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Biological Effects of Various Essential Oils on Citrus Decay Pathogens

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***Abstract* — *Penicillium digitatum* and *P. italicum* have been identified as important postharvest pathogens of citrus in world. In the present study, we evaluated the antifungal activity in the various essential oil against pathogens. Essential oils of *Syzygium aromaticum* and *Ferula assa-foetida* were found to possess strong antifungal activity against pathogens. The essential oils had inhibitory activities on the growth, conidia germination and germ tube elongation. The main components of clove oil were included Eugenol (82.8%), β-caryophyllene (4.9%), Eugenyl acetate (2.3%) that identified by GC-MS. The minimum inhibitory concentration (MIC) values of the clove oil ranged from 900 to 1000 ppm against the tested pathogens. Spore germination and germ tube elongation of the pathogens in PDA was strongly reduced in the presence of the clove oil. The clove oil at MIC concentration was a more effective approach to disease severity reduction on fruit than the application of other concentrations after incubation for 15 and 50 days at 20 °C and 4 °C, respectively. Activity of cellulose and pectinase decreased with increasing concentration of clove oil. However, this effect of clove oil reduced over time. The results of this evaluation of indicate that a compound found in clove oil was effective in reducing decay and enzymes activity.**

***Index Terms* — Essential oil, Citrus postharvest decay, *Penicillium digitatum*, *Penicillium italicum*, Pectinase, Cellulose.**

I. Introduction

Citrus is one of the most important and widely grown fruit crop in the world. The genus Citrus belongs to the sub-family Aurantioideae of the Rutaceae family. Citrus is a large genus that includes several major cultivated species, including *Citrus sinensis* (sweet orange), *C. reticulata* (tangerine and mandarin), *C. limon* (lemon), *C. grandis* (pummelo) and *C. paradisi* (grapefruit). They are widely grown in the tropical, subtropical, and borderline subtropical temperate areas of the world (1). In 2010, the global citrus acreage was 8.749 million hectares and citrus production was 122.976 million tons, which the top is ranked among all the fruit crops (2). Sweet orange is the largest citrus crop among fruit trees, accounting for 70% of total yield. It is one of the most economically important crop plants in the world (3).

Fungal infections are the main cause of postharvest rots of fresh fruit and vegetables during storage, transport and cause significant economic losses in the commercialization phase (4). Infections caused during postharvest conditions lowers the shelf life and adversely affect the market value of fruits. Postharvest fungal decay may cause significant losses to the citrus industry. *Penicillium digitatum* Sacc. and *P. italicum* Wehmer are the most common postharvest pathogens of citrus fruits. Injuries sustained to citrus fruit during harvest allow the entry of wound pathogens. These pathogens occur in almost all citrus growing regions of the world (5).

The synthetic fungicide Imazalil and Thiabendazole have been routinely used to control postharvest diseases including green and blue moulds (6). Traditionally, chemical fungicides provide the primary means for controlling postharvest fungal decay of fruit and vegetables. However, the alternative control methods are needed because of the negative public perceptions about the use of synthetic chemicals, increasing public concern regarding contamination of fruits and vegetables with fungicidal residues (7), proliferation of resistance in the pathogen populations, and high development cost of new chemicals (8). Research on plant-derived fungicides is now being intensified, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (9). Food safety is one of the major issues related to fresh fruit and vegetables (10). The use of plant extracts could be a useful alternative to synthetic fungicides in the management of rot fungi during postharvest handling of fruit and vegetables (4).

Essential oils (EOs) are natural volatile substances found in a variety of plants. They are complex mixture of mainly terpenoids, particularity monoterpenes and sequiterpenes, and a variety of aromatic phenols, oxides, ethers, alcohols esters, aldehydes and ketones that determine the characteristic aroma and odor of the plant (11). They were previously to have biological activities such as antiviral (12), antiparasitic (13), antifungal (14-16), bactericidal (17), insecticidal (18), and nematicidal (19, 20) effects.

A new approach to the control of postharvest pathogens, while maintaining fruit quality, has been implemented by the application of EOs. This approach eliminates the need for synthetic fungicides, thereby complying with consumer preferences, organic requirements and reducing environmental pollution (21) and also often active against a limited number of species, including the specific target species. They are also biodegradable and non-toxic. Although several EOs have been reported to have antifungal properties, few have been developed as commercial formulations for use in plant disease control, such as BM-608 containing 23.8% of *Melaleuca alternifolia* oil (22).

Ameziane *et al*. (23) reported that among the 21 plants tested the powders of *Thymus leptobotrys*, *Cistus villosus* and *Peganum harmala* plants totally inhibited the growth of *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum*, three postharvest fungal pathogens. Wang *et al*. (24) reported that the spore germination and mycelium growth of *P. italicum* and *P. digitatum* could be mildly

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stimulated by the Shatangju (*Citrus reticulata* Blanco) essential oil (EO) or did not show any obvious effect at a low concentration (<2.5 µl ml^{-1}), while could be strongly inhibited at a higher concentration. In addition, treatment with 20 µl ml^{-1} Shatangju EO could lead to a distinct irregular mycelium growth of *P. digitatum* and *P. italicum*, as well as to a reduction in cytoplasmic content of *P. italicum*.

Syzygium aromaticum (L.) Merr. & Perry, commonly known as clove, is an aromatic dried flowers bud of a tree in the family Myrtaceace. The exact geographical origin of the clove tree is unknown. It is probable that the tree originated in the warm and humid climate of tropical Asia, perhaps in the Moluccas Island (Indonesia) (25). The major component of clove oil is usually considered to be eugenol, with β-caryophyllene and eugenyl acetate, being present although in lower concentrations. On the chemical point of view, Politeo *et al*. (26) identified eugenol (80.5%) as the dominant volatile compound of the EO sample of dried clove buds from Croatia. Previous studies have reported antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as several food-borne fungal species (27, 28), antimicrobial (29), acaricidal (30) and insecticidal (31).

Many studies have reported on the antimicrobial property of *S. aromaticum* against various types of bacteria (27). However, until the present, no study has been conducted to reveal the antifungal property of the EO of the *S. aromaticum* against plant disease. Therefore, our study was carried out to reveal the potential of *S. aromaticum* EO.

P. italicum and *P. digitatum* produces various cellulose and pectinase. As a result, one of the most important pathogenetic mechanisms by *P. italicum* and *P. digitatum* secreted cellulase and pectinase enzymes for penetration in tissue of host. So change in value of these enzymes can be effective levels of pathogen virulence. Therefore, the objectives of the present investigation were (i) to screen and select effective EO of various plants against *P. italicum* and *P. digitatum* and (ii) identify the EO components of effective and (iii) evaluate activity of oils against postharvest decay *in vivo* and also (iv) in preventing or reducing the activity of cell wall degrading enzymes such as cellulase and pectinase, as a part of the infection process of theses fungi their host plants pathogenic mechanism.

II. MATERIALS AND METHODS

A. Fruit samples

Commercially harvested Thomson Navel oranges, [*Citrus sinensis* L. (Osbeck)], with healthy appearance were used within 2 weeks of storage at 4ºC.

B. Plant pathogenic fungi

The fungi used throughout the present study were *Penicillium digitatum* (causing green mould of citrus fruit) and *P. italicum* (causing blue mould of citrus fruit) obtained from culture collection of plant pathology laboratory, Abureihan Campus, University of Tehran were used in this study. The fungus isolates were maintained on potato dextrose agar (PDA) medium slants at 4˚C, and sub-cultured at monthly intervals. The spores of fungi was removed from the surface of 10 day-cultured and suspended in sterile-distilled water containing 0.05% (v/v) Tween80. Spore concentration of fungi was determined by haemocytometer and adjusted to 10^5 spores ml^{-1}.

C. Plant materials

All plant species were collected on the time interval October and November 2012 from Sistan and Baluchestan, Tehran and Kermanshah provinces of Iran (Table 1). The species were identified, and a voucher sample was kept in the laboratory of Botany (University of Tehran, Iran).

Table 1. List of plant species, family and part used in the preliminary screening for antifungal properties.

Plant species	Family	Plant part used
Azadirachta indica	Meliaceae	leaves
Acroptilon repens	Asteraceae	shoots
Descurainia sophia	Cruciferae	shoots
Ziziphus jujuba	Rhamnaceae	fruit
Capsella bursa-pastoris	Cruciferae	shoots
Quercus robur	Faguceae	fruit
Pinus eldarica	Pinaceae	leaves
Lavandula officinalis	Lamiaceae	leaves
Ferula assa-foetida	Umbelliferae	shoots
Euphorbia rigida	Euphorbiaceae	leaves
Syzygium aromaticum	Myrtaceae	flowers
Petroselinum sp.	Umbelliferae	leaves
Allium sativum	Liliaceae	fruit
Eucalyptus sp.	Myrtaceae	leaves
Rosmarinus officinalis	Lamiaceae	shoots
Allium heamanthoides	Liliaceae	shoots
Rhus coriaria	Anacardiaceae	shoots

D. Extraction of EO

All plants were washed with distilled water and dried at room temperature in the shade and away from direct sunlight. Then, using the mill was crushed and plant tissues were passed through a 10 mesh sieve. For isolation of the EOs, 100 g of dried plant materials were subjected to hydro-distillation for about 3 h, using a Clevenger apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in sealed glass bottles. It was protected from light by wrapping in aluminum foil and stored at 4 ˚C until used.

E. Screening for antifungal activity and fungal growth inhibition in vitro

The tests were performed using the agar medium assay described by Tatsadjieu et al. (32) with some modifications. For preliminary assessment and screening the efficacy of EOs obtained from various plants, the effect of each EO at 2000 ppm concentration on growth of the pathogens was investigated. Then, EOs with the highest levels of inhibition against the pathogens were selected for further experiments. PDA medium with different concentrations of EOs (i.e. 0-1500 ppm) were prepared by adding appropriate quantity of EO to melted medium, followed by addition of Tween-20 (100µL to 100 mL of medium) to disperse the oil in the medium. About 20 ml of the medium were poured into a Petri dish (9 cm × 1.5 cm). Each Petri-dish was inoculated at the center with a mycelial disc (10 mm diameter) taken at the periphery of pathogens colony grown on PDA for 72 h. Positive control (without EO) plates were inoculated following the same procedure. Plates were incubated at 25±1 ˚C for 5 days and the colony diameter was recorded each day. The mycelial growth inhibition (MGI)

percentage was calculated according to the following formula:

MGI (%) = $[(D_c-D_t)/D_c] \times 100$

Where, D_c = mean diameter of colony in the control (mm) and D_t = mean diameter of colony in the treatment (mm). Four replicate plates were used per treatment and the experiment was repeated three times.

F. Nature of toxicity of EOs

The nature of toxicity (fungistatic/fungicide) of the oils against fungi was determined as described by Thompson (33). The inhibited fungal mycelia plugs of the oil treated sets were reinoculated into fresh medium and revival of their growth was investigated.

G. Determination of minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and inhibitory concentration 50 (IC50)

MIC and MFC of EOs were determined as described by Plodpai et al. (34) and Tyagi and Malik (35) with few modifications. The PDA plates were amended with various concentrations of plant EO (i.e. 0-1500 ppm). For enhancing the EO solubility, Tween-20, 0.5% (v/v) was added. Each plate was inoculated with a mycelial plug (10 mm diameter) of pathogens. All plates were incubated in four replicates for each concentration at 25±1 °C for 120 h. Plates with Tween-20 but without any EO were used as control. Observation of fungal growth was done at a time interval of 24 h up to 120 h after incubation. The MIC values were determined as the lowest concentration of EO that completely prevented the visible fungal growth.

To determine MFC, the mycelial plugs were obtained from each Petri dish treated with the oil concentrations lower than MIC, cultured on PDA and incubated at 25±1 °C for 120 h. MFC was defined as the lowest concentration at which no colony growth was observed after subculturing into fresh PDA medium. IC50 values were graphically calculated from the dose-response curves based on measurement at various concentrations.

H. Comparing the fungitoxicity of EO with prevalent synthetic fungicides

The efficacy of the EO was compared with common fungicide, Thiabendazole (Tecto) and Imazalil (Fungaflor) by the agar medium assay.

I. Effect on conidia germination and germ tube elongation

The effects of the EOs on spore germination and germ tube elongation of pathogens were as described by Soylu et al. (36). A spore suspension (10^3 spores ml^{-1}) of pathogens were prepared from actively growing culture (7-8 days old) in sterile distilled water using a haemocytometer. Four different 50 µl aliquots of the spore suspension drops were spread on PDA medium supplemented with different concentrations of the EOs. Sterile distilled water alone and sterile distilled water with Tween-20 were used as controls. Plates were incubated at 25±1 °C for 10-12 hours, after which germination was stopped by applying a drop of lacto phenol-cotton blue to the inoculation sites on plates. The percentage of spore germination and the lengths of germ tubes were estimated under a microscope. The percent inhibition was calculated in relation to the respective control. Four replicates of each treatment were performed and the experiments were repeated twice.

J. GC-MS analysis

The chromatographic procedure was carried out using a Shimadzu QP 5000(FID). Gas chromatography-mass spectroscopy (GC-MS) analysis of the EO of *S. aromaticum* using fused silica capillary column HP5-MS (30m × 0.32 mm, film thickness 0.25 µm) was done. Helium was used as carrier gas, and a split ratio of 1/100 with injection volume of 1µL. The oven temperature used was maintained at 60 °C for 8 min. The temperature was then gradually raised at a rate of 3 °C per min to 180 °C per min and maintained at 180 °C for 5 min. The temperature at the injection port was 250 °C. The components of the EOs were identified by comparison of their retention indices with those published in the literature (37-39).

K. Efficacy of the EO on control of citrus postharvest decay

Fruits were surface-sterilized in 70% ethanol, and wound-inoculated with *P. digitatum* and/or *P. italicum* (10^5 spores ml^{-1}) using sterile needles. One wound, approximately 1 mm wide and 2 mm deep, was made in the middle of each fruit. About 5-6 h post-inoculation, fruit were dipped in a suspension of EO of *S. aromaticum* (at concentrations of IC50, 0.1×IC50 and 0.01×IC50) for 5 min. After air drying, treated fruit were stored in cardboard boxes at 4ºC (for 30 days) and 20ºC (for 20 days), and 70-80% RH. The controls consisted of infected fruit immersed in sterile distilled water with Tween-20, 0.5% (v/v) (positive control), non-infected fruit immersed in sterile distilled water with Tween-20, 0.5% (negative control) and infected fruit treated with Thiabendazole and Imazalil at a concentration of 1.5 g l^{-1}. The percentage of disease severity reduction (DSR%) was calculated by the equation:

DSR (%) = [(DSc - DSt)/DSc] × 100

Where DSc = average area with lesions on the positive control and DSt = area with lesions on the treated plants. Only the mechanically wounded region of the orange was used for the assessment of disease reduction.

L. Effect of EO on the activity of cellulase and pectinase

The efficacy of EO obtained from *S. aromaticum* at different concentrations in reducing the activity of cellulase and pectinase enzymes *in vitro* were determined using the methods described by Khairy et al. (40) and Abdel-Razik (41). The production of pectinase enzyme was carried out using a medium containing 4.6 g citrus pectin , 5.0 g yeast extract, 5.0 g peptone, and 5.0 g K_2HPO_4 in 1 liter of distilled water and pH 7.2±0.2 as described by MacMillan and Voughin (42). The same medium supplemented with 4.6 g carboxymethyl cellulose instead of citrus pectin was used for production of cellulase. Each concentration was added to the sterilized medium in each flask. Then, the flask was inoculated with a 1 cm diameter mycelial plug of each fungus. Cellulase and pectinase activity were determined after 10 days of incubation at 28 °C. The supernatants were obtained by filtration and centrifugation at 5000 rpm for 15 min at 4 °C. Then, the supernatants were used for crude enzyme preparation. Three flasks were used as replicates for each treatment as well as the control and the experiment was repeated three times.

- *Cellulase activity assay*

Cellulase activity was assayed following the method of Wood and Bhat (43). Briefly, 0.5 ml of cell free supernatant was incubated with 1 ml of 0.7% carboxymethyl cellulase in 0.05M acetate buffer with pH 4.8 and the reaction mixture was incubated at 50 °C for 60 minutes in static condition. After adding 2 ml of (3, 5-dinitrosalicylic acid) DNS reagent, the mixture was boiled for 10 min at 100 °C. The reaction was stopped by adding 1ml of 40% Potassium sodium tartrate. The absorbance was measured at 550 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity is defined as the amount of enzyme that catalyzed 1.0 µmol of glucose per minute during the hydrolysis reaction.

- *Pectinase activity assay*

Pectinase activity was determined based on the amount of reducing sugar (D-galacturonic acid) released in culture supernatant. The amount of D-galacturonic acid was determined by dinitrosalicilic acid colorimetric method of Colowich (43). Briefly, 0.5ml of cell free supernatant was incubated with 0.5ml of 1% pectin in 0.1M acetate buffer with pH 6.0 and the reaction mixture was incubated at 40 °C for 10 minutes in static condition. After adding 1ml of DNS reagent, the mixture was boiled for 5 min at 90 °C. The reaction was stopped by adding 1 ml of 1% Potassium sodium tartrate. Then the mixture was diluted by adding 2 ml of de-ionized water. The absorbance was measured at 540 nm. The unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of galacturonic acid per minute according to the standard curve. The standard curve was drawn based on the absorbance in different concentrations (µg/ml) of D-galacturonic acid.

M. Statistical analysis

All experiments were conducted on the basis of completely randomized designs. Data were subjected to analysis of variance (ANOVA) for a completely randomized design with four replicates using SPSS (version 21) software. The means were separated using Duncan's multiple range tests at $P < 0.05$, where the F-value was significant.

III. RESULTS

A. Inhibitory effect of EOs on mycelial growth of the fungal pathogens in vitro

The effects of concentration of 2000 ppm of the EOs on mycelial growth of pathogens are shown in Figure 1. EOs of *D. sophia*, *Z. jujube*, *C. bursa-pastoris*, *P. eldarica*, *L. officinalis*, *E. rigida*, *Petroselinum* sp., *A. indica*, *Eucalyptus* sp., *R. officinalis* and *R. coriaria* not have no inhibitory effect on mycelial growth of pathogens, and but EOs of *A. repens*, *A. heamanthoides*, *Q. robur*, *A. sativum*, *F. assa-foetida* and *S. aromaticum* inhibited mycelial growth of pathogens from 100% to 32.5% that the highest level of inhibition belonged to *S. aromaticum* and *F. assa-foetida* (Figure 1).

The effects of different concentrations of EO obtained from clove and asafetida on mycelial growth of pathogens are shown in Figure 2. The EOs inhibited the growth of pathogens in a dose dependent manner. Our results revealed that the antifungal activity of EOs increased with increasing the concentration.

For pathogens, the lowest IC50 was observed using the EO of *S. aromaticum* followed by *F. assa-foetida* (Table 2). Investigating fungistatic and/or fungicide activity revealed that the EOs of clove and asafetida had fungistatic properties against pathogens. Minimum concentration of the EOs required to completely inhibit the mycelial growth of fungi was different. The EO of *S. aromaticum* had the lowest MIC for pathogens among EOs tested. *P. digitatum* did not show any visible mycelial growth in presence of clove and asafetida oils at concentrations of 1000 and 1200 ppm, respectively. The EO of *S. aromaticum* had the lowest MIC for *P. italicum* among EOs tested. *P. italicum* did not show any mycelial growth in presence of EOs obtained from clove and asafetida at concentrations of 900 and 1350 ppm, respectively. At different concentrations lower than 1000 ppm, the EO of *S. aromaticum* was more effective against pathogens compared to the EO of *F. assa-foetida*. The MICs of synthetic fungicides Thiabendazole and Imazalil against pathogens were found to be 1500 and 1000 ppm, respectively, which were higher than that of the EOs tested in present study (Table 2).

Table 2. In vitro antifungal activity of the essential oils of *S. aromaticum* and *F. assa-foetida* compared to synthetic fungicides against mycelial growth of *P. italicum* and *P. digitatum.*

Fungi	***P. italicum***			***P. digitatum***		
Treatments	**IC50[a]**	**MFC[b]**	**MIC[c]**	**IC50[a]**	**MFC[b]**	**MIC[c]**
Essential oils						
Syzygium aromaticum	350	IN	900	350	IN	1000
Ferula assa-foetida	450	IN	1350	600	IN	1200
Fungicides						
Thiabendazole	750	1500	1500	750	1500	1500
Imazalil	500	1000	1000	500	1000	1000

[a] Inhibitory concentration with 50% inhibitory effect on the fungal growth (ppm)
[b] Minimum fungicidal concentration (ppm)
[c] Minimum inhibitory concentration (ppm)
IN: Ineffective

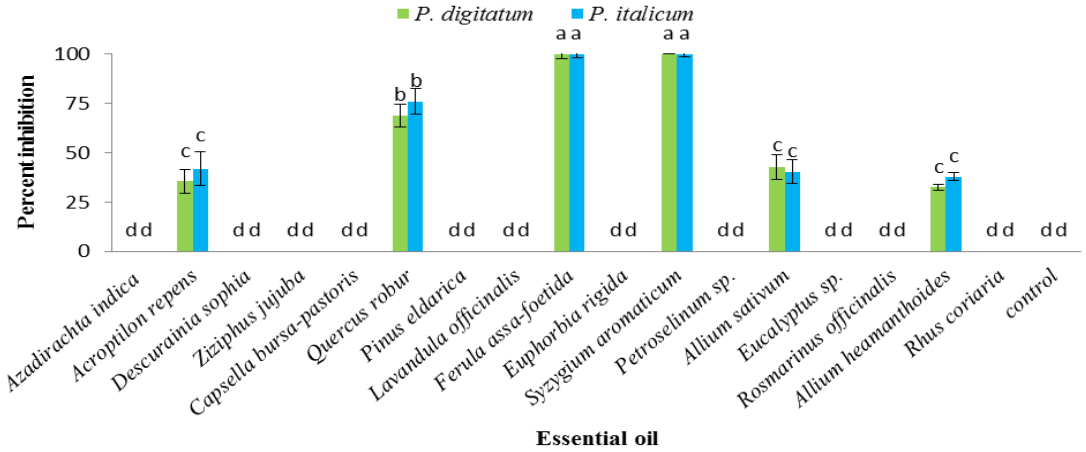

Figure 1. Effect of essential oils obtained from various plant species plants mycelial growth of *P. italicum* and *P. digitatum*. The means with the same letter do not have significant difference according to Duncan's Multiple Range test at $P < 0.05$.

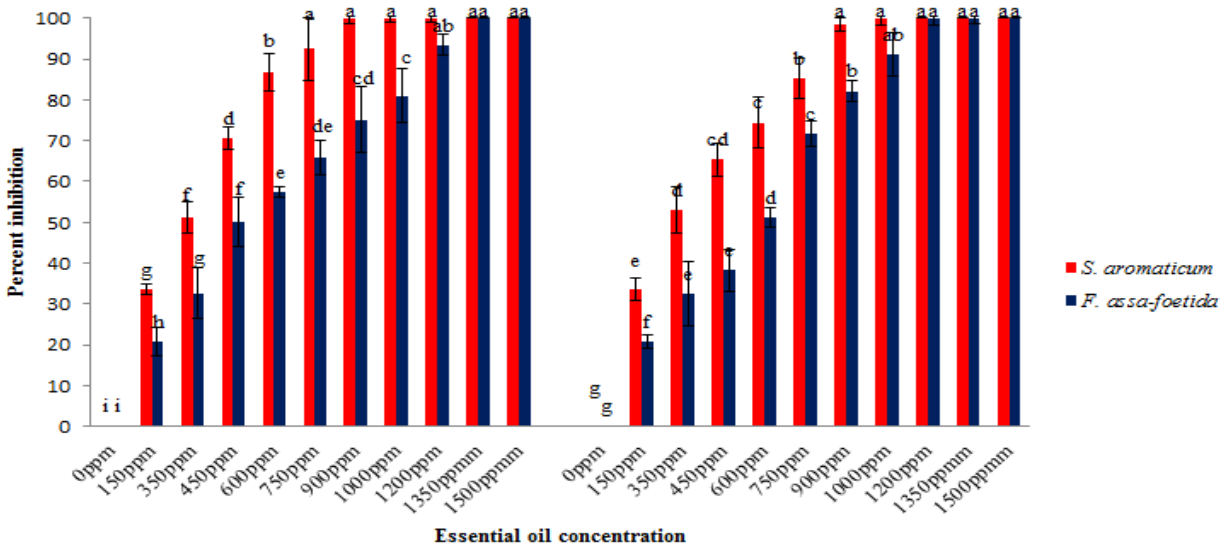

Figure 2. Effect of different concentrations of essential oils of *S. aromaticum* and *F. assa-foetida* on the mycelial growth of *P. italicum* and *P. digitatum*. The means with the same letter do not have significant difference according to Duncan's Multiple Range test at $P < 0.05$.

B. Composition of the EO of S. aromaticum

The result of GC-MS of EO of *S. aromaticum* is presented in Tables 3. Seventeen compounds were identified in the oil which constitutes about 96.6% of this oil. The EO was characterized by the presence of major compounds such as Eugenol (82.8%), β-caryophyllene (4.9%), Eugenyl acetate (2.3%), Myrcene (1.9%), α-Terpinene (1.4%) and Thymol (1.1%). In previous studies, Fayemiwo et al. (33) analyzed the EO of *S. aromaticum* originating from Nigeria, and they detected 28 compounds with Eugenol (2-Methoxy 4-(2-propenyl) phenol) (80.5%) and Eugenyl acetate (4-Allyl-2-methoxyphenyl acetate) (5.01%) constituting the major constituents.

Table 3. Chemical composition of *S. aromaticum* essential oil (%) determined by GC-MS.

No.	Compound name	RI[a]	Composition (%)
1	α-Pinene	921	0.6
2	α-Thujene	951	0.1
3	α-Terpinene	1019	1.4
4	ρ-Cymene	1023	0.5
5	Myrcene	1032	1.9
6	Limonene	1034	0.3
7	Methyl salicylate	1189	0.1
8	Linalool	1098	0.4
9	Terpinolene	1103	0.5
10	α-terpineol	1189	0.2
11	Thymol	1296	1.1
12	Eugenol	1361	82.8
13	β-caryophyllene	1412	4.9
14	Eugenyl acetate	1526	2.3
15	Caryophyllene oxide	1573	0.1
16	Terpinen-4-ol	1634	0.3
17	Heptadecane	1708	0.1
	Total	-	96.6

[a] RI: Retention index calculated on the basis of retention time of a mixture of n-alkanes (C8-C30).

Also Pinto et al. (34) analyzed the EO of *S. aromaticum* and they detected 19 components, representing 97.3% of the volatile oil that the oil was characterized by high amounts of a phenylpropanoid compound, eugenol (85.3 %). Our results were between the lowest and the highest values reported.

C. Effect of EO on conidia germination and germ tube elongation

The effects of the various concentrations of the EOs on conidia germination and germ tube elongation are shown in Figures 3. EOs of *S. aromaticum* and *F. assa-foetida* inhibited the spore germination of pathogens in a dose dependent manner. EO of *S. aromaticum* showed a significant activity against spore germination of *P. italicum* and *P. digitatum*. Although percentage germination and germ tube length were significantly reduced with each tested concentration, complete germination inhibition by clove oil was achieved at higher concentrations of 1000 ppm (Figures 3A). Complete germination inhibition was not achieved with the EOs although significantly lower percentage germination and germ tube lengths were associated with increased concentrations of the EOs (Figures 3B). EO of *S. aromaticum* which showed the highest efficacy in mycelial growth inhibition of pathogens was selected to be used in the rest of experiments.

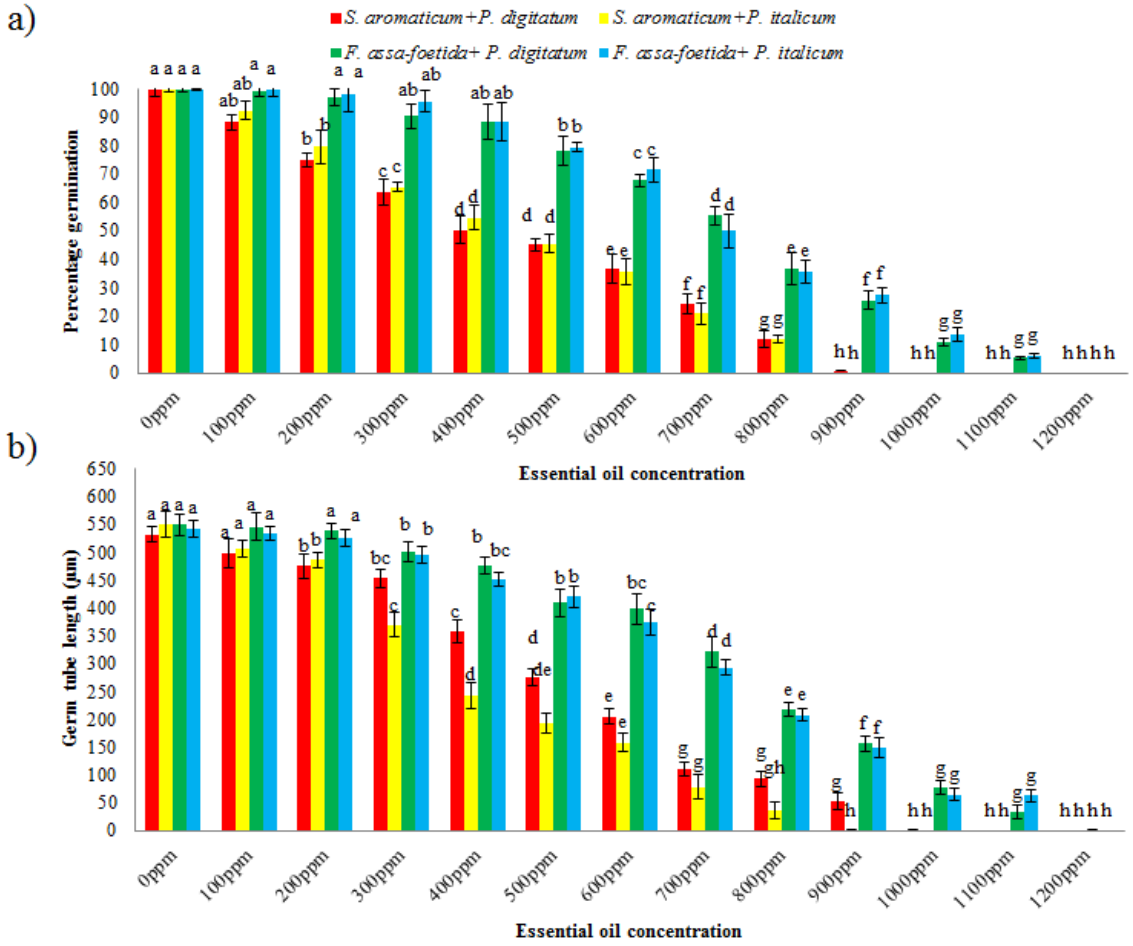

Figure 3. Effect of different concentrations of essential oils of *S. aromaticum* and *F. assa-foetida* on spore germination (a) and germ tube length (b) of *P. italicum* and *P. digitatum*. The means with the same letter do not have significant difference according to Duncan's Multiple Range test at $P < 0.05$.

D. Efficacy of the EO of S. aromaticum on control of citrus postharvest decay

The effects of the various concentrations of the EO of *S. aromaticum* and synthetic fungicides on fruit in conditions storage are shown in Figures 4. Significant differences were observed among the treatments and positive control (infected with pathogen but no antagonist) at both temperatures Results of the present study showed that treatment of fruit with EO of *S. aromaticum* with different concentrations decreased fungal decay development. EO of *S. aromaticum* treatment significantly reduced lesion area in fruits inoculated with *P. digitatum* and/or *P. italicum* after 20 and 30 days at 20 °C and 4 °C, respectively. These results suggest that EO of *S. aromaticum* may have the ability to control postharvest blue mold decay, but the ability is limited, and must be used with other control measures to obtain an improved treatment. As shown in our results, EO of *S. aromaticum* at MIC concentration could significantly control citrus postharvest decay under both storage conditions. Also the EO of *S. aromaticum*, controlled development of the pathogens, *P. digitatum* and/or *P. italicum*, as effectively as Imazalil and Thiabendazole (Figures 4). The EO can directly inhibit pathogen and also induction of disease resistance mechanisms.

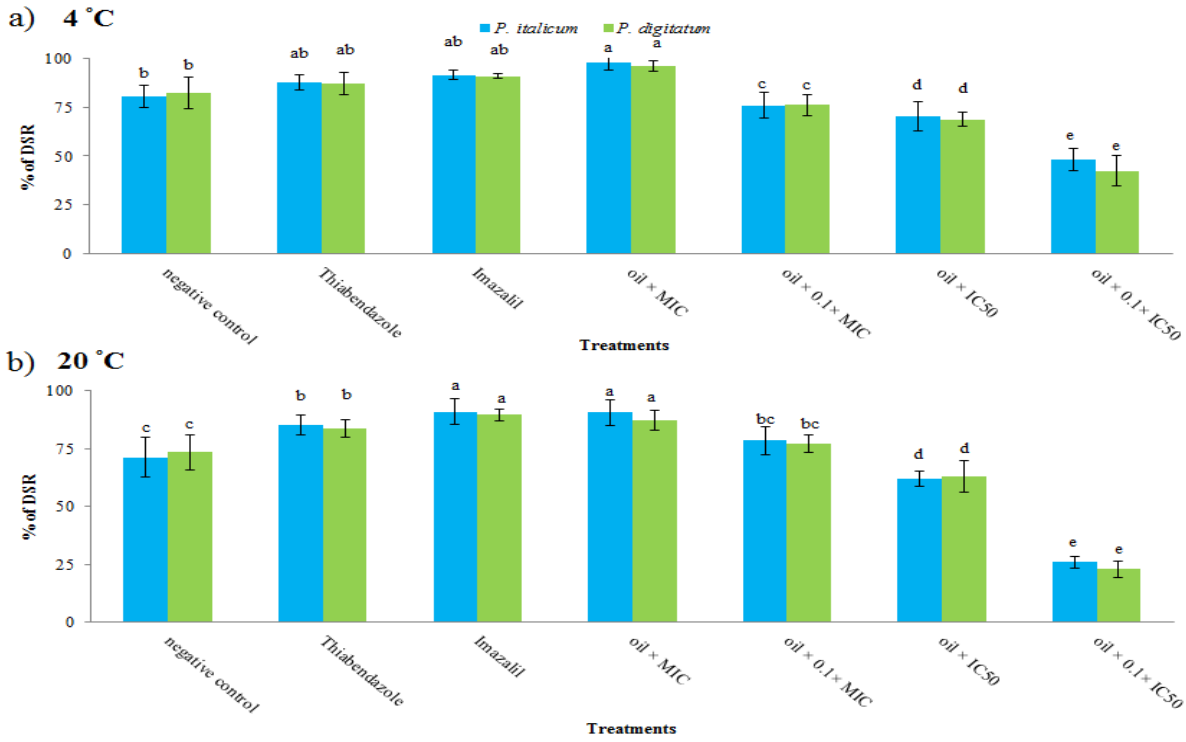

Figure 4. Percentage of disease severity reduction (% DSR) on fruit artificially infected with Percentage of fruit remaining uninfected after inoculation with *P. italicum* and *P. digitatum* at 10^5 spores ml^{-1}, followed by essential oil of *S. aromaticum* in comparison with Thiabendazole and Imazalil treatment. Fruits were stored at 4 and 20 ºC. There is significant difference between treatments, using Duncan's multiple range test (P<0.05) at both temperatures.

E. Effect of EO of S. aromaticum on enzymatic activity of P. digitatum and P. italicum

EO of *S. aromaticum* reduced the activity of cellulase and pectinase enzymes secreted by *P. digitatum* and *P. italicum* compared with the control. *In vitro*, *P. digitatum* showed maximum cellulase and pectinase activity after 168 and 216 hours post-culturing on liquid medium, respectively, and decreased afterward. During 10 days, enzyme activity was studied; EO of *S. aromaticum* at MIC concentration reduced the cellulase and pectinase enzyme activity secreted by *P. digitatum* from 44.2 to 18.2% (Figure 5a) and from 94.1 to 18.9% (Figure 5b), respectively. The cellulase and pectinase activity of *P. italicum* were reduced by treatment with *S. aromaticum*. During 10 days, enzyme activity was studied; EO of *S. aromaticum* at MIC concentration reduced the cellulase and pectinase enzyme activity secreted by *P. italicum* from 43.2 to 23.8% (Figure 5c) and from 96.9 to 18.6% (Figure 5d), respectively. *In vitro*, *P. italicum* showed maximum cellulase and pectinase activity after 144 and 216 hours post-culturing on liquid medium, respectively, and decreased afterward. So, the decrease in enzyme activity observed in this research may reflect a process elaborated of effective compounds of EO to reducing pathogenic.

IV. DISCUSSION

In the present study, the antifungal capability of EOs obtained from various plant species against *P. italicum* and *P. digitatum* was investigated using *in vitro* and *in vivo* assays. In our investigations, except EOs of *D. sophia*, *Z. jujube*, *C. bursa-pastoris*, *P. eldarica*, *L. officinalis*, *E. rigida*, *Petroselinum* sp., *A. indica*, *Eucalyptus* sp., *R. officinalis* and *R. coriaria*, other EOs tested had considerable antifungal capability against *P. italicum* and *P. digitatum*. EOs of *S. aromaticum* and *F. assa-foetida* which showed highest levels of inhibiting pathogens growth in preliminary evaluation were used for determining the capability of EOs.

The results showed that the antifungal activity of the EOs increased with an increase in concentration. The EO inhibited the growth of pathogens in a dose dependent manner. According to Imtiyaz Aslam and Roy (44), the extract of clove was potentially antifungal activities against *Penicillium notatum* and *Aspergillus niger*. Pinto et al. (34) indicates that clove oil and eugenol have considerable antifungal activity against *Candida*, *Aspergillus* and dermatophyte species, including fluconazole-resistant strains.

The MIC and IC50 of the EO various species plants against *P. italicum* and *P. digitatum* are shown in Table 2. EO of *S. aromaticum* showed the best activity against *P. italicum* with MIC value of 900 ppm followed by the EO of *F. assa-foetida* exhibiting MIC value of 1350 ppm. Also EO of *S. aromaticum* showed the best activity against *P. digitatum* with MIC value of 1000 ppm followed by the EO of *F. assa-foetida* exhibiting MIC value of 1200 ppm. According to Kouassi et al. (45), only *Cinnamomum zeylanicum*, *Cinnamomum verum* and *Eugenia caryophyllus* were active against the three pathogens (*Penicillium italicum*, *P. digitatum* and *Colletotrichum musea*) even at 100 ppm. The plant extracts reported effective against the fungi *P. digitatum* include *Allium sativum*, *Azadirachta indica*, *Withania somnifera* and *Acacia seyal* (46, 47).

Post-exposure of EO treatments on conidia germination and germ tube length (in µm) revealed differences among the treatments, implying that effects were fungistatic. The clove oil had inhibitory effect on germination and germ tube elongation of both *P. italicum* and *P. digitatum*, however, *P. italicum* appeared to be more sensitive to the inhibitory substances of clove oil.

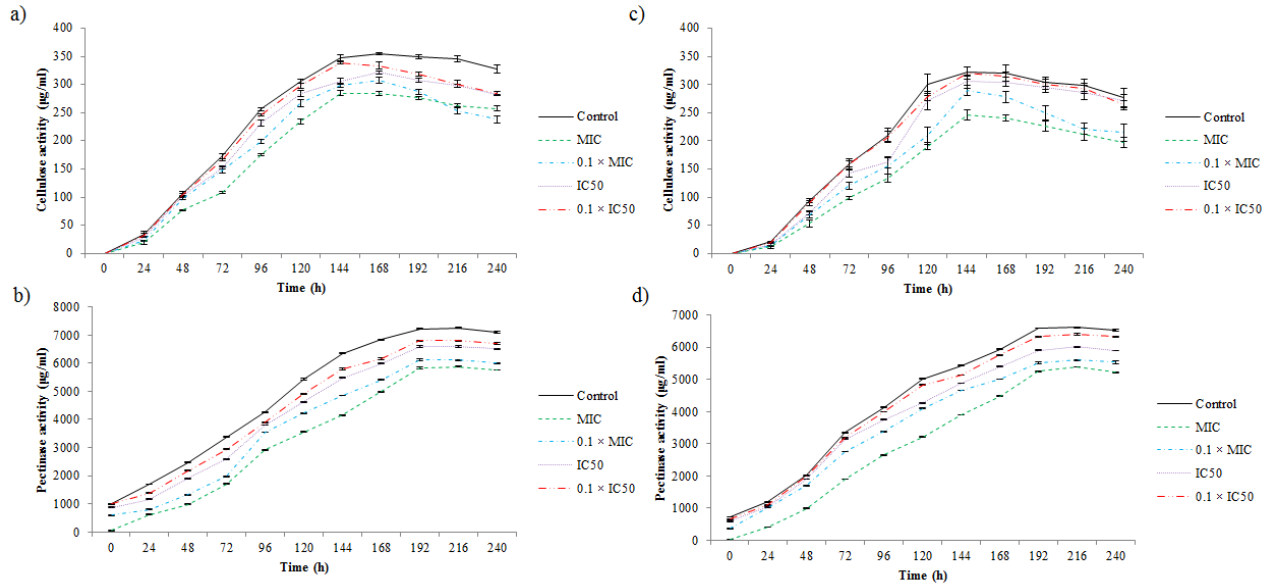

Figure 5. Effect of essential oil of *S. aromaticum* on cellulase (a) and pectinase (b) activity of *P. digitatum*. Effect of essential oil of *S. aromaticum* on cellulase (c) and pectinase (d) activity of *P. italicum*.

Conidia germination in the controls was >99.8% and germ tube growth was >532 µm. When pathogens exposed to clove oil (at 900 ppm), the percentage of germinated spores of *P. italicum* and *P. digitatum* was 0% and 0.35%, respectively. The most effective was clove oil which completely inhibited conidia germination and germ tube growth at the lowest concentration tested. Asafoetida and clove oils were found to inhibit the germ tube elongation in a dose-dependent manner. Soylu et al. (48) found that EOs from *Origanum syriacum* and *Foeniculum vulgare* plants possessed strong antimicrobial activity against conidial germination and germ tube elongation of *P. digitatum*.

Investigating fungistatic and/or fungicidal effects of the EO showed that the *S. aromaticum* and *F. assa-foetida* oils had fungistatic activity against pathogens. The minimum inhibitory concentration values obtained for EO used in this assay was almost lower than the values obtained for synthetic fungicides such as Thiabendazole and Imazalil. However, adverse effects caused by these fungicides are not comparable with EO.

As can be seen in Table 3, the main constituents of the *S. aromaticum* oil are phenylpropene, phenol, monoterpenoid, monoterpene, terpenes, terpenoids, aldehydes and alcohols (forming the largest constituent). The high percentage inhibition of these most effective EOs could be due to their high content of major compounds. The strong antifungal activity of clove oil could be due to their high content of phenolic compounds. Alitonou et al. (25) reported that the EO extracted from the seed of *S. aromaticum* is rich in eugenol with a strong content (>60.4%). It detains a high bactericide activity against *Escherichia coli* and *Staphylococcus aureus* and antifungal against *Aspergillus parasiticus*.

EOs are natural volatile substances found in a variety of plants. They are complex mixture of mainly terpenoids, particularity monoterpenes and sequiterpenes, and a variety of aromatic phenols, oxides, ethers, alcohols esters, aldehydes and ketones that determine the characteristic aroma and odor of the plant. Their chemical composition may vary considerably between aromatic plant species and varieties, and within the same variety from different geographic areas. In addition the effect of plant maturity at the time of oil extraction and the existence of chemotypic differences can also drastically affect their composition. On the other hand, it is well known that the chemical composition of plant EOs depend on various factors such as the plant variety, environmental conditions, and also plant-microbe interactions (49). EOs prevents the synthesis of DNA, RNA, proteins and polysaccharides in bacterial and fungal cells. This substances act Similar to the effects of antibiotics in fungi. The phenylpropenes constitute a relatively small part of EOs, and those that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde. The antimicrobial activity of phenylpropenes depends on the kind and number of substituents on the aromatic ring, selected microbial strains, and the experimental test parameters such as choice of growth medium, temperature and etc (50).

Eugenol is a major constituent in clove EO, and its antimicrobial activity is linked to its ability to permeabilize the cell membrane and interact with proteins. Eugenol's action on membranes occurs mainly by a non-specific permeabilization. The non-specific permeabilization of the cytoplasmic membrane by eugenol has been demonstrated in various studies as increased transport of potassium and ATP out of the cells (51). The hydroxyl group of eugenol is thought to bind to and affect the properties of proteins, thereby contributing to eugenol's inhibitory effect at sub-lethal concentrations. Consistent with this, eugenol has proven to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase, and protease. Inhibition of the ATPase may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired The antifungal mode of action of eugenol needs further investigation, but it is known to depend on cell proliferation (52).

Terpenes are hydrocarbons produced from combination of several isoprene units. The main terpenes are

monoterpenes and sesquiterpene. Examples of terpenes include p-cymene, limonene, terpinene, sabinene, and pinene. Terpenes do not represent a group of constituents with high inherent antimicrobial activity. Koutsoudaki et al. (53) compared the effect of α-pinene, β-pinene, p-cymene, β-myrcene, β-caryophyllene, limonene, and γ-terpinene against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, and their antimicrobial activity were low or absent. The p-Cymene and γ-terpinene were ineffective as fungicides against *Saccharomyces cerevisiae* (54). These in vitro tests indicate that terpenes are inefficient as antimicrobials when applied as single compounds. The antimicrobial activity of EOs can often be correlated to its content of phenolic constituents. Generally, the EOs possessing the strongest antibacterial properties against pathogens contain a high percentage of phenolic compounds such as eugenol, thymol and carvacrol are known antimicrobial agents (55).

The effectiveness of EO of *S. aromaticum* was comparable to that of Thiabendazole and Imazalil at commercial doses, indicating that this biocontrol agent could be used as a substitute for chemicals to control green and blue moulds of citrus fruit. Incidence of green and blue moulds were lowered to 87.13 and 90.63% when clove oil at MIC concentration were applied at 20°C, and to 95.93 and 97.64%, respectively, when applied at 4°C. The result for fruit stored at 4 °C for 30 days and 20 °C for 20 days showed that EO of *S. aromaticum* reduced growth of *P. digitatum* and *P. italicum*, and were effective material for control of pathogens. EO of *S. aromaticum* at MIC concentration appeared to be good effect have on green and blue moulds of citrus fruit in period storage. According to Azizi et al. (56), *T. vulgaris* and *M. piperita* EOs control green mould decay and postharvest quality of Valencia orange. Abd-Alla et al. (57) reported that Olive water extract (4%) gave a complete reduction of disease incidence and disease severity after the first storage period but after 45 days and 60 days of storage gave highly significant reduction of both disease incidence and disease severity by 86.8% and 80.0% and 73.3% and 89.5%, respectively. Results of the present study showed that treatment of fruit with clove oil with different concentrations decreased fungus decay development.

The plant cell wall is composed of polysaccharides and proteins. In addition, some cells have walls impregnated with lignin. In all cases, the polysaccharides constitute the major part of the wall. The wall polysaccharides are often classified into cellulase, hemicellulases, and pectin and these three types are represented in almost all cell walls in varying proportions. For the degradation of cell wall, exocellular enzymes like cellulolytic, hemicellulolytic, pectolytic and proteolytic enzymes are produced which are capable of attacking each of the major polymeric components (58). Pathogen fungi attack target cells by producing number of cell degrading enzyme which facilitates the entry and expansion of pathogen in the host tissue (59). Cellulolytic enzymes serve as invasive agents that enable the pathogen to penetrate the tissue of its host or as digestive agents that enable plant tissues to be penetrated by other enzymes and enable cellulase itself to be utilized as a carbon source (60). The current study revealed that EO of *S. aromaticum* was capable of decrease of mechanisms of pathogenesis of fungi. Over time effects of EO on the enzymes is reduced. The clove oil reduced the activity of cellulase and pectinase enzymes secreted by *P. digitatum* and *P. italicum* compared with the control. The present data revealed significant decrease in cellulase and pectinase enzyme activity secreted by *P. digitatum* and *P. italicum* of all treatments having EO compared to control. Among the studied clove oil different concentrations, oil at MIC concentration was caused the highest reduction in cellulase and pectinase activity. It can be suggested that a part of the inhibitory effect of EOs against *P. digitatum* and *P. italicum* might be related to indirect influence on their infection process by affecting the activity of cell wall degrading enzymes which are produced by the fungal pathogens associated with virulence in addition to the direct effect on the pathogens growth. Abd-Alla et al. (57) reported that Thyme oil at 1.0 and 2.0 concentrations gave a non-activity of pectinase enzyme secreted by *Penicillium digitutum* and result low activity of enzyme activity (10.5 U/ml) at low tested concentration was used if compared with control treatment, while, olive water waste at 0.5 and 1.0 concentrations caused low activity of pectinase enzyme (9.7, 10.9U/ ml). They also reported that at high plant extract concentration (2.0) for all tested extracts caused non-effect of enzyme activity. Asoufi et al. (61) also reported that the cellulase and pectinase activities associated with the virulence of indigenous *Sclerotinia sclerotiorum* isolates in Jordan valley. Among the studied clove oil different concentrations, oil at MIC concentration was caused the highest reduction in cellulase and pectinase activity. It can be suggested that a part of the inhibitory effect of EOs against *P. digitatum* and *P. italicum* might be related to indirect influence on their infection process by affecting the activity of cell wall degrading enzymes which are produced by the fungal pathogens associated with virulence in addition to the direct effect on the pathogens growth.

V. Conclusion

In conclusion, EO of *S. aromaticum* have good effect *in vivo* assay. According to the surveys conducted, clove oil contains effective compounds antifungal against *P. digitatum* and *P. italicum*. These results indicate that clove oil after suitable formulation could be used for the control of citrus postharvest decay.

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