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Research Article

Antimicrobial and Antioxidant Activities of Secondary Metabolites of an Endophytic fungus of *Azadirachta indica*

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Abstract

Background: Recent upsurge in the mortality rate and long hospital stays as a result of antimicrobial resistance caused by multi-drug resistant bacteria is a huge burden to public health sector. Intensive search for more effective and newer agents to deal with these problems. Endophytes are a novel source of potentially useful medicinal compounds. **Aim:** The study aimed at evaluating the antimicrobial and antioxidant activities of an endophytic fungus isolated from *Azadirachta indica* against multi drug resistant bacteria species. **Method:** Endophytic fungus was isolated from fresh leaves of Azadirachta indica. The fungus was fermented in rice medium, and the secondary metabolites were extracted. The antimicrobial activity of the extract against laboratory strains of *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Bacillus subtilis, Candida albicans, Aspergillus niger)* was determined. The fungal extract was also evaluated for antioxidant activity using the DPPH assay method. Using GC-FID analysis, some constituents of the fungal extracted were identified. **Result:** At 1 and 0.5 mg/mL, the fungal extract demonstrated antibacterial activity against Klebsiella pneumoniae but no activity against the other test microbes. However, the fungal extract of *A. indica* showed excellent antioxidant activity due to possession of bioactive secondary metabolites. **Conclusion:** The result of GC-FID showed the presence of Rutin, Lunamarin, Anthocyanin, Catechin, Naringenin, Flavan 3 ol, Sapogernin, Tannin, Spartein, Naringin, Flavonones, Steroids, Kaemferol, Cyanogenic glycoside, Phytate, Epicatechin, Reseveratol, Cardic glycoside and Epihedrine at different concentrations. This study reveals the dynamic potentials of the endophytic fungus of *A. indica* as the bedrock of naturally occurring bioactive agent.

Keywords: *Azadirachta indica, e*ndophytes, GC-FID, secondary metabolites, medicinal plants, phytochemicals.

INTRODUCTION

Endophytes are microbes, mostly fungi and bacteria present in plants asymptomatically. Endophytic microbes are often functional in that they may take up nutrients from the soil into plants, regulate plant development, increase stress resilience of plants, subdue virulence in pathogens, elevate disease resistance in plants, and subdue development of competitor plant species[1]. Over 60% anticancer drugs and 75% anti-infectious drugs approved by FDA are obtained from medicinal plants[2]. which these endophytes inhabit. Due to the promising capabilities of endophytes possessing phytochemical compounds for pharmaceutical, agricultural or industrial applications, the purpose of this study was to evaluate the antimicrobial and antioxidant activities of secondary metabolites of an endophytic fungus isolated from the leaves of *Azadirachta indica* growing in Anambra State, South-East Nigeria. *Azadirachta indica* is a tropical perennial tree, native to the Indian subcontinent and also found in Brazil[3]. It is used in folk medicine due to its antifungal, antiulcerogenic, antibacterial, and anti- genotoxic properties. The aqueous extract from the stem and bark, in addition to its anti-inflammatory action, has been used in traditional medicine for its stimulant, diuretic, tonic, antipyretic, as well as anti-tumor properties. It is fortified in antioxidant chemical compounds with analgesic and anti-inflammatory actions. It is also rich in chemical compounds that show hypoglycemic action and cardiovascular. Neem fruit are mostly used in the treatment of infections, fever, diarrhea, bronchitis, infected burns, skin diseases and hypertension[3]. *Azadirachta indica* is used for so many therapeutic purposes in different forms of preparations. This plant hosts different types of endophytic fungi[4]. Studies carried out on the endophytic fungal populations of some Nigerian medicinal plants have shown huge potentials possessed by these microbes as sources of novel compounds of pharmaceutical and industrial importance [5-13]. Possession of these beneficial activities that is eco-friendly and safe for human consumption justifies the study of endophytes from popular medicinal plants like *Azadirachta indica.*

MATERIALS AND METHODS

Isolation of Endophytic Fungus

Isolation of the endophytic fungus from *A. indica* was carried out using a previously described method by Abba *et al*[9]. Fresh healthy leaves of *A. indica* were collected from Awka, Anambra state, Nigeria. The leaves were distinctly washed thoroughly in running tap water, and cut into small fragments (about 1 cm2). The leaf fragments of the plant were surface-sterilized by immersion in 2% sodium hypochlorite solution for 2 min, 70% ethanol for nearly 2 min, before a final rinse in sterile water for 5 min. The sterilized leaf blade segments were transferred into prepared malt extract agar (MEA) plates supplemented with 0.05% w/v chloramphenicol. The plates were sealed with parafilms and incubated at a temperature of 28^{O}C and fungal growths from the leaf blade segments were observed. The fungal growths from distinct plants were subcultured onto freshly prepared agar plates to obtain pure cultures.

Fungal Fermentation and Extraction of Secondary Metabolites

The fungus was subjected to solid state fermentation in sterile rice medium. Erlenmeyer flasks (1000 mL) containing 100 g of rice + 100 ml of distilled were autoclaved at 15 psi at 121^{O}C for 1 h. The flasks were inoculated with 3 mm diameter agar blocks with the fungi and incubated at 28^{O}C for 21 days. At the end of fermentation, the fungal secondary metabolites were extracted with ethyl acetate and concentrated under pressure with rotary evaporator.

Chemical analysis/ Quantification by Gas Column – Flame Ionization Detection (GC-FID)

The analysis of phytochemical was performed on a BUCK M910 Gas chromatography equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150 °C with increasing rate of 3 °C/min and holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in an splitless mode. Relative quantity of the chemical compounds present in each of the extracts was expressed as percentage based on peak area produced in the chromatogram[14].

Determination of Antimicrobial activity of extracts

The antimicrobial assay for the crude extracts was carried out using the agar well diffusion assay as described by Akpotu et al with slight modifications[6]. The antimicrobial activity of the extracts of the plants under study were tested against six standard human pathogenic microbe species namely *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia*, *Bacillus subtilis, Candida albican and Aspergillus niger*. These were standard laboratory cultures whose susceptibility on commonly used antibiotics and fungi were already established. *Staphylococcus aureus and Bacillus subtilis* represent Gram positive bacteria while *Escherichia coli and Klebsiella pneumoniae* represents Gram negative bacteria. *Candida albican and Aspergillus niger represents fungi.*

The bacterial suspensions were adjusted to 0.5 McFarland turbidity standard and inoculated onto previously sterilized Mueller-Hinton Agar and Sabouraud Dextrose Agar plates (diameter: 90 mm) for bacteria and fungi respectively. A sterile cork- borer was used to make five wells (8 mm in diameter) on each of the MHA plates. Aliquots of 80 µl of each extract dilutions, reconstituted in DMSO at concentrations of 1.0, 0.5, 0.25, 0.125 and 0.063 mg/mL, were applied in each of the wells in the culture plates previously seeded with the test organisms and left on the bench to pre diffuse for 30mins. Ciprofloxacin (8 µg/mL) and Miconazole (50 µg/mL) served as the positive control against the test organisms. The cultures were incubated at 37^{o}C for 24 hours for bacteria and 28^{o}C for 3 days. The antimicrobial potential for the extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each of the crude extract, three replicates were conducted against each organism. Each of the sample was tested against all the test isolates.

Antioxidant activity

The free radical scavenging potentials of the endophytic fungal extracts were carried out as described by Chigozie *et al* [15], with some modifications. The free radical scavenging properties of the extracts against 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical were measured at 490 nm. The concentrations of the extracts and ascorbic acid used were 20, 40, 60, 80, and 100 µg/mL The reaction mixture consists of 25 µl of the stock, 25 µl of DPPH (0.1 mol/L) and 150 µl of methanol solution. These were added into their respective wells in the microtiter. The plate was incubated at 27oC for 30 min. The absorbance of the mixtures was measured at 490 nm using a UV-vis spectrophotometer (06452; USA). The experiment was done in triplicate for each fungal extract. Free radical scavenging activities were expressed as the percentage inhibition of each extract and calculated using the formula:

$$\%\text{DPPH Inhibition} = \frac{X-Y}{X} X \frac{100}{1}$$

Where X = control absorbance; Y = Sample absorbance

RESULT AND DISCUSSION

The endophytic fungus isolated from the leaves of A. indica was employed for the study as shown in Fig 1. The GC-FID results in Fig 2 revealed a broad range of phytochemical compounds that had been previously reported to have substantial antimicrobial and antioxidant activity. In this study, A. indica endophytic fungus was isolated and its potential was explored for its attributes to produce bioactive metabolites with pharmaceutical and biotechnology applications. Azadirachta leaves were subjected quantitatively using GC-FID for the phytochemical composition of the endophyte extracts. The GC-FID results in Fig 2 showed the extract is rich in alkaloids and flavonoids. These alkaloids and flavonoids have been used for various functions in human cells, including antimicrobial, anticancer, antioxidant, antimalarial and antidiabetic activities. Tannins, flavonoids, alkaloids, saponins have been revealed to possess broad range of pharmaceutical effects leading to some physiological changes and are used as active drug candidates in manufacturing drugs. The endophytic extract showed great reservoir of bioactive secondary metabolites such as tannins, flavonones, lunamarin, anthocyanin, catechin, naringenin, flavan 3 ol, sapogernin, rutin, kaempferol, steroids, cyanogenic glycosides, spartein which may have conferred medicinal activities on the plant through synergistic interactions between the plants and endophytes. The traditional use of *A. indica* has been justified by the results observed, as an antimicrobial and antioxidant agent and has further validated the fact that endophyte extract of the plant could have more potentials since these endophytes are inherent in the plant. Thus, sharing similar nutrients and possibly possess same bioactive secondary metabolites.

Figure 1: Leaves and Endophytic hyphal appearance of *A.indica* from the mid-ribs

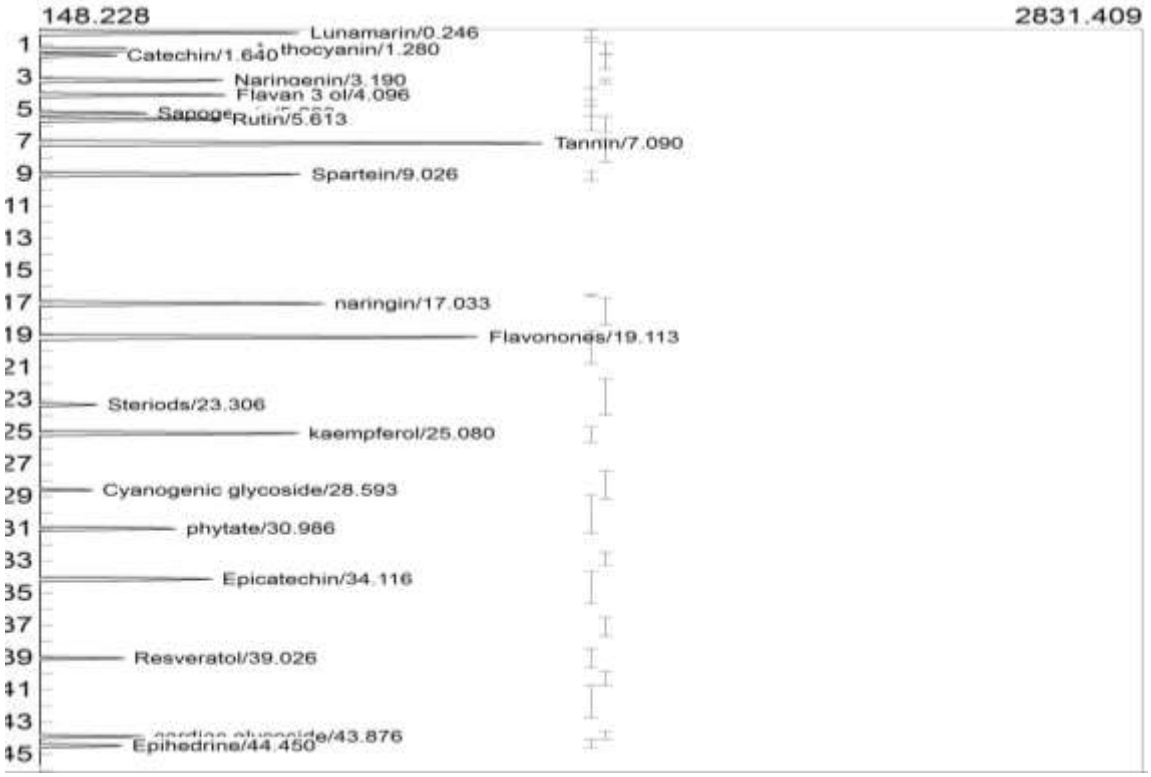

Figure 2: Chromatogram showing compounds detected in the endophytic fungal extract of *Azadirachta indica* using GC-FID

Endophytic fungi are popular to contribute plant fitness benefits, enabling adaptation of the plant host to biotic and abiotic stresses. These fungi have diverse secondary metabolites, some of which are bioactive compounds expressed as defensive weapons to protect the host plant from pests and diseases but also as metabolites for specific interactions and communications with the plant host[16]. In table 1, the GC-FID result of the endophytic fungus extract of *A. indica* revealed the presence of different alkaloids, flavonoids, steroids, etc. Various studies have shown that these compounds possess different biological activities. Tannins was found to be very high in the endophytic extract of *A. indica* and confirms its traditional use as an antimicrobial agent and have been extensively studied to be useful in external treatment of skin inflammation, injuries and may prevent onset of chronic diseases owing to its antimicrobial, antioxidant, radical scavenging, antiviral, antimutagenic effects[17]. Tannins have been also reported to possess antibacterial activity[18]. Sparteine is a quinoline alkaloid that possesses antimicrobial and antimalarial properties and it has been reported that both proanthocynin and spartein have antibacterial activity[19]. Sparteine has been studied to possess antimicrobial activity[20]. Ojukwu *et al* also stated that lunamarin, rutin, steroids, kampferol and flavone have antimicrobial properties[21]. In addition, it has been reported that flavon-3-ol, naringin and flavonone have anti-inflammatory and anticancer activities[22-24]. Cyanogenic glycoside has been attributed to own anticancer activity[24]. Sapogernin has anti-inflammatory and anticancer activities which is also found in the endophytic fungus extract of *A. indica*[25].

Table 1: Phytochemical compounds detected in the endophytic fungal extract of A. indica by GC-FID

Component	Retention time (mins)	Area	Height	External	Units
Lunamarin	0.246	9957.7768	782.312	5.4673	µg/ml
Anthocyanin	1.280	8342.6252	644.168	13.9878	µg/ml
Catechin	1.640	4279.6824	336.190	1.5033	µg/ml
Naringenin	3.190	7628.0534	596.491	7.6152	µg/ml
Flavan 3 ol	4.096	7704.1709	602.105	6.2654	ppm
Sapogernin	5.233	5224.3806	411.183	6.5641	µg/ml
Rutin	5.613	7595.2597	592.177	11.7829	µg/ml
Tannin	7.090	17990.4274	1376.280	10.4474	µg/ml
Spartein	9.026	10118.3940	786.357	5.0247	µg/ml
Naringin	17.033	10843.2774	843.163	10.0812	µg/ml
Flavonones	19.113	15862.0896	1220.495	12.2435	ppm
Steroids	23.306	3722.5277	291.728	5.9541	ppm
Kaemferol	25.080	10027.6642	781.331	9.0277	µg/ml
Cyanogenic glycoside	28.593	3564.6230	279.848	5.2703	ppm
Phytate	30.986	6172.2460	483.357	8.0886	µg/ml
Epicatechin	34.116	7295.8673	570.542	10.0604	µg/g
Resveratol	39.026	4534.6980	355.527	3.3941	ppm
Cardiac glycoside	43.876	5092.5632	401.247	1.9972	µg/ml
Epihedrine	44.450	4428.2046	349.075	3.2471	µg/ml
		150384.5314		138.0222	

Table 2: Antimicrobial activity of Endophytic fungal extract of *Neem*

Concentration (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)					
	S. aureus	***B. subtilis***	***E. coli***	***K. pneumoniae***	***C. albicans***	***A. niger***
1	0±0	0±0	0±0	**3±0**	0±0	0±0
0.5	0±0	0±0	0±0	**2±0**	0±0	0±0
0.25	0±0	0±0	0±0	0±0	0±0	0±0
0.13	0±0	0±0	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
Positive Ctrl	0	0	0	0	15	0

Key: *S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis; K. pneumoniae: Klebsiella pneumoniae; C. albicans: Candida albicans; A. niger: Aspergillus niger;* Positive controls: Cip: Ciprofloxacin (8 µg/mL); Mic: Miconazole (50 µg/mL).

Azadirachta indica has potential biological properties and effective against various bacterial, fungal infections, dental disorders, skin diseases, leprosy, syphilis, malaria and also has antiseptic property[2]. In the table 2, it was revealed that the fungal endophytic extract of *Azadirachta indica* had no antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Candida albicans and Aspergillus niger.* The results showed that the extract had activity only against *Klebsiella pneumoniae* (at a concentration of 0.5mg/mL and 1mg/mL) with an IZD ranging from 2-3mm, showing a dose dependent activity. The activity of the extract was resisted by the other test microbes. The endophytic fungal extracts demonstrated a minimal antimicrobial activity. Among the organism tested, *Klebsiella pneumoniae* is the most sensitive bacteria tested because it was susceptible to the fungal extract. In comparison with the control, the endophytic fungal extract of *Azadirachta indica* had more activity than the positive control against *Klebsiella pneumoniae.* This result confirmed previous report that endophytic bacteria from *A. indica* has antibacterial activity against *Klebsiella pneumoniae*[26]. In addition, a study carried out of 7 endogenous isolates from the neem, only 1 strain showed activity against methicillin-resistant *S. aureus* (MRSA) but did not against other pathogenic bacteria[27]. However, a study showed that Neem extract possess considerable antimicrobial activity against *Staphylococcus aureus*[28]. Similarly, a study showed that methanolic extract of *A. indica* exhibited moderate to low activity against *Staphylococcus aureus* though at a high

concentration, the concentration of plant extracts ranged from 0.78 mg/mL to 200 mg/Ml[29]. The antimicrobial activity of various solvent extracts of leaf and stem of neem against test microorganisms exhibited different sensitivities towards these extracts in a dose-dependent manner[30]. In the table 2, the endophytic fungal extract from *A. indica* showed no activity against *C.albicans* and *A.niger* which agrees with the report of Eze *et al.,* endophytic fungi from the leaves of *Newbouldia laevis, Ocimum gratissimum, Carica papaya* showed no antifungal activities on *C.albicans* and *A.fumigatus*[13].

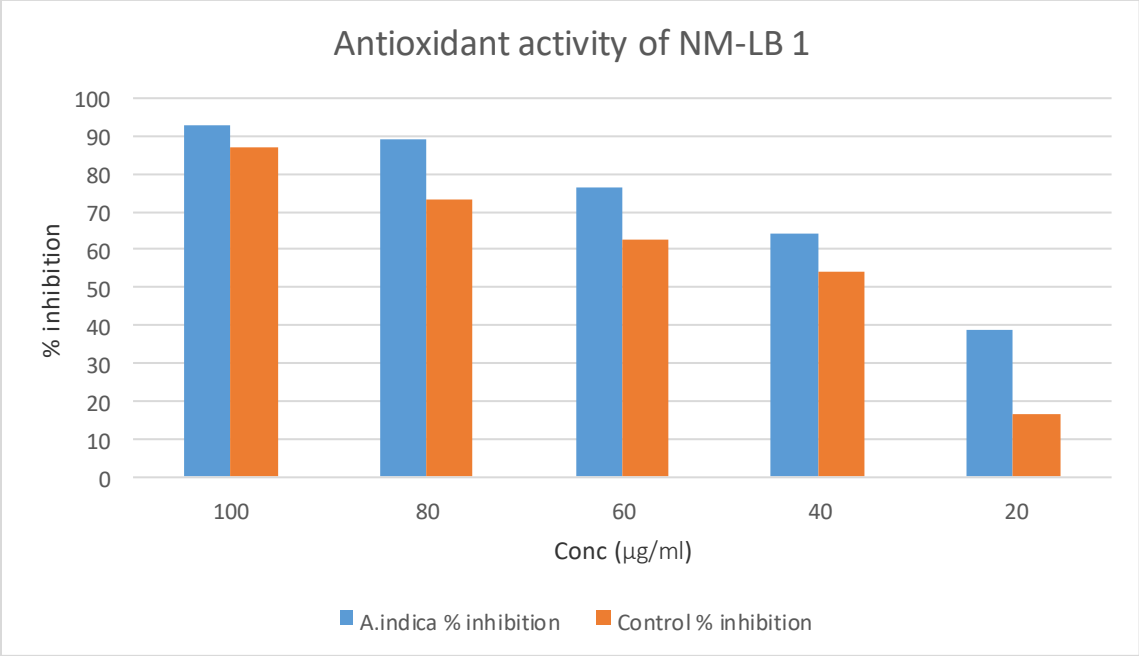

Figure 3: Antioxidant activity of NM-LB 1

The highest percentage of inhibition of antioxidant activities of NM-LB 1 (92.7) is seen at 100 µg/ml as revealed in Fig 3. The percentage of inhibition of antioxidant activities of NM-LB 1 showed concentration dependent. The endophytic fungal extracts of *A. indica* showed high antioxidant activities which fits in with the report of Xie and Schaich[31]. In addition, the antioxidant activities of these extracts is because of possession of secondary bioactive compounds and aid their antimicrobial activities against harmful microorganisms[32].

CONCLUSION

The results of this study showed that the extract of an endophytic fungus of *Azadirachta indica* possesses huge prospects as source of novel bioactive compounds with eco-friendly characteristics that would be of great importance to Pharmaceutical and Biotechnology industries.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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