INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

THE CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OIL OF *CYMBOPOGON NERVATUS* HOCHST. CHOIV.

MF. Abushama[1*], A. Hassan[2], H. Abdelgadir[3] and H. Khalid[3]

[1]Khartoum College of Medical Sciences, Department of Botany
[2]Ahfad University, Department of Health Sciences
[3]Medicinal & Aromatic Plants Research Institute, National Centre for Research

ABSTRACT

The antifungal activity of eight essential oils of some Sudanese medicinal plants, namely: *Cyperus rotandus* L., *Cymbopogon nervatus* Hochst. Choiv, *Ocimum basillicum* L., *Boswellia papyrifera* (del ex. Caill) Hochst., *Eucalyptus camaldulensis* Dehn., *Cymbopogon schoenanathu* L. Spring subsp Proximus, *Citrus paradise* Macf. and *Cuminim cyminum* was screened against *Aspergillus niger* and *Candida albicans* using Agar Well Diffusion Assay on Sabaroud Dextrose Agar (SDA). *C. nervatus* essential oil showed relatively high antifungal activity on the hyphal growth. Accordingly, the essential oil was qualitatively and quantatively investigated. Antifungal activity of the mentioned essential oil was studied on Potato Dextrose Agar (PDA). The 1:100 v/v dilutions of essential oil *C. nervatus* showed its maximum antifungal effect on *C. albicans* and *F. oxysporum.* Furthermore, the antifungal activity of essential oil's fractions obtained by Column Chromatography was investigated against Nystatin as a standard natural antifungal and Bifonazole as a standard synthesized antifungal using Disk Diffusion Method. The ether and benzene fractions showed high activity on *A. niger* and *C. albicans* cultures. Minimum Inhibition concentrations (MIC) of *C. nervatus* for A. *niger*, *A. flavus*, *A. spp*, *C. albicans* and *Fusarium oxysporum* were determined. The aflatoxins production by *A. flavus* was also investigated. The effect of different concentrations of the essential oil of *C. nervatus* on the toxins was analyzed by HPLC. The essential oil of aerial parts (0.303%) of *C. nervatus* was investigated by (GC-MS). The volatile oil consisted mainly of oxygenated monoterpenes. The antifungal activity of *C. nervatus* is reported for the first time.

Keywords: *Cymbopogon nervatus*, essential oil, antifungal, Aflatoxins, GCMS.

INTRODUCTION

The genus *Cymbopogon* contains 40 species, mostly native to the Old World Tropics, and constitutes an important proportion of Savannah grass. Although taxonomic classification is often complicated by hybridization and polyploidy, probably nine species are found in Sudan[1]. The extracts of some of these species are widely used in folk medicine for the treatment of digestive ailments and as flavoring. A commercial preparation is claimed to show antispasmodic activity, diuretic, an antihistaminic. In Sudanese traditional medicine, the plant inflorescence decoction is used to treat kidney pains and urethritis[2]. The plant contains a volatile oil[3].

C. nervatus species has been chemically investigated, the inflorescence consisted mainly of oxygenated monoterpenes[4], sesquiterpenes were also isolated[1].

In this work we are reporting the antifungal activity, as well as, the inhibitory effect of the essential oil of *Cymbopogon nervatus* on the production of Aflatoxins by *Aspergillus flavus*.

The antifungal activity of *C. nervatus* is reported for the first time.

MATERIALS AND METHODS

1. Plant materials

Plants were collected, identified and authenticated. Herbarium material was deposited at The Medicinal& Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan. The plant names, collection area and the plant parts used for the study are shown in (**Table 2).**

2. Method of Extraction

Plants Essential oils were extracted by Hydro-distillation using Clevenger Apparatus[5].

3. Preparation of essential oils for bioassay

Plant extracts were dissolved in sterile Dimethyl sulfoxide (DMSO) and kept as stock solutions. The extracts were filtered using Chromafil CA-45/ 25 S (MACHEREY-NAGEL postfach 10 13 52 D- 52313 Duren).

4. Tested Organisms

Table 1: Tested organisms

Organisms	Source
Aspergillus flavus	Egypt local strain isolated and identified at the natural and microbial chemistry research products department NRC By Prof. Dr. Mohamed Mabrook Atallah. Supplemented under the auspices of the author, Dr. Atallah.
Aspergillus niger	Serial number ATCC9763 (Filamentous Fungi)
Aspergilus sp.	Species unidentified
Candida albicans	Serial number ATCC9763 (Yeast Fungi)Personal
Fusarium oxysporum	Contact, Professor Mohamed Fareed, Egypt, NRC

5. Antifungal Activity

5.1. Agar Diffusion Assay

a. Screening of eight plant extracts for their antifungal activity against *Aspergillus niger* and *Candida albicans* was performed using Well Diffusion Method in SDA[6]. 10 ml aliquots of dextrose broth were inoculated with the test organisms and incubated at 30˚ C for 24 hours. Using a sterile pipette, 0.6 ml of the broth culture of the test organisms was added to 60 ml of molten agar which has been cooled to 45 ˚C, mixed well and poured into sterile Petri dishes. Duplicate plates of each organism were prepared. The agar was allowed to set and solidify and 4 wells were cut using a sterile cork borer and ensuring proper distribution of the wells. Using a 0.1 pipette and 100 micro liters of the plant extracts, concentrations were prepared by dissolving in ethanol. The diameter of the zones of inhibition was measured to the nearest mm.

b. Antifungal Activity of *C. nervatus* essential oil against *A. niger, A. flavus, A. spp., C.albicans* and *F. oxysporum* was studied using PDA[7]. Sterilized molten media (45 ml) at 45-50 ˚C were poured into 100 ml conical flasks. 0.1 ml of fungal suspensions was added to each flask, shaken, and then poured into 19cm Petri dishes. Essential oils extracts in propanol concentrations were prepared as follows; solution (1) **1:100** (0.1 ml oil + 4.45 ml 2- propyl alcohol + 4.45ml water). **1:250** (2.8 ml solution 1+ 3.6 ml alcohol + 3.6 ml water). **1:500**(1.6 ml solution 1 + 4.2 ml alcohol + 4.2 ml water). **1:1000**(1ml solution 1 + 4.5ml alcohol + 4.5 ml water). Inhibition zones were measured and recorded to the nearest (mm) after 48 hrs of incubation.

c. Antifungal activity of *C. nervatus* essential oil fractions on test organisms was studied using Disc Diffusion Method[8,9]. Discs were saturated with different amounts of crude oil and oil fractions obtained by column chromatography (ether, hexane and benzene extracts). Using micropipette Lipids, Saponified and Unsaponified compounds resulting from saponification process were also used. Nystatin was used as a standard natural antifungal and Bifonazole as a standard synthesized antifungal. Potato Dextrose agar was prepared for bioassay of fungal species. 0.1 ml from each suspension of the test organism was pipetted into 50 ml molten agar and poured into its respective Petri dish. The media was left to solidify and the discs were placed, aseptically, on the surface. The petri dishes were put in the refrigerator for 1 hour to ensure good diffusion of discs contents. Petri dishes with fungi were then incubated

at 30 ˚C for 24 hours. Inhibition zones in (mm) were measured for each disc.

5.2. Dilution Assay

The determination of (MIC) of some fungal spp using different concentrations of C.*nervatus* essential oil was performed using SDB[10]. Conical flasks (100 ml), each containing 9 ml broths, were prepared and autoclaved. 1ml of *C. nervatus* oil (1: 1000 oil diluted in propanol alcohol) was added to a flask under aseptic conditions to get 1: 10000 solution from which 1 ml is transferred to another flask to get 1: 1000000 dilutions. Same procedure was repeated to get 1: 40000 and 1: 400000 dilutions. These solutions were seeded by 0.05 ml suspensions of *A. niger, C. albicans*, and *F. oxysporum*. Flasks containing only broth were prepared as controls. These were also seeded with the three organisms. All Flasks were incubated at 30˚C for 24 hrs. Flasks containing the concentration of oil showing no fungal growth were designated as the minimum inhibition concentration.

6. The Inhibitory Effect of *C. nervatus* On Aflatoxins Produced by *Aspergillus Flavus*

Czapek Dox Broth was prepared in 500 c.c. Yeast extract (1.25 gm) were added to the broth. Each 50 ml of broth were poured into 250 ml conical flasks [11, 12]. Wheat seeds (250 gm) were added, so that 25 gm were added for each flask. Sterilized distilled water was added to the organisms slant to make suspension. 1ml of fungal suspension was added to each flask. Duplicate flasks were prepared for each concentration (0.1, 50, 20 and 10 micro liter of essential oil). Two flasks were set as control. The flasks were incubated in a shaking incubator, set at 28˚C and 150 rounds per minute for two weeks. 25 ml of water were added to each flask every time the media dries up. The media was mixed by a sterile spatula under aseptic conditions. At the end of the incubation period, the flasks contents were centrifuged (4500 round per minute for 15 minutes). Falcon tubes were used; each filled with 50 cubic centimeters.

7. Extraction of Aflatoxins

Filtrates of each concentrations as well as the control were subjected to extraction of Aflatoxins, by adding equal volumes of chloroform to culture supernatant. The mixture was placed in a separating funnel, shaken well and left to separate. The lower layer was taken and passed through filter paper containing 3 gm anhydrous sodium sulfate. The filtrate was received in a beaker, evaporated until dryness. 2 ml of Chloroform were then added and put in a vial and evaporated to dryness. The Aflatoxins (B1. B2, G1 and G2) measurement was done by HPLC technique[13].

RESULTS AND DISCUSSION

In the general screening of the eight essential oils measurements of inhibition zones, as shown in (**Table .2**, **Fig.1**) revealed that the essential oil of *C. nervatus* activity was the highest, followed by *C. cyminum* and *O. basilicum.* The data are in agreement with the published data for the essential oils of *C. cyminum* and *O. basilicum.* The essential oil *O. basilicum* has been shown *in vitro* to have antifungal activity against *C. albicans*, *Penicillium notatum*, and *Microsporeum gyseum* [14]. In another study of the antimicrobial activity of cumin essential oil against fungi namely (*Aspergillus* and *Penicillium* spp.) and yeasts (*Saccharomyces* and *Candida spp*.) the cultures were found to be more sensitive to cumin volatile oil and cuminaldehyde than bacteria and fungi had MIC values 10 to 20 times lower than those of bacteria [15].

The 1:100 v/v dilutions of essential oil *C. nervatus* showed its maximum antifungal effect on *C. albicans* and *F. oxysporum* with average inhibition zones of 20 mm and 19 mm respectively (**Fig.2**). No documented work is available on the antifungal activity of *C. nervatus* volatile oil. But the activity of *C. citratus* was studied as a potential antifungal agent. This oil reduced spore germination in *A. flavus*, *A. fumigatus*, *A. alternata*, *P. citrinum* and *T. harzianum*, with the effects dependent on oil concentration while *A. niger* was the most sensitive strain against the lemongrass oil[16].

The *C. nervatus* essential oil's fractions obtained by Column Chromatography were investigated for their antifungal activity using Disc Diffusion Method on PDA. Results in (**Table 3**) revealed high activity of the ether and the benzene fractions when compared to same concentrations of Nystatin and Biofonazole as standards for antifungal activity.

The two oil fractions showed high activity on *A. niger* and *C. albicans* cultures with inhibition zones (43 and 30 mm) and (40 and 30 mm) respectively.

Minimum inhibition concentration (MIC) of the essential oil of *C. nervatus* was investigated using Serial Dilution Method in (SDB), the results shown in (**Fig.3)** revealed that dilution 1: 40,000 v/v completely inhibited the growth of *A. niger*, *C. albicans* and *F. oxysporum*.

The inhibition of the hyphal growth of *A. flavus* led to the investigation the possibility to control

the production of aflatoxins produced by *A. flavus*. The implemented experiment proved to some extent the activity of the oil to decrease the toxins production. Aflatoxins produced by *A. flavus* were monitored using different essential oil concentrations of *C. nervatus.* HPLC analysis of the culture filtrates, showed a decrease in the production of B1, B2, G1 and G2 by the organism when compared to the standard. Maximum and minimum oil concentrations were compared to the control. Although a study [17] had suggested that inhibitory substances originating from plants' strategies have been shown to have limited effectiveness, more work on this volatile oil might open new avenues to control production of toxins by *A. flavus.* **(Fig. 4)**

The use of *C. nervatus* essential oil in drug industry as an antifungal is recommended. Furthermore, a broad research should be led towards the use of the essential oil of *C. nervatus* as a fungicide and a pesticide to prevent aflatoxin contamination as well as infections and diseases induced by *F. oxysporum*

The main constituents of the essential oil of aerial parts (0.303%) of *C. nervatus* were investigated by Gas Chromatography Mass Spectrometer (GC-MS) and are shown in (**Table 4).** The identified compounds in the volatile oil consisted mainly of oxygenated monoterpenes. Trans-p-Mentha-1(7), 8-dien-2-ol was identified as the major compound and presented 21.25%. Cycloheptane, 1, 3, 5-tris (Methylene), the second main compound, was also identified in the oil and presented 16.08%. Cis- p- Mentha- 1(7), 8- dien- 2- ol was the third main compound; its percentage was 9.83%, and P- mentha- Trans- 2, 8- Dien-1-ol constituted 9.83%. dl- Limonene was the fifth main compound constituting 7.37 %. Some of these results are in agreement with the findings of another study [3] of the chemical composition of the essential oil of the inflorescence (1.3%). The main constituents found, using GCMS technique, were Cis-p-mentha-1(7)8-dien-2-ol (25.2 %), Trans-p-mentha-1(7)8-dien-2-ol (22.9 %), 2-(1-methyl-propyl) cyclopentanone (11.3%), Trans-carveol (9.6 %), and Trans-p-2,8-mentha-dien-1-ol (8.4 %).

CONCLUSIONS

The current study verified the antifungal activity of *C. nervatus* on pathogenic organisms. The activity is proved by Microbiological Diffusion and Dilution Assays on different solid and liquid media. The antifungal activity of this oil is investigated for the first time in the current study.

The phytochemical investigation of *C. nervatus* essential oil accompanied with the bioassay analysis, especially the Disc Diffusion Assay of the fractions of the oil, ether and benzene, showed that the oxygenated compounds are responsible for the antimicrobial activity. The use of *C. nervatus* essential oil in drug industry as an antifungal is recommended.

The use of the essential oil of *C. nervatus* as a fungicide and a pesticide can prevent aflatoxin contamination as well as infections and diseases induced by *Fusarium oxysporum*. This indicates the development of strategies for economically feasible harvesting of crops and storage conditions of commodities which are affected by the organisms that produce these toxins.

More investigation should be done in this area, especially the determination of the essential oil concentrations that might have a clear impact on the production of these toxins.

Table 2: Plants Screened for their antifungal activity

Botanical Name	Vernacular Name	Family Name	Collection Area	Part used
Cuminim cyminum L.	Shamar	Apiaceae	Shimalia	Fruits
Boswellia Papyrifera (del ex. Caill) Hochst.	Luban	Burseraceae	Angasana	Aerial (secretion)
Cyperus rotandus L.	Syda	Cyperaceae	Shambat	Corms
Ocimum basillicum L.	Rehan	Lamiaceae	AlFaw	Aerial
Eucalyptus camaldulensis Dehn.	El- Ban	Myrtraceae	Khartoum	Leaves
Cymbopogon nervatus Hochst. Choiv	Nal	Poaceae	Butana	Aerial
Cymbopogon schoenanathu L. Spring subsp Proximus	Maharaib	Poaceae	Shambat	Aerial
Citrus paradise Macf.	Grape fruit	Rutaceae	Khartoum	Peel

Table 3: Inhibition zones (mm) of *C. nervatus* oil fractions using Disc diffusion Assay

Organisms	Crude Oil 100µ	Lipid 100µ	Ether 50µ	Benzene 50µ	Hexane 10µ	Bifonazole 50µ	Un-saponified 100µ	Saponified 20µ	Nystatin 50µ
A.niger	-	-	43	30	-	12	-	-	8
A.spp	-	-	45	40	-	12	-	-	-
C.albicans	-	-	40	30	-	-	-	-	14

Table 4: The chemical composition of essential oil of *Cymbopogon nervatus*

Compound	R.T.(min.)	Concentration (%)	Chemical Formula	Molecular weight	Base Peak
2-Oxabicyclo[2,2,2] oct-5-ene, 1,3,3,-trimethyl-,(+)	12.62	0.05	$C_{10}H_{16}O$	152	1o9.14
Cycloheptane,1,3,5-tris (Methylene)	13.36	16.08	$C_{10}H_{14}$	134	91.06
2-methylprop-1-enyl-cyclohexa-1,5,-diene	14.00	0.41	$C_{10}H_{14}$	134	91.06
Cycloheptane,1,3,5-tris (Methylene)	14.34	5.54	$C_{10}H_{14}$	134	91.06
dl-Limonene	14.50	7.36	$C_{10}H_{16}$	136	68.09
Benzene,1-methyl-4-(1-methylethenyl)	16.66	0.59	$C_{10}H_{12}$	132	132.06
Trans-D-Dihydrocarveol	16.80	0.38	$C_{10}H_{18}O$	154	109.12
Cycloheptan,1,3,5-tris(methylene)	17.48	3.87	$C_{10}H_{14}$	134	91.05
P-mentha-Trans-2,8-Dien-1-ol	19.09	3.13	$C_{10}H_{16}O$	152	109.04
Herboxide Second Isomer	19.37	0.17	$C_{10}H_{16}O$	152	67.07
Bicyclo [3,3,0] oct-2-EN-7-ON, 6-methyl	20.04	4.33	$C_9H_{12}O$	136	79.07
Bicyclo[3.3.0]octan-2-on, 6-methyl-7-methylene- oder 8-methyl-7-methylene-	20.46	3.63	$C_{10}H_{14}O$	150	135.04
Cis-p-Mentha-1(7), 8-dien-2-ol	21.63	9.83	$C_{10}H_{16}O$	152	109.04
[1,1-Bicyclopentyl]-2-one	21.84	3.35	$C_{10}H_{16}O$	152	84.04
Cis-Carveol	22.07	0.02	$C_{10}H_{16}O$	152	84.03
Carveol 1	22.23	1.97	$C_{10}H_{16}O$	152	84.04
Trans-(+)- Carveol	22.39	0.02	$C_{10}H_{16}O$	152	109.05
Trans-p-Mentha-1(7), 8-dien-2-ol	23.26	21.25	$C_{10}H_{16}O$	152	109.04
Cis-Limonene Oxide	23.59	0.02	$C_{10}H_{16}O$	152	67.17
1-cyclohexane-1-carboxaldehyde,4-(1-methyl ethenyl)	23.97	0.53	$C_{10}H_{14}O$	150	67.08
3-Dodecyne	24.19	0.07	$C_{12}H_{22}$	166	67.12
Pregnanol one Acetate	47.90	0.04	$C_{23}H_{36}O_3$	360	107.13

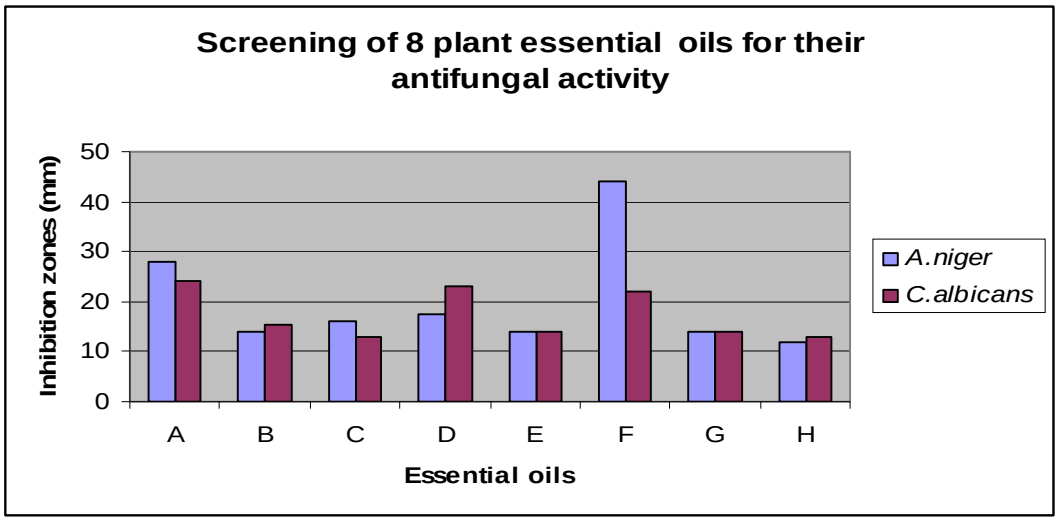


Fig. 1: A histogram showing the antifungal activity of the eight plant extracts against *A.niger* and *C. albicans*

A= essential oil of *Cuminum cyminum*
B= essential oil of *Boswellia papyrifera*
C= essential oil of *Cyperus rotandus*
D= essential oil of *Ocimum basilicum*
E= essential oil of *Eucalyptus camaldulensis*
F= essential oil of *Cymbopogon nervatus*
G= essential oil of *Cymbopogon schoenanathus* sp. Proximus
H= essential oil of *Citrus paradisi*

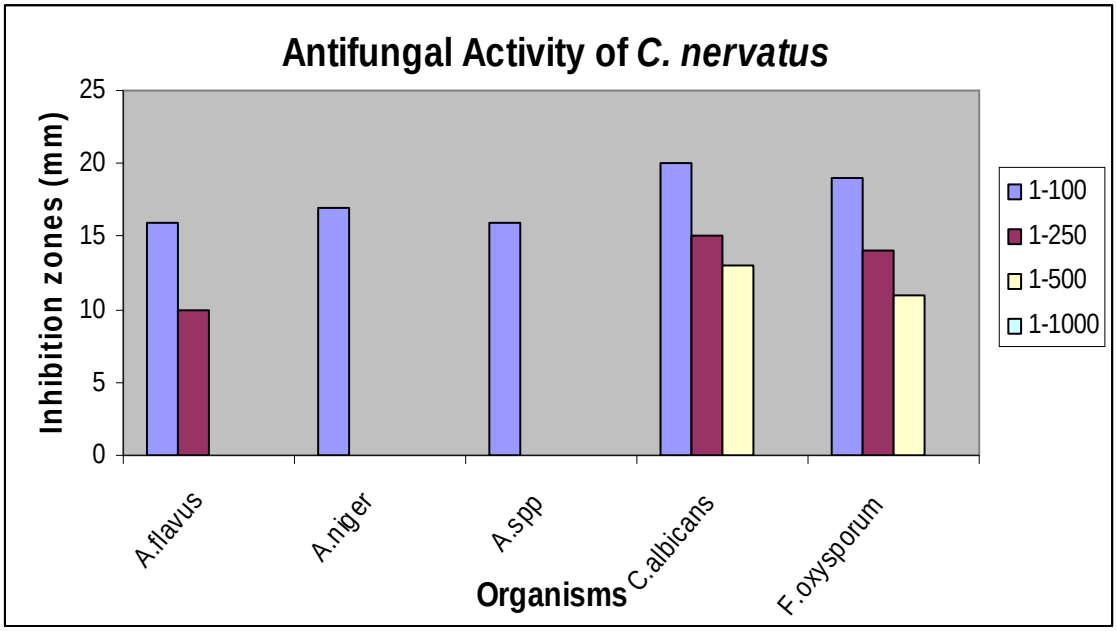


Fig. 2: A histogram showing the antifungal activity of *C. nervatus*

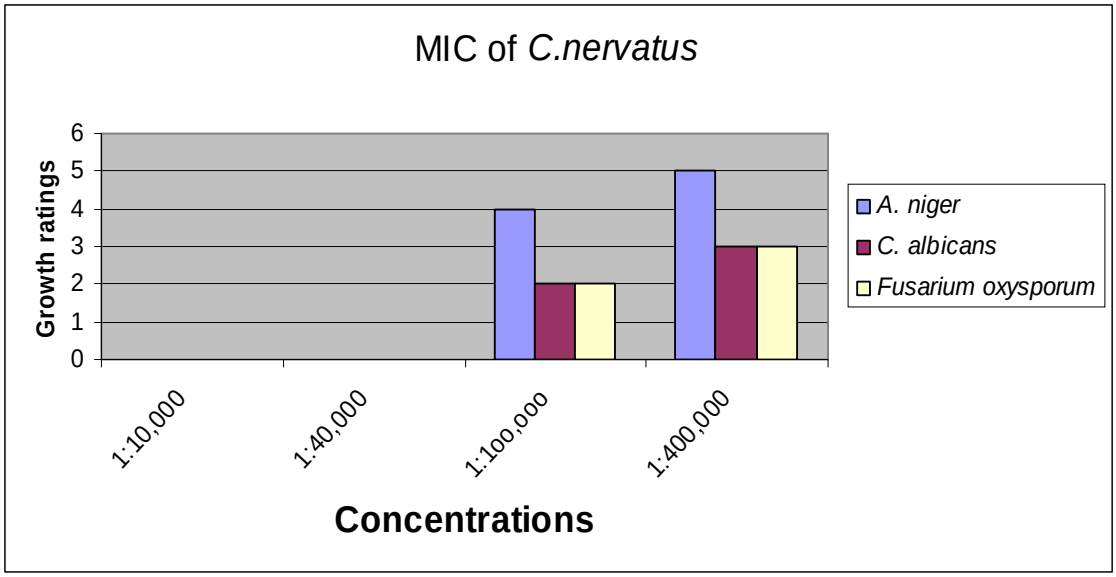
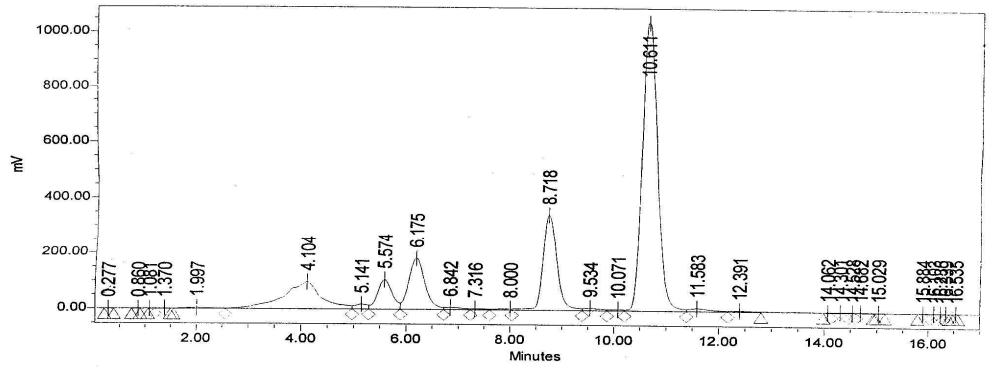


Fig. 3: A histogram showing the MIC of *C.nervatus* against *A.niger, C.albicans* and *F.oxysporum*

SampleName	std
Vial	1
Injection	7
Injection Volume	10.00 ul
Channel	SATIN
Run Time	17.0 Minutes

Sample Type	Unknown
Date Acquired	7/22/2007 2:27:23 PM
Acq Method Set	afs
Processing Method	afs210
Date Processed	7/22/2007 2:45:04 PM

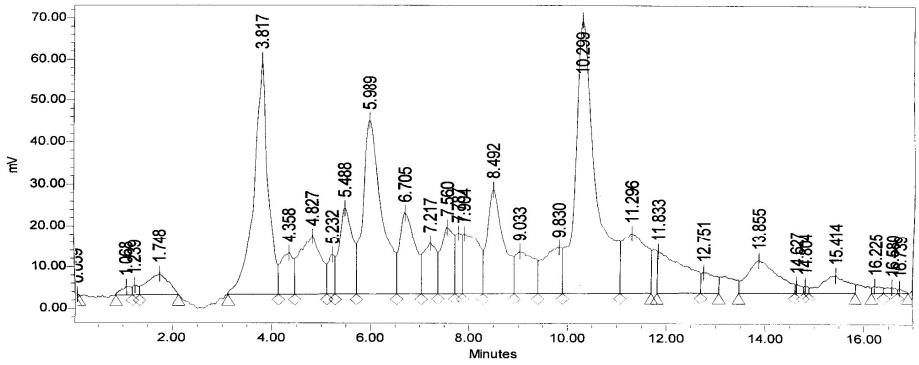
Auto-Scaled Chromatogram

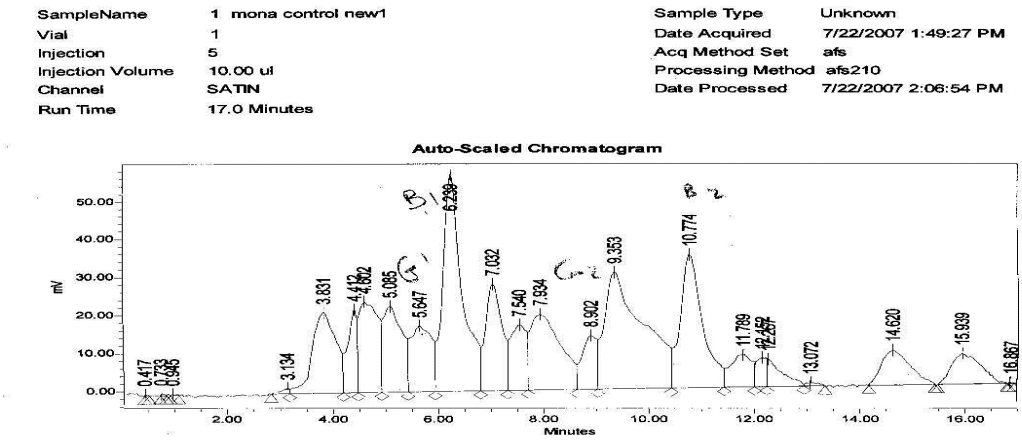


SampleName	2 Muna new
Vial	1
Injection	3
Injection Volume	10.00 ul
Channel	SATIN
Run Time	17.0 Minutes

Sample Type	Unknown
Date Acquired	7/10/2007 5:02:49 PM
Acq Method Set	afs
Processing Method	afs210
Date Processed	7/10/2007 5:20:36 PM

Auto-Scaled Chromatogram





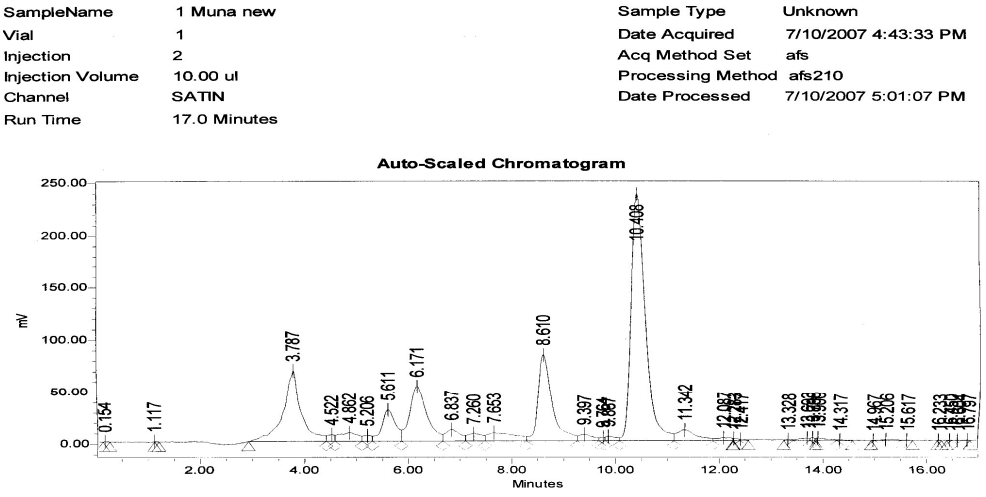


Fig. 4: HPLC analysis of *A. flavus* filtrates

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