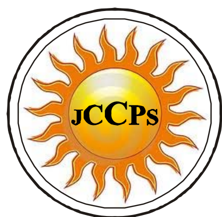

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BIOASSAY–GUIDED ISOLATION OF ANTIMICROBIAL COMPOUND AGAINST (*STAPHYLOCOCCUS AUREUS*) FROM *AZADIRACHTA INDICA* PLANT

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ABSTRACT

The antimicrobial potential of crude extracts, and fractions obtained from *Azadirachta indica* have been evaluated. Phytochemical screening of the extracts and metabolites revealed the presence of tannins, saponins, flavonoids, steroids/triterpenes, glycosides, phenols, carboxylic acids, esters, aldehydes and ketones. The antimicrobial activities of crude extracts and their secondary metabolites were tested against some human pathogens: *Staphylococcus aureus*, *Eschericha coli*, *Pseudomonas aeruginosa*, *Salmonella spp*., *Candida spp*., *Streptococcus spp*. and *Coliform spp*. Of all the samples tested, the extract from the leaves and its secondary metabolites showed antimicrobial activity against one or more species of the microorganisms. Acidic metabolite of leaves of *Azadirachta indica* (ALA), which has the highest activity against *Staphylococcus aureus* was further subjected to chromatographic purification and antimicrobial analysis. Isolate of the chromatographic separation (ALA2), which showed best antimicrobial activity was assayed using NMR and IR techniques. The result of the analyses revealed that the antimicrobial compound in *Azadirachta indica* (A. indica) was a flavonoid glycoside probably naringin.

Key words: *Azadirachta indica*, Crude extract, Metabolites, Antimicrobial activity, Phytochemicals, Naringin.

INTRODUCTION

Azadirachta indica (Neem) is a tree in the mahogany family Meliaceae. It is one of two species in the genus Azarachta, and is native to India, Burma, Bangladesh, Sri Lanka, Malaysia and Pakistan, growing in tropical and semi-tropical regions. In India, the tree is known as "Sacred Tree", "Heal All", "Nature's Drugstore", "Village Pharmacy" and "Panacea for all diseases". Products made from neem tree have been used in India for over two millennia for their medicinal properties. Neem products have been observed to be anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative[1]. Neem products are also used in selectively controlling pests in plants and particularly prescribed for skin disease[2]. All parts of the tree (seeds, leaves, flowers and bark) are said to have medicinal properties and are used for preparing many different medical preparations.

The antimicrobial properties of A. indica extracts have been investigated by several workers[3-16]. Anti-inflammatory and antimicrobial activities of the root, bark and leaves of A. indica was tested and found

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that 50% of the acetone extracts showed marked anti-inflammatory activity in carrageenan induced edema in rats[9]. They also discovered that the extracts possess more potent bactericidal activity than streptomycin, which was used as a standard. The report of investigations on antibacterial activity of leaf extracts *Azadirachta indica* A. Juss (Meliaceae) indicated that ethanolic and dichloromethane leaf extracts among other extracts of A. indica can be used as a potential source of antimicrobial agents[12]. Similarly,[4-7,10,12,14,16] it was found that A. indica possesses a very active against most the tested organisms. Antibacterial and phytochemical screening of the ethanolic leaf extract of A. indica showed that it contains pharmacologically active constituents that may be responsible for its activity against *S. aureus*, *E. coli*, and *S. typhi*[15]. In another study, a Neem cake extract in a broth model meat system was shown to counteract the growth of the gram-negative, gram-positive and microaerophilic bacteria[13].

Bioactive compounds from natural sources have been reported to be useful for treatment of various diseases[17]. The isolation and characterization of bioactive compounds begin with the general screening of plants to identify those with bioactivity against pathogenic organism[18]. The aim of this paper is to report on the isolation and partial characterization of a bioactive antimicrobial compound from ethanol extract of *Azadirachta indica*.

EXPERIMENTAL

Materials and methods

Plant materials

Roots, barks and leaves of the plant under investigation were collected from Ikot Abia Enin in Mkpat Enin Local Government Area of Akwa Ibom State, Nigeria. The plant was identified as *Azadirachta indica* of the family "Meliaceae" by Dr. (Mrs) M. E. Bassey, Department of Botany, University of Uyo, Uyo.

Crude extract preparation

Freshly collected leaves, barks and roots of *Azadirachta indica* were dried and ground to a semi-powder. About 30 g of each of the semi-powdered sample was extracted with 250 mL of ethanol for 12 hrs in a Soxhlet extractor. The ethanol was removed from the extract at room temperature to give a gel-like solid, which was dissolved in ethanol/water mixture (4:1) and filtered. The filtrate of each of the sample from the plant was used for preparation of acidic, basic and neutral metabolites and for preliminary phytochemical test and antimicrobial experiment. The acidic, basic and neutral metabolites were prepared following the method[19].

Antimicrobial tests

These experiments were carried out in Microbiology Department, Federal Medical Centre, Owerri, Imo State of Nigeria. Agar disc diffusion method was used and the microorganisms were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida spp*., *Salmonella spp*., and *Coliform spp*. An inoculating loop was touched to six isolated colonies of the pathogen on an agar plate and used to inoculate a tube of culture broth, which was incubated at 35-37°C until it became slightly turbid and was diluted to match the turbidity standard. Then sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of the agar plate. After the agar surface has dried for 5 min, the appropriate basic metabolite test disks were placed with a multiple applicator device. The agar plate was incubated at 35-37°C for 16-18 hrs, after which, the diameters of inhibition zones (areas showing little or no microbial growth) were measured to the nearest mm[20].

Determination of minimum inhibitory concentration (MIC)

The agar disc diffusion assay is a quantitative method based on the method of European Society of Clinical Microbiology and Infectious Diseases (2000) for evaluation of antimicrobial potentials. Standard solutions of the metabolites were prepared: 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.0625 mg/mL, in agar nutrient and distributed into sterile test tubes. 1 mL of each metabolite dilution was separately added into the plate and poured into Petri-plates. The test microorganism was spotted onto the surface of the solidified extract-agar mixture and the plates were inoculated, starting from the lowest concentration to the highest. After inoculation, the plates were allowed to dry for 30 min and incubated at 37°C for 18 hr, after which the samples were examined for microbial growth. The lowest concentration of metabolite, which showed no growth of microorganisms was taken as the MIC of the extract[20].

Thin layer chromatography (TLC)

A TLC (silica gel) was done using a mixture of mixture of ethanol and chloroform (1:1) as elution solvent and iodine for development.

Column chromatography

The gel-like acidic metabolite (ALA) was dissolved in ethanol and loaded on column packed with silica gel (60 g) and eluted with a mixture of ethanol/chloroform (1:1). The fractions were tested for antimicrobial activity against *S. aureus* and the fraction with the highest activity (ALA2) was selected for spectroscopic analysis.

NMR (^{1}H and ^{13}C) analysis

The NMR analysis of the fraction with the best bioactivity of acidic metabolite was performed using mercury 200BB varian (200 mH_z) NMR spectrometer (^{1}H at 200 mH_z and ^{13}C at 50 H_z) at the Central Research Laboratory, Obafemi Awolowo University of Ife, Osun State, Nigeria.

IR Analysis

The IR analysis of the fraction with the highest antimicrobial activity against *S. aureus* was carried out using FTIR Infra Red spectrophotometer BX-model by Pelkin Elmer, at the Central Research Laboratory, University of Ibadan, Oyo State, Nigeria.

RESULTS AND DISCUSSION

Data from Table 1 showed that extracts from leaves, barks and roots of *Azadirachta indica* contain all the phytochemicals tested with exception of alkaloids. Aldehydes, ketones and cardio-active glycosides were also absent in the bark extracts. The results of these phytochemical analyses are supported by the works of[16,21,22].

Table 1: Phytochemical analysis of crude extracts of plant parts

Phytochemicals	Roots of A. indica	Leaves of A. indica	Barks of A. indica
Tannins	++	+++	+++
Saponins	++	+++	+++
Fl avonoids	++	++	+++

Cont…

Phytochemicals	Roots of A. indica	Leaves of A. indica	Barks of A. indica
Steroid & triterpenes	+++	+++	++
Cardio-active glycosides	±	++	–
Phenols	+	+++	+++
Carboxylic acids	++	++	++
Esters	±	±	±
Aldehydes and ketones	±	±	-
Alkaloids	-	-	-

+++ = Strongly Positive, ++ = Positive, ± = weakly positive and - = not detected

A phytochemical analysis was also performed on acidic and basic metabolites of the leaves extract, which were found to have antimicrobial activity (Table 2). The acidic metabolite was found to contain tannins, saponins, flavonoids cardio-active glycosides, phenols, and carboxylic acids while the basic metabolite contains only three phytochemicals: saponin, steroids/triterpenes and aldehydes/ketones.

Table 2: Phytochemical analysis of metabolites

Phytochemical	ALA	BLA
Tannin	+++	-
Saponins	±	±
Flavonoids	+++	-
Steroids & triterpenes	-	++
Glycosides	++	-
Phenols	++	-
Carboxylic acids	+++	-
Esters	-	-
Aldehydes / Ketones	-	+++
Alkaloids	-	-

+++ = Strongly positive, ++ = Positive, ± = Weakly positive; - = Not detected

Results of the preliminary tests for antimicrobial activities of the plant extracts are shown in Table 3. Information from the table reveals that barks and roots of A. indica showed no antimicrobial activity against any of the six organisms tested while leaves of A. indica showed antimicrobial activity to four of the tested organisms. The use of this plant and others as antimicrobial agents for the treatment of microbial infection has been reported[4,7,10,11,14,21,23-25].

Results of Table 4 revealed that the bioactive compounds responsible for the antimicrobial activity of the plant extract are present in all the metabolites. BLA, ALA and NLA presented antimicrobial activity to at least one of the tested microorganism. Although the metabolites differ significantly in their activities against the microorganisms tested. Basic metabolites of the leaves extract showed activity to most of the tested microorganism while NLA showed activity to only one microorganism. Among the three metabolites, ALA exhibited greatest activity against S. aureus which is the organism of interest in this research work. Comparing the activities of metabolites obtained from the leaves extract of A. indica in Table 4 and crude

extract of leaves of A. indica in Table 3 to S. aureus, ALA brought about the greatest zones of inhibition in *S. aureus* while crude extract of leaves of A. indica, BLA and NLA brought about no inhibitory zones. This may be either because a more active site was developed in the acidic isolate or the constituent of the crude extract was inhibiting the antimicrobial activity. Information on comparative analysis of the susceptibility of other tested microorganism to different metabolites was also studied from Table 4.

Table 3: Antimicrobial activities of plants crude extracts

Test	Leaves of *Azadirachta indica*	Barks of *Azadirachta indica*	Roots of *Azadirachta indica*
Staphylococlus aureus	-	-	-
Escherichia coli	+	-	-
Pseudomonas aeruginosa	+	-	-
Candida Spp	+	-	-
Salmonella Spp	+	-	-
Coliform Spp	-	-	-

+ = Active, - = Not active

Table 4: Antimicrobial activity caused by plant metabolites through disk diffusion method

Test organisms	Concentration (mg)	BLA (IZ in mm)	ALA (IZ in mm)	NLA (IZ in mm)
Coliform Spp	100.00	15.0	15.0	-
	50.00	10.0	11.0	-
	25.00	6.5	6.5	-
	12.50	-	-	-
	6.25	-	-	-
E. coli	100.00	25.0	18.0	-
	50.00	20.0	14.0	-
	25.00	15.0	8.5	-
	12.50	10.0	-	-
	6.25	-	-	-
Staph. Aureus	100.00	-	35.0	-
	50.00	-	29.0	-
	25.00	-	22.0	-
	12.50	-	15.0	-
	6.25	-	10.0	-
Salmonella Spp	100.00	18.0	-	-
	50.00	14.0	-	-
	25.00	8.0	-	-
	12.50	-	-	-
	6.25	-	-	-

Cont…

Test organisms	**Concentration (mg)**	**BLA (IZ in mm)**	**ALA (IZ in mm)**	**NLA (IZ in mm)**
Candida Spp	100.00	18.0	30.0	14.0
	50.00	13.0	25.0	8.0
	25.00	7.5	18.0	-
	12.50	-	10.0	-
	6.25	-	-	-
Streptoco ccus Spp	100.00	20.0	-	-
	50.00	15.0	-	-
	25.00	10.0	-	-
	12.50	6.5	-	-
	6.25	-	-	-

ALA - Acidic metabolite, BLA - Basic metabolite, NLA – Neutral metabolite

The antimicrobial activity observed in this study was concentration dependent. In all the metabolites with antimicrobial activity, the inhibitory zones of the microorganisms increases with increase in the concentration of the metabolites. The susceptibility of *coliform spp* to different metabolite was studied. The study revealed that at all concentrations, coliform spp. was only sensitive to BLA and ALA. Though *coliform spp*. was susceptible to BLA and ALA, ALA is mostly preferred for the treatment of coliform causing ailment because it is the most sensitive metabolite to *coliform spp*. considering the effect of the metabolites on *E. coli*. BLA exhibited greater activity than ALA in all the concentrations with no observable activity in NLA. The study of susceptibility of *salmonella Spp* to BLA, ALA and NLA showed that all the metabolites except BBE showed no activity to the tested microorganism. Based on this result, it can be concluded that among the three metabolites used for the study, BLA is the most effective antimicrobial agent for inhibiting the growth of *salmonella Spp*. The results of the table showed that *candida spp* was susceptible to all the three metabolites. Among the metabolites, ALA showed the highest antimicrobial activity to *Candida spp* while NLA showed the least antimicrobial activity. The table was Analysis of the susceptibility of *streptococcus spp* to metabolites has also been presented. From the study, it was found out that *streptococcus spp* was sensitive to only one metabolite, BLA.

These observed antimicrobial activities exerted by the extracts and metabolites of different parts of the plant may be due to compounds in the plants such as the phenolics, tannins, flavonoids, glycosides, saponins and other phytochemicals[21,24,25]. The results of the investigation clearly indicate that the antimicrobial activity and the phytochemicals screened vary with the plant parts and metabolites used[8,14,15,23,26,30].

Table 5 presents data on the susceptibility of six microorganisms to the three chromatographic fractions of ALA. The three fractions of ALA showed almost equal antimicrobial activities to *staph. aureus* but ALA2 seems to show higher activity and was chosen for IR and NMR analysis. ALA3 showed higher antimicrobial activity to *candida albicans*, *salmonella spp* and *streptococcus spp* while ALA.2 also possessed better antimicrobial activity to *coliform spp*.

Result from the IR analysis of ALA.2 revealed the presence of: N-H_{str} in aliphatic and aromatic secondary amines or in primary amide or O-H_{str} in alcohol or in SO_3H or phenols (3437.00 cm^{-1}), C-H_{str} in methylene of alkanes and cycloalkanes except cyclopropane or in methane (2926.85cm^{-1}), C-H_{str} in alkanes or RCHO or O-H_{str} in carboxylic acids salt dimer (2853.19cm^{-1}), P-H_{str} in phosphite esters (2374.42cm^{-1}),

P=O$_{str}$ in triaryl phosphate esters (2079.79 cm^{-1}), C=N$_{str}$ in oximes (R_2C=NOH) or acyclic conjugated and cyclic imines or C=C$_{str}$ in isolated C=C or aromatics (1638.05 cm^{-1}), C-H$_{str}$ in CH_3-,CH_3CH_2-, $CH_3CH_2CH_2$-, $(CH_3)_2CH$, $(CH_3)_3C$, CH_3C=C (1460.22 cm^{-1}), C-H$_{str}$ in CH_3C=O, CH, CH_2=CH-, CH_2= C (1400.26 cm^{-1}), S=O$_{str}$ in amino sulphonic acid (1022.40 cm^{-1}), C-X$_{str}$ in alkylhalides and aromatic side chain halides e.g monobromo or monoiodide (629.08 cm^{-1}). The NMR (^{1}H) spectral studies of ALA2 showed a chemical shift, which indicated the presence of : RCH_3 δ0.8), R_2CH_2 or ROH or RHN_2 or R-SH (δ1.3), C=C-H or ArOH or H-C-F or $ArNH_2$ (δ4.7) and Ar-H (δ7.3), H-C-Br, H-C-I, C≡C-H (δ2.9) and H-C-OH, H-C-OR, RCOO-C-H (δ3.7). 14.0 (14H), 4.0 (4H), 4.0 (4H), and 9.0 (9H) were peak areas of the peaks at 0.50-1.60 ppm, 2.70-3.3 ppm, 3.4-3.9 ppm, and 4.3- 5.3 ppm, respectively. The NMR (^{13}C) spectrum of ALA2 fraction from column chromatography showed the following groups to be present in the sample. C≡C, C-O and C-N (77.879, 77.241, 76.604) ppm, respectively and C of alkane, C-O, C-N (32.152, 29.922, 14.346) ppm, respectively.

Table 5: Antimicrobial activity caused by fractions of ALA obtained from column chromatography

Test organisms	Concentration in mg	ALA 1 (IZ in mm)	ALA 2 (IZ in mm)	ALA 3 (IZ in mm)
Staph aureus	100.00	20.0	21.0	20.0
	50.00	16.0	16.0	16.0
	25.00	12.0	12.0	11.0
	12.50	8.5	7.5	6.5
	6.25	-	-	-
Candida albicans	100.00	20.0	20.0	30.0
	50.00	15.0	14.0	26.0
	25.00	7.0	8.0	20.0
	12.50	-	-	15.0
	6.25	-	-	8.5
E. coli	100.00	20.0	18.0	22.0
	50.00	14.0	13.0	15.0
	25.0	7.5	6.0	7.0
	12.50	-	-	-
	6.25	-	-	-
Samonella spp	100.00	22.0	20.0	25.0
	50.00	15.0	14.0	20.0
	25.0	14.0	7.0	14.0
	12.50	8.5	-	10.0
	6.25	-	-	5.5
Streptococcus spp	100.00	21.0	20.0	23.0
	50.00	15.0	13.0	19.0
	25.00	8.0	6.5	13.0
	12.50	-	-	8.5
	6.25	-	-	-

Cont…

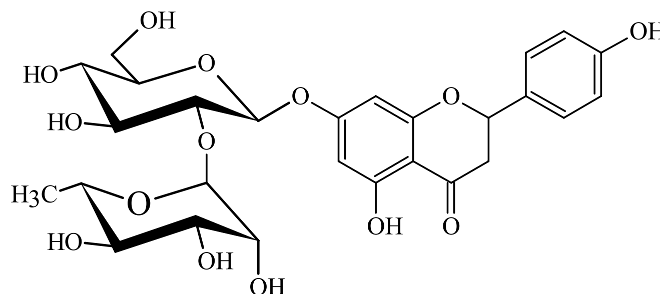
Test organisms	Concentration in mg	ALA 1 (IZ in mm)	ALA 2 (IZ in mm)	ALA 3 (IZ in mm)
Coliforn spp	100.00	15.0	24.0	17.0
	50.00	8.0	20.0	12.0
	25.00	-	14.0	7.5
	12.50	-	8.5	-
	6.25	-	-	-

ALA - Acidic metabolite, ALA_1, ALA_2 and ALA_3 are chromatographic fractions of acidic metabolite

Based on the data presented above, the Fluka library of spectroscopic analysis (supplied by Perkin Elmer) suggested that ALA.2 fraction obtained from column chromatography could be any of the following compounds; chitin, taurocholic acid (sodium salt), kappa-carrageenan, methylthymol blue (sodium salt), streptomycin sulfate, pectin, naringin, ρ-naphtholbenzein (alpha), or hydrocortisone acetate. Based on the chemistry of the compound observed in the laboratory, which includes:

- Solubility in dilute acids: the compound was insoluble in dilute mineral acids, suggesting it could be a carboxylic acid, phenol or sulphonic acid.
- Solubility in dilute alkali: the compound dissolved freely in dilute sodium hydroxide, suggesting it could be a carboxylic acid, phenol or sulphonic acid.
- Keto-enol tautomerism: the compound exhibited and always existed as an equilibrium mixture of the keto and enol forms.
- Colour: the compound is yellow in colour, suggesting that it could a flavonoid.

Hence, in view of the following laboratory observations, it was concluded that the compound herein referred to as ALA.2 could be methylthymol blue (sodium salt), ρ-naphtholbenzein (alpha) and naringin. However, in view of its colour, ALA.2 is probably a flavonoid, hence it is most likely to be the last named compound; Naringin. The identity of this compound as naringin was further substantiated by comparison of its spectral data with previously values[6,27,29].



Naringin or 7-[[2-o-(6-Deoxy-α-L-Mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one

CONCLUSION

In the final analysis, the results of the present investigation clearly indicate that *Azadirachta indica* possesses antimicrobial activities, which vary with plant materials and nature of metabolites. ALA (the

secondary metabolites of interest) which possesses activity against *S. aureus* was subjected to column chromatographic purification and antimicrobial. ALA.2 fractions possessed the best antimicrobial properties (against *staphylococcus aureus*). The IR and NMR analysis of the fractions showed that ALA.2 the antimicrobial agent was a flavonoid glycoside, probably naringin.

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