Original Research Article

Phytochemical, Antioxidant, and Antimicrobial Potentials of *Azadirachta indica* Leaf Extract using Bacteria Isolated fromPolluted Water as Test Organisms

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ABSTRACT

Aims: Globally, there is continuous emergence of infectious pathogens. Therefore, there is a need to search for new drug molecules of plant origin such as *A. indica* which is commonly grown in Nigeria. This study was aimed at evaluating the antioxidant and antimicrobial potentials of the leaf extract of *A. indica* and determining its bioactive molecules
Study design: This was an in-vitro laboratory study.
Place and Duration of Study: This research was carried out at the Microbial Resources Research Laboratory (MRRL), Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso Nigeria from April 2021 to January 2023.
Methodology: Collected fresh leaves of *A. indica* were destalked, air dried at 27 ^{o}C for 10 days, and finally grounded. The dried powders were extracted by maceration using four (4) solvents namely: water (aqueous), methanol, ethyl-acetate, and n-hexane. The leaf extracts were assessed for their antioxidant and antimicrobial potential. Bioactive molecules present in the leaf extract were determined byGas chromatography-mass spectrometry.
Results: It was observed that leaf extracts of *A. indica* possess bioactive molecules such as Phytol which is over 50% of the total biomolecules present. These biomolecules are responsible for the antimicrobial and antioxidant potentials of the extract.The ABTS and DPPH radicals scavenging activities ranged from 24.51 to 90.32 % and 31.79 to 79.59 % respectively.These extracts can be used to mop upfree radicals which have damaging effects on the cells of the body. The minimum inhibitory concentration (MIC) of the extract for each of the test bacteria isolates varied between 0.05 to 0.8 mg/ml. Thus, the leaf extract of *A. indica* is a reservoir of bioactive molecules which can be used for novel drug synthesis and discovery.
Conclusion: Thus, leaf extracts of *A. indica* contain potent biomolecules which can be harnessed for therapeutic use.

Keywords: Azadirachta indica, leaf extracts, antimicrobial, antioxidant, biomolecules, drug synthesis

1. INTRODUCTION

Globally, there is continuous emergence of infectious pathogens. Therefore, there is a need to search for new drug molecules of plant origin which are potent and have therapeutic implications in preventing and curing diseases.Plants extracts have been reported to show special roles in the prevention of diseases and treatment through their antioxidant activities, inhibition of microbial growth, and influencing other biological responses [1-2]. One such medicinal plant is

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Azadirachta indica which is commonly grown on Nigerian soil, especially in the Southwestern part. This tree grows very fast and can be as tall as 20 to 24 m. The plant parts are being used in traditional medicine to cure infectious and non-infectious diseases [3-5]. Several biological and pharmacological potentials of this plant have also been established which include: antibacterial [6], antifungal [7], and anti-inflammatory activities. Other uses include antiarthritic, antipyretic, hypoglycemic, anti-gastric ulcer, antifungal, antibacterial, and antitumor [8-10]. This study aimed to evaluate the antioxidant and antimicrobial potentials of n-hexane, ethyl acetate, methanol and aqueous extracts of a domesticated *A. indica* (LAU-VN-0264) leaves and assess the bioactive molecules present.

2. MATERIALS AND METHODS

2.1 Study Area

This study was done at the Microbial Resources Research Laboratory at the Biology Complex, Department of Pure and Applied Biology, LAUTECH Ogbomoso, Nigeria.

2.2 Plant Material

Fresh leaves of *Azadirachta indica* were collected from the premises of Sports Complex, Ladoke Akintola University of Technology (LAUTECH) Ogbomosho, Nigeria. Identification and authentication of the plant was done byProfessor ATJ Ogunkunle, a Botanist in the Department of Pure and Applied Biology, Ladoke Akintola University of Technology Ogbomosho Oyo State Nigeria.

2.3 Extraction of Plant Leaves Using Different Solvents

The collected fresh leaves of *A. indica* were destalked, air dried at room temperature for 10 days [11], and finally grounded using an electronic blending machine. It was stored in an air-tight plastic container at room temperature before extraction. The dried powders were extracted by maceration using four (4) solvents namely; water (aqueous), ethyl-acetate, n-hexane, and methanol. About 100g of the leaf powder was soaked in 700 ml of each solvent and agitated for 1 hour at 27 ^{o}C inside an Ultrasonic Sonicator. The plant extracts were decanted and filtered using Whatman filter paper and concentrated using a rotary evaporator at 40 ^{o}C.

2.4 Antioxidant screening of *A. indica* leaf extracts

2.4.1 DPPH scavenging assay

The antioxidant activity of the A. *indica* leaf extracts based on the scavenging activity of the 2, 2- diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to the method described by Brand-Williams *et al*[12]with slight modification.The scavenging ability of the plant on DPPH was calculated using the equation below [13-14].

$$\%\ Inhibition = \frac{Ac - Ao}{Ac}$$

Ac: absorbance of pure DPPH (control) in oxidized form

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Ao: absorbance of the extract after reaction with DPPH.

All measurements were carried out in triplicates.

2.4.2 Ferric-reducing power assay

Ferric reducing antioxidant potential (FRAP) was used to evaluate the total antioxidant power of the leaf extract [15-16]. About 100 µL of each concentration of the extracts (20–100 µg/mL) was added to 2.5 ml of 200 mM Phosphate buffer (pH 6.6) and 2.5 mL of 1% Potassium ferricyanide, incubated at 50 ^{o}C for 20 min. Thereafter, 2.5 mL of 10% Trichloroacetic acid was added and the tubes were centrifuged at 10,000 rpm for 10 minutes. Exactly 5 mL of the upper layer of the solution was mixed with 5.0 ml of distilled water and 1 mL of 0.1% Ferric chloride. The absorbance of the reaction mixtures was measured at 700 nm. The final results were expressed as mg ascorbic acid equivalent /100g of weight.

2.4.3 ABTS assay

The scavenging activity of the extract against *2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulfonic assay*(ABTS) radical was assessed as follows: The stock solutions of 7 mM ABTS and 2.4 mM Potassium persulphate in equal volumes were allowed to stand in the dark for 12 h at room temperature. The resultant ABTS solution was diluted by mixing 1 mL of freshly prepared ABTS solution to obtain an absorbance of 734 nm after 7 min. The percentage inhibition of ABTS by the extract was calculated and expressed as a function of the concentration of Trolox equivalent [17].

$$\%\ ABTS\ scavenging\ activity = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}$$

2.5 Qualitative Phytochemical Screening of Plant Extract

Phytochemical screening was performed to detect the presence of different classes of plant secondary metabolites such as tannins, saponin, terpenoids, steroids, phytate, flavonoid, alkaloid, cardiacglycoside, and anthraquinones.

2.6 Antimicrobial Potentials of *A. indica* Leaf Extracts

2.6.1 Collection and Maintenance of Test Bacteria Isolates

Already characterized bacteria were collected from the culture collection Bank of Microbial Resources Research Laboratory, Department of Pure and Applied Biology (Microbiology Unit), the Ladoke Akintola University of Technology Ogbomosho Oyo State, Nigeria. These are *Acinetobacter baylyi, Pseudomonas montelli, Stenotrophomonas maltophilia, Pseudomonas entomophila* and *Halomonas* sp. The bacteria were maintained on agar slants, at 37 ^{o}C.

2.6.2 Preparation of Culture Medium and Inoculum

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Nutrient agar and Muller Hinton agar used in this work were prepared according to the manufacturer's specifications following standard protocols. The media were sterilized at 121 ^{o}C for 15 min. About 20 ml of the medium was poured under aseptic conditions in a laminar flow. Active cultures were prepared by transferring a loopful of culture to 10 ml of nutrient broth and incubated at 37 °C for 24 hours in a rotary shaker [18].

2.6.3 Determination of Minimum Inhibitory Concentration (MIC)

This was achieved by the tube dilution method. Varying concentrations of the leaf extracts (0 - 0.4 mg/ml), were added to 2 ml of nutrient broth, with a loopful of the test bacteria organism and added into the tubes. Tubes containing bacteria cultures were incubated at 37 ^{o}C for 24 hours. The concentration at which there was no growth was regarded as a minimum inhibitory concentration[19].

2.7 Determination of Bioactive Molecules of *A. indica*Leaf Extracts by GC-MS

Gas chromatography-mass spectrometry (GC-MS) was used for the identification of the bioactive molecules present in the leaf extract of *A. indica*. The leaf extracts were analyzed using a Perkin Elmer GC–MS (Model Perkin Elmer Clarus 500, USA) equipped with a fused silica capillary coupled column, coupled with a Perkin Elmer Clarus 600C MS. The total GC running time was 36 min. The relative amounts (%) of each component were calculated by comparing its average peak area to the total area. The mass spectra were matched by computer matching with those of standards available in the existing computer library.

2.8Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed to test for differences between the groups' mean. Significant differences between the means were determined by Duncan's multiple range test and P values < 0.05 were regarded as significant.

3. RESULTS

The quantitative assessment of the phytochemicals of the aqueous, n-hexane, ethyl acetate, and methanol leaf extracts of *A. indica* areshown in Table 1. Ten (10) phytochemicals which are flavonoid, phenolic, tannin, phytate, steroid, cardiacglycosides, terpenoid, alkaloid, anthraquinone, and saponin were quantified in mg/100ml of each of the extracts. The methanol extract of *A. indica*showed significant differences in the quantity of each of the phytochemicals examined

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compared to the other three (3) solvents. The highest and lowest concentrations of flavonoid were 36.19±0.71 and 5.75±1.18 mg/100ml in methanol and n-hexane extracts of *A. indica* respectively.

Quantitative values of 88.84±2.60 and 95.50±0.65 mg/100ml for phenolic were observed to be higher in ethyl acetate and methanol extract of the plant while the lowest values which were 38.15±1.46 mg/100ml recorded for n-hexane leaf extracts. For tannin, quantitative values of 38.83±0.25 and 42.88±0.58 mg/100ml were found to be higher in aqueous and methanol extracts of *A. indica* respectively, although lower in ethyl acetate and n-hexane extracts. Phytate concentrations in the extract also showed the highest value (12.23±0.18 mg/100ml) for the ethanol extract, followed by 8.80±0.11 mg/100ml in the aqueous extract. However, there was no significant difference (P<0.05) in the quantitative values of 3.58±0.09 and 2.59±0.01 mg/100ml recorded in ethyl acetate and n-hexane extracts of *A. indica* respectively. Quantitative assessment of the steroid present showed that it was highest in methanol extract (23.14±0.71 mg/100ml) compared to 13.75±1.08, 11.01±0.89, 5.06±0.44 mg/100ml recorded for aqueous, ethyl acetate and n-hexane extract respectively. The amounts of glycoside were highest in methanol extract (29.52±1.26 mg/100ml) although relatively high in aqueous and ethyl extracts at 18.42±0.75 and 15.05±0.79 mg/100ml respectively but low in n-hexane extracts at 4.78±0.79 mg/100ml. Terpenoid concentration was also highest in the methanol extract at 17.23±0.34 mg/100ml, followed by 12.58±0.11 and 8.82±0.48 mg/100ml for aqueous and ethyl extracts respectively but lowest in n-hexane extracts at 2.74±0.56 mg/100ml.

Table 1: Quantitative assessment of the phytochemicals of aqueous, n-hexane, ethyl acetate, and methanol extracts of *A. indica*

Samples (Extract)	Flavonoid (mg/100ml)	Phenolic (mg/100ml)	Tannin (mg/100ml)	Phytate (mg/100ml)	Steroid (mg/100ml)	Cardiac Glycoside (mg/100ml)	Terpenoid (mg/100ml)	Alkaloid (mg/100ml)	Anthraquinone (mg/100ml)	Saponin (mg/100ml)
Az-aq	26.41±0.24b	59.30±2.77b	38.83±0.25a	8.80±0.11b	13.75±1.08b	18.42±0.75b	12.58±0.11b	0.07±0.01b	10.56±0.14a	44.02±0.54b
Az-ethyl	18.52±1.02c	88.84±2.60a	8.48±0.33b	3.58±0.09c	11.01±0.89b	15.05±0.79b	8.82±0.48b	0.06±0.01b	4.30±0.11b	11.44±1.64d
Az-hex	5.75±1.18d	38.15±1.46c	9.89±0.50b	2.59±0.01c	5.06±0.44c	4.78±0.79c	2.74±0.56c	0.03±0.01c	3.11±0.01b	23.85±1.34b
Az-meth	36.19±0.71a	95.50±0.65a	42.88±0.58a	12.23±0.18a	23.14±0.71a	29.52±1.26a	17.23±0.34a	0.12±0.01a	14.68±0.22a	61.18±0.88

Values are Mean±SEM of duplicate determinations.
Values along each column with different letters are significantly different from one another (P<0.05)
AZ-aq: aqueous extract of *A. indica*; AZ-ethyl: ethyl acetate extract of *A. indica*; AZ-hex: n-hexane extract of *A. indica*; AZ-meth: methanol extract of *A. indica*.

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For alkaloid, its quantitative value was highest in methanol extracts at 0.12±0.01 mg/100ml followed by 0.07±0.01 and 0.06±0.01b mg/100ml for aqueous and ethyl acetate extracts of *A. indica* respectively but low in the n-hexane extract at 0.03±0.01 mg/100ml. Methanol and aqueous extracts of *A. indica* had quantitative values of 14.68±0.22 and 10.56±0.14 mg/100ml for anthraquinone respectively while for the ethyl acetate and n-hexane extracts, it was 4.30±0.11 and 3.11±0.01 mg/ml respectively.Saponin quantification gave 61.18±0.88, 44.02±0.54, 23.85±1.34, and 11.44±1.64 mg/100ml for methanol, aqueous, n-hexane and ethyl acetate extracts of *A. indica* respectively as seen in Table 1.

Table 2: Antioxidant screening of aqueous, n-hexane, ethyl acetate and methanol extracts of *A. indica*

Sample	ABTS (% Inhibition)	DPPH (% Inhibition)	FRP (Aeg/100g)
Az-aq	76.03±0.57	49.42±2.29	1111.09±29.37a
Az-ethyl	57.09±1.89	74.03±1.16	629.53±16.52b
Az-hex	24.51±2.86	31.79±1.22	148.62±17.44c
Az-meth	90.32±1.79	79.59±0.84	741.81±41.30b

*Values are Mean±SEM of duplicate determinations
Az-aq: aqueous leaf extract of *A. indica*; Az-ethyl: ethyl acetate leaf extract of *A. indica*; Az-hex: n-hexane leaf extract of *A. indica*; Az-meth: methanol leaf extract of *A. indica*.

Table 2 shows the antioxidant potentials of each of the aqueous, n-hexane, ethyl acetate, and methanol extracts of *A. indica.* The ABTS inhibition was observed to be 90.32, 76.03, 57.09, and 24.51 % for methanol, aqueous ethyl acetate, and n-hexane extracts respectively. For DPPH, percentage Inhibition of 79.59, 74.03, 49.42, and 31.79 % was observed for methanol, ethyl acetate, aqueous, and n-hexane extracts respectively. The Ferric reducing power (FRP) was highest in the aqueous extract at 1111.09±29.37, followed by 741.81±41.30, 629.53±16.52 and 148.62±17.44 Aeg/100g for methanol, ethyl acetate, and n-hexane respectively.

The minimum inhibitory concentrations (MIC) of each of the leaf extracts were carried out using five *bacteria isolated from water samples which were A. baylyi, P. montelli,P. entomophila*, *Stenotrophomonas maltophilia*, and *Halomonas* sp. In Fig.1, the MIC for the aqueous leaf extracts of *A. indica* was 0.4, 0.2, 0.3, 0.05 and 0.8 mg/ml for *A. baylyi, P. montelli,P. entomophila*, *Stenotrophomonas maltophilia*, and *Halomonas* sp. respectively.

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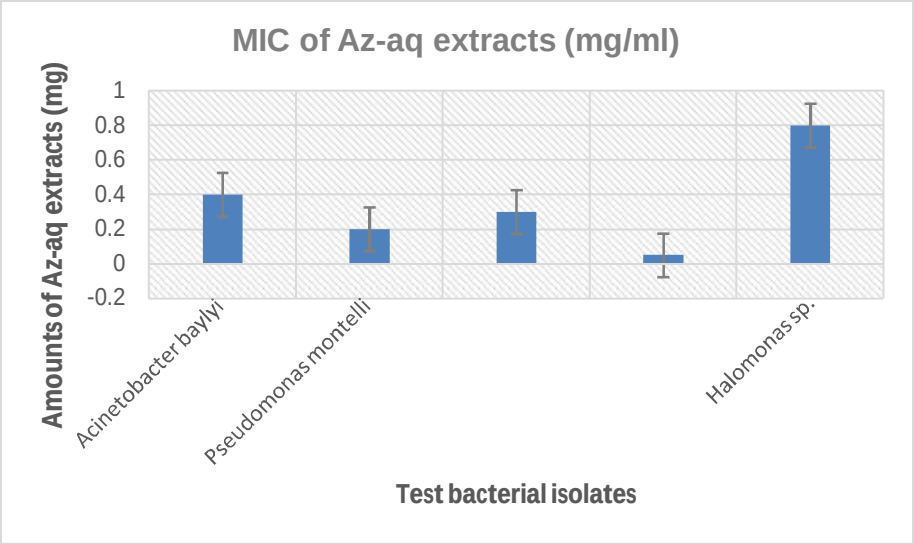

Fig.1: MIC of aqueous leaf extract of *A. indica* on test bacteria isolates

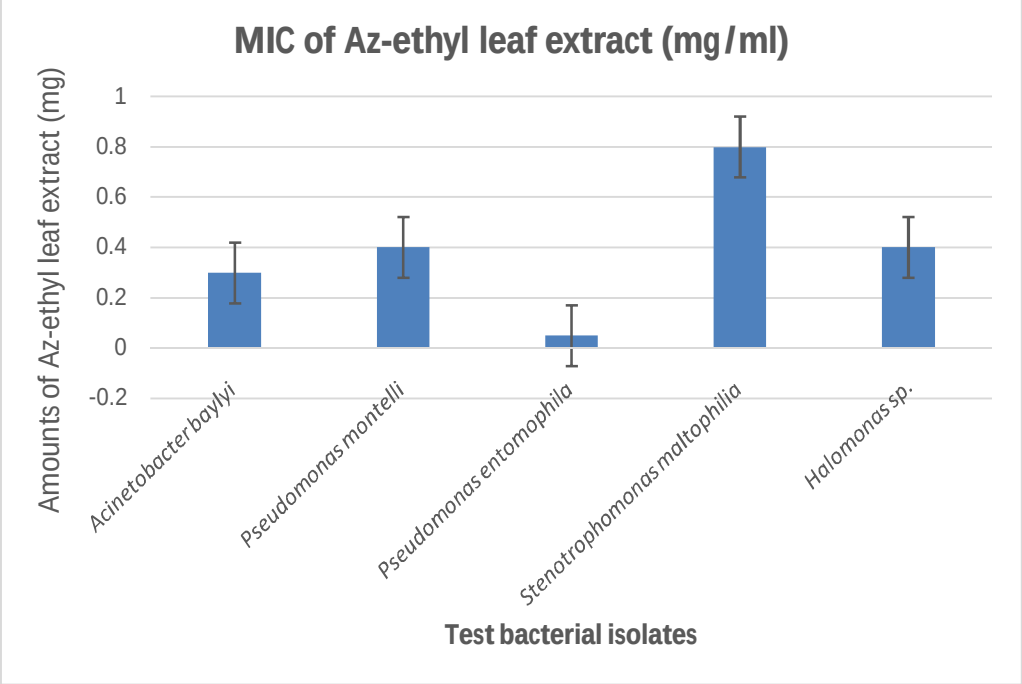

Fig. 2: MIC of ethyl acetate leaf extract of *A. indica* on test bacteria isolates.

Fig. 2 shows the MIC values for the ethyl acetate leaf extract of *A. indica* against the test bacteria isolates. The MIC values observed were; 0.3, and 0.4. 0.05, 0.8 and 0.4 mg/ml for *A. baylyi, P. montelli,P. entomophila*, *Stenotrophomonas maltophilia*, and *Halomonas* sp. respectively. Fig. 3 also shows the n-hexane leaf extracts of *A. indica*, in which the MIC values for each of the bacteria isolates were: 0.3, 0.4, 0.2, 0.05, 0.05 mg/ml for *A. baylyi, P. montelli,P. entomophila*, *S. maltophilia*, and *Halomonas* sp. respectively. Fig. 4 shows the MIC values for *A. baylyi, P. montelli,P. entomophila*, *Stenotrophomonas maltophilia*, and *Halomonas* sp. which were; 0.4, 0.8, 0.1, 0.1, 0.1 mg/ml respectively.

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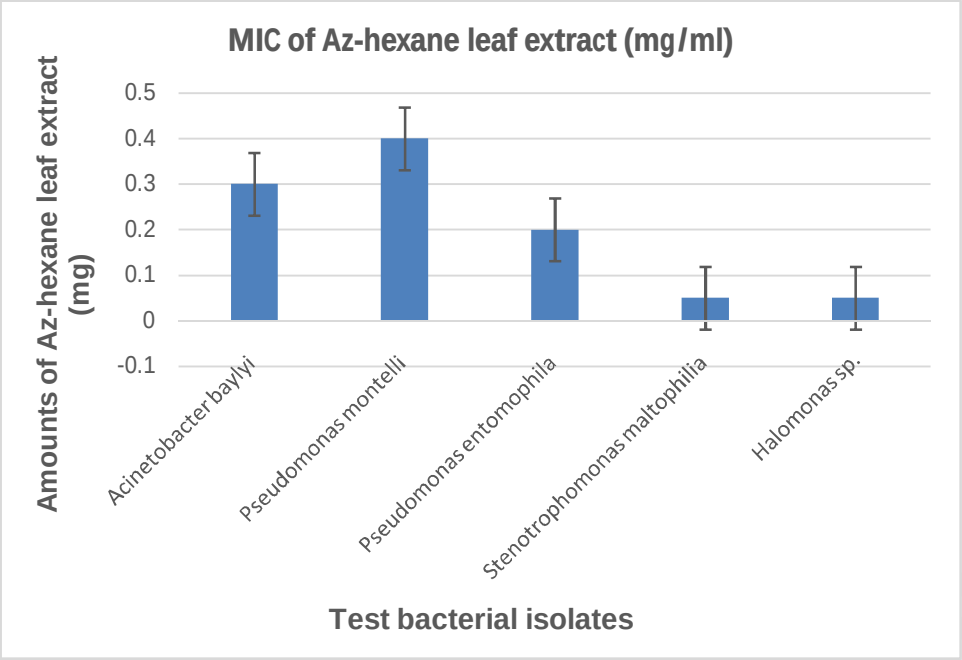

Fig.3: MIC of n-hexane leaf extract of *A. indica* on test bacteria isolates

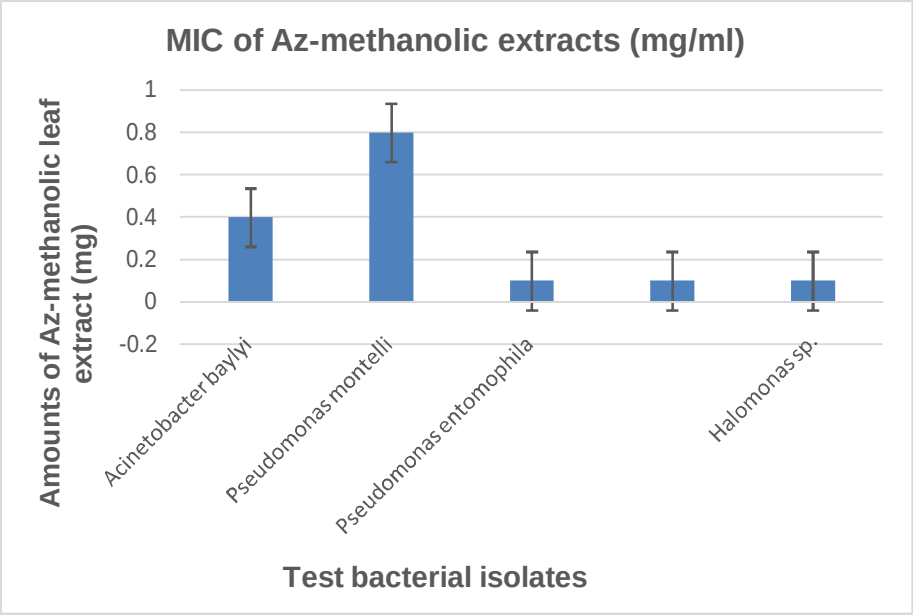

Fig. 4: MIC of methanol leaf extract of *A. indica* on test bacteria

Figures 5 and 6 show the GC-MS chromatogram of ethyl acetate and methanol leaf extract of *A. indica*. While thirteen (13) compounds were shown to be present in the ethyl acetate of *A. indica*, ten compounds (10) were revealed by that of methanol leaf extract as shown in Tables 3 and 4 respectively. Notably among all the compounds elucidated was Phytol which has the highest area coverage of 55.2 and 50.75 % in the ethyl acetate and methanol extracts respectively at about

16.47 min (Table 3 and 4). Other compounds showed very low area coverage ranging from 1.04 to 10.09 % in ethyl acetate and 3.01 to 9.65 % in methanol extracts as seen in Tables 3 and 4 respectively.

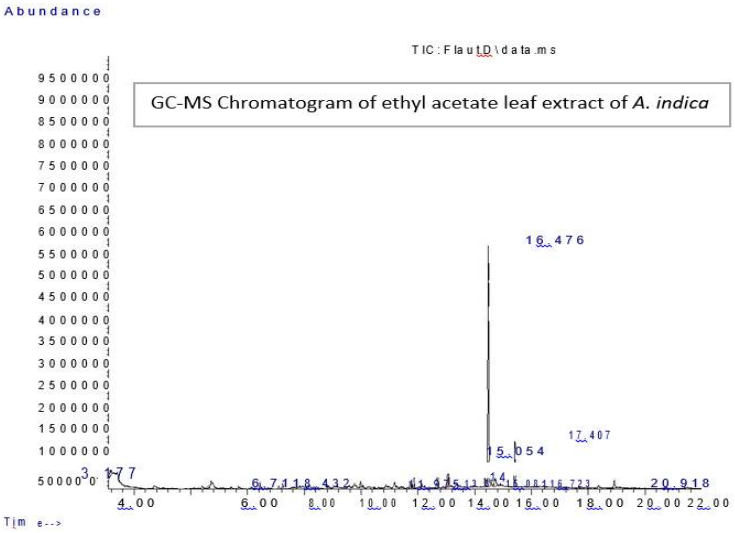

Fig.5: Chromatogram of ethyl acetate leaf extract of *A. indica*

Table 3: Compounds present in ethyl acetate leaf extract of *A. indica*

Peak	Retention time (min.)	Area (%)	Compound Name
1	3.175	2.85	Methanamine
2	6.714	1.04	Glycerol 1,2-diacetate
3	8.431	2.09	Methyl trifluoroacetate
4	11.975	1.84	Benzenemethanol,
5	13.869	2.34	2-Hexadecene,3,7,11,15 tetramethyl
6	14.678	4.54	Methyl ester, Hexadecanoic acid
7	15.052	6.68	n-Hexadecanoic acid, n-Tetracosanol-1
8	15.078	3.35	Didodecyl phthalate, Phthalic acid
9	16.359	2.23	9,12,15-Octadecatrienoicacid
10	16.476	55.2	Phytol
11	16.723	6.38	Cyclododecyne
12	17.408	10.09	11,13-Dimethyl-12-tetradecen-1-ol-acetate
13	20.920	3.46	2-Acetylbenzoic acid

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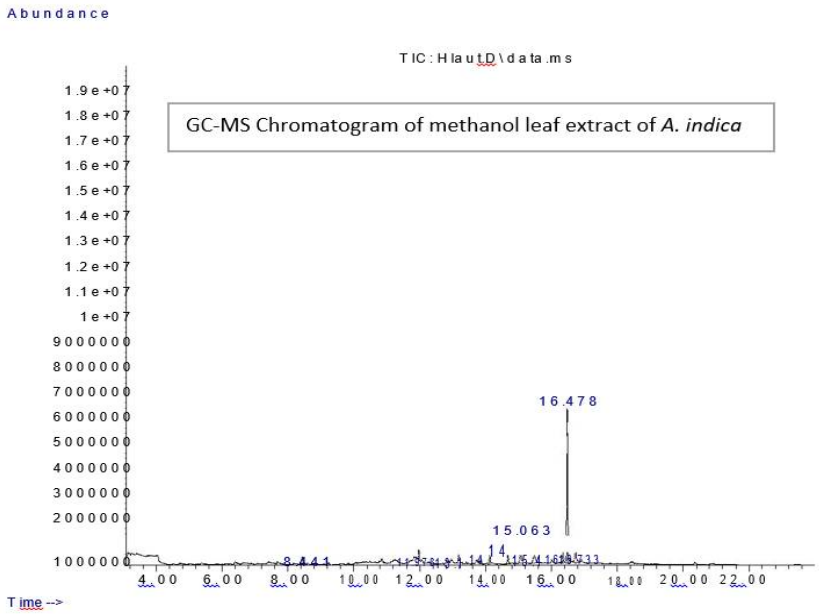

Fig. 6: Chromatogram of methanol leaf extract of *A. indica*

Table 4: Compounds present in methanol leaf extract of *A. indica*

Peak	Retention time (min.)	Area (%)	Compound Name
1	8.442	4.96	2-Pentenoic acid
2	11.98	3.01	Benzenemethanol
3	13.184	4.06	Hydrazinecarboxamide
4	14.128	3.51	3-(But-3-enyl)- cyclohexanone
5	14.678	5.16	Hexadecanoic acid, methyl ester
6	15.062	9.65	n-Hexadecanoic acid
7	15.483	7.17	Pentane, 3-ethyl-2-methyl
8	16.359	3.7	6,9-Octadecadienoic acid, methyl ester
9	16.479	50.75	Phytol, Oxirane
10	16.733	8.94	9,12,15-Octadecatrien-1-ol

4. DISCUSSION

According to Efferth [2], numerous pharmacologically active drugs are derived from natural resources including medicinal plants. These plant parts contain phytochemicals that have varying biochemical and pharmacological effects when consumed [20-21]. In this study, the presence of various medically important phytochemicals in *A. indica* was revealed. Our results are in line with that of Okwu and Josiah [22] who reported the chemical composition of two Nigerian medicinal plants. These phyto-compounds have been reported for their potential antibacterial, antifungal, and antiparasitic

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activities[23-24]. This aligns with the outcome of this work in which the leaf extracts of *A. indica* showed antibacterial activities against the test bacteria organisms [25]. This can also be supported by the work of Majolagbe *et al*[26], that metabolites from fungal endophytes possess antimicrobial properties due to the bioactive compounds present in them.

According to Farombi [27], African indigenous plants possess chemotherapeutic properties and produce bioactive prophylactic agents of medicinal importance [28-31]. The presence of several important phytochemicals is an indication that if *A. indica* is properly screened, it could serve as a natural drug resource of pharmaceutical significance [21,32]. Among the phytoconstituents present, the presence of tannin is an indication of the ability of *A. indica* to act as an antifungal, antidiarrheal, antioxidant, and anti-hemorrhoidal agent [33]. The presence of this compound could be responsible for the extract inhibiting bacteria that are resistant to antibiotics [34]. Tannin is known to be non-toxic and can generate physiological responses in animals that consume them.

The ABTS and DPPH radicals scavenging activities of the extracts show that it can be used to mop up free radicals which have damaging effects on the cells of the body. Already established is the fact that the reducing power of a natural compound may serve as a significant indicator of its potential antioxidant activity [32]. For example, the presence of flavonoids which are free radical scavengers and powerful anti-oxidants that prevents oxidative damage and possess anti-cancer properties is a good advantage to the use of the leaf extract of *A. indica*[35-36]. In addition, the occurrence of alkaloids which has been reported as one of the most efficient therapeutically significant plant substances is also proof of the biological activities demonstrated by the extract.

The percentage occurrence of the natural compounds was examined by GC-MS. Phytol was seen to have the highest coverage area of 50.75 % of all the bioactive compounds in the leaf extract of *A. indica.* Other compounds detected includes 2-Pentenoic acid (4.96 %), Benzenemethanol (3.01 %), Hydrazinecarboxamide (4.06 %), 3-(But-3-enyl)-cyclohexanone (3.51 %), Hexadecanoic acid (5.16 %), Pentane, 3-ethyl-2-methyl (7.17 %), 6,9-Octadecadienoic acid (3.7 %) and 9,12,15-Octadecatrien-1-ol (8.94 %). Thisis also indicative of the reasons for the antimicrobial and antioxidant potentials of the extract, which can be further explored for other biological functions.

5. CONCLUSION

The leaf extract of *A. indica* contains useful and bioactive molecules which are potential pharmaceutical precursors for novel drug synthesis and discovery. Thus, *A. indica* is a natural reservoir of bioactive molecules which can be considered for therapeutic purposes.

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