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In vitro and *in vivo* Antifungal Activities of Three Essential Oils Against Grey Mould Disease in Cucumber (*Cucumis sativus*)

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Abstract: The aim of this study was to find the effect of fungicide of *Foenicolom vulgare, Carum carvi* and *Carum copticum* essential oils in the control of fungal pathogen *Botrytis cinerea*, the casual agent of grey mould disease of cucumber (*Cucumis sativus* L.) *in vitro* and *in vivo* conditions. At first experiment, antifungal effects on essential oils were carried out with Solution Method (SM). In second experiment, fruits were infected artificially by *B. cinerea* spore and then treated by different concentrations of these essential oils. *In vitro* and *in vivo* experiment were conducted in a completely randomized factorial design with two factors; including five concentration treatments (0, 200, 400, 600 and 800 μL L^{-1}) as first factor and three using essential oils as second factor. The results of *in vitro* showed that *C. carvi* essential oil had the most of fungicidal effect in comparisons with other essential oils. The growth of *B. cinerea* was completely inhibited by *C. carvi*, *C. copticum* and *F. vulgare* essential oils at relatively low concentrations (400 and 600 μL L^{-1}). The results of *in vivo* experiment showed that *C. copticum*, *F. vulgare* and *C. carvi* essential oils at all applied concentrations inhibited *B. cinerea* growth on cucumber fruits completely in comparison to controls. Also these essential oils at all concentrations showed positive effects on some fruit quality characteristics e.g., titrable acidity, Total Soluble Solid, Ascorbic acid, pH and weight loss and these essential oils inhibited of infection fruits to grey mould and increased life storage fruits.

Key words: *Cucumis sativus*, essential oils, *Botrytis cinerea*, *C. carvi*

INTRODUCTION

Cucumber fruit (*Cucumis sativus* L.) is an important agricultural commodity in Iran. The conventional Cucumber fruit are classified as nonclimacteric (Biale and Young, 1981). It is 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 such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors 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). In addition, using synthetic antimicrobial chemicals could cause harm to agricultural workers when used continuously for a long period. Thus, research in the use of natural antimicrobials for disease control in agriculture is increasing. A number of naturally occurring compounds, including essential oils extracted from herbal plants, have been considered as natural antifungicides for controlling pathogens. There are many reports of the antifungal activity of essential oils from herbal plants. The activity varies widely, depending on the type of essential oil, test method and microorganism (Giese, 1994). In recent years, numerous studies have documented the antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* (Amiri *et al*., 2008; Anthony *et al*., 2004; Dikbas *et al*., 2008; Dubey *et al*., 2008; Feng and Zheng, 2007; Omidbeygi *et al*., 2007; Shahi *et al*., 2003; Tiana *et al*., 2011; Tzortzakis, 2007, 2009). In this study, the essential oils extracted from seeds of *Carum copticum*, *Foeniculum vulgare* and *Carum carvi* were tested for activity against *Botrytis cinerea in vitro* and *in vivo* conditions on cucumber fruits towards the development of a biological control system for the control of Botrytis rot of agricultural products.

MATERIALS AND METHODS

Plant materials and extraction of essential oils: The air-dried seeds of *Carum copticum*, *Foeniculum vulgare* and *Carum carvi* were supplied from agricultural research

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fields of Ferdowsi University of Mashhad (FUM). After the plant materials were authenticated the air-dried seeds (100 g) of medicinal plants were subjected to hydro distillation for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4°C until further analysis.

The first experiment: (*in vitro* experiment)

Antifungal effects of the essential oils on mycelial radial growth *in vitro* conditions: First, Antifungal activity was studied by using a contact assay (*in vitro*), which produces hyphal growth inhibition. Briefly, Potato Dextrose Agar (PDA) plates were prepared using 8 cm diameter glass Petri dishes. *In vitro* experiment was conducted in a Completely Randomized Factorial Design with two factors; including five concentration treatments (0, 200, 400, 600 and 800 μL L^{-1}) as first factor and three using essential oils as second factor, with four replications. The method was used for essential oils treatment on PDA medium Solution Method. In this method the essential oils were dissolved in tween 80-water solution (5% v/v) (Ozden and Bayindirli, 2002) and required amounts of the solutions/Petri dish were added to each of the PDA plates containing 20 mL of agar at 45°C. A 0.5 mm disk of mycelium was located on PDA medium. The treated medium was incubated in 24°C and mycelium growth was determined daily. Inhibitory percent was determined according to the following formula:

$$IP = \frac{dc - dt}{dc} \times 100$$

IP is Inhibitory percent, dc is mycelium growth diameter in control and dt is mycelium growth diameter in essential oil treated Petri dish.

Measurements

Spore germination assay: The effect of *Foeniculum vulgare*, *Carum copticum* and *Carum carvi* oils on spore germination were tested in Potato Dextrose Agar (PDA). The used oils were added to a 10 mL glass tube containing 5 mL PDA to obtain final concentrations 0, 200, 400, 600 and 800 μl L^{-1}. Spore suspension (10^5 spores mL^{-1}) of *B. cinerea* was prepared from actively growing culture (7-8 days old) in distilled sterile water. At the same time, aliquots (100 L) of spore suspensions (1×10^5 spores mL^{-1}) of *Botrytis cinerea* 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.

The second experiment (*In vivo* experiment)

Effect of these essential oils on postharvest decay and some qualities factors of *Botrytis cinerea* inoculation on cucumber fruits: *B. cinerea* Link was isolated from infected cucumber fruit. The culture was maintained on Potato-Dextrose Agar (PDA) at 4°C and fresh cultures were grown on PDA plates before use. Spore suspensions were prepared by removing the spores from the sporulation edges of a 7-8-days old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a hemocytometer and adjusted as required with sterile distilled water. In first, fruits were treated by Sodium hypochlorite (100 μl L^{-1}). Then fruits were dipped in prepared suspension and located in room temperature for 2 h in order to fixed fungal inoculation. According to the *in vitro* experiment, SM method was used. Fruits were treated by required essential oils and located in the packages separately. Experiment was conducted in a Completely Randomized Design, including five treatments (0, 200, 400, 600 and 800 μl L^{-1}) concentrations and three essential oils (*Foeniculum vulgare*, *Carum copticum* and *Carum carvi*), with three replications. Three numbers of fruits was used for each replicate. Essential oil-treated and untreated fruits together with controls were transferred into packages and were steeked in order preventing loss of essential oils and then put into the cold storage (7°C) (Asghari Marjanlo *et al*., 2009).

Measurements

Weight loss: In order to determine any weight loss during the storage of the fruits, both treated and untreated fruits were weighted previous and end day experiment.

Titrable Acidity (TA), Total Soluble Solids (TSS) and pH: Titrable acidity was measured using titration method. To do that, 10 mL fruit juice was added a few drops of phenolphethalein and titrated with 0.3 N NaOH up to pH 8.1. The results were expressed as g of citric acid per 100 g fresh weight. Total soluble solids content was measured using a digital Refractometer (model RFM340, Bellingham and Stanley, Kent and UK). The pH of fruit juice was measured using a Jenway 3320 pH meter calibrated by pH 4 and 7 buffer solutions.

Ascorbic Acid (AA): Ascorbic acid contents was measured by classical titration method using 2, 6-dichlorophenol indophenol solution and expressed as mg/100 mL (Bhaskara *et al*., 1998).

Decay rate: Decay rate was calculated by number of fruits with visible fungal infection for each package as percentage of decay.

Statistical analysis: The data was analyzed using SASS 9.1 statistical software. The results were calculated by one-way Analysis of Variance (ANOVA). The means were compared by Duncan tests.

RESULTS

Effect essential oils on radial growth *B. cinerea*: The radial growth of *B. cinerea* fungus inhibited by *C. carvi*, *C. copticum* and *F. vulgare* oils had strong activities, respectively (Fig. 1). All essential oils were found to inhibit the growth of *B. cinerea* in a dose-dependent manner. Result indicated that maximum radial growth except control, showed in 200 μl L^{-1} concentration of fennel essential oil and minimum radial growth were in 400, 600 and 800 μl L^{-1} concentrations of *C. carvi* and 600 and 800 μl L^{-1} concentrations of all used essential oils. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control that *C. carvi* oil had maximum percentage of inhibition with 74.85% inhibition. Results showed that 800 μl L^{-1} concentration these oils 100% inhibited of radial growth this fungus and 200 μl L^{-1} concentration these oils had lower inhibition percentage on radial growth of *B. cinerea* (Fig. 2).

Effects of essential oils on spore germination: Control of Germination spores of *B. cinerea* was totally inhibited by these essential oils at upper concentrations but between different essential oils no had significant effect on inhibition of germination spores of *B. cinerea*. In concentration of 800 μl L^{-1} all of essential oils were without Germination spores of *B. cinerea* (Fig. 3).

Effect of essential oils on postharvest quality factors of cucumber

Total Soluble Solids (TSS): The effect of these essential oils on Total soluble solids content fruit showed in Fig. 4. The result showed that no significant difference TSS content was observed in treated fruits among these essential oils, so *C. carvi* essence with 3.23° Brix more effective than other essences. In concentrations of 800 and 600 μl L^{-1} had lowest TSS change content than pre experiment to compare the controls. In The concentration 800 μl L^{-1} of *C. carvi* essence treatment had highest of TSS content (4.17° Brix) (Fig. 4).

Titrable acidity (TA)and TSS/TA: There was significant difference in titrable acidity content of treated fruits compared to the controls. The *C. carvi* essence was best essence with 0.453 (g/100 g fresh weight) TA content but no significant TA were observed with *C. copticum*

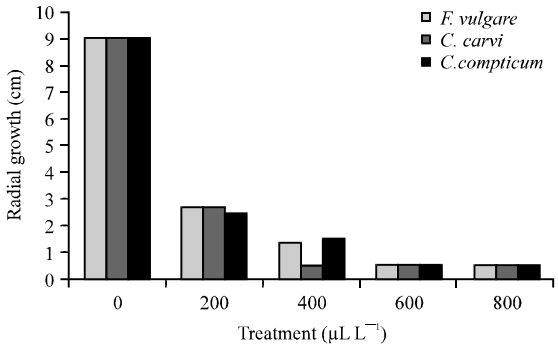

Fig. 1: Effect of different concentrations of essential oils on radial growth (cm) of *B. cinerea*

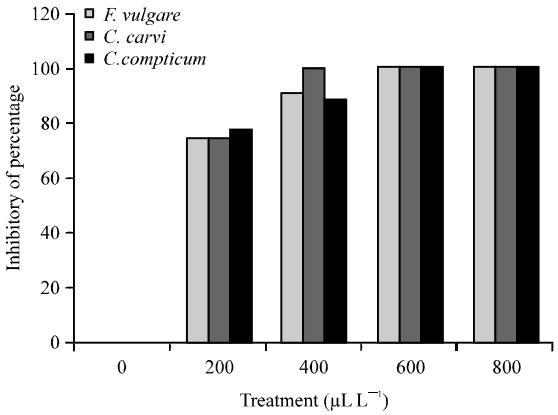

Fig. 2: Effect of different concentrations of essential oils on mycelia inhibition (%) of *B. cinerea*

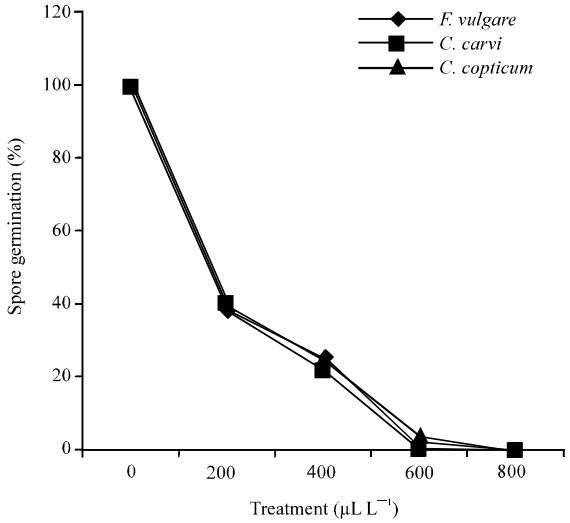

Fig. 3: Effect of different concentrations of essential oils on spore germination (%) of *B. cinerea*

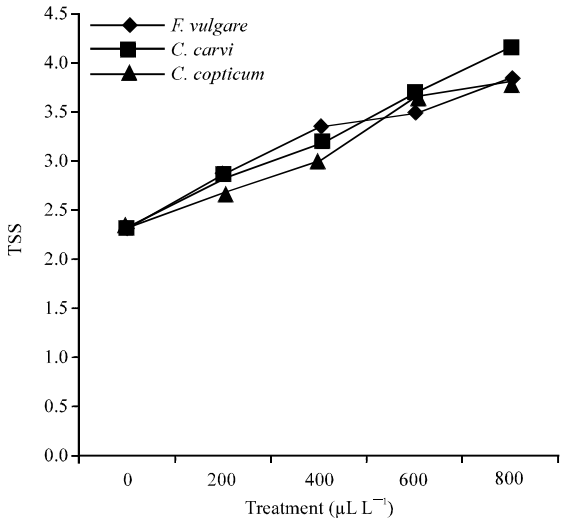

Fig. 4: Effect of different concentrations of essential oils on TSS content (°Brix)

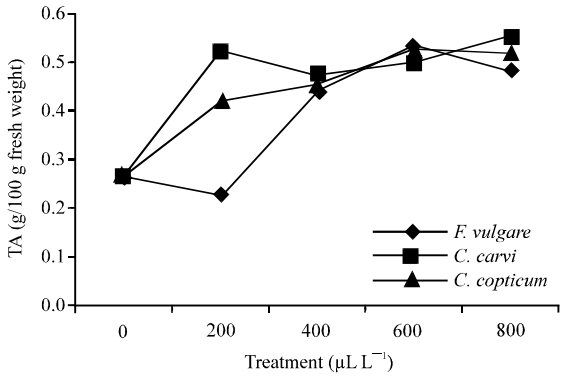

Fig. 5: Effect of different concentrations of essential oils on Titrable acidity (g/100 g fresh weight)

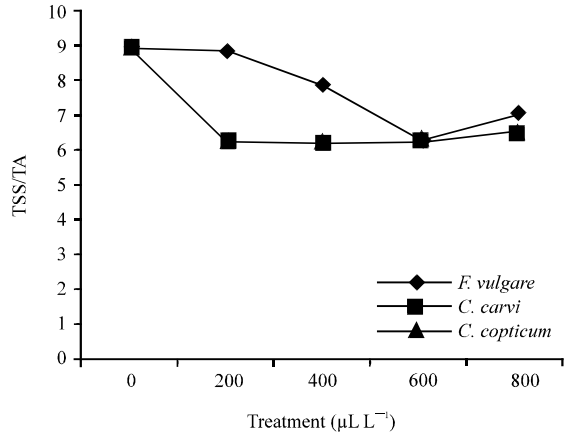

Fig. 6: Effect of different concentrations of essential oils on TSS/TA

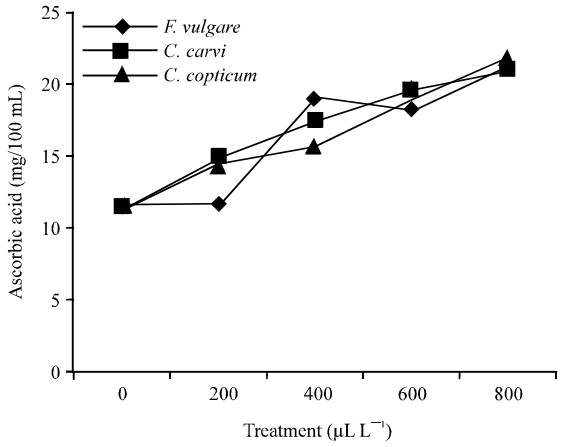

Fig. 7: Effect of different concentrations of essential oils on Ascorbic acid (mg/100 g)

essence (0.442 g/100 g fresh weight). In concentration 800 µl L^{-1}, TA content was more than other concentrations. *C. carvi* essence in concentration of 800 µl L^{-1} was best treatment (Fig. 5). The result showed fennel essence had more effective on TSS/TA content (7.78). There was highest TSS/TA content in control (8.89) and lowest content there were in *C. carvi* essence with the concentration 400 µl L^{-1} (Fig. 6).

Ascorbic acid content: There was in ascorbic acid content, significant difference among treatments with control. The *C. carvi* essence had more ascorbic acid (16.96 mg/100 mL) but no significant difference ascorbic acid content was observed in treated fruits among these essential oils. The *C. copticum* and *F. vulgare* essences in concentration of 800 µl L^{-1} had with 21.81 and 21.41 mg/100 mL, lowest change content than ascorbic acid value previous of treatment (Fig. 7). Probably ascorbic acid decreased by fungal infection due to cell wall break down by progress of time.

pH: The pH value of the treated cucumber juice was significantly different in among essential oils and control. The *F. vulgare* and *C. carvi* essences had heights and lowest pH value, respectively (5.17 and 4.84). However, the *C. copticum* essence in concentration of 600 µl L^{-1} had lowest pH value (4.59) (Fig. 8).

Decay rate: Essential oils-treated fruit better maintained ($p<0.01$) and have low severity of decay scores, whereas non-treated fruit showed increased fruit deterioration (Fig. 9). The *C. carvi* essence had lowest decay scores but no significant difference infection content was

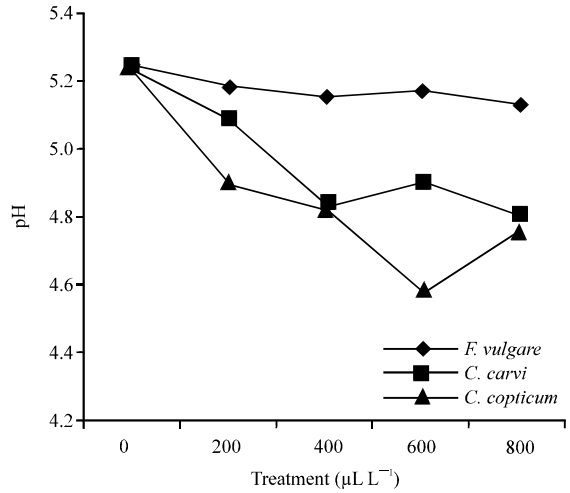

Fig. 8: Effect of different concentrations of essential oils on pH

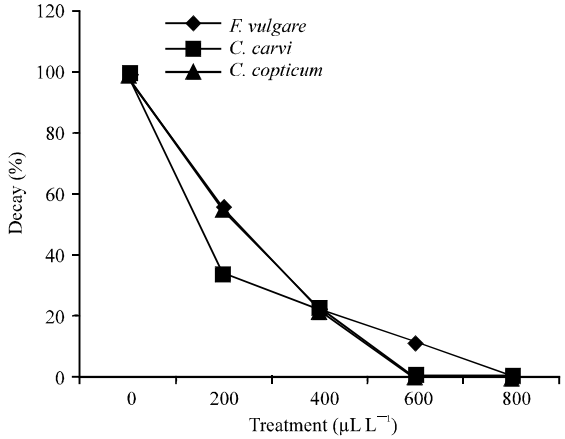

Fig. 9: Effect of different concentrations of essential oils on Decay rate

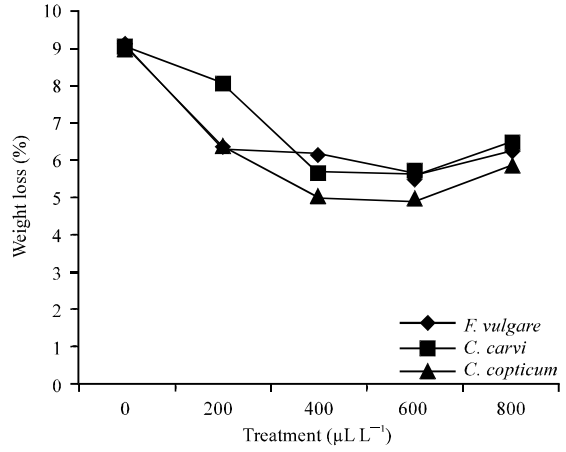

Fig. 10: Effect of different concentrations of essential oils on % weight loss

observed in treated fruits among these essential oils. No decay was observed in concentration of 800 µl L^{-1}.

Percentage of weight loss: The percentage of weight loss was very low for fruit treated by these essential oils and had significant difference compare to control ($p<0.01$), possibly due to increased respiration rates and fungi infection. But in concentration of 0 and 800 µl L^{-1} all of essences had the highest the percentage of weight loss. The *C. copticum* essence in concentration of 600 µl L^{-1} had lowest the percentage of weight loss (Fig. 10).

DISCUSSION

This study clearly showed that *Carum carvi, Carum copticum* and *Foeniculum vulgare* essential oils significantly reduced damage extent of the one of important cucumber postharvest pathogens namely *B. cinerea*. Data presented in this study indicated that *Carum carvi, Carum copticum* and *Foeniculum vulgare* oils had fungicide effective in concentrations of 400-800 µl L^{-1}, respectively, in similar results: The growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis subsp. michiganensis* fungus were completely inhibited by Oregano, thyme, dictamnus and marjoram essential oils at relatively low concentrations (85-300 mg mL^{-1}) (Daferera *et al*., 2003). Also, Bouchra *et al*. (2003) indicated that among essential oils used, *Origanum compactum* and *Thymus glandulosus* greatly inhibited the growth of the mycelium. So, spore germination and germ tube elongation were also inhibited by the essential oils tested. Although, the antimicrobial activity of essential oils attributed mainly to its major compounds, the synergistic or antagonistic effect of one compound in minor percentage in the mixture has to be considered. Each of the essential oil components has its own contribution on biological activity of the oil. For example, fennel, black zira and Ammi essential oils were characterized by the presence of cumin aldehyde, karvacrol and anetol. Cumin aldehyde was found as the main compound in black zira oil, while Anetol was found in fennel as the main compound and these compounds have more fungicide effect.

After exposure to the most effective oil concentrations determined *in vitro* studies, hyphae appeared degraded (Fig. 1, 2), large vesicles were visible within the cell walls. Shrivelled hyphal cells had either no cytoplasm or the cytoplasm was depleted of organelles. Under the effect of the oils, the growth of the fungus was suppressed. Complete absence of condition was also observed in oil treated Petri plates. These essential oils have positive effect on shelf life and reduce

decay content. For example, they had more TSS, TA, Ascorbic acid value and less decay to compare controls, which reported previously cinnamon and eucalyptus vapor had any significant effect on TSS on tomato but increased TSS level in strawberry (Tiana *et al*., 2011). In similar results: cumin essential oil spray on strawberry to control *B. cinerea* has significant effect on titrable acidity (Asghari Marjanlo *et al*., 2009). Previous experiments using eugenol, thymol or menthol vapors revealed benefits due to reduced weight loss in cherries and grapes (Serrano *et al*., 2005). Similar results were finding with eucalyptus and cinnamon oil in strawberry and tomato (Tiana *et al*., 2011). In fact, there was a linear correlation between ethylene and damage and thus the fungus was responsible for the majority of ethylene production, a part of the basal level typical of non-climacteric fruits. Accordingly, it has been reported that *B. cinerea* produced greater amounts of ethylene as the concentration of conidia inoculated *in vitro* or in the climacteric tomato fruit increased. The respiration rate was clearly affected by these essential oils concentrations and dimension of infection (Cristescu *et al*., 2002). Also, previous reports indicated that reduced fruit decay during postharvest treatments with volatile compounds for several produce including raspberry and kiwifruit (Wang, 2003; Williamson *et al*., 2007). Essential oils of *Carum copticum, Foeniculum vulgare* and *Carum carvi* mainly conjugated to compounds that have known as phenolic compounds, responsible for plants order and test that accumulate in some plants cells and had been shown useful effect of these compounds for pathogen control it is known that oxidation products of phlorsidzin (an orhto-dihidroxyphenolic compound) inhibit fungal are thought to inhibit the apple scab fungus *Venturia inaequalis*. It seems that similar role was done by phenolic compound of cumin essential oil such as γ-Terpinene, Cumin aldehyde and etc. These findings reveal that exogenous essential oil may have an antifungal function in cucumber fruits. Botrytis (grey mould) is the most important fungus that has been detected in cucumber fruit samples limiting its storage life (Asghari Marjanlo *et al*., 2009).

Further research is needed in order to obtain information regarding the practical effectiveness of essential oils to protect the plants or the plant products without phytotoxic effects but our study gives support for the application of certain essential oils to control *B. cinerea* under specific application conditions. This study demonstrates the *in vitro* activity of essential oils of some Apiaceae and their constituents against the grey mould disease and this study determine the effect of these oils on spore germination and *in vivo* testing, in order to evaluate their potential as preventive treatments. The data of presented that *Carum carvi* oil had high fungicide effective in control *B. cinerea* on cucumber fruits. However, more studies are required to recommendation of these essential oils as a commercial and natural antifungal for increase postharvest on horticultural crops.

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