Chemical Analysis And Antimicrobial Effects Of Petroleum Spirit Extract Of *Anogeissus Leocarpus*

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ABSTRACT

The plant Anogeissus leocarpus was extracted using petroleum spirit, ethyl acetate, chloroform and methanol. The petroleum spirit extract contains saturated carboxylic acids (tetradecanoic, hexadecanoic and octadecanoic acid), unsaturated acids (linoleic acid, and oleic acid) together with their esters (11, 14-eiocosadienoic acid methyl ester and its isomer). Also, long-chained hydrocarbons (tricosyne and octacosane), triglyceride and cholesterol were identified. These components were analysed by tlc, column chromatography, gc-ms, ir, 1D and 2D-nmr techniques. Antimicrobial effects of the extracts on the microorganisms are presented.

INTRODUCTION

Anogeissus leocarpus belongs to the family Combretaceae which is a family of species distributed in 16 genera as trees, shrubs, and woody climbers, and partly as lianas in tropical and sub-tropical regions especially in Africa[1]. The botanical description of the family has been given[2].

Many chemical compounds have been identified in plant species in the *Anogeissus* family. Examples include sugars and their derivatives from *A. schimperi* after partial acid hydrolysis[3-4], amino acids from gum exudates of *A. schimperi*[5], ellagic and flavellagic acids from the bark and wood extractives of *A. latifolia*[6-7], glycosides from *A. schimperi*[8], flavones and flavenoidal glycosides from *A. latifolia* and *Anogeissus acuminata*[9] and tannins from *A. acuminata*[10].

Plants in the *Anogeissus* family have been used in herbal medicine in many places such as Africa and India. For example, decoctions from the leaves of *A. latifolia* are used in the treatment of diarrhoea and gonorrhoea in India.[11] *A. leocarpus*, the subject of the current study, has numerous medicinal applications all over Africa[12, 13]. In Nigeria it is commonly called 'orin odan' or 'ayin' by 'Yorubas'; 'ganuwa' or 'farin gamji' by 'Hausas'; in 'Fulfude' it is called 'galaldi' while the 'Igbos' refer to it as 'abakalik atara' just to mention a few. In Nigeria and Ghana, the roots of the plant are used as a chewing stick.[13] In Ivory Coast and Upper Volta, pulped roots are applied to sores to promote healing.

Other than medicinal applications, *Anogeissus* species have been used for purposes that are very beneficial to man. The most common use of these plants is as glue. The stem exudate of the three species *A. leocarpus*[14], *A. latifolia*[15], and *A. schimperi*[16] are the most commonly used as gum. They are also a source of sugars, for example, L-arabinose was isolated in commercial quantity from stem exudate of *A. latifolia*[15]. Traditional tanners tan leather using leaves of A. *schimperii*[16] *A. latifolia*[15] and A. *leocarpus*[14].

A previous report[17] shows that 3,4,3'-tri-O-methylflavellagic acid and its glucoside were isolated from *Anogeissus leocarpus*. In a further search we hereby report the chemical analysis and antimicrobial effects of petroleum spirit extract of the plant.

EXPERIMENTAL

Chemicals and Instruments

All solvents used were redistilled on a vertical still and all nmr data were obtained from a 400 MHz Bruker NMR Spectrophotometer model ADVANCE DPX_{400} . IR spectra were obtained from Perkin-Elmer IR Spectrophotometer 1600 Series, with Hewlett Packard Color Pro Printer while GC-MS and UV spectra were respectively

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obtained from Hewlett Packard Gas Chromatographic Machine GC 5890 + TRIO-I Mass Spectrometer by VG Lab Ltd., and CE660 Multimude Computing UV Spectrophotometer 6000 Series. TLC was carried out on 0.2 mm layer of silica (Merck, Kieselgel $60F_{254}$) on aluminium sheets while column chromatography was carried out on silica gel (60-120 mesh size).

The plant material

Fresh bark of the plant *A. leocarpus* was collected at Grogoji village in Zaria Local Government area of Kaduna State. This was dried and a voucher specimen No.167 deposited in the herbarium of Botany Department of the Ahmadu Bello University, Zaria, Nigeria; the dried bark was powdered and used for extraction.

Extraction procedure

The extraction solvents used were petroleum spirit (40-60 ^{0}C), chloroform, ethanol and water in this order. Powdered bark (1,500 g) was weighed out and packed into the thimble of a Soxhlet extractor. Extraction was carried out until the solvent in the reservoir of the Soxhlet apparatus was colourless probably indicating the completion of extraction process. The extract was concentrated on a rotary evaporator. The extract was then dried completely under vacuum. The table below shows the solvents, percentage extraction and extract weights per 1,500 g of bark.

Table I Percentage extraction per 15OOg of bark

Solvents	Extract weight (g)	Percentage extraction
Petroleum Spirit	4.50	0.30
Chloroform	2.50	0.14
Ethanol	38.02	2.53
Water	50.13	3.34

Purification and analysis of the extractGC and spectroscopic data of crude extract

Analysis of the petroleum spirit extract gave: υ cm^{-1} (Neat) 3050-3500 (m -OH stretch), 1710 (s, -C=O stretch), 1428 (s), 1377 (m), 1172 (m). δH 0.9 (m, CH_3 groups), 1.35 (t + d, CH_3 groups), 1.65 (m, CH_2 groups), 2.08 (m,), 2.4 (m), 2.82 (m, unsaturated CH groups), 5.4 (m, -CH_2O-Acyl groups). [GC-MS. DB-17.49 ^{0}C (5 mins) x 15^{0}C/min, 325 ^{0}C (10 mins)] RT (mins) 17.05, 18.38, 18.53 and 18.70.

Alkaline hydrolysis of crude extract and isolation of the product

Crude petroleum spirit extract (200 mg) was dissolved in MeOH (50 cm^3) and NaOH (6M, 15 cm^3) was added. The mixture was refluxed for 20 hrs. After 16 hrs, tlc analysis of the reaction mixture showed complete hydrolysis with the absence of starting material spot.

The reaction mixture was cooled and extracted with Et_2O (2 x 75 cm^3) to remove the neutral component, washed with water, dried over anhydrous $MgSO_4$ and concentrated to give the product (160 mg). The spectral data are:

δH 0.63-1.4 (m, due to methyl groups), 1.4-1.53 (m), 1.53-1.74 (m), 1.75-1.90 (m), 1.9-2.12 (m), 2.12-2.42 (m, CH_2 groups), 2.43-2.85 (m), 3.20 (s), 3.5 (m, due to -CH_2-groups), 3.6 (t, J 6.6 Hz), 5.05 (m), 5.33 (m) 5.7-5.95 (m) and 6.08 (s).

The sample was compared with authentic cholesterol spectrum by 3-part tlc, it was found to contain cholesterol (R_f 0.35 in petroleum spirit:Et_2O, 1:1).

Purification of petroleum spirit extract by column chromatography

Thin-layer chromatography (tlc) was done on the crude petroleum spirit extract to determine a suitable solvent for its separation by column chromatography (C.C); petroleum spirit (60 - 80 ^{0}C) and Et_20 (1:1) gave the best separation and this medium was used as the mobile phase for column chromatography on silica.

The column was developed using the mobile phase starting with neat petroleum spirit (60 - 80 ^{0}C) and increasing the polarity in the sequence: Et_2O, ETOAc, chloroform, acetone, MeOH. Fractions were collected and analysed by tlc.

The combined fractions were concentrated at reduced pressure and dried under high vacuum. Table 2 gives the fractions, their weights (including percentages) and approximate R_f-values (petroleum spirit:Et_2O, 1:1).

Table2: **Weights and percentages of fractions from petroleum spirit extract**

Fraction	Weight of fraction (g)	percentage per 3,85g extract	R_f-values
1	0.72	18.70	0.97
2	1.30	33.76	0.97, 0.88
3	0.32	8.31	0.89, 0.78, 0.67
4	0.33	857	0.67, 0.62
5	0.56	14.54	0.57, 0.47
6	0.10	2.60.	047, 0.30
7	0.15	3.90	0.30, 0.20
8	0.28	7.27	0.16, 0.07
9	0.09	2.34	0.07, 0.03

Analvsis of the fractions

Further analysis was done only on fractions 1, 2 and 5, the main fractions.

Fraction I

δH 0.92 (m, overlapping methyl triplets), 1.30 (d, $-CH_2-$), 2.04 (m, $-CH_2-CH_2$), 2.30 (m, $-CH_2-$ groups), 2.80 (quartet), 5.10 (s), 5.38 (m, $-CH_2O$-Acyl group protons). [GC-MS DB-17 49 ^{0}C (5 min) x 15^{0}C/min, 325^{0}C(10 mins)] **RT** (min) 16.24, 17.50and 17.67.

Fraction 2

δH 0.9 (m, overlapping methyl triplets); 1.30 (m, $-CH_2-$), 1.60 (m, $-CH_2-CH_2-C$ (=0)), 1.94 (m, $-CH_2-CH_2-CH=$), 2.05 (m, $-CH_2C$ (=0)?), 2:75 (m, =CH- CH_2-CH-CH=), 4.08 (dd *J* 11.9, 6.0, - CH_2O Acyl), 4.22, (dd *J* 11.9, 4.3, - CH_2OAcy1), 5.28 (m, -CH=CH- & -*CH* (OAcyl)- CH_2OAcyl). [GC-MS DB-5, 50 ^{0}C (5 mins) x 15^{0}C/min, 325 ^{0}C (10 min)] RT (min) 18.23 and 24.90 with the following MS data, 219 (30), 218 (100), 203 (50), 173 (5), 133 (10), 105 (10).

Fraction 5

δH 0.9 (m, overlapping methyl triplets), 1.3 (d), *1.65* (m, $-CH_2-CH_2-C$=O), 2.8 (=CH-CH=CH-), 3.48 (m, $-CH_3O-$), 5.35 (d, -CH=CH-). [GC-MS DB-17 49 ^{0}C (5min) x 15 ^{0}C/min, 325 ^{0}C (10 min)] **RT** (min). 16.54 and 17'69.

Alkaline hydrolysis of fraction 1

Fraction 1 (80 mg) was dissolved in MeOH (10 cm^3), then NaOH (6 M, 2 cm^3) was added and the mixture heated under reflux for 20hrs. The solution was extracted into Et_2O (2 x 20 cm^3). washed with water (40 cm^3) and dried over anhydrous $MgSO_4$ and concentrated to give the neutral component (60 mg). The aqueous layer was neutralised with HCI (2M, 10 cm^3), and then extracted into Et_2O (2 x 30 cm^3) to give the acidic component in low yield (10 mg).

Acidic component δH 0.9 (m, $-CH_3$ groups), 1.85 (m, $-CH_2-$ groups), 2.2 (m, $-CH_2$-groups), 2.3 (t, $-CH_3$). [GC-MS DB-17 49 ^{0}C (5 mins) x 15^{0}C/min, 325 ^{0}C (10 min)] RT (mins). 15.62, 17.04, 19.90. R_f 0.40, 0.30 in petroleum spirit:Et_2O (1:1).

Neutral component δH 0.85 (m, $-CH_3$- groups), 2.3 (m, CH_2- groups), 2.8 (m, CH_2- groups). [GC-MS DB-17 49^{0}C (5min), x 15^{0}C/min, 325 ^{0}C (10 min)] RT (min.) 25.25, 15.68, 15.95, 17.13 and 18.46.

Alkaline hydrolysis of fractions 2 and 5

Each of fractions 2 and 5 was separately hydrolysed using a procedure similar to that of fraction 1 above to yield the acidic and neutral products. The data below gives the spectral data for the neutral and acidic components.

Acidic Component δH 0.9 (m, $-CH_3$ groups), 1.3 (m, $-CH_3$ groups), 1.6 (m, $-CH_3$ groups), 2.05 (m, $-CH_3$ groups), 2.31 (m, $-CH_2$ groups). [(GC-MS DB-5 50^{0}C (5 min), x 15^{0}C/min, 325^{0}C (10 min)] RT (min), 16.97, 18.17 and 18.65. R_f 0.23 and 0.10 in petroleum spirit Et_2O, (1:1).

Neutral Component δH 0.9 (m, $-CH_3$ groups), 1.3 (m, $-CH_3$ groups), 2.0 (m, $-CH_2$ groups), 2.3 (m, $-CH_3$ groups), 2.8 (m, $-CH_3$ groups), [GC-MS DB-5 50^{0}C (5mins) x 15^{0}C/min. 325 ^{0}C (10 min)] RT (min) 25.25.

Preliminary antimicrobial screening of crude extract

Materials: The test organisms used were *Pseudomonas aeruginosa* NCTC 6750 and *Bacillus subtilis* NCTC 8326. The concentration of the extract used is 1.0 g/cm^3 in MeOH.

Procedure: The screening was carried out by agar diffusion technique as follows: Sterile molten agar plates were inoculated with the diluted overnight cultures of the organisms i.e. 1:1000 for *Bacillus subtillis* and 1:5000 for *Pseudomonas aeruginosa.* The inoculation of the plate was by flooding. The plates were dried for 10 minutes and a sterile cork

borer (No. 4) of 8-mm diameter was used to bore wells into the agar bed. Sterile nutrient agar was used to seal the bottom of the wells and 0.3 cm^3 of the extract solution in methanol was placed in them. Also, 0.3 cm^3 of methanol was placed in one of the wells to serve as control.

One-hour pre-diffusion time was allowed after which the plates were incubated at 37°C for 18-24 hours. The zones of inhibition were then measured to the nearest millimeter.

The result obtained indicates that the crude petroleum spirit extract has growth inhibitory effects on Pseudomonas aeruginosa and *Bacilus subtillis* with inbition zones of 25 and 38 mm respectively.

RESULTS AND DISCUSSION

The petroleum spirit extract was analysed. Proton nmr, ir and gc-ms analyses of the crude extract gave spectra similar to those of long chain carboxylic acids. The broad OHstr and the -C=Ostr are evident in the ir spectrum. The broad CH_3- and -CH_2- signals at δ 0.9-1.7 ppm (characteristic of long chain alkanes), δ 5.4 (characteristics of the presence of —CH_2O-Acyl groups) and the (GC-MS gave four peaks with retention times (RT) of 17.05, 18.38, 18.53 and 18.70. The retention times for long chain alkanoic acids were determined using authentic samples and the same experimental conditions were adapted for the extract (see Figure 1). To release the free carboxylic acids by hydrolysis, the ester bonds in the sample were hydrolysed by alkali.

Based on this study, the likely alkanoic acid components in the petroleum spirit fraction of the plant are hexadecanoic acid, oleic acid and probably two C 18 alkenoic acids. The presence of alkanoic acids was further confirmed by the streaking nature of the sample on silica tlc plates, which is a result of H-bonding between the carboxylic acid O-H group and the siloxyl groups on the silica gel surface.

An attempt was made to further purify the crude extract by column chromatography. Nine fractions were collected but only fractions 1, 2, and 5 contained enough material to warrant further investigation. Proton nmr of the fractions indicated that fraction I contained mainly a long chain alkene with the alkenic proton appearing at δ 5.10 and 5.38ppm.

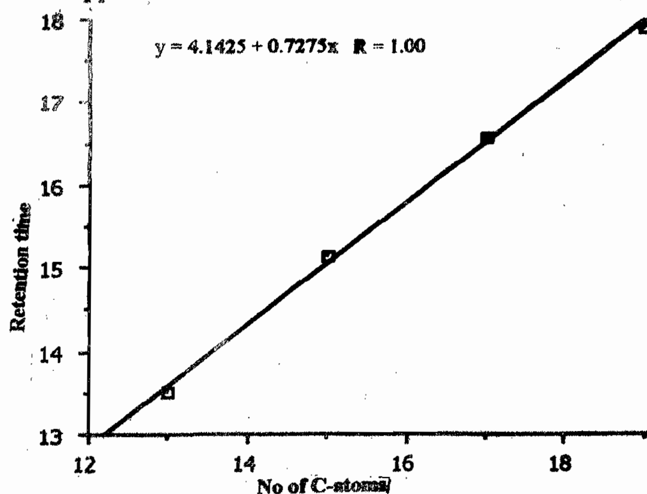


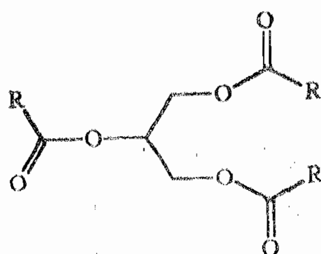
Figure 1: Graph showing retention time Vs no. of carbon atoms in the alkanoic acids.

Fractions 2 and 5 consisted mainly of esters of long chain alkenoic acids, similar to those of glycerides of hexadecenoic acids. Alkaline hydrolysis of fraction 1 gave an acidic component and a neutral component which consisted of alkenols. The retention time of the acid component was found to be similar to those of hexadecanoic acid and tetradecanoic acid (see Figure 1). Alkaline hydrolysis of fraction 2 and 5 gave a similar result to that obtained from fraction 1 with the acidic component consisting of unsaturated long chain carboxylic acids and the neutral component consisting of unsaturated alkenols.

The acidic component of fraction I gave a ^{1}H-nmr spectrum with peaks due to a long chain carboxylic acid from the multiplets at δ 1.85, 2.2 and 2.3, which are due to the presence of -CH_2- groups. The multiplets at δ 0.9 are due to -CH_3- groups. Gc-ms on DB-5 gave RT (min), 16.97 (hexadecanoic acid), 18.17 (linoleic acid) and 18.65 (octadecanoic acid). Comparison of RT of both hexadecanoic and octadecanoic acids agrees with those of standard acids run under the same conditions. Linoleic acid identification is supported by the peak match comparison of the spectra of authentic linoleic acid sample. ^{1}H-nmr spectrum of the neutral component gave peaks at δ 2.3 and 2.8 indicating unsaturation. Gc-ms on DB-5 gave RT (min) 25.25 with M^+ ion with m/z value of 425

which, from calculation, gave the formula $C_{31}H_{52}$ (i.e. an alkene with 4 double bond equivalents).

Fraction 2 gave a ^{1}H-nmr with peaks consistent with a triester of oleic acid (triolein) or of other triglyceride. The $-CH_2-$ groups absorb at δ 2.05 and 2.31, the multiplets at δ 2.80 are due to the unsaturation; the CH_3- groups absorb at δ 1.30 and 1.60. The connecting protons of the glycerol absorb at δ 4.15 (dd, *J* 11.9, 6.0 Hz) from CH_2O-, and δ 4.30 (d, *J* 1.9, 4.3 Hz), indicating a structure of the type:



R = alkyl group

(GC-MS on DB-5 gave RT (min) 18.23 (diunsaturated C_{18}-acid) and 24.90 (olean-12-ene, 3 methoxy-3).

Fraction *5* gave a ^{1}H-nmr spectrum with the skipped diene peaks at δ 2.8, methoxy peaks at δ 3.48 and CH_3 group peaks at δ 1.30. GC-MS gave RT (min), 16.54 (11, 14-eicosadienoic acid methyl ester) and 17.69 (eicosadienoic acid methyl ester).

Thus, the petroleum spirit extract contains carboxylic acids (tetradecanoic, hexadecanoic and octadecanoic acids) identification of which are made based on RT of the unknown and those of standards run under similar experimental conditions as shown in the graph given in figure 1. It also contains unsaturated acids (linoleic acid, and oleic acid) together with their esters (1 1,14-eiocosadienoic acid methyl ester and its isomer). Long-chained hydrocarbons (tricosyne and octacosane) were also identified. A triglyceride and cholesterol are confirmed present.

Preliminary antimicrobial screening of this extract using the agar diffusion method against standard *Bacillus subtilis* and *Pseudomonas aeruginosa* indicates that the extract possesses growth inhibitory effect on these organisms.

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