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Metabolomic Tools to Assess the Chemistry and Bioactivity of Endophytic Aspergillus Strain

Tawfike, A. F.¹²*, Tate, R.¹, Abbott, G.¹, Young, L.¹, Viegelmann, C.¹, Schumacher, M.³, Diederich, M.³, and Edrada-Ebel, R.A.¹*

¹ Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, The John Arbuthnott Building, 161 Cathedral Street, Glasgow G4 0NR, Scotland,
² Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt.
³ Laboratoire de Biologie Moleculaire et Cellulaire du Cancer, Fondation de Recherche Cancer et Sang, Hopital Kirchberg, 9 rue Edward Steichen, L-2540 Luxembourg

Endophytic fungi associated with medicinal plants are a potential source of novel chemistry and biology that may find applications as pharmaceutical and agrochemical drugs. In this study, a combination of metabolomics and bioactivity-guided approaches were employed to isolate anticancer secondary metabolites from an endophytic Aspergillus aculeatus. The endophyte was isolated from the Egyptian medicinal plant Terminalia laxiflora and identified using molecular biological methods. Metabolomics and dereplication studies were accomplished by utilizing the MZmine software coupled with the universal Dictionary of Natural Products database. Metabolic profiling, with aid of multivariate data analysis, was performed at different stages of the growth curve to choose the optimised method suitable for up-scaling. The optimised culture method yielded a crude extract abundant with biologically-active secondary metabolites. Crude extracts were fractionated using different high-throughput chromatographic techniques. Purified compounds were identified by HRESI-MS, 1D and 2D-NMR. This study introduced a new method of dereplication utilising both high-resolution mass spectrometry and NMR spectroscopy. The metabolites were putatively identified by applying a chemotaxonomic filter. We also present a short review on the diverse chemistry of terrestrial endophytic strains of Aspergillus, which has become a part of our dereplication work and this will be of wide interest to those working in this field.

Key words: Endophytic fungi; Aspergillus; dereplication; metabolomics; HRFTMS, NMR

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Introduction

Endophytic fungi associated with medicinal plants represent a potential source of novel chemistry and biology through the production of biologically-active natural products. Endophytes are microbes that inhabit living, internal tissues of plants without causing any immediate, apparent negative effects on their host plants. Endophytic fungi had previously been unobserved as potential sources of bioactive metabolites. This was most likely due to the absence of any sign of fungal colonization in the host plant. However, it was later recognized that plants contain countless, previously unnoticed, unrealized numbers of these microorganisms known as endophytes. This has driven an international scientific attempt to isolate endophytic fungi and to study their natural products. Scientists have since discovered that endophytes can be considered an important area for the discovery of new secondary metabolites. Each individual plant of about 300,000 higher plant species is host to one or more endophytes. It was found that the majority of endophytic species belong to the ascomycete and deuteromycete classes of fungi. In many cases endophytic fungi might be involved in the biosynthesis of plant products; however, they might also be the producers themselves of several groups of new pharmacologically-active and unique secondary metabolites. Moreover, plant-derived endophytes are considered to have an infinite undisclosed reservoir of metabolic diversity. Advanced methods in cultivation and natural products chemistry have provided access to a potentially rich source of novel drug leads with the advantage of efficient production with higher yields at low cost through large-scale cultivation. An area of major interest to us is to study these secondary metabolites as novel anticancer agents and antibiotics.

*Corresponding authors: RuAngelie Edrada-Ebel; Email: ruangelie.edrada-ebel@strath.ac.uk Phone: +44141 548 5968
Ahmed F. Tawfike; Email: ahmedtawfike@gmail.com Phone: +441582938120, Orchid ID: 0000-0003-2061-3093
However, there are other possibilities for these interactions. These include the possibility that the plant provides metabolites needed for the achievement of the life cycle of the microorganism or essentially for its growth and/or self-defense. Moreover, plants provide spatial structure, protection from dehydration, photosynthesis, and the opportunity for the endophyte to spread to the next generation of hosts in the case of vertical-transmission. Studies have shown that endophytes are more likely to be mutualistic when reproducing vertically (systemic) by growing into seeds, and more antagonistic to the host when transmitted horizontally (non-systemic) via spores. Endophytes interact mutually with their host plants mainly by increasing host resistance to herbivores as “acquired plant defenses.” In-vitro models have clearly established the antioxidant potency of extracts of mangrove plants and their associated endophytic fungi, which aids in understanding the mutual association of plants and endophytes against various biotic and abiotic stresses. Endophytes may also increase host fitness, strength, and competitive abilities by increasing nutrient uptake, germination success, resistance to dehydration, resistance to seed predators, tolerance to heavy metals and to high salinity, and growth rate by evolving biochemical pathways to produce plant growth hormones. Moreover, endophytes are able to promote the biological degradation of the dead host plant that starts the vital steps of nutrient recycling.

Aspergillus endophytes

The genus *Aspergillus* is one of the most common endophytic fungi found to be associated with both marine and terrestrial hosts. Endophytic *Aspergillus* have been described to occur in liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, plants and marine invertebrates from the arctic tundra to the tropics. In this paper, we present a short review of secondary metabolites that have been described for endophytic *Aspergillus* covering the period 2009-2015. Along with the short review, we have performed a metabolomics study of *Aspergillus aculeatus*, isolated from the leaves of *Terminalia laxiflora*, to demonstrate dereplication techniques in order to assess its chemistry that links to the biological activity of more specific secondary metabolites.

Bioactive secondary metabolites from Aspergillus

Taxol (1) is one of the most famous anticancer compounds isolated from natural sources. The diterpenoid was first isolated from the bark of *Taxus brevifolia* Nutt. and is still mainly extracted from the bark of yews even now. However, due to the slow growth and rarity of the trees, this method is unable to fill the increasing demand for taxol in the market. Alternative methods of taxol production have been studied, including complete chemical synthesis, semi-synthesis, and *Taxus* spp. plant cell culture. Since 1993, it has been discovered that fungi, such as *Taxomyces andreanae* and *Pestalotiopsis microspora*, also produce taxol. Currently, more than thirty taxol-producing fungi are known, the majority of which are *Taxus* endophytes belonging to ascomycetes and imperfect fungi. Recent examples of these strains are *A. candidus* MD3, isolated from the inner bark of *Taxus x media*, and *A. niger* var. *taxi*, from *Taxus cuspidata*.
A. niger MA-132, an endophytic fungus isolated from the fresh tissue of the marine mangrove 
Avicennia marina, yielded eight α-pyrone derivatives, namely, nigerapyrones A-E (2-6) and 
nigerapyrones F-H (7-9), as well as two congeners, asnipyrones B (10) and A (11). Nigerapyrone E (6) 
showed cytotoxicity against SW1990 (human pancreatic adenocarcinoma), MDA-MB-231 (breast 
cancer), and A549 (human lung carcinoma) cell lines (IC50 values of 38, 48 and 43 µM, respectively). 
It also possessed weak or moderate activity against MCF-7 (breast cancer), HepG2 (human liver 
cancer), Du145 (human prostate cancer) and NCI-H460 (human lung cancer) cell lines with IC50 
values of 105, 86, 86, 43, and 48 µM, respectively. Nigerapyrone B (3) showed selective activity 
against HepG2, asnipyrone A (11) exhibited activity against the A549 cell line, and nigerapyrone D (8) 
possessed moderate or weak activity against the MCF-7, HepG2, and A549 cell lines with IC50 values 
of 121, 81, and 81 µM, respectively. Aspergillus tubingensis was one of 49 endophytic fungi 
obtained from Dragon’s blood samples from Dracaena sp. They were investigated for antimicrobial 
and antitumor activity against six pathogenic microbes and five tumor cells. A. tubingensis showed 
activity against S. aureus and C. albicans, as well as having inhibition rates of 60-80% against breast 
cancer cells MCF7, 40-60% against ovarian cancer cells and 10-20% against human embryonic kidney 
cells 293-T. 9-Deacetoxyfumigaclavine C (12) was isolated from the culture of Aspergillus 
fumigatus from the stem of Cynodon dactylon, and showed selectively potent cytotoxicity against 
human leukemia cells (KS62) with an IC50 value of 3.1 µM.
A culture extract of *Aspergillus flavipes*, an endophytic fungus associated with *Acanthus ilicifolius*, gave upon fractionation five cytochalasins, Z16–Z20 (13-17), and three previously described compounds (18-20). Cytochalasin Z17 (14) and rosellichalasin (20) were cytotoxic against A549 cell lines, with IC₅₀ values of 5.6 and 7.9 µM, respectively. Moreover, 14 was isolated from the liquid culture of the endophytic fungus *Aspergillus terreus* IFB-E030 from the stem of *Artemisia annua* L. (Asteraceae), and also showed moderate cytotoxicity against human nasopharyngeal epidermoid tumor KB cell line with an IC₅₀ value of 26.2 µM.
Rubasperone D (21) a dimeric naphtho-γ-pyrone, together with the monomeric naphtho-γ-pyrone TMC-256 A1 (22), was isolated from the mangrove endophytic fungus *Aspergillus tubingensis* from the radix of *Pongamia pinnata* from the South China Sea in Guangxi Province, P. R. China. The cytotoxicity was tested against MCF-7, MDA-MB-435, Hep3B, Huh7 (human liver cancer cells), SNB19 (human glioblastoma cells), and U87 MG (human primary glioblastoma cells) \[25, 26\]. TMC 256 A1 (22) exhibited inhibitory activity against these six cancer cells with IC₅₀ values between 19.92 and 47.98 μM. However, Rubasperone D showed mild cytotoxicity to MCF-7 and U87MG cells with IC₅₀ 97.9 and 61.9 μM, respectively.

*Aspergillus ochraceus* ATCC 1009 resting cells were applied in the biotransformation of Sch-642305, a compound produced by *Phomopsis* sp. *CMU-LMA*, to yield three previously undescribed derivatives (23-25). Compound 23 showed antimicrobial activity against Gram-negative bacteria. Furthermore, all of the derivatives were cytotoxic against various cancer cell lines; however, it was notable that compound 24 possessed an IC₅₀ of 4 nM against human myelogenous leukemia K562, whereas the IC₅₀ of the parent compound Sch-642305 was 20 nM \[27\].

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One uncommon thiophene compound (26) was isolated from the mangrove endophyte *Aspergillus terreus* (No. GX7-3B), alongside anhydrojavanicin (27), 8-O-methylbostrycoidin (28), 8-O-methyljavanicin (29), botryosphaerone D (30), 6-ethyl-5-hydroxy-3,7-dimethoxynaphthoquinone (31), 3β,5α-dihydroxy-(22E,24R)-ergosta-7,22-dien-6-one (32), 3β,5α,14α-trihydroxy-(22E,24R)-ergosta-7, 22-dien-6-one (33), NGA0187 (34) and beaevericin (35). Compounds 27, 28, 34 and 35 inhibited α-acetylcholinesterase (AChE). Moreover, compounds 32 and 35 were cytotoxic against MCF-7, A549, HeLa (cervical cancer) and KB (oral cancer) cell lines with IC$_{50}$ values 4.98 and 2.02 (MCF-7), 1.95 and 0.82 (A549), 0.68 and 1.14 (Hela), and 1.50 and 1.10 µM (KB), respectively.

Malformin A1 (36) was characterized from the extract of *Aspergillus niger*, an endophytic fungus from the Chinese liverwort *Heteroscyphus tener* (Steph.) Schiffn. Malformin A1 showed significant cytotoxic activities against the human ovarian carcinoma A2780 (IC$_{50}$ 0.14 µM), lung cancer H1688 (IC$_{50}$ 1.02 µM), K562 (IC$_{50}$ 0.13 µM), M231 (IC$_{50}$ 0.45 µM), and prostate cancer PC3 (IC$_{50}$ 0.14 µM) cell lines in vitro.

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Asperfumigatin (37), isochaetominine (38), and 8'-O-methylasterric acid (39), together with other compounds, were purified from the culture of *Aspergillus fumigatus*, also an endophytic fungus from the Chinese liverwort *Heteroscyphus tener* (Steph.) Schiffn. Compounds 37 and 38 showed significant anticancer activity against PC3 (prostate cancer) cell line with IC$_{50}$ 30.6 and 32.2 μM, respectively. Among the isolated compounds was trypacidin (40) which displayed activity against PC3 with IC$_{50}$ 19.9 μM. Trypacidin also exhibited anticancer activity against PC3D (multiple drug resistance cells), A549 and NCI-H460 with IC$_{50}$ 39.6, 33.8 and 31 μM, respectively. The sweet potato endophyte *Aspergillus fumigatus* sp. isolate R7 yielded fumiquinazoline-F (41) and fumiquinazoline-D (42), both of which exhibited strong cytotoxic activity in brine shrimp assay. They were both also active against the Gram-positive *Bacillus subtilis* (12, 15 mm zone of inhibition), *Staphylococcus aureus* (12, 15 mm) and fungi (*Candida albicans* [11, 11 mm] and *Mucor miehi* [12, 13 mm]).
Nigerasterols A and B (43 and 44, respectively) were the first 5,9-epidioxy-sterol compounds to be isolated from a marine derived mangrove. Preliminary results showed that they possessed potent activity against tumor cell lines HL60 with IC\(_{50}\) 0.3 and 1.50 µM, respectively. Both 43 and 44 displayed potent activities against A549 cancer cell line with IC\(_{50}\) 1.82 and 5.41, respectively. They were isolated from the culture extract of Aspergillus niger MA-132, a fungal endophyte of the mangrove Avicennia marina [32]. The endophytic fungus Aspergillus terreus MHL-P22, isolated from the leaves of Malus halliana, produced two furandiones, asperterone B (45) and C (46), in liquid culture. Compounds 45 and 46 exhibited moderate cytotoxicity against human colorectal carcinoma SW1116 cells with IC\(_{50}\) values of 57.5 and 71.0 µM, respectively [33]. 6-Methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene-11-one-5,6,7,8-tetralene-7-acetamide (47), was isolated from an Aspergillus spp. endophyte from the seeds of Gloriosa superba and was found to possess anticancer activity comparable to the standard drugs Mitomycin-C and 5-FU when tested against six cancer cell lines. Compound 47 significantly inhibited MCF-7 and THP-1 (leukemia) cell lines with percent of inhibition 70% and 80%, respectively at 100 µg/mL. It also showed significant antimicrobial activity against S. aureus, B. subtilis, E. coli, S. cerevisiae, C. albicans, and C. gastricus with MIC 25, 50, 100, 25, 12.5, and 50 µg/mL, respectively [34]. Another endophyte, Aspergillus niger PN2, was isolated from the healthy tissues of Taxus baccata. It showed the ability to produce high quantities of lovastatin. The isolated fungal lovastatin exhibited strong cytotoxic activity in vitro against a test culture of human cancer cells (HeLa and HepG2) with IC\(_{50}\) 23.58 and 39.77 µM, respectively [35].
A cadinane-type sesquiterpenoid, aspergillusone D (48), was isolated for the first time from the endophytic fungus Aspergillus clavatus. Compound 48 showed cytotoxic activity against two cell lines (MCF-7 and A549) with IC$_{50}$ values of 5.9 and 0.2 µM, respectively. 12,13-Dihydroxyfumitremorgin C (49) and verruculogen (50) were produced by the fungal endophyte Aspergillus fumigatus and showed cytotoxic activity against the HepG2 cell line with IC$_{50}$ values of 4.5 µM and 9.8 µM, respectively. Terrephenols A and B (51 and 52), were isolated from the fungus Aspergillus terreus. Compounds 51 and 52 were cytotoxic against the A549 and MCF7 cell lines. 51 recorded IC$_{50}$ values of 4.2 and 3.6 µM against A549 and MCF7, respectively, and 52 showed IC$_{50}$ values of 3.9 and 4.8 µM against the same cell lines.
Aspergillus fumigatus Fresenius, was isolated from Juniperus communis L. Horstmann. It was found to be a novel source of deoxypodophyllotoxin (53), an anti-cancer pro-drug. It has displayed antimicrobial activity against the Gram-positive bacterium S. aureus subsp. aureus (DSM 799), and Gram-negative bacteria K. pneumoniae subsp. ozaenae (DSM 681) and P. aeruginosa (DSM 1128), but not E. coli (DSM 682) which was not at all susceptible to either podophyllotoxin or the fungal deoxypodophyllotoxin at the concentrations tested. Zone of inhibition, for deoxypodophyllotoxin, was recorded as 11.17 mm (DSM799), 8.34 mm (DSM681) and 7.67 mm (DSM1128).

Aspergillus fumigatus LN-4, an endophyte isolated from the stem bark of Melia azedarach, produced 39 fungal metabolites, among which were two previously undescribed alkaloids, 12β-hydroxy-13α-methoxyverruculogen TR-2 (55) and 3-hydroxyfumiquinazoline A (57). Sixteen of these compounds displayed potent antifungal activities; four of them, 12β-hydroxy-13α-methoxyverruculogen TR-2 (55), fumitremorgin B (56), verruculogen (50), and helvolic acid (54), showed antifungal MIC values of 6.25–50 μg/mL. These were similar to the MIC values of carbendazim and hymexazol, which were used as the positive controls. Compounds 56 and 50 exhibited significant toxicities toward brine shrimps and displayed the best antifeedant activity against armyworm larvae.

Aspergillus niger is one of thirteen different endophytes isolated from Tabebuia aregntea. Screening of the culture extract showed that Aspergillus niger yielded saponins, phenolics, anthraquinones, steroids, cardiac glycosides and tannins. The culture of A. niger showed the strongest antioxidant activity, correlating to its significant concentrations of phenolics, as well as...
significant antimicrobial activity against a variety of pathogenic fungi and bacteria [41]. The alkaloids fumigaclavine C (58) and pseurotin A (59) were obtained from endophyte Aspergillus sp. EJC08 isolated from the medicinal plant Bauhinia guianensis. Both are broad-spectrum antibacterial agents however fumigaclavine C was the most active against B. subtilis showing MIC of 7.81 mg/mL. While pseurotin showed MIC 15.62 mg/mL [41]. The mangrove endophyte Aspergillus sp. 16-5c produced a sesterterpenoid, asperterpenoid A (60). This compound inhibited the Mycobacterium tuberculosis protein tyrosine phosphatase B (mPTPB) with an IC₅₀ value of 2.2 μM [42].

Aspergillus tamarii was isolated from the leaves of Ficus carica L., and was found to produce several indolyl diketopiperazines (56, 50, 49, 61, 62 and 63). These compounds possessed antiphytopathogenic activity, implying that the endophyte may play a role in guarding the host through the production of these compounds. They were identified as fumitremorgin B (56), verruculogen (50), fumitremorgin C (49), cyclotryprostatin B (61), tryprostatin A (62) and tryprostatin B (63) [43]. Furthermore, compounds (49, 50, 56 and 61) and fumiquinazoline J (64) were also isolated from the extract of the culture of Aspergillus fumigatus, which was isolated from the root of Astragalus membranaceus. The purified compounds were assayed for antifungal and antibacterial activity. Compounds 49, 50, 56 and 61 possessed antimicrobial activity; however, the most significant finding was that compound 64 was comparable in activity to gentamicin with MIC 1 μg/mL against B. subtilis, S. aureus, E. coli and S. typhimurium. While it was more potent than nystatin with MIC 2, 4 and 2 μg/mL against fungi C. albicans, P. chrysogenum and F. solani, respectively. [44].
Aspergillus aculeatus, was one of fifteen bioactive fungal endophytes isolated from Garcinia species, showed anti-TB, antiplasmodium, antioxidant and anti-proliferation against human small-cell lung cancer cells (NCI-H187). Aspergillumarins A and B (65, 66), two dihydroisocoumarin derivatives, were produced by an Aspergillus sp., isolated from the fresh leaf of the mangrove Bruguiera gymnorrhiza from the South China Sea. They were weak antibacterial against S. aureus and B. subtilis at 50 µg/ml. The five compounds pseurotin A (59), 14-norpseurotin A (67), FD-838 (68), and pseurotin D (69), and fumoquinone B (70), were isolated from Aspergillus sp. strain F1544 that was isolated from a mature leaf of Guapira standleyana (Nyctaginaceae). The compounds were all active against Leishmania donovani with IC₅₀ 5.8, 4.4, 0.2, 0.5 and 0.5 µg/mL, respectively.

Aspernolides F (71) and G (72), two butyrolactones, were isolated for the first time from the endophyte Aspergillus terreus from the roots of Carthamus lanatus (Asteraceae). 71 was active against C. neoformans and MRSA with IC₅₀ values of 5.19 and 6.39 µg/mL, respectively. Five ergot alkaloids, fumigaclavines D-H (73-77), were isolated from the endophytic Aspergillus fumigatus. Compounds 75 and 77 were the most active against Veillonella parvula, an anaerobic bacterium, with an MIC of 16 µg/mL.
2-Phenylethanol was characterized from the cultural extract of *Aspergillus niger*. It constitutes approximately 4.06% of rose oil. It also has antiseptic, disinfectant, antimicrobial, and preservative properties [51]. The mycelial extract of *Aspergillus* sp. from the leaves of *Writhtia tinctoria* displayed a great antibacterial activity against *Pseudomonas fluorescens* and *P. aeruginosa*. FTIR analysis indicated the presence of alkaloids [52]. *Aspergillus* sp. JPY1, *Aspergillus* sp. JPY2, and *A. niger* were isolated from *Salvadora oleoides* Decne. The extracts of these endophytes were highly active against three pathogenic *Aspergilli* sp. [53]. *Aspergillus* sp. and 24 other endophytic fungi were isolated from nineteen plants from different areas in Panama. It was tested for anti-parasitic activity and showed > 70% inhibition toward *Leishmania donovani* [54].

Chitosan (78), a polysaccharide found within the cell walls of most species of fungi, has been extracted from endophytic *A. flavus* from different medicinal plants. It finds use in a wide range of applications, including cosmetics, agriculture, and the pharmaceutical and food industries. It also has natural antibacterial and antifungal properties, allowing it to be used in commercial disinfectants [55]. *As1-1*, a homogeneous exo-polysaccharide, was isolated from a mangrove endophytic fungus *Aspergillus* sp. Y16. It showed good *in vitro* antioxidant activity in 1,1-diphenyl-2-picrylhydrazyl...
(DPPH) and superoxide radical scavenging assays. As1-1 was primarily composed of mannose with small quantities of galactose

![Chemical structure of mannose and galactose](image)

**Huperzia serrata** produces the cholinesterase inhibitor (ChEI) huperzine A (HupA) (79). *A. niger* was one of 127 endophytic fungi isolated from the roots, stems, and leaves of *H. serrata* and screened for HupA. *A. niger* showed acetylcholinesterase (AChE) inhibition *in vitro* and was verified as a producer of HupA using various chromatographic techniques

![Chemical structure of huperzine A](image)

The endophytic *Aspergillus* sp. YXf3 from the leaf of *Ginkgo biloba* produced six p-terphenyl derivatives, 4"-deoxy-3-hydroxyterphenyllin (80), 4"-deoxy-5'-desmethylterphenyllin (81), 5'-desmethylterphenyllin (82), 4"-deoxycandidusin A (83), 4,5-dimethoxycandidusin A (84), and terphenolide (85), five previously known p-terphenyl compounds (86-90), and four previously undescribed diterpenoids with norcleistanthane (aspergiloid A (91) and aspergiloid B (92)), cleistanthane (aspergiloid C (93)), and isopimarane (aspergiloid D (94)) type skeletons. Compounds 83, 84, 85 and 88 exhibited moderate neuraminidase inhibitory activity and thus may work as antiviral agents

![Chemical structure of terphenyllin derivatives](image)
A. *tubingensis* (GX1-5E), a mangrove endophyte, produced three dimeric naphtho-γ-pyrones, named rubasperone A (95), rubasperone B (96), and rubasperone C (97). Moreover, two compounds, rubrofusarin (98) and rubrofusarin B (99), were also isolated. Rubrofusarin showed moderate tyrosinase inhibitory activity with an IC$_{50}$ value of 65.6 µM, whereas rubasperone C possessed mild α-glucosidase inhibitory activity with an IC$_{50}$ value of 97.3 µM$^{[25]}$. Asperterpenol A (100) and asperterpenol B (101), two sesterterpenoids, were isolated from a mangrove endophytic fungus *Aspergillus* sp. 085242, and significantly inhibited acetylcholinesterase with IC$_{50}$ values of 2.3 and 3.0 µM, respectively$^{[59]}$.

16α-hydroxy-5-N-acetylardeemin (102), a previously undescribed alkaloid, and seven other compounds (103-109), were isolated from the fermentation broth of an endophytic *A. terreus*. Compound 102 inhibited acetylcholinesterase. Compounds 103-109 were moderately or weakly...
cytotoxic against KB and HSC-T6 cell lines. Moreover, compounds 102, 103, 104 together with 110 were isolated from the fermentation broth of the endophyte *Aspergillus fumigatus* SPS-02 from *Artemisia annua* L. An in vitro chemosensitization assay revealed the varying activities of these ardeemins in the reversal of the multidrug-resistant (MDR) phenotype in three cancer cell lines, leukemia doxorubicin resistant cell K562/DOX, human lung adenocarcinoma cisplatin resistant cell A549/DDP, and ovarian cancer cisplatin resistant cell SK-OV-S/DDP. Compound 102 had the strongest MDR reversing effect at 5 μM concentration in K562/DOX and A549/DDP cell lines, while compound 104 showed the highest reversal capacity in the SK-OV-S/DDP cell line.

The methanol extract of *Aspergillus* sp. JPY2 isolated from *Salvadora oleoides* Decne (Salvadoraceae) significantly reduced blood glucose levels in glucose-loaded fasting and alloxan-induced diabetic Wistar albino rats. This was due to presence of phenol-2,6-bis (1, 1-dimethylethyl)-
4-Me in the active fractions. Compounds 111-115, isolated from culture extract of endophytic A. fumigatus from the leaves of Trifolium repens, significantly inhibited the secretion of IL-6 at 10 µM. Furthermore, compounds 112-115 significantly reduced collagen IV and fibronectin production at 10 µM. Anthraquinone derivatives may therefore be potential anti-DN (Diabetic Neuropathy) lead compounds.

Terrenolide S (116), a butenolide derivative, was isolated from the endophytic fungus Aspergillus terreus obtained from the roots of Carthamus lanatus (Asteraceae). 116 possessed anti-leishmanial activity against L. donovani with an IC₅₀ and IC₉₀ of 27.27 µM and 167.03 µM, respectively.

Avertoxin B (117) is an active inhibitor against human acetylcholinesterase with an IC₅₀ value of 14.9 µM. It was isolated from the fermentation culture of Aspergillus versicolor Y10, an endophytic fungus accompanied with Huperzia serrata. A previously described alkaloid, pseurotin A (59), was isolated from the endophytic fungus Aspergillus fumigatus. Pseurotin A inhibited the lipopolysaccharide-induced proinflammatory factors in BV2 microglial cells with an IC₅₀ indirectly resulting in anti-inflammatory activity. 1,7-dihydroxy-8-(methoxycarbonyl) xanthone-3-carboxylic acid (118) was a potent inhibitor of α-glucosidase (IC₅₀ value of 0.24 mM vs. 0.38 mM for acarbose). 118 was produced by Aspergillus versicolor, which was isolated from of Huperzia serrata. Aspergillines A-E (119-123) were isolated from the endophyte Aspergillus vesicolor. They were
anti-TMV and cytotoxic. Aspergillines A-E were tested for their cytotoxic activities against NB4 (promyelocytic leukemia), SH-SYSY (neuroblastoma), PC3, MCF7 and A549 cell lines. Compounds 119, 121 and 122 exhibited moderate cytotoxicity in all cell lines, with IC_{50} values ranging from 1.2 to 4.2 μM, whereas 120 and 123 displayed moderate cytotoxicity with IC_{50} values of <10 μM in the four cell lines.

Metabolomics

Metabolomics is the technology designed to provide general qualitative and quantitative profiles of metabolites in organisms exposed to different conditions. Plants and microorganisms produce a large number of metabolites with different activities and behaviors under stress conditions. Metabolomics provides extra information to analyse these complex relationships and explore the production of natural products to aid the search for novel compounds. Metabolomics provides a holistic overview of the biochemical status of biological systems. The metabolome is the complete set of small molecules found in a cell, tissue or organism at a certain point in time. Metabolomics is considered as the most functional approach in monitoring gene function and identifying the biochemical status of an organism.

Metabolomics applications and techniques

Metabolomics is applied in many aspects of natural drug discoveries, particularly in bioactivity screening to improve dereplication and identification procedures. Fast dereplication of known compounds and identification of lead bioactive metabolites is important in the primary stages of metabolomics profiling prior to intensive isolation work. Different analytical techniques are applied followed by multivariate data analysis (MVDA) to minimize the redundant analysis in isolation steps of active compounds from the natural source. Metabolomics has also been used to evaluate the bioavailability, toxicity and efficacy of natural compounds. This advancement in standard analysis...
associates chemical profile to bioactivity directly on the workflow; consequently, the activity of the individual compounds can be measured separately. The use of metabolomics is a good means to guide the isolation of compounds, as well as to help improve the productivity of downstream fermentation methods. Metabolomics provides statistical and computational tools to the standard approach of rapid HPLC fractionation, thus identifying the active entities at an earlier stage. The goal of HPLC fractionation is to obtain the maximum purity of active components which, however, is not achievable in the initial chromatographic isolation work. Metabolomics allows the determination of the active components during the initial fractionation. The functional groups contributing to bioactivity can also be identified, using an NMR-metabolomic PCA (principal component analysis) software such as ALICE. It also assists in prioritizing fractions for purification work. Metabolomics is used for quality control of the natural products and isolates to monitor different metabolic profiles; for example, the detection of herbal adulteration with similar species that have lower levels of the active compounds. It can also be used to monitor environmental alterations during growth and harvesting, post harvesting treatment, extraction and method of isolation, all of which can affect the efficacy of natural products. Metabolomics might help to get a better view of the mode of action of natural products and lead to the possibility of gaining proof of their pharmacological activity over different batches. This is based on considering the bulk of chemical constituents rather than focusing on certain individual components or groups of compounds. Metabolomics can be used to detect biomarkers and/or precursors to dereplicate the biosynthesis of the natural product at different development stages of their biological source as well as simultaneously screen for the bioactivity. By using combinations of different analytical methods, the bioassay-guided isolation route is getting shorter and rapid dereplication of known activities is rapidly delivered. Macros have been developed to facilitate the detection of the production of interesting secondary metabolites during microbial cultivation. This would assist in maintaining or enhancing biosynthesis of the desired compounds. These algorithms are coupled to differential expression analysis software like SIEVE and MZmine, web-based software developed by VTT (Technical Research Centre of Finland), which can then integrate the results to an in-house database that includes MarinLit and Antibase to further identify microbial secondary metabolites.

Metabolomics has a variety of applications and plays a significant role in systems biology, allowing the identification of potential lead compounds and the monitoring of the environmental conditions within fermentation systems to facilitate the expression and recovery of these metabolites.

Results and discussion

Plant materials, isolated endophytes and biological activity

Five endophytic fungi were isolated from four plant species (Table 1) collected from Al-Zohrya gardens, Zamalek, Cairo, Egypt. Four of the five endophytes belonged to Ascomycota; these were Aspergillus aculeatus, Curvularia sp., A. oryzae and A. flocculus, isolated from four Egyptian medicinal plants Terminalia laxiflora, Dracaena deremensis, Tabebuia argentea and Markhamia platycalyx, respectively. The endophytes were identified using molecular biology procedures by comparison of the isolated DNA sequence with those found in the gene bank. All of them showed 100% similarity, using a

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PCR program previously described by Rychlik and co-workers (Rychlik et al., 1990). For A. flocculus, which only showed 93% similarity, a touchdown PCR (Don et al., 1991) was employed. The touchdown PCR was used since impurities (DNA from other sources) were found. Thus, the primers avoided amplifying nonspecific sequences. By increasing the annealing temperature, only very specific base pairing between the primer and the DNA template was allowed. The primers were ITS and β-tubulin which are commonly used in the identification of the Ascomycetes. Moreover, specially designed primers AT1-F and AT1-R were used for A. oryzae due to the very close similarity in DNA sequence to A. flavus. Only one endophyte was found to belong to Zygomyctota, and that was Syncephalastrum racemosum, isolated from stem of M. platycalyx. S. racemosum was identified using an ITS primer and Zygomyctota primers V9D and LS266 employing the previously described PCR program (Rychlik et al., 1990).

The isolated endophytes showed remarkable anticancer activity against prostate cancer (PC3) and/or chronic myelogenous leukemia (K562) cell lines, except for Curvularia sp. (code: Ter-AL-F2a) which exhibited no significant anticancer activity against either cancer cell line. In addition, S. racemosum (code: Markh-ST-F3a, F3b) showed good activity against Trypanosoma brucei brucei.

Metabolomic and Dereplication studies for *Aspergillus aculeatus*

**Dereplication studies using NMR spectroscopy**

The $^1$HNMR spectra of *A. aculeatus* rice and liquid culture extracts (Fig1a, b) showed that the rice culture extract exhibited more resolved peaks in the aromatic region with the presence of a broad peak around 12 ppm indicating the presence of a hydrogen-bound phenolic moiety such as those found in flavonoids or anthraquinones (Fig1b).

**Dereplication studies using HR-MS**

The HRESI-MS data of both rice (RC) and liquid culture (LC) extracts of *A. aculeatus* have been subjected to a metabolomics analysis. This included data processing using MZmine 2.10 for peak detection and deconvolution using the local minimum search algorithm, deisotoping, alignment, gap filling, adduct and complex searching, molecular formula prediction and finally customized database search tools. Antibase® and/or Dictionary of Natural products (DNP) databases were employed to dereplicate the detected metabolites in both RC and LC extracts. Comparison of the total ion chromatogram of both showed presence of more peaks in the RC extract (Fig1c). The scatter plot (Fig1d) of the RC extract against the LC extract, showed more abundancy of the metabolites in the RC side of the diagonal, which was represented by the increase in density of the blue dots below the diagonal. This confirmed that *A. aculeatus* is producing more metabolites in RC culture than LC. The comparison showed the similarity and differences in the produced metabolites between both extracts. The only limitation of MZmine 2.10 was that the positive and negative ionization mode could not be combined. Hence a CSV file for each mode was exported from the MZmine and imported simultaneously into a macro written in MS-Excel which enables the combination of both modes, allowed further clean-up and database searching. Searching the Antibase® and DNP databases for the metabolites found in the RC and LC extracts of *A. aculeatus* as well as the ethyl acetate and BuOH extracts of *Terminalia* leaves, from which the endophyte was isolated, showed that most of the isolated metabolites have been previously identified (Table 2). Some of the metabolites produced by the
endophyte were also found at very low intensities in EtOAc and/or BuOH extracts of *Terminalia* leaves. Examples are the metabolites of ion peaks at m/z (retention time in minutes) 436.198 [M+H]+ (9.95 min), 381.155 [M+H]+ (9.57 min), 180.102 [M+H]+ (4.33 min) and 405.229 [M+H]+ (9.00 min) which were identified as neoxaline, dinaphtho[2,1-b:1',2'-d] furan-5,9-dion, phenylalanine-N-Me and phalarine respectively, as shown in Table 2. Neoxaline has been isolated previously from *Aspergillus japonicus* [78], (2,4,6a,10,12-pentahydroxy-6,6a-dihydrodinaphtho [2,1-b:1',2'-d]furan-5,9-dione was produced by *Sphaeropsis* sp. [79] and phalarine was characterized from the extract of the plant *Phalaris coerulescens* [80]. In Table 2, the peak areas of the produced metabolites were shown, and confirmed that metabolites produced at higher concentration in the fungal extracts were found at lower concentration in the plant extracts. This demonstrated two facts; first, the endophyte can produce the same metabolites as the host plant but in larger quantities and with the advantage of sustainability, and the second is allocation of the fungal metabolites in the plant extracts is proof of the biological source of the endophyte. Moreover, the metabolites at m/z 436.198 [M+H]+ (9.95 min) and 478.270 [M+H]+ (11.01 min) which were putatively identified as neoxaline and paraherquamide E respectively, were produced at higher concentrations by the LC. Meanwhile, metabolites at m/z 639.171 [M+H]+ (19.22 min), 235.119 [M+H]+ (3.21 min), 381.155 [M+H]+ (9.57 min), 180.102 [M+H]+ (4.33 min) and 405.229 [M+H]+ (9.00 min), putatively identified as secalonic acid D, JBIR-75, dinaphtho [2,1-b:1',2'-d] furan-5,9-dion, phenylalanine-N-Me and phalarine respectively, were found at larger quantities in the 7-day RC extract. This indicated that 7-day RC extract was richer in these metabolites than the other fungal extracts. The corresponding ion peak at m/z 475.325 which eluted at 37.42 min was found to be undescribed in the literature; however, MZmine was able to predict the molecular formula, which was C_{25}H_{46}O_{8}. Furthermore, a heatmap of the RC extracts, generated using the R 2.15.2 software (Fig 2) showed a significant difference in the produced metabolites, represented by the blue lines, between the 7- and 30-day RC extracts. This difference in metabolite pattern is an indication of the diversity in the chemical profile between the two extracts that may be reflected on the biological activity. Moreover, the 15-day RC extract showed a lower abundance of the produced metabolites than both the 7- and 30-day RC extracts. This indicated that the 15-day RC was an intermediate stage in which the metabolites were produced in 7-day culture and utilized by the fungi in the 15-day culture to produce other secondary metabolites in the 30-day culture as a survival mechanism.
<table>
<thead>
<tr>
<th>Plant species/PART</th>
<th>Fungi isolated</th>
<th>PCR program</th>
<th>Primers</th>
<th>Bioactivity of Fungal Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terminalia laxiflora</strong> (Combretaceae)</td>
<td><em>Aspergillus aculeatus</em></td>
<td>Normal</td>
<td>ITS1, ITS4</td>
<td>Prostate cancer cell line (PC-3)</td>
</tr>
<tr>
<td>Leaves</td>
<td><em>(Ter-YL-F1B, Ter-AL-F1)</em></td>
<td>100%</td>
<td></td>
<td>Chronic myelogenous leukemia (K562)</td>
</tr>
<tr>
<td></td>
<td><em>Curvularia</em> sp.</td>
<td>Normal</td>
<td>ITS1, ITS4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Ter-AL-F2, F2a)</em></td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus oryzae</em></td>
<td>Normal</td>
<td>ITS1, ITS4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Ter-AL-F2b)</em></td>
<td>100%</td>
<td>BT2a, BT2b</td>
<td>Prostate cancer cell line (PC-3)</td>
</tr>
<tr>
<td></td>
<td><em>Dracaena deremensis</em> (Agavaceae)</td>
<td><em>Aspergillus oryzae</em></td>
<td>Normal</td>
<td>ITS1, ITS4</td>
</tr>
<tr>
<td>Leaves</td>
<td><em>(Drac-MID, FROZ-F1)</em></td>
<td>100%</td>
<td>BT2a, BT2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Tabebuia argentea</em> (Bignoniaceae)</td>
<td><em>Aspergillus oryzae</em></td>
<td>Normal</td>
<td>ITS1, ITS4</td>
</tr>
<tr>
<td></td>
<td><em>(Tab-leaves F1a, F1b)</em></td>
<td>100%</td>
<td>BT2a, BT2b</td>
<td>Chronic myelogenous leukemia (K562)</td>
</tr>
<tr>
<td></td>
<td><em>Markhamia platycalyx</em> (Bignoniceae)</td>
<td><em>Aspergillus flocculosus</em> or <em>A. ochraceopetaliformis</em></td>
<td>Touch down</td>
<td>ITS1, ITS4</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td><em>(Markh-ST-F1, F2)</em></td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Syncephalastrum racemosum</em></td>
<td>93%</td>
<td>ITS1, ITS4</td>
<td>Anti-trypanosomal</td>
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</table>
**Fig 1a:** $^1$H NMR spectrum of *A. aculeatus* liquid culture (LC) extract, b: $^1$H NMR spectrum of *A. aculeatus* rice culture (RC) extract. The green circle indicates the broad peak which corresponds to a phenolic moiety, c: TIC of both RC (●) and LC (●), d: Scatter plot of RC (X-axis) and LC (Y-axis). The dots represent metabolites. Those lying on the diagonal are present in similar concentrations in both LC and RC extracts, whereas those lying above and below the diagonal are more intense in the LC and RC extracts, respectively.

**Table 2:** Peak area of the metabolites produced by *A. aculeatus* extracts including liquid culture fungal extract (LC), 7- 15-, and 30-day rice culture (RC) fungal extracts, and butanol extract (BuOH ext.) and ethyl acetate extract (EtOAc ext.) of plant leaves. The highlighted rows represent the metabolites produced at lower concentration in the plant extracts. All metabolites represented by [M+H]$^+$

<table>
<thead>
<tr>
<th>m/z</th>
<th>RT</th>
<th>Name/Formula /Source</th>
<th>LC</th>
<th>BuOH ext.</th>
<th>EtOAc ext.</th>
<th>7- day RC</th>
<th>15-day RC</th>
<th>30-day RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>235.119</td>
<td>3.21</td>
<td>C$<em>{12}$H$</em>{14}$N$_4$O$_2$</td>
<td>JBIR-75</td>
<td>7.11E+07</td>
<td>0</td>
<td>1.93E+09</td>
<td>3.03E+07</td>
<td>6.75E+08</td>
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<tr>
<td></td>
<td></td>
<td><em>Aspergillus</em> sp. fS14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180.102</td>
<td>4.33</td>
<td>C$<em>{10}$H$</em>{13}$NO$_2$</td>
<td>Phenyl-alanine-N-Me</td>
<td>1.37E+08</td>
<td>9.83E+03</td>
<td>5.45E+03</td>
<td>2.05E+09</td>
<td>6.27E+06</td>
</tr>
<tr>
<td>405.229</td>
<td>9.00</td>
<td>C_{23}H_{32}O_{6}</td>
<td>Phalarine</td>
<td>Phalaris coerulescens</td>
<td>2.45E+04</td>
<td>4.61E+03</td>
<td>2.02E+03</td>
<td>5.83E+08</td>
</tr>
<tr>
<td>340.233</td>
<td>9.35</td>
<td>C_{19}H_{27}N_{7}</td>
<td>Unknown</td>
<td>5.05E+08</td>
<td>0.00E+00</td>
<td>0.00E+00</td>
<td>8.75E+08</td>
<td>1.18E+09</td>
</tr>
<tr>
<td>203.118</td>
<td>9.39</td>
<td>C_{11}H_{18}N_{2}O</td>
<td>Nb-Acetyl-tryptamine</td>
<td>1.89E+07</td>
<td>0</td>
<td>0</td>
<td>2.97E+09</td>
<td>7.67E+06</td>
</tr>
<tr>
<td>381.155</td>
<td>9.57</td>
<td>C_{10}H_{38}O_{5}</td>
<td>Dinaphtho[2,1-b:1',2'-d] furan-5,9-dione</td>
<td>1.95E+05</td>
<td>2.85E+02</td>
<td>1.96E+03</td>
<td>7.24E+08</td>
<td>3.49E+06</td>
</tr>
<tr>
<td>436.198</td>
<td>9.95</td>
<td>C_{22}H_{29}NO_{8}</td>
<td>Neoxaline</td>
<td>A. japonicus</td>
<td>2.28E+09</td>
<td>0</td>
<td>6.96E+02</td>
<td>1.05E+09</td>
</tr>
<tr>
<td>478.27</td>
<td>11.01</td>
<td>C_{28}H_{32}N_{4}O_{4}</td>
<td>Paraherquamide E</td>
<td>A. aculeatus</td>
<td>1.38E+09</td>
<td>0</td>
<td>0</td>
<td>6.34E+08</td>
</tr>
<tr>
<td>230.248</td>
<td>17.99</td>
<td>C_{14}H_{31}NO</td>
<td>2-Amino-3-tetradecanol</td>
<td>Pseudodistoma obscurum</td>
<td>1.15E+07</td>
<td>0</td>
<td>0</td>
<td>1.10E+09</td>
</tr>
<tr>
<td>639.171</td>
<td>19.22</td>
<td>C_{12}H_{36}O_{14}</td>
<td>Secalonic acid D</td>
<td>A. aculeatus</td>
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<td>0</td>
<td>1.57E+09</td>
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<td>258.279</td>
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<td>0</td>
<td>0</td>
<td>1.84E+09</td>
<td>3.26E+07</td>
</tr>
<tr>
<td>475.325</td>
<td>37.42</td>
<td>C_{25}H_{40}O_{8}</td>
<td>Unknown</td>
<td>3.89E+08</td>
<td>1.54E+03</td>
<td>0</td>
<td>1.16E+09</td>
<td>1.12E+09</td>
</tr>
</tbody>
</table>
Fig2: Heatmap of *A. aculeatus* rice culture extracts in which the blue lines represent the produced metabolites. A greater quantity of blue lines indicates that more metabolites were produced.

**Metabolomic study using SIMCA-P**

*A. aculeatus* extracts vs *Terminalia* leaves extracts

Multivariate data analysis (MVDA) analysis of different culture extracts of *A. aculeatus* and the EtOAc extract of *Terminalia* leaves on SIMCA-P software discriminated the 7- and 30-day RC extracts from each other and from the other fungal and plant extracts as shown in the PCA score plot (Fig3a) which was indicative of the unique nature of the metabolites of both extracts. The PCA loading plot (Fig3b) shows the outlier metabolites expected to belong to each of the extracts, with those correlating to the 7-day RC extract highlighted in red. The 7-day extract of *A. aculeatus* showed significant anticancer activity compared to the other extracts when tested against prostate cancer cell line (PC3) and leukaemia cell lines (K562). The 15-day RC extract showed moderate activity. All other extracts including the 30-day RC showed no activity against both cancer cell lines. The OPLS-DA score plot (Fig3c) showed that both 7-day and 15-day RC extracts were distinguished from the other extracts. The OPLS-DA loading plot (Fig3d) showed the putative metabolites belonging to the 7-day RC extract which were identical to those exhibited in the PCA loading plot. These metabolites corresponded to ion peaks at *m/z* (retention time) 203.118 (9.38 min), 639.171 (18.55 min), 379.141 (10.18), 191.020 (1.94 min) and 235.119 (3.20 min), which are described in the dereplication table (Table 3) as *N*-acetyltryptamine, secalonic acid, dinaphthofurandione, citric acid and JBIR-75, respectively. The dereplication table (Table 3) has been accomplished by searching the DNP database which was built into MS-Excel using an *in-house* macro (Macintyre et al., 2014) using both positive and negative ionization modes in order to compare the predominant metabolites in the highly active 7-day RC extract with the other inactive extracts. The comparison showed that metabolites with corresponding ion peaks at *m/z* 203.118, 379.141 and 405.229 putatively identified as *N*-acetyltryptamine, dinaphthofurandione and phalarine, respectively, were found at higher...
concentrations in the 7-day rice culture extracts. This indicates that these metabolites may be ones responsible for the activity of the 7-day rice culture extract.

**MPLC fractions of 7 days RC extracts**

The most active extract (7-day RC) was fractionated using MPLC. The MPLC fractions were subjected to chemometric analysis using the SIMCA-P+ software. The PCA score plot (Fig4a) showed the outlier MPLC fractions while the PCA loading plot (Fig4b) showed the discriminated metabolites, which are unique and corresponding to these fractions. The putative metabolites obtained in Fig4b were similar to that obtained from the OPLS-DA loading plot of the extracts (Fig3d), confirming the validity of the previous metabolomics model used to compare the active with the inactive fungal extracts and showing the relationship between the discriminated metabolites and the exhibited bioactivity. The active fractions, resulting from the bioassay-guided fractionation of the 7-day RC extract, were compared to the inactive fractions to help in early prediction of the unique metabolites that could be responsible for the bioactivity. The OPLS-DA score plot (Fig4c) showed outlying and clustering of fractions 47-50 and 51-56 (colored in yellow in the plot), which indicated the same unique bioactive metabolites shown in the OPLS-DA loading plot (Fig4d). Both fractions were active against K562 cancer cell line. However, fraction 76, which was also active against K562, has not seen clustered with other fractions (Fig4c), emphasizing that it had different characteristic metabolites. Fractions 15-25, 26, 30-32 and 40-41 (colored in red in the plot), were active against PC3 cancer cell line, and so clustered together in OPLS-DA score plot (Fig4c). It is interesting to note, that fraction 40-41 was found to be singled out in the PCA analysis (Fig4a) which reflected its distinguishing metabolites. The loading scatter plot (Fig4d) showed the putative metabolites related to the active fractions of 7-days RC extract of *A. aculeatus*. They were represented by corresponding ion peak m/z 639.171 [M+H]+, 191.020 [M-H]+, 235.119 [M+H]+ and 379.141 [M-H]+ which were identified in Table 3 as secalonic acid, citric acid, JBIR 75 and dinaphtho[2,1-b:1′,2′-d]furan-5,9-dione, respectively. Fig5 shows the S-line plot overlaid with the structures of the metabolites that were isolated from the extract. These were identical to the metabolites putatively identified using metabolomics tools in the dereplication study and predicted to be responsible for the anticancer activity of the active extracts and/or fractions. Moreover, presence of the citric acid among the putative metabolites as well as the isolated metabolites indicated its importance as a precursor and/or a biomarker in the biosynthetic pathway of the secalonic acid compounds. Secalonic acid was the major anticancer compound in the 7-day rice culture extract of *A. aculeatus*. The compounds were isolated using chromatographic methods as described in supplementary documents and the structures were elucidated using HRESI-MS, MS-MS and 1D, 2D-NMR.
**Fig3a:** PCA score plot of *A. aculeatus* and *Terminalia* leaves extracts, b: PCA loading plot of *A. aculeatus* and *Terminalia* leaves extracts showing the outlier metabolites (●) belonging to 7-day RC extract, c: OPLS-DA score plot of *A. aculeatus* and *Terminalia* leaves extracts whereas (●) Active extracts (●) Moderately active extracts (●) Inactive extracts and d: OPLS-DA loading plot of *A. aculeatus* and *Terminalia* leaves extracts showing the putative metabolites (●) corresponding to 7-day RC extracts.

**Fig4a:** PCA score plot of the fractions from 7-day RC extract of *A. aculeatus* showing the discriminated fractions, b: PCA loading plot of the fractions showing the discriminated metabolites (●), c: OPLS-DA Score plot of the active vs inactive fractions whereas (●) Active against K562; (●) Active against PC3; (●) Active against both; (●) Inactive and d: OPLS-DA loading scatter plot of the active vs inactive fractions showing the predicted metabolites corresponding to the active fractions as (●) active against K562 (●) active against PC3.

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**Fig 5:** S-line of the active (pointed up) vs inactive (pointed down) fractions of 7-day RC extract of *A. aculeatus*.

**Table 3:** Dereplication table prepared using MS-Excel macro and DNP database involving both negative and positive ionization modes. The comparison is based on peak area.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Rt</th>
<th>Polarity</th>
<th>Formula/Name/Source</th>
<th>LC</th>
<th>EtOAc ext.</th>
<th>7-day RC</th>
<th>30-day RC</th>
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<tr>
<td>191.0201</td>
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<td>N</td>
<td>C₆H₆O₇</td>
<td>8.02E+06</td>
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<td>7.47E+07</td>
<td>3.55E+07</td>
</tr>
<tr>
<td>235.1189</td>
<td>3.21</td>
<td>P</td>
<td>C₁₀H₁₄N₂O₂</td>
<td>3.93E+06</td>
<td>0</td>
<td>1.09E+08</td>
<td>4.06E+07</td>
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<tr>
<td>365.1249</td>
<td>7.41</td>
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<td>C₁₃H₁₂O₈</td>
<td>3.13E+05</td>
<td>9.60E+02</td>
<td>7.28E+07</td>
<td>1.42E+08</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>Mass (m/z)</th>
<th>pKa</th>
<th>Charge</th>
<th>Molecular Formula</th>
<th>Common Name</th>
<th>Species</th>
<th>Log P</th>
<th>Log D</th>
<th>Log L</th>
<th>Log S</th>
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</thead>
<tbody>
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<td>405.2291</td>
<td>9.00</td>
<td>P</td>
<td>C$<em>{23}$H$</em>{32}$O$_{6}$</td>
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<td>7.41E+03</td>
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<td>C$<em>{12}$H$</em>{10}$N$_{2}$O</td>
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<td>1.31E+06</td>
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Biological activity

Anticancer activity of A. aculeatus

The cytotoxicity assays for all fungal extracts against the prostate cancer cell line (PC3) and chronic myelogenous leukaemia cancer cell line (K562) (Fig 6a, b) revealed significant anticancer activity against both cancer cell lines for sample numbers 5, 7, 12 and 23 which were represented by the codes Ter-AL-f2b, Ter-YL-F1b, Ter-AL-f1 and Markh-ST-F2a. Samples 5 was identified as A. oryaza, 7 and 12 are A. aculeatus and 23 is A. flocculus, respectively (Table 1). The percentage of cell viability was recorded as 5% and 7% only for A. aculeatus and A. flocculus respectively against the K562 cancer cell line. They achieved 7% and 18% cell viability respectively against the PC3 cancer cell line. The viability assays were accomplished for the various A. aculeatus extracts (Fig6c, d) and remarkable activity against both cancer cell lines was observed for the 7-day RC extract compared to the other with cell viability of 10% and 6% against PC3 and K562 respectively. Moreover, the 15-day RC extract showed good bioactivity toward K562 with 10% cell viability and a moderate activity against the PC3 cancer cell line with viability recorded 50%. Fig 7a and b show the active pooled fractions that resulted from the MPLC fractionation of the 7-day RC extract. These active fractions were further fractionated and purified to give 11 pure compounds. The cytotoxicity of the purified compounds was determined against the cancer cell lines PC3 and K562 as well as the normal epithelial cells (PNT2a) (Fig7c). The results showed that compounds 6, 7, 8, 9, 10 and 11 (homogenistic acid, secalonic acid D, C, B, uridine and JBIR-75, respectively) exhibited strong cytotoxicity against K562 cells, affording % cell viabilities of 40, 15, 15, 10, 7 and 20, respectively. However, compounds 7, 8, and 9 were found to be cytotoxic on normal cells recording % viabilities of 50, 70 and 60, respectively. On the other hand, compound 5 (secalonic acid F) showed no cytotoxicity against either normal or lymphoma cells, referring to the stereoselectivity of the active structure. The non-selective cytotoxicity of the secalonic acids, which upon modification at any of the stereocenters can produce improved therapeutic analogues, will potentially lead them to be considered as pro-drugs or drug lead compounds. Compounds 6 (homogenistic acid), 10 (uridine) and 11 (JBIR75) exhibited a strong cytotoxic effect on K562 cell line. They were less toxic to the normal cells exhibiting % cell viabilities of 70, 80 and 90, respectively. None of these metabolites have been previously reported in the literature as anticancer agents. Furthermore, secalonic acid derivatives (Compounds 7, 8, 9) and JBIR 75 (11) were earlier identified in this study through metabolomics tools as being among the putative metabolites in 7-days rice culture extract that were highly correlated with the anticancer activity of the crude extract. This confirmed the effectiveness of the metabolomics tools in the early identification of the active components.
**Fig6a:** Anticancer activity of all fungal extracts from MA plates against the K562 cancer cell line, b: Anticancer activity of all fungal extract from MA plates against the PC3 cancer cell line, c: Viability assay of different culture extracts of A. aculeatus against the PC3 cell line and d: Viability assay of different culture extracts of A. aculeatus against the K562 cell line.
Fig7a: Anticancer activity of MPLC fractions from 7-day rice culture extract of A. aculeatus against the K562 cancer cell line, b: Anticancer activity of MPLC fractions from 7-day rice culture extract of A. aculeatus against the PC3 cancer cell line and c: Activity of the purified compounds against both cancer cell lines and normal cells (●) normal cells, (●) PC3, (●) K562 and (●) NF-κB

Conclusion
Endophytes such as members of the genus *Aspergillus* have proven to be excellent sources of clinically relevant natural products. In this study, metabolomics tools were successfully employed to compare the metabolite pattern of solid and liquid culture extracts of *A. aculeatus* endophyte associated with *Terminalia laxiflora*. Natural products databases were efficiently used to de-replicate the detected metabolites in order to recognize either known or unknown compounds. Multivariate data analysis was helpful in highlighting the putative metabolites in the active fungal extracts and/or fractions. Metabolomics and dereplication studies using $^1$H NMR and HR-MS led to a faster and shorter method of putatively identifying the active metabolites at earlier stages. Moreover, metabolite fingerprinting was used to optimize the cultivation media that resulted in the maximum amounts of the active metabolites. Metabolite profiling aimed to highlight which extract and/or fraction will be prioritized for further fractionation. A metabolomic-guided protocol provided focus on the potency of metabolite present in a complex extract or fraction at micro- or nano-gram levels. This solves the problem of assuming the rely of bioactivity on the yield of respective metabolites in an active fraction or extract. Metabolomics has been shown to be a powerful facilitator in the discovery of natural products, which are considered an excellent source for novel leads, and even more, as a means to highlight active targets.
Acknowledgement:

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