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Research Article

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Screening of Metabolites of Endophytic Fungi Isolated from Leaves of *Azadirachta indica* for Antimicrobial and Cytotoxic Activities

Joy C. Nnanna[1], Peter M. Eze[1*], Ogechi O. Anyanwu[2], Treasure N. Ujam[3], Moses N. Ikegbunam[1], Festus B. C. Okoye[2], Charles O. Esimone[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria
[3]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Enugu State University of Sciences and Technology, Enugu, Nigeria

Abstract Two endophytic fungi (AIn-L4 and AIn-L5) were isolated from the leaves of *Azadirachta indica*. The fungi were subjected to solid state fermentation on rice medium and the fungal secondary metabolites were extracted using ethyl acetate. The fungal extracts were screened for possible antimicrobial and cytotoxic activities. Also, some of the bioactive compounds of the extracts were detected using high-performance liquid chromatography (HPLC) analysis. The result of the antimicrobial assay reveal that at 1 mg/mL, AIn-L5 extract showed mild antibacterial activity against *B. subtilis* and *S. typhi* with inhibition zone diameters of 4 and 5 mm respectively. Extract of AIn-L4 showed no activity against any of the test bacteria, and both fungal extracts showed no antifungal activity against the test fungi. Result of the cytotoxicity assay reveals that the extract of AIn-L4 showed 100% cytotoxic activity and completely inhibited the growth of mouse lymphoma cells L5178Y, while AIn-L5 showing no cytotoxic activity. The HPLC analysis of AIn-L4 extract revealed the presence of aureonitol, while AIn-L5 showed the presence of scytalone, cladosporin, and citreoisocoumarinol. The study showed that these endophytic fungi could be promising sources of novel bioactive compounds with pharmaceutical or industrial importance.

Keywords *Azadirachta indica*, endophytic fungi, secondary metabolites, antimicrobial and cytotoxic activities, HPLC analysis

Introduction

Endophytes are microorganisms that colonize asymptomatically the intercellular and/or intracellular parts of healthy plants and play an important role in drug discovery [1]. It has been proven that endophytic microbes have the ability to produce the same or similar bioactive chemicals as those originated from their host plants [2,3]. This confirms fungal endophytes as potential alternatives to plants for the industrial production of valuable natural products.

Nigeria's rich plant biodiversity presents an enormous platform for researchers to explore in the area of bioprospecting without the destructive harvesting of plants, but by exploring their associated endophytic organisms for pharmaceutically and industrially important molecules [4]. Several studies on the endophytic fungal populations of Nigerian medicinal plants have confirmed the enormous potentials which abound in these organisms as sources of novel bioactive molecules [2-11].

*Azadirachta indica (*Meliaceae), commonly known as Neem, is an evergreen tree indigenous to India and Africa, and found in tropical regions of the world [12]. The plant has been in use since ancient times to treat a number of human ailments and also as household pesticide. Extracts from the bark, leaves, fruits and roots *A. indica* have been used to control leprosy, intestinal helminthosis and respiratory disorders [13-16]. Biologically active compounds isolated from different parts of the plant include: azadirachtin, meliacin, gedunin, salanin, nimbin, valassin, and many other derivatives of these compounds [17].

A. indica have been studied for their endophytic microbial populations and several endophytes producing bioactive natural products have been reported from the plant [12, 18-25]. The search for microorganisms of biotechnological interest based on the ethnobotanical pharmacology of the plants they are associated with represents an alternative to discover new microorganisms and bioactive molecules [26]. Based on these principles, the exploration of microorganisms from new ecological niches, such as endophytes, constitutes an important strategy for obtaining novel bioactive molecules with pharmaceutical or industrial applications.

In our search for biologically active molecules from endophytic fungi associated with Nigerian plants, this study was carried out to investigate the metabolites of endophytic fungi isolated from *A. indica* for antimicrobial and cytotoxic activities, and identify its constituents using HPLC.

Methods

Collection of Plant Materials

Healthy leaves of *A. indica* leaves were collected from their trees growing in their natural environments at Asaba, Delta State, Nigeria. Isolation of endophytic fungi from the plant leaves, solid state fermentation of the fungi and extraction of the fungal secondary metabolites were carried out using standard methods as described by Eze *et al.* [4].

Antimicrobial Assay

Antibacterial and antifungal assays of the endophytic fungal extracts were carried out using the agar well diffusion method described by Akpotu *et al*. [9]. A concentration of 1 mg/mL of the extracts was tested against laboratory strains of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhi* in the antibacterial assay; and against *Candida albicans* and *Aspergillus fumigatus* in the antifungal assay. Gentamicin (10 µg/mL) and ketoconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal tests respectively, while DMSO was used as the negative control in both tests. The inhibition zone diameters (IZDs) produced by the extracts and controls against the test isolates were measured and recorded.

Cytotoxicity Assay

The cytotoxicity was tested against L5178Y mouse lymphoma cells using the MTT assay described by Ngwoke *et al.* [27]. Stock solutions of the endophytic fungal extracts were prepared in ethanol 96% (v/v). Exponentially growing cells were harvested, counted and diluted appropriately. From the cell suspension, 50 µL containing 3750 cells were pipetted into each of the 96-well microtiter plates. Subsequently, 50 µL of a solution of the test samples containing the appropriate concentration (10 µg/mL) was added to each well. The test plates were incubated at 37 ^{o}C with 5% CO_2 for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and, in the presence of mitochondrial dehydrogenases MTT is transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37^{o}C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min, 20^{o}C, 210 × g) with 200 µL DMSO, the cells were lysed to liberate the formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microliter spectrophotometer. The color intensity is correlated with the number of healthy living cells. All experiments were carried out in triplicates and repeated three times. Cell survival was calculated using the formula:

$$\text{Survival } \% = \frac{\text{Absorbance of treated} - \text{Absorbance of culture medium}}{\text{Absorbance of untreated} - \text{Absorbance of culture medium}} X\ 100$$

High performance liquid chromatography (HPLC)

HPLC analysis of the endophytic fungal extracts was carried out as described by Eze *et al*. [4]. This was done using a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. Detection was set at 235 nm and the absorption peaks of the fungal extract were analyzed by comparing with those in the HPLC-UV/Vis database.

Results

Two endophytic fungi (AIn-L4 and AIn-L5) were isolated from the leaves of *A. indica.* The result of the antimicrobial assay of the endophytic fungal extracts (Table 1) reveal that at 1 mg/mL, extract of AIn-L5 showed mild antibacterial activity against *B. subtilis* and *S. typhi* with IZD of 4 and 5 mm respectively.. Extract of AIn-L4 showed no activity against any of the bacteria, and both fungal extracts showed no antifungal activity against the test fungi *C. albicans* and *A. fumigatus*.

Result of the cytotoxicty assay (Table 2) reveals that the extract of AIn-L4 showed 100% cytotoxic activity and completely inhibited the growth of mouse lymphoma cell L5178Y, with AIn-L5 showing no cytotoxic activity.

The HPLC analysis of the extract of AIn-L4 revealed the presence of aureonitol, while AIn-L5 showed the presence of scytalone, cladosporin, and citreoisocoumarinol.

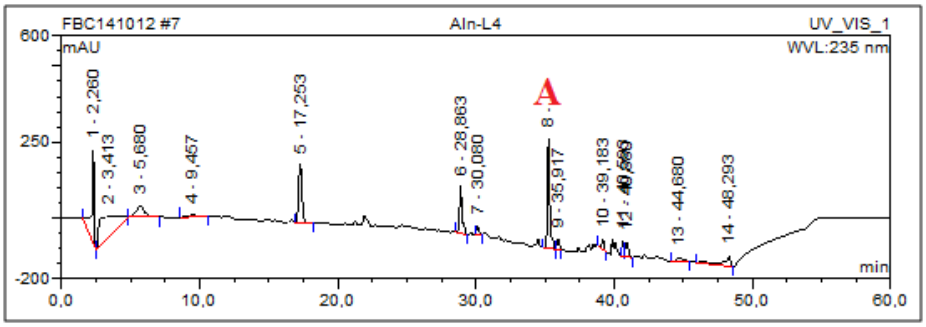
The HPLC chromatogram of the fungal extracts, as well as the UV-spectra and chemical structures of detected compounds are presented in Figures 1 and 2.

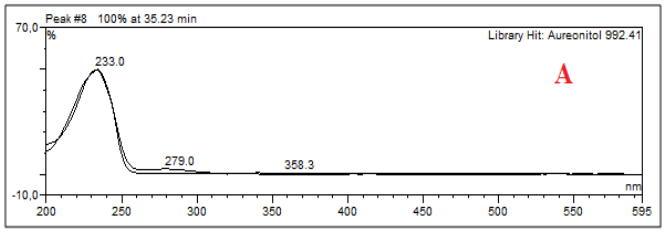
Table 1: Results of the antimicrobial evaluation of the fungal extract showing the inhibition zone diameters (IZD) (mm) produced against test organisms

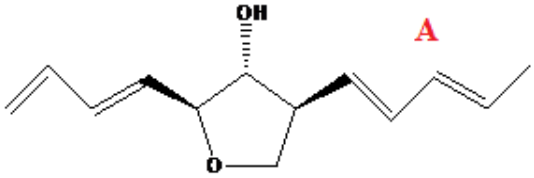
Test Organisms	AIn-L4	AIn-L5	Positive control	Negative control
			Gentamicin (10 µg/ml)	DMSO
S. aureus	0	0	17	0
S. typhi	0	5	21	0
B. subtilis	0	4	22	0
E. coli	0	0	16	0
			Ketoconazole (50 µg/ml)	DMSO
C. albicans	0	0	17	0
A. fumigatus	0	0	4	0

Table 2: Cytotoxic activity of endophytic fungal extracts against L5178Y mouse lymphoma cells

Endophytic fungal extracts	Concentration (µg/mL)	% inhibition
AIn-L4	10	100
AIn-L5	10	0

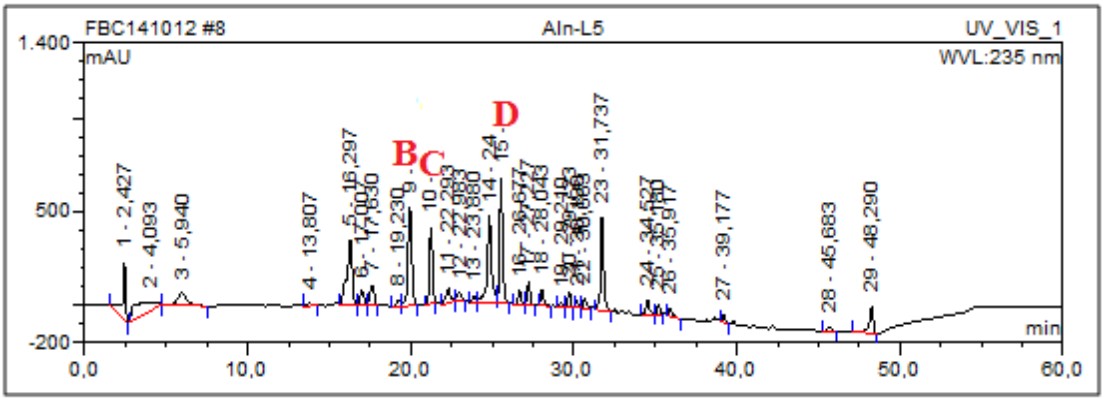


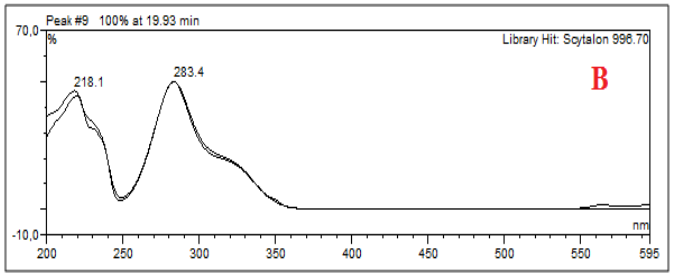


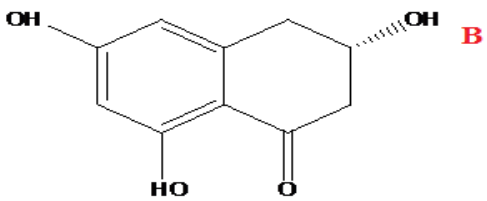


Aureonitol $C_{13}H_{18}O_2$, 206.28 g.mol^{-1}

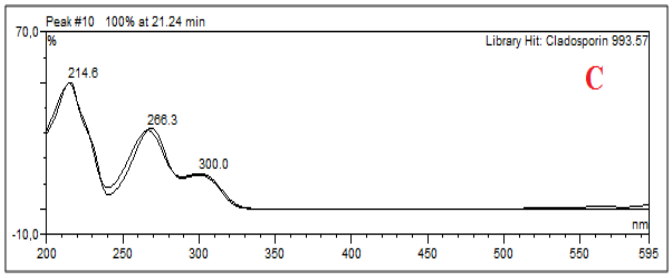
Figure 1: HPLC chromatogram of AIn-L4 extract, UV Spectra and chemical structure of the detected bioactive compound - aureonitol (A)

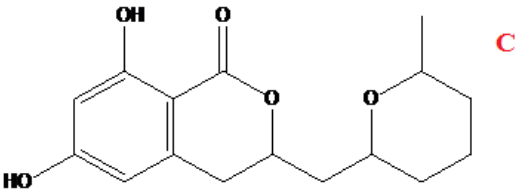




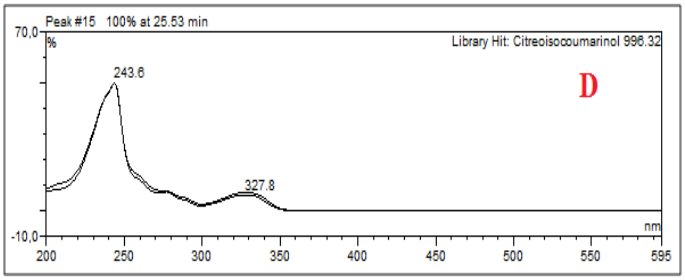


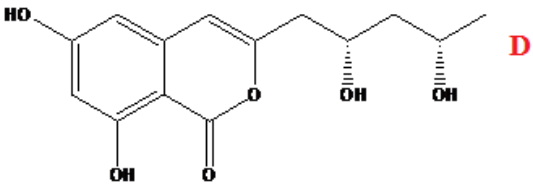
Scytalone $C_{10}H_{10}O_4$, 194 g.mol^{-1}





Cladosporin $C_{16}H_{20}O_5$, 292 g.mol^{-1}





Citreoisocoumarinol $C_{14}H_{16}O_6$, 280 g.mol^{-1}

Figure 2: HPLC chromatogram of AIn-L5 extract, UV Spectra and chemical structure of the detected bioactive compounds scytalone (B), cladosporin (C), and citreoisocoumarinol (D)

Discussion

From the result in Table 1, it can be seen that the extract of AIn-L5 showed mild antibacterial activity against *B. subtilis* and *S. typhi* with IZD of 4 and 5 mm respectively. This activity may be attributed to antimicrobial compounds present in the extract. Extract of AIn-L4 showed no antimicrobial activity against any of the test organisms. However, the extract of AIn-L4 demonstrated strong cytotoxic activity and completely inhibited the growth of mouse lymphoma cell L5178Y, with AIn-L5 showing no cytotoxic activity (Table 2).

HPLC analysis revealed the presence of several biologically active compounds in extracts of AIn-L4 and AIn-L5. Aureonitol, an antiviral compound, was detected in AIn-L4; while three other compounds (scytalone, cladosporin, and citreoisocoumarinol), with diverse biological properties, were detected in AIn-L5.

Aureonitol is a derivative of tetrahydrofuran derivative and has been shown to excellent antiviral activity against influenza A and B viruses [28]. Aureonitol has been isolated from different fungal species of the genus *Chaetomium* [28,29].

Scytalone has been previously isolated from several endophytic fungi which include *Cladosporium tenuissimum,* [30], *Annulohypoxylon* sp. [1], *Phomopsis* sp. [31], and *Scytalidium* sp. [32]. Scytalone has been known as an intermediate in the biosynthesis of melanin, the dark pigment of many phytopathogenic fungi [31,33,34]. Melanin is specifically involved with the hyphal (appresoria) penetration into leaves, and cell walls [34]. It was also observed that at low concentrations scytalone increased the growth rate of cells within the foliar lamina and roots of *Arabidopsis thaliana* [35].

Citreoisocoumarinol, an isocoumarin derivative has been previously isolated from different endophytic fungi such as *Penicillium corylophilum* [36], *Nectria sp*. [37], and *Fusarium tricinctum* [38], and it has been reported to show α-glucosidase inhibitory activity [37]. Isocoumarins are prevalent in most natural products that exhibit a wide range of biological activities including anti-diabetic [39], antimicrobial [40], insecticidal [41], antiparasitic [42,43], cytotoxic [44], anti-inflammatory [45], and anti-angiogenic [46].

Cladosporin, another isocoumarin derivative detected with citreoisocoumarinol in AIn-L5, has been isolated from several endophytic fungi including *Cladosporium cladosporioides* [47,48] and *Eurotium sp.* [49]. It has been reported to show antiplasmodial [50], antifungal [48], antibacterial [47,49], insecticidal [41], and antitumor properties [48].

Citreoisocoumarinol and cladosporin with known antimicrobial properties [40,47,49], together with other undetected compounds, may be responsible for the antimicrobial activity exhibited by the extract from AIn-L5. The excellent cytotoxic activity exhibited by AIn-L4 extract may be due to undetected cytotoxic compounds present in the extract. This cytotoxic activity indicates the potential of this fungus in the discovery of lead anticancer agents. Since the discovery of the anticancer compound paclitaxel (Taxol®) from a fungal endophyte, *Taxomyces adreanae* [51], the potentials of fungal metabolites as the source of anticancer agents has remained high as a whole lot of them are at different phases of clinical trial [52].

The expression of these bioactive chemical constituents by the fungal endophytes is an indication that endophytes associated with *A. indica* may be potential sources of precursors for drug development.

Conclusion

This study show that endophytic fungi associated with *A. indica* could be a promising source of novel bioactive compounds with pharmaceutical or industrial importance. It is recommended that further research on these endophytic fungi and their metabolites be carried out in order to confirm and expand the data provided in this preliminary investigation.

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References

1. Liu Y, Stuhldreier F, Kurtán T, Mándi A, Arumugam S, Lin W, Stork B, Wesselborg S, Weber H, Henrich B, Daletos G, Proksch P. Daldinone derivatives from the mangrove-derived endophytic fungus *Annulohypoxylon* sp. *RSC Adv.*, 2017, 7: 5381 –5393.
2. Ebada SS, Eze P, Okoye FBC, Esimone CO, Proksch P. The Fungal Endophyte *Nigrospora oryzae* Produces Quercetin Monoglycosides Previously Known Only from Plants. *Chemistry Select*, 2016, 1(11): 2767-2771.
3. Uzor PF, Ebrahim W, Osadebe PO, Nwodo JN, Okoye FB, Müller WEG, Lin WH, Liu Z, Proksch P. Metabolites from *Combretum dolichopetalum* and its associated endophytic fungus *Nigrospora oryzae*-Evidence for a metabolic partnership. *Fitoterapia*, 2015, 105:147-150.
4. Eze PM, Ojimba NK, Abonyi DO, Chukwunwejim CR, Abba CC, Okoye FBC, Esimone CO. Antimicrobial Activity of Metabolites of an Endophytic Fungus Isolated from the Leaves of *Citrus jambhiri* (Rutaceae). *Trop J Nat Prod Res.*, 2018, 2(3):145-149.
5. Okoye FBC, Lu S, Nworu CS, Esimone CO, Proksch P, Chaldi A. Debbab A. Depsidone and Diaryl Ether Derivatives from the Fungus *Corynespora cassiicola*, an Endophyte of *Gongronema latifolium*. *Tetrahedron Letters*, 2013, 54: 4210–4214.
6. Okoye FBC, Nworu CS, Akah PA, Esimone CO, Debbab A, Proksch P. Inhibition of inflammatory mediators and reactive oxygen and nitrogen species by some depsidones and diaryl ether derivatives isolated from *Corynespora cassiicola*, an endophytic fungus of *Gongronema latifolium* leaves. *Immunopharmacology and Immunotoxicology*, 2013, 35(6): 662-668.
7. Okoye FBC, Nworu CS, Debbab A, Esimone CO, Proksch P. Two new Cytochalasins from an endophytic fungus, KL-1.1 isolated from *Psidium guajava* leaves. *Phytochemistry Letters*, 2015, 14: 51-55.
8. Abba CC, Nduka I, Eze PM, Ujam TN, Abonyi DO, Okoye FBC. Antimicrobial Activity of Secondary Metabolites of Endophytic *Aspergillus Species* Isolated from *Loranthus micranthus*. *African Journal of Pharmaceutical Research and Development*, 2016, 8(2): 136-140.
9. Akpotu MO, Eze PM, Abba CC, Umeokoli BO, Nwachukwu CU, Okoye FBC, Esimone CO. Antimicrobial activities of secondary metabolites of endophytic fungi isolated from *Catharanthus roseus*. Journal of Health Science, 2017, 7(1): 15-22.
10. Chen H, Daletous G, Okoye FBC, Lai D, Dai H, Proksch P. A new cytotoxic cytochalasin from the endophytic fungus *Trichodama harzianum*. *Natural Products Communications*, 2015, 10(4): 585-587.
11. Okezie UM, Eze PM, Okoye FBC, Ikegbunam MN, Ugwu MC, Esimone CO. Biologically Active Metabolites of an Endophytic Fungus Isolated from *Vernonia Amygdalina. African Journal of Pharmaceutical Research and Development*, 2017, 9(1): 24-29.
12. Tenguria RK, Khan FN. Distribution of Endophytic Fungi in Leaves of *Azadirachta indica* A. JUSS. (Neem) of Panchmarhi Biosphere Reserve. *Current Botany*, 2011, 2(2): 27-29
13. Chattopadhyay RR, Chattopadhyay RN, Maitra SK. Possible mechanism of anti-inflammatory activity of *Azadirachta indica* leaf extract. *Indian J. Pharmacol.*, 1993, 25(2): 99-100.
14. Chattopadhyay RR, Bandyopadhyay M. Effect of *Azadirachta indica* leaf extract on serum lipid profile changes in normal and streptozotocin induced diabetic rats. *Afr. J. Biomed. Res.*, 2005, 8: 101-104.
15. Ketkar AY, Ketkar CM. The Neem tree: Source of unique natural products for integrated pest management, Medicine and other purpose. In: Schmutterer H (ed.), 1995: 518-525.
16. Mamman PH, Mshelia WP, Susbatrus SC, Sambo KW (2013). Antibacterial effects of crude extract of *Azadirachta indica* against *Escherichia coli*, *Salmonella* spp and *Staphylococcus aureus. International Journal of Medicine and Medical Sciences.* 5(1): 14-18.
17. Ogbuewu IP, Odoemenam VU, Obikaonu HO, Opara MN, Emenalom OO, Uchegbu MC, Okoli IC, Esonu BO, Iloeje MU (2011). The growing importance of Neem (*Azadirachta indica* A, Juss) in Agriculture, Industry, Medicine and Environment: A review. *Research Journal of Medicinal Plant.* 5 (3): 230-245

18. Singh AK, Sharma RK, Sharma V, Singh T, Kumar R, Kumari D (2017). Isolation, morphological identification and *in vitro* antibacterial activity of endophytic bacteria isolated from *Azadirachta indica* (neem) leaves. *Veterinary World.* 10(5): 510-516.
19. Mahesh B, Tejesvi MV, Nalini MS, Prakash HS, Kini KR, Subbiah V, Shetty HS (2005). Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss. *Current Science*, 2005, 88(2): 218-219.
20. Verma VC, Gond SK, Kumar A, Kharwar RN, Strobel GA. Endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss. (neem) from Varanasi (India). *Microb. Ecol.,* 2007, 54: 119-125.
21. Verma VC, Gond SK, Mishra A, Kumar A, Kharwar RN, Gange AC. Endophytic actinomycetes from *Azadirachta indica* A. Juss.: isolation, diversity and antimicrobial activity. *Microb. Ecol.,* 2009, 57: 749–756.
22. Li GH, Yu ZF, Li X, Wang XB, Zheng LJ, Zhang KQ. Nematicidal metabolites produced by the endophytic fungus *Geotrichum* sp. AL4. *Chem. Biodivers.*, 2007, 4: 1520- 1524.
23. Kharwar RN, Verma VC, Kumar A, Gond SK, Harper JK, Hess WM, Lobkovosky E, Ma C, Ren Y, Strobel GA. Javanicin, an antibacterial naphthaquinone from an endophytic fungus of neem, *Chloridium* sp. *Curr. Microbiol.,* 2009, 58: 233-238.
24. Wu SH, Chen YW, Shao SC, Wang LD, Li ZY, Yang LY, Li SL, Huang R. Tenmembered lactones from *Phomopsis* sp., an endophytic fungus of *Azadirachta indica*. *J. Nat. Prod.*, 2008, 71: 731–734.
25. Wu SH, Chen YW, Shao SC, Wang LD, Yu Y, Li ZY, Yang LY, Li SL, Huang R. Two new Solanapyrone analogues from the endophytic fungus *Nigrospora* sp. YB-141 of *Azadirachta indica*. *Chem. Biodivers*., 2009, 6: 79-85.
26. Conti R, Cunha IGB, Siqueira VM, Souza-Motta CM, Amorim ELC, Araújoc JM. Endophytic microorganisms from leaves of *Spermacoce verticillata* (L.): Diversity and antimicrobial activity. *Journal of Applied Pharmaceutical Science,* 2012, 2 (12): 017-022
27. Ngwoke KG, Nwoye OV, Eze PM, Nworu CS, Okoye FBC, Proksch P. *In Vitro* Cytotoxic Properties of *Cladosporium Cladosporioides* Extract against Human Ovarian Cancer Cells A2780 and Mouse Lymphoma Cells L5178Y. *African Journal of Pharmaceutical Research and Development,* 2017, 9(1): 80-85.
28. Sacramento CQ, Marttorelli A, Fintelman-Rodrigues N, de Freitas CS, de Melo GR, Rocha MEN, Kaiser CR, Rodrigues KF, da Costa GL, Alves CM, Santos-Filho O, Barbosa JP, Souza TML. Aureonitol, a Fungi-Derived Tetrahydrofuran, Inhibits Influenza Replication by Targeting Its Surface Glycoprotein Hemagglutinin. *Plos One*, 2015, 10(10): e0142246.
29. Jervis PJ, Cox LR. Total synthesis and proof of relative stereochemistry of (-)-aureonitol. *J Org Chem.*, 2008, 73(19): 7616–24.
30. Naseer S, Bhat KA, Qadri M, Riyaz-Ul-Hassan S, Malik FA, Khuroo MA. Bioactivity-Guided Isolation, Antimicrobial and Cytotoxic Evaluation of Secondary Metabolites from *Cladosporium tenuissimum* Associated with *Pinus wallichiana*. *Chemistry Select*, 2017, 2(3): 1311-1314.
31. Weber D, Gorzalczany S, Martino V, Acevedo C, Sterner O, Anke T. Metabolites from Endophytes of the Medicinal Plant *Erythrina crista-galli. Z. Naturforsch*, 2005, 60c: 467-477.
32. Findlay JA, Kwan D. Scytalone (3,6,8-Trihydrozytetralone), a metabolite from a *Scytalidium* Species. *Can. J. Chem.*, 1972, 51: 1617–1619.
33. Bell AA, Wheeler MH. Biosynthesis and function of fungal melanins. *Annu. Rev. Phytopathol.*, 1986, 24: 411-451.
34. Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet. Biol.,* 2003, 38: 143–158.
35. Abou-Mansour E, Couché E, Tabacchi R. Do fungal naphthalenones have a role in the development of esca symptoms. *Phytopathol. Mediterr.,* 2004, 43: 75–82.
36. Malmstrøm J, Christophersen C, Frisvad JC. Secondary metabolites characteristic of *Penicillium citrinum*, *Penicillium steckii* and related species. *Phytochemistry*, 2000, 54(3): 301-9.

37. Cui H, Liu Y, Nie Y, Liu Z, Chen S, Zhang Z, Lu Y, He L, Huang X, She Z. Polyketides from the Mangrove-Derived Endophytic Fungus *Nectria sp*. HN001 and their α-Glucosidase Inhibitory Activity. *Mar Drugs*, 2016, 14(5): pii E86.
38. Ola AR, Thomy D, Lai D, Brötz-Oesterhelt H, Proksch P. Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. *J Nat Prod.,* 2013, 76(11): 2094-2099.
39. Zhang H, Matsuda H, Kumahara A, Ito Y, Nakamura S, Yoshikawa M. New type of anti-diabetic compounds from the processed leaves of *Hydrangea macrophylla* var. thunbergii (Hydrangeae Dulcis Folium). *Bioorg Med Chem Lett.*, 2007, 17: 4972-4976.
40. Zhang W, Krohn K, Draeger S, Schulz B. Bioactive Isocoumarins Isolated from the Endophytic Fungus *Microdochium bolleyi. J Nat Prod.*, 2008, 71: 1078-1081.
41. Grove JF, Pople M. The insecticidal activity of some fungal dihydroisocoumarines. *Mycopathologia*, 1981, 76: 65–67.
42. Pal S, Chatare V, Pal M. Isocoumarin and Its Derivatives: An Overview on their Synthesis and Applications. *Current Organic Chemistry*, 2011, 15:782-800.
43. Peters W, Trotter ER, Robinson BL. The experimental chemotherapy of leishmaniasis. V. The activity of potential leishmanicides against *L. infantum* LV9' in NMRI mice. *Annals Trop Med Parasitol.,* 1980, 74: 289-298.
44. Varanda EA, Varella SD, Rampazo RA, Kitagawa RR, Raddi MSG, Vilegas W, dos Santos LC. Mutagenic and cytotoxic effect of planifolin: A naphthopyranone dimer isolated from *Paepalanthus planifolius. Toxicology In vitro*, 2006, 20: 664-668.
45. Stasi LC, Camuesco D, Nieto D, Vilegas W, Zarzuelo A, Galvez J. Intestinal anti-inflammatory activity of paepalantine, an isocoumarin isolated from the capitula of *Paepalanthus bromelioides*, in the trinitrobenzenesulphonic acid model of rat colitis. *Plant Medica*, 2004, 70: 315–20.
46. Yuan H, Junker B, Helquist P, Taylor RE. Synthesis of Anti-Angiogenic isocoumarins. *Curr Org Synt.*, 2004, 1: 1-9.
47. Xie QM, Huang JH. A preliminary study on Cladosporin. *J Fujian Coll Forest*, 1998, 8: 29-36.
48. Wang X, Radwan MM, Taráwneh AH, Gao J, Wedge DE, Rosa LH, Cutler HG, Cutler SJ. Antifungal Activity against Plant Pathogens of Metabolites from the Endophytic Fungus *Cladosporium cladosporioides*. *J Agric Food Chem*., 2013, 61(19): 4551–4555.
49. Anke H, Zähner H. Metabolic products of microorganisms. 170. On the antibiotic activity of cladosporin. *Arch. Microbiol.*, 1978, 116: 253–257.
50. Hoepfner D, McNamara CW, Lim CS, Studer C, Riedl R, Aust T, McCormack SL, Plouffe DM, Meister S, Schuierer S, Plikat U, Hartmann N, Staedtler F, Cotesta S, Schmitt EK, Petersen F, Supek F, Glynne RJ, Tallarico JA, Porter JA, Fishman MC, Bodenreider C, Diagana TT, Movva NR, Winzeler EA. Selective and specific inhibition of the *Plasmodium falciparum* lysyl-tRNA synthetase by the fungal secondary metabolite cladosporin. *Cell Host Microbe*, 2012, 11(6), 654-63.
51. Stierle A, Strobel G, Stierle D. Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. *Science*, 1993, 260 (5105):214-216.
52. Gomes GN, Lefranc F, Kijjoa A, Kiss R. Can some marine-derived fungal metabolites become actual anticancer agents? *Marine Drugs*, 2015, 13(6): 3950-3991.