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Effect of *Pistacia atlantica* Subsp. Kurdica essential oil and acetic acid on *Botrytis cinerea* growth in culture media, grape and cucumber fruits

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Abstract

The effect of *Pistacia atlantica Subsp. Kurdica* essential oil (EO) and acetic acid against *Botrytis cinerea* growth was investigated in synthetic media, cucumber and grape fruits. The antifungal assay was determined on PDA plates amended with thirty-three concentrations of this oil (250, 1000, 1500 to 26000 µl/l). This study was conducted for acetic acid of 0.1% alone at concentrations of 5, 15 and 25% .Also 0.1% of each dilutions of acetic acid were added to a series of sterile molten PDA that contain different concentrations of essential oil ranging from 50 to 3000 µl/l. EO inhibited radial growth on potato dextrose agar (PDA) in a dose-dependent manner. The growth was completely prevented by EO at 26000 ppm on PDA. In combination with 0.1% acetic acid 25% in fungal growth medium, minimum inhibitory concentration (MIC) of EO reduced to 3000 ppm. During 31 days of cold storage of cucumber and 60 days of grape at 4°C, the decay of these fruits caused by *B. cinerea* was reduced to 10% by using MIC of EO. Scanning electron microscopy (SEM) was done to study the mode of action of the oil in *Botrytis cinerea* and it was observed that treatment with the oil leads to distortion and thinning of the hyphal wall and the reduction in hyphal diameter. The results suggested the potential substitution of the antifungal chemicals by this EO and acetic acid as a natural inhibitor to control the growth of this mold in fruits such as cucumber and grape.

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Introduction

Cucumber (*Cucumis sativus* L.) and table grape (*Vitis vinifera*) are important agricultural commodities in Iran. They are susceptible to attack by various microorganisms such as *B. cinerea* fungus during storage. The protection of agricultural products from plant pathogens is necessary and has been achieved by various physical and chemical methods. Antimicrobial chemicals are often used in control of plant disease in agriculture (Moorman and Lease, 1992). However, there are some problems in utilizing the chemicals, for example, the high risk of toxic residues in the products and adapted fungi resistance to the chemicals (Sholberg and Conway 2004). Moreover, public concern over the indiscriminate use of synthetic fungicides has been growing. Thus, it is significant to develop new alternatives for disease control (Tian *et al.,* 2011).

Application of essential oil is a very attractive method for controlling postharvest diseases. 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 (Ormancey *et al.,* 2001). There is an idea that these essential oils play a significant role in plant defense mechanisms versus phytopathogenic microorganisms (Mihaliak *et al.,* 1991). Most of the essential oils have been reported to inhibit postharvest fungi under *in vitro* conditions (Bellerbeck *et al.,* 2001).

The genus *Pistacia* belongs to the family *Anacardiaceae*. Among 15 known species of pistachios, only 3 species grow in Iran, including *P. vera, P. Khinjuk* and *P. atlantica*. (Behboodi 2003). *Pistacia atlantica sub Kurdica* is widely spread around the Zagros Mountains and particularly in Western and Northern Iran and Eastern and Northern Iraq, Southern Turkey and Northern Syria so called Kurdistan. It shows a discontinuous pattern of distribution over the region and is an important constituent of the natural vegetation in this area. It is the major source of a gum that has not been known well to the world. The gum of this plant is obtained as an exudate after hurting the trunk and branches. It has been reported that the essential oil of this gum possess considerable *in vitro* antimicrobial activity (Tassou and Nychas 1995).

Acetic acid was commonly used as antimicrobial preservative or acidulent in a variety of food products by food manufactures (Davidson and Juneja 1990). Further studies have confirmed the antifungal activity of acetic acid (Corsetti *et al.,* 1998; Gourama 1997; Niku-Paavola *et al.,* 1999). However, interest in this subject remains high and significant research progress in this field has occurred in the meantime, although practical applications are still relatively few. The effect of *Pistacia atlantica sub Kurdica* essential oil as antimicrobial compounds against some microorganisms are investigated but the antifungal effect has not been studied so far. The objective of this study was to determine the efficacy of this essential oil alone and in combination with acetic acid against the gray mold incidence of cucumber and table grape fruits.

Materials and methods

Essential oil

The essential oil was obtained from Saqez Company of Kurdistan, Iran. It was filtered through 0.45 micron micro filter and then stored in the dark at 4°C with an air tight container.

Gas chromatography–mass spectrometry (GC–MS) analysis

In order to analyze and identify the combinations forming the essential oil,was analyzed by using a Hewlett-Packard 6890 series GC systems coupled to a mass spectrometer (model HP MSD 5973) equipped with a TRB-5MS capillary column (30m x 0.25mm, 0.25 µm film thickness). Helium was the carrier gas, at a flow rate of 1 mL/min. The \volume of injections was 1 microliter, injected in the splitless mode. Inlet temperature was adjusted to 260°C.

Preparation of spore suspension

B. cinerea was obtained from mycological collection of Department of Plant Protection, University of

Kurdistan, Iran. It was cultured on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slope for 10 days at 25 ± 1°C. Conidia were harvested by adding 10 ml of 0.05% Tween 80 solution (Merck, Darmstadt, Germany) to culture and gently scraping the mycelia with a sterile inoculating loop to free spores. Conidial concentration was determined by a haemocytometer and the suspension was diluted with 0.05% Tween 80 solution to give a final concentration of 10^6 ml^{-1} (Gandomi *et al.*, 2009).

In vitro antifungal assay

In our study the antifungal assay was determined on PDA plates amended with sixteen concentrations of this oil (500, 1000, 1500 to 26000 µl/l). The oil was added to sterile molten PDA to obtain the desired concentrations. Aliquots of 20 ml of the solution were immediately dispensed into petri plates which were seeded with 6 mm diameter mycelial plugs from the edge of 7 days old *B.cinerea*. Plates in three replicates were used for each treatment, and the inoculated plates were incubated in the dark at 25 ± 1°C. The same work was done for diluted glacial acetic acid at concentrations of 5, 15 and 25%. Each dilution of acetic acid was added in amount of 0.1% to fungal growth medium. Growth measurements were determined when the growth in the control plate reached the edge of the plate. The lowest concentration which inhibited the growth of the fungus was considered as minimum inhibitory concentration (MIC). The antifungal effect expressed as percent inhibition of radial growth by the following formula:

Inhibition of growth (%) $= \frac{Dc-Ds}{Dc} \times 100$

Where Dc is the diameter of colony in control sample, Ds is the diameter of colony in treated sample (Gandomi *et al.,* 2009).

In vitro synergistic assay

After dilution of glacial acetic acid with distilled water to concentrations of 5,15 and 25%, 0.1% of each dilutions was added to a series of sterile molten PDA that contain different concentrations of essential oil ranging from 50 to 3000 µl/l. A mycelial disc of approximately 6 mm in diameter cut from the periphery of a 7 days old culture, inoculated in the center of each petri dish, and then incubated at 25 ± 1°C.

Effect of essential oils on hyphal morphology

For scanning electron microscopy (SEM) analysis, mycelial discs (1 cm in diameter) from ten-day-old fungal cultures of *Botritys cinerea* exposed to the essential oil were used. After preparation of samples digital images captured using a VEGA TESCAN SEM at an accelerating voltage of 5 kV**.**

Fruits and inoculation

Cucumber and table grape were obtained from commercial market. The fruits of uniform size, free of physical damage and fungal infection were selected. They were surface sterilized with 2.5% sodium hypochlorite for 3 min, followed by washing with distilled water × 3. Fruits were arranged by groups of 10 in plastic containers. One group as control, another were dipped in fungi conidial suspension (10^6 ml^{-1}) for 1 min and one dipped in fungi conidial suspension flowed by spraying with minimum inhibitory concentration of essential oil. The last group was sprayed with MIC of EO. Fruits were stored at 4 and 25°C for 31days in Cucumber and 60 days in table grape.

Statistical analyses

SPSS statistic program (Ver.16, SPSS Inc., Chicago, IL, USA) was performed for all calculations. Analysis of variance was performed at the significance level of $P<0.05$. When appropriate, means were separated by using -way ANOVA and two-way ANOVA test ($P<0.05$).

Results and discussion

Chemical composition of essential oil

The identified chemical composition, retention time, and percentage composition by GC–MS analyses are given in Table 1. The oil mainly contained α-Pinene (91.47 %) and other components were present in amounts less than 4%.

Table 1. Chemical composition of essential oil of Pistacia atlantica subsp. Kurdica.

Number	Retention time (min)	Compound	Composition (%)
1	8.31	Tricyclene	0.14
2	9.06	Alpha Pinene	91.47
3	9.41	Camphene	0.94
4	9.63	Verbena	0.11
5	10.41	Sabinene	0.42
6	10.50	Beta Pinene	2.47
7	11.16	Beta Myrcene	0.48
8	11.80	Delta 3-Caren	0.36
9	12.33	Benzene, 1-methyl-4-(1-methylethyl)	0.24
10	12.49	1-limonene	0.6
11	12.56	1,8-Cineole	0.21
12	14.63	Alpha Terpinolene	0.42
13	14.91	Alpha – Pinene Oxide	0.18
14	15.19	Unknown	0.24
15	15.85	Alpha – Campholene Aldehyde	0.1
16	16.26	Trans-Pinocarveol	0.13
17	16.48	Unknown	0.47
18	17.02	Pinocarvone	0.08
19	17.76	Benzenemethanol,4-(1-methylethyl)	0.23
20	17.94	Alpha Terpineol	0.06
21	18.08	Bicycle[3.1.1]hept-2-ene-caboxal	0.01
22	18.47	Bicycle[3.1.1]hept-3-en-2-one	0.29
23	20.79	Bicycle[2.2.1]heptan-2-ol	0.12
24	21.13	Unknown	0.05
25	24.20	Unknown	0.12

Table 2. Effect of different concentrations of *Pistacia atlantica Subsp. Kurdica* essential oil on radial growth of *Botrytis cinerea*.

EO Concentration (µl/l)	Growth	
	Colony diameter (mm)*	Inhibition %
0	50±0	0
250	48±0.06	3.4
1250	42±0.15	15.4
1500	41±0.11	18.8
1750	39±0.10	22
2000	37±0.15	25.4
2250	36±0.10	28
2500	34±0.15	32.8
3500	31±0.10	38
5000	29±0.21	42.8
10000	25±0.25	49.4
11000	24±0.15	50.8
15000	21±0.11	58.8
19000	14±0.36	72
21000	5±0.36	89.4
24000	1±0.17	98
26000	0±0	100

Data are means ± SD of three replicates

In vitro antifungal activity assay

The efficacy of different concentrations of *Pistacia atlantica Subsp. Kurdica* EO on radial growth was studied in an agar medium. The results are presented in Table 2. All concentrations of EO exhibited significant reduction of fungal growth ($P < 0.05$) and the pattern of reduction was dose-dependent. At concentrations of 10000 and 11000 ppm, the radial growth reduced by 50%. %. In particular *Botrytis cinerea* did not show any mycelium growth at concentration of 26000 µl/l. Comparisons of average data by using one-way ANOVA (Table 3)showed significant differences among different groups.

The results shown in Table 4 indicate the effect of different concentrations of essential oil of Pistacia atlantica Subsp. Kurdica in combination with acetic acid on radial growth of Botrytis cinerea. The percent of growth inhibition by application of acetic acid alone at concentrations of 25, 15 and 5% were 32.4, 29.4 and 3.4%, respectively. In fungal growth medium with 0.1% of acetic acid at all its concentrations (25,

15 and 5%), by increasing the amount of essential oil (ranging from 50 to 3000 µl/l.), fungal colony diameter reduced significantly ($P<0.05$). The essential oil at concentration of 3000 ppm with 0.1% of acetic acid 25% was most effective against the growth of this phytopathogen and growth inhibition was 100% like when essential oil was used alone at concentration of 26000 ppm. Possible synergistic effect of acetic acid and essential oil plays an important role in fungi inhibition. In this test, the colony diameters (mm) of B. cinerea were measured on the plates treated following incubation. Two-way analysis of variance (ANOVA)was performed to test for significant differences between the colony diameters of B. cinerea from treated and control plates (Table 5).

Table 3. Analysis of variance concentrations of *Pistacia atlantica Subsp. Kurdica* essential oil on radial growth of *Botrytis cinerea*.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	123.318	30	4.111	4.302	.000
Within Groups	4.693	62	.076		
Total	128.012	92			

Table 4. Effect of different concentrations of *Pistacia atlantica Subsp. Kurdica* essential oil and acetic acid on radial growth of *Botrytis cinerea*.

EO Concentration (µl/l)	Growth at 25% con.		Growth at 15% con.		Growth at 5% con.	
	Colony Diameter (mm)*	**Inhibition (%)**	**Colony diameter (mm)***	**Inhibition (%)**	**Colony diameter (mm)***	Inhibition (%)
0	34±0.21	32.4	35±0.35	29.4	48±0.11	3.4
50	30±0.15	39.4	31.3±0.32	37.4	46±0.25	7.4
100	29±0.1	42	31±0.1	38	45±0.21	10.8
250	26±0.15	48.8	28±0.11	43.4	42±0.11	15.4
500	24±0.06	51.4	27.7±0.1	44.8	41.7±-.1	16.6
750	19±0.32	62.8	26±0.15	47.4	38±0.15	23.4
1000	17±0.3	66	25±0.25	50.8	35±0	30
1250	14±0.15	75.4	22±0.2	56	34.7±0.06	30.8
1500	12±0.15	75.4	21±0.3	58	34±0.1	32
1750	11±0.15	77.4	20±0.32	59.4	30±0.15	39
2000	10±0.11	78.8	19.9±0.3	60.2	29±0.21	42.8
2250	10±0.1	80	18±0.3	64.8	28±0.26	44
2500	7±0.15	86.8	15±0.13	69	27±0.11	46.4
2750	3±0.25	94.7	12±0.26	76	25±0.17	50
3000	0±0	100	10±0.25	80.4	23±0.15	54.4

Data are means ± SD of three replicates

Table 5. Analysis of variance of EO ,acetic acid AA and Their interaction.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
EO	42.612	4	10.653	357.754	.000
AA	18.117	2	9.059	304.209	.000
EO * AA	4.885	8	.611	20.506	.000
Error	.893	30	.030		
Corrected Total	66.508	44			

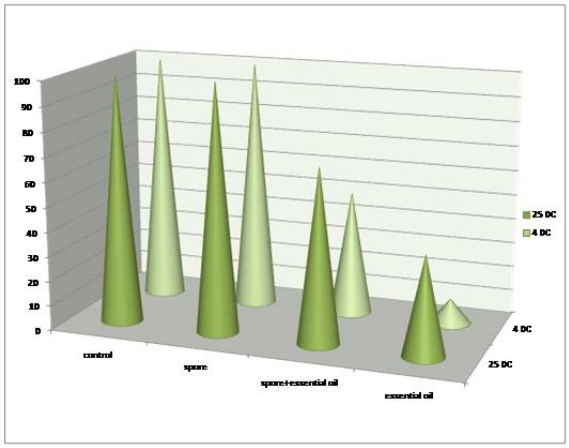

Fig. 1. The effect of essential oil of *Pistacia atlantica Subsp. Kurdica* on contamination level of cucumber fruits stored at 25 and 4°C after 31 days, respective.

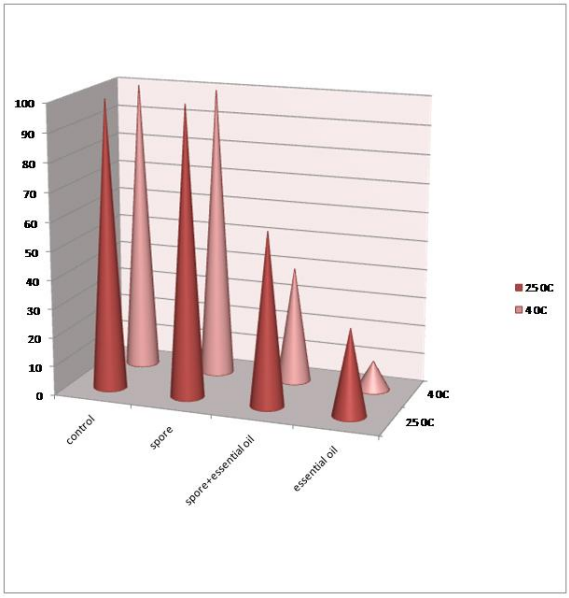

Fig. 2. The effect of essential oil of *Pistacia atlantica Subsp. Kurdica* on contamination level of grape fruits stored at 25 and 4°C after 60 days, respectively.

Effect on inoculated cucumber and grape fruits

The effect of essential oil of *Pistacia atlantica Subsp. Kurdica* on the control of fungal growth in cucumber and grape fruits stored at 4 and 25°C was shown in figures 1 and 2. After storage of cucumber for 31 days at 25°C, contamination levels at control, samples inoculated with fungi conidial suspension, samples with fungi conidial suspension and essential oil and samples that only sprayed with oil were 100%, 100%, 70% and 40%, respectively. With the same order, the percent of contamination levels for samples that stored at 4°C were 100%, 100%, 50% and 10%. At 25°C contamination levels of grape fruits were 100%, 100%, 60% and 30% after and at 4°C these amount were 100%, 100%, 40% and 10%, respectively after 60 days of storage. As it is expected, cold storage prolongs the shelf life of these fruits and postpones their decay. Reduction of contamination level at 4°C from 100% in control to 10% in samples with essential oil represents the fungistatic effect of this essential oil.

Effect of EO on hyphal morphology

Important morphological damage was detected in the hyphae exposed to EO compared to the hyphae in the controls. Hyphae of *B. cinerea* grown in the absence of EO showed typical features of the genus. The SEM micrographs showed important morphological damage due to EO (Figure 3).

Discussion

Recently, the exploitation of natural products to control decay and prolong storage life of perishable commodity has received more attention. Biologically, active natural products have the potential to replace synthetic fungicides (Tripathi and Dubey, 2004). The essential oil is one of the plant extracts applicable for the management of fungal rotting of fruit and vegetables, thereby prolonging shelf life (Meepagala *et al.,* 2002). Sensitivity of fungal species to plant essences is different and depends on essence type and

dose of application. Essential oil of *Pistacia atlantica Subsp. Kurdica* can possess antifungal activity against grey mold disease agent *B. cinerea* and can be exploited as a treatment for future plant disease management programs eliminating fungal spread. Large percentages of antifungal activities of this oil relate to α-pinene as the main compound (Xia *et al.,* 1999). Also, acetic acid was lethal at 0.1and 0.15% to *B. cinerea* and *Penicillium expansum*, respectively. At concentrations of 0.18–0.27% (vol/vol), acetic acid controlled *Botrytis* and *Penicillium* decay on two Canadian table grape varieties, to the same extent as SO_2, with no adverse effects on fruit composition (Roller 2003). Attempts to enhance the efficacy of natural compounds have led to the development of combined approaches based on additive and synergistic effect. To date, no reference data have been found about the effect of the combined application of *Pistacia atlantica Subsp. Kurdica* essential oil and acetic acid against the *B. cinerea*. Combination of essential oil and acetic acid provided a reduction of MIC of EO from 26000 to 3000 ppm.

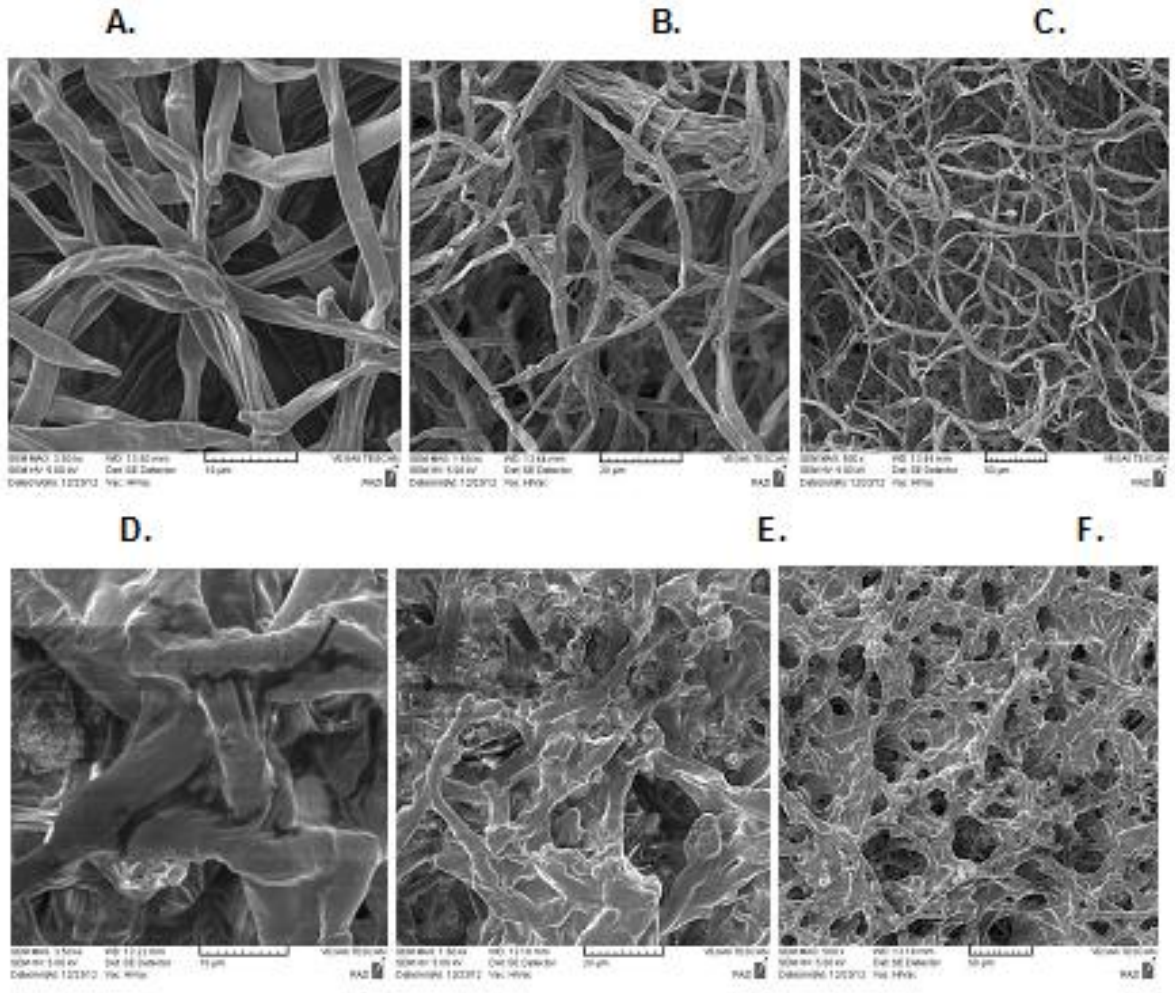

Fig. 3. The Pictures of Scanning electron microscopy (SEM) of B.cinerea (A,B,C) and the effect of *Pistacia atlantica* essential oil on *B.cinerea* (D,E,F) With dimensions [(A,D10 µm),(B,E:20 µm),(C,F:50µm)].

Conclusion

The effect of essential oil of *Pistacia atlantica Subsp. Kurdica* on the control of fungal growth in cucumber and grape fruits showed good potential to inhibit growth of *Botritys cinerea*.In conclusion, the results of the present work showed that the essential oil of *Pistacia atlantica Subsp. Kurdica* had an antifungal activity and can be effective in reducing corruption in fruits. Essential oils can be used as a source of sustainable eco-friendly fungicides. Also synergistic

effect of this oil and acetic acid can reduce the amount of oil consumption with the same effect. However, further studies are needed to evaluate the organoleptic effects of this EO application.

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