Antimicrobial, Cytotoxic and Phytochemical Analysis of Chloroform Extracts of *Andrographis paniculata* Root (Burm.F) and *Parquetina nigrescens* (Afzel) Aerial Part

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ABSTRACT: The menace of antibiotic resistance especially multi-drug resistant bacteria is increasing in the clinical setting. Therefore, the study investigated antimicrobial activity and phytochemical constituents of chloroform extracts of *Andrographis paniculata* root (Burm.F.) and *Parquetina nigrescens* (Afzel) aerial part. The extracts were screened for cytotoxic activity using brine shrimp (*Artemia salina*) lethality bioassay. The antimicrobial activity of the extracts was tested against *Citrobacter diversus, Klebsiella pneumoniae*, *Proteus vulgaris* and *Shigella sonnei* using tetrazolium microtitre-plate method. Analysis of chemical components of the extracts was done on Agilent Technologies 7890A gas chromatograph system s. GC-MS analysis of *A. paniculata* root extract and *P. nigrescens* aerial part extracts revealed a total of 32 and 22 compounds, respectively. The major components identified in these two extracts were fatty acid esters, alkaloids and terpenes. The lowest MIC observed with *A. paniculata* extract was 6.25 µg/ml for *Citrobacter diversus* and 12.5 µg/ml for *Klebsiella pneumoniae*. The lowest MIC recorded for *Parquetina nigrescens* extract was 3.125 µg/ml for *Citrobacter diversus, Shigella sonnei* and *Proteus vulgaris* while it was 12.5 µg/ml for *Klebsiella pneumoniae*. The susceptibility was compared with a clinical antibiotic (0.02 µg/ml) which served as the positive control. *P. nigrescens* with LC_{50} of 89.75 ± 0.18 µg/ml showed higher lethal toxicity than *A. paniculata* with LC_{50} of 167.5 ± 0.21 µg/ml. The observed toxicity might be due to presence of fatty acids and alkaloids in the extracts. The extract of the plants investigated in our study showed promising antimicrobial activity.

Key words: *Andrographis paniculata, Parquetina, nigrescens*, Spectroscopy, Toxicity, Antimicrobial

INTRODUCTION

The evolution, increasing prevalence and spread of pathogenic bacteria that are resistant to multiple antimicrobial agents is currently recognized as one of the most important problems in health sector globally.[1] Multidrug resistant bacterial strains pose major threat to clinical medicine and public health, internationally, specially to the tropical countries.[2-4] Despite the number of antibiotics available for the treatment of diseases in healthcare, the incidence of microbial infections has been increasing in the past few decades due to bacterial resistance to existing antibiotics. The search for new antibiotics especially against multi-drug resistant pathogenic bacteria like *Klebsiella* sp. has becomes a serious concern.

Historically, natural products have been important source of antibacterial agents especially those that are rich in alkaloids.[5] Correlation between pharmacological activity of medicinal plants and their phytochemicals constituents has increased the understanding of phytochemicals activity.[6-10] Therapeutic effects of isolated alkaloids have inspired researchers to synthesize many antibacterial drugs such as quinines, metronidazole, azomycin and bedaquiline.[5] Kabara (1984) reported antimicrobial activity of various fatty acids and their derivatives.[11]

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Phytomedicinal properties of plants make them relevant in drugs discovery within academia and pharmaceuticals.[12-16] The plants *Andrographis paniculata* and *Parquetina nigrescens* are good sources of antibacterial agents that can neutralize the ill effects of pathogenic microbes.[17-21]

Andrographis paniculata (Burm.F.) Wall. Ex Nees. (Family- Acanthaceae) is an annual herbaceous plant that grows well in the rain forest region of Nigeria. In folklore medicine, *A. paniculata* is widely used in Perso-Arabic medicine for treatment of astringent, antihelminthic, diuretic and leprosy.[22] *In vivo* and *in vitro* antibacterial and antiviral activities of the plant have been reported.[23-27] Phytochemicals, antimicrobial, anti-inflammatory, antiviral and antifungal effects of *A. paniculata* chloroform extract have been investigated and reported.[28-29] Antibacterial activity of polar and non-polar solvent extracts of *A. paniculata* were investigated against twelve skin diseases caused by bacteria strain. The minimum inhibitory concentration (MIC) of the extract depends on solvent used for extraction and microorganism.[22,30] Also, a similar report of MIC gram positive and gram negative bacteria was observed to be dependent on solvent.[28]

Parquetina nigrescens (*Asclepiadaceae*.) is an herbaceous plant common in West and Central Africa. It is a perennial plant that has twining stems with woody root at the base. Although the latex of *P. nigrescens* is very toxic, the leaves, bark latex and roots are used in treating various diseases.[31] Herbal preparation made from *P. nigrescens*, is marketed in Nigeria to treat anaemia.[32] An aqueous extract of *P. nigrescens* leaves exhibited moderate antimicrobial activity against a range of pathogenic bacteria.[21, 33] With these antecedents, it is of great interest to carry out a screening of chloroform extracts of these plants against some pathogenic bacteria and to reveal the active constituents by GC-MS analysis of the extracts.

MATERIALS AND METHODS

Isolation and identification of bacteria. Bacteria were isolated from urine using nutrient agar as general purpose medium and MacConkey agar as differential medium and then identified based on morphological and biochemical characteristics. The plates were incubated at 37^{o}C for 24-48 h. The appearance of the colonies on MacConkey agar were monitored and recorded. Distinct colonies were sub-cultured onto nnutrient agar in order to obtain pure culture of the bacterial isolates. Pure culture obtained was subjected to morphological and biochemical tests according to Cheeshbrough.[34]

Preparation of bacterial suspension. The inoculum density standardization for susceptibility test was matched to 0.5 McFarland standards (approximate bacterial suspension is 0.5 x 10^{8}). This standard was used strictly for the determination of turbidity in the bacterial suspension.

Chemical reagents. All the chemicals were analytical grade, and were purchased from AK Scientific, USA and Sigma-Aldrich (Germany).

Plant materials. The root of *P. nigrescens* and aerial parts of *A. paniculata* were collected from the vicinity of Oyo West Local Government Secretariat, Oyo, Nigeria in March 2019. The plant samples were identified and authenticated (261492FHI and 261501FHI for *P, nigrescens* and *A. paniculata* respectively) at Forest Research Institute of Nigeria, Ibadan, Nigeria.

Preparation of chloroform extracts. The dried aerial parts of *A. paniculata* were mechanically processed and then subjected to extraction with petroleum ether to eliminate lipophilic constituents.[35] The resulting extract (350 g) underwent a secondary extraction using chloroform for 24 hours. After concentration and suspension in water, the mixture formed two distinct layers. The chloroform layer, separated and concentrated under vacuum conditions, yielded the chloroform extract (8.3 g). This extract from *A. paniculata* was subsequently analyzed using GC-MS to identify its phytocomponents. The same extraction procedure was replicated to obtain the chloroform extract from *P. nigrescens* roots.

GC-MS analysis of chloroform extract. The phytochemical analysis was performed by gas Chromatography- mass Spectroscopy (GCMS-QP 2010) system coupled with a finigan MAT ion trap detector. The phytocomponents were separated in an RTX5MS column packed with 100% dimethylpolysiloxane. Helium was used as a carrier gas with a flow rate of 0.7 ml/min and injection volume of the sample was 0.5 µl, The analysis was performed under the following conditions; inlet temperature 250 °C, oven temperature was set at 50 °C for the first 1 min and latter raised to 160 °C with the flow rate of 20 °C/min. Temperature of 230 °C with the speed of 4 °C/min was attained at the last five minutes of analysis. Peak enrichment techniques were used to identify the components of the extract. The components of the extract were also confirmed by comparing their spectra with those of National Institute of Standards and Technology (NIST17) library mass spectra. GC-MS analyses of chloroform extracts of *P. nigrescens* and *A. paniculata* (Burm. F). Constituents were listed in order of elution from HP-5 capillary column. Santa Clara, CA, USA).

Identification of components. The components were identified by comparing the retention times and mass spectra of the chromatographic peaks with those of standards analysed under the same conditions. In addition, MassLib software (V9.3-106; 1996–2008) was used to match spectra of the components with those of National Institute of Standards and Technology (NIST17) library mass spectra and Wiley Registry of Mass Spectral Data (4th Ed.)

Determination of antimicrobial activity of chloroform extract. The bacterial suspension was swabbed on Mueller Hinton agar (Oxoid, England) and the antimicrobial activity of chloroform extract against test bacteria was determined according to the disk diffusion method. The plates were incubated aerobically at 35 ± 2ºC for 24 h. Diameter of the zones of inhibition (mm) was measured and interpreted. The readings were compared with neuropenem as clinical antibiotics using Clinical and Laboratory Standard Institute.[35]

Determination of minimum inhibitory concentration of the plant extracts. This test was performed in a sterile 96-well microtiter plates. For the evaluation of the active extract, two-fold dilution from stock solution of 200 µg/ml was prepared. Thus the working concentration were 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.125 µg/ml. The concentration of neuropenem (5 µg/ml) was prepared with distilled water as the positive control. The microdilution was performed in 96-well microtiter plates with U-shaped wells. Bacterial suspension was prepared using sterile water and adjusted to a 0.5 McFarland turbidity. Seven wells (B - H) were filled with 100 µl of the isolate in each well and thereafter, 100 µl of plant extract with varying concentration of 100 µg/ml – 3.125 µg/ml. The inoculated microtitre plates were sealed with paraffin and incubated at 37^{o}C for 24 h. The MIC of the samples was detected following addition of 50 µl of 0.20 mg/l p-iodonitrotetrazolium chloride (AK Scientific, Inc. USA) in all the wells and incubated at 37^{o}C for 30mins. Microbial growth was determined by observing the colour change from pinkish to red. Where there is no colour change, it indicates that there is no bacterial growth. Minimum inhibitory concentration (MIC) was defined as the lowest chloroform extract concentration that showed no colour change.[36]

Brine shrimp lethality test. Brine shrimp lethality test of the plant extracts was carried out using the procedure of Meyer.[37] brine shrimp eggs (*Artemia salina*) were hatched in artificial sea water. After, 48 h of incubation 10 larvae of *Artemia salina* were collected by pipette into calibrated 5 ml vial bottle.

The extracts were dissolved in tween 80 and seven different concentrations (5000, 2500, 1250, 625, 312.5, 156.25, 78.12 ppm) of each extract were prepared in artificial sea water. To each vial bottle that contain 10 *Artemia salina* in 4.5 ml of artificial sea water, 0.5 ml of the extract was added. The final concentration of the extract in each vial was one-fifth of its initial value. This was carried out in triplicate for each extract. Control experiment was tween 80

and artificial sea water without extract. After 24 h of incubation at room temperature each vial bottle was examined under a magnifying glass and the numbers of dead shrimps and survivors in each vial bottle were counted. Data obtained from this test was analyzed using the Graph pad prism computer program. The concentration with 50% lethality (LC_{50}), was calculated from a dose–response inhibition curve using nonlinear regression data analysis, expressed as mean ± SEM of three replicates, and subjected to student's t test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Antimicrobial assay and minimum inhibitory concentration. Bacteria investigated in this study were identified based on the morphological and biochemical characteristics. The bacterial isolates were identified to be *Citrobacter diversus*, *Shigella sonnei*, *Klebsiella pneumoniae* and *Proteus vulgaris* (Tables 1).

The susceptibility of bacterial isolates to extracts from *A. paniculata* and *P. nigrescens* was examined through the agar well diffusion technique. *Citrobacter diversus*, *Shigella sonnei* and *Proteus vulgaris* displayed susceptibility to *A. paniculata*, as evidenced by the observed zones of inhibition on the plates. However, *Klebsiella pneumoniae* showed resistance to *A. paniculata* extract. All the tested bacteria were observed susceptible to *P. nigrescens* (Table 2). Similar to the report of this study, other studies have reported antibacterial properties of *P. nigrescens* and *A. paniculata*.[20,28,33] The zones of inhibition of the extracts were compared with neuropenem (Clinical antibiotic) and interpreted based on standard criteria of Clinical and Laboratory Standards Institute.[36]

Table 1. Morphological and biochemical characteristics of bacteria isolates.

Characteristics	*Citrobacter diversus*	*Klebsiella pneuminiae*	*Proteus vulgaris*	*Shigella sonnei*
Gram reaction	-	-	-	-
Morphology	Rod	Rod	Rod	Rod
Catalase	+	+	+	+
Oxidase	-	-	-	-
Glucose	+	+	-	+
Lactose	+	+	-	-
Sucrose	+	+	-	+
Gas generated by microorganisms	-	-	-	+
Motility	+	-	+	-
Indole	-	-	+	-
H_2S	+	-	+	-
Citrate	+	+	+	+

Table 2. Antimicrobial susceptibility of bacterial isolates to chloroform extracts of *A. paniculata* and *P. nigrescens*.

Microorganisms	Chloroform extract (*A. paniculata*)	Chloroform extract (*P. nigrescens*)	Neuropenem
Citrobacter diversus	25 mm	15 mm	30 mm
Proteus vulgaris	20 mm	23 mm	32 mm
Klebsiella pneumoniae	NZI	21mm	26 mm
Shigella sonnei	20 mm	22 mm	30 mm

NZI – No Zone of Inhibition; mm – Millimeter.

The MIC of the extracts in two fold dilutions against the test bacteria revealed that 6.25 µg/ml of *P. nigrescens* was effective in inhibiting the *Shigella sonnei* while 100 µg/ml of *A. paniculata* was the minimum concentration that inhibited *Shigella sonnei* growth. Chloroform extract of *P. nigrescens* showed MIC at 6.25 µg/ml against *Proteus vulgaris* while extract of *A. paniculata* had 25 µg/ml as MIC against the same organism. Neuropenem was used as the positive control with the MIC of ≤ 0.1 µg/ml.[36] These extracts could be highly effective in the treatment and control of bacterial infections if the active compounds can be isolated. This is evidence, because the antimicrobial activity was obtained at very low concentration especially for *Klebsiella pneumoniae*, *Citrobacter diversus* and *Proteus vulgaris*. It was only *Shigella sonnei* that required 100 µg/ml of *A. paniculata* extract for its growth to be inhibited (Table 3).

Table 3. Minimum inhibitory concentration of chloroform extracts of *A. paniculata* and *P. nigrescens*.

Microorganisms	Chloroform extract (*A. paniculata*)	Chloroform extract (*P. nigrescens*)	Neuropenem
Citrobacter diversus	6.25 µg/ml	6.25 µg/ml	0.1 µg/ml
Shigella sonnei	100 µg/ml	6.25 µg/ml	0.05 µg/ml
Proteus vulgaris	25 µg/ml	6.25 µg/ml	0.1 µg/ml
Klebsiella pneumoniae	12.5 µg/ml	12.5 µg/ml	0.1 µg/ml

Table 4. Chemical composition of *P. nigrescens* chloroform extract.

SN	Name of the compound	Retention time	Mol. Formula	% composition
1	2-Furanmethanol, .alpha.-(2-nitropropyl)-, (R*,R*)-	13.272	$C_8H_{11}NO_4$	3.20
2	1-Nonadecene	14.302	$C_{19}H_{38}$	0.54
3	(2R,5S)-2-Butyl-5-propylpyrrolidine	14.399	$C_{11}H_{21}N$	4.75
4	n-Hexadecanoic acid	16.568	$C_{16}H_{32}O_2$	1.99
5	Hexadecanoic acid, ethyl ester	16.871	$C_{18}H_{36}O_2$	8.67
6	Eicosanal	17.191	$C_{20}H_{40}O$	0.23
7	5,5-Dimethyl-1,3-cyclohexanedione dioxime	17.300	$C_8H_{14}N_2O_2$	4.78
8	Heptadecanoic acid, ethyl ester	18.216	$C_{19}H_{38}O_2$	0.87
9	Linoleic acid ethyl ester	19.206	$C_{20}H_{36}O_2$	21.66
10	(E)-9-Octadecenoic acid ethyl ester	19.394	$C_{20}H_{38}O_2$	7.82
11	Octadecanoic acid, ethyl ester	19.709	$C_{20}H_{40}O_2$	4.45
12	Nonadecanoic acid, ethyl ester	21.162	$C_{21}H_{42}O_2$	0.44
13	Eicosanoic acid, ethyl ester	22.679	$C_{22}H_{36}O_2$	1.93
14	Hexadecane	24.207	$C_{16}H_{34}$	0.44
15	Docosanoic acid, ethyl ester	25.500	$C_{24}H_{48}O_2$	1.20
16	Heptadecane	26.633	$C_{17}H_{36}$	0.81
17	Octacosane	27.605	$C_{28}H_{58}$	0.92
18	Squalene	27.874	$C_{30}H_{50}$	1.47
19	Eicosane	28.498	$C_{20}H_{42}$	0.50
20	Carbonic acid, eicosyl vinyl ester	29.311	$C_{23}H_{44}O_3$	0.44
21	Tetracosane	30.072	$C_{24}H_{50}$	0.06
22	Campesterol	30.987	$C_{28}H_{48}O$	0.51
23	Stigmasterol	31.227	$C_{29}H_{48}O$	0.66
24	gamma.-Sitosterol	31.639	$C_{29}H_{50}O$	1.75
25	beta.-Amyrin	31.845	$C_{30}H_{50}O$	3.05
26	Olean-18-ene	31.891	$C_{30}H_{50}$	3.44
27	4,4,6a,6b,8a,11,12,14b-Octamethyl 1,4,4a,5,6,6a,6b, 7,8,8a, 9,10,11, 12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	31.983	$C_{30}H_{48}O$	0.85
28	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethy l-6-(1-methyl ethenyl)-	32.160	$C_{15}H_{22}O$	7.46
29	Lupeol	32.183	$C_{30}H_{50}O$	3.75
30	2-Myristynoyl-glycinamide	32.418	$C_{16}H_{28}N_2O_2$	5.52
31	9,19-Cyclolanostan-3-ol-11-one, acetate	32.526	$C_{32}H_{52}O_3$	3.40
32	Lup-20(29)-en 3-ol, acetate	32.824	$C_{32}H_{52}O_2$	2.44

The phytochemical components of the chloroform extract of *A. paniculata* and *P. nigrescens* were obtained through GC-MS analysis. The name and retention time of each component along with the chemical formula was presented in tables 4 and 5. GC-MS analysis of *P. nigrescens* extract resulted in the identification of thirty-two compounds while that of *P. nigrescens* resulted in the identification of twenty- two compounds. The major components of the two extracts were fatty acid esters, alkaloids and terpenes, although they were present in higher proportion in *P. nigrescens* than in *A. paniculata*. The percentage composition of alkaloids in *P. nigrescens* and *A. paniculata* are 13.5% and 8.6%, respectively. Higher activity exhibited by *P. nigrescens* extract might be as a result of higher proportion of alkaloids and fatty acid ester. The chromatograms of *P. nigrescens* and *A. paniculata* are presented in figures 1 and 2, respectively.

Table 5. Chemical composition of *A. paniculata* chloroform extract.

SN	Name of the compound	Retention time	Mol. formula	% composition
1	7-Oxabicyclo[4.1.0]heptan-3-ol, 6- (3-hydroxy-1-butenyl)-1,5,5-trimethyl-	13.066	$C_{13}H_{22}O_3$	2.71
2	Carbonic acid, hexadecyl 2,2,2-tri chloroethyl ester	14.319	$C_{19}H_{35}CI_3O_3$	2.05
3	Neophytadiene	14.851	$C_{20}H_{38}$	0.68
4	5-Eicosene, (E)-	16.791	$C_{20}H_{40}$	5.14
5	Phytol	18.479	$C_{20}H_{40}O$	11.76
6	9,12-Octadecadienoic acid (Z,Z)-	19.005	$C_{18}H_{32}O_2$	10.02
7	1-Heneicosanol	19.635	$C_{21}H_{44}O$	6.85
8	3-Eicosene, (E)-	22.633	$C_{20}H_{40}$	6.24
9	9-Eicosene, (E)-	24.212	$C_{20}H_{40}$	0.50
10	Trifluoroacetoxy hexadecane	25.460	$C_{18}H_{33}F_3O_2$	4.26
11	Heptadecane	26.627	$C_{17}H_{36}$	0.54
12	Octabenzone	27.377	$C_{21}H_{26}O_3$	5.42
13	10-Heneicosene	27.571	$C_{21}H_{42}$	6.29
14	Squalene	27.874	$C_{30}H_{50}$	9.28
15	Oleyl alcohol, trifluoroacetate	28.498	$C_{20}H_{35}F_3O_2$	2.20
16	Docosyl pentafluoropropionate	29.288	$C_{25}H_{45}F_5O_2$	2.23
17	1,2-Benzisothiazol-3-amine	29.751	$C_7H_6N_2S$	5.11
18	Octadecane	30.072	$C_{18}H_{38}$	2.84
19	Vitamin E	30.369	$C_{29}H_{50}O_2$	1.54
20	2,4-Cyclohexadien-1-one, 3,5-bis(1 ,1-dimethylethyl)-4-hydroxy-	31.233	$C_{14}H_{22}O_2$	1.36
21	.gamma.-Sitosterol	31.634	$C_{29}H_{50}O$	4.05
22	5-Methyl-2-phenylindolizine	33.516	$C_{15}H_{13}N$	3.58

Cytotoxicity assay. The brine shrimp lethality assay is a simple and inexpensive bioassay used for testing the cytotoxic efficacy of phytochemical present in the plant extracts. Here, LC_{50} value lower than 1000 µg/ml is considered bioactive.[38] The LC_{50} values of *P. nigrescens* and *A. paniculata* chloroform extracts were (89.75 ± 0.18) µg/ml and (167.5 ± 0.21) respectively. Hence, the two extracts were both toxic (active). This could suggest why the two plants are often used in medicinal practice. *P. nigrescens* extract was slightly more toxic against the brine shrimp than *A. paniculata*. This indicated why *P. nigrescens* extract had higher activity than *A. paniculata*. The result of bioassay correlated reasonably well with bioactivity test of the extracts. The potency could be as a result of higher percentage compositions of linoleic acid, alkaloid and a significant number of monoterpenes present in the

extract. The dietary, conjugated linoleic acid exerts anti-inflammatory effect by decreasing production of the inflammatory mediators such as prostaglandin E2, IL-6, IL-1β, TNFα, and nitric oxide.[39-40] Ester bond hydrolysis of membrane phospholipids by Phospholipase A2 and consequent release of fatty acids are the initiating steps of inflammation. Various findings revealed that the fatty acid, n-hexadecanoic acid, is an inhibitor of phospholipase A2, hence, an anti-inflammatory compound.[41]

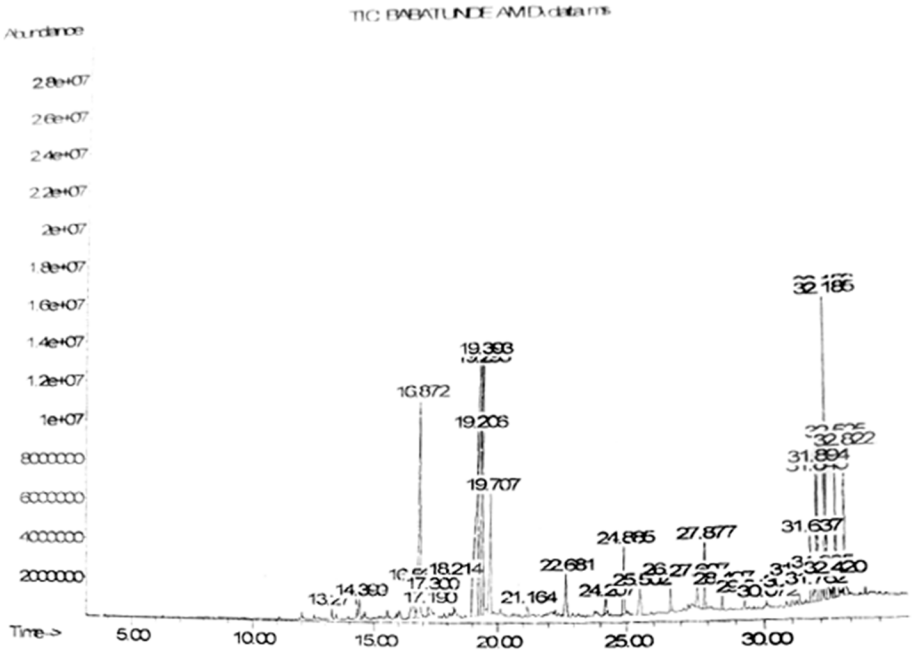

Figure 1. Chromatogram of *P. nigrescens.*

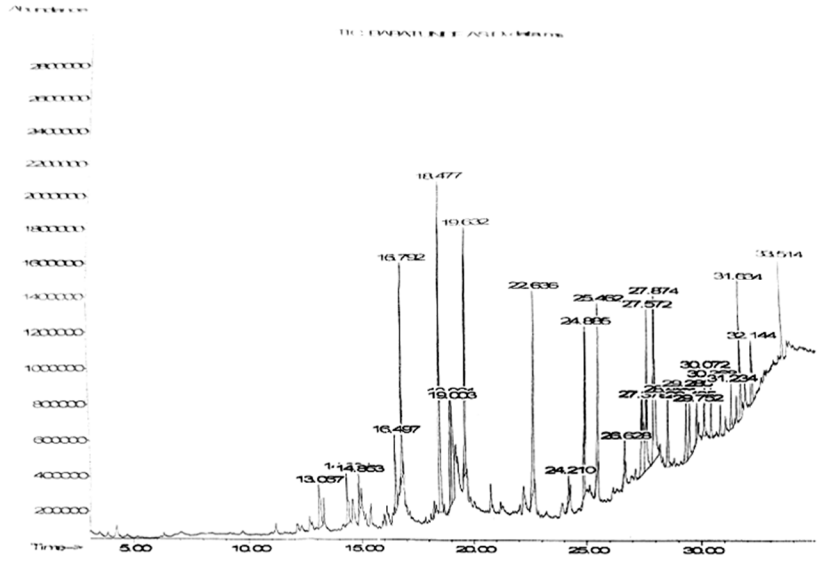

Figure 2. Chromatogram of *A. paniculata.*

CONCLUSION

The extracts of *P. nigrescens* and *A. paniculata* were found highly active antimicrobially due to the observed level of zones of inhibition in the agar well diffusion assay. minimum inhibitory concentration of the extracts was also observed to be very low especially in the *P. nigrescens* extract. Spectroscopic analysis revealed higher proportion of alkaloids in *P. nigrescens* than the occurrence in *A. paniculata*. The result of brine shrimp lethality test showed high level of toxic phytocomponents. Further research in the purification of the components of the extracts will provide a ray of hope in the discovery of novel antibiotics particularly in this period of global multi-drug resistance.

CONFLICT OF INTEREST

Authors declared that there is no conflict of interest and that this study was self-funded.

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