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Bioactivities Of Endophytic Fungi Metabolites From Salvia.

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ABSTRACT

An investigation of the endophytic fungi led to the isolation of Rhizopus microspores from Salvia aegyptiaca. To the best of our knowledge this is the first report for isolation of these fungi from Salvia. An investigation of the chemical constituents of the Ethyl acetate extract led to the isolation of ergosterol and sitosterol respectively. The Hexane, Ethyl acetate and n-butanol extract were evaluated for their cytotoxic activity against human Hepato cellular carcinoma (HepG-2), human breast cancer cells (MCF-7), Colon carcinoma (HCT-116), and prostate carcinoma cell lines (PC-3).Hexane extract exhibited potent selective cytotoxicity against four cell lines with IC_{50} values 15.2, 30.9 14.7 and 58.1 µg/mL, respectively. The crude extracts of Hexane, Ethyl acetate and n-butanol were teste for their antimicrobial activity against 3 Fungi, 3 Gram-negative bacteria and 3Gram-positive bacteria by the agar diffusion method. The result showed antibacterial activity against at least one pathogenic microorganism. The antioxidant activities were also estimated using 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH).

Keywords: Salvia aegyptiaca, Rhizopus microspores, Antimicrobial, antioxidant, Anticancer.

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INTRODUCTION

Endophyte fungal have an important source of bioactive compounds such as peptides, alkaloids, phenols and steroids, which have a range of medical applications (1). Medical plants were a known harbor for endophytic since they were able to produce biologically active compounds similar to those of the host. The spread of these endophytic organisms is obvious, but varied; host group and geographical distribution were not known. To date, only a few plants have studied for endophytic and the potential for production of biologically active metabolites (2).

Naturally biologically active chemicals produced by endophytic filamentous fungi *Fusarium* spp. and *Acremonium* spp show antimicrobial activity (3). Another endophytic fungus like *Pestalotiopsis* sp. have been isolated from the leaves of *Pinus caneriensis* and showed strong antimicrobial activity, inhibiting the growth of all tested Gram-positive and Gram-negative bacteria (4).

Thus, the ability to produce pharmacologically important natural products before known only from plant sources is sometimes also inherent in endophytic fungi, leading to more examples, including podophyllotoxin, which will certainly give to future studies of endophytic fungi. (5)

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants or animals. These products have used for human use for thousands of years, and plants have been a major source of compounds used for medicine. The search for plants or its endophytic fungi that contain secondary metabolites with antioxidant, antimicrobial and cytotoxic activity is important because they give green and renewable resources that can potentially replace the more harmful synthetic that are now involved (6).

The genus Salvia is biologically active and was used for civil medicines around the world. Several plants of this genus are supplemented with antioxidant, antibacterial, diuretic, antitumor, hypoglycemic effects and spasmolytic activity. Popular indications of the several species of this genus include treatment of psoriasis, eczema, flatulence, tuberculosis and other diseases (7-8).

Medicinal and aromatic plants are super organisms like all plant species naturally colonized by bacteria, and fungi. A wide range of natural biologically active compounds, obtained, in particular, from plant-related microbes, has not been largely studied. Fungal endophytes protect hosts from infectious agents and withstand under unfavorable conditions by the release of active metabolites. These creatures attracted much attention after the discovery of the fungi *Taxus brevifolia*, which produce antitumor Taxol. It was assumed that medicinal plants and their fungal endophytic communities produce similar therapeutic products. we know that medicinal plants contain endophytic fungi, which they are believed to associated with the production of pharmaceutical products. Endophytic organisms that exist in the tissues of living plants are potential resources of new natural products for use in the pharmaceutical and agricultural industries.

Moreover, the uses of fungal endophytes for the production of useful active metabolites are increasing. While microbial production of drugs, especially from endophytic fungi, will be of immediate interest for pharmaceutical importance than for plants. Therefore, it is necessary to investigate the fungal endophytic community in medicinal plants (9).

The genus *Salvia*, is one of the largest genera of the Lamiaceae family and includes more than 900 species widespread throughout the world, mainly in tropical and subtropical regions. The type includes herbaceous plants, drought dysentery or shrubby perennials, rarely biennial or annual, often highly aromatic. Sage species have been important since ancient times and they are used in a traditional way medicine all over the world. They have an antioxidant, antibacterial, antidiabetic, antineoplastic and anti-inflammatory effects. (10) *Salvia aegyptiaca* was distributed in northern border region of KSA (11).

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MATERIALS AND METHODS

Plant material

The aerial parts of ***Salvia aegyptiaca*** **(Sag)**was collect fromArar, Northern border, Kingdom of Saudi Arabia. The plant was identified by Department of Phytochemistry and Natural products, Northern Border University, Faculty of Pharmacy, Rafha.

Endophytes fungi Isolation (13, 14)

The plant samples were wash with de-mineralized water, dried and immersed in EtOH 70% for 60-120 s for surface sterilization. They were dried with sterile cotton cloth to stop the sterilization process and carefully struck over the surface of a first petri dish containing isolation medium with sterilized tweezers to ensure that no surface contaminating microbes were isolated under a laminar flow hood. A piece of tissue was then cut it into smaller segments with a sterile razor blade and put on a second petri dish containing isolation medium so that the freshly cut edges were in direct contact with the agar surface. After several days first fungal growth was observable. If different fungal strains developed from one sample, the individual strains were isolated by transferring hyphal tips growing out of the cut tissue pieces with a sterile loop onto a fresh malt agar dish. For purification of the fungal strains this step was repeated several times until the colony was deemed uniform.

Taxonomic identification of the obtained fungal strains through "The Regional Center for Mycology and Biotechnology", Al-Azhar University, Egypt. They were identify using morphological characters and using an Image Analysis System.

Fermentation

For produce of crude extract on a large scale, the pure fungal mycelium was inoculated into 1000 mL Erlenmeyer flasks containing 110 g of solid rice as the substrate with 110 mL of distilled water before autoclaving at 121 ºC for 20 minutes. Cultivation was performed at room temperature under static conditions and daylight. Depending on the fungal growth, cultures on rice medium were incubated for 4-6 weeks. The fermentation was brought to an end by adding 250 mL EtOAc to the culture flask and standing closed for at least 24 hours. Small scale fermentation was carried out in one Erlenmeyer flask to gain enough extract for first bioactivity screening. For mass growth to gain enough fungal extract for isolation of secondary metabolites, 20 flasks of rice were inoculated.

Extraction of solid rice medium

The fermented rice substrate was extracted repeatedly with EtOAc. The combined Ethyl acetate (EtOAc) phases were washed with 300 mL demine ralized water to eliminate remaining sugar and starch. All obtained extracts were taken to dryness under reduced pressure at 40°C and partitioned between 90% MeOH and n-hexane to remove the fatty acids.

The total methanolic extracts were concentrate under vacuum at 40°C to nearly dryness. The latter was fractionation by using ethyl acetate and Butanol. The ethyl acetate fraction was applied over Si gel column successively eluted with n-hexane and a gradient of n-hexane: ethyl acetate to afforded fractions F1to F7.

Phytochemical study

The chemical tests were carried out on the extract using the procedures sketched by Harborne and Evans. The tests were done to tannins, saponins, alkaloids, flavonoids, carbohydrates/glycosides, triterpenoids and anthraquinones. (14-16)

Antimicrobial Activity

Agar well diffusion method was used to investigate Antimicrobial activity. The test sample was used in concentration of 20mg/mL and zones of inhibition were measured mm ± standard deviation. The activity of tested samples was studied against the *Staphylococcus aureus* (AICC25923) and *Bacillus subtilis* (NCTC8236) while *klebsiella pneumonia*(AICC27853), *Escherichia coli* (AICC25922), and *Candida albicans* (AICC7596).

Antimicrobial tests were carried out by the agarwell‐diffusion method for tested bacteria and fungi spread on nutrient agar and malt extract agar, respectively. After the media had cooled and solidified, wells 6 mm were made in the solidified agar and loaded with 100 µL of tested sample solutions in 1 mL DMSO with concentrations of 10 mg/ml. Negative controls were prepared using DMSO employed for dissolving the tested samples while gentamycin, and amphotericin B were used as positive controls for Gram‐positive bacteria, Gram‐negative bacteria, and fungi, respectively. The inoculated plates were then incubated for24 h at 37°C for bacteria and 48 h at 28°C for fungi and the diameter of any resulting zones of inhibition of growth was measured in millimeter (mm).(17-18)

Evaluation of antioxidant activity

The stock solutions of extracts were prepared in methanol to achieve the concentration of 1 mg/ml. In brief, freshly prepared (0.004%w/v) methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10C in the dark. A methanol solution of the test compound was prepared. A 40 uL aliquot of the methanol solution was added to 3ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). Reference ascorbic acid was used as standard and experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity. (19-20)

The percent DPPH scavenging effect was calculated by using following equation: DPPH scavenging effect % inhibition = 1-(Absorbance of sample / Absorbance of control) x 100.

Cytotoxicity assays

The Hexane, EtOAc and n-butanol fractions were teste for cytotoxicity against three human tumor cell lines: Human Colon cancer cells (HCT-116), Human liver cancer hepatoma carcinoma cells (HepG-2), and Human lung carcinoma (A-549) cell lines. The cells were obtaine from the American Type Culture Collection (ATCC, USA). The cells were grown on Roswell Park Memorial Institute 1640 medium (Nissui Pharm., Japan) supplemented with inactivated fetal calf serum and gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub cultured 2–3 times a week. The cytotoxic activity was determined using cell viability assay method. The cell viability was calculated as the mean absorbance of control cells/mean absorbance of treated cells. Dose-response curves were prepared and the IC_{50} value was determined. (21-23)

The Mammalian cell lines: **MCF-7** cells (human breast cancer cell line), **HepG-2** cells (human Hepato cellular carcinoma), **PC-3** (prostate carcinoma) and **HCT-116** (colon carcinoma) were obtained from VACSERA Tissue Culture Unit.

The cells were propagating 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 sub cultured two times a week.

The cells were seed in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of the test sample was adding after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed micro titer plates (Falcon, NJ, USA) using a multichannel

pipette. The micro titer plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. After incubation of the cells for at 37°C, various concentrations of sample were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method.

In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates where rinse using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measure after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated**.** The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to find the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)]x100% 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 of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimate from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA).

Statistical analysis

All values were expressed as mean±SD and compared with the corresponding control values. P-Values are calculated by using oneway ANOVA followed by Tukey multiple comparison tests. $P < 0.05$ was regarded as statistically significant.

RESULT AND DISCUSSION

Phytochemical screening

The phytochemical screening of extract revealed that contains carbohydrates, alkaloids, tannins and triterpenes/sterols while Saponins was not present.

Isolation and identification of endophytes

The identification of endophytic fungi usually has been based on the characteristic of their macroscopic and microscopic morphology by using an Image Analysis System(Table 1 and Figure 1). One strain of endophytic fungi called *Rhizopus micro spores* was isolated and identified from *Salvia aegyptiaca*. (24-25)

Table 1 : Identification Report

Character	Examination
Culture Exam: Growth characteristics	Colonies hairy, gray; reverse yellowish brown.
Microscopic Exam.	
Sporangia	Globose, 85 µm in diameter.
Collumella	Collumellasubglobose to conical 50 µm in diameter
Sporangiophores	Sporangiophores single or in small groups 10.0 µm in diameter.
Sporangiospores	Elliposiodal 7.0X 3.5 µm.
Chlamydospores	Chlamydospores present.

N.B.: The data are expressed as means of multiple measurements & only the diagnostic features were given here.

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Figure 1: Photographic images of *Rhizopus microspores*. (A) Colonies of isolate after 4 days of incubation on malt agar medium, (B) and (C) microscopic view of the isolate (×100).

Isolation of the compounds

The ethyl acetate-soluble fraction was subjected to Si gel CC and eluted with n-hexane-EtOAc (90:10→0:100) to give sex fractions. Fraction F1 was chromate graphed using sephadex column chromatography and $CHCl_3$/MeOH, (9:1) as mobile phase to afforded Compounds 1 and 2(Figure 2).They were identified as sitosterol and ergosterol respectively using physic chemical and spectroscopic methods of spectral data (^{1}HNMR, ^{13}CNMR, HMQC, DEPT and COSY)as well as by comparison with literature data and with authentic samples. (26-27).The ^{1}H-and ^{13}C-NMRmeasurements were obtained with Bruker Avance spectrometers operating at 500 MHz (for 1H) and 125 MHz (for ^{13}C) inDMSO-d6 solution, and chemical shifts were expressed in δ (ppm) with reference to TMS, and coupling constant (J) in Hertz. ^{13}C multiplicities were determined by the DEPT pulse sequence (135°). HMBC and COSY NMR experiments were carried out using BrukerAV- 500 spectrometers.

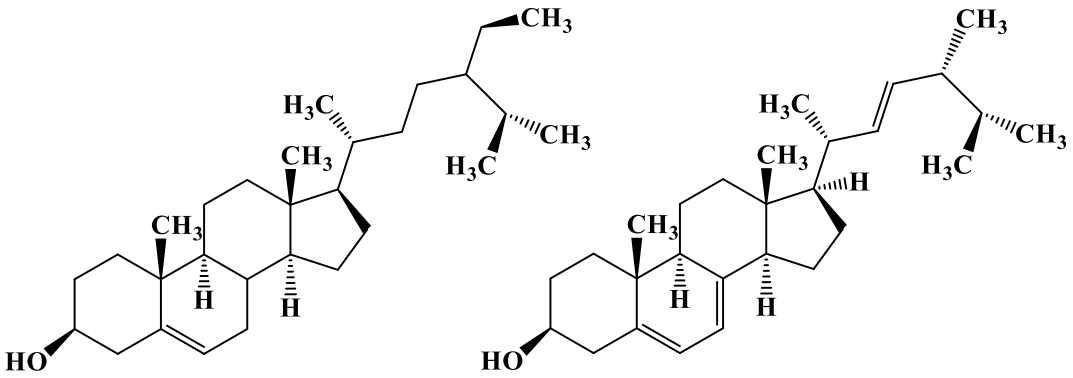

Figure 2: The structures of compounds 1and 2.

The antimicrobial activity

The in vitro studies in this work of all fractions i.e. hexane, ethyl acetate and n-butanol fractions were carried out against pathogenic gram-positive and negative bacterial and fungal strains. If 0.2 mg/mL concentration of test sample produce 6 mm zone against clinically pathogenic organism, then it is considered effective. All fractions except ethyl acetate fractions were found in effective Table 2. The zones of inhibition measured 15± 0.11mm, 11.0± 0.10mm, 14.0± 0.50mm, and 15.0± 0.20mm respectively against bacteria. The standard drug Gentamycin formed 12.0± 0.30mm of zone against *E. coli* and 10.0± 0.10mm against *k. pneumonia*. As far as Gram-positive organisms are concerned, good antibacterial activity was found with 11.0± 0.60mm zone inhibition against *S. aureus* and 15.0± 0.40mm against *B. Subtilis*, while the standard drug Gentamycin formed 12.0± 0.10mm and 8.40± 0.40mm zones of inhibition, respectively. Antifungal activity was screened for *three* Fungi which showed no activity (Table 2).

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Table 2: Antimicrobial activities of endophytic secondary metabolites

Endophytic Fungi / Tested microorganisms	*Rhizopus microspores*			Control
Fractions	Hexane	EtOAc	N. but	
FUNGI				***Ketoconazole***
Aspergillus fumigatus RCMB 002008	NA	NA	NA	17
Aspergillus flavus RCMB 002002	NA	NA	NA	16
Candida albicans RCMB 005003 (1) ATCC 10231	NA	NA	NA	20
Gram Positive Bacteria:				*Gentamycin*
Staphylococcus aureus (RCMB010010)	NA	NA	NA	24
Bacillus subtilis RCMB 015 (1)NRRL B-543	11	10	10	26
Streptococcus mutants RCMB 017 (1) ATCC 25175	NA	NA	10	20
Gram Negatvie Bacteria:				*Gentamycin*
Escherichia coli (RCMB 010052)ATCC 25955	NA	NA	NA	30
Proteus vulgaris RCMB 004 (1) ATCC 13315	NA	11	13	25
Enterobacter cloacae RCMB 001 (1) ATCC 23355	NA	NA	NA	27

The test was done using the diffusion agar technique, Well diameter: 6.0 mm (100 µl was tested), RCMB: Regional Center for Mycology and Biotechnology. Positive control for fungi was Ketoconazole (MIC)100 µg/ml while Positive control for bacteria was Gentamycin (MIC) 4µg/ml. *NA: No activity. The sample was tested at 10 mg/ml concentration.

The antioxidant activity

There are significant variations in the ability of hexane, ethyl acetate and n-butanol fractions of ***Rhizopus microspores*** metabolites to scavenge the DPPH radical with IC_{50} from 133.1, 123.6 and 88.9µg/mL respectively.

From the estimated IC_{50} values, the order of low potency is Hexane extract with IC_{50}133.1 µg/mL, ethyl acetate extract with IC_{50}123.6 µg/mL, followed by n-butanol extract with IC_{50}88.9 µg/mL (Figure 3). The all extracts showed a lesser DPPH scavenging activity to that of the positive control (Ascorbic acid 14.2 µg/mL).

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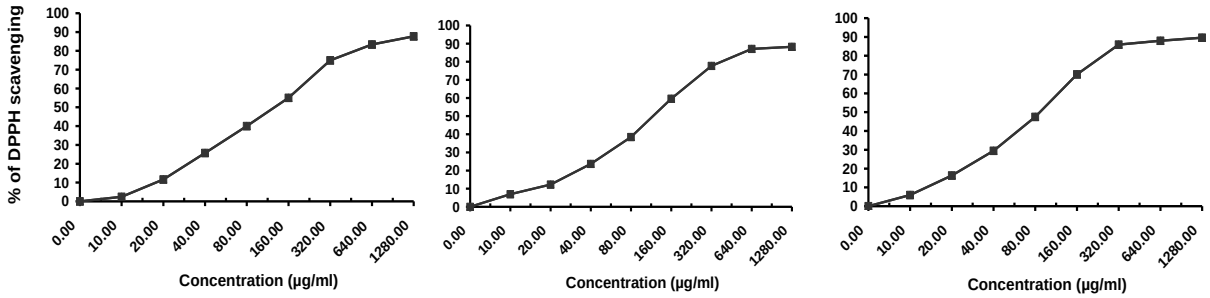

Figure 3: Antioxidant activity of *Rhizopus microspores* endophytic secondary metabolites of Hexane, EtOAc, n-butanol fractions respectively using DPPH scavenging.

The cytotoxic activity

Cytotoxic activities of the Hexane, EtOAc, n-butanol fractions were tested against four cancer cell lines Human liver cancer hepatoma carcinoma cells (HepG‑2), human breast cancer cells (MCF-7), colon carcinoma (HCT-116) and prostate carcinoma cell lines (PC-3). [Figures 4,5, 6 and7]. As a result, Hexane extract exhibited cytotoxic activity against the four cell lines with values of IC_{50}15.2, 30.914.7and 58.1µg/mL respectively. Also n-butanol extract exhibited cytotoxic activity against the four cell lines with values of IC_{50} 107, 45.9, 121 and 51.9 µg/mL respectively. On the other hand, Ethyl acetate extract exhibited a remarkable cytotoxic activity against the three cell lines with values of IC_{50}133, 101,121and189 µg/mL respectively.

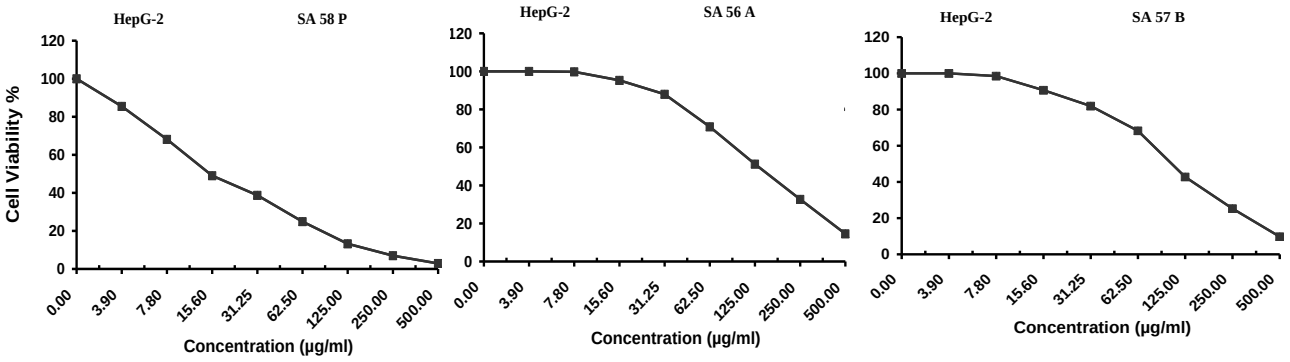

Figure 4:Cytotoxic activity of Hexane, EtOAc and n-butanol fractions against cancer cell lines HepG-2 cells (human Hepatocellular carcinoma) respectively.

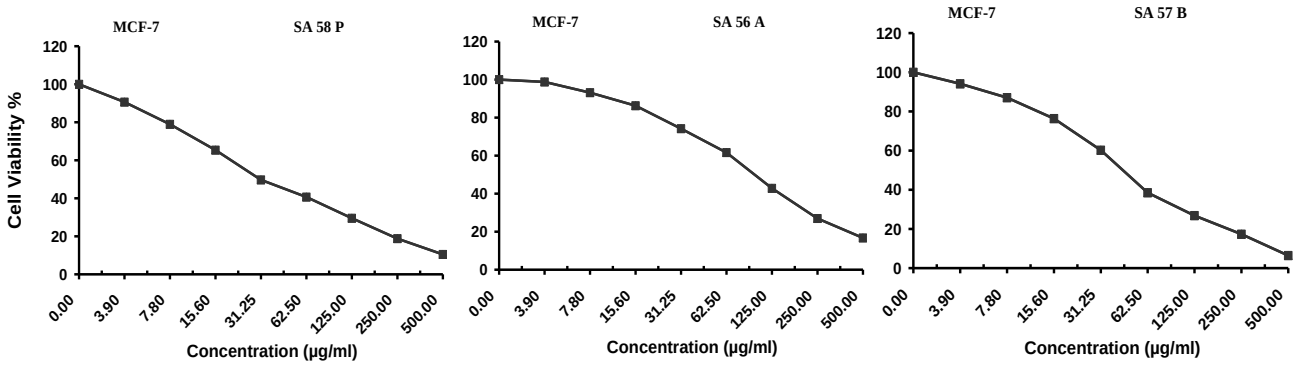

Figure 5:Cytotoxic activity of Hexane, EtOAc and n-butanol fractions against cancer cell lines MCF-7 cells (human breast cancer) respectively.

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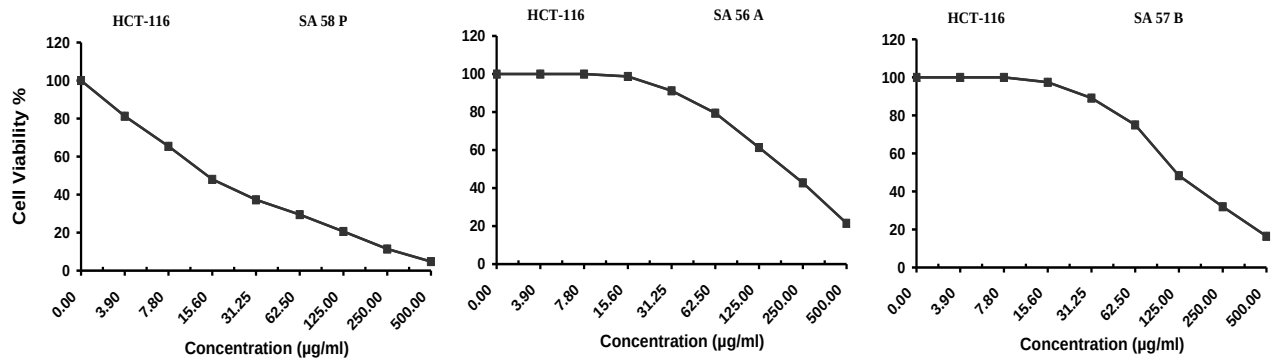

Figure 6: Cytotoxic activity of Hexane, EtOAc and n-butanol fractions against cancer cell lines HCT-116 (colon carcinoma) respectively.

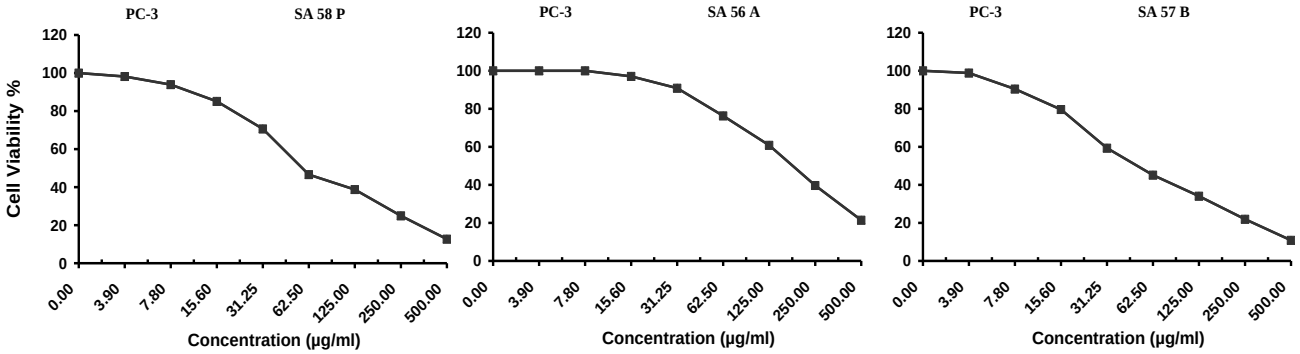

Figure 7: Cytotoxic activity of Hexane, EtOAc and n-butanol fractions against cancer cell lines PC-3 (prostatecarcinoma) respectively.

CONCLUSIONS

It was suggested that further work should be carried out to isolate, purify, and characterize formore active constituents responsible for the activity of these extracts. Also, additional work is encouraged to elucidate the possible mechanism of action of these constituents.

Thus, endophytic fungi play a significant role in the search for anticancer and might also represent an alternative source for the production of drugs that are not easily obtained by organic synthesis. The forthcoming of these fungi is of great interest and permits further investigation.

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