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BIO-CONTROL AND ULTRASTRUCTURE OF POST-HARVEST PATHOGENIC FUNGI OF APPLE FRUITS

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ABSTRACT

Post-harvest pathogenic fungi of fruits cause huge losses. Apples fruits are important for consumers. The antifungal activity of aqueous and ethanolic extracts of eleven medicinal plants at fives concentrations (0, 1, 3, 5, 10%; w/v) were tested against *Alternaria alplateternata*. Inhibition of fungal growth, along with the lower D_{50} values of the ethanolic extract of the tested plants compared to those of the aqueous extract exhibited more efficient antifungal activity.
The ultrastructure of the fungal hyphae without treatment (control) showed normal hyphae enclosed by a wall composed of three layers in which the middle layer is more electron- denser than the outer and inner layers. An intact plasma membrane was also observed. In addition, an electron-dense material was observed at the tip of the hyphae. The cytoplasm contained several organelles. On the other hand, the treated hyphae with the ethanolic extracts of the mixture of *E. citriodora* and *T. capitatus* exhibited many changes as noted in both T.S and L.S such as the increase of the electron density of the outer layer of the hyphal wall more than the control, also numerous big lipid bodies were almost occupied the cytoplasm. *Eucalyptus citridora* and *Thymus capitatus* had a potential as antifungal agent for biocontrol of post-harvest pathogenic fungi of tested apple fruits.

Keywords: Antifungal activity, Bio-control, *Alternaria*, Post-harvest Fungi, Apple Fruits, Medicinal Plants

INTRODUCTION

Post-harvest diseases are a significant issue for the agricultural sector, particularly in underdeveloped nations. According to estimates, post-harvest losses account for 10% to 40% of all agricultural product losses worldwide **(Enyiukwu et al. 2014; Sernait et al, 2020)**. The apple tree, *Malus* domestica Borkh.cv. Barkher, is a significant tree in every country and produces fruits that belong to the Rosaceae family. In wealthy nations, it was estimated that 20 to 25 percent of fruits were damaged after harvest **(Singh and Sharma, 2007)**. The fungal infections that cause diseases before and after harvest include *Botrytis cinerea*, *Alternaria alternata*, *Penicillium spp*., *Mucor spp*, and *Aspergillus spp* **(Wan and Tian, 2005)**.
Synthetic fungicides are the most popular technique of defending plants from fungal attack, but their overuse, coupled with high costs, the existence of residues, and the development of resistance, has had a detrimental impact on human health and the environment **(Paster and Bulllerman, 1988)**. The antibacterial effects of several therapeutic plant extracts could be beneficial. Alkaloids, tannins, saponins, glycosides, and flavonoids are potent secondary metabolites that these plants may possess that have antifungal properties **(Maswada and Elzaawely, 2013)**. One of the most prevalent diseases of various fruits is *Alternaria* **(Agrios, 1997)**. According to **Troncoso-Rojas and Tiznado-Hernández (2014)**, the *Alternaria alternata* (Kessler) genus is a significant disease that grows while quince fruits are stored, becomes visible during the marketing phase, and consequently causes significant post-harvest losses.
The major aim of this work was to conduct a survey of post-harvest harmful fungi. evaluation of some medicinal plants' aqueous preparations for eradicating these infections Additionally, Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) tests were done to determine whether this extract had any inhibitory effects or not.

MATERIAL AND METHODS

Plant samples collection

From New Damietta, Egypt's natural habitats, eleven different types of medicinal plants were collected (Table 1). These plants were deposited in the herbarium of the Botany and Microbiology Department, Faculty of Science, Damietta University, according to **Taeckholm (1974)** and **Boulos (2009)**.
The plant samples were washed with tap and then rinsed in distilled water and air dried at laboratory temperature (25-29 ºC) till they become crispy. Dried parts of the plants were ground using a blender and sieved to remove coarse particles and kept for further use.

Collection of spoiled fruits

The infected apple fruits (*M. domestica*) were collected from the local markets from Damietta Governorate. Spoiled fruits with fungal infections were chosen and transferred to laboratory in sterile plastic polyethylene bags and isolation of fungal pathogens was made.

Isolation and identification of fungal pathogens

According to the **Al-Hindi et al. (2011)** method, fungus pathogens were isolated from tthe fruits. The outermost portions of the diseased fruits were sliced into thin sections (2 mm in diameter), which were then surface sterilized in 0.1% mercuric chloride for 2-3 min before being rinsed three times with sterile distilled water. Sections were plated on water agar, and the mycelium was then transferred to sterile Potato Dextrose Agar (PDA) plates that were pre-mixed with penicillin (100,000 Units/L). The plates were incubated for 6-7 days at 27°C. Until pure cultures were produced, subcultures were created aseptically from the plates into comparable clean PDA plates and cultured under comparable circumstances. The isolated fungi were identified using macro- and microscopically methods. Macroscopic identification was based on mycelia color and culture development patterns. minuscule amounts of the fungus

Preparation of plant extracts

Each plant's used part weighed one gramme, which was then extracted on a shaker at 150 rpm for 24 hours at 25°C using either distilled water or 95% ethanol in a 10 ml volume of solvent. The mixture was centrifuged twice at 4000 rpm for 10 minutes after being filtered using sterile Whitman filter paper No. 1. For the ethanol extract, the dry residue was re-suspended in half of the original volume after the ethanol had completely evaporated, while the aqueous extract was concentrated into half of the original volume and DMSO was added (dimethyl sulfide). In order to prevent contamination, the supernatant was put into conical flasks and sterilized by being placed for 10 minutes in a digital water bath at 100°C. Various volumes (0, 1, 3, and 5)

Fungi as bio-control agents

Penicillium roqueforti, which was isolated from Roquefort cheese, and three entophytic fungi, *Chaetomium globosum*, *Emericella nidulans*, and *Sordaria fimicola*, which were kindly provided by the Arab Society for Fungal Conservation's Fungarium at Suez Canal University's Faculty of Science in

Ismaelia, Egypt, were among the five fungal species tested for their antifungal activity *Trichoderma herzianum* was graciously provided by Plant Pathology Department, Faculty of Agriculture, ,Mansoura University.

Table 1 List of plant species used for aqueous and ethanolic of extracts

Species	Common name	Family	Used Part
Moringa oleifera Lam.	Moringa	Moringaceae	Seeds
Ziziphus spina-christi (L.)	Sidr	Rhamnaceae	Leaves
Melia azedarach (L.)	Chinaberry tree	Meliaceae	Leaves
Nicotiana glauca Graham	Tobacco tree	Solannceae	Fruits
Cyperus rotundus (L.)	Purple nutsedge	Cyperaceae	Rhizomes
Schinus terebinthiofolius Raddi	Brazilian pepper	Anacardiaceae	Seeds
Lantana camara (L.)	Tickberry wild sage	Verbenaceae	Leaves
Zygophylleum aegyptium (L.)	Rotrate	Zygophyllaceae	Shoot
Delonix regia (Boj. ex Hook.) Raf	Royal poincina	Fabaceae	Bark
Eucalyptus citroidora L'Hér	Myrtle	Myrtaceaea	Shoot
Thymus capitatus (L.) Hoffmgg. et Link	Thyme	Lamiaceae	Shoot

Mixture of the best bio-control agents

E. citrodora and T. capitatus, the two most effective bio-control agents discovered, were put to the test singly or in combination; in the latter case, the two extracts were either given in half dose or full dose. The purpose of this experiment was to determine whether the combined effect of the two extracts is synergistic, additive, or antagonistic. It was decided to investigate the antifungal activity of five concentrations: 0, 1, 2, 3, and 5%.

Evaluation of antifungal activity *In Vitro*

According to **Baka (2014)**, the food poisoning technique was applied with modificatin. The produced bio-control agents in various doses were tested against an isolate of *Alternaria alternata* found in apple fruits. For *Alternaria alternata*, the solidified extract-amended media in the Petri plates were inoculated, each alone at the centre with 7 mm inoculums disc of each tested fungus. The fungal growth's diameter (in cm) and the percentage of inhibition of fungal growth compared to the control were assessed. The fatal concentration that inhibits fungal growth by 50% was used to determine the relative efficacy of both plant extracts and microbial filtrates (LC50).

Antifungal activity of *In Vivo*

With slight adjustments, **Badawy et al. (2012)**'s method for evaluating the antifungal activity in vivo was used. Fresh apple fruits that were in good health were cleaned with tap water before being sterilised by immersion in 70% ethanol for 1 minute, followed by three times in sterile distilled water, and then allowed to dry. A 0.7 cm disc of the margins of recently developed *A. alternata* that was 7 days old and freshly grown was used to inoculate each treatment. Fruits that were roughly equivalent in weight and volume were randomly divided into 6 equal groups, each with 3 fruits.
After 24 hours of *A. alternata* infection, six treatments were carried out as follows: spraying apple fruits with ethanol extracts of *T. capitatus* only at a concentration of 5%, *E. citriodora* only at a concentration of 10%, and a mixture of ethanol extracts of both *E. citriodora* and *T. capitatus* at a concentration of 2%. Two control sets were also prepared: a healthy set as a negative control, an infected
Fruits that had been treated were placed in plastic bags and incubated for 28 days at 25°C and >85% RH. Every week, the effectiveness of the therapy was evaluated. There were three trials with three fruits each in each replication. By measuring the black zone's diameter and calculating the percentage of disease incidence compared to the infected control, researchers were able to assess the effectiveness of the treatment. Alternaria infection manifests as a black zone around the infected location.

The inhibitory effect by electron microscopy

Scanning Electron Microscopy (SEM)

According to **Park et al., (2009)** the samples were prepared for SEM observation. Fungal hyphae prior to sporulation were handled in the following manner in order to examine the impact of plant extract on the hyphae of both *Alternaria alternata* using SEM. First, colonies on both control and treatment plates had hyphal discs (diameter 1 cm) cut from the actively expanding margin. These discs were then fixed for two hours at room temperature with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH7.2). The fixed hyphal discs were then passed through a graduated ethanol series of 70, 80, and 90%, once for ten minutes at each concentration, before being washed twice for ten minutes each in the same buffer (three times; 30 min at each concentration). The samples were critical point dried with CO2 in a Polaron CPD 7501 critical point drying machine (VG Microtech, East Grinstead, UK). Then, using a sputter coater system in a high-vacuum chamber (Polaron SC7620, VG Microtech), the fixed material was mounted on stubs using double-sided carbon tape and coated with gold/palladium for 150 s at 9 mA. Using a JEOL model JSM-6510LV scanning electron microscope (JEOL Ltd., Tokyo, Japan), the samples were examined and digital pictures were recorded.

Transmission Electron Microscopy (TEM)

samples (1 mm3) of a fungal culture were evaluated and processed by TEM using Hayat's method (2000) using extracts of a combination of *Eucalyptus citriodora* and *Thymus capitatus* (0.3%, 1.5%) for *A. alternata*. The samples were initially submerged for two hours at four degrees Celsius in a solution of 3% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.0. They were then washed in the same buffer and post-fixed in 1% (w/v) OsO4. They were then imbedded in Spurr's resin after being dehydrated using a graduated series of ethanol solutions. On Formvar-coated copper grids, ultrathin slices were obtained, stained with uranyl acetate (UA) and lead citrate (LC), and then analysed using a JEOL (JEM-2100) transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Statistical analysis

The inhibition zone of fungal growth estimated as percentage of the control was arcsine transformed before performing statistical analysis to ensure homogeneity of variance. Data were analyzed using SPSS version 22. Main separation was performed using the Duncan's multiple range tests at $p<0.05$.

RESULTS AND DISCUSSION

Isolation and identification of fungal pathogens

Three fungal infections were found to be infecting the apple fruit in the local markets of New Damietta, according to the preliminary examination into the occurrence of post-harvest deterioration of the fruit (Table 2). The following isolated fungi attacked the apple fruits used for testing. *Aspergillus niger*, *Penicillium expansum*, and *Alternaria alternata* were discovered to be the three most prevalent fungus species (Table 2).

Table 2 Number of colonies and the occurrence of isolated fungal species from spoiled fruits of apple. Each value is the mean of 5 replicates ± SE.

Disease	Isolated fungus	No. of colonies	Relative occurrence (% of total)
Alternaria rot	*Alternaria alternata* (Fr.)	13	4.48
Blue mold rot	Keissl	10	3.44
Black mold rot	*Penicillium expansum* Link *Aspergillus niger* van Tieghem	9	3.1

Antifungal activity of plant extracts

Five different concentrations (0, 1, 3, and 10%) of aqueous and ethanol extracts from eleven different plant species were used to investigate A's susceptibility to fungus development.*A alternata*. Table 3 showed that plant species and extract type had an impact on fungal growth as well as a highly significant variance in fungus susceptibility.

Table 3 Four-way ANOVA of the effect of the main factors (plant species, type of extract and concentration of the extract) and their interactions on the inhibition percentage of *Alternaria alternata.*

Source of variation	Df	F	Significant
Plant species	10	893.187	0.000
Extract	1	2101.675	0.000
Conc.	4	4044.657	0.000
Fungus * Plant species	10	163.753	0.000
Fungus * Extract	1	38.524	0.000
Fungus * Conc.	4	77.348	0.000
Plant species * Extract	10	70.280	0.000
Plant species * Conc.	40	114.209	0.000
Extract * Conc.	4	158.929	0.000
Fungus * Plant species * Extract	10	33.380	0.000
Fungus * Plant species * Conc.	40	17.574	0.000
Fungus * Extract * Conc.	4	6.930	0.000
Plant species * Extract * Conc.	40	15.317	0.000
Fungus * Plant species * Extract * Conc.	40	8.586	0.000

All of the examined plants' aqueous and ethanol extracts had an impact on A. alternata, although the ethanol extract had a stronger inhibitory effect on the test fungus' development than the water extract did. *Thymus capitatus* had the strongest inhibitory effect out of the 11 plant species, with average inhibition (for both aqueous and ethanol extracts) of 41% and 53%, respectively. With an average inhibition of 28.4% and 42%, respectively, compared to control, *Eucalyptus citriodora* placed second. *Nicotiana glauca*, on the other hand, demonstrated the least efficient one, with average inhibition of 6% and 4.8%, respectively. Depending on the type of extract and fungus, the remaining eight plant species displayed mild inhibition of varying degrees and ranks. The increase of the concentration of the plant extract led to a progressive inhibition of fungal growth; yet the concentration-response relationship differed in test fungus according to plant species and type of extract. For example, a saturable trend, with variable magnitude depending on the fungus, plant species and the extract was noticed in terms of both extracts of *Moringa oleifera*, *Ziziphus spina-christi* and *Delonix regia* on test fungus. Furthermore, both extracts of *Melia azedarach* on *A. alternata* (Figure 1).

In example A, the ethanol extract showed this saturable trend more frequently than the aqueous extract. Additionally, the test fungus was solely treated with aqueous extracts of *Cyperus rotundus* and *Lantana camara*. At the concentration where the difference between the two extracts is the greatest, the ethanol extract of the studied plant species had a higher inhibitory impact on fungal growth than the aqueous extract. In the case of the effects of *Melia azedarach*, *Schinus terebinthifolius*, *Eucalyptus citriodora*, and *Delonix regia* on test fungus, this difference increased with the concentration of plant extract. *Lantana camara* and *Thymus capitatus* on and *Nicotiana glauca* and *Zygophyllum aegyptium* on both displayed a pattern of maximal difference in the moderate quantities of the extract.

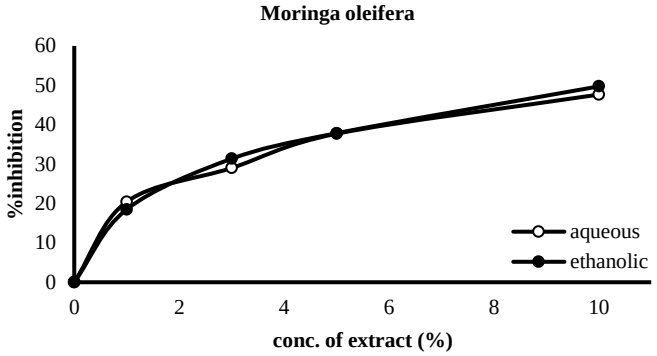
Table 4 Effect of plant species and type of extract on the growth of *A. alternata.* Each value is the mean of 5 replicates ± SE. Means with common letters are not significantly different at p<0.05.

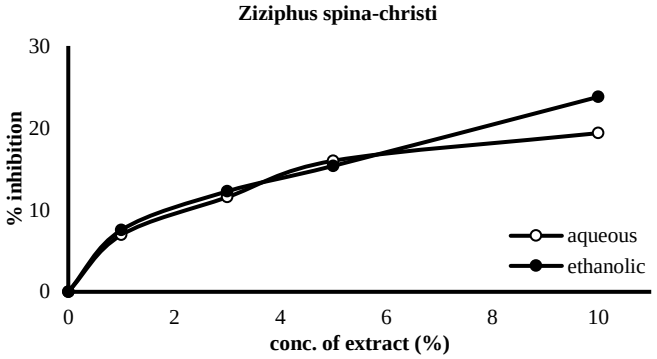
Scientific name	Inhibition of fungal growth (% of control)	
	Aqueous extract	Ethanolic extract
Nicotina glauca	5.23 ± 1.31^{a}	6.89 ± 1.76^{a}
Schinus terebinthiofolius	6.18 ± 1.44^{ab}	12.4 ± 2.25^{bcd}
Lantana camara	6.23 ± 2.22^{abc}	20.4 ± 3.78^{e}
Delonix regia	6.27 ± 1.21^{abcd}	10.7 ± 1.85^{b}
Ziziphus spina-christi	10.8 ± 1.85^{e}	11.8 ± 2.13^{bc}
Melia azedarach	12.1 ± 3.64^{ef}	34.5 ± 6.03^{i}
Eucalyptus citroidora	15.0 ± 3.86^{g}	41.8 ± 8.32^{j}
Zygophylleum aegyptium	18.2 ± 4.26^{h}	28.6 ± 5.04^{gh}
Cyperus rotundus	19.1 ± 3.65^{hi}	22.6 ± 4.45^{f}
Moringa oleifera	27.0 ± 4.37^{j}	27.5 ± 4.58^{g}
Thymus capitatus	35.6 ± 8.15^{k}	46.8 ± 9.37^{k}
Total of all species	14.7 ± 1.33^{a}	24.0 ± 1.79^{c}
Total of *A. alternata*	19.34 ± 1.14^{a}	

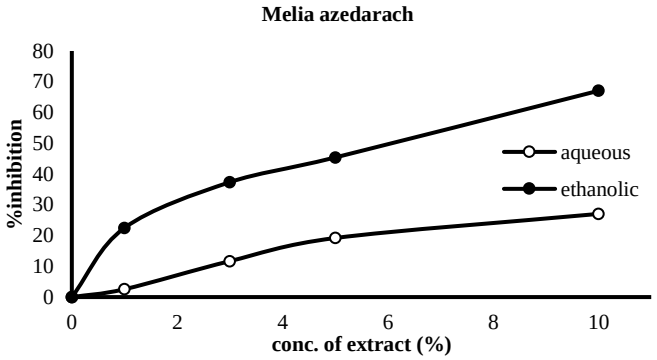
The concentration-response correlations of Figure 1 were used to compute the relative potency of the aqueous and ethanol extracts of the studied plant species on fungus growth in Table 5. In general, the LC50 value of the various species' ethanol extracts was significantly lower than that of the aqueous extract. The amount of fungal growth inhibition caused by the aqueous extract in the majority of *A. alternata* was too small to allow for the determination of the LC50. Only the most potent plannt species (*Thymus capitatus*), *Eucalyptus citriodora*, and *Schinus terebinthifolius'* LC50 of the aqueous extract could be estimated.

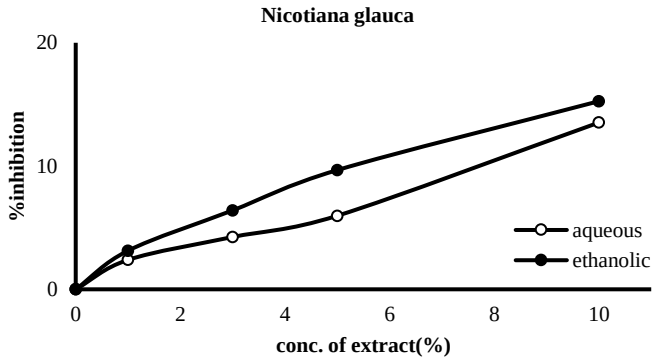
Table 5 Lethal concentration of plant extracts leading to 50% inhibition of fungal growth (LC_{50}).

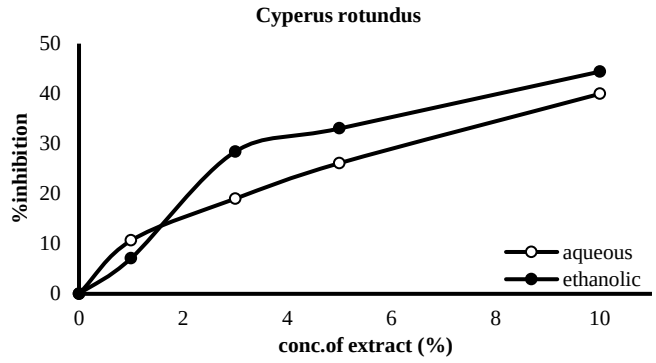
Plant species	LC_{50} (%) *Alternaria alternata*	
	Aqueous	Ethanolic
Nicotiana glauca	-	-
Schinus terebinthiofolius	-	-
Lantana camara	-	-
Delonix regia	-	-
Ziziphus spina-christi	-	-
Melia azedarach	-	6.2
Eucalyptus citriodora	-	3.7
Zygophyllum aegyptium	-	8.3
Cyperus rotundus	-	-
Moringa oleifera	-	10
Thymus capitatus	4.7	2.7











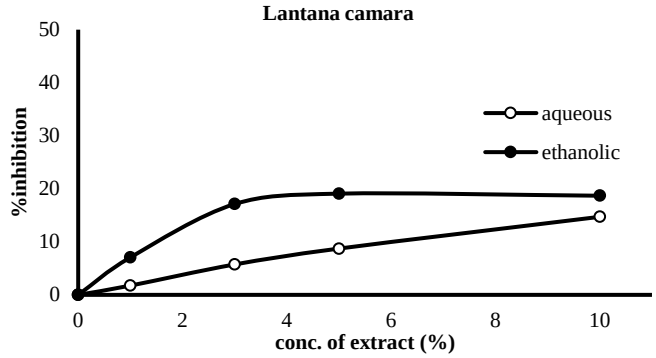
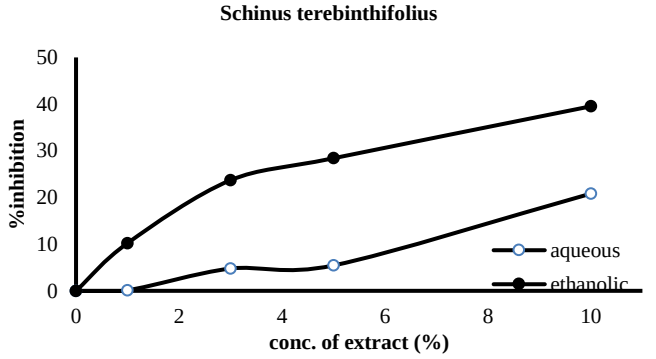
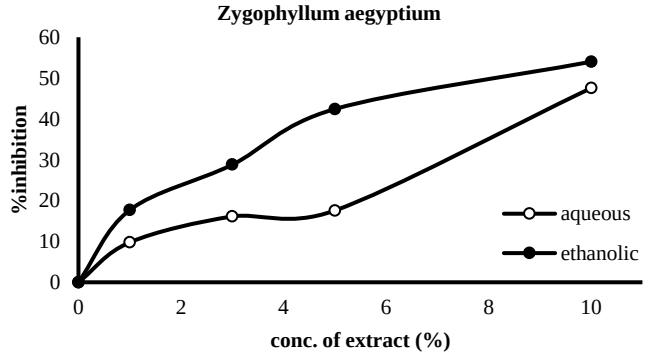
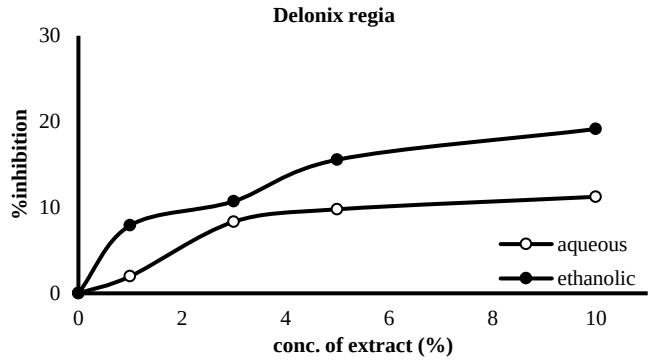
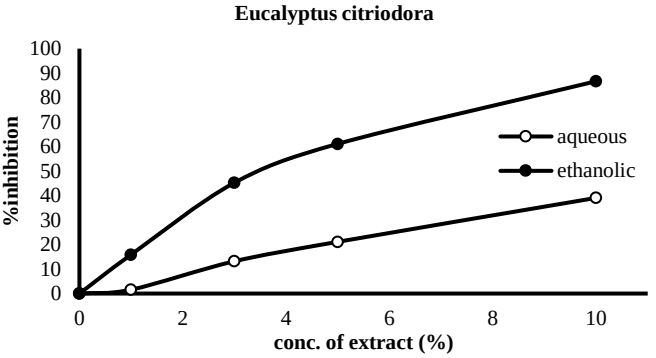


Figure 1.Effect of aqueous and ethanolic plant extracts of *Moringa oleifera, Ziziphus spina-christi*, *Melia azedarach*, *Nicotiana glauca*, *Cyperus rotundus* and *Lantana camara* on growth of *A. alternata*. Each value is the mean of three replicates ± S.E.









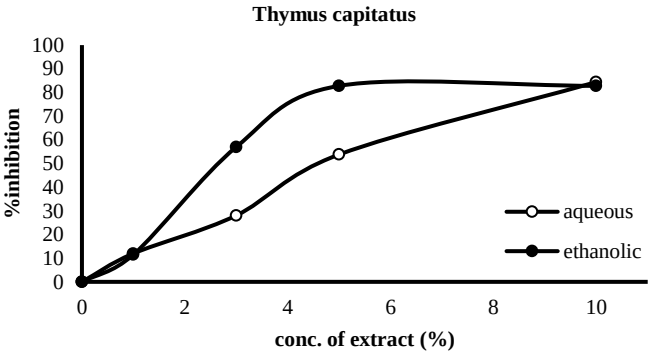


Figure 1 Cont. Effect of aqueous and ethanolic extracts of *Schinus terebinthifolius, Zygophyllum aegyptium*, *Delonix regia*, *Eucalyptus citriodora* and *Thymus capitatus* on growth of *A. alternata*. Each value is the mean of three replicates ± S.E.

***In Vivo* activity of plant extracts on fruits**

The most promising *In Vitro* results were used to guide the use of plant extracts in vivo, which was done to see whether there was a difference between the two types of research. Following infection by 24 hours, six treatments were administered, each lasting for 21 days, or until deterioration of the whole fruits in the infected control was noticed. According to Table (6), the type of treatment, the length of storage, and their interaction all had a highly significant impact on the fungal susceptibility.

Table 6 Two-way ANOVA of the effect of the main factors (Treatment, and storage period) and their interactions on the percentage inhibition of *Alternaria alternata*

Source of variation	df	F	Sig.
Treatment	4	9346.372	0.000
Time	3	11285.335	0.000
Treatment* Time	4	2217.396	0.000

The diameter of the infected area, which is an indication to disease spreading, increased by extending of storage period. Furthermore, the results indicated that all the treatments significantly decreased the infected area during the storage period (21 days), compared with the untreated control.

Trails of spraying the apple fruits with fungicide, *E. citriodora* only, *T. capitatus* only and the mixture of both showed a significant reduction in the infection of the fruit with different magnitude (Figure 2).

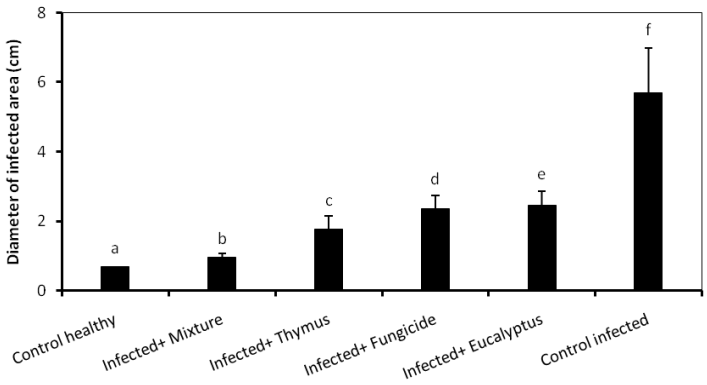


Figure 2 Activity of several antifungal agents in control of the process of infection of *Alternaria* rot in apple fruits. Each value is the mean of 12 measurements taken weekly over a period of 21 days ± SE. Means with common letters are not significantly different at p≤0.05.

It was clear that, at the end of the first week, it was noticed that the fungal infection was negligible in case of spraying by the mixture and *T. capitatus* only, after, 21 days the infection was 12% from the infected control by the mixture and 30% by *T. capitatus* (Figure 3) and (Plates 1, 2 and 3).

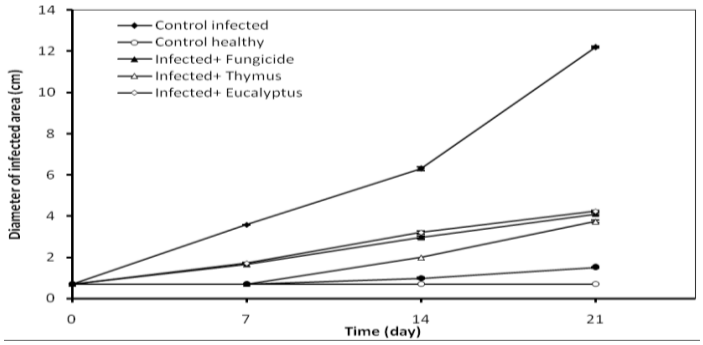


Figure 3 Time course of infection progress of apple fruits by *A. alternata* in response to application of several antifungal agents. Each value is the mean of 3 replicates ± SE.

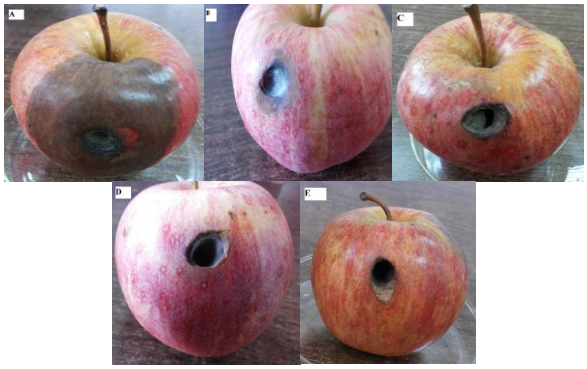


Plate 1 Apples previously infected with *A.alternata* after 7 days of treatments as A: infected control, B: treatment with *E.citriodora*, C: treatment with fungicide only, D: treatment with *T.capitatus* only and E: treatment with mixture of both *E.citriodora* and *T.capitatus*.

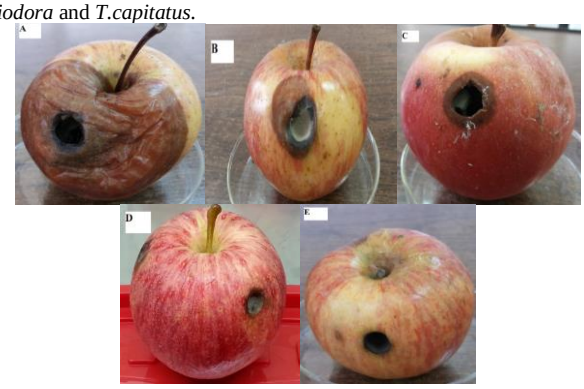


Plate 2 Aples previously infected with *A.alternata* after 14 days of treatments as A: infected control, B: treatment with *E.citriodora* only,C: treatment with fungicide only, D: treatment with *T.capitatus* only and E: treatment with mixture of both *E.citriodora* and *T.capitatus*.

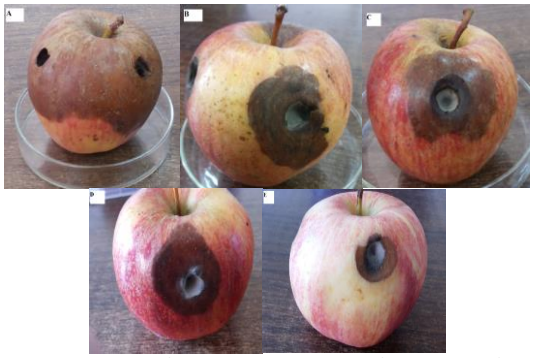
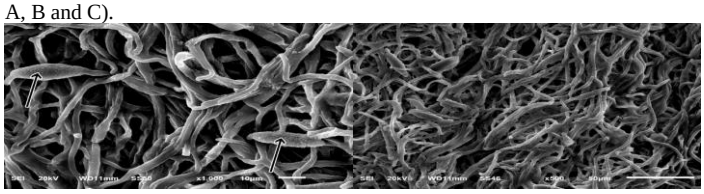
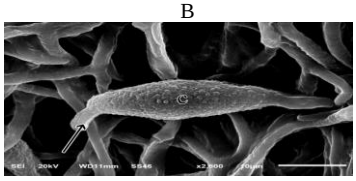


Plate 3 Apples previously infected with *A.alternata* after 21 days of treatments as A: infected control, B: treatment with *E.citriodora* only, C: treatment with fungicide only, D: treatment with *T.capitatus* only and E: treatment with mixture of both *E.citriodora* and *T.capitatus*.**Electron Microscopy**

Inhibitory effect of the mixture of *E. citriodora* and *T. capitatus* ethanolic extracts on *A. alternata*. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. Plates (4 A and B) showed that the untreated *A. alternata* hyphae as observed by SEM appeared normal, with no deformity and normal conidia were observed with verruculose surface ornamentation. A filiform peak with smooth surface was also noticed (Plates 4B and C). In contrast, *A. alternata* hyphae treated with ethanolic extract of the mixture of both *E. citriodora* and *T.capitatus* revealed a strong detrimental effect of the extract on both the hyphal and spore morphology. Deformity in hyphae was observed in the form of flattened hyphae, in addition affected and abnormal spores were noted (Plates 5 A, B and C). Furthermore, the ultrastructure of *A. alternata* hyphae and conidia without treatment (control) as observed by TEM showed normal hyphae enclosed by a wall composed of three layers in which the middle layer is more electron-denser than the outer and inner layers. An intact plasma membrane was also observed. In addition, an electron-dense material was observed at the tip of the hyphae. The cytoplasm contained several organelles such as the nucleus, lipid bodies and vacuoles (Plates 6 A and B). On the other hand, the hyphae of *A. alternata* treated with the ethanolic extracts of the mixture of *E. citriodora* and *T. capitatus* exhibited many dramatic changes as noted in both T.S and L.S such as the increase in the electron density of the outer layer of the hyphal wall more than the control, also numerous big lipid bodies were almost occupied the cytoplasm. In addition, vacuoles were filled with small particles were also observed (Plates 7 A, B and C).

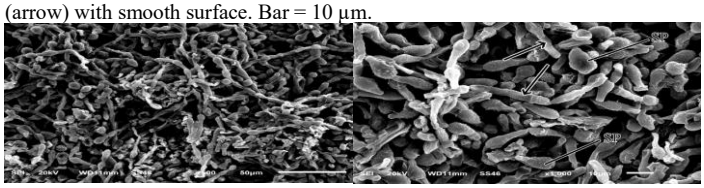


A B

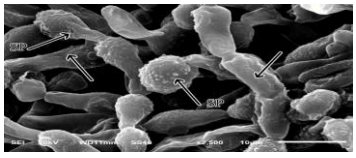


C

Plate 4 SEM micrographs showing untreated hyphae (control) of *Alternaria alternata*. **(A).** Low magnification of normal hyphae. No deformity was observed. Bar = 50 µm. **(B)**. A magnified part of A shownig normal hyphae and conidia (arrows). Bar = 10 µm. **(C)**. A magnified part of B showing normal hyphae. Note a conidium (C) with verruculose surface ornamentation. Note also filiform beak (arrow) with smooth surface. Bar = 10 µm.



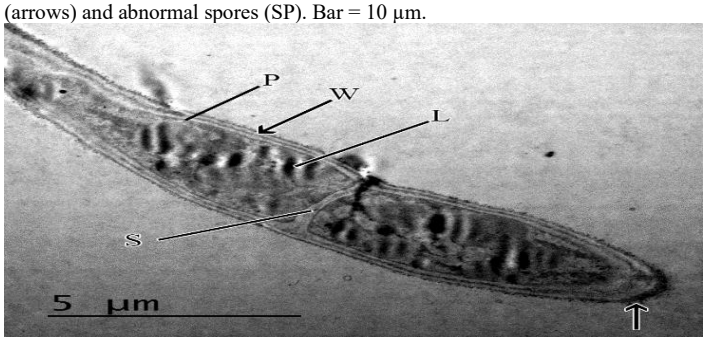
A B



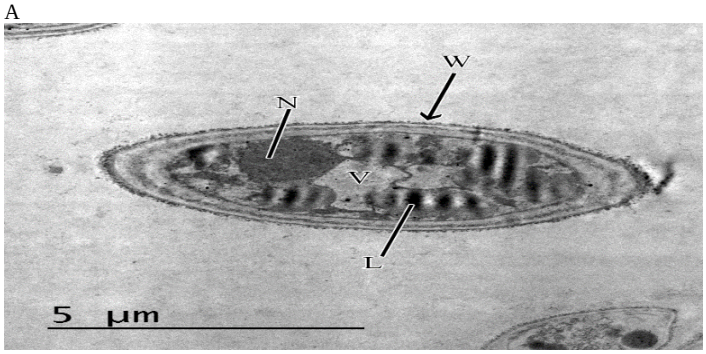
C

Plate 5 SEM micrographs showing treated hyphae of *Alternaria alternata* by concentration 1.5 % of mixture of both *Eucalyptus citriodora* and *Thymus capitatus*. **(A).** Low magnification showing deformed hyphae and spores. Bar = 50

µm. **(B).** A magnified part of A showing deformed hyphae (arrows) and affected spores (SP). Bar = 10 µm. **(C).** A magnified part of B showing flattened hyphae (arrows) and abnormal spores (SP). Bar = 10 µm.

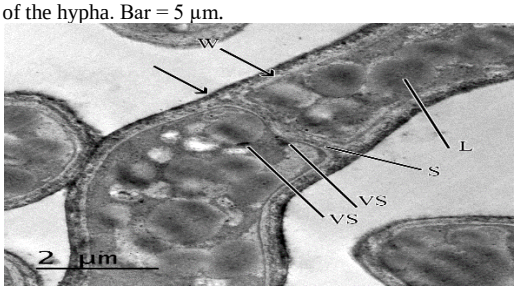


A

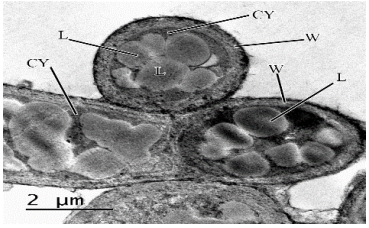


B

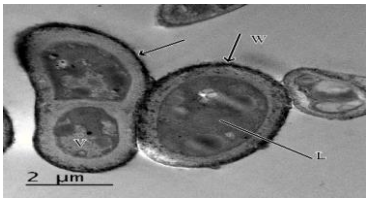
Plate 6 EM micrographs of *Alternaria alternata* hyphae. **A** and **B.** showing untreated (control) normal hypha. **(A)**. L. S. of a hypha enclosed by a wall (W) and intact plasma membrane (P). The hyphal cell contains lipid bodies (L). Note the septum (S). Note also the electron-dense material at the tip of the hypha (arrow). Bar = 5 µm. **(B).** Normal hypha. Note that the hyphal wall (W) is composed of three layers, the middle layer is more electron-denser than the outer and inner layers. Note also the nucleus (N), lipid bodies (L) and vacuoles (V) inside the cytoplasm of the hypha. Bar = 5 µm.



A



B



C

Plate 7 TEM micrographs of *Alternaria alternata* hyphae. **A, B** and **C** showing treated hyphae with concentration 1.5% of mixture of both *Eucalyptus citriodora* and *Thymus capitatus*. **(A)**. L. S. of a hypha showing an electron-dense layer (arrows) is deposited on the outer surface of hyphal wall (W). Note that the hyphal wall became more electron-denser than that of the control. Note numerous lipid bodies (L) and electron-dense vesicles (VS) inside the hyphal cytoplasm. A septum (S) can also be seen. Bar = 2 µm. **(B).** Showing two hyphae (T. S. and L.S). Note granulated cell walls (W) and electron-dense layer (arrows) deposited on the outer surface of the wall. Note also big lipid bodies (L) are almost occupy the electron-dense cytoplasm (CY). A hyphal septum can also be seen. Bar = 2 µm. **(C).** Showing two hyphae with thick granulated wall (W). Note an electron-dense layer deposited on the outer surface of the wall (arrows). Note also lipid bodies (L) and vacuoles (V) filled with small particles. Bar = 2 µm.

DISCUSSION

Post-harvest loss of fruits due to the fungal infection is considered a severe global problem in particular the developing countries **(Baka *et al*., 2015)**

In support to the present results, **Amiri and Bompeix (2005**) reported numerous *Penicillium* spp. associated with post-harvest fruit spoilage. Of these species, *Penicillium expansum*, *P. digitatum*, *P. crustosum*, and *P. solitum* had been recognized as the most frequent causative agents of apple spoilage **(Kim *et al*., 2005)**. *Alternaria* spp. is also a major fungal pathogen, which infect various local fruits such as apple.

Several factors control fruit invasion by fungal pathogens especially after harvest. The traditional measure to limit this problem is the use of chemical fungicides to increase the shelf life time of fruits. But, due to their dangerous consequences on human health, biological control of fruit spoilage is the current trend to solve this problem **(Tsoho, 2004; Enyiukwu *et al*., 2014)**. Many studies had reported the use of bio-control agents for post-harvest fruits diseases (**Manjula *et al*., 2005**).

The eleven plant species tested in the present study exhibited diverse antifungal activities which varied according to the fungal species, plant species and type of solvent. In general, *Eucalyptus citriodora* and *Thymus capitatus* exhibited the most effective effect against *A. alternata* whereas *Nicotiana glauca* was the least effective.

The differences in toxicity recorded between extracts are likely to be influenced by several factors such as the method of extraction, type of extracting solvent (the efficiency of the solvent to extract bioactive substances, variation in quantity of the active constituents and the difference in bioactive constituents between plants. The composition of bioactive compounds in turn vary from species to species, climatic conditions, and the physiological stage of plant development **(Pandey, 2007)**. In this study, ethanol had a greater capability for the extraction of active substances from tested plants than did water which is in agreement with the results obtained by **Stephan *et al.* (2005)** and the postulation of **Pandey (2007)** that the type of solvent and the ability of the solvent in extraction affect the inhibitory activity of the plant extracts.

The antifungal activity of *Eucalyptus citroidora* and *Thymus capitatus* can be related to the unique secondary bioactive compounds produced by the two species. In this respect, **Lee (2007)** reported the occurrence of several active antifungal compounds, like citronellal and isopulegol in *Eycalyptus citriodora* essential oil and ρ-cymene, γ-terpinene and thymol in *Thymus capitatus.* These active substances, because of their considerable lipophilicity, are subjected to extraction by ethanol to a greater extent than by water, which can partially explain the stronger antifungal efficiency of the ethanolic extract.

In agreement with **Shagal *et al*. (2012)** reported that aqueous extracts and ethanolic of *Eucalyptus* spp. share some components, but differ in others. Both the aqueous and ethanolic extracts contain high amounts of saponins, while the aqueous extract contains tannins, saponins, glycosides, steroids and anthraquinones but no alkaloids, flavonoids and terpenoids; however, the ethanolic extract contains tannins and steroids but no glycosides and anthraquinone. The presence of these phytochemicals in *Eucalyptus* spp. justifies manipulation of the plant in the management and bio control of various diseases or spoilage fruits.

Likewise, it had been reported that *Thymus capitatus* has a powerful antifungal activity by virtue of its high content of a wide range of bioactive compounds like essential oils which can act as biogenetic precursors of phenolic compounds such as ρ-cimene, γ-terpinene, and β-cariophyllene; in addition to its high content of phenols such as carvacrol **(Mariateresa et *al*., 2013)**. The mechanism of action of carvacrol and thymol as fungicides appears to be through the inhibition of ergosterol biosynthesis and disruption of membrane integrity of the fungus as reported by **Bouchra *et al*. (2003)** and **Ahmed *et al*. (2011)**.

In addition, phytochemical screening of *Thymus capitatus* revealed the presence of saponins, resins, flavonoids, essential and fixed oils; compounds of profound inhibitory effect fungi **(Kandil *et al*., 1994)**. Effective bioefficiency of thyme essential oils against *B. cinerea* as post-harvest fungi on apple fruits (**Banani *et al*.**, 2018).

Seed extract of *Moringa oleifera* showed pronounced inhibition of linear growth of *Alternaria alternata*, *A. solani*, *Fusarium oxysporum*, *F. solani* and *F. chlamydosporum*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina* **(Anwar *et al*., 2015)**.

The inhibitory activity of aqueous extracts of *Cyperus rotundus* rhizomes, Melia azedrach leaves and Lantana camara leaves against *Alternaria brassicae*. aqueous extracts of ginger, turmeric, and garlic have been effective in reducing growth of *A. alternata* growth and disease.The major chemical components of *C. rotundus* are essential oils, flavonoids, terpenoids, sesquiterpenes, acopaene, cyprotene, cyperene, aselinene, rotundene, valencene, cyperol, gurjunene, trans-calamenene, decadinene, gcalacorene, cadalene, amuurolene, gmuurolene, cyperotundone, mustakone, isocyperol and acyperone **(Imam *et al*. 2014)**

The present work revealed that *Nicotiana gluaca* exhibited the least antifungal activity against *A. alternata*. The low activity of *N. gluaca* was well-demonstrated by **Ochoa Fuentes *et al*. (2012)**.The antifungal activity of plant extracts may be related to the presence of many bioactive compounds such as flavonoids,

terpenoids, alkaloids, tannins, steroids, glycosides and phenolics The function of phenolics is due to their amphipathicity which facilitate their interactions with bio-membrane and thus induce the antimicrobial activity. The antifungal activity of alkaloids was already reported in several studies including different plant extracts **(Veldhuzien *et al.* , 2006)).**

Results indicated that the values of LC_{50} of the ethanolic extracts are more frequent and of lower magnitude than those of the aqueous extracts and that *Eucalyptus citriodora* and *Thymus capitatus* yielded the lowest LC_{50} among the studied species. Normally, the lower the LC_{50} the more potent is the antifungal activity of the extract; and whenever an extract has no value for LC_{50} this means that the antifungal activity of this extract is too weak to the extent that the relative inhibition of fungal growth never attained 50% even at the top concentration used (10% w/v).

The difference among pathogens in response to treatment with plant extracts may be attributed to their genetic or physiological differences **Shaukat *et al*. (1983)**. The present work revealed marked sensitivity of *A. alternata*. Similary, **Hadizadeh *et al*. (2009**) reported that *Ziziphus spina-christi* ethanolic extracts were effective on *A. alternata*.

In Vitro application of plant extracts against Post-harvest pathogenic fungi of fruits their result revealed that most of the concentrations of the extracts that were investigated were not efficient enough when assessed in the post-harvest assay, despite having demonstrated a high in vitro antifungal effect. Potentials of some plant extract was evaluated as biocontro against post-harvest fungi **(Sernit *et al,* 2020**)

Several *In Vitro* and *In Vivo* investigations suggested that the essential oils and plant extracts could be used as an effective antifungal bio-control agent against many phytopathogenic fungi **(El- Mohamedy *et al*., 2013)**. In line with this, many plants synthesize substances that are useful for controlling the growth of microorganisms with the advantage of being non-phytotoxic, systemic and biodegradable **(Kumari *et al*., 2015)**.

The severity of disease in *In Vivo* can be reduced by combination of both chemical and bio-control agents as stated by **Droby *et al* (1998)** who found that tests in citrus packinghouses indicated that bio-control alone cannot provide adequate control and must be combined with diluted fungicides or other methods to control post-harvest infection.

The *In Vivo* test to control *Alternaria* rot disease in apple, demonstrated that the infection was in the form of lesions. This is in agreement with **Vilanova *et al.* (2012)** who found that infection was in the form of lesions on fruits infected by *Penicillium digitatum* and lesions were not developed beyond the initial infection site. It was clear that all the treatments used in the present work showed a significant reduction in the infection of the fruits with different magnitude without completely destroying the pathogen. It is clear that, in general, the mixture of *E. citriodora* and *T. capitatus* was more potent. As far as the author is aware very little is known about the use of *Penicillium roqueforti* as a bio-control agent for the fungal post-harvest diseases of fruits. The antifungal activity of *P. roqueforti* against *A. alternata* explained on the basis of its ability to produce volatile terpenes such as, limonene, β-elemene and β-caryophyllene RI1494 **(Baka , 2015)**.

When the antifungal activity of ethanolic extracts of *E. citriodora* and *T. capitatus* were tested against *A. alternata*, either solitary or in combination at half or full dose, it was noticed that the full dose was more potent than the half one on *A. alternata*. Results indicated that the effect of the mixture of *E. citriodora* and *T. capitatus* was more effective on *A. alternata*.

SEM and TEM investigation revealed a strong detrimental effect of plant extracts on both fungal cells. Several studies demonstrated the effects of plant extracts on the ultrastructure of plant pathogenic fungi **(Soylu *et al.* 2006; Baka, 2014).**

The deformity of mycellium was observed in both fungi in the form of flattened hyphae particularly in *A. alternata* where deformity in spores was also observed. **Pârvu *et al*. (2010)** attributed such alterations and modifications to the enzymatic reactions regulating cell wall synthesis. Furthermore, an increase in the density of the outer layer of the hyphal cell wall and appearance of numerous lipid bodies occupying the cytoplasm was observed in *A. alternata*. In addition, vacuoles filled with small particles were observed, but highly vacuolation and collapsed cytoplasm were observed in *A. alternata*. These findings are in agreement with those of **Baka (2014)** who referred to the inhibitory activity of plant extracts on the late blight disease of tomato to the reduction of mycelial growth and inhibition in spore germination of the pathogen in varying degrees and to the increase of vacuolization and lipid contents with consequent reduction of cytoplasm and alteration of cell wall and plasma lemma.

The examination made with SEM are in accordance with those of **Soylu *et al.* (2006)** who verified that plant extracts caused the morphological alterations on the fungal hyphae of other plant pathogenic fungi. Generally, changes in the morphology of the hyphae could also be due to the loss of integrity of the cell wall. Consequently, plasma membrane permeability might be affected, which could explain the changes in the morphology and size of the internal organelles as suggested by **Nakamura *et al.* (2007)**.

Several studies attributed these abnormalities to the phenolic compounds since the amphipathicity of these compounds can explain their interactions with bio-membrane and thus lead to the antimicrobial activity **(Veldhuizen *et al*., 2006)**. Possible action mechanisms by which mycelial growth may be reduced or totally inhibited have been proposed. It is commonly accepted that the toxic effects of essential oils components of extracts on the functionality and structure of the cell membrane are responsible for the aforesaid activity **(Sikkema *et al*., 1995)**.

Omidbeygi *et al*. (2007) suggested that components of the essential oils and extracts cross the cell membrane, interacting with the enzymes and proteins of the membrane, and producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death.

In line with this, **Soylu *et al*. (2006)** and **Cristani *et al*. (2007)** reported that such antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. Similarily, **Lucini *et al*. (2006)** indicated that mycelial growth inhibition is caused by the monoterpenes present in essential oils. These components would increase the concentration of lipidic peroxides such as hydroxyl, alkoxyl and alkoperoxyl radicals and so bring about cell death.

The cyto-morphological modifications, particularly, the accumulation of lipid bodies and thickening of cell wall induced by the mixture of *T. capitatus* and *E. citriodora* extracts, is similar to those produced by some synthetic fungicides and other plant extracts **(Bianchi *et al*., 1997)**. Increase in the size and number of vacuols along with other alternations might also, in turn, modify the activity of membrane enzymes involved in the formation of cell wall, causing anomalous development. However, the response to extracts seems to be different depending on the target agent used and this was clear *A. alternata.*

CONCLUSION

In conclusion, the antifungal activity of aqueous and ethanollic extracts of some medicinal plants was tested. Out of the tested medicinal plants the *T. capitatus* and *E. citriodora* where the most effective in the antifungal test. The obtained results revealed that this technique is eco-friendly and cheap in cost might be applied to control the post-harvest pathogenic fungi of apple.

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