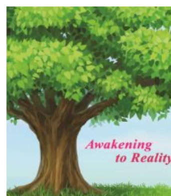


Available online at www.elixirpublishers.com (Elixir International Journal)

Natural Products Chemistry

Elixir Natural Products Chem. 117 (2018) 50565-50568

Elixir ISSN: 2229-712X

Biological Activity of Crude Extracts of Endophytic *Fusarium Oxysporum* and its Chemical Composition by Gas Chromatography–Mass Spectrometry

Hamdi M. D. Nasr[1,*], Ramadan O. Abdel-ghany[2], Sayed A. S. Mousa[1], Mostafa Alasmaey[1] and A. A. Atalla[1]

[1]Chemistry Department, Faculty of Science, Al-Azhar University, 71524 Assiut, Egypt.

[2]Microbiology Department, Faculty of Science, Assiut University, 71515 Assiut, Egypt.

ARTICLE INFO

Article history:

Received: 15 March 2018;
Received in revised form:
18 April 2018;
Accepted: 28 April 2018;

Keywords

Fusarium oxysporum,
Biological activity,
Ergosterol,
Gas chromatography-mass
Spectrometry (GC-MS)
Analysis.

ABSTRACT

In this study, *Fusarium oxysporum* AUMC9264 was isolated from *Lupinu stermis* L. which was identified by a morphological method and 18S rDNA sequence comparison. Potato Dextrose Agar (PDA) was used for activation and subcultures of the fungal isolate while Potato Dextrose Broth medium (PDB) used for metabolite production. The fungal extracts were tested *in vitro* for their Antimicrobial effects against some strains of bacteria and fungi. Ergosterol was isolated from the crude extract and elucidate according to ^{1}H-NMR and its mass spectrometry analysis, n-Hexan fraction was identified by gas chromatography– mass spectrometry.

© 2018 Elixir All rights reserved.

Introduction:

Fusarium oxysporum is a ubiquitous soil-borne fungus capable of causing wilt in several agricultural crops worldwide and is known to produce mycotoxins [1]. Conversely, it has been reported to produce compounds with antifungal [2] and anticancer [3] activities. Oxysporidinone was isolated from fermentations of *F. oxysporum* (CBS 330.95) and exhibited growth inhibitory activity against several plant pathogenic fungi [4]. While the liquid extract of Endophytic *F. oxysporum* 5-19 sp. Led to Beauvericin which have a potent inhibitory effect on *C. albicans, E. coli,* and *S. aureus* [5].

Crude extracts of *F. oxysporum* isolated from the *Smallanthus sonchifolius* exhibited cytotoxic activity against human cancer cells [6]. Different classes of organic compounds were isolated such as diketopiprazines [7], Vinblastine and vincristine, which are excellent anti-cancer [8].

Gas chromatography linked to mass spectrometry (GC-MS) has been widely used for a long time for structural analysis of fatty acids and terpenoid compounds [9]. Recent studies about the interaction between 3 species of date palm and fungal *Fusarium Oxyspourm* sp. by using GC-MS had been done [10]. At the same time this method used in analysis of liquid extract of *F. oxysporum* to lead phenyl acetic acid, which has antimicrobial, antifungal, phytotoxic properties [11] and detection of 38 VOCs produced by *F. oxysporum* which have a broad spectrum against *Meloidogyne incognita* and *Arthrobotrys conoides* [12].

In continuation of our search for isolation of bioactive natural products from fungi, we have isolated and identified *Fusarium oxysporum.* And its culture broth was subjected to detailed chemical composition.

The crude extract produced ergosterol, which consider the main fungal cholesterol present in the cell wall of the fungi [13]. Ergosterol was elucidate according to the ^{1}H-NMR analysis. Also, this study was interested in *vitro* bioassays for estimation of antimicrobial properties.

Experimental:

Materials and Methods:

The ^{1}H-NMR spectra were measured on Varian Inova 300 (75 MHz). Chemical shifts were measured relative to tetramethylsilane as internal standard. Mass spectra: ESI MS was recorded on a Finnigan LCQ. Column chromatography (CC): MN silica gel 60: 0.05-0.2 mm, 70-270 mesh (Macherey-Nagel & Co); silica gel (230-400 mesh) for flash chromatography: 30-60 μm (J. T. Baker); size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

Gas Chromatography–Mass Spectrometry Analysis (GC-MS):

Chemical constituents of the n-hexane fraction by using GC-MS analysis was performed by Assiut University GC-MS (7890 GC system/ 5975 Binert XL EI/CI MSD Column: DB-5 ms (30 m X 0.25 mm X 0.25 μm).

Fungal Isolation and Culture Conditions:

Fusarium oxysporum AUMC9264 was isolated from *Lupinus termis* L. and tested for Pathogenesis to lupine which has 60 % of wilting. The fungus metabolites play an important role in pathogenecity of study fungus. Potato Dextrose Agar (PDA) was used for activation and sub culturing of the fungal isolates. The medium containing potato extract (of 200g potato), 20g glucose, 20g agar, 250 mg Chloramphenicol and complete to 1 liter. The medium was autoclaved at 1.5 ATM pressure and 121°C.

Tele:
E-mail address: nasrhamdi@yahoo.com

© 2018 Elixir All rights reserved

The medium was poured into Petri dishes and cooled down to room temp. for solidification. Four Petri dishes were inoculated with *Fusarium oxysporum* incubated at 28±2 °C in static incubator.

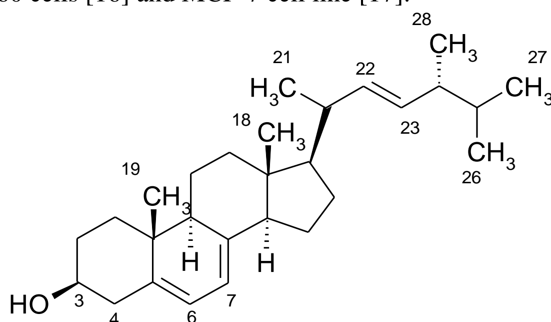
PDB medium (Potato Dextrose Broth; Potato extract (200 g), glucose (20 g), complete to one liter) the medium was used for preparations (subculturing and activation) of the inoculum (*F. oxysporum*). The fungus in Petri dishes was inoculated into three conical flask (500 ml) containing 100 ml broth medium and incubated at 28±2 °C and shocked at 155 RPM for one week for multiplication of the inoculums size. The inoculum was transferred to sterile large plastic bags (polyethylene) for the fermentation process. The bags contained synthetic fibers for enhancement of fungal growth and 10 Liter of sterile Broth PDB medium for fungal metabolite production (the bags were shacked twice a day). All the processes were done under aseptic conditions.

Extraction and Isolation of Metabolites:

After fermentation the fungal biomass was removed from the broth by filtration using cheese cloth and Whatmann No.1 filter paper. The broth medium extraction was carried out by adding an equal volume of ethyl acetate (v/v). After overnight extraction the organic layer was transferred to a round bottom flask and concentrated to dryness using a rotary vacuum Rotavapour (SINCO, China) with a heating water bath (< 40ºC) under reduced pressure, after which defatting was carried out using n-hexane solvent. The reduced volume of extract was then lyophilized and stored at 4°C for further use.

Results and Discussion:

Ergosterol was isolated as colorless solid, UV absorbing at 254 nm turned to violet coloration with anisaldehyde/sulfuric acid and heating and changed later to blue. Isolation and purification of ergosterol have been done by using sephadex LH-20 column chromatography (CH_2Cl_2/40% MeOH). The chemical structure of ergosterol was confirmed by ^{1}H-NMR and ESI mass spectroscopy, which displayed *pseudo*molecular ion peaks at *m/z* 419 $[M+Na]^+$ and 815 $[2M+Na]^+$, respectively. Compared with literature data [14,15] confirmed the structure. Ergosterol exhibited biological properties such as cytotoxicity against HL-60 cells [16] and MCF-7 cell line [17].



^{1}H NMR (CD_3OD, 300 MHz): δ 5.48 (dd, ^3J = 5.6, ^4J = 2.4 Hz, 1H, H-6), 5.34 (dd, ^3J = 5.4, ^4J = 2.7 Hz, 1H, H-7) 5.26 (d, J = 8.5 Hz, 1H, H-23), 5.24 (d, J = 8.5 Hz, 1H, H-22), 3.40 (m, 1H, H-3), 2.35 (m, 1H, H-4a), 2.17 (m, 1H, H-4b), 2.00-1.17 many protons overlapped, 0.95 (d, J = 6.7 Hz, 3H, H_3-21), 0.94 (s, 3H, H_3-19), 0.91 (d, J = 7.0 Hz, 3H, H_3-28), 0.85 (d, J = 6.8 Hz, 3H, H_3-26), 0.82 (d, J = 6.8 Hz, 3H, H_3-27), 0.62 (s, 3H, H_3-18). (+)-**ESIMS** *m/z* 419.3 $[M+Na]^+$, 815.7 $[2M+Na]^+$.

Chemical Composition (GC-MS) Analysis:

The gas chromatography mass spectrometry (GC-MS) analysis of the n-hexane fraction extract of *F. oxysporum* (Figure 1) revealed various important organic compounds, Two of them are fatty acids butanoic acid (1) and palmitic acid (4), Two terpenoid compounds, camphene (3) and dihydrophytol (5), And the others are fatty acid esters as shown in (Table 1). Fatty acid methyl esters are the most widely used derivatives for GC analysis. Indeed, they are simple in structure, volatiles, and have good chromatographic properties, and their preparation methods are now in routine at a smaller scale [18]. Fatty acids are ionized, forming fragments with certain mass and identified based on mass to charge ratio and compared with a library of known mass spectra which is stored on a computer database. Camphene (3) is a bicyclic monoterpene and it is nearly insoluble in water, but very soluble in common organic solvents. Camphene (3) is used in the preparation of fragrances and as a food additive for flavoring. Its mid-19th century use as a fuel for lamps was limited by its explosiveness. Also camphene (3) had anti-inflammation, analgesic and therapeutic properties, so it was decided that camphene had better opportunities in the pharmacological angle enabling it to find a place in the development of new therapeutic ideologies [19] also had a broad spectrum as the antifungal agent so it's used in the treatment of fungal skin infections, dysentery, athlete’s foot and dermatitis [20]. Palmitic acid or hexadecanoic acid (4) is the most common fatty acid (saturated) found in animals, plants and micro-organisms. It is a major component of the oil from palm trees (palm oil), but can also be found in meats [21]. Dihydrophytol (5) is Acyclic isoprenoid compounds with 20 carbon atoms are relatively abundant and widespread components of marine sediments [22,23]. The phytol classes are often used as biological markers [24]. Octadecyl-2-chloropropanoate (8) was identified by GC-MS from *Cinnamomum cassia* sp. [25].

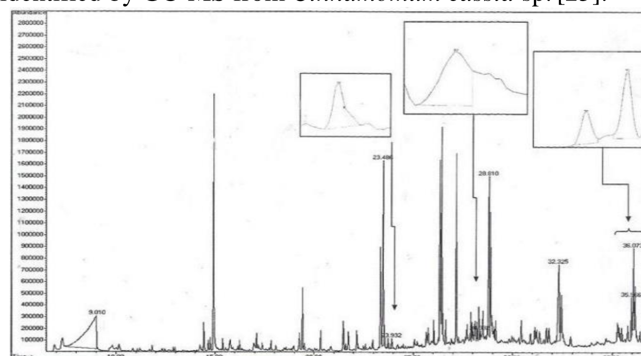
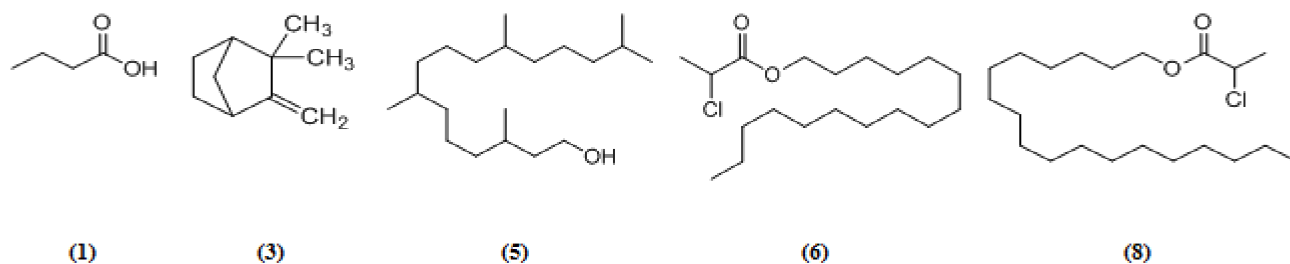


Figure 1. GC-MS spectrum of n-Hexane extract of *Fusarium oxysporum*.

Table 1. GC-MS analysis of the non-polar fraction of *Fusarium oxysporum*.

Compound	Value%	RT (min)	M. Formula	M. Weight
Butanoic acid (1)	13.669	9.013	$C_4H_8O_2$	88.10
Pentaflouropropanoic acid-4-hexadecyl ester (2)	3.055	23.363	$C_{19}H_{33}F_5O_2$	388.46
Camphene (3)	0.309	23.934	$C_{10}H_{16}$	136.24
Palmatic acid (4)	0.706	28.387	$C_{16}H_{32}O_2$	256.43
Dihydrophytol (5)	6.208	28.813	$C_{20}H_{42}O$	298.55
Hexadecyl 2-chloropropanoate (6)	4.735	32.327	$C_{19}H_{37}ClO_2$	332.95
Hexanedioic acid bis(2-ethylhexyl) ester (7)	1.354	35.97	$C_{22}H_{42}O_4$	370.56
Octadecyl-2-chloropropanoate (8)	2.792	36.075	$C_{21}H_{41}ClO_2$	361.00



Selected chemical structures of n-Hexan fraction

Biological Material:

Sabouraud's dextrose agar medium (g/l: peptone from meat, 10; glucose, 40; agar agar, 15) was used for determination of *F. oxysporum* extracellular metabolite effects on some of well identified fungal isolates (*Candida albicans* AUMC No. 1299, *Fusarium oxysporum* AUMC No. 5119, *Geotrichumcandidum* AUMC No. 226, *Aspergillus flavus* AUMC No. 1276, *Trichophytonrubrum* AUMC No. 1804) of AUMC (Assiut university Mycological center).

LB medium was used for testing the MIC of *F. oxysporum* metabolites on some of the recorded and well identified strains of bacteria (*Bacillus cereus* (+ve) AUMC No. B-52, *Serratiamarcescens* (-ve) AUMC No. B-55, *Staphylococcus aureus* (+ve) AUMC No. B-54, *Micrococcusluteus* AUMC No. B112, *Escherichia coli* (-ve) AUMC No. B-53, *Pseudomonas aerginosa* (-ve) AUMC No. B-73) used a concentration of 10mg/ml. DMSO was used as a solvent and negative control. The positive control used for antibacterial strains Chloramphenicol (0.1%, 50 µl), Clotrimazole (0.1%, 50 µl) was used as positive control for fungal isolates. The assisted extract of fungal biomass obtained from the PDB medium with DMSO was tested by agar well diffusion assay (NCCLS)[26].

Different concentrations of crude ethyl acetate extract ranging from 125 – 1000 *µg* were assessed against the test microorganisms. The crude extract of *Fusarium oxysporum* AUMC 9264 was effective against bacteria which varies depending on the strain used (**Table 2**). For bacterial strains there were two main MIC(s): 250 µg and 500 µg, respectively were observed against *Bacillus cereus* (+ve) AUMC No. B-52, *Staphylococcus aureus* (+ve) AUMC No. B-54, *Micrococcus luteus* AUMC No. B112; *Escherichia coli* (-ve) AUMC No. B-53, *Pseudomonas aerginosa* (-ve) AUMC No. B-73, *Serratia marcescens* (-ve) AUMC No. B-55). Whereas, the fungal metabolites did not record any effect on all tested fungal isolates (**Figure 2**).

Table 2. Antimicrobial assay for *F. oxysporum* AUMC9264 extract against some bacterial and fungal isolates.

		MIC	MIC		5000 *µg*
Bacteria	1000*µg*	500 *µg*	250*µg*	12*µg*	Control:Chlora-mphenicol
Bacillus cereus (+ve) AUMC No. B-52	24.33±0.33	10.67±0.33	0	0	26±0.57
Staphylococcus aureus (+ve) AUMC No. B-54	23.33±0.88	12±0.57	5.67±0.33	0	25±0.57
Micrococcusluteus AUMC No. B112	24.67±0.33	10.33±0.33	0	0	19.67±0.88
Escherichia coli (-ve) AUMC No. B-53	28.33±0.88	12.33±0.33	5.33±0.33	0	22±1
Pseudomonas aerginosa (-ve) AUMC No. B-73	26.67±0.88	12.33±0.33	0	0	21±0.57
Serratiamarcescens (-ve) AUMC No. B-55	27.67±0.88	13.33±0.67	5.33±0.33	0	18.33±0.33
Fungi					control: Clotrimazole
Candida albicans AUMC No. 1299	0	0	0	0	30.67 ±0.57
Geotrichumcandidum AUMC No. 226	0	0	0	0	26.66±0.774
Aspergillus flavus AUMC No. 1276	0	0	0	0	30.33±0.74
Fusarium oxysporum AUMC No. 5119	0	0	0	0	20.33±0.74
Trichophytonrubrum AUMC No. 1804	0	0	0	0	38.33±0.67

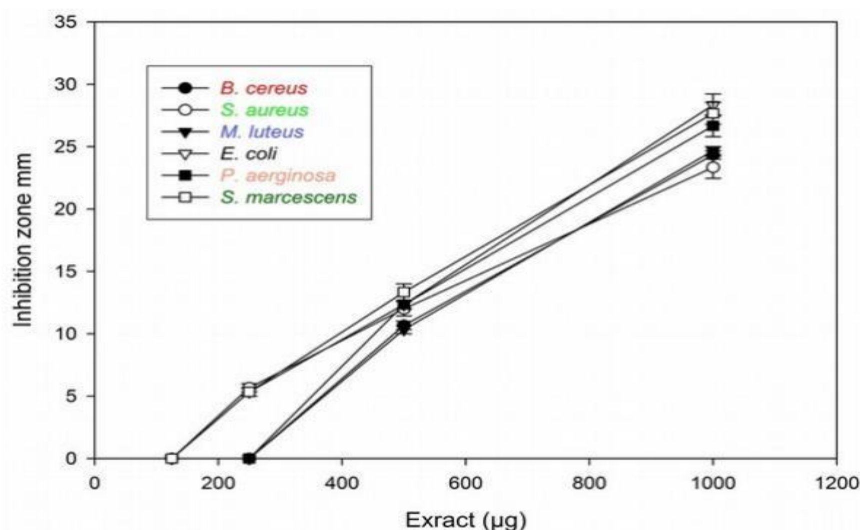


Figure 2. Antimicrobial assay for *Fusarium oxysporum* AUMC 9264 extract on some bacterial and fungal isolates showing two main MIC point for extracting metabolites at 250 and 500 µg.

Conclusion:

The present study found 8 chemical constituents from n-hexane fraction of *Fusarium oxysporum* AUMC9264 by Gas Chromatography Mass Spectrometry(GC-MS) analysis. The results of biological experimental study indicated that, the ethanolic extract of *F.oxysporum* AUMC9264 have antimicrobial activity against some bacterial spices and no result against studding fungal strains. And this attributes to contain several bioactive compounds with medicinal importance. However, further studies are needed to evaluate its bioactivity and toxicity profile.

References:

[1]Smith, S. N.; DeVay, J. E.; Hsieh, W.-H.; Lee, H.-J., Soil-borne populations of *Fusarium oxysporum* f. sp. *vasinfectum*, a cotton wilt fungus in California fields. *Mycologia* **2001**, 737-743.

[2]Jayasinghe, L.; Abbas, H. K.; Jacob, M. R.; Herath, W. H.; Nanayakkara, N. D., N-Methyl-4-hydroxy-2-pyridinone analogues from *Fusarium oxysporum*. *Journal of Natural Products* **2006**, 69 (3), 439-442.

[3]Zhan,J.; Burns, A. M.; Liu, M. X.;Faeth, S. H.; Gunatilaka, A. L.,Search for cell motility and angiogenesis inhibitors with potential anticancer activity:beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum.Journal of Natural Products* **2007**,70 (2),227-232.

[4]Breinholt, J.; Ludvigsen, S.; Rassing, B. R.; Rosendahl, C. N.; Nielsen, S. E.; Olsen, C. E., Oxysporidinone: a novel, antifungal N-methyl-4-hydroxy-2-pyridone from *Fusarium oxysporum*. *Journal of Natural Products* **1997**, 60 (1), 33-35.

[5]Zhang, H.; Ruan, C.; Bai, X.; Zhang, M.; Zhu, S.; Jiang, Y.,Isolation and Identification of the Antimicrobial Agent Beauvericin from the Endophytic *Fusarium oxysporum* 5-19 with NMR and ESI-MS/MS. *BioMed Research International* **2016**, 2016.

[6]Nascimento, A. M. d.; Conti, R.; Turatti, I. C.; Cavalcanti, B. C.;Costa-Lotufo, L. V.;Pessoa, C.; Moraes,M. O. d.; Manfrim, V.; Toledo, J. S.;Cruz, A. K., Bioactive extracts and chemical constituents of two endophytic strains of *Fusarium oxysporum*. *Revista Brasileira de Farmacognosia* **2012**, 22 (6), 1276-1281.

[7]Trigos, A.; Reyna, S.; Cervantes, L., Three diketopiperazines from the cultivated fungus *Fusarium oxysporum. Natural Product Letters* **1995**, 6 (4), 241-246.

[8]Kumar,A.;Patil, D.;Rajamohanan,P.R.;Ahmad,A., Isola tion, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. *PloS one* **2013**, 8 (9), e71805.

[9]Dubois, N.;Barnathan, G.; Gouygou, J. P.;Bergé, J. P., Gas chromatographic behavior of fatty acid derivatives for mass spectrometry on low-polarity capillary columns. *European Journal of Lipid Science and Technology* **2009**, 111 (7), 688-697.

[10]Boucenna-Mouzali, B.; Gaceb-Terrak, R.; Rahmania, F., GC–MS Analysis of Cell Wall-Bound Phenolic Compounds and Lignin Quantification in Date Palm Cultivars that are Resistant or Susceptible to *Fusarium oxysporum* f. sp. *albedinis.Arabian Journal for Science and Engineering* **2018**, 1-9.

[11]Ait, T. K.; Rahmania, F., Identification of phenylacetic acid produced by *Fusarium oxysporum* f. sp. *albedinis*, the causal agent of bayoud, using GC-MS. *Comptes Rendus Biologies* **2010**, 333 (11-12), 808-813.

[12]Freire, E.; Campos, V.; Oliveira, D.; Faria, M.; Pohlit, A.; Noberto, N.; Rezende, E.; Pfenning, L.; Silva, J., Volatile substances produced by *Fusarium oxysporum* from coffee rhizosphere and other microbes affect *Meloidogyne incognita* and *Arthrobotrys conoides*. *Journal of Nematology* **2012**, 44 (4), 321-328.

[13]Starratt, A.; Madhosingh, C., Sterol and fatty acid components of mycelium of *Fusarium oxysporum. Canadian Journal of Microbiology* **1967**, 13 (10), 1351-1355.

[14]Nagia, M. M.; El-Metwally, M. M.; Shaaban, M.; El-Zalabani, S. M.; Hanna, A. G., Four butyrolactones and diverse bioactive secondary metabolites from terrestrial *Aspergillus flavipes* MM2: isolation and structure determination.*Organic and Medicinal Chemistry Letters* **2012**, 2 (1), 9.

[15]Shirane, N.; Takenaka, H.; Ueda, K.; Hashimoto, Y.; Katoh, K.; Ishii, H., Sterol analysis of DMI-resistant and-sensitive strains of *Venturia inaequalis*. *Phytochemistry* **1996**, 41 (5), 1301-1308.

[16]Han, X.; Lin, Z.; Tao, H.; Liu, P.; Wang, Y.; Zhu, W., Cytotoxic metabolites from symbiotic fungus *Penicillium* sp. HK13-8 with *Rhizophora stylosa*. *Zhongguo Haiyang Yaowu* **2009**, 28 (5), 11-16.

[17]Ding, Y.; Bao, H. Y.; Bau, T.; Li, Y.; Kim, Y. H., Antitumor components from Naematoloma fasciculare. *Journal of Microbiology and Biotechnology* **2009**, 19 (10), 1135-1138.

[18]Spitzer, V., Structure analysis of fatty acids by gas chromatography—low resolution electron impact mass spectrometry of their 4, 4-dimethyloxazoline derivatives—a review. *Progress in Lipid Research* **1996**, 35 (4), 387-408.

[19]Quintans-Júnior, L.; Moreira, J. C.; Pasquali, M. A.; Rabie, S.; Pires, A. S.; Schröder, R.; Rabelo, T. K.; Santos, J.; Lima, P. S.; Cavalcanti, S. C., *Antinociceptive activity and redox profile of the monoterpenes*. ISRN toxicology **2013**, 2013.

[20]Marei, G. I. K.; Rasoul, M. A. A.; Abdelgaleil, S. A., Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. *Pesticide Biochemistry and Physiology* **2012**, 103 (1), 56-61.

[21]Gunstone, F. D.; Harwood, J. L.; Dijkstra, A. J., The lipid handbook with CD-ROM. CRC press: 2007.

[22]Brooks, P.; Maxwell, J., Early stage fate of phytol in a recently deposited lacustrine sediment. *Advances in Organic Geochemistry* **1973**, 977-991.

[23]Rontani, J.-F.; Bonin, P. C.; Volkman, J. K., Biodegradation of free phytol by bacterial communities isolated from marine sediments under aerobic and denitrifying conditions. *Applied and Environmental Microbiology* **1999**, 65 (12), 5484-5492.

[24]Volkman,J. K.,Acyclic isoprenoids as biological markers. *Biological Markers in the Sedimentary Record*. **1986**, 1817-1828.

[25]Bharath,M.;Azeem, M.;Basha,S.; Keerthan, H., Antim icrobial Activity of Cinnamonextracts against Foodborne Pathogens *E. Coli*, *S.Tyhimurium* and *S. Aureus & L. Monocytogens.Journal of Pharmacy and Biological Sciences*, **2016**, 11(6):66-72.

[26]Barry,A. L.; Craig,W. A.; Nadler, H.; Reller, L. B.; Sanders,C.C.;Swenson, J. M., Methods for determining bactericidal activity of antimicrobial agents: approved guideline. *NCCLS* document M26-A **1999**, 19 (18).