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Chioma Nnennaya Chinaka
Department of Medicinal and Pharmaceutical Chemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

Kenneth Maduabuchi Ezealisiji
Department of Medicinal and Pharmaceutical Chemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

Rukevwe Elohor Akpofure
Department of Medicinal and Pharmaceutical Chemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

Correspondence
Chioma Nnennaya Chinaka
Department of Medicinal and Pharmaceutical Chemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

Phyto-chemical Characterization of the Leaf extracts of *Terminalia catappa* L. (Combretaceae) Using Ultra violet-Visible, Fourier transform infrared and Gas chromatography-Mass Spectroscopic techniques

Chioma Nnennaya Chinaka, Kenneth Maduabuchi Ezealisiji and Rukevwe Elohor Akpofure

Abstract
Although the African herbal medicine have achieved unequivocal success as an ethno medicine yet they suffer many setbacks in their commercial value and global acceptance as a result of poor regulation and standardization. This study aims to characterize the phyto- chemical constituents present in the n-hexane and dichloromethane leaf extracts of *Terminalia catappa* using chromatographic and spectroscopic methods. The crude n-hexane and dichloromethane extracts were analyzed using ultra-violet spectroscopy (UV-2500PC series), Fourier Transform Infrared spectroscopy (FTIR) model 8400S and GC-MS (model –QP 2010 plus Spec). The identification of compounds was done using NIST Ver. 2.0 Year 2005 library. The biological activity are based on Dr Duke's phytochemical and ethno-botanical databases. Peaks corresponding to chalcones, simple phenols, carotenoids and chlorophyll were observed in the UV-Visible spectroscopy. The FTIR revealed functional groups such as alkanes, alkenes, alcohols and ethers. The GC-MS revealed phyto-compounds such as n-Hexadecanoic acid, octadecanoic acid, and chloroacetic acid present in the dichloromethane extract and stearic acid, oleic acid, n-Heptadenoic acid, in the n-hexane extract. The presence of these phytochemicals especially long chain fatty acids and their percentage occurrence may provide proper identification and explain their usage in ethno-medicine.

Keywords: *Terminalia catappa*, GC-MS, FTIR, phytochemical, database, phyto-chemical characterization

Introduction
Herbal medicine have been recognized as a source of bioactive phytochemicals which can be used to treat and prevent various human health challenges whether chronic or acute diseases [1–3]. Evidence of its ethno medicinal benefits has led about 80% of the population in the developing countries to depend on herbal medicine for their primary health needs [4]. Furthermore, herbal medicine have been fully integrated as part of complementary and alternative medicine because of their efficacy, safety and affordability. The global acceptance of herbal medicine is very obvious as the chemical principles isolated from plants are compounded as drugs, nutraceuticals and health supplements which are marketed worldwide [5, 6]. These many successes have been achieved by the herbal medicine from Asian nations such as Indian Traditional medicine (Ayurveda, Sidha, and Unani) and Traditional Chinese medicine (TCM) which though ancient in origin have been well researched, standardized and are properly regulated [7, 8]. In contrast, the African herbal medicine have neither achieved high commercial value nor received much interest in the international market due to adulteration, lack of standardization and poor regulation of the plant products and medicine [8–10]. There is therefore a need to standardize, harmonize and regulate plant products and medicine of African traditional system [11–14] to enhance its acceptance globally and to build confidence in their usage as medicine.

Terminalia catappa L. (Combretaceae) popularly known as Indian almond or tropical almond, is used in folk medicine as vermifuge, antioxidant, hepatoprotective, anti-inflammatory, antimicrobial, antidiabetic, and anticancer [15–18]. It is widely distributed in tropical and subtropical regions especially in coastal areas where it serves primarily as shade and as an ornamental tree. Attempts to ascertain the quality and to authenticate the *T. catappa* leaves has been done using pharmacognostic (microscopic and macroscopic) methods [19] while chemical characterization of the stem bark [20] and that of the nut [21] has been done using chromatography hyphenated with spectroscopy (GC-MS).

In this present research, characterization and standardization of *T. catappa* leaves using instrumentation methods such as GC-MS and FTIR and UV spectroscopy was done to evaluate the phyto-chemical-components, assuring quality and thereby validating their ethno-medicinal usage.

Materials and Method

Chemicals

Dichloromethane, n-hexane, methanol were all of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA)

Plant Materials and Extraction

The fallen, ripe leaves of the *Terminalia catappa* (Combretaceae) tree were collected in July 2016 from the University of Port Harcourt, Choba, Rivers State, Nigeria, identified and authenticated by Mr. Osuala of the department of Pharmacognosy, University of Port Harcourt. A voucher specimen (UPA 118507) of the leaf sample was deposited in the herbarium of the department.

The dried leaves of *T. catappa* was milled to powder using Willey mill (Thomas Willy mills, Swedesboro, NJ, USA). 500g of the pulverized leaves was defatted by macerating with n-hexane (2L) for 72h, filtered and the marc obtained was air dried before macerating in dichloromethane (2L) for 72h and filtered. The different filtrates obtained were concentrated under vacuum and reduced pressure (BUCHI, Rotavapour R-205, BUCHI Labortechnik AG CH-9230, Flamil, Switzerland) at 40°C.

UV-VIS and FTIR spectroscopic analysis

10mg of the individual extract was diluted with 50ml of the corresponding solvent, centrifuged at 3000rpm for 10min and filtered using Whatmann No 4 filter paper using vacuum pump. A further 1: 10 dilution of the centrifuged solution was made with the solvent. The extract obtained was scanned in the wavelength ranging from 200-800nm using the UV-2500PC series Ver. 2.30 spectrophotometer and the characteristic peaks were noted. The diluted extract above was used in carrying out the FTIR analysis using FTIR-8400S spectrometer system. The characteristic peaks were also detected. The peak values of the UV-VIS and FTIR were recorded. The same process was carried out using the dichloromethane extract of the plant.

GC-MS analysis

Preparation of extract for GC-MS analysis

The different dried extracts (n-hexane and dichloromethane) were re-dissolved in their respective solvents, vortexed and filtered through 0.45µm syringe filter. 1µl aliquot solution of the sample was injected into the GC-MS equipment.

Instrumentation and Chromatographic conditions

GC-MS analysis was carried out on a GC-MS (Model: QP2010 plus Shimadzu, Tokyo, Japan) which comprises of AOC-20i auto-sampler and gas-chromatography hyphenated to a mass spectrometer (GC-MS) instrument equipped with a VF 5ms fused silica capillary column of 30m length, 0.25mm diameter and 0.25 µm film thickness. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was applied. The carrier gas was Helium (99.99%) used at a constant flow rate of 1.58 ml/min. The injector and mass transfer line temperature were set at 250 to 200 °C respectively, and an injection volume of 1µl was employed (split ratio 10:1). The oven temperature was programmed from 80 °C (isothermal for 1min), with an increase of 10 °C/min to 200°C for 4min, 10°C/min to 280° C ending with a 5min isothermal at 280 °C. The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 200 °C, solvent cut time, 2.5min, relative detector gain mode, scan speed 1666 µ/sec; scan range 40-800 µm, the interface temperature is 250 °C. The total running time of GC-MS was 30min. The relative percentage of the extract was expressed as percentage with peak area normalization.

Identification of component

The relative percentage amount of each phyto-component was calculated by comparing its average peak area to the total areas. The detection employed the NIST (National Institute of Standard and Technology) Ver. 2.0 Year 2005 library. The compound's biological activity prediction is based on Dr. Duke's phytochemical and Ethno-botanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA [22]. Interpretation of GC-MS was conducted using the database of NIST having more than 62,000 patterns. The spectrum of the unknown phyto-components was compared with the spectrum of the known components stored in the NIST library. The name and molecular weight of the phyto-components of the test materials were ascertained.

Results

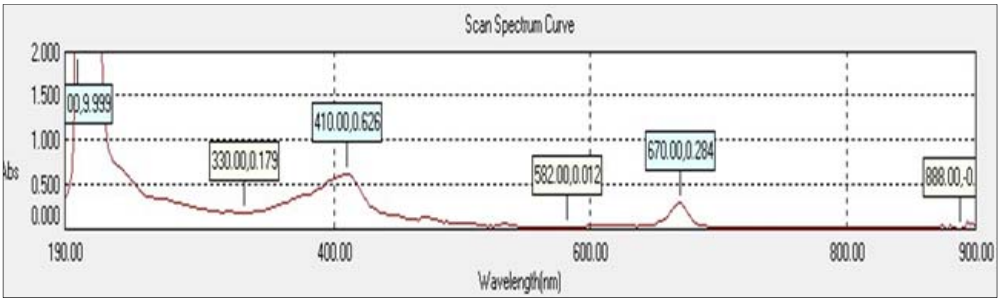

Fig 1: UV-VIS Spectrum of n-hexane leaf extracts of Terminala catappa

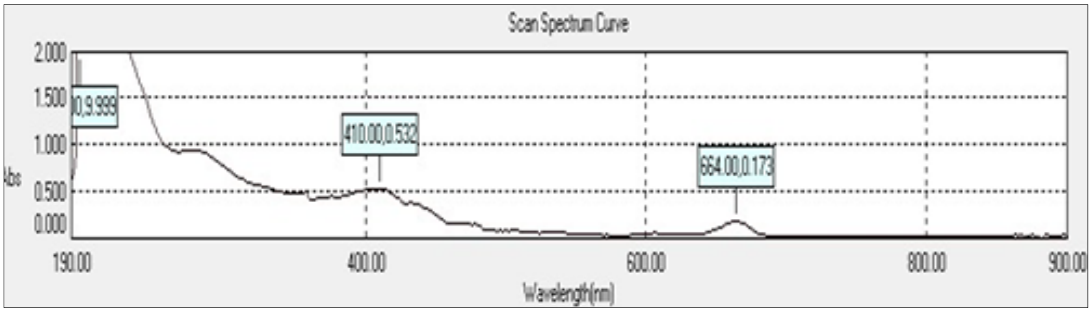

Fig 2: UV-VIS Spectrum of dichloromethane extract of *Terminala catappa*

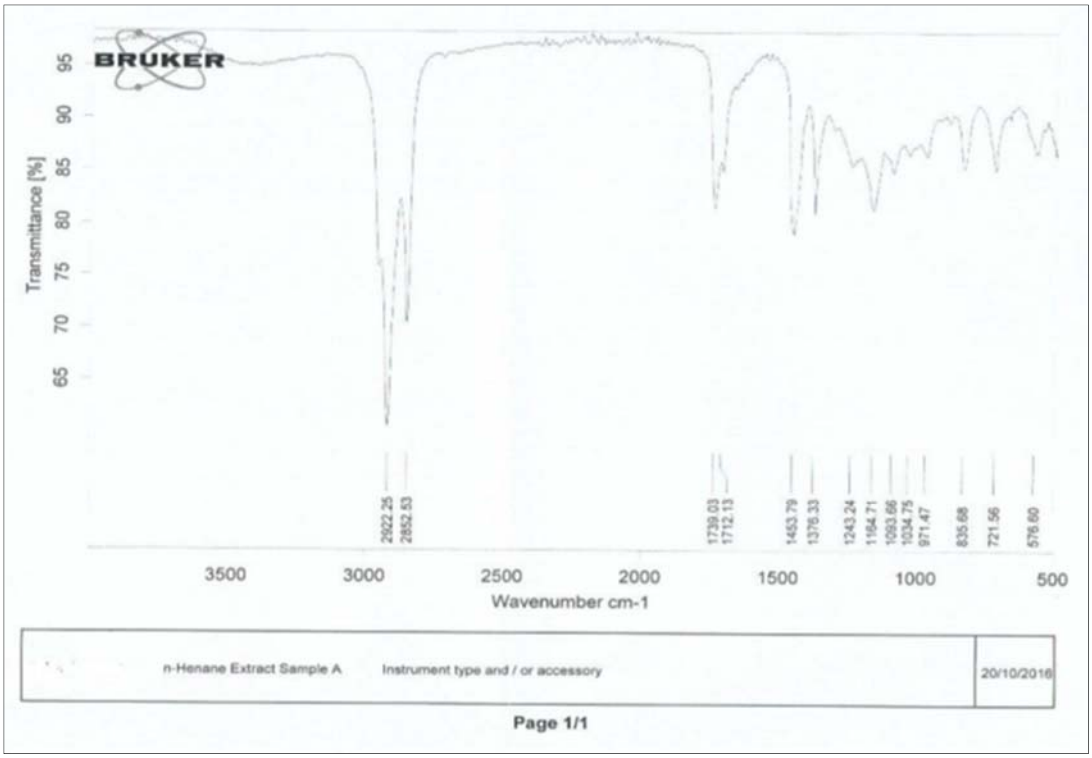

Fig 3: FTIR spectrum of the n-hexane extract of Terminalia catappa

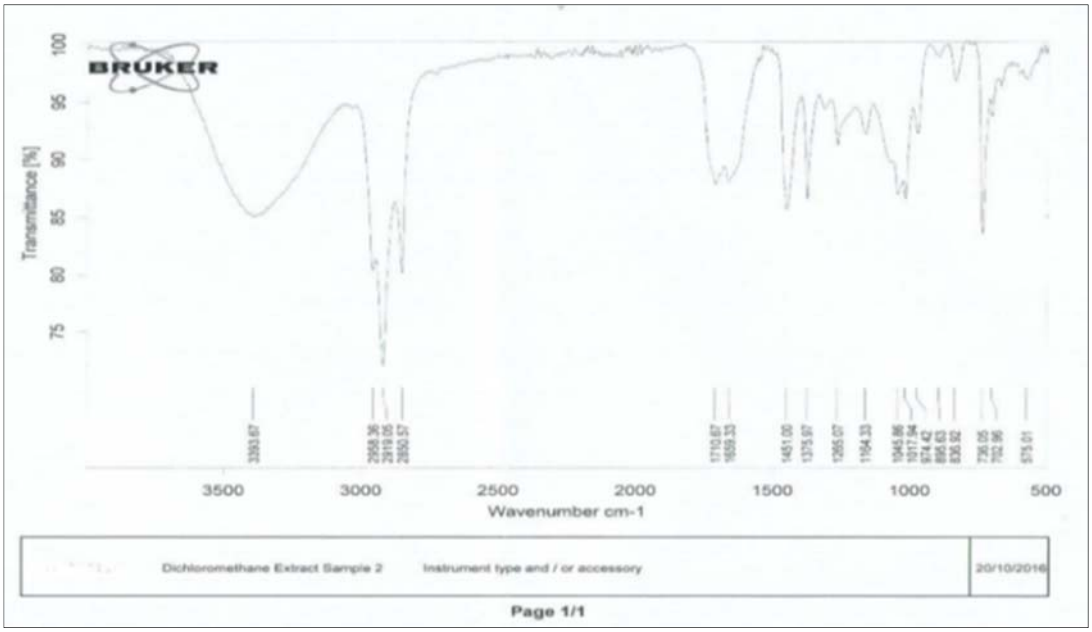

Fig 4: FTIR Spectrum of dichloromethane extract of *Terminala catappa*

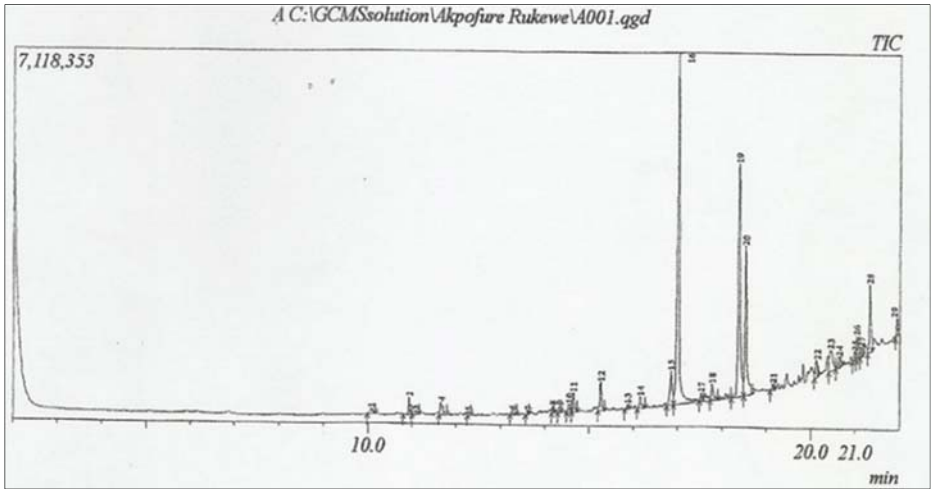

Fig 5: Gas Chromatograph of n-hexane extract of *Terminalia catappa*

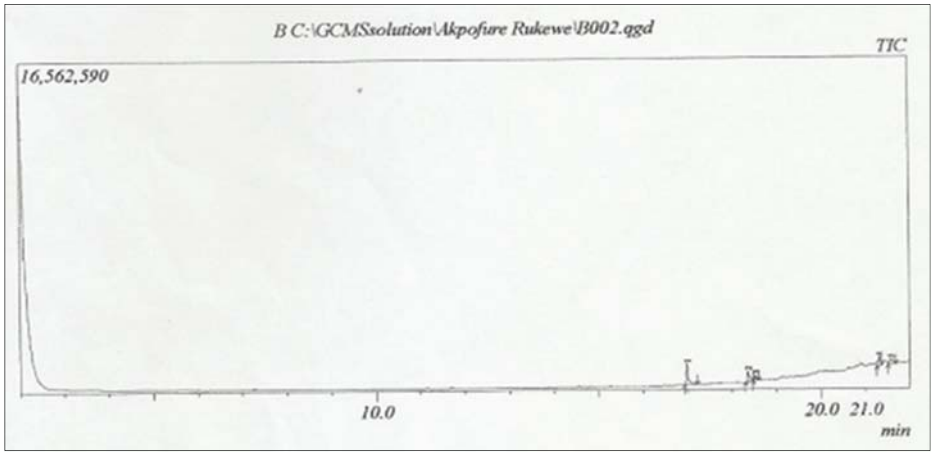

Fig 6: Gas Chromatograph of dichloromethane extracts of *Terminalia catappa*

Table 1: Percentage yield of the n-hexane and dichloromethane extracts from 500g of *T. catappa*

Type of extract	n- hexane extract	Dichloromethane extract
% yield	2.56	1.87

Table 2: FTIR peak values and Functional groups of the extracts of different of *T. catappa*

n-Hexane		Dichloromethane	
Peak Values (cm^{-1})	Functional group	Peak Values (cm^{-1})	Functional group
2922.25	C-H stretching	3393.67	O-H (broad and strong)
2852.53	C-H Terminal of an alkyl or alkene	2958.36	C-H, terminal of an alkyl or alkene
1453.79	C-H bending	2850.57	C-H stretching
1243.24	C-O stretching	1659.33	C=C stretching
835.68	=C-H bending	1164.33	C-O stretching
721.56	C-H rocking		

Table 3: Phyto-components identified in the n-Hexane extract of the leaves of *T. catappa* by GC-MS

S/N	Retention time (min)	Name of Compound	Molecular Formula	Molecular Weight ($gmol^{-1}$)	Peak area (%)
1	10.08	1,8- Nonadien-3-ol	$C_9H_{16}O$	140	0.73
2	10.93	Alpha bergamotene	$C_{15}H_{24}$	204	0.96
3	11.65	Chloroacetic acid octyl ester	$C_{10}H_{19}ClO_2$	206	2.52
4	12.27	1,3-Diformal-1-rhamnitol acetate	$C_{10}H_{12}O_8$	260	0.24
5	13.26	Decanynoic acid	$C_{10}H_{16}O_2$	168	0.41
6	13.69	Pipridine	$C_7H_{12}ClNO$	161	0.28
7	14.03	Tetrahydrofuran-2-one, 3-[1 fluoroethyl]-5{[2] hydroxypropyl] benzeneethyyl	$C_{17}H_{23}FO_3$	294	1.45
8	14.23	Phthalofyne	$C_{14}H_{14}O_4$	246	0.78
9	14.65	Benzenesulfonamide	$C_6H_7NO_2S$	157	1.60
10	15.26	Tetradecanoic acid (Myristic acid)	$C_{14}H_{28}O_2$	228	2.25

11	15.87	Chloroacetic acid, decyl ester	$C_{12}H_{23}ClO_2$	234	6.95
12	16.15	n-Pentadecanoic acid	$C_{15}H_{30}O_2$	242	1.02
13	16.85	cis-9- Hexadecanoic acid (Palmitoleic acid)	$C_{16}H_{30}O_2$	254	3.66
14	17.01	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256	35.63
15	17.53	Chloroacetic acid, 4-Pentadecyl ester	$C_{17}H_{33}ClO_2$	304	0.52
16	17.77	n-Heptadecanoic acid (Margaric acid)	$C_{17}H_{34}O_2$	270	1.45
17	18.38	Oleic acid	$C_{18}H_{34}O_2$	282	21.14
18	18.53	Stearic acid	$C_{18}H_{36}O_2$	284	11.44
19	20.44	1,2-Dipalmitin	$C_{35}H_{68}O_5$	568	3.18
20	20.65	Chloroacetic acid 3-tridecyl ester	$C_{15}H_{29}ClO_2$	276	0.97
21	20.84	Adipic acid, beta-citronellyl heptyl ester	$C_{23}H_{42}O_4$	384	1.88
22	20.99	Cyclohexanecarboxylic acid	$C_{18}H_{32}O_2$	280	0.55

Table 4: Phyto-components identified in the dichloromethane extract of the leaves of T. catappa by GC-MS

S/N	Retention time (min)	Name of Compound	Molecular Formula	Molecular Weight ($gmol^{-1}$)	Peak area (%)
1	16.98	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	35.18
2	18.35	6-Chlorododecanoic acid	$C_{13}H_{24}ClO_2$	282	20.36
3	18.51	Octadecanoic acid	$C_{18}H_{36}O_2$	284	8.32
4	21.30	Glycerol-1-palmitate	$C_{19}H_{38}O_4$	330	18.51
5	21.57	Chloroacetic acid	$C_{18}H_{35}ClO_2$	318	17.63

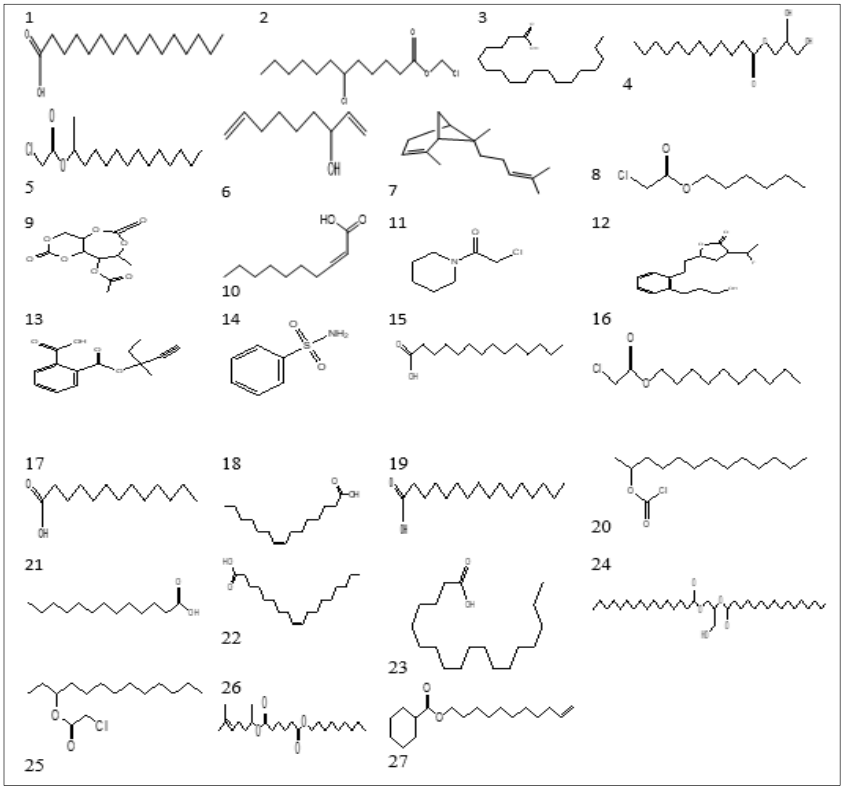

Fig 7: Phyto-components identified in n-hexane and dichloromethane extracts of T. catappa leaves. 1: n-Hexadecanoic acid; 2: Chloromethyl-6-chlorododecanoate; 3: Octadecanoic acid (Stearic acid); 4: Glycerol -1-palmitate; 5: Chloroacetic acid, 4-hexadecyl ester; 6: 1,8-Nondien-3-ol; 7: alpha-Bergamotene; 8: Chloroacetic acid, octyl ester; 9: 1,3:2,5-Diformal-1-rhamnitol acetate; 10: Decanynoic acid; 11: Piperdine; 12: Tetrahydrofuranone; 13: Phthalofyne acid; 14: Benzenesulfonamide; 15: Tetradecanoic acid (myristic acid); 16: Chloroacetic acid, decyl ester; 17: n-Pentadecanoic acid; 18: cis-9-Hexdecanoic acid; 19: n-Hexadecanoic acid (Palmitic acid); 20: Chloroacetic acid, 4-pentadecyl ester; 21: n-Heptadecanoic acid (Margaric acid); 22: Oleic acid; 23: Stearic acid; 24: 1,2-Dipalmitin; 25: Chloroacetic acid 3-tridecylester; 26 Adipic acid, beta-citronellyl heptyl ester; 27: Cyclohexanecarboxylic acid.

Table 4: Biological activities of some active principles present in the n-hexane and dichloromethane extracts of the leaves of *T. catappa*

Phyto-components	Nature of Compound	Biological Activities
n-Hexadecanoic acid	Saturated fatty acid	Hypercholesterolemic, hemolytic, 5-Alpha-reductase inhibitor, antialopecia, antioxidant, antifibrinolytic, lubricant, nematicide
Phthalic acid, mono (1-ethyl-1-methyl-2-propynyl) ester (Phthalofyne)	Aromatic dicarboxylic ester	Anthelmintic especially whipworm
Tetradecanoic acid (Myristic acid)	Saturated fatty acid	Cancer-protective, cosmetic, nematicide, lubricant, hypercholesterolemic
n-Pentadecanoic acid	Saturated fatty acid	Antioxidant, Lubricant
Cis-9-Hexadecanoic acid (Palmitoleic acid)	Unsaturated fatty acid	Increases insulin sensitivity and inhibits destruction of insulin secretory pancreatic beta cells [23]

Heptadecanoic acid (margaric acid)	Saturated fatty acid	Antioxidant, it can be used to detect elevated insulin, associated with high ferritin as well resolve metabolic syndrome [24]
Cis-9-Octadecanoic acid (Oleic acid)	Unsaturated fatty acid	5-Alpha reductase inhibitor, allergenic, cancer preventive, antialopecia, hypercholesterolemic and anti-inflammatory
n-Octadecanoic acid (Stearic acid	Saturated fatty acid	Propecic, hypercholesterolemic, lubricant,5- Alpha reductase inhibitor

Source: Dr Duke's phytochemical and Ethno-botanical Databases {Online Database}

Discussion

The research on the chemical characterization of the n-hexane and the dichloromethane leaf extracts of *T. catappa* have been conducted to identify some bioactive phytochemicals and their relative amounts which may provide a chemical fingerprint for standardization and also provide a basis for its ethno-medicinal usage. The solvent of extraction often determine the polarity of compounds isolated and as such, non-polar solvents such as n-hexane and dichloromethane has the ability to isolate non-polar, volatile constituents from the leaves of *T. catappa*.

The UV-VIS spectroscopic probing of the phyto-chemical constituents between 200- 800nm provide a simple identification of unsaturated and conjugated systems. Different compounds are identified because of their characteristic wavelength of maximum absorption in the UV spectra, for example peak values of 670 and 410 nm in the n-hexane extract correlate with that of chlorophyll and chalcones /aurones respectively while for the dichloromethane extract a shoulder between 250 and 260 nm is indicative of a simple phenol, a peak at 410 nm shows a carotenoid and a third peak at 664nm corresponds to chlorophyll [25, 26].

The FTIR spectra (Fig. 3 and 4) showing the wavelength and the intensities of absorption of the extracts were registered and these were used to identify the functional groups present in the extracts. The n-hexane extracts revealed the presence of alkanes from the appearance of sharp, strong absorption band at 2922.25cm^{-1}. The sharp intermediate peak at 2852.53 cm^{-1} indicates a C-H stretching at the terminal position of an alkyl or alkenes while very weak absorption band around 1243cm^{-1} and 835.68 cm^{-}1 shows the presence of C-O stretching in ether, and alcohol and ester group (Table 2). The dichloromethane extract however showed strong absorption band in the region of 3393.67 cm^{-1} indicating the presence of O-H (hydroxyl) group of an alcohol or phenol. The strong and sharp band at 2958.36 cm^{-1} indicates the C-H stretching of an alkyl or alkene group. Variable bands around 1659.35 cm^{-1} also indicates the C=C stretching due to conjugated double bond. Finally the presence of intermediate peak at 1164 cm^{-1} shows the presence of alkoxy (C-O) group. There has been the isolation of functional groups such as alkanes, alkenes, alcohols, and alkoxy groups from non-polar extracts of some other plant researches [27, 28]

The GC-MS data: Fig.5 and 6, combines the retention characteristics, peak intensities and integrated mass spectra obtained to reveal the complexity of the component mixtures of extracts. These active phyto-components of the n-hexane and dichloromethane extracts with their respective retention time, molecular formula, molecular weight and relative percentages (peak area %) are presented in Table 4 and 5. The n-hexane extract shows the presence of twenty two compounds of which the major components include: n-Hexadecanoic acid (35.6%), Oleic acid (21.14%), Stearic acid (11.44%), Chloroacetic acid, decyl ester (6.95%) 1,2-dipalmitin (3.18%), Chloroacetic acid, octyl ester (2.52%), Tetradecanoic acid (myristic acid) (2.25%), Benzenesulfonamide (1.60%), n-Heptadecanoic acid (Margaric acid) (1.45%), Adipic acid, beta-citronelly heptyl ester (1.88%) while the dichloromethane extract of *T. catappa* leaf contains five components namely: n-Hexadecanoic acid (35.18%), 6-Chlorododecanoic acid (chloromethyl ester) (20.36%), Glycerol-1- palmitate (18.51%), chloroacetic acid, 4-hexadecyl ester (17.63%) and octadecanoic acid (8.32%). n-Hexadecanoic acid is the major component for n-hexane and dichloromethane extracts (35.63% and 35.18% respectively) while chloroacetic acid esters are present in both extracts. The isolation of long chain hydrocarbon and fatty acids may be due to the method of isolation of components and the use of GC-MS which is temperature dependent and thus volatile, non-polar compounds are broken into other smaller fragments or derivatives. Therefore, there is the obvious presence of compounds belonging to the same homologous series such as Tetradecanoic acid, Pentadecanoic acid, Hexadecanoic acid, Heptadecanoic acid and stearic acid. There is also the presence of chloroacetic acid and its different esters. These fragmentations could be due the to temperature of the GC - MS and volatility of the chemical constituents. The biological and medicinal uses of the components (Table 5) reveals compounds with antioxidant, anticancer, anti-inflammatory, antidiabetic, and antihelmentic activity which corresponds with some previous research results on the leaves of *T catappa* [29–31]. All of these validates the use of *T. catappa* in ethnomedicine and add to its chemical characterization in view of its proper quality documentation and standardization.

Conclusion

This research could provide a phyto-chemical characterization for the proper identification of non-polar extracts of *Terminalia catappa* which may suggest some evidence for its usage in herbal medicine in different clime. Effort should also be given to analyze the methanolic extracts using LC-MS as this would provide most of the polar compounds which is non-volatile and could not be detected with the GC-MS.

Conflict of interest

The authors declare that they have no conflict of interests.

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