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# Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*.

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## ABSTRACT

The aim of this study was to identify the endophytic fungi of the medicinal plant *Kigelia africana* (Lam) Benth. (Bignoniaceae) and to investigate their potential antimicrobial activity. Seven species of endophytic fungi were successfully isolated from *K. Africana* for the first time: including *Cladosporium sp.*, *Aspergillus flavus, Aspergillus sp., Curvularia lunata* as well as three unknown species. The fungal extracts were assessed for antibacterial activity against three standard pathogenic bacterial strains: *Bacillus subtilis, Staphylococcus aureus and Escherichia coli.* Most of the extracts showed *in vitro* inhibition of bacterial growth. The phytochemical screening revealed the existence of a diverse group of secondary metabolites in the crude extracts of the endophytic fungi that resemble those in the host plant extracts.

**Keywords:** Natural products; host-endophytes relationship; TLC screening; antibacterial assays; traditional medicine; fungal diversity

## INTRODUCTION

Endophytes are symbiotic microbial organisms that inhabit the interior of plants without causing an apparent harm to the host (Hirsch & Braun, 1992). Endophytes belong to diverse groups of bacteria and fungi (Bandara *et al.,* 2006). Endophytic fungi are extremely ubiquitous; it is thought that the vast majority of plant species in natural ecosystems (if not all of them) harbor fungal endophytes (Rodriguez *et al.,* 2009). Endophytic fungi are estimated to be represented by at least one million species residing in plants (Dreyfuss & Chapela, 1994). Research on endophytes dates back to over one hundred years (Petrini, 1986). During this period, several aspects of endophyte biology were thoroughly studied, including the diversity, taxonomy, reproduction, host ecology and effects on the host (e.g. Saikkonen *et al.,* 1998). Because natural selection favors the evolution of beneficial endophytic strains, several endophytes were found to secret secondary metabolites that protect plants against insect pests, pathogenetic organisms as well as herbivores (Saikkonen *et al*., 2004), thus, endophytes represent a promising source of novel, biologically active metabolites for pharmacological and agricultural applications (Dreyfuss & Chapela, 1994; Schulz *et al.,* 2002).

Biochemical research revealed that a wide variety of natural products can be obtained from endophytic microbes (Schulz *et al.,* 2002; Strobel & Daisy, 2003). Natural products from endophytic fungi were observed to inhibit many pathogenetic organisms including bacteria, fungi, viruses and protozoans. For example, effective antifungal agents were obtained from *Cryptosporiopsis quercina*, an endophytic fungus isolated from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia (Strobel *et al.,* 1999). The endophyte *Phomopsis sp.* was found to produce Phomopsichalasin, a metabolite that shows significant antibacterial activity in disk diffusion

assays (Horn *et al.,* 1995). Cercosporin, an effective anti-parasitic agent, has been isolated from the endophytic fungus *Mycosphaerella sp* associated with the plant *sychotria horizontalis* in panama (Moreno *et al.,* 2011). Many antiviral agents were reported from endophytic fungi; two novel compounds cytonic acid A and B have been isolated from the endophytic fungus *Cytonaema sp.* These compounds are inhibitor of human cytomegalovirus (hCMV) protease (Guo *et al.,* 2000).

Due to host-endophyte coevolution, some plants that produce bioactive natural products have associated endophytes that produce the same natural products (Tan & Zou, 2001). Since the microbial sources of bioactive compounds are easier and more economical for large-scale production than plant sources, the discovery that rare, valuable plant products might also be produced by their endophytic microorganisms is of special pharmacological interest (Strobel & Daisy, 2003). A famous example is the anticancer agent 'Taxol' that is found in yew tree species (*Taxus sp.*). Stierle and Strobel (1993) have isolated and characterized a novel taxol producing fungus *Taxomyces andreanae*, from the yew *Taxus brevif*olia. Thus, when searching for novel, endophyte-based drugs, a particularly fruitful approach would be to survey traditional medicinal plants for the bioactive metabolites that may be produced by their associated endophytes (e.g. Verma *et al,* 2007; Huang *et al.* 2008; de Siqueira, 2011).

*Kigelia* is a genus of flowering plants that belongs to the Bignoniaceae; a tropical family that contains 120 genera and 800 species. The species *Kigelia africana* (Lam) Benth. (syn. K. pinnata) is widely distributed throughout tropical Africa (Burkill, 1985; Coates-Palgrave, 1988; Joffe, 2003). *K. africana* is an important component of the traditional African medicinal practices, and, more recently, is used as a commercially manufactured drug. It is used as a cure for a variety of skin complaints including sunburn, chafing, psoriasis, itchy scalp and nappy rash. Moreover, the stem bark of *K. africana* is used in the production of an antimicrobial cream which is applied for the treatment of a variety of common microbial infections. Bark extracts (and sometimes extracts from leaves and fruits as well) are also used to treat fungal infestations such as ringworm, mycosis and athlete's foot. *K. africana* is reputedly effective against malignant neoplasms, including melanoma, tumors and breast cancer and is widely used as an anticancer treatment both locally and commercially (El Kamali & Khalid, 1996; El Ghazali *et al.,* 1997; Van Wyk *et al.,* 1997; Jackson *et al.,* 2000; Musa *et al.,* 2011).

Studies on the pharmacological properties of *K. africana* supported its traditional uses in African medicinal practices. Extracts of *K. africana* contains two active compound groups, naphthoquinones and iridoids that are responsible for most of its antimicrobial activity. Extracts of the bark, wood, roots and fruits have both antibacterial and antifungal properties. The extracts of *K. Africana* were found to exert *in vitro* inhibitory influence against Gram-negative and Gram-positive bacteria. Moreover, the growth of the yeast *Candida albicans* is inhibited by these extracts (Akunyili *et al.,* 1991; Akunyili & Houghto, 1993; Binutu *et al.,* 1996; Fabry *et al.,* 1998). Laboratory assessment for the renowned anti-cancer properties of *K. africana* has detected in-vitro activity against melanoma. Variable chemical constituents might be responsible for the effect against melanoma cell lines including naphthoquinones, lapachol, isopinnatal, sterols and iridoids (Jackson *et al.,* 2000).

In this paper, we investigate the diversity and the antibacterial properties

of the endophytic fungi associated with the medicinal plant *K. Africana*.

## MATERIALS AND METHODS

### *Samples collection*

Tissue samples of leaves, fruits, bark and stem were collected from healthy *Kigelia Africana* trees at the University of Khartoum Main Campus, Khartoum, Sudan.

### *Sample processing*

All selected parts were washed thoroughly in running tap water followed by double distilled water before processing. The samples were cut into small pieces. Bark and stem samples were cut into 1.0 × 1.0 cm pieces; leaves were cut into small discs using sterile cork borer. To eliminate epiphytic microorganisms, all the samples were initially surface treated. The samples were immersed in 70% ethanol for 1-3 min. and then sterilized with aqueous sodium hypochlorite (4% available chlorine) for 3-5 min. and then rinsed in 70% ethanol for nearly 2-5 min, before a final rinse in sterilized double distilled water. Each sample was then dried under aseptic conditions. Segments (a total of 30 at three to six segments per Petri plate) of samples were placed on potato dextrose agar (PDA) amended with chloramphenicol 500mg/L. The parafilm sealed Petri dishes were then incubated at 27 C˚ the plates were checked on alternate days and hyphal tips of actively growing fungi were then subcultured.

### *Endophyte identification*

The identification procedure of endophytic fungi was based on morphology. The seven isolated species were described according to their macroscopic features (i.e. the colour, shape and growth of cultured colonies) as well as microscopic characteristics (i.e. the structure of hyphae, conidia and conidiophores). Obtained data were then compared with the descriptions of endophytic fungi species in the literature and matches were recorded. When the morphological investigation fails to reveal the identity of the isolated fungus, the species is marked as 'unknown' and given a number. Analysis of the anti-bacterial activity, as well as the phytochemical screening were carried out on all species, identified and unidentified.

### *Endophyte cultivation*

Seven fungal strains dominant from all parts, were selected for large scale cultivation. Each fugal strain was inoculated in 15 Petri dishes containing PDA media. Fungal biomass, including the medium were cut into small and the mixture was soaked with 500 ml ethyl acetate in 1L conical flasks for 6 days and the flasks were shake on alternate days, and then filtered using whatman no. 1 filter paper. The filtrates were evaporated to dryness using rotatory evaporator. On the other hand about 10 g of powdered dried leaves of the plant material were soaked in sufficient amount of ethyl acetate for 6 days using the same method of extraction, and then the crude extract was left to evaporate to dryness.

### *Antibacterial activity*

In order to assess the anti-bacterial activity of the endophytic fungi, three standard bacterial strains were used: *Escherichia coli*, *Staphyllococcus aureus* and *Bacillus subtilis*.

A lope full of the inoculum of each bacterial strain was suspended in 5ml of nutrient broth and incubated overnight at 37º c. The overnight cultures were diluted 1/10 with sterile normal saline to produce a suspension containing about 810-910 CFU (colony forming unit) per ml. The suspension was stored in a refrigerator at 4º C till used. Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained. The cup-plate agar diffusion method (Kavanagh, 1972) was adopted,

with some minor modifications. One hundred microliters of the standardized bacterial stock suspension were thoroughly mixed with sterile melted nutrient agar which was maintained at 45ºC. The inoculated nutrient agar was distributed into sterile Petri dishes and the agar was left to set. Wells (10mm) were cut out in each of these plates using sterile cork borer (No.6). Using sterilized Pasteur pipettes 0.2 ml of the extract of concentration 10% (0.1mg/ml) was carefully added into the wells and allowed to diffuse at room temperature for 2 hrs. The plates were then incubated at 37º C for 18-24 hrs. and the diameter of the zones of inhibition around each well measured, averaged and recorded at the end of the incubation period. Each fungal extract was applied against all the bacterial strains. Three replicates were carried out for each antibacterial activity test.

***TLC Screening***

About 1 mg of each extract was used for chromatography. A solvent system of 4:1 CHCl3: MeOH was prepared and placed in a tank and the lid was replaced. The plant extract and the fungi extracts were spotted, each one on separated origin on the plate. Chromatographic separations were carried out using silica gel (E.Merck, type 60), precoated silica gel GF254. The plates was placed carefully into the tank and covered with the lid. After development, the plate was removed and the solvent front was marked with pencil and allowed to dry.

TLC plates were viewed under UV light at 254 nm for fluorescence quenching spots and at 366nm for fluorescent spots. Ceric sulphate solution was used as spraying reagent to detect the spots.

## RESULTS

***Identification of endophytic fungi***

Cultivation of the different tissues of *Kigelia Africana* has led to the isolation of seven species of endophytic fungi. Four species were successfully identified as *Aspergillus flavus*, *Aspergillus sp.*, *Curvularia lunata* and *Cladosporium sp*. Three species remained unknown.

***Antibacterial activity***

Preliminary antibacterial screening was carried out on the crude extracts of the seven fungi species against the three standard bacterial pathogens. The antibacterial effect of the fungi extracts is shown in Table 1. The inhibition zone values were highly variable, ranging between 14-37 mm. The extracts from *Cladosporium sp*. *Aspergillus sp.* and two of the unknown species were effective (I. Z. D. > 20 mm) against all the bacterial strains.

Plate 1: Pure culture of isolated *Apergillus flavus*

Plate 2: Pure culture of *isolated A. sp.*

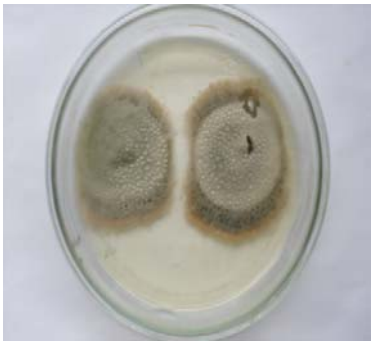
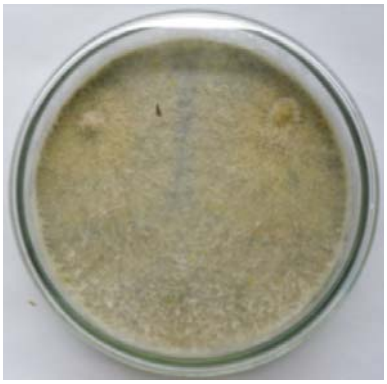


Plate 3: Pure culture of isolated *Cuvularia lunata*

Plate 4: Pure culture of isolated *Cladosprium. sp.*



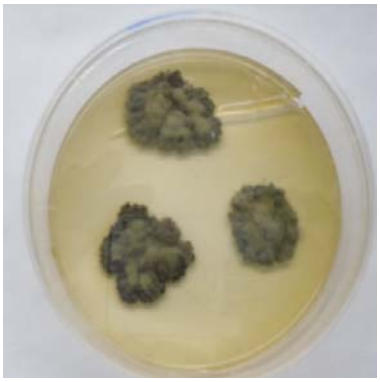


Plate 5: Pure cultures of unidentified fungi species (sterile mycelia).

Table 1: Antibacterial inhibitory activity of endophyte fungi extracts

| Bacterial strains | BS | SA | EC |
|---|---|---|---|
| Fungi Extracts | I. Z. D. (mm) | | |
| 1 | 32 | 33 | 34 |
| 2 | 25 | 26 | 35 |
| 3 | 14 | 20 | 16 |
| 4 | 15 | 17 | 16 |
| 5 | 33 | 26 | 32 |
| 6 | 35 | 28 | 37 |
| 7 | 14 | 18 | 16 |

BS: *Bacillus subtilis*; S.A: *staphylococcus aureus;* EC: *Escherichia coli*
I.Z.D.: Mean diameter of growth inhibition zones in mm average of three replicates.
Fungi crude extracts in order 1=Unknown; 2= *Cladosporium* sp.; 3= *Curvularia lunata*; 4= *Aspergillus flavus*;5= *Aspergillus* sp.; 6 & 7 unknown

### *Phytochemical screening*

TLC separation of crude extracts using the solvent system, CHCl3: MeOH in a 4:1 ratio visualized under UV365nm and detected with ceric sulphate spray reagent, revealed the presence of many spots in all samples except sample 1 and 7 prepared from two of the unknown fungi. The obtained pattern might be attributed to the presence of lipophilic, sterols and/or triterpenes compounds in addition to different classes of compounds of intermediate polarity. The pattern of spots in the plant crude extracts resembles that is shown by the endophytic fungi extracts, thus suggesting the presence of similar chemical constituents in the two sources.

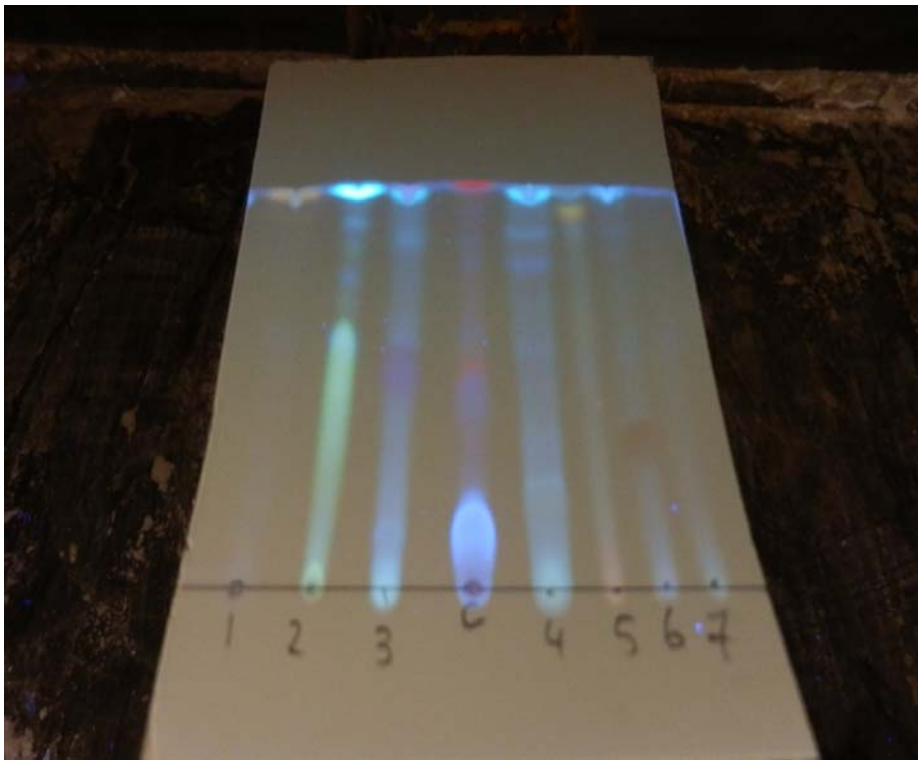



Fig. 1: Thin layer chromatogram under $UV_{254}$ of endophyte and plant crude extracts Fungi crude extracts in order 1=Unknown; 2= *Cladosporium* sp.; 3= *Curvularia lunata*; 4= *Aspergillus flavus*;5= *Aspergillus* sp.; 6 & 7 unknown and C= EtOAc plant crude extract. Solvent system: $CHCl_3$: MeOH (4:1)

## DISCUSSION

During this study, a survey has been conducted for the diversity of endophytic fungi associated with the medicinal plant *Kigelia africana*. A total of seven fungal endophytes were isolated and cultured in the laboratory. Morphological investigations, using both macroscopic and microscopic features, have resulted in the identification of four fungal species: *Aspergillus flavus*, *A. sp.*, *Curvularia lunata* and *Cladosporium sp*. Three fungal species, although subjected to the same morphological investigations, remains unidentified. Overall, these preliminary results suggest that *K. africana* hosts many species of endophytic fungi. This study represents the first attempt to isolate and identify endophytes from *K. africana* and thus it provides a research base for further investigations.

In this study, an initial assessment was performed for the antibacterial activity of the isolated endophyte species. The crude extracts from the culture of endophytic fungi grown aerobically in PDA medium displayed anti-bacterial activity. Some extracts were effective against all the bacterial strains included in the study. These results might be attributed either to the antimicrobial potency of the extract or to the high concentration of unidentified active principle in the extracts. Other endophytic fungal extracts which showed low anti-microbial activity in the bioassay may have active compounds but probably in smaller amounts and/or the screened crude extracts could yield more potent compounds once they had undergone some purification (Fabry *et al.,* 1998).

Given that protecting the plant host against pests and pathogens is in the interest of the endophyte, just as it is in the interest of the host genome itself, natural selection is expected to favor those endophytic strains that produce similar defensive chemicals to that of their hosts. True enough, some plants and their associated endophytes were found to produce the same natural compounds (Saikkonen *et al.,* 2004). It has been suggested that the mechanism by which this metabolic similarity is produced is through genetic transformation between the host and the endophyte that might have occurred during the evolutionary time (Tan and Zou, 2001). Phytochemical screening using the TLC technique was carried out to assess the diversity of chemical compounds produced by *K. africana* associated endophytic fungi. Interestingly, similarity was observed between plant crude extract and fungi extracts, thus implying the existence of cooperative production of some secondary metabolites.

Further research on the endophytic fungi of *K. africana* is required in order to confirm and expand the data provided in this preliminary investigation. Assessment of the fungal diversity in *K. africana* should be conducted using the molecular tools of identification. Since our study was the primary screening for the antibacterial activity of these extracts, assaying minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of them are suggested in order to strengthen the findings of the current study. Moreover, attempts should be made, using analytical chemistry procedures, in order to isolate and identify the bioactive compounds responsible for the antibacterial activity reported here. These obtained antibacterial compounds should then be evaluated against wider range of bacterial strains as well as *in vivo*, and tested for their safety and efficacy as therapeutic principles against infectious disease.

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