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Organic Compounds in the GCMS Analysis of Methanolic Leaf Extract of *Anogeissus leiocarpus*

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Abstract: GCMS of n-butanolic leaf extract of *anogeissus leiocarpus* showed eleven peaks, indicating the presence of eleven compounds. Five of these compounds are fatty acids and their derivatives. Among these are palmitic acid, palmitic acid methyl ester, oleic acid, oleic acid methyl ester and stearic acid. Many fatty acids are known to have antibacterial and antifungal properties. Other compounds seen are phthalic acid and di-n-octyl phthalate. The GCMS result of anogeissus leiocarpus leaf extract validates the use in traditional medicine as antibacterial.

Keywords: Anogeissus Leiocarpus Leaf, Traditional Medicine, Fatty Acids, Antibacterial, GCMS

Introduction

Anogeissus leoicarpus is a tree belonging to the family combretaceae. It is common in tropical and sub tropical countries [1]. The plant is distributed throughout Central and West Africa where it is used as medicinal herb and fodder. In Nigeria, it is used to treat respiratory and other human diseases [2]. The leaf infusion and decoction is used as cough medicine. The powdered root is used to treat wounds and ulcers while the powdered stem bark is rubbed on gums to reduce toothache [3]. *A. leiocarpus* is also used in the treatment for diarrhoea, syphilis chancres, stimulant, aphrodisiac and tanicide for horses and donkeys [4]. This plant is shown to be active as antimicrobial agent against gram positive and gram negative bacteria [5&6], antimycobacterial activity [7,8,9] and trypanacidal [10]. It has demonstrated activity against *Candida albicans* [11,12,13]. Phytochemical screening of Anogeissus leiocarpus shows the presence of several secondary metabolites such as tannins, saponins, alkaloids and flavonoids in the root, bark and leaf extracts of the plant [14,3].

The picture of *Anogeissus leiocarpus* tree

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Materials And Methods

Leaves of *Anogeissus leiocarpu,* was freshly collected around September from Vom in Jos south L.G.A of Plateau state, Nigeria and were identified at the Federal Department of Forestry, Jos.

Assay For Active Principles In The Plants' Extracts

The fresh leaves of *Anogeissus leiocarpus,* (marke) was oven dried at 60°C (degrees centigrade) for 5 days. The plant sample was pulverized and 500 g of the powdered leaf was soaked overnight in n-Hexane to defat (remove the fats and oil). The n-Hexane was squeezed out of the plant material and the solvent recovered by distillation. The extract from n-hexane was dried down on a water bath. The solid mass obtained was weighed, labeled and stored in a cool dry place. The defatted plant material (marc) was then re dissolved with 500 ml of distilled water and extracted with 2 liters of methanol in an air tight clean flat bottom container for 7 days. This was done at room temperature with occasional stirring and shaking. The extract was then first filtered through a fresh plug of cotton wool and finally through a Whatman No 4 filter paper. The methanol was recovered by distillation using soxlet apparatus and the methanolic extract dried down on a water bath and the solid mass obtained weighed and stored appropriately. 5g of the crude methanolic extract was dissolved in distilled water and solvent-solvent partitioning (Figure 1) was done according to the protocol designed by Kupchan [15] and modified by Van-Wagenen [16]. The methanolic solution was fractionated successively using solvents of increasing polarity, ethyl acetate and n-butanol. The solvents were recovered by distillation and the fractions evaporated to dryness using a water bath. The fractions were weighed, labelled appropriately and stored in a cool dry place.

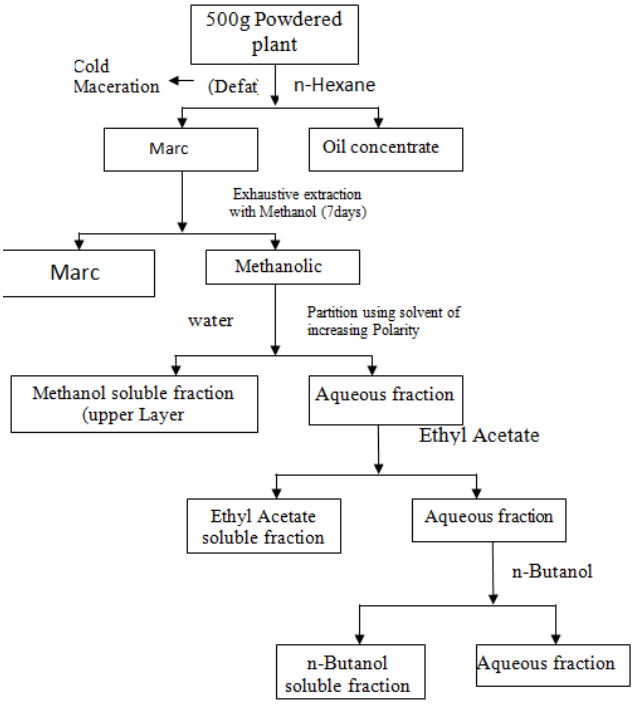

Figure 1: A flow chart describing the extraction and partitioning of the plant in various solvents

Column Chromatography of the n-Butanolic Fraction of the Plant Extracts

The leaf extract in n-hexane, methanol, ethyl acetate and n-butanol fractions were tested for antibacterial activities. The n-butanolic fraction of the leaf extract was found to have the highest antibacterial activities. The n-butanolic fraction of the leaf extract was subjected to column chromatography as follows. Qualikems silica gel 60-120 mesh was dissolved with ethyl acetate to get a slurry. The column (40cm high and 3cm in diameter) was lined with cotton wool at the base. The slurry was gradually (avoiding air bubbles) poured into the column until the column is well packed. The top of the gel is lined with cotton wool. The column is allowed to equilibrate for one hour. The n-butanolic extract was loaded into the column and a layer of cotton wool used to line the extract on top, such that the extract is sandwiched between the layers of cotton wool. 200ml of ethyl acetate was gradually poured into the column. Ethyl acetate (EA) and methanol are mixed in ratios of increasing order of polarity according to the protocol shown below. Ethyl acetate is fraction 1, 90ml of EA and 10ml of methanol is fraction 2 etc. Fraction 11 is 100ml of methanol. The flow rate is 18 minutes per 100ml. Each fraction was collected into a porcelain evaporating dish and dried down using a water bath and transfered into an appropriately labeled bijour bottle. The bottles are well stoppered and stored in the refridgerator.

Table 1
Protocol showing the ratio of EA and methanol used

	1	2	3	4	5	6	7	8	9	10	11
EA(ml)	200	90	80	70	60	50	40	30	20	10	-
Methanol(ml)	-	10	20	30	40	50	60	70	80	90	100

Thin Layer Chromatography of Anogeissus leoicarpus Fraction 7.

The different fractions of the column chromatography of the leaf extract were subjected to antibacterial testing and fraction 7 was found to be most active. Fraction 7 was therefore subjected to TLC. The TLC plates were prepared using 30g of silica gel dissolved with 60ml of water to get a slurry. The 20×20 glass plates were loaded in the TLC plate maker. The slurry was spread on the plates using the plate maker machine. The plates were allowed to dry at room temperature before transferring them to the oven at 110°C for 1hour. Prior to use the plates were activated in the oven at 110°C for 30 minutes. The solvents used were chloroform, acetic acid and distilled water in the ratio of 7:2:1. The solvents were mixed (elutant) and poured into the electrophoretic tank such that the depth of the elutant was not more than 1cm. 20µl of the samples (ie fraction 7 of anogeissus leoicarpus leaf) was spotted on the plate 2cm from the bottom of the plate (ie the origin). After spotting, it was allowed to dry for 5minutes. The plate was immersed into the tank such that 1cm of plate was inside the elutant. The plate was vertically placed in the tank and the lid placed over it to make airtight. Development was for 1 hour 45 minutes. The retardation factors (Rf) of the sample was recorded and the picture of the TLC plate documented in appendix 1.

Gas Chromatography Mass Spectrometry (GCMS) of the TLC Spots

The TLC gave a picture of a single spot as the sample did not separate into different components. The single spot of each sample was scraped into the elutant and subjected to GCMS. The results were recorded.

Table 2 Showing the compounds identified in the GC-MS analysis of n-butanolic leaf extract of anogeissus leiocarpus.

S/No.	Peak No.	Peak area	Peak area %	Retention time	Compound name	Common name	Molecular formular	Molecular weight
1	4	7000933	6.20	18.442	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	phthalic acid	$C_{16}H_{22}O_4$	278
2	5	2790326	2.47	19.137	Hexadecanoic acid methyl ester	Palmitic acid methyl ester	C17H34O2	270
3	6	22567807	20.00	20.534	n-Hexadecanoic acid	Palmitic acid	C16H32O2	256
4	7	7368344	6.53	22.366	9-Octadecenoic acid methyl ester	Oleic acid methyl ester	$C_{19}H_{36}O_2$	296
5	8	42012627	37.22	23.408	9-Octadecenoic acid	Oleic acid	$C_{18}H_{34}O_2$	282
6	9	15490049	13.72	23.636	Octadecanoic acid	Stearic acid	$C_{18}H_{36}O_2$	284
7	11	2220968	1.97	27.507	1,2-Benzenedicarboxylic acid, diisooctyl ester	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390

Table Showing the compounds identified in the GC-MS analysis of n-butanolic leaf extract of anogeissus leiocarpus. Seven compounds matched 100% with those from the NIST library (Appendix 2). Five of these compounds are found to be fatty acids or their derivatives. Among these are (1) palmitic acid methyl ester (hexadecanoic acid methyl ester) (2) palmitic acid (n-hexadecanoic acid) (3) oleic acid (9-octadecenoic acid) (4) Oleic acid methyl ester (9-Octadecenoic acid methyl ester) and (5) stearic acid (octadecanoic acid).

Discussion

Methanolic extract of *Anogeissus leiocarpus* leaf was produced and further subjected to exhaustive sequential extraction along polarity gradient of solvents using n-hexane, ethylacetate, n-butanol, methanol and water.

The n-butanolic fraction of the plant extract was subjected to column chromatography and eleven fractions were obtained for the plant under study. These fractions were subjected to antibacterial screening and the results recorded in appendix 3. The results showed that fraction 7 of *A.leiocarpus* had the highest antibacterial activity. This fraction was then subjected to thin layer chromatography. The single spot obtained (Appendix 1) was used for GC- MS analysis.

GCMS chromatogram of the n-butanolic leaf extract of *A.leiocarpus* (Appendix 2) showed eleven peaks indicating the presence of eleven compounds. Identification was based on the molecular structure, molecular mass and calculated fragments. The spectrum of the unknown component was compared with the spectrum of the component stored in the database of National Institute Standard and Technology (NIST). The name, molecular weight and structure of the components of the test materials were ascertained. The relative percentage amount of each component was given. The GCMS was done in order to determine whether this plant species contain any individual compound or group of compounds, which

may substantiate its traditional use as an herbal medicine in the treatment of bacterial infections. The chemical constituents present in herbs are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body than synthetic drugs. Seven compounds matched 100% with those from the NIST library (Table 2). Five of these compounds are found to be fatty acids or their derivatives. Among these are (1) palmitic acid methyl ester (hexadecanoic acid methyl ester) (2) palmitic acid (n-hexadecanoic acid) (3) oleic acid (9-octadecenoic acid) (4) Oleic acid methyl ester (9-Octadecenoic acid methyl ester) and (5) stearic acid (octadecanoic acid). Fatty acids (FAs) are ubiquitous molecules typically found bound to other compounds such as glycerol, sugars or phosphate head groups to form lipids. FAs can be released from lipids, typically by enzyme action, to become free fatty acids (FFAs), which have diverse and potent biological activities. Fatty acids are the primary constituents of edible oils and medicinal herbs and are reported to possess the ability to interfere with bacterial growth and survival [17]. Many fatty acids are known to have antibacterial and antifungal properties [18]. Palmitic acid, stearic acid, oleic acid and many other fatty acids have been reported to have potential antibacterial and antifungal activities [19]. Indeed, FFAs are often identified as the active ingredients in ethnic and herbal medicines [20,21]. Hexadecanoic acid methyl ester is an antioxidant, flavor and hypocholesterolemic [22]. n-Hexadecanoic acid (palmitic acid) is the most common saturated fatty acid that occur in nature, it is an antioxidant, hypocholesterolemic, nematicide and pesticide. Stearic acid is a saturated fatty acid, it is the most common saturated fatty acid that occur in nature following palmitic acid. Stearic acid has been reported to have antimicrobial activities [21,23]. Oleic acid is a long chain monounsaturated acid and a potent antibacterial [24,25]. Antimicrobial activity of fatty acids was stated to be dependent on chain length and unsaturation degree [17,18]. Long-chain unsaturated fatty acids exhibit inhibitory activity against many bacteria [26]. Examples of long chain unsaturated fatty acids are linoleic and oleic acids, and are reported as potent antibacterials [24,25]. Some studies have been undertaken to understand the mechanism of antimicrobial action of fatty acids and it was concluded that fatty acids and their esters exhibited non-specific modes of action. Davidson[27] and Lunde [28] stated that while the antibacterial mechanisms of fatty acids and their esters may be unknown, these compounds resemble the bipolar membrane of the bacterial cell wall in having both a hydrophilic and hydrophobic tail. This similarity suggests that the fatty acids could possibly target bacterial and fungal cell walls thus killing them by penetrating and disrupting normal function of the cell wall. Zheng[25] in their work found that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, which has served as a promising target for antibacterial drugs. The bacterial fatty acid synthesis is carried out by a set of individual enzymes in conjunction with acyl carrier protein (ACP)-associated substrates. FabI catalyzes the final, rate-limiting step of the chain elongation process in bacterial fatty acid synthesis; it has been validated as an excellent target for antibacterial drug development [29]. Linoleic acid is a model compound of unsaturated fatty acids which selectively inhibits FabI of *S. aureus* and *E. coli*. [30]. Other long-chain unsaturated fatty acids also inhibit FabI, whereas long-chain saturated fatty acids were not active. In the antibacterial assay using whole cells, unsaturated fatty acids showed greater inhibition than saturated fatty acids, which is consistent with the results seen by several other investigators [31,32]. In addition, two phthalates namely1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester also known as phthalic acid and 1,2-Benzenedicarboxylic acid, dioctyl ester also called Di-n-octyl phthalate were identified in the chromatogram. These are aromatic dicarboxylic acids and their ester. Dibutyl phthalate (DBP) is a bioactive ester. It has antimicrobial activity against gram positive and gram negative bacteria and also against unicellular and filamentous fungi[33]. DBP is however an enzyme endocrine disruptor with estrogenic activity [34]. The GCMS chromatogram of anogeissus leiocarpus leaf extract with high percentage concentration of fatty acids and their derivatives which have been established by many scientific researches as potent antimicrobials may validate its use as a herbal treatment for many microbial diseases.

Summary Of Findings

The methanolic crude extract of *Anogeissus leiocarpus* leaf is rich in many phytochemical compounds and have antibacterial efficacies. The n-butanolic fraction of these plants' extracts have more antibacterial efficacy than the other solvents used for extraction in this study, suggesting that this solvent extracted more of the antibacterial bioactive agents.
The bioactive agent of this plant extract are phthalates, fatty acids and their esters as seen in the GC-MS analysis.

Conclusion

This study has validated the ancient uses of this plant materials in the treatment of many diseases especially bacterial infections in Nigeria. The use of this plant as a herbal medicine should be encouraged.

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.

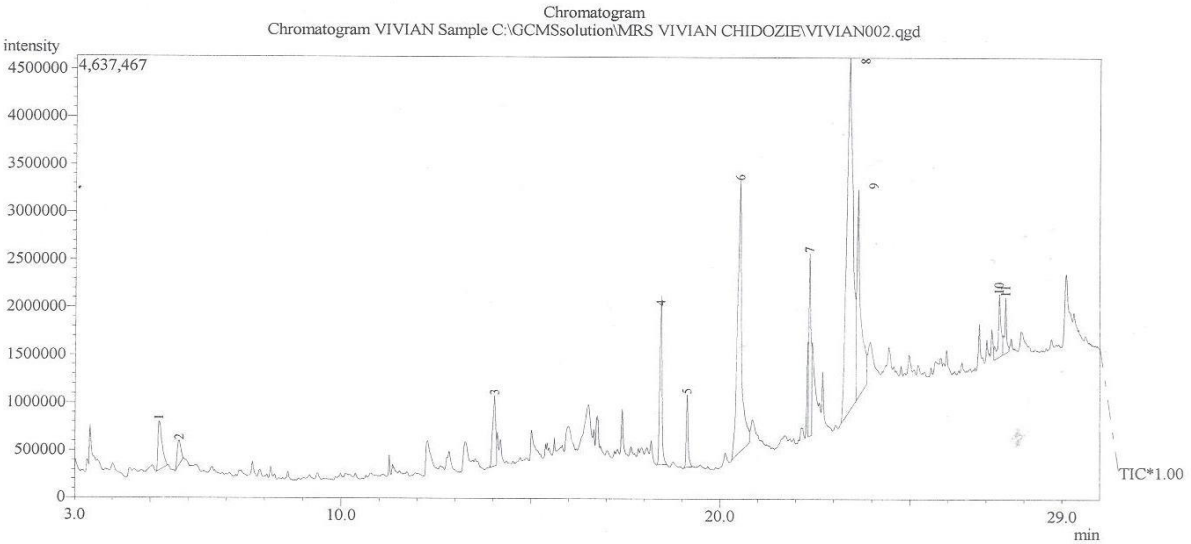
APPENDIX

Appendix 1 Picture of TLC

GCMS-QP2010 PLUS
SHIMADZU,JAPAN

NARICT, ZARIA
GCMS ANALYSIS

MRS VIVIAN CHIDOZIE (SAMPLE - K1)



Method

[Comment]

Analytical Line 1

[AOC-20i]

# of Rinses with Presolvent	:4
# of Rinses with Solvent(post)	:4
# of Rinses with Sample	:3
Plunger Speed(Suction)	:High
Viscosity Comp. Time	:0.2 sec
Plunger Speed(Injection)	:High
Syringe Insertion Speed	:High
Injection Mode	:Normal
Pumping Times	:5
Inj. Port Dwell Time	:0.3 sec
Terminal Air Gap	:No
Plunger Washing Speed	:High
Washing Volume	:8uL
Syringe Suction Position	:0.0 mm
Syringe Injection Position	:0.0 mm
Use 3 Solvent Vial	:1 vial

[GC-2010]

Column Oven Temp.	:80.0 °C
Injection Temp.	:250.00 °C
Injection Mode	:Split
Flow Control Mode	:Linear Velocity
Pressure	:108.0 kPa
Total Flow	:6.2 mL/min
Column Flow	:1.58 mL/min
Linear Velocity	:46.3 cm/sec
Purge Flow	:3.0 mL/min
Split Ratio	:1.0
High Pressure Injection	:OFF
Carrier Gas Saver	:OFF
Splitter Hold	:OFF

Oven Temp. Program

Rate	Temperature(°C)	Hold Time(min)
-	80.0	2.00
9.00	200.0	4.00
10.00	280.0	5.00

APPENDIX 2: GCMS Analysis of the TLC Spot of Anogeissus leiocarpus Fraction 7