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In vitro and *in vivo* antifungal activity of botanical oils against *Alternaria solani* causing early blight of tomato

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Abstract

In vitro as well as *in vivo* activities, it was investigated to examine if different botanical oils have any effects on the radial growth of *A. solani* and are able to reduce early blight incidence and severity in the field condition. Oils from various plant sources such as Jojoba (*Simmondsia chinensis*), Ginger *(Zingiber officinale* Roscoe), Garlic (*Allium sativum*), Clove (*Syzygium aromaticum*), Sesame (*Sesamum indicum*), Eucalyptus (*Eucalyptus glabulus*), Cinnamon (*Cinnamon zylanicum*) and Castor (*Ricinu communis)*, lemon (*Citrus limon*) and mustard (*Brassica nigra*) were tested at concentration of 0.1%, 1% and 3% to determine their effects on the mycelial growth of *A. solani*. The 3% dosage of oil of ginger, lemon and castor inhibited the maximum radial growth of *A. solani* by 29.6%, 29% and 27% respectively. Meanwhile, the 1% concentration of lemon oil was also recorded the maximum growth inhibition of the pathogen by 27%. Moderate to lowest inhibition of the fungal growth was observed with cinnamon oil (21.6%) followed by oil of mustard (21.6%), jojoba (21%), sesame (21%) and garlic 20%. In *vivo*, at 1% concentration, the least disease incidence of 29.7% and 29.8% were achieved on plants treated with oil of garlic and lemon, respectively. Severity of early blight was significantly reduced by 34.9% of clove oil followed by 34.3% and 34.2% of eucalyptus and garlic, respectively. Fruit yield of tomato was totally increased with all oil treatment, clove oil significantly improved plant height and increased fruit yield by 58.3Kg/plot.

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Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the popular vegetable and a major horticultural crop grown all over the world and occupies a prominent position in the world's vegetable economy. It is the second most consumed vegetable after potato, ranking first among the processing crops (FAO, 2007). About 152,956,115 tons of tomatoes were produced in the world in 2009 (FAO, 2009). In 2011, the cultivated area under tomato plants in Saudi Arabia was 14,175 hectares, which produced 483,588 tons, where the annual imports of tomato in Saudi Arabian was 340,000 tons at cost of $20 million (FAOSTAT© FAO, 2013).

Tomato products are important part of human diets. Currently, tomato has a higher consumption rate in developed countries and is often referred to as a luxury crop. In developing countries, tomato has become important part of the food basket as well. It is also the most widely consumed vegetable crop in Saudi Arabia.

Tomatoes consumed fresh in salads or cooked within sauces, meat or fish and soup dishes. They can be processed in purées, juices and ketchup. Dried and canned tomatoes are economically important processed products (Shankara *et al*., 2005). They are an important source of daily nutrients contain vitamin C, A and E (Beecher, 1998), providing around 20 mg of vitamin C per 100g of edible product (Wilcox *et al*., 2003). Farmers receive lower yield mostly due to diseases, pests and sub-optimal fertilization. The most important factors responsible for the low productivity of tomato are diseases and insect pests. Among those diseases, early blight is one of foliar diseases of tomato caused by *Alternaria solani* is the most destructive and widespread in temperate, tropical and subtropical regions of the world, which causes reduction in quality and quantity of tomato yield (Hijmans *et al*., 2000). It can severely damage incurring a loss of 50 to 80% on tomato susceptible hybrids varieties. *A. solani* can infect each part of the plant (causing foliage blight, fruit lesions and stem collar rot) and can damage during all stages of plant development (Abada *et al*., 2008).

The primary symptom of early blight is small dark brown spots on the lowest and oldest leaves. The tissue around the primary lesions may turns bright yellow, and if lesions are numerous, the entire leaves may become necrosis and chlorotic. The spots get enlarged, they develop concentric rings which give them a bull's eye. In favorable weather conditions, disease develop, lesions can become numerous and plants defoliate, which damage the quantity and quality of tomato fruits (Kouyoumjian, 2007).

The most common method for controlling effectively and extensively early blight tomato disease is the use of fungicides. Though they are costly, fungicide treatments are generally the most effective control measures. However, they are not only costly but also capable of creating problems on the environment, human health in all areas of the world and may lead to the development of resistance in pathogenic fungi to common fungicides. The natural plant products, which known as botanical pesticides, have been used to control microorganisms causing plant and human diseases. Several researchers have worked the various aspects of biological control of plant pathogens (Cook, 1993; Jayaraj and Ramabadron, 1996; Ashwani *et al*., 2004; Mukhopadhyay, 1994; Dube, 2001; Shalini and Dohroo, 2005; Sendhilvel *et al*., 2005; Harman, 2006). Plant products are environmentally safe, cost effective and easily biodegradable. This makes them useful for the management of fungal diseases in plants as alternative to synthetic fungicides. Therefore, to study the efficiency of botanical oil against early blight (*A. solani*) on tomato the objectives for this research was conducted to screen the efficacy of some botanical oils for its inhibitory effect as well as to determine the impacts of natural botanical products on the controlling of tomato early blight under open field condition

Materials and methods

Isolation and culture preparation

Tomato leaves which showing typical early blight symptoms were collected in the early 2015 from growing tomato plants from different fields of Hada al sham area of Jamoom, Saudi Arabia. The infected leaves were brought to laboratory and the diseased

leaves with *A. solani* were cut into small bits measuring about 5mm and surface sterilized with mercuric chloride solution for 1 min, wash twice by sterile distilled water. Pieces were then put on Potato Dextrose Agar (PDA) medium and incubated under 12 h light and 12 h dark at 28±2°C according to Naik *et al*., 2010. Pure culture of the *A. solani* was obtained by Hyphal Tip Isolation Method. Pathogen was identified following the cultural and morphobiometric characteristics criteria (Ellis, 1971). The pathogenicity test was confirmed by Koch's postulates. The isolated fungus was grown on PDA slants, stored at 5°C in refrigerator and sub culturing will be done subsequently at intervals of 30 days for further research studies. Laboratory experiment was conducted at the Plant Protection laboratory, Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid land agriculture, King Abdulaziz Univesity.

In vitro, efficacy of plant botanical oils on colony growth of A. solani

Commercial grade of the botanical oils from Jojoba (*Simmondsia chinensis*), Ginger (*Zingiber officinale* Roscoe), Garlic (*Allium sativum*), Clove (*Syzygium aromaticum*), Sesame (*Sesamum indicum*), Eucalyptus (*Eucalyptus glabulus*), Cinnamon (*Cinnamon zylanicum*) and Castor (*Ricinus communis)*, lemon (*Citrus limon*) and mustard (*Brassica nigra*) were obtained from the local market Al- Ahlam for seeds oil production - Jeddah. The botanical oils were stored in bottles under dark condition at 4 °C. The botanical oils were chosen on the basis of the previous studies with documented antimicrobial activity.

For the antifungal activity of the botanical oils using the poisoned food technique according to Soad and Abdel Galeil, (2008), required amount of the stock solutions from botanical oils into Tween 20 were incorporated to enhance oil solubility into the sterile PDA medium and then poured in the Petri dishes (9.0 cm) at 45 °C to obtain final concentrations 0.1, 1.0, and 3 per cent and the control plates were carrying out only Tween 20 in PDA without adding of the oils.

All the treatments were inoculated by 5 mm plugs from 7 days old culture of the pathogen. Four plates were used per each treatment. Plates were incubated for 7 days in a growth chamber under alternative of 12 h light and 12 h dark at 28±2°C. The diameter of developed radial growth of the pathogen was observed after 7 days from inoculation and the percentage of reduction in the colony diameter compare to control was calculated using the formula suggested by Taskeen *et al*., (2011).

$$PGI = \frac{DC-DT}{DC} \times 100$$

Where: PGI = Percentage growth inhibition

DC = Average growth diameter of fungal colonies obtained from control plates.

DT = Average growth diameter of fungal colonies obtained from treated plates.

Field evaluation of botanical oils for the management of tomato early blight

Location for conducting of experiment

The study was conducted on the agricultural research station, Hada Al Sham, department of arid land agriculture, faculty of meteorology, environment and arid land agriculture, King Abdul Aziz University, Jeddah, Saudi Arabia in 2015/2016.

Design and layout of the experiment

For experiments a randomized complete block design with three replicates was used. The layout was done as per experimental design on 20 November 2015. The field was divided into three blocks each of which representing a replication. The unit plots contained of single rows 14m long and with 1m between plants, 1m between rows and block to block distance was 2m.

Seven treatments were T_1= lemon oil, T_2= Clove oil, T_3= Garlic oil, T_4= Jojoba oil, T_5= Eucalyptus oil, T_6= Positive control, T_7= Negative control

Filed trial

Tomato seeds of the Supper Star variety were obtained from the DIGI AGRI EST. COM, Jeddah, Saudi Arabia in 2015 and sown in the commercial substrate for seedlings which were kept inside the greenhouse in 25-30°C. In this stage, the seedlings were receiving daily irrigation to keep the substrate moist. In the field, soil was amended with the required amount of organic manure and fertilizer (NPK) was applied once prior to transplanting of seedling to the soil and as well as in all growing stages according to the plant needs, plants were properly daily irrigated and weeds were controlled using manual removal throughout the season in the experiment. Four weeks after sowing, when two pairs of true leaves were present, the seedlings were transplanted to the open field.

The inoculation of the tomato plants with *A. solani* spore suspension at concentration level of 10^6 spores mL^{-1} (the inoculum density was measured by hemocytometer) were carried out by spraying it on tomato seedlings 20 days after transplanting using manually hand sprayer. Inoculated seedlings were covered by polyethylene bags for 30 h to keep the plant surface under high humidity level at 30-32°C for inoculums of the pathogen to be germinated.

All the treatments were applied to the plants at the concentrations of 10ml L^{-1} of water as foliar treatment and amount of 10ml of Tween 20 was used as a solvent for all the treatments. In the negative control, 10ml of Tween 20 L^{-1} was applied in the water solution, whereas, the tomato plants in positive control treatment were sprayed by 10 ml Tween 20 and standard protective fungicide (mancozeb) in recommended dose.

The initial application of the treatment was made, when, typical spots symptoms of the early blight was appeared on the leaves and was continued throughout the end of growing season in 15 day interval. Each plot for each treatment was consisted of 14 plants from which five plants in the replicate were randomly chosen for assessment of early blight incidence, severity and fruit yield.

Assessment of disease severity and data analysis

Disease incidence and severity were measured with canopy position (upper, middle and lower canopy). Twenty five leaves from the selected five plants for each plot were used to record the disease parameters according to the method of Awad (1980). Incidence of early blight was assessed by counting the number of leaves that are diseased and percentage was calculated from the total twenty five leaves observed in the five randomly selected plants in each replication. The observation was recorded individually using 0-5 rating scale (Table 1) based on leaf area, stem and fruit covered by blight symptoms following the rating scale described by Pandey *et al.*, (2003).

The per cent incidence for each plot was calculated as:-

$$\text{Disease Incidence (\%)} = \frac{\text{Number of diseased Leaves} \times 100}{\text{Total Number of Leaves observed}}$$

Percent disease index (PDI) was calculated as follows:

$$\text{PDI} = \frac{\text{sum of all rating} \times 100}{\text{total number of observations} \times \text{maximum rating grade}}$$

The efficacy for each treatment was calculated. % Efficacy = (DSC-DST)/DSC ×100.

Where, DST: Disease severity under treatment, and DSC: Disease severity under control.

Statistical analysis

In vitro, Statistical analysis of the data was carried out in CRD and field trial in RCBD layout. Where, significant differences for the means were calculated based on ANOVA, the mean comparison of different treatments was performed using LSD at $P \leq 0.05$ level of probability (El-Nakhlawy, 2010).

Results

In vitro antifungal activity of botanical oils against A. solani

The antifungal activity of ten botanical oils from different plant sources (clove, lemon, garlic, jojoba, eucalyptus, cinnamon, mustard, ginger, sesame and castor) was assayed at three levels of 0.1%, 1% and 3% concentration in the laboratory against *A. solani* using food poisoned technique. All of the tested botanical oils significantly inhibited the mycelial growth at all concentration levels compared to the control (Tween 20) (Table 2).

The fungal growth was reduced significantly with the increasing in concentrations of the botanical oils and recorded least colony growth at the highest doses applied. 100 percent reduction in the radial growth of the pathogen was not recorded in at any doses of the tested oils.

Table 1. Rating Scale for assessment of early blight on tomato plants.

Rating	Reaction description
0	Free from infection
1	< 10% surface area covering leaf, stem and fruit
2	11-25% foliage of plant covered with few isolated spots
3	Many spots coalesced on the leaves, covering 25-50% surface area of plant
4	51-75% area of the plants infected, fruits also infected at peduncle end, defoliation and blightening started. Sunken lesions with prominent concentric rings on stems, petioles and fruits
5	<75% area of plant part blighted, sever lesions on stem and fruit rotting on peduncle end.

For all botanical oils tested, mycelial growth inhibition showed significant differences at 3% concentration as compared to the 0.1%. The botanical oil from lemon at 3% concentration exhibited the maximum inhibition of radial growth of *A. solani* (31.6%) followed by ginger oil and castor oil at 3% concentration (29.6% and 27% respectively). Among the tested concentration (0.1, 1.0 and 3%) the lemon oil exhibited excellent antifungal effects (31.6%, 29% and 27%, respectively) whereas, there as significantly differences at each oil concentration from castor and ginger (27%, 20% and 19%) and (29.6%, 25.6% and 15%) respectively.

Table 2. *In vitro*, the efficacy of botanical oils on the radial growth of *A.solani*

No.	Botanical oil	Concentration (%)	Colony diameter (cm)	% of inhibition
1	Clove oil	0.1	4.42 c *	11.6
		1	4.25 d	15.0
		3	4.15 de	17.0
2	Lemon oil	0.1	3.65 gh	27.0
		1	3.55 hk	29.0
		3	3.42 k	31.6
3	Garlic oil	0.1	4.65 b	7.0
		1	4.20 de	16.0
		3	3.97 ef	20.0
4	Jojoba oil	0.1	4.25 d	15.0
		1	4.07 e	18.6
		3	3.92 f	21.6
5	Eucalyptus oil	0.1	4.47 bc	10.6
		1	4.30 cd	14.0
		3	4.10 e	18.0
6	Cinnamon oil	0.1	4.17 de	16.6
		1	4.02 ef	19.6
		3	3.92 f	21.6
7	Mustard oil	0.1	4.6 b	8.0
		1	4.20 de	16.0
		3	3.92 f	21.6
8	Ginger oil	0.1	4.25 d	15.0
		1	3.72 g	25.6
		3	3.52 hk	29.6
9	Sesame oil	0.1	4.15 de	17.0
		1	4.07 e	18.6
		3	3.95 ef	21.0
10	Castor oil	0.1	4.05 ef	19.0
		1	4.00 ef	20.0
		3	3.65 gh	27.0
	Control		5.0 a	-
	LSD (0.05%)		0.14	-
CV (%)			2.41	-

(*) Mean values within the same column followed by the same letter are not significantly different at $p < 0.05$.

The least inhibition percent of colony growth of the pathogen was shown by garlic oil at 0.1% concentration (7%) followed by mustard oil at 0.1 concentration (8%).

Meanwhile, the clove oil at each three concentration had the weak antifungal effect on the radial growth (17%, 15% and 11%) inhibition followed by eucalyptus oil with 18%, 14% and 10% of inhibition.

Table 3. Effects of foliar spray with botanical oils on early blight incidence and severity of tomato plants under field condition.

No.	Treatment	Incidence (%)	Reduction of disease incidence compared to control (%)	Severity (%)	Reduction of disease severity over control (%)
1	Lemon oil	29.8 d *	38.0	18.46 c *	29.8
2	Clove oil	35.7 c	25.8	17.33 cd	34.9
3	Garlic oil	29.74 de	38.2	17.3 cd	34.2
4	Jojoba oil	39.6 b	17.7	21.4 b	18.7
5	Eucalyptus oil	33.6 cd	30.1	17.28 cd	34.3
6	Positive control (Mancozeb)	23.9 e	50.3	13.26 d	49.6
7	Negative control (Water)	48.13 a	--	26.33 a	---
LSD at 0.05%		2.149	--	0.548	--
CV%		2.79	--	1.64	--

(*) Mean values within the same columns followed by the same letter are not significantly different at $p < 0.05$.

Field evaluation of botanical oils for the management of tomato early blight

The efficiency of botanical oils as foliar application on early blight incidence, severity and plant height and fruit yield of tomato was screened in the field condition. All treatments significantly reduced incidence and severity of disease as well as improved the yield and growth of tomato plants (Table 3 and 4).

In vivo, at 1% concentration of botanical oil from various plants *viz.*, lemon, clove, garlic, jojoba and eucalyptus, the least disease incidence of 29.7% and 29.8% was noted on garlic and lemon treatment, respectively. Oils of jojoba, clove and eucalyptus were showed maximum disease incidence by 39.6%, 35.7% and 33.6%, respectively. Meanwhile, garlic oil exhibited the highest reduction of disease incidence (38.2%) followed by lemon oil (38%). Oils from Eucalyptus and clove recorded the moderate reduction of early blight incidence (301% and 25.8% respectively). However, the lowest reduction in disease incidence was shown (17.1%) with jojoba oil compared to negative control. In case of early blight severity garlic oil also found to be the best treatment to reduce disease severity up to (34.2 %).

Moreover, 49.6% reduction of the disease severity was recorded by positive control with mancozeb. Clove oil was next to garlic for controlling early blight disease and reduced about 34.1% disease severity over control. Among the applied oil treatment, the maximum disease severity of 21.4% showed on plants that treated by jojoba oil followed by lemon oil by 18.4%.

There was significant difference in total tomato fruit yield per plot in all botanical applications compared to the control (Table 3). Result revealed that, maximum effective treatments improved tomato fruit yield. Maximum fruit yield for clove oil was (58.3Kg/plot) followed by mustard oil (52.2Kg/plot), Garlic oil (50.8Kg), Jojoba oil (47.6Kg) and eucalyptus oil (44.3Kg). The lowest increase of tomato fruit yield of 43.4Kg was recorded for treatment of negative control amended with Tween 20 and water, while, tomato fruit yield of 54Kg was observed by positive control which was amended with mancozeb and water. Meanwhile, among all of the tested treatments, application of clove oil was the best treatment that significantly increased fruit yield up to

58.3Kg even compared to positive control (54Kg). Growth of tomato plants was also significantly induced with clove oil treatment, in which the plant height was ranged 82.6 cm compared to positive control (79 cm).

The oil treatments from lemon, jojoba and eucalyptus were not significantly different in their effectiveness for increasing of plant growth. Meanwhile, subsequent application of eucalyptus oil resulted yellowing of tomato plants.

Table 4. Effect of botanical oils on height and fruit yield of tomato plants under field condition.

No.	Treatment	Plant height (cm)	Fruit yield (Kg)/plot
1	Lemon oil	73.0 cd *	52.2 bc *
2	Clove oil	82.6 a	58.3 a
3	Garlic oil	73.4 cd	50.8 bc
4	Jojoba oil	72.8 cd	47.6 c
5	Eucalyptus oil	68.2 d	44.3 d
6	Positive control (Mancozeb)	79.0 b	54.0 b
7	Negative control (Water)	74.7 c	43.4 de
LSD at 0.05%		3.28	2.43
CV%		2.46	2.72

(*) Mean values within the same columns followed by the same letter are not significantly different at $p < 0.05$.

Discussion

In this study, we investigated the antifungal activities of essential oils of Jojoba (*Simmondsia chinensis*), Ginger (*Zingiber officinale Roscoe*), Garlic (*Allium sativum*), Clove (*Syzygium aromaticum*), Sesame (*Sesamum indicum*), Eucalyptus (*Eucalyptus glabulus*), Cinnamon (*Cinnamon zylanicum*) and Castor (*Ricinus communis*), lemon and mustard against *A. solani* the causal agent of tomato early blight, *in vitro*. All of these botanical oils had the inhibitory effects on mycelial radial growth of *A. solani*. As recorded, the antifungal effects of essential oils were dependent on the oil concentration and the type of essential oil. *In vitro* tests observed that the essential oil of lemon oil at concentration of 3% had the highest inhibition activity. The weakest inhibition effect was observed on 0.1% concentration of garlic oil but there was a significantly difference in all three doses of garlic oil for their radial growth inhibition. Ginger oil was the second most effective treatment among the tested oils for their effectiveness regarding of pathogen growth inhibition. Several studies have explored the potential of essential oils as antifungal agents (Abdolahi *et al.*, 2010; Tanović *et al.*, 2005; Lee *et al.*, 2007; Fawzi *et al.*, 2009).

The antimycotic activity of essential oils and ethanol extracts of garlic, galangal, cinnamon, eucalyptus, elecampane, basil and clove against *Aspergillus flavus, A. niger, A. ostianus, Alternaria alternata* and *Fusarium* sp. had broad-spectrum activity against all tested fungi (Youssef, *et al.*, 2013). The efficiency of clove (*Syzygium aromaticum* L.) essential oil for the control of *Fusarium oxysporum* f. sp *radicis lycopercisi*, *F. commune* and *F. redolens* revealed, moderate to high level antifungal activities (Hamini-Kadar *et al.*, 2014). The inhibitory effects of coriander extract, cinnamon extract and lemon essential oil against (*Cladosporium cladosporioides, Aspergillus parasiticus, Penicillium chrysogenum, Eurotium herbariorum* and *Aspergillus carbonarius*) showed a complete inhibition of growth of the tested fungi at ≥1.25 µL/mL.

The antifungal activity of essential oils as foliar application has been proved by earlier researchers. Foliar application of essential oils from caraway, carnation and thyme had a srong effect on the early blight of potato caused by *A. solani* compared to the fungicide Ridomil MZ 72.

The reduction in disease incidence and yield increase was showed with 1% of carnation, thyme and caraway oils (Nehal El-Mougy, 2009). Moreover, the essential oil of *Eucalyptus globules* Labill can be used as a fumigant during the storage and transportation of fruits and vegetables to prevent the post-harvest fungal decay and spoilage (Riddhi and Yogesh, 2015). Eucalyptus essential oil at different concentrations was evaluated to control *Alternaria solani*. At 1% and 2% concentration enhanced the growth of plants and exhibited inhibitory effect on development of disease. Moreover, at 3 and 4% of eucalyptus essential oil becomes toxic to the growing plants and plants showed yellowing and stunted growth (Ravi *et al.*, 2014).

Conclusion

Alternaria solani is a field pathogen that causes severe losses of tomato as well as potato plants. So the present results of the investigation revealed that use of botanical oils is safe, applicable and cost effective method for managing of foliar diseases. Due to the fact that chemical compounds are going to be ineffective and are harmful for consumers, control strategy for this disease in the future is applying biological method of disease management. The application of botanical oils in agriculture is suitable alternative method in plant disease control program and could act as main antifungal compounds without leaving any toxic residue on the products. Application of the botanical oils from different plant species is one of the important management strategies. Based on the current study, we can confirm this statement and encourage coming research of implementations of these and other potential botanical oils *in vitro* as well as *in vivo*.

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