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ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF PLANT-DERIVED ENDOPHYTIC METABOLITES FROM NORTHERN BORDER REGION, KSA.

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Abstract:

Indigenous plants in Northern border region of Saudi Arabia are still virgins; the majority of them are still not markedly investigated for their phytoconstituents and/or their endophytic metabolites. The present work deals with the isolation, identification of endophytic fungal strains and investigation of their antimicrobial and cytotoxic activities from two indigenous plants, Polygonum aviculare and Achillea fragrantissima, three fungal strains have been isolated and identified as Pseudeurotium oval, Aspergillus sydowii, and Trichoderma longibrachiatum. These fungal strains were cultivated on solid nutrient media (rice media), their secondary metabolites were extracted and fractionated. The antimicrobial and Cytotoxic activities of these fractions were evaluated. Sub-fractions of both A. sydowii, and T. longibrachiatum showed a comparable antimicrobial activities while those of P. oval were inactive. In addition, Sub-fractions of both A. sydowii, and T. longibrachiatum showed a promising cytotoxic activities against three Mammalian cancer cell lines: MCF-7 cells (human breast cancer cell line), HepG-2 cells (human Hepatocellular carcinoma) and HCT-116 (colon carcinoma) while the most promising results obtained from T. longibrachiatum sub-fractions.

Key words: *Achillea fragrantissima, Polygonum aviculare, Pseudeurotium oval, Aspergillus sydowii, and Trichoderma longibrachiatum, Cytotoxic and antimicrobial activity*

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INTRODUCTION:

The flora of Saudi Arabia is one of the richest biodiversities in the Arabian Peninsula and comprises very important genetic resources of crops and medicinal plants. The components of this flora are admixture of Asian, African and Mediterranean regions' plants. This flora comprises 2250 species belonging to 835 genera and about 142 families. 147 species are classified as "endemic", 721 species as "endangered" and about 22 species are believed as completely extinct [1]. *Achillea fragrantissima* [Forssk] Sch. Bip is a wild herbaceous shrub belongs to the Asteracea family. It has been used for many years in traditional medicine in Middle Eastern countries for the treatment of respiratory diseases, skin diseases, gastro- intestinal disturbances, high blood pressure, stomach aches and diabetes [2, 3]. Traditionally, herbal sample, extract and /or volatile oil of *Achillea fragrantissima* have been used for its antiseptic antipyretic, analgesic, and antiviral activities. [4,5,6]. Furthermore, ethanolic extract of *A. fragrantissima* was tested for its anti-inflammatory effects on lipopolysaccharide (LPS)-activated primary cultures of brain microglial cells. It was found that phytochemicals present in the *A. fragrantissima* extract could be beneficial in preventing/treating neurodegenerative diseases [7]. The extract shows also anti-inflammatory, and antiulcerogenic effects, antilukaemic, antioxidants [8,9,10]. Recent report demonstrated the antisickling effect of *Achillea fragrantissima* extract. [11]. Polygonum aviculare L. [P. aviculare], a member of the Polygonaceae family, is distributed across Asia, Africa, Latin America and the Middle East, where it is used as a traditional medicine. It has antimicrobial [12] and anti-inflammatory activities [13], inhibit the precontraction of airway smooth muscle [ASM]. [14] Many active constituents mainly flavonol glucuronides have been reported, their biological activities have been also investigated for their Smooth muscle relaxants, antioxidants, and anti-leukaemic activities [15,16,17,18,19]. Endophytes, microorganisms that reside in the internal tissues of living plants without causing any immediate overt negative effects, have been found in every plant species examined to date and recognized as potential sources of novel natural products for exploitation in medicine, agriculture, and industry with more and more bioactive natural products isolated from the microorganisms [20]. Conservative estimates suggest that there are more than 1.5 million fungal species, of which only approximately 5% (~72, 000 known species) have been identified by taxonomists [21]. Endophytes are the chemical synthesizers inside plants, this means the opportunity to find new and targeting natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great[22]. Although fungi have proven to be prolific producers of diverse bioactive secondary metabolites, a great many fungi remain to be chemically not explored [23]. These observations argue strongly for continued exploration of fungal chemistry in order to help meet the increasing demand for new medicinally and agriculturally beneficial agents. [21]. The present work deals with the bioactive secondary metabolites from endophytic fungi located inside the plant tissues of northern border region of KSA. A targeted biological evaluation includes cytotoxicic and antimicrobial activities**.**

MATERIALS & METHODS:

Plant material

Achillea fragrantissima, *Polygonum aviculare*, were collected from the northern border region, Kingdom of Saudia Arabia and stored at 4° C until isolation procedures were instituted.

Purification of fungal strains

In aseptic condition, Plant leaves were cut into small pieces, washed with sterilized demineralized water, then thoroughly surface treated with 70% ethanol for 1-2 minutes, rinsed in sterilized demineralized water and ultimately air dried under a laminar flow hood. With a sterile scalpel, outer tissues were removed from the plant samples and the inner tissues were carefully dissected under sterile conditions and placed onto modified malt agar plates (to which 1% original plant powder for each plant sample is added before sterilization of the medium, to support growth of the young fungal colonies), antibiotics (chloramphenicol/streptomycin) were also added to exclude the bacterial contamination. After 2 weeks of incubation at room temperature, hyphal tips of the fungi were transferred to fresh malt agar medium. Plates are prepared in triplicates to eliminate the possibility of contamination. Pure strains were obtained after repeated inoculation. Two fungal strains (*Pseudeurotium oval* and *Aspergillus sydowii*) were obtained from *Achillea fragrantissima* leaf sample, and another one fungal strains (*Trichoderma longibrachiatum*) was obtained from *Polygonum aviculare* leaf sample.

Malt agar (MA) medium

MA medium was used for isolation and purification of the fungal strain and composed of Malt extract (15.0 g), agar-agar (15.0 g), distilled water (to 1000 mL). pH was adjusted with NaOH/HCl to 7.4 - 7.8). For the isolation of endophytic fungi from plant tissues 0.1 g of chloramphenicol /streptomycin (1:1)

were added to the medium to suppress bacterial growth, 10 g powder from the original plant were also added to facilitate and support the growth of the endophytic fungi.

Malt Extract Agar (MEA)
Ingredients in g/l: malt extract, 20.0; peptone, 1.0; glucose 20.0; agar, 20.0 and distilled water 1L. The pH medium was adjusted at 5.5. This medium was used for cultivation of the tested fungi for anti-fungal activity tests..

Sabouraud's dextrose agar (SDA)
The medium was used for cultivation of test pathogenic fungi and has the following composition (g/l): Glucose, 20; peptone, 10; agar, 25 and distilled water, 1L. The pH was adjusted at 5.4. The medium was autoclaved at 115°C for 15 min.

Nutrient agar (NA)
The medium was used to cultivate tested pathogenic bacteria. It contains (g/l): Beef extract, 3; peptone, 5 and distilled water 1L.

Rice solid medium
The medium was used to mass cultivation of endophytic fungal strains. (to 100 g commercially available rice was added 100 mL of distilled water and kept overnight prior to autoclaving, 10 flasks for each fungal strain)

Identification of fungal strains
The fungal strains were identified at "*the regional centre for mycology and biotechnology"*, Faculty of Science, Al-Azhar university, Cairo, Egypt. Identification was based on current universal keys; mainly: Domsch, K.H., Gams, W. & Anderson, T.-H. Compendium of Soil Fungi, second edition, taxonomically revised by W. Gams. IHW-Verlag, Eching, 2007, Atlas of Clinical Fungi, 2nd Edition by G.S. deHoog H. Gene, and M.J. Figueras. Centraalbureau voor Schimmelcultures, 2000, 1126 pp.; & Using Scanning Electron Microscope.

Cultivation
Mass growth of the fungal strains for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks (1 L each). The fungi were grown on rice solid medium (to 100 g commercially available rice was added 100 mL of distilled water and kept overnight prior to autoclaving, 10 flasks for each fungal strain) at room temperature under static conditions for 30 days.

Extraction and fractionation of Endophytic metabolites
The solid cultures after 30 days were extracted with absolute methanol several times till exhaustion, and the concentrated residues were (197.3 g, 85.6 g, 109.4 g, for *Pseudeurotium oval, Aspergillus sydowii, and Trichoderma longibrachiatum* respectively). Total alcoholic extract of each endophytic fungal strain was collected and dried under reduced pressure, then 10 g. of each (*A. sydowii* and *P. oval* extracts) solid extract was separately diluted with distilled water and fractionated successively using N. Hexane (N.Hex), Ethyl acetate (EtOAc), and Butanol (But) while the aqueous residue was discarded. The obtained subfractions were subjected to antimicrobial and cytotoxic activity testing. The total extract of *T. longibrchiatum* was fractionated using Vacuum liquid chromatography (VLC) into 7 subsequent subfractios (F1-F7) using N. Hex, (**F1,** 0.45 g**)**; N.hex /EtOAc (50:50), (**F2,** 4.61 g**)**; $CHCl_3$, (**F3, 0.**820 g**)**; $CHCl_3$/MeOH (96:4), (**F4,** 4.70 g**)**; $CHCl_3$/MeOH (90:10), (**F5,** 28.16 g**)**; $CHCl_3$/MeOH (80:20), (**F6,** 15.83 g**)**; $CHCl_3$/MeOH (70:30), (**F7,** 70.65g**)**.

Biological screening of the secondary metabolite subfractions
Two procedures were applied for Biological screening of the secondary metabolite subfractions

Antimicrobial Activity
Antimicrobial activity of endophytic secondary metabolite subfractions was carried out at antimicrobial activity unit in the regional centre for mycology and biotechnology, Faculty of Science, Al-Azhar University, Cairo, Egypt. Antibacterial and antifungal activities were expressed as the diameter of inhibition zones; agar well diffusion method was used. Holes (1 cm diameter) were digger in the agar using sterile cork borer in sterile malt agar plates for fungi and sterile nutrient agar plates for bacteria, which had previously been uniformly seeded with tested microorganisms. The holes were filled by test samples (100 µl). Plates were left in a cooled incubator at 4 °C for one hour for diffusion and then incubated at 37°C for tested bacteria and 28°C for tested fungi. Inhibition zones developed due to active antimicrobial metabolites were measured after 24 hours of incubation for bacteria and 48 hours of incubation for fungi. The antibiotic gentamycin was used as the antibacterial positive control. Also, ketoconazole was used as antifungal positive control [24].

Cytotoxicity Assay
Cytotoxicity Assay of endophytic secondary metabolite subfractions was carried out through the regional centre for mycology and biotechnology, Faculty of Science, Al-Azhar University, Cairo, Egypt.

Mammalian cancer cell lines
MCF-7 cells (human breast cancer cell line, HepG-2 cells (human Hepatocellular carcinoma) and HCT-116 (colon carcinoma) were obtained from VACSERA Tissue Culture Unit.

Chemicals Used
Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA).
Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza.

Crystal violet stain (1%)
It composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper.

Cell line Propagation
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.

Cytotoxicity evaluation using viability assay

For cytotoxicity assay, the cells were seeded 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 added 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 microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter 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. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. 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. Adopted from [25,26]. 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 were rinsed 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 measured 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 determine the number of viable cells and the percentage of viability was calculated as [1-(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 estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA)

RESULTS:

Antimicrobial activity
The antimicrobial activity testing of each subfraction was carried out against pathogenic organisms, Gram Positive Bacteria: Staphylococcus aureus (RCMB 010010) & Bacillus subtilis RCMB 015 (1) NRRL B-543; Gram Negatvie Bacteria: *Escherichia coli* (RCMB 010052) ATCC 25955 & *Proteus vulgaris* RCMB 004 (1) ATCC 13315; Fungi: *Aspergillus fumigatus* (RCMB 002008),& *Candida albicans* RCMB 005003 (1) ATCC 10231. The antimicrobial agents are used as reference, Positive control for fungi: Ketoconazole (MIC) 100 g/ml; Positive control for bacteria Gentamycin (MIC) 4g/ml. Mean zone of inhibition in mm beyond well diameter (6 mm) produced on a range of pathogenic microorganisms Results are depicted in table 1.

Table 1: Antimicrobial activities of endophytic secondary metabolites

Sample / Tested microorganisms	*Pseudeurotium oval*			*Aspergillus sydowii*			*Trichoderma longibrachiatum*			Control
	n. but	EtOAc	n. hex	n. but	n. hex	EtOAc	F_2	F_4	F_5	
A. fumigatus	-	-	-	-	-	-	-	-	-	17
C. albicans	-	-	-	-	-	-	-	-	20	20
S. aureus	-	-	-	-	-	10	-	16	16	24
B. subtilis	-	8	-	-	9	12	-	15	14	26
E. coli	-	-	-	-	9	10	-	17	17	30
P. vulgaris	-	-	-	-	10	14	12	16	15	25

- 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
- - = No significant activity.
- The sample was tested at 10 mg/ml concentration.

Cytotoxic activity

The cytotoxic activity evaluation was carried out for *A .sydowii* and *T .longibrachiatum* subfractions against three Mammalian tumor cell lines :MCF-7 cells (human breast cancer cell line, HepG-2 cells (human Hepatocellular carcinoma) and HCT-116 (colon carcinoma .(The 50% inhibitory concentration (IC ,($_{50}$the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve] Fig 2 ,1 . and 3 [for each conc. using Graphpad Prism software (San Diego, CA. USA .(The %50 inhibitory concentration (IC ($_{50}$of each fraction against each cell line was summarized in Table 2.

Table 2: Antimicrobial activities of endophytic secondary metabolites

Sample	HepG-2 IC_{50} ±.SD [µg/ml]	MCF-7 IC_{50} ±.SD [µg/ml]	HCT-116 IC_{50} ±.SD [µg/ml]
A. Sydowii N.Hex	103.7 ± 7.1	137.6 ± 9.8	76 ± 5.8
A. Sydowii EtOAc	48.7 ± 4.7	72.6 ± 3.1	26.5 ± 2.3
A. Sydowii N.But	94.3 ± 3.6	120 ± 9.4	89.1 ± 7.2
T. longbrachiatum F2	24.1 ± 1.9	28 ± 2.4	14.9 ± 1.2
T. longbrachiatum F4	7.9 ± 1.3	11.81 ± 0.9	7.3 ± 0.7
T. longbrachiatum F5	11.3 ± 0.8	14.8 ± 1.4	7.61 ± 0.5

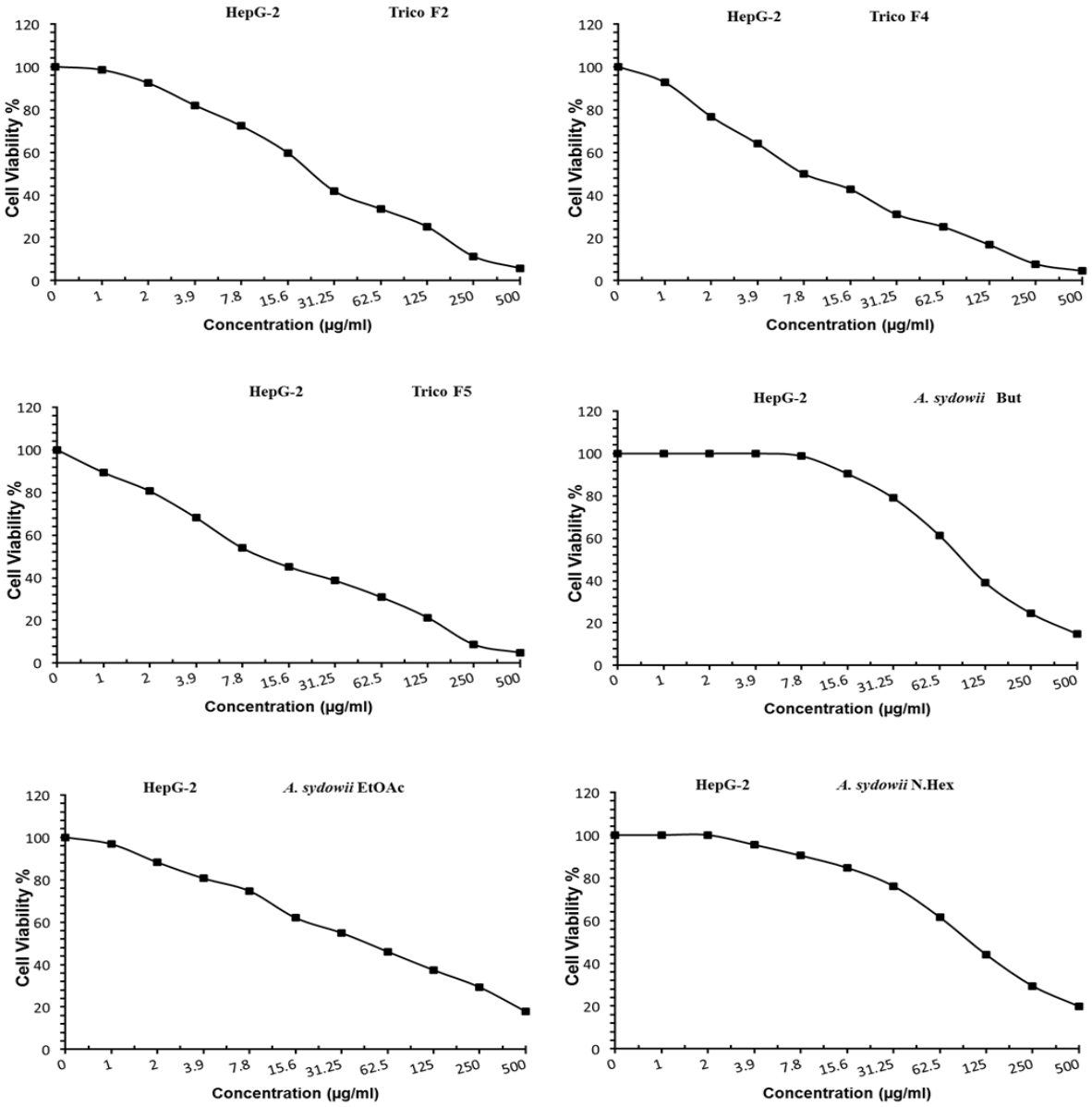

Fig. 1: cytotoxic effect of *A. sydowii* and *T. longibrachiatum* subfractions against HepG-2 cell lines

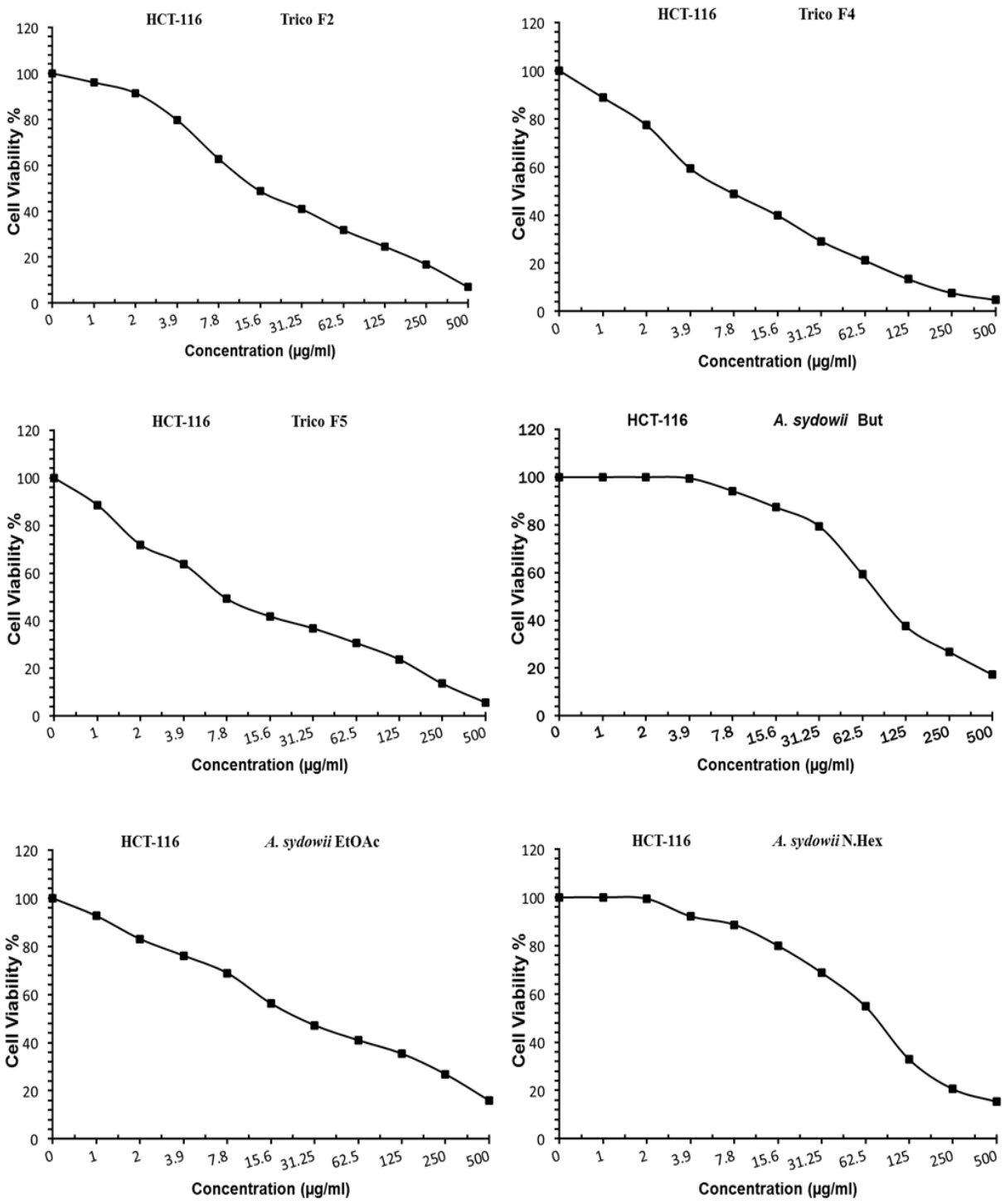

Fig. 2: cytotoxic effect of *A. sydowii* and *T. longibrachiatum* subfractions against BCT-116 cell lines

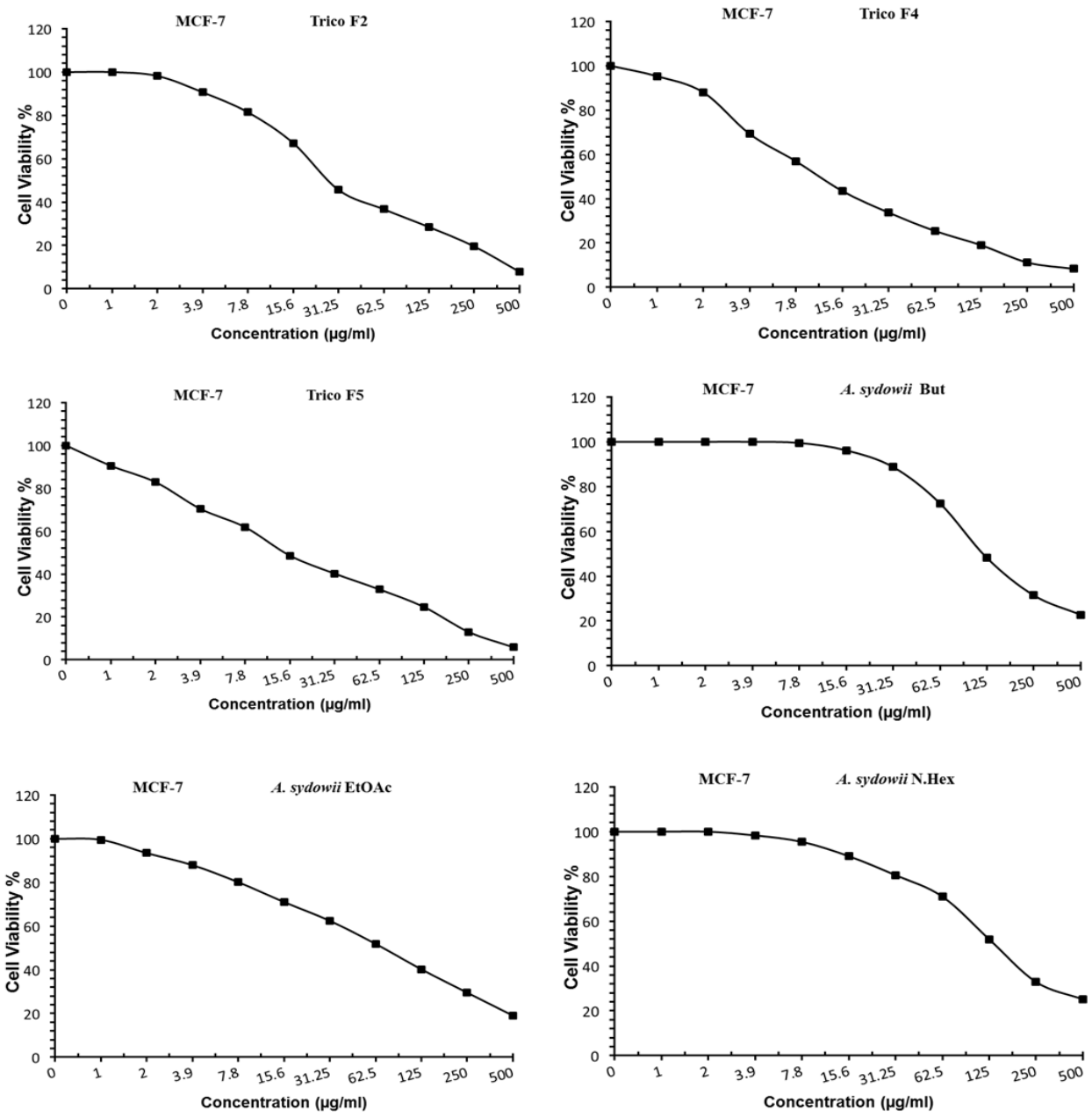

Fig. 3: cytotoxic effect of *A. sydowii* and *T. longibrachiatum* subfractions against MCF-7 cell lines

DISCUSSION:

Although *A. fragrantissima* and *P. aviculare* from the same geographical area have been studied previously for their active constituents and biological activity, this is the first report for their bioactive endophytic metabolites.

The antimicrobial activity testing showed that, *T. longibrachiatum* and *an A.sydowii fraction shows* considerable antimicrobial activities while *P. oval* fractions showed no activities. However F4 and F5 of *T. longibrachiatum* were comparatively the most active fractions.

According to cytotoxic activity results represented statistically in figure 4, it is clear that *T. longibrachiatum* fractions were more active than *A.sydowii* fractions, while F4 and F5 were comparatively the most acive fractions against the three types of cancer cell lines.

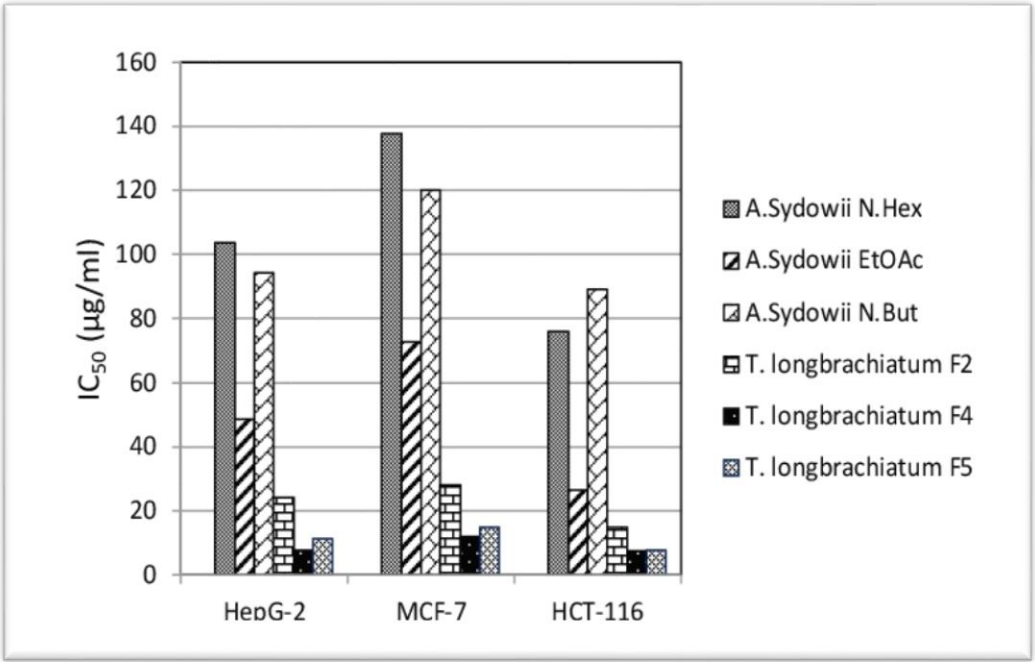

Fig. 4: cytotoxic effect of *A. sydowii* and *T. longibrachiatum* subfractions against three different cell lines

A. sydowii have been reported from both marine and terresterial sources and produce unique and biologically active secondary metabolites. When fermented in an oligotrophic medium, the EtOAc extract of A. sydowii ZSDS1-F6 showed significant antimicrobial activity against Klebsiella pneumonia and Aeromonas hydrophila, and richer chemodiversity than those from the nutritive media [27]. Different strains of *Aspergillus Sydowii* are previously reported from both terrestrial and marine habitats. *A. sydowii* is capable of producing large numbers of secondary metabolites, but most are detectable in only trace levels. Currently, >30 metabolites from A. sydowii strains have been published in the literature; however the role and bioactivity of these are largely undescribed. [28-31].

T. longibrachiatum is a member of the genus *Trichoderma*. Many *Trichoderma* species have long been used as biocontrol agents against many plant pathogens, and have capability to enhance plant growth and modify the rhizosphere and capability to grow under adverse conditions [32-36]. *T. longibrachiatum* is capable to produce diverse secondary metabolites according to the media composition and the environmental conditions in which they are grown. Many endophytic secondary metabolites have been previously isolated from *T. longibrachiatum* belonging to different chemical classes some of them showed promising antifungal and antibacterial activities. [37-41]. However it is the first time to isolate *A. sydowii* and *T. longibrachiatum* from plants indigenous to the Arabic peninsula

CONCLUSION:

This research focus on the isolation and identification of endophytic fungal strains from two indigenous plants, *Polygonum aviculare* and *Achillea fragrantissima*, in Northern border region of Saudi Arabia as well as investigation of their antimicrobial and cytotoxic activities. Three fungal strains have been isolated and identified as *Pseudeurotium oval, Aspergillus sydowii,* and *Trichoderma longibrachiatum.* These fungal strains were cultivated on solid nutrient media [rice media] , their secondary metabolites were extracted and fractionated. The antimicrobial and cytotoxic activities of these fractions were evaluated. Sub-fractions of both *A. sydowii,* and *T. longibrachiatum* showed a comparable antimicrobial activities while those of *P. oval* were inactive. In addition, Sub-fractions of both *A. sydowii,* and *T. longibrachiatum* showed a promising cytotoxic activities against three Mammalian cancer cell lines: MCF-7, HepG-2 and HCT-116, while the most promising results obtained from *T. longibrachiatum* sub-fractions. These promising results encouraged us to continue studying in the future with the aim of isolation and identification of individual secondary metabolites which may be considered as effective drug leads in the near future.

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