



Phytochemical and SDS-dissociated proteins of pathogenic and nonpathogenic *Fusarium oxysporum* isolates

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Abstract: The aim of this study to characterize and compare the phytochemical and protein pattern of pathogenic and non-pathogenic *Fusarium oxysporum* isolates.

Fusarium oxysporum is considered one of the most distributed species in soil-borne fungi communities, particularly in plant rhizospheres, where pathogenic and nonpathogenic strains may be found. Higher phenolic and flavonoid content was found in the extract of the nonpathogenic *F. oxysporum* (F.o.-T5) isolate, as the total phenol and total flavonoid were 56.0 and 27.0 mg /g respectively. Whereas, the total phenol and flavonoid content in culture filtrates of the pathogenic *F. oxysporum* (F.o.-T2) were lower by 12.5% and 29.63% respectively than the nonpathogenic isolate.

Results of SD-PAGE protein showed that the pathogenic *F. oxysporum* (F.o.-T2) detected only six bands at R_f values ranged from 5 to 35 KDa, while the nonpathogenic *F. oxysporum* (F.o.-T5) showed bands at R_f ranged from 5 to 245 KDa. The differences in protein patterns were sufficient to allow comparison of the fungal isolates.

Twenty two compounds were identified by GC/MS analysis of culture filtrate of the nonpathogenic *F. oxysporum* isolate and these compounds were varied in their chemical and molecular weight than that compounds detected in culture filtrate of the pathogenic isolate.

Keywords: *Fusarium oxysporum*, protein pattern, SDS-dissociated, GC/MS, nonpathogenic, phytochemical analysis.

Introduction:

Fusarium species are important pathogen of vascular wilt, a disease that affects a large variety of economically important crops, especially in the temperate regions of the world^{1,2}. Infection with *Fusarium* commonly results in reduction of the quality and yield of the crop. Additionally, many of these fungi are capable of producing phytotoxic secondary metabolites that cause wilting, necrosis, growth inhibition and inhibition of seed germination in some plants^{3,4}. The endophytic nonpathogenic isolates of *F. oxysporum* have the same characteristic as pathogenic, except that they are not disease causing and hence are important because these organisms can sustain up to the crop duration². Some strains of the nonpathogenic *F. oxysporum* isolates have shown the ability to suppress the growth of several fungal plant pathogens such as *Phytophthora erythroseptica* and *Pythium ultimum*^{5,6} and to affect the germination of *Sclerotium sclerotiorum* sclerotia⁷. A little is known about the antagonism related with antifungal metabolite production by non-pathogenic *F. oxysporum*⁸ but several investigators reported that the endophytic fungi residing some plants are able to produce bioactive compounds such as saponins, phenol, flavonoid, tannins, alkaloids, anthroquinones and terpenoids^{9,10,11}. Rasekhi¹² reported that GC/MS results revealed different metabolites in culture filtrates of *F. proliferation* and some of them are toxic compounds against fungi and bacteria and possess many biological activities.

Biological control of wilt diseases caused by *Fusarium oxysporum* on several crops, has been attained with strains of nonpathogenic *Fusarium oxysporum*^{13,14}. Nawar, Lubna¹⁵ reported that the nonpathogenic isolate of *F. oxysporum* isolate was highly antagonistic to the pathogenic isolate *in-vitro* and *in-vitro* tests.

It was therefore, necessary to implement an easy time saving and practical technique, to distinguish between pathogenic and nonpathogenic *Fusarium oxysporum*^{16,17}. The implications of gel electrophoresis in fungal characterization and taxonomy have been reviewed by^{18,19} for phytopathogenic fungi. Techniques that aid identification (gel electrophoresis) have made possible the examination of inherent genetic variation among a number of pathogenic and nonpathogenic *Fusarium* spp. in the rhizosphere soil¹⁷. Mycelial differences in protein pattern among *Fusarium* spp. and their isolates have been reported by various workers^{20,21,22,23}.

This investigation was done to characterize and compare the protein pattern (SDS_PAGE) from mycelia and the variation between phytochemical composition of the two culture filtrates of pathogenic and nonpathogenic *Fusarium oxysporum* isolates.

Materials and Methods:

1. Fungal isolates: An isolate of pathogenic *Fusarium oxysporum* f.sp. *lycopersici* (F.O.-T2) and isolate of nonpathogenic *F. oxysporum* (F.o.-T5) used in this investigation were previously isolated and identified by the author¹⁵. The pathogenic isolate was isolated from the roots of wilted tomato plant, while the non-pathogenic isolate was isolated from the rhizosphere of symptomless and healthy tomato root collected near Jeddah, Saudi Arabia. *Fusarium oxysporum* isolates were stored on slants of potato dextrose agar (PDA) medium at 4°C until used in the current research. Greenhouse test was done to evaluate the pathogenic or nonpathogenic ability of the two tested isolates (F.o.T2 and F.o.T5 respectively) for infection using tomato seedlings as the host plant (unpublished data).

2. Growing fungal isolate: Preparation of culture filtrate and extraction of mycelial proteins for gel electrophoresis were carried out according to¹⁷. Mycelium was produced in 250ml Erlenmeyer flasks containing 100ml of potato dextrose broth (PDB) {200g of peeled fresh potatoes and 20g of dextrose/l of distilled water}. and the pH was adjusted to 6.5. Small mycelial plugs on PDA of individual isolate was aseptically transferred into flasks containing sterilized PDB and incubated in shaker at 27±2°C and at 50rpm/min. Mycelium mats from two flasks for each isolate were combined and filtered through five layer of sterile cheese cloth, washed three times with phosphate buffer (pH,7.0) and then frozen at -20°C until use.

3. Determination of total Phenol content. Total phenolic contents in the culture filtrates of pathogenic and nonpathogenic *Fusarium oxysporum* were estimated using the Folin-Ciocalteu colorimetric method described by Taga *et al.* (1984) with some modification. Briefly, the appropriate dilutions of the samples (0.2 ml) were oxidized with 0.5N Folin-Ciocalteu reagents for 4 min at room temperature. Then the reaction was neutralized with saturated sodium carbonate (75 g/l). The absorbance of the resulting blue color was measured at 750 nm with the spectrophotometer against blank. The total phenol content was calculated on the basis of the standard curve of gallic acid. Phenol contents were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

4. Determination of total flavonoid content: Total flavonoid content in the culture filtrates was determined by a colorimetric method reported by²⁵. Extract samples (0.25ml) was mixed with deionized water (1.25 ml). A sodium nitrite solution at 5% (0.75 ml) was added and samples were incubated for 6 min at room temperature. AlCl₃ at 10% (0.15ml) was aggregated and the mixture was incubated (5 min). Finally, 0.5 ml of sodium hydroxide (1 M) was added. Made the volume of the mixture to 2.5 ml with distilled water and incubated at 25°C for 30 min. Absorbance was measured at 510 nm against blank. The content of flavonoid was calculated on the basis of the standard curve of quercetin and the results were expressed as mg of quercetin equivalent per g of extract.

5. SDS PAGE for total soluble proteins:

The Total soluble protein profiles of the pathogenic and nonpathogenic *F. oxysporum* isolates subjected to the above mentioned condition were analyzed by SDS- PAGE according to¹⁷. Protein sample were dissolved in 100µl buffer (0.125M Tris pH 6.8, 20% glycerol, 2% SDS and 14.4mM β- Mercaptoethanol) for 10 minutes in a boiling water bath at 100°C. The samples were cooled to ambient temperature and 50µl of protein samples

were loaded on Tris-glycine gels (5% stacking and 15% resolving). Electrophoresis was performed on biotech vertical gel electrophoresis unit. Current of 50/100 volts for stacking and separating was applied. The gels were stained with 0.25% coomassie blue (0.25gms G250, 10ml acetic acid, 45ml methanol and 45 ml DD water) and visualized in the same solution excluding G250. The gels were photographed and scored for protein bands using Bio-rad Image Lab analysis Gel documentation system.

6. Gas chromatography-mass spectrometry (GC/MS) analysis: Culture filtrates of the pathogenic and nonpathogenic *F. oxysporum* isolates subjected to the above mentioned conditions were analyzed using a Thermo Scientific, Trace GC Ultra/ ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness).

GC-MS Separation of compounds was achieved using a GC-MS system from Agilent company model 7890. An Hp-5MS fused silica capillary column (Hewlett- Packed, 30 m, 0.25 mm i.d., 0.25 μ m film Thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by MS Chem Station software (Hewlett- Packed, version A.01. 01). Electron impact mass spectra were recorded at 70 eV. Ultra-high purity helium (99.999%) was used as the carrier gas at flow rate of 1mL/min. The injection volume was 1 μ L and all the injections were performed in a split less mode. Injector temperatures was 250 $^{\circ}$ C. Temperature program was used: 60 $^{\circ}$ C (2 min)–30 $^{\circ}$ C /min–170 $^{\circ}$ C (5 min)–7 $^{\circ}$ C /min–250 $^{\circ}$ C (10 min)²⁶. The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system. All used chemicals and solvents were HPLC-grade and obtained from Merck Company.

Results and Discussions:

Determination of total phenolic and flavonoid contents of culture filtrates:

Fusarium oxysporum is considered one of the most distributed species in soil-borne fungi communities, particularly in plant rhizospheres²⁷, where pathogenic and nonpathogenic strains may be found. Variation in the total phenolic and flavonoid contents among the pathogenic and nonpathogenic *F. oxysporum* isolates were observed in the present study (Table, 1). The results showed that the higher phenolic and flavonoid contents were found in the extract of the nonpathogenic *F. oxysporum* (F.o.-T5) isolate, as the total phenol and total flavonoid were 56.0 and 27.0 mg /g respectively. Whereas, the total phenol and flavonoid content in culture filtrates of the pathogenic *F. oxysporum* (F.o.-T2) were lower by 12.5 and 29.63% respectively than the nonpathogenic isolate.

Table (1):Total phenol and flavonoid contents of culture filtrates of the pathogenic and non-pathogenic *F. oxysporum*

Extract	Total phenols (mg eq GA/g extract)	Total flavonoids (mg eq qu/g extract)
Non -pathogenic <i>F. oxysporum</i> (F.o.-T5)	56.00 \pm 7.81	27.00 \pm 3.7
Pathogenic <i>F. oxysporum</i> (F.o.-T2)	49.00 \pm 2.92	19.00 \pm 3.21

The more phenol and flavonoid content in the nonpathogenic isolate may have contributed to their antifungal activity. High phenolic and flavonoid contents found in the extract of *Fusarium* imply the contribution of these compounds to antifungal activities which was consistent with early studies^{11,28,29}. Some strains of *F. oxysporum* have shown the ability to suppress the growth of several fungal plant pathogens such as *Phytophthora erythroseptica* and *Pythium ultimum*^{5,6} and to affect the germination of *S. sclerotiorum* sclerotia⁷.

SD-PAGE protein. Analysis of proteins of the mycelium of the pathogenic *F.oxysporum* f.sp. *lycopersici* (F.O.-T2) and isolate of the nonpathogenic *F. oxysporum* (F.o.-T5) by one dimensional gel electrophoresis (SD-PAGE) revealed heterogenicity in protein by location and intensity (Fig. 1).

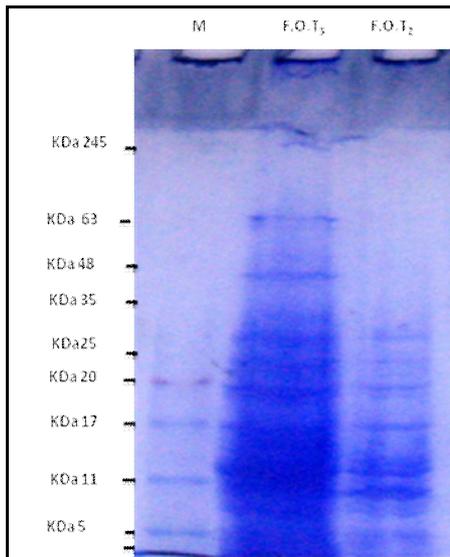


Fig. (1): SD-PAGE proteins of the pathogenic *F.oxysporum* f.sp. *lycopersici* (F.O.-T2) and the nonpathogenic *F. oxysporum* (F.o.-T5) by one dimensional gel electrophoresis.

The SDS-PAGE technique used for analyzing the proteins from the isolates of *F. oxysporum* 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^{30,31}. The results also showed that the pathogenic *F.oxysporum* f.sp. *lycopersici* (F.O.-T2) and the nonpathogenic *F. oxysporum* (F.o.-T5) isolates had their own unique protein profiles. The molecular weight of all protein bands ranged from 5.0 to 245 KDa. The differences in protein patterns were sufficient to allow comparison of the fungal isolates. Data revealed the occurrence of three common band groups in these two *Fusarium* isolates. The first group spans the R_f values range 5.0-35 KDa. The second band group is within the R_f values range 48-63 KDa, while the third group is within R_f values range ≥ 63 to ≤ 245 KDa. Results also showed that the pathogenic *F. oxysporum* (F.o.-T2) detected only six bands at R_f valued ranged from 5 to 35 KDa, while the nonpathogenic *F. oxysporum* (F.o.-T5) showed bands at R_f ranged from 5 to 245 KDa. The present data indicate that it is possible to differentiate between different *Fusarium* isolates as indicated by differences in banding pattern. These results disagree with those obtained by^{15,32,33} as no differences were found among mycelium protein profiles (SDS-PAGE) of different species and formae specialis of *F. oxysporum*.

GC/MS analysis of *Fusarium oxysporum* culture filtrate.

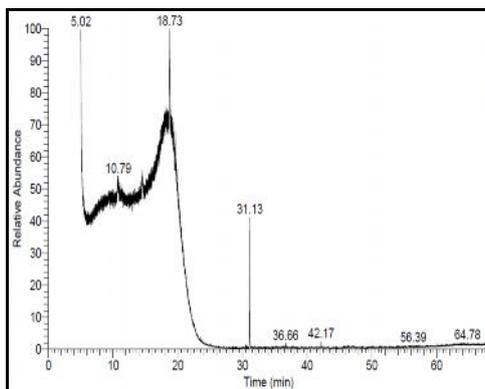


Fig 2. GC-MS chromatogram of nonpathogenic *Fusarium oxysporum* (F.O.-T5) after 21 days Incubation in a PDB medium at 28°C.

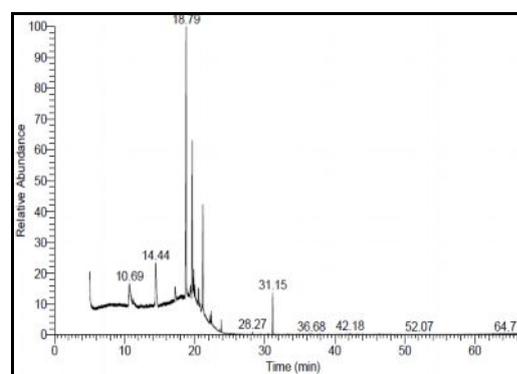


Fig 3. GC-MS chromatogram of pathogenic *Fusarium oxysporum* (F.O.-T2) after 21 days incubation in a PDB medium at 28°C .

Table(2): Components of culture filtrate of the pathogenic *Fusarium oxysporum*f.sp. *lycopersici* performed by GC/MS analysis

No.	Probability	Compound name	Molecular weight	Retention time (Min)	Peak area %	Molecular formula
1	6.91	2'Acetamido3'chloro5'(2(2,4ditertpentylphenoxy)butyrylamino)6'hydroxybenzanilid	621	5.02	1.04	C ₃₅ H ₄₄ ClN ₃ O ₅
2	10.76	3,3'''Dibromo2,2',2'',2'''tetramethoxy5,5',5'',5'''tetramethylquarter phenyl	642	6.34	1.07	C ₃₂ H ₃₆ Br ₂ O ₄
3	87.14	Dodecachloro3,4benzo phenanthrene	636	7.62	1.00	C ₁₈ Cl ₁₂
4	36.73	Cadmium chloride porphine derivative	607	8.69	1.17	C ₂₉ H ₄₀ CdClN ₅
5	87.25	5,11,17,23Tetrabutyl25,26,27,28tetrahydroxycalix4arene	648	9.45	1.51	C ₄₄ H ₅₆ O ₄
6	50.09	(4Bromophenyl) bis(2,4dibromophenyl) amine	635	11.23	1.04	C ₁₈ H ₁₀ Br ₅ N
7	96.32	Dodecachloro3,4benzophenanthrene	636	11.33	1.19	C ₁₈ Cl ₁₂
8	38.20	Penitrem A	633	11.50	1.02	C ₃₇ H ₄₄ ClNO ₆
9	60.73	2,7,12,17tetrabrom(allàs) cyclotetrathiophen	640	12.73	0.98	C ₁₆ H ₄ Br ₄ S ₄
10	97.56	Tetrakis(1,1dimethylethyl)penta Cyclo (octacosal(dodecaene)	646	14.22	1.35	C ₄₄ H ₅₄ O ₄
11	55.48	Dodecachloro3,4benzophenanthrene	636	14.43	5.95	C ₁₈ Cl ₁₂
12	67.37	3,4,5,6Tetrakis (pchlorophenoxy)1,2dicyanobenzene	632	15.96	1.04	C ₃₂ H ₁₆ C ₁₄ N ₂ O ₄
13	37.04	Argon (CAS)	40	16.15	0.95	Ar
14	44.19	1,2Propadiene (CAS)	40	17.25	2.46	C ₃ H ₄
15	63.90	Ethylene oxide	44	17.53	1.46	C ₂ H ₄ O
16	62.07	1(4Anisyl)2,5di(4(1,3dioxolan2yl)phenyl)3,4diphenyl1,3cyclopentadiene	620	17.69	1.84	C ₄₂ H ₃₆ O ₅
17	51.95	2,2'Dibromo5,5'di(4methoxyphenyl)4,4'ditertbutylbiphenyl	634	17.95	1.10	C ₃₄ H=Br ₂ O ₂
18	23.01	Ditungsten, tris(cyclooctatetraene)	680	18.21	1.02	C ₂₄ H ₂₄ W ₂
19	77.35	MesoTetraphenyl2,3 cisdihydroxy2,3chlorin	648	18.26	0.95	C ₄₄ H ₃₂ N ₄ O ₂
20	56.27	{1',2'bis(Methoxycarbonyl)1,1,6,7,11,12hexamethylbenzo[16,17d]phthalocyanine }zinc	624	19.37	1.78	C ₃₄ H ₃₂ N ₄ O ₄ Zn
21	23.27	Methyl 5hydroxy5methyl5,6,7,8Tetrahydronaphthalene1carboxylate	220	31.12	16.46	C ₁₃ H ₁₆ O ₃
22	32.84	6Acetylbenzo[b]naphtho[2,1d]thiophene	276	42.16	1.03	C ₁₈ H ₁₂ OS

Table(3): Components of culture filtrate of the nonpathogenic *F. oxysporum* isolate performed by GC/MS analysis.

No.	Probability	Compound name	Molecular weight	Retention time (R.T.)	% area	Molecular formula
1	98.86	5,10 bis (3aminophenyl)15,20diphenyl por phyrin	644	6.32	1.16	C ₄₄ H ₃₂ N ₆
2	53.83	(2Methoxyethoxy)methyl2,12Dibromo7 phenyl5,6,8,9tetrahydrobenz[a,j]anthracene14c	646	6.37	0.15	C ₃₃ H ₂₈ Br ₂ O ₄
3	31.00	N,N'Bis[3methoxy4hydroxy5bromobenzylidene(cyano)acetyl]1,4butanediamine	646	6.37	0.15.	C ₂₆ H ₂₄ Br ₂ N ₄ O ₆
4	20.84	PALLADIUM	568	6.75	0.19	C ₂₈ H ₃₈ N ₄ O ₂ Pd
5	35.29	YGRKKRRQRRRGV VKRRLDL/5	2598	7.79	0.14	NA
6	38.89	2,7,12,17tetrabrom(allàs)cyclotetrathiophen	640	8.05	0.17	C ₁₆ H ₄ Br ₄ S ₄
7	92.66	(4Bromophenyl)bis(2,4dibromophenyl)amine	635	8.16	0.14	C ₁₈ H ₁₀ Br ₅ N
8	26.10	(4Bromophenyl)bis(2,4dibromophenyl)amine	635	8.78	0.13	C ₁₈ H ₁₀ Br ₅ N
9	87.49	Dodecachloro3,4benzophenanthrene	635	9.19	0.14	C ₁₈ Cl ₁₂
10	56.04	56.04 (Z)13Bromo8,8"(Ethene1,2diyl)bis(2terbutyl4,5,9,10tetrahydropyrene)	630	10.67	4.17	C ₄₂ H ₄₇ Br
11	45.68	{1',2'bis(Methoxycarbonyl)1,1,6,7,11,12 hexamethylbenzo[16,17d]phthalocyanine }zinc	630	11.64	0.12	C ₃₄ H ₃₂ N ₄ O ₄ Zn
12	90.12	Dodecachloro3,4benzophenanthrene	645	11.77	0.14	C ₁₈ Cl ₁₂
13	72.88	8Oxo5,6dihydro6(methoxycarbonyl)5,10,15,20tetraphenyl 8H ₇ oxaphyrin	628	12.38	0.14	C ₄₅ H ₃₂ N ₄ O ₄
14	9.42	Hexamethyl2,7anthraquinono[26,27b]phthalocyanine}zinc	607	12.92	0.12	C ₃₈ H ₃₀ N ₄ O ₂ Zn
15	8.16	2,2Bis[4[(4,6dichloro1,3,5triazin2yl)oxy]phenyl]1,1,1,3,3,3hexafluoropropane	630	13.61	0.21	C ₂₁ H ₈ C ₁₄ F ₆ N ₆ O ₂
16	44.62	1,8Cineole, Eucalyptol, 1,8Cineole	154	14.44	6.99	C ₁₀ H ₁₈ O
17	13.11	Mo(CO) ₂ [(C ₄ H ₉ CP) ₂]Mo(CO) ₄ [(C ₄ H ₉ CP) ₂]	726	15.57	0.30	C ₂₁ H ₂₇ Mo ₂ O ₆ P ₅
18	13.23	1,2Dihydropyridine,1(1oxobutyl)	151	17.22	1.73	C ₉ H ₁₃ NO
19	36.58	2,2'Dibromo5,5'di(4methoxyphenyl)4,4'ditertbutylbiphenyl	634	17.67	0.19	C ₃₄ H ₃₆ Br ₂ O ₂
20	16.48	psi.,psi.Carotene,3,3',4,4'tetrahydro1,1',2,2'tetrahydro1,1'dimethoxy2,2'dioxo	624	18.00	0.18	C ₄₂ H ₅₆ O ₄
21	38.28	(4Bromophenyl)bis(24dibromophenyl)amine	635	18.46	0.17	C ₁₈ H ₁₀ Br ₅ N
22	30.00	Camphor (CAS)	152	18.78	36.59	C ₁₀ H ₁₆ O
23	11.57	Pinocarvone	150	19.41	2.16	C ₁₀ H ₁₄ O
24	27.06	endoBorneol	154	19.66	18.38	C ₁₀ H ₁₈ O
25	19.63	Cyclohexanone,2methyl5(1methylethenyl)	152	19.87	2.91	C ₁₀ H ₁₆ O
26	17.73	3Cyclohexene1methanol,	154	20.57	2.19	C ₁₀ H ₁₈ O
27	6.30	Dodecane,2,2,4,9,11,11hexamethyl(CAS)	254	23.02	0.18	C ₁₈ H ₃₈
28	28.55	(4Bromophenyl)bis(2,4dibromophenyl)amine	635	23.59	0.21	C ₁₈ H ₁₀ Br ₅ N
29	58.75	Butyl hydroxy Toluene	220	31.15	3.21	C ₁₅ H ₂₄ O
30	40.26	7,9Ditertbutyl1oxaspiro(4,5)deca6,9dione2,8dione	276	42.18	0.15	C ₁₇ H ₂₄ O ₃

Present study detected a variation in the chemical composition of culture filtrate of the pathogenic and nonpathogenic *F. oxysporum* isolates. Culture filtrate of the nonpathogenic *F. oxysporum* isolate contained some compounds varied in their chemical composition and their molecular weights. These compounds were differed than that compounds detected in culture filtrate of pathogenic isolate (Figs.,2 and 3 and Tables, 2and 3).The identified compounds from culture filtrate of the nonpathogenic *F. oxysporum* isolate are illustrated in Fig.(2) and Table (3), whereas, thirty compounds were detected by GC/MS. Data also showed that there is a predominance of alkanes in this extract and the main constituents were dodecaene (97.56), Dodecachloro3,4benzophenanthrene (96.32), 28 tetra hydroxycalix4arene (87.25), Dodecachloro3,4benzo phenanthrene (87.14) and mesoTetraphenyl2,3 cisdihydroxy2,3chlorin (77.35).

While, the culture filtrate of the pathogenic *F. oxysporum* isolate detected twenty two compounds and the main constituents were 5,10 bis (3aminophenyl)15,20 diphenyl porphyrin (98.86), (4Bromophenyl)bis (2,4dibromophenyl)amine (92.66), Dodecachloro3, 4benzophenanthrene (90.12), and (methoxycarbonyl) tetraphenyl 8H₇oxaporphyrin (77.88).

This study demonstrated that the pathogenic and nonpathogenic of fungal isolates identified as the same species and isolated from the same rhizosphere soil of the same plant, may vary considerably in the composition of secondary metabolites produced. A little is known about the antagonism related with antifungal metabolite production by nonpathogenic *F. oxysporum*⁸ but several investigators reported that the endophytic fungi residing plants are able to produce bioactive compounds such as saponins, phenol, flavonoid, tannins, alkaloids, anthroquinons and terpenoids^{9,10,11,12}.

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