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Antifungal activity of the cyanobacterium *Microcystis aeruginosa* against mycotoxigenic fungi

Diaa A. Marrez*, Yousef Y. Sultan

Marine Toxins Laboratory, Toxicology and Food Contaminants Department, National Research Centre, Cairo, Egypt.

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ABSTRACT

Cyanobacteria are rich sources of bioactive compounds. In the present study, the antifungal activity of different solvent extracts of *Microcystis aeruginosa* was evaluated. Among these extracts, diethyl ether extract showed the highest antifungal activity against all tested mycotoxigenic fungi and gave inhibition zone ranged from 10.3 to 21.6 mm. In this direction, the fraction (F4) was the best fraction in antifungal activity against all tested fungi. Sub-fraction F4-10 was chosen as the highest sub-fraction had bioactivity. Using TLC bioautography, GC-MS and NMR; two antifungal compounds were isolated, purified and identified as Butylated Hydroxytoluene (BHT) and Hexadecanoic acid, methyl ester.

INTRODUCTION

Cyanobacteria are prokaryotic cells use chlorophyll for photosynthesis. Therefore, many species have capability for nitrogen fixation (Chronakis, 2000). They are found in a wide range of different habitats, including marine, hot springs, soils and hyper-saline environments (Colyer *et al.*, 2005; Tiwari *et al.*, 2005). Cyanobacteria appeared to be rich source of many useful products, which include various types of substances ranging from organic acids, carbohydrates, amino acids, peptides, vitamins, growth substances, antibiotics, enzymes and toxic compounds (Codd, 1997 and Carmichael, 2001). They can be also used as an animal feed and biofertilizer (Bano and Siddiqui, 2004). The biotechnological applications of cyanobacteria have been reported in diverse areas, such as agriculture, aquaculture, bioremediation, biofuels, pharmaceutical and nutraceuticals (Abed *et al.*, 2009 and Pandey *et al.*, 2013). A large number of cyanobacterial strains are known to produce intracellular and extracellular metabolites with various biological activities, *i.e.* antibacterial, antifungal, antiviral, antialgal and antiprotozoal activity (Burja *et al.*, 2001; Chlipala *et al.*, 2009 and Sultan *et al.*, 2016). These identified substances include fatty acids, phenolics, terpenoids, N-glycosides, lipopeptides, cyclic peptides and isonitrile-containing indole alkaloids (Mundt *et al.*, 2003; Neuhof *et al.*, 2005 and Mo *et al.*, 2009). Moreover, cyanobacteria are produced toxins associated with water blooms. About 40 genera of cyanobacterial species are responsible for production of cyanobacterial toxins (Grindberg *et al.*, 2008). These toxins can be classified according to their toxic mechanism in vertebrates as hepatotoxins, neurotoxins, irritants and dermatotoxins (Stewart *et al.*, 2006). The genus *Microcystis* belong to the most common bloom forming cyanobacteria in many freshwater bodies worldwide and also occur in coastal brackish areas (Rybicka, 2005; Mazur *et al.*, 2003). *Microcystis aeruginosa* is known to produce a large numbers of toxic or bioactive metabolites in freshwater environment. The majority of these compounds have ecological roles as allelochemical (Berry *et al.*, 2008). These allelochemicals play a role in defense of *M. aeruginosa* against predators and gazers, especially aquatic invertebrates and larvae.

* *Corresponding Author*
Diaa A. Marrez, Researcher at Marine Toxins Laboratory, Toxicology and Food Contaminants Department, National Research Centre
Email:diaamm80 @ hotmail.com

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Several foods and agricultural products are subject to contamination with mycotoxigenic fungi which cause varied troubles in both human and animal health. Therefore, there are many efforts towards finding new sources of antifungal metabolites as a natural fungicidal agent. The aim of the present work was to study the antifungal activity of *M. aeruginosa* extracts against several species of mycotoxigenic fungi. Furthermore, the work aimed to isolate, purify and structure elucidate of these antifungal compounds.

MATERIALS AND METHODS

Organism and growth culture

Pure strain of *Microcystis aeruginosa* was obtained from Marine Toxins lab., National Research Centre, Egypt (Marrez *et al*., 2016). The culture media used for cultivation of *M. aeruginosa* was BG-11 medium (Rippka *et al*., 1979). It composed of 1.5 g $NaNO_3$; 0.004 g K_2HPO_4; 0.075 g $MgSO_4.7H_2O$; 0.036 g $CaCl_2.2H_2O$; 0.006 g citric acid; 0.02 mg Na_2CO3; 0.001 g Na_2EDTA; 0.63 g ferric ammonium citrate and 1.0 ml trace elements (TE) in 1000 ml distilled water. TE (g/l) is combined of 2.86 g H_3BO_3; 1.81 g $MnCl_2$. $4H_2O$; 0.222 g $ZnSO_4.7H_2O$; 0.39g Na_2MoO_4. $2H_2O$; 0.079g $CuSO_4$. $5H_2O$ and 0.0494g $Co(NO_3)_2.6H_2O$. After autoclaving and cooling, pH was adjusted to 7.1.

Preparation of *M. aeruginosa* extracts

At the stationary phase of growth (approximately 25 days), *M. aeruginosa* biomass was harvested and dried in a hot air oven at 50°C over night. The dried biomass (5g) extracted with different solvent of aqueous, methanol, ethanol, acetone, chloroform, diethyl ether, ethyl acetate and hexane. The extracts were sonicated for 20 min using ultrasonic microtip probe of 400 watt and centrifuged at 4500 rpm for 10 min. Supernatant was retained and the pellet re-extracted as before three times. Combined supernatant was evaporated to dryness at 40°C using rotary evaporator. Dried extracts were stored in labeled sterile vials in a refrigerator till further use (Chauhan *et al*., 2010).

Antifungal assay

Test microorganisms

Nine fungal species were used for antifungal assay, *Aspergillus flavus* NRRL 3357, *A. parasiticus* SSWT 2999, *A. westerdijikia* CCT 6795, *A. steynii* IBT LKN 23096, *A. ochraceus* ITAL 14, *A. carbonarius* ITAL 204, *Fusarium verticillioides* ITEM 10027, *F. proliferatum* MPVP 328 and *Penicillium verrucosum* BFE 500. The fungal strains were obtained from Applied Mycology Dept., Cranfield Unvi., UK. The stock cultures were grown on potato dextrose agar slant at 25˚C for 5 days and then kept in refrigerator till use.

Media used for antifungal assay

Yeast extract sucrose medium (YES) composed of 20g yeast extract, 150g sucrose and 20g agar in 1000 ml distilled water (Tsubouchi *et al*., 1987) was used for fungal disc diffusion test. Potato dextrose agar medium (PDA) composed of potato 200g, dextrose 15g and agar 20g in 1000 ml distilled water. The pH was adjusted to 7.0 (ATCC, 1984), for determination of minimum inhibitory concentration.

Disc diffusion technique

The fungal strains were plated onto potato dextrose agar (PDA) and incubated for 5 days at 25°C. The spore suspension of each fungus was prepared in 0.01% Tween 80 solution. The concentration of spore suspension of each strain was adjusted by comparison with the 0.5 McFarland standard, the turbidity of the inoculum suspension represented approximately 2 x 10^8 cfu ml^{-1}. Sterilized filter paper discs (6 mm) were loaded with the extracts and dried completely under sterile conditions. Petri dishes of YES medium were inoculated with 50 µl of each fungal culture and uniformly spread using sterile L- glass rod. The extract loaded discs were placed on the seeded plates by using a sterile forceps. Negative control was prepared by using DMSO and the commercial fungicide Nystatin (1000 Unit ml^{-1}) was used as a positive control.

The inoculated plates were incubated at 25°C for 24 - 48 h. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the tested fungus (Medeiros *et al*., 2011). All treatments consisted of three replicates and the averages of the experimental results determined.

Determination of minimum inhibitory concentration (MIC)

MIC against fungi was performed using the technique of Perrucci *et al*. (2004). Crude extracts at different concentrations were separately dissolved in 0.5 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany), then mixed with 9.5 ml of melting, 45^{0}C, PDA and poured into Petri dish (6 cm). The prepared plates were centrally inoculated with 3µl of fungal suspension. The plates were incubated at 25°C for 24-48h. At the end of the incubation period, mycelial growth was monitored and MIC was determined.

Fractionation, isolation and structure elucidation of bioactive compounds

Column chromatography

The diethyl ether extracts (DEE) was fractionated using column chromatography technique. Glass column (30 x 500mm) initially packed with 5g anhydrous sodium sulphate followed by 30g of silica gel (0.06 - 0.2 mm, 70 - 230 mesh ASTM) using chloroform as a carrier solvent to create slurry. The stopcock was opened to allow the silica gel packing to settle, while the excess chloroform was drained. During draining, another 5g anhydrous sodium sulphate was added to the top of silica gel to prevent column from drying.

A portion of DEE (500 mg) in 10 ml chloroform was loaded to the column and allowed to flow at a rate of a drop sec^{-1}. The order of solvent of elution in column chromatography was fixed as chloroform: methanol (98:2), (95:5), (90:10), (80:20),

(50:50), (25:75) and finally methanol 100% to give 7 fractions. The column fractions (50 ml each) were evaporated under vacuum and analysed for bioassay tests. The active fractions in bioassay tests were divided to sub-fractions by submitted to column chromatography to develop by its elution and divided in 10 sub-fractions (5 ml each).

Thin layer chromatography (TLC)

TLC was performed on aluminum plate 10 x 10 cm, silica gel 60 (Merck, Darmstadt, Germany). Sub-fractions were spotted 2 cm from the base of the plate and 1.5 cm intervals between spots. Fifty microliter of each active sub-fraction (20 mg ml^{-1}) was spotted onto the silica gel plate and allowed to dry for a few minutes. Afterwards, the plate was developed and run to 6 cm distance from the spotting base line with toluene: methanol: acetone: acetic acid (15:2:1:1) in a previously saturated glass chamber at room temperature.

The developed plate was dried under normal air and the spots were read at 254 nm and 366 nm using UV chamber. The R_f (retention factor) values of isolated compounds were determined by the following formula: R_f= Distance traveled by extract/distance traveled by solvent system.

TLC bioautography for bioactivity screening

Bioautographic evaluation was conducted in order to check the antimicrobial activity of separated compounds on TLC plate. Previous developed TLC plates were sterilized by UV lamp for 30 min and placed on 15 ml potato dextrose agar plate. Molten potato dextrose agar 20 ml seeded with 1 ml of spore suspension was poured on TLC plate. After agar got solidified the petri plates were kept at 4˚C for 3 h and incubated at 25°C for 24 - 48 h. The inhibition zones were detected by staining with iodonitrotetrazolium chloride reagent 2% (INT). After spraying with INT, the inhibition zones appear as clear spots against the red background (Hamburger and Cordell, 1987). The inhibition zones were compared with the R_f values of the control TLC plate so that the active compounds were located on TLC.

Active bands were scratched from several TLC plates, dissolved in diethyl ether and filtrated to discard silica gel. Its purity as a single spot was confirmed by re-chromatographed on TLC with the same elution solvent system to make sure that is one single compound.

Structure elucidation of the isolated bioactive compounds

GC-MS analysis

The GC/MS analysis was performed 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). For GC/MS detection an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1ml min-1. The injector and MS transfer line temperature was set at 280 C.

The oven temperature was programmed at an initial temperature 50 C (hold 2 min) to150˚C at an increasing rate of 7˚C min^{-1}, then to 270 C at an increasing rate 5˚C min^{-1} (hold 2min) then to 310 as a final temperature at an increasing rate of 3.5˚C min^{-1} (hold 10 min).

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.

Nuclear magnetic resonance (NMR)

NMR spectra were acquired in DMSO-d6 on a Jeol ECA 500MHz NMR spectrometer, at 500 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{1}H and ^{13}C. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d6 and were converted to TMS scale by adding 29.8.

RESULTS AND DISCUSSION

Microcystis aeruginosa crude extracts

Antifungal activity of *M. aeruginosa* extracts against different species of mycotoxigenic fungi is shown in Table (1). The diethyl ether extracts showed the best antifungal activity against all tested fungi achieving inhibition zone in range between 10.3 mm against *A. flavus* to 21.6 mm against *F. verticillioides*. *A. flavus* was the most sensitive fungus affected by all extracts with exception of methanol extract. However, *A. ochraceus* and *P. verrucosum* were tolerant of resistance to all *M. aeruginosa* extracts with exception of diethyl ether extract which had inhibition zone 20.0 and 16.8 mm, respectively.

Several studies examined the activity of *M. aeruginosa* extracts against fungi. Khalid *et al*. (2010) reported that *Microcystis aeruginosa* methanolic extract exhibited strong antifungal activity against 20 fungal species included 7 human pathogens and 5 plant pathogens.

Madhumathi *et al*. (2011) found that *M. aeruginosa* methanol, ethanol, acetone and diethyl ether extracts had the largest antifungal activity against *C.ablicans* and *S. cerevisiae*. Also, *Salem et al.* (2014) indicated that *Microcystis* sp. acetone extract showed antifungal activity against *A. niger*. Shrivastava (2014) found that *M. aeroginosa* methanolic extract significantly inhibited the mycelial growth of *Aspergillus fumagatus*, *Candida albicans* and *Rhizoctonia solani*. Jaiswal *et al*. (2011) revealed that *M. aeruginosa* dichloromethane and ethyl acetate extracts had antifungal activity against the phytopathogenic fungus *Rhizoctonia solani*. Perveen and Alwathani (2013) found that methanol extract *M. aeroginosa* showed antifungal activity against *Fusarium* sp., *F. oxysporum*, *F. solani* and *A. flavus*, while acetone had antifungal against *F. solani*. Moreover, Kulik (1995) reported that cyanobacterial culture filtrates or cell extracts were applied to seeds for protect them against *Fusarium* sp. and *Rhizoctonia solani*.

Table 1: Antifungal activity of *Microcystis aeruginosa* crude extracts.

Fungi	Inhibition zone mm (Mean ± S.E.)								
	-ve control	+ve control	MeOH	EtOH	Acetone	CH_3Cl	DEE	EtOA	Hexane
A. flavus	0	16.1±0.74	0	7.2±0.28	9.3±0.89	8.7±0.28	10.3±1.25	8.0±0.5	9.8±1.15
A. niger	0	7.5±0.35	8.0±0.50	7.5±0.50	8.2±0.28	0	13. 7±1.56	11.3±1.04	0
A. ochraceus	0	10.9±0.54	0	0	0	0	20.0±1.50	0	0
A. parasiticus	0	11.8±2.01	0	7.7±0.58	0	8.2±0.76	13.3±1.25	9.3±0.76	0
A. westerdijikia	0	10.5±0.35	0	0	0	0	10.2±0.76	8.0±0.50	0
A. Carbonarius	0	10.4±0.41	9.8±1.15	0	0	0	10.5±1.80	7.7±0.28	0
F. verticillioides	0	11.0±0.35	0	0	0	0	21.6±0.75	7.2±0.78	0
F. Proliferatum	0	11.0±0.61	0	0	10.2±1.04	9.8±1.04	17.8±1.25	0	0
P. verrucosum	0	9.9±1.43	0	0	0	0	16.8±0.76	0	0

n=3, *S.E: standard error, 0: No inhibition, MeOH: methanol, EtOH: ethanol, DEE: diethyl ether, EtOA: ethyl acetate, negative control: DMSO, positive control: Nystatin.

Table 2: Antifungal activity of *Microcystis aeruginosa* diethyl ether fractions.

Fungi	Inhibition zone mm (Mean ± S.E.)							
	Control	F1	F2	F3	F4	F5	F6	F7
A. flavus	0	8.3±1.15	9.0±2.00	0	8.0±0.00	0	0	0
A. steynii	0	7.0±0.00	0	0	7.7±0.58	0	0	0
A. ochraceus	0	9.3±1.52	0	7.3±0.58	10.7±0.58	0	0	0
A. parasiticus	0	0	9.3±1.52	0	7.2±0.76	8.3±1.15	7.7±0.15	0
A. westerdijikia	0	0	0	0	9.7±1.15	0	8.0±0.00	8.3±0.58
A. Carbonarius	0	0	0	0	8.3±0.58	0	8.0±0.00	0
F. verticillioides	0	0	7.0±0.00	7.3±0.58	7.7±0.28	0	0	0
F. Proliferatum	0	0	0	0	8.0±1.00	9.3±1.08	0	0
P. verrucosum	0	8.7±0.58	8.7±0.28	8.3±1.52	8.3±0.58	0	7.3±0.58	0

n=3, *S.E: standard error, 0: No inhibition

Table 3: Antifungal activity of *Microcystis aeruginosa* sub-fraction F4

Fungi	Inhibition zone mm (Mean ± S.E.)									
	F4-1	F4-2	F4-3	F4-4	F4-5	F4-6	F4-7	F4-8	F4-9	F4-10
A. flavus	0	0	0	9.7 ±1.15	8.7±1.52	10.3±2.31	0	8.3±0.58	7.0 ±0.00	8.0±1.00
A. steynii	0	0	7.3±0.58	0	7.7 ±1.15	8.0 ±1.00	0	0	7.7 ±1.15	7.0±0.00
A. ochraceus	7.7±1.15	8.0±1.00	0	9.0 ±1.64	8.0 ±1.73	9.3 ±1.52	0	0	8.3 ±0.58	9.3±1.15
A. parasiticus	0	0	8.7±0.58	0	8.3 ±1.15	7.0 ±0.00	7.7±1.15	8.3±0.58	--	8.7±0.58
A. westerdijikia	0	7.7±0.58	0	0	9.0 ±1.73	8.7±1.15	10.0±1.52	0	8.3 ±1.15	10.3±2.08
A. Carbonarius	0	0	0	8.3±0.58	9.3 ±1.52	8.7 ±0.58	8.33 ±1.15	0	8.3±0.58	10.0 ±2.00
F. verticillioides	8.0±1.73	0	8.0±0.00	7.7 ±1.15	7.0 ±0.00	8.3 ±1.15	0	0	6.8 ±0.28	7.7±0.58
F. Proliferatum	0	0	0	8.0 ±1.00	0	0	7..7±0.58	8.7±1.52	7.0 ±0.00	10.3±1.21
P. verrucosum	0	0	0	7.7 ±1.15	7.3 ±0.58	7.7 ±1.15	8.0±1.00	7.7±1.15	8.0 ±1.00	8.3±1.15

n=3, *S.E: standard error, 0: No inhibition.

Microcystis aeruginosa diethyl ether fractions

The antifungal activity of *M. aeruginosa* DEE fractions against nine mycotoxigenic fungi is represented in Table (2). Fraction F4 had antifungal activity against all tested fungi. In contrast, F7 showed no activity against tested fungi except *A. westerdijikia* with inhibition zone of 8.3 mm. The highest antifungal activity was observed using F4 against *A. ochraceus* with inhibition zone of 10.7 mm followed *A. carbonarius* with inhibition zone of 9.7 mm. Perveen and Alwathani (2013) indicated that methanol: acetone: diethyl ether (5:2:1) extract observed strong inhibitory effect against *F. solani* and *Cladosporium* sp., and moderate activity against *Fusarium* sp. and *F. oxysporum*. Kamble *et al*. (2012) revealed that the diethyl ether extract of *M. aeroginosa* had the largest inhibition zone against *S. cerevisiae* and *C. albicans* on the agar.

Microcystis aeruginosa diethyl ether sub-fractions

Based on the results of antifungal activity of *M. aeruginosa* diethyl ether fractions, the active fractions against all tested fungi were fractionated into sub-fractions to increase the probability of bioactive compounds isolation and by disposing the sub-fractions that had no activity against the tested microorganisms. F4 was separated into 10 sub-fractions (F4-1 to F4-10). All obtained sub-fractions were evaluated for their antifungal activity.

Table (3) shows the antifungal activity of *M. aeruginosa* DEE fraction F4. Sub-fraction F4-10 showed antifungal activity against all tested fungi followed by F4-5 and F4-6 which had antifungal activity against all tested fungi except *F. Proliferatum*. The highest antifungal activity was recorded in F4-6 against *A. flavus* and F4-10 against *A. westerdijikia* and *F. Proliferatum* with inhibition zone of 10.3 mm.

Identification of compounds in Sub-fraction F4-10 using GC-MS

Totally 7 compounds were identified by GC-MS from *M. aeruginosa* sub-fraction F4-10. These compounds were 9,12-Octadecadienoic acid (Z,Z)- with peak area percent 7.57%, Butylated hydroxytoluene 13.40%, Pentadeconic acid,4- hexadecyl ester 5.70%, Hexadecadienoic acid, methyl ester 27.44%, 9-

Octadecadienoic acid Z-(CAS) 10.77%, Heptadecane 5.37% and 3-Methyl-2-butanol 2.11% (Fig. 1 and Table 4).

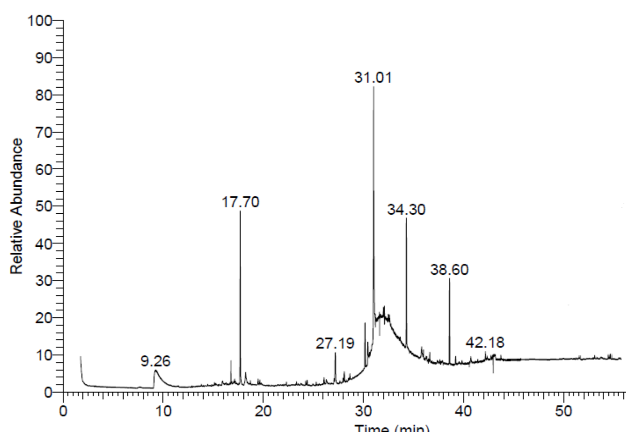

Fig. 1: GC-MS chromatogram of *M. aeruginosa* DEE extract sub-fraction F4-10.

Table 4: Components detected in sub-fraction F4-10 of *M. aeruginosa* DEE extract.

No	RT	Compound	Area %	Molecular formula	MW
1	9.26	9,12-Octadecadienoic acid (Z,Z)-	7.57	$C_{18}H_{32}O_2$	280
2	17.70	Butylated hydroxy toluene	13.40	$C_{15}H_{24}O$	220
3	27.19	Pentadeconic acid,4- hexadecyl ester	5.70	$C_{15}H_{30}O_2$	242
4	31.01	Hexadecadienoic acid, methyl ester	27.44	$C_{15}H_{30}O_2$	270
5	34.30	9-Octadecadienoic acid Z-(CAS)	10.77	$C_{18}H_{34}O_2$	282
6	38.60	Heptadecane	5.37	$C_{17}H_{36}$	240
7	42.18	3-Methyl-2-butanol	2.11	$C_5H_{12}O$	88

Marimuthu *et al*. (2014) indicated that the fatty acids Hexadecanoic acid methyl ester, 9-Octadecenoic acid and 9,12-Octadecadienoic acid (Z,Z) extracted from dry Christmas lima bean had antifungal activity against *A. flavus* and *A. niger*. Also in this direction, Ahmed *et al*. (2012) revealed that 9-Octadecenoic acid from the lipid extract of medicinal plant *Acacia modesta* had antifungal activity against *A. flavus* and *F. solani* as well as cytotoxic activity against *Artemia salina*. Demirel *et al*. (2011) and Mavi *et al*. (2011) indicated that Butylated hydroxytoluene from medicinal plants methanolic extract had antifungal activity against *C. albicans*. Mubarak *et al*. (2012) found that the fatty acid 9-Octadecenoic acid from lipid extract of *Scenedesmus bijugatus* had antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*. Udgire and Pathade (2013) reported that Pentadeconic acid, 4- hexadecyl ester which isolated from the medicinal plant *Valeriana wallichii* showed had antimicrobial activity against *K. pneumonia*, *E. coli*, *S. aureus*, *P. aeruginosa* and *A. niger*. Kumar *et al*. (2011), Jain *et al*. (2012) and Govindappa *et al*. (2014) reported that 9-Octadecenoic acid, methyl ester and 9,12-Octadecadienoic acid, methyl ester isolated from some plants and *Spirulina platensis* display antibacterial and antifungal activity against several human pathogenic microorganisms.

Detection of bioactive compounds in sub-fraction F4-10 by TLC bioautography

Five clear and distinct bands were isolated from sub-fraction F4-10 using the solvent elution system toluene: methanol: acetone: acetic acid at ratio of 15:2:1:1 with retention factor values (R_f) of 0.48, 0.55, 0.62, 0.66 and 0.71. The bioautography of separated bands on TLC was used to detect the bioactivities of these bands against tested fungal strains. Table (5) illustrates TLC bioautography of *M. aeruginosa* sub-fraction F4-10 bands against 9 mycotoxigenic fungi. The spots of R_f 0.55 and 0.71 showed clear inhibition zone against all tested fungi followed by spot of Rf 0.66 which had antifungal activity against tested fungi except *A. ochraceus* and *F. proliferatum*. No antifungal activities were observed in bands of R_f values 0.48.

Table 5: Inhibition of fungal growth on bioautographic TLC plates by *M. aeruginosa* diethyl ether sub-fraction F4-10.

Fungi	R_f (Retention factor)				
	0.48	0.55	0.62	0.66	0.71
A. flavus	--	++	++	+	+++
A. steynii	--	++	+	+	+++
A. ochraceus	--	+++	--	--	++
A. parasiticus	--	+++	+	++	++
A. westerdijikia	--	+++	++	+	+
A. carbonarius	--	++	--	+	+++
F. verticillioides	--	++	--	+	+++
F. proliferatum	--	+++	--	--	++
P. verrucosum	--	++	++	++	+++

(--): No inhibition, (+): inhibition zone 2-5 mm, (++): inhibition zone 5-7 mm, (+++): inhibition zone > 7mm.

Identification of antifungal compounds

Based on bioautography assay, Spots of R_f 0.55 and 0.71 in sub-fraction F4-10 were selected according to their high activity against all tested fungi.

Structure elucidation of compound A

Compound R_f 0.55 A (R_f 0.55) had a molecular weight of 220 Dalton and chemical structure $C_{15}H_{24}O$ based on GC-MS analysis (Fig. 2) (Saittagaroon *et al*., 1984 and Ruan *et al*., 2014).

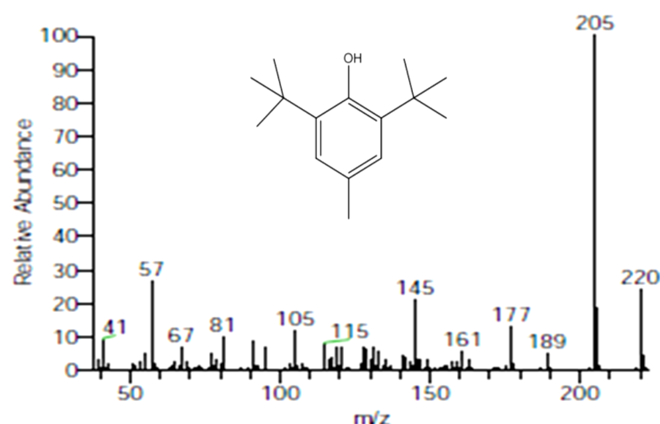

Fig. 2: GC-MS spectrum and chemical structure of Butylated Hydroxytoluene.

^{1}H-NMR spectrum of compound A was measured in $CDCl_3$ at 500 MHz and revealed characteristic of Butylated Hydroxytoluene (BHT) as follow; two aliphatic singlet resonances at δ ppm 1.52 (s, 18H) attributed to two t-butyl groups, δ ppm 2.28 (s, 3H) belonging to CH_3 attached to phenyl moiety. In addition to one aromatic signal at δ ppm 7.03 (s, 2H) attributed to two

aromatic protons and another signal δ ppm 5.01 (s, 1H) which disappeared on addition of drop of D_2O suggesting that it is belonging to the phenolic OH. So, the structure of compound A is confirmed to be (BHT) 2,6-di t-butyl-4-methyl phenol (Silva *et al*., 2009 and Hwang *et al*., 2014). Passone *et al*. (2007) found that phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl paraben (PP) caused inhibition to *Aspergillus* growth at in vitro level. Also, Passone *et al*. (2008) found that a mixture of BHA, PP and BHT was more effective than the individual components in controlling the growth and AFB1 accumulation by *Aspergillus* section *Flavi* on peanuts. Demirel *et al*. (2011) and Mavi *et al*. (2011) indicated that Butylated hydroxytoluene from medicinal plants methanolic extract had antimicrobial activity against *E*. coli, *P*. *aeruginosa*, *S*. *aureus* and *C*. *albicans*.

Structure elucidation of compound B

Fig. (3) illustrates the chemical structure of compound B (R_f 0.71). According to GC-MS spectrum analysis the peak exhibited at 26.98 min retention time had a molecular weight 270 Dalton and chemical structure $C_{17}H_{34}O_2$ (Abubacker and Deepalakshmi, 2013; Li *et al*., 2013; He *et al*., 2014). ^{1}H-NMR confirmed the structure by diagnostic signals at δ=2.4 – 2.7 ppm (2H, d), δ=1.53 ppm (2H, m), δ=1.2 ppm (26H, m) and δ=3.54 ppm (3H, s). So, the structure of compound B was Hexadecanoic acid methyl ester (Saxena *et al*., 2007 and Oyugi *et al*., 2011). Most studies done to isolate and identify this compound were from plants. Prabhadevi *et al*. (2012) and Mujeeb *et al*. (2014) reported that hexadecanoic acid methyl ester which isolated from different plants showed antioxidant, anti-inflammatory, antibacterial, anticandidal, antifungal and cancer preventive activities. Also, Senthilkumar *et al*. (2013) reported that hexadecanoic acid methyl ester isolated from leaf extract of medicinal plant *Ruellia tuberosa* had antimicrobial activity against *E*. *coli*, *P*. *aeruginosa*, *K*. *pneumonia*, *B*. *subtilis*, *Penicillium* sp. and *Aspergillus* sp. Lakshmi and Rajalakshmi (2011) indicated that hexadecanoic acid methyl ester possessed antioxidant, nematicidal and pesticidal activities. However, in similar study Khalid *et al*. (2010) isolated hexadecanoic acid methyl ester from toxic cyanobacteria *Microcystis aeruginosa*. They found that it had antibacterial activity against *B*. *cereus*, *E*. *coli*, *K*. *pneumonia*, *S*. *aureus*, *S*. *typhi* and *V*. *choleriae*.

Minimum inhibitory concentration (MIC) values

As shown in Table (6) the highest activity of *M*. *aeruginosa* DEE was recorded against *F*. *verticillioides* followed by *F*. *proliferatum* with MIC value of 0.62 and 0.66 mg ml^{-1}medium, respectively. Whereas, the lowest activity was showed against *A*. *ochraceus* with MIC values 1.28 mg ml^{-1}. *M*. *aeruginosa* F4 showed highest activity against *A*. *westerdijikia* with MIC of 0.8 mg ml^{-1}. The highest activity of sub-fraction F4-10 was showed against *A*. *flavus* with MIC value of 0.75 mg ml^{-1}. MIC values of BHT (compound A) against tested fungi ranged from 1.98 to 3.78 mg ml^{-1}. While, the MIC values of Hexadecanoic acid methyl ester (compound B) ranged from 0.82 to 1.3 mg ml-^{1}. It should be noticed that MIC values of diethyl ether crude extract were lower than fraction, sub-fraction and both purified compounds. This activity might be attributed to the synergistic effect of different compounds in crude extract ether than each compound alone. No available studies measured MIC of extracts, fractions, sub-fractions and pure compounds from *M*. *aeruginosa*, whereas other organisms were studied. Jassbi *et al*. (2013) indicated that Hexadecanoic acid methyl ester from a red algae *Hypneaflagelli formis* and two brown algae *Cystoseiramyrica* and *Sargassum boveanum* had antimicrobial activity against *E*. *coli*, *K*. *pneumonia*, *S*. *typhi*, *S*. *aureus*, *S*. *epidemidis*, *B*. *subtilis*, *A*. *niger* and *C*. *albicans* with MIC values ranged from 3 to 45.5 μg ml^{-1}. Al-Rekabi (2011) reported that *Oscillatoria irrigua* aqueous fraction had antifungal activity against *A*. *flavus* with MIC value 1.25 mg ml^{-1}.

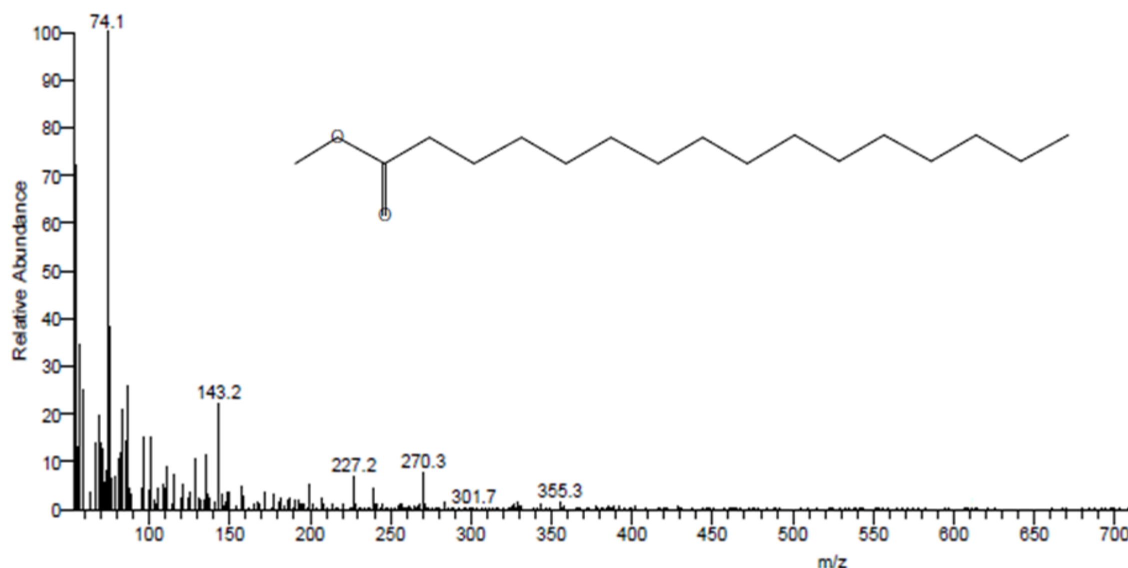

Fig. 3: GC-MS spectrum and chemical structure of Hexadecanoic acid, methyl ester.

Table 6: MIC values (mg ml^{-1}) of *M. aeruginosa* DEE, fraction F4, sub-fraction F4-10, compound A and B.

Fungi	MIC values mg ml^{-1}(Mean ± S.E.)				
	DEE*	F4	F4-10	Compound A	Compound B
A. flavus	0.74±0.21	1.60±0.28	0.75±0.14	2.24±0.28	0.94±0.14
A. steynii	0.82±0.14	1.35±0.21	1.15±0.21	1.98±0.14	0.91±0.11
A. ochraceus	1.28±0.14	1.40±0.14	1.32±0.14	3.12±0.21	1.04±0.08
A. parasiticus	0.88±0.28	1.00±0.11	0.92±0.11	2.44±0.28	1.22±0.28
A. westerdijikia	0.82±0.08	0.80±0.28	0.84±0.21	3.02±0.14	1.26±0.21
A. carbonarius	1.04±0.14	1.12±0.11	1.25±0.21	2.28±0.14	1.30±0.28
F. verticillioides	0.62±0.21	1.25±0.14	1.00±0.14	2.84±0.28	0.93±0.14
F. proliferatum	0.66±0.14	0.88±0.11	0.82±0.11	3.78±0.21	0.82±0.11
P. verrucosum	0.82±0.21	1.22±0.21	1.78±0.28	3.22±0.28	1.24±0.28

n=3, S.E: standard error, DEE*: Diethyl ether crude extract.

CONCLUSION

The current investigation highlights the potential use of *M. aeroginosa* as a source of antifungal compounds for application in agriculture and food industry as bio-product. Diethyl ether was the favorable solvent to extract compounds had antifungal activity from *M. aeroginosa*. BHT and Hexadecanoic acid, methyl ester were the main identified compounds responsible for this activity. Finally, it is recommended to use BHT form bio-source like *M. aeroginosa* as a preservative in food industry instead of synthetic BHT.

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