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Antifungal Activity of Essential Oils from Some Medicinal Plants of Iran against *Alternaria alternate*

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Abstract: Problem statement: Increasing public concern over the level of pesticide residues in food especially fresh produce has built up adequate pressure for scientists to look for less hazardous and environmentally safer compounds for controlling post harvest diseases. Essential oils as registered food grade materials have the potential to be applied as alternative anti-fungal treatments for fresh fruits and vegetables. **Approach:** We present in this study, the identification of the essential oils with antifungal activity from some medicinal plants of Iran (nettle (*Urtica dioica* L.), thyme (*Thymus vulgaris* L.), eucalyptus (*Eucalyptus* spp.), Rue (*Ruta graveolens* L.) and common yarrow (*Achillea millefolium* L.)), and their potential application as "generally regarded as safe" antifungal compounds against *Alternaria alternate* on tomato as a model pathosystem. **Results:** Both the nettle and the thyme oils exhibited antifungal activity against *A. alternata.* The thyme oil exhibited a lower degree of inhibition 68.5 and 74.8% at 1500 and 2000 ppm, respectively. Spore germination and germ tube elongation of the pathogens in potato dextrose broth was strongly reduced in the presence of 1500 ppm of the nettle oil. The same concentration of this oil reduced the percentage of decayed tomatoes. The experiments on reducing the development of natural tomato rot gave similar results. **Conclusions:** Application of essential oils for postharvest disease control of fresh produce, as a novel emerging alternative to hazardous anti-fungal treatments will allow a safer and environmentally more acceptable management of postharvest diseases.

Key words: Nettle, thyme, eucalyptus, rue, common yarrow, *Alternaria* rot

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is an important commercial crop in the world. Nutritional values of tomato make it a widely accepted vegetable by consumers. Nevertheless, tomato is a very perishable vegetable with a short shelf-life and high susceptibility to fungal disease. During prolonged storage, tomato is susceptible to postharvest disease caused by various pathogenic fungi. *Alternaria alternata* is a saprophytic pathogen of tomato causing post harvest losses at high frequency [2]. The main method to control *Alternaria* rot and other postharvest diseases is based on application of synthetic chemical products. However, nowadays consumers demand less use of synthetic chemicals and still expect food to be free from blemishes, microbial growth, toxins and other quality deteriorating factors [9].

Application of essential oil is a very attractive method for controlling postharvest diseases. Production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests and indeed, essential oils have been shown to possess antimicrobial and antifungal properties [1]. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use [12]. Essential oils are made up of many different volatile compounds and the composition of the oil quite often varies between species[10]. It is difficult to

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associate the antifungal activity to single compounds or classes of compounds. It seems that the antifungal and antimicrobial effects are the result of many compounds acting synergistically [4]. Thus, there would be negligible chance of development of resistant races of fungi after application of essential oils to fruit and vegetables. As a consequence essential oils are one of the most promising candidate groups of natural compounds for the development of safer antifungal agents.

The objective of this study was to investigate the inhibitory effects of essential oils extracted from five plant species against *A. alternata* and to evaluate the potential application of essential oils to control *Alternaria* rot of stored tomatoes.

MATERIALS AND METHODS

Extraction of essential oil: Steam distillation was used for the extraction of the essential oil. The air-dried plants material weighing 200 g was placed in round bottom flask containing 150 mL water and distilled. The distillate was saturated with NaCl and transferred to a separator funnel where it was extracted with diethyl ether. The organic phase was recovered and concentrated on a steam bath to yield 2.8 g/100 g plants material. The essential oils were stored in bottles at 4°C.

Plant material: The plant materials were collected from Research Institute of Forests and Rangelands, Ahwaz, Iran, in 2007. Voucher specimen was deposited in the Botany Department of Science College Shahid Chamran University. Plant material were freed from foreign materials and carefully rubbed between soft cloths to remove dust.

Isolation of fungi: Fungi were isolated from deteriorating tomato fruits purchased from the open market using Potato Dextrose Agar (PDA) containing 100 mg chloramphenicol per ml and identified with the aid of the appropriate taxonomic keys[3]. The isolates were maintained on PDA slants at 4°C till needed.

***In vitro* antifungal assay:**

***In vitro* contact assay:** PDA was autoclaved and cooled in a water bath to 40°C. The essential oils were mixed with sterile molten PDA to obtain final concentrations 0, 100, 500, 1000, 1500 and 2000 ppm. The PDA was poured into 90 mm Petri plates (15 mL plate^{-1}) that were then inoculated with 6 mm plugs from 7-days-old cultures. Three replicates were used per treatment. Plates were incubated for 7 days at 28°C. Fungal growth was recorded after 7 days. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control. Experiments were performed three times.

Spore germination assay: The effect of nettle oil on spore germination and germ tube elongation of the pathogens were tested in Potato Dextrose Broth (PDB). Nettle oil was added to a 10 ml glass tube containing 5 mL PDB to obtain final concentrations 0, 100, 500, 1000, 1500 and 2000 ppm. At the same time, aliquots (100 μL) of spore suspensions (1×10^7 spores mL^{-1}) of *A. alternata* were added to each tube. After 20 h of incubation at 28°C on a rotary shaker (200 rpm), at least 100 spores per replicate were observed microscopically to determine germination rate and germ tube length[8]. Experiments were repeated three times.

***In vivo* applicability of nettle oil in control of post harvest fungal rot of tomatoes:**

Effects of nettle oil on decay development in artificially inoculated and wounded fruits: Fruits were selected for freedom of injuries and infections and were placed in 1.5 L plastic boxes. Fruits were dipped in the solution of 1% sodium hypochlorite for 2 min, rinsed with tap water and air-dried before wounding. Tomatoes were wounded with a sterile puncher to make one uniform 2 mm deep by 5-mm wide wound on their peel at the equatorial region. Aliquots of 20 μL of 500, 1000, 1500 and 2000 ppm nettle oil and sterile distilled water (control) was pipetted into each wound site. After 0.5 h, 10μL of conidial suspension of *A. alternata* (5×10^4 spores mL^{-1}) was pipetted into each wound. Treated tomatoes were stored at 20°C. The percentage of infected fruits was recorded after 5 days of incubation. Each treatment was replicated three times with 20 fruits per replicate.

Effects of nettle oil on naturally infected development in unwounded fruit: The concentration of the nettle oils were prepared by dissolving the requisite amounts in 25 mL of 0.05% Tween-80 and then mixing with 475 mL of sterile distilled water. The control sets were prepared similarly using equal amounts of sterilized water in place of the essential oil. Tomatoes were dipped into the solutions for 1 min at room temperature and air dried. Fruits were dipped into sterile distilled water, which served as control and air dried. Treated tomatoes were stored at 20°C for 21 days. The percentage of infected fruits was recorded when about 50% tomatoes of the control had decayed. Each treatment was replicated three times with 20 fruits per replicate.

Statistical analysis: Statistical analysis of the data obtained in the present study was carried out in a

completely randomized design layout with three replicates using Statgraphics plus 2.0. Where a significant difference between means was verified based on ANOVA, the comparison of means of different treatments was performed using Tukey's test at p = 0.05.

RESULTS

***In vitro* antifungal assay:**

***In vitro* contact assay:** The inhibitory effects of nettle, thyme, eucalyptus, Rue and common yarrow oils against *A. alternata* are shown in Table 1. Nettle oil at 1500 ppm showed potent and completely inhibitory effect on the radial growth of *A. alternate*. The oil of thyme exhibited a moderate to high antifungal activity against the pathogen tested, ranging from 68.5-74.8% at 1500 and 2000 ppm, respectively.

Low antifungal effects of Rue and eucalyptus oil were observed against on *A. alternata in vitro* contact assay with fungal mycelial growth inhibition percentage from 7.3-30.6 and 8.7-20.7%, respectively. Common yarrow oil did not affect the growth of *A. alternata*.

Spore germination assay: According to the results given in the Table 2, spore germination of pathogens in PDB was strongly inhibited in the presence of nettle oil. There was a significant inhibition (*p<0.05*) of fungal spore germination by different concentrations of essential oil.

Table 1: The inhibitory effects of essential oils on *A. alternate*

Essential oil	Percentage of inhibition				
	100	500	1000	1500	2000 (ppm)
Nettle oil	14.3±1.5a[a]	43.9±0.7b	65.9±2.3c	100±0d	100.0±0d
Thyme oil	10.6±1.8a	31.5±2.2b	43.6±1.6b	68.5±0.4c	74.80±0.84c
Eucalyptus oil	-[b]	-	7.30±0.3a	16.0±1.4a	30.60±2.6b
Rue oil	-	-	8.70±2.3a	15.9±0.8a	20.70±1.3a
Common yarrow oil	-	-	-	-	-

[a]: Values are the mean of three replicates ± *SE*; [b]: Indicates ineffective on microorganism

Table 2: Effect of different concentrations of nettle oil on spore germination and germ tube elongation of *A. alternata*

Treatments	Spore germination (%)	Germ tube length (µm)
Control	100±0a[a]	-
100 ppm	59.6±1.5b	98.1±2.3a
500 ppm	31.5±3.2c	83.9±0.8b
1000 ppm	12.6±0.6d	53.2±1.7c
1500 ppm	6.4±1.3e	30.7±2.3d
2000 ppm	6.1±0.9e	28.2±2.7e

[a]: Values are the mean of three replicates ±SE. Where the letters are the same, there is no significant difference between the means of different concentrations

Above 93% inhibition of fungal spore germination was observed at 1500 ppm concentration of nettle oil. The germ tube length was only 2.8 µm at 1500 ppm. Spores of *A. alternata* all germinated after 20 h incubation at 28°C in PDB without nettle oil. The germ tubes were long and entwined so that germ tube length could not be determined.

***In vivo* applicability of nettle oil in control of post harvest fungal rot of tomatoes:**

Effects of nettle oil on rot development in artificially inoculated and wounded fruits: The results shown in Fig. 1 indicate that when wounded tomatoes were treated with nettle oil, all concentrations (except 100 ppm) significantly inhibited *A. alternata* on tomatoes stored at 20°C for 5 days ($p<0.05$). The percentage of decayed tomatoes treated by 1500 ppm nettle oil was reduced by 46.42% compared to the control. Treatment with nettle oil did not cause any visible disorders and off-odor to the fruits after 5 days of storage.

Effects of nettle oil on naturally infected development in unwounded fruit: The results show that when unwounded tomatoes were treated with nettle oil, all concentrations significantly inhibited *A. alternata* on tomatoes stored at 25°C for 5 days ($p<0.05$) (Fig. 2). The percentage of decayed tomatoes treated by 1500 ppm nettle oil was reduced by 41.6% compared to the control.

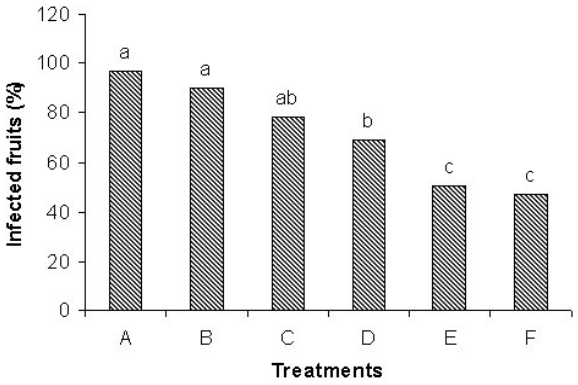

Fig. 1: Inhibition of *A. alternata* on artificially inoculated and wounded tomatoes by nettle oil: (A) Control, (B) 100 ppm, (C) 500 ppm, (D) 1000 ppm, (E) 1500 ppm and (F) 2000 ppm significant differences ($p<0.05$) between means are indicated by letters above histogram bars. Where the letters are the same, there is no significant difference between different treatments

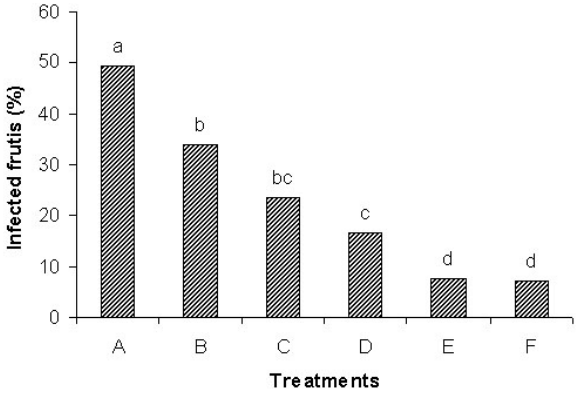

Fig. 2: Effects of nettle oil on naturally infected development in unwounded tomatoes: (A) Control, (B) 100 ppm, (C) 500 ppm, (D) 1000 ppm, (E) 1500 ppm and (F) 2000 ppm significant differences ($p<0.05$) between means are indicated by letters above histogram bars. Bars with the same letters show are not significantly difference.

DISCUSSION

In an attempt to reduce the use of synthetic pesticides, over the past two decades extensive investigations have been made into the possible exploitation of plant compounds as natural commercial products, that are safe for humans and the environment[7] Although essential oils from various wild plants have been reported in the past to be effective against a wide range of micro-organisms *in vitro*[6], these essential oils failed to inhibit pathogen growth *in vivo*[5] and failed to control diseases under field conditions in most cases. Although *in vitro* screening of plant extracts is an important first step in identifying plants with potential application in agriculture, *in vivo* confirmation of this potential is essential in the search for plant derived preparations with the potential to be commercialized [13].

The ability of five essential oils to inhibit *A. alternata* was evaluated *in vitro* contact assays. The most active oil was nettle oil followed by thyme oil. The antifungal efficacy appears to relate to the chemical composition of essential oils. The spore germination assay demonstrated the inhibitory effect of nettle oil on the spores of *A. alternata*.

The *in vivo* efficacy of nettle oil was studied in this study. The results showed nettle oil significantly reduced the rot not only in artificially inoculated and wounded fruits but also in unwounded fruit. Although the percentage of infected fruits in tomatoes treated by nettle oil was significantly lower than those in control, the inhibition by nettle oil in tomatoes was not as dramatic as that in plates. In general, 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 [11]. Because essential oils are made up of many different volatile compounds, the post storage residual levels are anticipated to be low. Whether the effective concentrations of nettle oil would affect the flavor of tomatoes, calls for more work on studying the quality parameters of tomatoes after storage.

CONCLUSION

In conclusion, examination of various concentrations of thyme and nettle oils on *A. alternata* in this study showed promising prospects for the utilization of natural plants or their oils and extracts in post harvest disease control. *In vivo* experiments showed that nettle oil could reduce postharvest diseases on tomatoes caused by *A. alternata*. So essential oils can be used as a potential source of sustainable eco-friendly botanical fungicides, after successful completion of wide range trials.

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