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Potentiality of some Isolated Compounds from Halfa Barr (*Cymbopogon proximus* Stapf.) against the Toxigenic Fungi *Fusarium verticillioides* and *Aspergillus flavus*

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The *n*-hexane extract of *Cymbopogon proximus* Stapf. showed a potent antifungal effect against two of the toxigenic fungi, namely *Fusarium verticillioides* and *Aspergillus flavus*. The chromatographic analysis of the extract yielded the potent ethyl acetate fraction, from which 8 compounds could be isolated and identified by spectroscopic methods. Three compounds of them are 2-methyl undec-2-en-10 al, eudesm-11 ol and elemol were found to be the most potent compounds against both pathogens.

Keywords: *Aspergillus flavus, Cymbopogon proximus, Fusarium verticillioides*, halfa barr and toxigenic fungi.

Nowadays, it is well known that many of medicinal and indigenous plants have allelopathic effects on the growth of various fungi and bacteria (Yen and Chen, 1994; Inderejit and Spencer, 1995; Nakamura *et al.*, 1999; El-Sayed *et al.*, 2000; El-Kazzaz *et al.*, 2003). Moreover, Some reports have emphasized that certain plant diseases could be controlled by using plant materials. Fahmy and Mahmoud (2001) controlled the late-wilt disease of maize by amending soil with dried leaves of *Eucalyptus rostrata* L. They found also that soaking maize seeds in the alcoholic-eucalyptus leaf extracts caused significant reduction in the disease incidence. El-Assiuty *et al.* (2006) screened different extracts from various plant species for their effect in inhibiting the radial growth of some plant pathogens *in vitro* and controlling the grain rot of maize in the field as well. They found that the *n*-hexane extract of *Cymbopogon proximus* Stapf. (halfa barr) was superior to other extracts of the evaluated plant species in inhibiting the growth of *Fusarium verticillioides* and *Aspergillus flavus* and reducing the production of mycotoxins; fumonisin and aflatoxin. Thus, this study was carried out to identify the principle(s) responsible for the poisonous effect of *C. proximus-n*-hexane extract on these toxigenic fungi.

Materials and Methods

1. *Plant materials:*

Cymbopogon proximus Stapf. (halfa barr) material was purchased from Harraz Herbal Drug Store, Cairo, Egypt in 2005. It was kindly verified by Prof. M. Abdel-Ghany, The Herbarium, Fac. of Sci., Cairo Univ.

2. *Chromatographic materials*:

All used solvents were chemically pure grade:

- Silica gel 60 for column (E. Merck).
- Silica gel G plates for T.L.C. (E. Merck).
- Solvent system:- Toluene : Ethyl Acetate : Methanol (94:3:3). Spray reagent was vanillin-sulphuric acid reagent (Wagner *et al.*, 1984).

3. *Extraction and isolation:*

The powder of the *n*-hexane extract of the plant material was prepared by percolation at room temperature, then the solvent was driven off to give a residue (10gm). The residue was evaluated for the biological inhibitory effect on the target fungi.

4. *Chromatographic fractionation:*

Ten gm of the residue of *n*-hexane extract was chromatographed on a silica gel column and elution was carried out using *n*-hexane 100%, *n*-hexane+ethyl acetate (10, 20, 30 and 50%), ethyl acetate 100 %, ethyl acetate+metahnol (10, 20, 30 and 50%) and finally with methanol 100%. Fractions (100ml each) were collected and tested for their biological actions.

As the 100% ethyl acetate fractions were only active in inhibiting the growth of target fungi, they were collected and dried. Following the method adopted by Wagner *et al.* (1984), the residue (7gm) was re-chromatographed on silica gel column and fractionation was done by elution with toluene 100%, toluene-ethyl acetate (1, 2, 3, 4, 5, 10, 50 and 100%). Fractions (100ml each) were collected and screened for their biological activity. Fraction was obtained, and by T.L.C. it showed several spots (Tables 4 and 5), then the spots were separated by preparative T.L.C. using silica gel G 0.25mm thickness. The isolated compounds were purified and subjected to mass-spectrum analysis.

Mass-spectrum analysis:

Shimadzu QP.1000 EX e.I.70 e.v., mass-spectrum (M.S.) apparatus was used. The analysis was carried out in Microanalitical Unit, Fac. of Sci., Cairo Univ. Amount of 10µg of each sample was direct injected. Isothermal at 250°C under controlled conditions. Ion source temp., 250°C. High vacuum ($9x10^{-6}$) and low vacuum ($2x10^{-3}$)

Toxigenic fungi:

Fungi selected for this study were kindly obtained from the Culture Type Collection of the Plant Pathology Research Institute, ARC, Giza, Egypt. They are: 1) *Fusarium verticillioides* Sacc. (syn. *F. moniliforme*), st.77; the incitant of grain and stalk rot of maize, sorghum and other graminacious species and fumonisin producer. 2) *Aspergillus flavus* L.K., st.15; the mould of many field and food crops and feed commodities and aflatoxin producer.

Bioassay:

To evaluate the chemical fractions for their efficiency in reducing the radial growth of target fungi, two methods of screening the fractions obtained throughout

this work versus the target fungi were followed: 1) A known volume of the extract was incorporated into PDA to give the final concentration of 2500 ppm and poured into Petri dishes (6-cm-diameter). Then, the medium was inoculated at the centre of the dishes with 8mm-diameter discs from 6-day-old cultures of fungi under study and incubated at 27°C. Each treatment was quadruplicated and extract-free PDA acted as control. Radial growths of both fungi were measured periodically and the efficiency of each treatment was calculated as percentage of reduction in colony diameter over the control. 2) Two ml of spore suspensions (10^6 conidia/ml) of fungi under study were seeded into250 ml PDA just prior to solidifying the medium. Media were poured into 6-cm-diameter plates and 8-mm-diameter wells were made at the centre of plated media by the aid of a cork borer. Residues of each fraction were dissolved in methanol to give the final concentration of 5000ppm, then 100µl were incorporated into the prepared wells and plates were incubated at 27°C. Four replicate plates were used and methanol acted as control.

Results

N-hexane extract was fractionated by subjecting to column chromatography and each fraction was evaluated against the radial growth of target fungi. Data presented in Table (1) show that different quantities of extracted fractions were obtained by using the various solvent systems.

Table 1. Chromatographic *n*-hexane fractions of *n*-hexane extract (10gm)

Solvent	Fraction (F)	Weight of Residue (mg)
N-hexane	F1 10*X100ml	210
	F2 20X100ml	190
	F3 10X100ml	160
	F4 20X100ml	140
N-hexane + ethyl acetate	10% FE1 10X100ml	10
	20% FE2 10X100ml	10
	30% FE3 10X100	10
	50% FE4A 10X100 ml	20
Ethyl acetate	100% FE5 40X100 ml	8250
Ethyl acetate + methanol	10% FEM1 10X100ml	350
	20% FEM2 10X100 ml	150
	30% FEM3 10X100ml	100
	50% FEM4 10X100ml	70
Methanol	100% FM1 40X100ml	300

* Number of fractions collected together.

Data in Table (2) indicate that all fractions have the potential to retard the growth of the two tested pathogens on PDA. They differ quantitatively in their effect in this respect, where the fraction of 100% ethyl acetate (FE5) gave the highest effect on both fungi (100 and 75% reduction on *F. verticillioides* and *A. flavus* respectively) comparable to the other fractions. It was found also that 100% ethyl acetate was the most efficient solvent in this process, giving more than 80% (8250 mg extract) of the total obtained *n*-hexane extracts (10000mg). For this reason, this extract was subjected to fractionation by toluene / ethyl acetate.

Table 2. Efficiency of different fractions of *n*-hexane extracts on reduction in growth diameter of *F. verticillioides* and *A. flavus*

Fraction	Reduction (%) *	
	F. verticillioides	*A. flavus*
F1	0	38
F2	8	7
F3	24	24
F4	8	20
FE1	0.9	100
FE2	62	73
FE3	68	71
FE4	68	69
FE5	100	75
FEM1	58	62
FEM2	86	71
FEM3	50	58
FEM4	59	62
FM1	65	100

* Growth reduction (%) was calculated in relative to the linear growth in control.

A total number of 236 fractions were collected throughout this trial and running was done for each on T.L.C. as mentioned before. After running, all similar fractions according to the number of bands, colour and *Rf* were gathered in one fraction. However, a number of 81 fractions were evaluated for their efficiency on reducing the fungal growth of the target fungi (Table 3).

Bioassay was carried out in PDA seeded with spore suspensions of the pathogens and100 µl of each fraction at the concentration of 5000ppm was introduced into the wells done in the centre of each PDA plate using the bioassay method (No. 2) as mentioned before. After the incubation period of 48 h, the inhibition zones around the wells were measured and recorded for each fraction.

Data in Table (3) indicate that only four fractions (100% toluene and 99% toluene: 1% ethyl acetate) were potent inhibitors to the growth of both of the target fungi. Whereas, other fractions had no effect in inhibiting the fungal growths.

Table 3. Efficiency of toluene/ethyl acetate fractions on growth of *F. verticillioides* and *A. flavus*, measured as inhibition zones

Fraction	Toluene: Ethyl acetate	Weight (mg)	Inhibition zone (mm)	
			F. verticillioides	*A. flavus*
F1(1-14)	100 : 0	40	0	0
F2 (15)	100 : 0	130	5	5
F3 (16)	100 : 0	170	5	10
F4(17)	99 : 1	180	15	20
F5 (18)	99 : 1	160	4	10
F6 (19-25)	99 : 1	640	0	0
F7 (26-32)	98 : 2	460	0	0
F8 (33-38)	97 : 3	370	0	0
F9 (39-63)	96 : 4	940	0	0
F10 (64-75)	95 : 5	280	0	0
F11 (76-186)	90 : 10	1940	0	0
F12 (187-217)	50 : 50	830	0	0
F13 (218-236)	0 : 100	90	0	0

Therefore, the four potent fractions (F2, F3, F4 and F5) were gathered and mixed in one fraction. This fraction was evaporated, and the residue was weighted and re-dissolved in methanol. Running was done on T.L.C. to all amount of the obtained residue. Eight clear different bands appeared, isolated separately by scratching, were solved in small amount of methanol, filtered twice by using Whatman No.1 filter paper, evaporated, and weighted. All compounds obtained from bands were amorphous powders. Mass spectrum was used to identify the compound(s) consisted in each band.

Bioassay of the eight isolated compounds was done against *F. verticillioides* and *A. flavus* by seeding into PDA at concentrations differ according to the sharing proportion of each in the total fraction residue (240mg). Plates were incubated at 27°C for 48 h. Four replicates were used. After the elapse of the incubation period, the results were recorded as inhibition zones around the wells.

Results in Table (4) show that all of the 8 isolated compounds were effective in retarding the growth of one or two of the target fungi, in general. It was found that compounds number; 1& 2 were effective only on *A. flavus* but not effective on *F. verticillioides*. Whereas, compound No. 5 inhibited the growth of *A. flavus* only. The rest compounds were effective in inhibiting the growth of both fungi with different degrees of effect as shown in Table (4). It is obvious from the results that compound No. 8 gave the best effect in this respect followed by compounds Nos. 6 and 7. It is worth mentioning that the conidiation of *A. flavus* has been retarded due to the poison effect of compounds Nos. 3, 6, 7 and 8, up to about 7 days of incubation.

Properties of the 8 compounds isolated by T.L.C. following the system used in this study are shown in Table (5).

Table 4. Efficiency of isolated compounds in inhibiting the radial growth of *F. verticillioides* and *A. flavus*

Compound No.	Weight (mg)	Concentration (ppm)	Inhibition zone (mm)	
			F. verticillioides	*A. flavus*
1	10	208	0.0	7.0
2	20	417	0.0	7.0
3	70	1458	6.0	6.0
4	10	208	7.0	0.0
5	40	834	0.0	5.0
6	60	1250	15.0	10.0
7	20	417	10.0	8.0
8	10	208	18.0	13.0

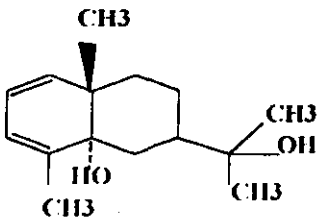
Table 5. Chromatographic properties of the isolated compounds

Compound	Weight (mg)	*Rf*	Colour
1	10	0.04	Canary yellow
2	20	0.08	Blue
3	70	0.133	Pink
4	10	0.200	Paige
5	40	0.286	Blue
6	60	0.586	Dark blue
7	20	0.660	Light blue
8	10	0.713	Light blue

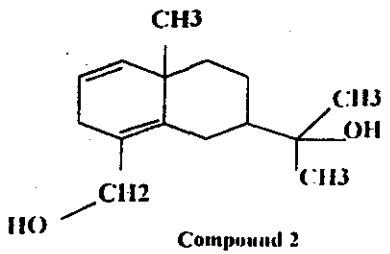
Since all the compounds were obtained in small amounts and in an amorphous state, M.S. analysis was resorted for their identification. The results obtained are compiled in Table (6).

Table 6. M.S. data of the isolated compounds

Compound No.	Molecular ion (M+)	Base peak (bp)	Other major peaks
1	236	59	221, 218, 67, 55
2	236	59	205, 219, 132, 107
3	238	59	221, 137, 69, 57
4	218	163	203, 134, 57, 55
5	252	59	236, 220, 219, 218
6	168	55	139, 111, 83, 89
7	224	59	205, 189, 161, 87
8	222	59	204, 161, 121, 93



Compound 1



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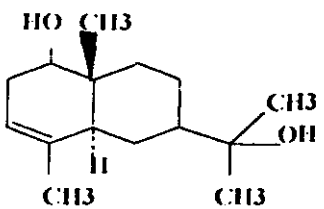
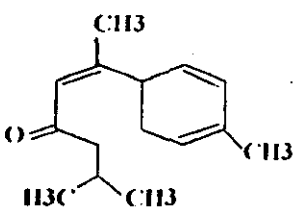
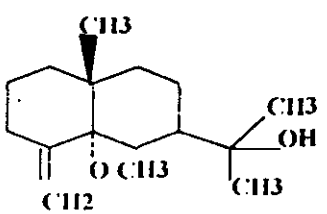


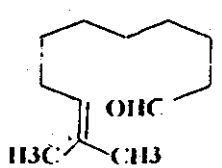
Compound 3



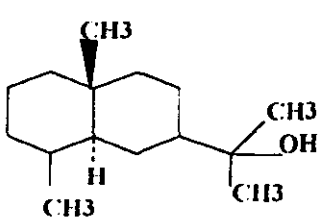
Compound 4



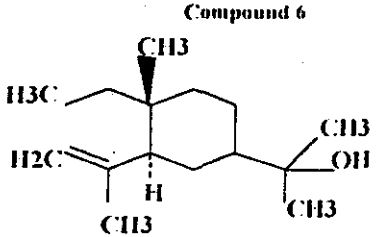
Compound 5



Compound 6



Compound 7



Compound 8

Fig (1). Structure formulae of the isolated compounds

From these results and Fig. (1), it could be concluded with the aid of available literatures of Wagner *et al.* (1984), Robert (1995) and El-Askary *et al.* (2003) that these compounds have the following structures:

Compound 1 Eudesm 5,11 dihydroxy -1,3- diene
Compound 2 Eudesm 4 hydroxy methyl-11- hydroxy 1,4, diene
Compound 3 Eudesm 7,11 dihydroxy 3ene
Compound 4 Atlantone
Compound 5 Eudesm 4 methylene -5-methoxy-11- ol
Compound 6 2 methyl undec-2-ene-10-al
Compound 7 Eudesm-11- ol
Compound 8 Elemol

Discussion

In a recent study of El-Assiuty *et al.* (2006), *n*-hexane-*Cymbopogon proximus* (halfa barr) was found to have an impressive effect on the growth of some plant pathogens *in vitro*. Of these pathogens, the toxigenic fungi; *F. verticillioides* and *A. flavus;* the cause of maize ear rots, which affect seriously the man and animal health (Widstrom, 1996; Cardwell *et al.*, 2000). The present work proved that ethyl acetate and methanol fractions affected dramatically the target fungi when added to the fungal growing media. On the other hand, when these fractions were chromatographed by using silica gel-toluene/ethyl acetate, only four fractions were found to be effective in inhibiting the growth of both tested fungi.

Eight compounds were isolated from these fractions by preparative T.L.C. They were found to have potent effect in retarding the growth of one or two of the target fungi. The isolated compounds were found to be sesquiterpenes. Five of them (compounds 1, 2, 3, 5 and 7) are derivatives of eudesmol. While, compound 4 is a ketonic monocyclic sesquiterpene and compound 8 is an alcoholic monocyclic sesquiterpene. But, compound 6 was the only straight chain aliphatic unsaturated aldehyde. It is worth mentioning that many sesquiterpenes were previously isolated from the unsaponifiable fraction of the petroleum ether and hexane extracts of *C. proximus* (Radwan 1975; Evens *et al.*, 1982 and El-Askary *et al.*, 2003). Terpenes are widespread in nature, mainly in plants as constituents of essential oils, and many of them are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones. Besides the antispasmodic properties of such discovered sesquiterpenes such as proximadiol, some of these compounds are known in the time being as natural antibiotics produced by many plant species and mushroom fungi that may affect microorganisms and plant pathogens (Krol-Bogomilski, 2006). Therefore, the antifungal effect of the *n*-hexane extract observed while screening some plant extracts on the growth of plant pathogens in the Lab. may be regarded to the poisonous effect of the antibiotic nature of the compounds exist in this fraction.

Further study on the possibility of using this fraction from *C. proximus* in such a way to control plant diseases in the field and/or the market is suggested. Applying natural products extracted from plants to protect food commodities rather than the hazardous pesticides is suggested by the government.

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قدرة بعض المركبات المعزولة من نبات حلفا برعلى إيقاف نمو الفطريات المفرزة للسموم الفيوزاريوم فيرتيسيلويدز والأسبرجيلاس فلافاس

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أظهرت الدراسة أن مستخلص الهكسان الناتج من عشب نبات الحلفا بر كان له القدرة على إيقاف نمو الفطرين المفرزين للسموم الفطرية تحت الدراسة وهما " الأسبرجيلاس فلافاس والفيوزاريوم فيرتيسيلويدز" فى المعمل. وقد أنتج التحليل الكروماتوجرافى لهذا المستخلص الجزء الناتج عن الإستخلاص بواسطة الأيثايل أسيتات والذى كان له تأثير مضاد للفطرين المذكورين وقد أمكن عزل عدد ثمانية من المركبات من هذا المستخلص بواسطة طريقة تحليل الكتلة كانت جميعها لها تأثير فى تثبيط نمو الفطرين تحت الدراسة بدرجات متفاوتة. حيث كان أكثرها تأثيرا فى هذا المجال ثلاثة مركبات منها وهى تتبع مجموعة المركبات التى تعرف باسم مجموعة السيسكويتربينات المعروفة فى مجال البحوث الصيدلية والطبية والتى تقاوم بعض الأمراض عند الإنسان. وقد كان لهذا البحث السبق فى تعريف بعض المركبات ذات التأثير المضاد للفطريات الممرضة للنباتات والمفرزة للسموم المؤثرة على صحة الإنسان.