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Full Length Research Paper

Cytotoxic Effects of *Emericella nidulans* EGCU 312-Derived Secondary Metabolites on Cancer Cells

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The fungus, *Emericella nidulans* was isolated from soil. The ITS region of 5.8S rRNA of the isolated fungus was amplified and sequenced. *E. nidulans* EGCU312 was given an accession number: KC511056 in the NCBI GenBank. Twenty one (21) fractions were obtained from the ethyl acetate extract of fungal filtrate. Fraction no. 12 showed the highest antioxidant activity with 81.54% at 200 µg/ml. High anticancer activities (against EACC cell line) ranging between 64.3 and 87.7% at 200 µg/ml, were exhibited by fractions no. 1, 2, 4, 9, 12 and 20. The mode of action of anticancer activity was studied by measuring activities of lactate dehydrogenase (LDH) and caspase-3. Fraction no. 12 gave the highest effect (2249.2 U/l) in LDH released as compared to control cells (1127.7 U/l) and caused a 1.56-fold increase in caspase-3 activity. Interestingly, fraction no. 12 caused 100% inhibition of *Staphylococcus aureus* and *Escherichia coli* at 50 µg/ml, and *Aspergillus fumigatus* at 100 µg/ml. The minimum bactericidal concentrations (MBC) of this fraction were 4 and 10 µg/ml for *S. aureus* and *E. coli*, respectively, while the minimum inhibitory concentration (MIC) was 45 µg/ml against *A. fumigatus*. GC-MS profile of fraction no. 12 showed 21 compounds, six of which, that is, 2-methylbenzylamine, N-heptyl-N-octyl; naphthalene, 2,3,6-trimethyl-; octadecanoic acid, ethyl ester; 1,2-benzenedicarboxylic acid, butyl octyl ester; tributylacetylcitrate; 1,2- and benzenedicarboxylic acid, diisooctyl ester, were of known biological activities.

Key words: *Emericella nidulans* EGCU 312, antioxidant, anticancer, antimicrobial.

INTRODUCTION

Secondary metabolites are natural products distinguished from primary metabolites, which are small compounds of intermediary metabolism needed for growth, development and reproduction of a living organism. On the other hand, secondary metabolites play non-essential roles (Vining, 1992). They are often used in defense against predation and habitat encroachment, or even used in communication. Therefore, these natural compounds endow the organisms that produce them, survival advantage over non-producing species.

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Secondary metabolites are largely found in bacteria, fungi, plants, dinoflagellates, mollusk sponges and insects. The fungal kingdom, encompassing many species, is a rich source of natural products with important medicinal properties. So far, 1,500 compounds in fungi have already been isolated, and more than half of these natural products have antibacterial, antitumor or antifungal activity (Pelaez, 2005; Wang et al., 2013; Shen et al., 2014). Many well-known drugs have been isolated from a variety of fungal species, such as penicillin, an antibiotic, lovastatin, cholesterol lowering drug, and cyclosporine, an immunosuppressant (Hoffmeister and Keller, 2007). Due to the structural diversity of these fungal secondary metabolites, discovery of novel fungal natural products may lead to a variety of new medicines. There are four major classes of fungal secondary metabolites, categorized by their biosynthesis pathway: non ribosomal peptides (NRP), polyketides (PK), terpenes and indole alkaloids (Keller et al., 2005).

Soil has the largest population of microbes. Cultured soil microbes have been an incredibly productive source of drugs, for example the cancer chemotherapeutics doxorubicin hydrochloride, bleomycin, daunorubicin and mitomycin. Aspergilli represent a group of filamentous fungi that plays a key role in industrial biotechnology. *Emericella nidulans* (teleomorph of *Aspergillus nidulans*) serves as a working horse in industrial production of enzymes and chemicals. Although, studies related to the biopotential activities of antimicrobial, antioxidant, and anticancer metabolites from this fungus based on drug discovery are limited.

Antimicrobial agents have been widespread and largely in effective therapeutic use since their discovery in the 20th century. However, the emergence of multi-drug resistant pathogens now presents an increasing global challenge to both human and veterinary medicine. It is now widely acknowledged that there is a need to develop novel antimicrobial agents to minimize the threat of further antimicrobial resistance (Hearst et al., 2009).

Free radicals are implicated in the pathogenesis of various human diseases such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damages, coronary heart diseases, and arthritis (Moon et al., 2006). Antioxidants serve as the defensive factor against free radicals in the body. Synthetic antioxidants such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are usually used as food additives by the food industry to prevent lipid peroxidation. However, their application has been limited because of possible toxic and carcinogenic components formed during their degradation. In view of these health concerns, finding safer, more effective and economic natural antioxidants is highly desirable (Mathew and Abraham, 2006). A number of microorga-nisms are commonly known to produce antioxidants, these include *Penicillium roquefortii*, *Aspergillus candidus*, *Mortierella* sp., *Emericella falconensis*, *Acremonium* sp., *Colletotrichum gloeosporioides* (Rios et al., 2006), *Mycelia sterilia* (Mathew and Abraham, 2006), *Antrodia camphorata*(Song and Yen, 2002), *Chaetomium* sp., *Cladosporium*s p., *Torula* sp*., Phoma* sp. etc. (Huang et al., 2007). A lot of fungi still needs to be explored as the production, downstream processing of actual bioactive phytochemicals from plants is quite tougher as compared to microbes.

During the last decades, more and more work have been done by researchers in the search for drugs against cancer, seeing that the disease is becoming a major cause of death among the population of developed countries (Szekeres and Novotny, 2002). The various forms of cancer require multiple approaches for their treatment, which opens a wide field of research that has to be explored. Natural products have therefore been recognized as one promising source for antitumor compounds. A substantial amount of research into cytotoxic natural products has been carried out in the last 50 years, and significant advances in cancer treatment have been achieved (da Rocha et al., 2001). Filamentous fungi can be considered as a useful source for production of antitumor secondary metabolites which therefore can be used as a novel therapeutic strategy for treatment of cancer.

Keeping the above in mind, the present study was planned to screen and expand the spectrum of *E. nidulans* having antioxidant, anticancer and antimicrobial compounds.

MATERIALS AND METHODS

Isolation and identification of fungal isolate

Soil samples, obtained from an agricultural soil from Cairo University, Giza, Egypt, were used as inocula for soil dilution plate method (Johnson et al., 1960). They were plated on modified solid Jackson s medium containing: glycerol 1.5%, sucrose 1.5%, peptone 0.6%, yeast extract 0.15%, NaCl1.5%, KH_2PO_4 0.06%, $MgSO_4.7H_2O$ 0.5%, $CuSO_4.5H_2O$ 0.0001%, $FeSO_4.7H_2O$ 0.0003%, and agar, 1.5%. Streptomycin (30 µg/ml) was added to the above medium after sterilization by autoclaving at 121°C and 1.5 bars for 15 min. Plates were incubated at 28°C for 4 days. Fungal colonies were purified by sub-culturing on modified solid Jackson s medium.

Identification of one of the developed fungal isolates was carried out by morphological and microscopic examinations (such as color, texture of mycelia, spore formation pattern, etc.). This was followed by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. Genomic DNA was isolated using Qiagen kit. Internal transcribed spacer (ITS) region of 5.8SrRNA was amplified using the primer ITS5 with sequence 5'- GGA AGT AAA AGT CGT AAC AAG G. Sequencing of PCR amplified product was performed at Macrogen (South Korea). The resulting sequence was entered into the BLAST algorithm of National Centre of Biological Information (NCBI) database to obtain closely related phylogenetic sequences and a phylogenetic tree was constructed. The obtained sequence was then submitted to the GenBank of NCBI database.

Fungal production of secondary metabolites

A two-step culture was performed for secondary metabolites

Table 1. Fractions collected from ethyl acetate crude extract of *E. nidulans* EGCU 312 using column chromatography.

Fraction no.	Hexane (%)	Chloroform (%)	Ethyl acetate (%)
1	100	0	
2	90	10	
3	80	20	
4	70	30	
5	60	40	
6	50	50	
7	40	60	
8	30	70	
9	20	80	
10	10	90	
11	0	100	0
12		90	10
13		80	20
14		70	30
15		60	40
16		50	50
17		40	60
18		30	70
19		20	80
20		10	90
21		0	100

production from *E. nidulans* EGCU 312. First, cultivation was carried out in 250-Erlenmeyer flasks each containing 100 ml modified Jackson's medium used as seed medium. Inocula were prepared by harvesting spores from 7-day-old PDA slants of *E. nidulans* EGCU 312 in sterilized solution containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2 x 10^6 spores/ml. Each flask was inoculated with 1 ml spore suspension. The flasks were incubated for 24 h at 30°C in a shaking incubator (180 rpm). The produced culture was used as 10 % inocula for second step of cultivation. Fermentation was performed in 500-Erlenmeyer flasks, each containing 250 ml modified Jackson's medium. Flasks were incubated for seven days at 30°C in a shaking incubator (180 rpm). The fermented whole broth was filtered through cheesecloth to separate into supernatant and mycelia. The former was used in the following step.

Preparation of fungal extracts

Fifteen liters of the fungal culture filtrate were subjected to extraction with ethyl acetate, three times and solvent layer was separated. Collected solvent extract was evaporated under vacuum using rotary evaporator (40°C) to dryness and then weighed.

Separation of active gradients (secondary metabolites) from fungal extract

Ten grams of the *E. nidulans* EGCU 312crude ethyl acetate extract were fractionated over a Vacuum Liquid Chromatographic Column (VLC, 15 x10 cm, i.d packed with VLC silica gel H (100 g). Gradient elution was carried out with hexane, chloroform and their mixture with an increased polarity pattern with ethyl acetate (100% hexane to 100% chloroform and finally 100% ethyl acetate)). Fractions (200 ml of each) were collected (as shown in Table 1). The biological activities of each fraction were processed as antioxidant, anticancer and antimicrobial.

Antioxidant activity

DPPH method

The 2,2 diphenyl-1-picrylhydrazyl (DPPH) test was carried out for the 21 fractions as described by Burits and Bucar (2000). 1 ml of fungal extract/fractions (100 and 200 µg/ml) was mixed with 1 ml DPPH reagent (0.002% (w/v) /methanol solution). After an incubation in the dark at room temperature for 30 min, the absorbance was measured at 517 nm (using Jenway 6130 spectrophotometer). Butylated hydroxyl toluene (100 and 200 µg/ml) was used as positive control. This test was carried out in triplicate and the antioxidant activity was calculated as follows:

Activity (%) = A_c-A_t / A_c x 100

Where, A_t is the absorbance of samples and A_c the absorbance of methanolic DPPH solution.

Anticancer activity (viability test)

Induction of tumor cell line

Female Swiss albino mice (kept under environmental and nutritional conditions for two weeks) were injected intraperitoneal (i.p) by Ehrlich

ascites carcinoma cells (EACC), for preparation of tumor cell line. EACC resistant to endoxan were used. The parent line was first supplied, courtesy of Dr. G. Klein, Amsterdam, Holland. The tumor line was maintained in the National Cancer Institute, Egypt in Female Swiss Albino mice by weekly transplantation of 2.5 x 10^6 cells which were centrifuged at 1000 xg for 5 min at 4°C. The pellet was washed with saline (0.9% NaCl), then the needed number of cells was prepared by suspending the cells in the appropriate volume of saline.

The viability percentage of tumor cells was measured by the modified cytotoxic trypan blue exclusion technique (Bennet and Catovsky, 1976). The culture medium used was prepared using RPMI medium, 10% fetal bovine serum and 10% l-glutamine. Trypan blue (0.4%) was prepared then kept in brown closed glass bottle. The viability percentage (V %) of tumor cells was measured after incubation with the tested fungal extracts as well as DMSO as control. 2 ml of cells (4x10^6 cells) were transferred into a set of tubes, then different fungal extract/fraction (100 and 200 µg/ml) were added into the tubes as well as DMSO. The tubes were incubated at 37°C for 2 h. Then, in a test tube containing 80 µl saline and 10 µl trypan blue, 10 µl of cell suspension were added and mixed then the number of living cells was calculated using a hemocytometer.

The mechanisms of tested fungal extracts as anticancer were subjected to the promising fractions only, that is, the fractions which showed high cytotoxicity to EACC (Fractions 1, 2, 4, 9, 12 and 20). Each of these six fractions was evaluated as membrane destructors for cancer cells, that is, release of lactic acid dehydrogenase (LDH) out of cells. In addition, the action of fungal extracts (6 promising fractions) as apoptosis compounds against cancer cells was tested using caspase activity assay.

LDH activity

In EACC samples, cells were counted microscopically and the lactic dehydrogenase (LDH; EC 1.1.1.27) activity was determined in the ascites solution by method of Kaplan and Pesce (1996).

Caspase activity assay

After centrifugation of the treated cells as previously described, caspase-3 enzymatic activity was determined in treated and untreated cancer cells using Caspase Apoptosis Assay Kit (Cat. #-786-200/50) (Geno Technology Inc. St. Louis MO, USA). Prior to use, caspase kit reagents were first prepared, followed by lysis of the treated cells according to a modification of the manufacturer's protocol. In this study, cells were lysed with a sonicator (Misonix, Farmingdale, NY, USA), and caspase-3 enzymatic activity in the lysates was determined as described. Briefly, microtiter wells were set up in duplicates for controls, blank, and test cells (lysates). Then 50 pL of 2 x Caspase assay buffer were transferred into each well followed by addition of 50 ~tL of the cell lysate to the wells, and addition of 5 pL of the caspase substrate, Ac-DEV-AFC. A few minutes were allowed for reaction, and the plate was read (at a zero initial time) on ELIZA micro-plate reader (NX 1001 multi-font) at 405 nm. The plate was then incubated at 37°C for 2.5 h and the absorbance read again at 405 nm wavelength. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the color reaction. Therefore, to quantify the enzyme in the lysates, the fold increase in caspase-3 protease activity was determined by comparing the absorbance from the treated samples with the non-treated controls. To further confirm, compare and establish non-specific protease activity, control experiments were repeated and run with or without caspase-3 specific inhibitor, ZVAD- FMK. Briefly, reaction wells of the MTP were prepared to contain the following: a) 5 pL lysate + 50 }.tL of 2 x assay buffer + 1 btL of Z-VAD-FMK + 5 gL Ac-DEVAFC conjugate; b) 50 pL of 2 x assay buffer + 5 btL Ac- DEV-AFC +1 pL distilled water; and c) 5 btL cell lysate + 50 btL of 2 x assay buffer + 1pL distilled water. The plate was incubated at 37°C for 2.5 h and the absorbance read at 405 nm as described above. The inter-treatment data were compared to ascertain and confirm the effect of ZVAD- FMK on caspase-3 enzymatic activity.

Antimicrobial activity

Each of 21 fractions previously mentioned was tested as antibacterial and antifungal agents as follows:

Antibacterial assays

The method of Jiang et al. (2005) was adopted with some modifications. One loopful of fresh bacteria (*Staphylococcus aureus* ATCC6538 or *Escherichia coli* ATCC8739) was suspended in an appropriate amount of sterilized saline solution, forming a bacterial cell suspension. The viable cell number in the suspension was controlled *via* the turbidity comparison method. This suspension was diluted to a prescribed cell concentration with sterilized distilled saline solution, thus preparing a bacterial cell suspension that was directly used for the antibacterial tests for the fractionated extract. In a 96-well microtiterplate, 150 µl of bacterial cell suspension were added per well.

The fractions (dissolved in 1% DMSO) were tested at a final concentration of 50 µg/ml. The negative control system contained 1% DMSO instead of the fraction tested. Streptomycin (at a final concentration of 50 µg/ml) was tested as positive control. The microtiter plate was incubated for 2 h at 30°C. 100 µl of the suspension was pipetted and quickly mixed with sterilized saline solution. The viable cell number in each of the tested fraction/-bacterial suspension systems at the contact time was determined by conventional spread-plate method. Replicates were made and colonies were counted after 24 h of incubation at 37°C on nutrient agar medium (0.5% peptone, 0.3% beef extract, 0.5% NaCl, 1.5% agar). The percentage of growth inhibition was counted as follows:

$$\text{Inhibition (\%)} = \frac{\text{Negative control} - \text{Treatment}}{\text{Negative control}} \times 100$$

The minimum bactericidal concentration (MBC) of fraction no. 12 or streptomycin against *S. aureus* or *E. coli* was determined. The above mentioned antibacterial assay was followed. The MBC values were determined as the lowest concentration that inhibited colony formation.

Antifungal assays

Spore suspensions of *Aspergillus fumigatus* were obtained from their respective 7-day-old PDA slants in sterilized solution containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2 x 10^6 spores/ml. The spore germination assay was conducted. 50 µl of spore suspension were transferred to each well of amicrotiter plate containing 100 µl liquid Czapek-Dox medium with fraction to yield a final concentration of 100 µg/ml.

DMSO (1%) and amphotericin B replaced tested fractions were used as negative and positive controls, respectively. The plate was incubated at 30°C for 16 h. All tests were conducted in replicates. Spores were considered to be germinated when the germ tube extended to at least twice the length of the spore itself (Griffin,

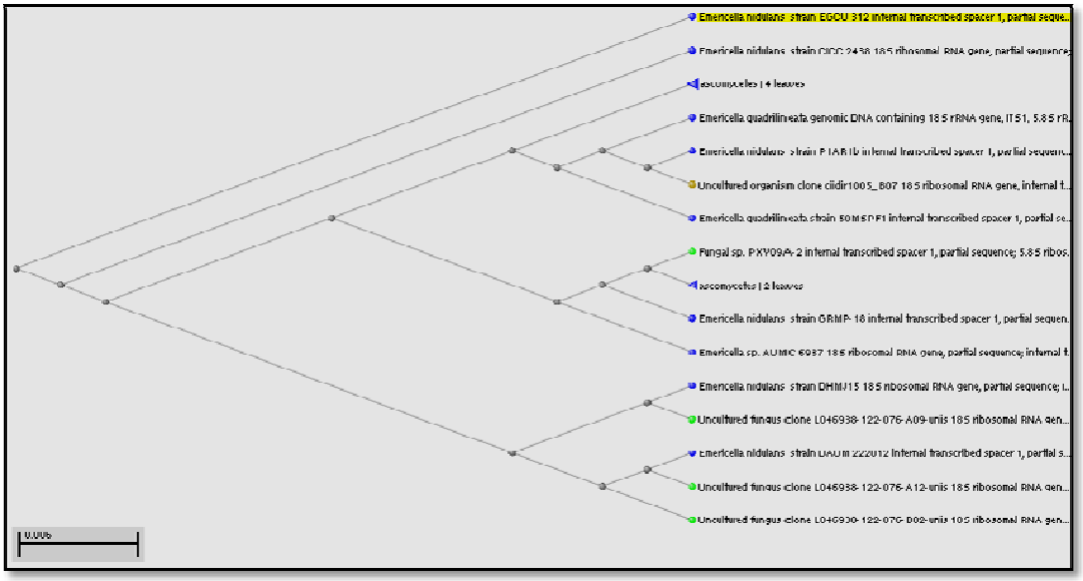

Figure 1. Phylogenetic tree showing genetic relationship between the isolate *E. nidulans* EGCU 312 (in highlight) and other closely related reference microorganisms.

1994). Germinated spores were counted using a hemocytometer. About 100 spores per replicate were observe d to detect s pore germination. The percentage of inhibition of spore germination was counted as mention ed before. This antifungal assay was then used to find the minimum inhibitory concentration (MIC) of fraction no. 12 oramphotericin B a gainst *A. fumigatus*. The minimum concentration that inhibited spore germination was considered as the MIC value.

Inocula of *Candida parapsilosis* were prepare d from cultures on malt agar slants (3% malt, 1.5% agar) incubated at 37°C for 16-18 h. The yeast cells were washed in sterile salin e, centrifuged and resuspended in saline. The number of blastospores/ml of suspension was determined by h emocytometer. A suitable volume of suspension was seeded into 250 ml Erlenmeyer flasks containing 100 ml molten malt agar medium and poured in sterilized P etri plates. A sterilized cork borer (6 mm diameter) was used to punch wells in solidified medium and filled with extracts of 40 µl of 100 µg/ml final concentration of fractions. DMSO (1%) was used as negative control. The efficacy of extracts against *C. parapsilosis* was compared with the antibiotic amphotericin B (positive control). The plates were incubated at 37°C for 24 h. Replicates were assayed for each sample. The anticandidal activity was interpreted from the size of diameter of inhibition zone measured to the nearest millimetre (mm) as observed from the clear zones surrounding the wells.

GC-MS analysis

Fro m the six promising fractions, fraction no. 12 which was obtained by successive extraction (90% chlo roform and 10% ethyl acetate) of eth yl acetate crude fungal extract of *E. nidulans* EGCU 312 gave the highest anticancer and antioxidant activities at the two concentration test. In addition, the same fraction showed very strong antimicrobial activity. Therefore, the identification of the active principles of this fraction was taken into consideration using GC-MS analysis. GC/MS analysis was performed on a Thermoquest -Finnigan trace GC-MS equipped with a DB-5 (5% (w/v) phenyl) methylpolysiloxane column (60 m \ 0.25 mm i.d., film thickness 0.25 µm). The injection temperature was 220°C and the oven temperature wa s raised from 40°C (3 min hold) to 250°C at a rate of 5°C/min, then held at 2 50°C for 2 min; transfer line tempe rature was 250°C. Exactly 1 µ l of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned ove r the 40 to 500 m/z range with an ionizing voltage of 70 eV and identification was based on standard mass library of National Institute of Standards and Techn ology (NIST Version 2.0) to detect the possible extract components.

RESULTS AND DISCUSSION

Isolation and identification of fungal isolate

In th e present study, five different fungal isolates were obtained. Purification and iden tification was performed for fungal isolate no. 1. It was identified as *E. nidulans* through morphological and microscopic examinations. Identification was further confirmed by molecular technique. ITS re gion is the mo st widely sequenced DNA region in fungi. It is most useful for molecular systematics at the species level, and even within species (Meenupriya and Thangaraj, 2011).

In this study, DN A was isola ted from isolate no. 1 and the ITS region of 5.8SrRNA was amplified using the specific primer ITS5. The sequence was determined using automated sequencers. Amplifi-cation and sequencing of fungal rRNA gene resulted in 381 bp long nucleotide sequence. NCBI Blast search sequence similarity was conducted against the existing non-redundant nucleotide sequence database. This showed maxi mum identity (88%) with *E. nidulans* species. The phylogenetic tree was constructed (Figure 1) to show sequence alignment with available sequences from NCBI

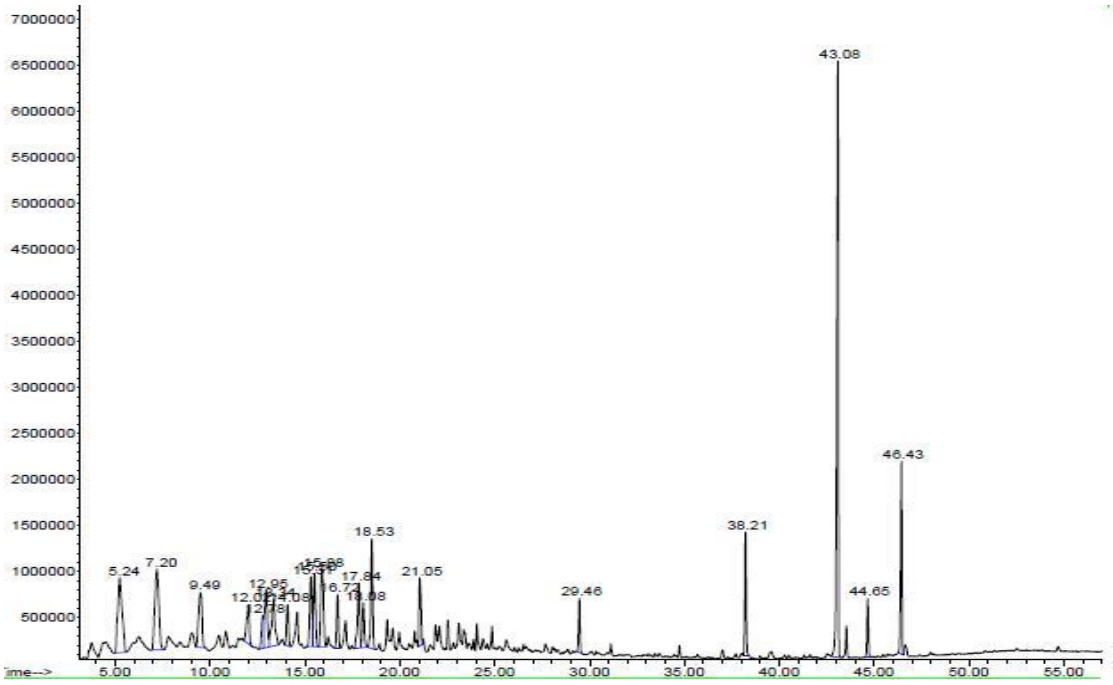

Figure 2. GC/MS chromatogram of fraction no. 12 of ethyl acetate extract from *E. nidulans* EGCU 312.

data bank (first 15 hits in Blast results). The 381 bp long nucleotide sequence from this work was deposited in NCBI GenBank and was given a strain identifier, *E. nidulans* EGCU 312, with accession number: KC511056.

The ethyl acetate extract of *E. nidulans* EGCU 312 was fractionated on silica gel column. Twenty-one fractions (Fr.1-21) were obtained (Table 1). All fractions were tested for their antioxidant, anticancer and antimicrobial activities.

GC-MS analysis

GC-MS profile of fraction no.12 is shown in Figure 2 and its retention time (RT) and percentage of peak of individual compounds are presented in Table 2. Fraction no. 12 showed eight major compounds, that is, tributylacetylcitrate (22.95%), hexadecane (7.87%), pentadecane (7.81%), 1, 2-benzene dicarboxylic acid, diisoctyl ester (6.43%), 2-methyl benzyl amine, N-heptyl-N- octyl (5.23%), benzene, (1-methyl undecyl) (4.81%), heptadecane (4.56%) and 1, 2-benzene dicarboxylic acid, buty (4.05%). These compounds could be responsible for different biological activities.

Tributylacetylcitrate was detected in *Casimiroa edulis* leaf extract and showed insecticidal activity against *Spodoptera littoralis* larvae (Barakat, 2011). Also, 1,2-benzene dicarboxylic was reported to possess anti-inflammatory (Li et al., 2004) and antibacterial activity (Modupe et al., 2010). Moreover, Senthilkumar et al. (2011) found that 1,2-dicarboxylic acid anddiisooctyl ester along with other compounds would have suppressed the growth of *F. oxysporum*.

Antioxidant activity

The DPPH scavenging assay was performed to test the percentage of antioxidant activity of the twenty-one separated fractions of *E. nidulans* EGCU 312 (Table 3). The separated fraction no. 12 showed the highest antioxidant activity with 60.47 and 81.54% at 100 and 200 µg/ml and followed by fractions no. 1, 2, 4 and 20 (with activity ranged 77.0, 64.30, 76.80 and 79.41% at 200 ug/ml, respectively) when compared with butylated hydroxyl anisole as standard antioxidant (88.75% at 200 ug/ml).

Twenty-one (21) compounds were identified in the promising fraction (fraction no. 12) of *E. nidulans* EGCU by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 2. The spectrum of the unknown components of fraction no. 12 was compared with the spectra of known components stored in the NIST library. Six compounds with their biological activities were found in *E. nidulans* EGCU (Figure 2 and Table 2). Moreover, two from these compounds (benzenedicarboxylic acid, butyl octyl ester and 1,2-benzenedicarboxylic acid, diisooctyl ester, with concentration 4.05 and 6.43%, respectively) had high antioxidant activity as reported by Senthilkumar et al. (2011) and Shanab et al. (2010, 2011).

Table 2. List of major components and their biological activities of promising fraction (12) obtained from *E. nidulans* EGCU 312 through GC-MS study.

RT	Compound name	Relative concentration	Reported activities
5.0-12.02	Alkane derivatives	22.83	No activity
12.78	Benzene, (1-pentylhexyl)-	1.72	
12.95	Benzene, (1-butylheptyl)	3.77	
13.34	Benzene, (1-propyloctyl)-	3.77	No activity
14.08	Benzene, (1-ethylnonyl)-	2.01	
15.31	Benzene, (1-pentylheptyl)-	3.79	
15.50	Benzene, (1-butyloctyl)-	3.69	
15.88	2-Methylbenzylamine, N-heptyl-N-octyl	5.23	Antimicrobial
16.72	Benzene, (1-ethyldecyl)-	2.36	
17.83	Benzene, (1-pentylheptyl)-	3.54	No activity
18.08	Benzene, (1-butylnonyl)-	2.12	
18.53	Benzene, (1-methylundecyl)-	4.81	
21.05	Naphthalene, 2,3,6-trimethyl-	3.12	Antimicrobial, Anticancer
29.47	Octadecanoic acid, ethyl ester	1.88	Antioxidant, Anticancer
38.22	1,2-Benzenedicarboxylic acid, butyl octyl ester	4.05	Antimicrobial, antifouling
43.09	Tributyl acetylcitrate	22.95	Antimicrobial activity
44.65	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	1.93	No activity
46.43	1,2-Benzenedicarboxylic acid, diisooctyl ester	6.43	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenase-inhibitor and pesticide

Anticancer activity

Viability test assay was used to assess the anticancer activity of the 21 derived fractions against EACC cell line. Fractions no. 1, 2, 4, 9, 12 and 20 showed acceptable potency against EACC cell line with high anticancer activity ranging 77.0, 64.3, 84.1, 87.7, 77.5 and 76.7% at 200 µg/ml, respectively (Table 3).

From the identified chemical constituents of fraction no. 12 as a promising fraction in most biological activities, three compounds were known to have anticancer activity namely, naphthalene, 2, 3 6-trimethyl (3.12%); Octadecanoic acid, ethyl ester (1.88%) and 1,2-Benzenedicarboxylic acid, diisooctyl ester (6.43%). These results were in agreement with the results obtained by Senthilkumar et al. (2011) and Shanab et al. (2010, 2011).

LDH activity

The cytotoxic effect of fungal fractions was tested *via* the lactate dehydrogenase (LDH) release assay, based on the extent of LDH leakage into the medium. The augmented release of LDH into the media is reflective of cell membrane damage. Therefore, we conducted this experiment in order to estimate the release of LDH after treatment with various concentrations of fungal extract/fraction. As expected, fungal promising fractions (1, 2, 4, 9, 12 and 20) caused cytotoxicity in a dose dependent manner. Fraction no. 12 gave the highest effect by a 2249.2 U/L increase in LDH-release when compared with control cells (1127.7 U/L) as shown in Table 4.

Caspase-3 activity

Caspase-3 is an effector caspase that plays a central role in the mitochondrial-mediated cell death pathway and is responsible for the breakdown of several cellular components involved in DNA and its repair and regulation. The caspase-3 activityin cell line was measured after 24 h of incubation with the six promising fractions (Table 4). The obtained data revealed that, the highest activity of caspase enzyme was obtained by fractions no. 2 and 12 (1.92 and 1.56 relative to control 1.0, respectively).

The results revealed that these compounds enhance cancer cell damage and death. Therefore, from the results of LDH and caspase-3 we can suggest that the active compounds separated from the isolated fungus have anticancer activity by damaging the cancer cells by programmed cell death (apoptosis).

Table 3. Antioxidant and anticancer activity (%) of different fractions from *E. nidulans* EGCU 312.

Fractions no.	Conc. (µg/ml)	Antioxidant activity (%)	Anticancer activity %	
1	100	50.56 ± 2.30	39.5 ± 1.85	
	200	77.0 ± 3.45	88.5 ± 6.45	
2	100	45.97 ± 2.0	50.3± 2.4	
	200	64.30 ± 4.65	81.5 ± 0.89	
3	100	15.64 ± 0.5	41.6 ± 2.15	
	200	24.61 ± 1.50	75.9 ± 1.78	
4	100	51.62 ± 1.89	60 ± 5.12	
	200	76.80 ± 3.65	84.1 ± 3.65	
5	100	52.64 ± 5.42	27.65± 0.15	
	200	54.67 ± 2.10	55 ± 3.25	
6	100	12.60 ± 0.54	20.3± 2.0	
	200	34.61 ± 2.63	41.65± 4.89	
7	100	27.0 ± 1.45	31.65± 1.78	
	200	29.01 ± 2.0	48.6± 3.6	
8	100	24.65 ± 1.62	25.8± 2.5	
	200	44.0 ± 1.78	52.5± 3.9	
9	100	35.80 ± 2.15	64.2± 2.5	
	200	55.74 ± 2.68	87.7± 3.9	
10	100	34.62 ± 1.54	32.2 ± 1.87	
	200	59.60 ± 2.69	58.6± 2.8	
11	100	23.4 ± 1.54	27.6± 0.8	
	200	45.72 ± 2.45	57.9± 2.8	
12	100	60.47 ± 2.63	48.9 ± 1.74	
	200	81.54 ± 4.58	77.56 ± 2.6	
13	100	24.60 ± 1.20	13.6± 0.8	
	200	39.87 ± 1.47	66.2± 2.0	
14	100	12.7 ± 1.02	16.98 ± 1.7	
	200	28.64 ± 2.8	62.5± 3.6	
15	100	27.64 ± 1.25	14.52 ± 2.4	
	200	45.67 ± 2.47	48.6± 3.7	
16	100	19.0 ± 0.65	28.3± 2.0	
	200	27.89 ± 1.45	57.6 ± 1.45	
17	100	45.65 ± 2.98	36.5± 3.6	
	200	55.87 ± 3.65	55.9± 2.5	
18	100	34.61 ± 1.45	22.4 ± 1.65	
	200	59.87 ± 3.67	58.7± 3.8	
19	100	22.16 ± 1.48	10.32 ± 1.6	
	200	37.89 ± 2.78	55.65 ± 4.9	
20	100	53.97 ± 1.52	21.65 ± 1.2	
	200	79.41 ± 6.98	76.7± 3.7	
21	100	33.02 ± 4.3	33.5± 2.0	
	200	56.65 ± 2.45	62.2± 3.8	

Data are mean ± standard error.

Antimicrobial activity

Antimicrobial activities of the 21 fractions are shown in Table 5. The Gram positive bacterium *S. aureus* ATCC6538, the Gram negative bacterium *E. coli* ATCC8739, the human pathogenic fungus *A. fumigatus*

Table 4. Lactate dehydrogenase and caspase-3 enzymes activities as U/L in treated (with promising fractions) and untreated cells.

Fraction no.	LDH (U/L)	Caspase-3 (U/L)
Control (untreated cells)	1127.7 ± 20.6	0.27 ± 0.05
1	1161.7 ± 12.5	0.38 ± 0.02
2	1103.9 ± 10.6	0.52 ± 0.07
4	1096.7 ± 32.5	0.25 ± 0.0
9	816.9 ± 12.8	0.26 ± 0.0
12	2249.2 ± 23.0	0.42 ± 0.03
20	889.3 ± 21.4	0.28 ± 0.01

Data are mean ± standard error

Table 5. Bioassay monitoring of antimicrobial metabolite production by *E. nidulans* EGCU 312.

Fraction no.	Test organism			
	S. aureus **(% inhibition)**	***E. coli*** **(% inhibition)**	***A. fumigatus*** **(% inhibition)**	***C. parapsilosis*** **(inhibition zone, mm) (well diameter, 8 mm)**
-ve Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+ve Control	100±0	100±0	100±0	27 ± 1.02
1	100±0	6 ± 1.30	0 ± 0	21 ± 1.34
2	10 ± 1.14	0 ± 0	26 ± 1.77	19 ± 1.23
3	93 ± 2.32	0 ± 0	31 ± 2.17	16 ± 1.58
4	0 ± 0	0 ± 0	90 ± 3.01	18 ± 1.31
5	8 ± 1.22	8 ± 1.91	87 ± 2.14	0 ± 0
6	95 ± 2.18	60 ± 1.99	14 ± 2.33	22 ± 1.68
7	100±0	0 ± 0	52 ± 1.91	17 ± 1.45
8	75 ± 1.43	0 ± 0	0 ± 0	16 ± 1.73
9	0 ± 0	0 ± 0	100±0	12 ± 1.05
10	60 ± 1.76	47 ± 2.43	0 ± 0	16 ± 1.53
11	100 ±0	52 ± 2.08	100±0	0 ± 0
12	100 ±0	100±0	100±0	0 ± 0
13	100±0	0 ± 0	99 ± 0.58	0 ± 0
14	60 ± 1.68	0 ± 0	30 ± 1.94	0 ± 0
15	80 ± 2.02	15 ± 1.82	0 ± 0	0 ± 0
16	15 ± 1.39	0 ± 0	0 ± 0	17 ± 1.27
17	51 ± 2.17	22 ± 1.62	21 ± 1.53	0 ± 0
18	99 ± 1.00	58 ± 1.57	0 ± 0	0 ± 0
19	100±0	0 ± 0	0 ± 0	0 ± 0
20	98 ± 1.15	65 ± 2.13	0 ± 0	0 ± 0
21	90 ± 2.11	63 ± 2.21	0 ± 0	0 ± 0

Data are mean ± standard error; -ve Control: 1% DMSO. +ve Control: Streptomycin in the case of *S. aureus* and *E. coli*; amphotericin B in the case of *A. fumigatus* and *C. parapsilosis*.

and the human pathogenic yeast fungus *Candida parapsilosis* were used as test organisms. The inhibitory effect of these fractions was compared with standard antibiotics. The isolated fractions showed different degrees of growth suppression of the tested microorganisms. A hypothesized mechanism of antimicrobial activity could be change in cell permeability due to interaction between the fungal secondary metabolites in the tested fractions and the electronegative charges on the cell surfaces. The interaction leads to leakage of intracellular electrolytes and proteinaceous constituents. Another mechanism is interaction of the extracted secondary metabolites with the microbial DNA, leading to inhibition of mRNA and protein synthesis. From the results in Table 5, it appeared that the Gram positive bacterium *S. aureus* was more sensitive to the tested fractions when compared with the Gram negative bacterium *E. coli*. This could be attributed to their markedly different cell wall structure (Feng et al., 2000). The peptidoglycan in the cell walls of the Gram positive *S. aureus* is much thicker than that in *E. coli*, while the lipopolysaccharide (LPS) layer is much thicker in *E. coli.* The LPS layer is thought to provide protection to the cell wall against antibiotics. Concentrations of the tested frac-

Table 6. Antimicrobial activity (MBC or MIC) of fraction no. 12 against pathogenic microorganisms.

Antimicrobial activity	MBC (µg/ml)		MIC (µg/ml)
	S. aureus	*E. coli*	*A. fumigatus*
+ve Control	9	20	70
Fraction no. 12	4	10	45

+ve Control: Streptomycin in the case of *S. aureus* and *E. coli*; amphotericin B in the case of *A. fumigatus.*

tions were doubled in the case of fungi. Generally, fungi were more resistant to the tested fractions than bacteria. The structure of fungi and bacteria differ in very significant ways (such as the diploid nature of most fungi and the longer generation time of fungi as compared to bacteria), and the available antibacterial and antifungal agents target structures and functions most relevant to the organisms to be inhibited (Ghannoum and Rice, 1999). Another difference between bacteria and fungi lies in the cell wall structure, where the fungal cell wall is primarily composed of chitin, glucan, mannans and glycoproteins (Bowman and Free, 2006).

Obviously, fraction no. 12 was the most potent inhibitor. This fraction (50 µg/ml) caused complete inhibition of cell viability of *S. aureus* and *E. coli* (100 %). Interestingly, at 100 µg/ml, it showed 100% inhibition of spore germination of *A. fumigatus*. However, it showed no effect on the growth of *C. parapsilosis*.

The antimicrobial test was performed with different concentrations of fraction no. 12 (from 1 to 50 µg/ml in the case of *S. aureus* and *E. coli*, and from 1 to 100 µg/ml in the case of *A. fumigatus*). The results of MBC and MIC values of fraction no. 12 were very promising (Table 6). The MBC values by this fraction were 4 and 10 µg/ml, as compared to 9 and 20 µg/ml by streptomycin for *S. aureus* and *E. coli*, respectively. While the MIC values were 45 and 70 µg/ml by fraction no. 12 and amphotericin B, respectively, against *A. fumigatus*. It can be speculated that one or more of the active ingredients found in fraction no. 12 might mediate the observed inhibitory activities.

The data in Table 2 reveals that, the current fraction was rich in antimicrobial agents e.g: 2-methyl-benzylamine, N-heptyl-N-octyl (5.23%), naphthalene, 2,3,6-trimethyl- (3.12%), 1,2-benzenedicarboxylic acid, butyl octyl ester (4.05%), tributyl acetyl citrate (22.95 %) and 1,2-benzenedicarboxylic acid, diisooctyl ester (6.43%). The cytotoxicity of these compounds appeared also, by the anticancer effects as previously mentioned.

From the foregoing, it can be concluded that valuable secondary metabolites could be extracted from the soil fungus *E. nidulans* EGCU 312. The applicable importance of such metabolites lies in the fact that they showed distinguishable antioxidant, anticancer and anti-microbial activities.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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