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Etudes phytochimique, cytotoxique et antibactérienne de champignons endophytes issus de plantes médicinales du Soudan

Phytochemical study, cytotoxic and antibacterial potentialities of endophytic fungi from medicinal plants from Sudan

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Résumé

Pour la première fois, l'étude de la flore fongique endophytique de cinq plantes médicinales soudanaises : *Calotropis procera* (Ait.), *Catharanthus roseus* (L.), *Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.), and *Vernonia amygdalina* (Del.) a été réalisée. Un total de 23 souches de champignons endophytes ont été isolées à partir des plantes après la stérilisation de surface puis les différentes analyses biologiques ont été effectuées. Les extraits bruts d'acétate d'éthyle de 21 endophytes ainsi que de leurs plantes hôtes ont été évalués pour leur teneur en phénols totaux et leur activité antioxydante en utilisant respectivement la méthode colorimértrique Folin-Ciocalteu et le piégeage des radicaux libres par la méthode 1,1,-diphényl-2-picrylhydrazil (DPPH) *in vitro*. Une évaluation générale de la cytotoxicité de 16 endophytes sélectionnés ainsi que de leurs plantes hôtes a été réalisée selon le test MTT sur trois types de cellules cancéreuses : carcinome du sein humain (MCF7), adénocarcinome du côlon (HT29 et HCT116). Ces extraits ont été aussi testés, selon la méthode de dilution en bouillon, sur deux souches bactériennes représentatives, *Escherichia coli* et la souche résistante à la méthicilline de *Staphylococcus aureus*. La teneur en phénols totaux (89,9 ±7,1 mg Equivalent d'Acide Gallique EAG/g) ainsi que l'activité antioxydante (IC_{50}: 18±0,1 µg/mL) les plus élevées ont été observées pour l'endophyte, *Aspergillus terreus* 2 isolé à partir des graines de *T. foenum-graecum*. *Byssochlamys spectabilis* a montré l'activité cytotoxique la plus importante (1,51 ± 0,2 µg/mL), suivi par *Cladosporium cladosporioides* 2 (10,5 ± 1,5 µg/mL), puis par *Alternaria* sp. (13,5 ± 1,8 µg/mL). Seules six souches ont montré une activité contre *S. aureus* avec des valeurs de MIC qui se situent entre 0,125 et 2 mg/mL dont: *Alternaria alternata* (0,125 mg/mL), *Alternaria* sp. (0,250 mg/mL), *Byssochlamys spectabilis* (0,5 mg/mL). 10 composés purs (0,3 à 40 mg) ont été isolés à partir des extraits bruts d'acétate d'éthyle de *Curvularia papendorfii.* Le nouveau composé pur (AFB) 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (acide Khartomique) a montré une activité antibactérienne modérée contre *S. aureus* avec une CIM de 62,5 µg/mL et une faible activité cytotoxique sur les cellules MCF7 avec une IC_{50} > 100 µM. Le composé pur AF1 a montré une activité cytotoxique modérée sur les cellules HT29 avec une IC_{50} de 29,78 µM et une très faible activité antibactérienne contre *S. aureus*. Ces deux composés ne présentent pas d'activité antioxydante.

Mots-clés : Plantes médicinales soudanaises, champignons endophytes, *Vernonia amygdalina*, *Curvularia papendorfii*, activité antibactérienne, cytotoxicité.

Abstract

This study investigated, for the first time, the endophytic fungi flora of five Sudanese medicinal plants: *Calotropis procera* (Ait.), *Catharanthus roseus* (L.), *Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.) and *Vernonia amygdalina* (Del.). A total of 23 endophytic fungal strains were isolated from the plants after surface disinfection and different biological tests were performed. Total phenolic content (TPC) and total antioxidant activity of ethyl acetate crude extracts of 21 endophytes and their host plants were estimated using respectively the Folin-Ciocalteu colorimetric method and 1,1,-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging *in vitro* method. General evaluation of the cytotoxicity of 16 selected endophytes and their host plants was performed by the MTT assay using cancer cells type: Human breast carcinoma (MCF7) and Colon adenocarcinoma (HT29 and HCT116). Preliminary antibacterial screening was done for the 16 endophytes. These extracts were also tested against two representative bacterial strains, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*, by broth dilution tests. The endophyte, *Aspergillus terreus* 1 from *T. foenum-graecum* seeds had the highest TPC in term of Gallic Acid Equivalent (89.9 ± 7.1 mg GAE/g) and antioxidant activity (IC_{50}: 18±0.1µg/mL). *Byssochlamys spectabilis* showed strong cytotoxicity (1.51 ± 0.2 µg/mL) followed by *Cladosporium cladosporioides* 2 (10.5 ± 1.5 µg/mL), then *Alternaria* sp. (13.5 ± 1.8 µg/mL). Only six strains showed activity against methicillin-resistant *S. aureus* with MIC values ranging between 0.125-2 mg/mL, *Alternaria alternata* (0.125 mg/mL) *Alternaria* sp. (0.250 mg/mL) and *Byssochlamys spectabilis* values (0.5 mg/mL). Ten pure compounds (0.3 to 40 mg) were isolated from ethyl acetate crude extract of *Curvularia papendorfii* .The new pure compound (AFB) 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (Khartoumic acid) revealed moderate antibacterial activity against *S. aureus* with MIC value 62.5 µg/mL and weak cytotoxicity with a $IC_{50} > 100$ µM against MCF7 cells. The pure compound AF1 showed moderate cytotoxic activity with IC_{50} value of 29.78 µM against HT29 and weak antibacterial activity with MIC 250 µg/mL against *S. aureus.* Both compounds displayed no antioxidant activity.

Keywords: Sudanese medicinal plants, endophytic fungi, *Vernonia amygdalina*, *Curvularia papendorfii* , antibacterial activity, cytotoxicity.

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List of Abbreviation

ABTS: 2,2'-azino- bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt

ACN: acetonitrile

CC: column chromatogarphy

CDCL3: deuterated chloroform

CD_3OD : deuterated methanol, methanol *d4*

C_5D_5N: deuterated pyridine, pyridine *d4*

$CHCl_3$: chloroform

^{13}C NMR: carbon-13 nuclear magnetic resonance

CMA: corn meal agar

COSY: correlation spectroscopy

DCM: dichloromethane

DMSO: dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EtOAC: ethyl acetate

EtOH: ethanol

FA: formic acid

GC/MS: gas chromatography–mass spectrometry

HMBC: heteronuclear multiple-bond correlation

HSQC: heteronuclear single-quantum correlation spectroscopy

^{1}H NMR: proton nuclear magnetic resonance

IC_{50}: half maximal inhibitory concentration

IR: infra-red

MBC: minimum bactericidal concentration

MeOD: deuterated methanol

MeOH: methanol

MIC: minimum inhibitory concentration

MS: mass spectrometry

NMR: Nuclear magnetic resonance

NOESY: nuclear overhauser effect spectroscopy

PDA: potato dextrose agar

REA: rice extract agar

R_f: retardation factor or retention factor

Si: silica

TAC: total antioxidant capacity

TLC: thin layer chromarography

TPC: total phenolic content

WHO: world health organization

UV: ultraviolet

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Introduction Générale

Introduction générale

I. Contexte

Les endophytes sont des organismes qui vivent dans les espaces intercellulaires des végétaux sans causer de dommage apparent à leur hôte (Arnold, 2007). Ils jouent un rôle important chez la plante hôte; ils améliorent la résistance à différents stress, aux maladies (Naik *et al*., 2009), aux insectes et ils dissuadent les herbivores (Riedell *et al*., 1991, *Li et al*., 2004); ils augmentent aussi la biomasse (Tudzynski et Sharon, 2002). Les endophytes sont des sources très intéressantes pour la découverte de nouveaux médicaments. Le nombre de brevets concernant les endophytes dans un but de production de métabolites secondaires dotés d'activités biologiques importantes a augmenté de façon spectaculaire au cours des 20 dernières années (Priti *et al*., 2009). Les différentes classes de métabolites bioactifs obtenus à partir d'endophytes comprennent des alcaloïdes, des cytochalasines, des polykétides, des terpènes, des flavonoïdes et des stéroïdes (Guo *et al.,* 2008). Un des exemples les plus connus est celui du paclitaxel, le premier anticancéreux avec un chiffre d'affaire d'un billion de dollars (Strobel et Daisy, 2003). Le Paclitaxel a été un tel succès dans les années 1990 que la survie de la plante source, l'if *Taxus brevifolia*, a été menacée par sa surexploitation. Un endophyte isolé de *T. brevifolia*, *Taxomyces andreanae*, a été découvert et il produisait du paclitaxel. Cette observation pouvait être liée au transfert de gènes de l'hôte vers le champignon (Stierle *et al.,* 1993).

Le cancer est une maladie caractérisée par la prolifération anarchique de cellules anormales. Si la prolifération cellulaire n'est pas contrôlée, elle peut entraîner la mort. Le cancer est la deuxième cause de décès aux États-Unis. En 2015, environ 1.658.370 nouveaux cas de cancer devraient être diagnostiqués (ACS, 2015). Ainsi, il est essentiel de développer de nouvelles sources d'obtention de nouveaux composés thérapeutiques.

Les microorganismes sont la source d'obtention de nombreux médicaments utilisés dans le traitement des infections bactériennes et fongiques (par exemple la pénicilline, l'érythromycine, la streptomycine, les tétracyclines, la vancomycine, l'amphotéricine), mais aussi dans le traitement du cancer (par exemple la daunorubicine, la doxorubicine, la mitomycine), dans le traitement du rejet de greffe (par exemple la cyclosporine, FK-506, la rapamycine) et aussi pour diminuer le taux de cholestérol sanguin (par exemple les statines

comme la lovastatine et la mévastatine retrouvées dans la levure de riz rouge). Les champignons endophytes sont considérés comme une source prometteuse de nouveaux composés montrant une activité anticancéreuse (Kharwar, 2011). Dans ce travail, il est envisagé de rechercher des produits naturels anticancéreux et/ou antibactériens à partir de champignons endophytes isolés de plantes médicinales soudanaises : *Vernonia amygdalina* Del. (Asteraceae), *Calotropis procera* Ait. (Asclepiadaceae), *Catharanthus roseus* L. (Apocynaceae), *Euphorbia prostrata* Ait. (Euphorbiaceae) et *Trigonella foenum-graecum* L. (Fabaceae).

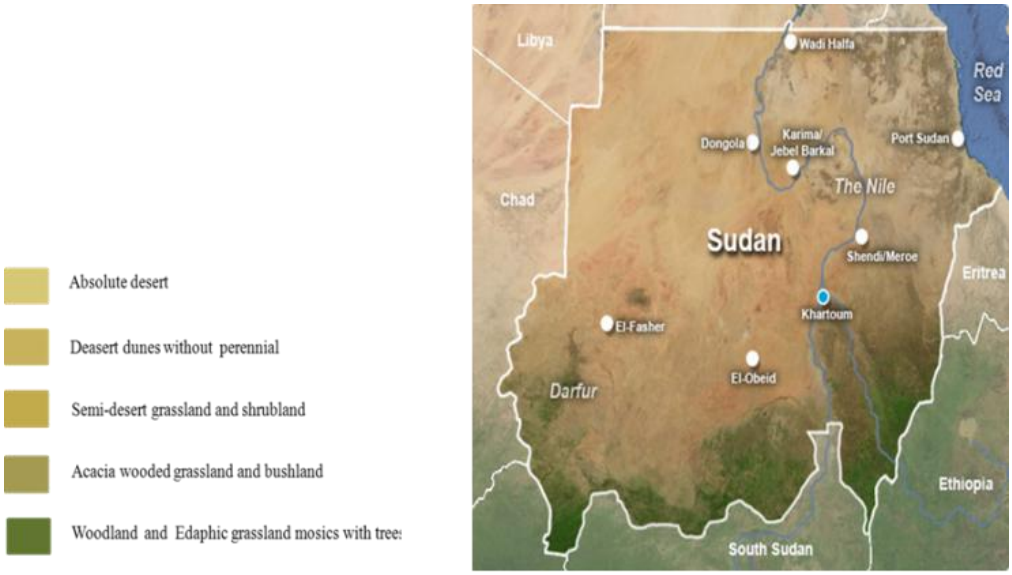


Figure I. Soudan couverture végétale (Wikipedia, 2011).

II. Présentation du problème

Un problème important, rencontré dans la recherche de produits naturels biologiquement actifs, est lié à la faible concentration de ces composés dans la drogue. Par exemple, on estime que l'on a besoin de 38000 ifs pour produire 25 kg de paclitaxel en vue de traiter 12 000 patients. L'abattage d'un arbre âgé de 100 ans permet de récolter environ 3 kg d'écorces contenant environ 300 mg de paclitaxel (Cragg et Snader, 1991). Avec les endophytes, il serait possible de trouver une solution alternative d'approvisionnement de cette molécule. En effet certains endophytes peuvent produire du paclitaxel ou les mêmes composés que leur plante hôte. Ainsi il est possible de produire les composés actifs en conditions *in vitro*. Cette technique permettrait d'augmenter la productivité en quelques

semaines alors qu'il est nécessaire d'attendre des années, notamment pour les arbres, avant de pouvoir récolter les drogues en vue de procéder à l'extraction de la molécule d'intérêt. Par ailleurs, cette approche permettrait non seulement de préserver l'environnement et la biodiversité mais aussi une diminution du prix de revient des médicaments.

III. Objectifs de l'étude

Dans le but d'identifier de nouveaux agents anticancéreux et antibactériens d'origine naturelle, nous avons étudié, pour la première fois, les champignons endophytes isolés à partir de cinq plantes médicinales soudanaises. Ce travail a nécessité différentes étapes qui sont décrites ci-dessous :

- Isolement de champignons endophytes à partir de cinq plantes médicinales du Soudan qui sont traditionnellement utilisées au Soudan et dans plusieurs pays d'Afrique pour leur propiété antitumorale.
- Identification moléculaire des souches d'endophytes isolées.
- Mise en évidence des activités cytotoxique et antibactérienne des extraits d'endophytes à l'acétate d'éthyle.
- Purification, isolement et identification de composés purs produits par les champignons endophytes.
- Recherche des activités biologiques des composés purs isolés.
- Optimisation des conditions de culture des champignons endophytes dans le but d'augmenter l'accumulation des produits d'intérêt.

IV. Intérêt de l'étude

Cette étude constitue une approche tout à fait novatrice sur l'identification d'agents cytotoxiques et antibactériens à partir de champignons endophytes issus de plantes médicinales en provenance du Soudan. L'originalité de ce travail est d'étudier la flore fongique de plantes indigènes soudanaises, qui sont peu connues d'un point de vue phytochimique, et qui sont traditionnellement employées au Soudan ainsi que dans certains pays africains pour leur activité antitumorale. Ce travail offre l'opportunité d'isoler de nouveaux produits bioactifs qui sont compatibles avec le respect de l'environnement et conduit à la découverte de nouvelles espèces fongiques.

V. Organisation de l'étude

Cette étude est présentée sous la forme de trois chapitres. L'introduction générale présente non seulement les champignons endophytes isolés de plantes médicinales mais aussi leur potentialité à produire des substances biologiquement actives. L'introduction annonce également les différentes étapes menées pour cette étude

Le chapitre I est une revue de la littérature qui porte sur les champignons endophytes en général, sur la classification de ces endophytes, sur l'obtention de composés bioactifs à partir des endophytes et sur la découverte de substances naturelles à activités anticancéreuse, antioxydante et antimicrobienne à partir de champignons endophytes. Enfin, une description botanique et des études phytochimiques sur les cinq plantes médicinales sélectionnées sont versées.

Le chapitre II comporte la description du matériel et des méthodes utilisées, en particulier la collecte des plantes, l'isolement des champignons endophytes, l'identification de ces champignons et leur culture, la préparation des extraits à l'acétate d'éthyl, les essais biologiques et enfin l'étude phytochimique de la souche sélectionnée.

Le chapitre III, intitulé Résultats et Discussion, est divisé en quatre sections. La première section porte sur la taxonomie des champignons endophytes, leurs identifications morphologique et moléculaire. La section deux concerne les propriétés organoleptiques des extraits bruts d'endophytes ainsi que le screening phytochimique par TLC des extraits d'endophytes et des extraits des plantes hôtes. Dans la section trois, sont présentés les résultats des analyses chimiques et biologiques des extraits fongiques et végétaux (évaluation de l'activité antioxydante, de la cytotoxicité et de l'activité antibactérienne). La quatrième section est consacrée à l'étude du champignon endophyte du genre *Curvularia* isolé de *Vernonia amygdalina*, en particulier les résultats rapportent la purification et la caractérisation de composés purs ainsi que leurs activités biologiques. Enfin, pour *Curvularia papendorfii* une optimisation du milieu de culture a été réalisée en vue d'améliorer la production des composés actifs.

Une conclusion et des perspectives terminent ce manuscrit et récapitulent les résultats les plus intéressants obtenus durant ces années de thèse. Les références bibliographiques et des annexes sont présentées à la fin du document.

General Introduction

I. Background

Endophytes are organisms that colonize internal plant tissues without causing apparent harm to their host (Arnold, 2007). They influence greatly the physiological activities of host plants by enhancement of stress and disease resistance (Naik *et al*., 2009), insects and mammalian herbivores deterrence (Riedell *et al.,* 1991, Li *et al*., 2004) and an increase of biomass (Tudzynski and Sharon, 2002). Endophytes are treasure chest of drug discovery; the number of patents that use endophytes for the production of secondary metabolites with biologically important activities has increased dramatically in the past 20 years (Priti *et al*, 2009). Classes of bioactive metabolites obtained from endophytes include alkaloids, cytochalasins, polyketides, terpenoids, flavonoids, and steroids (Guo *et al*., 2008). One of the best-known and most interested examples is that of paclitaxel, the first billion dollar anticancer drug (Strobel and Daisy, 2003). Paclitaxel was so successful in the 1990s that the survival of the source plant, the yew *Taxus brevifolia*, was threatened by overharvesting. An endophyte of *T. brevifolia*, *Taxomyces andreanae*, was also found to produce paclitaxel; presumably the result of lateral gene transfer from host to fungus (Stierle *et al*., 1993).

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is the second most common cause of death in the US. In 2015, about 1,658,370 new cancer cases are expected to be diagnosed (ACS, 2015). Thus, the needs to develop novel resource of novel therapeutic compounds are urgent.

Microorganisms are the source of lifesaving treatments for bacterial and fungal infections (e.g., penicillin, erythromycin, streptomycin, tetracycline, vancomycin, amphotericin), cancer (e.g., daunorubicin, doxorubicin, mitomycin, taxol), transplant rejection (e.g., cyclosporin, FK-506, rapamycin), and high cholesterol (e.g., statins such as lovastatin and mevastatin). The uniqueness of the endophytic community of fungi is stressed as a promising source of novel compounds with anticancer activity (Kharwar, 2011). The researcher plans to establish microbial natural products anticancer and antibacterial agents from endophytic fungi isolated from five medicinal plants grow in Sudan: *Vernonia amygdalina* Del. (Asteraceae), *Calotropis procera* Ait. (Asclepiadaceae), *Catharanthus roseus* L. (Apocynaceae), *Euphorbia prostrata* Ait. (Euphorbiaceae), and *Trigonella foenum-graecum* L. (Fabaceae). These plants had different uses in folk medicin in Sudan and some

African contries including cancer (Haider Abdalgader, Personal communication, 2010; Steenkamp, 2003). They have been selected for three major reasons. They grow in an adverse environment in Sudan where the climate is arid and they are employed by traditional remedies in Sudan as anticancer plants Their extracts have shown some biological activities including antiproliferative activity and antioxidant potential (Kenny *et al*., 2003; Amin *et al*; 2005; El Ghazali, 2007; Gesham *et al*., 2008; Pigares and Narendhirakannan, 2013).

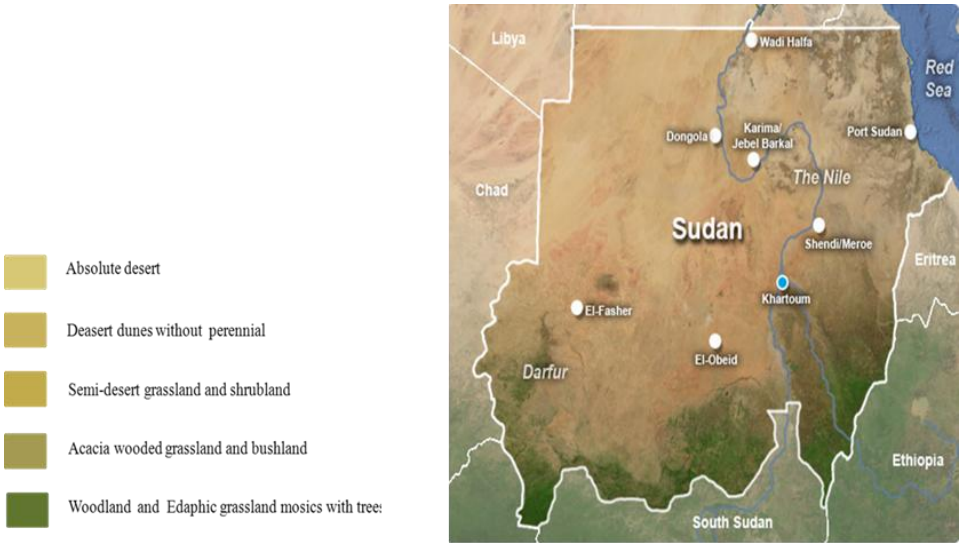


Figure I. Sudan vegetation cover (Wikipedia, 2011)

II. Statement of the problem

One significant problem associated with natural product drug research that nature only produces a relatively small amount of these phytochemicals. For example, it is estimated that 38,000 yew trees must be harvested to generate 25kg of Taxol to treat 12,000 patients. The sacrifice of a 100-year old tree yielding about 3 kg of bark containing some 300 mg of paclitaxel is required to obtain approximately a single cancer chemotherapy drug dose (Cragg and Snader, 1991). The endophytes could be an alternative solution, because some of them can produce same compounds as their host plants, then the active compounds might be produce *in vitro*. This could lead to an increase of the productivity within few weeks instead of years as with the medicinal plants. Furthermore, we can save the environments and the biodiversity, in addition to the decrease of drug prices.

III. The Objectives of the Study

As part of our ongoing efforts towards finding novel anticancer and antibacterial agents from natural resources we investigated, for the first time, the endophytic fungi flora of the five selected Sudanese medicinal plants, and the potentiality of being a resource of novel active compounds. This objective was achieved through these steps:

- Isolation of endophyte fungi from five Sudanese medicinal plants, which are traditionally employed plants in Sudan and some African countries for antitumor activity.
- Biological screening for cytotoxic, antioxydant, and antibacterial activities of ethyl acetate fungal culture extracts
- Verification of the endophyte strains of fungi which will contain bioactive agents.
- Isolation, separation and identification of the active compounds produced by endophytic fungus isolates.
- Confirmation of the biological activity of the isolated pure compounds.
- Improvement and optimization of culture techniques and conditions in order to increase the productivity of the desired compounds.

IV. The Significance of the Study

This study had provided the first report on anticancer and antibacterial agent from fungal endophytes of five medicinal plants from Sudan. The significance of this work is to study the mycoflora from native Sudanese medicinal plants which are traditionally employed in Sudan and some African countries for antitumor activities. This work provided the best opportunities of isolation of novel bioactive products which could be reliable, economical and environmentally safe, as well as isolation of new fungal species.

V. The Organization of the Study

This study is presented in three major chapters. Firstly, general introductions, presenting the general back ground about endophytic fungi of medicinal plants and their potentiality for producing active agents. Then, the statement of the problem, followed by the purpose of the study, the significance of the study, research design and methodology, and the organization of the study are successively presented.

Chapter one is a literature review divided into 8 major reports: introduction to endophytic fungi, classification of endophytic fungi, fungi as source of bioactive compounds, anticancer compounds from fungi, anticancer, antioxidant, antimicrobial compounds from endophytic fungi, and finally the five selected medicinal plants.

Chapter two is about Materials and Methods which are ordered into 7 major divisions: collection, identification and authentication of the medicinal plants, isolation of endophytic fungi, identification of endophytic fungi, cultivation of endophyte strains, preparation of ethyl acetate extracts from endophytic fungi, biological assays and finally phytochemistry of the selected endophyte.

Chapter three consists of results and discussions part, which is divided into four sections: section one, taxonomy of endophyic fungi, morphological and molecular identification of the endophytes. Section two: organoleptic properties and extractive value of endophytes fungal crude extracts and preliminary screening of chemical constitute of the 5 medicinal plants and their endophytes by TLC. Section three: chemical and biological assay for endophytic fungi crude extracts and their host plants (general evaluation of antioxidant capacity, cytotoxicity, and antibacterial). Section four: *Curvularia papendorfii* endophytic fungus of *Vernonia maygdalina*, this section contains phytochemistry, isolation and characterization of pure compounds beside their biological activity. Finally, *Curvularia papendorfii* medium optimization for the production of the active compound **AFB** is reported**.**

A conclusion and perspectives were written to recap all the findings of this study. The references and appendix section were added at the end of the thesis.

Chapter One

Literature Review

Chapter One

1. Literature Review

1.1. Introduction to endophytic fungi

The term "endophyte" is derived from the Greek, *endon* = within and *phyte* = plant. It was first introduced in 1866 by de Bary. It was used broadly to refer to any organism found within tissues of living plants; including everything from virulent foliar pathogens to mycorrhizal root sombionts; subsequent re-definitions led to confusion regarding the meaning of the term. Modern mycologists generally agree that endophytes (Fig. 1) are organisms that colonize internal plant tissues without causing apparent harm to their host. Different groups of organism such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants (Arnold, 2007).

Collectively, more than 100 years of research suggest that most, if not all, plants in natural ecosystems are symbiotic with mycorrhizal fungi and/or fungal endophytes (Petrini, 1986). Unlike mycorrhizal fungi that colonize plant roots and grow into the rhizosphere, endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves, emerging to often occur sparsely as hypha in the intercellular fluids and wall spaces of their plant hosts, sporulate at plant or host-tissue senescence (Bacon and White, 2000). Studies of endophytic fungi were initiated nearly 200 years ago, when Person in 1772 described the species *Sphaeria typhena*, now known as *Epichloe typhina* (Pers.) Tul. (Khan, 2007). Fossils, in a 400-million-year-old, indicated that plants have been associated with endophytes. Krings *et al*. (2007) studied petrographic thin sections of the Rhynie chert plant *Nothia aphylla*, they found that three fungal endophytes occur in prostrata axes of this plant.

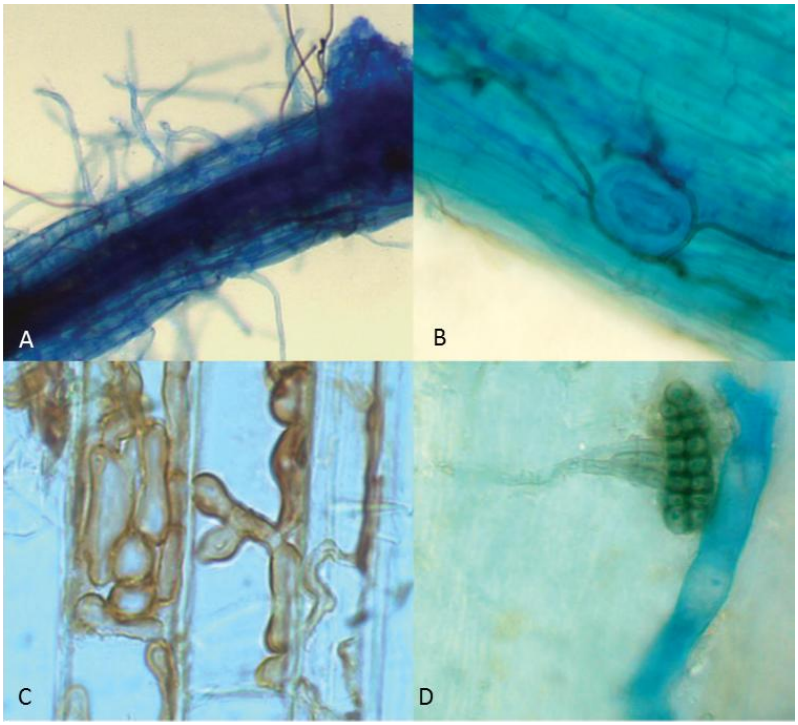


Figure 1. Dark septate endophytes (DSEs). (a) Stained root section of the grass *Bouteloua gracilis* showing internal and extraradical hyphae; (b) DSE hyphae encircling a mycorrhizal vesicle (AMF) in a *Geum rossii* root; (c) hyphal proliferation in plant cells; and (d) germinating DSE spore in *G. rossii* root. (Porras-Alfaro and Bayman, 2011).

1.1.1. Relationships between fungal endophytes and their host plants

A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic (Arnold, 2007) (Fig. 2). Results from grass-endophyte systems suggest that endophytes are herbivore antagonists and enhance plant growth (Clay, 1990). Correspondingly, mutualistic antagonism towards insects and pathogens has been claimed also for forest endophytes (Faeth, 2002).

A review of the literature suggests that a significant number of fungi exhibit multiple ecological roles, such as the human pathogen and soil saprotroph *Coccidioides posadasii*. Similarly, fungi such as *Chaetomium globosum* are known as endophytes, saprotrophs, and pathogens (Arnold and Engelbrecht, 2007). Although it is not yet clear whether the same genotypes can play each of these roles with equal success, the ecological lability of these species is remarkable. Understanding the mechanisms behind that lability represents one among many frontiers in endophyte biology (Anrold, 2007).

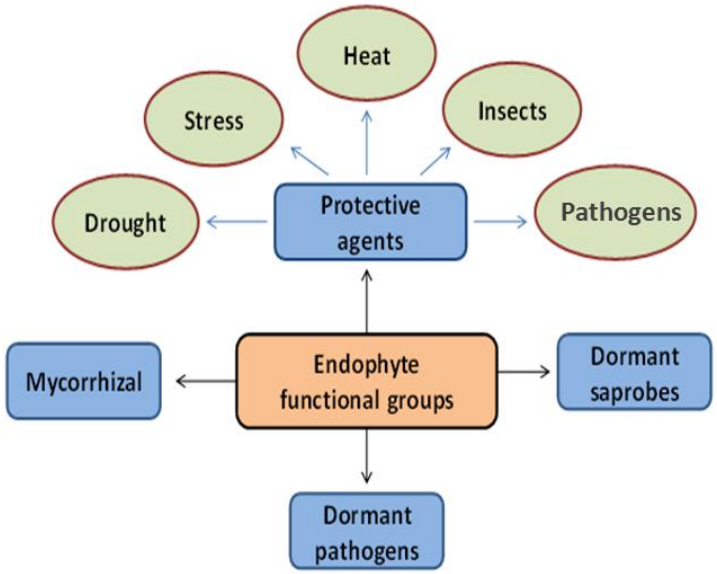


Figure 2. The mycobiome. As part of a fungal community, endophytic fungi may have one or multiple functional roles during their life cycles or in response to plant or environmental cues (Porras-Alfaro and Bayman, 2011).

1.1.2. Diversity of endophytic fungi host plants

Endophytic fungi have been recovered from plants in hot deserts, Arctic tundra, mangroves, temperate and tropical forests, grasslands and savannas, and croplands. They are known from mosses and other nonvascular plants, ferns and other seedless plants, conifers, and flowering plants. Their biological diversity is enormous, especially in temperate and tropical rainforests. The fungi are hosted in nearly 300,000 land plant species, with each plant hosting one or more of these fungi (Arnold, 2008)

1.1.3. Occurrence of endophytes within the host plants tissues

The relationships of endophytes with single or multiple plant host can be described in terms of host-specificity, host-recurrence, host-selecticity or host-preference (Zhou and Hyde, 2001).

Host-specificity is the relationship in which a fungus is restricted to a single host or a group of related species, but does not occur in other unrelated plants in the same habitat (Holliday, 1998). The frequent predominant occurrence of an endophytic fungus on a particular host or a range of plant host is often defined as host recurrence, but the fungus can

also occur infrequently on other host plants in the same habitat (Zhou and Hyde, 2001). Bagchi and Banerjee (2013) studied the tissue specificity symbiosis; they isolated endophytic fungi from leaf, petiole and stem of *Bauhinia vahlii*. They found that, the colonization frequency of endophytic fungi is much higher in petiole (86.67%) in comparison to stem (77.33%) and leaf (70.67%). Whereas, some researchers reported that endophytic fungal colonization is higher in leaf segments rather than stem segments of some tropical medicinal plants (Raviraja, 2005; Banerjee and Mahapatra, 2010).

The phenomenon, which is categorized as host-selectivity is a one endophytic fungal species, may form relationships with two related plant species, but demonstrate a preference for one particular host (Cohen, 2006). The term host-preference, however, is more frequently used by mycologists to indicate a common occurrence or uniqueness of the occurrence of a fungus on a particular host. The differences in endophyte assemble from different hosts might be related to the chemical differences of the host (Paulus *et al*., 2006).

1.1.3.1. Effect of climate on endophytic population

Chareprasert *et al.* (2006) studied the leaves of two different plants which were collected during January to December for investigation of seasonal effects. They found that the lower number of isolates recovered from trees during the dry season indicated that environmental factors, such as rainfall and atmospheric humidity might influence the occurrence of some endophytic species. Rodrigues (1994) suggested that the lower number of isolates recovered during the dry season could be related to the effects of water stress. It is known that under water deficit, some plants may accumulate non-structural carbohydrates. This accumulation generally leads to build up of carbon-based defences such as tannins, making the plant less susceptible to fungal endophyte colonization during the dry season.

1.2. Classification of endophytic fungi

Schaechter (2011) stated that endophytic fungi have frequently been divided into two major groups based on differences in taxonomy, host range, colonization transmission patterns, tissue specificity and ecological function. Group one is the clavicipitaceous endophytes (C-endophytes) which infect some grasses. Group two is the nonclavicipitaceous

endophytes (NC-endophytes). Rodriguez *et al*. (2008) stated another point of view of the classification of the endophytic fungi, they classified them into four classes.

1.2.1. Clavicipitaceous endophytes (Class I)

The Clavicipitaceae is a family of fungi (Hypocreales; Ascomycota) including free living and symbiotic species associated with insects and fungi or grasses, rushes and sedges (Bancon and White, 2000). Many of its members produce alkaloids toxic to animals and humans. Clavicipitaceous endophytes of grasses were first noted by European investigators in the late 19th century in seeds of *Lolium temulentum*, *Lolium arvense*, *Lolium linicolum*, and *Lolium remotum* (Guerin, 1898; Vogl, 1898). From their earliest discovery, investigators hypothesized a link to toxic syndromes experienced by animals that consume infected tissues. And these hypotheses were tested when Bacon *et al.* (1977) linked the endophyte *Neotyphodium coenophialum* to the widespread occurrence of 'summer syndrome' toxicosis in cattle grazing tall fescue pastures (*Festuca arundinacea*).

Mycelium of clavicipitaceous endophytes occurs in intercellular spaces of leaf sheaths, culms, and rhizomes, and may also be present, if sparsely, on the surface of leaf blades (White *et al*.,1996; Moy *et al*., 2000; Dugan *et al*., 2002; Tadych *et al*., 2007).

1.2.1.1. The effects of clavicipitaceous endophytes on host plant

- **Insects deterrence**

Most clavicipitaceous endophytes enhance resistance of hosts to insect feeding; the benefits arise in part from the production of alkaloidic mycotoxins loline and peramine which are generally associated with resistance to insects (Rowan and Gaynor, 1986; Clay, 1990; Patterson *et al*., 1991; Riedell *et al*., 1991).

- **Mammlian herbivores deterrence**

Some clavicipitaceous endophytes have been reported to deter feeding by mammalian herbivores, because they produced mycotoxins such like ergot and lolitrem alkaloids (White, 1987; Gentile *et al*., 1999).

- **Reduction of nematodes**

Also some studies indicated that clavicipitaceous endophytes had anti-nematode activity; Kimmons *et al*. (1990) stated that infection of tall fescus (*Festuca arundinacea*) with an endophytic fungus (*Acremonium coenophialum*) has been shown to reduce nematode population's in field soils.

- **Increase resistance of host disease**

Some studies indicated that clavicipitaceous endophytes produced indole derivative compounds, a sesquiterpene, and a diacetamide from *Epichloë festucae*, that inhibit the growth of other pathogenic fungi (Yue *et al*., 2000; Lee, 2010).

- **Enhance the ecophysiology of host plants**

Clavicipitaceous endophytes enhance the ecophysiology of host plants and enable plants to counter abiotic stresses such as drought (Arechavaleta *et al.*, 1989) and metal contamination. For example, *Neothyphodium coenophialum* infection leads to the development of extensive root systems that enable plants to better acquire soil moisture and absorb nutrients, resulting in drought avoidance and faster recovery from water stress. In some cases, endophytes stimulate longer root hairs and enhance exudation of 'phenolic-like compounds' into the rhizosphere, resulting in more efficient absorption of soil phosphorus and enhanced aluminum tolerance via chelation (Malinowski and Belesky, 2000).

1.2.2. Nonclavicipitaceous endophytes (Class II)

Traditionally NC-endophytes treated as a single functional group but Rodriguez *et al*. (2008) who showed that NC-endophytes represent three distinct functional groups based on host colonization and transmission *in planta* biodiversity and fitness benefits conferred to hosts.

Class II endophytes include the hyperdiverse endophytic fungi associated with leaves of tropical trees (Lodge *et al*., 1996; Fröhlich and Hyde, 1999; Arnold, *et al*., 2000; Gamboa and Bayman, 2001), as well as the highly diverse associates of above-ground tissues of nonvascular plants, seedless vascular plants, conifers, and woody and herbaceous angiosperms in biomes ranging from tropical forests to boreal and Arctic/Antarctic communities (Carroll and Carroll, 1978; Petrini, 1986; Stone, 1988).

Most fungal endophytes species belong to Ascomycetes, with a minority of Basidiomycetes. Fungal group 'dark septate endophytes' (DSE) are distinguished as a functional group based on the presence of darkly melanized septa.

1.2.2.1. The effects on host plant

- **Avoiding abiotic stress**

One attribute that appears unique to Class II NC-endophytes is the ability of individual isolates to asymptomatically colonize and confer habitat-adapted, fitness benefits on genetically distant host species representing monocots and eudicots (Rodriguez *et al*., 2008). This phenomenon was discovered by comparing fitness benefits conferred by Class II endophytes in plants growing in geothermal soils (*Curvularia protuberata*) coastal beaches (*Fusarium culmorum*) and agricultural fields (*Colletotrichum* spp.) (Redman *et al*., 2002; Márquez *et al*., 2007)

- **Increase of biomass**

Most of class II endophytes examined have increased host shoot and/or root biomass. Tudzynski and Sharon (2002) stated that this was a result of the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi.

- **Protection from fungal pathogens**

Many of class II endophytes protect hosts to some extent against fungal pathogens (Danielsen and Jensen, 1999; Narisawa *et al*., 2002; Campanile *et al*., 2007). By different strategies for example, production of secondary metabolites (Schulz *et al.,* 1999), few studies revealed interactions with host defenses; fungal parasitism (Samuels *et al*., 2000); induction of systemic resistance (Vu *et al*., 2006); or to compete with endophytes for resources or niche space.

1.3. Endophytes versus epiphytes

Endophytes are often contrasted with epiphytes, which live on external plant surfaces (Santamarı´a and Bayman, 2005). In practice, the distinction is that epiphytes can be washed off plant surfaces or be inactivated by surface disinfection, usually with sodium hypochlorite

and ethanol to break surface tension, whereas endophytes cannot. Thus, an epiphyte that survives surface disinfection and grows in culture might be assumed to be an endophyte (Arnold and Lutzoni, 2007). Although there are few studies comparing phylloplane and endophytic fungal communities of the same leaves, comparisons within pine and coffee leaves indicate that endophytic communities are distinct from epiphytic ones, even though they may live less than a millimeter apart (Santamarı´a and Bayman, 2005). Temporally as well as practically, the distinction between endophytes and epiphytes is often arbitrary. Many horizontally transmitted endophytes presumably start growing on the surface of the leaf before penetration. Also, endophytes may become epiphytes when internal tissues are exposed, and may protect the exposed tissues from the environment. In shoot tip–derived tissue cultures of *Pinus sylvestris*, calli were found to be covered by hyphae of the endophytes *Hormonema dematioides*, *Rhodotorula minuta*, and associated biofilms (Pirttila *et al*., 2002). How such endophytes coordinate function, interact with other microbiome biofilm components, and affect plant fitness needs further exploration.

1.4. Fungi as source of bioactive compounds

1.4.1. Fungal metabolites

Fungal metabolites are many and diverse. In addition, to those associated with proteins synthesis and respiration, many special secondary metabolites have been isolated and, frequently, chemically defined. Some of these are waste products while others such as pigments, toxins, and antibiotics clearly have biological function. Because of their synthetic abilities, fungi are used in industry for the production of alcohol, citric acid and other organic acids, various enzymes, riboflavin, etc (kirk *et al.*, 2008).

1.4.2. Bioactive compounds from endophytic fungi

Through 12 years studying endophyte metabolites, Schulz *et al.* (2002) found a correlation between biological activity of fungal metabolites and biotope. They reported a higher proportion of the endophytic fungi exhibited biological activity than the soil isolates did; whereas 83% of the algal isolates and 80% of endophytic fungi from plants inhabited at least one of the test organisms for antibacterial, fungicidal, algicidal or herbicidal activities,

only 64% of those from soil did. Also they had isolated compounds belonged to diverse structural groups: terpenoids, steroids, xanthones, chinones, phenols, isocoumarines, benzopyranones, tetralones, cytochalasines and enniatines.

1.4.3. Some type of isolated natural products from endophytic fungi

1.4.3.1. Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. Alkaloids are quite common secondary metabolites in endophytes, and some of them showed also anti-microbial activities (Souza *et al*., 2004). It has been of great interest that some of the most potent of these plant-derived antitumor alkaloids have also been reported as isolates from endophytic fungi. These endophytes have usually been associated with a host organism that has also been reported to produce the compound of interest. Camptothecin (CPT) was isolated in 2005 from a fungal endophyte isolated from the inner bark of *Nothapodytes foetida* identified as *Entrophosphora infrequens* (Puri *et al*., 2005). Vincristine (Oncovin®), also known as leurocristine, is a vinca alkaloid originally isolated from *Catharanthus roseus* It has been isolated by different researchers from the *Catharanthus roseus* endophyte *Fusarium oxysporum* (Zhang *et al*., 2000). Chaetoglobosin U is a cytochalasin-based alkaloid isolated from *Chaetomium globosum*, an endophytic fungus residing within the stem of healthy *Imperata cylindrical* (Ding *et al*., 2006). Chaetoglobosins A and C were characterized from the culture of an endophytic *Chaetomium.globosum* isolated from the leaves of *Ginkgo biloba* with antibacterial activity (Fig. 3).

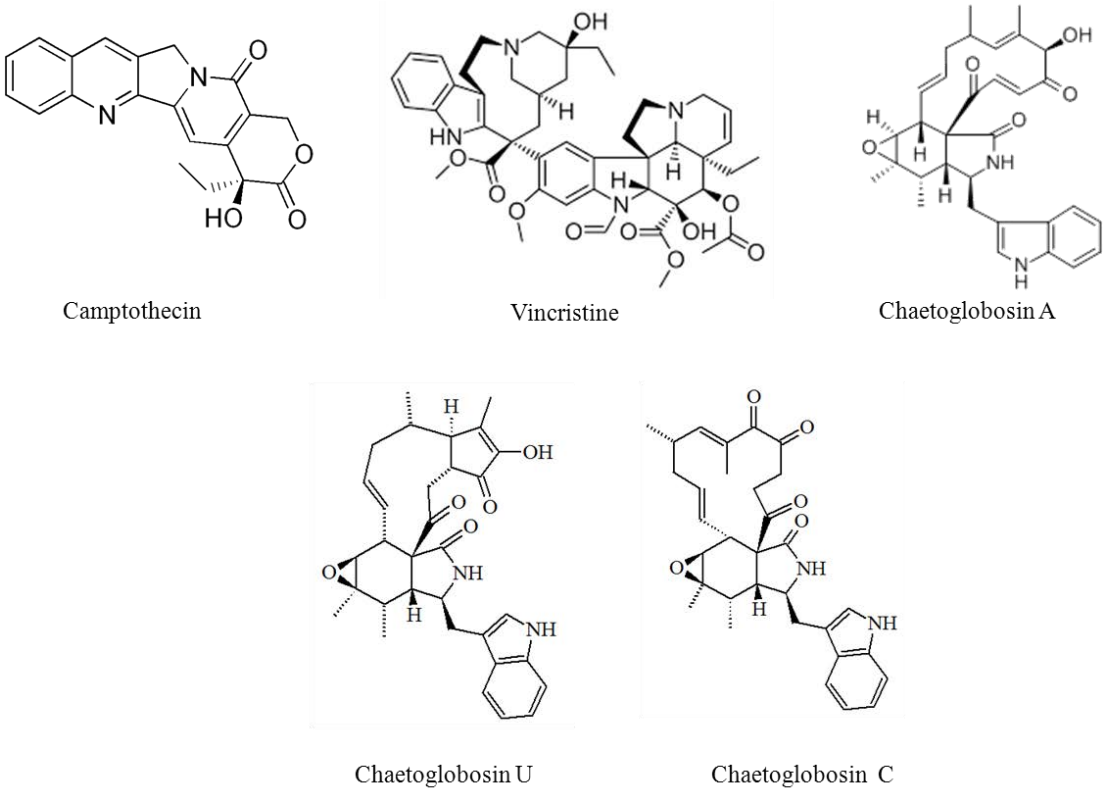


Figure 3. Some alkaloids isolated from endophytic fungi.

1.4.3.2. Phenols

Phenols and phenolic acids have often been isolated from some endophyte cultures originating from a variety of the host plants (Yu *et al*., 2010). Pestalachloride A and B had a significant anti-fungal activity against three plant pathogens (Li *et al.*, 2008). Pestalachloride C and D showed moderate antibacterial activity. Furthermore, two isomeric novel tridepsides cytonic acids A and B were reported as human cytomegalovirus (an ubiquitous opportunistic pathogen) protease inhibitors from the culture of the endophytic fungus *Cytonaema* sp. isolated from *Quercus* sp. Tricin and related flavone glycosides 1 to 3 were toxic to mosquito larvae, have been isolated from endophyte-infected blue grass *Poa ampla* (Tan and Zou, 2001). Two antimicrobial flavonoids were isolated from the culture extract of endophytic fungus *Nodulisporium* sp. from *Juniperus cedre* on Gomera Island (Dai *et al*., 2006) (Fig. 4).

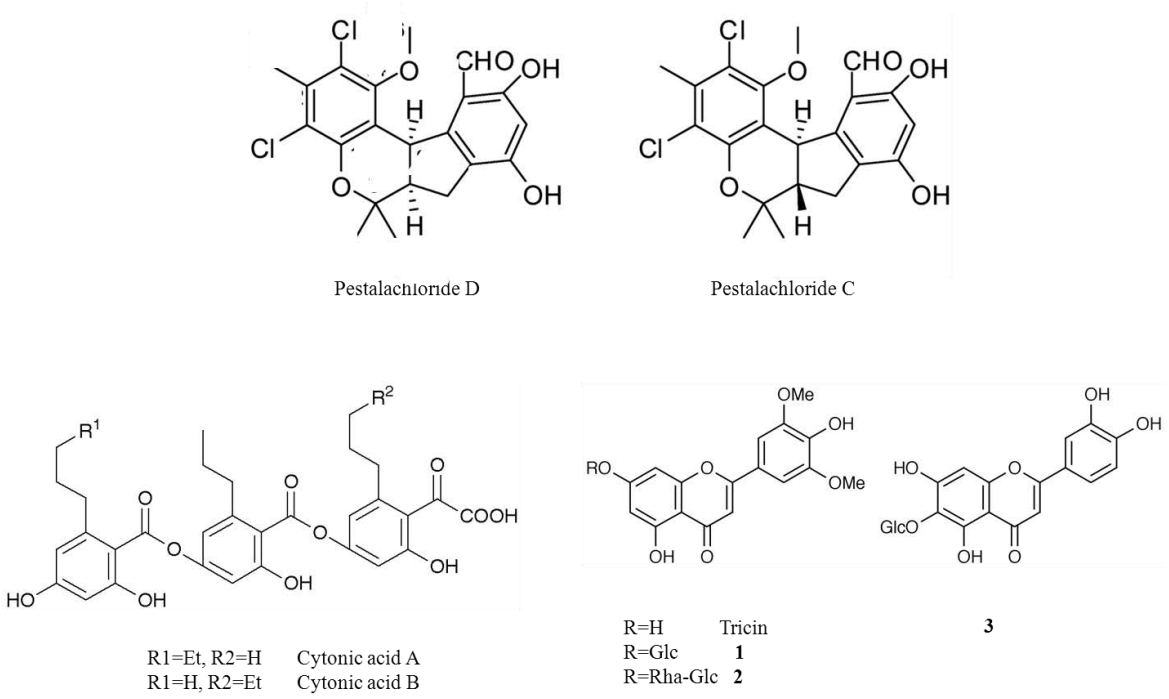


Figure 4. Some phenols isolated from enfophytic fungi.

1.4.3.3. Steroids

Many steroids are produced by endophytes, but most of the isolated compounds showed moderate antimicrobial activities. Along with ergosterol, 3β,5α,6β-trihydroxyergosta-7,22-diene **1**, 3β-hydroxyergosta-5-ene **2**, 3-oxoergosta-4,6,8(14), 22-tetraene **3**, 3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene **4**, 3β-hydroxy-5α,8α-epidioxyergosta-6,9(11),22-triene **5** and 3-oxoergosta-4-ene **6**, two new steroids, 3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene **7** and 3β,5α-dihydroxy-6β-phenyl- acetoxyergosta-7,22-diene **8** were characterized from the liquid culture of an fungal endophyte *Colletotrichum* sp. of *Artemisia annua*. Metabolites **3**, **4**, **7** and **8** were shown to be antifungal against some crop pathogens *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum* and *Phytophthora capisici* (Yu *et al*., 2010) (Fig. 5).

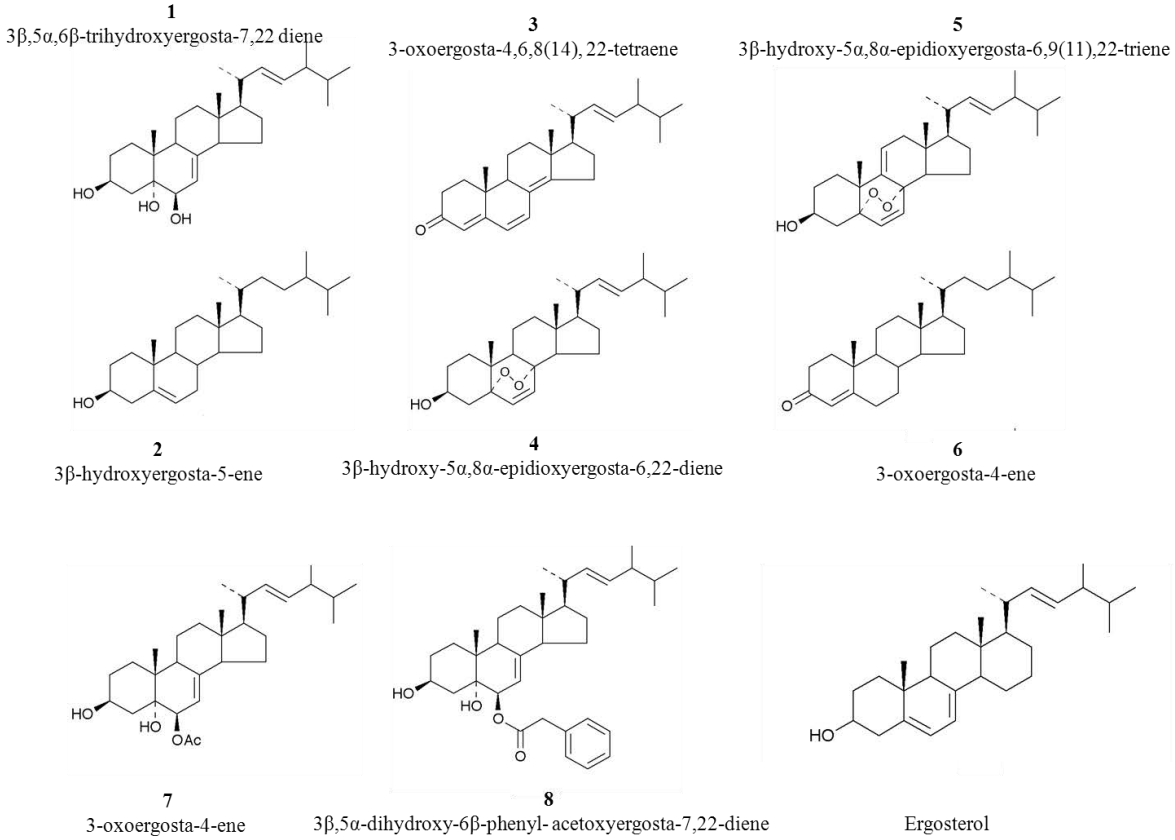


Figure 5. Some steroids isolated from endophytic fungi.

1.4.3.4. Terpenoids

Sesquiterpenes, diterpenoids and triterpenoids are the major terpenoids isolated from endophytes (Yu *et al*., 2010). In period of 2006 to 2010, sixty five sesquiterpenes, fourty five diterpenes, five monoterpenes and twelve other terpenes, amounting to 127 terpenoids were isolated from endophytic fungi and all have biological activity such as anti-microbial, anti-cancer and anti- protozoa (Souza *et al*., 2004).

1.4.3.4.1. Sesquiterpenes

Three novel eremophilane-type sesquiterpenes were isolated from the endophyte *Xylaria* sp. associated with *Licuala spinosa*. The three compounds, eremophilanolide 1, 2 and 3 exhibited moderate cytotoxic activity with IC_{50} values of 3.8–21 mM against cancer cell lines KB, MCF-7, and NCI-H187 (Isaka *et al.,* 2010). A new eudesmane sesquiterpene, *ent*-4(15)-eudesmen-11-ol-1-one, was isolated from the endophytic fungus *Eutypella* sp. associated with *Etlingera littoralis* (Earth ginger). Four cytotoxic sesquiterpene compounds, 8-deoxytrichothecin, trichothecolone, 7α-hydroxytrichodermol and 7α-hydroxyscirpene, were isolated from fungal isolate KLAR 5, a mitosporic Hypocreales found in a healthy twig of the

Thai medicinal plant *Knema laurina*. Also, tauranin, merulin A and C were sesquiterpene compounds isolated from endophytic fungi with cytotoxic activity (Kharwar *et al*., 2011) (Fig 6).

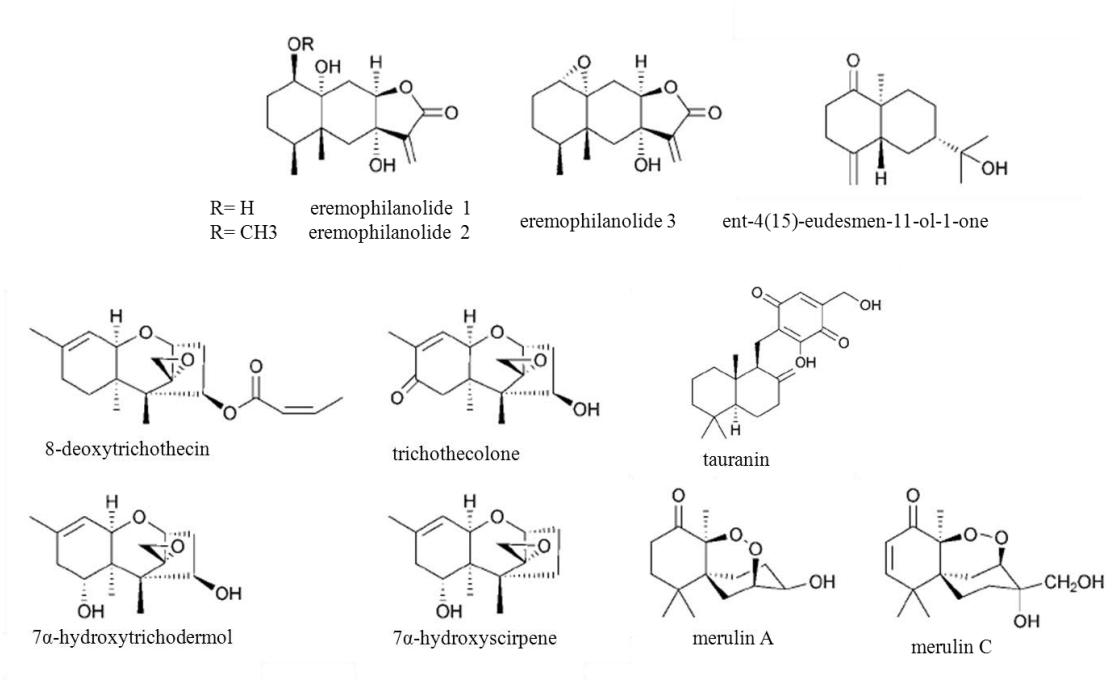


Figure 6. Some sesquiterpenes isolated from endophytic fungi.

1.4.3.4.2. Diterpenes

Two new insect toxins **1** and **2** of a pimarane diterpene framework were isolated from the broth of an unidentified endophyte from a needle of the balsam fir *Abies balsamea*. Subglutinol A and B, immunosuppressive but noncytotoxic, were produced by *Fusarium subglutinans*, an endophytic fungus from the perennial twining vine *Tripterygium wilfordii*. Guanacastepene, a novel diterpenoid produced by an unidentified fungus from the branch of *Daphnopsis americana* growing in Guanacaste, Costa Rica, was shown to be antibacterial against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Tan and Zou, 2001).Taxol originally characterized from the inner bark of the Pacific yew, *Taxus brevifolia*, is an efficacious anticancer diterpene found in extremely small quantities in slowly growing *Taxus* species (Stirele *et al*., 1993) (Fig. 7).

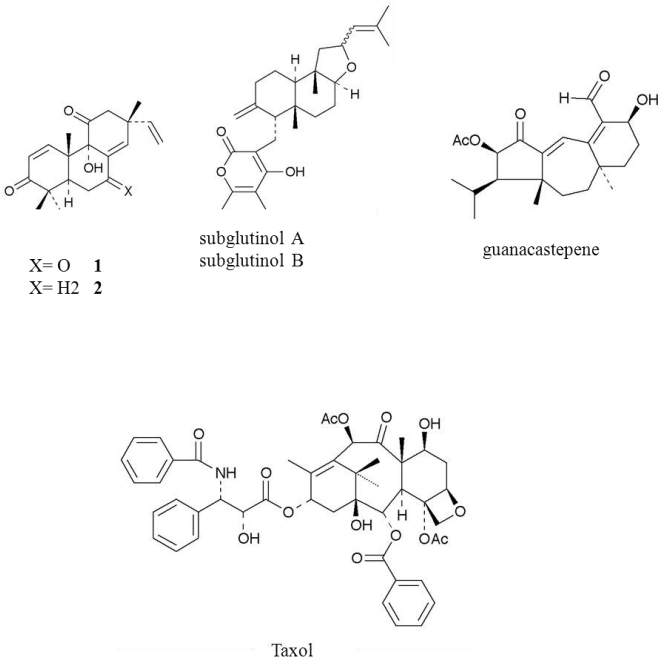


Figure 7. Some diterpenes isolated from endophytic fungi.

1.4.3.5. Quinones

Some endophytes produced quinones displaying significant growth inhibition against phytopathogens such as spiroketals from *Edenia gomzpompae*. Torreyanic acid is an unusual dimeric quinone isolated from *Pestalotiopsis microspora*, an endophyte of *Torreya taxifolia* (Lee *et al*., 1996). Insecticidal rugulosin was characterized from *Hormonema dematioides*, an endophytic fungus of balsam fir. From cultures of an unidentified endophyte obtained from an eastern larch (*Larix laricina*) needle, 8,1',5'-trihydroxy-3',4'dihydro-1'*H*-[2,4']binaphthalenyl-1,4,2'-trione was characterized as a toxin to spruce budworm larvae. Preussomerin N_1, palmarumycin CP_{4a}, and palmarumycin CP_5 were new ras farnesyl-protein transferase inhibitors produced by an endophytic *Coniothyrium* sp. (Tan and Zou, 2001). A highly hydroxylated quinone altersolanol A, characterized from phytopathogenic *Alternaria* spp., was reisolated from an endophytic *Phoma multirostrata* with its antibacterial activity disclosed (Yang *et al*., 1994). (Fig. 8).

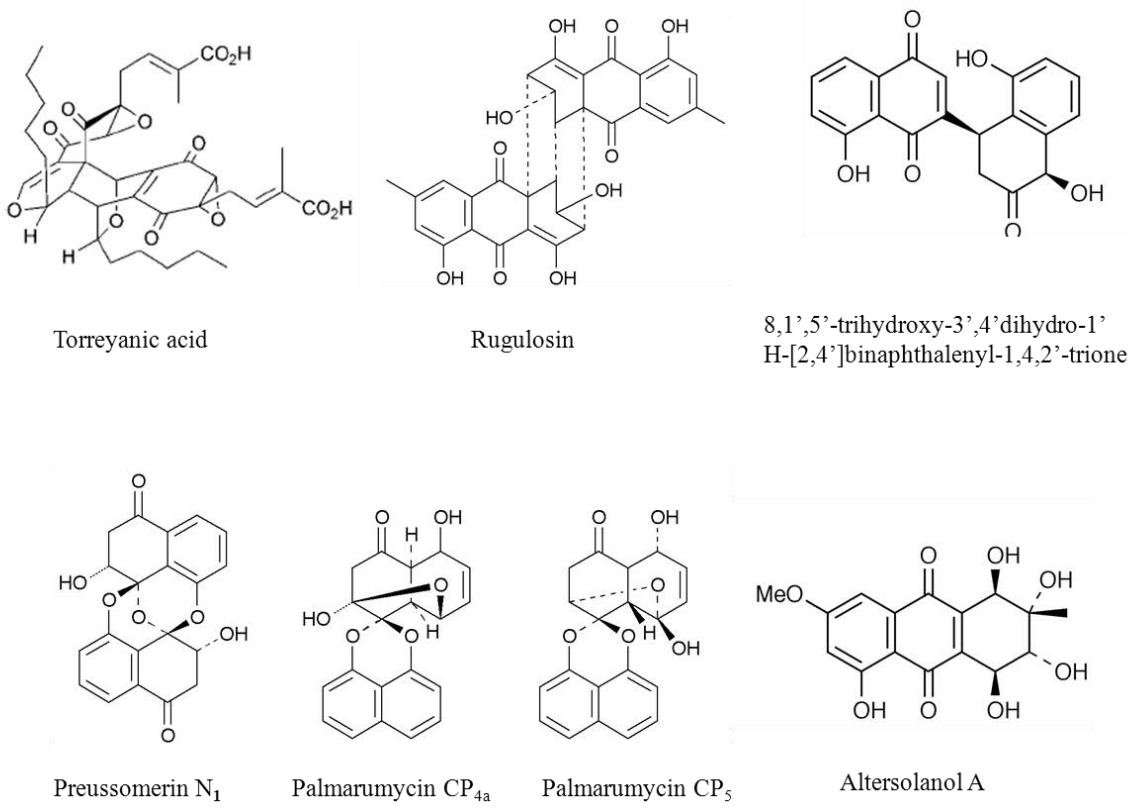
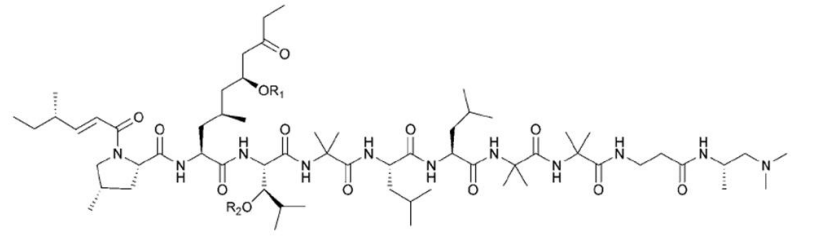


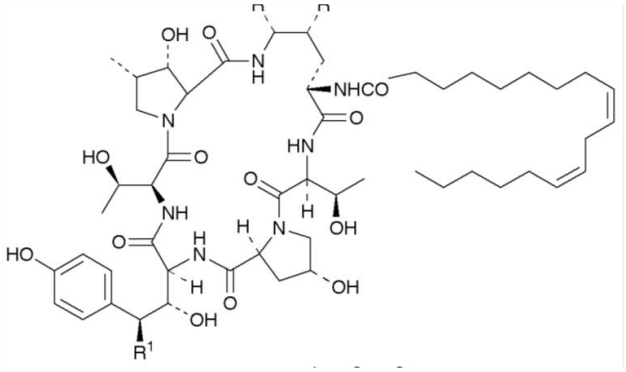
Figure 8. Some quinones isolated from endophytic fungi.

1.4.3.6. Peptides

Many peptides produced by endophytes displayed significant antimicrobial activities, such as Leucinostatin A produced by *Acremonium* sp. (Strobel *et al*., 1997a). Leucinostatin A was isolated almost forty years ago from cultures of *Penicillium lilacum*. It has received much attention over the years because of its potent biological activity against several different cell lines. *Acremonium* sp., isolated from *Taxus baccata,* was also shown to produce Leucinostatin A and leucinostatin A di-O-b-glucoside when grown in liquid culture (Kharwar *et al.*, 2011). The cyclopeptides echinocandins A, B, D and H, produced by *Aspergillus rugulosus* and *A. nidulans* var. *echinulatus*. Further they were isolated from endophytes *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus sylvatica* respectively, and shown to be antimicrobial. Cryptocandin, a cyclopeptide with potent antifungal activities, is a metabolite of endophyte *Cryptosporiopsis* cf. *quercina* of red wood (Tan and Zou, 2001).



R1=R2=H leucinostatin A
R1=R2= Glucose leucinostatin A di-O-b-glucoside



R1=H. R2 =R 3=OH leucinostatin B
R1=R2= R3=H leucinostatin D
R1=R2= OH, R3= OMe leucinostatin H

Figure 9. Some peptides isolated from endophytic fungi.

Several other compounds belonging to different chemical classes have been reported such as: aldehydes, chromones, cyclohexanones, esters, lactones, xanthones (Kharwar *et al*., 2011).

1.4.4. Effects of plant's environment on the bioactive components of endophytes

The environment of the endophytic fungi inside the plants plays a significant role into their secondary metabolites. Yu *et al*. (2010) screened the presence of endophytes that produce antimicrobial agents into medicinal plants and different plants in special environments frequently. They found that 35% which isolated from the medicinal plants had an antimicrobial activity, while 18% from plants in special environment (Fig. 10).

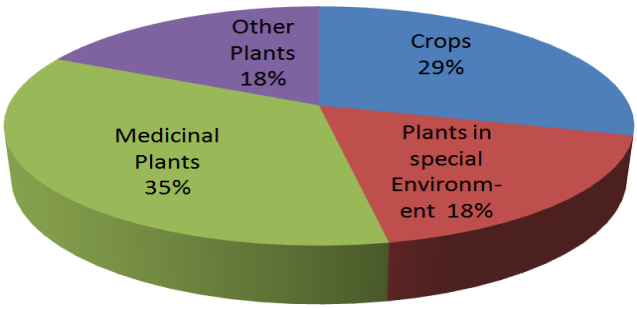


Figure 10. Proportion of biologically active endophytic fungal isolates from different sources with antimicrobial activities. (Yu *et al*., 2010).

1.5. Anticancer agents from fungi

Cancer is a disease characterized by unregulated cell proliferation, and leads to spread of abnormal cells and uncontrolled tissue growth (ACS, 2015).

Fungi produce metabolites belonging to highly diverse structural classes, including aromatic compounds, amino acids anthracenones, butanolides, butenolides, cytochalasans, macrolides, naphthalenones, pyrones, terpenes,… etc. Surprisingly, however, no fungi-derived agent has been approved as an anticancer drug so far, despite the tremendous amount of research aimed at the identification of fungal metabolites with promising anticancer activities (Evidente *et al.,* 2014).

The anticancer metabolites from fungi were classified into three categories by Evidente *et al.* (2014) based on the fungal source from which they are isolated. These include metabolites produced by: (i) phytopathogenic, (ii) toxigenic, and (iii) non-toxigenic fungi.

1.5.1. Phytotoxins produced by phytopathogenic fungi

Many researchers reported several fungal plant pathogens producing phytotoxins with antitumor and anticancer activities.

Cytochalasins are part of a larger group of metabolites named cytochalasans and they incorporate diverse polyketide–amino acid hybrid structures with a wide range of distinctive

biological functions. More than 60 different cytochalasans from several species of fungi have been purified, identified and sub-grouped. And for example not exclusively: Cotylenin A, Fusicoccin A, Ophiobolins, Alternethanoxins A and B, Phyllostictines A–D, Nectriapyrone, Macrosporin, Alternasolanol A, Sphaeropsidin A and related metabolites, such as isopimarane diterpenes, represented by smardaesidin A (Fig.11) (Evidente *et al*., 2014, Lallemand *et al*., 2012)

1.5.2. Metabolites produced by toxigenic fungi

Toxine producing fungi such as *Neosartorya* and *Eurotium* genera are respectively sexual and imperfect stage forms of *Aspergillus* species. Like *Aspergillus*, they produce a variety of mycotoxins, such as gliotoxin and pyripyropenes.

Two metabolites, namely bislongiquinolide and 2′, 3′-dihydrotrichodimerol (bisorbibutenolide) (Fig.11), were found with IC_{50} < 100 μM in six cancer cell lines analyzed. They were isolated from the biomass of fungus *Trichoderma citrinoviride*.

The terpenoid sequiterpene and the trypthopan-derived terpene alkaloid eurochevalierine were isolated from the organic extract of rice culture of *N. pseudofischeri*. Eurochevalierine additionally was obtained from *Eurotium chevalieri* (Kanokmedhakul *et al.*, 2011; Eamvijarn *et al*., 2012).

In addition, 12 compounds displayed weak cytotoxic activity. These 12 (Fig. 12) metabolites include phyllostin, seiricardines B and C, cavoxin, cyclopaldic acid, flufuran, fusapyrone, scytolide, seiricuprolide, seiridin, verrucarin E and 16, 17-dihydrobislongiquinolide (Balde *et al*., 2010).

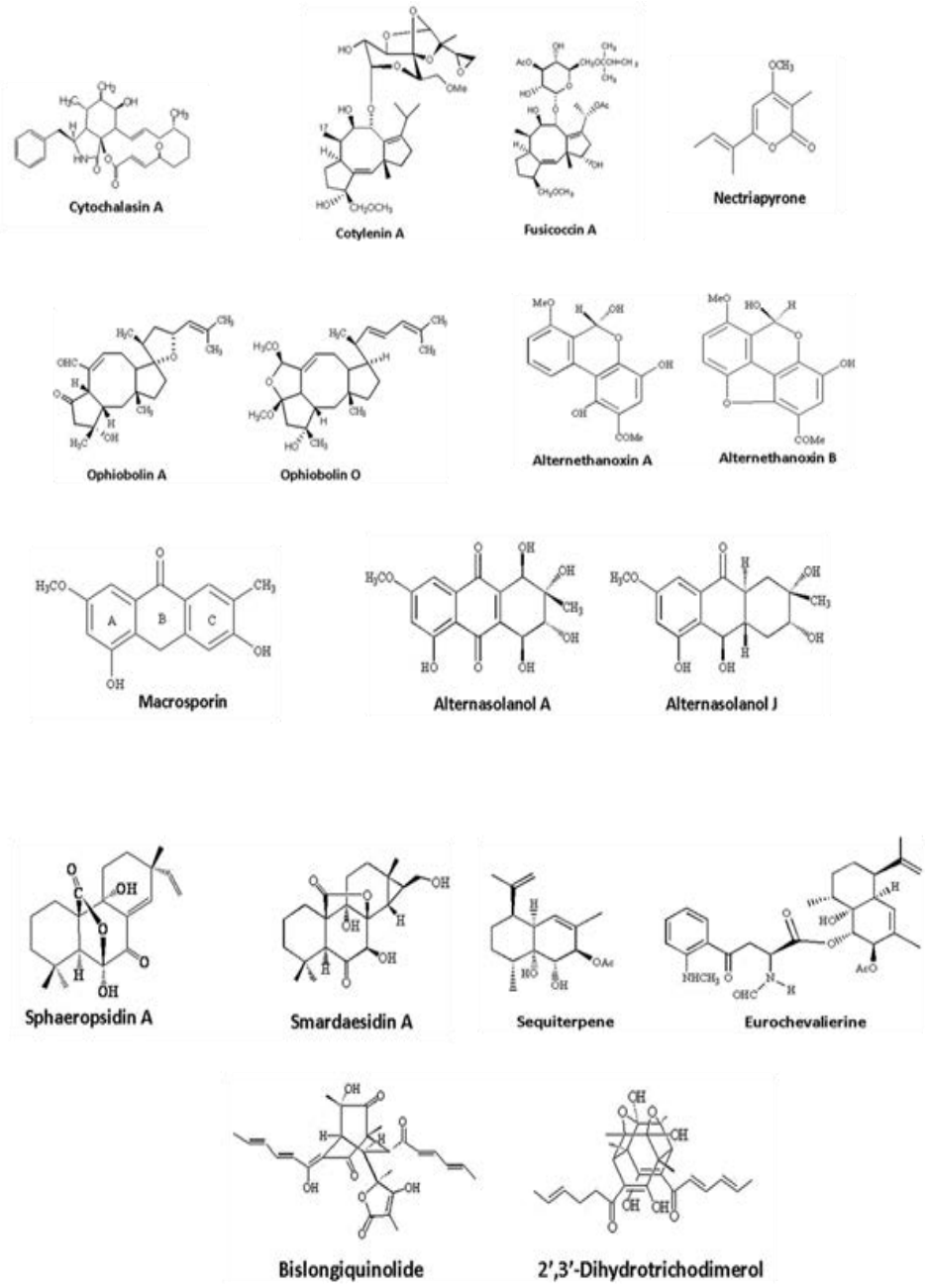


Figure 11. Some phytotoxins as cytotoxic agents from fungi.

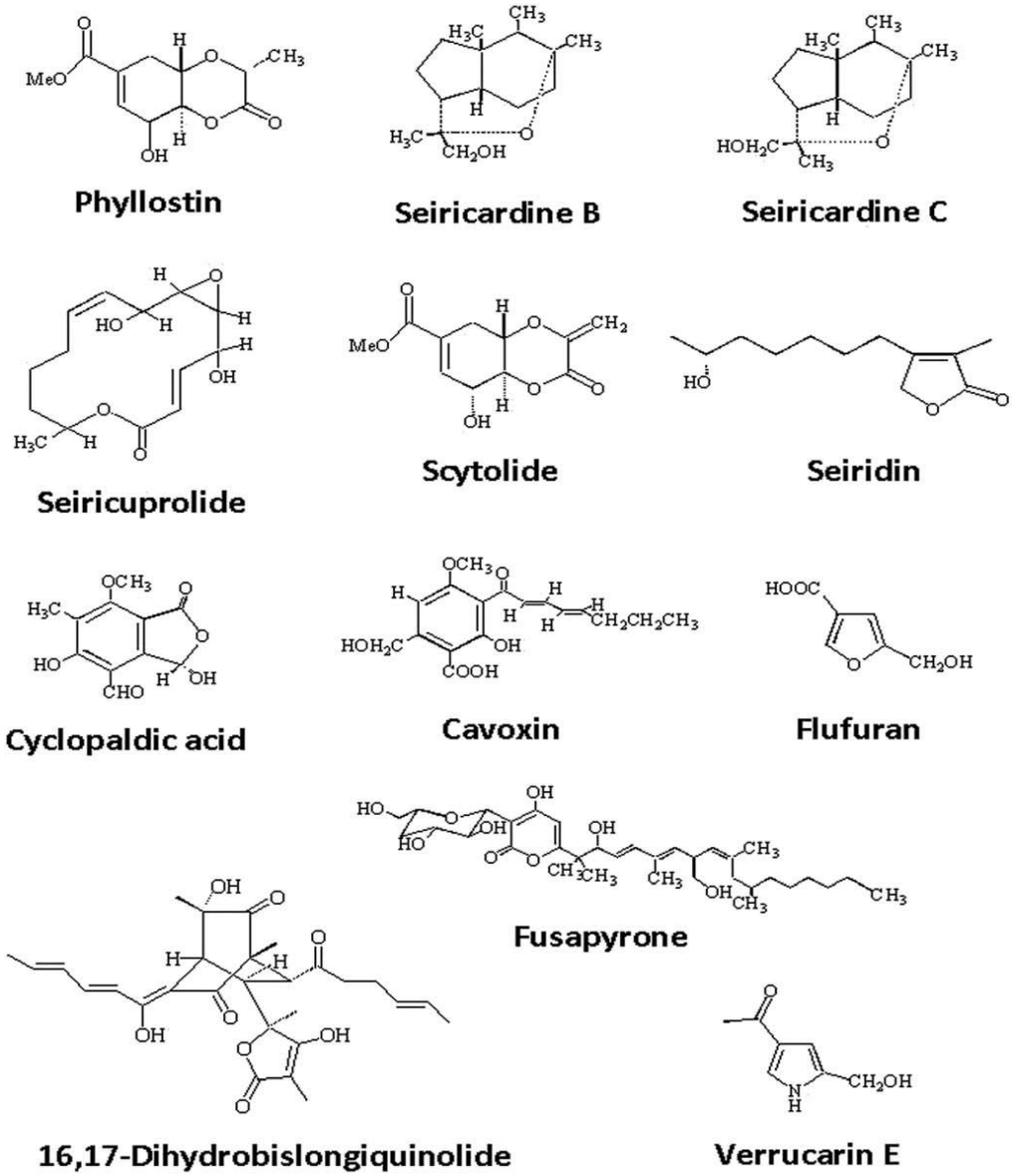


Figure 12. Some cytotoxic compounds isolated from fungi.

1.5.3. Metabolites produced by nontoxigenic fungi

Tryprostatins A and B (Fig.13) are indole alkaloidal fungal products isolated from *Aspergillus fumigatus* (Cui *et al*., 1995). Halenaquinone (Fig.13) was isolated from two Indo-Pacific collections of the sponge *Xestospongia carbonaria*. Additional metabolites isolated from this sponge include tetrahydrohalenaquinone B, 14-methoxyhalenaquinone, xestoquinolide A, xestoquinolide B, halenaquinol, halenaquinol sulfate, xestoquinone, and tetrahydrohalenaquinone A (Alvi *et al*., 1993).

Three epi-oligothiaketopiperazines, named TAN-1496 A, C and E (Fig.13), were isolated together with the known TAN-1496 B and D from the culture filtrates of *Microsphaeropsis* sp. FL-16144, a strain isolated from a soil sample in Ibaragi prefecture, Japan (Funabashi *et al*., 1994).

Added to that Pintulin; gliotoxin, methylthiogliotoxin, perybisin, macrosphelide, fusarisetin A, and oxaspirodion (Fig.13) (Evidente *et al*., 2014).

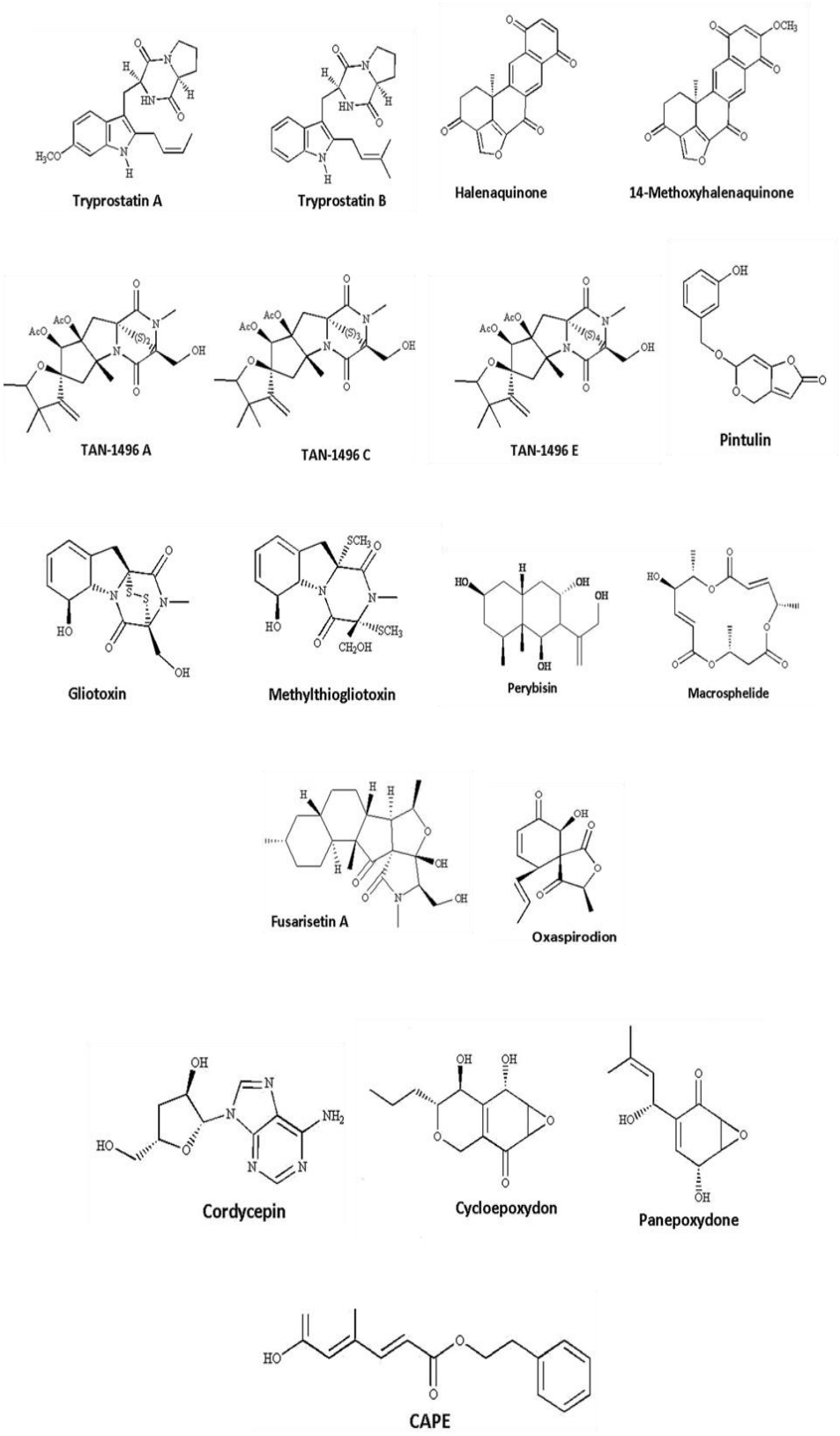


Figure 13. Some cytotoxic compounds isolated from nontoxigenic fungi.

1.5.5. Anticancer agents from endophytic fungi

Endophytic fungi are proving to be prolific producers of anticancer compounds from many different chemical classes. In the past ten years, 100 compounds with significant cytotoxicity were reported from endophytic fungi, and the isolation of anticancer compounds has been increasing over five year intervals – it is interesting to note from 1990–1995, only a single novel anticancer agent was reported from endophytic fungi. The most exciting discovery in this area of research during this period was an endophyic fungus *Taxomyces andreanae* which was isolated from *Taxus brevifolia* capable of producing paclitaxel, an anticancer agent associated with a higher plant host, the Northwest Pacific yew tree (Stierle *et al*. 1993). This discovery spurred interest not only in fungal endophytes as a source of novel anticancer agents, but also in endophytes as an alternative source of valuable higher-plant metabolites. In the next five-year interval, 1996–2000, only two novel compounds were discovered, but a fungal source of vincristine was reported. From 2001–2005, nine novel anticancer agents were reported, as well as the first fungal source of the important anticancer agent camptothecin. From 2006–2010, 75 compounds with significant cytotoxicity were reported. Of these, 43 were novel structures. During this same period, fungal sources of the plant-derived anticancer compounds podophyllotoxin and hypericin were discovered, as well as new fungal sources of camptothecin and paclitaxel.

These divers compounds isolated from endophytic fungi were belonged to: Aldehydes, Alkaloids and nitrogen-containing heterocycles, Benzo[j]fluoranthenes, Chromones, Cyclohexanones, Depsidones, Depsipeptides, Ergochromes, Esters, Lactones, Lignans, Peptides, Polyketides, Quinones, Spirobisnaphthalenes, Diterpenes, Sesquiterpenes, Xanthones.

Kharwar *et al.* (2011) stated that one hundred anticancer compounds belonging to 19 different chemical classes with activity against 45 different cell lines have been isolated from over 50 different fungal species belonging to 6 different endophytic fungal groups 57% of these compounds were novel or were analogues of known compounds. Some of these compounds are shown in Table 1. There has been a significant increase in the number of anticancer compounds isolated from endophytic fungi following the first report of the production of paclitaxel by a fungus (Stierle *et al*., 1993).

Table 1. Some anti-cancer compounds isolated from endophytic fungi.

Host plant	Fungal endophyte	Compounds	Chemical nature	References
Polysiphonia urceolata	*Chaetomium globosum*	Chaetopyanin	Aldehyde	(Wang *et al*., 2006)
Nothapodytes foetida	*Entrophospora infrequens*	Camptothecin	Alkaloid	(Puri *et al*., 2005)
Camptotheca acuminata	*Neurospora crassa*	Camptothecin	Alkaloid	(Rehman *et al*., 2008)
Catharanthus roseus	*Fusarium oxysporum*	Vincristine	Alkaloid	(Zhang *et al*., 2000)
Imperata cylindrica	*Chaetomium globosum*	Chaetoglobosin U Chaetoglobosin C Chaetoglobosin F Chaetoglobosin E Penochalasin A	Alkaloid Alkaloid Alkaloid Alkaloid Alkaloid	(Ding *et al*., 2006)
Tripterygium wilfordii	*Rhinocladiella* sp.	Cytochalasin 1 Cytochalasin 2 Cytochalasin 3 Cytochalasin E	Alkaloid Alkaloid Alkaloid Alkaloid	(Lee *et al*., 1995)
Artemisia annua	*Hypoxylon truncatum*	Daldinone C Daldinone D	Benzo[*j*]fluoranthene Benzo[*j*]fluoranthene	(Gu *et al*., 2007)
Camellia sinensis	*Pestalotiopsis fici*	Pestaloficiol I Pestaloficiol J Pestaloficiol K Pestaloficiol L	Chromone Chromone Chromone Chromone	(Liu *et al*., 2009)
Ephedra fasciculata	*Fusarium oxysporium*	Beauvericin	Depsipeptide	(Zhan *et al*., 2007)
Dicerandra frutescens	*Phomopsis longicolla*	Dicerandrol A	Erochrome	(Wagenaar and Clardy, 2001)
Ephedra fasciculata	*Chaetomium globosum*	Globosumone A Globosumone B	Ester Ester	(Bashyal *et al*., 2005)
Taxus mairei	*Aspergillus clavatus*	Brefeldin A	Lactone	(Kharwar *et al*., 2011)
Torreya grandis	*Paecilomyces* sp.	Brefeldin A	Lactone	(Kharwar *et al*., 2011)
Podophyllum hexandrum	*Trametes hirsuta*	Podophyllotoxin	Lignan	(Puri *et al*., 2006)
Taxus baccata	*Acremonium* sp.	Leucinostatin	Peptide	(Strobel *et al*., 1997b)
Polygonum senegalense	*Alternaria* sp.	Alternariol Alternusin	Polyketide Polyketide	(Aly *et al.,* 2008)
Sequoia sempervirens	*Aspergillus parasiticus*	Sequoiatones A Sequoiatones B	Polyketide	(Stierle *et al*., 1999)
Torreya taxifolia	*Pestalotiopsis microspora*	Torreyanic acid	Quinone	(Lee *et al.,* 1996)
Salvia officinalis	*Chaetomium* sp.	Cochliodinol Isocochliodinol	Quinone Quinone	(Debbab *et al.,* 2009)
Mangrove plant	*Halorosellinia* sp. *Guignardia* sp.	Anthracenedione	Quinone	(Zhang *et al*., 2010)
Taxus brevifolia	*Taxomyces*	Paclitaxel	Terpene	(Stierle *et al*., 1993)

	andreanae			
Xylopia aromatica	*Periconia atropurpurea*	Periconicin	Terpene	(Stierle *et al*., 1993)
Platyclocarpus granatum	*Phyllosticta spinarum*	Tauranin	Terpene	(Wijeratne *et al.,* 2008)
Xylocarpus grantum	XG8D (a basidiomycete)	Merulin A Merulin C	Terpene Terpene	(Chokpaiboon *et al*., 2010)
Tectona grandis	*Phomopsis* sp.	Phomoxanthone Phomoxanthone	Xanthone	(Isaka *et al.,* 2001)

1.6. Antioxidant agents from endophytic fungi

Reactive oxygen species (ROS, e.g., O2− and OH−) and free radical-meditated reactions can cause oxidative damage to biomolecules e.g., lipids, proteins and DNA eventually contributing to, for example, aging cancer, atherosclerosis, coronary heart ailment, diabetes, Alzheimer's disease, and other neurodegenerative disorders (Finkel and Holbrook 2000; Halliwell, 1994).

The interest in antioxidants has increased because of their high capacity in scavenging free radicals referred to various diseases (Silva *et al*., 2007) and synthetic antioxidants were subsequently generated and produced. However, synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene used in foods, may accumulate in the body and cause liver damage and carcinogenesis (Valentao *et al*., 2002). Therefore, natural non-toxic and plant-source antioxidants have attracted more attention to protect the human body against chronic diseases (Hu and Kitts, 2000).

Many studies have shown that fungal endophytes are ubiquitous in plant species. Endophytic fungi are a potential source of natural antioxidants and some novel bioactive compounds (Huang *et al*., 2007).

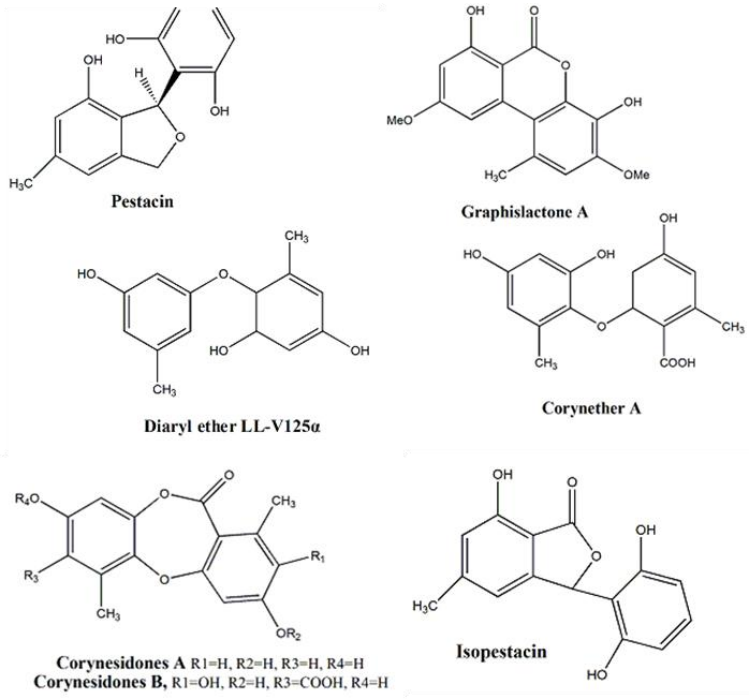


Figure 14. Antioxidants compounds isolated from endophytic fungi

Pestacin and isopestacin are two compounds produced by *Pestalotiopsis microspora* from *Terminalia morobensis* with antioxidant and antifungal properties (Harper *et al.,* 2003). Corynesidones A, B and Diaryl ether (LL-V125α) were isolated from endophytic fungus *Corynespora cassiicola* (Chomcheon *et al*., 2009). Graphislactone A was produced by *Cephalosporium* sp. and *Microsphaeropsis olivacea* which were isolated from and *Trachelospermum jasminoides* and *Pilgerodendron uviferum* respectively (Fig. 14) (Hormazabal *et al*., 2005, Song *et al*., 2005).

1.7. Antimicrobial agent of endophytic fungi

The first natural fungal antibiotic is Penicillin discovered by Fleming in 1928. Many fungi, when growth under appropriate conditions, are now known to produce antibiotics, such as: Antiamoebin, Alternaric acid, Calvacin, Cephalosporins, Dendrochin, Flammulin, Fumigillin, Fumigatin, Fusidic acid, Gliotoxin, Griseofulvin, Helenin, Lepiochlorin, Patulin, Penatin, Penicillic acid, Penicillin, Phomin, Poricin, Proliferin, Sparassol, Statolin,

Trichomycin, Trichothecin, Trypacidin, Ustilagic acid, Variecolin, Viridin and Wortmannin (Krik *et al.*, 2008).

A great number of novel natural products possessing antimicrobial activities have been isolated from endophytes (Fig. 15). It is believed that screening for antimicrobial compounds from endophytes is promising source to overcome the increasing threat of drug resistant strains of human and plant pathogen. Antimicrobial classes include: alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids (Yu *et al*., 2010).

Since the first endophytic fungus was identified, a lot of attention has been given to the potential of exploitation of these fungi for the production of novel antibiotics. This niche should be meticulously investigated and used as a base for sustainable research and development of new antibacterial substances that can respond to current antimicrobial resistance.

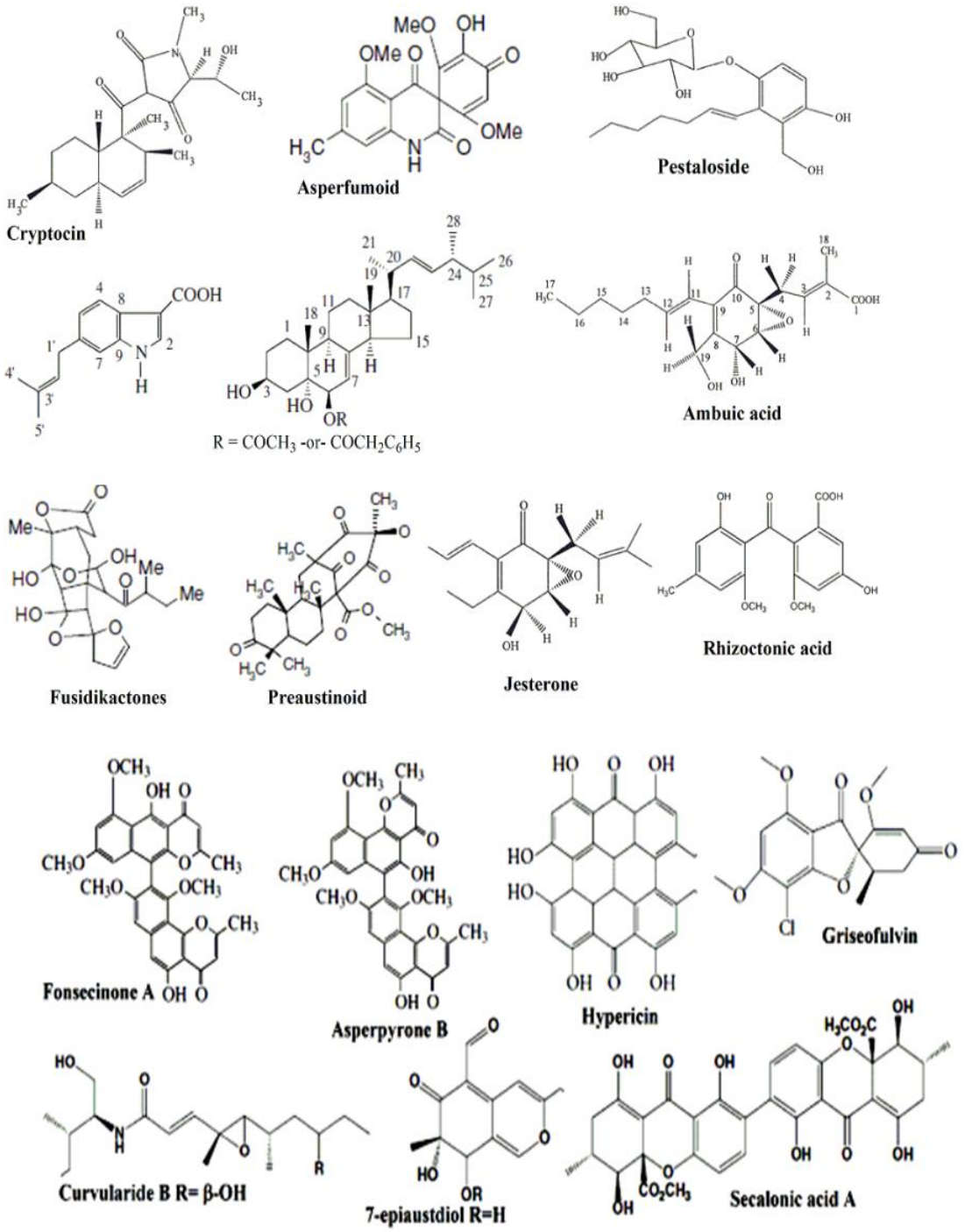


Figure 15. Some antimicrobial metabolites isolated from endophytic fungi (Yu *et al*., 2010).

1.8. Medicinal plants and their endophytes

Some endophytes can produce the same rare and important bioactive compounds originally characteristic of the host plant (Tan and Zou, 2001). This ability is of great importance that it provides an alternative strategy for reducing the need to harvest slow-growing and possibly rare plants and also help to preserve the world's ever diminishing biodiversity. Moreover, the production of a high value phytochemical by exploiting a microbial source is easier and more economical and it leads to increased availability and the reduced market price of the product (Strobel *et al*., 2004).

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalin, and quinine. According to World Health Organization (WHO) estimation, 80% of the population of some Asian and African countries presently use herbal medicine for some aspect of primary health care (Alves and Rosa, 2007). In recent years a lot of efforts have been employed to identify novel molecules derived from natural sources that exhibit a range of clinical and pharmacological activities. This thus led to an extensive research on organic substances synthesized by various plants and microorganisms growing in diverse habitats and displaying a range of habit.

1.8.1. Traditional medicine in Sudan

People, in Sudan, have developed their own unique traditional medical culture. They knew the secrets of many areas of the country depend on herbal medicines. It is important to note that only 11% of the population has access to formal medical care; the majority of the population's main medical source is traditional of herbal medicine. Thus, home remedies are available in virtually every Sudanese home including those of cities where access to modern medical care is available. So herbal medicine represent an integral part of the health care system (Yagi, 2011)

Vernonia amygdalina Del. (Asteraceae), *Euphorbia prostrata* Ait (Euphorbiaceae), *Trigonella foenum-graecum* L. (Fabaceae), *Calotropis procera* Ait. (Asclepiadaceae) and *Catharanthus roseus* L. (Apocynaceae) are medicinal plants that have several uses in Sudanese folk medicine (El Gazali, 2007). They have been selected for three major reasons. They grow in an adverse environment in Sudan where the climate is arid and they are

employed by traditional remedies in Sudan as anticancer plants (there is no well documented history in Sudan on the use of medicinal plants for cancer treatment) also other African countries including South Africa (Steenkamp, 2003). However, the endophytes mycoflora of these five plants have not been investigated.

1.8.2. *Vernonia amygdalina* Del.

Vernonia amygdalina Delile, which common name is bitter leaf and vernacular names is Gulb Elwadi. (Fig. 16) (Synonyms Gymnanthemum amygdalinumn (Delile) Walp. 1843) belongs to the family Asteraceae. *Vernonia* is the largest genus in the tribe Vernoniae with close to 1000 species (Keeley and Jones, 1979). The genus *Vernonia* is known for having several species with food, medicinal and industrial uses.

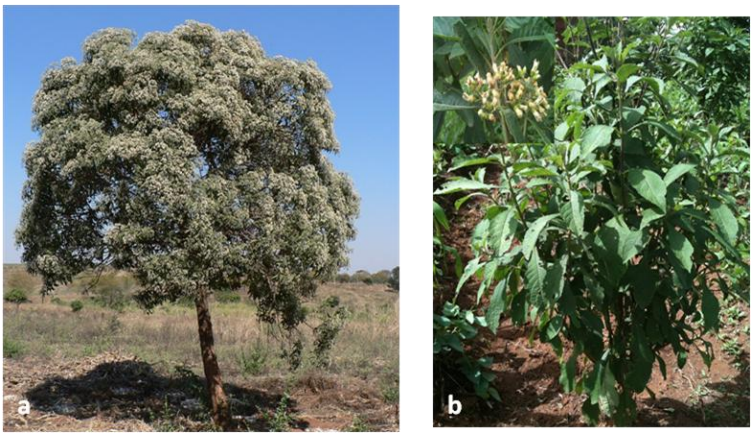


Figure 16. *Vernonia amygdalina* Del. **A.** Tree and **B.** Young tree (Toyang and Verpoorte, 2013).

1.8.2.1. Botanical description

V. amygdalina is a small tree up to 10 m tall; bark light grey or brown; fissured, brittle branches. Leaves lanceolate oblong; up to 28 x 0 cm, but usually 10-15 x 4-5 cm. Leaves, medium to dark green, with or without sparse hairs above, with fine, soft, pale hairs below and conspicuous red-veining; apex and base tapering, base always almost symmetric, margin entire or very finely toothed; petiole usually very short but may be 1-2 cm long. Flower heads thistle like, small, creamy white, 10 mm long, grouped in dense heads, axillary and terminal, forming large flat clusters, 15 cm in diameter, sweetly scented (Fig. 17).

Figure 17. *Vernonia amygdalina* 1, leaf; 2, flowering branch; 3, flowering head; 4, fruit. (Fomum, 2004).

1.8.2.2. Origin and geographic distribution

V. amygdalina occurs wild in most countries of tropical Africa, from Guinea east to Somalia and South to North-Eastern South Africa, and in Yemen. It is commonly grown as a vegetable in Benin, Nigeria, Cameroon, Gabon and DR Congo, and to a lesser extent in their neighboring countries.

1.8.2.3. Traditional uses

V. amygdalina is commonly used in traditional medicine. Leaf decoctions are used to treat fever, malaria, diarrhoea, dysentery, hepatitis and cough, as a laxative and as a fertility inducer. They are also used as a medicine for scabies, headache and stomach-ache. Root extracts are also used as treatment against malaria and gastrointestinal disorders. In Nigeria leaves are placed on a wound as a substitute for iodine. One of the most common medicinal uses of *V. amygdalina* is as a treatment against intestinal worms including nematodes. Not only humans but also chimpanzees ingest the bitter pith of *V. amygdalina* for the control of

intestinal nematode infections. In Zimbabwe, a root infusion is used to treat sexually transmitted diseases. Bark infusions are also taken to treat fever and diarrhoea, dried flowers against stomach disorders. *Vernonia amygdalina* is also useful as a control agent against diseases in plants. The ash from burnt branches is used to control seed-borne fungi (*Curvularia*, *Aspergillus*, *Fusarium* and *Penicillium* spp.) thus ameliorating seed viability and germination capacity. It has also been used for brewing beer as a substitute for hop. *Vernonia amygdalina* is a well-known bee plant (Fomum, 2004).

1.8.2.4. Phytochemistry

Members of the genus *Vernonia* are good sources of sesquiterpene lactones. This class of compounds has been reported to be insect antifeedant, antifungal, cytotoxic and antitumoral (Wedge *et al.,* 2000; Kumari *et al*., 2003). Several sesquiterpenes include vernolide, vernolepin, vernodalin, vernodalol, vernodalin and hydroxyvernolide were isolated (Kupchan *et al*., 1969; Jisaka *et al*., 1993a; Koshimizu *et al*., 1994; Erasto *et al*. 2006). The occurrence of steroidal saponins and Stigmastane-type saponins such as Vernoniosides A1, A2, A3, A4, B1, B2, B3, C, D and E was also have been reported. Furthermore, flavonoids like luteolin, luteolin 7-*O*-*β*-glucoroniside, luteolin 7-*O*-*β*-glucoside have also been reported in this plant (Rwangabo *et al.*, 1986; Ohigashi *et al*., 1991; Jisaka *et al*., 1992, 1993b; Igile *et al.*, 1994). Other compounds like terpens, coumarins, phenolic acid, lignans, xanthons, anthraquinones and edoties (peptides) have been also isolated (Wall *et al*., 1998; Tona *et al*., 2004).

1.8.3. *Calotropis procera* Ait.

Calotropis procera Ait. (Fig.18) which common name is Giant milk weed and vernacular name is Sodom apple and Usher belongs to the family Asclepiadaceae. It is distributed mainly in the tropical and subtropical regions of the world. *Calotropis* species are commonly known as milkweeds because of the latex they produce. *Calotropis* species are considered common weeds in some parts of the world. These species are drought-resistant and salt-tolerant to a relatively high degree.

Figure 18. *Calotropis procera* Ait. (A) Shrub and (B) Inflorescence and fruit (Alsagarden, 2015).

1.8.3.1. Botanical description

Calotropis procera (Fig. 19) occurs as a single or many stemmed soft-wooded shrub up to 6m. All parts of the plant exude white milky latex when cut. Leaves are opposite-decussate, simple, ovate to obovate with 4-6 pairs of subopposite nerves prominent on the abaxial surface, an acute apex, sessile which is about 30x25 cm. Inflorescences arise from the base of the leaves in pedunculate (7 cm) cymes of 3-20. Flowers consist of 5 small triangular dirty white sepals, 5 thick ovate petals (1cm x 1cm) which are white at the base and purple at the tips and 5 purple tipped stamens, which surround a white 5 lobed stigma 11. Fruits consist of green, spongy ovoid fruits (follicles), up to 15cm long by 10cm wide. They split open to release plumed, papery light brown seeds with a pappus of white filaments up to 6cm long on one side. The main flowering period would be from March to October 12 (Sharma *et al.,* 2011).

1.8.3.2. Geographic distribution

C. procera is native to India, Pakistan, Nepal, Afghanistan, Algeria, Iran, Iraq, Israel, Kenya, Kuwait, Niger, Nigeria, Oman, Saudi Arabia, Sudan, United Arab Emirates, Vietnam, Yemen and Zimbabwe (Chatterjee and Chandra, 1995).

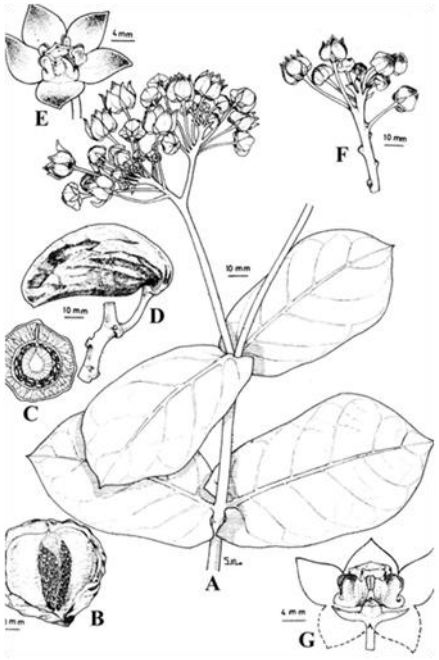


Figure 19. *Calotropis procera* A: Flowering twig; B: Dehisced fruit; C: Transverse section of fruit; D: Intact fruit; E: Flower; F: Inflorescence; G: Corna and gynostegium (Efloras, 2015).

1.8.3.3. Traditional uses

The milky juice of *C. procera* is used as purgative, while the flowers are used as digestive, stomachic, and tonic, and in the treatment of cough, asthma, and loss of appetite. The root bark promotes secretion and is useful in treating skin diseases, enlargement of abdominal viscera, intestinal worms, ascites, and anasarca. The alcoholic root extract also has analgesic, anticonvulsant, and sedative properties (Argal and Pathak, 2006).

1.8.3.4. Phytochemistry

Phytochemical studies on *C. procera* have afforded several types of compounds such as cardenolide, triterpinoids, alkaloids, resins, anthocyanins and proteolytic enzymes in latex, flavonoids, tannins, sterol, saponins, cardiac glycosides. Flowers contain terpenes, multiflorenol, and cyclisadol (Al-Yahya *et al*., 1990). The flower contains also the flavonoids, quercetin -3- rutinoside, sterol, calactin, calotoxin, calotropagenin, calotropin, polysaccharides with D-arabinose, glucose, glucosamine and L-rhamnose. Flowers also contain enzymes, 3-proteinase and calotropain (protease). Other chemical constituents of *C.*

procera flowers are lupeol, uscharin, proceroside, proceragenin (cardenolide), syriogenin, taraxast-20(30)-en-3-(4-methyl-3-pentenoate), 3-thiazoline cardenolide, gigantin, giganteol, isogiganteol, uscharidin, uzarigenin voruscharin α-calotropeol, 3-epimoretenol, alactuceryl acetate and α-lactuceryl isovalerate.

Root bark of *C. procera* contains triterpenes, a new norditerpenyl ester, named calotropterpenyl ester, and two unknown pentacyclic triterpinoids, namely calotropursenyl acetate and calotropfriedelenyl acetate, akundarol isovalerate, mundarol isovalerate and quercetin -3- rutinoside (Ansari and Ali, 2001).

The leaves contain mainly the amyrin, amyrin acetate, ß-sitosterol, urosolic acid, cardenolides, calotropin, calotropagenin.The latex contains caoutchouc, calotropin, calotoxin 0.15%, calactin 0.15%, uscharin 0.45%, trypsin, voruscharin, uzarigenin, syriogenin and proceroside (Atef *et al.,* 1999).

1.8.4. *Trigonella foenum-graecum* L.

Plant *Trigonella foenum-graecum* (Fig.20) which common name is Fenugreek and vernacular name is Helba. Synonyms: *Trigonella gladiata* Ldb. belongs to family Fabaceae. It is a common leguminous plant used as condiments and medicinal purposes.

1.8.4.1. Botanical description

Stems 10-50 cm are sparsely pubescent. Leaflets 20-50 X 10-15 mm, obovate to oblong-oblanceolate are denticulate. Flowers, solitary or paired, are subsessile. Calyx 6-8 mm, the teeth about as long as tube; corolla 12-18 mm, yellowish-white tinged with violet at the base. Legume (excluding beak) 60-110 X 4-6 mm, erect or patent are linear, somewhat curved, glabrous or glabrescent, with longitudinal veins; beak (10-)20-30 mm. Seeds 5 X 3 mm are quadrangular, somewhat compressed, yellow or pale brown, finely tuberculate (Fig. 21).

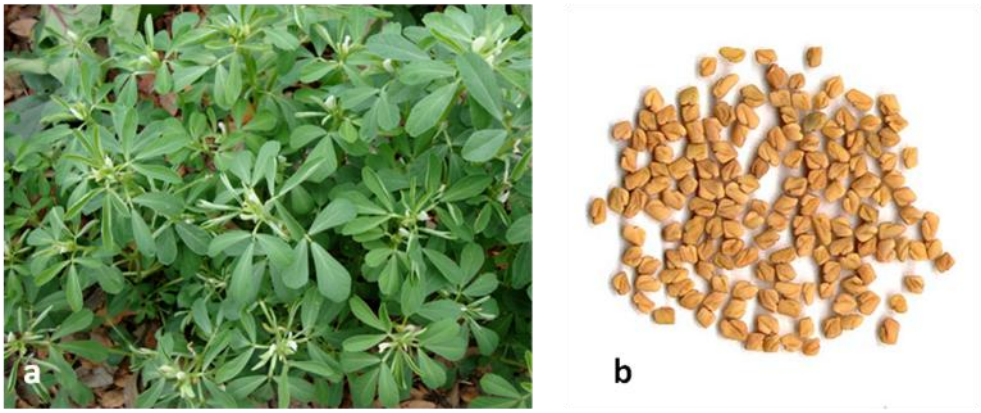


Figure 20. *Trigonella foenum-graecum* L. a: Plant, b: seeds (Nikoletta, 2014).

1.8.4.2. Geographical distribution

Indigenous to countries on the eastern shores of Mediterranean, fenugreek is widely cultivated in India, Egypt, Ethiopia, Morocco and occasionally in England. *T. foenum-graecum* is extensively grown in the tropical and subtropical regions of India.

Figure 21. *Trigonella foenum-graecum* L. (Nikoletta, 2014)

1.8.4.3. Traditional uses

Medical papyri from ancient Egyptian tombs report that it was used to reduce fevers and as a food. In religious rites it was one of the numerous components of the celebrated kuphi (holysmoke), an Egyptian compound of incense used in fumigation and embaming. Its seeds possess a significant place in the Ayurvedic system of medicine as these are carminative, antipyretic and anthelmintic, and used in colic flatulence, dysentery, diarrhea dyspepsia with loss of appetite, chronic cough, dropsy, enlargement of liver and spleen, rickets, gout and diabetes besides the insect repellent properties. Subsequently, its seeds have also been reported to possess various significant activities such as antifertility and antitumor (Jain *et al*., 1995).

1.8.4.4. Phytochemistry

Chemically, its seeds are reported to contain 28% mucilages. As Guar gum, improving the strength characteristics of commercial coating-bases. Dried mucilage has remarkable swelling properties and may find application as adjuvant in pharmaceutical preparations, as tablet disintegrator it is more effective than alginic acid. It contains 5% of a stronger-smelling bitter fixed oil, 22-30% proteins. Also it contains carbohydrates, ascorbic acid, nucleic acid, lipids, phenols, essential oils and volatile oils. The seeds also contain various pharmacologically important bioactive compounds such as flavonoids – like kaempferol, luteolin, apigenin, quercetin, vitexin, isovitexin, vicenin-I and vicenin-II, as alkaloid like strigonelline which exhibited the hypoglycaemic activity, carotenoids, coumarins, steroid sapogenin peptide ester. Seeds are a rich source of saponins and sapogenins, both of triterpenic and steroidal types. The work of Fazli and Hardman (1968) brought fenugreek seeds into prominence as a potential source for the production of corticosteroids due to the presence of higher level of diosgenin up 2.2%. Subsequently, extensive work was carried out on the isolation and identification of saponins and steroidal sapogenins in *T. foenum-graecum* (Jain *et al*., 1995).

1.8.5. *Euphorbia prostrata* Ait.

Euphorbia prostrata Ait. (Fig. 22) which common name is Prostrata sandmat and trailing red spurge and vernacular name is Um lubaina Synonyms: *Chamaesyce prostrata* (Aiton), belongs to the family Euphorbiaceae. It is an annual herb occurring in two forms, red

and green, recognised as two ecotypes; the former being tolerant to high calcium levels in the soil.

Figure 22. *Euphorbia prostrata* Ait. (Flickr, 2012).

1.8.5.1. Botanical description

E. prostrata is prostrata or erect annual forb to 40 cm; herbage with milky sap. Leaves are opposite, inequilateral at base, 5-10 mm long, margins entire. Inflorescences are generally on short, lateral branches. Flowers are monoecious borne in cyathia, petaloid appendages minute; Staminate flowers 4, generally in 5 clusters around pistillate flower, each flower a stamen; Pistillate flower: 1, central, stalked; ovary chambers 3, ovule 1 per chamber, styles 3, divided 1/2 length. Fruits are capsule, 1.5 mm long, bearing hairs on external angles, spheric, lobed; Seeds are ± 1 mm, ovoid, white to grey with sharp transverse ridges (Fig. 23).

1.8.5.2. Geographic distribution

E. prostrata is native to the West Indies, but is now widely distributed throughout the tropics and subtropics. It occurs throughout tropical Africa and the Indian Ocean islands (Mosango, 2008).

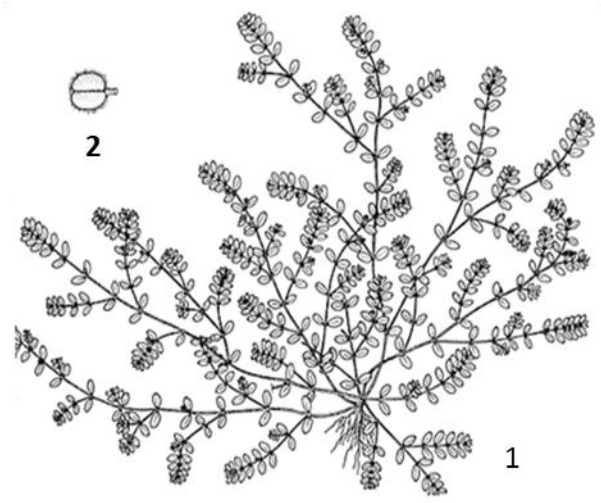


Figure 23. *Euphorbia prostrata* Ait. 1, plant habit; 2, fruit (Mosango, 2008).

1.8.5.1. Traditional uses

E. prostrata is used in Ayurvedic medicine for bronchial asthma. In Australia, the latex has been used as an application to sores and, in North America, the plant is a snake bite remedy. According to Indian folklore, the plant has anti-inflammatory properties and is also considered as a blood purifier (Sharma and Sharma, 1972). Leaves of *E. prostrata* are one of the traditional Chinese medicines for treating diarrhoea, dysuria and rheumatism (Yoshida, *et al*., 1990). All parts of *Euphorbia prostrata* are widely used in African traditional medicine. In Burkina Faso the leaves are rubbed onto wasp stings and scorpion stings. In Togo a leaf decoction is drunk to treat threatened abortion. Small balls of ground plants are inserted into the vagina to treat female sterility and painful menstruation. In Benin the pounded aerial parts with pounded shells are taken to treat irregular menstruation. Ground leaves in water are administered against difficult childbirth. In Nigeria a plant decoction is taken for its astringent, vulnerary and anthelmintic properties, and crushed plants are used by the Igbo people as a poultice for broken arms. In Cameroon crushed leaves are eaten to treat amoebic dysentery. In Gabon a leaf extract is applied as an enema to treat inflammations. Leaf powder mixed with palm oil is rubbed on the head to treat headache (Mosango, 2008).

1.8.5.7. Phytochemistry

Three new ellagitannins named prostratins A-C have been isolated from *E. prostrata*. Ten compounds have been isolated from *E. prostrata* and identified as gallic acid, corilagin, 1,2,3-tri-O-galloyl-D-glucose,geraniin,tellimagradin I, II, rugosin A, rugosin E, rugosin D and rugosin G on the basis of physicochemical and spectroscopic methods (Yoshida *et al.*, 1990).

The active principles in *E. prostrata* are chiefly flavonoids, phenolic acid and tannins. Flavonoids and phenolic acid have been reported to have anti-inflammatory, analgesic, antioxidant, haemostatic, and antithrombotic. The chemical analysis of *E. prostrata* revealed that it contains phenolic compounds like gallic acid which activates Hageman factor which causes hypercoagulability and ellagic acid which suppress histamine release. It also contains flavonoids like apigenin, and luteolin°. Tannins are known to possess astringent and haemostatic properties. Preclinical studies carried out on the extract have confirmed its wound healing and anti-hemorrhoidal activity (Gupta, 2011). The latex is irritant and blistering to the skin and mucous membranes and is reported to cause blindness. From different fractions of extracts of the dried leaves, a range of hydrolyzable ellagitannins were isolated, including prostratins A, B and C, euphorbins G and H, tellimagradin I and II, and rugosins A, D, E and G. Flavonoids isolated from the aerial parts include: kaempferol, cosmosiin (apigenin-7-glucoside), rhamnetin-3-galactoside, quercetin and quercetin-3-rhamnoside. Other constituents of the aerial parts include the sterols β-amyrine acetate, β-sitosterol, campesterol, stigmasterol and cholesterol. The aerial parts also contain the terpene alcohol β-terpineol, gallic acid, corilagin, 1,2,3-tri-O-galloyl-D-glucose, geraniin, and various amino acids, including n-valeramide and N,N-dimethyl-4-benzoxybutylamine. From the roots, a myricylic alcohol and two triterpenes, taraxerol and tirucallol, have been isolated. Both flavonoids and tannins have been reported to have anti-inflammatory, analgesic, haemostatic, antithrombic and vasoprotective actions. The flavonoids furthermore have antiviral, anti-allergic, antiplatelet, anti-tumour and antioxidant properties.

The ethanol and water extracts of the whole plant showed significant antifungal activity against the dermatophytes *Trichophyton mentagrophytes*, *Trichophyton simii* and *Microsporum gypseum in vitro* and *in vivo* in goats and rabbits. The cured extracts were tested on lesions caused by these fungi in 3–4 weeks and the activity of *E. prostrata* extracts were as effective as benzoic acid. A water extract inhibited growth, spore formation, and

enterotoxin production of *Clostridium perfringens* type A. Ethanolic extracts from the aerial parts showed significant antibacterial activity against *Escherichia coli* and *Bacillus subtilis*. An aqueous ethanol extract showed significant antibacterial activity *in vivo* against *Shigella dysenteriae* in tests with rats. A methanol extract of the leaves showed considerable inhibitory effects against HIV-1 protease, and a water extract against hepatitis C virus protease (Mosango, 2008).

1.8.6. *Catharanthus roseus* (L) G. Don

Catharanthus roseus (Fig. 24) which common names are Madagascar periwinkle and rosy periwinkle, vernacular name is Vinca belongs to the family Apocynaceae. Synonyms: *Vinca rosea* L. (1759), *Lochnera rosea* (L.) Rchb. ex Endl. (1838). The antimitotic properties of some of its alkaloids were discovered accidentally in the late 1950s during searches for antidiabetic substances.

Figure 24. *Catharanthus roseus* (L) G. Don (USDA, 2009).

1.8.6.1. Botanical description

C. roseus is an evergreen subherb or herbaceous plant growing to 1 m. tall. The leaves are oval to oblong, 2.5- 9.0 cm long and 1- 3.5 cm broad glossy green hairless with a pale midrib and a short petiole about 1- 1.8 cm long and they are arranged in the opposite pairs. The flowers are white to dark pink with a dark red centre, with a basal tube about 2.5- 3 cm long and a corolla about 2-5 cm diameter with five petal like lobes. The fruit is a pair of follicles about 2-4 cm long and 3 mm broad (Sain and Sharma, 2013) (Fig. 25).

1.8.6.2. Geographical Distribution

C. roseus originates from Madagascar, but for centuries it has been cultivated as an ornamental throughout the tropics and occasionally in the subtropics; it has become naturalized in many regions. It was brought under cultivation in the first half of the 18th century in Paris, from seeds collected in Madagascar, and was later distributed from European botanical gardens to the tropics. *C. roseus* is also cultivated and common in Sudan.

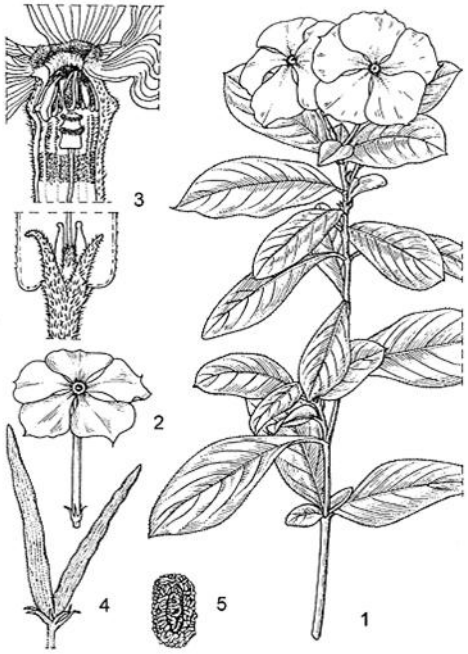


Figure 25. *Catharanthus roseus* 1, flowering twig; 2, flower; 3, base and top of corolla tube in longitudinal section; 4, fruit; 5, seed (Schmelzer, 2007).

1.8.6.3. Traditional uses

In Africa, especially in the Indian Ocean islands, medicinal uses of *C. roseus* are manifold and are similar to those in Asia. A decoction of all parts of *C. roseus* is well known as an oral hypoglycaemic agent. The decoction is also taken to treat malaria, dengue fever, diarrhoea, diabetes, cancer and skin diseases. Extracts prepared from the leaves have been applied as antiseptic agents for the healing of wounds, against haemorrhage and skin rash and as a mouthwash to treat toothache. The aerial parts are also considered diaphoretic and

diuretic, and decoctions are taken to relieve indigestion, dyspepsia, dysentery, toothache and the effects of wasp stings, and as an emetic, purgative, vermifuge and depurative. In Uganda, an infusion of the leaves is taken to treat stomach ulcers. In Botswana, the leaves ground in milk are applied to mature abscesses. In Togo a root decoction is taken to treat dysmenorrhoea.

The aerial parts of the plant are used for the extraction of the medicinal alkaloids vincristine and vinblastine. The alkaloids are prescribed in anticancer therapy, usually as part of complex chemotherapy protocols. The dried root is an industrial source of ajmalicine, which increases the blood flow in the brain and peripheral parts of the body. Preparations of ajmalicine are used to treat the psychological and behavioural problems of senility, sensory problems (dizziness, tinnitus), cranial traumas and their neurological complications (Schmelzer, 2007).

1.8.6.4. Phytochemistry

C. roseus has been found to contain as many as 130 constituents with an indole or dihydroindole structure. The principal component is vindoline (up to 0.5%); other compounds are serpentine, catharanthine, ajmalicine (raubasine), akuammine, lochnerine, lochnericine and tetrahydroalstonine. Ajmalicine and serpentine are essentially present in the roots, whereas catharanthine and vindoline accumulate in aerial parts. The aerial parts contain 0.2–1% alkaloids. The substances of pharmacological interest are the bisindole alkaloids, most of them containing a plumeran (vindoline) or an ibogan (catharanthine) moiety. Several of these alkaloids have cytostatic properties, but occur in very small amounts: vincristine (leurocristine) in up to 3 g/t of dried plant material and vinblastine (vincaleucoblastine) in a slightly larger amount. Other active compounds are leurosidine (vinrosidine) and leurosine. Some of the alkaloids (e.g. catharanthine, leurosine and vindoline) exhibit a moderate hypoglycaemic action. The fresh leaf juice though shows considerable hypoglycaemic activity. Vinblastine markedly inhibits the *in vitro* reproduction of *Trypanosoma cruzi*, the organism causing Chagas' disease. Antiviral activity has been reported *in vitro* for some *Catharanthus* alkaloids, e.g. leurocristine, perivine and vincristine. Extracts of the plants have shown fungicidal activity (e.g. against *Fusarium solani* that causes wilt e.g. in aubergine and *Sclerotium rolfsii* that causes diseases such as southern blight in tomato) and nematicidal activity (e.g. against *Meloidogyne incognita* and *Meloidogyne javanica*). Extracts

of the dried flowers, dried leaves or fresh roots have shown antibacterial activity against some human pathogens. Callus tissue of *C. roseus* can be cultured on various media, and can produce a variety of monomeric alkaloids. The alkaloid spectra *in vitro* roots and shoot cultures are similar to those of roots and aerial parts, respectively (Schmelze, 2007).

Chapter Two

Materials and Methods

Chapter Two

2. Materials and Methods

2.1. Collection, identification and authentication of the plant material

The selected medicinal plants: *Vernonia amygdalina* Del. *Calotropis procera* Ait., *Catharanthus roseus* L. and *Euphorbia prostrata* Ait., were collected from the natural populations from Sudan in two locations: Khartoum (15°38 N 32°32 E) and Shendi (16°41 N 33°26 E). Seeds of *Trigonella foenum-graecum* L. were collected from the local market in Khartoum. The plant materials were identified and authenticated on the basis of botanical characteristics by an expert botanist, Dr. Haider, a taxonomist in Medicinal and Aromatic Plants Research Institute (MAPRI).

Healthy leaves and stems were collected randomly from different plants for the study. Plant material samples were brought to the laboratory in bags, washed thoroughly in running tap water followed by deionized (DI) water. Then, sample were stored at 4 °C before to be processed within few hours after sampling.

2.2. Isolation of endophytic fungi

The leaf and stem samples were cut into small pieces using a blade. Samples were surface disinfected as described by Petrini and Dreyfuss (1981) with modifiations. Sequential immersion of samples in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min and sterile distilled water for 1 min, two times. Finally the samples were blotted in sterile blotted paper. The surface-sterilized samples were cut using a sterile blade. Leaves were cut into 0.5-1 cm with and without midrib. Stem samples were cut into 0.5-1.5 cm. Four to five sterile pieces were placed on the surface of PDA medium amended with chloramphenicol 500 mg/L to inhibit bacterial growth (Alexander and Strete 2001; Wiyakrutta *et al.*, 2004). The efficiency of the surface disinfection procedure was confirmed by plating 1 mL of the final rinse water on PDA plates. The Petri dishes were incubated at 28 ºC for 7 days. At the end of the incubation, the fungal colony was subcultured on PDA plate. Each fungal culture was checked for purity and subcultured, to another PDA plate. The fungal isolates were numbered

and stored on slant of PDA plate at 4ºC or as spores and mycelium in 15% (v/v) glycerol at -80 ºC (Zhang *et al*., 2009).

2.3. Identification of endophytic fungi

2.3.1. Fungal morphological characterization

Identification of the fungal strains was based on different criteria: morphology of culture or hyphae, the characteristics of the spores and the reproductive structures if the features were discernible using lactophenol or lactophenol cotton blue stains (Carmichael *et al*., 1980; Webster, 1980; Barnett and Hunter, 1998; Watanabe, 2002; Watanabe, 2010). Photos were taken using Olympus C-5060 Wide Zoom camera.

2.3.2. Molecular characterization

The taxonomic characterization of fungal isolates was confirmed by ITS sequencing for 16 fungal strains.

2. 3. 2. 1. ITS Amplification

Fungal strain was cultivated on a PDA plate, covered with a sheet of sterile cellophane on the surface to collect fungal mycelium as described by Larena *et al.* (1999). The plates were then incubated at 28°C for 7 – 15 days.

For each fungal strain, 0.5 g of mycelium were weighted and immediately frozen in liquid nitrogen. Then, mycelium was ground with liquid nitrogen in a mortar using a pestle. Genomic DNA was extracted according to the method described by Ranjard *et al*. (2003). Briefly, the mycelium powder was suspended in extracting buffer (Tris HCl 100 µM, EDTA 100 µM, NaCl 100 µM, SDS 2%, pH 8) (1:1, w/v). After centrifugation at 16 100 g for 5 min, the supernatant was collected and proteins were precipitated with sodium acetate 5 M, pH 5.5 (1/10, v/v) for 1h at 4°C. After centrifugation at 16 100 g, 5 min, DNA was precipitated with isopropanol (v/v) for 18h at -20°C. After centrifugation at 16 100 g for 30 min, DNA was washed with cold ethanol 70% and was suspended in sterile UP H_2O. DNA was kept frozen at -20°C.

Fungal isolates were identified by sequencing of internal transcribed spacer (ITS) regions of rDNA (Eurofins Genomics ITS1-F and ITS4). 2,5 µl of 10x Taq Polymerase buffer (MP Biomedicals, France), 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 0.5 µM of each fungal primer, 0.625 U of Taq Polymerase (MP Biomedicals, France), 25 ng of fungal DNA were combined in a final volume of 25 µl. PCR amplifications were carried out with the primers ITS1 and ITS4 (Larena *et al*. 1999) as follows: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a 15 min final elongation at 72°C. PCR products were commercially sequenced (GATC Biotech, Konstanz, Germany). DNA sequences were analyzed using BLASTn (http://www.ncbi.nlm.nih.gov/) and the most closely related species were determined. Nucleic acid sequences of the ITS region of fungal strains were deposited in Gene Bank (accession numbers KR673900 to KR673909).

2.4. Cultivation of fungi for the production of metabolites

The fungi were cultured in appropriate media for the production of secondary metabolites. Solid phase fermentation was performed. In most cases fungi were cultured on Potato Dextrose Agar (PDA) for the primary screening and for the extraction. Secondary metabolites were extracted from the culture media and mycelium using EtOAc.

2.4.1. Small scale cultivation

Small scale cultivation was carried out primarily to perform bioassays for the detection of active metabolites. The fungal strains were cultivated on PDA in 20 petri dishes, each plate containing 20 mL of PDA media (0.5 L), at 28°C for 7-10 days depending on the fungal strain growth rate.

2.4.1.1 Extraction of metabolites from endophytic fungi

Ethyl acetate was used for metabolite extraction according to Campos *et al*. (2008). Each 20 plate culture of endophytic fungus was crushed using a blender, and then macerated with EtOAc for 24 h. The sediment was extracted 3 times with EtOAc to obtain the organic phase. Finally, the crude extract was obtained after evaporation of EtOAc and kept at 4°C.

2.5. Extraction of metabolites from the host medicinal plants

Ethyl acetate was used for extraction of secondary metabolites. 20 g of dry leaves and stems from each plant, *i.e*., *C. procera, V. amygdalina, E. prostrata*, *C. roseus* and seeds of *Trigonella foenum-graecum* were used. The secondary metabolites of the endophytic fungi and their host plants were compared using TLC.

2.6. Chemical and biological assays

2.6.1. Evaluation of antioxidant activities of endophytes and their host plants

2.6.1.1. Total antioxidant capacity assay

2.6.1.1.1. DPPH radical-scavenging test

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a commercially available stable free radical, which is purple in color. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyle hydrazine, which is yellow in color (Fig. 19). The degree of discoloration of purple to yellow was measured at 520 nm. This wave length measures the scavenging potential of plant extracts.

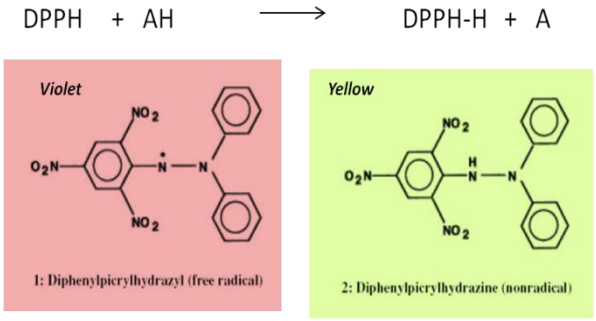


Figure 26. DPPH reaction when incubated with an anti-oxidant molecule.

Antioxidant activity of the extracts was estimated using DPPH *in vitro* method. Test samples were dissolved separately in methanol to get test solution of 1 mg/mL. Series of extract/isolated compound solutions of different concentrations (10, 20 and 100 µg/mL) were prepared by diluting with methanol. Assays were performed in 96-well, microtiter plates. 140

µL of 0.6×10^6 mol/L DPPH was added to each well containing 70 µL of sample. The mixture was shaken gently and left to stand for 30 min in dark at room temperature. The absorbance was measured spectrophotometrically at 517 nm using a microtiter plate reader (Synergy HT Biotek, logiciel GEN5). Blank was done in the same way using methanol and sample without DPPH and control was done in the same way but using DPPH and methanol without sample. Ascorbic acid was used as reference antioxidant compound. Every analysis was done in triplicate.

The ability to scavenge DPPH radicals was calculated by the following equation:

DPPH radical scavenging activity (%) =

$$\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}} \times 100$$

where;

Abs_{sample} is the absorbance of DPPH radical+sample;

Abs_{blank} is the absorbance of sample+methanol;

$Abs_{control}$ is the absorbance of DPPH radical+methanol.

The IC_{50} value was calculated from the linear regression of plots of concentration of the test sample against the mean percentage of the antioxidant activity. Results were expressed as mean±SEM and the IC_{50} values obtained from the regression plots (Sigma PlotsR 2001, SPSS Science) had a good coefficient of correlation (R^2=0.998).

2.6.1.1.2. Determination of total phenolic content (TPC)

Total phenolics were determined by using the Folin–Ciocalteu method. 20 µl of diluted extract (50 µl extract in 150 µl water) were mixed with 100 µl Folin-Ciocalteu reagent

and then 80 µl of Sodium Carbonate 75% was added in all wells of the assay plate. The plate was incubated in the microplate reader as soon as the 75% sodium carbonate was added according to the following protocol: temperature 25°C for 30 minutes with 10 minutes average stirring for 5 s. After incubation the absorbance of the resulting blue color was measured with a spectrophotometer at 760 nm using a microtiter plate reader (Synergy HT Biotek, logiciel GEN5). Analysis was done in triplicate for each extract. Quantification was based on the standard curve of gallic acid. The results were expressed as gallic acid equivalent (GAE), *i.e.*, mg gallic acid /g and calculating the contents is as follows:

$$\text{Total phenolic content (mg/g)} = \frac{Abs_{sample} - Abs_{blank} - b}{a \times (50 \times DF)}$$

Where;

Abs_{sample}: absorbance for a test with the extract to 1/4

Abs_{blank}: absorbance for a test with water

a: slope of the "standard range"

b: Director of right "standard range" coefficient

50: For the amount in 1 ml of extract from the amount in 20 µl

DF: Dilution factor normally 4, except samples (not/too) concentrated,

so out of range.

2.6.1.1.4. Determination of correlation coefficient R^2 for the analysis of total antioxidants capacity and total phenolic content.

The correlation coefficient R^2 values were determined between total antioxidant capacity and total phenolic content.

2.6.2. Cytotoxicity assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of the extracts of 16 selected endophytes against human breast carcinoma (MCF7) and colon adenocarcinoma (HT29, HCT116) cells and human hepatocarcinoma (HepG2) only against *Vernonia amygdalina* and its endophytes as described by (Tzanova *et al*., 2009; Revelant *et al.*, 2015), following 48h exposure. Cell survival was detected by MTT reduction. This assay was used as a preliminary screening method in order to select one endophytic fungus for the phytochemical screening.

2.6.2.1. Crude extract preparation for biological assay

Each crude extract obtained by ethyl acetate extraction was diluted in DMSO to obtain a final concentration of 25 mg/mL.

2.6.2.2. Cytotoxicity evaluation (IC_{50})

The cytotoxicity assay was performed as described by Mosman (1983) using the thiazolyl blue tetrazolium bromide (MTT) procedure. Four cell lines were used, they derived from human colon adenocarcinoma (HT29 and HCT116), human breast carcinoma (MCF-7) and human hepatocarcinoma (HepG2). HT29 and HCT116 cells were cultivated in Dulbecco's minimum essential medium (DMEM, Eurobio, Courtaboeuf, France) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (Eurobio), 1% Penicillin/streptomycin (Eurobio) and 2 mM L-Glutamine (Eurobio). MCF7 and HepG2 cells were cultivated respectively in RμMI and Eagle's minimum medium, instead of DMEM, containing the same cell culture additives. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

In brief, HT29, HCT116, and HepG2 cells were seeded in 96-wells-plate at 5.000 cells/well, whereas MCF7 were seeded at 10,000 cells/well (Greiner-Bio-One GmbH, FrieKenhanusen, Germany). 24 hours after seeding, 100 µL of medium containing increasing concentrations of each extract (range from 0.01 to 100 µg/mL) were added to each well for 72 hours at 37 °C. After incubation, the medium was discarded and 100 µL/well of MTT solution (0.5 mg/ml diluted in DMEM, MEM or RμMI medium) were added and incubated for 2

hours. Water-insoluble formazan blue crystals were finally dissolved in DMSO. Each plate was read at 570 nm. IC_{50} was calculated using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data are expressed as IC_{50} ± S.D. obtained from quadruplicate determination of two independent experiments (n = 8).

2.6.3. Evaluation of antimicrobial activity

In this part two complementary technics were used: Agar disc diffusion test and broth dilution test.

2.6.3.1. Preparation of the bacterial suspension

17 strains of bacteria were used in this study:

- Gram negative bacteria: *Pseudomonas aeruginosa* (CIP82118). *Salmonella abony*, *Escherichia coli* (ATCC 8739).
- Gram positive bacteria: *Staphylococcus aureus* (ATCC 6538), methicillin (oxacillin) resistant *S. aureus*, *S. arlettae*, *S. capitis*, *S. hominis*, *S. auricularis*, *S. epidermidis*, *S. haemolyticus*, *S. xylosus*, *S. lugdunensis, S. sciuri. Enterococcus faecalis*, *E. faecium*, *Bacillus cereus*, *Kytococcus sedentarius.*

Bacteria were kept at −80°C in brain heart infusion broth (BioRad) supplemented with 20% glycerol. Before each experiment, the bacterial strains were subcultured and were incubated at 37°C overnight to have fresh colonies. Bacterial inoculum was monitored by setting the culture optical density (measured in distilled water at an absorbance wavelength of 620 nm) at 0.156 for *E. coli* and *P. aeruginosa* and 0.300 for *S. aureus* and *Enterococcus faecalis*, corresponding to 10^8 CFU mL^{-1}. Bacterial suspensions were further adjusted to 10^6 CFU mL^{-1} in sterile water. Petri dishes with Muller-Hinton agar (MH) medium were treated with each bacterial suspension separately. The agar disc diffusion test was then performed.

2.6.3.2. Agar disc diffusion test

Tested samples were dissolved in 5% DMSO at a concentration of 5 mg/mL for extracts and 1 mg/mL for fractions. 40 µL of the solutions were pipetted onto a sterile filter disk (Schleicher and Schuell, 6 mm), then were left to dry at 40ºC for 2h. The discs were placed onto treated plates with the suspension of the test organisms. Furan was used as positive control. The plates were incubated at 37ºC overnight. The inhibition zones were then measured (Schulz *et al*., 1995).

2.6.3.3. Determination of minimum inhibitory concentration (MIC)

MIC of ethyl acetate extract and active fractions was determined based on a broth microdilution method in a 96-well microplate (Al-Bayati, 2008). *Staphylococcus aureus* and methicillin-resistant *S. aureus* were cultured overnight at 37°C on Mueller Hinton (MH) broth and adjusted to a final density of 10^8 $CFUmL^{-1}$ as mentioned above. The crude extract, fractions and pure compounds (2 mg/mL and 250 µg/mL respectively) was dissolved in 5% DMSO and twofold serial dilutions were made in the concentration range from 0.015 to 2 mg/mL and 1.9 to 250 µg/ml respectively. In the 96- well plate, each well had 50 µL of MH broth, 100 µL of bacterial inoculum and 50 µL of different concentrations of fungal extract. The plate was incubated at 37ºC for 24 h. After incubation, the bacterial growth was visually inspected and the lowest concentration of fungal extract at which no observable bacterial growth or observable turbidity was taken as the MIC value. The experiments were carried out in triplicate.

2.6.3.4. Determination of minimum bactericidal concentration (MBC)

- **First day**

100 µL of bacterial suspension 10^6 UFC/mL were transferred into a tube containing 900 µL of sterile water. Therefore, the bacterial concentration was 10^5 UFC/mL. The same was done to obtain a population of about 10^3 UFC/mL. Then, streaking on a track 4 MH agar, a 10^3 CFU/mL, 10^4 CFU/mL, 10^5 CFU/mL, and 10^6 UFC/mL. This will be the witness box that will be incubated 24 h at 37°C (Fig. 27).

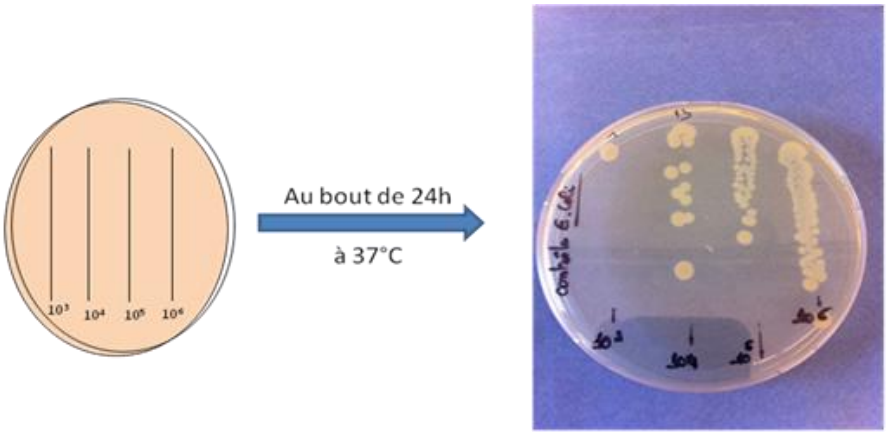


Figure 27. Streaking of bacteria with different concentrations 10^6, 10^5, 10^4 and 10^3 as a CFU/mL control.

The MBC is equal to a 3 log reduction of the initial bacterial population or 0.1%. In our case it is necessary that the number of colonies grown in the presence of the isolated compounds correspond to the same development colonies in dilution 10^3 CFU/mL in order to have a bactericidal effect.

- **Second day**

The control box is recovered and placed in a refrigerator at 2°C to stop bacterial growth. After determination of MIC, a streaking on MH agar was performed for all wells which had no bacterial growth (Fig. 28)

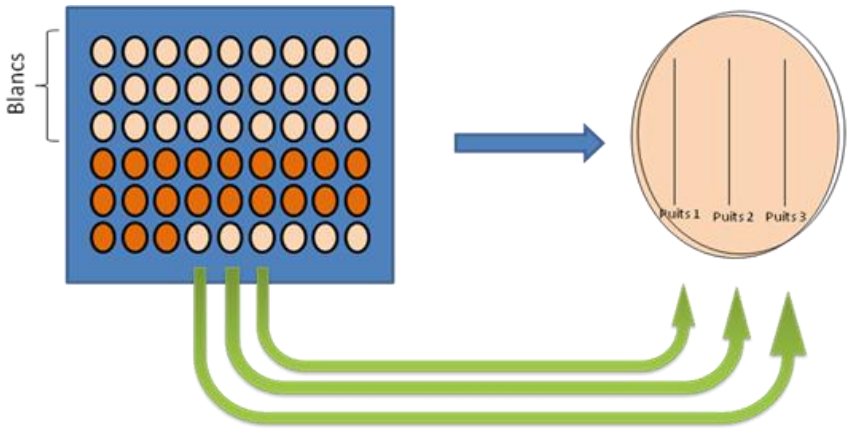


Figure 28. Streaking of wells which had not obvious bacterial growth.

- **Results: MBC determination**

The MBC is in our case, the first streaking which was noticed a lack of development of colonies. To have a bactericidal effect it is necessary that the streaks correspond to that of the 10^3 CFU/mL. Since the MBC 3 log corresponds to a reduction of the MIC.

2.7. Large scale cultivation

The growth condition and media requirements for the production of secondary metabolites were adjusted at small scale cultivation for *Curvularia papendorfii* endophytic fungus of *Vernonia amygdalina*. Selected medium was used for the large scale cultivation. The fungal strain was cultivated on 15 L of PDA at 30º C for 7-10 days. Cultivation was done for the identification of active compounds.

2.8. Phytochemical Methods

2.8.1. Chromatographic Techniques

All crude extracts were initially analyzed by thin layer chromatography. One selected crude extract of *Curvularia papendorfii* was further analyzed by the mean of chromatographic techniques (TLC, CC, GC and HPLC) and spectroscopic analyses for pure compounds.

2.8.1.1. Thing layer chromatography TLC

TLCs were carried out using aluminum precoated TLC plates with Silica gel 60 F_{254} (0.2 thickness) TLC plates (Merck). The chromatographs were developed by the ascending method at room temperature. After dryness, the plates were sprayed using different reagents. Solvent system used in this study were: Petroleum ether: Ethyl acetate (8:2 v/v)

- Petroleum ether: Acetone (7:3 v/v)
- Hexane: Ethyl acetate (9:1 v/v)
- Hexane: Acetone (3:2 v/v)
- Ethyl acetate: Methanol: Acetic acid (9:0.5:0.5 v/v)
- Dichloromethane: Methanol: Acetic acid (8: 1.5:0.5 v/v)

- Dichloromethane: Methanol: Formic acid (9:0.5:0.5 v/v)
- Dichloromethane: Ethyl acetate: Formic acid (2.5:2.5:1 v/v)

Detection of compounds on TLC chromatography was viewed under UV light 254 and 366 nm for fluorescence or quenching spots. TLC plates were sprayed with one of the following reagents (Table 2) as described by Reich and Schibli (2007).

Table 2. Reagents for phytochemical detection.

Reagent name	Preparation and use	Examination	Detection of natural compounds
Aluminum chloride	1 g aluminum chloride was dissolved in 100 mL methanol. The plates were, sprayed and dried.	UV 366 nm	Flavonoids and anthraquinones
Acetic anhydride-sulfuric acid (Liebermann-Buchard's)	1 mL sulfuric acid and 20 mL acetic acid anhydride were carefully diluted to 100 mL with chloroform. The plates were sprayed and dried.	White light	Sterols, terpenoids.
Anisaldehyde-sulfuric acid	10 mL sulfuric acid were carefully added to an ice-cooled mixture of 170 mL methanol and 20 mL acetic acid. To this solution 1 mL anisaldehyde was added.	White light and UV 366 nm	Terpenoids, saponins, sterols, iridoids, most lipophilic compounds.
Dragendorff's reagent	Solution A: 0.85 g basic bismuth nitrate was dissolved in 10 mL acetic acid and 40 mL water under heating. Solution B: 8 g potassium iodide	White light	Alkaloids, heterocyclic nitrogen compound.

	were dissolved in 30 mL water. Just before spraying, 1 mL of each solution was mixed with 4 mL acetic and 20 mL water.		
Natural product/ polyethylene glycol (NP/PEG) spraying solutions (Neu's reagent)	Solution A: 1 g of diphenylborinic acid aminoethylester was dissolved in 100 mL methanol. Solution B: 5 g of polyethylene glycol 400 (marcrogol) were dissolved in 100 mL ethanol. The plates were heated at 100°C for 3 min then sprayed while still hot with solution A and B consecutively. In some cases solution A was only sufficient.	UV 366 nm	Flavonoids, carbohydrates, anthocyanines, plant acids.
Sulfuric acid	20 mL sulfuric acid were carefully added to 180 mL ice-cold methanol. The plates were immersed in reagent for 1 s then heated at 100 °C for 5 min°.	White light, UV366 nm	General reagent
Vanillin/H_2SO_4	2 mL sulfuric acid were added carefully to an ice-cold mixture of 1 g vanillin and 100 mL ethanol. Plates were sprayed then heated at 100 °C for 5 min°.	White light, UV366 nm	Terpenoids, sterols, salicin, ergot, alkaloids, most lipophilic compounds.

R_f values were calculated as follows:

R_f=Distance moved by the solute/ Distance moved by the solvent.

TLC was used to monitor the identity of each of the crude extracts, fractions and the qualitative purity of the isolated compounds. Also it was utilized to optimize the solvent system that would be applied for column chromatography.

2.8.3. Column Chromatography (CC)

Column chromatography was performed on opened glass column. Two different sizes: for crude extract (25x4.5 cm) and for the fractions (25x2 cm) with different stationary phases:

- Silica gel 60 (40-63µm) (Merck, 113905)
- Sephadex LH 20

2.8.3.1. Flash chromatography by Combiflash

Analyses were performed on Combiflash Rf TELEDYNE Isco. using Redisep Rf TELEDYNE Isco repached column. Crude extract was subjected to flash column chromatography using silica gel stationary phase and solvent system previously determined by TLC.

2.8.3.2 Analytical HPLC

Analytical HPLC was used to identify interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. Peaks were detected by UV-VIS diode array detector. HPLC was performed on a Merck Hitachi HPLC system which consisted of The analytical column used is a thermo ODS Hypersil C18 250x2.4 Sn 10264142 lot 13232 5u. L-7100 HPLC pump, a programmable L-7400 UV detector (Merck Hitachi), an injection needle (M27050) for L7200 autosampler (Merck Hitachi), and a Merck Hitachi diode array detector L-7453. Data recording was carried out by D7000-HSM software (Merck).

The eluent consisted of (A) water 2% Formic acid and (B). MeoH 2% Formic acid gradient profile was: 0–15 min from 67% B, 15–25 min from 73% B, 25–30 min 67% B. The flow-rate was 1 mL/min. The wavelength was 254 nm.

2.8.3.3. Semi preparative HPLC

The semi preparative HPLC was used for the isolation of pure compounds from fractions previously separated using flash column chromatography. Semi preparative was performed using analytical column thermo ODS Hypersil C18 250x10 Sn 10264142 lot 13232 5u. Gilson 321 HPLC pump, a programmable Shimadzu SPD-10A UV/Vis detector, a manual injection needle 1mL, and a fractions collector SV type MC3 (SELI). Data recording was carried out by Winilab III software. Each injection consists of 10 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system pumped through the column at a rate of 2 mL/min. The eluted peaks which were detected by the online UV and detector were collected separately in tube.

2.8.4. Analytical Techniques

2.8.4.1. Melting Points

Melting points were measured using electro thermal melting point apparatus model No. 1A6304.

2.8.4.2. IR spectroscopy

IR recorded spectra were performed on Perkin-Eelmer model 1650 FTIR spectrophotometer using 150 mg KBr and 1 mg of the isolated compounds.

2.8.4.3. NMR spectroscopy

All NMR experiments were performed with a Bruker Avance DRX-400 instrument (Bruker Spectrospin, Rheinstetten, Germany) operating at a proton frequency of 400.13 MHz.

2.8.4.4. Gas Liquid Chromatography/Mass Spectroscopy analysis (GC/MS)

Analyses were performed on a Shimadzu QP2010-device operating in EI mode (Electronic Impact) at 70 eV. The column used was DB-5ms (30 m x 0.25 µm x 0.25mm). which comes from Sigma-Aldrich. The elution program lasted 38.5 min. Temperature of the injector was set at 250 °C. The mobile phase used was helium with a flow 0.8 mL/min. The injection volume was 1 µL and the split ratio was 5. The interface temperature was set at 310 °C and that of the source was 200 °C. The SIM mode was used and started from 3.5 to 35.5 min.

2.8.4.5. HPLC (LC-ESI-MS)

The LC system consisting of a U3000- Dionex LC the LC Part is dionex the mass part is bruker daltonics equipped with an injector comprising a loop of 5 .µL and a UV detector at 210 and 300 nm. The analytical column used is a MIXED MODE Acclaim HILIC-1 ID 2.1 mm (150 mm x 5 µm x 120 Å). Column oven temperature was 30°C. Solvent A is water/HCOOH and solvent B is pure acetonitrile. The elution parameters are as follows: gradient profile was: 0–30 min from 50% B, 30–32 min from 70% B, 32–35 min 50% B. The micropump flow-rate was 0.05 ml /min.

The mass spectra HR-ESI-MS (High Resolution Electrospray Ionization Mass Spectrometry) in the positive ionization mode were obtained on a Q-TOF (Bruker Daltonics micrOTOF-QTM). This mass spectrometer combines a quadrupole (Q) and a time of flight analyzer with a reflector (TOF-R, Time Of Flight). The principle of time of flight analyzer is based on the relationship between the mass m and the velocity v of the ions. The instrument measures the time required for ions to travel in a vacuum (10-5 mbar) a distance L without field. The capillary voltage set at 4.5 kV, the source voltage 150 V. Desolvation gas and nebulizing gas used was nitrogen at a flow rate of 4 L/min (for desolvation) and a 2 bar pressure for nebulisation. The temperature of the ESI source and the desolvation of the gas was set at 190°C. Bruker Daltonics DataAnalysis 4.1 software was used for data acquisition and processing of data.

2.9. Isolation of pure compounds from *Curvularia papendorfii* crude extract.

The initial method of fractionation of *C. papendorfii* crude extract was done using opened glass column on silica gel. Successive elution were performed from the column with gradients of Hexane/EtOAc, (9:1 to 2:8) then EtOAc/MeOH/Acetic acid (8:1:1) and finally a column wash step with methanol. 13 fractions (A to M) were obtained after combining the eluates according to their similarity behavior on TLC. Fraction M (433 mg) was further purified on silica gel CC and eluted with CH_2Cl_2: Methanol: Acetic acid (9.5:0.5:0.1) in mixture of increasing polarity. 6 sub-fractions were obtained (M1 to M6). Then sub-fraction M4 (352.8 mg) was subjected to repeated eluent mixture of CH_2Cl_2: Methanol: Acetic acid (95:4:1). Six sub-fractions (4a to 4f) were obtained on combining the eluates. Sub fraction 4d

(236 mg) was subjected to repeated CC with mixture of CH_2Cl_2: Methanol: Acetic acid (96:4:2). Three sub-fractions were obtained (Di to Diii) (Fig. 29).

Second method of purification of *C. papendorfii* crude extract was done using flash column combiflash. The first gradient was EtOAc: MeOH: FA; 50:50: 0:1; v/v. 75 fractions of 15 mL each were collected. They were pooled into 8 groups (A to H) based on their similarity, as assessed by thin layer chromatography on silica gel plates. The second gradient was (CH_2Cl_2: MeOH: FA; 80:10:10; v/v) followed by MeOH. A total of 155 fractions of 15 mL each were collected. They were pooled into 4 groups (I to L) based on their similarity, as assessed by TLC. In Fraction (E) colorless crystals precipitated. They were filtrated and were subjected to repeated combiflash eluted with EtOAC: Cycl Hexane; 80:20; v/v to afford pure compound **AF1** (40 mg), semi pure E1 and sub fraction E2. Semi pure E1 was subjected to semi preparative HPLC using (MeOH: water) (67: 33: 2% FA), to obtain **AF2**, **AF3** and **AF4** pure compounds (Fig. 30).

Fraction (L), the last fraction of *C. papendorfii* crude extract, was subjected to repeated combiflash with gradient (CH_2Cl_2: MeOH: FA; 80:10:10; v/v). Four fractions were obtained (Bi, Bii and Biii). Fraction (Biii) was subjected to semi preparative HPLC using MeOH: water 67: 33: 2% FA. 7 pure compounds were isolated **AFT2** (1.0 mg), **AFT3** (2.6 mg), **AFP1** (1.6 mg), **AFP2** (0.9 mg)**, AFP3** (0.9 mg)**, AFP4** (10 mg).

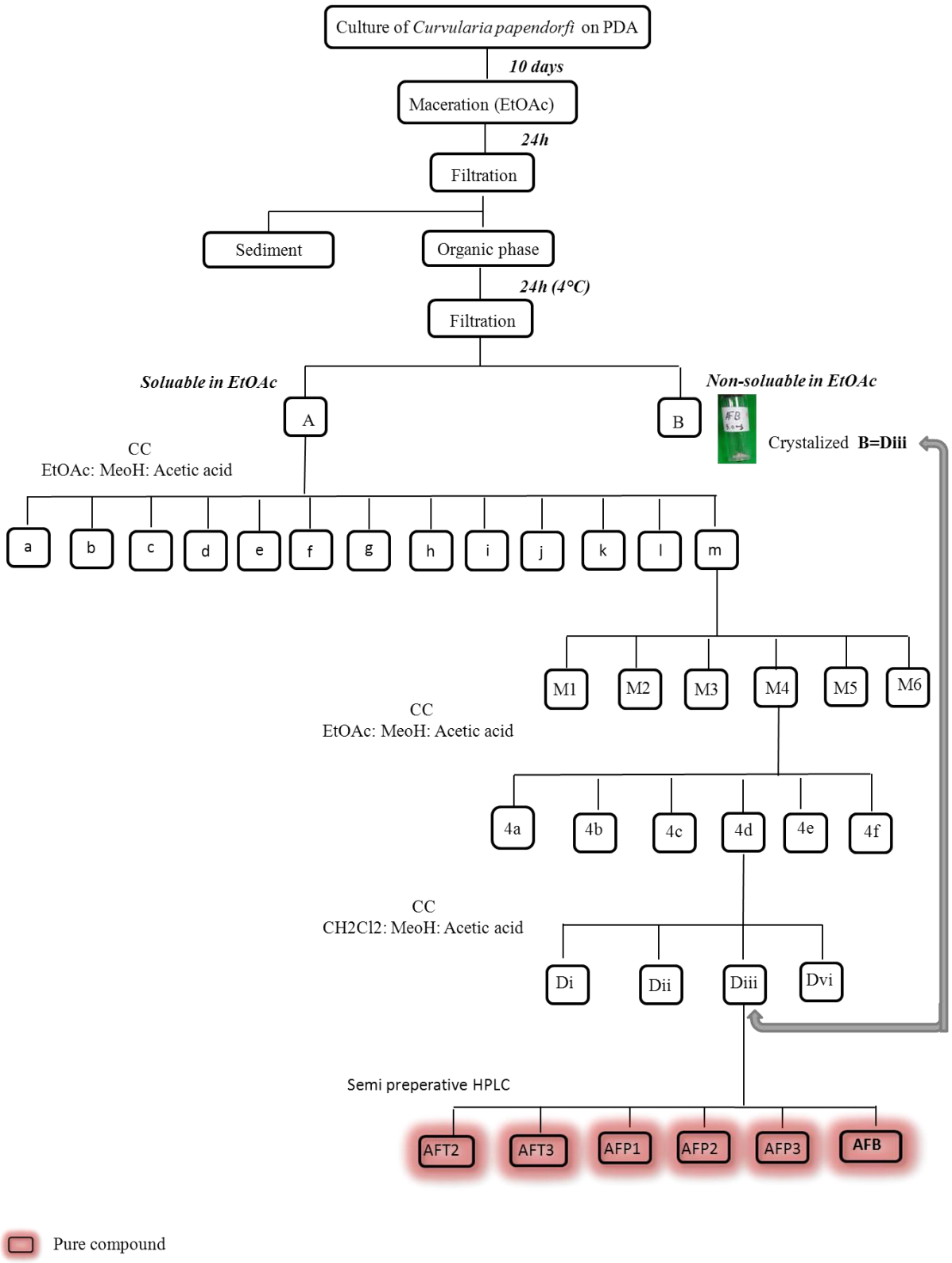


Figure 29. Scheme of extraction of *Curvularia papendorfii* crude extract and flew chart of purification by open column and semi preparative HPLC.

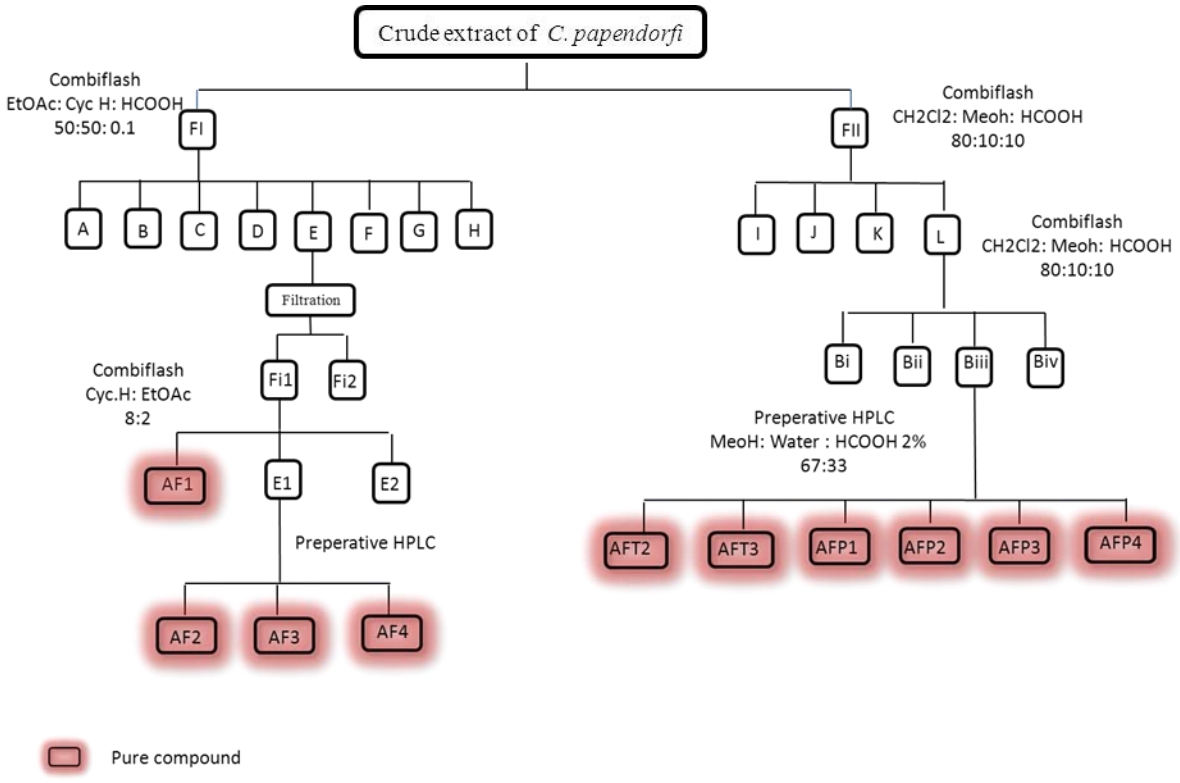


Figure 30. The flew chart of pure compounds of *Curvularia papendorfii* by combiflash and semi preparative HPLC

2.9.1. Evaluation of pH effect on production of *Curvularia papendorfii* metabolites.

Initial pH ranges were adjusted from 3.5 to 9.5. 250 mL of PDA, were used for each pH. *C. papendorifi* was cultured and incubated for 7 days at 30°C. The growth rate of each colony was measured daily during the seven days. Crude extract of each pH was prepared as described at the beginning of this chapter (2.4.1.1), and dry weight was measured. Fraction B was obtained by CC with eluent (EtOAc: Cyclo Hexane: Acetic acid; 80:15:5; v/v) and a final wash column step using MeOH.

Chapter Three

Results and Discussion

Section One

Résumé Résultats et discussion

La section 1 concerne l'identification et la taxonomie de champignons endophytes isolés de cinq plantes tropicales originaires du Soudan : *Calotropis procera* (Ait.), *Cathranthus, roseus (L.), Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.) et *Vernonia amygdalina.* Au total, 23 souches de champignon endophytes ont été isolees à partir des cinq plantes médicinales soudanaises, après la stérilisation de surface des explants. Cinq endophytes ont été isolés de *c. procera*, six de *c. roseus*, quatre de *E. prostrata*, cinq de *T. foenum-graecum* et trois de *V.amygdalina*. Ces souches fongiques ont été classées en 12 taxons différents. Dix neuf souches appartiennent aux Ascomycètes, alors que trois souches appartiennent à la classe fongique des Deutéromycètes et une seule appartient aux Basidiomycètes. Trois souches n'ont pas sporulé; elles ont été regroupées sous le nom de mycélium stérile. Ce groupe de champignons est répandu chez les endophytes. Les genres *Alternaria, Cladosprium, Phoma, Chaetomium, Curvularia, Emericella, Pleosporales* et *Aspergillus* sont très courants chez les endophytes. Les genres *Byssochlamys, Trametes,* et *Hansforia* ont été rapportés aussi chez les endophytes, La diversité fongique chez les plantes médicinales soudanaises n'est pas très élevée par rapport à celle qui est trouvée en Chine, en Inde et au Pakistan. Le climat extrêmement aride du Soudan pourrait expliquer ce phénomène. Dans de nombreuses régions, la pluie tombe environ neuf mois par an, mais toujours moins que cinq mm. Par ailleurs, la majorité des champignons endophytes pourrait correspondre à des souches non cultivables *in vitro*.

La section 2 concerne les aspects organoleptiques des extraits isolés de 21 souches de champignons endophytes isolés des plantes précédentes. *Aspergillus terreus* et *Alternaria alternata* issus de *C. procera* se sont révélés avoir des rendements d'extraction élevés (242 et 206 mg respectivement). Les analyses chromatographiques par CCM ont montré que tous les extraits d'endophytes renfermaient des terpènes, des composés phénoliques et rarement des alcaloïdes. Seuls deux extraits bruts de *Cladosporium cladosporioides* 2 et de *Curvularia papendorfii* isolés de *Vernonia amygdalina* renfermaient des alcaloïdes.

La section 3 a pour but d'évaluer les activités biologiques des extraits des endophytes, en particulier des activités antioxydantes, des activités anticancéreuses et des activités antibactériennes. Parmi les endophytes, *Aspergillus terreus* 1 issu des graines de *Trigonella foenum-graecum* a montré la plus haute teneur en polyphénols (89,9 ± 7,1 mg GAE / g) et la

plus haute activité antioxydante (CI_{50}: 18 ± 0,1 µg / ml). Par ailleurs, une évaluation de la cytotoxicité de 16 extraits d'endophytes et de leurs plantes hôtes a été réalisée par le test MTT utilisant trois souches de cellules cancéreuses: cancer du sein (MCF7), deux souches pour l'adénocarcinome du colon (HT29, HCT116). *Byssochlamys spectabilis* a montré une activité significative (1,51 ± 0,2 µg / ml) suivi par *Cladosporium cladosporioides* 2 (10,5 ± 1,5 µg / ml), puis par *Alternaria* sp. (13,5 ± 1,8 µg / ml). Trois plantes médicinales, *C. procera*, *V. amygdalina* et *C. roseus* ont montré une activité cytotoxique significative contre au moins une lignée cellulaire (2,19 ± 0,1, 5,6 ± 0,4 et 7,00 ± 0,43 µg / ml respectivement). Les activités antibactériennes ont été évaluées sur deux souches bactériennes, l'une à Gram négatif (*E. coli*) et l'autre à Gram positif (*Staphylococcus aureus* résistant à la méthicilline (SARM)). Parmi les 16 souches de champignons, seulement quatre présentaient une activité faible contre *S. aureus* résistant à la méthicilline (SARM) : *Alternaria alternata* (125 µg / mL), *Alternaria* sp. et *Byssochlamys spectabilis* (500 ug / mL) et *Aspergillus terreus* 1 (1000 µg / mL). La section quatre est centrée sur l'étude phytochimique du champignon *Curvularia papendorfii*. L'extrait à l'acétate d'ethyl de cet endophyte a été soumis à un fractionnement bio-guidé par une activité antibactérienne contre *Staphylococcus aureus* résistant à la méthicilline (SARM). Dix composés purs (0,3 à 40 mg) ont été isolés. Un ensemble de méthodes spectroscopiques, ID et 2D-RMN, IR, UV et spectrométrie de masse à haute résolution (HR-ESI-MS) a été utilisé pour la détermination structurale de deux composés, AFB et AF1. Les tests biologiques ont été effectués pour ces deux composés. Le nouveau composé pur (AFB) 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (acide Khartomique) a montré une activité antibactérienne modérée contre le *Staphylococcus aureus* résistant à la méthicilline (SARM) avec une valeur de MIC 62,5 µg / mL et une faible cytotoxicité avec une valeur de IC_{50} > 100 uM contre les cellules MCF7. Cependant AF1 a montré une activité modérée (IC_{50}: 29,78 uM) contre MCF7 et une faible activité (MIC 250 µg / ml) contre *Staphylococcus aureus* résistant à la méthicilline (SARM). Il est à noter que ces deux composés ne possèdent aucune activité anti-oxydante. Par ailleurs, l'effet du pH a été étudié sur la production du composé AFB. L'endophyte *C. papendorfii* a été cultivé sur un milieu PDA avec différents pH allant de 3,5 à 9,5. Les résultats ont montré que la production d'AFB la plus élevée a été obtenue avec un pH de 6,5.

Chapter Three

3. Results and Discussion

Section One

3.1. Isolation and taxonomic characterization of endophytic fungi isolated from five Sudanese plants

Endophytic fungi are group of fungi living into plant tissues without any apparent symptoms (Guntilaka, 2006). Endophytic fungi are considered as an outstanding source of novel biologically active compounds with wide-ranging applications such as anti-cancer and antibacterial activity, or as an alternative source of compounds originally isolated from higher plants such as Taxol (Strobel *et al*., 2004).

It has been hypothesized that plants from unique environment and with an ethnobotanical history could be good candidates for endophytes producing novel bioactive compounds. Therefore, study of endophytic fungi of five medicinal plants used in Sudan was engaged in order to evaluate their capacity to produce anti-cancer and antibacterial substances. Five plant species used in the traditional Sudanese medicine were selected: *Calotropis procera* Ait., *Catharanthus roseus* L., *Euphorbia prostrata* Ait., *Trigonella foenum-graecum* L., and *Vernonia amygdalina* Del.

A culture-dependent based approach was developed in order to isolate fungal endophytes colonizing different organs of these plants. For that, the non-selective medium Potato Dextrose Agar (PDA) was used, since this common medium favor the growth of many fungi, even if sporulation can be reduced on this medium (Krik *et al*. 2008).

3.1.1. Isolation of fungal endophytes

Plants were collected as described in chapter two Materials and Methods. Fungal endophytes were isolated considering aerial parts of the plants *i. e*. leaves, stems and seeds. Fungal endophytes were isolated on PDA medium, after surface disinfection of plants samples. Morphological characterization of fungal endophytes was done for those which produced obvious spores or conidia, and then further molecular characterization was performed.

A total of 23 endophytic fungal strains were isolated from the five medicinal plants: five isolates from *C. procera,* five isolates from *T. foenum-graecum*, three isolates from *V. amygdalina*, six isolates from *C. roseus* and four isolates from *E. prostrata* (Table 3).

Table 3. Endophytic fungi isolated from different organs of five medicinal plants: *Calotropis procera*, *Trigonella foenum-graecum, Vernonia amygdalina, Catharanthus roseus*, and *Euphorbia prostrata.*

Plant species	Plant Organ	Isolate n°.	Morphological identification	°Molecular identification	
				Closest species (% identity)	Gene bank number
C. procera	Leaves	1	*Alternaria*	*A. alternata* (99%)	-
	Leave	2	*Aspergillus*	*A. terreus* 1(100%)	KR673900
	Leaves	3	*Cladosporium*	*C. cladosporioides* 1(99%)	KR673902
	Stems	4	*Trametes*	*T. versicolor* (99%)	KR673903
	Stems	5	*Phoma*	-	-
T.foenum-graecum	Seeds	6	*Chaetomium*	*C. globosum* (99%)	KR673904
	Seeds	7	*Aspergillus*	*A. terreus* 2(100%)	KR673901
	Seeds	8-10	Mycilia sterilia	-	-
V. amygdalina	Leaves	11	*Cladosporium*	*C. cladosporioides* 2(99%)	-
	L+S	12	*Curvularia*	*C. papendorfi* (99%)	KR673909
	Stems	13	*Hansfordia*	*H. sinuosae* (99%)	KR673905
C. roseus	Leaves	14	*Curvularia*	*C. aeria* (99%)	KR673906
	Stems	15	*Chaetomium*	-	-
	Stems	16	*Phoma*	*P. multirostrata* (99%)	-
	Leaves	17	*Pleosporales*	*Pleosporales* sp.	-
	Leaves	18	*Emericella*	-	-
		19	*Apergillus niger*	-	-
E. prostrata	Aerial	20	*Curvularia*	*C. australiensis* 1 (99%)	KR673907
	part	21	*Curvularia*	*C. australiensis* 2 (99%)	KR673908
	L+S	22	*Byssochlamys*	*B. spectabilis* (98%)	-
		23	*Alternaria*	*Alternaria* sp. (99%)	-

3.1.2. Taxonomic characterization of fungal endophytes of each plant

Fungal strains that have been isolated were further characterized by morph typing. The taxonomic affiliation of each strain was confirmed by a molecular approach. Three fungal

isolates failed to sporulate on different media and were classified as Mycilia sterilia spp. The lack of sporulation is a common problem associated to endophytic fungi (Gamboa and Bayman, 2001; Promputtha, *et al*., 2005). The morphological characterization of each fungal strain was described and affiliated to one particular genus. Further molecular identification was done for 16 fungal strains which were survived during the study years after the isolation from the host plant. These fungal strains were identified by sequencing of internal transcribed spacer (ITS) regions of rDNA with universal primers ITS1 and ITS4.as described in chapter two Materials and methods. The sequences obtained were compared with those submitted sequences in Genbank (http://www.ncbi.nlm.nih.gov/).

3.1.2.1. *Calotropis procera* Ait.

Five endophytic fungi were isolated from both leaves and stems (Table 3). Four isolates belonged to Ascomycotina and were affiliated with four genera. One isolate was belonging to Basidiomycotina:

- **Strain n°. 1. *Alternaria alternata* (Ascomycotina)**

This strain was isolated from leaves of *C. procera*. This strain produced grey colonies with light pinkish edges on PDA plates. Reverse side of the colonies were brown (Fig. 31). Conidiophores were pale brown, simple or branched, bearing catenulate conidia at the apex and apical fertile parts. Conidia were catenulate, mostly in a chain, often branched. Conidia were porosporous 0.8-1.5×1-2.5 µm, acropetally developed, dark brown, cylindrical or spindle-shaped, often with cylindrical beaks, muriform composed of 3–4 (8) transverse walls and 1–2 longitudinal walls. This strain belonged to Ascomycotina and was affiliated to *Alternaria* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 2. *Aspergillus terreus* 1 (Ascomycotina)**

This strain was isolated from leaves of *C. procera.* On PDA plates colonies of this strain were brown reverse side was brown (Fig. 32). Conidiophores were 13.8-14.5 µm, hyaline, simple, occasionally thick-walled, inflated globosely or ellipsoidally at the apex (called vesicles 1.5-1.8 µm), bearing spore heads composed of catenulate conidia borne on

uniseriate phialides on vesicles: conidial heads light brown, loosely columnar. Conidia were phialosporous 0.3 µm, pale brown to yellowish brown, globose, delicately rough at the surface. This strain was affiliated to *Aspergillus terreus* and then the taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 3. *Cladosporium cladosporioides* 1 (Ascomycotina)**

Fungal strain n°. 3 was isolated from leaves of *C. procera*. Colonies on PDA plates were dark grey reverse side was grey (Fig. 33). Conidiophores were pale brown, erect, branched 2–3 times, at the apical parts, bearing catenulate conidia in each branch. Conidia were blastosporous, often not well differentiated from branches, hyaline or pale brown, ovate, ellipsoidal, cylindrical, subglobose, irregular in shape, apiculate at one end, often truncate at another end. This strain was affiliated to *Cladospotium* sp. and then the taxonomic characterization was confirmed by ITS sequencing (Table 3).

Strain n°. 4. *Trametes versicolor* (Basidiomycotina)

This strain was isolated from stems of *C. procera*. Colonies on PDA plates were white with pale white reverse side. Only hypha and some chlamydospores were observed even on other media PSA and CMA. The taxonomic characterization was analyzed by ITS sequencing (Table 3). This fungal isolate that belongs to Basidiomycotina was affiliated with *Trametes versicolor*.

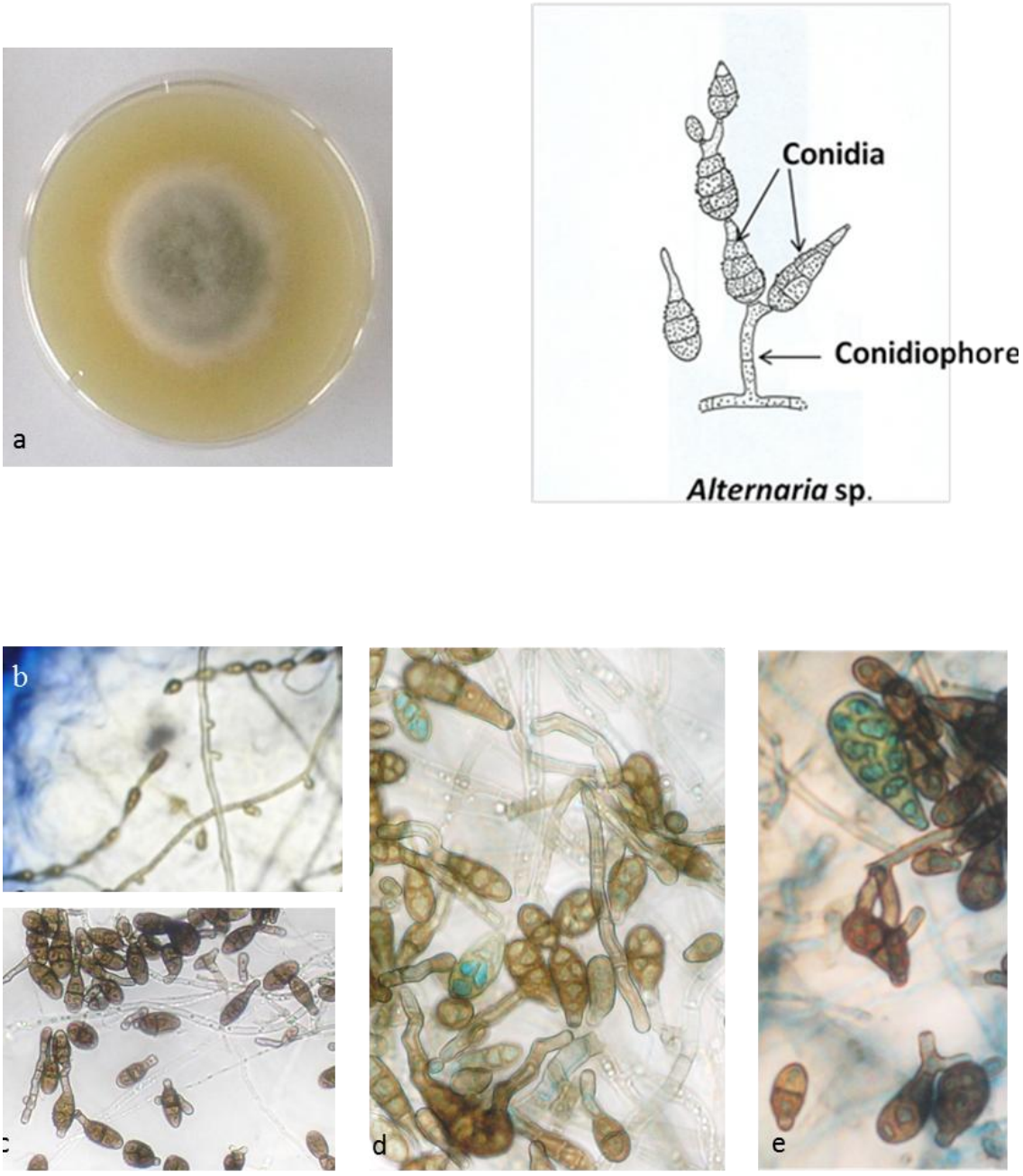


Figure 31. *Alternaria alternate* a: culture on PDA plate; b and c: conidia chains and conidia ×100; d and e: conidiophores and conidia ×400.

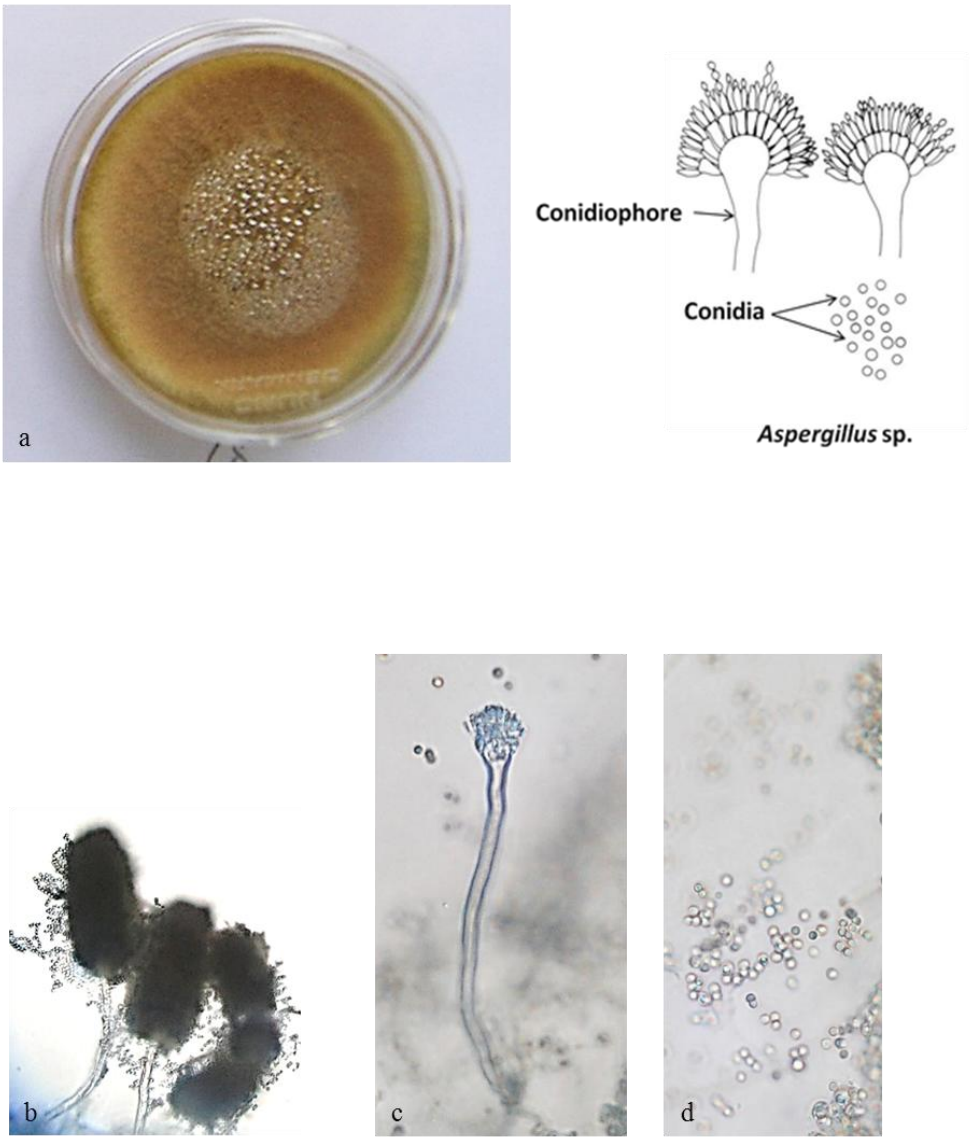


Figure 32. *Aspergillus terreus* 1 a: culture on PDA plate; b: conidiophore and spore mass ×100; c and d: vesicle, phialides, and conidia ×400.

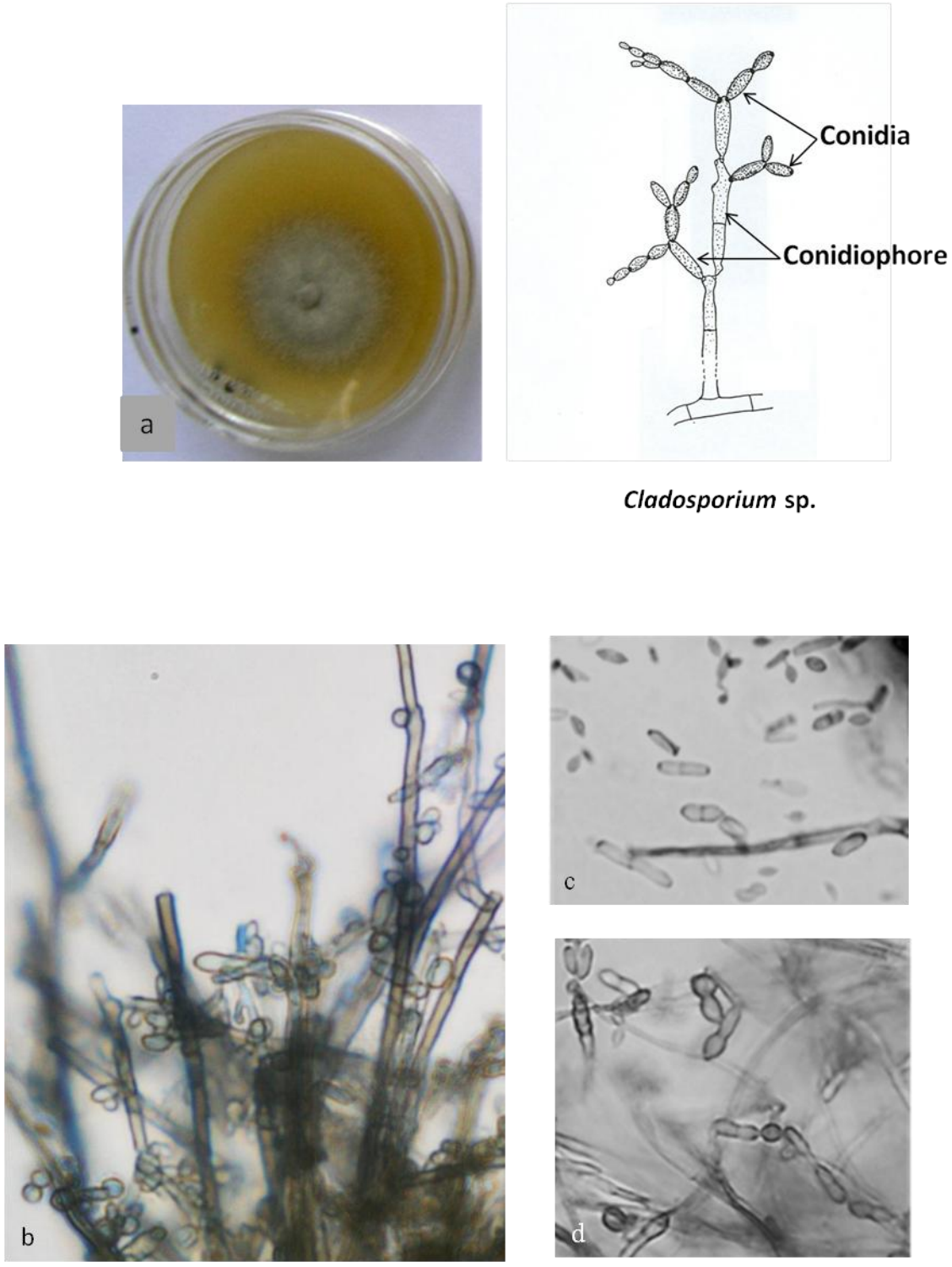


Figure 33. *Cladosporium cladosporioides* 1 a: culture on PDA plate; b, c and d: conidiophores and conidia ×400.

Strain n°. 5. *Phoma* sp. (Ascomycotina)

Fungal strain n°. 5 was isolated from stems of *C. procera*. It produced irregular, olivaceous grey colonies with darker patches on PDA plates. The reverse side of the colonies was blackish brown°. Mycelia were immersed, branched, septate and brown in color. Conidiomata were pycnidial, dark brown in color, semi-immersed and thin-walled. Pycnidia were developed separately or aggregately on medium. Pycnidia were sometimes become erumpent, unilocular and globose. Conidia were straight cylindrical with 1-4 guttules. Chlamydospores were multicellular. Pseudosclerotia were formed by aggregated clamydospores which were pale to dark brown in color. This fungal strain belonged to Ascomycotina and was affiliated with *Phoma*. However, the strain failed to survive during the study, so we did not have the possibility to confirm the taxonomic affiliation using molecular approach.

3.1.2.1.2. *Trigonella foenum-graecum* (L.)

Five endophytic fungi were isolated from seeds. Three belonged to Deuteromycetes and were grouped as sterile fungi. Two belonged to Ascomycotina and were affiliated to two genera:

Strain n°. 6. *Chaetomium globosum* (Ascomycotina)

Isolate n°. 6 produced colonies on PDA plates that pale white with darker patches. The reverse side of the colonies was brown (Fig 34). Perithecia were 16.3-17.5×18.8-21.3 µm globose, subglobose, or barrel-shaped, covered with terminal hairs in the upper surface, rhizoidal basally: terminal hairs dark yellowish green, straight, wavy, curved, loosely curled spirally. Asci were hyaline, clavate, 8-ascosporous. Ascospores were 0.8×1-1.1 µm, hyaline, lemon-shaped, apiculate at both ends. This strain was affiliated to *Chaetomium* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

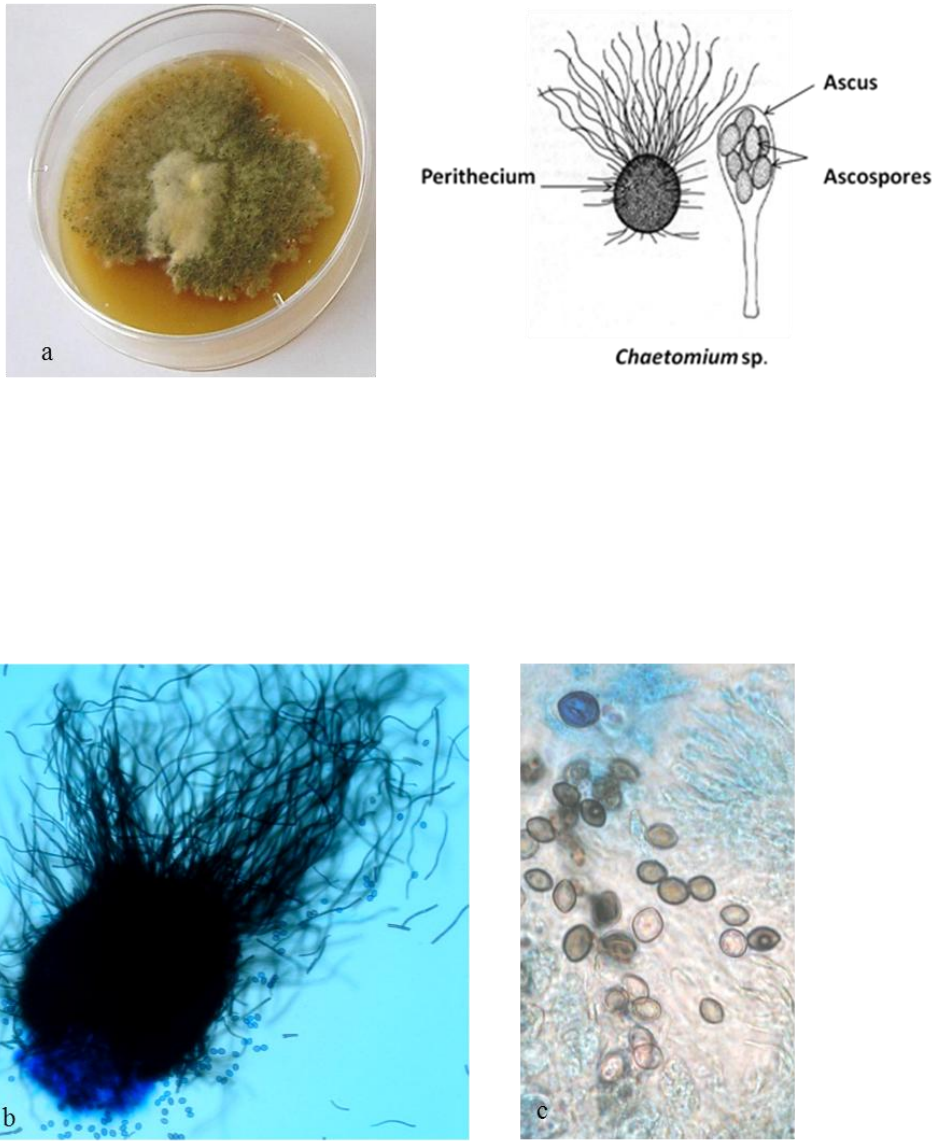


Figure 34. *Chaetomium globosum* a: culture on PDA plate; b: terminal hairs on perithecium ×100; c: asci and ascospores ×400.

- **Strain n°. 7. *Aspergillus terreus* 2 (Ascomycotina)**

Colonies on PDA plates were brown on both sides (Fig. 35). Conidiophores were 13.8-16.3 µm, hyaline, simple, occasionally thick-walled, inflated globosely or ellipsoidally at the apex (alled vesicles 1.8-2 µm), bearing spore heads composed of catenulate conidia borne on uniseriate phialides on vesicles: conidial heads light brown, loosely columnar. Conidia were phialosporous 0.3 µm, pale brown to yellowish brown, globose, delicately rough at the surface. This isolate belonged to Ascomycotina and was affiliated with *A. terreus*. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strains n°. 8, 9 and 10. Mycelia sterilia spp (Deuteromycetes)**

On PDA plates colonies were grey with pale white reverse side. Only hypha and some chlamydospores were discernable, even on other media such as PSA and CMA. These fungal strains failed to survive during the study, so we did not have the possibility to analyze the taxonomic affiliation using molecular approach.

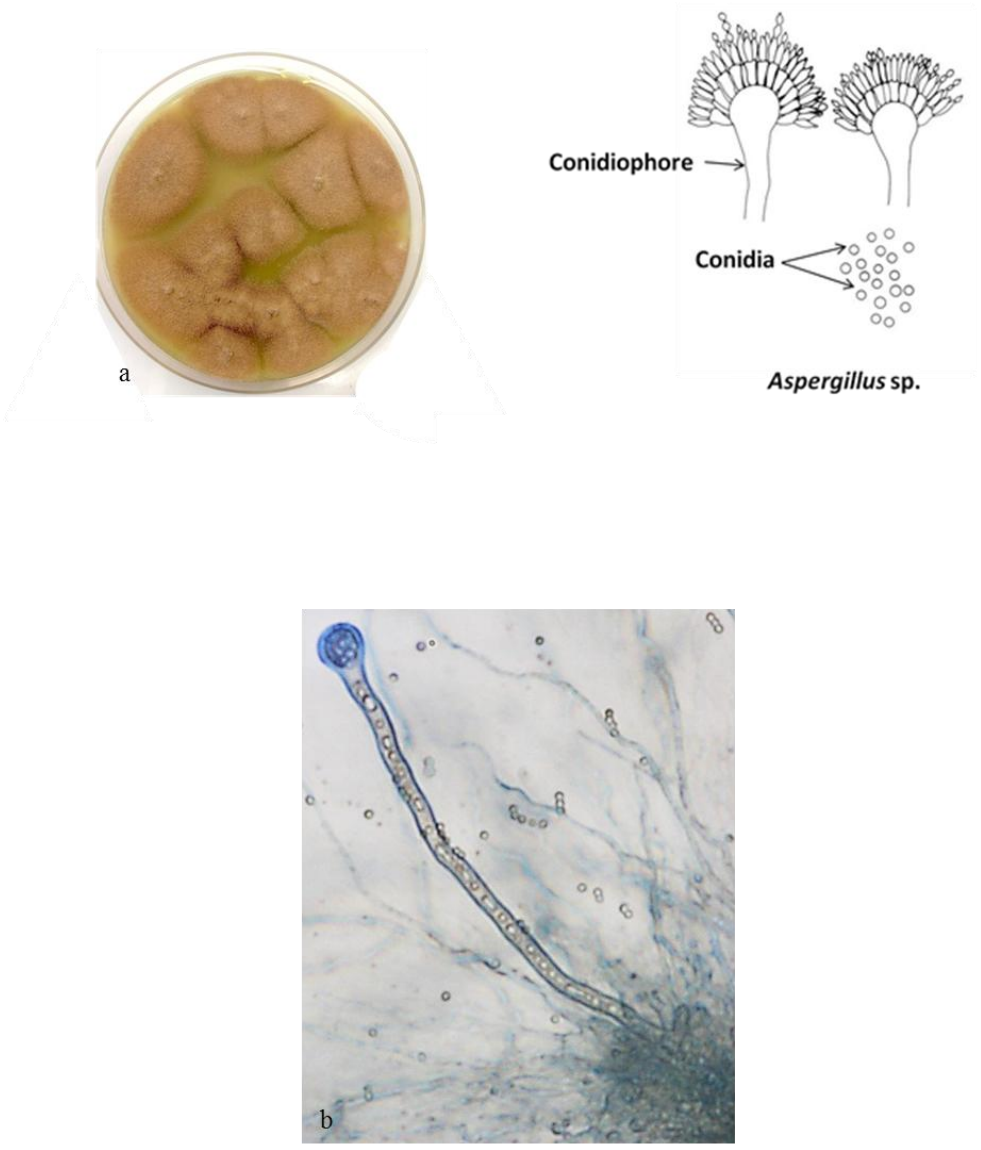


Figure 35. *Aspergillus terreus* 2 a: culture on PDA plate; b: vesicle, phialides, and conidia ×400.

3.1.2.2. *Vernonia amygdalina* (Del.)

Three endophytic fungi were isolated from leaves and stems of *V. amygdalina.* All the isolates belonged to Ascomycotina and were affiliated with 3 genera.

- **Strain n°. 11. *Cladosporium cladosporioides 2* (Ascomycotina)**

This fungus was isolated from leaves of *V. amygdalina*. Colonies on PDA plates were dark grey with grey reverse side (Fig. 36). Conidiophores were pale brown, erect, branched 2–3 times, at the apical parts, bearing catenulate conidia in each branch. Conidia were blastosporous, often not well differentiated from branches, hyaline or pale brown, ovate, ellipsoidal, cylindrical, subglobose, irregular in shape, apiculate at one end, often truncate at another end. This isolate belonged to Ascomycotina and was affiliated with *Cladosporium* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 12. *Curvularia papendorfii* (Ascomycotina)**

Fungus n°. 12 was isolated from both stems and leaves of *V. amygdalina***.** Colonies on PDA plates were dark grey with grey reverse side (Fig. 37). Conidiophores were erect, brown, simple or branched, straight or curved, bearing conidia apically and laterally, conspicuous pores left after detachment of conidia. Conidia were porosporous, subellipsoidal, mostly 4-celled, darker brown in 2-central cells, especially curved, larger in the penultimate cells, with indistinct hilum basally. Chylamidospoes were intercellular 1.3-1.8×1.8-2.5 µm. This isolate belonged to Ascomycotina and was affiliated with *Curvularia* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 13. *Hansfordia sinuosae* (Ascomycotina)**

This fungus was isolated from stems of *V. amygdalina***.** Colonies were black with darker patches on PDA plates with a black reverse side (Fig. 38). Perithecia were black 0.6-0.9×0.7-1 µm, ovate, subglobose or barrel-shaped, covered with terminal hairs on almost all the upper surface; terminal hairs dark brown, simple, straight, or curved, branched dichotomously. Asci were hyaline, clavate, 8-ascosporous. Ascospores were 0.3-0.6×0.5-0.8 µm pale brown, ovate or ellipsoidal, apiculate at one or both ends. This isolate belonged to

Ascomycotina and was affiliated with *Hansfordia sinuosae*. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

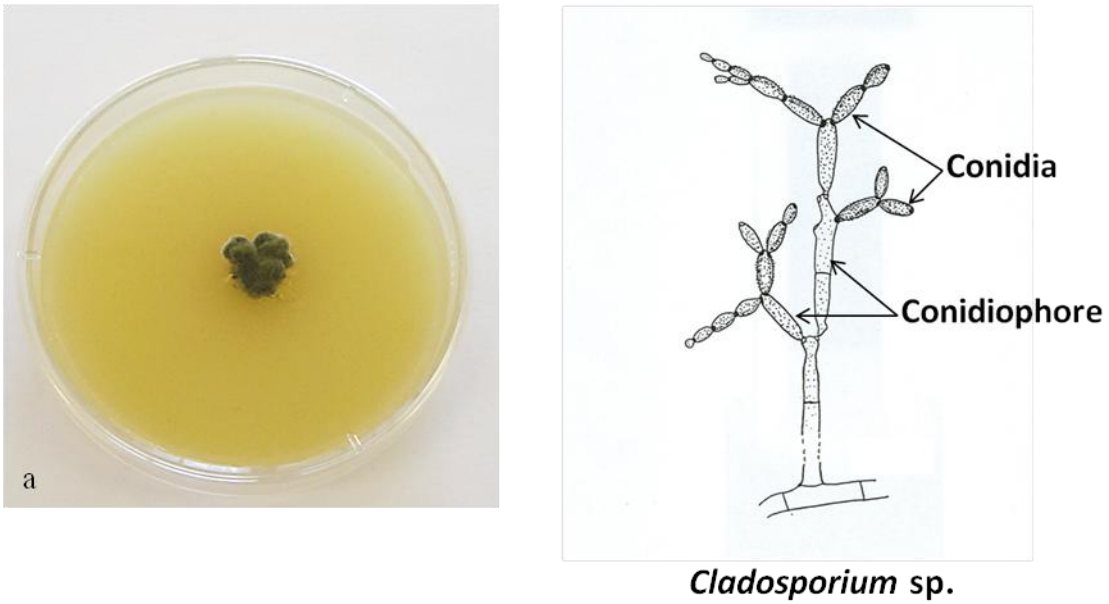
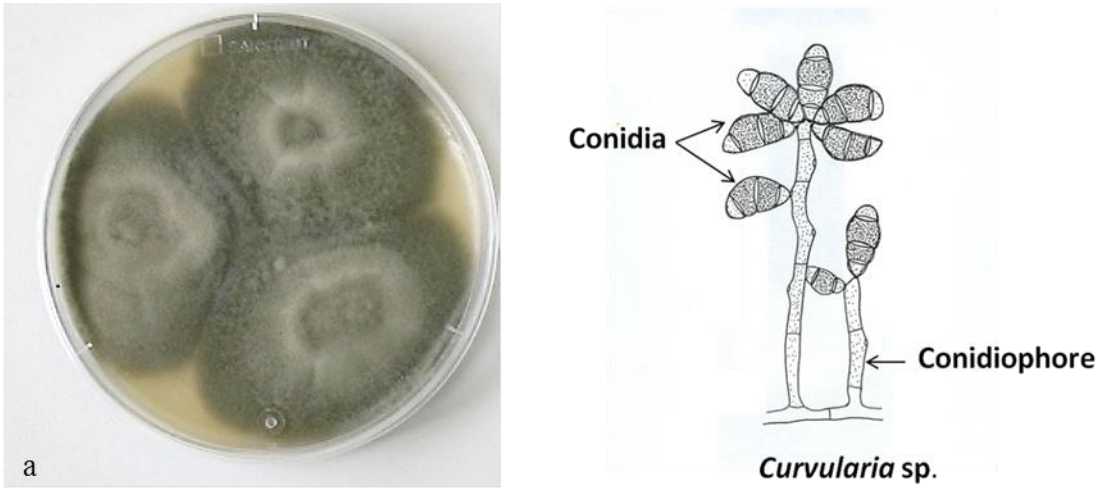


Figure 36. *Cladosporium cladosporioides* 2 a: culture on PDA plate; b, c and d: conidiophore, conidia and hypha ×400.



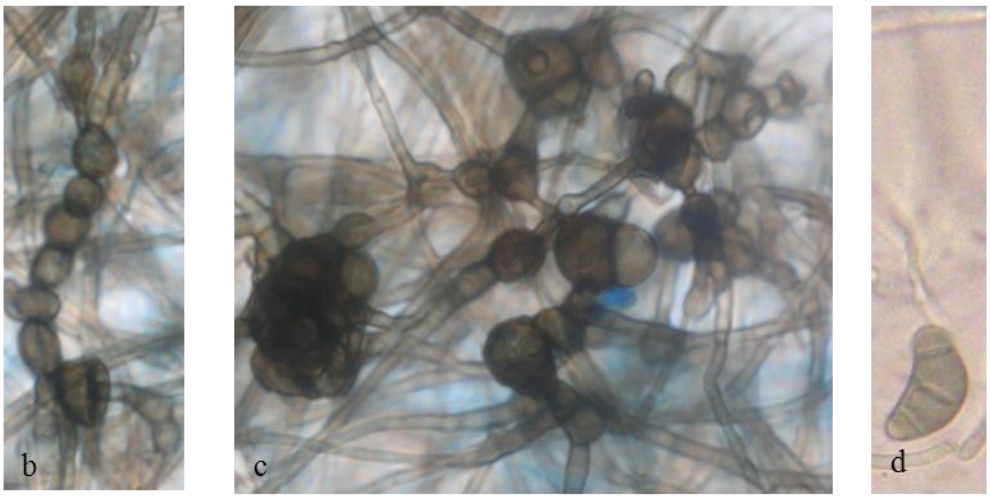


Figure 37. *Curvularia papendorfii* a: culture on PDA plate; b and c chlamydospore ×400; d: conidia ×400.

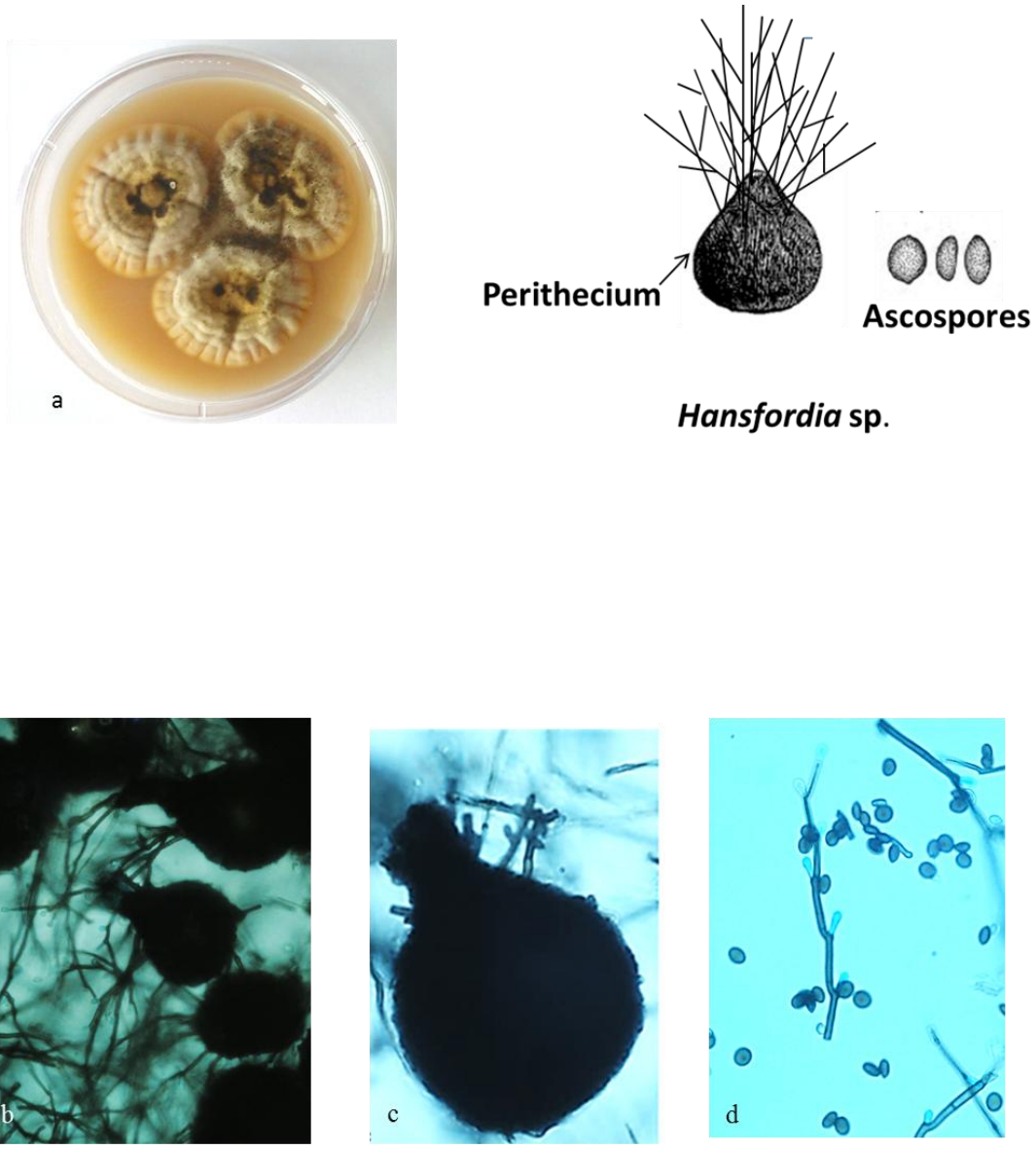


Figure 38. *Hansfordia sinuosae* a: culture on PDA plate; b: terminal hairs on perithecia ×100; c: perithecium and d: ascospores ×400.

3.1.2.3. *Catharanthus roseus* (L.)

Six endophytic fungal isolates were obtained from leaves and stems of *C. roseus.* All the isolates belonged to Ascomycotina and were affiliated with 6 genera.

- **Strain n°. 14. *Curvularia aeria* (Ascomycotina)**

Strain n°. 14 was isolated from leaves of *C. roseus*. Colonies on PDA plates were on both sides (Fig. 39). Conidiophores were erect, brown, simple or branched, straight or curved, bearing conidia apically and laterally, conspicuous pores left after detachment of conidia. Conidia were porosporous, subellipsoidal 0.8-1×2-2.5 µm, mostly 4-celled, darker brown in 2-central cells, especially curved, larger in the penultimate cells, with indistinct hilum basally. This isolate belonged to Ascomycotina and was affiliated with *Curvularia aeria*. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 15. *Chaetomium* sp. (Ascomycotina)**

Fungal strain n°. 15 was isolated from stems of *C. roseus*. Colonies were brown with darker patches on PDA plates whereas reverse side was brown (Fig. 40). Perithecia were globose, subglobose, or barrel-shaped, covered with terminal hairs in the upper surface, rhizoidal basally: terminal hairs dark yellowish green, straight, wavy, curved, loosely curled spirally often 4 times or undulate. Asci were hyaline, clavate, 8-ascosporous. Ascospores were hyaline, lemon-shaped, apiculate at both ends. This strain affiliated with *Chaetomium* sp. was failed to survive during the study. We did not have the possibility to confirm the taxonomic affiliation using molecular approach.

Strain n°. 16. *Phoma multirostrata* (Ascomycotina)

This strain was isolated from stems of *C. roseus*. It produced irregular, black colonies with darker patches on PDA plates (Fig. 41). Pycnidia were 11.3×15.8 µm, globose, subglobose, or disc-shaped, conspicuously ostiolate: peridium dark brown, pseudoparenchymatous. Conidia were 0.3×0.4-0.5 µm hyaline, ellipsoidal, 1-celled. Chlamydospores solitary, dark brown, granulate, thick-walled This isolate belonged to Ascomycotina and was affiliated with *Phoma* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

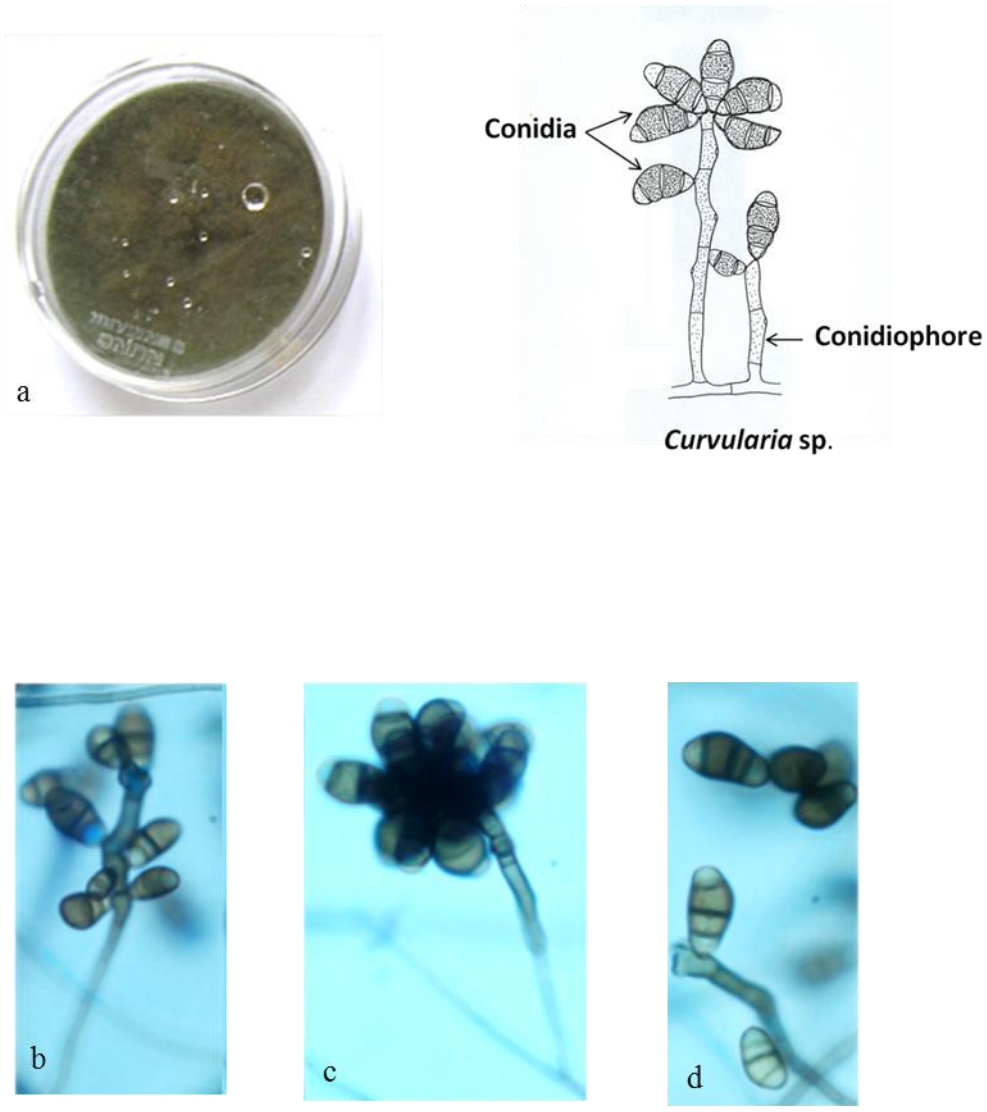


Figure 39. *Curvularia aeria* a: culture on PDA plate; b, c and d: conidiophore and conidia ×400.

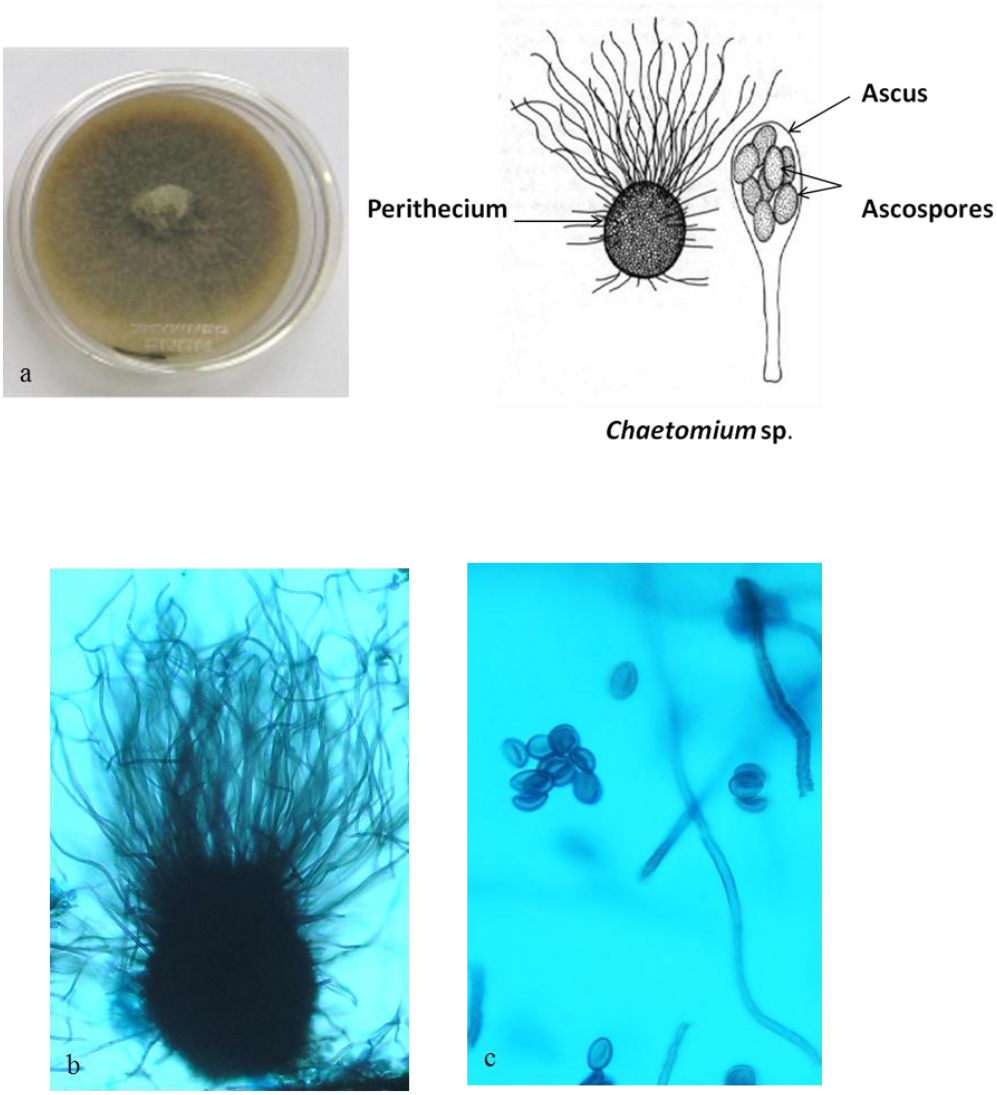


Figure 40. *Chaetomium* sp. a: culture on PDA plate; b: terminal hairs on perithecium ×100; c: ascospores ×400.

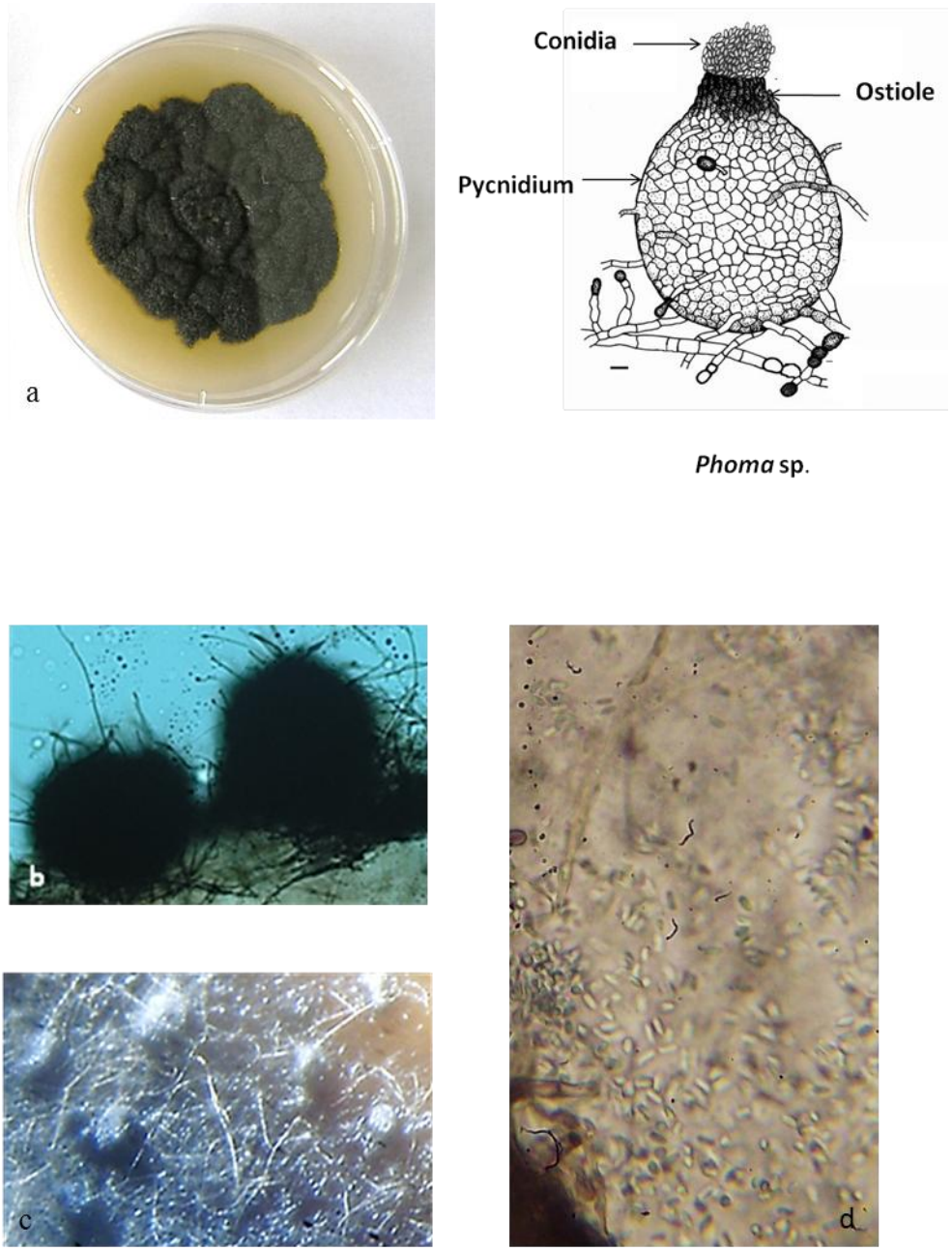


Figure 41. *Phoma multirostrata* a: culture on PDA plate; b: pycnidia ×100; c: pycnidial ostiole ×100; d: conidia ×400.

- **Strain n°. 17. *Pleosporales* sp. (Ascomycotina)**

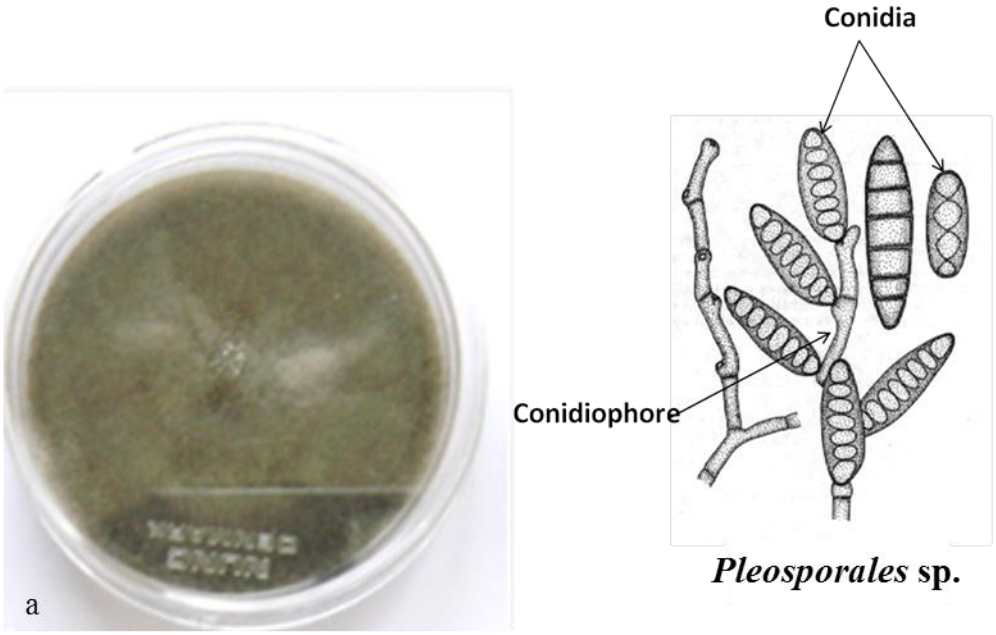
Isolate n°. 17 was isolated from leaves of *C. roseus*. Colonies were dark grey on PDA plates with a black reverse side (Fig. 42). Conidiophores were brown, erect, simple or branched, bearing conidia apically and laterally on apical fertile parts. Conidia were porosporous, solitary, pale brown, ellipsoidal 0.8-1.3×1.8-3.8 µm, usually 4-celled, occasionally inflated apically, without hilum basally. This isolate belonged to Ascomycotina and was affiliated with *Pleosporales* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 18. *Emericella* sp. (Ascomycotina)**

Fungal strain n°. 18 was isolated from leaves of *C. roseus*. On PDA plates colonies were typically olivaceous green in color with dark red-brown (Fig. 43). Reverse side was reddish brown°. Cleistothecia were developing within and upon the conidial layer. Conidial heads were short, columnar and biseriate. Conidiophores were usually short, brownish and smooth-walled. Conidia were globose and rough-walled. Thick-walled globose Hülle cells were present also red ascospores. This fungal strain affiliated with *Emericella* was failed to survive during the study. It was not possible to confirm the taxonomic affiliation using molecular approach.

Strain n°. 19. *Aspergillus niger* (Ascomycotina)

This strain was isolated from leaves of *C. roseus*. This strain grew rapidly on PDA plates and colonies were carbon black in color. The strain produced abundant submerged mycelia in the medium. Conidiophores were smooth with thick walls, unseptate. They were uncoloured near the vesicle. Conidial heads were fuscous black, globose. Vesicles were colorless and globose, thick walled. Conidial chains were present over the entire surface of vesicles. Conidia were rough, globose. This fungal strain was failed to survive during the study. We did not have the possibility to confirm its taxonomic affiliation with *Aspergillus*.



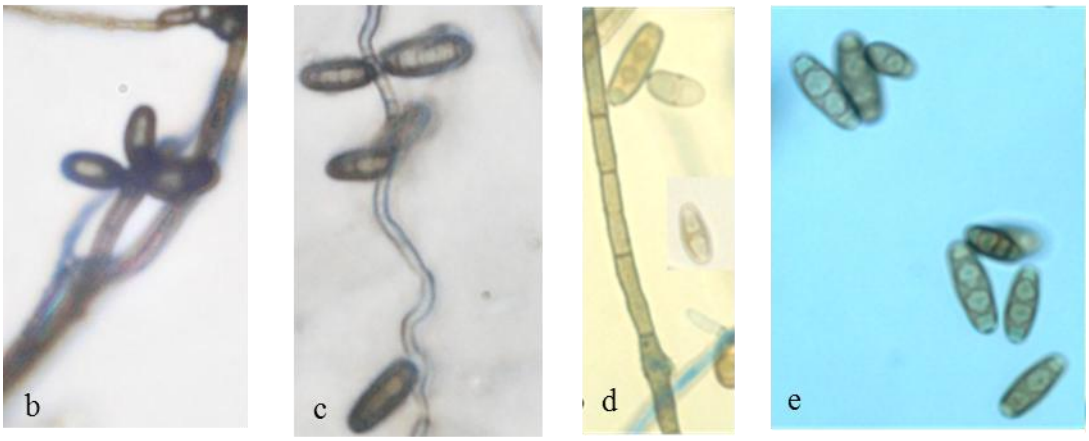
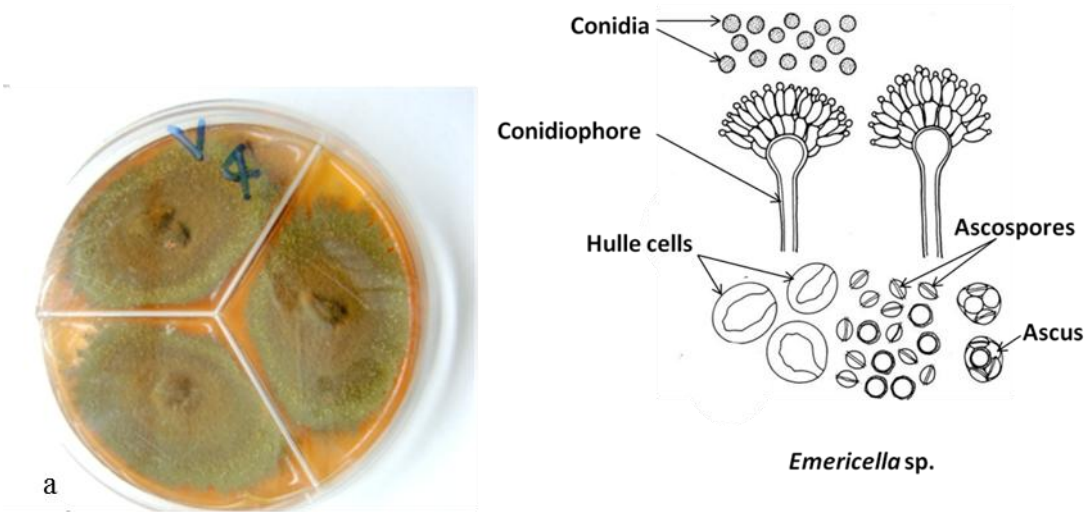


Figure 42. *Pleosporales* sp. a: culture on PDA; b, c, d and e: conidiophore, conidia and hypha ×400.



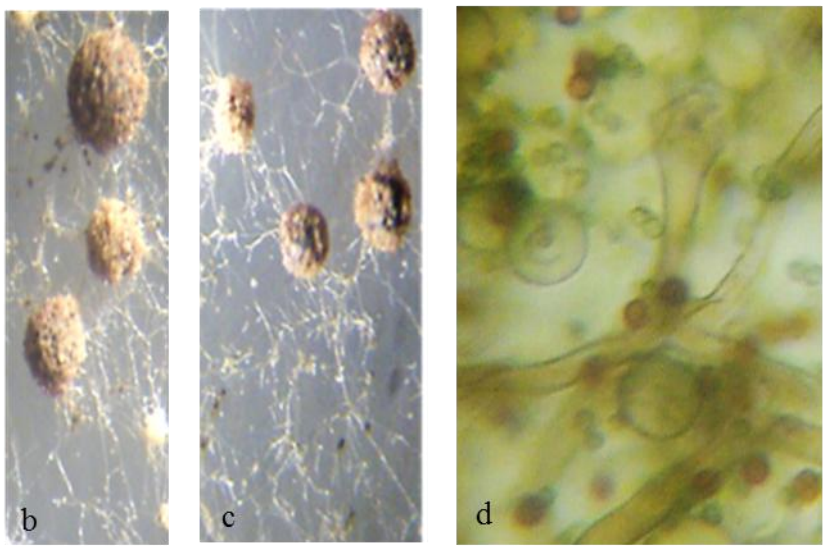


Figure 43. *Emericella* sp. a: culture on PDA plate; b and c: cleistothecia ×100; d: conidiophore, conidia, Hulle cells and red ascospore ×400.

3.1.2.4. *Euphorbia prostrata* (Ait.)

Four endophytic fungal strains were isolated from the aerial part (leaves and stems) *E. prostrata*. The four strains belonged to Ascomycotina and were affiliated with three genera.

- **Strains n°. 20 and 21. *Curvularia australiensis* 1 and 2 (Ascomycotina)**

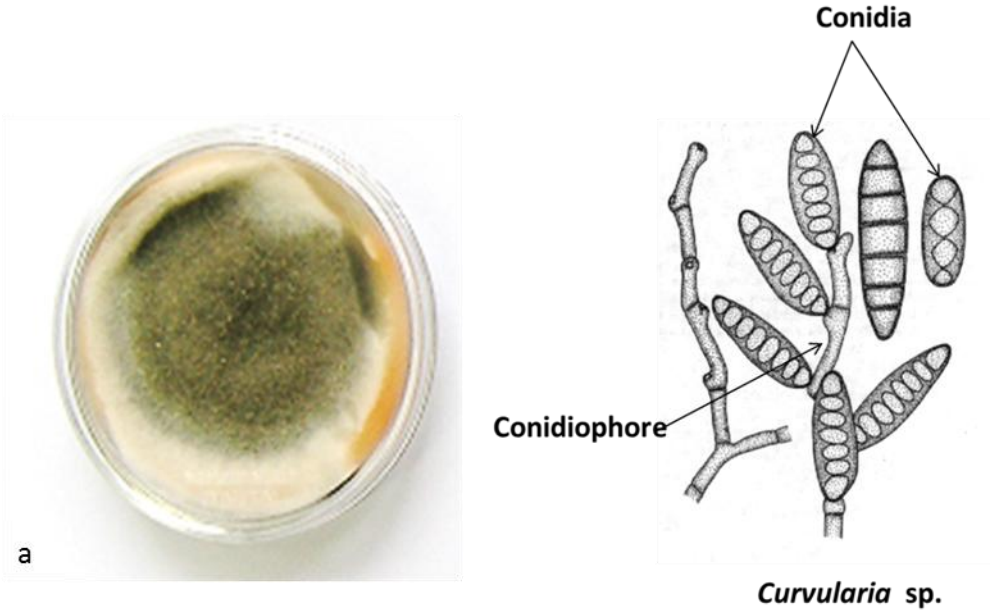
Two fungal strains with the same morphological characteristics were isolated from the aerial part (leaves and stems) of *E. prostrata*. Isolate n°. 20 produced dark grey colonies with black reverse side on PDA plates (Fig. 44). Isolate n°. 21 produced pink colonies at the begging then become grey colonies with pinkish edges reverse side was brown on PDA plates (Fig. 45). Conidiophores were brown, erect, simple or branched, bearing conidia apically and laterally on apical fertile parts. Conidia were porosporous, solitary, pale brown 0.8-1.3×1.8-2.8 µm, ellipsoidal, usually 4-celled, occasionally inflated apically, without hilum basally. This isolate belonged to Ascomycotina and was affiliated with *Curvularia* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 22. *Byssochlamys spectabilis* (Ascomycotina)**

This fungal strain was isolated from the aerial part of (leaves and stems) *E. prostrata*. Colonies of this strain were green with white edges on PDA plates. The reverse side was pale white (Fig. 46). Conidiophores were hyaline, erect, branched apically, bearing catenulate conidia on terminal phialides: phialides opposite, or occasionally verticillate, ampulliform with cylindrical base and acutely pointed in the median°. Conidia were phialosporous 0.3×0.4-0.5 µm terminal, subglobose or broadly ellipsoidal, slightly rough or minutely echinulate on the surface. This isolate belonged to Ascomycotina and was affiliated with *Byssochlamys* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).

- **Strain n°. 23. *Alternaria* sp. (Ascomycotina)**

This fungal strain was isolated from the aerial part of *E. prostrata* (leaves and stems) Colonies were grey with light grey patches on PDA plates. The reverse side was black (Fig. 47). Conidiophores were brown, erect and simple or branched, bearing one to several conidia apically or subapically. Conidia were porosporous 1.3-2×2.8-5 µm, brown to dark brown, ellipsoidal, ovate, muriform composed of usually 3- transverse septa and 1–3 longitudinal septa, constricted at or near septa, rough marginally. This isolate belonged to Ascomycotina and was affiliated with *Alternaria* sp. The taxonomic characterization was confirmed by ITS sequencing (Table 3).



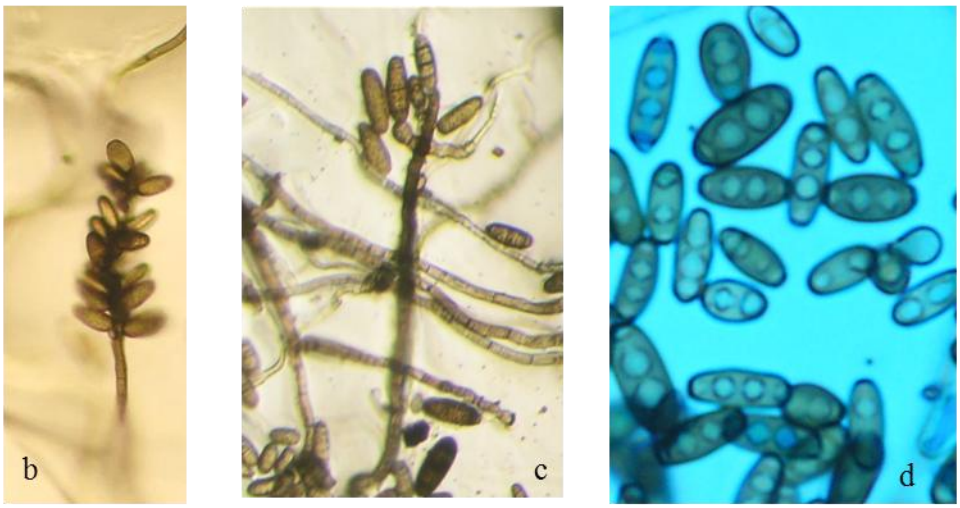


Figure 44. *Curvularia australiensis* 1 a: culture on PDA plate; b and c: conidiophore and conidia ×100; d: conidia ×400.

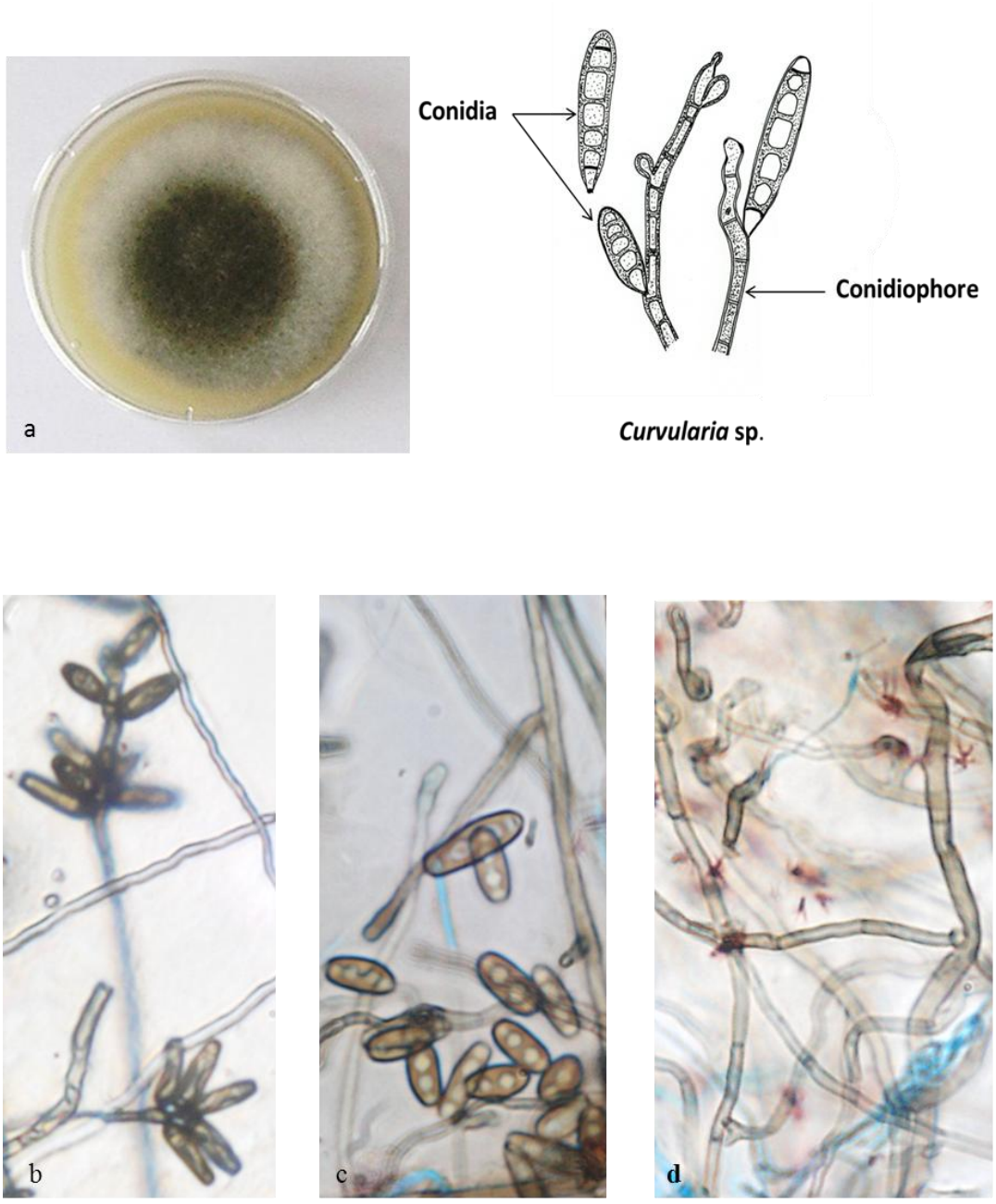


Figure 45. *Curvularia australiensis* 2 a: culture on PDA plate; b: conidiophore and conidia ×400; c: conidia and d: red crystals ×400.

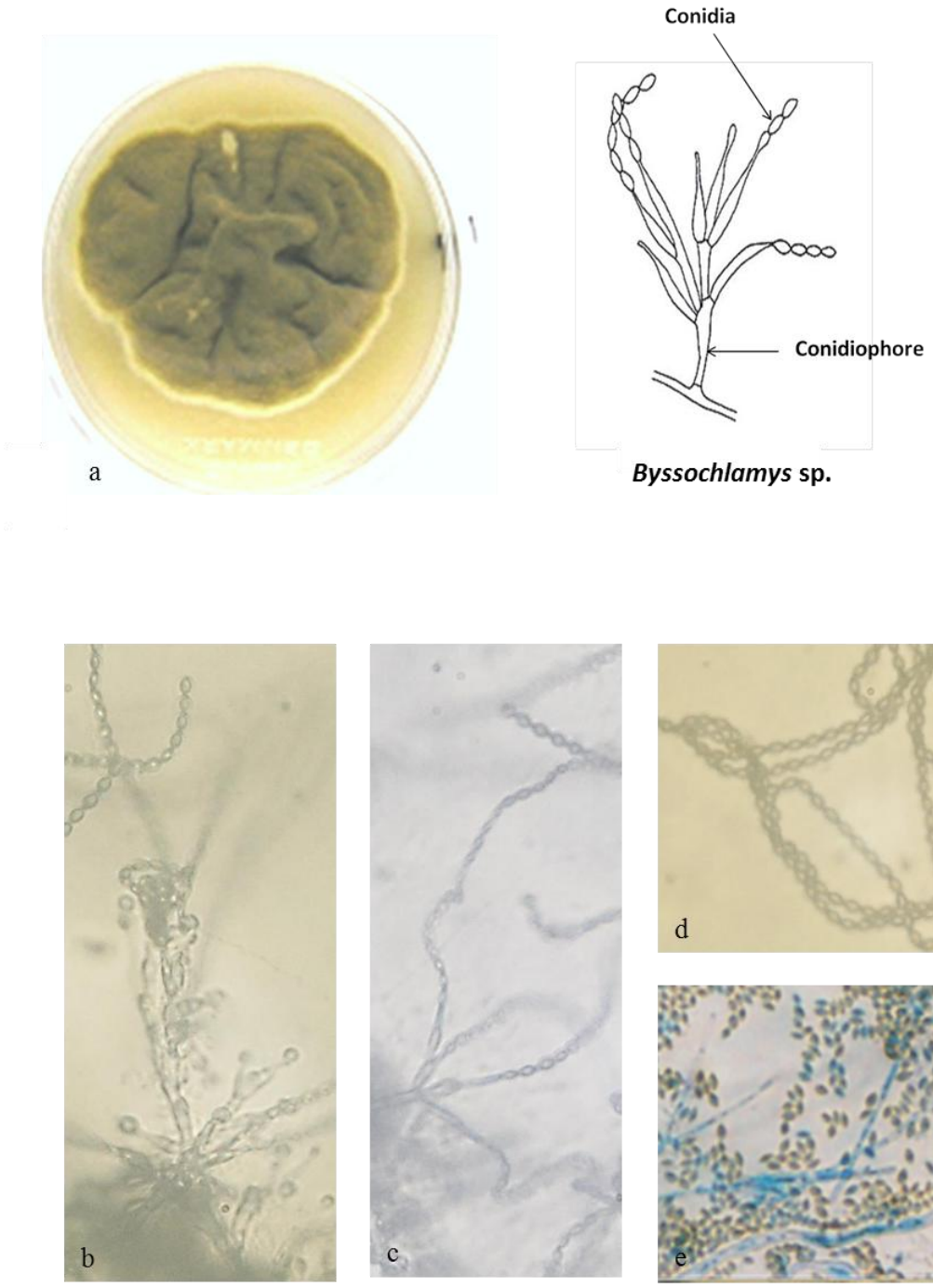


Figure 46. *Byssochlamys spectabilis* a: culture on PDA plate; b and c: conidiophores. d: conidia chains; e: conidia ×400.

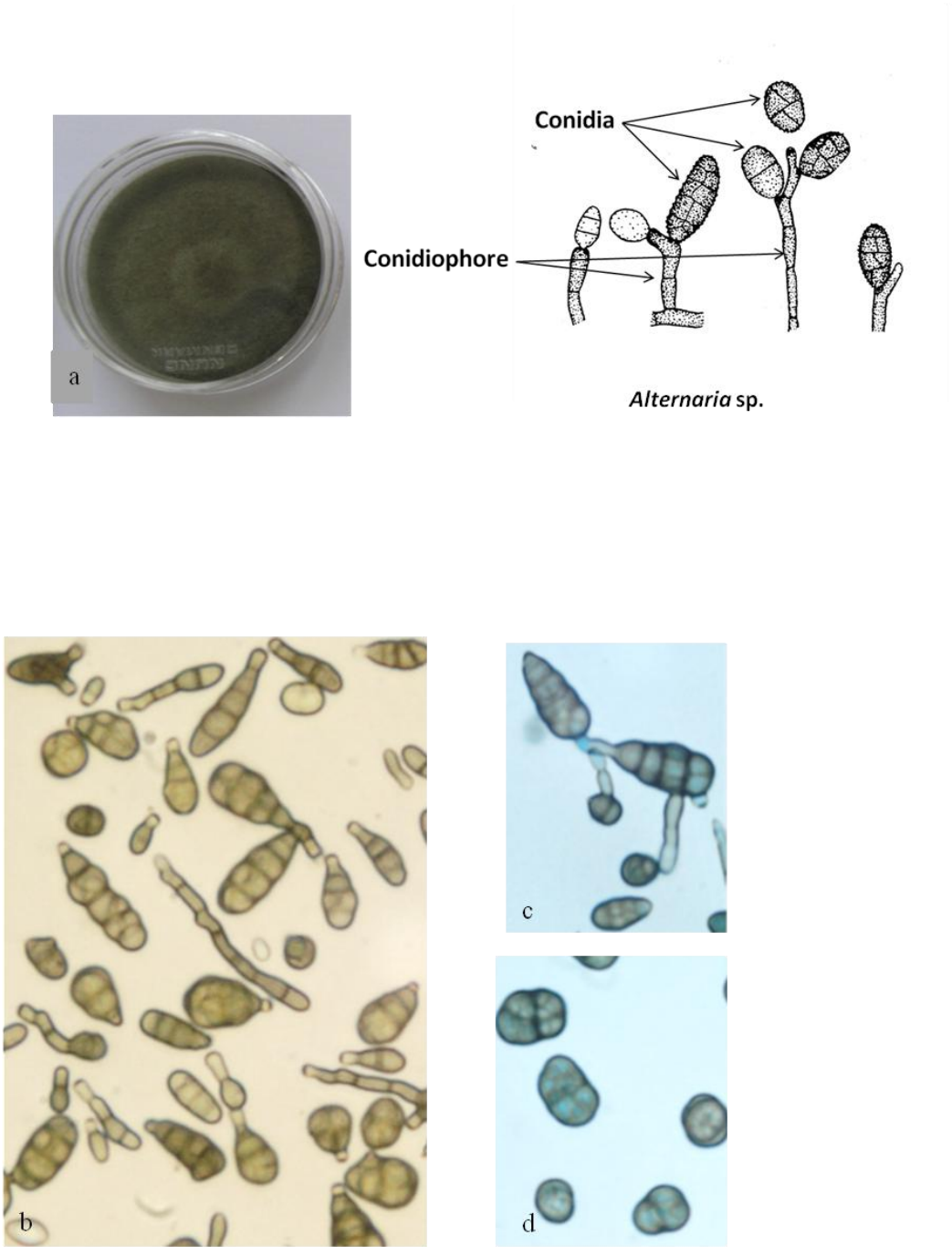


Figure 47. *Alternaria* sp. a: culture on PDA plate; b and c: conidia and conidiophore ×400.

3.1.3. Biodiversity of endophytic fungi from Sudanese medicinal plants

Endophytic fungi play an important role in physiological activities of their host plants, such as influencing stress and disease resistance, insects and mammalian herbivores deterrence, increase of biomass. Diverse endophytic fungi reside in medicinal plants, representing a rich resource of bioactive natural products with potential for exploitation in pharmaceutical and agricultural arenas (Schullz *et al*., 2002).

In our study, we attempted to analyze the diversity of culturable fungal endophytes in 5 plant species that are used in the traditional medicine in Sudan. Between three and six strains were isolated from the leaves, stems and/or seeds of each plant. As compared to other studies, the number of culturable endophytic fungi isolated from Sudanese medicinal plants is really low (Khan, 2007; Huang *et al*., 2007; Selvanathan *et al*., 2011). Environmental factors such as rainfall and atmospheric humidity could affect endophytic fungal communities within their host plants (Petrini, 1991; Selvanathan *et al.,* 2011). In this study, the density of endophytic microorganisms in the five plants could be influenced by climate conditions that are extremely arid for most of the year in Sudan (with about nine months with average rainfall lower than five mm), especially because the isolation of the endophtyes was performed during the dry months (October to January).

The 23 strains that have isolated on PDA plates were classified into 12 different taxa. 19 strains belong to Ascomycotina, whereas three strains belong to fungal class Deuteromycetes and only one belongs to Basidiomycotina.Three strains were failed to sporulate and were grouped as mycelia sterilia, a group of fungi that has been shown to be prevalent in some studies considering endophytic communities (Lacap *et al.*, 2003). Ascomycetes are known to well grown on artificial media such as PDA, as compared to Basidiomycetes. Among Ascomycetes, the genera identified were: *Alternaria*, *Aspergillus*, *Byssochlamys*, *Cladosporium*, *Chaetomium*, *Curvularia*, *Emericella*, *Phoma; Pleosporales* and *Hansfordia*. Among Basidiomycotina, one genus *Trametes* was identified. Most of the genera identified in this study were previously found as endophytes in tropical, sub-tropical or temperate plants (Dai *et al*., 2006; Huang *et al*., 2008). Some of these fungal genera have also been characterized in soil and in the phyllosphere of different plants (Watanabe, 2010).

In this study, most of the fungi were isolated either from leaves or stems of each plant species. Several previous reports showed that endophytic fungi could have tissues specificity and host preference (Gamboa and Bayman, 2001). However, in our study, because of the low number of strains that have been isolated from each plant, no significant evidence of both

tissue specificity and host preference was observed. Further studies using molecular tools are needed to have a better characterization of the diversity of endophytic fungal communities in those five Sudanese medicinal plants and to evaluate the influence of plant species and plant organs on this diversity.

In conclusion, this section was focused on the fungal endophytes strains, which isolated from five Sudanese plants: *Calotropis procera* Ait, *Catharanthus roseus* L, *Euphorbia prostrata* Ait, *Trigonella foenum-graecum* L and *Vernonia amygdalina* Del. A total of 23 endophytic fungal strains were isolated from the five plants after surface disinfection. Five isolates from *C. procera,* six isolates from *C. roseus*, four isolates from *E. prostrata,* five isolates from *T. foenum-graecum* three isolates from *V. amygdalina*. These fungal strains were classified into 12 different taxa. 19 strains belong to Ascomycotina, whereas three strains belong to fungal class Deuteromycetes and only one belongs to Basidiomycotina. Three strains were failed to sporulate and were grouped as mycelia sterilia. The diversity of the fungal endophytes of the five medicinal plants was not high. May be the majority of the endophytic fungi were non-culturable. Further studies using molecular approach (such as pyrosequencing) are needed to analyze the diversity of fungal endophytes in these 5 medicinal plants.

Section Two

Section Two

3.2. General physical and phytochemical screening of endophytes's crude extracts

3.2.1. Organoleptic properties and extractive values of endophyte fungal crude extracts

Ethyl acetate extracts of 21 endophytic fungi isolated from *Vernonia amygdalina*, *Calotropis procera*, *Catharanthus roseus*, *Euphorbia prostrata* and *Trigonella foenum-graecum* were prepared as described in chapter two Material and Methods. In Table 4 are shown the extractive values and organoleptic properties of the 21 endophytes. Among them, *Aspergillus terreus* 1 and *Alternaria alternata* (242 and 206 mg respectively) from *C. procera* were found to have the highest extractive yields followed by *Byssochlamys spectabilis* (155.4 mg) from *E. prostrata* then *Chaetomium* sp. (142.5 mg) from *C. roseus.* Whereas, *Curvularia australiensis* 1 (36.6 mg) from *E. prostrata* gave the lowest quantity of extract.

In addition, the color and texture of the endophytic fungi extracts were observed and shown in Table 4. The majority of the dried extracts were brown (light brown, or reddish brown) and few were pinkish orange or reddish black. The texture generally appeared to be ranged from viscous, sticky, stearic or powder.

3.2.2. Preliminary screening of chemical constitutes of the medicinal plants and their endophytes by thin layer chromatography

Ethyl acetate crude extracts of leaves and stems of *V. amygdalina*, *C. procera*, *C. roseus*, *E. prostrata* and seeds of *T. foenum-graecum* and their endophytes were subjected to preliminary phytochemical screening using thin layer chromatography technique. TLC plates of all extracts were developed using two different solvent systems. Petroleum ether: Ethyl acetate (8:2/v:v) and Petroleum ether: Acetone (7:3; v:v).

Table 4. Organoleptic properties and dry weight (mg per 20 plates of culture) of ethyl acetate extracts of endophytes from *Calotropis procera* (1-4), *Trigonella foenum-graecum* (5-9), *Vernonia amygdalina* (10-12), *Catharanthus roseus* (13-17) and *Euphorbia prostrata* (18-21).

Sample n°.	Crude extract of endophyte	Color	Texture	Dry weight (mg)
1	*Aspergillus terreus* 1	Dark brown	Viscous	**242**
2	*Cladosporium cladosporioides* 1	Brown	Viscous	67.0
3	*Alternaria alternata*	Brown	Viscous	**206**
4	*Trametes versicolor*	Brown	Viscous	81.6
5	*Chaetomium globosum*	Brown	Viscous	67.2
6	Mycelia sterilia sp. 1	Brown	Viscous	66.2
7	Mycelia sterilia sp. 2	Brown	Viscous	68.8
8	Mycelia sterilia sp. 3	Brown	Viscous	69.1
9	*Aspergillus terreus* 2	Dark brown	Viscous	85.9
10	*Cladosoprium cladosporioides* 2	Dark brown	Viscous	82.4
11	*Curvularia papendorfii*	Dark brown	Viscous	82.7
12	*Hansfordia sinuosae*	Brown	Viscous	104.9
13	*Curvularia aeria*	Reddish brown	Viscous	69.8
14	*Chaetomium* sp.	Dark brown	Viscous	**142.5**
15	*Phoma multirostrata*	Brown	Stearic	**125.7**
16	*Pleosporales* sp.	Reddish brown	Sticky	66.8
17	*Emericella* sp.	Brown	Sticky	107
18	*Curvularia australiensis* 1	Pinkish Black	Powder	36.6
19	*Curvularia australiensis* 2	Pinkish orange	Powder	121.9
20	*Byssochlamys spectabilis*	Brwon	Sticky	**155.4**
21	*Alternaria* sp.	Brwon	Viscous	61.3

The plates were detected with different reveling reagents: Sulfuric acid 20% as general reagent; Dragendorff's reagent for alkaloids; Natural product reagent for flavonoids, carbohydrates, anthocyanines and plant acids; Aluminum chloride for flavonoids and anthraquinones and Liebermann-Buchard's reagent for sterols and terpenoids as described by (Reich and Schibli, 2006). Chromatograms of all ethyl acetate crude extracts of endophytic fungi isolated from the fifth medicinal plants as well as crude extracts of the plants revealed

spots with different color characteristics and polarities, also sparing with specific reagents showed that all crude extracts were rich with different secondary metabolites such as flavonoids, polyphenols, terpenoids, sterols and alkaloids.

3.2.3. Thin layer chromatography of endophytic fungi crude extracts of *Calotropis procera*

Four endophytic fungi were isolated from *C. procera*: *Aspergillus terreus* 1 (N°.1), *Cladosporium cladosporioides* 1 (N°. 2), *Alternaria alternata* (N°. 3) and *Trametes versicolor* (N°. 4).

One non-polar R_f value: 0.95 was found in three endophytic fungi *Aspergillus terreus* 1 (N°.1) *Cladosporium cladosporioides* 1 (N°.2) and *Alternaria alternate* (N°.3) and leaves extract, whereas one polar spot R_f 0.15 was shared both with *Aspergillus terreus* 1, leaf and stem extracts. *Trametes versicolor* (N°. 4) had one non-polar spot R_f value 0.91 common also on leaves extract (Fig. 48-A). One spot R_f 0.9 was shared with all the endophytes and *C. procera* leaves and stems (Fig. 48-B).

For flavonoids only stem extract shared some spots with the endophytes. One blue spot with R_f 0.93 shared with *Aspergillus terreus* 1 (N°.1), *Cladosporium cladosporioides* 1 (N°.2) and *Alternaria alternata* (N°.3). Another spot was observed R_f 0.6 with *Aspergillus terreus* 1 (N°.1) and stem, also one spot with R_f 0.46 was found on both *Cladosporium cladosporioides* 1 (N°. 2) and stem extract (Fig. 49).

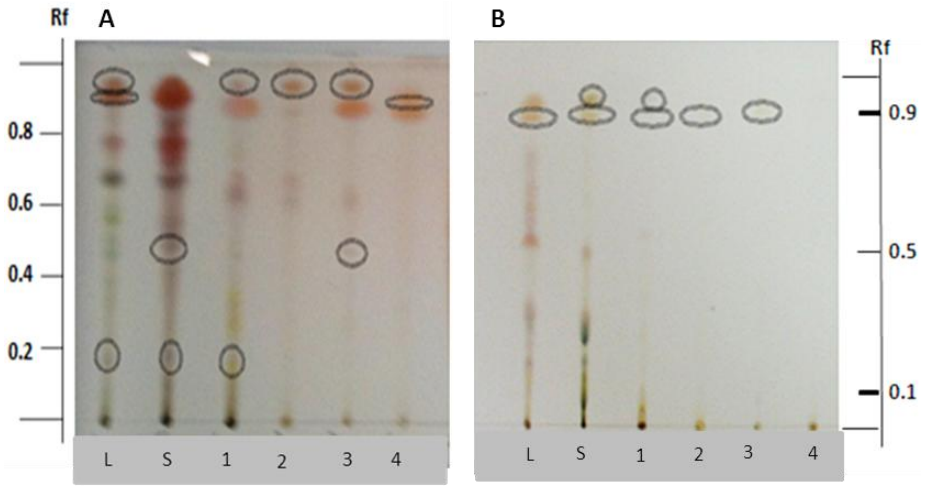


Figure 48. A. General TLC: *Calotropis procera* (L: leaves. S: stem) and its endophytic fungi (1, 2, 3 and 4) Solvent: Petroleum ether: Acetone (7:3); Reveling reagent's: Sulfuric acid 20%. B. Triterpenes and Sterols TLC. Solvent: Petroleum ether: Ethyl acetate (8:2). Reveling reagent's Liebermann-Buchard's.

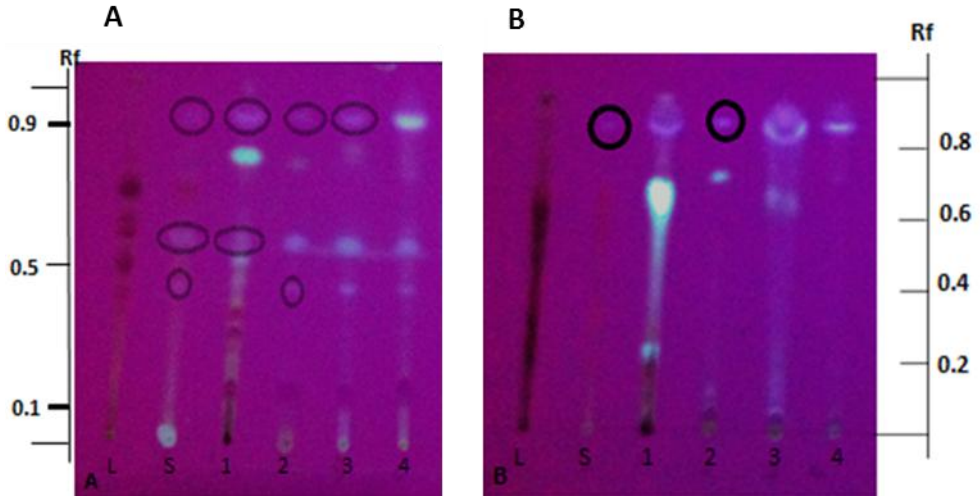


Figure 49. Flavonoids TLC: *Calotropis procera* (L: leaves. S: stems) and its endophytic fungi (1, 2, 3 and 4). **A**. Solvent: Petroleum ether: Acetone (7:3); Reveling reagent's: Natural product, under UV light (365nm). **B**. Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Aluminum chloride, under UV light (365nm).

3.2.4. Thin layer chromatography of endophytic fungi crude extracts of *Trigonella foenum-graecum*

Five endophytic fungi were isolated from *T. foenum-graecum*: *Chaetomium globosum* (N°. 5), three Mycelia sterilia spp. (N°. 6, 7 and 8 respectively) and *Aspergillus terreus* 2 (N°. 9).

One brown spot R_f 0.95 and yellow spot R_f 0.87 were displayed in both seed extract and Mycelia sterilia sp. (N°. 8) (Fig. 50-A). Also only non-polar spot with R_f 0.93 was shared with seeds extract and three endophytes *Chaetomium globosum* (N°. 5) and two Mycelia sterilia spp. (N°. 6 and 8) (Fig. 50-B). Concerning flavonoids one blue spot with R_f 0.95 existed in seed extract and Mycelia sterilia sp. (N°. 8) (Fig. 51-A).

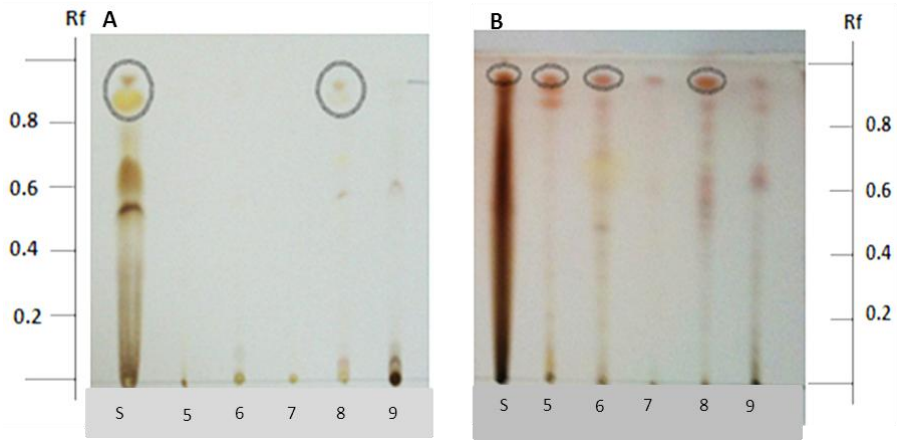


Figure 50. A. General TLC *Trigonella foenum-graecum* (seeds) and its endophytic fungi (5, 6, 7, 8 and 9); Solvent: Petroleum ether: Acetone (7:3); Reveling reagent's: Sulfuric acid 20%. **B.** Triterpenes and Sterols TLC. Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's Liebermann-Buchard's.

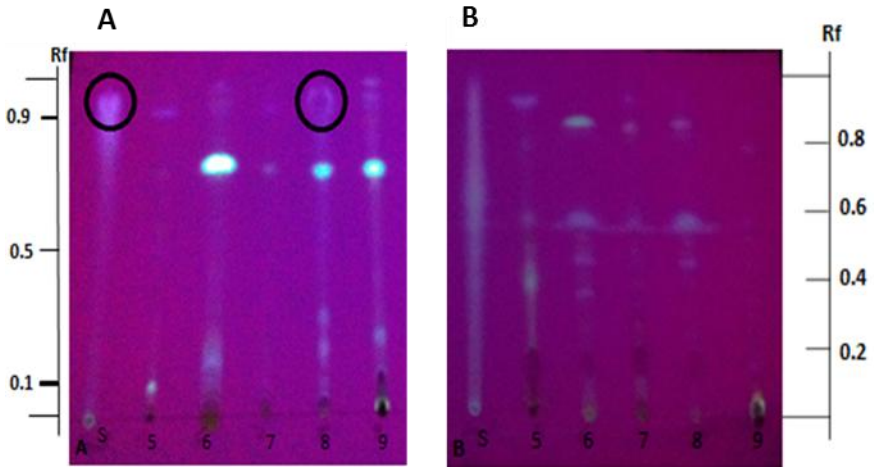


Figure 51. Flavonoids TLC: *Trigonella foenum-graecum* (seeds) and its endophytic fungi (5, 6, 7, 8 and 9); **A.** Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Aluminum chloride, under UV light (365nm). **B**. Solvent: Petroleum ether: Acetone (7:3). Reveling reagent's: Natural product, under UV light (365nm).

3.2.5. Thin layer chromatography of endophytic fungi crude extracts of *Vernonia amygdalina*

Three endophytic fungi were isolated from *V. amygdalina* leaves and stems: *Cladosporium cladosporioides* 2 (N°.10), *Curvularia papendorfii* (N°.11) and *Hansfordia sinuosae* (N°.12). Three spots with R_f values: 0.36, 0.47 and 0.95 respectively were shared by *Cladosporium cladosporioides* 2 (N°.10), *Curvularia papendorfii* (N°.11) and leaves extract of *V. amygdalina*. Two violet spots with R_f values: 0.77 and 0.84 respectively were found in *Cladosporium cladosporioides* 2 (N°.10) and stem extract (Fig. 52-A). Chromatogram sprayed with Dragendorff's reagent displayed two yellow spots, R_f values: 0.39 and 0.48 respectively were shared in *Cladosporium cladosporioides* 2 (N°.10), *Curvularia papendorfii* (N°.11) and leave extract of the host plants (Fig. 52-B). Dragendorff's reagent displayed normally red to orange color in the presence of alkaloids. Here the yellow color could be in relation with the low concentration of alkaloids at least in the leaves and stems of *V. amygdalina* which has been described by Adebayo *et al*. (2014).

Two chromatograms sprayed with Natural product and Aluminum chloride reagents showed no common spots with the endophytic fungi and leaf and stem extracts of their host plant for the flavonoid and polyphenol compounds (Fig. 53). *Hansfordia sinuosae* (N°.12). shared with plant stem one violet spot with R_f 0.9. (Fig. 54)

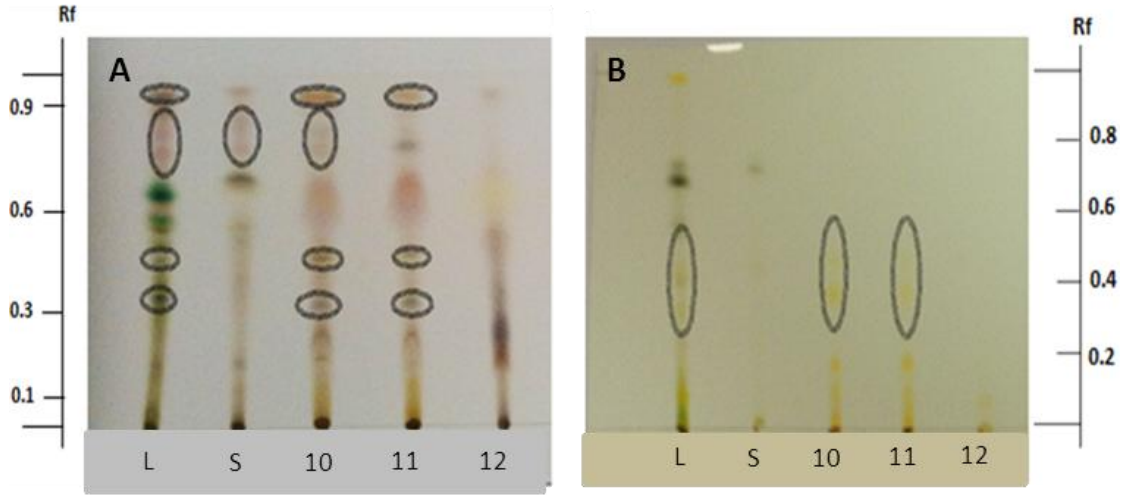


Figure 52. A. General TLC: *Vernonia amygdalina* (L: leaves. S: stems) and its endophytic fungi (10, 11 and 12). Solvent: Petroleum ether: Acetone (7:3), Reveling reagent's: Sulfuric acid 20%. B. Alkaloids TLC. Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Dragendorff.

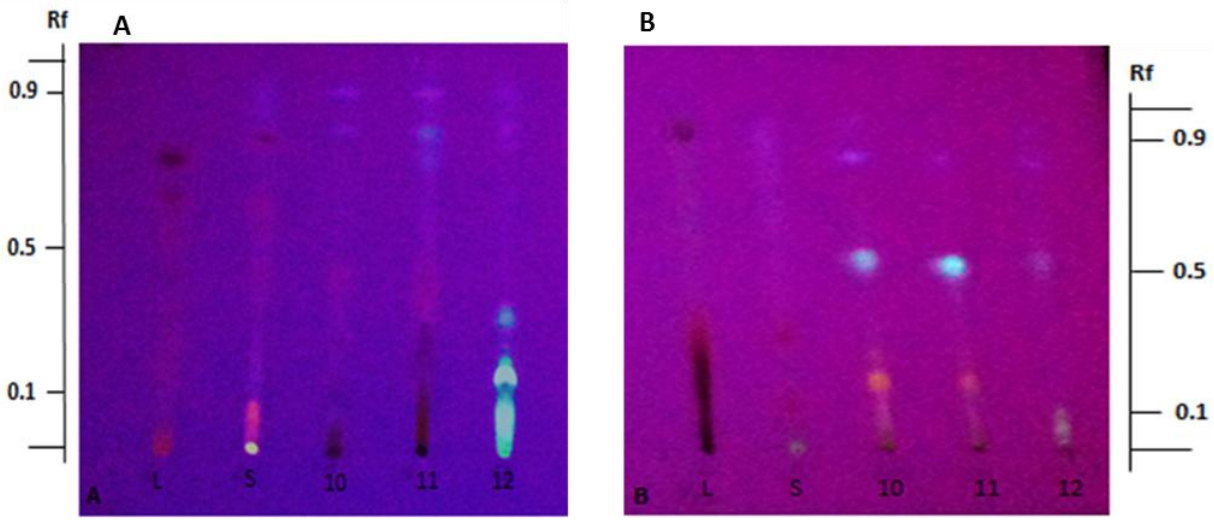


Figure 53. Flavonoids TLC: *Vernonia amygdalina* (L: leaves. S: stems) and its endophytic fungi (10, 11 and 12). **A.** Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Natural product, in UV light (365nm). **B.** Reveling reagent's: Aluminum chloride, under UV light (365nm).

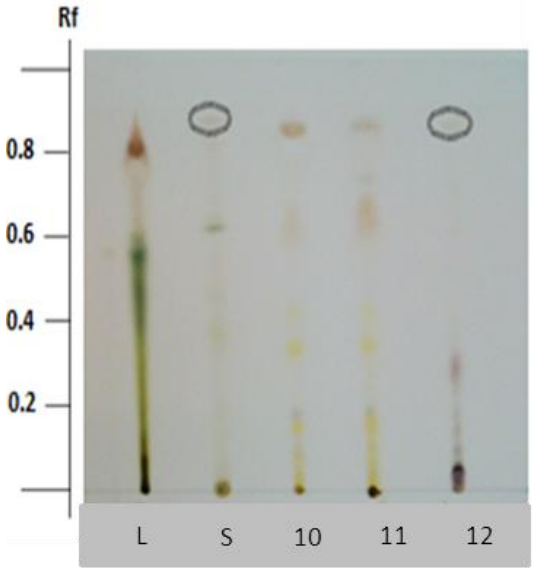


Figure 54. Triterpenes and Sterols TLC: *Vernonia amygdalina* (L: leaves and S: stems) and its endophytic fungi (10, 11 and 12) Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Liebermann-Buchar

3.2.6. Thin layer chromatography of endophytic fungi crude extracts of *Catharanthus roseus*

Five endophytic fungi were isolated from *C. roseus*: *Curvularia aeria* (N°.13), *Chaetomium* sp. (N°.14), *Phoma multirostrata* (N°.15), *Pleosporales* sp. (N°.16) and *Emericella* sp. (N°.17).

Phoma multirostrata (N°.15) revealed two violet spots R_f values: 0.56 and 0.8 respectively. They were also existing in the stem extract, the non-polar spot was also shared with endophyte (N°. 16) *Pleosporales* sp. (Fig. 55-A). One violet spots R_f value 0.77 was exhibited in both leaves and *Pleosporales* sp. (N°. 16). While one spot R_f value 0.6 was display in *Emericella* sp. (N°.17) and similar to one in leaves extract (Fig. 55-A). One brown spot R_f value 0.95 was shared with endophytes (N°. 15 and 16) *Phoma multirostrata*, *Pleosporales* sp. and plant stem. While leaves extract shared two spots with R_f (0.6 and 0.8) *Pleosporales* sp. (N°.16) and *Emericella* sp. (N°. 17) (Fig. 55-B). No shared spots with the endophytic fungi, leaf and stem extracts of the host plant for the flavonoid and polyphenol compounds were observed (Fig. 56).

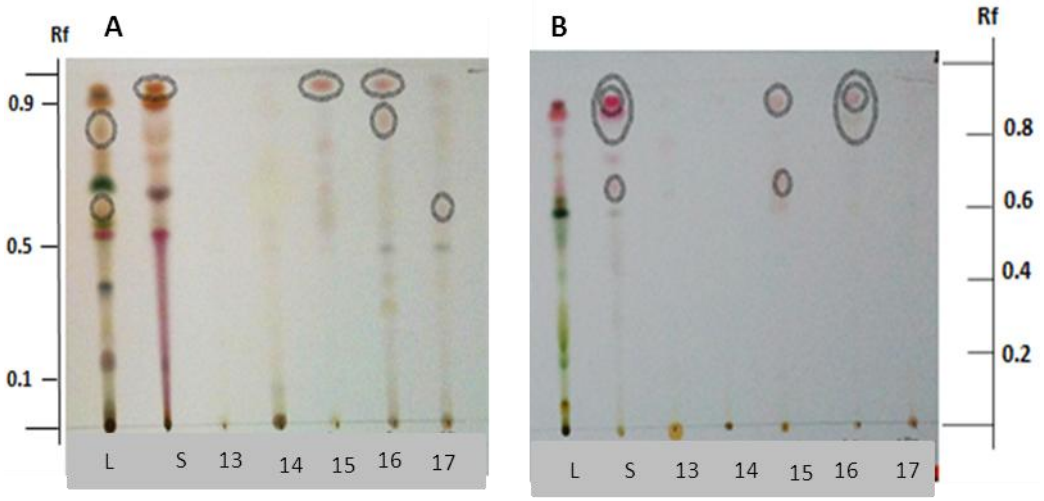


Figure 55. A. General TLC: *Catharanthus roseus* (L: leaves. S: stems) and its endophytic fungi (13, 14, 15, 16 and 17). Solvent: Petroleum ether: Acetone (7:3), Reveling reagent's: Sulfuric acid 20%. B. Triterpenes and Sterols TLC. Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Liebermann-Buchar.

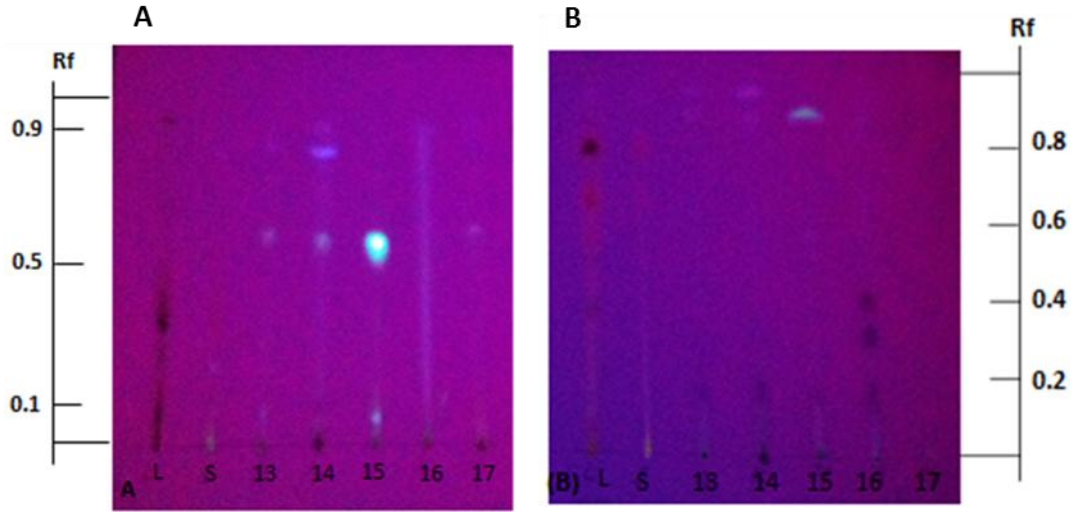


Figure 56. Flavonoids TLC *Catharanthus roseus* (L: leaves. S: stems) and its endophytic fungi (13, 14, 15, 16 and 17). **A.** Solvent: Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Aluminum chloride, under UV light (365nm). **B.** Solvent: Petroleum ether: Acetone (7:3), Reveling reagent's: Natural product, under UV light (365nm).

3.2.7. Thin layer chromatography of endophytic fungi crude extracts of *Euphorbia prostrata*

Four endophytic fungi were isolate from *E. prostrata*: *Curvularia australiensis* 1 (N°.18), *Curvularia australiensis* 2 (N°.19), *Byssochlamys spectabilis* (N°. 20) and *Alternaria* sp. (N°.21).

Only one non-polar reddish brown spots R_f value: 0,95 was displayed in both aerial part extract of *E. prostrata* and endophytic fungus (N°.19) *Curvularia australiensis* 2 (Fig. 57-A). While one violet spot R_f value 0,95 was common on all extracts *E. prostrata* and its endophytes (Fig. 57-B). No shared spot with the endophytic fungi and *E. prostrata* extract for the flavonoid and polyphenol compounds was observed (Fig. 58).

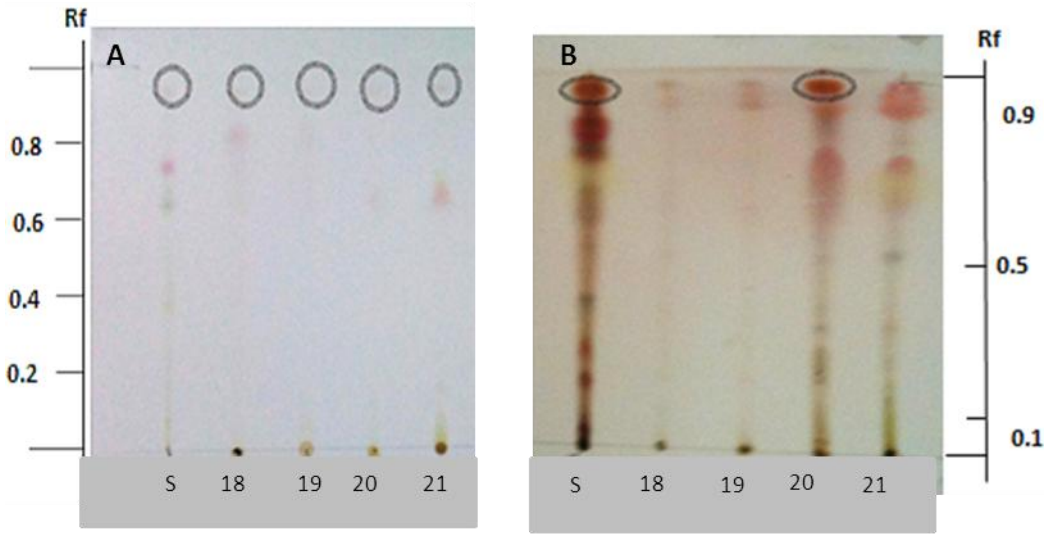


Figure 57. A. General TLC. *Euphorbia prostrata* (L: leaves. S: stems) and its endophytic fungi (18, 19, 20 and 21); Solvent: Petroleum ether: Acetone (7:3), Reveling reagent's: Sulfuric acid 20%. **B.** Triterpenes and Sterols TLC. Petroleum ether: Ethyl acetate (8:2), Reveling reagent's: Liebermann-Buchar.

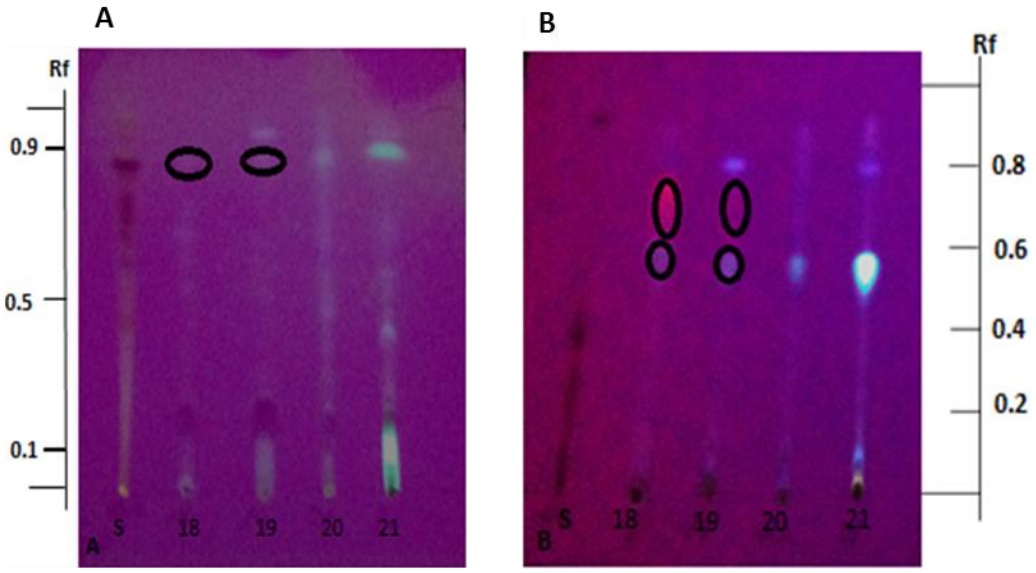


Figure 58. Flavonoids TLC in *Euphorbia prostrata* (L: leaves and S: stems) and its endophytic fungi (18, 19, 20 and 21) **A.** Solvent: Petroleum ether: Acetone (7:3); Reveling reagent's: Natural product, under UV light (365nm). **B.** Solvent: Petroleum ether: Ethyl acetate (8:2); Reveling reagent's: Aluminum chloride, under UV light (365nm).

On the pilot TLC screening it was clear that endophytic fungi isolated from *C. procera*, *T. foenum-graecum*, *V. amygdalina*, *C. roseus*, and *E. prostrata* were rich in distinct secondary metabolites similar or different from that of their host plants (Table 5). All extract of endophytes were very rich with terpenoids. This result is in accordance with Yu *et al*. (2010) who reported that endophytic fungi were rich in sesquiterpenes, diterpenoids and triterpenoids. The last group contained the major terpenoids isolated from endophytes. Souza *et al*. (2011) stated that 127 terpenoids were isolated from endophytic fungi and all have biological activity anti-microbial, anti-cancer and anti- protozoa. Among the five medicinal plants studied, only two endophytes of *V. amygdalina* revealed orange spots of alkaloids. Souza *et al*, (2011) described that alkaloids are quite common secondary metabolites in endophytes. While all the endophyte crude extracts are very rich in flavonoids and phenolic compounds. Yu *et al*. (2010) reported that phenols and phenolic acids have often been isolated from some endophyte cultures originating from a variety of the host plants.

Endophytic fungi have received increased attention because they can produce similar or same compounds as their host plant. Therefore, it can be used as potential source of novel natural products for food, industrial, medicinal and agricultural industries (Zhao *et al*., 2014).

Notable shared spots between endophytes and their host plant extracts could be observed in the chromatograms indicative of their resemblance type of constituents. Plant-derived compounds have played an important role in the development of several clinically

useful anticancer drugs. vinblastine, vincristine, camptothecin and taxol are some of the clinically useful anticancer drugs. Some endophytic fungi were reported to produce these compounds as their host plants such as *Alternaria* sp. isolated from *Catharanthus roseus* was an endophyte vinblastine-producing (Guo *et al*., 1998). And the first taxol-producing fungus *Taxomyces andreanae* endophyte was isolated from *Taxus brevifolia* (Stierle *et al*. 1993). Whereas, several active compounds such as camptothecin was isolated from endophyte *Nothapodytes foetida* only and not from its host plant *Entrophospora infrequens* (Amna *et al*., 2006).

In conclusion, this section is intended to evaluate the extractive values and organoleptic properties of 21 endophytes isolated from *Vernonia amygdalina*, *Calotropis procera*, *Catharanthus roseus*, *Euphorbia prostrata*, and *Trigonella foenum-graecum.* Each crude extract was prepared from fungal strain cultured on 20 plates of PDA and extracted with ethyl acetate. Among the 21 endophytes, *Aspergillus terreus* 1 and *Alternaria alternata* from *C. procera* were found to have the highest extractive yields (242 and 206 mg respectively) followed by *Byssochlamys spectabilis* (155.4 mg) from *E. prostrata.* Whereas, *Curvularia australiensis* 1 from *E. prostrata* gave the lowest quantity (36.6 mg). Then a general screening on the chemical constitute of ethyl acetate extracts of 21 endophytic fungi and their host plants was performed by TLC using different reagents. All extracts of endophytes were very rich with terpenoids, phenolic compounds and rarely with alkaloids. Among all the endophytes tested, only two crude extracts of *Cladosporium cladosporioides* 2 and *Curvularia papendorfii* endophytes of *Vernonia amygdalina* were wealthy with alkaloids. *Vernonia amygdalina* endophytes were chosen for further study depending on the chemical constitute of the host plant and the TLC profile of its endophytes. Beside no published work on the fungal endophytes from *Vernonia amygdalina* was reported.

Table 5. Evaluation of major constituents in ethyl acetate extracts of 21 isolated endophytic fungi and their host plants by TLC. (+) 1 to 2 spots. (++) 3 to 4 spots. (+++) 5 spots. (++++) > 5 spots.

Extracts	Alkaloids	Terpenoids and Sterols	Flavonoids and Phenols
Calotropis procera			
Leaves	-	+ +	+ + + +
Stems	-	+ + +	+ +
Aspergillus terreus 1	-	+ +	+ + + +
Cladosporium cladosporioides 1	-	+ + +	+ +
Alternaria alternata	-	+ +	+ + +
Trametes versicolor	-	+	+ + +
Trigonella foenum-graecum Seeds	-	+	+
Chaetomium globosum	-	+ + +	+ + +
Mycelia sterilia sp.1	-	+ + +	+ ++
Mycelia sterilia sp.2	-	+ +	+ +
Mycelia sterilia sp.3	-	+ + + +	+ + +
Aspergillus terreus 2	-	+ +	+ + + +
Vernonia amygdalina			
Leaves	-	+ +	-
Stems	-	+	-
Cladosporium cladosporioides 2	+	+ +	+ +
Curvularia papendorfii	+	+ + +	+ +
Hansfordia sinuosae	-	+ +	+ +
Catharanthus roseus			
Leaves	-	+ + + +	+ + + +
Stems	-	+ + + +	++
Curvularia aeria	-	+	+
Chaetomium sp.	-	++	++
Phoma multirostrata	-	+ + + +	+
Pleosporales sp.	-	+ ++	-
Emericella sp.	-	+++	-
Euphorbia prostrata			
Aerial part (Stems+Leaves)	-	+ +	+ + + +

Curvularia australiensis 1	-	+ + +	+ + + +
Curvularia australiensis 2	-	+ + +	+ + +
Byssochlamys spectabilis	-	+	+ +
Alternaria sp.	-	+	+ + +

Section Three

Section Three

3.3. Chemical and biological test of endophytic fungi crude extracts

3.3.1. Determination of total phenolic content (TPC)

Ethyl acetate extraction was used for the isolation of the endophytes and their host plant secondary metabolites. This method of extraction is the most efficient method of isolating fungal secondary metabolites (Gao *et al*., 2012). Total phenolic contents of ethyl acetate crude extracts of 21 endophytes and different parts of their host plants were estimated using the classical Folin-Ciocalteu colorimetric method as shown in Fig. 59. It was found that the 5 medicinal plants contained TPC values ranging from 0.5±0.1 (*T. foenum-graecum* seed extract) to 32.6±2.9 mg Gallic Acid Equivalents (GAE)/g) (*V. amygdalina* stem extract). While TPC values of 21 endophytes revealed variations ranged from 13.6±1.0 to 89.9±7.1 mg GAE/g. Two *Aspergillus terreus* strains of both *C. procera* and *T. foenum-graecum* showed the highest TPC values (77.2±7.5 and 89.9±7.1 mg GAE/g respectively), followed by *Pleosporales* sp. from *C. roseus* (51±3.8 mg GAE/g) and *Trametes versicolor* from *C. procera* (50.7±13 mg GAE/g); then *Hansfordia sinuosae* from *V. amygdalina* (39.4±4.6 mg GAE/g). *Chaetomium globosum* from *T. foenum-graecum* recorded 36.8± 16.3 mg GAE/g. While the lowest TPC values were exposed by two endophytes *Cladosporium cladosporioides* 1 from *C. procera* (14.2± 2 mg GAE/g), and *Curvularia australiensis* 1 (13.6± 1 mg GAE/g) from *E. prostrata*.

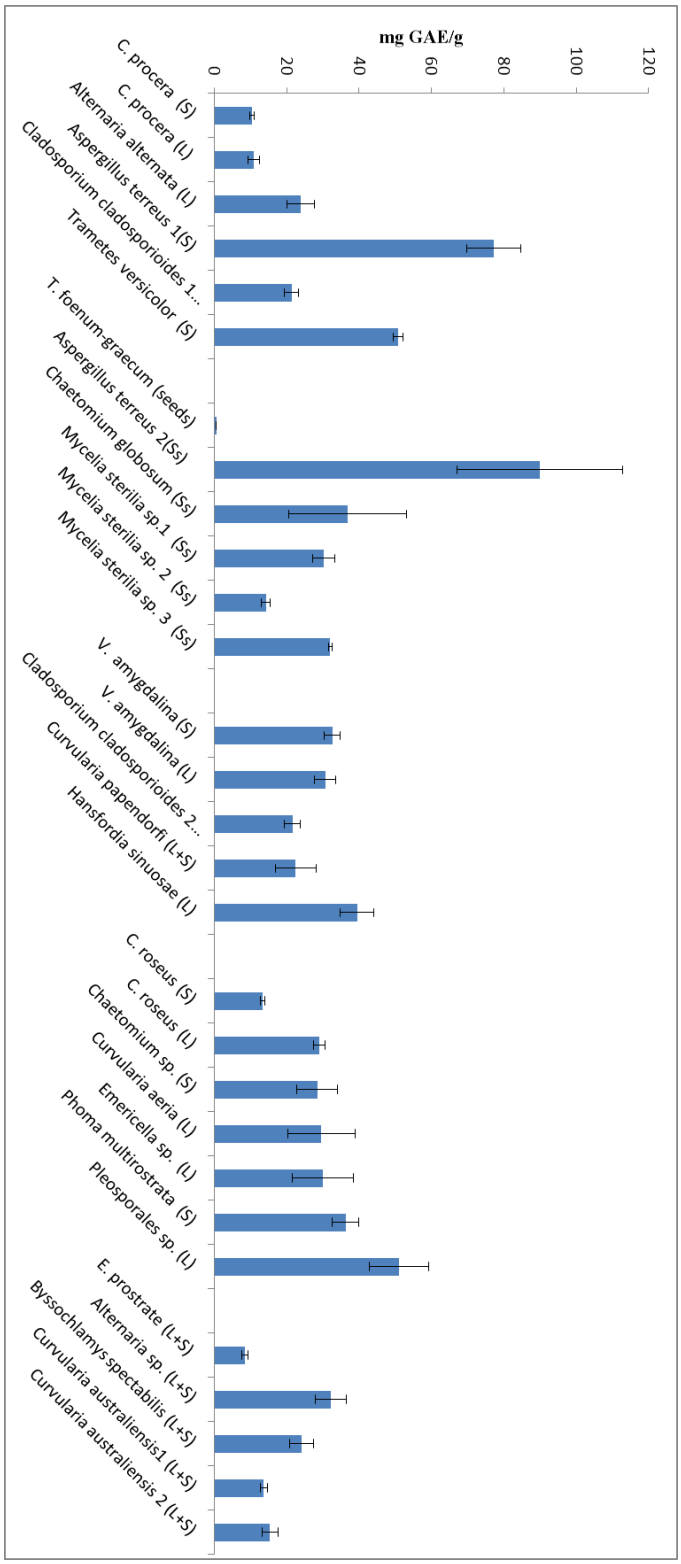


Figure 59. Total phenolic content in ethyl acetate extracts of endophytes and their host plants. (S) Stem, (L) leaves (Ss) Seeds, (S+L) stem and leaves. Values are means ± SD of three determinations.

3.3.2. Total antioxidant capacity assay (TAC)

The antioxidant potentiality was investigated using DPPH radical scavenging assay for the 21 ethyl acetate extracts of the endophytic fungi and their host plants. The total antioxidant capacity (TAC) IC_{50} values are shown in Table 6. In comparison with positive control Ascorbic acid (5±0.1 µg/mL), the TAC IC_{50} of the medicinal host plants ranged from 50±1.7 µg/mL for *V. amygdalina* leaves to non-activity of *T. foenum-graecum* seeds. The endophyte extracts revealed extremely wide range of IC_{50} values, from 18±0.1 µg/mL for *Aspergillus terreus*1 isolated from *T. foenum-graecum* to 2686±51.7 µg/mL for *Phoma multirostrata* isolated from *C. roseus*. Despite the high TAC of stems and leaves of *V. amygdalina* (IC_{50}: 63±1.8 and 50±1.7 µg/mL respectively), their endophyte extracts showed low TAC (IC_{50}: 252±5.1 to 480±3.9 µg/mL). In contrast the seed extract of *T. foenum-graecum* had no antioxidant activity while *Aspergillus terreus* 2, isolated from the seeds showed powerful TAC (18±0.1 µg/mL). These results indicated that no correlation between the TACs of the endophytes and the host plants can be established. The main factor is the fungal genus, indeed *Aspergillus terreus* strains were recorded the highest TAC.

The highest TAC and the highest TPC were obtained with *Aspergillus terreus* 1 isolated from *C. procera* (IC_{50}: 58±4.0 µg/mL, TPC: 77.2±7.5 mg GAE/g) and *Aspergillus terreus* 2 from *T. foenum-graecum* (IC_{50}: 18±0.1 µg/mL, TPC: 89.9±7.1 mg GAE/g). These results are in accordance with Yadav *et al*. (2014) who reported that various species of *Aspergillus* strains showed the highest TPC with 58 to 60 mg GAE/g. It is noted that crude extract of *T. foenum-graecum* seeds from Sudan revealed no antioxidant activity that could be explained by the low concentration of TPC (0.5±0.1 mg GAE/g). The *Aspergillus terreus* 1 isolated from *T. foenum-graecum* seeds with strong antioxidant activity and high phenolic content is recommended for further investigations. In contrarast previous works reported that seed ethyl acetate crude extract of *T. foenum-graecum* demonstrated strong antioxidant activity in relation with high phenolic content (106.316 mg GAE/g) (Kenny *et al*, 2013).

Table 6. IC_{50} values of DPPH radical scavenging activity of the ethyl acetate extracts of endophytic fungi and their host plants Values are means ± SD of three analyses.

Crude extract	DPPH (µg/mL)
Ascorbic acid	5±0.1
***C. procera* (S)**	668±8.1
***C. procera* (L)**	388±7.2
Alternaria alternata (L)	236±8.3
Aspergillus terreus (S)	58±0.4
Cladosporium cladosporioides 1 (L)	1142±1.3
Trametes versicolor (S)	1030±3.0
***T. foenum-graecum* (seeds)**	*
Aspergillus terreus (Ss)	**18±0.1**
Chaetomium globosum (Ss)	70±0.3
Mycelia sterilia sp.1 (Ss)	1013±4.2
Mycelia sterilia sp.2 (Ss)	933±5.3
Mycelia sterilia sp.3 (Ss)	1070±3.2
***V. amygdalina* (S)**	63±1.8
***V. amygdalina* (L)**	50±1.7
Hansfordia sinuosa (L)	252±5.1
Cladosporium cladosporioides 2 (L)	480±3.9
Curvularia papendorfii (L+S)	461±5.5
***C. roseus* (S)**	1119±2.6
***C. roseus* (L)**	113±0.4
Chaetomium sp. (S)	405±5.2
Curvularia aeria (L)	105±2.7
Emericella sp. (L)	137±1.3
Phoma multirostrata (S)	2686±51.7
Pleosporales sp.(L)	1556±1.5
***E. prostrata* (L+S)**	203±7.6
*Curvularia australiensis*1 (L+S)	2305±23.4
Curvularia australiensis 2 (L+S)	1074±7.7
Alternaria sp. (L+S)	1348±5.6
Byssochlamys spectabilis (L+S)	122±0.4

* indicated not active, (L) leaves, (S) stem, (Ss) seeds and (L+S) leaves and stem.

3.3.2.1. Correlation analyses of total antioxidant capacity and phenolic content

Previous studies have concluded that there is a linear correlation between total phenolic content and antioxidant potential of most samples (Sultana *et al.,* 2007). Correlation coefficients (R^2) values of DPPH scavenging assay and total phenolic content of 21 endophytic fungi extracts were measured and are presented in Table 7. A high positive linear correlation (R^2 = 0.9986) was found between total phenolic content and DPPH assay of endophytic fungi extracts isolated from *V. amygdalina*. Endophytic fungi extracts isolated from *C. procera* and *T. foenum-graecum* showed moderate correlation (R^2 = 0.6808 and 0.5145, respectively). Whereas very weak correlation was observed (R^2 = 0.2469 and 0.1544) for *C. roseus* and *E. prostrata* endophyte extracts, respectively. The results of the positive linear correlation of endphytes from *V. amygdalina*, *T. foenum-graecum* and *C. procera* indicated that the phenolic compounds in the endophytic fungi significantly contributed to their antioxidant activity. Previous studies also revealed that phenolic compounds are major antioxidant constituents in medicinal plants, vegetables, fruits, and spices (Cai *et al.,* 2004; Surveswaran *et al*., 2007). The highest positive correlation of *V. amygdalina* endophytes strongly contribute to the high antioxidant capacity of the leaf and stem extracts IC_{50} values of 50±1.7and 63±1.8 µm/mL, respectively. The antioxidant activity of *V. amgdalina* leaves was reported, several flavonoids such as: luteolin, luteolin 7-O-β-glucoroniside and luteolin 7-O-β-glucoside were isolated from *V. amygdalina* (Igile *et al*., 1994; Udensi *et al*., 2002 and Tona *et al*. 2004).

Table 7. Correlation coefficients (R^2) values of DPPH scavenging assay and total polyphenol content of endophytic fungi of the five medicinal plants

Endophyte source	***Correlation coefficient (R^2) values***
Vernonia amygdalina	0.9991
Calotropis procera	0.6808
Trigonella foenum-graecum	0.5156
Catharanthus roseus	0.2469
Euphorbia prostrata	0.1544

3.3.3. Cytotoxicity assay of 16 selected endophytic fungi extracts and their host plants

Cytotoxicity studies are useful initial step in determining the potential toxicity of plant extracts or endophytes of the medicinal plants. This part is intended to assess the cytotoxic activity using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) of 16 selected endophytic fungi from the five medicinal plants *C. procera T. foenum-graecum*. *V. amygdalina, C. roseu*s and *E. prostrata*. Ethyl acetate crude extract of the endophyes and their host plants were tested against three cell lines: human breast carcinoma (MCF7), and colon adenocarcinoma (HT29 and HCT116) cells. Beside human hepatocarcinoma (HepG2) against only *V.amygdalina* and its endophytes.

Cytotoxic activity was evaluated from 1 to 100 µg/mL as described in chapter two Materials and Methods. The majority of the 16 endophytic fungi revealed cytotoxic activity at least against one cancer cell line. The cytotoxic activity ranging between 1.51 ± 0.2 to 91.7 ± 6.9 µg/mL for extract of *Byssochlamys spectabilis,* isolated from *E. prostrata,* and *Pleosporales* sp., isolated from *C. roseus* respectively. While some medicinal plants showed strong activity against at least one cancer cell line ranging from 2.19 ± 0.12 for stems of *C. procera* to 7.83 ± 1.28 µg/mL for leaves of *C. roseus*. Besides that, some endophytes such as *Aspergillus terreus* 1 isolated from *C. procera*, *Phoma multirostrata* isolated from *C. roseus* and some medicinal plants such as *E. prostrata* and *T. foenum-graecum* displayed very weak activity, more than 100 µg/mL (Table 8).

Among the 16 tested endophytes, *Byssochlamys spectabilis* isolated from *E. prostrata* showed strong activity (1.51 ± 0.2 µg/mL) followed by *Cladosporium cladosporioides 2* isolated from *V. amygdalina* (10.5 ± 1.5 µg/mL), then *Alternaria* sp. isolated from *E. prostrata* (13.5 ± 1.8 µg/mL).Whereas, *Curvularia papendorfii* isolated from *V. amygdalina* revealed modest cytotoxicity (21.5 ± 5.9 µg/mL) against MCF7 cells. Some endophytes showed only moderate toxicity against HCT116 cell line such as *Curvularia australiensis* 1, *Alternaria* sp., *Byssochlamys spectabilis* from *E. prostrata* and *Aspergillus terreus* 2 from *T. foenum-graecum* (25.6 ± 1.9, 29.1 ± 2.5, 30.4 ± 1.1 and 30.7 ± 1.01 µg/mL respectively). While the cytotoxic activity of the 16 tested endophytes against HT29 cell line were ranging from moderate to weak, among them only *Alternaria* sp. from *E. prostrata* displayed moderate activity (28.4 ± 5.2 µg/mL).

Concerning the medicinal plants, *C. procera* (Stems) showed strong activity (IC_{50} value of 2.19 ± 0.12 µg/mL) against HCT116 cells, followed by *V. amygdalina* (leaves) (IC_{50} value of 5.6 ± 0.4 µg/mL), and then *C. roseus* (leaves) (IC_{50} value of 7.83 ± 1.28). Only two of these medicinal plants revealed powerful cytotoxicy against HT29 cells, *C. roseus* (leaves) (IC_{50} value of 7.00 ± 0.43 µg/mL) and *V. amygdalina* (Stems) (IC_{50} value of 15.3 ± 3.6 µg/mL). *V. amygdalina* (leaves) displayed moderate activity (IC_{50} values of 27.5 ± 5.731 and 31.9 ± 5.1µg/mL) against MCF7 and HT29 cells respectively. *E. prostrata*, *T. foenum-graecum*, *C. roseus* (Stems) and *C. procera* (leaves) showed no activity against all the cancer cell lines tested.

Surprisingly, *E. prostrata* (leaves and stems) revealed no activity against all the cancer cell lines tested in our study. In contrast, Pigares and Narendhirakannan (2013) reported efficient antitumor activity of *Euphorbia prostrata* extract. Despite the none cytotoxic host plant *E. prostrata*, the most toxic endophyte *Byssochlamys spectabilis* was isolated from this plant. Also *Alternaria* sp., isolated from *E. prostrata* showed also powerful activity.

Fungal endophytes of *V. amygdalina* showed wide range in their cytotoxicity. The most toxic endophytic fungus extract, against MCF7 and HepG2 cell line, was *Cladosporium cladosporioides* 2 (IC_{50} value of 10.5 ± 1.5 and 30.3 ± 4.4 µg/mL respectively). This fungus was isolated from the leaves of *V. amygdalina* and exhibited a cytotoxicity higher than the leaves. Previous study on endophytes revealed that *Cladosporium oxysporum* produced taxol. The taxol extracted from the fungus suppressed growth of cancer cell line HCT15 with an IC_{50} value of 3.5 µM concentration by 24 h treatment (Kathamuthu *et al.*, 2014). On the other hand, *Cladosporium cladosporioides* 2 showed no cytotoxic activity against both colon adenocarcinoma. Then *Curvularia papendorfii* showed cytotoxic effect against MCF7 and HepG2 cell lines (IC_{50} values of 21.5 ± 5.9 and 38.7 ± 8.2 µg/mL respectively). A worthy notice of this fungus, that it was present into both leaf and stem tissues. Whereas, *Curvularia papendorfii* showed no cytotoxic activity against HT29 and HCT116 cells. *Hansfordia sinuosae* was isolated from the stem of *V. amygdalina*. This fungus exhibited cytotoxic activity only against colon adenocarcinoma HT29 cell line (IC_{50} 47.6 ± 4.1 µg/mL). Also no toxicity against the other cancer cells.

Table 8. Cytotoxicity IC_{50} values of ethyl acetate crude extracts of 16 selected endophytes and their host plants.

Crude extract	IC_{50} (µg/mL)*		
	MCF7	**HT29**	**HCT116**
***Calotropis procera* (Leaves)**	**-**	>100	>100
***Calotropis procera* (Stems)**	**-**	**7.25 ± 2.21**	**2.19 ± 0.123**
Aspergillus terreus 1	>100	> 100	>100
Alternaria alternata	>100	> 100	52.5 ± 3.0
Cladosporium cladosporioides 1	>100	77.7 ± 9.2	45.6 ± 1.5
Trametes versicolor	48.3 ± 4.7	53.9 ± 11.7	84.3 ± 6.8
***Trigonella foenum-graecum* (Seeds)**	**-**	>100	>100
Chaetomium globosum	>100	>100	75.2 ± 2.8
Aspergillus terreus 2	**-**	>100	30.7 ± 1.01
***Vernonia amygdalina* (Leaves)**	27.5 ± 5.7	31.9 ± 5.1	**5.6 ± 0.4**
***Vernonia amygdalina* (Stems)**	49.6 ± 4.4	15.3 ± 3.6	>100
Cladosporium cladosporioides 2	**10.5 ± 1.5**	>100	>100
Curvularia papendorfii	21.5 ± 5.9	>100	>100
Hansfordia sinuosae	> 100	47.6 ± 4.1	>100
***Catharanthus roseus* (Stems)**	-	>100	>100
***Catharanthus roseus* (Leaves)**	**-**	**7.00 ± 0.43**	**7.83 ± 1.28**
Curvularia aeria	>100	74.5 ± 12.5	53.9 ± 4.53
Phoma multirostrata	>100	>100	>100
Pleosporales sp.	91.7 ± 6.9	69.4 ± 3.8	36.7 ± 8.3
***Euphorbia prostrata* (Leaves+Stems)**	-	>100	>100
Curvularia australiensis 1	46.4 ± 2.5	54.3 ± 6.9	25.6 ± 1.9
Curvularia australiensis 2	>100	>100	59.7 ± 4.4
Alternaria sp.	**13.5 ± 1.8**	28.4 ± 5.2	29.1 ± 2.5
Byssochlamys spectabilis	**1.51 ± 0.2**	56.3 ± 5.7	30.4 ± 1.1

* Viability was determined by the MTT procedure using human breast carcinoma (MCF7) and colon adenocarcinoma (HT29, HCT116) cells. IC_{50} values are means ±S.E.M calculated from results obtained from quadruplicate determination of two independent experiments (n= 8).

Stem extract of *V. amygdalina* showed cytotoxic effect against HT29 and MCF7 (IC_{50} values of 15.3 ± 3.6 and 49.6 ± 4.4µg/mL respectively), while leaf extracts had strong cytotoxicity (IC_{50} value of 5.6 ± 0.4 µg/mL) against HCT116 and moderate cytotoxicity (IC_{50} value of 27.5 ± 5.7 and 31.9±5.1 µg/mL) against MCF7and HT29 respectively. These findings corroborate the previous work reported by earlier investigators (Gresham *et al*., 2008). The significant activity of *V. amygdalina* may be due to its different compounds which were isolated in previous studies including coumarins, flavonoids, sesquiterpene lactons and edotides (Izevbigie, 2003).

Despite the powerful activity of *C. procera* (Stems) (IC_{50} values of 7.25 ± 2.21 and 2.19 ± 0.123 µg/mL), fungal endophytes of this plant displayed weak cytotoxicity (ranging IC_{50} values of 45.6 ± 1.5 to 84.3 ± 6.8 µg/mL) even *Trametes versicolor* which was isolated from the stem.

C. roseus (leaves) revealed strong cytotoxicity (IC_{50} value of 7.00 ± 0.43 and 7.83 ± 1.28 µg/mL). This results was expected because *C. roseus* produces vinblastine, vincristine two natural alkaloids, which are used as major drugs in the treatment of lymphoma and leukemia, respectively (Chandra, 2012).

Trigonella foenum-graecum seeds showed no cytotoxicity. Only chemo preventive effect was reported of the seeds. Amin *et al*. (2005) reported a significant chemo preventive effect of fenugreek seeds *T. foenum-graecum* against breast cancer. *Aspergillus terreus* 2, isolated from *T. foenum-graecum,* showed moderate activity (IC_{50} value of 30.7 ± 1.01 µg/mL). *Chaetomium globosum* displayed weak cytotoxicity (IC_{50} value of 75.2 ± 2.8µg/mL). Although *Chaetomium globosum* revealed weak activity, Globosumone A, an ester with strong cytotoxicity was isolated from *Chaetomium globosum*, an endophtye of Mormon tea *Ephedra fasciculata* (Bashyal *et al.*, 2005).

Kharwar *et al*. (2011) stated that one hundred anticancer compounds with activity against 45 different cell lines have been isolated from over 50 different fungal species. 57% were novel or were analogues of known compounds. Therefore, endophytic fungi isolated in this study could be promising source of novel analogues of anticancer substances.

3.3.4. Antibacterial assay of 16 selected endophytic fungi

A preliminary screening was performed for the 16 selected endophytic fungi from the five medicinal plants. Ethyl acetate crude extracts of the endophytes were tested against two bacterial strains Gram-negative *Escherichia. coli* and Gram-positive methicillin-resistant *Staphylococcus aureus*, by broth dilution methods using micro dilution tray (96 wells). Methicillin-resistant *S. aureus* is one of the pathogen strains causing the majority of hospital infections and effectively escape the effects of antibacterial drugs (Rice, 2008). Among the 16 tested fungal strains, only six extracts of endophytes: *Alternaria alternata*, *Aspergillus terreus*1 isolated from *C. procera*, two isolated from *E. prostrata*: *Alternaria* sp.: *Byssochlamys spectabilis* and two other isolated from *T. foenum-graecum Aspergillus terreus* 2, *Chaetomium globosum* showed an activity against *S. aureus* ranging between 0.125-2 mg/mL. However none activity (>2 mg/mL) was observed for the 16 endophytes against *E. coli* (Table. 9). In this screening the genus *Alternaria* isolated from *C. procera* and *E. prostrata* showed promising antibacterial activity with MIC values of 0.125 and 0.250 mg/mL respectively. Several studies showed no activity of the endophyte extracts against *E.coli*. For example, Buatong *et al*. (2011) determined MIC values of a total of 385 Hexane and Ethyl acetate extracts from 150 fungal endophytes isolated from leaves and branches of 12 mangrove species. They found that 92 extracts produced inhibitory compounds. Most of the extracts (28–32%) inhibited *S. aureus* (MIC 4–200 µg/ml). None of the extracts inhibited *E. coli*. Gong and Guo (2009) studied the endophytic fungi of two plants *Dracaena cambodiana* and *Aquilaria sinensis* and obtained 300 isolates. 172 were from *Dracaena cambodiana* and 128 from *Aquilaria sinensis*. 21 isolates showed antimicrobial activity, however, none of these isolates was active against *E. coli*, and only 15 were active against *B. subtilis* and/or *S. aureus*. Previous studies in which endophytic extracts were prepared using different solvents like hexane methanol, ethanol, and ethyl acetate showed that methanol, ethanol and most often ethyl acetate have always antibacterial activity, whereas extracts prepared with nonpolar solvents display no antibacterial activity (Radic and Strukelj, 2012).

Table 9. MIC values of 16 endophytic fungi ethyl acetate extracts, isolated from *C. procera*, *C. roseus*, *E. prostrata T. foenum-graecum*, and *V. amygdalina* against two bacterial strains *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*.

Crude extract of endohytes	**MIC (mg/mL)**	
	S. aureus	***E. coli***
Alternaria alternata	**0.125**	>2
Aspergillus terreus1	0.5	>2
Cladosporium cladosporioides 1	>2	>2
Trametes versicolor	>2	>2
Aspergillus terreus2	1	>2
Chaetomium globosum	2	>2
Hansfordia sinuosae	>2	>2
Cladosporium cladosporioides 2	>2	>2
Curvularia papendorfii	>2	>2
Pleosporales sp.	>2	>2
Curvularia aeria	>2	>2
Phoma multirostrata	>2	>2
*Curvularia autraliensis*1	>2	>2
Curvularia autraliensis 2	>2	>2
Alternaria sp.	**0.25**	>2
Byssochlamys spectabilis	0.5	>2

Several published works showed strong activity of ethyl acetate extract of endophytic fungi (Buatong *et al*., 2011; Radji *et al*., 2011). In this study ethyl acetate was used for extraction. The majority of the extracts had no activity against both Gram-negative and Gram-positive bacteria. The crude extracts were prepared in 2015 after three years of isolation from the host plants and, during the three years more than 6 sub-cultures were performed. The effect of losing the activity after several sub-cultures was obvious during our research study especially in antibacterial activity of *Curvularia papendorfii* crude extract between 2013 and 2015. It was observed that the activity was decreased from 0.312 mg (in 2013) to >2 mg (in 2015).

In conclusion, this section was intended to evaluate anti-oxidant, cytotoxic and antibacterial potentiality of endophyte crude extracts isolated from the five plants. Total phenolic contents TPC of ethyl acetate crude extracts of 21 endophytes and different parts of

their host plants were estimated using the classical Folin-Ciocalteu colorimetric method, alongside total antioxidant capacity TAC was estimated using (1,1,-diphenyl-2-picrylhydrazl) DPPH free radical scavenging *in vitro* method. Among the endophytes, *Aspergillus terreus* 1. from *Trigonella foenum-graecum* seeds demonstrated the highest both total phenolic content in term of mg Gallic Acid Equivalent/g (89.9±7.1 mg GAE/g) and antioxidant activity for DPPH radical scavenging assay (IC_{50} value of 18±0.1 µg/mL). A high positive linear correlation ($R^2 = 0.9991$) was found between TAC and TPC of endophytic fungi isolated from *V. amygdalina*. General evaluation of the cytotoxicity of ethyl acetate extracts of endophytes 16 and their host plants was performed by the MTT assay using three cancer cell type: Human breast carcinoma (MCF7), and Colon adenocarcinoma (HT29, HCT116) cells. 14 endophytes displayed cytotoxic activity ranging between strong to weak. *Byssochlamys spectabilis* showed powerful (IC_{50} value of 1.51 ± 0.2 µg/mL) followed by *Cladosporium cladosporioides* 2 (IC_{50} value of 10.5 ± 1.5 µg/mL), then *Alternaria* sp. (IC_{50} value of 13.5 ± 1.8 µg/mL). Astonishingly, the most two cytotoxic fungi were isolated from *E. prostrata* which showed weak toxicity >100 µg/mL. Three medicinal plants *C. procera* stem, *V. amygdalina* leaves and *C. roseus* leaves revealed strong cytotoxicity against at least one cell line (IC_{50} value of 2.19 ± 0.1, 5.6 ± 0.4 and 7.00 ± 0.43 µg/mL respectively). Further preliminary screening was done for the 16 endophytic fungi, extracts of the endophytes were tested against two bacterial strains Gram- negative *E. coli* and Gram-positive methicillin-resistant *Staphylococcus aureus* by broth dilution methods using micro dilution tray (96 wells). Some fungal endophyte extracts revealed activity; among 16 fungal strains only six showed activity against methicillin-resistant *S. aureus* with MIC values ranging between 0.125-2 mg/mL, *Alternaria alternata* (0.125 mg/mL) *Alternaria* sp. (0.250 mg/mL) and *Byssochlamys spectabilis* values (0.5 mg/mL). It was noticed in assessment that all endophytes crude extracts lack the activity against *E. coli* even those who were active against methicillin-resistant *Staphylococcus aureus*. The findings of this section revealed that some endophytic fungi of the five Sudanese medicinal plants studied, could be a potential source of novel natural anti-oxidant and anti-cancer compounds.

Section Four

Section Four

3.4.1. *Curvularia papendorfii* endophytic fungus of *Vernonia amygdalina*

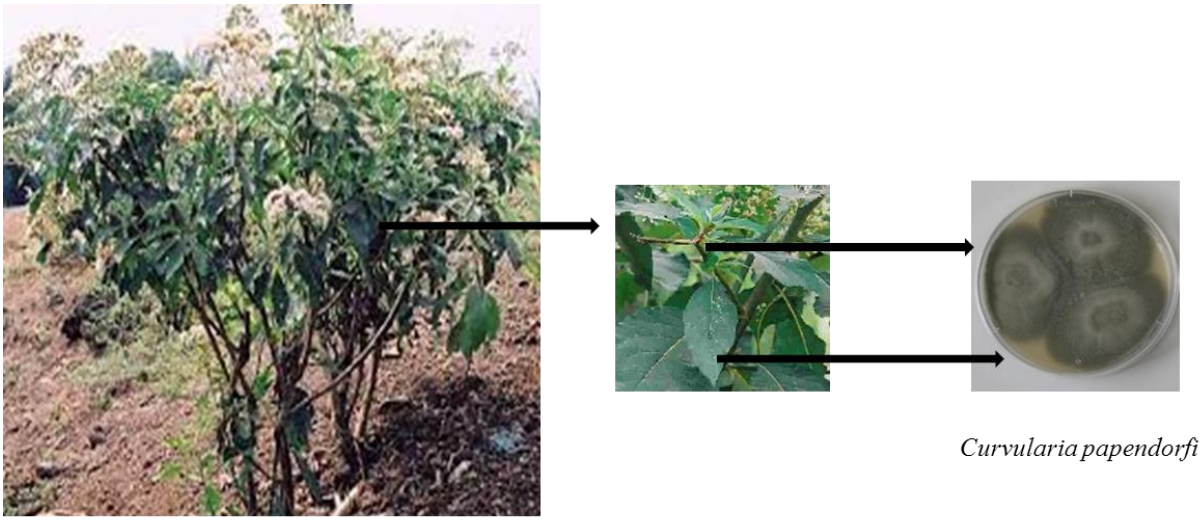


Figure 60. *Vernonia amygdalina* tree and its endophytic fungus *Curvularia papendorfii*

This section focused only on *Curvularia papendorfii* (Fig. 60), the biological activity and the phytochemical analysis of the crude extract. The selection of *C. papendorfii* as a major fungus for the bioassay-guided fractionation was based on several reasons. Firstly, the host plant *Vernonia amygdalina* showed a powerful cytotoxicity and it is known containing several compounds with anticancer activity such as sesquiterpene lactones and steroidal saponins. Beside that endophytic fungi flora of this plant was not studied before. Secondly, *C. papendorfii* was isolated several times from both leaves and stems. Thirdly, the crude extract of *C. papendorfii* had a selected activity against methicillin-resistant *Staphylococcus aureus* and this bacterial strain is one of the pathogen strains that cause the majority of hospital infections and effectively escape the effects of antibacterial drugs (Rice, 2008). Fourthly, this fungus has a moderate cytotoxic activity against human breast carcinoma (MCF7) cells as the

host plant, and the TLC profile revealed that its crude extract is very rich with terpenoids, flavonoids and alkaloids. Therefore, it would be of interest to investigate its antibacterial or anticancer potentialities as well as its secondary metabolite constituents.

3.4.2. Antibacterial-assay of *Curvularia papendorfii* crude extract

Antibacterial potentiality of *C. papendorfii* ethyl acetate crude extract against several Gram-positive and Gram-negative bacterial strains was performed using agar diffusion and broth dilution test. *C. papendorfii* crude extract revealed a selected antibacterial activity against *Staphylococcus aureus* as well as methicillin-resistant *S. aureus* (MRSA). The results presented in this section were obtained with crude extract of *C. papendorfii* prepared in 2013.

The results obtained by the disk diffusion method indicated that the ethyl acetate crude extract of *C. papendorfii* had an effective antimicrobial activity against most of Gram positive bacteria and no effect against three Gram negative bacterial strains (Table 10). Furan was used as positive control. The crude extract exhibited maximum inhibition zone of 13 mm against *Staphylococcus. aureus*, methicillin-resistant *S. aureus* and *S. capitis*, 12 mm against *S. epidermidis*, 10 mm against *S. lentus*, *S. warneri, S. sciuri, S. xylosus, S. haemolyticus* and *S. lugdunensis,* 9 mm against *Enterococcus faecalis*, *Kytococcus sedentarius* and *S. arlettae.* In contrast, no inhibitory effect against *Enterococcus faecium, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella abony* was observed. Then, these results were confirmed by broth dilution method and the MIC values were 312 µg/ml for *Staphylococcus aureus* as well as for MRSA.

The *C. papendorfii* extract showed selected antimicrobial activity against most Gram positive bacterial strains, especially *Staphylococcus* spp. and no activity against Gram-negative bacteria. However, previous studies on the host plant revealed that, the aqueous extract of the leaves of *V. amygdalina* inhibited the growth of Gram positive bacterium *Staphylococcus aureus* and the Gram negative bacterium *Escherichia coli* (Adetunji *et al*., 2013).

Table 10. Antimicrobial activity of crude extract of *Curvularia papendorfii* from *Vernonia amygdalina* against several Gram-positive and Gram-negative bacterial strains in an agar diffusion assay. The inhibition zone was measured in mm and derived from experiments in triplicates.

Bacterial strains	**Crude extract**	**Furan**
Pseudomonas aeruginosa	6	-
Escherichia coli	6	-
Salmonella abony	6	-
Staphylococcus aureus	13	27
methicillin-resistant *S. aureus*	13	-
S. arlettae	9	-
S. lentus	10	-
S. epidermidis	12	-
S. haemolyticus	10	-
S. xylosus	10	-
S. sciuri	10	-
S. warneri	10	-
S. capitis	13	-
S. lugdunensis	10	-
Enterococcus faecalis	9	-
E. faecium	6	-
Bacillus cereus	6	-
Kytococcus sedentarius	9	-

3.4.3. Bioassay-guided fractionation

Ethyl acetate crude extract of *C. papendorfii* was subjected to bioassay-guided fractionation by antibacterial assay against methicillin-resistant *Staphylococcus aureus*. First a precipitation was obtained spontaneously from the crude ethyl acetate extract which was filtered and thus two fractions namely, A (soluble one) and B (precipitate) were obtained and were subjected to antibacterial test to identify the active fraction. Results showed that fraction A displayed antibacterial activity against methicillin-resistant *S. aureus*, with MIC value of 312 µg/mL whereas, fraction B was not active. Furthermore, fraction A was subjected to fractionation by CC and 13 subfractions (a-m) were collected and subjected to antibacterial test. Results revealed that fractions f, g and m showed antibacterial activity with MIC 250, 125 and 78 µg/mL respectively. Fraction f was purified by combiflash to give a pure compound (**AF1**) with MIC value of 250 µg/mL. Fraction m was further subjected to CC and 6 subfractions (M1-M6) were obtained and only the fraction M4 possessed antibacterial activity with MIC 78 µg/mL. This fraction was rechromatographed and the 6 subfractions (4a-4f) obtained were subjected to antibacterial test where the fraction 4d was active with MIC 78 µg/mL. Again this fraction (4d) was subjected to bioassay-guided fractionation and 4 subfractions (Di-Div) were collected. Diii was purified through semi preparative HPLC to get the compound **AFB** which was found to exhibit an intresting antibacterial activity with MIC value of 62.5 µg/mL. Summary of the bioassay-guided fractionation is given in Fig. 61.

The results obtained showed that upon fractionation the antibacterial activity increased by 1.2-fold, 2.5-fold and 4-fold for fractions f, g and m respectively. Moreover, the antibacterial activity of the fraction m remained the same under further fractionations (78 µg/mL) and was slightly increased when the pure compound (**AFB**) was obtained (62 µg/mL). Thus, it could be suggested that the antibacterial activity of ethyl acetate extract of *C. papendorfi* could be mainly due to presence of active constituents rather than active fractions where synergy might exist.

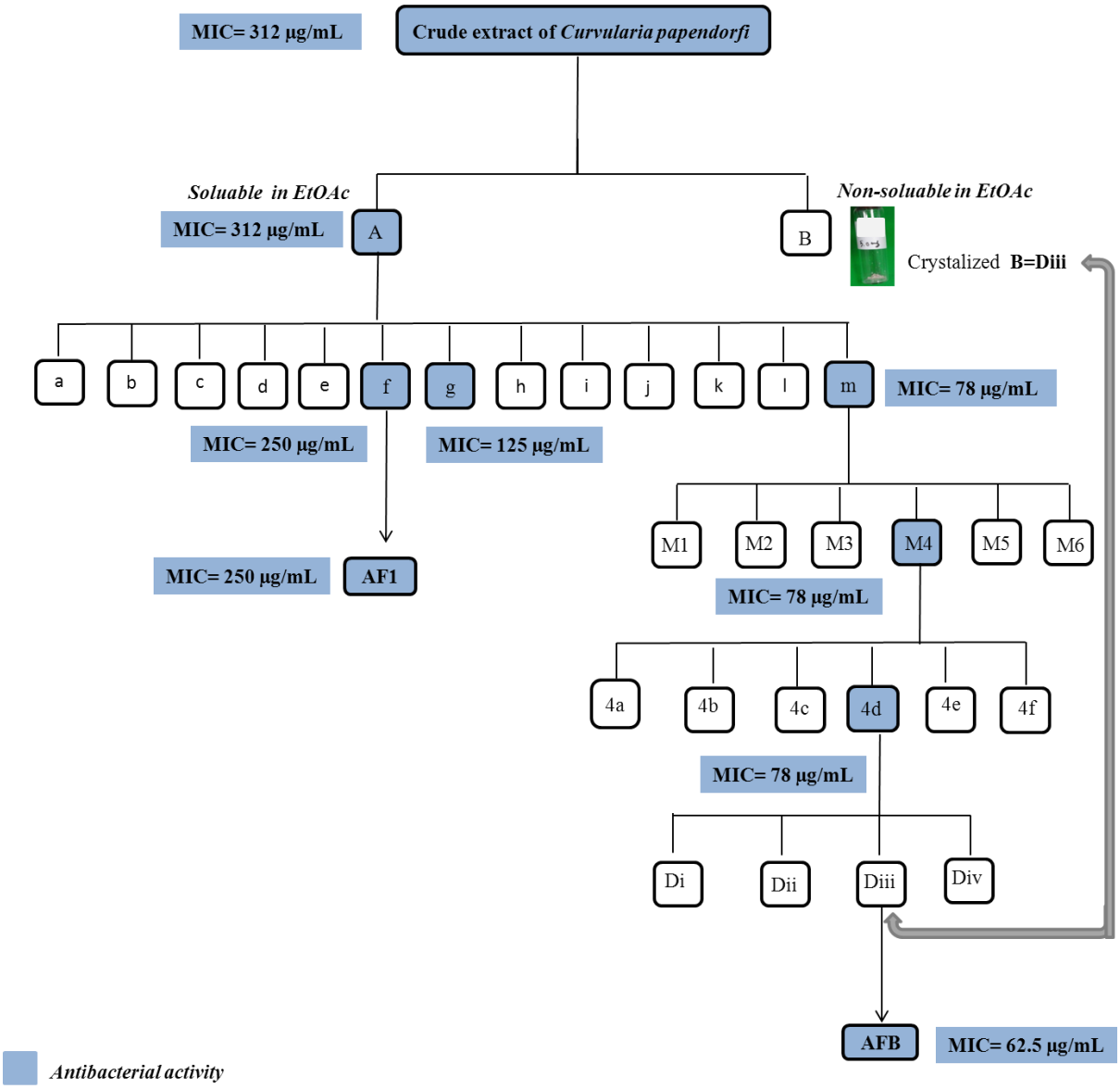


Figure 61. Bioassay-guided fractionation of *Curvularia papendorfii* crude extract by antibacterial assay against methicillin-resistant *Staphylococcus aureus*.

3.4.4. Cytotoxicity of selected fractions

Ethyl acetate crude extract of *C.papendorfii* as well as some of fractions obtained from the bioassay-guided fractionation by antibacterial assay were selected for the cytotoxicity test against MCF7 cell line (Fig. 62). Interestingly, fraction B, which did not show any antibacterial activity, was found to have potent cytotoxic activity with IC_{50} 5.3 µg/mL, a value higher than that obtained from the crude extract by 4-fold. The subfractions from fraction A were also found to reveal anticancer activity, where fraction f and the pure compound **AF1** gave IC_{50} value 16 and 29.78 µg/mL respectively. Fraction Diii (Fig. 63) displayed potent

anticancer activity with the same IC_{50} value (5 µg/mL) as that obtained by fraction B (5.3 µg/mL). In fact, it has the same R_f value and present the same crystal form as that of B suggesting their identical structure.

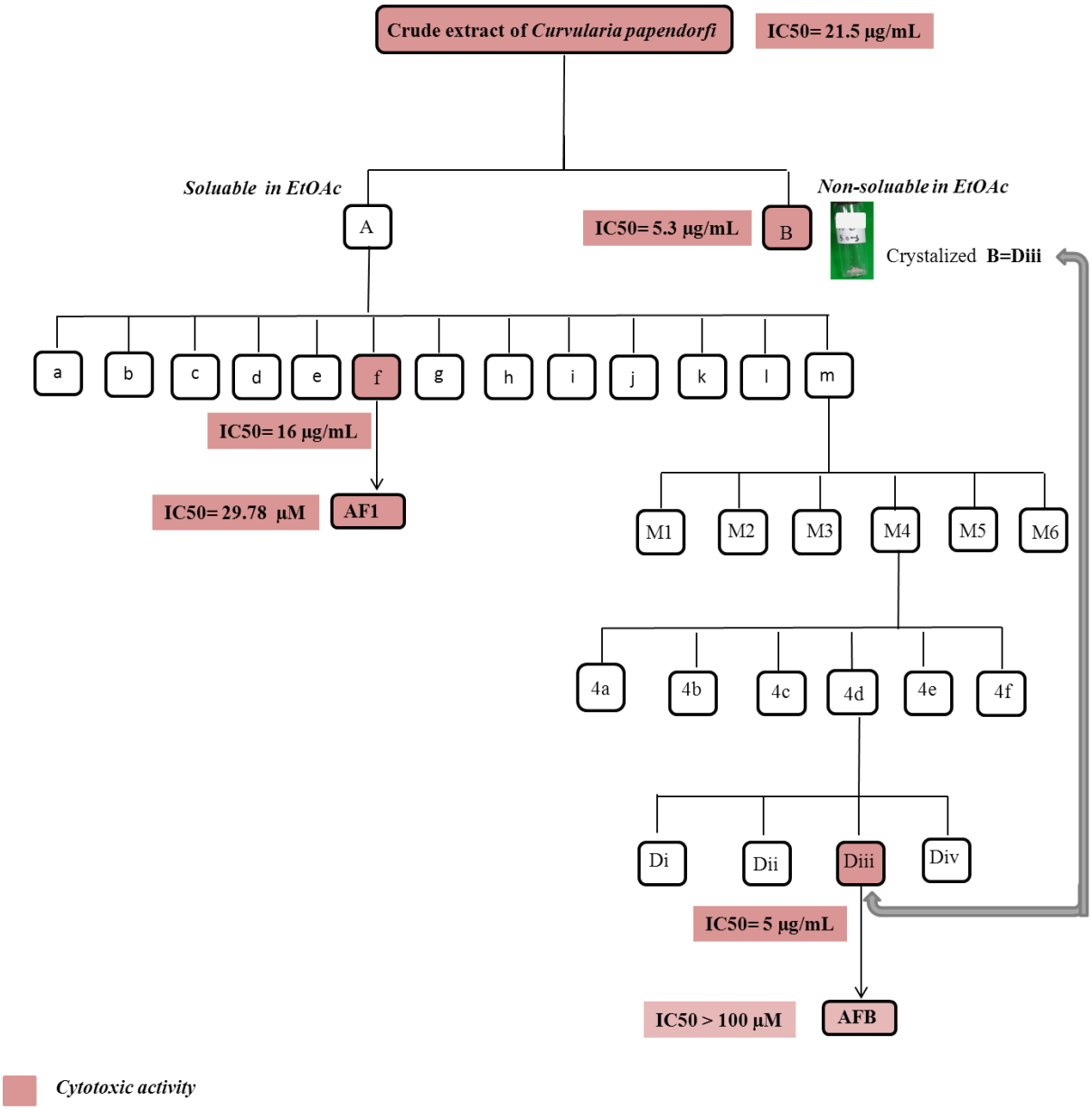


Figure 62. Cytotoxicity against MCF7 cell line of selected fractions and pure compounds of *Curvularia papendorfii* crude extract.

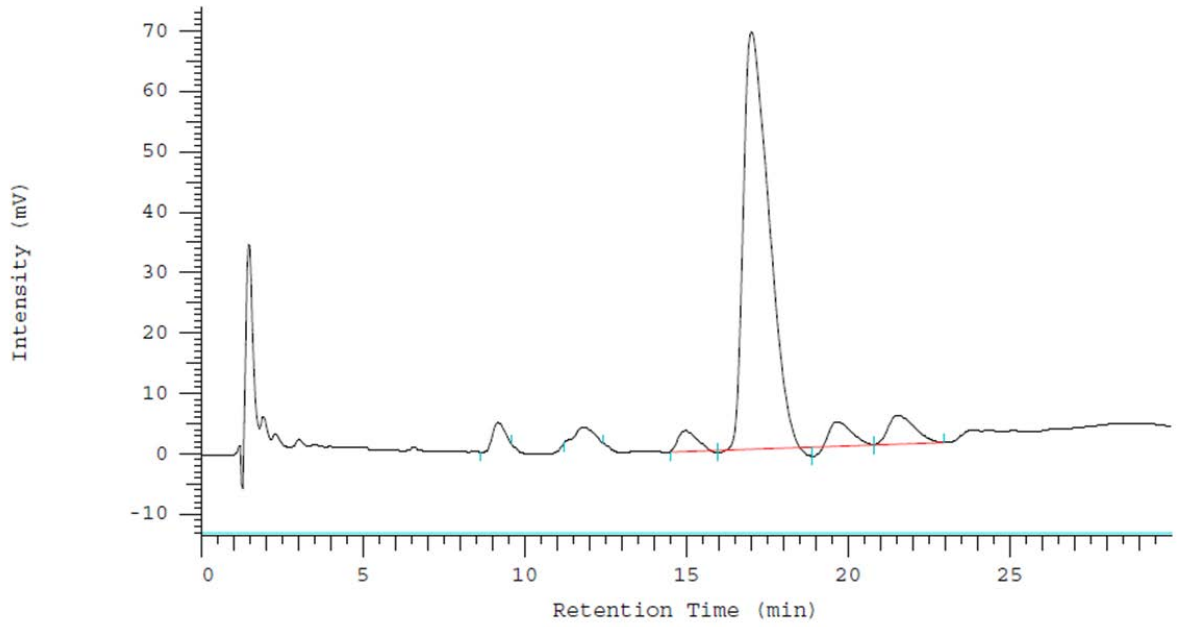


Figure 63. HPLC profile of fraction Diii.

Ten pure compounds (0.3 - 40 mg) were isolated from ethyl acetate crude extract of *C. papendorfi* including **AFB** (15 mg) and **AF1** (40 mg). The flow chart of the pure compounds is summarized in Fig. 64. And their HPLC profile in Fig. 65.

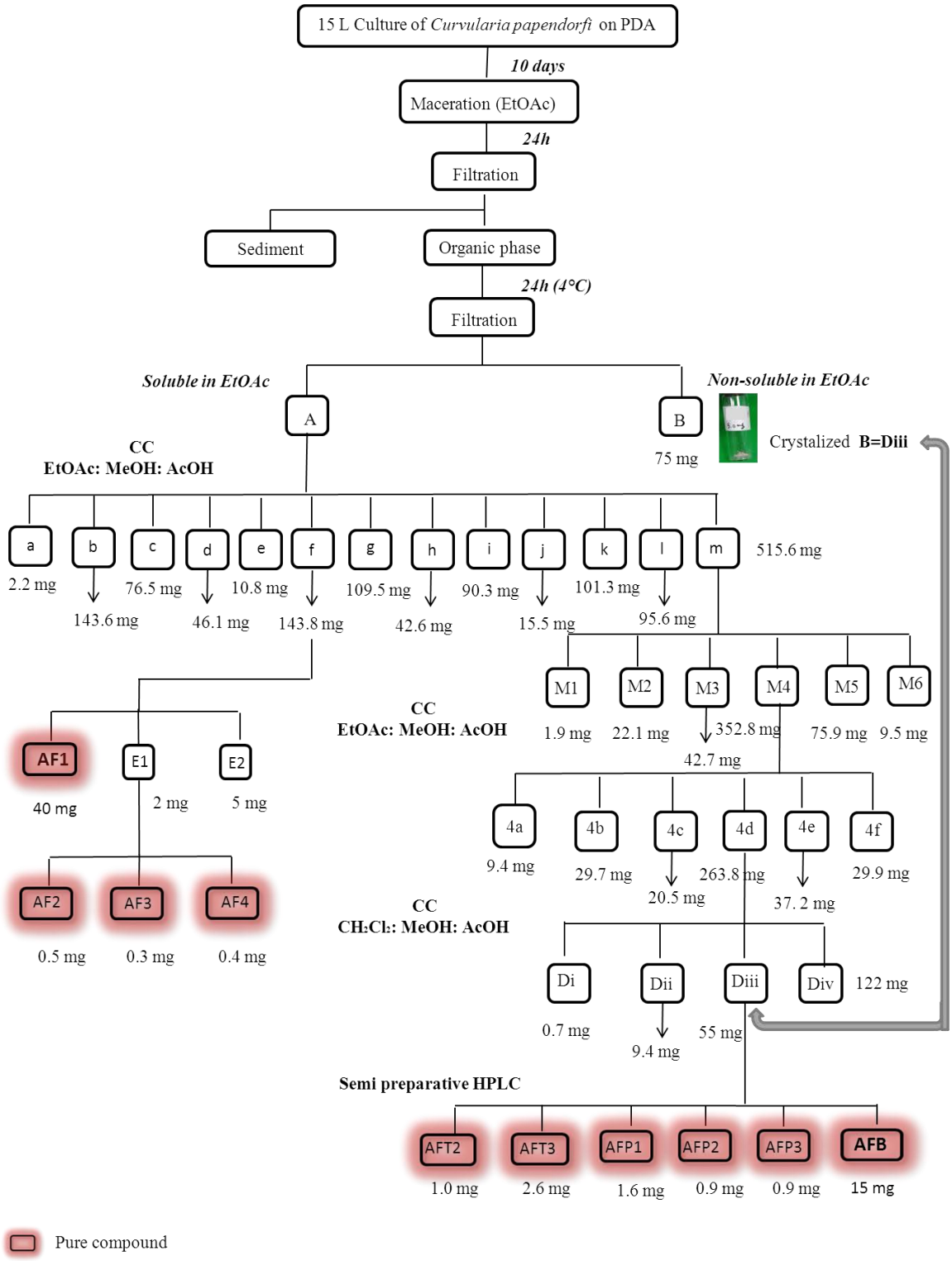


Figure 64. The flow chart of the ten pure compounds from ethyl acetate crude extract of *Curvularia panpendorfii* endopytic fungus of *Vernonia amygdalina.*

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3.4.5. Characterization of compound AFB

Compound **AFB** was obtained as white powder (15 mg). It gave R_f= 0.52 in 90%EtOAc, 0.5% Methanol and 0.5% Acetic acid developing solvent. A grey color after spraing with both Vanillin/H_2So_4 and Anisaldehyde-sulfuric acid was observed. HPLC profile of **AFB** is shown in Fig. 65.

An ion peak at *m/z* = 573.3790 $[M+Na]^+$ in HR-ESI^+MS and an ion peak at *m/z* 549.3793 $[M-H]^-$ in HR-ESI^- MS indicated that the molecular mass was 550 corresponding to the formula $C_{32}H_{54}O_7$. (Fig. 67-A and B).

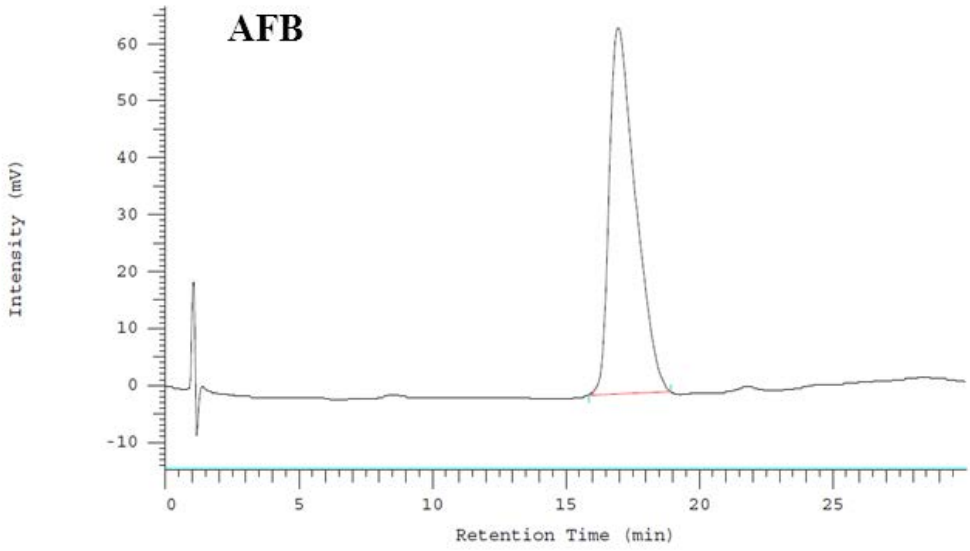


Figure 65. HPLC profile of **AFB**.

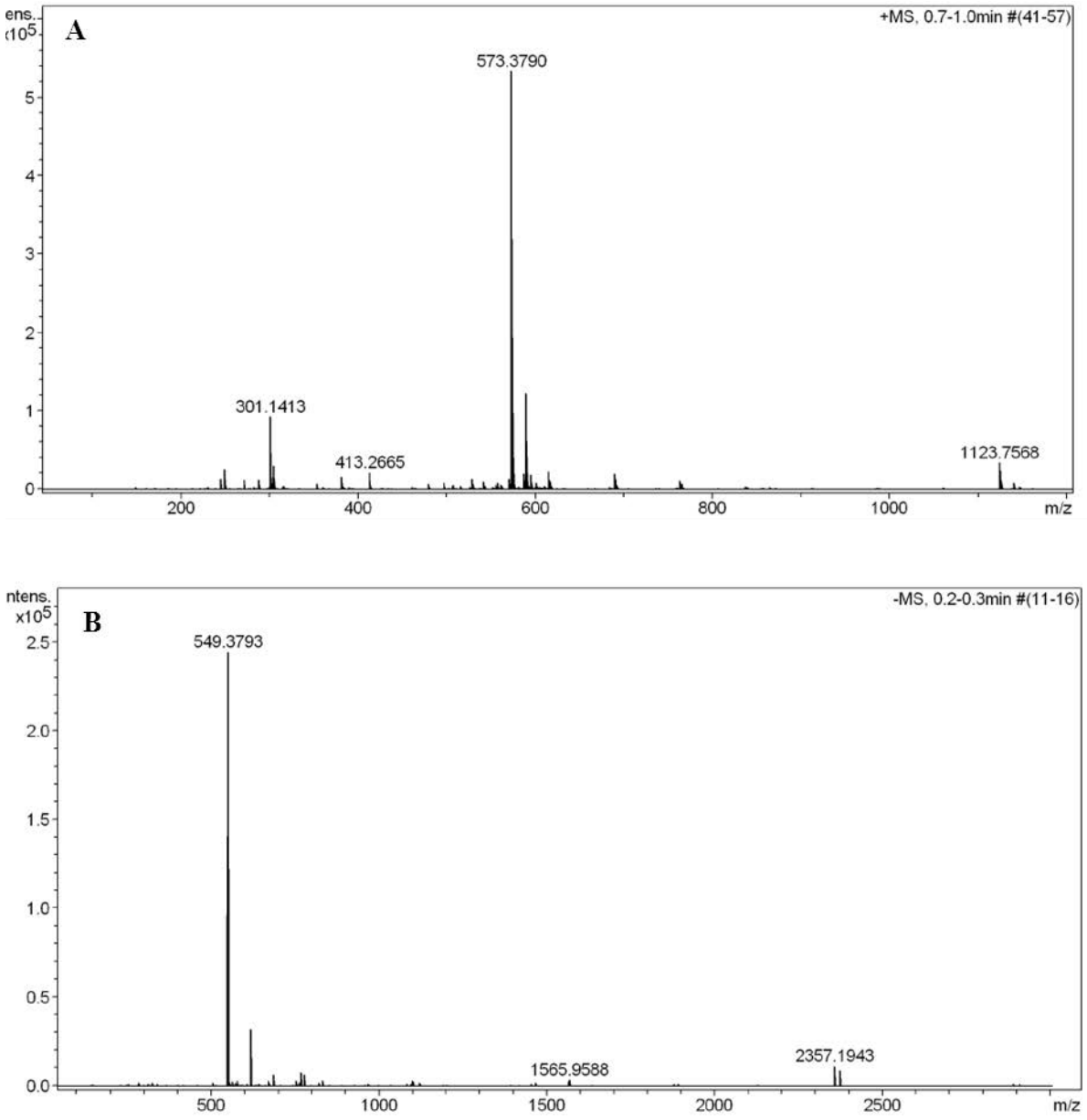


Figure 66. A. HR-ESI-MS positive mode of compound **AFB**, and **B.** HR-ESI-MS negative mode of compound **AFB**.

The IR spectrum (Fig. 67) showed a carboxylic group at 2914 cm^{-1}, an ester group at 1714 cm^{-1}, weak absorption band of alkene groups at 1666 cm^{-1} and strong absorption band at 3383 cm^{-1} indicated many hydroxyl groups.

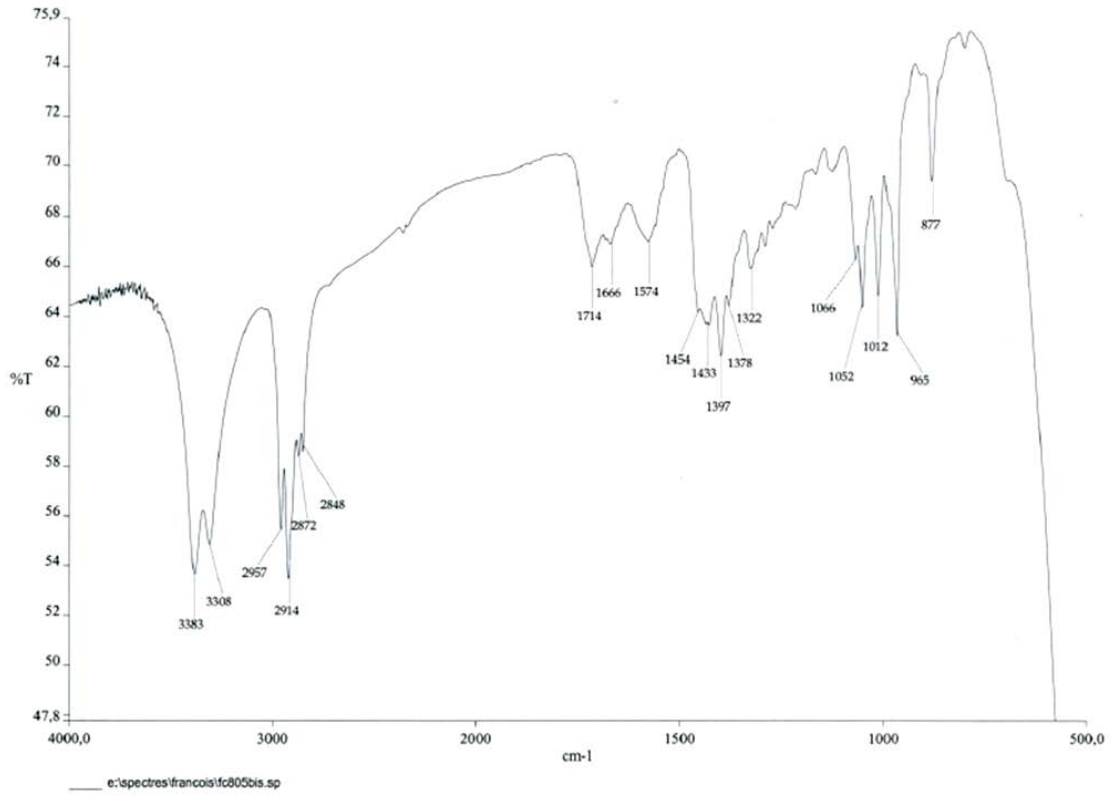


Figure 67. IR spectrum of **AFB**

The NMR data obtained from compound AFB include ^{1}H NMR and ^{13}C NMR spectra, in addition to proton – proton correlated spectroscopy (HH-COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond coherence (HMBC) (Fig. 68, 69, 70, 71 and 72) showed signals assignable to:

- Six methyl groups: 22.9 (C-30), 20.7 (C-32), 20.3 (C-31), 17.9 (C-27), 13.5 (C-28), 11.7 (C-26) ppm.
- Ten olefinic carbons: 140.0 (C-19), 139.0 (C-16), 136.3 (C-12), 136.0 (C-9), 135.9 (C-18), 135.6 (C-4), 134.1 (C-8), 129.9 (C-17), 128.9 (C-5), 128.8 (C-13) ppm.
- Five carbons with a carbon-oxygen single bond: 83.7 (C-15), 73.4 (C-11), 73.7 (C-7), 70.3 (C-3), 61.2 (C-29) ppm. The last one was characteristic of primary alcohol. The others were secondary hydroxyl functions.
- The deshielding of the carbon C-1 at 175.3 ppm suggested the presence of carbonyl function of carboxylic acid.
- The methylene groups H-2 and H-2 at 2.47 ppm were correlated in HMBC with acid carbon C-1 (175.3 ppm).
- Also the methylene group at 2.47 ppm was correlated with two others signals in HMBC: C-3 (70.3 ppm) and C-4 (135.6 ppm).
- It was observed a H-C coupling between H-4 and C-5 corresponding at one double bond.
- The ^{1}H NMR spectra showed a section comprising two repeated 4 carbon units respectively a methylene (H-6 and H-10), a hydroxymethine (H-7 and H-11) and a disubstituted double bond (H-8/H-9 and H-12/H-13). This section presented a set of twinned NMR signals.
- The presence of 3 double bonded carbons with coupling constant of 15 Hz (H-4/H-5), (H-8/H-9), (H-12/H-13) was confirm the configuration *trans*.
- Two trisubstituted unsaturated bonds were observed C-16/C-17 and C-18/C-19.
- One of methyl group was located on the second trisubstituted unsaturated bonds (d, 1.77 ppm, CH_3-28 at C-16).
- The COSY experiment showed a correlation between CH_3-28 and H-17.
- The primary alcohol CH_2OH (AB system for H-29 and H-29' at C-18) was located on one of the two trisubstituted unsaturated bonds.
- The methyl CH_3-30 presented H-C correlation with C-20 and C-21.

Summary of the ^{1}H and ^{13}C NMR spectra was given in Table 11.

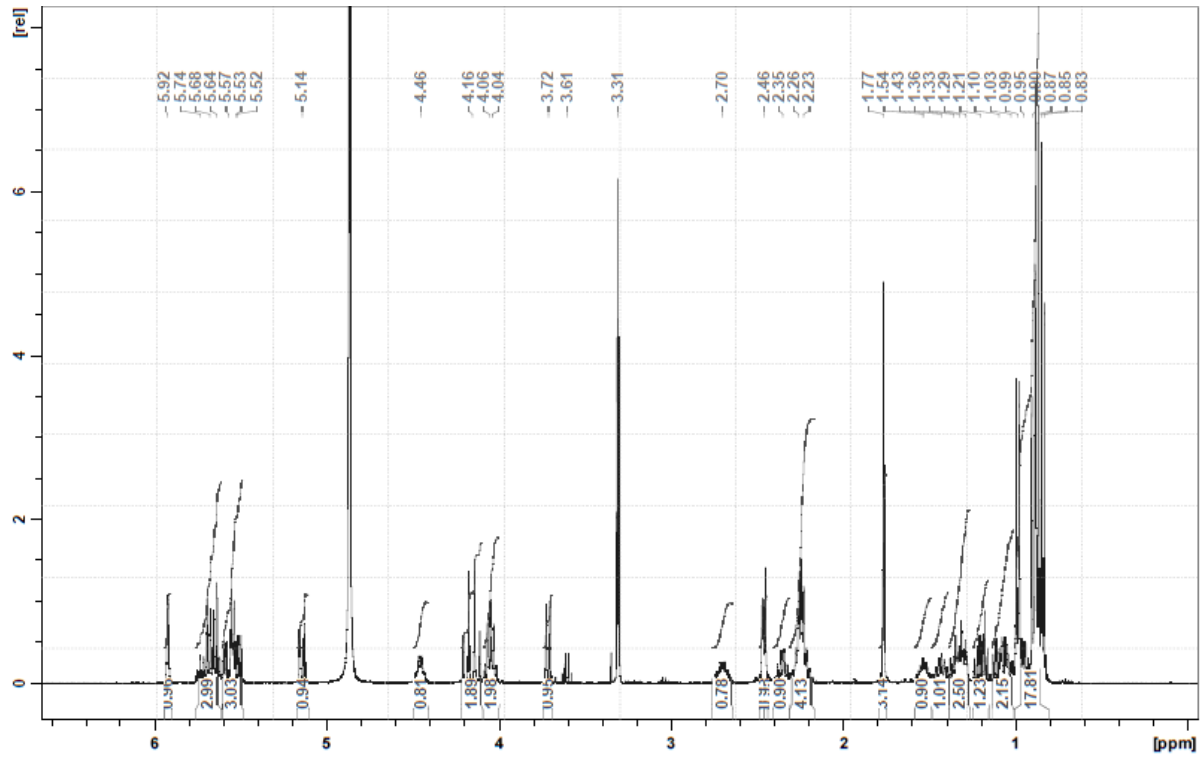


Figure 68. ^{1}H NMR spectrum of **AFB** in MeOH d_4 at 400 MHz

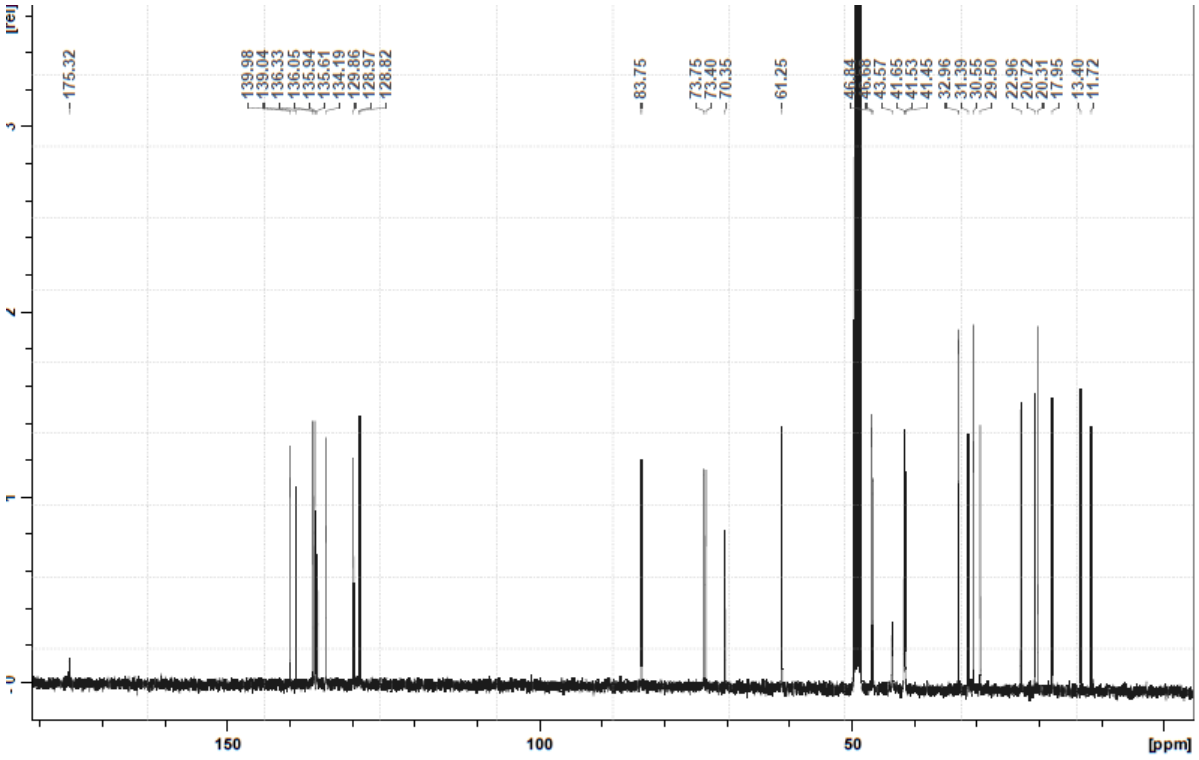


Figure 69. ^{13}C NMR spectrum of **AFB** in MeOH d_4 at 100 MHz

Table 11. ^{1}H and ^{13}C-NMR data for **AFB** in methanol d_4

Position	^{13}C	^{1}H
C-1	175.3	-
C-2	43.5	2.47 m (2H, H-2, H-2')
C-3	70.3	4.47 m (1H, H-3)
C-4	135.6	5.59 dd (1H, H-4, *J* =6.6Hz, 18Hz)
C-5	128.9	5.73 dd (1H, H-5, *J* =7Hz, 15Hz)
C-6	41.4	2.26 m (2H, H-6, H-6')
C-7	73.7	4.05 m (1H, H-7)
C-8	134.1	5.48 dd (1H, H-8, *J* =7Hz, 15Hz)
C-9	136.0	5.64 dd (1H, H-9, *J* =7Hz, 15Hz)
C-10	41.5	2.23 m (2H, H-10, H-10')
C-11	73.4	4.03 m (1H, H-11)
C-12	136.3	5.54 dd (1H, H-12, *J* =7Hz, 18Hz)
C-13	128.8	5.70 dd (1H, H-13, *J* =7Hz, 15Hz)
C-14	41.6	2.35 m (1H, H-14)
C-15	83.7	3.72 d (1H, H-15, *J* =8.6Hz)
C-16	139.0	-
C-17	129.9	5.92 s (1H, H-17)
C-18	135.9	-
C-19	140.0	5.14 d (1H, H-19, *J* =10Hz)
C-20	31.4	2.70 m (1H, H-20)
C-21	46.7	1.30 m (1H, H-21) 1.05 m (1H, H-21')
C-22	32.9	1.43 m (1H, H-22)
C-23	46.8	1.20 m (1H, H-23) 0.95 m (1H, H-23')
C-24	29.5	1.52 m (1H, H-24)
C-25	30.5	1.09 m (1H, H-25) 1.39 m (1H, H-25')
C-26	11.7	0.86 t (3H, CH3, H-26, *J* =7Hz)
C-27	17.9	0.89 d (3H, CH3, H-27, *J* =7Hz)
C-28	13.5	1.77 d (3H, CH3, H-28, *J* =1.3Hz)
C-29	61.2	4.16 AB system d (1H, H-29, *J* =12Hz) 4.16 AB system d (1H, H-29', *J* =12Hz)
C-30	22.9	0.99 d (3H, CH3, H-30, *J* =6.6Hz)
C-31	20.3	0.84 d (3H, CH3, H-31, *J* =6.6Hz)
C-32	20.7	0.87 d (3H, CH3, H-32, *J* =6.6Hz)

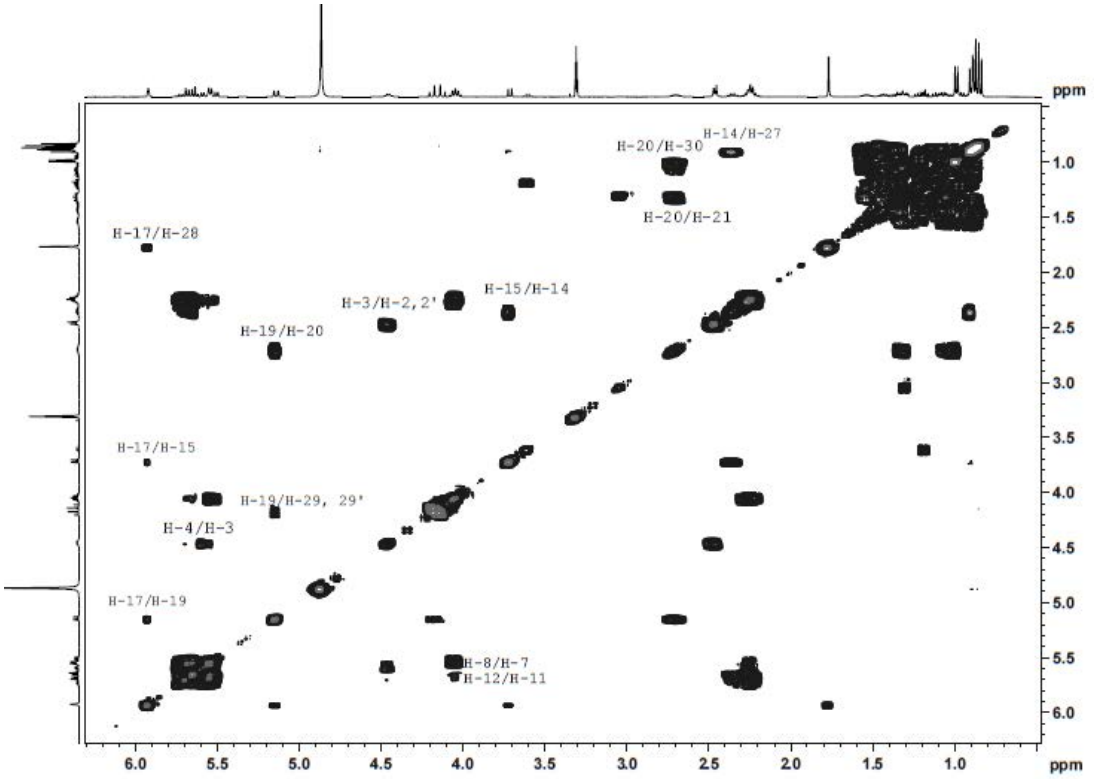


Figure 70. COSY spectrum of **AFB** in methanol d_4

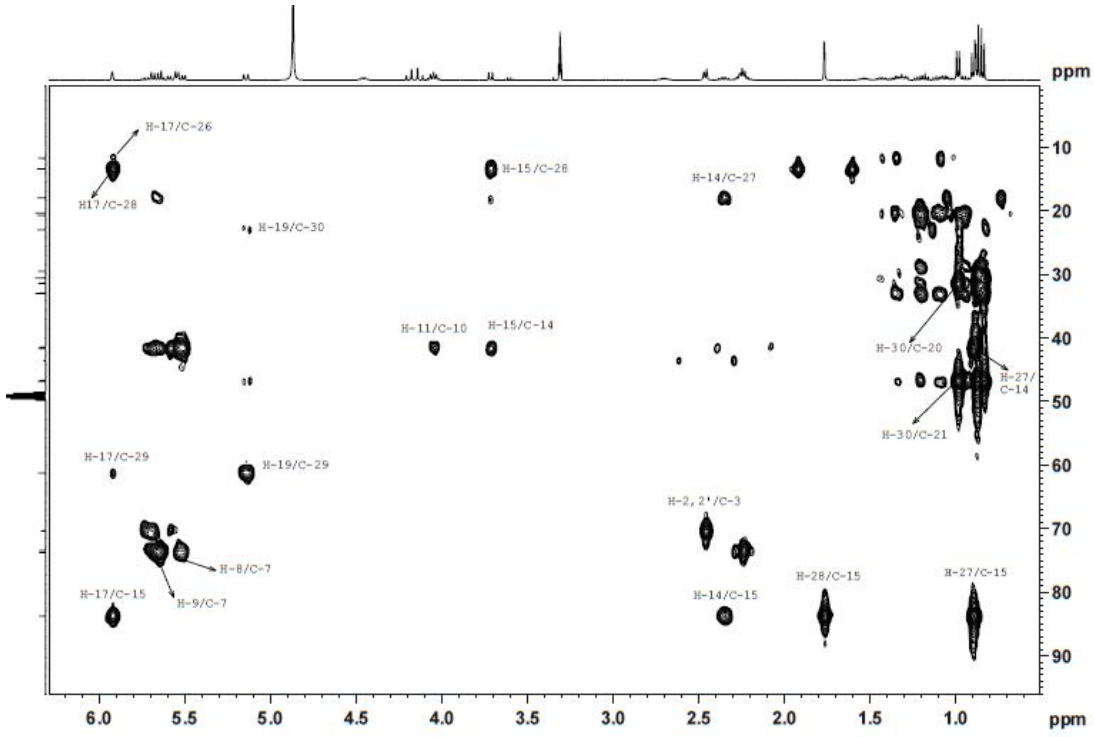


Figure 71. HMBC (1) spectrum of **AFB** in methanol d_4

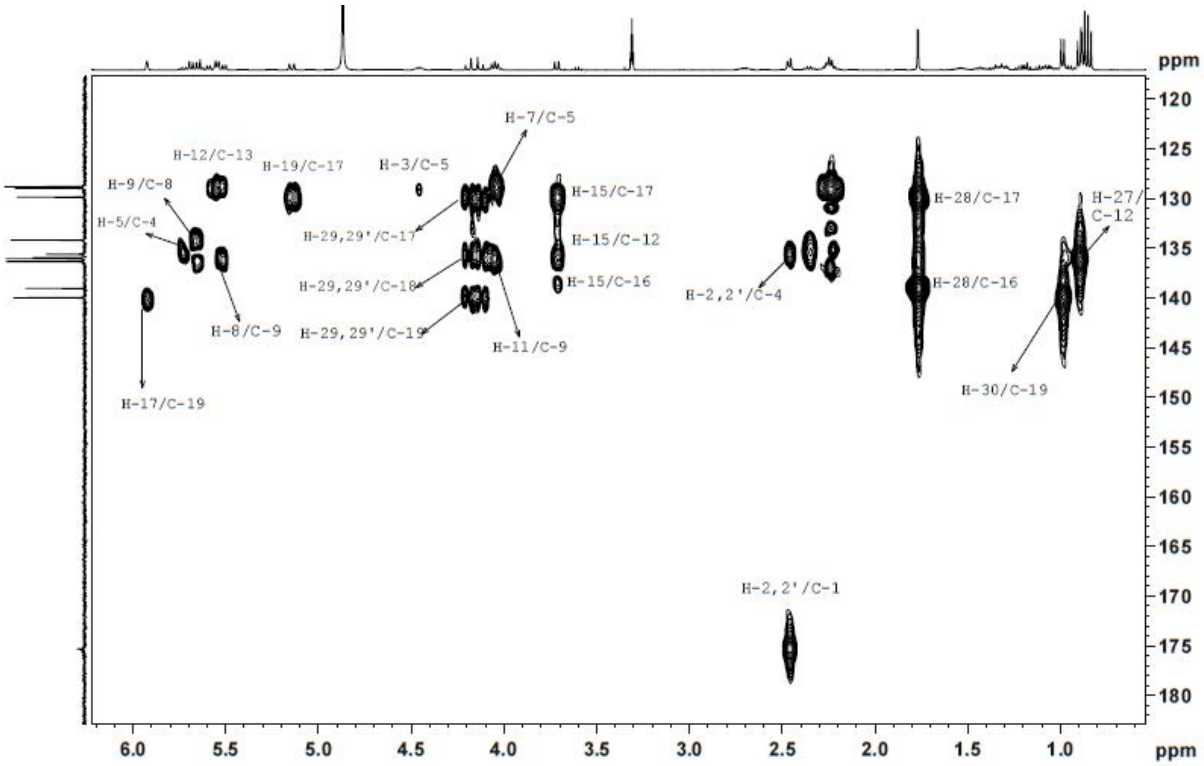


Figure 72. HMBC(2) spectrum of **AFB** in methanol d_4

Another analysis was performed in pyridine C_5D_5N (pyridine *d5*), because of the presence of the overlapped signals in NMR spectra performed in CD_3OD. The ^1H NMR spectrum of **AFB** in pyridine d_5 showed a good separation in the region of double bonds (6.4 ppm - 5.9 ppm). Also it was possible to observe the presence of five methyl groups (doublets respectively a 2.17, 1.16, 1.06, 0.91, 0.84 ppm) and one other CH_3 (triplet at 0.85 ppm). Four methylene protons at 2.58 ppm and 2.64 ppm were determined (Fig. 73 and 74). Summary of both ^1H and ^{13}C NMR spectra are shown in the Table 12.

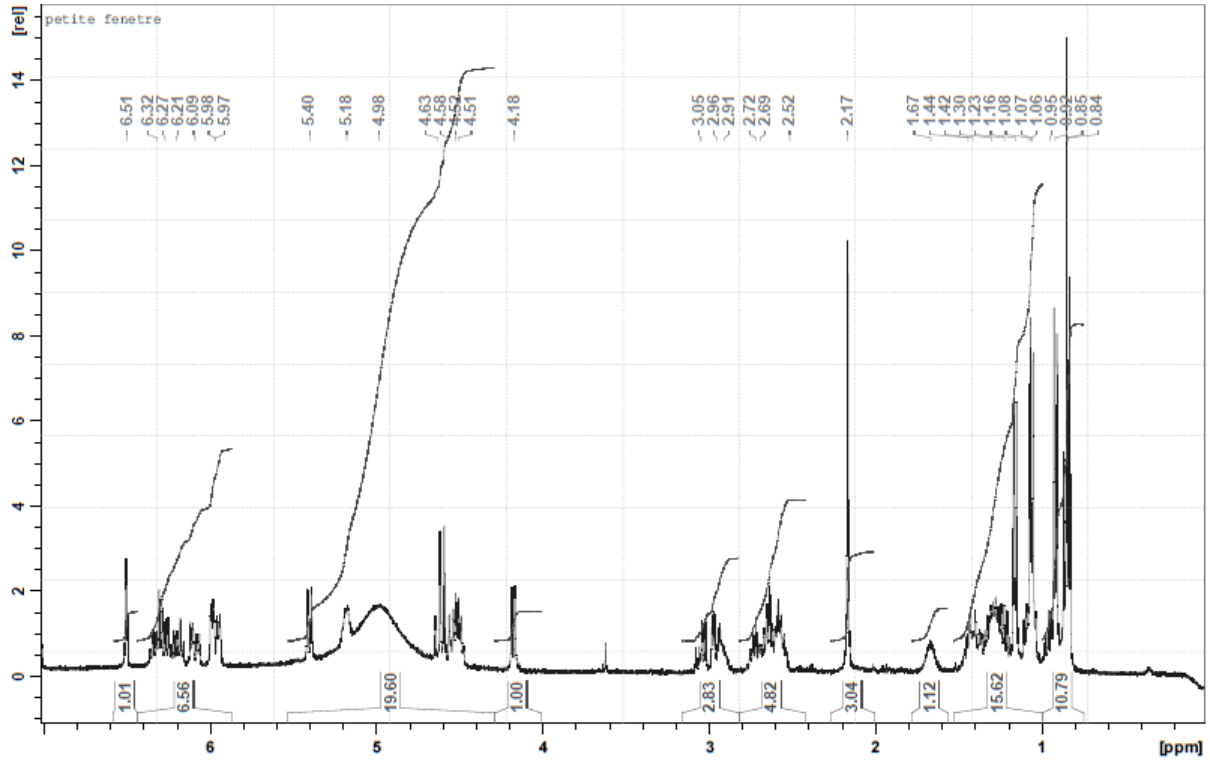


Figure 73. ^{1}H NMR spectrum of **AFB** in pyridine d_5 at 400 MHz

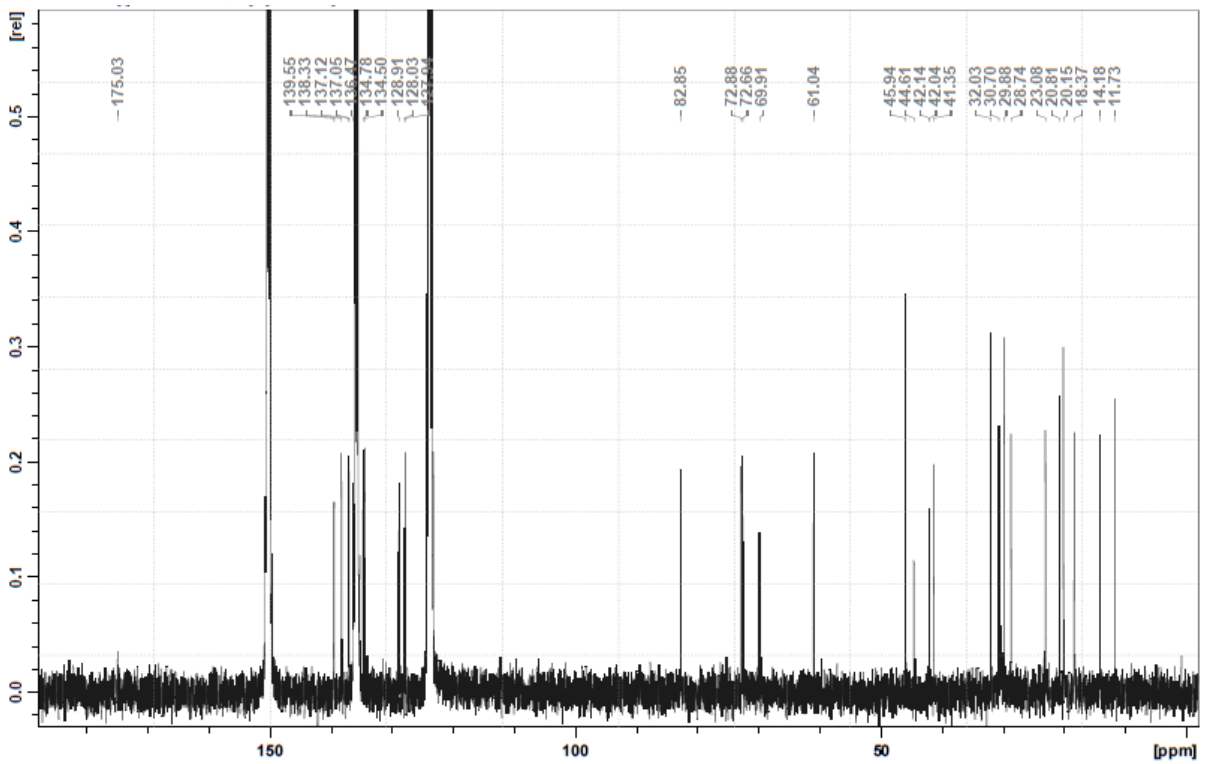


Figure 74. ^{13}C NMR spectrum of **AFB** in pyridine d_5 at 100 MHz

Table 12. ^{1}H and ^{13}C-NMR data for **AFB** in pyridine *d5*

Position	^{13}C	^{1}H
C-1	175.0	-
C-2	44.6	3.05 dd (1H, *J* =15Hz, *J* =8.5Hz, H-2') 2.96 dd (1H, *J* =15Hz, *J* =5Hz, H-2)
C-3	69.9	5.18 m (1H, H-3)
C-4	136.5	6.10 dd (1H, H-4, *J* =6Hz, 15Hz)
C-5	128.0	6.32 dd (1H, H-5, *J* =7Hz, 15Hz)
C-6	42.0	2.58 m (2H, H-6, H-6')
C-7	72.7	4.52 m (1H, H-7)
C-8	137.0	5.97 dd (1H, H-8, *J* =6Hz, 15Hz)
C-9	127.9	6.21 dd (1H, H-9, *J* =7.6Hz, 15Hz)
C-10	42.1	2.64 m (2H, H-10, H-10')
C-11	72.9	4.51 m (1H, H-11)
C-12	134.8	5.98 dd (1H, H-12, *J* =6Hz, 15Hz)
C-13	134.5	6.27 dd (1H, H-13, *J* =7.6Hz, 15Hz)
C-14	41.35	2.72 m (1H, H-14)
C-15	82.8	4.18 d (1H, *J* =8Hz, H-15)
C-16	139.5	-
C-17	128.9	6.51 bs (1H, H-17)
C-18	137.1	-
C-19	138.3	5.40 d (1H, *J* =10Hz, H-19)
C-20	30.7	2.91 m (1H, H-20)
C-21	45.9	1.42 m (1H, H-21') 1.07 m (1H, H-21)
C-22	32.0	1.43 m (1H, H-22)
C-23	45.9	1.22 m (1H, H-23') 0.95 m (1H, H-23)
C-24	28.7	1.66 m (1H, H-24)
C-25	29.9	1.29 m (1H, H-25') 1.07 m (1H, H-25)
C-26	11.7	0.85 t (3H, CH_3, *J* =7Hz, C-26)
C-27	18.4	1.16 d (3H, CH_3, *J* =6.6Hz, C-27)
C-28	14.2	2.17 d (3H, CH_3, *J* =0.9Hz, C-28)
C-29	61.0	4.60 d (1H, *J* AB =12Hz, H-29') 4.58 d (1H, *J* AB =12Hz, H-29)
C-30	23.1	1.06 d (3H, CH_3, *J* =6.6Hz, C-30)
C-31	20.1	0.84 d (3H, CH_3, *J* =6Hz, C-31)
C-32	20.8	0.91 d (3H, CH_3, *J* =6.6Hz, C-32)
-	-	4.98 broad m (5H, 5-OH)

Compound **AFB** was identified as (3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid), since it was never described in the literature it is new compound. The presence of 5 doubles bonds and one insaturation of carboxylic acid suggest that the structure is a long chain. This compound is a polyfunctionalized long-chain carboxylic compound (Fig. 75).

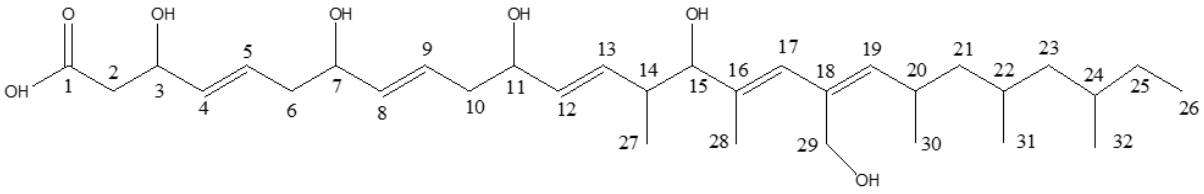


Figure 75. 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (Khartoumic acid).

3.4.6. The pure compound AF1

Compound **AF1** was obtained as white powder (40 mg) It gave $R_{f=}$ 0.56 in 50% EtOAc an 50% Cyclohexane developing solvent. A yellow to brown color after spraing with Vanillin/H_2So_4 and a grey color with Anisaldehyde-sulfuric acid were observed. HPLC profile is shown in Fig. 76.

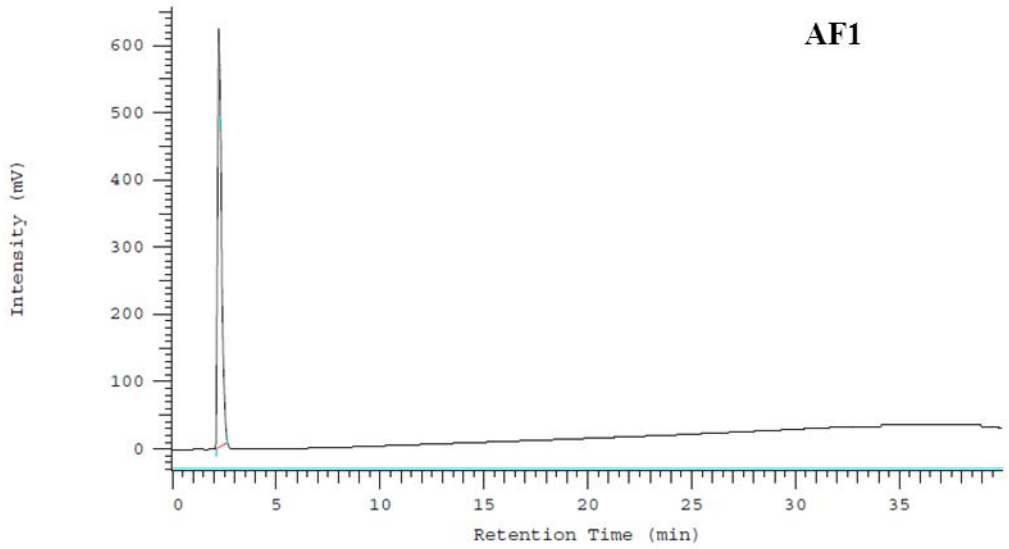


Figure 76. HPLC profile of **AF1**.

3.4.7. Biological assay of pure compounds

3.4.7.1. Antibacterial assay of pure compounds

General screening was performed in order to evaluate the antibacterial activity of **AFB** and **AF1** against Gram-positive methicillin resistant *Staphylococcus aureus* and Gram-negative *E.coli.* **AFB** revealed moderate activity (62.5 µg/mL) against methicillin-resistant *S. aureus* and no activity against *E. coli*. While **AF1** showed weak activity against *S. aureus* and it was inactive against *E. coli* (Table 13). This antibacterial activity of the pure compounds is the same as the crude extract of *C. papendorfii* which has shown selected activity against all the strains of *S. aureus* and lack activity against all tested Gram negative bacterial strains. Similar behavior (selective activity against Gram-positive bacteria) of two sesquiterpene lactones from leaves of *V. amygdalina* was reported by Erasto *et al*. (2006) who stated that

vernolide and vernodalol exhibited a significant bactericidal activity against five Gram positive bacteria while lacking efficacy against the Gram negative strains.

Table 13. Antibacterial activity of two pure compounds, **AFB** and **AF1**, isolated from *Curvularia papendorfii* crude extract, against Gram-negative *Escherichia coli* and Gram-positive methicillin-resistant *Staphylococcus aureus*.

Compounds	***Staphylococcus aureus***		***Escherichia coli***	
	MIC (µg/mL)	**MBC**	**MIC(µg/mL)**	**MBC**
AFB	62.5	125	>2000	>2000
AF1	250	>250	>2000	>2000

3.4.7.2. Cytotoxic assays and anti-oxidant activity of pure compounds

Cytotoxic assays of the pure compounds **AFB** and **AF1** against MCF7, HT29 and HCT116 cell lines were performed. Surprisingly, very weak cytotoxicity of **AFB** against MCF7 cells and no activity against HT29 and HCT116 cell lines were observed. Despite **AFB** is the major compound in the most cytotoxic fractions B and Diii (Fig. 65). More investigations are needed for the other five pure compounds within fraction Diii. **AF1** revealed only moderate cytotoxicity against HT29 and HCT116. The purity of **AF1** was 88%. Furter purification needs to verify the real activity of the pure compound. Both pure compounds (**AFB** and **AF1**) did not exert any antioxidant activity from the DPPH radical scavening assays (Table 14).

Table 14. Cytotoxicity of **AFB** and **AF1** against MCF7, HT29 and HCT116 cell lines, anti-oxidant activity by DPPH radical-scavenging test.

Compounds	**MIC (µM)**			**IC_{50} (µg/mL)**
	MCF7	HT29	HCT116	DPPH
AFB	>100	NA	NA	NA
AF1	-	29.78 ± 5.16	32.43 ± 2.38	NA

NA not active.

3.4.8. *In vitro* culture optimization of crude extract of *Curvularia papendorfii*

The yield of bioactive compounds can sometimes be substantially increased by the optimization of physical factors (temprature, salinity, pH value, and light) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes (Calvo *et al*., 2002; Llorens *et al*., 2004). This part of Section Four focused on the effect of the physical factor pH of the culture media on the production of the compound **AFB** from *C. papendorfii.* This experiment was brought about to give the fungus environment closed to that in the host plant. During our research we used to cultivate the fungus on PDA at pH 6.5. *C. papendorfii* was cultivated on PDA media with serial pH values, initial pH ranges were adjusted from 3.5 to 9.5. The dry weight was measured and a quantative calibration of percentage of **AFB** was measured using analytical HPLC.

The effect of pH on the biomass and production of **AFB** are shown in Table 15 and Fig. 77. It was observed that pH 6.5 was the optimal pH for growth and **AFB** production (40%), followed by a pH 5.5 and 8.5 (33%), and the lowest percentage of **AFB** production was observed at pH 4.5 (9%). The highest dry weight was obtained at pH 9.5. The pH of the culture medium is one of the determining factor for the metabolism and hence for the biosynthesis of secondary metabolites. The pH is related to permeability characteristic of the cell wall and membrane and thus has got effect on either ion uptake or loss to nutrient medium (Hansen, 1968).

Table 15. The effect of different pH values on *Curvularia papendorfii* growth rate and percentage of AFB.

Medium pH	Dry weight mg	Percentage % of AFB	Growth rate (mm/day)
3.5	107	21%	12
4.5	88	9%	13
5.5	82	33%	10
6.5	97	**40**%	14
7.5	105	34%	13
8.5	98	33%	12
9.5	**135**	30%	12

Most fungi grow best at approximately pH 7, but tolerate a wide range from 3-10 (or even 11) (Kirk *et al.,* 2008). It was found that *C. papendorfii* grows in all media with different pH values from acidic, neutral to alkaline with almost similar growth rate, mainly in the production of secondary metabolites. It was observed that *C. papendorfii* produced the highest percentage of **AFB** at pH 6.5 (40%). Merlin *et al*. (2013) stated that medium with pH 6 was found to be optimal for growth and bioactive metabolite production of *Fusarium solani*.

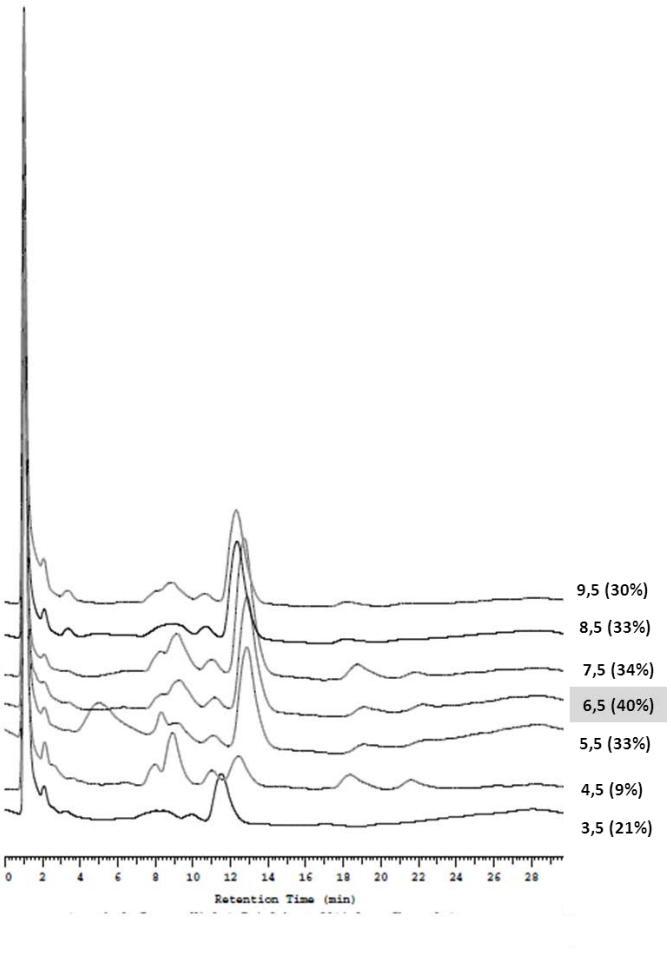


Figure 77. Different HPLC profiles for fraction B from *Curvularia papendorfii* crude extract cultivated on serial pH (3.5 -9.5).

Digrak and Ozcelik (2001) investigated the effect of media pH on the biomass and the inhibition activity of 5 fungi they reported significant retarded at pH 3.0. The growth of all the tested species was greatest at pH 8.0, however the highest quantities of active metabolite production were observed at pH 5.0. Considering the finding of this preliminary evaluation of pH effect on production of **AFB**, *C. papendorfii* showed good production of **AFB** on pH

range between 5.5 to 8.5.Further investigations are needed for the effect of pH on the biological activity. On the other hand, more physical and chemical conditions should be verified in order to optimize the best condition of production of **AFB**.

To recap this section, ten pure compounds (0.3 to 40 mg) were isolated from ethyl acetate crude extract of *C.papendorfii*. A combination of spectroscopic methods ID and 2D NMR, IR, UV and high-resolution mass spectrometry (HR-ESI-MS) was used for structural determination of two compounds **AFB** and **AF1**. Biological and chemical tests were performed for the two compounds. **AFB** revealed moderate antibacterial activity against MRSA with MIC value 62.5 µg/mL and weak cytotoxicity with MIC value >100 µM against MCF7 cell. While **AF1** showed moderate activity IC_{50} value 29.78 µM against HT29 cells and very weak activity with MIC value 250 µg/mL against MRSA. Both compounds displayed none antioxidant activity. Furthermore, the effect of pH on the production of **AFB** was investigated where *C. papendorfii* was cultivated on PDA with different pH ranged between 3.5 to 9.5. The findings revealed that the highest production of **AFB** was obtained on pH 6.5.

Conclusion and Perspectives

Conclusion

Ce travail a porté sur l'étude de champignons endophytes issus de cinq plantes médicinales soudanaises: *Calotropis procera* (Ait.), *Catharanthus roseus* (L.), *Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.) et *Vernonia amygdalina* (Del.). Cette recherche a pour objectifl'objectif d'identifier de nouveaux agents anticancéreux et anti-bactériens d'origine naturelle. Les résultats sont présentés en quatre sections avec un résumé à la fin de chacune d'entre elles. La première section présente l'identification morphologique et moléculaire des champignons endophytes isolés. La section deux montre les propriétés physiques des extraits à l'acétate d'éthyle des champignons ainsi qu'une étude chromatographique par CCM des extraits bruts des endophytes et de leurs plantes hôtes. La troisième section est centrée sur les résultats des tests chimiques et biologiques des extraits d'endophytes. Enfin, la section quatre rapporte les fractionnements bioguidés et l'isolement de composés purs à partir d'extraits du champignon endophyte *Curvularia papendorfii* isolé de *Vernonia amygdalina*.

Au total 23 souches de champignons endophytes ont été isolées à partir des cinq plantes après avoir réalisé une stérilisation de surface des explants. Cinq endophytes issus de *C. procera*, six de *C. roseus*, quatre de *E. prostrata*, cinq de *T. foenum-graecum*, et trois de *V. amygdalina* ont été isolés. Ces souches fongiques ont été classées en 12 taxons différents. 19 souches appartiennent aux Ascomycètes, alors que trois souches appartiennent à la classe fongique des Deutéromycètes et une seule appartient aux Basidiomycètes. Trois souches n'ont pas sporulé, elles ont été regroupées sous le nom de mycélium stérile. La diversité des endophytes, dans les plantes étudiées, s'est montrée peu élevée en comparaison avec d'autres pays comme la Chine, l'Inde et le Pakistan. Cela peut être dû au climat du Soudan, il est extrêmement aride, la plupart du temps avec une pluviométrie moyenne inférieure à cinq mm pendant neuf mois par an. La faible diversité fongique peut aussi s'expliquer par la présence de champignons endophytes non cultivables *in vitro*.

Parmi les 21 endophytes, *Aspergillus terreus* 1 et *Alternaria alternata* chez *C. procera* se sont révélés avoir des rendements d'extraction élevés avec l'acétate d'éthyle (242 et 206 mg respectivement) suivie de *Byssochlamys spectabilis* (155,4 mg) chez *E. prostrata*. Les analyses chromatographiques par CCM des extraits ont montré que tous les extraits des endophytes renfermaient des terpènes, des composés phénoliques et rarement des alcaloïdes.

Seuls deux extraits bruts de *Cladosporium cladosporioides* 2 et de *Curvularia papendorfii* isolés de *Vernonia amygdalina* ont montré la présence d'alcaloïdes.

Le contenu en polyphénols totaux (PTC), dans les extraits bruts à l'acétate d'éthyle de 21 souches d'endophytes et dans les extraits de différentes drogues des plantes hôtes, a été estimé en utilisant la méthode colorimétrique de Folin-Ciocalteu. Par ailleurs l'activité antioxydante (TAC) a été estimée à l'aide de 1,1, diphényl-2 -picrylhydrazl (DPPH) par piégeage des radicaux libres *in vitro*. Parmi les endophytes, *Aspergillus terreus* 2 isolé des graines de *Trigonella foenum-graecum* a montré le taux de PTC le plus élevé (89,9 ± 7,1 mg GAE / g) ainsi que l'activité antioxydante la plus élevée (IC_{50}: 18 ± 0,1 µg/mL). Une corrélation linéaire positive élevée (R^2 = 0,9991) a été observée entre TAC et TPC des champignons endophytes isolés à partir de *V. amygdalina*. L'évaluation de la cytotoxicité des extraits de 16 endophytes et de leurs plantes hôtes a été effectuée avec le test au MTT en utilisant trois souches cancéreuses; le carcinome du sein (MCF7), et l'adénocarcinome du colon (HT29 et HCT116). 14 endophytes ont montré une activité cytotoxique. *Byssochlamys spectabilis* a montré l'activité la plus élevée (1,51 ± 0,2 µg / mL), suivi de *Cladosporium cladosporioides* 2 (10,5 ± 1,5 µg / ml), puis *Alternaria* sp. (13,5 ± 1,8 µg / ml). Il est à noter que la plupart des champignons cytotoxiques ont été isolés à partir de *E. prostrata* qui a montré une faible activité cytotoxique (> 100 µg / mL). En outre, les extraits de 16 champignons ont été testés sur deux souches bactériennes, l'une à Gram négatif (*Escherichia coli*) et l'autre à Gram positif (*Staphylococcus aureus* résistant à la méthicilline SARM). Seules six souches ont montré une activité contre *S. aureus* avec des valeurs de MIC situées entre 0,125 et 2 mg / mL, parmi lesquelles *Alternaria alternata* (0,125 mg / mL), *Alternaria* sp. (0,250 mg / mL) et *Byssochlamys spectabilis* (0,5 mg / mL).

Le travail de phytochimie a porté sur la souche *Curvularia papendorfii* en raison de la détection d'alcaloïdes dans l'extrait à l'acétate d'éthyle par CCM. L'extrait brut a été soumis à un fractionnement bioguidé en mesurant l'activité antibactérienne vis-à-vis de la souche *Staphylococcus aureus* résistante à la méthicilline (SARM). Dix composés purs (0,3 à 40 mg) ont été isolés. Un ensemble de méthodes spectroscopiques, RMN ID et 2D, IR, UV et spectrométrie de masse à haute résolution (HR-ESI-MS), a été utilisé pour la détermination de la structure de deux composés, **AFB** et **AF1**. Les tests biologiques et chimiques ont été effectués pour les deux composés. Le nouveau composé pur (**AFB**) 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (acide Khartomique) a montré une activité antibactérienne modérée contre *S. aureus* résistant

à la méthicilline (SARM) avec la valeur de IC_{50} = 62,5 µg/mL et une faible cytotoxicité avec la valeur de IC_{50} > 100 µM contre les cellules MCF7. **AF1** a montré une activité cytotoxique modérée avec une valeur de IC_{50} = 29,78 µM sur la lignée HT29 et une très faible activité contre *S. aureus* résistant à la méthicilline (SARM) avec une valeur de CMI 250 µM. Les deux composés ne présentent aucune activité anti-oxydante. Enfin l'effet du pH sur la production du composé **AFB** a été étudié. *C. papendorfii* a été cultivé sur PDA avec différents pH compris entre 3,5 à 9,5. Les résultats ont montré que la plus forte production d'**AFB** a été obtenue à pH 6,5.

Plusieurs perspectives découlent de ce travail:

- L'approche moléculaire des souches d'endophytes (comme le pyroséquençage) doit être poursuivie pour analyser la diversité des champignons endophytes dans les 5 plantes médicinales étudiées. En effet, on a remarqué que peu de souches de champignons ont été isolées à partir des plantes soudanaises.
- A partir de *Curvularia papendorfii*, le composé pur **AF1** a montré une cytotoxicité contre les cellules MCF7, cependant d'autres études sont nécessaires pour vérifier le mécanisme d'action de ce composé. Par ailleurs, au niveau de la fraction B qui a montré une cytotoxicité significative, d'autres études sont nécessaires pour caractériser les cinq composés mineurs présents dans cette fraction et pour déterminer leurs effets biologiques. Il faudrait aussi poursuivre l'optimisation de la culture de *C. papendorfii* en vue d'augmenter la production des composés bioactifs.
- L'endophyte *Aspergillus terreus* 1, isolé à partir de graines de *Trigonella foenum-graecum,* a montré la plus haute teneur en composés phénoliques totaux et la plus forte activité antioxydante. Ce champignon pourrait être une source prometteuse de nouvelles substances antioxydantes.
- La plupart des endophytes ont montré une cytotoxicité contre au moins une lignée cellulaire cancéreuse, en particulier *Byssochlamys spectabilis* et *Alternaria* sp. isolés de *Euphorbia prostrata* et *Cladosporium cladosporioides* 2 isolées de *Vernonia amygdalina.* Ces endophytes pourraient être une source intéressante pour l'obtention de nouveaux composés cytotoxiques.

Conclusion

This work was established to study the endophytic fungi flora of five Sudanese medicinal plants *Calotropis procera* Ait., *Catharanthus roseus* L., *Euphorbia prostrata* Ait., *Trigonella foenum-graecum* L., and *Vernonia amygdalina* Del. In this research we investigated for the first time, the endophytic fungi of these five plants, as a part of our ongoing work towards finding novel anticancer and antibacterial agents from natural resources. The results were divided into four sections. Section one summarizes the morphological and molecular identifications of the isolated endophytic fungi. Section two recaps physical properties of the ethyl acetate crude extracts and general phytochemical screening on crude extracts of the endophytes and their host plants by TLC. Section three presents results of chemical and biological tests of the endophyte crude extracts. Section four includes bioassay-guided fractionations and isolation of pure compounds from the endophytic fungus *Curvularia papendorfii* isolated from *Vernonia amygdalina*.

Section one is focused on identification and taxonomy of endophytic fungi isolated from the five plants. A total of 23 endophytic fungal strains were isolated after surface disinfection. Five endophytes from *C. procera,* six from *C. roseus*, four from *E. prostrata,* five from *T. foenum-graecum*, and three from *V. amygdalina* were isolated. These fungal strains were classified into 12 different taxa. 19 strains belong to Ascomycotina, whereas three strains belong to fungal class Deuteromycetes, they were failed to sporulate and were grouped as mycelia sterilia. Only one belongs to Basidiomycotina. The diversity of the fungal endophytes of the five medicinal plants was not high. May be the majority of the endophytic fungi were non-culturable. Further studies using molecular approach (such as pyrosequencing) are needed to analyze the diversity of fungal endophytes in these 5 medicinal plants.

The second section is intended to evaluate the extractive values and organoleptic properties of 21 endophytes isolated from the five medicinal plants. Each crude extract was prepared from fungal strain cultured on 20 plates of PDA and extracted with ethyl acetate. Among the 21 endophytes, *Aspergillus terreus* 1 and *Alternaria alternata* from *C. procera* were found to have the highest extractive yields (242 and 206 mg respectively) followed by *Byssochlamys spectabilis* (155.4 mg) from *E. prostrata, w*hereas, *Curvularia australiensis* 1 from *E. prostrata* gave the lowest quantity (36.6 mg). Then a general screening on the chemical constitute of ethyl acetate extracts of 21 endophytic fungi and their host plants was

performed by TLC using different reagents. All extracts of endophytes were very rich with terpenoids, phenolic compounds and rarely with alkaloids. Among all the endophytes tested, only two crude extracts of *Cladosporium cladosporioides* 2 and *Curvularia papendorfii* endophytes of *Vernonia amygdalina* were wealthy with alkaloids. *Vernonia amygdalina* endophytes were chosen depending on the chemical constitute of the host plant and the TLC profile of its endophytes. Beside no published work on the fungal endophytes from *Vernonia amygdalina* was reported.

Section three was intended to perform chemical and biological assay to evaluate the crude extract of the endophytes from the five medicinal plants. Total phenolic contents (TPC) of ethyl acetate crude extracts of 21 endophytes and different parts of their host plants were estimated using the classical Folin-Ciocalteu colorimetric method, alongside total antioxidant capacity (TAC) was estimated using 1,1,-diphenyl-2-picrylhydrazl (DPPH) free radical scavenging *in vitro* method. Among the endophytes, *Aspergillus* sp. from *Trigonella foenum-graecum* seeds demonstrated the highest both total phenolic content in term of Gallic Acid Equivalent (89.9±7.1 mg GAE/g) and antioxidant activity for DPPH radical scavenging assay (IC_{50}: 18±0.1µg/mL). A high positive linear correlation (R^2 = 0.9991) was found between TAC and TPC of endophytic fungi isolated from *V. amygdalina*. General evaluation of the cytotoxicity of the ethyl acetate extracts of endophytes 16 and their host plants was performed by the MTT assay using three cancer cells type; Human breast carcinoma (MCF7), and Colon adenocarcinoma (HT29 and HCT116) cells. 14 endophytes displayed cytotoxic activity ranging between strong to weak. *Byssochlamys spectabilis* showed powerful (1.51 ± 0.2 µg/mL) activity followed by *Cladosporium cladosporioides* 2 (10.5 ± 1.5 µg/mL), then *Alternaria* sp. (13.5 ± 1.8 µg/mL). Astonishingly, the most two cytotoxic fungi were isolated from *E. prostrata* which showed weak activity >100 µg/mL. Three medicinal plants *C. procera* stem, *V. amygdalina* leaves and *C. roseus* leaves revealed strong cytotoxicity at least against one cell line (2.19 ± 0.1, 5.6 ± 0.4 and 7.00 ± 0.43 µg/mL respectively). Further preliminary screening was done for the 16 endophytic fungi, extracts of the endophyes were tested against two bacterial strains Gram- negative *Escherichia coli* and Gram-positive methicillin-resistant *Staphylococcus aureus* by broth dilution methods using micro dilution tray (96 wells). Among 16 fungal strains tested, only six showed activity against methicillin-resistant *S. aureus* with MIC values ranging between 0.125-2 mg/mL: *Alternaria alternata* (0.125 mg/mL), *Alternaria* sp. (0.250 mg/mL) and *Byssochlamys spectabilis* (0.5 mg/mL). It was noticed in this assessment that, all endophytes crude extract lack the activity against *E.*

coli even those who were active against methicillin-resistant *Staphylococcus aureus*. The findings of this section revealed that some endophytic fungi of the five Sudanese medicinal plants studied, could be a potential source of novel natural anti-oxidant, anti-cancer and antibacterial compounds.

The last section was focused on *Curvularia papendorfii* the selected fungus for detailed phytochemical and biological activity analyses. Ethyl acetate crude extract of *C. papendorfii* was subjected to bioassay-guided fractionation by antibacterial assay against *Staphylococcus aureus* methicillin resistant Ten pure compounds (0.3 to 40 mg) were isolated from ethyl acetate crude extract of *C. papendorfii.* A combination of spectroscopic methods; ID and 2D NMR, IR, UV and high-resolution mass spectrometry (HR-ESI-MS) was used for structural determination of the two compounds **AFB** and **AF1**. Biological and chemical tests were performed for the two compounds. The new compound (**AFB)** 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (Khartoumic acid) revealed moderate antibacterial activity against methicillin-resistant *S. aureus* with MIC value 62.5 µg/mL and weak cytotoxicity with IC_{50} value >100 µM against MCF7 cells. **AF1** showed moderate activity IC_{50} value of 29.78 µM against MCF7 and very weak activity with MIC value 250 µg/mL against methicillin-resistant *S. aureus.* Both compounds displayed no anti-oxidant activity. Furthermore, the effect of pH on the production of **AFB** was investigated; *C. papendorfii* was cultured on PDA with different pH ranging between 3.5 to 9.5. The findings revealed that the highest production of **AFB** was obtained at pH 6.5.

Several perspectives stem from this work:

- Further studies using molecular approach (such as pyrosequencing) are needed to analyze the diversity of fungal endophytes in these 5 medicinal plants. Indeed, it was noticed that only few fungal strains were isolated from the Sudanese plants, however other endophytes could be present in the Sudanese plants but not able to be cultivated *in vitro*.
- From *Curvularia papendorfii*, the major endophyte fungus of this study, the pure compound **AF1** has shown cytotoxicity against MCF7 cells however more investigations are needed to verify the mechanism of this compound. Beside that the fraction B, which had shown a significant cytotoxicity, more researches are needed to characterize the five minor compounds present in this fraction and their biological effects. Add to that, to

continue the culture optimization of *Curvularia papendorfii* in order to improve the production of the bioactive compounds.

- Endophyte *Aspergillus terreus* 1, isolated from *Trigonella foenum-graecum* seeds, demonstrated the highest both total phenolic content and antioxidant activity. Further investigations are required, this fungus could be a promising source of novel antioxidant substances.
- Most of the endophytes showed cytotoxicity against at least one cancer cell line, in particular *Byssochlamys spectabilis* and *Alternaria* sp. isolated from *Euphorbia prostrata* and *Cladosporium cladosporioides* 2 isolated from *Vernonia amygdalina.* They could be an auspicious source of novel cytotoxic compounds.

References

References

A

Adebayo, L. O., James, A., Kasim, S. B. and Jagri O. P. (2014). Leaf extracts of *Vernonia amygdalina* Del. from northern Ghana contain bioactive agents that inhibit the growth of some beta-lactamase producing bacteria *in vitro*. *B.J.P.R.*, **4**(2): 192–202.

Adetunji, C. O. 1, Olaniyi, O. O. and Ogunkunle, A. T. J. (2013). Bacterial activity of crude extracts of V*ernonia amygdalina* on clinical isolates. *J. Microbiol. Antimicrob*., **5**(6): 60–64.

Alexander, S. K. and Strete, D. (2001). Microbiology- A Photographic Atlas for The Laboratory. Publisher: Benjamin Cummings - Sanfransisco USA.

Alsagarden. (2015) http://www.alsagarden.com/fr/506-calotropis-procera-pommier-de-sodome-arbre-%C3%A0-couilles-graines.html#sthash.e5q55hcJ.dpbs (06/06/2015)

Alves, R. R. N. and Rosa, I. L. (2007). Biodiversity, traditional medicine and public health: where do they meet?. *J. Ethnobiology. Ethnomedicine*, **3**(14): 1–9.

Alvi, K. A., Rodr´ıguez, J., Diaz, M. C., *et al.* (1993). Protein tyrosine kinase inhibitory properties of planar polycyclics obtained from the marine sponge *Xestospongia* cf. *carbonaria* and from total synthesis. *J. Org. Chem*., **58**: 4871–4880.

Aly, A. H., Edrada-Ebel, R., Wray, V., *et al*. (2008). Bioactive metabolites from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*. *Phytochemistry*, **69**(8): 1716–1725.

Al-Yahya, M. A., Al-Meshal, I. A., Mossa, A., *et al*. (1990). Saudi Plants, A Phytochemical and Biological Approach. General directorate of research grant sprograms. KACST, Riyadh. Saudi Arabia.

American Cancer Society (ACS). (2015). Cancer Facts and Figures. Atlanta, Georgia.

Amin, A., Alkaabi, A., Al-Falasi, S. and Daoud, S. A. (2005). Chemopreventive activities of *Trigonella foenum graecum* (Fenugreek) against breast cancer. *Cell Biol. Int.,* **29**: 687–694.

Amna, T., Puri, S. C., Verma, V., *et al*., (2006). Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin. *Can. J. Microbiol.* **52**: 189–196.

Ansari, S. H., and Ali, M. (2001). Norditerpenic ester and pentacyclic triterpenoids from the root bark of *Calotropis procera* (Ait.) R. Br. *Pharmazie*, **56**: 175–177.

Arechavaleta, M., Bacon, C. W., Hoveland, C. S. and Radcliffe, D. E. (1989). Effect of the tall fescue endophyte on plant response to environmental stress. *Agron. J.*, **81**: 83–90.

Argal, A. and Pathak, A. K. (2006). CNS activity of *Calotropis gigantea* roots. *J. Ethnopharmacol.*, **106**: 142–145.

Arnold, A. E. (2008). Endophytic Fungi: Hidden Components of Tropical Community Ecology. In: Schnitzer S, Carson W, eds. Tropical Forest Community Ecology. Malden, MA, USA: Blackwell Scientific, Inc.

Arnold, A. E. and Engelbrecht, B. M. J. (2007). Fungal endophytes double minimum leaf conductance in seedlings of a tropical tree. *J. Trop. Ecol*., **23**: 369–372.

Arnold, A. E. and Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots?. *Ecology*, **88**: 541–549.

Arnold, A. E., Maynard, Z., Gilbert, G. S., *et al*. (2000). Are tropical fungal endophytes hyperdiverse?. *Ecol. Lett*., **3**: 267–274.

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal. Biol*., **21**: (2–3) 51–66.

Atef, G. H., Elgamal, M. H. A., Morsy, N. A. M., *et al.* (1999). Two cardenolides from *Calotropis procera*. *J. Magn. Reson. Chem.*, **17**: 754–757.

B

Bacon, C. W. and White, J. F. J. (2000). Physiological adaptations in the evolution of endophytism in the Clavicipitaceae. In: Bacon, C.W., White, J. F. J., eds. Microbial Endophytes. New York, NY, USA: Marcel Dekker Inc.

Bagchi, B. and Banerjee, D. (2013). Diversity of fungal endophytes in *Bauhinia vahlii* (a lianas) from different regions of *Paschim medinipur* district of west Bengal. International. *J. Sci. Environ. Technol*., **2**(4): 748–756.

Balde, E. S., Andolfi, A., C., Bruy`ere, D., *et al*. (2010). Investigations of fungal secondary metabolites with potential anticancer activity. *J. Nat. Prod*., **73**(5): 969–971.

Banerjee, D. and Mahapatra, S. (2010). Diversity and screening for antimicrobial activity of endophytic fungi from *Alstonia scholaris* L. (Apocynaceae). *Acta. Microbiol. Imm. H.*, **57** (3): 211–219.

Barnett, H. L. and Hunter, B. B. (1998). Illustrated genera of imperfect fungi. APS Press: St. Paul, Minnesota.

Bashyal, B. P., Wijeratne, E. M., Faeth, S. H. and Gunatilaka, A. A. (2005). Globosumones A-C, cytotoxic orsellinic acid esters from the Sonoran desert endophytic fungus *Chaetomium globosum*. *J. Nat. Prod*., **68**: 724–728.

Buatong, J., Phongpaichit, S., Rukachaisirikul, V. and Sakayaroj, J. (2011). Antimicrobial activity of crude extracts from mangrove fungal endophytes. *World J. Microbiol. Biotechnol.*, **27**: 3005–3008.

C

Cai, Y. Z., Luo, Q., Sun, M. and Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*., **74**: 2157–2184.

Calvo, A. M., Wilson, R. A., Bok, J. W. and Keller, N. P. (2002). Relationship between secondary metabolism and fungal development. *Micrbiol. Mol. Biol. Rev*., **66**: 447–459.

Campanile, G, Ruscelli, A. and Luisi, N. (2007). Antagonistic activity of endophytic fungi towards *Diplodia corticola* assessed by in vitro and in planta tests. *Eur. J. Plant Pathol.*, **117**: 237–246.

Carmichael, J. W., Brycekendrick, W., Conners, I. L. and Lynne, S. (1980). Genera of Hyphomycetes. The University of Alberta Press: Edmonton, Alberta, Canada.

Carroll, G. C. and Carroll, F. E. (1978). Studies on the incidence of coniferous needle endophytes in the pacific northwest, USA. *Can. J. Botany*, **56**: 3034–3043.

Chandra, S. (2012). Endophytic fungi: novel sources of anticancer lead molecules. *Appl. Microbio. Biotechnol.,* **95**: 47–59.

Chareprasert, S., Piapukiew, J., Thienhirun, S. *et al*. (2006). Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr *World J. Microbiol. Biotechnol.*, **22**(5): 481–486.

Chatterjee, A. and Chandra, P. S. (1995). The treatise of Indian medicinal plants. Vol. 4, CSIR, New Delhi.

Chokpaiboon, S., Sommit, D., Teerawatananond T., *et al*. (2010). Cytotoxic nor-chamigrane and chamigrane endoperoxides from a basidiomycetous fungus. *J. Nat. Prod.*, **73**: 1005–1007.

Chomcheon, P., Wiyakrutta, S., Sriubolmas, N., *et al*. (2009). Aromatase inhibitory, radical scavenging and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus *Corynespora cassiicola* L36. *Phytochemistry,* **70**: 407–413.

Clay, K. (1990). Fungal endophytes of grasses. *Annu. Rev. Ecolo*. S*yst*., **21**: 275–297.

Cohen, S. D. (2006). Host selectivity and genetic variation of *Discula umbrinella* isolates from two oak species: Analyses of intergenic spacer region sequences of ribosomal DNA. *Microbol. Ecol.,* **52**: 463–469.

Compos, F. F., Rosa L. H., Cota B. B., *et al.* (2008). Leishmanicidal metabolites from *Cochliobolus* sp. an endophyte fungus isolated from *Piptadenia adiantoides* (Fabaceae). *PLOS. Negl. Trop. Dis.,* **2**: e348.

Cragg, G. M. and Snader, K. M. (1991). Taxol: the supply issue. *Cancer Cells*, **3**: 233–235.

Cui, C. B., Kakeya, H., Okada, G., *et al*. (1995). Tryprostatins A and B, novel mammalian cell cycle inhibitors produced by *Aspergillus fumigatus*. *J. Antibiot*., **48**: 1382–1384.

D

Dai, J., Krohn, K., Floerke, U., *et al*. (2006). Metabolites from the endophytic fungus *Nodulisporium* sp. from Juniperus cedre. *Eur. J. Org. Chem.,* **15**: 3498–3506.

Danielsen, S. and Jensen, D. F. (1999). Fungal endophytes from stalks of tropical maize and grasses: isolation, identification, and screening for antagonism against *Fusarium verticillioides* in maize stalks. *Biocontrol. Sci. Technol.,* **9**: 545–553.

Debbab, A. H., Aly, R.; Edrada-Ebel, A. *et al.* (2009). Bioactive metabolites from endophytic fungus *Stemphylium globuliferum* isolated from *Mentha pulegium*. *Biotechnol. Agron. Soc. Environ.*, **13**: 229–234.

Digrak, M. and Ozcelik, S. (2001). Determination of some fungal metabolites as influenced by temperature, time, pH, and sugars by bioassay method. *Turk. J. Biol*. **25**: 197–203.

Ding, G., Song, Y. C., Chen, J. R., *et al*. (2006).Chaetoglobosin U, a Cytochalasan alkaloid from endophytic *Chaetomium globosum* IFBE019. *J. Nat. Prod.*, **69**: 302–304.

Dugan, F. M., Sitton, J., Sullivan, R. F. and White, J. F. J. (2002). The neotyphodium endophyte of wild barley (*Hordeum brevisubulatum* subsp *violaceum*) grows and sporulates on leaf surfaces of the host. *Symbiosis*, **32**: 147–159.

E

Eamvijarn, A., Kijjoa, A., Bruy`ere, C., *et al.* (2012). Secondary metabolites from a culture of the fungus *Neosartorya pseudofischeri* and their *in vitro* cytostatic activity in human cancer cells. *Planta Med.*, **78**(16): 1767–1776.

Efloras. (2015) http://www.efloras.org/object_page.aspx?object_id=86625 (06/06/2015)

El Ghazali, G. E., (1997). The promising medicinal plants of the Sudan. National Council for Research, Khartoum University Press, Sudan.

Erasto, P., D., Grierson, S., *et al.* (2006). Bioactive sesquiterpene lactones from the leaves of *Vernonia amygdalina*. *J. Ethnopharmacol.,* **106**(1): 117–120.

Evidente, A., Kornienko, A., Cimmino, A., *et al*. (2014). Fungal metabolites with anticancer activity. *Nat. Prod. Rep*., **31**: 617–627.

F

Faeth, S. H. (2002). Are endophytic fungi defensive plant mutualists?. *Oikos,* 98: 25–36.

Fazli, F. R. Y. and Hardman, R. (1968). The spice fenugreek (*Trigonella foenum-graecum* L.). Its commercial varieties of seed as a source of diosgenin. *Trop. Sci.,* **10**: 66–78.

Finkel, T., N. and Holbrook, J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, **408**: 239–247.

Flickr. (2012). https://www.flickr.com/photos/68444386@N07/13293022783 (06/06/2015)

Fomum, F. U. (2004). *Vernonia amygdalina* Delile In: Grubben GJH, Denton OA (Editors). PROTA 2: Vegeta-bles/Légumes. [CD-Rom]. PROTA, Wageningen, Netherlands.

Fröhlich, J. and Hyde, K. D. (1999). Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic?. *Biodiver. Conserv.,* **8**: 977–1004.

Funabashi, Y., Horiguchi, T., Iinuma, S., *et al*. (1994), TAN-1496 A, C and E, diketopiperazine antibiotics with inhibitory activity against mammalian DNA topoisomerase I. *J. Antibiot*., **47**: 1202–1208.

G

Gamboa, M. A. and Bayman, P. (2001). Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). *Biotropica*, **33**: 352–360.

Gao, Y., Zhao, J., Zu, Y., *et al*. (2012). Antioxidant properties, superoxide dismutase and glutathione reductase activities in HepG2 cells with a fungal endophyte producing

apigenin from pigeon pea [*Cajanus cajan* (L.) Millsp]. *Food Res. Int.,* **49**(1): 147–152.

Gentile, A. M., Rossi, S., Cabral, D., *et al.* (1999). Origin divergence and phylogeny of Epichloë endophytes of native argentine grasses. *Mol. Phylogenet. Evol*., **35**: 196–208.

Gong, L. J., and Guo, S. X. (2009). Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. *Afr. J. Biotechnol*., **8**: 731–736.

Gresham, L. J., Ross, J. E. and Izevbigie. B. (2008). *Vernonia amygdalina*: Anticancer Activity, Authentication, and Adulteration Detection. *Int. J. Environ. Res. Public Health.*, **5**: 342–348.

Gu, W., Ge, H. M., Song, Y. C., *et al*. (2007). Cytotoxic benzo[j]fluoranthene metabolites from *Hypoxylon truncatum* IFB-18, an endophyte of *Artemisia annua. J. Nat. Prod.,* **70**: 114–117.

Guerin, D. (1898). Sur la presence d'un champignon dans l'lvraie. *J. Bot*., **12**: 230–238.

Gunatilaka A. A. L. (2006). Natural products from plant associated microorganisms: distribution, structural diversity, bioactivity and implications of their occurrence. *J. Nat. Prod.*, **69**: 509–526.

Guo, B., Li, H. and Zhang, L. (1998) Isolation of the fungus producing vinblastine. *J Yunnan Univ.* (Nat Sci Edit), **20**: 214–215.

Guo, B., Wang, Y., Sun, X. and Tang, K. (2008). Bioactive natural products from endophytes. *Appl. Biochem. Microbiol.,* **44**: 136–42.

Gupta, P. J. (2011). The efficacy of *Euphorbia prostrata* in early grades of symptomatic hemorrhoids - a pilot study. *Eur. Rev. Med. Pharmaco.*, **15**: 199–203

H

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: Curiosity, cause, or consequence?. *Lancet.*, **344**: 721–724.

Hansen, O. H. (1968). Ecology, physiology, and biochemistry of blue green algae. *Ann. Rev. Microbiol*., **22**: 47–57.

Holliday P, (1998). A Dictionary of Plant Pathology. Cambridge. University Press, Cambridge, UK.

Hormazabal, E., Schmeda-Hirschmann, G., Astudillo, L., *et al.* (2005). Metabolites from *Microsphaeropsis olivacea*, an endophytic fungus of *Pilgerodendron uviferum*. *Zeitschrift für Naturforschung*, **60**c: 11–21.

Hu, C. and Kitts, D. D. (2000). Studies on the antioxidant activity of Echinacea root extract. *J. Agr. Food Chem*., **48**: 1466–1472.

Huang, W., Cai, Y., Xing, J. and Corke, H. (2007). A Potential antioxidant resource: endophytic from medicinal plant. *Econ. Bot*., **61**(1): 14–30.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., *et al.* (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Divers*., **33**: 61–75.

I

Igile, G. O., Olezek, W., Jurzysata, M., *et al*, (1994). Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *J. Agr. Food Chem*., **42** (11): 2445 – 2448.

Isaka, M. Chinthanom, P. Boonruangprapa, T., *et al*., (2010). Eremophilane-type sesquiterpenes from the fungus *Xylaria* sp. BCC 21097. *J. Nat. Prod*., **73**: 683–687.

Isaka, M., Jaturapat, A., Rukseree, K., *et al*. (2001). Phomoxanthones A and B, novel xanthone dimers from the endophytic fungus *Phomopsis* species. *J. Nat. Prod*., **64**(8): 1015–1018.

Izevbigie, E. B. (2003). Discovery of water-soluble anticancer agents (edotides) from a vegetable found in Benin City, Nigeria. *Exp. Biol. Med*., **228**: 293–298.

J

Jain, V., Jain, P., Sharma, S. and Kakani, R. (1995). Hypolipidaemic activity of syndrex, a hydroalcoholic extract of fenugreek seeds Single blind clinical study. *Int. Med. J.*, **89**: 1–41.

Jisaka, M., Ohigashi, H., Takagaki, T., *et al.* (1992). Bitter steroid glucosides, vernoniosides A1, A2, A3 and related B1 from a possible medicinal plant *Vernonia amygdalina* used by wild chimpanzees. *Tetrahedron*, **48**: 625–632.

Jisaka, M., Ohigashi, H., Takegawa, K., *et al.* (1993a). Steroid gluccosides from *Vernonia amygdalina*, a possible chimpanzee medicinal plant. *Phytochemistry*., **34**: 409–413.

Jisaka, M., Ohigashi, H., Takegawa, K., *et al.* (1993b). Antinutritional and antimicrobial activities of bitter sesquiterpene lactones of *Vernonia amygdalina* a possible medicinal plant used by wild chimpanzee. *Biosci. Biotech. Biochem.*, **57**: 833–834.

K

Kanokmedhakul, K., Kanokmedhakul, S., Suwannatrai, R., *et al*. (2011). Bioactive meroterpenoids and alkaloids from the fungus *Eurotium chevalieri*. *Tetrahedron.* **67**: 5461–5468.

Kathamuthu, G. R., Shanmugam, S., Ramar, M., *et al*. (2014). Fungal taxol extracted from *Cladosporium oxysporum* Induces apoptosis in T47D human breast cancer cell Line. *Asian Pac. J. Cancer Prev.*, **15**(16): 6627–6632.

Keeley, S. C. and Jones, S. B. (1979). Distribution of pollen types in *Vernonia* (Vernonieae: Compositae). *Systematic Bot.*, **4**: 195–202.

Kenny, O., Smyth, T. J., Hewage, C. M. and Brunton, N. P. (2013). Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. *Food Chem*., **141**(4)15: 4295–4302.

Khan, R. (2007). Isolation, Identification and Cultivation of Endophytic Fungi from Medicinal plants for the production and Characterization of Bioactive Fungal Metabolites. Ph. D. Thesis University of Karachi.

Kharwar, R. N, Mishra, A., Gond, S. K., *et al*. (2011). Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Nat. Prod. Rep*., **28** (7): 1208–28.

Kimmons, C. A. Gwinn, K. D. and Bernard, E. C. (1990). Nematode reproduction onendophyte-infected and endophyte-free tall fescue. *Plant Dis*., **74**: 757–761.

Kirk, P. M., Cannon, P. F., Minter, D.W. and Stalpers, A. J. (2008). Dictionary of The Fungi. 10th edition. Cromwell Press. Trowbridge. UK.

Koshimizu, K., Ohigashi, H. and Huffman, M. A. (1994). Use of *Vernonia amygdalina* by wild chimpanzee: possible roles of its bitter and related constituents. *Physiol. Behav*., **56**: 1209–1216.

Krings, M., Taylor, T. N., Hass, H., *et al* (2007). Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *New Phytologist*., **174**: 648–657.

Kumari, K.G. N., Masilamani, S., Ganesh, M. R., *et al*. (2003). Zaluzanin D: a fungistatic sesquiterpene from *Vernonia arborea*. *Fitoterapia*, **74**: 479–482.

Kupchan, S. M., Hemingway, R. J., Karim, A. and Wermer, D. (1969). Tumour inhibitors XLVII vernodalin and vernomygdin, two new cytotoxic sesquiterpene lactones from *Vernonia amygdalina* Del. *J. Org. Chem.*, **34**: 3908–3911.

L

Lacap, D. C., Hyde, K. D. and Liew, E. C. Y. (2003). An evaluation of fungal 'morphotype' concept based on ribosomal DNA sequences. *Fungal Divers.,* **12**: 53–66.

Lallemand, B., Masi, M., Maddau, L., M., *et al.* (2012). Evaluation of *in vitro* anticancer activity of sphaeropsidins A-C, fungal rearranged pimarane diterpenes, and semisynthetic derivatives. *Phytochem. Lett.,* **5**: 770–775.

Larena, I., Salazar, O., Gonzalez V., *et al*. (1999). Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *J. Biotechnol*., **75**: 187–194.

Lee, J. C. (1995). Isolation and structure determination of bioactive compounds from endophytic and insect-associated fungi (Ph. D. Thesis), Cornell University, New York.

Lee, J. C., Strobel, G. A., Lobkovsky, E. and Clardy, J. C. (1996). Torreyanic acid: a selectively cytotoxic quinone dimer from the endophytic fungus *Pestalotiopsis microspora*. *J. Org. Chem*., **61**: 3232–3233.

Lee, K. (2010). The Effects of Endophytic *Fusarium verticillioides* on the Intractions of Maize and Its Fungal Pathogen *Ustilago maydis*. Ph. D. Thesis. University of Minn Eesota.

Larena, I., Salazar, O., Gonzalez, V., *et al*. (1999). Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *J. Biotech*. **75**: 187-194.

Li, E. W., Jiang, L. H., Guo, H., *et al.* (2008). Pestalachlorides A-C, antifungal metabolites from the plant endophytic fungus *Pestalotiopsis adusta. Bioorgan. Med. Chem.,* **16**: 7894–7899.

Liu, L., Liu, S., Niu, S., *et al*. (2009). Isoprenylated chromone derivatives from the plant endophytic fungus *Pestalotiopsis fici*. *J. Nat. Prod*., **72**: 1482–1486.

Llorens, A., Matco, R., Hinojo, M. J., *et al.* (2004). Influence of the interactions among ecological variables in the characterization of Zearalenone producing isolates of *Fusarium* spp. *Syst. Appl. Microbiol*., **27**: 253–260.

Lodge, D. J., Fisher, P. J. and Sutton, B. C. (1996). Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia*, **88**: 733–738.

Lu, H., Zou, W. X., Meng, J. C. *et al*. (2000) New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. *Plant Sci*., **151**: 67–73.

M

Malinowski, D. P. and Belesky, D. P. (2000). Adaptations of endophtye-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci*., **40**: 923–940.

Márquez, L. M., Redman, R. S., Rodriguez, R. J. and Roossinck, M. J. (2007). A virus in a fungus in a plant – three way symbiosis required for thermal tolerance. *Science*, **315**: 513–515.

Merlin, J. N., Christhudas, I. V. S. N., Kumar, P. P. and Agastian, P. (2013). Optimization of growth and bioactive metabolite production: *Fusarium solani. Asian J. Pharm. Clin. Res.*, **6**(3): 98–103.

Mosango, D. M., (2008). *Euphorbia thymifolia* L. [Internet] Record from PROTA4U. Schmelzer, G. H. & Gurib-Fakim, A. (Editors). PROTA (Plant Resources of Tropical Africa/Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands. Accessed 20 May 2015.

Moy, M., Belanger, F., Duncan, R., *et al.* (2000). Identification of epiphyllous mycelial nets on leaves of grasses infected by Clavicipitaceous endophytes. *Symbiosis*, **28**: 291–302.

N

Naik, B. S., Shashikala, J. and Krishnamurthy, Y. L. (2009). Study on the diversity of endophytic communities from rice (*Oryza sativa* L.) and their antagonistic activities in vitro. *Microbiol. Res.*, **164**: 290–296.

Narisawa, K., Kawamata, H., Currah, R. S. and Hashiba, T. (2002). Suppression of *Verticillium* wilt in eggplant by some fungal root endophytes. *Eur. J. Plant Pathol*., **108**: 103–109.

Nikoletta, V (2014) http://antalvali.com/gorogszena-trigonella-foenum-graecum-l-a-gyogynovenyek-kiralynoje.html (28/05/2015)

O

Ohigashi, H., Jisaka, M., Takagaki, T., *et al*. (1991). Bitter principle and a related steroid glucoside from *Vernonia amygdalina* a possible medicinal plant for wild chimpanzees. *Agric. Biol. Chem*., **55**: 1201–1204.

P

Patterson, C. G., Potter, D. A. and Fanin, F. F. (1991). Feeding deterrence of alkaloids from endophyte-infected grasses to Japanese beetle grubs. *Entomologia Experimentalis et Applicata*., **61**: 285–289.

Paulus, B., Kanowski, J., Gadek, P. and Hyde, K. D. (2006). Diversity and distrubtion of saprobic microfungi in leaf litter of an Austrailian tropical rainforest. *Mycol. Res*., **110**: 1441–1454.

Petrini, O. (1986). Taxonomy of Endophytic Fungi of Aerial Plant tissues. In Microbiology of the Phyllosphere (ed.N.j.Fokkema. Van den Heuvel), Cambridge. University Press: Cambridge.

Petrini, O. (1991). Fungal Endophytes of Tree Leaves. In Andrews, J. A., Hirano, S. S., eds. Microbial Ecology of Leaves. Springer-Verlag: New York, USA.

Petrini, O. and Dreyfuss, M. (1981). Endophytische Pilze in epiphytischen Araceae, Bromeliaceae and Orchidaceae. *Sydowia*, **34**: 135–145.

Pigares, E. and Narendhirakannan, R. T. (2013). Evaluation of *in vivo* anti-oxidant activity of *Euphorbia prostrata* o DMBA induced skin cancer. Souvernir of 3rd International Science Congress.Coimbatora, India, 8th-9th Dec.

Pirttila, A. M., Laukkanen, H. and Hohtola, A. (2002). Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes?. *Planta*, **214**: 848–852.

Porras-Alfaro, A. and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annu. Rev. Phytopathol*., **49**: 291–315.

Priti, V., Ramesha, B. T., Singh, S., *et al*. (2009). How promising are endophytic fungi as alternative sources of plant secondary metabolites?. *Curr. Sci*., **97**: 477–78.

Promputtha, I., Jeewon, R., Lumyong, S., *et al*. (2005). Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Divers*, **20**: 167–186.

Puri, S. C., Nazir, A., Chawla, R., *et al*. (2006). The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *J. Biotechnol.* **122**: 494–510.

Puri, S. G., Verma, V., Amna, T., *et al*. (2005). An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *J. Nat. Prod.,* **68**(12): 1717–1719.

R

Radi´ca, N. and ˇStrukeljc, B. (2012). Endophytic fungi−The treasure chest of antibacterial substances. *Phytomedicine* **19**: 1270– 1284.

Radji, M., Sumiati, A., Rachmayani, R. and Elya, B. (2011). Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity. *Afr. J. Biotechnol.*, **10**: 103–107.

Ranjard, L., Lejon, D. P. H., Mougel, C., *et al.* (2003). Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities, *Environ. Microbiol.*, **5**: 1111–1120.

Raviraja, N. S. (2005). Fungal endophytes in five medicinal plant species from Kudremukh Range, Western Ghats of India. *J. Basic Microbiol*., **45**: 230–235.

Redman, R. S., Sheehan, K. B., Stout, R. G., *et al* (2002). Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science*, **298**: 1581.

Rehman, S., Shawl, A. S., Kour, A., *et al*. (2008). An endophytic *Neurospora* sp. from *Nothapodytes foetida* producing camptothecin. *Appl. Biochem. Microbiol*., **44**(2): 203–209.

Reich, E and Schibli, A. (2007). Hight-performance thin-layer chromatography for the analysis of medicinal plants. Thieme Medical Publishers, Inc. New York.

Revelant, G., Huber-Villaume, S., Dunand, S., *et al*. (2015). Synthesis and biological evaluation of novel 2-heteroarylimino-1,3-thiazolidin-4-ones as potential anti-tumor agents. *Eur. J. Med. Chem.*, **94**: 102–112.

Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infec. Dis*., **197**: 1079–1081.

Riedell, W. E., Kieckhefer, R. E., Petroski, R. J. and Powell, R. G. (1991). Naturally occurring and synthetic loline alkaloid derivatives: insect feeding behavior modification and toxicity. *J. Entomol. Sci*., **26**: 122–129.

Rodrigues, K. F. (1994). The foliar fungal endophytes of the Amazonian palm Euterpe oleracea. *Mycologia*, **86**: 376–385.

Rodriguez, R. J., Henson, J., Van Volkenburgh, E., *et al*. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *I. S. M. E.*, **2**: 404– 416.

Rowan, D. D. and Gaynor, D. L. (1986). Isolation of feeding deterrent against stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. *J. Chem. Ecol*., **12**: 647–658.

Rwangabo, P. C., Laekman, G., Claeys, M., *et al.* (1986). Phytochemical and pharmacological investigation of the active fraction from the flowers of *Vernonia amygdalina* Del. *Planta Med*., **5**: 431.

S

Sain, M., and Sharma, V. (2013). *Catharanthus roseus* (an anti-cancerous drug yielding plant) - a review of potential therapeutic properties. *Int. J. Pure Applied Biosci*., **1**(6): 139–142.

Samuels, G. J., Pardo-Schultheiss, R., Hebbar, K. P., *et al.* (2000). *Trichoderma stromaticum* sp. nov., a parasite of the cacao witches broom pathogen. *Mycol. Res.,* **104**: 760–764.

Santamaría, J. and Bayman, P. (005). Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). *Microbial Ecol*., **50**: 1–8.

Schaechter, M. (2011). Eukaryotic Microbes. Academic Press. E-book.

Schmelzer, G. H., (2007). *Catharanthus roseus* (L.) G. Don. In: Schmelzer, G. H. & Gurib-Fakim, A. (Editors). PROTA (Plant Resources of Tropical Africa/ Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands. Accessed 6 mai 2015.

Schulz, B., Guske, S., Dammann, U. and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis*, **25**: 213–227.

Schulz, B., Rommert, A. K., Dammann, U., *et al.* (1999). The endophyte-host interaction: a balanced antagonism?. *Mycol. Res*., **10**: 1275–1283.

Schulz, B., Sucker, J., Aust, H., *et al*. (1995). Biologically active secondary metabolites of endophytic *Pezicula* species. *Mycol Res*., **99**: 1007–1015.

Schulz, B., Boyle, C., Draeger, S. and Rommert, A. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol. Res.*, **106**(9): 996–1004.

Selvanathan, S., Indrakumar, I. and Johnpaul, M. (2011). Biodiversity of the endophytic fungi isolated from *Calotropis gigantean* (L) R. BR. Rec. Res. Sci. Technol., **3**(4): 94–100.

Sharma, A. K. (2011). Pharmacognostical aspects of *Calotropis procera* (Ait) R. Br. *Int. J. Pharma. Bio. Sci.*, **2**(3): 480–8.

Sharma, G. P. and Sharma, P. V. (1972). Experimental studies on antispasmodic and bronchodilator actions of Dugdhika (*E. thymifolia* Linn and *E, prostrata* W. Ait). *J. Research in Indian Medicine*, **7**: 24–28.

Silva, E. M., Souza, J. N. S., Rogez, H., *et al.* (2007). Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. *Food Chem.,* **101**(3): 1012–1018.

Song, Y. C., Huang, W. Y., Sun, C. *et al*, (2005). Characterization of graphislactone A as the antioxidant and free radical-scavenging substance from the culture of *Cephalosporium* sp. IFB-E001, an endophytic fungus in *Trachelospermum jasminoides*. *Biol. Pharmac. Bull*., **28**: 506–509.

Souza J. J., Vieira I. J. C., Rodrigues-Filho E. and Braz-Filho R. (2011). Terpenoids from endophytic fungi. *Molecules*, **16**: 10604–10618.

Souza, A. Q. L., Souza, A. D. L., Filho, S. A., *et al*. (2004) Atividade antimicrobiana de fungos endofíticos isolados de plantas tóxicas da Amazônia: Palicourea longiflora (aubl.) riche Strychnos cogensbentham. *Acta. Amaz*., **34**:185–195.

Steenkamp, V. (2003). Traditional herbal remedies used by South African women for gynaecological complaints. *J. Ethnopharmacol,*, 86: 97–108.

Stierle, A, Strobel, G. A. and Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*. an endophytic fungus of Pacific Yew. *Science*, **260**: 214–16.

Stierle, A. A. Stierle, D. B and Bugni, T., (1999). Sequoiatones A and B: Novel antitumor metabolites isolated from a redwood endophyte. *J. Org. Chem.*, **64**: 5479–5484.

Stone, J. K. (1988). Fine structure of latent infection by Rhabdocline parkeri on Douglas fir, with observation on uninfected epidermal cells. *Can. J. Bot*., **66**: 45–54.

Strobel G. A, Hess, W. M., Li, J. Y., *et al*. (1997). *Pestalotiopsis guepinii*, a taxol-producing endophyte of the wollemi pine, *Wollemia nobilis*. *Aust. J. Bot.*, **45**(6): 1073–1082.

Strobel, G. A. Torzynski, R. and Bollon, A. (1997). *Acremonium* sp. a leucinostatin A producing endophyte of European yew (*Taxus baccata*). *Plant Sci.*, **128**: 97–108.

Strobel, G. A. and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev*., **67**: 491–502.

Strobel, G., Daisy, B., Castillo, U. and Harper, J. (2004). Natural products from endophytic microorganisms. *J. Nat. Prod*., **67**: 257–268.

Sultana, B., Anwar, F. and Przybylski, R. (2007). Antioxidant potential of corncob extracts for stabilization of corn oil subjected to microwave heating. *Food Chem.*, **104**: 997–1005.

Surveswaran, S., Cai, Y. Z., Corke, H. and Sun, M. (2007). Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chem*., 102: 938–995.

T

Tadych, M., Bergen, M., Dugan, F. M. and White, J. F. (2007). Evaluation of the potential role of water in the spread of conidia of the *Neotyphodium* endophyte of *Poa ampla*. *Mycol. Res*., **111**: 466–472.

Tan, R. X. and Zou, W. X. (2001) Endophytes: a Rich Source of Functional Metabolites. *Nat. Prod. Rep.*, **18**: 448–459.

Tona, L., Cimanga, R. K., Mesia, K., *et al*. (2004). In vitro antiplasmodial activity of extracts and fractions of seven medicinal plants used in the Democratic Republic of Congo. *J. Ethnopharmacol*., **93**: 27–32.

Toyang, N. J. and Verpoorte, R. (2013). A review of the medicinal potentials of plants of the genus *Vernonia* (Asteraceae). *J. Ethnopharmacol.*, **146**(3): 681–723.

Tudzynski, B. and Sharon, A. (2002). Biosynthesis, Biological Role and Application of fungal phyto-hormones. In: Osiewacz H D, ed. The Mycota X. Industrial Applications. Berlin, Germany: Springer-Verlag.

Tzanova, T., Gerova, M., Petrov, O., *et al.* (2009). Synthesis and antioxidant potential of novel synthetic benzophenone analogues. *Eur. J. Med. Chem*. **44**(6): 2724–30.

U

Udensi, E. A., Ijeh, I. I. and Ogbonna, U. (2002). Effect of traditional processing on the phytochemical and nutrient composition of some local Nigerian leafy vegetables. *J. Sci. Technol*., **8**: 37–40.

USDA. (2009). http://plants.usda.gov/core/profile?symbol=Caro14

V

Valentao, P., Fernandes, E., Carvalho, F., *et al.* (2002). Antioxidative properties of cardoon (*Cynara cardunculus* L.) infusion against superoxide radical. Hydroxyl radical, and hypochlorous acid. *J. Agr. Food Chem*., **50**: 4989–4993.

Vogl, A. (1898). Mehl und die anderen mehlprodukte der cerealien und leguminosen. Zeitschrift Nahrungsmittle Untersuchung Hyg. *Warenkunde*, **12**: 25–29.

Vu, T., Hauschild, R. and Sikora, R. A. (2006). *Fusarium oxysporum* endophytes induced systemic resistance against *Radopholus similis* on banana. *Nematol.*, **8**: 847–852.

W

Wagenaar, M. M. and Clardy, J. (2001). Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an endangered mint. *J. Nat. Prod*., **64**: 1006–1009.

Wall, M. E., Wani, M. C., Manikumar, G., *et al*. (1998). Plant antimutagenic a gents, flavonoids. *J. Nat. Prod*., **51**: 1084–1089.

Wang, S., Li, X. M., Teuscher, F. *et al.* (2006). Chaetopyranin, a benzaldehyde derivative, and other related metabolites from *Chaetomium globosum*, an endophytic fungus derived from the marine red alga *Polysiphonia urceolata*. *J. Nat. Prod.,* **69**: 1622–1625.

Watanabe, T. (2002). Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species. 2nd ed. CRC Press LLC. Washington, D.C.

Watanabe, T. (2010). Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species. 3ed ed. CRC Press LLC. Washington, D.C.

Webster, J. (1980). Introduction to Fungi. 2nd ed. Cambridge University Press. Cambridge.

Wedge, D. E., Galindo, J. C. G. and Macias, F. A. (2000). Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. *Phytochemistry*, **53**: 747–757.

White, J. F. J. (1987). The widespread distribution of endophytes in the Poaceae. *Plant Dis.*, **71**: 340–342.

White, J. F. J., Martin, T. I., and Cabral, D. (1996). Endophyte-host associations in grasses. III. Conidia formation by *Acremonium* endophytes in thephylloplanes of *Agrostis hiemalis* and *Poa rigidifolia*. *Mycologia*, **88**:174–178.

Wijeratne, E. M. K., Paranagama, P. A., Burns, A. M., *et al.* (2008) Sesquiterpene quinones and related metabolites from *Phyllosticta spinarum*, a fungal strain endophytic in platycladus orientalis of the sonoran desert. *J. Nat. Prod*., **71**: 218 – 222.

Wikipedia. http://en.wikipedia.org/wiki/Geography_of_Sudan#/media/File:Sudan_sat.jpg (01/08/2011).

Wiyakrutta, S., Sriubolmas, N., Panphut, W., *et al.* (2004). Endophytic Fungi with Anti-microbial, Anti-cancer and Anti-malarial Activities Isolated from Thai Medicinal Plants. *World J. Microbiol. Biotechnol*., **20**: 265–272.

Y

Yadav, M., Yadav, A. and Yadav, J. P. (2014) In vitro antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pac. J. Trop. Med.*, **7**: S256–S261.

Yagi, S. (2011). Phytochemical and biological of study of Sudanese Plants: *Hydnora johannis* Beccari (Hydnoraceae) and *Citrullus lanatus* (Thunb.) Matsum. Et Nakai var. citroides (Bailey) Mansf. (Cucurbitaceae). Ph. D Thesis. Université de Lorraine.

Yang, X., Strobel, G. A., Stierle, A., *et al.* (1994). A fungal endophyte - tree relationship - *Phoma multirostrata* in *Taxus wallachiana*. *Plant Sci.*, **102**:1–9.

Yoshida, T., Namba, O., Chen, L., *et al.* (1990). Ellagitannin monomers and oligomers from *Euphorbia prostrata* Ait. and oligomers from *Loropetalum chinense* Oliv. *Chemical and Pharmaceutical Bulletin*, **38**(12): 3296–3302.

Yu, H., Zhang, L., Li, L., *et al*. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiol. Res.,***165**(6): 437–449.

Yue, C., Miller C. J., White, J. F. J. and Richardson, M. (2000). Isolation and characterization of fungal inhibitors from *Epichloë festucae*. *J. Agr. Food Chem*., **48**: 4687–4692.

Z

Zhan, J., Burns, A. M., Liu, M. X., *et al*. (2007). Search for cell motility and angiogenesis inhibitors with potential anticancer activity: beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum*. *J. Nat. Prod*., **70**(2): 227–232.

Zhang, J. Y., Tao, L. Y., Liang, Y. J., *et al*. (2010). Anthracenedione derivatives as anticancer agents isolated from secondary metabolites of the mangrove endophytic fungi. *Mar. Drugs*., **8**: 1469–1481.

Zhang, L. B., Gou, L. H. and Zeng, S. V. (2000). Preliminary study on the isolation of endophytic fungus of *Catharanthus roseus* and its fermentation to produce product of therapeutic value. *Chin. Tradit. Herbal Drugs*., **11**: 805–807.

Zhang, P., Zhou, P. P. and Yu, L. J. (2009). An endophytic taxol-producing fungus from *Taxus media*, *Cladosporium cladosporioides* MD2. *Curr. Microbiol*., **59**(3):227–232.

Zhao, J. T. D., Ma, D. H., Luo, M., *et al.* (2014). In vitro antioxidant activities and antioxidant enzyme activities in HepG2 cells and main active compounds of endophytic fungus from pigeon pea [*Cajanus cajan* (L.) Millsp.]. *Food Res. Int*., **56**(0): 243-251.

Zhou, D. Q. and Hyde, K. D. (2001). Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. *Mycol. Res*., **105**: 1449–1457.

Annexes

Annexes

I. Fungal media.

II. Calibration plot of total phenolic content.

III. NMR spectra of isolated compounds.

- **AFB**

IV. Scientific Production.

- **Poster**
- **International publication**

I. Fungal media

Corn Meal Agar (CMA)

Maize meal	30 g
Agar	20 g
water	1L

Potato Dextrose Agar (PDA)

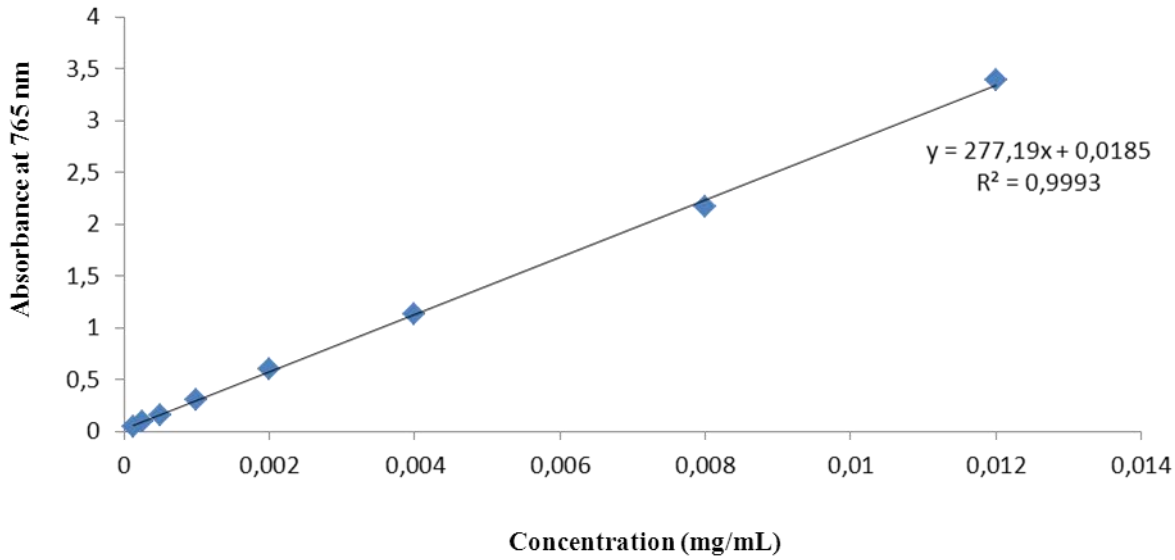
Potatoes	200 g
Dextrose	15 g
Agar	20 g
water	1L

Potato Sucrose Agar (PSA)

Powdered potato	5 g
Sucrose	15 g
Agar	20 g
water	1L

II. Calibration plot of total phenolic content

The standard curve of gallic acid



III. NMR spectra of isolated compound

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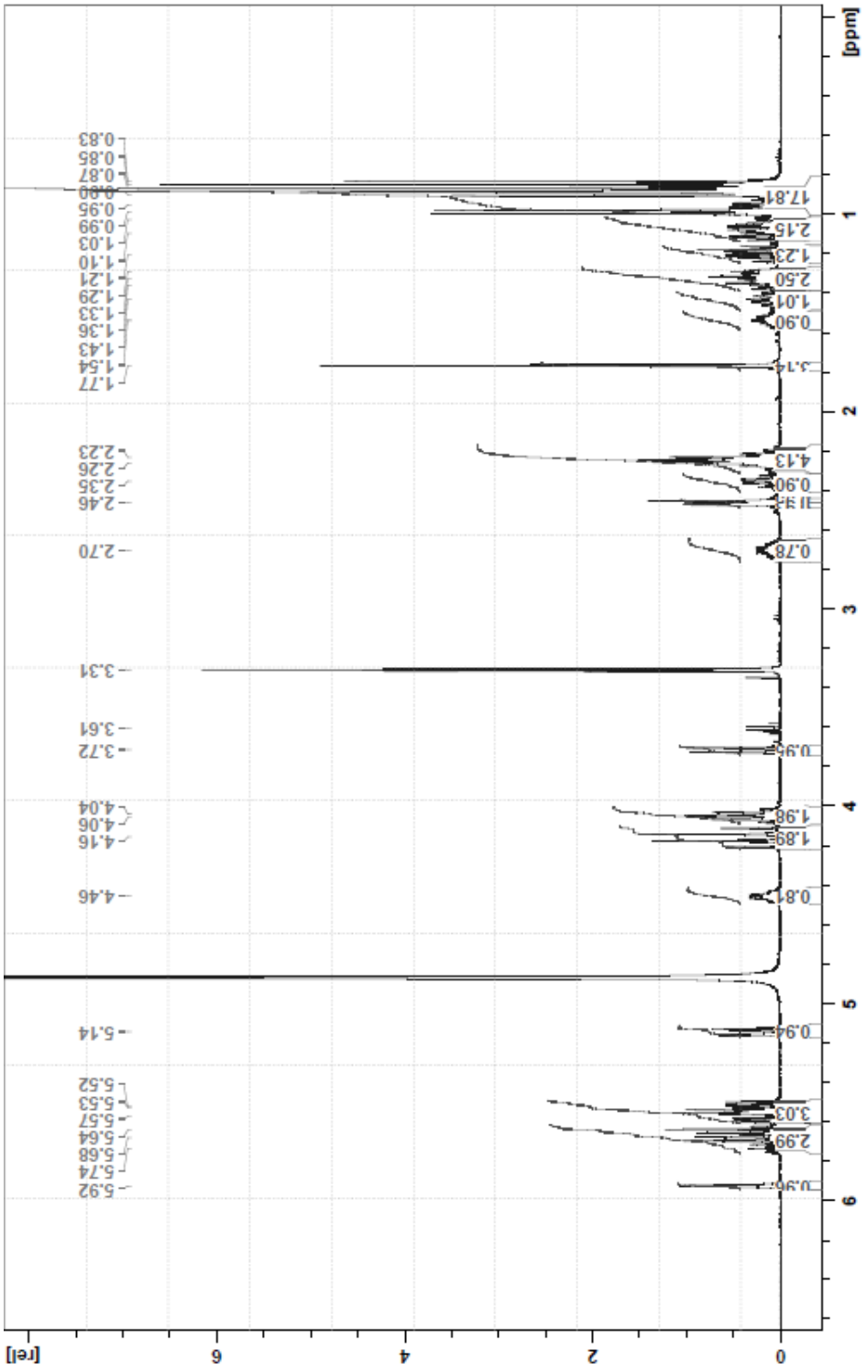

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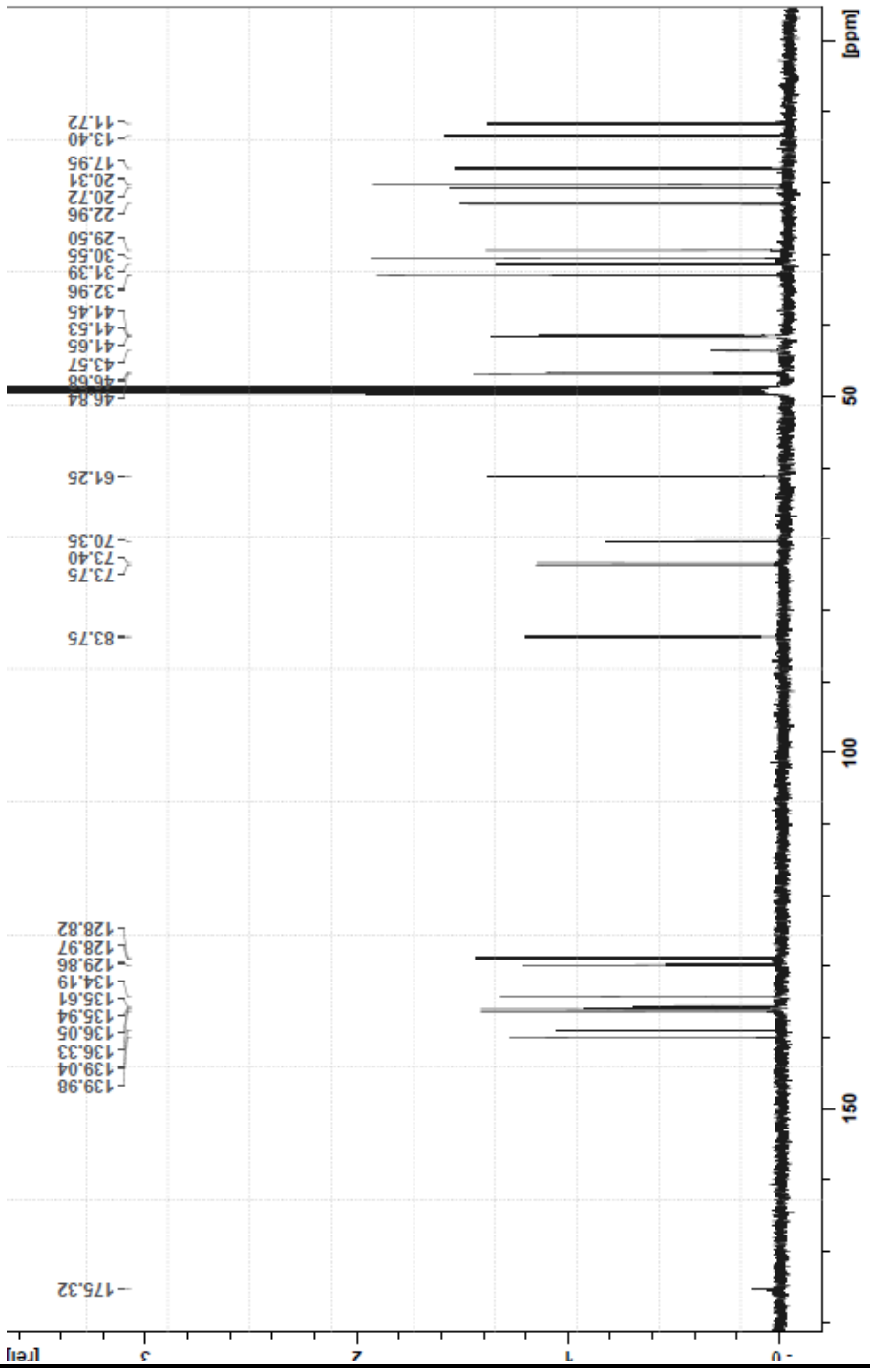

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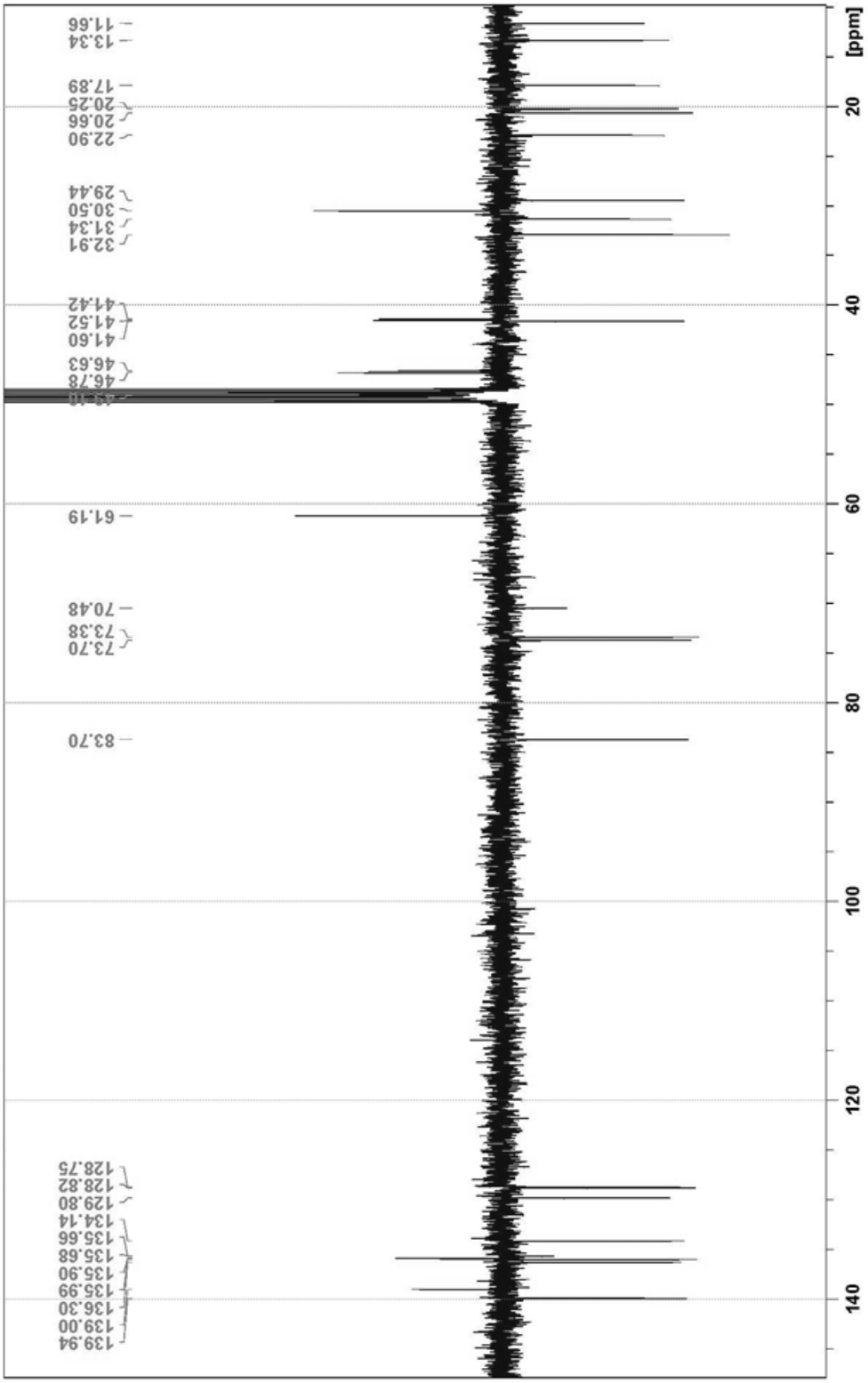

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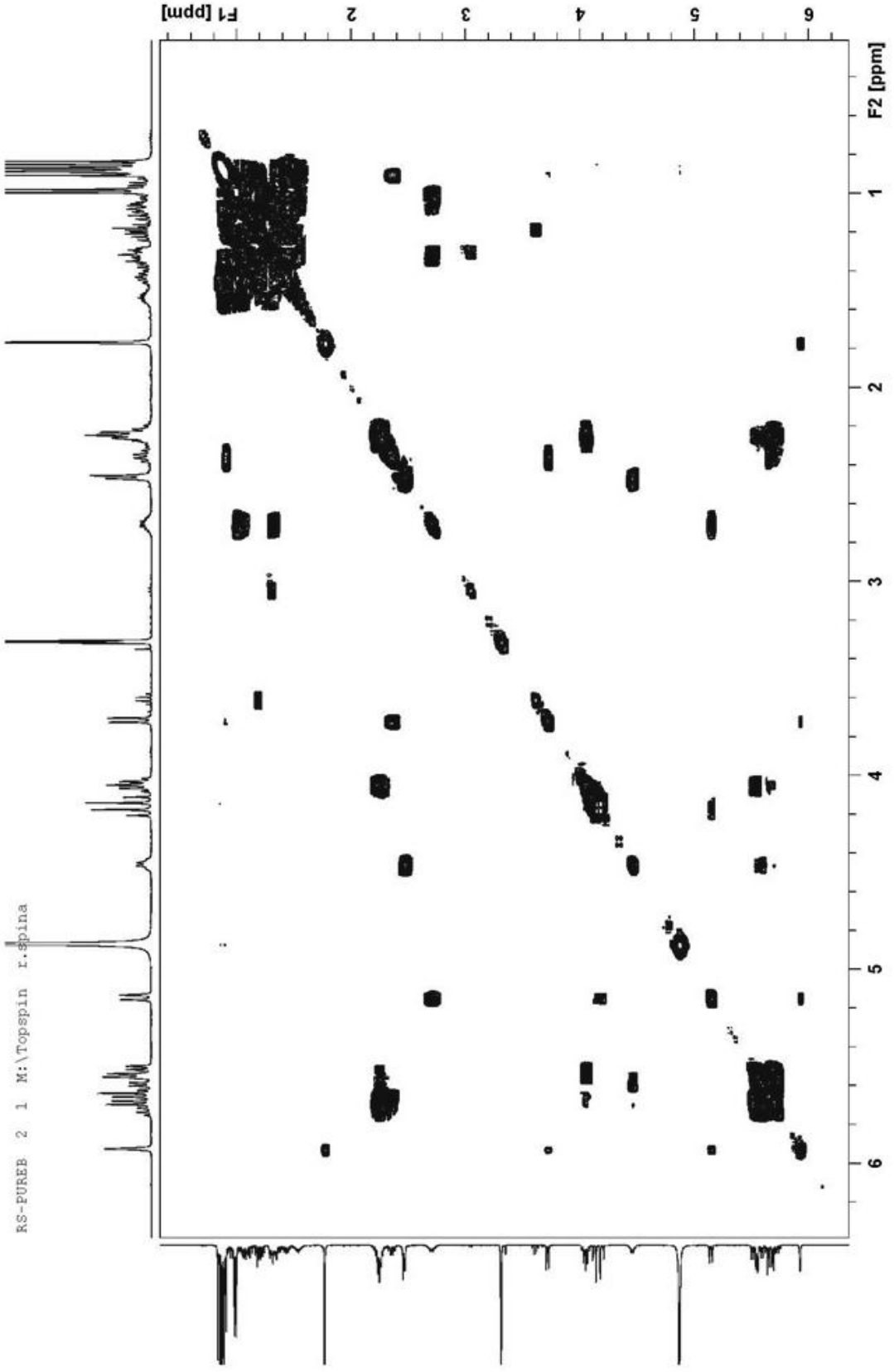

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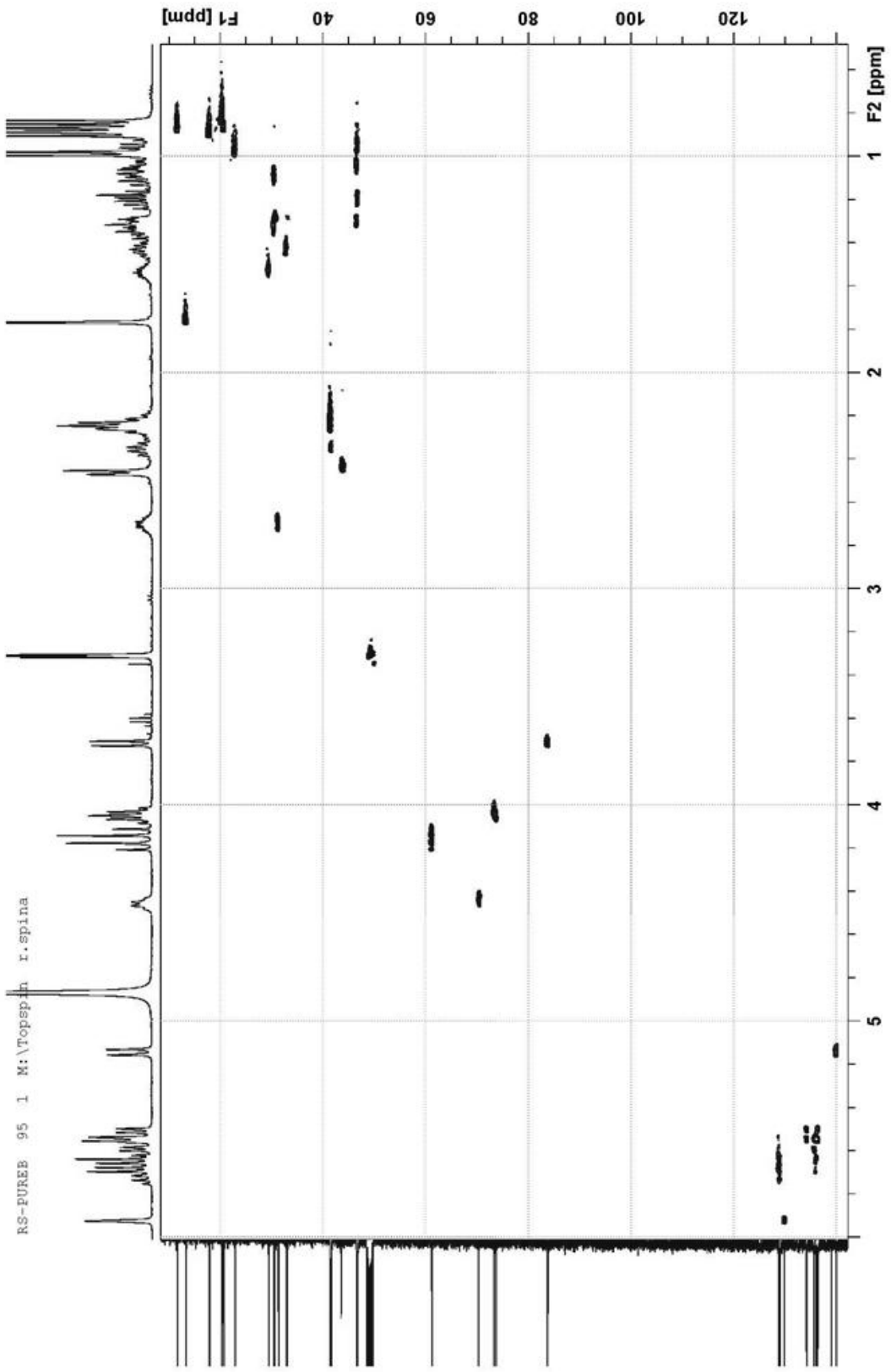

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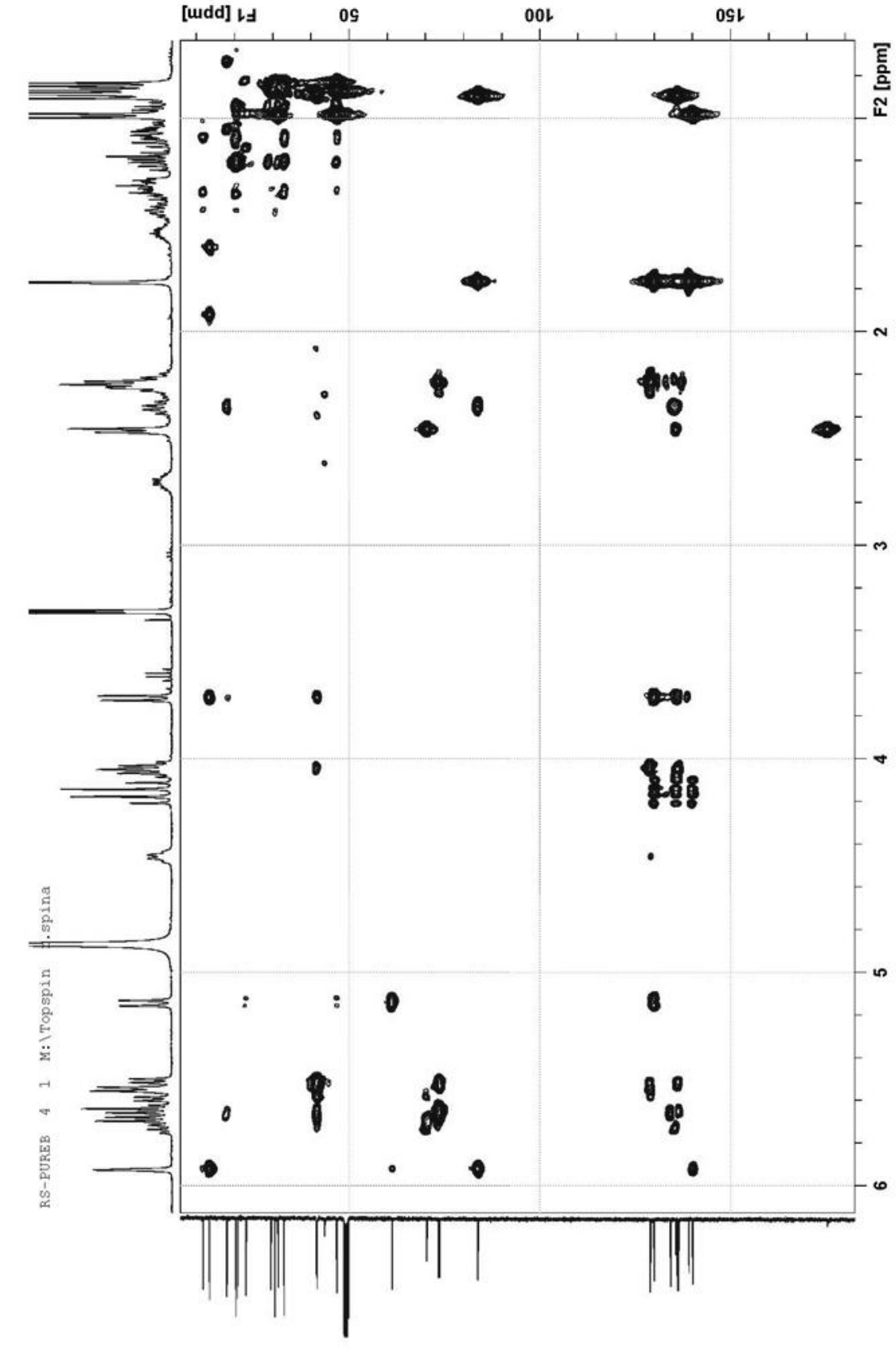
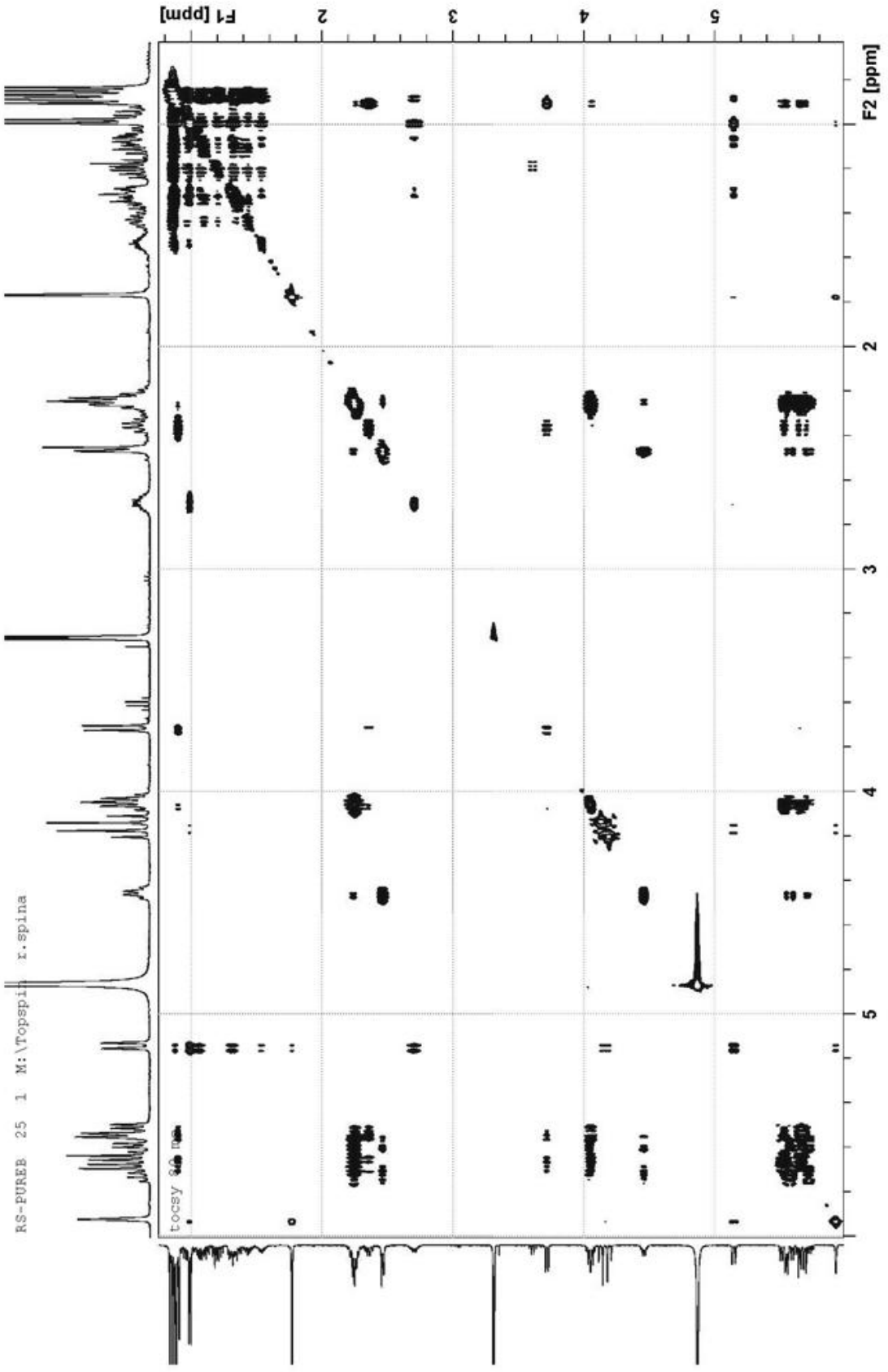


AFB 400 MHz: TOCSY



IV. Scientific Production

a. Poster

- Potential antioxidant resource of endophytic fungi from some Sudanese medicinal plants. Phytoday 2015 Congrès. Le 20 avril 2015 -Faculté de Pharmacie, Université de Strasbourg.

b. Publication

- Khiralla, A. Mohamed, I., Thomas, J, Mignard, B., Spina, R., Yagi, S., Laurain-Mattar, D. (2015). **A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants**. Asian Pacific Journal of Tropical Medicine. Asian Pacific Journal of Tropical Medicine, 8(9): 701–704.

- Afra Khiralla, Ietidal Mohamed, Tzvetomira Tzanova, Hervé Schohn, Sophie Slezack-Deschaumes, Alain Hehn, Philippe Andre; Gaëlle Carre, Rosella Spina, Denyse Bagrel, Annelise Lobstein, Sakina Yagi, Dominique Laurain-Mattar**. Promising cytotoxic and antibacterial potentialities of endophytic fungi associated with medicinal plants from Sudan.** *In preparation.*

C. Conference paper

- Biological Activity of Endophytic Fungi Assciated with Medicinal Plants From Sudan. The 16th NAPRECA Symposium on Natural Products, 2015. Arusha, Tanzania.

Potential antioxidant resource of endophytic fungi from some Sudanese medicinal plants

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Introduction

Endophytes are organisms that colonize internal plant tissues without causing apparent harm to their host. Endophytic fungi from medicinal plants are a potential antioxidant resource [1]. *Vernonia amygdalina* Del. (Asteraceae), *Calotropis procera* Ait. (Asclepiadaceae), *Catharanthus roseus* L.(Apocynaceae), *Euphorbia prostrata* Ait. (Euphorbiaceae), and *Trigonella foenum-graecum* L. (Fabaceae) are medicinal plants that have several uses in Sudanese folk medicine (Fig.1). Their extracts have shown some biological activities including antiproliferative activity and antioxidant potential [2].

Fig.1. A. *Catharanthus roseus* L. (Apocynaceae) B. *Calotropis procera* Ait. (Asclepiadaceae) C. *Euphorbia prostrata* Ait. (Euphorbiaceae) D. *Vernonia amygdalina* Del. (Asteraceae) E. *Trigonella foenum-graecum* L. (Fabaceae).

Objectives

Evaluation of the total phenolic content and antioxidant activity of ethyl acetate extracts of 5 medicinal plants and their endophytic fungi.

Materials and methods

Fresh leaves and stems of the five plants were collected from Khartoum (15°38 N 32°32 E). Endophytic fungi were isloated after surface sterilization. Ethyl acetate extraction was done for each fungal strains from culture on Potato Dextrose Agar, alongside ethyl acetate extract was performed for each plant. Total phenolic content (TPC) of ethyl acetate extracts of endophytic fungi and their host plants was tested by classical Folin-Ciocalteu colorimetric method, also total antioxidant capacity (TAC) was estimated using (1,1,-diphenyl-2-picrylhydrazl) DPPH free radical scavenging in vitro method.

Results

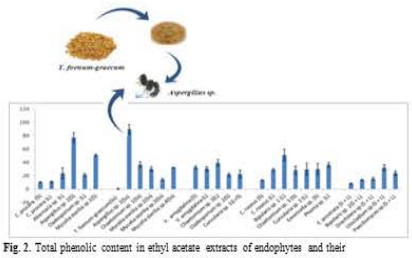

Fig. 2. Total phenolic content in ethyl acetate extracts of endophytes and their host plants. (S) Stem, (L) leaves (Sa) Seeds and (S+L) stem and leaves. Values are means ± SD of three determinations.

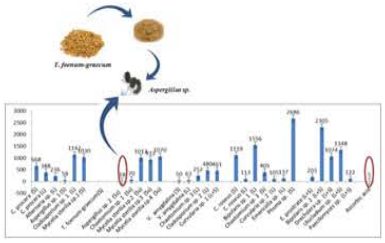

Fig. 3. IC50 values of 21 endophytic fungi and their host plants by DPPH radical scavenging assay. Values are means ± SD of three analyses.

Conclusion and perspectives

The present study revealed that some endophytic fungi of Sudanese medicinal plants could be a potential source of novel natural antioxidant compounds. *Aspergillus* sp. endophyte from *T. foenum-graecum* revealed significant antioxidant activity (IC50: 18 ± 0.1 µg/mL) and it contained the highest quantity of phenolic compounds (89.9±7.1 mg GAE/g), this fungus strain is recommended for further investigations.

References

[1] Huang W, Cai Y, Xing J, Corke H. A Potential antioxidant resource: endophytic from medicinal plant. Econ Bot 2007; 61(1): 14-30.

[2] El Ghazali GE. The promising medicinal plants of the Sudan. National Council for Research, Khartoum University Press, Sudan.1997.

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A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants

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ABSTRACT

Objective: To evaluate the total phenolic content and total antioxidant capacity of ethyl acetate extracts of 21 endophytic fungi isolated from five Sudanese medicinal plants: *Calotropis procera*, *Catharanthus roseus*, *Euphorbia prostrate*, *Vernonia amygdalina* and *Trigonella foenum-graecum*.

Methods: Crude extracts of endophytic fungi and their host plants were tested by classical Folin–Ciocalteu colorimetric method to determine the total phenolic content, also total antioxidant capacity was estimated using 1,1-diphenyl-2-picrylhydrazyl free radical scavenging *in vitro* method.

Results: Among the endophytes, endophytic fungus *Aspergillus* sp. from *Trigonella foenum-graecum* seeds demonstrated the highest both total phenolic content in term of gallic acid equivalent [(89.9 ± 7.1) mg GAE/g] and antioxidant activity for 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay [IC_{50}: (18.0 ± 0.1) μg/mL]. A high positive linear correlation (R^2 = 0.999 1) was found between total antioxidant capacity and total phenolic content of endophytic fungi isolated from *Vernonia amygdalina*.

Conclusions: The present study revealed that some endophytic fungi from the five Sudanese medicinal plants could be a potential source of novel natural antioxidant compounds.

1. Introduction

Endophytes are organisms that colonize internal plant tissues without causing apparent harm to their host [1]. Endophytic fungi from medicinal plants are a potential antioxidant resource [2]. *Vernonia amygdalina* (*V. amygdalina*) Del. (Asteraceae), *Calotropis procera* (*C. procera*) Ait. (Asclepiadaceae), *Catharanthus roseus* (*C. roseus*) L. (Apocynaceae), *Euphorbia prostrata* (*E. prostrata*) Ait. (Euphorbiaceae), and *Trigonella foenum-graecum* (*T. foenum-graecum*) L. (Fabaceae) are medicinal plants that have several uses in Sudanese folk medicine. Their extracts have shown some biological activities including antiproliferative activity and antioxidant potential [3]. However, the endophytes mycoflora of these five plants have not been investigated. As part of our ongoing efforts towards finding novel antioxidant agents from natural resources we investigated, for the first time, total phenolic content and total antioxidant capacities of some endophytic fungi from these medicinal plants.

2. Materials and methods

Fresh leaves and stems of *C. procera*, *C. roseus*, *E. prostrata*, *V. amygdalina* were collected from Khartoum (15°38′ N 32°32′ E) and *T. foenum-graecum* seeds were obtained from Khartoum local market. The plants were identified by Dr. Haider Abdal-gadir, taxonomist in the Medicinal and Aromatic Plants Research Institute in Khartoum (Sudan).

Endophytic fungi were isolated from different parts of the collected medicinal plants after surface sterilization as described

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by Zhang *et al.* [4]. The sterilized pieces were cultivated on potato dextrose agar medium which was amended with chloramphenicol (500 mg/L) to suppress bacterial growth. The efficiency of the surface sterilization procedure was confirmed by plating the final rinse water. Furthermore, the endophytic fungi were subcultured in order to obtain pure cultures, numbered and reserved at 4 °C. Identification of the fungal strains was based on the morphology of cultures or hyphae, the characteristics of the spores, and reproductive structures if the feature were discernible [5]. The cultures which failed to sporulate were grouped as mycelia sterilia [6].

Each fungal strain was cultivated on 20 petri dishes potato dextrose agar, and was incubated at 30 °C for 7–15 d. The solid fungal culture was crushed and extracted with ethyl acetate overnight, filtered, evaporated and preserved at 4 °C.

Dry leaves and stems of *V. amygdalina*, *C. procera*, *C. roseus*, *E. prostrata* and seeds of *T. foenum-graecum* were ground into fine powder. Each sample (20 g) was extracted with ethyl acetate overnight, filtered, evaporated and stored at 4 °C.

Total phenolic contents were determined using the Folin–Ciocalteu method as described by Wolfe *et al.* [7]. The absorbance of the resulting was measured with spectrophotometer at 760 nm using a microtiter plate reader (Synergy HT Biotek, logiciel GEN5). Analysis was done in triplicate for each extract. Quantification was based on the standard curve of gallic acid. The results were expressed as gallic acid equivalent (GAE), *i.e.*, mg gallic acid/g.

Total antioxidant capacity (TAC) of the extracts was estimated using DPPH *in vitro* method as described by Yagi *et al.* [8]. The absorbance was measured spectrophotometrically at 517 nm using a microtiter plate reader (Synergy HT Biotek, logiciel GEN5). Ascorbic acid was used as reference antioxidant compound. Each analysis was done in triplicate. The IC_{50} value was calculated from the linear regression of plots of concentration of the test sample against the mean percentage of the antioxidant activity. Results were expressed as mean ± SEM and the IC_{50} values obtained from the regression plots (Sigma PlotsR 2001, SPSS Science) had a good coefficient of correlation, (R^2 = 0.998).

3. Results

A total of 21 endophytic fungal strains were isolated from 5 Sudanese medicinal plants: three endophytic fungi from *V. amygdalina*, five from *T. foenum-graecum*, four from *C. procera*, five from *C. roseus*, and four from *E. prostrata*. The isolated fungal strains were classified into 12 different taxa (Table 1). Ten strains belong to Ascomycetes, whereas seven strains belong to fungal class Deuteromycetes, four strains were failed to sporulate and were grouped as mycelia sterilia. This group of fungi is a common problem concerning the identification of endophytic fungi [9].

TPC of ethyl acetate crude extracts of 21 endophytes and different parts of their host plants were estimated using the classical Folin–Ciocalteu colorimetric method as shown in (Figure 1). It was found that the five medicinal plants contained TPC values ranged from (0.5 ± 0.1) (*T. foenum-graecum* seeds extract) to (32.7 ± 2.9) mg GAE/g (*V. amygdalina* stem extract). TPC values of 21 endophytes revealed variations ranged from (13.6 ± 1.0) to (89.9 ± 7.1) mg GAE/g. Two *Aspergillus* spp. of both *C. procera* and *T. foenum-graecum* showed the highest TPC values [(77.2 ± 7.5) and (89.9 ± 7.1) mg GAE/g respectively].

The antioxidant potential using DPPH radical scavenging assay was investigated for the 21 ethyl acetate extracts of endophytic fungi and their medicinal host plants. Table 2 showed that TAC IC_{50} values of the medicinal host plants ranged from (50.0 ± 1.7) μg/mL (*V. amygdalina* stem) to no activity (*T. foenum-graecum* seeds). The endophyte extracts revealed extremely wide range of IC_{50} values, from (18.0 ± 0.1) μg/mL for *Aspergillus* sp. isolated from *T. foenum-graecum* to (2 686.0 ± 51.7) μg/mL for *Phoma* sp. in *C. roseus*.

A high positive linear correlation (R^2 = 0.999 1) was found between TAC and TPC of endophytic fungi isolated from *V. amygdalina*. Endophytic fungi isolated from *T. foenum-*

Table 1

Taxonomic identification of the endophytic fungi isolated from various organs of *V. amygdalina*, *C. procera*, *C. roseus*, *E. prostrata* (stems, leaves) and *T. foenum-graecum* (seeds).

	Fungal taxon	Host plant name	Host plant organs
Ascomycetes	*Alternaria* sp.	*C. procera*	Leaves
	Bipolaris sp. 1	*C. roseus*	Leaves
	Bipolaris sp. 2	*E. prostrata*	Leaves + Stem
	Curvularia sp. 1	*C. roseus*	Leaves
	Curvularia sp. 2	*V. amygdalina*	Leaves + Stem
	Chaetomium sp. 1	*C. roseus*	Stem
	Chaetomium sp. 2	*T. foenum-graecum*	Seeds
	Chaetomium sp. 3	*V. amygdalina*	Leaves
	Drechslera sp.	*E. prostrata*	Leaves + Stem
	Emericella sp.	*C. roseus*	Leaves
Deuteromycetes	*Aspergillus* sp. 1	*C. procera*	Stem
	Aspergillus sp. 2	*T. foenum-graecum*	Seeds
	Cladosporium sp. 1	*C. procera*	Leaves
	Cladosporium sp. 2	*V. amygdalina*	Leaves
	Paecilomyces sp.	*E. prostrata*	Leaves + Stem
	Phoma sp.	*C. roseus*	Stem
	Mycelia sterilia sp.1	*C. procera*	Stem
	Mycelia sterilia sp. 2	*T. foenum-graecum*	Seeds
	Mycelia sterilia sp. 3	*T. foenum-graecum*	Seeds
	Mycelia sterilia sp. 4	*T. foenum-graecum*	Seeds

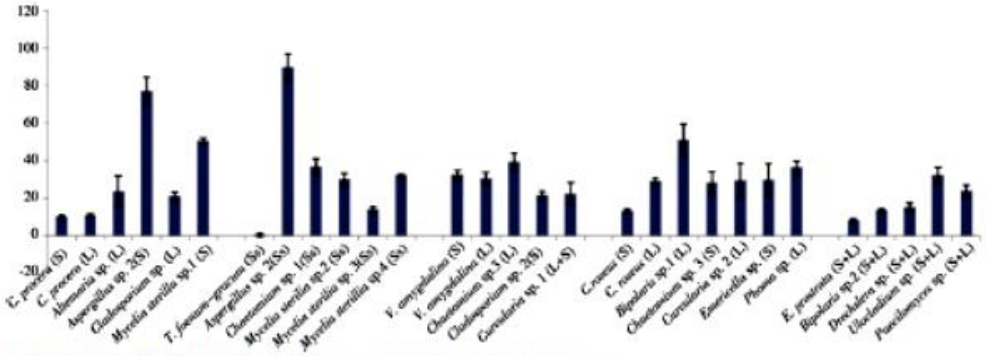

Figure 1. Total phenolic content in ethyl acetate extracts of endophytes and their host plants. (S) Stem, (L) leaves, (Ss) Seeds and (S + L) stem and leaves.

Table 2

IC_{50} values of 21 endophytic fungi and their host plants by DPPH radical scavenging assay (mean ± SD).

Crude extract	DPPH (μg/mL)
Ascorbic acid	5.0 ± 0.1
C. procera (S)	668.0 ± 8.1
C. procera (L)	388.0 ± 7.2
Alternaria sp. (L)	236.0 ± 8.3
Aspergillus sp. 1 (S)	58.0 ± 0.4
Cladosporium sp. 1 (L)	1142.0 ± 1.3
Mycelia sterilia sp. 1 (S)	1030.0 ± 3.0
T. foenum-graecum (seeds)	[a]
Aspergillus sp. 2 (Ss)	18.0 ± 0.1
Chaetomium sp. 1 (Ss)	70.0 ± 0.3
Mycelia sterilia sp. 2 (Ss)	1013.0 ± 4.2
Mycelia sterilia sp. 3 (Ss)	933.0 ± 5.3
Mycelia sterilia sp. 4 (Ss)	1070.0 ± 3.2
V. amygdalina (S)	50.0 ± 1.7
V. amygdalina (L)	63.0 ± 1.8
Chaetomium sp. 3 (L)	252.0 ± 5.1
Cladosporium sp. 2 (L)	480.0 ± 3.9
Curvularia sp. 1 (L + S)	461.0 ± 5.5
C. roseus (S)	1119.0 ± 2.6
C. roseus (L)	113.0 ± 0.4
Bipolaris sp. 1 (L)	1556.0 ± 1.5
Chaetomium sp. 3 (S)	405.0 ± 5.2
Curvularia sp. 2 (L)	105.0 ± 2.7
Emericella sp. (L)	137.0 ± 1.3
Phoma sp. (S)	2686.0 ± 51.7
E. prostrate (L + S)	203.0 ± 7.6
Bipolaris sp. 2 (L + S)	2305.0 ± 23.4
Drechslera sp. (L + S)	1074.0 ± 7.7
Ulocladium sp. (L + S)	1348.0 ± 5.6
Paecilomyces sp. (L + S)	122.0 ± 0.4

[a] Indicated not active, (L) leaves, (S) stem, (Ss) seeds and (L + S) leaves and stem.

graecum and *C. procera* showed moderate correlation (R^2 = 0.680 8 and 0.515 6, respectively).

4. Discussion

The majority of the fungal genera isolated from the Sudanese plants were common endophytes (*Alternaria*, *Cladosporium*, *Phoma*, *Chaetomium*, *Drechslera*, *Curvularia*, *Bipolaris*, *Paecilomyces*, *Emericella* and *Aspergillus*). However *Ulocladium* were reported only few times as endophytes [10]. The low diversity of the endophytes of the Sudanese medicinal plants may be due to the climate where it is extremely arid for most of the year with about nine months with average rainfall lower than five mm. Some authors [11] reported that a significant variation was detected in the colonization frequency of endophytic species in relation with the environmental factors such as rainfall and atmospheric humidity.

Ethyl acetate is selective solvent which extract low and high molecular weight polyphenols. Despite the high TAC of stems and leaves of *V. amygdalina* [IC_{50}: (50.0 ± 1.7 μg/mL and (63.0 ± 1.8) respectively)], their endophyte extracts showed low TAC [(IC_{50}: (252.0 ± 5.1) to (480.0 ± 3.9) μg/mL). In contrario the seed extract of *T. foenum-graecum* had no antioxidant activity while *Aspergillus* sp. 2, isolated from the seeds, showed significant TAC [(18.0 ± 0.1) μg/mL]. These results indicated that no correlation between the TACs of the endophytes and the host plants can be established. The main factor is the fungal genus, indeed *Aspergillus* spp. were recorded the highest TAC. The highest TAC and TPC were obtained with *Aspergillus* spp. extracts isolated from both *C. procera* [IC_{50}: (58.0 ± 4.0) μg/mL, TPC: (77.2 ± 7.5) mg GAE/g] and *T. foenum-graecum* [IC_{50}: (18.0 ± 0.1) μg/mL, TPC: (89.9 ± 7.1) mg GAE/g]. It is noted that crude extract of *T. foenum-graecum* seeds from Sudan revealed no antioxidant activity that could be explained by the low concentration of TPC [(0.5 ± 0.1) mg GAE/g]. In contrario previous works reported that seed ethyl acetate crude extract of *T. foenum-graecum* demonstrated strong antioxidant activity in relation with high phenolic content (106.316 mg GAE/g) [12].

In conclusion, in this study we investigated the diversity of endophytic fungi of 5 Sudanese medicinal plants. The 21 endophytes were identified and classified. *Mycelia sterilia*, and *Chaetomium*, were the dominant fungal taxa isolated. The endophyte diversity was poor in comparison with the results obtained with plants growing in other countries. Our findings revealed the first report on endophytic fungi of 5 Sudanese medicinal plants. Some of them were worthy with phenolic compounds and may serve as potential source of natural antioxidants. The *Aspergillus* sp. endophyte of *T. foenum-graecum* was revealed significant antioxidant activity alongside this strain was rich with phenolic compounds, this fungus strain is recommended for further investigations.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Arnold AE. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biol Rev* 2007; **21**(2–3): 51-66.

[2] Huang W, Cai Y, Xing J, Corke H. A potential antioxidant resource: endophytic from medicinal plant. *Econ Bot* 2007; **61**(1): 14-30.

[3] El Ghazali GE. *The promising medicinal plants of the Sudan*. National Council for Research: Khartoum University Press; 1997.

[4] Zhang P, Zhou P, Yu L. An endophytic taxol-producing fungus from *Taxus media*, *Cladosporium cladosporioides* MD2. *Curr Microbiol* 2009; **59**: 227-232.

[5] Watanabe T. *Pictorial atlas of soil and seed fungi: Morphologies of cultured fungi and key to species*. 2nd ed. Washington, D.C: CRC Press LLC; 2002.

[6] Promputtha I, Jeewon R, Lumyong S, Mckenzie EHC, Hyde KD. Ribosomal DNA fingerprinting in the identification of non sporulating edophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Divers* 2005; **20**: 167-186.

[7] Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. *J Agric Food Chem* 2003; **51**: 609-614.

[8] Yagi S, Drouart N, Bourgaud F, Henry M, Chapleur Y, Laurain-Mattar D. Antioxidant and antiglycation properties of *Hydnora johannis* roots. *S Afr J Bot* 2012; **84**: 124-127.

[9] Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Divers* 2008; **33**: 61-75.

[10] Marquez SS, Bills GF, Acuna LD, Zabalgogeazcoa I. Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. *Fungal Divers* 2010; **41**: 115-123.

[11] Selvanathan S, Indrakumar I, Johnpaul M. Biodiversity of the endophytic fungi isolated from *Calotropis gigantean* (L) R. Br. *Rec Res Sci Techol* 2011; 3(4): 94-100.

[12] Kenny O, Smyth TJ, Hewage CM, Brunton NP. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. *Food Chem* 2013; **141**: 4295-4302.

Promising cytotoxic and antibacterial potentialities of endophytic fungi associated with medicinal plants from Sudan

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Abstract

A study on endophytic fungi isolated from five medicinal plants from Sudan: *Calotropis procera* Ait., *Catharanthus roseus* L., *Euphorbia prostrata* Ait., *Trigonella foenum-graecum* L. and *Vernonia amygdalina* Del. was performed to identify the culturable endophytic fungal strains by sequencing of internal transcribed spacer (ITS) regions of rDNA. The evaluation of the cytotoxicity of the ethyl acetate extracts of the endophytic fungal strains by the MTT assay was done on three cancer cell type: Human breast carcinoma (MCF7) and Colon adenocarcinoma (HT29, HCT116) cells. Antibacterial screening was carried out against two bacterial strains: Gram-negative *Escherichia coli* and Gram-positive methicillin-resistant *Staphylococcus aureus*, by broth dilution method. Fungal endophyte *Byssochlamys spectabilis* showed strong cytotoxicity with IC_{50} value of 1.51 ± 0.2 µg mL^{-1} followed by *Cladosporium cladosporioides* 2 with IC_{50} value of 10.5 ± 1.5 µg mL^{-1}, then *Alternaria* sp. with IC_{50} value of 13.5 ± 1.8 µg mL^{-1}. While *Alternaria alternata* displayed antibacterial activity with MIC value of 0.125 mg mL^{-1}.

The findings of this work revealed auspicious approach of endophytic fungi associated with medicinal plants from Sudan, could consider as an attractive source for new therapeutic compounds.

Keywords

Sudan medicinal plants. Endophytic fungi. Cytotoxicity. Breast carcinoma (MCF7). Colon adenocarcinoma (HT29, HCT116). Antibacterial activity.

Introduction

Endophytic fungi are hidden fungi which colonized within the living tissues of plants, for some or all of their life cycle, without causing apparent harm to their hosts. These fungi that are ubiquitous in plant species have significant ecological benefits to their host plants (Rodriguez *et al*., 2009). These effects are assumed to be the result of the production of a broad range of active metabolites influencing plant tolerance to biotic and abiotic stresses (Strobel *et al*., 2004; Aly *et al*., 2013). However, even if fungal endophytes have been described for a long time, these microorganisms only received much more attention in the last decades. Indeed, this group of fungi represents a source of novel bioactive compounds with potential applications for medicine or as well as an alternative source of pharmacologically important drugs originally obtained from higher plants (Aly *et al*., 2010; Kharwar *et al*., 2011, Lingqi *et al*., 2000; Strobel, 2003). Therefore, the use of fungal endophytes and their bioactive compounds offer a unique opportunity to discover novel therapeutic compounds to combat cancers and antibiotic resistant bacteria, that is highly needed.

In the present work, we investigated the presence of endophytes in five plant species used in Sudanese traditional medicines: *Calotropis procera* Ait., *Catharanthus roseus* L., *Euphorbia prostrata* Ait., *Trigonella foenum-graecum* L. and *Vernonia amygdalina* Del. These plant species have been selected for different reasons. They grow in an adverse environment in Sudan where the climat is arid and they are traditionally employed in Sudan and in some other african countries as anticancer plants. We managed to isolate and taxonomically characterize fungal endophytes from the different candidates. Ethyl acetate extracts were assessed both on three cancer cell lines using MTT assays and on two bacterial strains (the Gram-negative *Escherichia coli* and the Gram-positive methicillin-resistant *Staphylococcus aureus*) using a broth dilution method.

Materials and Methods

Collection, identification and authentication of the plant material

Fresh healthy leaves and stems of *Calotropis procera* Ait., *Catharanthus roseus* L., *Euphorbia prostrata* Ait., *Vernonia amygdalina* Del and seeds of *Trigonella foenum-graecum*. L were randomly collected from Khartoum, Sudan. The plant samples were washed thoroughly in running tap water and were processed within few hours after sampling.

Isolation of the fungal endophytes

Plant samples were surface disinfected as described by Petrini and Dreyfuss (1981) with minor modifications. Briefly, segments were surface disinfected by sequential immersions in 70% (v/v) ethanol for 1 min and 5% sodium hypochlorite solution for 5 min and then rinsed two times for 1 min with sterile distilled water. The surface sterilized 240 segments were incubated on Potato Dextrose Agar medium amended with chloramphenicol 500 mg L^{-1} at 28°C, until fungal growth emerged from the plant segments. The efficiency of the surface sterilization procedure was confirmed by plating the final rinse water on PDA medium. the absence of any micro-organism was assessed after for 15 day long incubation at 28 °C. The endophytic fungi were isolated from the surface sterilized plants by serial sub culturing on PDA plates. Fungal isolates were stored as slants at 4 °C. The colonization frequency (CF%) of endophytes species in their host tissues was calculated by using the formula $CF\% = N_{col} / N_t \times 100$, where, N_{col} is the number of segments colonized by endophytes and N_t: the total number of segments.

Identification of fungal strains

Sporulating fungi were identified based on the characteristics of the fungal colony, the morphology of hyphae, the characteristics of spores and reproductive structures after

lactophenol or lactophenol cotton blue staining by Olympus IX 50 microscope. Photos were taken using Olympus C-5060 Wide Zoom camera.

Sequencing of internal transcribed spacers (ITS) regions of rDNA was done to confirm morphological observations. Each fungal isolate was cultured on PDA plate covered with a sheet of sterile cellophane at 28 °C for 7 to 15 day, in order to collect fungal mycelium (Larena *et al.*, 1999). Then, 0.5 g of fresh mycelium was collected and ground with liquid nitrogen in a mortar using a pestle. DNA was extracted according to the method described by Ranjard *et al*. (2003).

PCR was performed using ITS1 and ITS4 primers. 2.5 µl 10x *Taq* DNA Polymerase buffer (MP Biomedicals, France), 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.625 U of *Taq* DNA Polymerase (MP Biomedicals, France), 25 ng of fungal genomic DNA were combined in a final volume of 25 µl. PCR amplifications were carried out as follows: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72°C for 2 min, with a 15 min final elongation at 72 °C. PCR products were sequenced (GATC Biotech, Konstanz, Germany). DNA sequences were compared to sequences available in public databases (GenBank) using the BLASTn tool (http://www.ncbi.nlm.nih.gov/).

Preparation of fungal ethyl acetate extracts

Ethyl acetate extracts were prepared according to Campos *et al*. (2008), with minor modifications. Each 20 fungal mycelium on PDA medium was ground together using a blender and then transferred to an Erlenmeyer containing 1 L of ethyl acetate. After 24 h of incubation at room temperature, the mix was filtered and dried under vacuum in a rotary-evaporator at 40 °C. The dry organic extracts were stored at 4 ºC.

Preparation of ethyl acetate extracts from the host medicinal plants

Dry leaves and stems from *C. procera, V. amygdalina, E. prostrata*, *C. roseus* and seeds of *T.*

foenum-graecum were ground into fine powder using a mortar. Each plant sample (20 g) was macerated in 100 mL ethyl acetate overnight at room temperature and filtered by filter paper. Then, the crude extract was evaporated and the dry crude extracts obtained were stored at 4°C.

Cytotoxicity assay

The cytotoxicity assay was performed with the crude extracts of the five medicinal plants and their endophytes using the thiazolyl blue tetrazolium bromide (MTT) procedure as described by Mosman (1983). Three cell lines deriving from human colon adenocarcinoma (HT29 and HCT116) and human breast carcinoma (MCF-7)) were tested. HT29 and HCT116 cells were cultivated in Dulbecco's minimum essential medium (DMEM, Eurobio, Courtaboeuf, France) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (Eurobio), 1% Penicillin/streptomycin (Eurobio) and 2 mM L-Glutamine (Eurobio). MCF7 cells were cultivated respectively in RμMI and Eagle's minimum medium instead of DMEM containing the same cell culture additives. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

The cells were seeded in 96-wells-plate at a density of 5.000 cells/well. Twenty hours after seeding, 100 µL of medium containing increasing concentrations of the extracts (range from 0.01 to 100 µg mL^{-1}) were added to each well and further incubated for 72 hours at 37 °C. After incubation, the medium was discarded and 100 µL/well of MTT solution (0.5 mg mL^{-1} diluted in DMEM, MEM or RμMI medium) were added and incubated for 2 additional hours. The water-insoluble formazan blue crystals generated were dissolved in DMSO and each plate was observed at 570 nm. IC_{50} was calculated using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data are expressed as IC_{50} ± SD obtained from quadruplicate determination of two independent experiments (n = 8).

Antibacterial activity

1. Preparation of the bacterial suspension

Bacteria were stored at −80 °C in brain heart infusion broth (BioRad) supplemented with 20% glycerol. Before each experiment, the bacterial strains were streaked on plates and incubated at 37 °C overnight in order to have fresh colonies. Bacterial inoculum was monitored by setting the culture optical density (measured in distilled water at an absorbance wavelength of 620 nm) at 0.156 for *Escherichia coli* and 0.300 for methicillin-resistant *Staphylococcus aureus* corresponding to 10^8 CFU mL^{-1}. Bacterial suspensions were further diluted to 10^6 CFU mL^{-1} in sterile water.

2. Determination of the minimum inhibitory concentration (MIC)

MIC of ethyl acetate extracts was determined based on a broth microdilution method in a 96-well microplate. Each crude extract (2 mg mL^{-1}) was dissolved in 5% DMSO and twofold serial dilutions were made in the concentration range from 0.015 to 2 mg mL^{-1}. In the 96- well plate, each well had 50 µL of MH broth, 100 µL of bacterial inoculum (10^6 CFU mL^{-1}) and 50 µL of serial concentrations of fungal extract. The plate was incubated at 37 ºC for 24 h. The plates were scanned at 620 nm each two hours using a micro titer reader VersaMax™ Microplate Reader from Molecular Devices. After incubation, the lowest concentration of extracts which showed no observable bacterial growth or observable turbidity was taken as MIC values were calculated. The experiments were carried out in triplicate.

Results and discussion

Plants from unique environment and with an ethnobotanical history have been suggested to be good candidates to identify novel endophytic microorganisms producing novel bioactive

compounds (Strobel, 2003). Therefore, a study was engaged to investigate the cytotoxic and antibacterial activities of endophytic fungi associated with five plant species commonly used in Sudanese traditional medicine.

A total of 23 endophytic fungal strains were isolated from the five plants: *C. procera, C. roseus*, *E. prostrata*, *T. foenum-graecum*, and *V. amygdalina*. Despite the high total fungal colonization frequency 95.8% the same fungal species were isolated several times from the plant segments. The diversity of the number of endophytes which were recovered from the five plants was so low, this could be influenced by climate conditions that are extremely arid for most of the year in Sudan with about nine months with average rainfall lower than five mm, especially because the isolation of the endophytes was performed during the dry months (October to January). Colonization of host plants by endophytic fungi is believed to contribute to host plant adaptation to biotic and a biotic stress factors (Aly *et al*., 2010).

Fiveteen fungal endophytes were selected from the isolated endophytes from the five medicinal plants, they were identified using ITS-rDNA sequences, the 15 isolates were classified into 10 different taxa. 15 strains belong to Ascomycotina and one to Basidiomycotina (Table 1) (Figure 1 and 2).

Ethyl acetate was used for the preparation of organic crude extracts from both fungal endophytes and medicinal plants in order to evaluate their cytotoxic and antibacterial activities. Ethyl acetate was chosen because this solvent is known with its average polarity and minimum toxicity.

Considering cytotoxic activity of ethyl acetate extracts from fungal endophytes. It was observed that 14 endophytes of 15 revealed cytotoxic activity (IC_{50} < 100 µg mL^{-1}) at least against one cancer cell line. The IC_{50} values ranged from 1.51 ± 0.2 for extract of *Byssochlamys spectabilis,* to 91.7 ± 6.9 µg mL^{-1} for extract of *Pleosporales* sp. The MCF7 cell line was the most sensitive since three fungal extract displayed cytotoxicity with IC_{50} <

20 µg mL^{-1} *Byssochlamys spectabilis, Cladosporium cladosporioides 2* and *Alternaria* sp. Only two isolates, *Aspergillus terreus* 1 isolated from *C. procera* and *Phoma multirostrata* isolated from *C. roseus* showed very weak cytotoxic activity with IC_{50} >100 µg mL^{-1} against all cancer cell lines. Among the 14 fungal endophytes showing cytotoxic activity, *Byssochlamys spectabilis* isolated from *E. prostrata* showed outstanding cytotoxicity (IC_{50} value of 1.51 ± 0.2 µg mL^{-1}), followed by *Cladosporium cladosporioides 2* isolated from *V. amygdalina* (IC_{50} value of 10.5 ± 1.5 µg mL^{-1}), then *Alternaria* sp. isolated from *E. prostrata* (IC_{50} value of 13.5 ± 1.8 µg mL^{-1}). Some endophytes showed only moderate toxicity against HCT116 cell line such as *Curvularia australiensis* 1, *Alternaria* sp., *Byssochlamys spectabilis* from *E. prostrata* and *Aspergillus terreus* 2 from *T. foenum-graecum* (IC_{50} values of 25.6 ± 1.9, 29.1 ± 2.5, 30.4 ± 1.1 and 30.7 ± 1.01 µg mL^{-1} respectively). While the cytotoxic activity of the 15 tested endophytes against HT29 cell line were ranging from moderate to weak, among them only *Alternaria* sp. from *E. prostrata* displayed moderate activity (IC_{50} value of 28.4 ± 5.2 µg mL^{-1}).

Concerning the plant extracts, two plant extracts revealed strong cytotoxicity. Their cytotoxicity ranged from 2.19 ± 0.12 to 7.8 ± 1.28 µg mL^{-1} (Table 2). These values were within the cutoff point of the National Cancer Institute criteria for cytotoxicity (IC_{50} < 20 µg mL^{-1}) in the screening of crude plant extracts (Lee *et al*., 2005). The HCT116 cell line was the most sensitive since, three plant extracts *Calotropis procera* (Stems), *Vernonia amygdalina* (Leaves), *Catharanthus roseus* (Leaves) displayed powerful.

The medicinal plant, *C. procera* (stems) showed powerful activity (IC_{50} value of 2.19 ± 0.12 µg mL^{-1}) against HCT116 cells, followed by *V. amygdalina* (leaves) (IC_{50} value of 5.6 ± 0.4 µg mL^{-1}), and then *C. roseus* (leaves) (IC_{50} value of 7.83 ± 1.2). Only two of these medicinal plants are toxic for HT29 cells, *C. roseus* (leaves) (IC_{50} value of 7.00 ± 0.43 µg mL^{-1}) and *V. amygdalina* (stems) (IC_{50} value of 15.3 ± 3.6 µg mL^{-1}). *V. amygdalina* (leaves) displayed

moderate activity (IC_{50} values of 27.5 ± 5.7 and 31.9 ± 5.1 µg mL^{-1}) against MCF7 and HT29 cells respectively. *E. prostrata*, *T. foenum-graecum*, *C. roseus* (stems) and *C. procera* (leaves) showed low cytoxicity > 100 against all the cancer cell lines tested. It is noteworthy that *E. prostrata* (leaves and stems) extracts revealed weak activity against all the cancer cell lines tested in this study while the most toxic endophyte *Byssochlamys spectabilis* was isolated from this plant.

Another remarkable notice of the cytotoxicity screening that an inverse relationship was observed between medicinal plants and their endophytes, such as *C. roseus* and *C. procera*, which showed high cytotoxicity and the endophytes revealed low cytotoxicity, such as *Aspergillus terreus* 1, *Alternaria alternata*, *Curvularia aeria* and *Phoma multirostrata*. On the other hand, some medicinal plants, such as *E. prostrata* and *T. foenum-graecum*, were displayed low cytotoxicity and in contrario, their endohytes were exhibited strong cytotoxicity. The same observation was stated by Khiralla *et al*. (2015) on the antioxidant activity, an inverse relationship was observed between the same five medicinal plants and their endophytes.

Ethyl acetate crude extracts of the endophytes were tested against two bacterial strains Gram-negative *Escherichia coli* and Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA), Using the broth dilution methods in micro dilution tray (96 wells). MRSA is one of the pathogen strains causing the majority of hospital infections and escape the effects of antibacterial drugs (Rice, 2008). Here we make evidence that six (40%) fungal extracts displayed antibacterial activity against MRSA: *Alternaria alternata*, *Aspergillus terreus*1 isolated from *C. procera*, two strains isolated from *E. prostrata*: *Alternaria* sp., *Byssochlamys spectabilis* and two other strains isolated from *T. foenum-graecum, Aspergillus terreus* 2, *Chaetomium globosum* showed an activity against MRSA ranging between 0.125-2 mg mL^{-1}. However none activity (>2 mg mL^{-1}) was observed for the 16 endophytes against *E. coli*

(Table 3). In this general screening, the genus *Alternaria* isolated from *C. procera* and *E. prostrata* displayed the interesting antibacterial activity with MIC values of 0.125 and 0.250 mg mL^{-1} respectively. Several studies showed no activity of the endophytic extracts against *E. coli*, such as Gong and Guo (2009) which studied the endophytic fungi of two plants *Dracaena cambodiana* and *Aquilaria sinensis* and obtained 300 isolates. Only 21 isolates showed antimicrobial activity, however, none of these isolates was active against *E. coli*.

Conclusion

In this report, we identified and characterized at the molecular level 15 endophytes which were isolated from five medicinal plants collected in Sudan. The cytotoxicity and antibacterial properties of ethyl acetate extracts prepared from these fungi were assessed on different cancer cell lines and bacterial strains respectively. We made evidence that extracts prepared from *Byssochlamys spectabilis* showed a strong cytotoxicity (IC_{50} value of 1.51 ± 0.2 µg mL^{-1}) followed by *Cladosporium cladosporioides* 2 (IC_{50} value of 10.5 ± 1.5 µg mL^{-1}) and *Alternaria* sp. (IC_{50} value of 13.5 ± 1.8 µg mL^{-1}). These fungal strains associated with medicinal plants could be considered as promising cytotoxic source in the future for the discovery of new therapeutic compounds.

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References

1. Aly AH, Debbab A & Proksch P (2013) Fungal endophytes – secret producers of bioactive plant metabolites. *Pharmazie*, **68**: 499-505.
2. Gong LJ & Guo SX (2009) Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. *Afr J Biotechnol*, **8**: 731–736.
3. Kharwar RN, Mishra A, Gond SK, Stierle, A & Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Nat Prod Rep*, **28** (7): 1208-28. doi: 10.1039/c1np00008j.
4. Khiralla' A, Mohamed I, Thomas J, Mignard B, Spina R, Yagi S & Laurain-Mattar D (2015). A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants. *Asian Pac J Trop Med*, **8** (9): 701-704.
5. Larena I, Salazar, O, Gonzalez V, *et al*. (1999) Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *J Biotechnol*, **75**: 187–194.
6. Lee CC & Houghton P (2005) Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *J Ethnopharmacol*, **100**:237-243.
7. Lingqi Z, Bo G, Haiyan L, Songrong Z, Hua S, Su G & Rongcheng W (2000). Preliminary study on the isolation of endophytic fungus of Catharanthus roseus and its fermentation to produce products of therapeutic value. *Chinense traditional and Herbal drugs*, **31**: 805-807.
8. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth,* **65**, 55–63.
9. Petrini O & Dreyfuss M (1981) Endophytische Pilze in epiphytischen Araceae, Bromeliaceae and Orchidaceae. *Sydowia*, **34**: 135–145.

10. Ranjard L, Lejon DPH, Mougel C, Schehrer L, Merdinoglu D & Chaussod R (2003) Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. *Environ Microbiol*, **5**: 1111-1120.
11. Rice LB (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infec Dis*, **197**: 1079–1081.
12. Rodriguez RJ, White Jr JF, Arnold AE & Redman RS (2009) Fungal endophytes: diversity and functional roles. *New Phytol*, **182**: 314-330.
13. Strobel GA (2003). Endophytes as sources of bioactive products. *Microbes Infect*, **5**: 535-544.
14. Strobel GA, Daisy B, Castillo U & Harper J (2004) Natural products from endophytic microorganisms. *J Nat Prod*, **67**: 257-268.
15. Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sun P & Qin L (2010) Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiol Res*, **165**: 437-449.

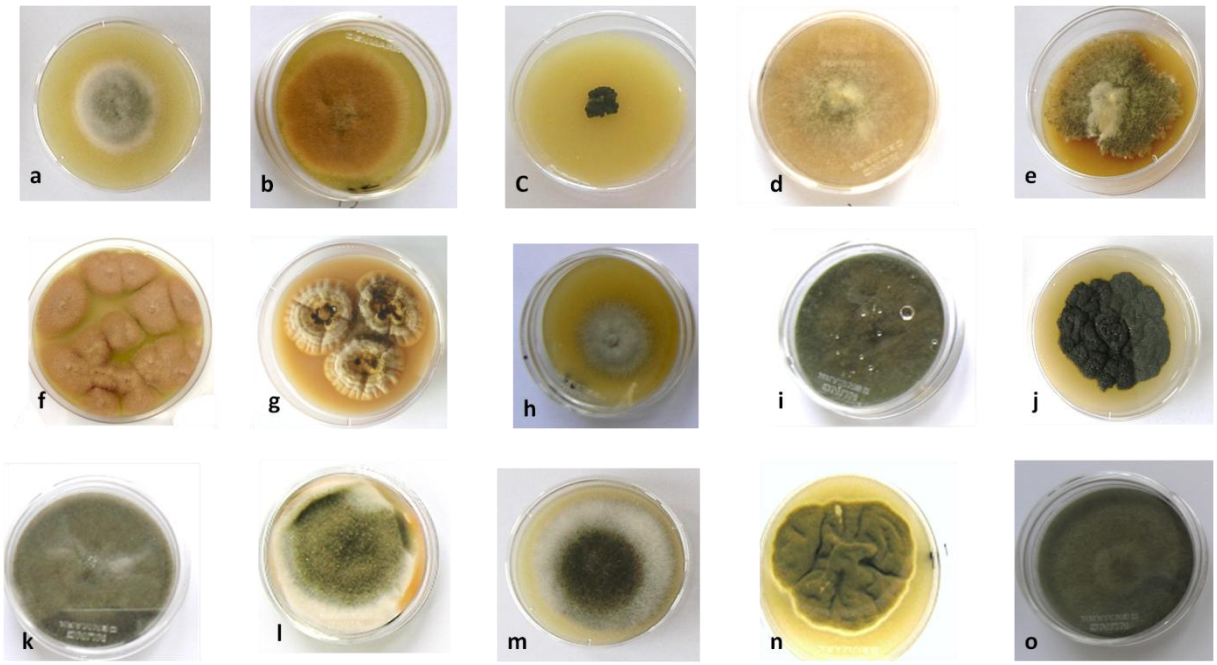
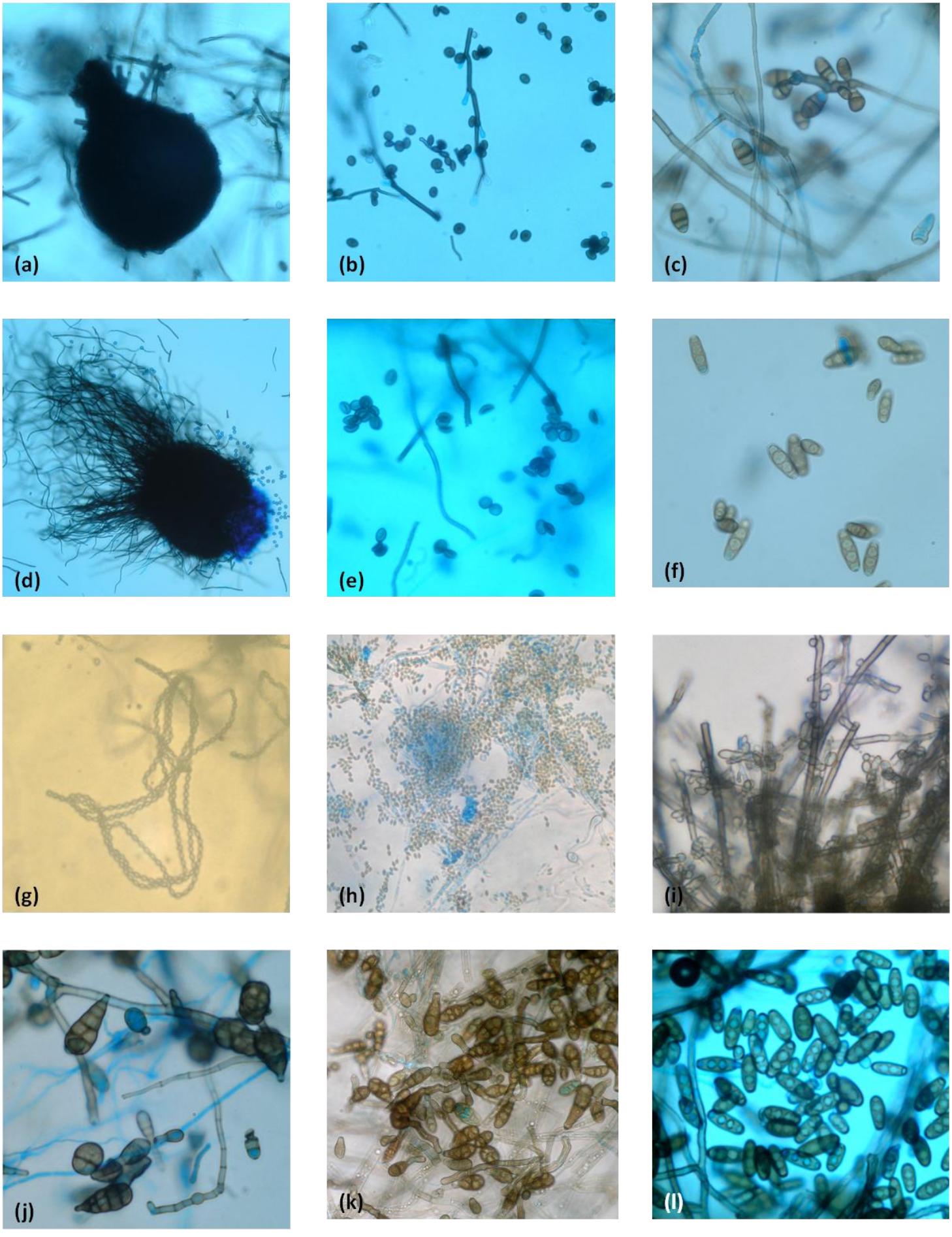


Figure 1. Fungal endophyte cultures: a. *Alternaria alternata.* b. *Aspergillus terreus* 1. c. *Cladosporium cladosporioides* 1. d. *Trametes versicolor*. e. *Chaetomium globosum*. f. *Aspergillus terreus* 2. g. *Hansfordia sinuosae*. h. *Cladosporium cladosporioides* 2. i. *Curvularia aeria*. j. *Phoma multirostrata*. k. Pleosporales sp. l. *Curvularia australiensis* 1. m. *Curvularia australiensis* 2. n. *Byssochlamys spectabilis*. o. *Alternaria* sp.


(a)
(b)
(c)
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(f)
(g)
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Figure 2 . Some morphology of fungal endophyte isolates: **(a)** and **(b)** *Hansfordia sinuosae* perithecium and ascospores ×400. **(c)** *Curvularia aeria* ascospores ×400. **(d)** and **(e)** *Chaetomium globosum* terminal hairs on perithecium ×100 and ascospores ×400. **(f)** *Pleosporales* sp conidia ×400. **(g)** and **(h)** *Byssochlamys spectabilis* conidia chains and conidia ×400 *Curvularia australiensis* 1 conidia ×400. **(i)** *Cladosporium cladosporioides* 1 conidiophores and conidia ×400. **(j)** *Alternaria* sp. conidia ×400. **(k)** *Alternaria alterna* conidia ×400. **(l)** *Curvularia australiensis* 2 conidia ×400.

Table 1. Endophytic fungi isolated from different organs of five medicinal plants: *Calotropis procera*, *Catharanthus roseus*, *Euphorbia prostrata*, *Trigonella foenum-graecum*, and *Vernonia amygdalina*.

Host plant	**Plant Organ**	**Isolate n°.**	**Molecular identification**	**Closest species (% identity)°**
C. procera	Leaves	1	*Alternaria alternata*	99%
	Leave	2	*Aspergillus terreus* 1	100%
	Leaves	3	*Cladosporium cladosporioides* 1	99%
	Stems	4	*Trametes versicolor*	99%
T. foenum-graecum	Seeds	5	*Chaetomium globosum*	99%
	Seeds	6	*Aspergillus terreus* 2	100%
V. amygdalina	Leaves	7	*Cladosporium cladosporioides* 2	99%
	Stems	8	*Hansfordia sinuosae*	99%
C. roseus	Leaves	9	*Curvularia aeria*	99%
	Stems	10	*Phoma multirostrata*	99%
	Leaves	11	*Pleosporales* sp.	99%.
E. prostrata	Aerial part	12	*Curvularia australiensis* 1	99%
	L+S	13	*Curvularia australiensis* 2	99%
		14	*Byssochlamys spectabilis*	98%
		15	*Alternaria* sp.	99%

Table 2. Cytotoxicity IC_{50} values of ethyl acetate crude extracts of 15 selected endophytes and their host plants.

Crude extracts	Cytotoxicity IC_{50} (µg mL^{-1})*		
	MCF7	HT29	HCT116
Calotropis procera (Leaves)	-	>100	>100
Calotropis procera (Stems)	-	**7.25 ± 2.2**	**2.19 ± 0.1**
Aspergillus terreus 1	>100	> 100	>100
Alternaria alternata	>100	> 100	52.5 ± 3.0
Cladosporium cladosporioides 1	>100	77.7 ± 9.2	45.6 ± 1.5
Trametes versicolor	48.3 ± 4.7	53.9 ± 11.7	84.3 ± 6.8
Trigonella foenum-graecum (Seeds)	-	>100	>100
Chaetomium globosum	>100	>100	75.2 ± 2.8
Aspergillus terreus 2	-	>100	30.7 ± 1.0
Vernonia amygdalina (Leaves)	27.5 ± 5.7	31.9 ± 5.1	**5.6 ± 0.4**
Vernonia amygdalina (Stems)	49.6 ± 4.4	15.3 ± 3.6	>100
Cladosporium cladosporioides 2	**10.5 ± 1.5**	>100	>100
Hansfordia sinuosae	> 100	47.6 ± 4.1	>100
Catharanthus roseus (Stems)	-	>100	>100
Catharanthus roseus (Leaves)	-	**7.00 ± 0.4**	**7.8 ± 1.2**
Curvularia aeria	>100	74.5 ± 12.5	53.9 ± 4.5
Phoma multirostrata	>100	>100	>100
Pleosporales sp.	91.7 ± 6.9	69.4 ± 3.8	36.7 ± 8.3
Euphorbia prostrata (Leaves+Stems)	-	>100	>100
Curvularia australiensis 1	46.4 ± 2.5	54.3 ± 6.9	25.6 ± 1.9
Curvularia australiensis 2	>100	>100	59.7 ± 4.4
Alternaria sp.	**13.5 ± 1.8**	28.4 ± 5.2	29.1 ± 2.5
Byssochlamys spectabilis	**1.51 ± 0.2**	56.3 ± 5.7	30.4 ± 1.1

* Viability was determined by the MTT procedure using human breast carcinoma (MCF7) and colon adenocarcinoma (HT29, HCT116) cells. IC_{50} values are means ± S.E.M calculated from results obtained from quadruplicate determination of two independent experiments (n= 8).

Table 3. MIC values of 15 endophytic fungi ethyl acetate extracts, isolated from *C. procera* , *C. roseus* , *E. prostrata T. foenum-graecum*, and *V. amygdalina* against two bacterial strains: *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*.

Crude extract of endophytes	**Antibacterial activity MIC (mg mL^{-1})**	
	S. aureus	*E. coli*
Alternaria alternata	**0.125**	>2
Aspergillus terreus 1	0.5	>2
Cladosporium cladosporioides 1	>2	>2
Trametes versicolor	>2	>2
Aspergillus terreus 2	1	>2
Chaetomium globosum	2	>2
Hansfordia sinuosae	>2	>2
Cladosporium cladosporioides 2	>2	>2
Pleosporales sp.	>2	>2
Curvularia aeria	>2	>2
Phoma multirostrata	>2	>2
Curvularia autraliensis 1	>2	>2
Curvularia autraliensis 2	>2	>2
Alternaria sp.	**0.25**	>2
Byssochlamys spectabilis	0.5	>2

Résumé

Pour la première fois, l'étude de la flore fongique endophytique de cinq plantes médicinales soudanaises : *Calotropis procera* (Ait.), *Catharanthus roseus* (L.), *Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.), and *Vernonia amygdalina* (Del.) a été réalisée. Un total de 23 souches de champignons endophytes ont été isolées à partir des plantes après la stérilisation de surface puis les différentes analyses biologiques ont été effectuées. Les extraits bruts d'acétate d'éthyle de 21 endophytes ainsi que de leurs plantes hôtes ont été évalués pour leur teneur en phénols totaux et leur activité antioxydante en utilisant respectivement la méthode colorimértrique Folin-Ciocalteu et le piégeage des radicaux libres par la méthode 1,1,-diphényl-2-picrylhydrazil (DPPH) *in vitro*. Une évaluation générale de la cytotoxicité de 16 endophytes sélectionnés ainsi que de leurs plantes hôtes a été réalisée selon le test MTT sur trois types de cellules cancéreuses : carcinome du sein humain (MCF7), adénocarcinome du côlon (HT29 et HCT116). Ces extraits ont été aussi testés, selon la méthode de dilution en bouillon, sur deux souches bactériennes représentatives, *Escherichia coli* et la souche résistante à la méthicilline de *Staphylococcus aureus*. La teneur en phénols totaux (89,9 ±7,1 mg Equivalent d'Acide Gallique EAG/g) ainsi que l'activité antioxydante (IC_{50}: 18±0,1 µg/mL) les plus élevées ont été observées pour l'endophyte, *Aspergillus terreus* 2 isolé à partir des graines de *T. foenum-graecum*. *Byssochlamys spectabilis* a montré l'activité cytotoxique la plus importante (1,51 ± 0,2 µg/mL), suivi par *Cladosporium cladosporioides* 2 (10,5 ± 1,5 µg/mL), puis par *Alternaria* sp. (13,5 ± 1,8 µg/mL). Seules six souches ont montré une activité contre *S. aureus* avec des valeurs de MIC qui se situent entre 0,125 et 2 mg/mL dont: *Alternaria alternata* (0,125 mg/mL), *Alternaria* sp. (0,250 mg/mL), *Byssochlamys spectabilis* (0,5 mg/mL). 10 composés purs (0,3 à 40 mg) ont été isolés à partir des extraits bruts d'acétate d'éthyle de *Curvularia papendorfii.* Le nouveau composé pur (AFB)3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (acide Khartomique) a montré une activité antibactérienne modérée contre *S. aureus* avec une CIM de 62,5 µg/mL et une faible activité cytotoxique sur les cellules MCF7 avec une IC_{50} > 100 µM. Le composé pur AF1 a montré une activité cytotoxique modérée sur les cellules HT29 avec une IC_{50} de 29,78 µM et une très faible activité antibactérienne contre *S. aureus*. Ces deux composés ne présentent pas d'activité antioxydante.

Mots-clés : Plantes médicinales soudanaises, champignons endophytes, *Vernonia amygdalina*, *Curvularia papendorfii*, activité antibactérienne, cytotoxicité.

Abstract

This study investigated, for the first time, the endophytic fungi flora of five Sudanese medicinal plants: *Calotropis procera* (Ait.), *Catharanthus roseus* (L.), *Euphorbia prostrata* (Ait.), *Trigonella foenum-graecum* (L.) and *Vernonia amygdalina* (Del.). A total of 23 endophytic fungal strains were isolated from the plants after surface disinfection and different biological tests were performed. Total phenolic content (TPC) and total antioxidant activity of ethyl acetate crude extracts of 21 endophytes and their host plants were estimated using respectively the Folin-Ciocalteu colorimetric method and 1,1,-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging *in vitro* method. General evaluation of the cytotoxicity of 16 selected endophytes and their host plants was performed by the MTT assay using cancer cells type: Human breast carcinoma (MCF7) and Colon adenocarcinoma (HT29 and HCT116). Preliminary antibacterial screening was done for the 16 endophytes. These extracts were also tested against two representative bacterial strains, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*, by broth dilution tests. The endophyte, *Aspergillus terreus* 1 from *T. foenum-graecum* seeds had the highest TPC in term of Gallic Acid Equivalent (89.9 ± 7.1 mg GAE/g) and antioxidant activity (IC_{50}: 18±0.1µg/mL). *Byssochlamys spectabilis* showed strong cytotoxicity (1.51 ± 0.2 µg/mL) followed by *Cladosporium cladosporioides* 2 (10.5 ± 1.5 µg/mL), then *Alternaria* sp. (13.5 ± 1.8 µg/mL). Only six strains showed activity against methicillin-resistant *S. aureus* with MIC values ranging between 0.125-2 mg/mL, *Alternaria alternata* (0.125 mg/mL) *Alternaria* sp. (0.250 mg/mL) and *Byssochlamys spectabilis* values (0.5 mg/mL). Ten pure compounds (0.3 to 40 mg) were isolated from ethyl acetate crude extract of *Curvularia papendorfii* .The new pure compound (AFB) 3,7,11,15-Tetrahydroxy-18-hydroxymethyl-14,16,20,22,24-pentamethyl-hexacosa-4E,8E,12E,16,18-pentaenoic acid (Khartoumic acid) revealed moderate antibacterial activity against *S. aureus* with MIC value 62.5 µg/mL and weak cytotoxicity with a IC_{50} > 100 µM against MCF7 cells. The pure compound AF1 showed moderate cytotoxic activity with IC_{50} value of 29.78 µM against HT29 and weak antibacterial activity with MIC 250 µg/mL against *S. aureus.* Both compounds displayed no antioxidant activity.

Keywords: Sudanese medicinal plants, endophytic fungi, *Vernonia amygdalina*, *Curvularia papendorfii*, antibacterial activity, cytotoxicity.