzadeh *et al*., 2005 and Gachkar *et al*., 2007) showed that *Cuminum cyminum L* oil prevents the growth of *Escherichia coli (E. Coli)*, *Listeria monositogenes* and *Salmonella aureus* bacteria. According to the study of (Romano *et al*., 2008), *Rosmarinus officinalis L* essential oil prevents the growth of *Escherichia coli* and *Salmonella aureus* bacteria. Soft rot and tuber and bacterial wilting are among dangerous diseases in potatoes which are caused by *Pectobacterium carotovorum* (*P. carotovorum*) and *Ralstonia solanacearum (R. solanacearum)* bacteria, respectively. These diseases greatly reduce the quality and quantity of products. These two diseases are some of the most important diseases of tuber, seedlings and bushes of potato and are easily transmitted through cultivation of infected tubers (Murray *et al*., 1995).

MATERIAL AND METHODS

Collection of herb samples and extraction of essential oils from them

The leaves of *Thymus vulgaris L., Rosmarinus officinalis L.* and *Eucalyptus Globules L.* were collected from the Agricultural Research Institute of Tehran University and leaves of *Coriandrum sativum L.* and *Cuminum cyminum L* collected from Research Farm of Food Science and Technology Institute of Tehran University. Then, they were dried in the shade and exposed to air flow. They were then placed in a cool place to prevent their deterioration and decay (Asgari, 1999; Smsam Shariat, 1993). Essential oil extraction from the herb's new organs (vegetative form with flower bed) was carried out through Clevenger distillation[1] for 4 hours and immediately absorbed by dry sulfate and kept in a dark place at 4 ° C until analysis time.

Preparation of bacteria

R. solanacearum and P. carotovorum bacteria were supplied from the Agricultural School of Tehran University.

Growth inhibition test

First, *R. solanacearum and P. carotovorum* bacteria were cultured in broth nutrient culture medium[2] and used in the experiment after 24 hours. Disc diffusion method was used for growth inhibition test (Murray *et al*., 1995). Plates containing agar medium (for *E. coli*) and sucrose peptone agar (for *R. solanacearum*) were prepared and 100 µl of suppository of 10^8 cfu / ml of bacteria was poured on each plate and dried. Filter paper (Sartorius) was prepared by punching in 6 mm pieces and sterilized in the autoclave. The essential oil of *T. vulgaris* was prepared in 99.6% ethanol with dilutions of 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 75 and 100%. Each paper disk was incubated with 10 µL of each dilution and placed in the middle of plate containing the bacteria after drying. Ethanol solvent was used as a negative control and erythromycin (µg / disc10) and streptomycin (µg / disc15) antibiotics were used as a positive control. In negative control treatment, 10 µl of ethanol solvent 99.6% was added to paper disk. Samples were stored in incubator at 30 ° C. After 48 hours, the inhibition hole was marked with a millimeter-based ruler.

Determining the lowest inhibitory concentration and the lowest bactericidal concentration

For this purpose, tubular dilution method was used (Kivanc & Akgul, 1986). In this method, *E. coli, R. solanacearum and P. carotovorum b*acteria were separately cultured in test tubes containing 5 milliliters of broth culture medium. Twenty-four hours after 10^8 cfu suspension per milliliter of each bacterium, 50 µL of each 0.01, 0.05 and 0.1% dilutions of essential oil were added to 99.6% ethanol solvent. Samples were stored in an incubator at 30 ° C. After 48 hours, treatments were macroscopically examined and the lowest concentration with no visible inhibitory effect on bacteria was considered as the minimum inhibitory concentration (MIC). In the control treatment, 50 milliliters of 99.6% ethanol solvent were used.

Greenhouse studies

Fontane type of potato containing prepared buds was supplied from Varamin region. The mixture of sand, clay and soil litter (duff) was prepared with a 1: 1: 1 ratio and sterilized by autoclave at 121 ° C for 1 hour (Irikiin *et al*., 2006). In each pot, a germinated tuber of potato was planted and stored in a greenhouse at a temperature of 28-30 ° C and a humidity of 60%. Every 7 days, the bushes were treated with nitrogen, phosphate and potassium fertilizers in a ratio of 3 per thousand and iron chelate in a ratio of 3 per thousand using spray method. *Thymus vulgaris L* essential oil was used in greenhouse stage, because it showed the highest level of inhibitory effect on both bacteria in the laboratory stage. For this purpose, 100 milliliters of *T. vulgaris L* oil were prepared with 1% concentration at a ratio of 100: 1: 1 (Essential Oil: Alcohol: Water) and poured into each pot two months after potato cultivation. Twenty-four-hours cultivation of *P. carotovorum* and *R. solanacearum* bacteria was used based on the Kempe & Sequeira method (1983) to carry out inoculation of stems and roots. Forty-eight hours after applying essential oil on each pot, 20 µL of 10^8 cfu suspension of bacteria was injected at the angle of leaf and stem using insulin syringe. In infecting the roots of herbs, roots were first a little scratched with a sterilized knife and then; 100 milliliters of 10^8 cfu suspension of bacteria was poured onto the soil. Forty-eight hours later, a concentration of 0.5% *T. vulgaris L* essential oil was prepared with a ratio of 0.5: 0.5: 0.5 (essential oil: alcohol: water) and sprayed on the plant foliage to dissolve thyme oil on the herb. The experiments included four treatments of bacteria-infected herbs, bacteria-infected and oil-impregnated herbs, completely healthy herbs and completely healthy and oil-impregnated herbs. Recording of bushes was carried out twenty days after inoculation. Bacterial wilt disease index was measured based on Kempe and Sequeira method (1983) and according to the scale of 4.1-1 as following: Zero score indicated no symptoms, score 1: 1-25% wilt of foliage, score 2: 26-50% wilting of foliage, score 3: 75-51% wilting of foliage and score 4: 100-76% wilting of foliage. Rot and tuber disease index was measured according to the scale of 1-8. Zero score was considered for seedlings without symptoms of yellowish, wilting, blackening and rotting of stem and score 8 was considered for seedlings that had been completely destroyed (Bagheri & Zafari, 2005).

Determining disease incidence, biological control and biomass increase

The disease incidence was calculated through counting the number of affected bushes in relation to total number of bushes in each treatment. Biological control efficiency and biomass increase in fresh weight were calculated using (Xue *et al*., 2008) method through following formulas:

Biomass increase= [average fresh weight of control/ (average fresh weight of control - average fresh weight of treatment] *100

Biological control efficiency= [disease of control/ (disease of control - disease treatment] *100

RESULTS AND DISCUSIONS

Laboratory and greenhouse tests were carried out in three replications and three samples of each treatment were used in each replication. The results were analyzed using a completely randomized design with MSTAT-C software. The mean comparison was performed by Duncan's multiple-range test.

Laboratory results

All five essential oils showed antibacterial effects on tested bacteria. The size of inhibitory shaft was different according to the concentration and types of essential oil, so that 100% concentration of *T. vulgaris L* showed the most antibacterial activity by creating a diameter of 34.8 mm inhibition in *R. solanacearum* which was more than measured shaft in streptomycin sample (22 mm) (Table-1). *Rosmarinus officinalis L* had the highest antibacterial effect on both *R. solanacearum* and *P. Carotovorum* (both equal to 11.8 mm) after *T. vulgaris L* (Tables 1 and 2). Negative control had no bactericidal activity on any of the pathogenic bacteria. According to laboratory results, it was identified that *R. solanacearum* is resistant to erythromycin antibiotics. However, the bactericidal effect of essential oils has a direct relationship with their concentration; so that higher values of inhibitory shaft were measured at concentrations of 50, 75 and 100%. The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) varied in different oils from 1 to 1000 μg / μL. The minimum bacterial concentration of under study bacteria was related to *T. vulgaris L* essential oil which was equaled to 1 microgram per milliliter on *R. Solanacearum* bacterium and 5 micrograms per milliliter on *E. coli* and *P. carotovorum* bacteria.

Greenhouse results

The results of biological control of *T. vulgaris L* essential oil on bacterial wilting and soft rot and tuber diseases in greenhouse condition have been presented in Tables (3), (4) and (5). In general, it was observed a significant decrease in incidence of wilting and soft rot and tuber diseases and a significant increase in stem and root weights of potato plant. No change was observed in stem and root weights of healthy plant treated with essential oil in negative control. In this experiment, positive control showed more than 80% of symptoms of wilting and soft rot and tuber. While none of negative control treatments showed disease symptoms. In addition, there was no abnormal indication on the leaves or roots of healthy plants treated with essential oil. According to Table-3, the incidence of disease in plant infected with P. carotovorum and treated with thyme oil of 40.67% was higher than positive control (77.93%). Dry and fresh weights were significantly increased with potato leaf litter ($p < 0.05$), so that the fresh weight of root was 24.30% and the stem dry weight was 1.95 g, in comparison with positive control (18.66% And 1.33 g, respectively) (Table-3). The results of dry and fresh weights of plant contaminated with *R. solanacearum* had a significant difference with positive control Table-4. Also, the dry and wet weights of root and stem of bacterial wilt disease was significantly reduced in positive control. The incidence of disease was 34.73% in treatment infected with *R. solanacearum*, which was significantly in comparison with positive control (80.67%) as has been shown in Table-4. As it can be seen from Table-5, the highest amount of disease controlled by *Thymus vulgaris L* essential oil in *R. solanacearum* infected plant was 54.15%, while applying this essential oil *in P. carotovorum* infected plant could control 51.11% of soft rot and tuber disease. The biomass of root and branch of infected potatoes with R. solanacearum and P. carotovorum increased up to 21.35% (Table-5).

Table-1. Antibacterial effect of various concentrations of tested essential oils on *R. solanacearum* bacteria.

Essential oil	Concentration (%)											
	0	**0.01**	**0.05**	**0.1**	**0.5**	**1**	**5**	**10**	**25**	**50**	**70**	**100**
Coriandrum sativum L	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.6 l	7.1 k	7.8 j	h9/1
Cuminum cyminum L	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.5 l	7.1 k	8 j	9 h	9.6 g
Thymus sativum L	0 m	0 m	0 m	6.5 l	7.8 j	11.8 e	10 if	11.8 e	13.1 d	22.8 c	29.6 b	34.8 a
Rosmarinus officinalis	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.5 l	8 j	8.8 hi	11.8 e
Eucalyptus globules L	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.5 l

The size of inhibition shafts includes diameter of the paper disc (6 millimeters).
In each column, non-similar letters with Duncan's test are significant at the level of 5%
Concentration of zero is related to ethanol treatment (negative control).
Values of t inhibition shaft are in millimeters.

Table-2. Antibacterial effect of various concentrations of tested essential oils on *P. Carotovorum* bacteria.

Essential oil	Concentration(%)											
	0	**0.01**	**0.05**	**0.1**	**0.5**	**1**	**5**	**10**	**25**	**50**	**70**	**100**
Coriandrum sativum L	0 m	0 m	0 m	0 m	0 m	0 m	8 hi	9.1 f	10 f	10 f	10.6 e	d 11.17
Cuminum cyminum L	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.6 m	7.1 kl	7.1 kl	7.5 jk	jk 7.5
Thymus sativum L	0 m	0 m	0 m	0 m	7.1 kl	7.6 ij	10.5 g	10.5 e	14 c	16.6 b	16 b	a 16.5
Rosmarinus officinalis	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.5 m	8 j	7.1 kl	h 8.1
Eucalyptus globules L	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	0 m	6.8 lm	ij 7.6

The size of inhibition shafts includes diameter of the paper disc (6 millimeters).
In each column, non-similar letters with Duncan's test are significant at the level of 5%
Concentration of zero is related to ethanol treatment (negative control).
Values of t inhibition shaft are in millimeters.

Table-3. Pathogenicity percentage of *P. carotovorum* in the potato plant treated with *Thymus vulgaris L* essential oil in greenhouse condition.

Treatment	Incidence (%)	Root fresh weight (g)	Stem fresh weight (g)	Dry weight (g)	Stem dry weight (g)
Positive control*	77.93 a	18.66 b	12.84 b	1.18 b	1.33 b
Plant infected with bacteria treated by essential oil	40.67 b	24.30 ab	15.51 b	1.50 b	1.95 ab
Healthy plant treated by essential oil	0 c	32.08 a	18.98 a	1.97 a	2.14 a
Negative control (healthy plant)	0 c	32.09 a	18.47 a	1.91 a	2.01 a

* Bacteria infected plant and untreated with essential oils
In each column, non-similar letters with Duncan's test are significant at the level of 5%

Table-4. Pathogenicity percentage of *R. Solanacearum* in the potato plant treated with *Thymus vulgaris* L essential oil in greenhouse condition.

Treatment	Incidence (%)	Root fresh weight (g)	Stem fresh weight (g)	Dry weight (g)	Stem dry weight (g)
Positive control*	80.67 a	14.47 c	12.79 c	1.31 c	1.84 b
Plant infected with bacteria treated by essential oil	34.73 b	18.88 b	16.55 b	2.61 b	2.69 b
Healthy plant treated by essential oil	0 c	22.82 b	23.55 a	3.0 a	3.26 a
Negative control (healthy plant)	0 c	22.82 b	23.55 a	3.0 a	3.26 a

* Bacteria infected plant and untreated with essential oils
In each column, non-similar letters with Duncan's test are significant at the level of 5%

Table-5. Bio-control efficiency and biomass increase of root and branch.

Disease	Biological control efficiency (%)	Root biomass increase (%)	Branch biomass increase (%)
Soft rot and tuber	51.11 a	18.55 b	17.33 b
Bacterial wilting	54.15 b	23.25 ab	21.35 b

In each column, non-similar letters with Duncan's test are significant at the level of 5%

Several reports have ever been provided on anti-bacterial properties of Thymus vulgaris L essential oil (Bhaskara *et al*., 1998). In greenhouse conditions, antibacterial properties of *T. vulgaris L* on Xanthomonas citri PV Citri bacterium has been reported equal to 69% (Samavi *et al*., 2009). Also, (Lucas *et al*., 2012) observed a decrease in the disease caused by the *Xanthomonas vesicatoria bacteria* as a result of using *Thymus vulgaris L* essential oil with concentration of 0.1%. Recently, many researchers have tested a variety of herbal essential oils on *R. solanacearum* and *P. carotovorum* and showed the importance of the problem by the results they got. (Jeong *et al*., 2009) tested essential oil of *Cymbopogon sp.* at a concentration of 0.5% on the growth of *P. carotovorum* bacteria and observed complete inhibition of bacterial growth. (Vukovic *et al*., 2007) proved that *Teucrium sp.* has bactericidal effects on *P. carotovorum* bacteria. In addition, (Biavati *et al*., 2004) showed the antibacterial activity of *Satureja sp.* and *Thymbra sp.* on *P carotovorum bacterium*. In another study, (Vokou *et al*., 1993) proved the bactericidal effects of *Rosmarinus officinalis L* against *P. carotovorum*. (Ji *et al*., 2005) tested the thymol on *R. solanacearum* under field conditions and observed prevention of wilting disease in the plants. The results of (Pattnak *et al*., 1996) and other studies have shown that essential oils of medicinal herbs are rich in biological and antifungal substances and can control a limited number of herbal pathogenic fungi. Antimicrobial properties of 37 herbal extracts from 23 plant species were investigated against 7 plant pathogens and 5 fungi responsible for food corruption under laboratory conditions and favorable results were obtained (Pattnaik *et al*., 1996). Study of (Moreno *et al*., 2006) also proved that Thymus vulgaris L plant from the mint family, has airborne substances that have secondary metabolites including thymol and carvacrol. *Rosmarinus officinalis L* plant belongs to the mint family containing a rich source of phenolic compounds with anti-bacterial and anti-oxidant properties and its antimicrobial property is related to the presence of Rosmaric Acid, Carnoside and Carnosol (Moreno *et al.*, 2006). After *Thymus vulgaris L*, *Coriandrum sativum L* had a proper antibacterial effect on all three bacteria. *Coriandrum sativum L* has no phenolic compounds, but it contains 60-70% linalool[2]. (Wan *et al*., 1998) found that Bactericidal properties of Basil's essential oil are mainly due to the presence of linalool (Wan *et al*., 1998). *Rosmarinus officinalis L* essential oil does not contain phenol antibacterial compounds. In a study by (Daferera *et al*., 2002), *Rosmarinus officinalis L* essential oil had very weak antibacterial effect on the pathogenicity of *Fusarium sp.* ‹*Botrytis cinerea* and *Michiganensis clavibacter* compared to *Thymus vulgaris L* essential oil. Also, it has been shown that antibacterial effect of *T. vulgaris L* is due to the presence of phenolic compounds, especially thymol. Terpenic phenols are bonded with amine and hydroxylamine groups of bacteria membrane and cause bacterial cell proliferation and death by destroying wall structure and releasing lipids into cytoplasm (Juven *et al*., 1994). More than 60 components have been identified in *T. vulgaris* essential oil, which often have antioxidant and anti-bacterial properties (Baranauskiene *et al*., 2003). The most important compounds in this essential oil are thymol (60-44%) and carvacrol (2-2.2-4.2%) which have the most and least antibacterial activity, respectively (Bounatoriro *et al*., 2007). Therefore, it seems that partial compounds in *Thymus vulgaris L* essential oil play a synergistic role in antibacterial activity (Bounatoriro *et al*., 2007). In 2011, extracts of several plants including garlic were used to prevent the growth of penny-silica storage fungus in apple fruits (Hirpomi *et al*., 2011). Garlic extract was also used alone and once with vegetable oil to control the blue and green molds of orange. The results showed that antifungal

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effect of the extract was 100% more effective in combination with vegetable oil (Obagwu and Korsten *et al*., 2003). Even utilizing essential oils of *Thymus vulgaris L*, Peppermint and Indian chamomile against some cellar fungus of avocado and peach completely prevents mucilage growth of these fungi and leads to non-caries in fruits after harvesting and *T. vulgaris L* essential oil can definitely be used as a natural fungicide to control post-harvest fungal diseases (Sellamuthu *et al*., 2013).

CONCLUSIONS

Among essential oils of five medicinal herbs used in the present study, *Thymus vulgaris L* essential oil showed the highest antibacterial properties on under study bacteria in laboratory and in greenhouse conditions. In greenhouse investigations of the study, a significant decrease was observed in the incidence of both wilting and soft rot and tuber diseases as a result of Thymus vulgaris L essential oil activity in potato plant. The combination of essential oil components in the soil and in vicinity of root within 48 hours may induce systemic resistance to the plant and as a result has reduced the incidence of disease. It has been proven that plants have defense mechanisms against attack of pathogens and some of these mechanisms are induced by living or non-living matter. The induced resistance has been detected by observing the limitation or inhibition of disease growth symptoms. Also, it would be possible that *Thymus vulgaris L* essential oil has had a bactericidal effect on the soil and resulted in loss of a percentage of tested bacteria in the soil. The greenhouse and laboratory results of present study indicated that *Thymus vulgaris L* essential oil has a high antibacterial capacity and can be used in combination control with other non-chemical methods against bacterial wilt and soft rot and tuber diseases of potato. However, farm research is needed to confirm the effectiveness of using this essential oil in natural conditions and to calculate the concentration of essential oil for achieving an acceptable and affordable disease control.

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