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Antimicrobial Activity and Cytotoxicity of Endophytic Fungi Associated with Four Medicinal Plants from Sudan

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ABSTRACT

In this study four medicinal plants, used in Sudan to cure different types of health problems, were selected for isolation of endophytic fungi, fungi reside within the plant host tissues without causing disease symptoms. A total of 20 distinct endophytic fungi species were isolated from surface- sterilized parts of the plants, *Kigelia africana*, *Cymbopogon proximus*, *Balanites egyptiaca* and *Solenostemma argel*. Nine of the isolated fungi were identified according to their microscopic and macroscopic characteristics. The remaining strains which did not have spores or have sterile mycelium were given codes as unidentified species (UID). The ethyl acetate extracts of the pure isolated endophytic fungi were prepared from fungal material and culture media. Antimicrobial activity using agar diffusion well against different pathogenic microbes at 500 µg/ml concentration and MTT cytotoxicity bioassay using African green monkey kidney normal cell line (vero cell line) were performed for all extracts. The extracts were then screened for the presence of Taxol, a diterpene anticancer drug, using different chromatographic and spectroscopic techniques. The antimicrobial activity against the most common pathogenic bacteria and fungi revealed significant activity with inhibition zone range between 14- 37 mm compare to antimicrobial controls 17-26 mm at 40µg/ml. The cytotoxicity assay results showed that the IC_{50} values were quite diverse, ranging from 3.5 μg/ml to more than 1000 μg/ml, whereas that of the positive control podophyllotoxin, a well-known cytotoxic lignan, was 2.72 μg/ml. Taxol was detected in reference to standard one from the extract of unidentified fungi No. 10 of *Kigelia africana*,. Future work will focus on the bioassay guided-fractionation of potent bioactive extracts to isolate the active compounds responsible for their activities. Improvement of culture techniques and the application of genetic engineering to increase taxol production will be carried out, followed by isolation of pure Taxol from the active crude extract.

Keywords: Sudan, Endophytic fungi, Cytotoxicity, Antimicrobial activity.

INTRODUCTION

Medicinal plants have provided mankind for decades with essential sources of new drug leads. The medicinal property of these plants might be due to the microorganisms they hosted. Some plants have precisely been endangered with extinction due to pressure brought upon them as an effect of their numerous therapeutic uses, this has inspired the pharmaceutical companies as well as scientists to consider the possibilities of using endophytic microorganisms as substituent sources for the production of novel effective drugs [1]. Endophytic fungi are fungi that living in the inner tissues of the plants, carry out part or all of their life cycle, and their host plants never undergo any damage or apparent disease as an effect of their subsistence there. Many of the endophytic fungi characterize with its potentiality to synthesized pharmacologically active metabolites that are similar to structures of their host plants metabolites [2]. Endophytic fungi have been evaluated for many biological activities for example; antibiotics, antiviral, anticancer and antioxidants [3]. Fungal endophytes show remarkably different types of bioactive natural compounds with distinctive skeleton, including many aliphatic and phenolic compounds, alkaloids, phenylpropanoids, peptides, polyketides, and terpenoids, many of them are used in the form of antibiotics, antitumor[4]. The study is aimed to screen four important medicinal plants from Sudan for possible isolation of potent endophytes fungal strains which have the ability to produce antimicrobial and cytotoxic compounds, followed by screening their tendency to produce antitumor drug Taxol.

MATERIALS AND METHODS

Plant Material

Healthy plant samples were selected and were collected or purchased from around the University of Khartoum or Khartoum market, Khartoum state (Central Sudan), to avoid contamination with pathogens; plants with no visible symptoms of disease were carefully selected after physical examination. Four mature plants material were authenticated at the Dept. of Botany, University of Khartoum using voucher specimens at the Dept. herbarium. The specimens were identified as; *Kigelia africana* (Lam.) Benth. (Kig),

Cymbopogon proximus (Hochst. ex A. Rich.) Stapf (Prox), *Balanites egyptiaca* (L.) Delile (Bal) and *Solenostemma argel* (Delile) Hayne (Arg). Collected plant samples were brought to the laboratory within 24 hours after sample collection following guidelines of Monnanda *et al*. [5]. Fresh leaves, stems, barks of the selected medicinal plants were separated for further isolation of endophytes.

Isolation, Identification and Preservation of Endophytic Fungi

The fungal endophytes were isolated according to the reported methods [6, 7]. The endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruiting structures and spore morphology. All the purified endophytes were maintained at 4 ºC till further used.

Antimicrobial activity

Test microorganisms

Standard strains of microorganisms were tested in this study and were obtained from Medicinal and Aromatic Institute of research, National Research Center, Khartoum. The strains used including*; Bacillus subtilis* (NCTC 8236), *Escherichia coli* (ATCC 25922**),** *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (NR) *Candida albicans (*ATCC 7596), *Aspergillus niger* (ATCC 27853).

Antimicrobial activity assay

The cup-plate agar diffusion method was adopted with some minor modifications[8]. Two replicates were carried out for each extract against each of the test organisms. The diameters of the resultant growth inhibition zones were measured, averaged and the mean values were tabulated.

MTT Cytotoxicity test

The method used in this study was developed by Mosmann [9]. The test was carried out at Medicinal and Aromatic Institute of Research, National Research Center, Khartoum as was reported previously by Elnour *et al*. with some minor modification [10]. The Percentage of survival cells was calculated using the following formula: % cell survival = [(At − Ab)/ (Ac − Ab)]x 100

Where, At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells). % cell inhibition = 100 − % cell survival

Determination of IC_{50}

IC_{50}, the concentration of compound required to inhibit 50% cell growth, was determined by plotting a graph of Log (concentration of Extract) vs % cell inhibition. A line drawn from the 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of the compound). The antilog of that value gives the IC_{50} value. Data interpretation Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation.

Detection of Taxol

The crude fungi extracts were screened for the presence of Taxol, using different chromatographic and spectroscopic techniques [11].

Thin layer Chromatography (TLC)

Fungal extracts and standard Taxol were spotted and Chromatographic separations were carried according to method of Stierle *et al*. reported previously [12].

High performance Liquid Chromatography (HPLC)

For further confirmation of the presence of Taxol, only the fungal extracts detected to contain spot similar to standard Taxol in TLC, were analyzed by HPLC using reported method [13].

UV Spectroscopy

The samples were analyzed by UV absorption dissolved in 100% methanol at 273 nm in a Beckman DU-40 Spectrophotometer and compared with the spectra of standard Taxol [13].

Liquid chromatography -Mass Spectroscopy (LCMS) technique

The data were recorded on a Bruker Daltonics Esquire 3000 Plus Ion Trap mass spectrometer attached to an Agilent 1000 series HPLC system. The samples were infused into the mass spectrometer through a reverse-phase C18 column (UltraPac, 4.0 × 250 mm, LKB, Sweden) by the application of a gradient elution using a binary solvent delivery system (solvent A – 0.1% formic acid in water and solvent B – 0.1% formic acid in acetonitrile) at a flow rate of 0.3 ml/min. The data were acquired over a m/z range of 500–1000 in positive ion mode and analyzed using the Esquire analysis software [11].

Results and Discussion

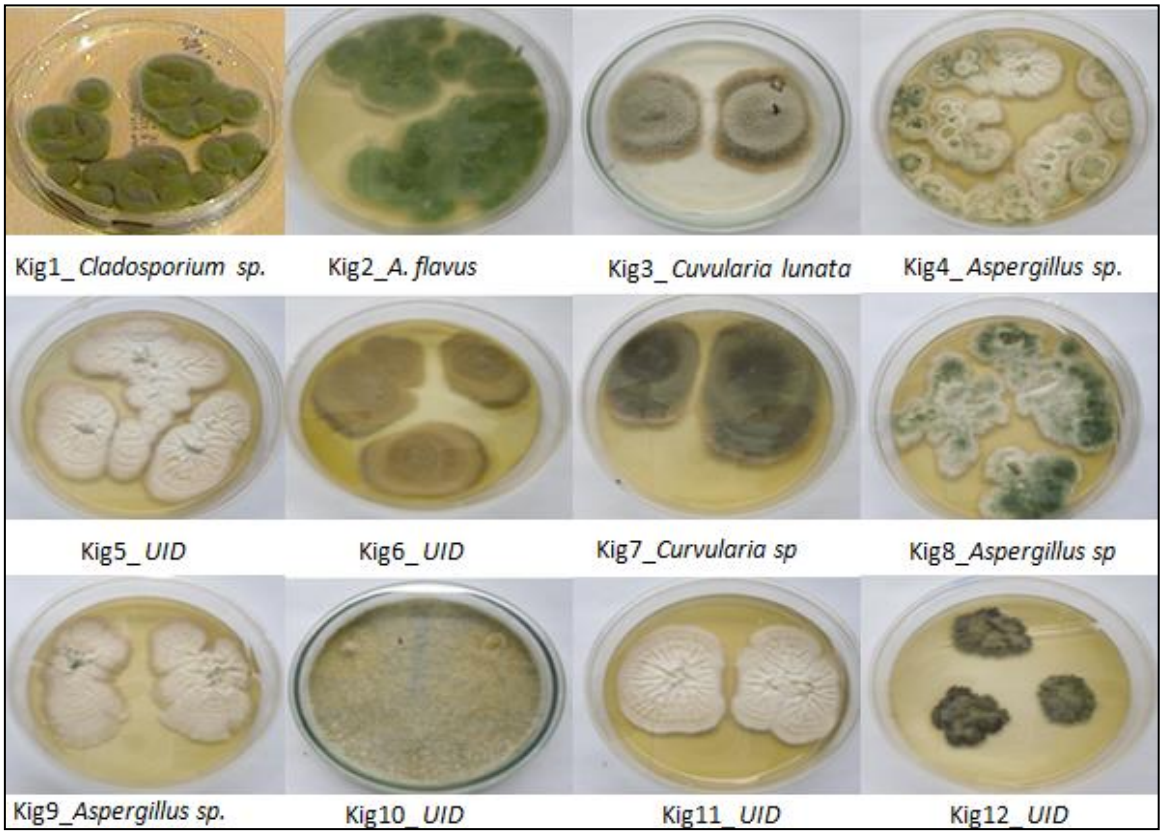
Four plants material were collected, identified and were processed for the isolation of endophytic fungi. The fungi were isolated from surface sterilized tissues of healthy stem, leaves, and bark of the plant species. A total of 20 isolates were obtained from the different parts of the plant samples (Table 1, Fig.1). Among them, nine strains were fully identified. The potential endophytic fungi have been identified for at least exact genus base on macroscopic colony and spore or conidia characteristic. The remaining fungi (11strains) were not identified because it was non sporulating species (sterilia mycelia). Potentially all fungi are capable of producing a non-sporulating state, many fungi so not adapt well to routine mycologic media and growth conditions and therefore may not sporulate [14]. Specialized media, light-dark cycles, UV light, and low or high temperatures may be required to stimulate sporulation. Sterile mycelium consists of various morphological fungal types, but not forming true spores. This group of fungi is considerably prevalent in endophyte studies [15]. A total of 12 endophyte fungi were isolated from *Kigelia africana* (Kig 1-12) and then identified as four different species of *Aspergillus*, one of them; *A. flavus*, two species of *Curvularia*; one is *C. lunata*, *Cladosporium* sp. and five unidentified species**.** In the meantime, five species were isolated from *Cymbopogon proximus* (Prox 1-5) two of them were identified as *Curvularia sp*., and *Pleospora sp*. whereas, screening of *Balanites aegyptiaca*, and *Solenostemma argel* revealed three unidentified species of endophyte fungi (Bal 1-2, and Arg). (Table1, Fig.1). Some studies have been conducted about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effects on host [16]. The results obtained in this work agree with several reports, Edson *et al*. reported that seven endophytic fungi identified as *Penicillium chloroleucon*, *Myrothecium gramineum*, *Phomopsis* sp., *Alternaria brassicae*, *Cercospora chrysanthemi*, *Cladosporium uredinicola*, and *Aureobasidium leucospermi* were isolated from *Kigelia africana*[17]. An endophyte

BAK-I was isolated from the bark of *Kigelia africana* and was characterized morphologically and on the basis of ITS-5.8S rDNA sequences as *Botryosphaeria dothidea* strain [18]. To the best of our knowledge, no systematic studies have investigated the endophyte microorganism from medicinal plants *Cymbopogon proximus*, *Balanites aegyptiaca*, and *Solenostemma argel*, this is the first report, since these plants have been reported to be part of the important medicinal plant used intensively in the Sudan.

Table 1: The isolated endophytic fungi from the selected medicinal plants

No.	Fungus code	Colony Colour	Fungus name
1	Kig 1	Green-Olive	*Cladosprium sp.*
2	Kig 2	Green	*Aspergillus flavus*
3	Kig 3	Black- grey	*Curvularia lunta*
4	Kig 4	White +green	*Aspergillus sp.*
5	Kig 5	Pink	UID
6	Kig 6	Brown	UID
7	Kig 7	Black- dark green	*Curvularia sp.*
8	Kig 8	White –green	*Aspergillus sp.*
9	Kig 9	Pink	*Aspergillus sp.*
10	Kig 10	Yellow - dark grey (mix.)	UID
11	Kig 11	Off white	UID
12	Kig 12	Black- dark green	UID
13	Prox 1	Red	UID
14	Prox 2	Pink- grey	*Curvularia sp.*
15	Prox 3	Black-dark green	*Pleospora sp.*
16	Prox 4	White	UID
17	Prox 5	Pink	UID
18	Bal 1	Grey	UID
19	Bal 2	White	UID
20	Arg 1	Pink- black	UID

UID ≡ UIdentifieD



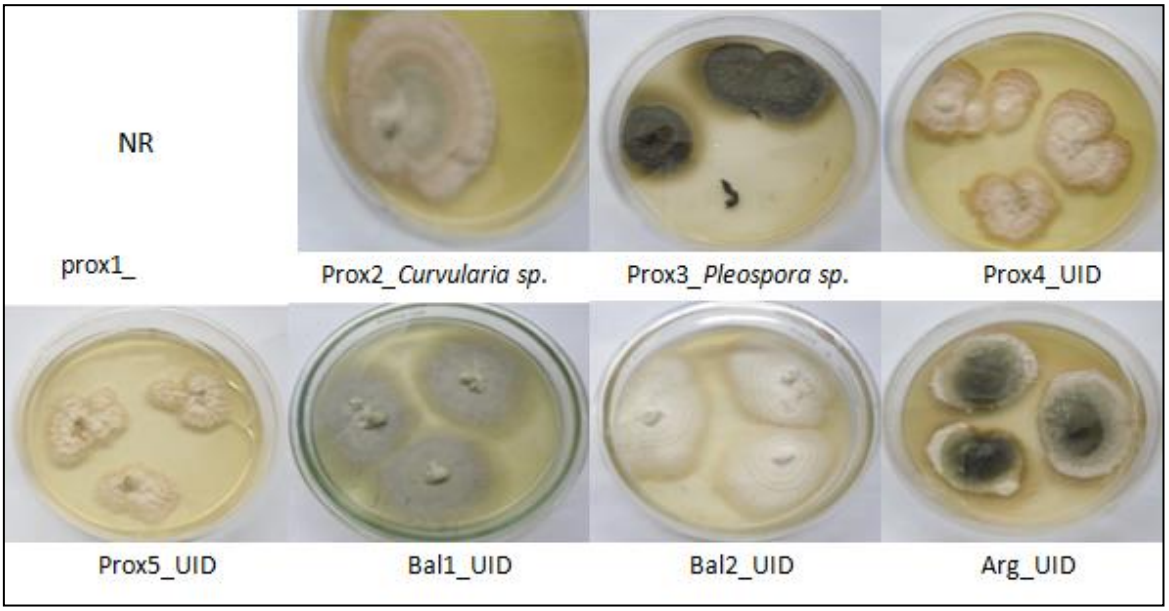


Figure 1: Pure cultures of the isolated endophytic fungi from the four medicinal plants

Antimicrobial activity

Ethyl acetate crude extracts were prepared from the cultures of endophytic fungi grown aerobically in PDA medium. The resulted extracts were screened for antimicrobial activity against two fungi; *Aspergillus niger* and *Candida albicans* and four bacterial pathogens; *Bacillus subtilus, staphylococcus aureus, Escherichia coli, Salmonella sp.,* the justification of choosing these bacterial species has been based on their different cell wall structure the Gram-positive bacteria compared with Gram-negative bacteria. Cup plate agar diffusion assay was adopted. It is clear from the results in Table 2, the most of the crude extracts demonstrated a broad spectrum activity against the four tested bacteria, *Candida albicans* and *Aspergillus niger,* with the diameters of inhibition zones ranging from 14 to 37 mm for the tested bacteria, and from 14 to 35 mm for the tested fungi at concentration 500 µg/ml. In contrast, antibacterial activity of the crude extracts seemed to be higher than that of the standard antibiotics and antifungal at 40 µg/ml. Among all extracts tested about 94% revealed antimicrobial activity against at least three strains. The activities of the extracts against fungi may be due to the similarities in eukaryotic characteristics between the endophytic fungi and the tested organisms[19]. Crude extracts of *Kigelia africana* endophyte fungi samples demonstrated significant activity against all bacterial pathogens with inhibition zone 15-37 mm. and moderate activity (12-25 mm.) against pathogenic fungi and yeast used in this study. The maximum antibacterial activity of 37 mm were obtained when tested Kig 11 against *E. coli* and minimum activity recorded when treated pathogenic strains; *Bacillus* and *staphylococcus* with Kig 1 and Kig 3 extracts. Extracts of *Cymbopogon proximus* endophytic samples displayed activity against all tested organisms. The extracts were prepared from the *Balanites* (Bal1 and 2) and *Solenostemma* (Arg) endophytic fungi indicated significant antibacterial activity against all tested pathogens, only Bal1 extract exhibited no activity against *Candida albicans*.

These results suggest the presence of either good antimicrobial potency of the extracts or of a high concentration of an active principle in the extracts of strains showing positive biological activities. Metabolites produced by endophytes are being recognized as a versatile arsenal of antimicrobial agents. Some endophytes have been known to possess superior biosynthetic capabilities, owing to their presumable gene recombination with the host, while residing and reproducing inside the healthy plant tissues [20]. A high proportion of endophytic fungi (80%) produce biologically active compounds in tests for antibacterial, fungicidal and herbicidal activities [21]. The continued development of new antimicrobial compounds is important to overcome the difficulties related to the treatment of infections caused by resistant pathogens in accordance with Petersen *et al* [22]. Thus, endophytic fungi have emerged as an alternative source for the production of new antimicrobial agents. The observation that antimicrobial, although in crude extract, were detectable in several isolates may indicate, but not prove, that these isolates produce bioactive substances.

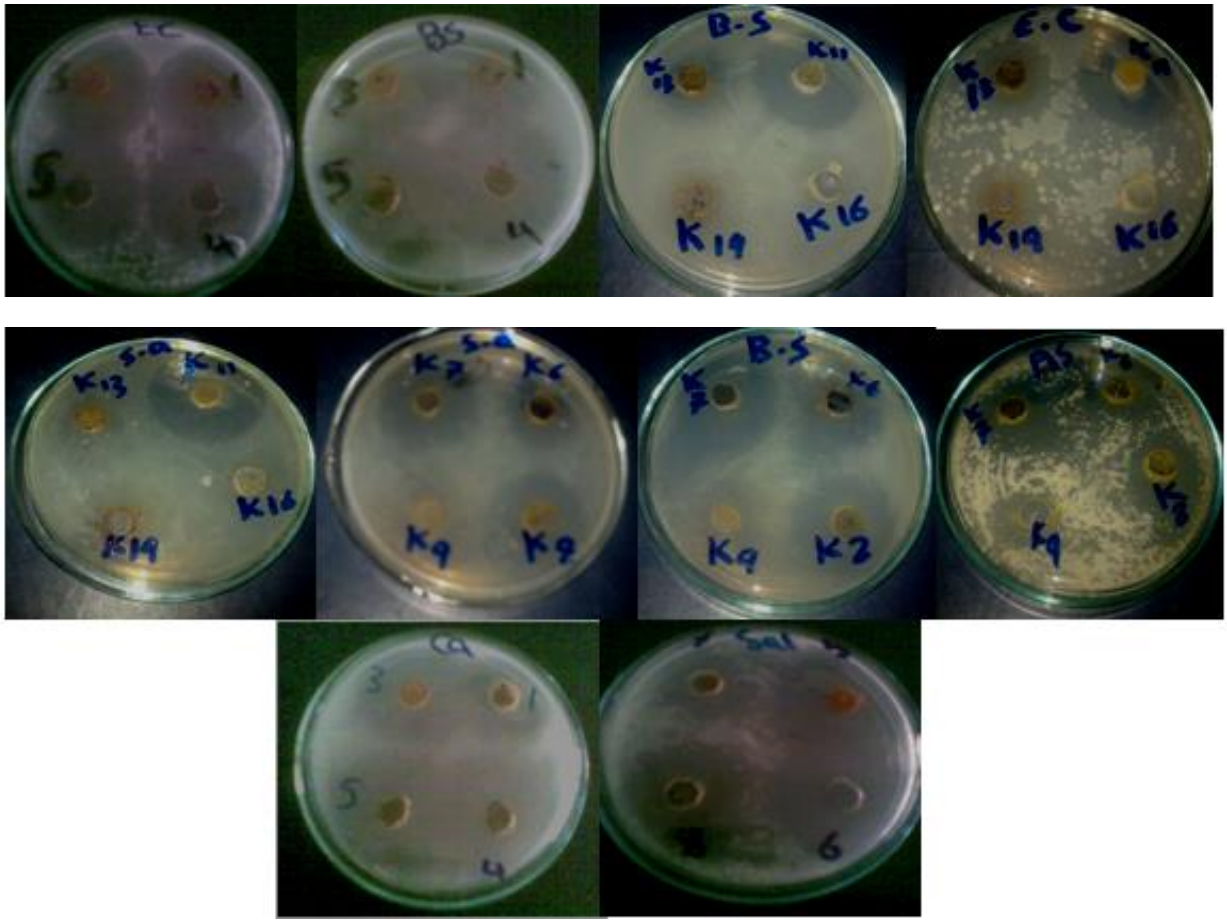


Figure 2: Antimicrobial activity of the most active extracts. *Bs: Bacillus subtilus; Sa: staphylococcus aureus.; Ec: Escherichia coli; Sal: Salmonella sp.; As: Aspergillus niger; Ca: Candida albcans;*

Table 2: Antimicrobial activity of crude ethyl acetate extracts of endophytic fungi cultures (diameter of zone of inhibition in mm)

Ex. code	Zone of inhibition (diameter in mm)					
	Bs	Sa	Ec	Sal.	As	Ca
Kig 1	14±0.03	14±0.00	20±0.05	21±0.04	14±0.03	14±0.01
Kig 2	15±0.01	17±0.03	16±0.03	16±0.02	14±0.01	12±0.00
Kig 3	14±0.00	20±0.01	16±0.04	17±0.04	25±0.03	16±0.04
Kig 4	16±0.02	16±0.00	20±0.04	20±0.03	14±0.03	16±0.01
Kig 7	31±0.06	32±0.07	30±0.02	32±0.05	16±0.01	17±0.02
Kig 10	32±0.03	33±0.06	34±0.05	31±0.05	16±0.05	31±0.03
Kig 11	35±0.03	28±0.04	37±0.02	33±0.05	14±0.01	29±0.05
Kig 12	14±0.07	18±0.01	16±0.03	16±0.02	14±0.02	14±0.02
Prox 1	25±0.01	26±0.04	35±0.04	33±0.01	14±0.00	26±0.08
Prox 2	33±0.07	26±0.04	32±0.03	35±0.03	15±0.03	21±0.01
Prox 3	30±0.05	30±0.02	31±0.06	32±0.04	14±0.02	26±0.03
Prox 4	28±0.02	31±0.05	31±0.03	33±0.02	14±0.01	24±0.03
Prox 5	31±0.07	31±0.01	33±0.06	36±0.04	14±0.01	20±0.03
Bal 1	21±0.04	25±0.03	29±0.01	30±0.00	14±0.02	NR
Bal 2	30±0.02	31±0.00	31±0.05	32±0.03	23±0.01	31±0.00
Arg	37±0.05	34±0.04	35±0.01	35±0.03	28±0.00	33±0.00
C1	20±0.03	17±0.00	26±0.03	20±0.06	----	----
C2	----	----	----	----	NR	25±0.01

Bs: Bacillus subtilus; Sa: staphylococcus aureus.; Ec: Escherichia coli; Sal: Salmonella sp.; As: Aspergillus niger; Ca: Candida albcans; Kig: Kigelia africna ; Prox :Cymbopogon proximus ; C1: antibiotics standard; C2: antifungal standard; NR: Not recorded

Cytotoxicity

In this study, the crude extracts of 14 isolated endophyte fungi species from the four medicinal plants, were tested for cytotoxicity in MTT cytotoxicity assay using normal cell of African green monkey kidney (vero cell line). The MTT test was carried out at concentrations 500, 250 and 125 µg/ml. The ethyl acetate extract was considered potential cytotoxic, if the treatment with the extract showed percentage viability cells less than 50 %, which means that the extract can inhibit cell growth more than 50% at these concentrations. The MTT assay is a well-documented cell viability assay and has been modified by several investigators since it was first developed by Mosmann[9] and Monks *et al*. [23]. The assay is extensively used for measuring cell survival and proliferation. This assay is based on the transformation of tetrazolium salt, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinic dehydrogenases in viable cells yielding blue to purple formazan crystals that are not soluble in aqueous solution. There is a direct proportionality between the formazan produced and the number of viable cells. The cytotoxicity assay results showed that the IC_{50} values were quite diverse, ranging from 3.5 μg/ml to more than 1000 μg/ml, whereas that of the positive control podophyllotoxin, a well-known cytotoxic lignan, was 2.72 μg/ml (Table 3). According to Meyer *et al*. [24], if an extract has an IC_{50} less than 1000 μg/ml, it is considered to be active. Most of the *Kigelia* endophytes extracts showed some cytotoxic activity at least at one concentration except for Kig 10. Almost all crude extracts of *Cymbopogon* endophytes exhibited null activity, only Prox 5 exhibited cytotoxicity at lower concentration used. The extracts of *Balanites* endophytes were demonstrated moderate activity at higher concentration. Additionally, it was observed that Kig 3 displayed meaningful toxicity (IC_{50}: 75.8 μg/ml) compare to that of podophyllotoxin (IC_{50}: 2.72 μg/ml). This is the first report on the assessment of the extracts of these endophytic fungal strains isolated from these sources, for their cytotoxicity towards the *in vitro* vero cell line. From this study, it can be speculated that extracts of some endophytic fungi isolated from their host possess some anticancer potential. Screening of endophytic fungi as anti-cancer was conducted using extracts of secondary metabolites such as study conducted by Kumala *et al*. [25] and Karrosi *et al*. [26]. Meanwhile, Lin *et al*. [27] performed screening based on the sequence of bases from endophytic fungi that have a homologous sequence with primer that produces polyketide synthase (PKS). However, according to Tan and Zou [3], anti-cancer compounds from endophytic fungi is not only polyketide, but could also be alkaloids, terpenoids, or phenol.

Detection of Taxol

The extracts of the fungi culture were examined for the presence of Taxol, a diterpene compound and a potent antimitotic agent with excellent activity against a range of cancers, using chromatographic and spectroscopic analyses. From a total of 12 extracts tested, one fungal extract was observed to produce the compound by TLC, HPLC, LCMS and UV spectrum. The chromatographic properties of the one spot detected in crude extract Kig 10, were found identical to standard taxol in solvent system chloroform: methanol 7:1 ratio, and it gives a bluish spot fading to dark gray after 24 hours, with the spray reagent vanillin (Fig.3a). In HPLC analysis, the fungal extract gave a peak with similar retention time as standard taxol (Fig.3b). The presence of taxol in the fungal extract was also detected by UV spectroscopy (Fig.3c) and LCMS (Fig.3d). Trace amount of Taxol was detected from the Kig 10 extract. However, the isolation of this trace amount is not possible. The improvement of the fungus growth conditions will be of great significance to increase the amount of desired substance. There have been many reports on the isolation of Taxol-producing endophytic fungi demonstrating that organisms other than *Taxus* sp. could produce Taxol [28, 29]. Thus, fermentation processes using Taxol-producing microorganisms may be an alternative promising way to produce Taxol.

Table 3: MTT cytotoxicity assay of the isolated endophytic fungi crude extracts

S. code	Conc. µg/ml	Average Optical Density ± SD	IC_{50} µg/ml	Inhibition %
-ve control		3.41± 1.48		-0.17
+ve control		0.13± 0.06	2.72	96.22
Kig 1	500	0.33± 0.14		90.33
	250	3.24± 1.41	260.1	5.05
	125	1.99± 0.86		41.64
Kig 3	500	0.40± 0.22		88.35
	250	0.28± 0.14	75.8	91.82
	125	1.20± 0.95		64.76
Kig 4	500	0.21± 0.09		93.83
	250	1.87± 1.52	171.9	45.14
	125	1.53± 1.20		55.11
Kig 8	500	1.55± 1.06		54.55
	250	2.92± 1.30	257.9	14.17
	125	0.27± 0.12		92.21
Kig 10	500	2.77± 1.20		18.94
	250	2.24± 0.98	917.9	34.26
	125	2.99± 1.30		12.23
Kig 12	500	2.29± 0.99		32.93
	250	0.34± 0.15	252.2	90.13
	125	2.76± 1.22		19.08

Prox 1	500	3.19± 1.39		6.23
	250	3.37± 1.46	116.5	1.12
	125	1.88± 1.63		44.7
Prox 3	500	3.63± 1.59		-6.05
	250	3.62± 1.57	3.5	-6.48
	125	3.19± 1.38		6.62
Prox 4	500	3.16± 1.37		7.39
	250	3.33± 1.45	121.6	2.2
	125	3.51± 1.52		-2.91
Prox 5	500	167.7± 144.34		16.37
	250	85.43± 72.18	126.1	-24.03
	125	**0.164± 0.07**		95.16
Bal 1	500	2.80± 1.21		18.01
	250	3.38± 1.47	192.2	0.87
	125	**0.93± 0.74**		72.67

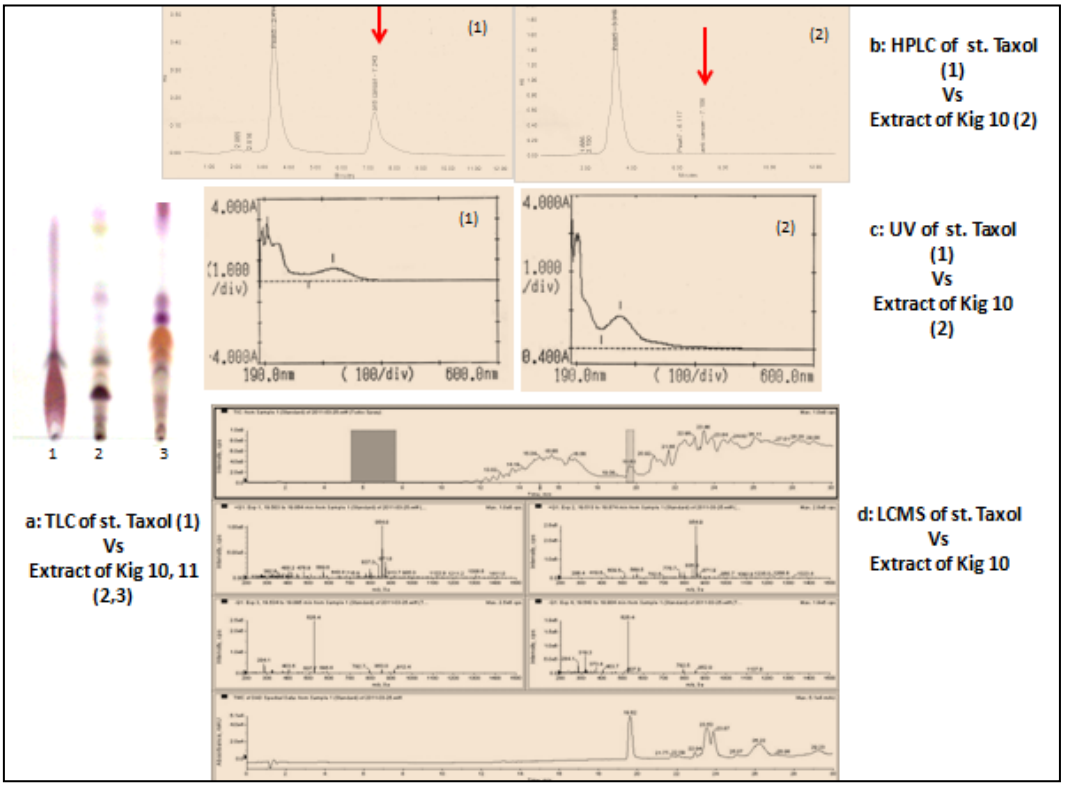


Figure 3: The chromatographical and spectroscopical comparison between standard taxol Vs crude extract of the fungus Kig 10

Interestingly, our results revealed that the extract in which taxol was detected, was non cytotoxic to normal cells (vero cells), and previous studies reported that paclitaxel lacks selectivity toward cancer cells it is harm normal cells [30], our detected natural Taxol or Taxol derivative it might be possess selectivity and safe to normal cells. Recently, the main target in anticancer researches for novel and effective drugs is to detect hits with selective cytotoxicity, hits evince strong cytotoxic effect on malignant cells with a minimal effect on normal cells [31, 32].

CONCLUSION

The results of this study indicated that medicinal plants which grow in the Sudan harbour different strains of endophytic fungi. The isolated endophytic fungi have exhibited promising antitumour and antimicrobial activity. These findings can form the basis for further phytochemical studies to isolate active compounds, elucidate the structures, evaluate them against a wider range of pathogenic microbe strains and *in vivo* models. The study recommended the importance of searching new sources to obtain safety and effective new therapeutic principles against cancer with selective properties against malignant cells with a minimal effect on normal cell. Using endophytic fungi as substitutions will help greatly in conserve the biodiversity of medicinal plants.

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Conflict of interest

The authors declare no conflict of interests

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