GC-MS Determination of Bioactive Constituents of the methanolic fractions of *Cnidoscolusaconitifolius*

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ABSTRACT

Background:

Diabetes mellitus is a major metabolic disorder affecting a huge population all over the world. *Cnidoscolus* species have been extensively used for the management of diabetes in folkloric medicine. The presence of diverse secondary metabolites has been reported from species of the genus*Cnidoscolus*. However, there has not been much information available on phytochemical components and biological activity in the leaf methanol extract of *Cnidoscolusaconitifolius*

Objective:

This study was designed to extract and identify some bioactive compounds in the leaf methanol fractions of *C. aconitifolius*which may provide insight on its pharmacological properties and its use in traditional medicine.

Place and Duration of Study:

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National Cereals Research Institute Zaria, Nigeria between June 2012 and July 2013

Methodology:

Twenty grams of the powdered sample were subjected to column chromatography over silica gel (60-120 mesh) and eluted with 100ml each of n-hexane, petroleum ether, chloroform, methanol and respectively at the rate of 1mL/min. The eluates were concentrated and labeled as F_I, F_2, F_3 and F_4.The percentage yields of the fractions were 7.55(%),6.00(%), 15.5(%), and 65.00(%)(w/w) respectively. n-hexane and petroleum etherdid not elute much of the compounds. The active methanol fraction of *C. aconitifolius*extract(F_4) which showed the highest hypoglycaemic effect as identified by Oral Glucose Tolerance Test (OGTT) in rats wastaken for Gas Chromatography Mass Spectroscopy (GC-MS) analysis for separation of the bio-active components. GC-MS analysis was performed using a GC-MS (Model: QP2010 PLUS SHIMADZU, JAPAN) comprising a AOC-20i auto-sampler and gas-chromatograph interfaced to a mass spectrometer.

Results:

The ~~biologically active~~organic components of the GC-MS analysis provided peaks of six different phytochemical compounds, with their retention time (RT) and peak area (PA) in addition to minor constituents. The major compounds aredodecanoic acid-1, 2, 3-propanetriyl ester (RT:25.74, PA: 51.18%), cyclotetradecane (RT: 23.39, PA:15.59%), eicosanoic acid (RT:20.61, PA:18.47%); octadecanoic acid (RT: 16.82, PA:1.21%), 4-nitrosophenyl-beta-phenyl propionate (RT: 11.53, PA: 4.38%), benzene acetic acid, phenyl malonic acid and 3-oxo-4-phenylbutyronitrile (RT:10.34, PA: 9.17%). The presence of these compounds in the plant extract may at least be responsible for one of the pharmacological properties of *C. aconitifolius*and thus recommended as plant of phyto-

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pharmaceutical importance

KEYWORDS:*Phytochemials, Bioactive compounds, GC-MS analysis, Cnidoscolusaconitifolius*

1. **INTRODUCTION**

Use of plants as a source of medicine has been inherited and is animportant component of the health care system. Herbal medicines derived from plant extracts are being utilized increasingly to treat a wide variety of clinical diseases, although relatively little is known regarding their modes of action. Studies have shown that commonly consumed medicinal plants are good sources of polyphenols, saponins, flavonoids and phenyl propanoids(1).These compoundsdisplay a vast variety of pharmacological activitiessuch as anti-inflammatory, anticancer, anticarcinogenic,antibacterial, antioxidant, antifungal, antiviral activities etc. In 2002, World Health Organization (WHO) estimated that more than 80% of the world's population depends on traditional herbalmedicine for the treatment of different ailments (2).

Diabetes mellitus is one of the most severe and incurable metabolic disorders characterized by increased blood glucose level as a result of an absolute or relative lack of insulin and failure of insulin to act on its targets tissue (3).According to the World Health Organization (WHO), almost 70% of the diabetic patients use plants as a primary source of antidiabetic agents in order to satisfy their principal health needs (4).

. With the increasing demands for herbal medicinal products in healthcare all over the world, medicinal plant extract manufacturers have started using the most appropriate extraction technologies in order to identify and isolate the chemical entities present in them.The purpose of identification of phytochemicals in plants is to attain the therapeutically desired active portion and to eliminate unwanted materials (5). A special feature of higher plants istheir capacity to produce a large number of organic chemicalsof

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high structural density called secondary metabolites. A knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agent, but also for disclosing new sources of economic phyto-compounds for the synthesis of complex chemical substances and for discovering the actual significance of folklorics.

C. aconitifolius (Miller) of family Euphorbiaceae is commonly referred to as *' Chaya', ' Tree spinach'* in Mexico, *' Efo-Iyanaipaja'* or *'* E*fo-Jerusalem'* in southwest Nigeria and *' Hospital Too Far'* in eastern part of Nigeria. (6). It is an ornamental evergreen drought deciduous shrub of about 5m tall with 32cm long and 30cm wide palmate leaves alternately arranged (7). The leaves are commonly eaten as vegetable, serve as blood builder (8) and possess most essential amino acids thus, making the leavea potential panacea for kwashiorkor and other related protein-deficiency diseases (9). A wide variety of the folkloric use of this herb in ethno medicine includes treatment for alcoholism, diabetes, kidney stone, insomnia, gout, scorpion stings and as cure for brain and vision improvements (10). Meanwhile it has been utilized extensively as a major component for the treatment of noninsulin-dependent diabetes mellitus. It has also been reported recently that the use resulted in a satisfactory hypoglycemic effect in diabetic animal models(11). Basic research involving animal models have shown that this herb attenuates renal dysfunction caused by ethanol toxicity, and also exhibits insulinogenic property in inbred type-2 diabetic mice (12). It also reported toelicit hepatoprotective activity in rats intoxicated with mega dose of paracetamol (13). The intrinsic potency of medicinal plantsis attributable to the chemical constituents present. Evaluating the biological potency provides a direct assessment of its pharmacological quality.

In order to validate the pharmacological properties of this plant, there is need to identify the chemical components and bioactive principles present. Thereforethe present study was aimed at identification of the phytochemical constituents present in the methanol fraction of *Cnidoscolusaconitifolius*leaves using GC-MS analysis.

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2. MATERIALS AND METHODS

2.1 Collection and identification of Plant Material

Fresh leaves of *Cnidoscolusaconitifolius* (CA) were collected with hand in glove from Amaekpu in Ohafia Local Government Area of Abia State, Nigeria, in the morning hours between the month of October and December 2012. Samples were identified and authenticated by Mr. IbeNdukwe, a taxonomist in the Herbarium Section of the Department of Forestry and Environmental Management, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria. A voucher specimen was kept at the herbarium in the same Department (Specimen No. FHI-107727).

2.1.1 Preparation of plant material

The leaves of *C. aconitifolius*were sorted, washed thoroughly with distilled water to remove dirt and debris, cut into smaller pieces before it was shade dried for 3 weeks at room temperature (28 ± 3^0C).. The dried leaves were pulverized into fine powder using electric blender (CORONA-REF. 121, Landers and Qlink blender, Model No. OBL-15L40). The powdered materials were stored in air tight polyethene bags protected from direct sunlight until required for use.

2.1.2 Plant Sample Extraction

One hundred grams of the powdered leaves was extracted with 500mL of 40% ethanol overnight in a stopped bottle and with occasional stirring at room temperature (28± 3^0C). The sample was first sieved using muslin cloth and then filtered using Whatman No.1 filter paper. This process was repeated three times. The filtrate was concentrated under reduced pressure at 40^0C for 45min in a rotary vacuum evaporator, and then lyophilized to get a brown aromatic solid extract (ethanol extract of CA). The yield of the extract was expressed in terms of the percentage of the dry weight of initial plant material used (yield 35.37% w/w). The dry extract obtained was kept in a refrigerator at 4^oC until required for use.

2.1.3 Column Fractionation of Ethanol extract

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The dry crude extract was subjected to column chromatographyaccording to standard methods(14,15). The sample for the column was prepared by adsorbing 20g of the ethanol extract of *C. aconitifolius*with 60g of silica gel G (60-120 mesh). The mixture was air dried and carefully layered on top of the packed silica gel in the column (14cm length) using a glass funnel. The extract in the column was eluted with 100ml each of petroleum ether, chloroform, methanol and n-hexane respectively at the rate of 1mL/min. The eluates were concentrated and labeled as F_1, F_2, F_3 and F_4. The percentage yields of the fractions were 7.55%, 6.00%, 15.5%, and 65.00% (w/w) respectively. Petroleum ether and hexane did not elute much of the compounds. The methanol fraction of *C. aconitifolius*leaves which showed the highest hypoglycaemic effect as identified by Oral Glucose Tolerance Test (OGTT) in rats was further analyzed by Gas Chromatography-Mass Spectrometry (GC-MS).

2.1.4 Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis was carried out on a GC-MS (Model: QP2010 PLUS Shimadzu, Japan) comprising a AOC-20i auto-sampler and gas-chromatograph interfaced to a mass spectrometer (GC-MS) The instrument is equipped with a VF 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 µm film thickness. The temperatures employed were; column oven temperature 80^{o}C, Injection Temp 250^{0}C at a pressure of 108.0kPa, with total flow and column flow of 6.20 ml/min and 1.58ml/min respectively. The linear velocity was 46.3 cm/sec and a purge flow of 3.0mL/min. The GC program ion source and interface temperature were 200.00^{0}C and 250.00^{0}C respectively with solvent cut time of 2.50min. The MS program starting time was 3.00min which ended at 30.00min. with event time of 0.50sec, scan speed of 1666 µL/sec, scan range 40-800u and an injection volume of 1 µL of the plant extract (split ratio 10:1). The total running time of GC-MS was 30 min. The relative percentage of the extract was expressed as percentage with peak area normalization.

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2.1.5 Identification of phytocompounds

Interpretation on the mass spectra was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The fragmentation pattern spectra of the unknown components were compared with those of known components stored in the NIST library (NIST Ver. 2.0 of 2005). The compound bioactivity prediction is based on Dr. Duke‟s Phytochemical and Ethnobotanical Databases (Dr. Duke Database, 2014 http:www.ars-grin.gov/cgi-bin/duke/ethnobot..(16).The relative percentage amount of each phyto-component was calculated by comparing its average peak area to the total area. The name, molecular weight and structure of the components of the test materials were ascertained.

3. RESULTS AND DISCUSSION

Chromatographic purification of the ethanol extract of the leaves of *C. aconitifolius*with different solventsproduced four fractions (F_1-F_4) with the methanol fraction producing the highest yield and hypoglycaemic effect as observed by oral glucose tolerance test in rats. This may suggest higher proportion of the polar plant component were extracted in the methanol fraction.The compounds present in the methanol extract of leaves of *C. aconitifolius*were identified by GC-MS analysis as shown in Fig.1.

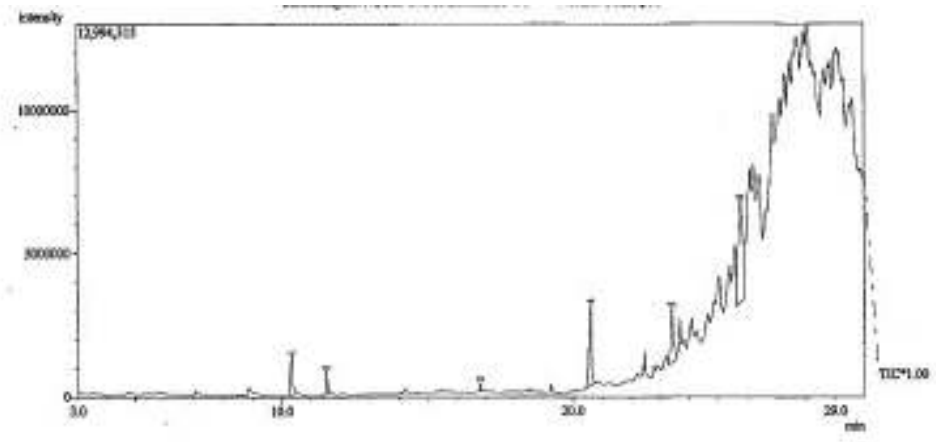

Fig. 1: GC-MS Chromatogram of methanol fraction of *Cnidoscolusaconitifolius*leaves

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The active principle, area of peak concentration (%), retention time ((RT) molecular weight (MW), and molecular formula (MF) in the methanol extract as identified through the NIST database are listed in Table 1.

Table 1: Phyto-components identified in the methanol extract of *Cnidoscolusaconitifolius*by GC-MS analysis

Peak No.	Retention time(s) (RT)	Name of Compound (MF)	Molecular Formula (MW)	Molecular Weight (g/mol)	Peak Area (%)
1	1249	Phenylmalonic acid	$C_9H_8O_4$	180	9.17
	1641	Benzene acetic acid	$C_8H_8O_2$	136	9.17
	1473	3-Oxo-4-phenylbutyronitrile	$C_{10}H_9NO$	159	9.17
	718	Spiro (2, 4) hepta-4, 6-diene	C_7H_8	92	9.17
2.	000	4 Nitrosophenyl-β-phenyl propionate	$C_{15}H_{13}NO_3$	255	4.38
	1349	Benzene propanoic acid	$C_9H_{10}O_2$	150	4.38
	1394	3-Phenyl-Propionic acid	$C_{12}H_{16}O_2$	192	4.38
	31769	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.21
	2167	Octadecanoic acid	$C_{18}H_{36}O_2$	284	1.21
	1968	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.21
4.	2366	Eicosanoic acid	$C_{20}H_{40}O_2$	312	18.47
	1869	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	18.47
5.	1679	Cyclo-tetradecane	$C_{14}H_{28}$	196	15.59
	18185	5-Octadecene	$C_{18}H_{36}$	252	15.59
	1620	7-Hexadecene	$C_{16}H_{32}$	224	15.59
	2017	5-Eicosene	$C_{20}H_{40}$	280	15.59
	64336	Do-decanoicacid-1, 2, 3-propane-triyl ester	$C_{39}H_{74}O_6$	638	51.18
	3218	Do-decanoicacid, 1-hydroxy Methyl-1, 2-diyl ester	$C_{27}H_{52}O_5$	456	51.18
	1570	Do-decanoicacid, ethenyl ester	$C_{14}H_{26}O_2$	226	51.18

The main component of the methanol extract of the leaves wasrecognized as the metabolitesresponsible for its antidiuretic effect. Theorganic compounds in methanol extract of the leaves whichwere identified through their fragmentation patterns include 4-dodecanoic acid-1, 2 3- propanetriyl ester (RT: 25.744, PA: 51.18%),

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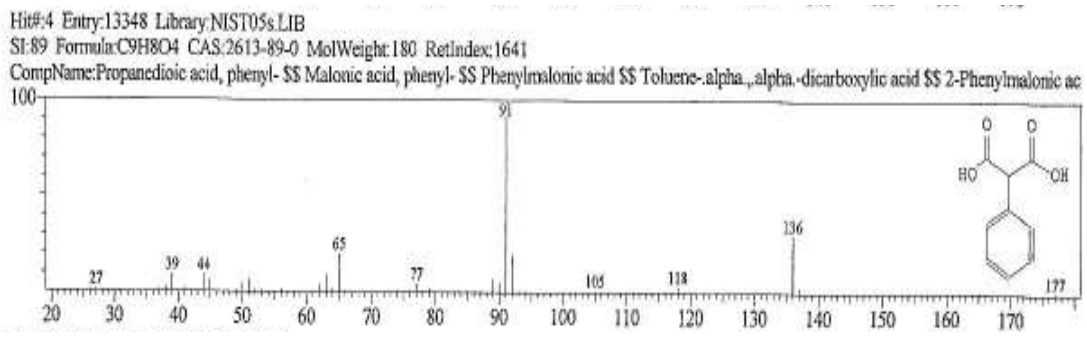
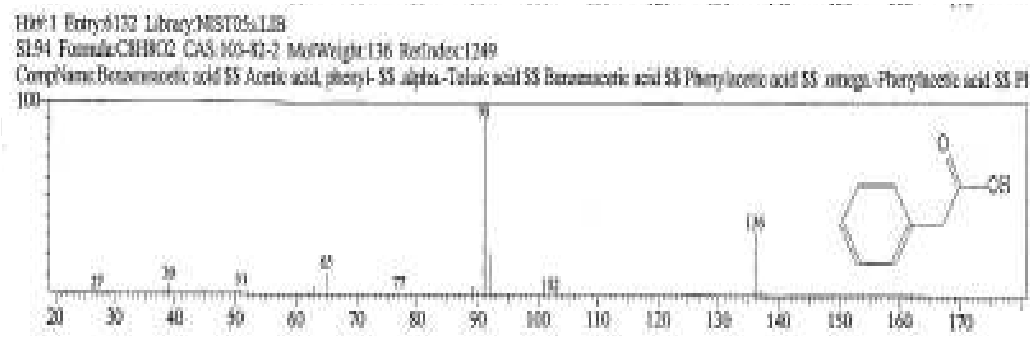
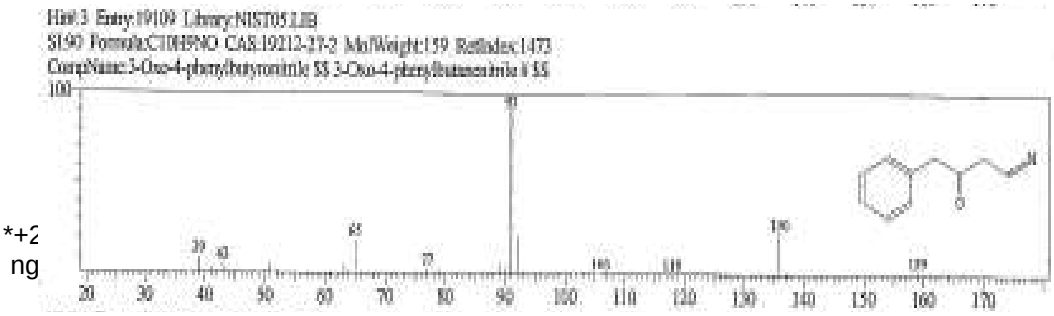
cyclotetradecane(RT: 23.39, PA: 15.59%), eicosanoic acid (RT: 20.61, PA: 18.47%) and octadecanoic acid (RT: 16.82, PA: 1.21%). Others are 4-Nitrosophenyl-beta-phenyl propionate (RT: 11.53, PA: 4.38%), Benzene acetic acid,phenylmalonic acid and 3-Oxo-4-phenylbutyronitrile (RT: 10.34, PA: 9.17). dodecanoic acid-1, 2, 3- propanetriyl ester have high retention time (25.74min) and molecular weight (638), while benzene acetic acid is of low molecular weight (136) and retention time (10.34 min). A similar trend of the presence of fatty acids in extracts of dichloromethane and hexane of*C.aconitifolius*has been reported(17), particularly in studies correlatingthe comparative analysis of the nature of the solvent of extraction and chemical composition of the leaves(18)..The mass spectrum of the individual components are shown in Figure 2 (a-h)



a. Phenylmalonic acid



b.



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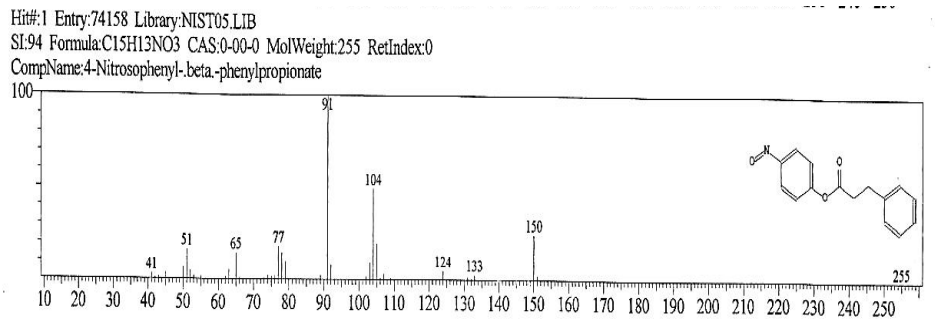
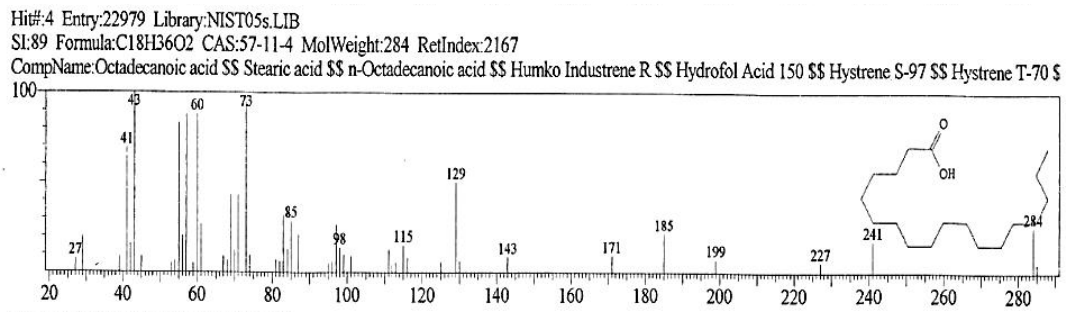
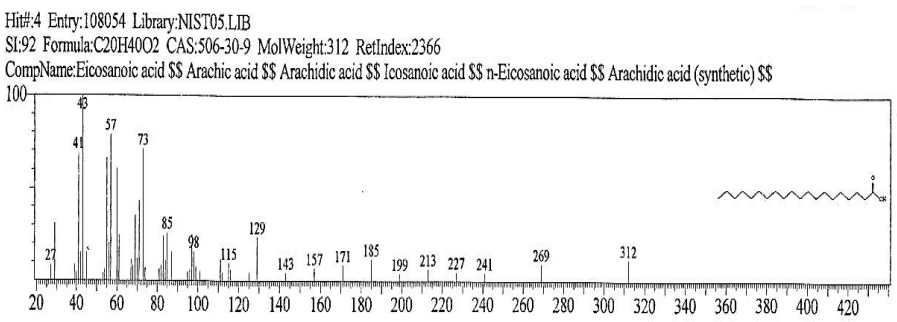
c.3-Oxo-4-phenylbutyronitrile



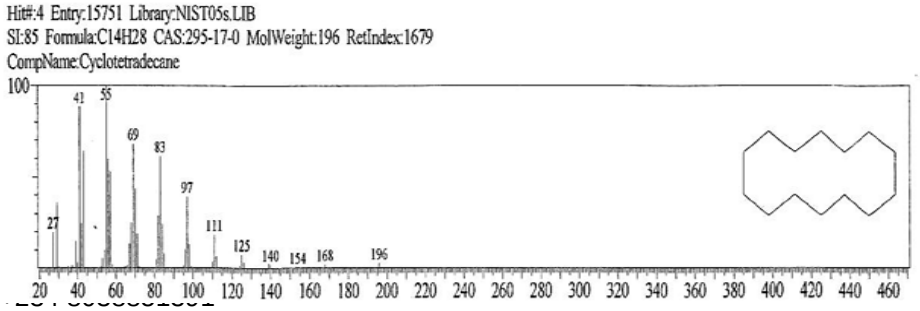
d..4 Nitrosophenyl-β-phenyl propionate



e.Octadecanoic acid



f.Eicosanoic acid



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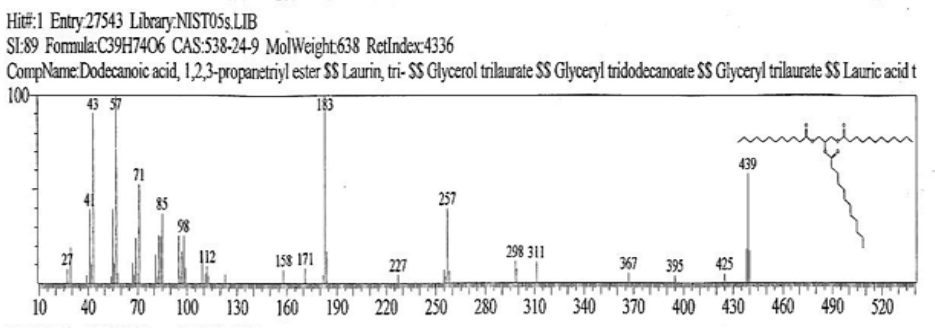
g.Cyclo-tetradecane



h.Do-decanoicacid-1,2,3-propane-triyl ester

The peaks retention time ranged from 10.33 -25.00 while the peak percentage area ranged from 9.17–15.80.The compounds prediction and biological activities is based on Dr Duke'Phytochemical and ethno botanical databases as tabulated in Table 2.

Table 2: Biological activities of some active principles present in methanol fraction of *Cnidoscolusaconitifolius* (20

Phyto-components	**Nature of compound**	**Activities (16)**
Tetradecanoic acid	Fatty acid	Antioxidant, cancer preventive, nematocide, hypercholesterolemic, lubricant
Do-decanoic acid,-1,2,3 –propane -triyl ester	Fatty acid	Hypercholesterolemic, antiarthritic, nematocide, hepatoprtective,
Octadecanoic acid	Fatty acid	b5-α reductase inhibitor, Cosmetic, Flavour, Hypocholesterolemic, Lubricant, Perfumery, Propecic and Suppository.
Eicosanoic acid	Fatty acid	Antinflamtory, anti- therogenic
9-Octadecenoic acid	Fatty acid	Anti-inflammatory, Anti-alopecic, Anemiagenic, 5-α reductase inhibitor, α-reductase inhibitor lubricant, Antitumour, Choleretic,

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		Dermatitigenic, Immunostimulant, Anti-leucotriene-D4, Antiandrogenic, Lipoxygenase inhibitor, Allergenic, Flavour, Hypocholesterolemic, Insectifuge, Irritant, Percutaneo-stimulant, Perfumery and Propecic
n-Hexadecanoic acid	Fatty acid	Anti-alopecic, Anti-androgenic, Antioxidant, Haemolytic, Hypercholesterolemic, Lubricant, Nematicide, Pesticide, Propecic, Flavour 5-α reductase inhibitor. Anti-inflammatory,
Dodecanoic acid -ethyl ester	Fatty acid ester	Hypercholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge, Antihistamine, Anti-eczemic, Anti-acne, 5-Alpha reductase inhibitor, Antiandrogenic, Anti-arthritic, Anti-coronary.
Hexadecanoic acid methyl ester	Fatty acid ester	Antioxidant, Hypercholesterolemic, Lubricant, Nematicide, Pesticide, Hemolytic 5-Alpha reductase inhibitor, Flavour, Antiandrogenic
5-Octadecene	Olefins	

In the present study, six major compounds have been identified from the ethanol extract of the leaves of *C.aconitifolius*by Gas Chromatography-Mass Spectrometry analysis. Authentication of medicinal plants at genetic and chemical level is a critical step in the use of these botanical materials. Gas Chromatography-Mass Spectrometry (GC-MS) is a valuable tool for reliable identification of phytocompounds (19).

The GC-MS analysisshowed a fragmentation pattern characteristic ofthe presence of fatty acids such as octadecanoic acid, tetradecanoicacid,and do-decanoic acid. These phytochemicals are known to have antimicrobial activity, antioxidant, hypercholesterolemic, cancer preventive and hepatoprotectiveactivity(20, 21). Similar compounds were observed in methanol fractions of C. *aconitifolius*with 9-octadecanoic acid as the prevailing compound (17).

In addition, the methanolic fraction of *C. aconitifolius* also showed the presence of phenolic compounds, saturated and unsaturated fatty acids including eicosanoic acid, a

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component of membrane and precursor of a group of hormones like prostaglandins, thromboxanes and prostacy-clins which are important in regulation of diverse physiological processes(22). The eicosanoids in the extract is reported to have antiinflammatoryproperties(23).

This action correlates with the active components isolated from this rhizome such as The study on the active principle of methanol fraction of *C. aconitifolius*revealed that the plant contains a wide range of phytochemicasl which may contribute to its therapeutical value. The bioactivities of these compounds may depend on the lipophilic properties of their functional groups. Many metabolites have been found to possess interesting biological activities and find applications such as pharmaceuticals, insecticides, dyes, flavors and fragrances (20).

4. CONCLUSION

From the present study, GC-MS analysis of the methanol fraction of *C. aconitifolius extracts* afforded active compounds which were easily distinguishable fatty acids that contribute to the therapeutic potential of the plant. This may justify its use as herbal therapy for the treatment of various diseases by traditional medical practitioners. It also suggests that further investigation on these phytochemicals will pave the way for the venture of cost effective drug with less side effect.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

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This study will provide additional insights on the use of traditional herbal medicine and to being able to develop new cure for some ailments like diabetes as one of the leading diseases causing numerous deaths worldwide.

Authors' contributions

This work was carried out in collaboration between both authors. Author NKA participated in all operations of this manuscript. Author NKA wrote the first draft of the manuscript. Authors oo designed the study and wrote the protocol performed. Author NKA managed the analyses of the study and managed the literature searches. Author OO revised the manuscript and Author NKA has the final responsible for all information presented. Both authors read and approved the final manuscript

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