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Antibacterial Effect of Effective Compounds of *Satureja hortensis* and *Thymus vulgaris* Essential Oils against *Erwinia amylovora*

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ABSTRACT

Use of essential oils as pesticides or fungicides is safer than chemicals, but it requires plenty of the plant to be effective. Hence, finding the effective compounds of essential oils and their synthesis decreases the problem of preparing the natural compound. The antibacterial effect of *Satureja hortensis* L., *Thymus vulgaris* L. essential oils, and their major constituents were determined using the disc diffusion method. These essential oils prevented *Erwinia amylovora* growth (that causes fire blight disease). The essential oils were fractionated using preparative column chromatography (Silica column) and all fractions were tested for their antibacterial activities on this bacterium. Effective fractions were analyzed by GC-MS. Results showed that carvacrol is the effective compound in *Satureja hortensis* essential oil and has strong antibacterial effect. The effective compounds in *Thymus vulgaris* essential oil are thymol and carvacrol. Thymol and carvacrol showed a strong antibacterial effect against *E. amylovora* in the disc diffusion method. These compounds prevented the growth of *E. amylovora* in sucrose and nutrient agar media.

Keywords: Carvacrol, Column chromatography, *Satureja hortensis,* Thymol, *Thymus vulgaris.*

INTRODUCTION

Fire blight, which is caused by *Erwinia amylovora* (Burrill), is a highly destructive and economically important bacterial disease that can decimate apple and pear orchards in a single season. *E. amylovora*, which is kind of gram-negative bacteria, infects the host through natural openings in flowers (Wilson *et al.,* 1989) or wounds occurring in young organs, often following hailstorms. *E. amylovora* is a necrogenic phytopathogenic bacterium and infects a wide variety of landscape plants in the rosaceous family including apple, crab apple, pear, pyracantha, etc. (Bonn and van der Zwet, 2000). Fire blight is the most important bacterial disease of *Maloideae*. The major economic impact of the disease has been reported in 40 countries, (Bonn and van der Zwet, 2000). Causative pathogen early outbreaks in the 20th century have been reported for Japan and New Zealand (Bonn and van der Zwet, 2000) around 1960, and the disease has become distributed to Europe and countries of the Mediterranean region. In the southern hemisphere, a transient occurrence of *E. amylovora* on plants in the Botanic Gardens of Melbourne was reported for 1997 (Jock *et al.*, 2000). The pathogen infects all plant parts, including blossoms, fruit, leaves, shoots, limbs, and trunks. The bacteria colonize the intercellular spaces of bark,

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causing the death of the plant cells associated with distortion of cell walls and the formation of lysogenic cavities. In the susceptible, succulent shoots, the necrosis spreads downwards from the apex with browning of the tissues (Vanneste and Eden-Green, 2000). The exopolysaccharides amylovoran and levan and the protein harpin have been demonstrated to be pathogenicity factors for the colonization of host plants by the pathogen. Fire blight symptoms result in extended necrosis of infected tissues, as a consequence of the massive colonization of parenchyma intercellular spaces. In susceptible hosts, bacteria can spread through the apoplast leading to systemic infection. The death of woody tissue leads to the formation of cankers, where bacteria can eventually over winter (Vanneste, 1995). The production of virulence factors of phytopathogenic *Erwinia* species is regulated by global systems which respond to stimuli like cell density (quorum sensing) or to other unknown signals resulting in the control by the Rsm global regulatory system (Whitehead *et al.,* 2002).

The chemical control of *E. amylovora* relies primarily on copper compounds. Antibiotics, such as streptomycin, can also be very effective. However, copper compounds can be toxic to plants, and can cause fruit rust that negatively affects fruit finish (Steiner, 2000). The antibacterial effects of essential oils of plant origin are attractive candidates for pear (*Pyrus communis L.*) to increase resistance to fire blight. Numerous preventive measurements have been applied to decrease the chance of introduction of the disease in countries without fire blight. On the other hand, the worldwide complex trades of plants and plant products as well as practical limitations on controlling all tourist activities with plant material make it impossible to completely avoid the importation of contaminated plant tissue. Therefore, the absence of fire blight in South America, South Africa, Australia and also Southeast Asia raises the question of intrinsic conditions unfavorable to establishment of the disease. In fire blight countries, control of the plant disease is a pressing need for apple and pear production in the 21st century.

In this paper, the effects of separated fractions of *Thymus* and *Satureja* essential oils on *E. amylovora* causes of fire blight disease were studied under in vitro conditions. In recent years, two consumer-driven demands have arisen in the food industry. The first is the provision of fresh, natural foods requiring minimal preparation while the second is the control of food safety (Knobloch *et al*., 1989). Only a few studies have evaluated the potential role of essential oils and their components as food preservatives. Purified compounds derived from essential oils such as carvacrol, eugenol, linalool, and thymol inhibit a variety of microorganisms (Hulin *et al*., 1998). Volatile Plant oils have been recognized to possess biological activities (Deans and Waterman, 1993); they have been reported to possess antibacterial (Deans *et al*., 1994), antifungal (Carlton *et al*., 1992; Mehdi *et al*., 2008) and antioxidant properties (Dorman and Hiltunen, 2004; Piccaglia *et al*., 1993). Most of the antimicrobial activity in essential oils from spices and culinary herbs appears to be associated with phenolic compounds (Davidson and Naidu, 2000). The properties of essential oil of thyme are particularly linked to the phenolic metabolites thymol and carvacrol (Shetty *et al*., 1996; Horvath *et al*., 2002; Exarchou *et al*., 2002). In general, the levels of essential oils and their compounds necessary to inhibit microbial growth are higher in foods than in culture media. This is due to interactions between phenolic compounds and the food matrix (Nychas and Tassou, 2000). Partitioning of the hydrophobic antibacterial essential oil components into the fat content of the food may prevent them from coming into contact with bacterial cells growing in the hydrophilic regions in the food (Gill *et al*., 2002). In general, gram-positive bacteria are more sensitive than gram-negative bacteria to essential oils or their compounds (Hulin *et*

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al., 1998; Nychas and Tassou, 2000). The genus *Thymus* includes about 350 species worldwide and is distributed mainly in temperate Eurasia (Asfaw *et al*., 2000). In *Thymus*, the phenolic fraction (thymol and carvacrol) is the main component of essential oil (McGimpsy *et al*., 1994). Phenolic compounds are widely distributed in plants but their functions are not clearly known (Wilkins, 1969), also thymol and carvacrol is a major compound of *S. hortensis* essential oil (Guenther, 1970; Ghannadi, 2002; Güllüce *et al*., 2003; Sahin *et al*., 2003; Sefidkon, *et al*., 2006). For separation of these phenolic compounds on fire blight disease, column chromatography and TLC have been used earlier (Runti *et al*., 1960; Sugisawa *et al*., 1988; Pothier *et al*., 2001). There are a few reports on the antimicrobial activity of essential oils or their major constituents towards *E. amylovora*. In this research we investigated the antibacterial effect of stable components of essential oils of *Satureja hortensis* L. and *Thymus vulgaris* L. on *E. amylovora* at 40℃. We have developed a simple and convenient method employing column chromatography and GC-MS for separation and determination of essential oil compounds and their effect on *E. amylovora* bacterium has been studied to find natural antibacterial components. Solvent selection for achieving a good separation was very critical in our work. The chosen solvents were non-toxic with viscosity <2cP (Snyder, 1978). Those solvents or mixtures of solvents having suitable adsorbtion strength were preferred.

MATERIALS AND METHODS

Instrumentation

A Finnigan MAT (Austin, USA) GCQ system of GC–MS with an ion trap detector was used for quantification and confirmation of the essential oil's effective compounds. The detector was applied in the electron impact (EI) mode, equivalent to electron energy of 70 eV. Operating conditions were as follows: the capillary column was a 25 m×0.32 mm i.d; the carrier gas was helium 99.999% purity at a flow rate of 1.0 ml min^{-1}. The injector in split less mode (valve 1 minnute closed) was 260ºC, the column temperature following an initial period of 50ºC for 0.2 minute was 10ºC min^{-1} to 230ºC. The data system used was Xcalibur 1.1, USA. Full scan data were monitored and multiple ion chromatograms were built for quantification.

Plant Material

Aerial parts of flowering *T. vulgaris* and *S. hortensis* grown in the open air were collected from sites in Tehran Province from May to July 2005. Collected plant materials were dried in the shade; the leaves of the plants were separated from the stem and ground in a grinder with a 2 mm in diameter mesh.

Isolation of the Essential Oil

Twenty g of *T. vulgaris* was hydro distilled with 300 ml water for 2 hours in a Clevenger-type apparatus according to the European Pharmacopoeia (1975) (yield 1.14% v/w) (Saez, 1995, Sefidkon *et al*., 1999). In order to obtain *S. hortensis* essential oil 40 g the plant material was hydro distilled for 4 hours (yield 1.32 % v/w) (Rojas and Usubillaga, 2000). The essential oils obtained were dried over anhydrous sodium sulphate and, after filtration, stored at 4ºC until tested and analyzed.

Solvent Selection

Solvent selection for achieving a good separation was very critical to our work. Four solvents were chosen as better mobile

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phase, out of 15 solvents that were used for separation of these compounds. The chosen solvents were non-toxic with a viscosity <2cP. Solvents or mixtures of solvents having suitable adsorbtion strength were preferred. The TLC spot test was used to determine the adsorbtion strength (Table 1) (Snyder, 1978).

Table 1. The strength of mobile phases for elution of compounds in a column.

Mobile Phase	Solvent Strength
Hexane	0.00
Hexane +20% Toluene	0.09
Toluene	0.23
Toluene+20% Ethyl acetate	0.28
Toluene+60% Ethyl acetate	0.34
Ethyl acetate	0.38
Ethyl acetate+20% Ethanol	0.48
Methanol	0.73

Chromatography

For performing the separation of essential oil compounds, the column was packed with 20 g silica (0.2-0.5 µm size). 0.1 g of essential oil was dissolved in 3 ml hexane and injected on the column. Elution of compounds in the column started with the first solvent as a mobile phase that has the lowest strength between other solvents (hexane); two fractions were obtained with this solvent. Elution was followed with other solvents as given in Table 1 and with each solvent 4 fractions were obtained. This experiment was repeated three times. The solvent of each fraction was evaporated until to dryness at low temperature (40-50ºC) then kept in a dark vial at 5ºC.

Microorganisms

E. amylovora were obtained from the IAU Research Lab North Tehran Branch. The bacterium had been isolated from pear trees (*Pyrus communis* L.), from Karadj using the European Plant Protection Organization (EPPO) method (1992). The pathogenicity tests had been carried out using (Ritchie and Klos, 1974) method. Bacterium was cultivated in Nutrient Broth at 26–27°C for 24 hours in a shaker. A conclusive cell concentration was 108 CFU ml^{-1}, which was numbered in Nutrient Broth using serial dilution method.

Determination of Antibacterial Activities

The paper disc diffusion method was used to reveal the antibacterial activity of essential oils and fractions. Sterilized filter paper discs (5 mm) (Whatman No1) were soaked with 20 µl of essential oil, their related fraction, their solvents as control and also thymol and carvacrol standard solutions dissolved in toluene, and put in the middle of plates containing 20 ml of nutrient agar inoculated with 100 µl containing 10^8 CFU ml^{-1} of the bacterial suspension. For a negative control, pure mobile phase was used. The plates were incubated at 27ºC for 48 hours. The inhibition zones around the paper disc were measured by compass. The experiment was done in triplicate. (Basim *et al*., 2005)

RESULTS

The effect of essential oils of phenolic compounds and their constituents has not been specially investigated on *E. amylovora*. The objective of the present study was to evaluate the antibacterial effect of *S. hortensis* and *T. vulgaris* essential oils and their major constituents to inhibit the growth of *E. amylovora* a pathogen for which few data are available. The antibacterial activities of essential oils in vitro tested against *E. amylovora* showed that *S. hortensis* and *T. vulgaris* could inhibit growth of this bacterium (Table 4). GC-MS was employed to identify the essential oils compounds (Table 2). After 48 hours out of

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Table 2. The major compounds in *T. vulgaris* and in *S. hortensis* essential oil.

Compound	Peak area % in *Satureia*	Peak area % in *thymus*
α-Pinene	3.63	3.15
β -Pinene	1.18	-
α –Myrcene	3.04	0.86
β-Cymene	9.56	20.04
1,8-Cineol	-	1.27
γ-Terpinene	31.34	0.92
Linalool	-	2.36
Borneol	-	0.02
4-Terpineol	-	0.12
Anisole	-	0.94
Carvacrol methyl ether	-	4.49
Thymol	-	49.5
Carvacrol	35.82	7.68
Trans-caryophyllene	1.16	6.84
Caryophyllene oxide	-	2.2
Bisabolene	2.01	-

Table 4. Inhibition of *E. amylovora* by *T. vulgaris* and *S. hortensis* essential oils and their effective fractions.

Essential oil and fraction	Inhibition (mm)
Thymus vulgaris L.	25
Satureia hortensis	25
Fraction 6 in *Thymus vulgaris*	10
Fraction 7 in *Thymus vulgaris*	8
Fraction 6 in *Satureia hortensis*	5
Fraction 7 in *Satureia hortensis*	1

Table 6: Inhibition of *E. amylovora* by standard solutions of thymol and carvacrol.

Standard solution	Inhibition (mm)
Pure carvacrol	25
Carvacrol 5% (v/v)	6
Carvacrol 3% (v/v)	4
Thymol 5 % (w/v)	7

28 fractions and its solvents that affected on *E. amylovora* (Table 3), it showed that only the two toluene fractions 6 and 7 in *S. hortensis* and *T. vulgaris* inhibit the growth of *E. amylovora* (Table 3) and solvents do not have any effect on the bacterium. GC-MS was employed to identify the effective compounds, fraction 6 of *T. vulgaris* plant contained 84.7% thymol and 8% carvacrol, while fraction 7 contained 72% thymol (Table 5) (Figures 1 and 2). In case of *S. hortensis,* plant fractions 6 and 7 contained 99% and 22% carvacrol respectively and no thymol was observed (Table 5) (Figures 3 and 4). The antimicrobial effects of thymol and carvacrol standard solutions against *E. amylovora* were also studied. Results have been shown in Table 6.

DISCUSSION

Plant essential oils are a potentially useful source of antimicrobial compounds. Essential oils are natural products extracted from vegetal materials which, because of their antibacterial, antifungal, antioxidant and anti-carcinogenic properties, can be used as natural additives in many foods (Teissedre and Waterhouse, 2000). Results

Table 3. Fractions of *T. vulgaris* and *S. hortensis* essential oils and their effects.

Mobile Phase	Number of fractions	Effective fraction (s)	Effect of solvents (mm)
Hexane	1,2	--	0
Hexane+20%Toluene	3,4,5,6	----	0
Toluene	7,8,9,10	7,8,--	0
Toluene+20 % Ethyl acetate	11,12,13,14	----	0
Toluene+60% Ethyl acetate	15,16,17,18	----	0
Ethyl acetate	19,20,21,22	----	0
Ethyl acetate +20% Ethanol	23,24,25,26	----	0
Methanol	27,28	--	

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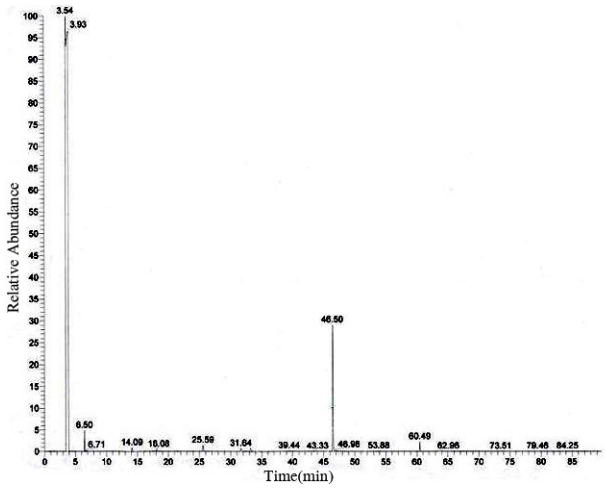

Figure 1. GC-MS of fraction 6 in *T. vulgaris* essential oil.

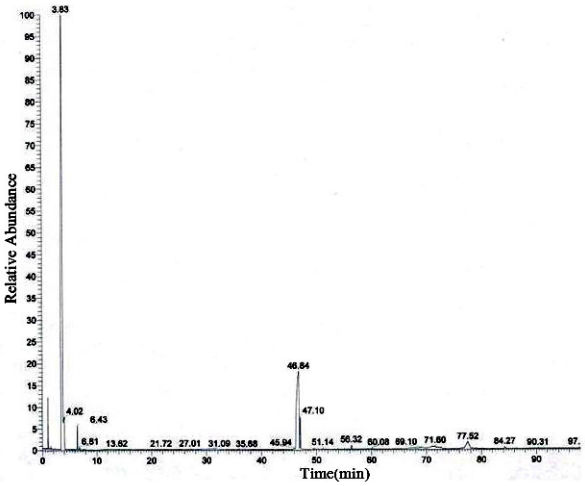

Figure.2 GC-MS of fraction 7 in *T. vulgaris* essential oil.

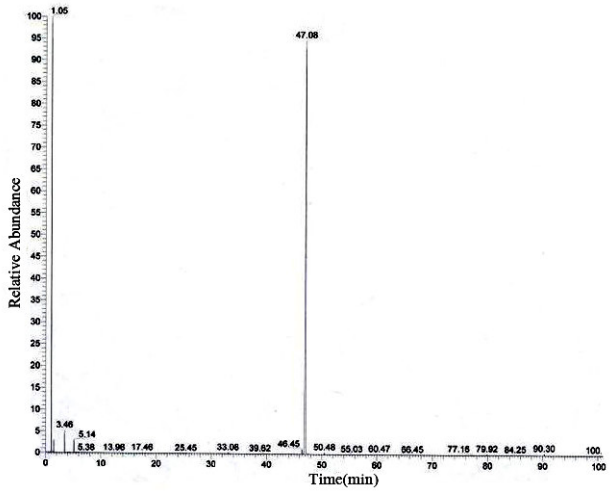

Figure 3. GC-MS of fraction 6 in *S. hortensis* essential oil.

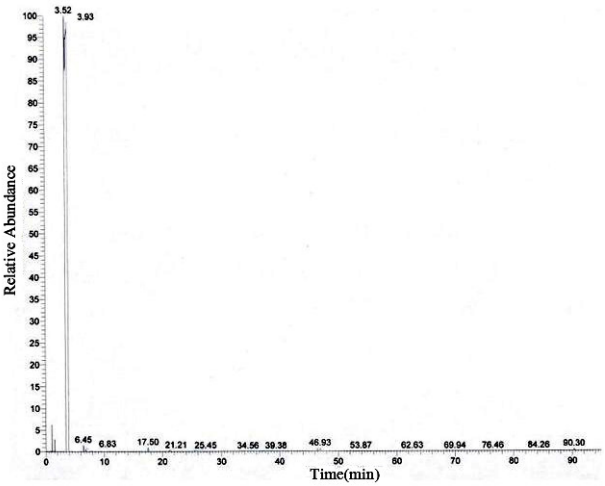

Figure 4. GC-MS of fraction 7 in *S. hortensis* essential oil.

reported from different studies are difficult to compare, presumably because of the different test methods, bacterial strains and sources of antimicrobial samples used. The composition of the essential oil of herbs and spices can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the method of extraction of the oil (Jerkovic *et al*., 2001). It is recommended to use a synergistic combination of essential oils and their compounds, e.g. carvacrol in combination with p-cymene (Ultee *et al*., 2000b), thus enabling to decrease their concentrations and minimize adverse sensorial effects. Evidently, more studies are needed on the antimicrobial properties of essential oils and their compounds before they can be used as food preservatives. Numerous studies have demonstrated that the essential oil of thyme is among the most potent essential oils with regard to antimicrobial properties (Manou *et al*., 1998). Thymol and carvacrol were among the most active components against multiple food-borne pathogens (Friedman *et al*.,

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Table 5: The major compounds in first toluene fractions (6 and 7) of *T. vulgaris* and *S. hortensis* essential oil.

Fraction no.	Compound	Peak area in *T. vulgaris* %	Peak area in *S. hortensis* %
6	Thymol	84.7	-
6	Carvacrol	8.05	99
6	Trans-Caryophyllene	0.061	-
7	β-Cymene	0.55	-
7	1,8-Cineol	1.08	-
7	Linalool	5.41	-
7	Borneol	2.64	-
7	Thymol	70.04	-
7	Caryophyllene oxide	5.31	-
	Carvacrol	-	22

2002; Sahin *et al*., 2003; Boyraz and Ozcan, 2006). The results show that *S. hortensis* and *T. vulgaris* essential oils have the same effect against *E. amylovora* and the major compounds in each essential oil are thymol and carvacrol that were found to be effective compounds as a main compound. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different location on the phenolic ring. Both substances appear to make the cell membrane permeable (Lambert *et al*., 2001). Among seven individual oil components tested against bacterial strains, thymol was a component with the widest spectrum followed by carvacrol (Dorman and Deans, 2000). Carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride has been shown to have no influence on this action, suggesting a mechanism other than chelation of cations in the outer membrane (Helander *et al*., 1998). Carvacrol has been characterized as an inhibitor of growth of different pathogens (Ultee *et al*., 2000a) and was shown to have a bactericidal effect towards *Salmonella* sp. in pieces of fish stored at 4°C (Hulin *et al*., 1998). Ultee and Smid (2001) observed a sharp decrease of the toxin production by *Bacillus cereus* in the presence of carvacrol in BHI. Carvacrol also inhibited toxin production of *B .cereus* in soup but approximately 50-fold higher concentrations were needed to reach the same effect as in broth. Studies with *B. cereus* have also shown that carvacrol interacts with the cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains (Ultee *et al*., 2000 a). This distortion of the physical structure would cause expansion and destabilization of the membrane, increasing membrane fluidity which, in turn, would increase passive permeability (Ultee *et al*., 2002). Measurement of the average phase transition temperature of the bacterial lipids confirmed that membranes instantaneously became more fluid in the presence of carvacrol (Ultee *et al*., 2000 a). The passage of *B. cereus* cell metabolites across the cell membrane on exposure to carvacrol has also been investigated. Measurements of the membrane potential (Du) of exponentially growing cells revealed a sharp decrease on the addition of carvacrol and indicated a weakening of the proton motive force. The pH gradient across the cell membrane was weakened by the presence of carvacrol and was completely dissipated in the presence of 1 mM or more. Furthermore, intracellular levels of potassium ions dropped whilst extra cellular amounts increased proportionately, the total amount remaining constant (Ultee *et al*., 1999). It was concluded that carvacrol forms channels through the membrane by pushing apart the

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fatty acid chains of the phospholipids, allowing ions to leave the cytoplasm (Ultee *et al*., 2000 b). Several essential oils of aromatic plants including, *T. vulgaris* (thyme) and *Ocimum basilicum* (basil), have totally inhibited fungal development on maize kernels (Montes-Belmont and Convajal, 1998). Mustard essential oil has been used for inhibition of fungal growth on bread (Nielsen and Rios, 2000; Mourgues *et al*., 1998) showed antibacterial activity of several peptides on *E. amylovora*. The result indicated Cecropins had the highest bactericidal efficiency, followed by T4 lysozyme. Pollen (a fine, powder-like material produced by flowering plants and gathered by bees) and propolis or bee glue extracts have antibacterial activity towards *E. amylovora* (Basim *et al*., 2005). Bagamboula *et al.* (2004) have shown that thyme essential oil, thymol, and carvacrol can inhibit of *Shigella* sp. in the agar well diffusion method.

CONCLUSIONS

The main purpose of the present study was to determine the antimicrobial effectiveness of *S. hortensis* and *T. vulgaris* essential oils and their active compounds (carvacrol and thymol) as a decontaminating agent for fresh producE. The use of these natural compounds may improve food safety and overall microbial quality. The application of essential oils as antimicrobials in foods is often discouraged because of the potential loss of antimicrobial action due to their volatility and lipophilicity. The use of thyme essential oil, carvacrol and thymol as decontamination agents rather than as preservatives in foods and the application of decontamination treatment to a carbohydrate-rich food type may explain their antimicrobial effectiveness. Thymol or carvacrol and their mixture can be used to stop the growth of *E. amylovora* under *in vitro* conditions and may be suitable for preventing the fire blight disease especially in fruits such as apple and pear.

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اثر ضد باکتریایی اجزای اصلی اسانس های آویشن و مرزه بر روی باکتری اروینیا آمیلوورا

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چکیده

استفاده از اسانس های گیاهی بعنوان آفت کش یا قارچ کش ایمن تر از سموم شیمیایی مورد استفاده در این زمینه است، اما نیاز به تهیه مقادیر زیاد گیاه برای تهیه اسانس مشکلاتی به همراه دارد بنابراین ترکیبات موثر بر روی آفات و قارچ ها و ساخت مصنوعی آنها میتواند مشکل تهیه اسانس بصورت طبیعی را برطرف نماید. اثر ضد باکتریایی اسانس های آویشن و مرزه و همچنین اجزای اصلی آنها با استفاده از روش پخش بر روی صفحه مشخص شد. این اسانس ها از رشد باکتری اروینیا آمیلوورا (عامل اصلی آتشک گلابی) جلوگیری کردند. با استفاده از کروماتوگرافی ستونی تهیه ای (ستون سیلیکاژل) اجزای اصلی هر یک از اسانس ها از یکدیگر جدا شده و تمامی اجزا بر روی باکتری اثر داده شدند، اجزای موثر با کمک کروماتوگرافی گازی – طیف سنج جرمی شناسایی شدند.پس ازاین مرحله استاندارد های خالص خریداری شدند و با اثر بر روی باکتری صحت آزمایش را تایید کردند. این تحقیق نشان داد که کارواکرول که قسمت اصلی اسانس مرزه است خاصیت ضد باکتریایی قوی دارد و همچنین در اسانس آویشن تیمول و کارواکرول اجزای اصلی اسانس را تشکیل می دادند و به خوبی از رشد باکتری اروینیا آمیلوورا در شرایط آزمایشگاه جلو گیری کردند.

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