Chemical Composition and Antifungal Activities of Essential Clove Oil (*Syzyguim Aromaticum*) Against Soil Born Plant Pathogenic Fungi

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Abstract: This study aimed to identify the chemical composition of the essential oil extracted from the *Syzygium aromaticum* flower buds, and investigate its antifungal activities against soil born pathogenic fungi. The extraction yield oil percentage was about 6.7% for grounded flower buds. The GC-MS analysis identified 22 compounds, including Eugenol (37.41%), as major compound, followed by Eugenol acetate (15.57%), β-Caryophyllene (13.83%), Phenol,5-allyl-2-methoxy (8.75%), Cis-13-Eicosenoic acid (3.65%), Cholecalciferol (3.25%), Cis-Retinal (2.92%), Longifolene (2.53%) and other compounds (12.09%), respectively. The efficacy of essential clove oil from against soil born pathogenic fungi: *Botrytis cinerea, Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium semitectum* was assayed by food poisoned technique, after exposure to oil concentrations 25, 50, 100, 150 and 200 ppm. High concentration showed the highest inhibition of the fungal mycelium growth and vice-versa. Complete inhibition in mycelium growth occurred for *R. solani* and *S. rolfsii* at 200 ppm while the inhibition percent reached 88.5% and 82.3% for *B. cinerea* and *F. semitectum,* respectively compared with dimethomorph (50% WDG). The *B. cinerea* showed the highest response to essential clove oil followed by *S. rolfsii, R. solani* and *F. semitectum.* The EC_{50} values were 29.3, 37.2, 46.2 and 64.4 ppm, respectively for the above species. In this study the effect of essential clove oil using EC_{50} for *R. solani* or *S. rolfsii* on enzymes (amylase, catalase, cellulase and protease) activities were evaluated. Potato dextrose (PD) liquid media treated with clove oil induced significant decrease in all enzymes in each fungus, expect catalase enzyme. So, it can be concluded that clove EO might have antifungal activity against soil borne fungi.

Keywords: Clove oil, GC-Ms identification, Efficacy soil born fungi, extracellular enzymes**.**

1.Introduction:

In the past few decades, pathogenic fungi alone cause nearly 30% reduction in the yield of major food and cash crops **(Abad *et al.,* 2007)**. For long time, traditional agricultural practices have been replaced by synthetic chemicals for the management of plant pathogens, pests and weeds. That would increase crop production but with some deterioration of environmental quality and human health, were induced **(Harris *et al.,* 2001; Pathak *et al.,* 2022)**. The increasing incidence of resistance among pathogens towards synthetic chemicals is also a cause for serious concern. In recent years, medicinal plants were used as novel antimicrobials and to control insects, nematodes and vertebrates **(Alinezhad *et al.,* 2011; Maleki *et al.,* 2011; Slamet *et al.,* 2016)**. Plants are rich with beneficial secondary metabolites. Essential oils have long been used as spices and additives in the food flavoring industry **(Pandey *et al.,* 2017)**. Their essential oils and extracts have some widely biological activities, especially antimicrobial effects against different pathogenic organisms **(Bakkali *et al.,* 2008; Tolouee *et al.,* 2010)**.

Clove (*Syzygium aromaticum*) belongs to the family Myrtaceae, is an aromatic flower buds and it is the most important and second valuable spice in world trade **(Batiha *et al.* 2020; Giannenas *et al.,* 2020).** Oil from clove bud is known for its antioxidant, antifungicidal, anticarcinogenic, and anesthetic activities and for its antiprotozoal effects, attributing to it contains of sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds **(Immacolata *et al.,* 2017; Sharifi-Rad *et al.,* 2017; de Oliveira et al., 2021)**. Monoterpenes are the main components of many EOs. They are originated from secondary metabolites of plants and other organisms and have several biological activities **(Da Silva *et al.,* 2020)**.

The primary active compounds found in clove oil and extracts are eugenol, eucalyptol, and β-caryophyllene. These compounds have been shown to possess insecticidal, antimicrobial, and nematocidal activities against various plant pathogens **(Kacániová *et al.*, 2021; Elnabawy *et al.*, 2022)**.

Eugenol is the main volatile compound of extracted oil from clove bud (*S. aromaticum* L.) that is used in traditional medicine, as a bactericide, fungicide and plant pathogenic fungi such as *F. moniliforme*, *F. oxysporum*, *Aspergillus* sp. **(Ayoola *et al.*, 2008)**. The propanol and acetone clove extracts showed cent per cent inhibition of *S. rolfsii* compared to the untreated control **(Ranapratap *et al.*, 2023)**.

1.1. Aim of the work:

1.The present study aimed to clove EOS isolation and identification procedures by GC-MS.

2. Investigate antifungal activity against soil born pathogenic fungi.

3.Study the effect of EOS using EC_{50} on extracellular enzymes produced by soil born fungi.

2. Materials and Methods:

2.1. plant material

The flower buds of clove family Myrtaceae were collected from Research Station Qanater (Qalyubia), Research Department of Medicinal and Aromatic Plants-Horticulture Research Institute, Agriculture Research Center (A.R.C.), Giza, Eygpt. The flower buds were powdered after drying by using electrical blender to get fine powder for further use.

2.2. Fungal strains used

Fungi, *Botrytis cinerea* and *Rhizoctonia solani* brought from Plant Pathology Institute, Agricultural Research Center (A.R.C.), Giza, Egypt.

Sclerotium rolfsii and *Fusarium semitectum* was supplied from the Department of Fungicides, Bactericides and Nematicides, (Central Agricultural Pesticides Laboratory (CAPL)), Agricultural Research Center (A.R.C.) Giza, Egypt.

2.3. Fungicide used

Trade name: Agromorf 50% WDG

Common name: Dimethomorph

Chemical name: $C_{11}H_{22}ClNO_4$

IUPAC name: 4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl) acryloyl] morpholine

Recommended rate of application: 50g/100L, it was provided by Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center (A.R.C.) Giza, Egypt.

2.4. Essential oil (EO) isolation

Samples of powder flower buds' parts for each replicate (100g of each sample) of clove (*Syzyguim aromaticum*) were subjected to hydro-distillation for 6 hrs using Clevenger-type apparatus (**Frohlich *et al.*, 2019)** to extract and calculate oil percentage according to **Guenther (1961)**. The essential oil kept in brown tube for each treatment dried over anhydrous sodium sulphate and stored in a refrigerator at 4°C until identify the chemical constituents and examined bioassay testing. The yield of the essential oils was expressed in percentage and was calculated using the following formula:

The yield (%) = EO weight(g) / Sample weight (g) *100

2.5. Chemical composition of EO using by GC/MS

To identify the chemical composition of the EOS using gas chromatography at the Micro Analytical Center- Cairo University. the gas chromatography with mass spectrometer detector (Shimadzu Qp-2010 plus) with the following specifications. Instrument: capillary GC-2010 plus Gas Chromatographs (Shimadzu Corp., Japan), coupled with a Shimadzu Qp 2010 Plus detector (Flame Ionization Detector). The GC system was equipped with a Stabilwax column (30 m x 0.25 mm i.d., 0.25 μm film thickness). Analysis was carried out using helium as carrier gas at a flow rate of 1.0 mL/min at a split ratio of 1:10 and the following temperature program: 40 ºC for 1 min; rising at 4.0 ºC/min to 150 ºC and held for 6 min; rising at 4 ºC/min to 210 ºC and held for 1min. The injector and detector were held at 210 ºC and 250 ºC, respectively. Diluted samples (1:10 hexane, v/v) of 0.2 μL of the mixtures were always injected. Most of the compounds were identified using GC standards.

2.6. Effect of EO against soil born pathogenic fungi *in vitro*

The efficacy of EO from Clove against soil born disease fungi: *B. cinerea*; *R. solani*; *S. rolfsii* and *F. semitectum* was determined by food poisoned technique **(Mohanty *et al.*, 2012)**. The concentrations EO were 25, 50, 100, 150 and 200 ppm mixed with 50ml of sterilized Potato dextrose agar (PDA) medium. The media was allowed to solidify. At seven days old fungal culture disk of 5-mm diameter was taken and inoculated to the center of Petri dishes containing EO. Fungicide dimethomorph

at recommended concentration (as a positive control) and PDA medium without oil served as negative control. All dishes were incubated at 25 ± 2°C and radial growth of colony was measured when the mycelia of control had almost filled the Petri dishes. Each test was performed in triplicate.

The fungal growth inhibition was calculated for treatment against control using the following formula **(Taisan *et al.*, 2014)**.

Inhibition of growth (%) = R-r/R *100

R is the radial growth of fungal mycelia in the control plate.

r is the radial growth of fungal mycelia in the treated plate.

EC_{50} and EC_{90} values were determined by the linear regression Lpd line on computer program of the probit of the tested fungus percentage inhibition vs. Logs the concentration (mg/L) of the EO according to the method of **Finney (1971)**.

2.7. Effect of essential oil (EC_{50}) on extracellular enzymes produced by soil born fungi

Essential oil (EO) at EC_{50} of each fungus was added to 50 ml sterilized (PD) liquid medium inoculated with 3 discs (5 mm) of any fungus *R. solani* or *S. rolfsii.* Control liquid medium inoculated with each fungus without EO. Three flasks were used as replicates. All flasks were incubated at 25 ±2 ºC. When the mycelial growth covered the surface media in control flask (check), the mycelial matrix was excluded by filtration. The mycelial mates were homogenized and taken for quantification.

2.7.1. Determination of amylase activity

One gram of each fungus was homogenized in a mortar with 4 ml (0.01 M) Tris-HCl buffer pH 8.0 containing 0.02 M NaCl and $CaCl_2$. The supernatant was used for total amylase activity according to the method described by **Dewez *et al.* (2005)**. The total amylase activity is expressed as mg starch consumed/ 15min/1g fungus.

2.7.2. Determination of catalase activity

One gram of each mycelial was homogenized two times with 0.01M phosphate buffer (pH 7.0). Catalase activity was determined according to the method described by **Dewez *et al.* (2005).** The enzyme was determined as the change in absorbance at wave length 240 nm per one gram fungus/min.

2.7.3. Determination of cellulase activity

Cellulase activity was determined by measuring the loss in viscosity of carboxymethyl cellulose (CMC) solution at 37 ºC as described by **Tian *et al.* (2011).**

2.7.4. Determination of protease activity

Protease activity was determined as described by **Dewez *et al.* (2005).** The blue color developed was determined after 5min. at wave length 625nm.

2.8. Statistical analysis

The test data were submitted to analysis of variance (ANOVA) with SAS software. The significance level of p≤ 0.05 (low), p≤ 0.01 (moderate), p≤ 0.001(high) were accepted as the level of probability and compared of means by Tukey's test.

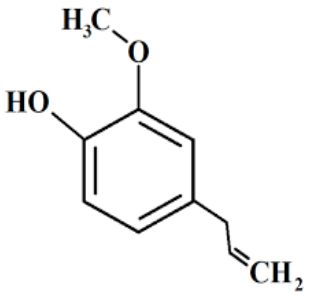
3. Results:

3.1. The yield of essential oil

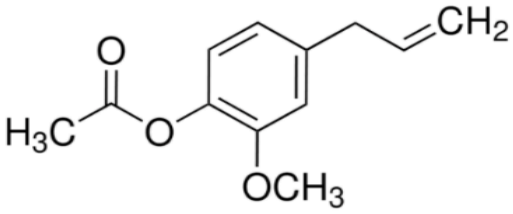
In this present study the EO using hydro-distillation technique in a Clevenger-type apparatus from dried flower buds of clove gave a value of volatile oil percentage (6.7%) and the oil appeared as light yellow This result is in agreement with (**Paramita *et al*. 2020; Dorsaf *et al*. 2021)** obtained the highest extraction yield essential clove oil was (7.1 ± 0.8%). Also, **Evariste *et al.* (2024)** found that the yield of Eos from flower buds was found to be 11.13%.

3.2. Chemical composition of Essential oil

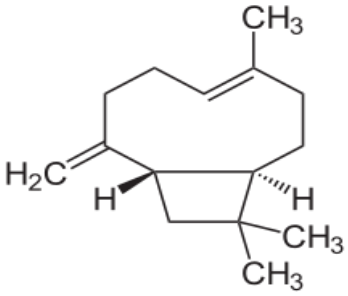
Data presented in Table (1) and Fig. (1) indicate the analysis of essential clove oil showed twenty- two compounds which identified components by GC-Ms. The EO contained eugenol (37.41%) was the principal constituent followed by eugenol acetate (15.57%), β-Caryophyllene (13.83%), Phenol,5-allyl-2-methoxy (8.75%), Cis-13-Eicosenoic acid (3.65%), Cholecalciferol (3.25%), Cis-Retinal (2.92), Longifolene (2.53%) and other compounds (12.09%), This result is in agreement with **Paramita *et al*. (2020)** where clove buds' oil contained two major components eugenol and eugenol acetate in extracted with values 85.01 and 13.06%, respectively. However, **Dorsaf *et al*. (2021)** showed GC-MS analysis allowed the identification of 17 heterogeneous compounds, including eugenol (68.7-87.4%), as major compound, cyperene (20.5-7.2%), phenethyl isovalerate (6.4-3.6%), and cis-thujopsene (1.9-0.8%), respectively, for grounded and ungrounded seeds.



Eugenol



Eugenol acetate



β-Caryophyllene

Fig. (1). Chemical structure of main compounds of essential clove oil

Table (1). Chemical composition of essential clove oil identified components by GC-MS

No. of peak	Compounds	Abundance %
1	Longifolene	2.53
2	Phenol,5-allyl-2-methoxy	8.75
3	β-Caryophyllene	13.83
4	α-Humulene	0.40
5	Eugenol	37.41
6	Cis- Thujopsene	0.71
7	Cholecalciferol	3.25
8	Trans-m-propenyl guaiacol	0.40
9	Longifolen aldehyde	0.57
10	Humulene-1,2-epoxid	0.62
11	Cis,α-Santalol	1.61
12	Eugenol acetate	15.57
13	(-)Spathulenol	1.86
14	Cedrenol	1.40
15	Geranyl-α-terpinene	1.64
16	α-Bisaolol	0.66
17	Cyanidincation	1.45
18	Hexadecanaic acid ethyl ester	0.64
19	Cis-Retinal	2.92
20	Cis-13-Eicosenoic acid	3.65
21	Methyl Salicylate	0.02
22	Caryophyllene Oxide	0.11

GC-MS: Gas chromatography using mass spectrometry detection.

3.3. Efficacy of EOs *in vitro* assays

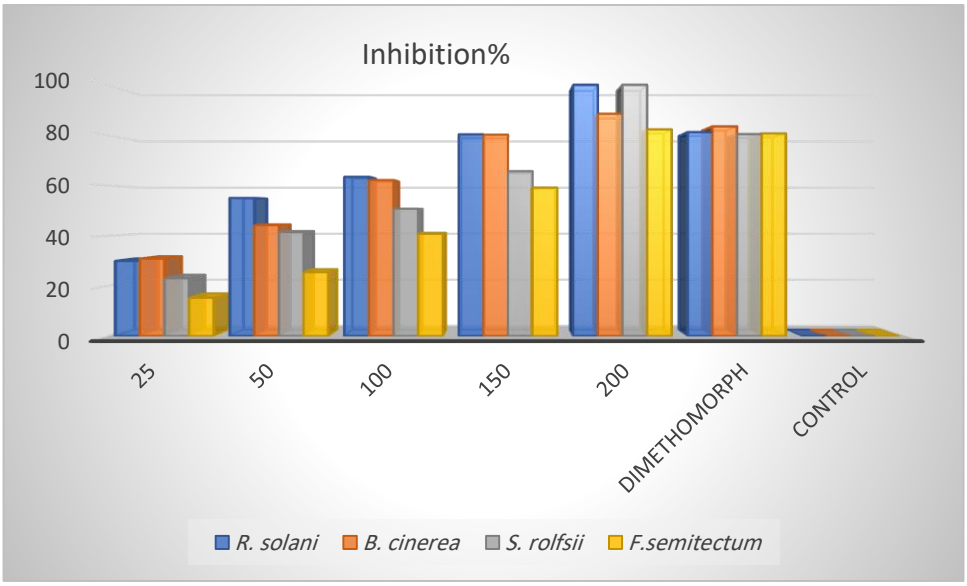
Data in Table (2) and Fig. (2) indicate the effect of clove oil against the tested fungi *R. solani*, *B. cinerea*, *S. rolfsii* and *F. semitectum* under laboratory conditions. High concentration of EO showed the highest inhibition of the fungal mycelium growth and vice-versa. Complete inhibition in mycelium growth occurred for *R. solani* and *S. rolfsii* at 200 ppm. While the inhibition percent reached 88.5 and 82.3 % for *B. cinerea* and *F. semitectum* respectively compared with dimethomorph.

The data indicated that the *B. cinerea* showed the highest response to EO, followed by *S. rolfsii*, *R. solani* and *F. semitectum* the EC_{50} values were 29.3, 37.2, 46.2 and 64.4 ppm, respectively. These results showed that the EO reduced the mycelial growth of the pathogenic fungi which are in agreement with those of **(Lopez *et al.* 2008; Inder *et al.* 2011)** who showed that clove oil have considerable antifungal activity against pathogenic fungi and analysis of the structures of eugenol suggests that its ability to eradicate fungi independent on the existence of an aromatic ring.

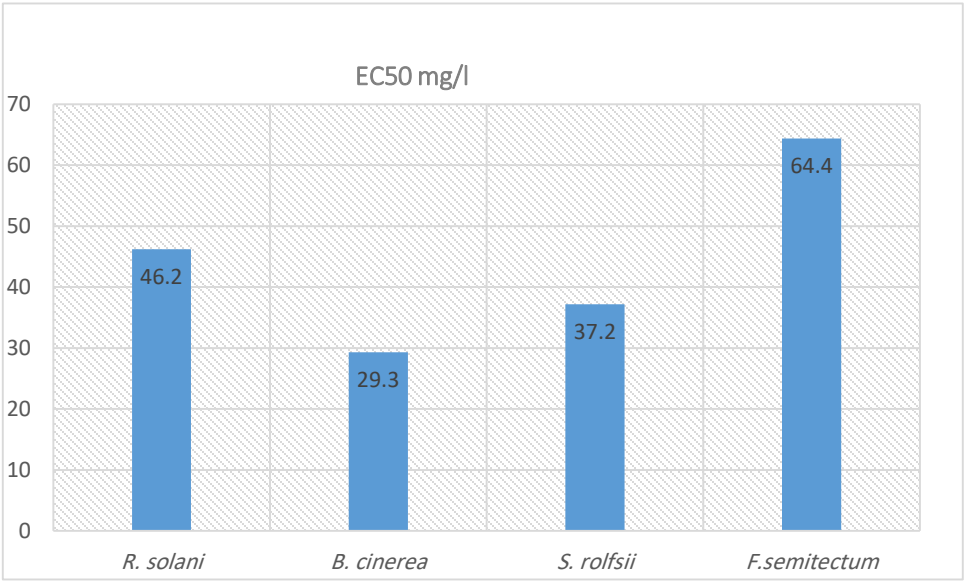
Table (2). The inhibition percent of EOS on the tested pathogenic fungi

Concentration (ppm)	% of inhibition			
	R. solani	***B. cinerea***	***S. rolfsii***	***F.semitectum***
25	30.0	30.9	23.1	15.3
50	55.1	44.3	41.5	25.5
100	63.5	62.2	50.8	40.8
150	80.3	80.2	65.7	59.2
200	100	88.5	100	82.3
****Positive control**	81.2	83.5	80.4	80.7
***Control**	0.0	0.0	0.0	0.0
EC_{50}	46.2	29.3	37.2	64.4
EC_{90}	207.4	198.2	188	401.3
Slope	1.9±0.20	1.6±0.19	1.8±0.20	1.9±0.20

*EOS: Essential oil **Positive control: Dimethomorph (50% WDG) ppm: parts per million
***Control: (PDA medium without clove oil and discs were cut from the pathogen only).
Each number represents the mean of 3 replicates.
EC_{50} and EC_{90}: indicate the effective concentrations (mg/l) that cause 50% and 90% growth inhibition.



(A)



(B)

Fig. (2). (A) The mean growth inhibition percent in tested *B. cinerea, R. solani, S. rolfsii* and *F. semitectum* mycelia exposed to concentrations 25, 50, 100, 150 and 200 ppm of essential clove oil. (B) Evaluation of the 50% inhibitory concentration (EC_{50}) of clove oil on the growth of the above-mentioned fungi

3.4. Effect of essential oil (EC_{50}) on extracellular enzymes produced by soil born fungi

Data presented in table (3) indicated the effect of the treatment liquid PD media with essential clove oil at EC_{50} of each fungus on enzyme activities of *Rhizoctonia solani* and *Sclerotium rolfsii*.

Data revealed that the amylase activity was moderately inhibited in both *Rhizoconia solani* (11.6 ±3.1) and *Sclerotium rolfsii* (1.7 ± 0.01 mg/ 1g) compared to untreated media they reached 15.6 ± 2.3 and 3.1 ± 0.1 mg/1g respectively.

Table (3). Effect of essential oil at (EC_{50}) level on extracellular enzymes produced by *R. solani* and *S. rolfsii*

Enzymes	Treated (EO)	Control (Untreated)
	R. solani	
Amylase activity (mg starch/g)	11.6 ±3.1**	15.6 ± 2.3
Catalase activity(OD/min)	0.63 ± 0.02	0.71± 0.01
Cellulase activity(RA units/ml)	35.0 ± 3.5***	56.0 ± 4.2
Protease activity(OD/5min)	0.26 ± 0.01**	0.53 ± 0.03
	S. rolfsii	
Amylase activity(mg starch/g)	1.7 ± 0.01**	3.1 ± 0.1
Catalase activity(OD/min)	0.80 ± 0.04	0.83 ± 0.02
Cellulase activity(RA units/ml)	28.0 ±6.3*	31.0 ± 4.3
Protease activity(OD/5min)	0.32 ± 0.02***	0.53 ± 0.03

*Significant P<0.05 **P<0.01 ***P<0.001
EO: Essential clove oil Each number represents the mean of 3 replicates.
Control: (PD liquid medium without clove oil and discs were cut from the pathogen only).

Also, data showed the treatment with EO exerted non-significant effect on catalase activity for *R. solani* (0.63 ± 0.02) OD/ min and *S. rolfsii* (0.80 ± 0.04) OD/min compared to untreated media 0.71± 0.01 OD / min and 0.83 ± 0.02 OD / min respectively.

The reduction of cellulase activity was highly significant (P< 0.001) in case of *R. solani* treated with EO

showing 35.0 ± 3.5 RA units/ ml compared to untreated media being 56.0 ± 4.2 RA units/ ml. On the other hand, the reduction of the enzyme was significantly low ($P<0.05$) for *S. rolfsii* treated with EO showing 28.0 ±6.3 RA units/ ml compared to untreated media 31.0 ± 4.3 RA units/ml.

Protease activity in *S. rolfsii* showed significant reduction when treated with EO (0.32 ± 0.02) OD/5min compared to 0.53 ± 0.03 OD/5min for the untreated control. In *R. solani* the enzyme activity was 0.26 ± 0.01 OD/5min compared to 0.53 ± 0.03 OD/5min in the untreated media. These results agree with **Yasmin *et al.* (2006)** which illustrated that using clove oil has an inhibitory effect on the enzymes pectinase, cellulase, protease and lipase *on R. solani, F. solani, F. moniliorme, F. semitectum, F. gruminearm* and *C. acremonium*.

Monoterpenes are nonpolar with hydrophobic and lipophilic characteristics, allowing them to interact with fungal cell membranes. This interaction is considered essential in disrupting cellular and energy homeostasis, leading to cell membrane damage and metabolic alterations **(Viriato, 2014; Da Silva *et al.*, 2020)**.

The deficiency of these components may lead to weakening the fungi activities and their inability to infect the plant. It seems that the main target of clove oil is the cell membrane in fungi causing increased permeability, disruption of membrane integrity and key enzymes is important for energy regulation of synthetic pathways **(Judit *et al.*, 2011)**.

Conclusions:

The results of our study showed that the clove oil gave value of volatile oil percentage (6.7%) and a rich source of monoterpenes and sesquiterpenes such as eugenol, eugenol acetate and β-Caryophyllene. This clove oil exhibited a good *in vitro* antifungal activity against soil born fungi: *B. cinerea, S. rolfsii, R. solani* and *F. semitectum.* Using of clove oil has an inhibitory effect on the enzyme's amylase, catalyse, cellulase and protease produced by the fungi mentioned above.

The main target of this oil is its effect on the cell membrane in fungi causing increased permeability, disruption of membrane integrity and key enzymes which are considered an important for energy regulation.

The study imply that terpenoid compounds might be promising candidates for developing innovative antifungal agents which serving as natural fungicides in the agricultural strategies. Nevertheless, additional studies will be needed to justify the potential use of this oil as a synthetic compound for traditional purposes.

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التركيب الكيميائى والنشاط المضاد للفطريات لزيت القرنفل ضد فطريات التربة المسببة للأمراض النباتية

عزة رسمى عمارة

قسم بحوث المبيدات الفطريه والبكتيريه والنيماتوديه - المعمل المركزي للمبيدات- مركز البحوث الزرعيه - الدقي - الجيزة - مصر

الملخص العربى:

تهدف هذه الدراسة إلى دراسة التركيب الكيميائى للزيت العطرى المستخرج من براعم أزهار نبات القرنفل بإستخدام التقطير المائى بالإضافة إلى تقييم النشاط الإبادى المضاد للفطريات. كانت نسبة إنتاج الزيت المستخرج من مطحون براعم الأزهار حوالى 6.7%. تم التعرف على 22 مركب من خلال التحليل بجهاز كروماتوجراف الغاز ومطياف الكتلة GC-Ms. يعد Eugenol (37.41%) المركب الرئيسى يليه

Eugenol acetate (15.57%), β-Caryophyllene (13.83%), Phenol,5-allyl-2-methoxy (8.75%), Cis-13-Eicosenoic acid (3.65%), Cholecalciferol (3.25%), Cis-Retinal (2.92), Longifolene (2.53%) and other compounds (12.09%),

على التوالى. تم تقييم فعالية زيت القرنفل الطيار ضد فطريات التربة :

B. cinerea, R. solani, S. rolfsii and *F. semitectum*

بإستخدام تقنية السم الغذائى . إستخدم تركيزات 25 ، 50، 100، 150، 200 جزء فى المليون. أظهر التركيز العالى لزيت القرنفل أعلى تثبيط لنمو الميسيليوم الفطرى. حدث تثبيط كامل لنمو الميسيليوم فى فطرى *R. solani* and *S. rolfsii* عند تركيز 200 جزء فى المليون بينما وصلت نسبة التثبيط

82.3 % ، 88.5 % فى فطرى *B. cinerea* and *F. semitectum* على التوالى مقارنتا بالمبيد الفطرى dimethomorph 50% WDG وقد أظهر فطر *B. cinerea* أعلى إستجابة لزيت القرنفل يلية *S. rolfsii, R. solani* and *F. semitectum* حيث كانت قيمة EC_{50} 29.3, 37.2, 46.2 and 64.4 جزء فى المليون على التوالى. فى هذه الدراسة تم تقييم تأثير زيت القرنفل بإستخدام EC_{50} على نشاط إنزيمات (الأميليز ، الكتاليز ، السيليوليز ، البروتيز) التى تفرزها فطريات:

R. solani or *S. rolfsii*. أدى زيت القرنفل إلى إنخفاض نشاط جميع الإنزيمات المختبرة من خلال بيئه ديكستروز البطاطس السائلة ما عدا إنزيم الكتاليز. لذلك يمكن الإستنتاج أن زيت القرنفل المحتوى

على مركبات التربينات له تأثيرمضاد لفطريات التربة المسببه للأمراض النباتية.