



## Efficiency of Essential Oils as Antifungal Agents against *Aspergillus fumigatus* KY026061 causing Allergic Bronchopulmonary Aspergillosis (ABPA)

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**A**LLERGIC bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis are often found in severely immuno-suppressed patients and are characterized by invasion of conidia of *Aspergillus fumigatus* into blood vessels which can result in dissemination to other organs. Aspergilloma is a fungal ball that develops in previous cavity lung lesions. Essential oils have been used as antifungal agents. Inhalation of vapors of the essential oils kill invaders attached to the inner respiratory lining and work synergistically with the body defenses. In this study, (Ginger) and *Cinnamomum camphora* (Camphor) with inhibition zones ranging from 11 to 31mm. Treatment of *A. fumigatus* with cinnamon essential oil leads to external changes, irregular cell shape and disintegration of fungus cell wall as detected under transmission electron microscope and then make analysis by GC-MS.

**Keywords:** Antifungal, Aspergillosis, Essential oils, *A. fumigatus* KY026061.

### Introduction

The genus *Aspergillus* includes over 180 species which are ubiquitous and are especially common in soil and decaying vegetation. Around 20 species of the genus *Aspergillus* have been reported as the causative agents of opportunistic infections in human beings. Among these, *Aspergillus fumigatus* is the most commonly isolated species. Manifestation of disease range from allergic to cavitary disease with systemic invasion. Aspergillosis is an opportunistic infection which can attack the lungs, ears, eyes, digestive system, kidney and brain (Chakraborty et al., 2006; Dubey et al., 2006; Schauwvlieghe et al., 2018). It develops mainly in individuals who are immunocompromised either from disease or from immunosuppressive drugs and is a leading cause of death in acute leukemia. Conversely, it may develop as an allergic response. Aspergillosis develops in the body either by inhalation or by penetration of (conidia) through surgical interventions and colonization

of wounds. Resistance of *Aspergillus* to some clinically used antifungal compounds brings a worrying clinical prognostic in people attacked by aspergillosis (Canuto & Rodero, 2002; Curtis et al., 2005; Shah et al., 2018). Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries.

The oils cover a broad spectrum of biological activity which has led to an increased interest among researchers. Essential oils and their volatile vapors were investigated for their inhibitory effect on spore germination and mycelial growth of *Aspergillus fumigatus* (Rios & Recio, 2005; Verweij et al., 2016; Cavayas et al., 2018). The major non immune evasive *Aspergillus* virulence factor include polysaccharides in cell wall component, in which cell wall is the first and important barrier that protect fungal cell wall components especially polysaccharides which are key of fungal cell to bind and invasion of host epithelium.

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The aim of this study was to evaluate the antifungal activity of five plant essential oils.

## Materials and Methods

### Maintenance of culture

*Aspergillus fumigatus* was isolated from a patient complaining of cough, breathing problems and bronchial asthma. *A. fumigatus* was maintained on 2% Potato Dextrose Agar (PDA) medium for 7 days at  $28^{\circ}\pm 2^{\circ}\text{C}$  with adding 0.05g/L chloramphenicol as antibacterial agent (Mahmoud et al., 2011), with minor modification. The isolate was identified microscopically according to Moubasher (1993).

### Molecular identification of the tested fungal isolate

#### DNA extraction

Fifty mg of the mycelium of the tested fungus was picked off from the surface of 7 days-old colony grown on 2% PDA medium. Fungal mycelium was ground in liquid nitrogen using a mortar and pestle. DNA was extracted using i-genomic DNA extraction Mini Kit (INTRON Biotechnology, Inc, Cat. No. 17371) according to manufacturer's instructions. The eluted DNA was stored at  $-20^{\circ}\text{C}$ .

#### PCR conditions

Amplification of internal transcribed spacer (ITS) region of rDNA gene was conducted in an automated thermal cycler (C1000TM Thermal Cycler, Bio-RAD) using ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG-3') primers (White et al., 1990). The following parameters were used: 35 cycles of  $94^{\circ}\text{C}$  for 30 sec,  $51^{\circ}\text{C}$  for 1min,  $72^{\circ}\text{C}$  for 1.5min, and a final extension at  $72^{\circ}\text{C}$  for 3min. Each PCR mixture (25 $\mu\text{l}$ ) was prepared as follow, (1 $\mu\text{l}$ ) of 25ng nucleic acid, 1 $\mu\text{l}$  of each primer (10pmol), (12.5 $\mu\text{l}$ ) of GoTag® Colorless Master Mix (Promega Corporation, USA) and 9.5 $\mu\text{l}$  of nuclease free water (Promega). The experiment was carried out at Plant Pathology and Biotechnology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Kafr- Elsheikh University, Egypt.

#### Sequence analysis of the ITS region

PCR product was sent by City of Scientific Research and Technology Applications, New Borg El Arab City, Alexandria, Egypt to Macrogen Company (Seoul, Korea) to be sequenced. The

DNA nucleotide sequence was analyzed using DNA BLASTn (NCBI). Pairwise and multiple DNA sequence alignment were carried out using Clustal W (1.82) (Thompson et al. 1994).

### Essential oils

Essential oils of cinnamon, fennel, lavender, camphor and ginger were purchased from Captain Company in the local market. Different concentrations of the oils were prepared (1:1, 1:3, 1:5 and 1:10v/v) using Dimethyl sulphoxide (DMSO).

### Antifungal assay

Preliminary screening of antifungal activity was conducted using agar well diffusion assay as described by Smania et al. (1995). Fungal inoculum was prepared in sterile saline solution. Each 0.5ml of fungal suspension was poured into the sterilized Petri plates. After molten Sabouraud dextrose agar (SDA) was poured into Petri plates which were rotated to mix the inoculum and the medium uniformly. After solidification wells of 6mm diameter were bored with the help of sterilized cork-borer. 20 $\mu\text{l}$  of essential oils of different concentrations and 10% DMSO was also used as a control. Amphotericin B, itraconazole, fluconazole, ketoconazole and nystatin were used as positive controls. The plates were incubated at  $25^{\circ}\text{C}$  for 2-3 days. The results were expressed in terms of the diameters of inhibition zone around each well and the mean were then calculated. All experiments were carried out in triplicates, and the mean of 3 readings was calculated.

### Protein assay

Protein content of 0.1gm from mycelia of *Aspergillus fumigatus* KY026061 was determined by the method of Bradford (1976) and the reaction mixture was measured at 595nm by spectrophotometer using bovine serum albumin protein as standard.

### Preparation of polysaccharide

Samples were carried out according to the method described by Lung & Tasi (2009). Intra polysaccharide (IPS) was isolated from cultured mycelia of *A. fumigatus* KY026061. The fresh mycelia were washed with ethanol (95%) followed by distilled water, ground with distilled water and put in a conical flask containing distilled water. The mycelia were extracted with boiling water for 1hr and autoclaved at  $121^{\circ}\text{C}$

for 15min and then filtered through filter paper (Whatman No. 1). Filtrates were treated with two volumes of 95% (v/v) ethanol and left over night at 4°C. The resultant precipitate was recovered by centrifugation at 3000rpm (2g) for 20min (Wu et al., 2008) and then the supernatant was discarded. The contents of precipitated polysaccharides were estimated spectrophotometrically at 490nm (Dubois et al., 1956).

#### *Effect of cinammon oil on mycelia and conidia of Aspergillus fumigatus KY026061*

In this experiment, Transmission Electron Microscope (TEM) was applied to follow the mode of action of cinnamon oil on 20% DMSO. The morphology of conidial cell walls and mycelia of *A. fumigatus* were compared with control (without cinammon essential oil). Preparation of fungal sample for TEM examination was done according to Spuur (1969) and Ellis & Griffiths (1974) at the TEM Unit in Faculty of Medicine, Tanta University, Egypt.

#### *GC-MS analysis*

Cinnamon oil components were determined by gas chromatography, mass spectroscopy in Claurs 580/560S. Using a column 30.0m x 250µm, Rtx-5MS (cross bond 5% diphenyl 95% dimethyl poly siloxane), Perkin Elmer Company in equipped with heated FID. The GC conditions were employed using Helium as carrier gas (0.8ml/min) and the temperature program was 100°C for 1min, followed by an increase of 120°C/min to 220°C for the remainder of the run. Detector and injection point heaters were 275 and

250°C, respectively, and typically 0.1 or 1.0ul was injected at a 20:1 split. The identification of oil components was based on their retention time with volatile literature values (Uniyal et al., 2012). Experiments were done in Central lab, Tanta University,

#### *Statistical analysis*

The inhibitory zones of essential oils were expressed as the mean, Standard deviation and compared using Student Waller Ducan test at P <0.05.

## **Results**

#### *Molecular identification of the fungal strain*

As shown in Fig. 1, the ITS sequence analysis of the tested strain RES1 revealed 100% similarity with other strains of *Aspergillus fumigatus* accessed from GenBank *A. fumigatus* RES1 (Accession No.: KY026061). Fungal strains that belong to section fumigati such as *A. lentulus*, *A. viridinutans* and *Neosartorya spinosa* exhibited slightly lower similarity (98%) and appeared in a separate clade.

#### *Sensitivity of Aspergillus fumigatus KY026061 to antifungal agents*

Results of in Table 1 showed that itraconazole gave high inhibition zone (19mm) against *A. fumigatus* KY026061, while, ketoconazol gave low inhibition zone 11mm. In case of fluconazole, nystatin and amphotericin b, no activity was observed.

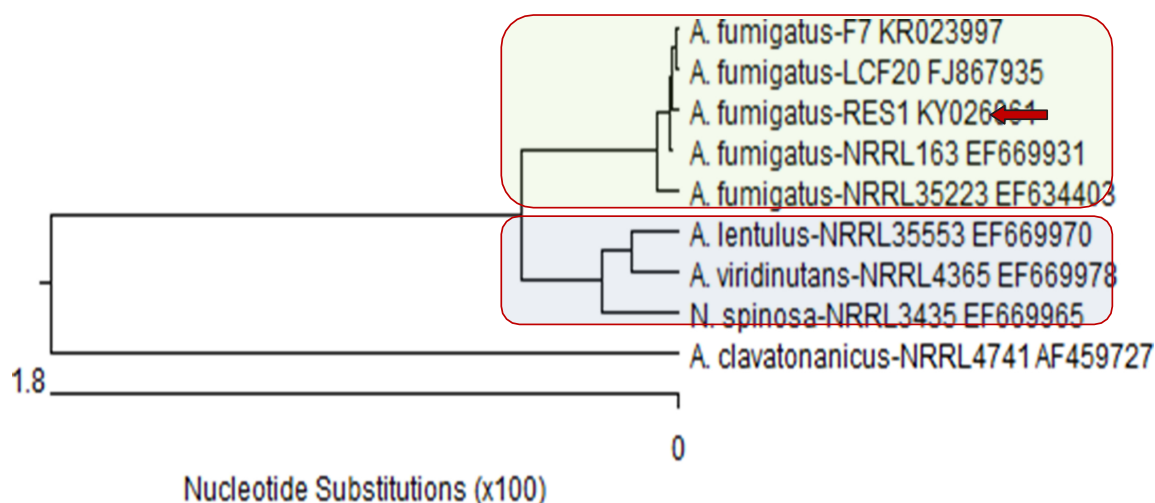


Fig. 1. Phylogenetic tree of the ITS sequence of *A. fumigatus* RES1 (Accession No.: KY026061) aligned by clustal W method with the closely related sequences accessed from the GenBank.

**TABLE 1.** Activity of different standard commercial antifungal agents against *A.fumigatus* KY026061.

Standard antifungal agent	Concentration ( $\mu\text{g}/\text{disk}$ )	Mean of inhibition zone (mm)
Amphotericin B (AP)	50	0 $\pm$ 0.0
Fluconazole (FLC)	25	0 $\pm$ 0.0
Itraconazole (IT)	30	19 $\pm$ 0.20
Ketoconazole (KT)	10	11 $\pm$ 0.10
Metronidazole (MT)	5	0 $\pm$ 0.0
Nystatin (NS)	100IU	0 $\pm$ 0.0

#### *Sensitivity of Aspergillus fumigatus* KY026061 to essential oils

*Aspergillus fumigatus* was sensitive to the five essential oils dissolved in 10% DMSO at concentrations of 1:1 and 1:3v/v with the highest inhibition being observed with cinnamon oil (25mm) as shown in Table 2 and Fig. 2. There was a slight decrease in the activity of cinnamon oil as the dilution rate 1:5v/v. Essential oils of ginger and camphor were still active at 1:5v/v but they were no longer active at 1:10 v/v. The remaining oils of lavender and fennel lost their activity at 1:5 and 1:10v/v.

#### *Estimation of protein and polysaccharide after treatment of Aspergillus fumigatus* KY026061 by different concentrations of cinnamon oil

Figure 3 showed that at concentration (1:1v/v) of cinnamon oil, the protein and polysaccharides were 0.087 and 0.0mg/ml, respectively. By diluting oil, the protein and polysaccharides contents were 0.526 and 0.03mg/ml, respectively, at (1:3, v/v) concentration of cinnamon oil. It can be concluded that the concentration of cinnamon oil (1:1v/v) affected the protein and polysaccharides contents which were responsible for virulence of

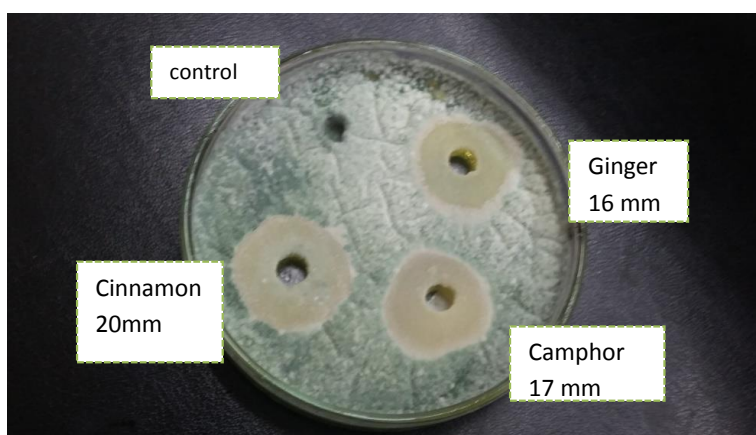
#### *A. fumigatus* KY026061.

#### *Effect of cinammon essential oil on the cell wall of A. fumigatus* KY026061 as seen by TEM

It was noticed at concentration (1:5v/v) of cinnamon essential oil some changes on fungal cell wall, such as forming some protrusion and pores in conidia and mycelia. Also, it was noticed that there were degenerated intercellular septa in mycelia. Irregular and torned cell wall in conidia and mycelia. Treated *A. fumigatus* KY026061 with this concentration of cinnamon essential oil cause shrinkage of cell cavity (Fig. 4).

#### *Gas liquid chromatography of essential oil constituents*

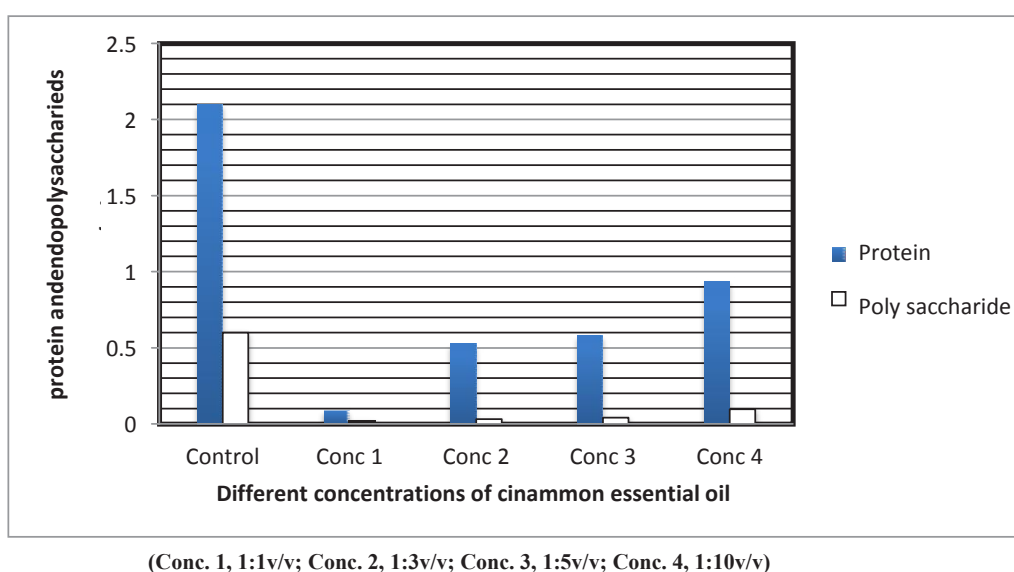
In the present work, a fairly wide range of different types of compounds was detected (Fig. 5), including hydrocarbons, alcohols and aldehydes. From Fig. 6 (a, b) and (Table 3) the cinnamon oil consists of Cinnamic aldehyde (28.716%) which represented the major components, Heneicosane, Heptadecane, Eicosane (5.639%) in addition to Heptacosanol, Coumarin at percentage (1.516, 2.190% , respectively).

**Fig. 2.** Effect of different essential oils against *A. fumigatus* KY026061 at concentration (1:5v/v).

**TABLE 2.** Mean diameters of inhibition zones (mm) at different concentrations of essential oils affecting *Aspergillus fumigatus* KY026061.

Essential oils	Conc. 1 (1:1v/v)	Conc. 2 (1:3v/v)	Conc. 3 (1:5v/v)	Conc. 4 (1:10v/v)
Ginger	11±0.01	23±0.0	16±0.0	0±0.0
Cinnamon	25±0.05	22±0.05	20±0.0	18±0.02
Lavender	20±0.01	17±0.03	0±0.0	0±0.0
Camphor	21±0.072	22±0.076	17±0.057	0±0.0
Fennel	20±0.057		11±0.07	0±0.0

Value is the mean±SD of three replicates



**Fig. 3.** Estimation of protein and polysaccharide after treatment of *A. fumigatus* KY026061 by different concentrations of cinnamon oil.

### Discussion

Bansod & Rai (2008) reported antifungal activity of some essential oils against pathogenic *Aspergillus fumigatus* and *A.niger*. Plant oils are important source of antifungal compounds and they may provide a renewable source of useful fungicides that can be utilized in antimycotic drugs against *A. fumigatus* and *A. niger* infection in patients suffering from respiratory diseases. In our study essential oils extracted from *Cinnamomum verum* (Cinnamon), *Lavandula angustifolia* (Lavender), *Foeniculum vulgare* (Fennel), *Zingiber officinale* (Ginger) and *Cinnamomum camphora* (Camphor) showed high antimycotic activity. These results support that plant essential oils have a role as

pharmaceuticals. It was concluded that volatile vapors of essential oils possessed fungicidal activity at high dose level, preventing to resume growth after removal of essential oils. Therefore, volatile vapors of essential oils could be widely applicable in treatment and prevention of fungal infections; the cell walls of fungus mainly consist of polysaccharides, which is glycosidic-linked with glucose and mannose (Wessels & Sietsma, 1981). When mycelia were exposed to cinnamon oil, the content of protein and polysaccharide was reduced. It may be concluded that the mode of oil action increasing was due to the lipophilic and low molecular weight components of essential oils that pass easily through the plasma membrane of the fungal cell leading to



disruption of membrane permeability and osmotic balance of the cell, causing cell death (Chao et al., 2005). Essential oils negatively affect the cellular membrane by reacting with active sites of enzymes or cellular ions, thereby, depleting ATP (Mohamed et al., 2018). Ergosterol is specific to fungi and is the major sterol component of the fungal cell membrane. It is also responsible for maintaining the cell function and integrity (Rodriguez et al., 1985 ; Jenks & Hoenigl, 2018). It has been shown that essential oils can also cause a considerable reduction in the quantity of ergosterol. The antimicrobial components of the essential oils cross the cell membrane, interact with the enzymes and proteins of the membrane, thus producing a flux of protons towards the cell exterior which induces disruption to the fungal cell organization and, ultimately, their death as has been supported by the transmission electron

microscope there were changes in tested fungal cell wall with external disintegration and irregular cell wall (Pinto et al., 2009; Rodriguez et al., 2018). The results of GC/MS analysis of cinnamon essential oil showed that cinnamaldehyde was the major component of this essential oil. This finding was according to studies carried out by several investigators (Li et al., 2013; Rahemi et al., 2015; Kaskatepe et al., 2016; Brnawi et al., 2019).

### Conclusions

It can be concluded that cinnamon essential oil has a good antifungal potential against *A. fumigatus* KY026061. The present results suggest the possibility of treatment of microbial respiratory diseases by cinnamon essential oil, after several studies.

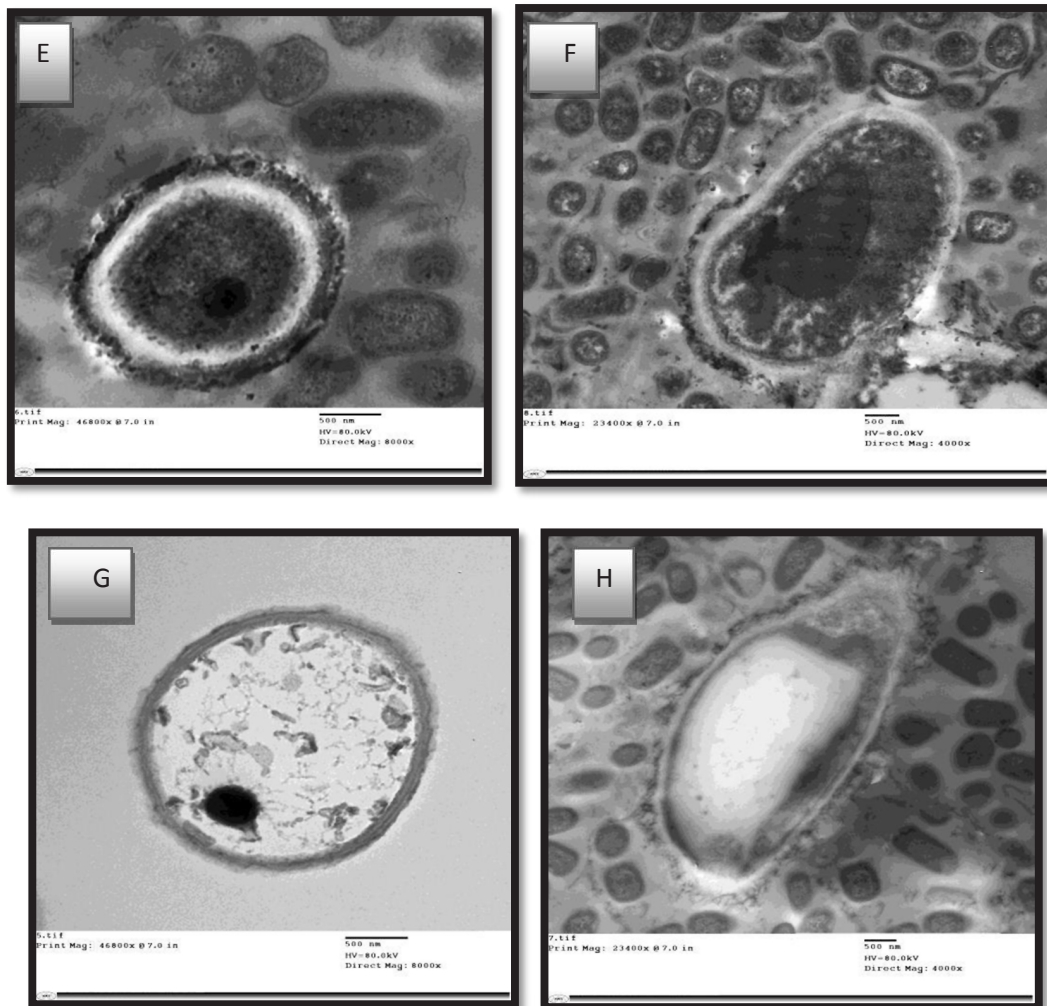


Fig . 4. Transmission electron microscope of *A. fumigatus* KY026061 conidia and mycelia (E & F), control (G & H) at concentration of 1:5v/v of cinnamon essential oil in 10% DMSO.

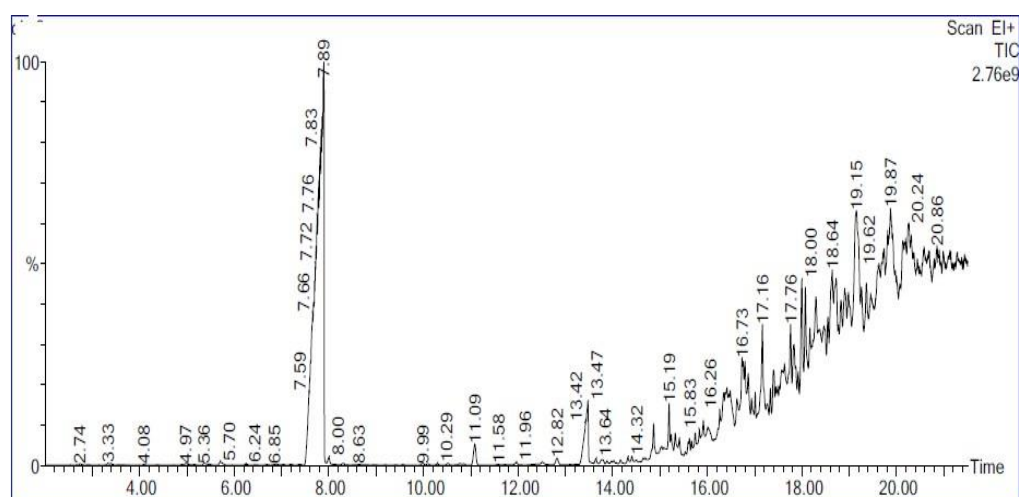


Fig. 5. GC-MS chromatogram of cinnamon essential oil.

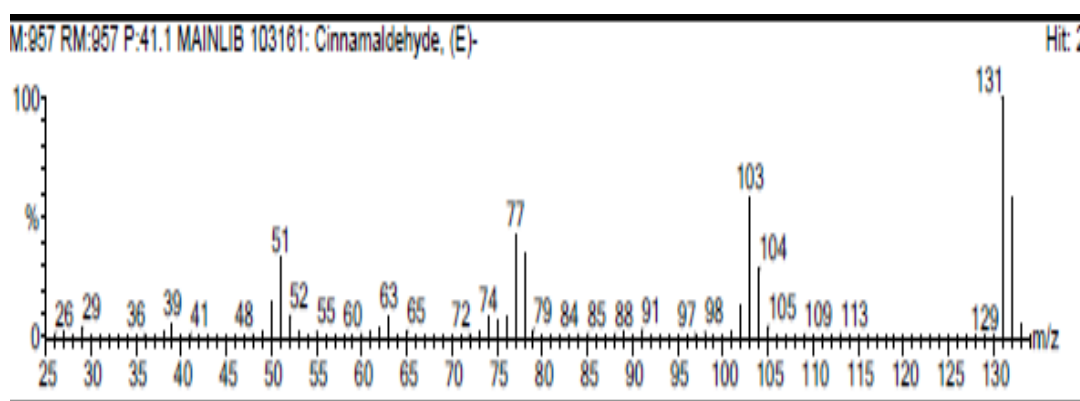
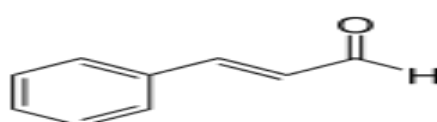


Fig. 6 a. Mass spectrum chromatogram of cinnamaldehyde.



Cinnamaldehyde(C<sub>9</sub>H<sub>8</sub>O)

Fig. 6 b. The molecular formula of the active component of cinnamon essential oil (Roya et al., 2017).

TABLE 3. GC-MS analysis of important cinnamon essential oil components.

Peaks	Retention time (min)	Area %	Name compound cinnamon oil
1	7.896	28.716	Cinnamaldehyde
2	13.473	2.190	Coumarin
10	18.295	1.516	Heptacosanol
15	19.151	5.639	Heneicosane, Heptadecane, Eicosane
18	19.811	1.320	Eicosane, heneicosane
19	19.871	3.782	Eptadecane, Dodecanol, Ethanol
20	20.261	1.769	Heneicosane

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## الزيوت الأساسية كمضاد للفطريات ضد الاسبرجلس فيومجائس KY026061ABPA المسبب لداء رشاشيات الجهاز التنفسي ABPA

ايمان حسن فتحى عبد الظاهر محمد، وجيه عبد الفتاح الشونى، سامية شبانة، علا عبد السلام  
قسم النبات - كلية العلوم - جامعة طنطا - طنطا - مصر.

داء رشاشيات الفصبة الهوائية والرئتين والالتهاب الرئوى الفطرى بفطر الاسبرجلس فيومجائس ويصاب به المرضى الذين لديهم نقص مناعة باختراق الكونديا للأوعية الدموية ومنها لباقي الاعضاء. استخدام الزيوت الطيارة كمضادة لهذا الفطر واستنشاق بخارها يقلل من التصاق الفطر بالبطانة الداخلية للجهاز التنفسي كما أنه يعمل مع وسائل دفاع الجسم.

فى هذه الدراسة تم استخدام خمس زيوت عطرية وهى هما زيت القرفة، زيت اللافندر، زيت الشمر، زيت الزنجبيل وزيت الكافور واعطوا تأثيرا مثبتا تراوح بين 11-31 مم. وتم دراسة الفرق بالميكروسكوب الألكترونى بين فطر الاسبرجلس فيومجائس بعد معالجة بزيت القرفة واخر لم يعالج وقد لوحظ وجود تأثيرا على جدر خلايا الفطر حيث نتج شكل غير منظم وتشوه فى جدر الفطر. تلى ذلك إجراء تحليل كيميائى بالمعمل المركزى بطنطا للتعرف على بعض المركبات الكيميائية الموجودة فى زيت القرفة والتي كان لها أفضل تأثير على الفطر. وقد تم اختبار تأثيرها على فطر التجارب.