Mycelial Growth, Protein Patterns and Molecular Characterizations by RAPD-PCR of *Fusariumoxysporum* Fungus Affected by of 1,8-Cineol, Eugenol, Linalool, Methyl Cinnamate and Thymol

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Abstract: A study was undertaken to investigate the antifungal activities of naturally occurring compounds namely; thymol, eugenol, methyl cinnamate, linalool and 1,8-cineol against *Fusariumoxysporum* which was economically important phytopathogic fungus in the Egyptian environment. The LC_{25} and LC_{50}'s of the tested compounds against were determined according to the relationship drawn between the logarithm of concentration and the percent of growth inhibition (ldp lines), and the toxicity indexes and relative potencies were calculated. Thymol was the most effective compound followed by eugenol, methyl cinnamate and linalool, descendingly. The LC_{50}'s of thymol, eugenol and methyl cinnamate against *F. oxysporum* were 81.89, 214.74 and 290.66 µg/ml, respectively. The 1,8-cineol compound didn't show any fungicidal activity. The present study is also attempted to determine the differences between treated and untreated samples at the molecular level based on the analysis of protein analysis and RAPD-PCR.

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Key words: antifungal, *Fusariumoxysporum,* thymol, eugenol, methyl cinnamate, linalool, 1,8-cineol, protein profile, RAPD-PCR.

1. Introduction:

Food safety is an increasingly important public health issue. Nearly, 30% people in the world suffer from food borne diseases every year caused by microbes **(Burt, 2004; Shephard, 2008).**

Moulds or microscopic filamentous fungi are ubiquitous microorganisms with a great capacity to colonize many kinds of substrates and to proliferate under extreme environmental conditions **(Singh *et al.,* 1991; Nickelsen and Jakobsen, 1997)**. Poor storage management can lead to rapid deterioration in nutritional quality of food commodities with production of volatile metabolites giving off-odours. Moulds also produce mycotoxins that can be teratogenic, carcinogenic or cause feed refusal and emesis **(Yu *et al.,* 2003; Magan *et al.,* 2004)**.

Fusarium spp. are a widespread cosmopolitan group of fungi and commonly colonize aerial and subterranean plant parts, either as primary or secondary invaders. Some species are common in soil and it is rare to find necrotic root of a plant in most agricultural soils that is not colonized by at least one *Fusarium* sp. **(Nelson *et al.,* 1983)**. Of all diseases caused by *Fusarium*, probably the most important are the vascular wilt diseases caused by formaespeciales of *Fusariumoxysporum*. These fungi attack a diverse group of plants including crops, ornamentals and trees **(Nelson *et al.,* 1981)**.

Restrictions imposed by the food industry and regulatory agencies on the use of some synthetic food additives have led to renewed interest in searching for alternatives, as natural antimicrobial compounds, particularly those from plants **(Delaquis and Mazza, 1995; Hammer *et al.,* 1999)**. Essential oils as well as compounds derived from them possess a wide range of activities of which the antimicrobial activity is moststudied **(Hammer *et al.,* 2003; Ngueфack *et al.,* 2004a, b, 2007).** Their applications as preservatives in food or antiseptics and disinfectants are widely studied **(Nielsen and Rios, 2000; Pauli, 2001; Burt, 2004).**

The wide and indiscriminate use of chemical fungicides has been the cause of the appearance of resistant plant pathogens, leading to the occurrence of serious diseases. Due to this, there is an increasing interest to obtain alternative antimicrobial agents for using in plant disease control systems. One of the main procedures used in the research of biologically active substances is a systematic screening for the interaction between microorganisms and plant products. This procedure has been a source of useful agents to control the microbial survival **(Salvat *et al.,* 2001).** Plant products of recognized antimicrobial spectrum could appear in food conservation systems as main antimicrobial compounds or as adjuvants to

improve the action of other antimicrobial compounds **(Kaur and Arora, 1999).**

Among other chemical products, aromatic plants possess essential oils resulting from secondary metabolism. These substances have a great economic potential, especially in the food, pharmaceutical and perfumery sectors. Thus, the number of studies on the chemical composition and biological properties of these oils, as well as the taxonomic, environmental and cultivation factors that lead to variation in their quantity and quality, has been increasing **(Simõs *et al.*, 2003).** The objectives of this work were to evaluate the antifungal activity of some naturally occurring compounds. Considering these aspects, this thesis will do throw some light upon the following criteria; (1) studying the antimicrobial activity of some naturally occurring compounds on *Fusariumoxysporum*, (2) studying the mode of action of effective compounds against the tested fungi through the following points: (2.1.) protein banding patteren through using protein electrophoresis techniques and (2.2.) characterization of fungal isolates by using polymerase chain reaction based molecular method (RAPD-PCR Randomly Amplified Polymorphic-Polymerase Chain Reaction).

Material and Methods:

I. Materials:

I.1. Fungal isolates:

The fungi used in this thesis were kindly supplied by Plant Pathology Institute, Agricultural Research Center. They were as follows; *Fusariumoxysporum, Rhizoctoniasolani, Botrytis cinerea, Alternariasolani*and *Sclortiumrolfsii*.

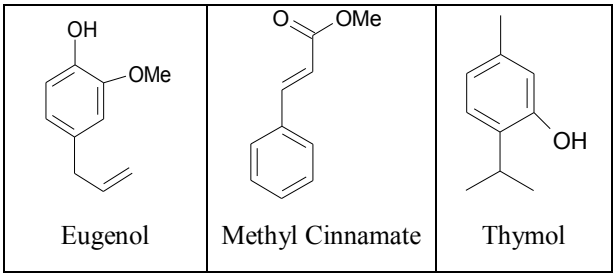
I.2. Chemicals:

I.2.1. Solvents:

Xylene was purchased from El Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt. Dimethyl sulfoxide (DMSO) and Dimethyl formamide (DMF) were purchased from Merck Company, Germany.

I.2.2. Pure Tested Compounds:

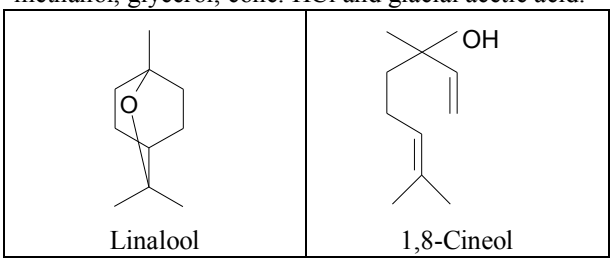
Thymol, methyl cinnamate, eugenol, linalool and 1,8- cineole were purchased from Merck Company, Germany.



Eugenol | Methyl Cinnamate | Thymol

1.2.4. Protein SDS-PAGE chemicals:

Acryl**a**mide, N, N, Methyl-bis acrylamide, 2-Mercaptoethanol, Tris (hydroxymethyl) amino methelene, N,N,N,N-tetramethylethylenediamine (TEMED), glycine, Ammonium persulphate (APS) $(NH_4)_2S_2O_8$, SDS (sodium dodecyl sulphate), Coomassie brilliant blue R-250, Bromophenol blue, methanol, glycerol, conc. HCl and glacial acetic acid.



Linalool | 1,8-Cineol

1.2.5. DNA chemicals:

Tris (hydroxymethyl) amino methelene, sodium salt of ethylenediaminetetraaceticacid (Na-EDTA), sodium dodecyl sulphate (SDS), 2-Mercaptoethanol, phenol, chloroform, RNase, proteinase K, sodium acetate, isopropanol, *Taq*DNA polymerase, agrose, Tris-base (2-Amino-2-hydroxymethyl-propane-1,3-diol), boric acid and ethidium bromide.

II. Methods:

II.1. Bioassay techniques:

Mycelial growth inhibition technique:

The antifungal compounds were prepared in dimethyl sulfoxide (DMSO) and tested for mycelial growth inhibition activity against *Fusariumoxysporum* using the food poison technique **(Joong – Hyeop *et al.*, 2005)**. A potato dextrose agar medium was used as the basal medium for all tested fungi. To test the antifungal activities of the selected compounds, sterile Petri dishes containing the compound dissolved in DMSO/Tween 80 emulsifier (80/20 v/v) diluted in PDA medium were prepared. Tween 80 alone as a control (0.025% by volume) was added to PDA medium as an emulsifier control. Plates containing media mixed with DMSO (0.1% by volume) were included as a solvent control. Also, DMSO and Tween 80 (4/1 v/v) were added to PDA medium as a solvent/emulsifier control. Finally, PDA plates treated with distilled water were served as a negative control.

Agar disks (5 mm in diameter) of the tested fungi were cut from completely grown cultures and placed at the center of the plates containing antifungal substances of the used concentration in ppm (µg/ml**).**

Four replicates of each concentration (50, 100, 150, 200 and 250 µg/ml for thymol, eugenol and methyl cinnamate compounds) (500, 1000, 2000, 4000 and 8000 µg/ml for linalool and 1,8-cineol compounds) of each fungus were incubated at 28ºC for all tested fungi. Radial growth was measured from the centers of the dishes sides by caliper every 48

hours and the mean was calculated of two perpendicular colony diameters in each replicate. Inhibition of growth was calculated in relation to the growth in the control, according to the equation of **Sztejnberg *et al.*, 1983:**

$$\%\text{ of inhibition} = 1 - \left(\frac{\text{Diameter of treated colony}}{\text{Diameter of control colony}}\right) \times 100$$

The corrected percentage of growth inhibition was used to calculate the LC_{50} values according to **Finney (1971)**. The toxicity lines were drawn for evaluating LC_{10}, LC_{25}, LC_{50} and LC_{90} and the slope for every treatment was estimated. The toxicity indexes and relative potencies were calculated according to **Sun (1950).**

$$\text{Toxicity Index} = \frac{LC_{50}\text{ of the most effective compound}}{LC_{50}\text{ of the other compound}} \times 100 = ...\%$$

$$\text{Relative Potency} = \frac{LC_{50}\text{ of the least effective compound}}{LC_{50}\text{ of the other compound}} = ...\text{Fold}$$

II.2. Protein Electrophoresis (SDS-PAGE):

Total cellular proteins of *Fusariumoxysporum* fungus, as an example of the tested five fungi both untreated and treated with thymol, eugenol and methyl cinnamate at LC_{10} concentrations were analyzed by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) as adopted by **Laemmli (1970).**

II.2.1. Preparation of protein samples:

Proteins were prepared according to the methods described by **Guseva and Gromova (1982).** Different volumes of prepared solutions to obtain the LC_{10} concentrations for each compound were added to conical flasks containing sterilized 50 ml of potato dextrose broth medium. A separate PDB flask free of the tested compounds was used as check (control) treatment. The flasks were inoculated with fungal disks (about 5 mm) and incubated at 28ºC. The mycelia of each treatment were harvested and filtered through cheese cloth, washed with distilled water several times, and freeze-dried. These frozen mycelia were ground in liquid nitrogen to a fine powder and suspended in phosphate buffer (0.5M) pH 8.3 (1-3 ml/g mycelium). The mixture was centrifuged at 47800 g for 20 min. The supernatant containing the soluble proteins was dispensed into Eppendorfs for storage at -20° C until use in electrophoretic analysis **(Bielenin *et al.*, 1988).**

I.2.2. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Preparation of gel plates:

The concentration of the gel was estimated according to the formula:

% of gel = V x C/T

Where V = volume of acrylamide solution used in ml.

C = concentration of acrylamide solution.

T = total volume of gel mixture in ml.

Stock solution:

1. Monomer solution (polyacrylamide 30%):

It was prepared by dissolving 30 gm of polyacrlamide and 0.8 gmbisacrylamid (N, N-Methelene-bisacrylamid) in suitable amount of distilled water and completed to 100 ml. The solution was filtered and kept ready for use about 30 day maximum at 4ºC in the dark.

2. Running gel buffer (Tris buffer 1.5*M*, pH 8.8):

18.15 gmTris [Tris (hydroxymethyl) amino methelene] were dissolved in 60 ml distilled water and the pH was adjusted to pH 8.8 with conc. HCl and the volume was completed to 100 ml by distilled water. The solution was filtered and stored at 4ºC.

3. Stacking gel buffer (Tris buffer 0.5*M*, pH 6.8):

6 gmTris were dissolved in 60 ml distilled water and pH was adjusted to 6.8 with conc. HCl and the volume was completed to100 ml by distilled water .The solution was filtered and stored at 4°C.

4. SDS solution (10%):

Dissolve 10 gm SDS in 90 ml distilled water with gentle stirring and up to 100 ml stored at room temperature.

5. Initiator (10% Ammonium persulfate, APS):

dissolve 100 mg APS in 1ml distilled waterto make the 10% ammonium persulfate solution (freshly prepared).

6. Activator TEMED (N,N,N,N-tetramethylenediamine).

7. Sample buffer : (0.125M Tris, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol and (0.002% bromophenol blue):

1.51 gm of Tris and 20 ml of glycerol were dissolved with 35 ml of water. The pH was adjusted to 6.75 with conc. HCl and 4 gm SDS, 10 ml 2-mercaptoethanol, 0.002 gmbromophenol blue were added. The final volume was completed to 100 ml with distilled water. The solution was shaken to dissolve bromophenol blue and stored at -20°C.

8. Tank buffer (electrode buffer pH 8.3, 0.05 M Tris, 0.192 M Glycine and 0.1% SDS):

It was prepared by dissolving 15 gm of Tris and 72.2 gm of Glycine and 5 gm SDS in suitable amount of distilled water. The final volume was completed to 5 liter with distilled water and store at 4°C.

9. Staining solution (0.025% CBB R- 250, 40% methanol , 7% glacial acetic acid):

0.125 gm of Coomassie Brilliant Blue (CBB) was dissolved in 200 ml of methanol and 35 ml of glacial acetic acid, distilled water was added to make

final volume of 500 ml, the solution was filtered and stored at room temperature.

10. Destaining solution I (50% methanol, 10% glacial acetic acid):

500 ml of methanol was mixed with 100 ml of glacial acetic acid and 400 ml of distilled water.

11. Destaining solution II (5% methanol, 7% glacial acetic acid):

50 ml of methanol was mixed with 70 ml of glacial acetic acid and 880 ml of distilled water.

12. Protein marker:

Wide range molecular weight protein marker produced by (Fermentas). The protein marker is a mixture of 9 recombinant, highly purified proteins ranged from 10-250 KDa.

Procedure:

1.The separating gel solution (12 %, 1.5mm thick):

It consists of 20 ml (30%) polyacrlamide for (15% acrylamide), 12.5 ml (1.5M) Tris buffer pH 8.8, 0.5 ml of 10% SDS solution, 0.5 ml of (10%) ammonium persulphate solution and 0.02 ml TEMED. The final volume was completed to 50 ml by distilled water. The mixture was transferred to assembled sandwich into the dual gel casters. Immediately overly the monomer solution with a thin layer of water – saturated n-butanol or water to prevent gel exposure to oxygen. Allow the gel to polymerize for 45 minutes to 1 hour. After polymerization, the overlay was poured off and the surface was rinsed several times with distilled water.

2. Stacking gel (4% acrylamide):

It consist of 1.7 ml (30%) polyacrlamide, 1.25 ml (0.5M) Tris buffer pH 6.8, 0.1 ml of 10% SDS solution, 0.1 ml (10%) ammonium persulphate and 0.01 ml TEMED. The final volume was completed to 10 ml by distilled water. The mixture was transferred into the sandwich and poured over the separation gel. A comb was placed completely into assembled gel sandwich and the gel was allowed to be polymerized for 30-45 minutes. The comb was removed by pulling it straight up slowly and gently. The wells were completely filled with tank buffer and the gels were ready for loading the samples to run.

3. Preparation of the samples for fractionation on SDS – PAGE:

To prepare the samples for loading on SDS gel, solution contains 75 µg protein from supernatant of fungal soluble protein was mixed with equal volume of sample buffer and boiled for 5 min on water bath.

4. Refraction process:

After electrophoresis apparatus was prepared for running, the boiled samples were loaded on wells. To determine the MW of subunits of the refractionated sample protein 40 µl of a molecular weight protein marker were loaded on the gel in first well. Loading tips were used for loading the samples under the tank buffer in the wells. The gel sandwiches were removed from the casting stand and were inserted into the electrophoresis chamber. The upper and lower electrophoresis chambers were filled with tank buffer and refractionation process was carried out at room temperature (20 to 25ºC) , 25 mA and 80-90 V (for each 1.5 mm thick gel) until the dye reached the bottom of the separating gel after ˜ 5 hours.

5. Staining of SDS refractionated samples protein

For visudization of the subunits of the refractionated samples protein, the gel was stained for = 24 hrs in 0.1 Coomassie Brilliant Blue reagent R-250 with gentle shaking .It was destained for 1 h in destaining solution (I) and then in destaining solution (II) until the gel between the protein bands become colorless. After staining the gel was photographed. Scanning molecular weight, using Lab Image version. 2.6 program.

II.3. Molecular Techniques:

1. DNA extraction:

The extraction of DNA from untreated (control) and treated *Fusariumoxysporum* fungus at the LC_{10} concentration of the promising compounds was performed according to **Lee and Taylor (1990)** with some modifications.

Lyophilized mycelium of *Fusariumoxysporum* (60 mg) was ground in the presence of liquid nitrogen and mixed with 400 µl lysis buffer (50 mM TrisHCl, pH 7.2, 50 mM Na-EDTA, pH 8.0, 3% SDS and 1% 2-mercaptoethanol) at the rate of 1 gm/20 ml and vortexed gently. The suspension was then incubated at 65ºC for 1h in a water bath followed by addition of 400 µl of phenol/ saturated with Tris-HCl (pH 8.0) and centrifuged in a microfuge (Eppendorf, USA) at 10,000 rpm for 15 min. at 4ºC. The aqueous phase containing the DNA was transferred into a fresh tube, and an equal volume of phenol:chloroform (1:1) was added. After that, the tube was inverted gently for 2 -3 times, then centrifuged at 10,000 rpm for 15 min. at 4ºC. This step was repeated 3 times, and then the aqueous phase was extracted with an equal volume of chloroform by centrifugation at 10,000 rpm for 5 min. To remove RNA contamination, 50 µl/ml RNase (10 mg/ml) (Sigma Co., USA) were added to the upper clear phase and incubated for 1 h at 37 ºC in a water bath. The extracted DNA was deproteinized by adding 200 µl/ml proteinase K (10 mg/ml) (Sigma) and incubating at 35ºC in a water bath overnight **(Gurr and Mcpherson, 1992)**. Phenol:chloroform (1:1 v/v) extraction was repeated until the interface between the aqueous and phenol:chloroform phase became clear. The aqueous phase was extracted with an equal volume of chloroform by centrifugation at 10,000 rpm for 5 min. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 6.5) plus 1 volume of isopropanol and incubated at -20ºC for 2 h or

overnight, followed by centrifugation at 10,000 rpm for 10 min. at 4ºC. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 500-1000 µl of TE buffer pH 6.5 (10 mM Tris-HCl and 0.1 mM Na-EDTA pH 8.0) depending upon the size of pellet. DNA concentration was determined spectrophotometerically at 280/260 nm and adjusted to 10 ng/µl.

2. Randomly Amplified Polymorphic - Polymerase Chain Reaction (RAPD-PCR):

RAPD-PCR was carried out according to the procedure given by **Williams *et al.*, (1990)** with minor modifications.

Amplified reaction was carried out in a volume of 50 µl. Each reaction mixture contained 50 ng genomic DNA (as a template), 0.5 µM decamer oligonucleotide primer from OPERON Technologies, Alameda, CA. (Kit A, C, D, E and Z), 2 units of *Taq* DNA polymerase (Promega Corp., Madison, WI, USA), 5 µl of 10X buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0) and 1% Triton X-100], 3 mM $MgCl_2$, 0.2 mM dNTPs (dATP, dCTP, dTTp, dGTP) and deionized dd H_2O. The reaction was overlaid with a drop of mineral oil. PCR amplified was performed in a Perkin-Elemer/DNA Thermal cycler 480 (Norwalk, CT) for 40 cycles after initial denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min, extension at 72ºC for 2 min. The primer extension segment was extended to 5 min at 72ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agrose gel at 60 volts for three and half hrs with 1X TBE buffer (10 gmTris-base, 5.5 gm boric acid and 4 ml 0.5 M Na-EDTA pH 8.0 in 1 liter). PCR products were visualized by staining gel in ethidium bromide (0.5 µg/ml) and photographed under UV light using a polarized camera. Amplified products were visually examined and the presence or absence of each size class was scored as 0 and 1, respectively.

Nucleotide Sequence (5'-3') of Operon 10-mer Primers used in RAPD-PCR

	Primer	Nucleotide Sequence 5'-3'
1	**OPA-04**	AATCGGGCTG
2	**OPA-06**	GGTCCCTGAC
3	**OPA-11**	CAATCGCCGT
4	**OPA-17**	GACCGCTTGT
5	**OPC-07**	GTCCCGACGA
6	**OPD-14**	CTTCCCCAAG
7	**OPE-05**	TCAGGGAGGT
8	**OPE-17**	CTACTGCCGT
9	**OPZ-13**	GACTAAGCCC
10	**OPZ-19**	GTGCGAGCAA

3. RAPD analysis:

The banding patterns generated by RAPD-PCR analysis were compared to determine the genetic differences of treated and untreated fungi. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The similarity coefficient (F) between the control and the treatment was defined by the formula of **Nei and Li (1979)**, $F=2N_{XY}/N_X+N_Y$, where N_{XY} is the number of common bands between the control and treatment and N_X and N_Y are the number of bands in the control X and the treatment Y, respectively. Dendrogram was derived from the distance by the unwighted paired-group method, arithmetic mean (UPGMA) algorithm contained in the computer program package NTSYS 1.5 **(Rohlf, 1990).**

Results and Discussion:

I.Antifungal activity of the selected compounds against *Fusariumoxysporum*:

The antifungal activity of the tested compounds upon *F. oxysporum* after 6 days of incubation and at the end of incubation period is given in table (1). From the obtained results, the inhibition percentage when using thymol compound after 6 days of incubation at 50 µg/ml was 16.93%, at 250 µg/ml was 98.41% and concentrations 100, 150 and 200 µg/ml gave 65.08, 87.98 and 95.82% growth inhibition, respectively. In case of using eugenol, 50 µg/ml concentration gave 3.91% growth inhibition; 250 µg/ml gave 57.29% while the concentration 100, 150 and 200 µg/ml gave 17.78, 33.23 and 46.57% growth inhibition, respectively. For methyl cinnamate, the lowest concentration (50 µg/ml) gave 0.06% growth inhibition, approximately middle concentration (150 µg/ml) had 11.14% linear growth inhibition while the highest concentration (250 µg/ml) had 39.05% growth inhibition. For the lowest effective compound, linalool 500 µg/ml concentration gave 1.36%; the 8000 µg/ml gave complete growth inhibition whereas 4000 µg/ml concentration gave 80.64% growth inhibition. The 1,8-cineol compound showed no antifungal activity for concentrations 500, 1000, 2000 and 4000 µg/ml and gave only 10.67% growth inhibition at 8000 µg/ml concentration.

From the LC_{50} values of the tested natural occurring compounds given in the table we concluded that the most effective compound was thymol followed by eugenol, methyl cinnamate and finally linalool where the 1,8-cineol compound showed no antifungal activity on the tested fungus. The LC_{50}'s were 81.89, 214.74, 290.66 and 1357.4 µg/ml for thymol, eugenol, methyl cinnamate and linalool, respectively.

According to the LC_{50} values, the toxicity indexes of the tested compounds, eugenol, methyl

cinnamate and linalool were 38.14, 28.16 and 6.03%, respectively when comparing with the highest effective compound thymol which had recorded the highest toxicity index 100%. The relative potencies of the selected compounds, thymol, eugenol and methyl cinnamate were 16.58, 6.32 and 4.67 folds, respectively when compared with the lowest effective linalool compound.

The Ldp-lines of the selected compounds were plotted on a logarithmic paper (log concentration) in relation to percentage of fungal growth inhibition as given in figure (10). The highest slope was for linalool compound (5.14) and then after, when using thymol (4.47). The lowest slope was for eugenol compound (2.78), after that, the slope of methyl cinnamate (4.24).

It can be concluded that compounds can be arranged descendingly according to their effectiveness upon *F. oxysporum* as follows; thymol>eugenol> methyl cinnamate> linalool

Table (1): Effect of the selected compounds on *Fusariumoxysporum* after 6 days of incubation.

Compounds tested	Concentrations in ppm (µg/ml)					LC_{25}	LC_{50}	Slope	Toxicity Index	Relative Potency
	A	B	C	D	E					
Thymol*	16.93	65.08	87.98	95.82	98.41	57.84	**81.89**	4.47	**100**	**16.58**
Eugenol*	3.91	17.78	33.23	46.57	57.29	122.90	214.74	2.78	38.14	6.32
Methyl cinnamate*	0.06	2.5	11.14	24.54	39.05	201.58	290.66	4.24	28.16	4.67
Linalool**	1.36	24.76	80.64	99.21	100	1003.42	1357.4	5.14	6.03	1
1,8-Cineol**	0.0	0.0	0.0	0.0	10.67	-	-	-	-	-

A, B, C, D and E concentrations are:
* 50, 100, 150, 200 and 250 µg/ml; ** 500, 1000, 2000, 4000 and 8000 µg/ml, respectively.

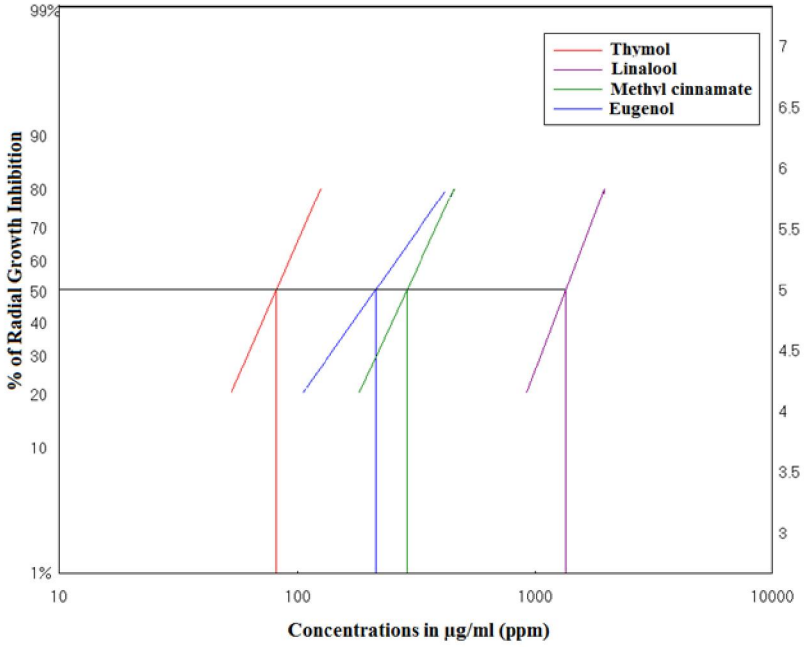


Fig.(1): Ldp lines of the selected compounds against *F. oxysporum*.

III.1. SDS-PAGE analysis:

The effect of the tested naturally occurring compounds namely, thymol, eugenol and methyl cinnamate on the protein banding pattern of *Fusariumoxysporum* mat, as an example of the five tested fungi was investigated. This investigation was carried out by SDS-PAGE electrophoresis.

Among the protein banding patterns of untreated and treated *F. oxysporum* mats, at the LC_{10} concentrations incubated for 6 days at 25°C, showed the presence of 8 different protein bands that ranged in molecular weight between 51.209 and 15.927 KDa in untreated fungus (Fig. 2 and Table 2) and 12 different protein bands ranging between 51.347 and

16.195 KDa with the fungus treated with eugenol. The highest molecular weight bands were recorded in the fungal mat treated with methyl cinnamate (328.333 and 311.667 KDa), while the lowest molecular weight bands were recorded when the fungus was treated thymol (14.854 KDa). The untreated fungus produced number of protein bands lower than the treated fungi. Two bands which have molecular weights of (32.76 and 30.938 KDa.) were characterized the untreated fungus.

The dendrogram in figure (22) was divided into two clusters. Cluster 1 represented the untreated sample and cluster 2 represented the treated samples with different tested compounds (phenolic and ester compounds). Cluster 2 was divided into 2 groups. Group 1 represented the sample treated with methyl cinnamate and group 2 represented the samples treated with the thymol and eugenol.

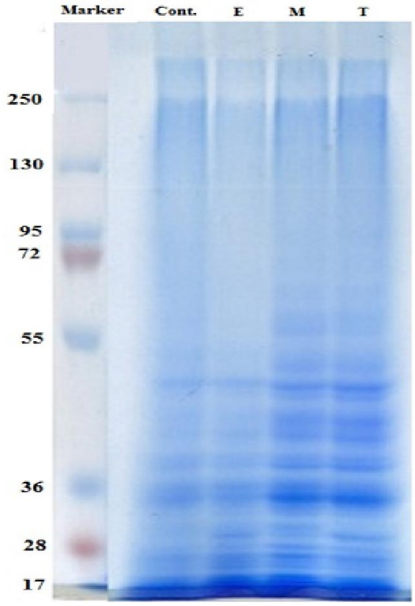


Fig. (2): SDS-PAGE analysis of *Fusariumoxysporum* matstreated with three different organic compounds at LC_{10}. **Marker**: standard protein marker (Fermentous). **Cont**: Total protein extract of untreated fungal mat. **E, M and T**: Total protein extracts from fungal mats under the action of eugenol**(E)**, methyl cinnamate**(M)** and thymol**(T)**.

III.2. Randomly Amplified Polymorphic-Polymerase Chain Reaction (RAPD-PCR)*:*

The RAPD-PCR technique was used as a tool for setting up a convenient and standard protocol to determine the genetic variation between untreated and treated *F. oxysporum*.

Five (OPA-04, OPA-11, OPA-17, OPC-07 and OPZ-19) out of ten primers gave clearly differences between treated and untreated fungi on the basis of the amplified product patterns as shown in Figures 23, 24, 25, 26 and 27. The comparison between the untreated and treated samples showed differences in the sizes and numbers of the amplified fragments per primer, indicating a high degree of variability between untreated and treated samples.

The result in table (18) showed that a total of 12 DNA fragments were produced with OPA-04 primer between untreated fungus and treated fungi with eugenol. One band at 1750 bp was characterized the untreated fungus. Furthermore, eight different discriminating fragments were found to distinguish the fungus treated with Eugenol from untreated fungus.

In OPA-11, the total DNA amplified fragments between untreated and treated samples was seven, one band at 2450 bp was characterized the sample treated with Eugenol from the untreated sample. In OPA-17, OPC-07 and OPZ-19 no discriminating bands could be considered specific to differentiate between untreated and treated Eugenol samples.

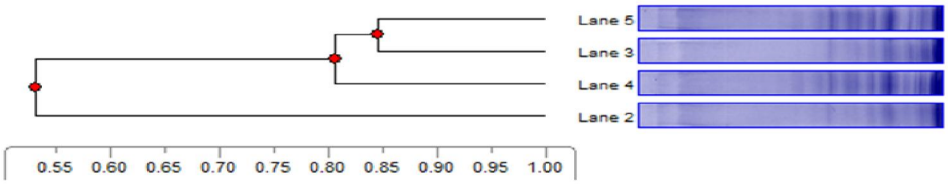


Fig. (3): Dendrogram demonstrating the relationships between untreated *F. oxysporum* and treated *F. oxysporum* with three different organic compounds (Eugenol, Methyl cinnamate and Thymol separately) based on the protein banding patterns. Lane 2: untreated fungus, lanes 4, 3 and 5: treated fungus with methyl cinnamate, eugenol and thymol, respectively.

Table (2): Molecular weights (KDa) of protein peptides detected by SDS-PAGE in protein profiles of *Fusariumoxysporum* treated with eugenol, methyl cinnamate and thymol at concentration of LC_{10} compared to untreated fungus (control):

Ref Band Number	Control		Eugenol		Methyl cinnamate		Thymol	
	Band No	MW	BandNo	MW	Band No	MW	Band No	MW
1	-	-	-	-	1	328.333	-	-
2	-	-	-	-	2	311.667	-	-
3	-	-	1	51.347	-	-	1	51.362
4	**1**	**51.209**	-	-	3	51.209	-	-
5	-	-	2	48.09	-	-	-	-
6	-	-	-	-	4	47.951	-	-
7	-	-	-	-	-	-	2	47.678
8	-	-	3	45.071	-	-	-	-
9	-	-	-	-	5	44.905	-	-
10	-	-	-	-	-	-	3	44.453
11	-	-	4	40.375	-	-	-	-
12	-	-	-	-	6	39.844	-	-
13	-	-	-	-	-	-	4	39.641
14	-	-	5	39.325	-	-	-	-
15	-	-	-	-	7	38.983	-	-
16	**2**	**38.815**	-	-	-	-	-	-
17	-	-	-	-	-	-	5	38.795
18	-	-	6	35.629	-	-	-	-
19	-	-	-	-	8	35.284	-	-
20	**3**	**35.172**	-	-	-	-	-	-
21	-	-	7	34.623	-	-	-	-
22	-	-	-	-	9	34.433	-	-
23	**4**	**34.332**	-	-	-	-	6	34.339
24	**5**	**32.76**	-	-	-	-	-	-
25	-	-	-	-	10	31.068	-	-
26	**6**	**30.938**	-	-	-	-	-	-
27	-	-	-	-	11	30.094	-	-
28	-	-	8	29.794	-	-	7	29.788
29	-	-	-	-	12	27.796	-	-
30	-	-	9	27.587	-	-	-	-
31	-	-	-	-	-	-	8	27.157
32	-	-	-	-	13	25.516	-	-
33	**7**	**24.757**	10	24.753	-	-	9	24.756
34	-	-	-	-	14	22.598		
35	-	-	11	22.313	-	-	-	-
36	-	-	-	-	-	-	10	22.032
36	-	-	-	-	15	16.463	-	-
37	-	-	12	16.195	-	-	-	-
38	**8**	**15.927**	-	-	-	-	11	15.927
39	-	-	-	-	-	-	12	14.854

Result in table (19) showed the genetic variation between the untreated and treated sample with methyl cinnamate at LC_{10}. OPA-04 and OPC-07 were found to distinguish untreated from the treated fungus, which the total number of amplified bands was 11. The unique band at 1750 bp was characterized untreated fungus while seven distinguished fragments were amplified, from 1350 bp to 1650 bp and from 2130 to 2500 bp, and distinguished the treated fungus from untreated one. In OPC-07 the total number of amplified bands was 12, the band at 380 bp was characterized the untreated sample and the band at 650 bp was characterized the treated fungus.

OPA-11, OPA-17 and OPZ-19 were unable to discriminate the untreated sample from treated sample. The result in table (20) revealed the comparison between the untreated and treated samples with Thymol at LC10. OPC-07 and OPZ-19 no discrimination was found between untreated and treated samples, while with OPA-04 one amplified fragment at 1750 bp was characterized the untreated sample from the treated one but nine distinguished bands was characterized the sample treated with thymol, these bands was ranged from 1350 bp to 1650 bp and from 2130 bp to 2500 bp.

With OPA-11 and OPA-17 there was one band in each characterized the sample treated with thymol at 2450 bp and 300 bp, respectively.

Primer OPA-04 showed potential for discriminating the treated samples with three compounds in compared with untreated fungus. OPA-11 and OPA-17 were characterized the sample treated with Thymol, while OPZ-19 and OPC-07 were characterized methyl cinnamate.

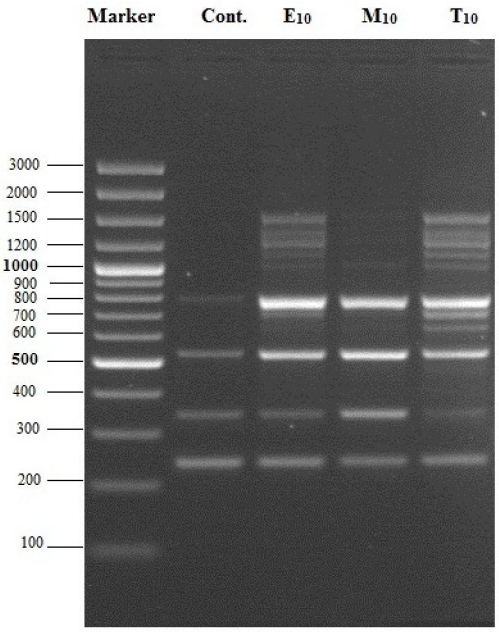


Fig. (4): RAPD fragments amplified from genomic DNA of untreated and treated *F. oxysporum* generated by OPA-04. **Marker**: standard DNA. **Cont.**: untreated fungus. **E_{10}**, **M_{10}** and **T_{10}**: treated fungus with Eugenol (E_{10}), Methyl cinnamate (M_{10}) and Thymol (T_{10}) separately at LC_{10}.

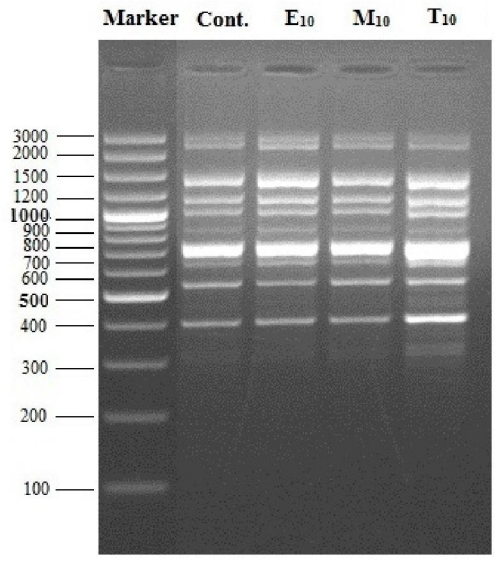


Fig. (5): RAPD fragments amplified from genomic DNA of untreated and treated *F. oxysporum* generated by OPA-17. **Marker**: standard DNA. **Cont.**: untreated fungus. **E_{10}**, **M_{10}** and **T_{10}**: treated fungus with Eugenol (E_{10}), Methyl cinnamate (M_{10}) and Thymol (T_{10}) separately at LC_{10}.

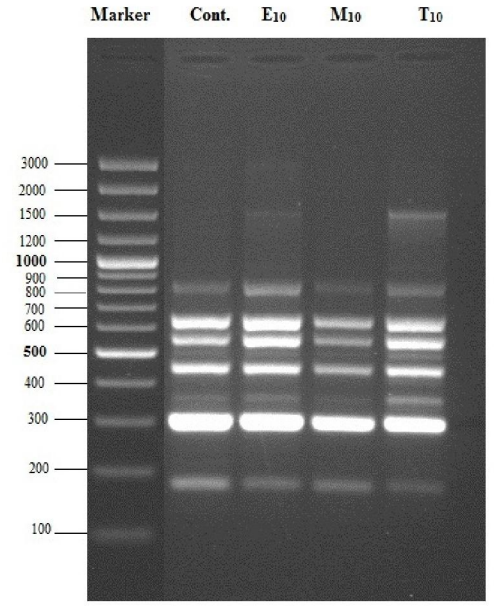


Fig. (6): RAPD fragments amplified from genomic DNA of untreated and treated *F. oxysporum* generated by OPA-11. **Marker**: standard DNA. **Cont.**: untreated fungus. **E_{10}**, **M_{10}** and **T_{10}**: treated fungus with Eugenol (E_{10}), Methyl cinnamate (M_{10}) and Thymol (T_{10}) separately at LC_{10}.

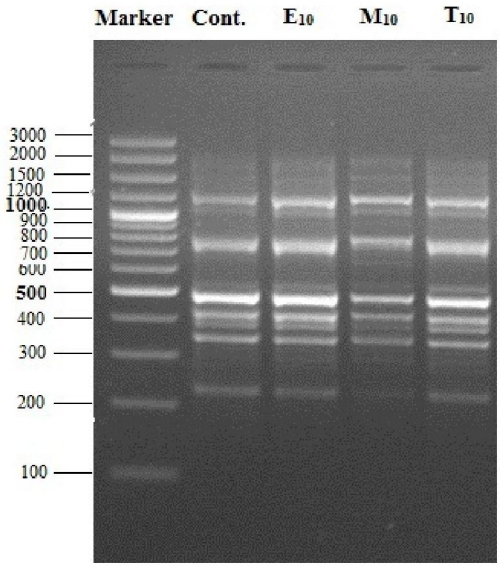


Fig. (7): RAPD fragments amplified from genomic DNA of untreated and treated *F. oxysporum* generated by OPC-07. **Marker**: standard DNA. **Cont.**: untreated fungus. **E_{10}**, **M_{10}** and **T_{10}**: treated fungus with Eugenol (E_{10}), Methyl cinnamate (M_{10}) and Thymol (T_{10}) separately at LC_{10}.

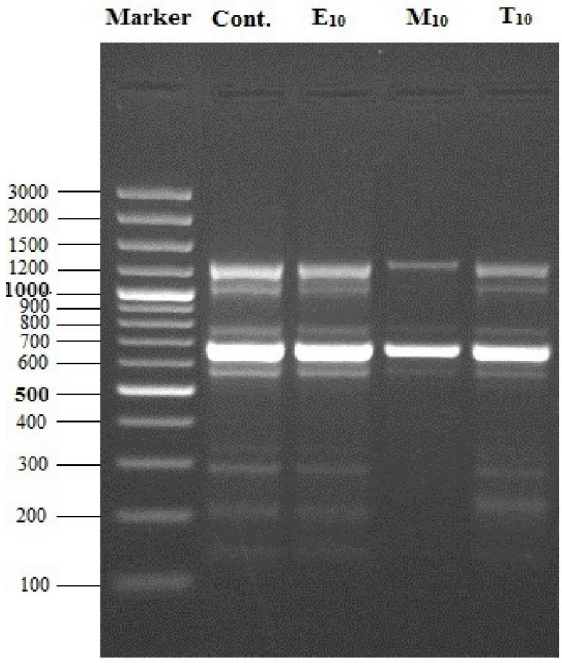


Fig. (8): RAPD fragments amplified from genomic DNA of untreated and treated *F. oxysporum* generated by OPZ-19. **Marker**: standard DNA. **Cont.**: untreated fungus. **E_{10}**, **M_{10}** and **T_{10}**: treated fungus with Eugenol (E_{10}), Methyl cinnamate (M_{10}) and Thymol (T_{10}) separately at LC_{10}.

Table (3): Oligonucleotide primers used for generating random amplified polymorphic DNA patterns from untreated and treated *F. oxysporum* with Eugenol:

Primer	Total No. of bands in treated and untreated	Control		Eugenol	
		NAB	NDB	NAB	NDB
OPA-04	12	4	1	11	8
OPA-11	7	6	0	7	1
OPA-17	9	9	0	9	0
OPC-07	11	11	0	11	0
OPZ-19	9	9	0	9	0

Table (4): Oligonucleotide primers used for generating random amplified polymorphic DNA patterns from untreated and treated *F. oxysporum* with Methyl cinnamate:

Primer	Total No. of bands in treated and untreated	Control		Methyl cinnamate	
		NAB	NDB	NAB	NDB
OPA-04	11	4	1	10	7
OPA-11	6	6	0	6	0
OPA-17	9	9	0	9	0
OPC-07	12	11	1	11	1
OPZ-19	9	9	5	4	0

Table (5): Oligonucleotide primers used for generating random amplified polymorphic DNA patterns from untreated and treated *F. oxysporum* with Thymol:

Primer	Total No. of bands in treated and untreated	Control		Thymol	
		NAB	NDB	NAB	NDB
OPA-04	13	4	1	12	9
OPA-11	7	6	0	7	1
OPA-17	10	9	0	10	1
OPC-07	11	11	0	11	0
OPZ-19	9	9	0	9	0

NAB = No. of Amplified Bands
NDB = No. of Differentiation bands

RAPD analysis:

The combined data from all samples were analyzed using SAHN program to produce a

dendogram (figure 28). Variation in intensity was observed with some bands in the developed RAPD patterns, but this was not considered in the computer analysis.

The only factor considered was presence or absence of any particular bands. The resulting dendogram showed the relatedness and genetic variation between the untreated and treated samples and also among the treated samples with the three tested compounds.

The dendogram was divided into clusters. The first cluster was represented the untreated fungus while the second cluster was divided into two groups. Group no.1 was represented the samples treated with methyl cinnamate and group no.2 included the samples treated with thymol and eugenol.

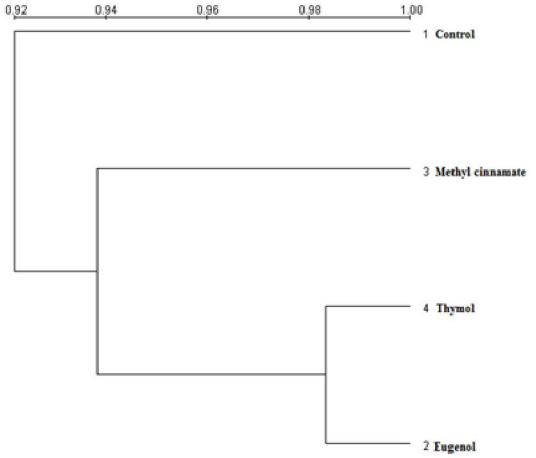


Figure (9): Dendogram showing relationships between the untreated and treated samples (*F. oxysporum*). Based on the RAPD polymorphisms. **1**: Control (untreated sample). **2**: Treated fungus with eugenol. **3**: Treated fungus with methyl cinnamate. **4**: Treated fungus with thymol.

4. Discussion:

Pathogenic fungi could decrease the growth of many economically important crops **(Fletcher *et al.*, 2006)**. Synthetic fungicides are currently the main solutions for fungal pathogenic diseases. Conversely, resistance of fungicides by various plant pathogenic fungi is the primary cause with regard to poor disease control of agriculture **(Steffens *et al.*, 1996; Aguin *et al.*, 2006; Ishii, 2006)**. Moreover, the use of synthetic fungicides could be dangerous to humans and the environment. Recently, the use of synthetic fungicides is diminishing, thus the substitutions of their materials by natural products has increased significantly with regard to research, especially when considering the health and environmental benefits **(Duke, 1993; Daayf *et al.*, 1995)**.

Alternative natural pesticides are necessary for use in the control of pathogenic fungal diseases in plants. Essential oils and extracts from various parts of plants is one of the most promising groups of natural compounds which may be developed for use as natural fungicides substitute the synthetic pesticides due to the presence of terpene constituents within differing functional groups found in the oils. There exists much evidence indicating that the essential oils and various extracts of plants were employed as fungicides **(Manohar *et al.*, 2001; Chang *et al.*, 2008 and Yen *et al.*, 2008).**

Recently, several investigations have been conducted into the antifungal actions of essential oils against phytopathogenic fungi **(Shimoni *et al.*, 1993; Zygadlo *et al.*, 1994; Prudent *et al.*, 1995; Zygadlo *et al.*, 1995; Carta *et al.*, 1996 and Bishop and Thorton 1997).**

Accordingly, an investigation was carried out to elucidate the effect of five naturally occurring compounds namely eugenol, thymol, methyl cinnamate, linalool and 1,8-cineol on *F. oxysporum*.

Biological activity of natural compounds in relation to their chemical structure:

In the antimicrobial action of essential oil components, the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of the main importance. The activity rank of essential oil components is as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. The highest activity was reported for phenols – thymol, carvacrol and eugenol, which is explained by the acidic nature of the hydroxyl group, forming a hydrogen bond with an enzyme active center **(Kalemba and Kunicka 2003)**. Therefore, essential oils with phenols as main compounds express the highest activity against microorganisms, and their activity spectrum is the broadest.

The antifungal activity of 47 kinds of essential oils and several related compounds were examined against seven fungi. The results suggest that secondary alcohols (e.g., 2-octanol, L-menthol, borneol) and tertiary alcohols (e.g., linalool) possess a markedly lower antifungal activity as compared to primary alcohols such as cinnamyl alcohol, geraniol, and citronellol. The antifungal activity of eugenol (4-allyl-guaiacol), a phenolic compound, was found to be 8-10 times higher than that of guaiacol (o-methoxyphenol) and 3-4 times higher than that of creosol (4-methylguaiacol). From the molecular structure, it is clear that the addition of alkyl or alkenyl groups to the benzene ring of either phenol or guaiacol enhanced the antimicrobial activity. The activity of these phenolic compounds appeared to depend on the size of the added alkyl or alkenyl

group, where the larger the size of the alkyl or alkenyl group, the stronger the antimicrobial activity **(Kurita *et al.*, 1981, Knobloch *et al.*, 1989 and Pelczar *et al.*, 1993).** Because alkyl or alkenyl groups are hydrophobic, these results indicate that hydrophobicity above a minimum extent was required for phenolic compounds to show a potent antimicrobial effect.

The exact cause-effect relation for the mode of action of phenolic compounds has not been determined yet, but **Davdison (1993)** indicated that, it may deactivate essential enzymes, reacting with the cell membrane or disturbing material functionality.

Biochemical and Molecular Studies:

SDS-PAGE analysis:

When the fungus subjected to the three treatments, eugenol, methyl cinnamate and thymol, new protein bands were produced. Two protein bands (32.76 and 30.938 KDa) were produced in the untreated fungus and characterized it. The appearance of new protein bands in the treated samples refer to the synthesis of the new protein which is controlled by the structural gene, while the variability in the band intensity may be associated with the effect of the organic compounds on the gene expression **(Mendhulkar, 1993; Georgiou & Petropoulou, 2001; Georgiou *et al*., 2003).**

Bai *et al.* (2004) and Eichholzer *et al.* (2006) reported that the stress proteins are formed by microorganisms as a result of a change in the environment such as exposure to heat, radiation or chemicals, so-called stress-susceptible genes are expressed. According to current insights, such proteins can contribute to protect it against environmental changes.

From dendrogram, the untreated sample was completely different from the treated samples. Cluster 2 is divided into two groups, group 1 represented the sample treated with methyl cinnamate and group 2 represented the samples treated with eugenol and thymol. This result may explain that, the thymol and eugenol may have the same genetic variation and accordingly, may have the same mode of action on the fungal growth while, the methyl cinnamate may has mode of action differs from thymol and eugenol on the fungal growth. Since, the electrophoresis of proteins appeared to be a useful tool for identification and characterization the genetic differences between untreated and treated fungi and also among the treated samples.

Burgess *et al.* (1995) and Aly *et al.* (2003) reported that the SDS-PAGE technique is relatively simple and inexpensive for differentiation and identification of isolates and has been used previously for studying variation in a number of fungal populations.

Randomly Amplified Polymorphic-Polymerase Chain Reaction (RAPD-PCR) between treated and untreated *F. oxysporum* fungus*:*

Although identification and differentiation between fungal strains or untreated and treated samples on the basis of morphological and pathological characteristics was used by many workers for a long time, it faced problems in identification and also caused confusion **(Brayford, 1989).**

The Identification and differentiation between organisms based on morphology and electron microscopy is difficult and tedious process. Therefore, biochemical and molecular technique were used in an attempt for differentiation between untreated and treated *F. oxysporum*.

The main goal of this research was to use RAPD-PCR and protein analysis as genetic markers to differentiate between untreated fungus and treated fungus with three different organic compounds.

In RAPD-PCR analysis, five decameric random oligonucleotide primers (OPA-04, OPA-11, OPA-17, OPC-07 and OPZ-19) out of ten primers tested, easily distinguished the untreated fungus from the treated fungi.

OPA-04 shows potential for discriminating the treated samples with three compounds from the untreated fungus while, OPA-11 and OPA-17 were characterized the sample treated with Thymol, while OPZ-19 and OPC-07 were characterized the sample treated with methyl cinnamate.

The result of the RAPD analysis indicated the close relationship between the Eugenol and Thymol. The two components (phenolic compounds) were in the same group in dendrogram, while Methyl cinnamate (ester compound) was in separate group but under the same cluster. The untreated sample was separated completely from the treated samples and represents cluster 1 and the treated samples represent cluster 2. RAPD analysis is extremely powerful and can separate individuals having intra- and inter-specific variability **(Khalil *et. al.*, 2003).** It gives more comprehensive information regarding the genetic variability among the pathogen populations as it is based on the entire genome of a microorganism (**Achenback *et al.*, 1996).**

This result was similar to SDS-PAGE analysis. The RAPD-PCR analysis indicated that the Eugenol and Thymol may have the same mode of action on the fungal growth; otherwise methyl cinnamate has a different mode of action.

RAPD-PCR is a powerful tool in differentiation between untreated and treated samples and also among the three treatments.

The aforementioned discussion clarifies the suitability of RAPD-PCR and protein markers to

distinguish between the untreated and treated samples and also among the treated samples.

Biochemical and molecular markers are being increasingly used to characterize fungal plant pathogens. They are versatile and highly informative tools for fungal pathogen identification and diagnosis **(Majer *et al.*, 1996).**

The electrophoretic separation of protein profiles, SDS-PAGE, is a useful tool in differentiation. This method is relatively easy and many samples can be analyzed at the same time. It is also cheaper than other fingerprinting methods. Moreover, the results obtained by SDS-PAGE of whole-cell proteins can discriminate at much the same level as DNA fingerprinting **(Priest and Austin, 1993)** in some cases.

Conclusion

According to the hypothesis relationship between chemical structure of natural compound and antifungal activity; thymol and eugenol (phenolic compound) were the most predominant antifungal activity towards the tested fungi followed by methyl cinnamate (as a part of keto compound) where the lowest antifungal activity were linalool and 1,8-cineol (tertiary alcohol and ether). Besides using the morphological and pathological characteristics, the present work aims to employ SDS-PAGE and RAPD-PCR as a genetic variation tools in differentiation between untreated and treated samples.

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