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RESEARCH ARTICLE

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EXPLORING THE ANTIMICROBIAL POTENTIAL OF LOCAL MARINE FUNGI

ABSTRACT:

In search for bioactive compounds, 88 fungal isolates were collected from three Egyptian marine habitats. Crude extracts of 17 marine derived fungal isolates out of the 88 isolates showed variable activity against some human pathogenic bacterial strains (*Salmonella enterica, Proteus sp., Klebseilla pneumonia, Staphylococcus aureus* and *Escherichia coli*). The most potent fungal isolate that showed the highest antimicrobial activity was molecularly identified as *Aspergillus welwetsichiae.* Optimization of culture conditions of *A. welwetsichiae* revealed that the highest antimicrobial activity was obtained in shaking conditions (150rpm), using Czapek- dox medium, at pH 9 and incubation at 25°C for 3 days. *Salmonella enterica* was the most sensitive test organism regarding the highest inhibition zone through all the optimization tests. Ethyl acetate extract of *A. welwetsichiae* was subjected to chemical analysis and declared that the active antibacterial metabolite is a mixture of acid (Mono (2-ethylhexyl) phthalate) and ester (bis (2-ethylhexyl) phthalate). Retesting the two compounds separately, they showed no activity which confirmed the synergistic antibacterial effect of the two compounds together. Upon testing the cytotoxic effect of the active antibacterial metabolite (mixture of acid and ester) against human lung fibroblast normal cell (WI-38 cell line), the results showed that there was a very weak inhibitory activity suggesting the possibility of considering it as a safe compound for further steps as a pharmaceutical product.

KEY WORDS:

Marine derived fungi, Antimicrobial compounds, *Aspergillus*

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INTRODUCTION:

There are two major types of biologically important environments in which the salt factor will interact with microbial populations, soil and water (Mansuma *et al*., 2001). The oceans and seas cover 70% of the earth's surface and possess a wide diversity of natural flora and fauna. Among the 36 known living phyla, 34 are found in marine environments with more than 0.3 million known species of flora and fauna (Faulkner, 2001).

New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral, bacterial and fungal diseases (Nazar *et al.*, 2009). Because of the highly chemical and physical harsh condition in marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities (Ravikumar *et al.*, 2010).

Marine fungi are prolific resources of natural products (Ebel, 2010; Jensen and Fenical, 2002). However, the potential of marine fungi has only been investigated to a limited extent. In the last few decades, marine-derived fungi have been recognized as one of the most recent barely tapped sources for new biologically active secondary metabolites (Jensen and Fenical, 2002) including antitumor, antibacterial, antiviral, antifungal, anti-inflammatory and enzyme inhibitor compounds. This is probably because marine fungi have been explored to a lesser extent than their terrestrial counterparts, which have been known for a long time as a very important source of biologically active and economically important natural products, such as those for use in treatment of human diseases as well as other biotechnological applications (Tan and Zou, 2001; Strobel, 2002).

Aspergillus fungi have received the most of the attention among all the marine-derived fungi, which accounted for 31% of the marine fungal origin. The marine natural products have diverse chemical structures mainly including; polyketides, fatty acids, sterols and terpenoides, 96% of which displayed bioactivities such as cytotoxicity, antimicrobial activity, antioxidant and insecticidal activity (Zhao, 2016).

Bacterial resistance is spreading throughout the world; especially in all health care associated pathogens revealing the steadily decreasing potentiates of prevalent antibiotics (Gould, 2008); thus, necessitating the discovery of novel compounds, modification of already existing antimicrobial stock, be it from fungi, actinomycetes or any natural resources. Because of huge expenditure on synthetic molecule with effective antimicrobial properties, natural products are still a worth promise. Although, more such organisms for their antimicrobial potential to solve the problem of emerging strains of resistant microorganisms (Kaur and Arora, 2015.

The potential of marine fungi to produce a vast array of secondary metabolites that are gaining importance for their biotechnological as well as medical and pharmaceutical applications has attracted our attention. However, the aim of this study is to highlight the occurrence of local marine fungal isolates having the chemical potential of bioactive metabolites with antimicrobial activity.

MATERIAL AND METHODS:

Marine fungi are an ecologically rather than physiologically or taxonomically defined group of microorganisms (Hyde *et al.*, 1998).

Collection of water samples:

Water samples were collected from three different sites in Egypt; Red Sea (Sharm El-Sheikh), Mediterranean Sea (Alexandria) and Bitter lakes (Ismailia). From each location, samples were collected in clean sanitized bottles at a depth of 1.5 meter using LaMotte water sampler (model JT-1 code 1077). These samples were collected (during March and April, 2012), transferred to the laboratory and stored in refrigerator for further analysis.

Isolation of Marine Fungi:

Three different culture media were used for isolation of marine fungi; Sabouraud dextrose yeast extract agar medium (Feng *et al.*, 1990), Czapek- Dox agar medium (Thom and Church, 1926), and Yeast extract medium (Wickerham, 1951).

Isolation media were prepared with sea water and supplemented with chloramphenicol (250 mg/L) as antibacterial agent. The agar plates were inoculated with water samples (0.2 ml) and incubated for three weeks. The agar plates were regularly examined to verify the growing colonies. Distinct fungal colonies on the isolation media were then transferred to new plates for further purification. The single purified colonies were picked up and subcultured on slants prepared from the same contents of the isolation media and maintained for further investigations (Samuel *et al.*, 2011).

Assessment of Antimicrobial Activity of Marine Fungal Isolates:

Preparation of the aqueous crude extract:

Each 50 ml of Czapek-Dox broth medium (pH 6.5) in Erlenmeyer flasks (250 ml) was inoculated with one plug of each fungal isolate and incubated in shaking incubator at 150 rpm, at 28°C for 7 days (Zainuddin *et al.*, 2010). The fungal mycelia were harvested by filtration through Whatman filter paper No. 1, the supernatant was then filtered through two types of Millipore Syringe filters (0.45 µm) and *(*0.22 µm) to get cell free extract (Manimegalai *et al.*, 2013). The sterilized cell free filtrate was used for screening of antibacterial and antifungal activities against the tested bacterial and fungal pathogens.

Antimicrobial activity test:

Agar well diffusion method was used to evaluate the antimicrobial activity of the sterilized cell free fungal extracts (Mathan *et al.*, 2013) against some human bacterial and fungal pathogens. The bacterial pathogens; *Salmonella enterica* ATCC 25566*, Proteus sp.* (clinical isolate), *Klebseilla pneumonia* ATCC 10031*, Staphylococcus aureus* ss. *aureus* ATCC 6538 *and Escherichia coli* ATCC 51659 were purchased from Microbiological Resources Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University. While fungal pathogens; *Aspergillus flavus* (kf 028197)*, Aspergillus niger* (kf 358715) and *Aspergillus fumigatus* (kf 201647) were kindly

supplied by personal contact. *Candida albicans* was obtained from culture collection, Department of Microbiology, Faculty of science, Ain-shams University.

The sterilized fungal aqueous extracts (200 µl) were loaded in the wells punched in agar plates seeded with different dilutions of spore suspension (concentration equivalent to standard McFarland 0.5) of the different tested pathogens. For bacterial pathogens, nutrient agar plates (APHA, 1917) were seeded with spore suspension (10^{8}) and incubated at 37°C for 24hrs. Czapek-Dox agar plates were seeded with spore suspension of (10^{4}) of the fungal pathogens and incubated for 7 days. Malt extract agar plates were seeded with spore suspension of yeast pathogens (10^{6}) and incubated for 48 hrs. Fungal and yeast plates were incubated at 28°C.The mean diameter of inhibition zones were measured in millimeters and recorded (Powthong *et al.,* 2012).

Identification of the active marine fungal isolates:

Identification was determined based on their macroscopic and microscopic morphological characteristics using the universal manual **(**De Hoog *et al*., 2000). The identification of the most potent isolate was confirmed using the molecular techniques.

Molecular identification of the most potent isolate:

Molecular identification was carried out in Sigma Company. DNA extraction was made by using protocol of Gene Plant genomic DNA purification kit (Thermo) # K0791. Then PCR was made by using Maxima Hot Start PCR Master Mix (Thermo) # K0221: Forward primer ITS1: (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse ITS 4: (5'- TCC TCC GCT TAT TGA TAT GC-3').

Initial denaturation at 95°C for 10 minutes. Denaturation 95°C for 30 seconds. Annealing at 55°C for 1 minute. Extension at 72°C for 1 minute. Final extension at 72°C for 15 minute. Number of cycles, 35. The PCR was clean up to the product using Gene JET™ PCR purification Kit (Thermo) # K0701. Finally sequencing to the PCR product was made in GATC Company using forward and reverse primers, ABI 3730xl DNA Sequencer.

Extraction and elucidation of the structure of the antimicrobial metabolite from the most potent marine fungal isolate:

Extraction of antimicrobial metabolite (Atalla *et al.,* 2008):

Czapek- dox broth medium (100 ml) was inoculated with plugs of the most potent marine fungal isolate (No. 9) and incubated in a shaking incubator (150 rpm) at 25°C, for 7 days. After incubation days, fungal mycelium was separated from culture broth by filtration through Whatman No.1 filter paper. For extraction of antimicrobial metabolites, the filtrate was treated with equal volumes of four different organic solvents namely hexane, chloroform, dichloromethane and ethyl acetate. Then, shaking the mixture vigorously for 10 minutes in a separating funnel. The miscible mixture was allowed to stand for 5 minutes and the solvent phase containing the fungal antimicrobial metabolites was collected in a flask and concentrated in a rotator evaporator at 30°C. The obtained residue was dissolved in 2% dimethy sulfoxide (DMSO) and stored at 4°C. Assessment of antimicrobial activity was carried out using agar well diffusion method. In nutrient agar plates with 0.5 McFarland suspension of *Salmonella enterica*, a volume of 200 µl of concentrated fungal extract dissolved in 2% DMSO was loaded in wells punched in agar plates and incubated at 37°C for 24 hrs. Similarly, 200 µl of 2% DMSO was used as a negative control and Chloramphenicol (10 µg) was used as positive control.

Extraction and chemical characterization of the antimicrobial metabolite from the most potent marine fungal isolate:

Purification of the antimicrobial metabolite:

The active components in the ethyl acetate crude extract were separated using silica gel thin layer chromatography precoated aluminum sheets $60F_{254}$ (Merck). Two mobile phases were used for extraction; ethyl acetate: chloroform (1:1) (v/v) and diethyl ether: petroleum ether (1:1) (v/v). The crude extract was spotted at 1.5cm from the bottom of the sheets and allowed to dry, then developed in an ascending order for an hour. The produced spots were located by their fluorescence on chromatograms under U.V light and R_f (retardation factor) values were determined (Atalla *et al.*, 2011).

Elucidation of structure of the antimicrobial active metabolite via spectroscopic analysis:

It is difficult to access the original compound of interest present in natural products which is usually a complex mixture of several compounds. Three important techniques were undertaken to reach the structure of antimicrobial metabolite: GC- MS, IR and NMR spectroscopy.

- Fourier Transform Infrared Resonance spectroscopy (FTIR):

The infrared spectra were recorded using potassium bromide disks on FTIR Thermo Electron Nicolet 7600 (USA) infrared spectrometer at the Central laboratory of Faculty of science, Ain shams university.

- Gas chromatography–mass spectrometry (GC-MS)

The mass spectra were recorded on Aglient Technologies GC-MS 5977A mass spectrometer operating at 70 ev at the Central laboratory of Faculty of science, Ain shams

- Nuclear Magnetic Resonance spectroscopy (NMR):

The ^{1}H-NMR spectra were measured on Varian Gemini 400 MHz spectrometer, with chemical shift (δ) expressed in ppm downfield with tetramethylsilane (TMS) as internal standard, in DMSO-d6 and coupling constants J in Hz. at the main chemical warfare laboratories.

Evaluation of cytotoxicity against WI-38 cell line:

The purified antimicrobial compound was evaluated for cytotoxicity against WI-38 cell line (human Lung Fibroblast normal cell). The cell line was obtained from VACSERA Tissue Culture Unit. Test was carried out at the Regional Center for Mycology & Biotechnology, Al-Azhar University.

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

The cells were seeded in 96-well plate at a cell concentration of 1 × 10^4 cells per well. Tested extracts dissolved in DMSO were added to the wells in triplicates with concentrations of 0, 15.60, 31.25, 62.50, 12.50, 250, and 500 µg/mL for 48 hrs. The cytotoxic activity was determined using viability assay. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)] x 100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve after treatment with the specified compound (Mosmann, 1983; Gomha *et al.,* 2015).

Statistical Results:

Statistical analysis of experimental data was performed using IBM SPSS 2011; to detect the optimum conditions for maximum antibacterial activity of the most potent marine- derived fungal isolate. The probability of error (P value) at 0.05 or less was considered significant, while at 0.01 and 0.001 highly significant.

RESULTS AND DISCUSSION:

Isolation of marine fungi:

In this study, eighty-eight marine fungal isolates were collected from the three different isolation sites in Egypt; 42 isolate (48%) from Red Sea, 32 isolate (36%) from Bitter lakes and 14 isolate (16%) from Mediterranean Sea. The distribution of marine fungal isolates collected from different sites in Egypt.

Data of this study revealed that the yield of the collected samples was higher from Red sea than other sites. Nadeem *et al.* (2015) reported that, Red sea was recognized as a rich source of microbial diversity with unique metabolites with pharmaceutical and medicinal importance.

Assessment of antimicrobial activity of the isolates:

All marine- derived fungal isolates in this study were subjected to antimicrobial activity test against a group of pathogenic bacteria, mold and yeast. As shown in table 1, the crude extracts of only seventeen (19%) marine- derived fungal isolates showed activity against the tested bacterial pathogens. This data coincides with other literatures (Zhang *et al.*, 2012 & 2013; Qin *et al.*, 2015), where it was reported that 38% – 59% of the test extracts from marine fungi exhibited antibacterial or antifungal activities. Also, Suay *et al.* (2000) reported that about 70% of fungal strains were active against bacteria.

Table 1. Assessment of antibacterial activity of the active marine fungal isolates (17 isolate).

	Diameter of inhibition zones in mm				
Isolate number	*Escherichia coli*	*Klebseilla pneumonia*	*Staphylococcus aureus* ss. *aureus*	*Salmonella enterica*	*Proteus sp.*
1	-	18	-	25	-
2				20	19
3	-	17	-	-	17
4	-	-	-	30	
5	-	-	-	-	17
6	-	25	-	-	-
7	-	-	-	25	22
8	-	-	-	25	18
9	19	-	-	24	19
10	13	-	-	20	18
11	-	-	-	21	18
12	-	30	-	-	-
13	-	27	-	-	-
14	-	24	-	20	-
15	-	-	-	30	25
16	14	-	-	22	17
17	-	-	18	21	-

Isolates number 9, 10 from (Red sea) and No. 16 from (Bitter lakes) showed activity against most of the tested bacterial pathogens, where the diameter of inhibition zones ranged from 13mm in case of *E. coli* to 24 mm in case of *Salmonella enterica*. Accordingly, isolate No. 9 had been selected for further investigations as being the most potent isolate with the highest antibacterial activity (inhibition zones ranged from 19 mm to 24 mm).

In this study, antibacterial activity was found to be more effective towards Gram-negative bacteria than Gram-positive bacteria.

These results were different from that of Christophersen *et al.* (1999), Höller *et al.* (2000), and Suay *et al.* (2000) where they reported that antibacterial activity is more common towards Gram-positive bacteria than Gram-negative bacteria. These differences in susceptibility towards antibiotically active secondary metabolic extracts have been repeatedly attributed to differences in cell wall structure of Gram-positive bacteria compared to Gram-negative bacteria. The cell walls of Gram-positive bacteria are less complex and lack the natural sieve effect against large molecules (Hawkey, 1998), whereas the outer membrane and the periplasmic space that is present in Gram-negative bacteria is thought to provide an additional degree of protection against antibiotics targeting the cell wall (Basile *et al.*, 1998). By that fact, in this study, the marine- derived fungal isolates from those regions in Egypt are of great importance since they are more active against Gram-negative bacteria.

Identification of the active marine fungal isolates:

The seventeen marine-derived fungal isolates which gave variable activity against the tested bacterial pathogens were identified traditionally by their macroscopic and microscopic characteristics (Table 2). While the identification of the most potent fungal isolate (No. 9) was confirmed using the molecular techniques. In the current study, out of the 17 active isolates 14 (82%) belonged to genus *Aspergillus*, two *Penicillium* species (11%) and one *Cladosporium* species (Table 2). In accordance to our results, Das *et al.* (2009) reported that, *Aspergillus* was the most dominant genus representing 33% followed by *Penicillium* 13% of the marine fungal isolates collected in his study. Xu *et al.* (2015) in their study, reported that dominant genera of the 105 marine fungi with antibacterial and antifungal activity were species of the genus *Aspergillus* (31 strain) and the genus *Penicillium* (16 strain), and considered *Aspergillus* as one of the dominant marine fungi and strains related to this genus producing more new antibacterial and antifungal compounds than any other genus. Also, they reported that, over 700 compounds in total were purified from 105 fungal strains that can produce antimicrobial compounds and were investigated for their activities. There are 285 compounds (approximately 40% of the total) showed antibacterial and antifungal activities and 116 (15% of the total) are new antibacterial and antifungal compounds. *Aspergillus* fungi have received the most of attention among all marine derived fungi, which accounted for 31% of the marine fungal origin (Zhao *et al*., 2016).

As shown in the results of table 2, members of *Aspergillus niger* (group) were represented by 12 (82%) out of 14 marine fungal isolates belonging to *Aspergillus* genus. *Aspergillus niger* as an example of one of the species related to *Aspergilllus niger* (group) is one of the best pharmaceutical friendly organism that produces a lot of industrially important enzymes as well as some other products. Related to antimicrobial activity, it shows high potency in producing antimicrobial compounds such as tensyuic acid, nigerazine B, tensidol A and ochratoxin (Nielsen *et al.*, 2009).

Table 2. List of the active marine fungal isolates and their antibacterial activity

Isolate number	Identification	Isolation site	Active against
1	*Penecillium waksmanii*	Meditteranean Sea	K, Sa
2	*Aspergillus niger* (group)	Meditteranean Sea	Sa, P
3	*Aspergillus niger* (group)	Meditteranean Sea	K, P
4	*Aspergillus flavus* (group)	Meditteranean Sea	Sa
5	*Aspergillus niger* (group)	Meditteranean Sea	P
6	*Aspergillus niger* (group)	Red Sea	K
7	*Aspergillus niger* (group)	Red Sea	Sa, P
8	*Aspergillus niger* (group)	Red Sea	Sa, p
9	*Aspergillus niger* (group)	Red Sea	E, Sa, P
10	*Aspergillus niger* (group)	Red Sea	E, Sa, P
11	*Aspergillus niger* (group)	Red Sea	Sa, P
12	*Aspergillus flavus* (group)	Bitter Lakes	K
13	*Cladosporium sphaerospermum*	Bitter Lakes	K
14	*Aspergillus niger* (group)	Bitter Lakes	K, Sa
15	*Penecillium waksmanii*	Bitter Lakes	Sa, P
16	*Aspergillus niger* (group)	Bitter Lakes	E, Sa, P
17	*Aspergillus niger* (group)	Bitter Lakes	St, Sa

Note: K for *Klebseilla pneumonia*, Sa for *Salmonella enterica*, E for *Escherichia coli*, P for *Proteus sp*. and St for *Staphylococcus aureus* ss. *aureus.*

Molecular identification of the most potent isolate (No.9):

A sequence database search using the BLAST search program analysis of the National Center for Biotechnology Information (NCBI, http://www. ncbi. nlm.nih.gov/BLAST/) against various sequences was used to identify the phylogenetic similarities among the *Aspergillus niger* (group) isolates and the published DNA sequences in GenBank. The BLAST search demonstrated that this isolate was closely related to *Aspergillus welwitschiae* with sequence similarity of more than 98% to the ITS1-5.8S-ITS2 regions of the rRNA genes using ITS1 and ITS4 primers and this was sufficient to indicate that this isolate belongs to the same species (*Aspergillus welwitschiae*) (Figs 1 & 2).

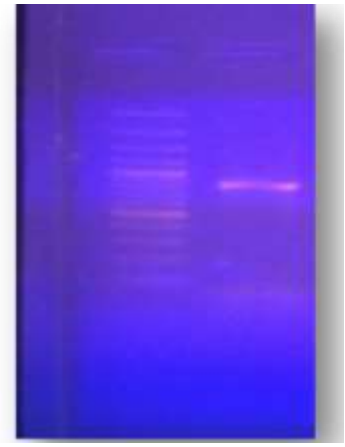

Fig. 1. Amplified PCR product of the most potent isolate

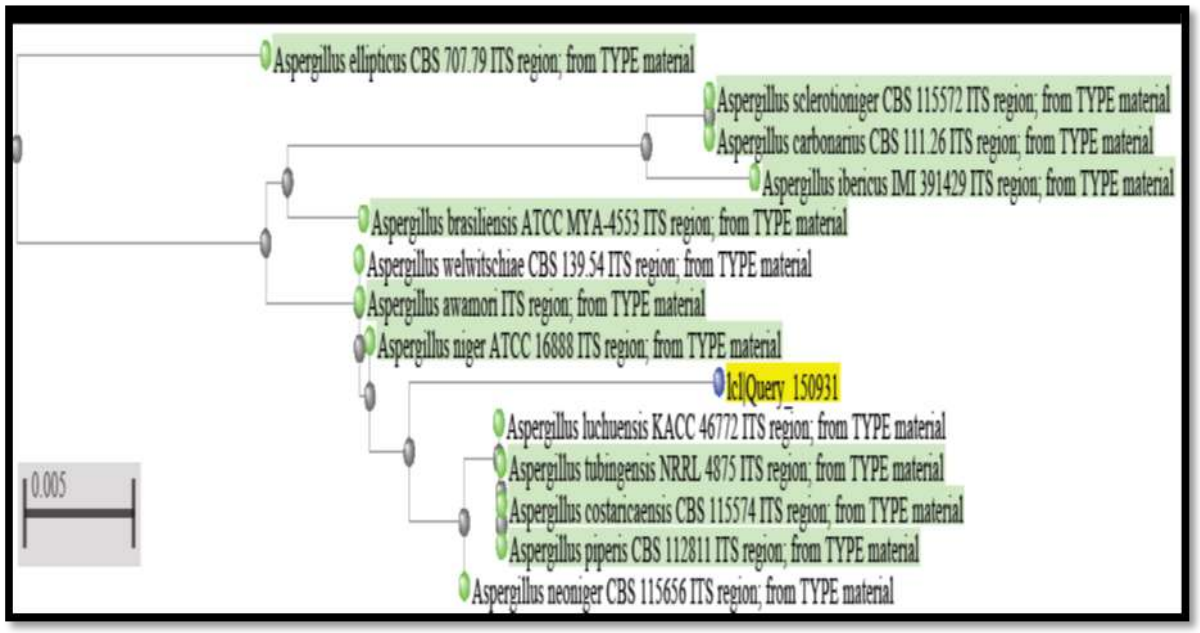

Fig. 2. The phylogenetic tree of *Aspergillus twelwitschiae* (98% similarity).

Optimization of culture conditions of *Aspergillus welwitschiae*:

The yield of bioactive compounds can sometimes be substantially increased by the optimization of physical (temperature, salinity, pH and light) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes (Miao *et al*., 2006; Ritchie *et al*., 2009; Jain and Pundir, 2011; Sudarkodi *et al.,* 2012).

Antimicrobial activity was first tested in both static and shaken conditions (150 rpm). The obtained results indicated that, antimicrobial activity of shaken culture was higher than static one. For this reason, experiments in this study were carried out in shaken condition at 150 rpm. Duplicates of Erlenmeyer flasks (250 ml) containing 50 ml broth media used to determine the physiological and physical conditions that would affect the antibacterial activity of *Aspergillus welwitschiae.* Parameters of culture conditions were changed one at a time while all other cultivation parameters remained unchanged (Bhattacharyya and Jha, 2011; Ramos and Said, 2011). In all tests, antibacterial activity was measured as diameter of inhibition zone in mm.

Effect of medium composition on antibacterial activity of *Aspergillus welwitschiae*:

Selection of a suitable medium for antimicrobial production is an important step; such a medium was a prerequisite for further studies (Shukla *et al.*, 2014). Three different media namely; Czapek-Dox broth, Glucose Peptone Yeast extract broth and Potato Dextrose broth (Murray *et al.,* 2007) were tested. The antimicrobial activity was assessed after 7 days of incubation at 28°C.

In this study, the antibacterial activity of *Aspergillus welwitschiae* was recorded only on Czapek-Dox broth medium and the highest inhibition zone (30 mm) was obtained against *Salmonella enterica* (Table 3). This result indicated the suitability of

Czapek-Dox broth medium in the achievement of the highest antibacterial activity of *A. welwitschiae.* While, other studies reported that potato dextrose broth was the best medium for the growth and enhanced production of secondary metabolites from *Aspergillus terreus* (Mathan *et al.,* 2013). Karen *et al.* (2013) tested the importance of different media types, including six liquid and five solid media on the secondary metabolite production of three fungal strains in their drug-discovery screening process. They found a surprising result that the amount of extract produced in liquid cultures was often so drastically smaller than those produced in solid media cultures. Also, production varied widely between the types of the tested media, but generally, rice appeared to be consistently producing high quantities of the active metabolites.

Effect of temperature on the antibacterial activity of *Aspergillus welwitschiae*:

In this study, three sets of flasks containing Czapek-dox broth (pH 6.5, in shaking condition 150 rpm, for 7 days) were incubated at three different temperatures (25, 30, and 35°C). One-way ANOVA of normal data was carried out and as shown in table 3, there was a significance difference between diameters of inhibition zones by *Aspergillus welwitschiae* against *Salmonella enterica, E. coli* and *Proteus sp.* and incubation temperatures at 25°C, 30°C, and 35°C. By Post- hoc test, it was proved that temperature 25°C was the optimum temperature with 0.006 level of significance (P= 0.006).

Bhattacharyya and Jha (2011) reported that incubation temperature ranging from 25°C to 35°C to be optimum for antimicrobial activity of *Aspergillus* strain. Suja *et al.* (2013) found that incubation temperature (25°C) enhanced the antimicrobial compound production of *Aspergillus terreus*, also Kalyani *et al*. (2016) recorded 30°C as the best incubation temperature for *Aspergillus niger* (MTTC- 961). The recorded results agree with our results.

Effect of pH on the antibacterial activity of *Aspergillus welwitschiae*:

The initial pH of Czapek-Dox broth media was adjusted at three different pH values (pH 5, pH7 and pH 9) using 1N NaOH and 1N HCL. Flasks were incubated under shaking conditions (150rpm) for 7 days at 28°C (Smitha and Rosamma, 2014). One- way ANOVA of normal data was carried out and as shown in table 3, that there was a significance difference between diameters of inhibition zones by *Aspergillus welwitschiae* against *Salmonella enterica* (p = 0.001) and *Proteus sp.* (p = 0.003) and pH values (5, 7, and 9) and by Post-hoc test, it was proved that pH 9 was the optimum pH value against both *Salmonella enterica* and *Proteus sp.* There was no significance difference between diameters of inhibition zones by *Aspergillus welwitschiae* against *E. coli.* (p = 0.270) under different pH value tested.

The pH of a culture medium is usually not constant throughout fermentation and the changes that occur are highly dependent on composition of the medium (Shukla *et al.*, 2014). Thongwai and Kunopakarn (2007) pointed out that most of microorganisms can synthesis antimicrobial compounds at pH ranging from 5.5 to 8.5. Jain and Pundir (2011) reported that maximum bioactive metabolite production in their study was at pH 6.0, also Mathan *et al.* (2013) recorded pH 5.5 was optimum for *Aspergillus terreus*, these studies suggest the acidophilic characteristics of the active isolates. Likewise, optimum pH for the growth and secondary metabolite production of *Fusarium solani* was found to be 6.0 by Merlin *et al.* (2013). On the contrary, in the current study, the highest antibacterial activity was observed when *A. welwitschiae* was grown at pH 9 and inhibition zone reached 32mm against *Salmonella enterica* (Table 3). Inhibition zones were significantly less when the fungus was grown at neutral and acidic pH.

Effect of agitation speed on the antibacterial activity of *Aspergillus welwitschiae*:

In this study, inoculated flasks with Czapek-Dox broth medium were incubated at 28°C for 7 days at three different rpm (100,150, and 200).

For agitation speed; one- way ANOVA of normal data was carried out and as shown in table 3, there was a significance difference between diameters of inhibition zones by *Aspergillus welwitschiae* against *E. coli* (p = 0.029) and *Proteus sp.* (p = 0.045) and different agitation speeds (100, 150, and 200) and by Post- hoc test, it was proved that 150 rpm was the optimum agitation speed against both *E. coli* and *Proteus sp.* No significant difference was observed between diameters of inhibition zones by *Aspergillus welwitschiae* against *Salmonella enterica* (p = 0.100) under different agitation speeds.

It has been reported previously that a strain of *Aspergillus fumigatus* produced bioactive metabolites on growth medium, maize and commercial animal feed with rotary shaker at 150 rpm (Wenehed *et al.*, 2003; Liu *et al.*, 2004). This result also corroborated with those mentioned by Atalla *et al.* (2008) who incubated the marine fungus named *Varicosporina ramulosa* on a rotary shaker at 150 rpm for biologically active compounds production. Different from our results and the other mentioned studies, Kaur and Arora (2015) reported highest antimicrobial activity at 200 rpm and remained same till 250 rpm.

Effect of incubation periods on the antibacterial activity of *Aspergillus welwitschiae*:

Time duration required for growth and metabolite production by different fungi varies significantly. Thus, proper determination of specific incubation requirement is of high importance for maximum harvesting of the metabolite (Alberts, 1990). Inoculated flasks of Czapek-Dox broth medium were incubated at 28°C for different incubation periods (3 days, 5 days, 7 days, 9 days, 11 days, and 13 days) in shaking conditions at 150 rpm. Antibacterial activity was recorded every two days for each set of flasks.

One- way ANOVA of normal data was carried out and as shown in table 3, there was a significant difference between diameters of inhibition zones by *Aspergillus welwitschiae* against *E. coli, Salmonella enterica* and *Proteus sp.* and different incubation periods and by Post- hoc test, it was proved that 3-days incubation period was the optimum incubation period with 0.001 level of significance (P = 0.001).

As shown in table 3, the production of antibacterial metabolite varied significantly with incubation days. Maximum antibacterial activity was recorded after three days of incubation where inhibition zones reached 39 mm, 37mm, and 21 mm against each of *E. coli, Salmonella enterica*, and *Proteus sp.*, respectively. Antibacterial activity decreased gradually by increasing the incubation period and no activity was observed by the 13th day of the incubation period. Kalyani *et al.* (2016) reported maximum antibacterial metabolite production by *Aspergillus niger* when grown for 144 h (6 days). Kaur and Arora (2015) reported the maximum antibacterial activity on 5th day of incubation of *Aspergillus terreus* which remained more or less stable till 9th day and then declined.

The culture starts going in decline phase which results in reduced bioactivity and that may be due to accumulation of some inhibitory/toxic residues against secreted bioactive compounds (Singh *et al.,* 2014). The time course for antimicrobial agent production differs according to the strain and cultivation conditions. For instance, the maximum antimicrobial agent production was achieved after 5 days of incubation of *Penicillium corylophilum* (Silva *et al.*, 2004) and 4 days of incubation of *Cladosporium sp*. (Miao and Qian, 2005).

Table 3. Effect of some factors on the antibacterial activity of the crude extract *Aspergillus welwitschiae*

Tested factors	Tested Bacterial Pathogens			
	Conditions	*Salmonella enterica*	*Escherichia coli*	*Proteus sp.*
1- Medium Composition	CDB	27.50* ± 2.8**	24.25 ± 2.2	24.50 ± 0.5
	GPY	0	0	0
	PDB	0	0	0
2- Temperature	25°C	39.75 ± 4.6	29.75 ± 0.9	24.25 ± 1.5
	30°C	25.25 ± 2	23.00 ± 2.4	19.00 ± 0.8
	35°C	0	0	0
3- pH	pH 5	23.25 ± 0.957	20.25 ± 1.258	19.50 ± 0.577
	pH 7	24.25 ± 2.217	21.50 ± 0.577	24.25 ± 1.258
	pH 9	32.25 ± 2.062	23.75 ± 4.787	21.50 ± 1.915
4- Agitation speed	100 rpm	24.50 ± 8.103	19.75 ± 2.217	18.75 ± 0.957
	150 rpm	35.00 ± 0.816	23.25 ± 3.096	26.25 ± 1.258
	200 rpm	28.25 ± 6.850	21 ± 2.708	23.75 ± 3.775
5- Incubation days	3 days	36.50 ± 0.577	39.00 ± 0.816	21.00 ± 0.816
	5 days	19.25 ± 0.957	20.00 ± 0.816	18.25 ± 1.708
	7 days	29.25 ± 0.957	19.50 ± 1.291	18.00 ± 0.816
	9 days	22.50 ± 0.577	20.00 ± 0.00	20.50 ± 0.577
	11 days	18.50 ± 0.577	0	0
	13 days	0	0	0

* Results are given as mean value of diameters of inhibition zones measured in mm,
** ± standard deviation.

Extraction and chemical characterization of the antimicrobial metabolite from *Aspergillus welwitschiae*:

Extraction of antimicrobial metabolite:

Organic solvents also play an important role in extraction of bioactive natural products in the form of crude compounds from broth media (Goutam *et al.*, 2014). Among four tested organic solvents for extraction of antibacterial metabolites from *Aspergillus welwitschiae*, ethyl acetate was the only solvent which extracted antibacterial metabolite giving 29 mm diameter zone of inhibition against *Salmonella enterica* upon antimicrobial activity test (Fig. 3). Negative control (2%DMSO) showed no antibacterial activity and positive control (chloramphenicol 10 µg) showed 57 mm diameter zone of inhibition. The same results from the study of Lu *et al*. (2000) and Sun *et al*. (2011) have used ethyl acetate as a solvent for extraction, while Jain and Pundir (2011) found that chloroform was the best solvent for extracting *Aspergillus* metabolites.

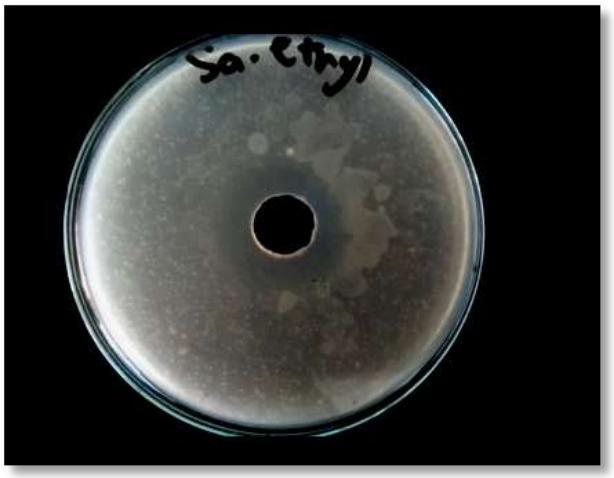

Fig. 3. Antibacterial activity of ethyl acetate crude extract of *Aspergillus welwitschiae* against *Salmonella enterica*

Chemical characterization of extracted antimicrobial metabolite from *Aspergillus welwitschiae*.

- **Purification of the antimicrobial metabolite:**

When the brown colored crude metabolite extracted by ethyl acetate was spotted on silica gel TLC sheets and examined under U.V light for purification, the result showed that the ethyl acetate contained two fluorescent spots under U.V light; one band travelled through silica gel sheet with an R_F value of 0.82 and the other remained at the baseline (Fig. 4).

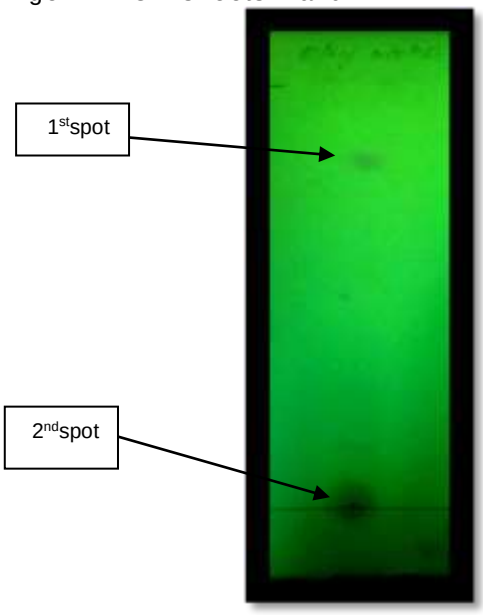

Fig. 4. TLC sheet with dark fluorescent spots produced from extracted antibacterial metabolite

- **Structure elucidation of the antimicrobial active metabolite via spectroscopic analysis**

To identify the extracted active compounds in the separated organic layer, the FTIR spectrum of the extracted metabolite showed the appearance of broad peak at (3500 - 3000 cm^{-1}) characteristic for OH of carboxylic acid, peaks at 3020, 2929, and 2855 cm^{-1} characteristic for sp^2 and sp^3 CH and peaks characteristic for carbonyl groups at 1741, 1727 and 1645 cm^{-1} (Fig. 5). The above data indicated that there is a mixture of acid and ester.

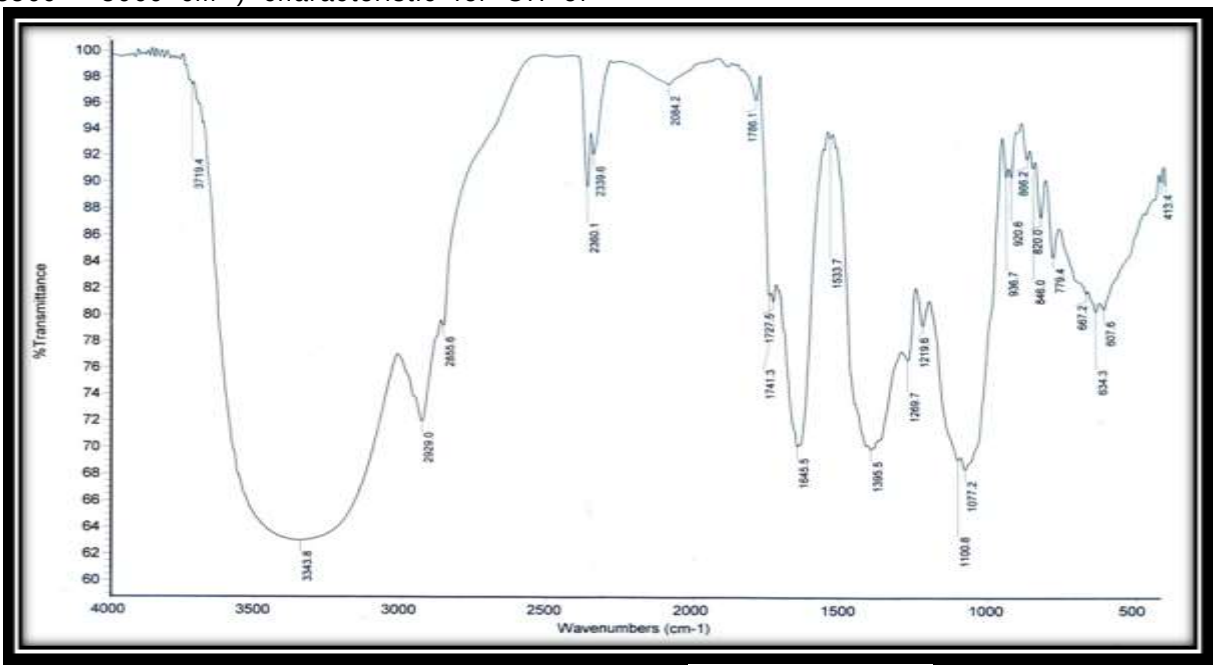

Fig. 5. Infrared spectrum of the purified antibacterial metabolite from *Aspergillus welwitschiae*

To separate the acid, the mixture was dissolved in sodium carbonate solution (50%) followed by extraction by ethyl acetate. The organic layer gave a pure solid which was separated and identified by spectroscopic data as (bis-2-(ethylhexyl) phthalate). The IR spectrum (Fig. 6) showed peaks at 2955, 2929, 2852 cm^{-1} characteristic for sp3 CH and peaks characteristic for carbonyl ester at 1731 cm^{-1}. The assigned structures were supported by ^{1}H-NMR and mass spectra. The ^{1}H-NMR spectra revealed the existence of triplet peak corresponding to 4 CH_3 groups at δ 0.81 - 0.84 ppm, a multiplet signals for 8CH_2 at δ1.19-1.70 ppm, a multiplet signals for 2CH at δ 3.70 - 3.75 ppm, a multiplet signals for 2 -OCH_2 at δ4.01 - 4.12 ppm and multiplet signals for aromatic protons at δ7.65 - 7.69 ppm (Fig. 7). The MS spectrum showed the correct molecular ion peak at 391 corresponding to M+1, beside some of abundant peaks.

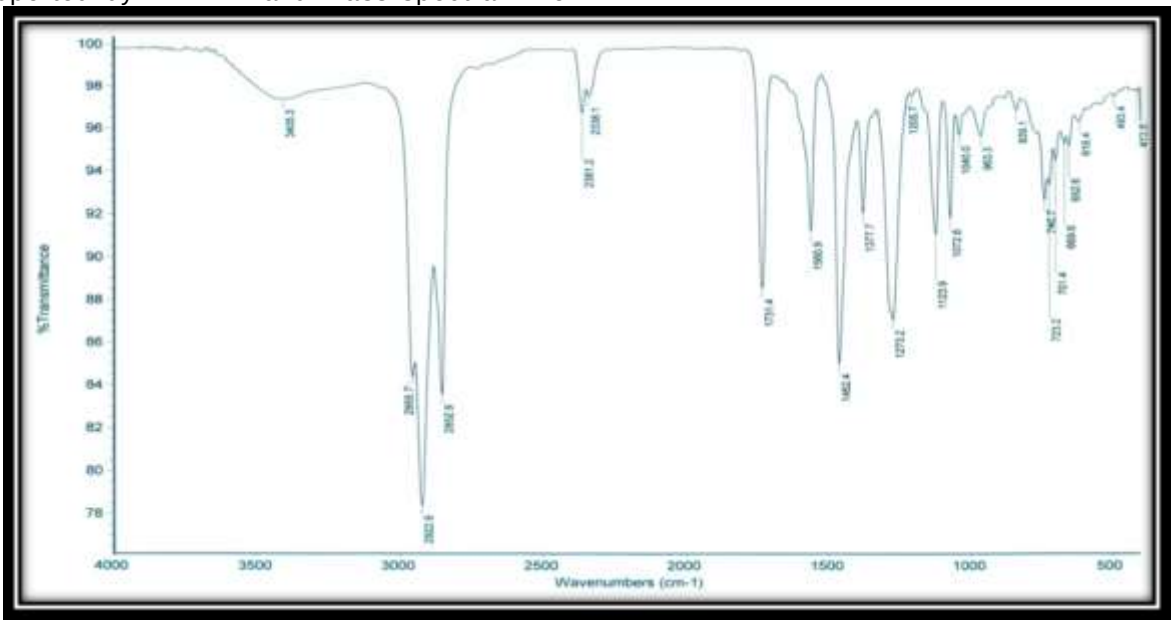

Fig. 6. Infrared spectrum of the ester portion (bis-2-(ethylhexyl) phthalate) of the antibacterial metabolite from *Aspergillus welwitschiae*

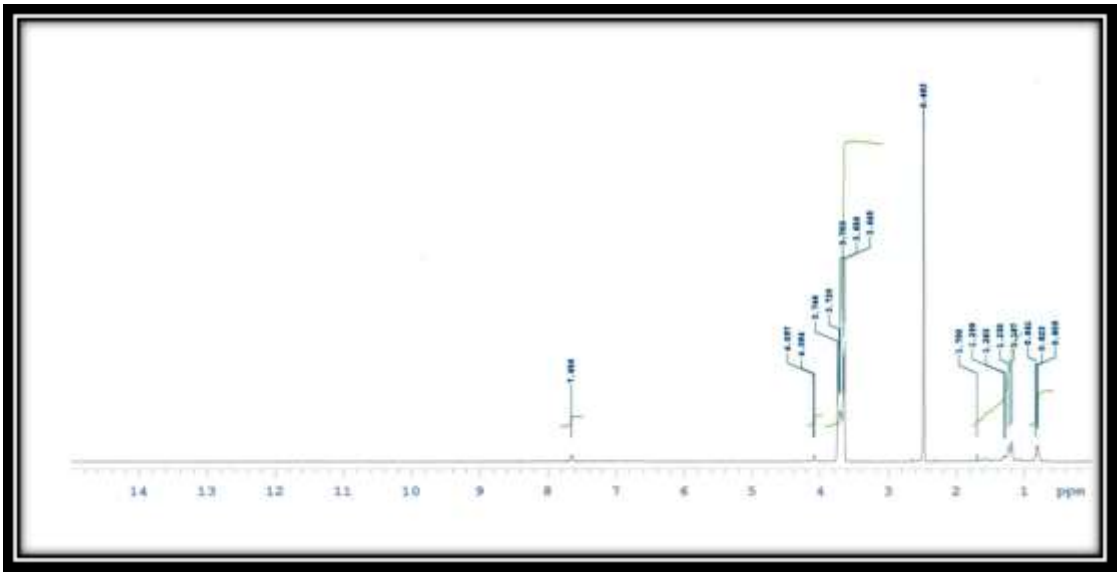

Fig. 7. The ^{1}H-NMR spectra of the ester portion (bis-2-(ethylhexyl) phthalate) of the antibacterial metabolite from *Aspergillus welwitschiae.*

The chemical characterization of the separated acid after acidification of the aqueous layer was determined based on elemental analysis and the MS spectral data (Fig. 8) as Mono (2-ethylhexyl) phthalate with the molecular formula $C_{16}H_{22}O_4$; molecular weight 279 corresponding to M+1, beside some of abundant peaks at m/z(%): 279(12), 239(10), 167(30), 149(65), 97(57), 85(57), 71(80), and 57(100).

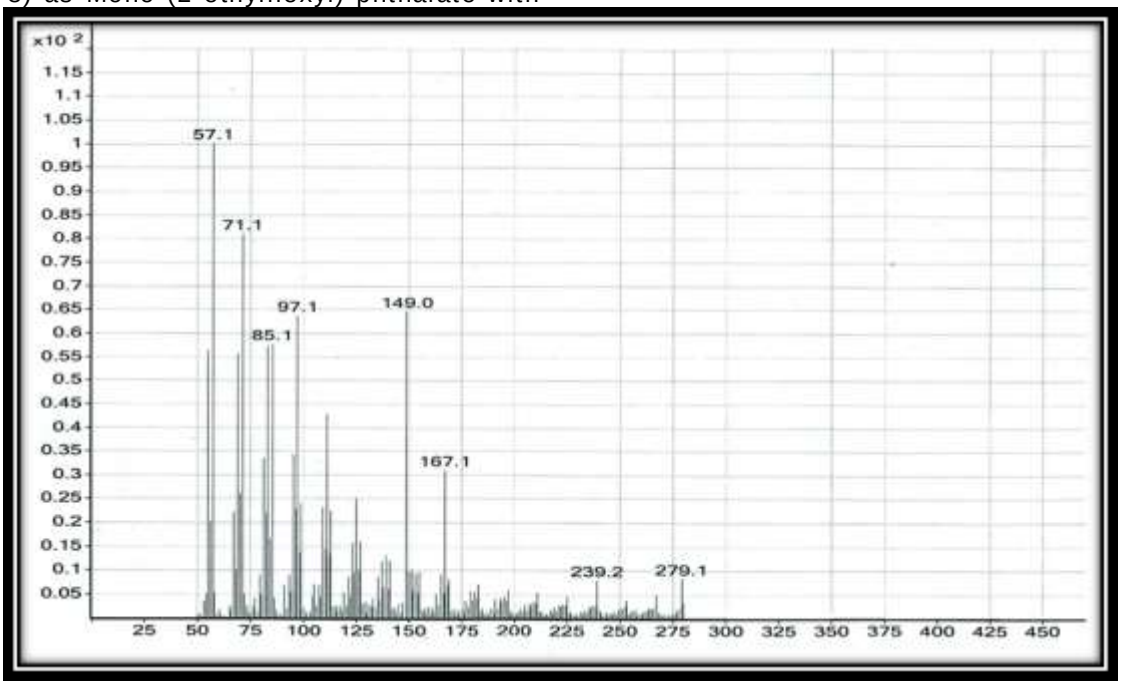

Fig. 8. Mass spectrum of the acid portion (Mono (2-ethylhexyl) phthalate) of the antibacterial metabolite from *Aspergillus welwitschiae.*

The chemical characterization of active fraction extracted from the marine fungus *Phoma herbarum* VB7 was determined based on the GC- MS and spectral data as Mono (2ethylhexyl) phthalate (Bhimba *et al.,* 2012). Atalla *et al.* (2011) in his study on marine-derived fungus *Penicillium brevicompactum* have suggested that the isolated compounds which had an antimicrobial effect may be (di "2- ethyl hexyl" phthalate) and fungisterol or one of its isomers. These extracted compounds from both studies resemble the compounds extracted from the marine- derived fungus *Aspergillus welwitschiae* in this study.

Upon testing the antibacterial activity of each of bis-2-(ethylhexyl) phthalate and (Mono (2-ethylhexyl) phthalate) separately by agar good diffusion method against *Salmonella enterica,* they didn't exert any antibacterial activity against the test pathogen unlike testing them in mixture (acid and ester together) which was active against *Salmonella enterica* confirming the synergistic antibacterial effect of the two components together.

Evaluation of cytotoxicity against WI-38 cell line:

Upon investigating cytotoxicity of extracted antibacterial metabolite (mixture of acid and ester) towards WI-38 cell line (human lung fibroblast normal cells), it was shown from the obtained data (Fig. 9) that there was a very weak inhibitory activity against human Lung Fibroblast normal cells detected under these experimental conditions suggesting the possibility of considering it as a safe compound for further steps as a pharmaceutical product.

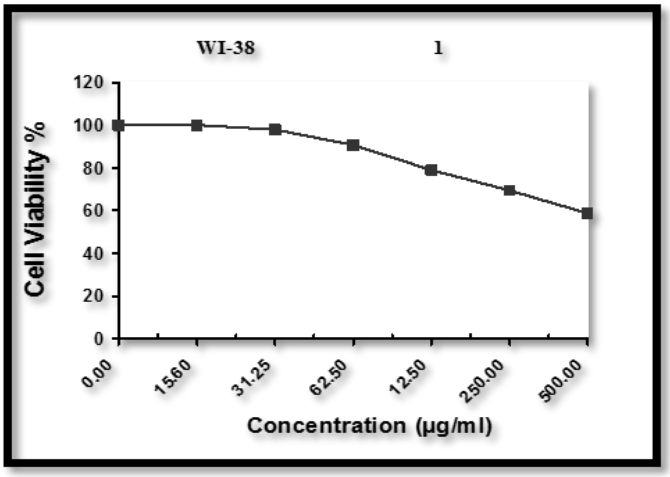

Fig. 9. Relation between viable cells (%) and drug concentration (µg/ml) extracted from *A. welwitschiae*

CONCLUSION:

This current study suggests *Aspergillus welwitschiae* as a potential candidate offering a better scope for the production, purification and isolation of broad spectrum antibacterial compound (especially against Gram negative bacteria). These findings in addition to those reported for other active isolates in this study (16 isolate) may facilitate the scale up and further purification to ascertain the compound responsible for antimicrobial activity and pharmaceutical applications.

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استكشاف القدرة الضد ميكروبية للفطريات البحرية المحلية

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في البحث عن المركبات النشطة بيولوجيا تم عزل ٨٨ فطر من مياه بحرية مصرية (البحر الأحمر، البحر المتوسط والبحيرات المرة) وعند عمل مسح للنشاط الضد ميكروبي لمستخلصات ال ٨٨ فطر المعزولين، أوضحت النتائج أن المستخلصات الخام ل ١٧ فطر فقط أظهرت نشاط متفاوت ضد بعض سلالات بكتيرية ممرضة للانسان (*Salmonella enterica, Proteus sp., Klebseilla pneumonia, Staphylococcus aureus* and *Escherichia coli*). وكان اقوى فطر معزول والذي اعطي اعلي نشاط ضد ميكروبي تم تعريفه بطرق البيولوجيا الجزيئية هو *Aspergillus welwetsichiae* . عند دراسة أفضل الظروف المؤثرة علي هذ الفطرللحصول علي أعلي إنتاجية للمركبات، أوضحت النتائج ان أعلي نشاط مضاد للبكتيريا تم الحصول عليه باستخدام الوسط الغذائي السائل زابكس دوكس في سرعة اهتزاز مساوية ل ١٥٠rpm، عند الأس الهيدروجيني ٩ودرجة حرارة ٢٥ درجة مئوية وفترة نمو لمدة ثلاث أيام. *Salmonella enterica* كان الأكثر حساسية من بين العزلات الممرضة المختبرة وسجلت اعلي مناطق تثبيط خلال هذه التجارب. تم تعريض مستخلص الايثيل اسيتات من *Aspergillus welwitschiae* للتحليل الكيميائي. أوضحت النتائج ان المركب المسئول عن هذا النشاط هو مزيج من حمض Mono (2 ethylhexyl) phthalate واستر bis (2-ethylhexyl) phthalate وعند اختبار كلا منهما علي حدة لم يظهرأي نشاط لأي مركب منهم مفردا مما يؤكد وجود تأثير متناغم بينهم في احداث النشاط المضاد للبكتيريا. عند اختبار سمية هذا المركب المستخلص الضد بكتيري ضد السلالة الخلوية WI-38 (خلايا ليفية طبيعية من رئة الإنسان) أوضحت النتائج أن هذا المركب لديه نشاط مثبط ضعيف جدا ضد تلك الخلايا مما يشير الي امكانية اعتبار هذا المركب المضاد للبكتيريا آمن وواعد كمنتج صيدلاني في دراسات مستقبلية.