

STANDARDISATION AND ANTIMALARIAL EVALUATION OF THE METHANOL AND
ENDOPHYTIC FUNGI EXTRACT ISOLATED FROM THE AZADIRACHTA INDICA
(MELIACEAE) MICE MODEL

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Abstract

Background: Endophytes can synthesis bioactive compounds responsible for plants' defence against pathogens, thus evaluating *Azadirachta indica* antimalarial potential.

Objectives: The study aimed to standardise and evaluate the antimalarial properties of the methanolic, and endophytic fungi extract of *Azadirachta indica* using *Plasmodium berghei*, rice media and mice (laboratory animal).

Methods: The study followed standard methods in the extraction, phytochemical and physicochemical evaluation of the powdered leaves of *A. indica*. The study isolated endophytic fungi using malt extract agar. Fungal fermentation was done (21 days), and ethyl acetate was extracted. Acute toxicity tests were performed on the crude and endophytic fungal extracts. The antimalarial curative study was done for both extracts, with artemether-lumefantrine as a positive control to evaluate the percentage curative effect (PCE). HPLC analysis was based on the retention time and UV absorption spectra.

Results: Phytochemical and physicochemical analyses showed the existence of alkaloids, saponins, and tannins and low moisture content. Seven endophytic fungi were isolated. Fungal fermentation yielded an appreciable amount of endophytic fungi. The oral acute toxicity study of the crude extract in mice revealed lethality reaction at a 4472.14 mg/kg dose, while the seven endophytic fungi extracts were above 5000 mg/kg. The crude extract had its highest PCE (83.3 0%) at 125 mg/kg. AMRc and AMRy endophytic extracts gave the highest PCE at 125 mg/kg (95 and 95.72 %). Artemeter-lumefantrine showed PCE of 98.62 % at 0.9 mg/kg. HPLC analysis of the endophytic extracts identified methyl- 2- (4-hydroxyphenyl) acetate, neuroleulin B, aniquinazoline, palitantin, genistein, procyanidin, and pipernonaline.

Conclusion: The findings of this study demonstrated the antimalarial potentials of the endophytes of *A. indica*, which may be exploited further.

Keywords: Endophyte, microscopy, artemether-lumefantrine, percentage curative effect.

Introduction

The necessity for novel and therapeutic compounds to provide improved quality of life in all aspects of human health conditions is ever-growing. Bacterial drug resistance, the emergence of life-threatening viruses, the recurrent problems of diseases in persons with organ transplants, and the incredible rise in the occurrence of infections due to fungal pathogens, the advent of resistant strains of malaria parasites in the world's population all underscore our inadequacy to cope with all these medical problems.^[1] Natural products are chemical compounds derived from plants and living organisms, including plants, animals, aquatic life, and microorganisms. The success of various medicinal drugs from the microbial origin, such as the penicillin antibiotics from *Penicillium hrysogenum*, the discovery of cyclosporine, an immunosuppressant from *Tolypocladium inflatum*, among others., has dramatically shifted the focus of drug discovery from plants to microorganisms. Among the most significant eukaryotic organisms, fungi produce many secondary metabolites that serve as drugs or lead

compounds for synthetic modifications.^[2] Endophytes possess a resistance mechanism against pathogenic invasion via the production of secondary metabolites.^[3] Various studies have described many beneficial and safe antimicrobial compounds isolated from endophytes belonging to several chemical classes like phenols, peptides, terpenoids, flavonoids, alkaloids, quinines, and steroids. The quantitative phytochemical analyses of the endophytic extract of this plant have shown the abundant reservoir of alkaloids and flavonoids in the endophytic extract of the plant *Azadirachta indica*.^[4] This study evaluated the antimalarial activity of the methanolic extract, and endophytic fungi extract isolated from *Azadirachta indica*.

Materials and Methods

Plant materials collection

Healthy leaves of *Azadirachta indica* were collected from their trees growing in the natural environment at the Faculty of Pharmaceutical Sciences Agulu, Nnamdi Azikiwe University on the 2nd of December, 2015. The plants were identified, examined, and authenticated in the Pharmacognosy and Traditional Medicine Department, Nnamdi Azikiwe University, Awka.

Experimental Animals

The study utilised Swiss Albino mice (25 – 30g). All the animals were obtained from the animal house of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Agulu, Nigeria. The animals were kept in standard laboratory conditions of 12 hr light, room temperature, 40-60 % relative humidity, and fed with rodent feed (Guinea Feeds Nigeria Ltd). The animals were fed ad libitum.

Parasite

The parasite, *Plasmodium berghei* used for this study was obtained from the Department of Pharmaceutics, University of Nigeria Nsukka. The strain was maintained in the laboratory by serial blood passage from mouse to mouse.

Experimental Procedures

Phytochemical analysis of the crude plant leaves

The quantitative and qualitative analyses of the extracts were carried out using standard methods.^[5]

Methanol Extract Preparation

One hundred grams (100 g) of the dried, pulverised plant leaves were weighed using a weighing balance (Camry EK350 Model, China) and cold macerated in 500 ml of methanol for 72 h with intermittent shaking. The resulting solutions were filtered with Whatman filter paper. The filtrate was concentrated *in vacuo* using a rotary evaporator (RE300 Model, United Kingdom) at four °C.

Isolation of endophytic fungi

The plant leaves were rinsed thoroughly with sterile water to remove dust and debris in the laboratory before being disinfected by immersion in 70 % ethanol for 3 min and 2 % sodium hypochlorite for 2 min. Then, they were washed using distilled water (sterile) and blotted dry on sterile blotting paper in a lamina flow cabinet. A sterile scalpel was used to cut the leaf blade and mid-rib approximately 1 cm in length. About 5-6 segments were placed aseptically on malt agar media, prepared with 250 mg chloramphenicol per litre of the media to suppress bacterial growth. The cut end of the leaf blade and midrib was made to be in contact with the media. The plates were then incubated at 25-28 °C for five days but monitored every day. Most of the fungal growth was initiated within five days of inoculation. The hyphae growing from the plant materials were sub-cultured repeatedly until a pure culture was obtained.

Fungal fermentation and extraction of metabolites

Fungal fermentation was done using solid-state fermentation. A 100 g of unpolished rice was sterilised with 200 ml of water at 121 °C for 30 min. After cooling, blocks of actively growing pure fungal isolates were transferred onto the rice media under aseptic conditions, plugged with cotton wool and foil, and left on the fermentation shelf

for 21 days. On the 21st day, the fermentation was stopped by adding 500ml of ethyl acetate until the rice media was covered entirely. A sterile aluminium rod was used to break apart the mycelia growth on the rice media and to loosen the media. The flasks were allowed to stand for two days with intermittent agitation. Then, filtration was done using a sterile cheesecloth to remove the mycelia, the rice media, and finally, through Whatman filter paper. Using a rotary evaporator, the filtrates (extracts) were concentrated at the speed of 7 rpm at 50°C. The filtrates were used for the biological assay.

Acute toxicity study

Acute toxicity tests were performed using a modified method of OECD- 423 guidelines.^[6]

A total of 13 mice were employed and done in two phases:

Phase I: In this phase, the animals were grouped into three groups of three mice per group-

Group 1 – 10 mg/kg of the extract was administered per oral; Group 2- 100 mg/kg; Group 3- 1000 mg/kg.

The animals were constantly monitored for two hours, intermittently for the next 6 hours, and after 24 hours for behavioural changes and mortality. From the result of the 1st phase, the 2nd phase was carried out.

Phase 2: Four mice were used and grouped into four groups of one mouse per group in this phase. The animals received treatment as follows:

Group 1- 2000 mg/kg; Group 2-3000 mg/kg; Group 3- 4000 mg/kg; Group 4- 5000 mg/kg

The animals were monitored as in phase 1 for behavioural changes and mortality,

The LD₅₀ was calculated by the formula:

$$LD_{50} = \sqrt{D_0 \times D_{100}}$$

D₀ = Highest dose that gave no mortality; and D₁₀₀ = Lowest dose that produced mortality.

Antimalarial curative study

Antimalarial curative studies of endophytic extract and methanol leaf extract were carried out according to a standard method.^[7] In this model, a total of seventy-two mice were used. They were grouped into twenty-four groups of three mice per group. Blood was collected from donor mice infected with the parasite (*Plasmodium berghei*) by oculus puncture and diluted with normal saline such that 0.2 ml contains approximately 1x10⁷ infected red cells. The animals in each group were infected with the parasite by single oral administration of 0.2 ml diluted blood and left for 72 hours to establish the infection. After infection, the animals were treated as elaborated in the table below. A thin blood film was made from the tail blood stained with Giemsa stain and was examined for parasitemia on day 4 and 8 post-treatment, respectively. The percentage curative effect was obtained with the formula shown below:

$$\frac{D_0 - DA}{D_0} \times 100$$

$$\frac{1}{1}$$

Where: D₀ = day 0 mean parasitemia and DA = Treated day mean parasitemia

HPLC analysis

Each dried fungal metabolite extract (2 mg) was reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10mins and after that centrifuged at 3000 rpm for 5 min. Then, 100 µL of each dissolved sample was transferred into HPLC vials containing 500 µL of HPLC grade methanol. HPLC analysis was carried out on the sample using a Dionex P580 HPLC system coupled to a photodiode array detector (UV3440S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280, and 340 nm, respectively. Eurospher-10 C18 (Knauer, Germany) was used to refill the column for separation (125 x 4 mm; length x internal diameter), and a linear gradient of nano pure water was adjusted to pH two by addition of formic acid, and methanol was used as eluent.

Results

Result of isolation of endophytic fungi:

A total of seven fungal endophytes designated AMRc – Azadirachta midrib c; AMRY– Azadirachta midrib y; AMRd – Azadirachta midrib d; AMRe – Azadirachta midrib e; ALBg – Azadirachta leaf blade g; ALBd - Azadirachta leaf blade d; ALBx - Azadirachta leaf blade x were isolated. Their respective yields are indicated in the table below. Three endophytic extracts showed the highest antimalarial activity. See Tables 1 and 2

Table 1: Antimalarial Curative Study Groups and Treatment

S/N	Group	Treatment (mg/kg)
1	A	500 AMRe
2	A	250 AMRe
3	B	500 ALBx
4	B	250 ALBx
5	C	500 AMRc
6	C1	250 AMRc
7	C2	125 AMRc
8	C3	62.25 AMRc
9	D	500 ALBg
10	D	250 ALBg
11	E	500 AMRy
12	E1	250 AMRy
13	E2	125 AMRy
14	E3	62.25 AMRy
15	F	500 AMRd
16	F	250 AMRd

17	G	500 ALBd
18	G	250 ALBd
19	H	500 CEA
20	H1	250 CEA
21	H2	125 CEA
22	H3	62.25 CEA
23	I	ACT 0.9
24	J	10 ml/kg 5% Tween 80

Table 2: Yield of secondary metabolites obtained from endophytic fungi

S/N	Endophytic Fungi	Metabolite yield (mg)	% Yield
1	AMRc	495	4.95
2	AMRy	520	5.20
3	AMRe	205.9	2.06
4	AMRb	150	1.50
5	ALBg	450	4.50
6	ALBd	400	4.00
7	ALBx	370	3.70

KEYS: A= *Azadirachta indica*, LB=Leaf Blade, MR= Mid-rib

The result of extraction and phytochemical screening of *Azadirachta indica* leaves

The quantitative and qualitative phytochemical screening results of the crude extract of *Azadirachta indica* leaves show a low yield of 2.5 % and appropriate saponin content of 9.5 %. The quantified phytoconstituents in the plant's leaves showed saponins to be the highest, followed by alkaloid, tannin, and flavonoid. See Tables 3- 5

Table 3: Yield of methanol extract of *Azadirachta indica* leaves

Plant	Weight of plant powder (g)	Weight of crude methanol extract (g)	Percentage yield (% w/w)
<i>Azadirachta indica</i>	100	2.5	2.5

Table 4: Qualitative phytochemical constituents of methanol extract of *Azadirachta Indica*

Phytochemicals	Observation
Tannins	+
Saponins	+
Flavonoids	+
Steroids	-
Alkaloids	+
Cardiac glycosides	-
Carbohydrates	-
Proteins	-
Starch	-
Triterpenoids	-

Key: (-) absent; (+) present

Table 5: *Quantitative phytochemical constituents of Azadirachta indica leaves*

Constituent	Percentage yield (% w/w)
Alkaloid	5.00
Tannin	1.17
Saponin	9.50
Flavonoid	0.13

HPLC results

The results of HPLC screening of the endophytic fungal extract from *Azadirachta indica* are presented in Table and Figures, showing the identified phyto-constituents of the endophytic fungal extract of *Azadirachta indica* leaves. In addition, the reported pharmacological activities are also shown. See Table 6, Figures 2-8

Table 6: *HPLC result of the endophytic fungal extract from Azadirachta Indica*

Endophytic Fungal Extract	Comparable Peak
AMR _B	Aureonitol, Halenadinon, Dipiperamide, Pestaliopyrone, Aniquinazoline, chloramphenicol.
AMR _C	Dragmacidonamine, Rubrofusarin, Antibiotic 3192C, Callyaerin, Dicaffeoylchina
AMR _e	Dehydropiperonaline, (E) – 4 – [(2 – Methylpropyl) amino] – 4 – oxo – 2 – butenoic acid, Aerophobin, Monocerin.
AMR _y	Aniquinazoline, Bastadin 4, A new aflavinine derivative, Pestaliopyrone, Genistein, tetrahydroaltersolanol, (2)-5-4- hydrobenzylidene) – hydantoin, Questin, Palitantin,.
LBx	Amin – Chlor-Phe-Assigr, Neurolnin, Ethyl 2 – (4 – hydroxyphenyl) acetate.

ALB _g	Ethyl 2 – (4 – hydroxyphenyl) acetate, PAI, Citreodimene, Ruspolinon
ALB _D	PAI, Citreodimene, Carbonarone.

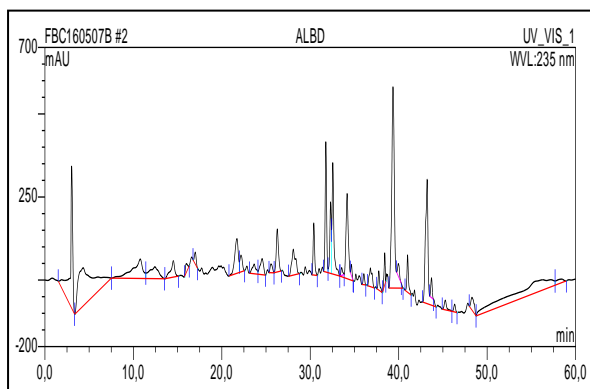


Figure 2: HPLC chromatogram of ALB_D of endophytic extract of *Azadirachta indica* showing A - citreodrimene (R_t= 26.27 min), B - carbonarones (R_t=30.40 min), C - Plasminogen activator inhibitor type 1 (R_t= 31.77 min), D- Plasminogen activator inhibitor type 1 (R_t= 32.56 min)

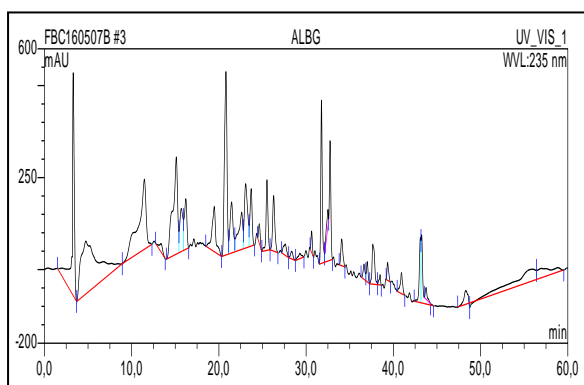


Figure 3: HPLC chromatogram of ALB_G of endophytic extract of *Azadirachta indica* showing E - ethyl 2- (4- hydroxyphenyl) acetate (R_t = 15.12 min), F-Ruspolinon (R_t=20.81 min), G - PAI-1 (R_t=31.76 min), H – Citreodrimene (R_t= 32.75 min)

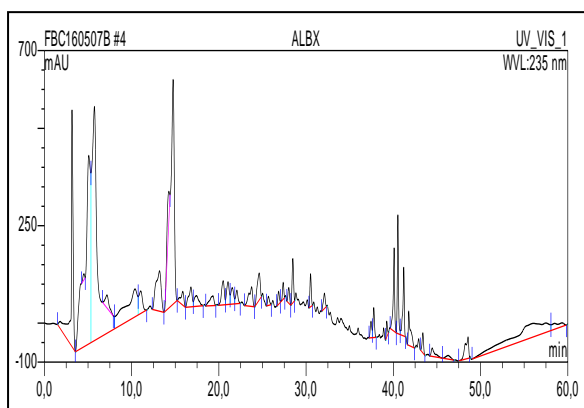


Figure 4: HPLC chromatogram of ALBx of endophytic extract of *Azadirachta indica* showing I - Ethyl 2 - (4 - hydroxyphenyl) acetate ($R_t=14.26$ min and 14.75 min), J - amin-chlor-phe-Essigsr. ($R_t= 28.48$ min), K-neurolenin ($R_t=30.51$ min).

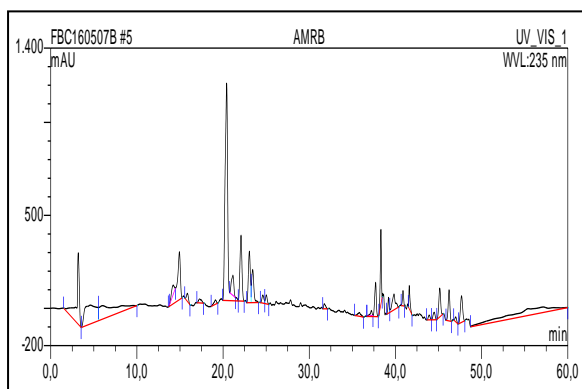


Figure 5: HPLC chromatogram of AMRb of endophytic extract of *Azadirachta indica* showing L - halenadinon ($R_t= 20.42$ min), M- dipiperamide ($R_t= 22.08$ min), N-pestaliopyrone ($R_t= 23.05$ min) , O- aniquinazoline ($R_t=23.44$ min), P- aureonitol ($R_t= 38.32$ min)

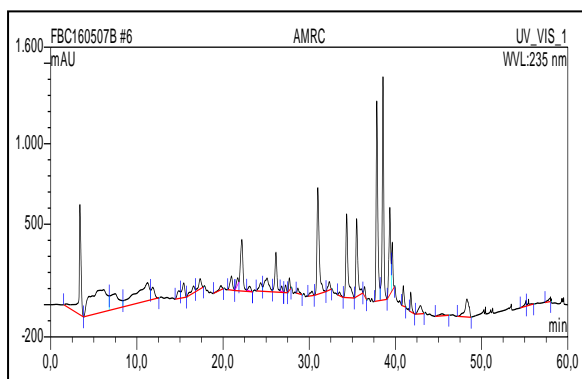


Figure 6: HPLC chromatogram of AMRe of endophytic extract of *Azadirachta indica* showing Q- Methyl -2- (4- hydroxyphenyl) acetate ($R_t=14.86$ min), R- Dicaffeoylchina ($R_t= 22.18$ min), S- callyaerin ($R_t=26.13$ min), T- antibiotic 3192C ($R_t= 31.00$ min), U – Dragmacidonamine ($R_t= 34.34$ min), V – Rubrofusarin ($R_t= 35.50$ min).

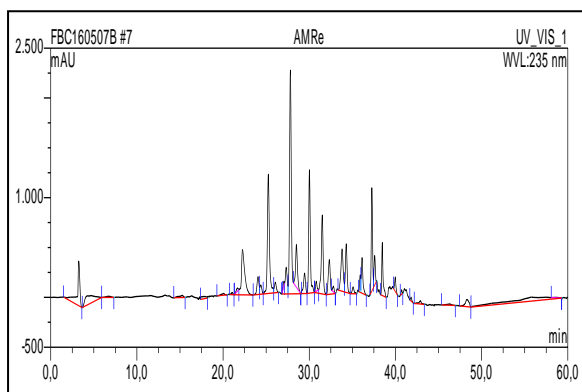


Figure 7: HPLC chromatogram of AMRe of endophytic extract of *Azadirachta indica* showing W- Monocerin (Rt= 22.26 min), X- Dehydropiperonaline (Rt= 25.25 min), Y- (E) – 4 – [(2 – Methylpropyl) amino] – 4 – oxo – 2 – butenoic acid (Rt= 27.81 min), Z- Aerophobin (Rt= 29.46 min), A₁- Monocerin (Rt= 34.51 min).

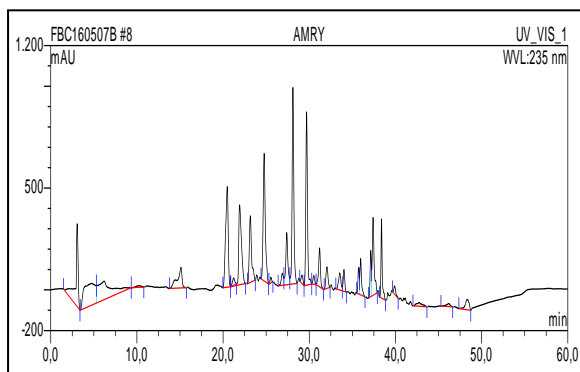


Figure 8: HPLC chromatogram of AMRy of endophytic extract of *Azadirachta indica* showing A2- Bastadin 4 (Rt= 20.48 min), A3- A new aflavinine derivative (Rt= 21.92 min), A4- Pestaliopyrone (Rt= 23.16 min), A5- Genistein (Rt= 24.76 min), A6- Aniquinazoline (Rt= 27.39 min), A7- tetrahydroaltersolanol (Rt= 28.11 min), A8- Aniquinazoline (Rt= 29.69 min).

Result of physicochemical, organoleptic, and microscopical evaluations

Results of physicochemical, organoleptic, and microscopically evaluations are shown and adequately defined. See Tables 7-10 and Figure 1

Table 7: Reported Biological Activities of some of the Chemical Compounds present in the *Azadirachta indica*

Chemical Compounds	Previously isolated from	Biological Activities
Dipiperamide C ₃₄ H ₃₈ N ₂ O ₈	An alkaloid from white pepper	Nutrient ²⁸
Aureonitol	<i>Helichrysum</i> sp	Antiviral ²⁹
Dragmacidonamine	<i>Dysidea</i> sp	Antibacterial ³⁰
Aniquinazoline (alkaloid)	<i>Aspergillus nidulans</i>	Antimalarial ¹⁷ , Cytotoxic ¹⁷ , Antibacterial ³¹
Callyaerine	Marine sponge, <i>Callyspongia aerizusa</i>	Cytotoxic ¹⁴

Cynarine (Dicaffeoylchina)	<i>Cynara cardunculus</i>	Inhibits taste receptors
Piperonaline		Mosquito Larvicidal ¹⁸
Monocerin	<i>Dreschleramonoceras</i>	Insecticidal ¹¹
Dehydropiperonaline	<i>Piper langum L.</i>	Vasodilating activity ³²
Neurolenin B		Antimalarial ¹⁰
Carbonarones	<i>Aspergilluscarbonarices</i>	Antibacterial ³³
Procyanidin	<i>Quercus petraea</i> – heartwood	Antibacterial, ⁹ Antioxidant ⁹
Pavetannin		Antiviral ³⁴
Bastadin 5	Marine sponge	Antiangiogenic ¹³
PAI – 1 (Plasminogen activator inhibitor type 1)	Wheat plant, <i>Triticumaestivum L.</i>	
Genistein	<i>Genista tinctoria</i>	Antioxidant ³⁵
Palitantin C ₁₄ H ₂₂ O ₄	<i>Trichophytonharzianum</i>	Antiprotozoal, ¹⁶ Antifungal ¹⁶
Questin C ₁₆ H ₁₂ O ₅	<i>Eurotiumsp</i>	Antidiabetic ³⁶
Methyl – 2- (4-hydroxyphenyl) acetate		Antimalarial activity ¹²

Table 8: Physicochemical analysis of *Azadirachta indica* crude extract

Parameter	Yield (%w/w)
Moisture content	9.0±0.68
Total Ash	11.5±0.12

Acid insoluble ash	2.0±0.03
Water-soluble ash	7.5±0.05
Water-soluble extractive	0.20±0.19
Alcohol soluble extractive value	4.57±0.12

Table 9: Organoleptic evaluation of *Azadirachta indica* leaf powder

Features	Observation
Colour	Green
Odour	Non-characteristic
Taste	Slightly bitter
Texture	Coarse powder

Table 10: Microscopical examination of *Azadirachta indica* leaf powder

Features	Observation
Epidermal trichomes	Present
Calcium oxalate	Absent
Starch	Absent
Fibres	Present
Lignified vessel	Present



Figure 1: Picture of *Azadirachta indica* plant showing the seed, leaf, and stem

Discussion

The selection of the plant materials was based on numerous reports on their ethnomedicinal uses. These ethnomedicinal effects are thought to be mediated through their various bioactive metabolites. Natural products have continued to be the foundation for managing different ailments affecting humanity, including malaria. The phytochemical screening of the methanolic extract of *Azadirachta indica* indicated the existence of flavonoids, alkaloids, saponin, and tannins. The plant's age, geographical location, and climatic condition affect the quantity and quality of the saponins, alkaloids, tannin, and flavonoids. The appreciable amount of alkaloid present implies possible excellent antimalarial activity. It has been recorded that alkaloids possess antimalarial activity, which must have contributed to the plant's experimental antimalarial (antiprotozoal) activity.^[8] The presence of these chemical constituents within endophytes is an indication that they may be a potential source of precursors for drug development.

The HPLC chromatogram of the endophytic extract of *Azadirachta indica* leaf displayed the occurrence of many compounds; these compounds were identified based on similarity with data (UV spectra) in an inbuilt library and literature comparison. The chromatogram of the endophytic extract showed that major compounds were present. Some of these preliminarily identified compounds may have contributed to the antimalarial activity of the plant, given their reported activities.^[9-15] These compounds identified also confirmed some of the plant's ethnomedicinal claims, including antibacterial, antimalarial, antidiabetic, antioxidant, antifungal, and antioxidant activities.

Oral acute toxicity study in mice revealed no lethality or toxic reaction at any of the administered doses between (100 mg/kg and 5000 mg/kg). This implies that the endophytic extract of the plant is not toxic and has a good safety profile, and will be administered in very high doses orally before the toxic level can be reached. However, the LD₅₀ of methanol extract of *Azadirachta indica* leaf shows that toxicity will be reached at a very high concentration.

The HPLC analysis showed that these three extracts had compounds with a very potent antimalarial activity which may have contributed to their very high percentage curative effects (PCE). These compounds include; palitantin, neurolepin B, aniquinazoline, piperonaline, and methyl-2- (4-hydroxyphenyl) acetate.^[10,12,16-18] The high alkaloid content of the crude and other undetected compounds may have contributed to its high antimalarial activity recorded. The findings of this study demonstrated the antimalarial potentials of *Azadirachta indica*, which may be exploited in the management of acute malaria infections. The effective dose at 50 % curative effect (ED₅₀) was calculated from the dose-response relationship curve. At 38 mg/kg AMRy and AMRcendophytic extract, a 50 % reduction in parasitemia was achieved. The ED₅₀ of the crude extract was 40 mg/kg. The least dose that achieved 50 % parasitemia reduction was obtained; hence the dose of crude extract was higher than the endophytic extract. Therefore, results from the research work have shown that the endophytic extract is more effective than the methanol leaf extract of *A. indica* both in dose, antimalarial effect, and toxicity profile.

Estimating the physicochemical parameters of crude drugs is essential in detecting adulteration or improper handling of crude drugs. The moisture content of crude drugs determines their shelf-life. Reduced moisture content discourages bacteria, fungi, or yeast growth and ensures safe storage. The general requirement of moisture content in the crude drug is that it should not be more than 14 %, ^[19] and the value obtained in this research work (9 %) is within the accepted range. The result of the moisture content of the *Azadirachta indica* leaf suggests that

it could be stored safely for an extended period. The ash value is the residue remaining after incineration of the plant material. This residue represents foreign organic matter such as metallic salts or silica naturally occurring in the crude drug or sticking to it or deliberately added as a form of adulteration. Ash that is insoluble in acid is part of total ash that is not soluble in hydrochloric acid (e.g. sand and siliceous earth), while water-soluble ash is the water-soluble portion of the total ash.^[20] The total ash value (11.5 %) of crude powder of *Azadirachta indica* leaf was found to be approximately five times more than the acid insoluble ash (2 %) and water-soluble ash (7.5 %). The crude powder of *Azadirachta indica* leaf was higher in alcohol soluble extractive (4.57 %) than in water-soluble extractive (0.20 %).

Unwanted parts of medicinal plants may sometimes possess a character that will increase the ash value. Ash value of crude drugs varies within a wide range, and any fixed difference in these values indicates a change in quality.^[21, 22] The extractive value of a medicinal plant is the amount of active phytochemical constituents in a given quantity of that plant when extracted with a particular solvent(s). The higher alcohol extractive value of *Azadirachta indica* leaf suggests that the constituents may be more soluble in alcohol than water. These values could be helpful for the compilation of a suitable monograph for the proper identification of this plant.

Organoleptic and macroscopic evaluations are the first steps towards establishing the identity and purity of crude drugs.^[23] Microscopy of powdered drugs is essential since once the plant is dried and ground, it is likely to lose its morphological distinctiveness and thus prone to adulteration. Organoleptic evaluation is based on sensory characters such as colour, odour, taste, and texture.^[24] Organoleptic evaluation of *Azadirachta indica* powdered leaf revealed a coarse green powder with an aromatic odour and slightly bitter taste, which are useful diagnostic characters. Microscopical examination of this plant showed the absence of calcium oxalate crystals and starch granules. Microscopic characters observed include lignified spiral vessel stained pink, group of phloem fibres, and covering trichome (non-glandular trichome), which previous workers also reported.^[25, 26] These are diagnostic characters that could be used to differentiate *Azadirachta indica* leaf from other closely related species, and varieties thus are helpful in proper identification of this plant part.

The HPLC analysis of these endophytic fungal metabolites showed the presence of compounds belonging to alkaloids, coumarins, phenols, cyclic dipeptides, and other structural types, indicating that they could be valuable precursors in drug development. It has been suggested that virtually all plants in natural ecosystems form an intimate symbiotic relationship with one or more types of endophytic fungi.^[27] The current standard techniques for isolating fungal endophytes only account for non-fastidious endophytes. Therefore, the obligate biotrophs are yet to be routinely cultured. Hence, the true diversity of endophytes associated with these plants is underestimated.

Conclusion

The results from this study have revealed that the medicinal plant *Azadirachta indica* harbours diverse species of endophytic fungi. These endophytic fungi can be made to produce their secondary metabolites under controlled solid-state fermentation. Some of these endophytic fungal extracts displayed good antiplasmodial activity against *P. berghei*. The crude methanolic extract of the plant also showed high antimalarial activity. The very high antimalarial activity of the endophytic fungal extract could suggest that the endophytes conferred that activity on the plant. Furthermore, these endophytic fungal extracts displayed strong antimalarial activity, which was very close to that of Artemether-lumefantrine, thus could have a high effect in treating acute malaria infections. The endophytic fungal extract AMRy demonstrated the most potent antimalarial activity with a PCE of about 95.72 %; this shows the potential of this endophyte in discovering lead antimalarial agents.

Recommendations

1. Isolation and characterisation of chemically active components of the methanolic leaf extract and endophytic extract to elucidate their specific mechanisms of action
2. Further investigation of the toxicity of this plant to evaluate its effect during long-term administration.

References

1. Strobel G., Daisy B., Castillo U., and Harper J. Natural products from endophytic microorganisms. *Journal of Natural Products*, 2004; 67: 257-268.
2. Bode H.B., Bethe B., Hofs R., Zeek A. Big effects from small changes: possible ways to explore nature's chemical diversity. *Chem Bio Chem.*, 2002; 3: 619-627.
3. Tan R.X., Zou W.X. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* 2001; 18: 448-459.
4. Nwankwo OL, Chukwuebuka OC, Collins OO, Samuel BJ, Obasi JC, Iloh ES, Nwankwo EO, Quantitative phytochemical analysis of the fungus endophytic extracts isolated from *Azadirachta indica* using gas chromatography- flame ionisation detector, *Journal of Drug Delivery and Therapeutics*. 2021; 11(5):80-83. DOI: <http://dx.doi.org/10.22270/jddt.v11i5.4999>
5. Edoga H.O., Okwu D.E., Mbaebie B.O. (2005). Phytochemicals constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol.* 2005; 4(7): 685- 688.
6. OECD guidelines. Available from: https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocus/oecd/oecd_gl423.pdf
7. Knight DJ, Peters W. The antimalarial action of N-benzoyloxydihydrotriazines: The action of cycloguanil (BRL50216) against rodent malaria and studies on its mode of action. *Ann Trop Med Parasitol.* 1980; 74:393-404.
8. [Dua H.S.](#), [Faraj L.A.](#), [Said D.G.](#), [Gray T.](#), [Lowe J.](#) Human corneal anatomy redefined: a novel pre-Desemet's layer (Dua's layer). *Ophthalmology*, 2013; 120(9): 1778-85.
9. Souquet J.M, Cheynier V., Brossaud F., Moutounet M. Polymeric proanthocyanidins from grape skins. *Phytochemistry*, 1996; 43, 509-512.
10. Blair S, Mesa J, Correa A, Carmona-Fonseca J, Granados H, Saez J. Antimalarial activity of neurolepin B and derivatives of *Eupatorium inulaefolium* (Asteraceae). *Pharmazie*, 2002; 57: 413-415.
11. Axford L.C., Simpson T.J., Willis C.L. Synthesis and incorporation of the first polyketide synthase free intermediate in monocerin biosynthesis. *Angewandte Chemie International Edition*, 2004; 43: 727-730.
12. Bischoff T.A., Kelley C.J., Karchesy Y., Laurantos M., Nguyen-Dinh P., Arefi A.G. Antimalarial activity of lactucin and lactucopicrin: sesquiterpene lactones isolated from *Cichorium intybus* L. *Journal of Ethnopharmacology*, 2004; 95 (2-3): 455-7.
13. Kotoku N., Hiramatsu A., Tsujita H., Hirakawa Y., Sanagawa M., Aoki S., Kobayashi M. Structure-activity relationships study of bastadin 6, an anti-angiogenic brominated tyrosine derived metabolite from a marine sponge. *Arch Pharm Chem Life Sci.* 2008; 341: 568-577.
14. Sabrin R.M.I, Edrada-Ebel R, Mohamed G.A., Youssef D.T.A., Wray V., Proksch P. Callyaerin G, a new cytotoxic cyclic peptide from the marine sponge *Callyspongia aerizusa*. [ARKIVOC - Online Journal of Organic Chemistry](#), 2008; (12): 164-171
15. Sacramento C.Q., Martorelli A., Fintelman-Rodrigues N., de Freitas C.S., de Melo G.R., Rocha M.E.N., et al. (2015) Aureonitol, fungi-derived tetrahydrofuran, inhibits influenza replication by targeting its surface glycoprotein hemagglutinin. *PLoS ONE* 10(10): e0139236.
16. [Hong B.C.](#), [Wu M.F.](#), [Tseng H.C.](#), [Huang G.F.](#), [Su C.F.](#), [Liao J.H.](#) (2007). Organocatalytic asymmetric Robinson annulation of alpha, beta-unsaturated aldehydes: applications to the total synthesis of (+)-palitantin. [J Org Chem.](#); 26: 72(22): 8459-71.
17. Chun-Yan Z., Xiao G., Bao-Kang H. (2013). Chemical Constituents from an Endophytic Fungus *Chaetomium globosum* Z1. *Nat. Prod. Comm.* 8(9):1217-1218
18. Lee S.E. (2000). Mosquito larvicidal activity of piperonaline, a piperidine alkaloid derived from long pepper, *Piper longum*. *J. Am. Mosq. Control Assoc.*, 16 (2000), pp. 245-247.
19. Jhade D., Ahirwar D., Jain R., Sharma N.K., Gupta S. (2011). Pharmacognostic Standardisation, Physico and Phytochemical Evaluation of *Amaranthus spinosus* Linn. Root. *J. Young Pharm.* 3:221- 225.
20. Mritunjay K., Prodyut M., Sudarshana B., Kabita M. (2013). Physico-Chemical Evaluation, Preliminary Phytochemical Investigation, Fluorescence And Tlc Analysis Of Leaves Of The Plant *Lasia spinosa* (Lour) Thwaites. *Int. J. Pharm. Pharm Sci.*, 5(2):306-310
21. Odoh R., Adebayo K.S. (2011). Assessment of Trace Heavy Metals Contaminations of some Selected Vegetables Irrigated with Water from River Benue within Makurdi Metropolis, Benue State, Nigeria. *Adv. Appl. Sci. Res.*, 2(5): 590- 601.
22. Nzelu C.O., Gomez E.A., Caceres A.G., Sakurai T., Martini-Robles L., Uezato H., et al. (2014). Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for *Leishmania* infection. *Acta Trop.*, 132: 1-6.
23. WHO (2002). World Health Organization Fact Sheet Number 271. World Health Organisation, Geneva.
24. Kokate, K.K., Purohit, A.P., Gokhale, S.B., (2008). *Pharmacognosy*, Forty-second edition, VallabhPrakashan, India (pp. 13-44).

25. Kumari R.P., Sivakumar J., Thankappan B., Kumarasamy A. (2013). C-Phycocyanin Modulates Selenite-Induced Cataractogenesis in Rats. *Biological Trace Element Research*, 151(1): 59–67.
26. Ugbabe G.E. (2013). Scanning electron microscopy of the leaf epidermis and pollen grains in the family Bignoniaceae Juss. in Nigeria. Available from: <http://www.omicsgroup.com/conferences/pharmacognosy-phytochemistry-natural-products-2013/> Accessed 13/11/2015.
27. Petrini O. (1991). Fungal endophyte of tree leaves. In Andrews J.H. and Hirano S.S. (eds.), *Microbial Ecology of Leaves*. Springer- Verlag, New York. USA; pp 179-197.
28. Yannai S. (2004). *Dictionary of Food Compounds with CD-ROM: Additives, flavours, and ingredients*. Boca Raton: Chapman & Hall/CRC.
29. Carolina Q. S., Andressa M., Natalia F., Caroline S., Gabrielle R., Marco E., Carlos R. K., Katia F. R., Gisela L. , Cristiane M. , Osvaldo S., Jussara P., Thiago M. (2015). Aureonitol, a Fungi-Derived Tetrahydrofuran, Inhibits Influenza Replication by Targeting Its Surface Glycoprotein Hemagglutinin. *PLOS ONE* | DOI:10.1371/journal.pone.0139236
30. Shen R., Wei M., Tunhai X., Xiuping L. (2010). Two novel alkaloids from the South China Sea marine sponge *Dysidea* sp. *The Journal of Antibiotics*; 63:699–701.
31. Singh B., Negi S. S. (1987). Evaluation of peanut, mustard, linseed, and cottonseed meals for wool production in Angora rabbits. *J. Appl. Rabbit Res.*, 10 (1): 30-34.
32. Shoji N., Umeyama A., Saito N., Takemoto T., Kajiwara A., Ohizumi Y. (1986). Dehydro-piperonaline, an amide possessing coronary vasodilating activity, isolated from *Piper longum* L. *J Pharm Sci.*; 75(12): 1188-9.
33. <http://pubchem.ncbi.nlm.nih.gov>
34. Onn M.L. (2013). Screening of mangrove endophytic fungi for bioactive compounds. pp 88 – 96.
35. Fedoreyev A.S., Pokushalova T.V., Veselova M.V., Glebko L.I., Kulesh N.I., Muzarok T.I., Seletskaya L.D., Bulgakov V.P., Zhuravlev Y.N. (2000). Isoflavonoid production by callus cultures of *Maackiaamurensis*. *Fitoterapia*, 71: 365-372.
36. <http://www.ncbi.nlm.nih.gov/mesh/67048944>