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Original Research Article

Phytochemical Analysis Of n- Hexane and Ethylacetate Extracts Of Diodia scandens Sw and Spectroscopic Identification Of an Omega- 6 Fatty acid and a Glyceryl trilinoleoate.

ABSTRACT

Traditional Medicine Practitioners in South East Nigeria use Diodia scadens Swartz for the treatment of different diseases such as veneral,cutaneous ,subcutaneousfungal infections,inflammatory, antiabortion, fertility management ,antibiotic and as antidotes for snake bite

Cold maceration of n- hexane and Ethyl acetate solvents extracts for 48 hours yielded brownish oily solids which were subjected to phytochemical analysis. Column Chromatographic fractionation of the crude extracts using n-hexane:ethyl acetate in different v/vratio of 95:05:90:10:8020; down to 100cm^3 of ethyl acetate yielded different fractions. Eluent fractions with same or similar Rf values were pooled togetherand evaporated . These were subjected to spectroscopic analysis. They were labelled as CHDS 11 and CHDS12 from the 95:05 n- hexane:ethyl acetate solvents mixture. The result of the Phytochemical analysis of the crude n- hexane extract showed the presence of steroids, glycoside, alkaloids, and saponins . Crudeethyl acetate extract showed the presence of steroid and saponin . The fraction CHDS11, was identified as cis, cis-9, 12 - octadecadienioc acid(linoleic acid).The 1H NMR chemical shift of 5.05(t) confirmed the hydrogens attached to the double bonds at C-9 and 10; C-12 and 13 adjacent to the methylene group; CHDS12 was identified as 1, 2, 3-propanetriyltris (cis, cis - 9, 12-octadecadienoate), known as glyceryltrilinoleoate withthe chemical shifts 5.28ppm showing the vinyl protons at C-9 and 10; C-12 and 13; 5.19ppm showed protons in between trilinoleoate chains. The presence of these compounds justified the use of this plant for treating arthritis and the diseases earlier mentioned.. To the best of our knowledge, this is the first time these compounds are isolated from this plant.

Keywords: Diodiascandens,n-hexane,ethylacetate,phytochemical analysis,1HNMR.

INTRODUCTION

Human beings from antiquity depended on plants either directly for foods and beverages, or indirectly as feed for animals or the flavoring of foods. Plants are also the source of beverages produced either by infusion, such as coffee and tea; by fermentation, such as beer and wine; or by distillation, such as whisky, vodka, rum, and other alcoholic spirits.

Plants are the source of many natural products such as essential oils, natural dyes, pigments, waxes, resins, tannins, alkaloids, amber and cork. Products derived from plants include soaps, shampoos, perfumes, cosmetics, paint, varnish, turpentine, rubber, latex, lubricants, linoleum, plastics, inks, and gums. Renewable fuels from plants include firewood, peat and many other

UNDER PEER REVIEW

biofuels. Coal and petroleum are fossil fuels derived from the remains of plants. Olive oil has been used in lamps for centuries to provide illumination [7].

Man has been engaged in the use of the plants and their different parts for treatment of various ailments. Generally, plants have been used throughout the world in folk medicine and as local cure for common ailments. Medicinal plants in particular have been in use for centuries as remedies for human diseases like malaria, dysentary, diarrhoea, typhoid, arthritis, infertility etc because they contain components of therapeutic value [11]. Folk medicine gave rise to traditional system of medicine in various diseases.

Medicinal herb is considered to be a chemical factory as it contains multitude of chemical compounds like alkaloids, glycosides, saponins, resins, oleoresins, sesquiterpene, lactones and oils (essential and fixed). India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. According to an estimate, 120 or so plant based drugs prescribed for use throughout the world come from just 95 plant species [14].Natural antimicrobials can be derived from plants, animal tissues and microorganisms. The shortcoming of the drugs available today propelled the discovery of new pharmacotherapeutic agents from medicinal plant research [14].

DESCRIPTION OF PLANT

Diodia scandens Sw(Rubiaceae) is an evergreen perennial herb, which has an alternate leaf arrangement, petiole is present. It has compound leaves, ovate to lanceolate in shape, reculate venation, entire in margin, its apex is acute, its base is cuneate, it has glabrous surface and its texture is charteceous. *Diodia scandens* Sw has a dark green coloration, tasteless, odorless and has solitary inflorescence. It is a straggling herb, which has been in use in the western Africa system of medicine. It has enormous usefulness and importance; whole parts of the plants are useful in curing various ailments [6].

The plant's medicinal value includes its use as antidotes, painkiller, treatment of venereal diseases and cutaneous and subcutaneous fungal infections. The different parts of the plants- sap, leaf, stem and root, are used for various medical purposes. The leaf is used for treating arthritis, rheumatism, cutaneous and subcutaneous parasitic infection, diarrhoea, dysentery and antiabortifacients. The leaf plus roots are used for dropsy, swellings, oedema, and gout and as lactation stimulants; while the sap is used for treating ear infections, paralysis, epilepsy, convulsions, spasm and pulmonary troubles [6]. The whole plant of *D. scandens Sw* is used for treating fibroid and uterine disorder [10].

MATERIALS AND METHODS

MATERIALS

The solid compounds used in this work were purified and solvents redistilled before use. The spectroscopic equipment were;
Infraredspectrometer, transmittance 4000-650.
Ultraviolet/ Visible spectrometer, 4.20(468).
Nuclear Magnetic Resonance, 400 MHz

METHODS

UNDER PEER REVIEW

Sample collection and preparation.

Diodia scandens Sw was collected at St. Mary's Pro-Cathedral Parish Udi and the plant was identified by a qualified taxonomist, Prof. J C. Okafor, at No. 7 Dona drive, Off Ihiala Street, Independence Layout Enugu, Enugu state, Nigeria.

The plant leaves were washed and air dried.. The dried leaves were ground with a mechanical grinder. It was stored in large sample bottle and labeled.

Extraction of the plant material.

Using cold maceration method n-hexane and ethylacetate were used. The pulverised sample (200 g) was macerated in 500 $cm^3$of n-hexane, stirred vigorously and was left for 48 hours. It was filtered and the filtrate was allowed to dry at room temperature .. This was repeated using ethylacetate.

Preparation of reagents

Meyer's reagent: Meyer's reagent was prepared by adding 1.3 g of mercuric iodide in 10 $cm^3$of water and 5.0 g of potassium iodide in 20 $cm^3$of water and making it up to 100 cm^3 solution in a flask [1].

Marquis reagent: Marquis reagent was prepared by mixing concentrated tetraoxosulphate (VI) acid and formalin in the ratio of 10:1 v/v respectively [1].

Draggendorf's reagent: Draggendorf's reagent was prepared by dissolving 0.6 g bismuth subnitrate in 2 cm^3concentrated hydrochloric acid and 10 cm^3 of water and was mixed with 6 g of potassium iodide in 10 cm^3 of water. Then 7 cm^3 of concentrated hydrochloric acid 15 $cm^3$of water was added and the whole was diluted with 400 $cm^3$of water [1].

PHYTOCHEMICAL ANALYSIS [1], [12]

Tests for Alkaloids

a. **Test with Meyer's reagent (potassium – mercuric iodide):**Meyer's reagent (2cm^3) was added to 2cm^3 of the each extract. A white precipitate was formed in n-hexane and methanol extracts, showing the presence of alkaloid.

b. **Test with Draggendorf's reagent (potassium bismuthic compound):**Draggendorf's reagents (2cm^3) was added to 2 cm^3 of the each extract. A red precipitate was formed in methanol extract only, showing the presence of alkaloid.

c. Test with Maquis reagent: Marquis reagent 1 cm^3 was added to 1 cm^3 of the each extract. A reddish brown colour was formed in n-hexane and methanol extracts, showing the presence of alkaloid.

Tests for Steroids

a. Lieberman - Buchard test : Chloroform (5 cm^3) was added to 2 cm^3 of each extract. Acetic anhydride (2 cm^3) was added to the mixture and concentrated tetraoxosulphate (VI) was added drop wise. A blue green colouration was formed in each of the extract, showing the presence of steroid.

UNDER PEER REVIEW

b. **Salkowski test :** Chloroform (2 cm^3) was added to 2 cm^3 of the each extract, and concentrated tetraoxosulphate (VI) acid was added drop wise. A red blue colouration was formed in each of the extract, showing the presence of steroid.

c. Formaldehyde *test:* Chloroform (2 cm^3) was added to 2 cm^3 of the each extract, 2 cm^3 of formaldehyde reagent was added to it. Acetic anhydride (1 cm^3) was added to the mixture. A blue colour was formed in each of the extract, showing the presence of steroid.

Test for Flavonoids [8]

a. **Test with 10% NaOH:**Solution of 10% sodium hydroxide was added to 2 cm^3 of the each extract. A yellow colour was formed in methanol extract, showing the presence of flavonoid.

b. **Test with Tetraoxosulphate (VI) acid:** Concentrated tetraoxosulphate (VI) acid (1 cm^3) was added to 3 cm^3 of the extract. A yellow colour was formed in methanol extract, showing the presence of flavonoid.

c. **Test withMg-HCl:**Mg-HCl (1 cm^3) was added to 2 cm^3 of the extract. There was absence of yellow and red colour in each of the extract.

Test for Cardiac glycoside

Keller – Killinai test: Glacial acetic acid (2 cm^3) was added to 2 $cm^3$of the each extract, 2 drops of aqueous ferric chloride solution was added to the mixture and 1.5cm^3 of concentrated H_2S0_4 was also added [9]. A brown ring was formed in n-hexane extract, showing the presence of glycoside.

Tests for Saponins

a. **Frothing test:** Each of the extract (1 cm^3) was dissolved in 1cm^3 of water, the mixture was stirred [9]. Foams was formed in each of the extract, showing the presence of saponin.

b. **Emulsion test:** Olive oil (5 drops) was added to 1 $cm^3$of the extract, the mixture was stirred vigorously [9]. Emulsion was formed in ethylacetate and methanol extracts, showing the presence saponin.

Tests for Tannins

a. **Test with 5% ferric chloride:**Solution of 5% ferric chloride (2 cm^3) was added to 2 cm^3 of the each extract [12]. Black precipitate was formed in methanol extract, showing the presence of tannin.

b. **Test with freshly prepared potassium hydroxide solution:**Solution of 10% freshly prepared potassium hydroxide (2 cm^3) was added to 2 cm^3 of the each extract [12]. Dirty white precipitate was formed in methanol extract, showing the presence of tannin.

c. **Phlobatanin test:** Solutionof 1% HCl (1 cm^3) was added to 2 $cm^3$of the each extract, it was boiled [12]. There was absence of red precipitate in each of the extract.

Tests for Anthraquinones

UNDER PEER REVIEW

a. **Bornthrager's test:** Benzene (5 cm^3) was added to 5 cm^3 of the extract, it was stirred and filtered, 5 cm^3 of 10% NH_4OH solution was added to the filtrate, it was shaken properly [12]. There was absence of pink, red or violet colour in each of the extract.

b. **Combined Anthraquinone:**Each of the extract (5 cm^3) was boiled with 10cm^3 of 1M H_2S0_4 and was filtered while hot, to this 5cm^3 of benzene was added and it was shaken. The benzene layer was separated, and to it, 2 cm^3 of 10% $NH_3$was added [12]. There was absence of pink and red colour in each of the extract.

COLUMN CHROMATOGRAPHY

The crude n- hexane extract was column chromatographed on silica gel adsorbent using n-hexane:ethyl acetate v/v in the ratio of 95:05, 90:10, 80:20,70:30 up to 100 cm^3 of ethyl acetate.Fractions with same or similar Rf values were pooled together. Fractions CHDS11 and CHDS12 were collected from 95:05 n-hexane:ethyl acetate.

RESULTS AND DISCUSSION

Mass and percentage yield of crude extracts

Table .1 showed the mass and percentage yield of crude extracts from the solvents. Ethylacetate had a mass yield of 7.15 g (3.58%) and n-hexane had weight 3.18 g (1.59%). From the result, a more polar solvent ethylactetate extracted more than a non polar solvent. This shows that polar solvents are better extraction solvent than non-polar solvents

TABLE 1: MASS and percentage yield of the crude extracts from the solvents

Extracting solvents	Mass of crude extract (gram)	Percentage yield
Ds ethylacetate	7.15	3.58
Ds n-hexane	3.18	1.59

Ethylacetate extracted a higher percentage mass than n-hexane

Phytochemical analysis of the crude extracts of *D. scandens Sw*

Table 2. showed the phytochemical result for crude extract of *D. scandens Sw*. The phytochemical analysis of n-Hexane crude extract showed that glycoside, steroid, saponin and alkaloid were present. In the ethyl acetate, steroid and saponin were present . Properties of saponin containing herbs are many and varied and may include diuretic, expectorant, anti-catarrhal, anti-inflammatory, antispamodic, aphrodisiac, antioxidant, emmenagogue, cardiac stimulant, hormone modulating, hepatoprotective, and adrenal adaptogenic effects [16]. Steroids have been reported to have antibacterial properties and they are very important compounds especially due to their relationship with compounds such as sex hormones. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity. Several workers have reported the analgesic, antispasmodic and antibacterial properties of alkaloids. Glycosides are known to lower the blood pressure according to many reports [16]. The results obtained in this study thus suggest that the identified phytochemical compounds may be the bioactive constituents, and this plant is proving to be an increasingly valuable reservoir of bioactive compounds of substantial medicinal merit.

UNDER PEER REVIEW

TABLE2: PHYTOCHEMICAL RESULT FOR CRUDE EXTRACT OF *Diodia scandens Sw*

	Ds n-Hexane	Ds ethylacetate
Glycoside	+	–
Steroid		
i) Salkowski test	+	+
ii) Lieberman-Buchard test	+	+
iii) Formaldehyde test	+	+
Saponin		
i) Emulsion test	+	+
ii) Frothing test	–	+
Alkaloids		
i) Maquis test	+	–
ii) Meyer's test	+	–
iii) Draggendorff's test	–	–
Tannins		
i) Test with 5% $Fecl_3$	–	–
ii) Test with 10% K0H	–	–
Phlobatanintest	–	–
Anthraquinonetest	–	–
Flavanoids		
Test with concentrated H_2SO_4	–	–
Test with 10% NaOH	–	–
Test with Mg - HCl	–	–

Key: + = present, - = absent.

SPECTROSCOPIC ANALYSIS OF THE PURE SAMPLES

Table 3:. Pure samples were analysed for structural elucidation using ^{1}H NMR (400 MHz, $CDCl_3$).
Compounds were got from CHDS11,and CHDS12.
The compound CHDS11 was oily solid and has a wine red colour. Fraction CHDS12 was an oily solid.

Table 3: SPECTROSCOPIC ANALYSIS OF THE PURE SAMPLES

Fraction	Colour and nature
CH DS_{11}	Wine red oil
CH DS_{12}	Orange oil

Isolated fractions and their colours.

^{1}H NMR spectrum of CHDS11 as (cis,cis-9,12-octadecadienoic acid) in $CDCl_3$ compared to literature

UNDER PEER REVIEW

Table 3 showed the experimental chemical shifts and multiplicity of CHDS11 compared to literature values..

The chemical shift at 0.8(qd) showed the presence of methyl group at C-18; 5 .05(t) confirms the hydrogens attached to the 2 double bonds at C – 9 and 10; C-12 and 13 of the compound.

The chemical shift 2.3 (t) at C-11 shows the methylene hydrogen linking the two double bonds, 1.97 (t) at C-2 indicates the methylene hydrogen attached to carboxylic acid group. The chemical shift 1.61(s) at C-14 shows methylene hydrogen preceding the vinyl carbons. The chemical shift 1.20 (m) indicates methylene groups in between the CH_2 following the vinyl carbon and the carboxylic acid group and the one ($3(CH_2)$) in between the methyl group and the $-CH_2-$ group attached to vinyl carbon, given a total of 14 hydrogens. These chemical shifts as compared with literature confirm the presence of linoleic acid in the sample from *D. scandens Sw*.

The presence of fatty acids such as linoleic acid which balances female reproductive hormones and also helps to lubricate the mucous membrane.

Growing evidence indicates that the design and delivery of supplemental fatty acids to the lower gut may target reproductive tissues to improve reproductive function and fertility. Improvement in embryo survival may be associated with suppression of uterine prostaglandin secretion via linoleic acid or other longer chain unsaturated fatty acids. Changes in follicular dynamics can be affected by fat supplementation and may lead to a more fertile ovulation. This improvement may be due to alterations in metabolic hormones and growth hormone or hormonal clearance [5]. Thus, the fatty acid present in *D. scandens Sw* must be responsible for reduction of swelling and nourishing of the uterine wall.

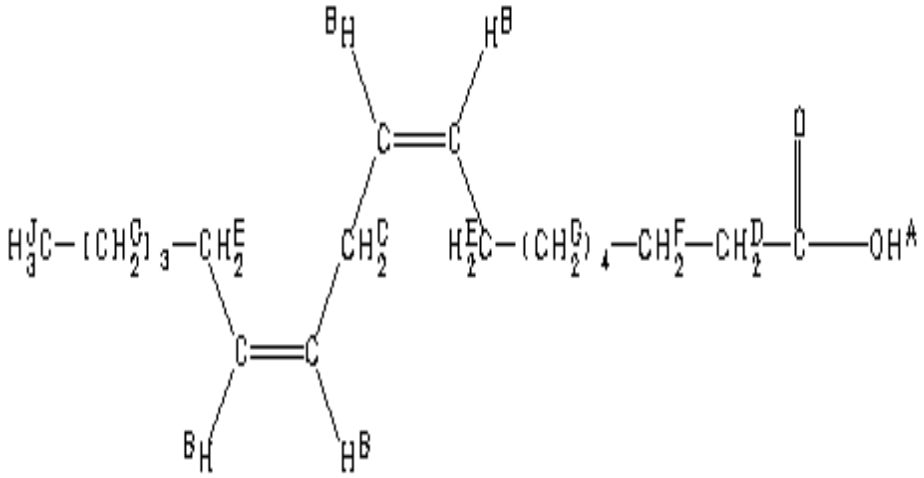
Table 4: Experimental chemical shifts and multiplicity of CHDS11 compared to literature.

Proton	Experimental chemical shift δ and multiplicity	SDBS literature	Anatoli et al Literature
CH – 9,10,12,13	5.05 (t)	5.58-5.12	5.35 (m)
CH – 8,14	2.23 (t)	2.764	2.77 (t)
CH_2 – 2	1.97 (t)	2.33	2.33
CH_2 – 3	1.54 (d)	1.63	1.61
CH –11	1.61 (s)	2.02	2.07 (q)
CH – 15	1.20 (m)	-	1.30 (m)
CH – 16	1.20 (m)	–	1.30 (m)
CH – 17	1.20 (m)	1.10–1.53	1.31(m)
CH_2 – 4,5,6,7	1.20 (m)	1.10 – 1.53	1.33(m)
CH_3 – 18	0.80 (qd)	0.9	0.9 (t)

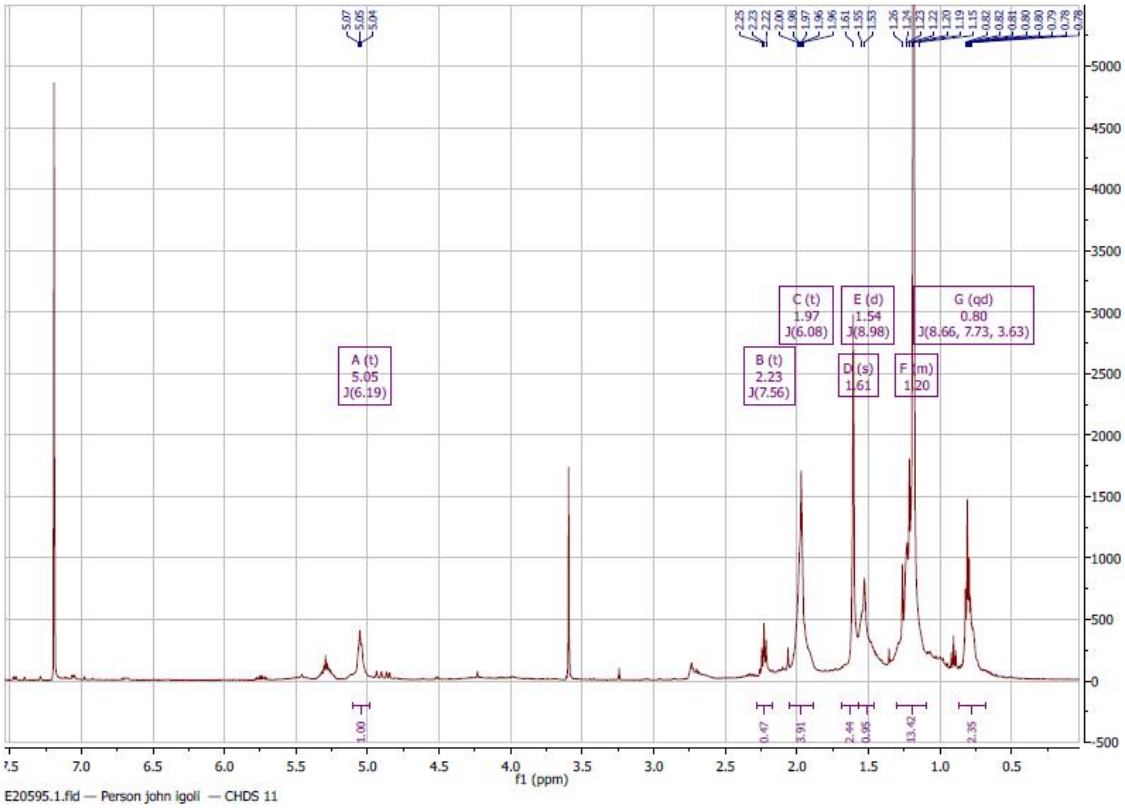
Experimental NMR data showing the protons and chemical shifts in ppm

Figure 1: CIS, CIS-9,12-OCTADECADIENOIC ACID, $C_{18}H_{32}O_2$ (linoleic acid)(CHDS11)

UNDER PEER REVIEW



299
300
301
302
303 **^{1}H NMR spectrum of cis,cis-9,12-octadecadienoic acid**
304



305
306
307
308 **^{1}H NMR spectrum of CHDS12 as 1,2,3-propanetriyltris(cis,cis-9,12-**
309 **octadecadienoate) in $CDCl_3$ compared to literature.**

UNDER PEER REVIEW

Table 4: showed the experimental chemical shift and multiplicity of CHDS12. It was orange oil and gave an R_f value of 0.68.

The chemical shifts 5.28(m) indicates vinyl protons at C – 9, 12 in the compound. The chemical shift 5.19(t) shows protons in between trilinoleoate chains. The chemical shift 4.22(dd) and 4.07(dd) indicates the proton glyceride group. The chemical shift 2.72(dq) shows methylene protons in between the double bonds. The chemical shift 2.24(td) shows $–CH_2–$group following the ester group. The three chemical shifts 1.99(m), 1.54(d) and 1.21(m) are methylene group in between the vinyl carbon and the $– CH_2–$ group attached to the ester carbon. 0.81(td) shows a methyl group. The chemical shifts as compared to literature confirm the presence of triglyceride, 1, 2, 3 – propanetriyltris (cis, cis – 9, 12 – octadecadienoate) is present in *D. scandens* Sw.

This compound 1,2,3-propanetriyltris(cis,cis-9,12-octadecadienoate) increases the blood permeability in the brain. The blood brain barrier forms a structural and functional barrier between the blood circulation and brain parenchyma, regulates the transport of molecules, and prevents blood cells accessing brain tissues. Triolein emulsion transiently increased vascular permeability with interstitial edema in the cat brain when administrated via the carotid artery [4]. Triglycerides are esters of glycerol combined with three chains of fatty acids. Elevated triglyceride are strong indicator of biliary function, fat metabolism, the function of the liver and hereditary. There is generally a sugar-handling issue with elevated triglyceride or adult onset diabetes. Decrease triglyceride suggests poor release of fatty acid, endocrine hyperfunction, and immune problem [15]. Triglyceride insulates the body from extreme temperature changes. They absorb and transport vitamin A, D, E and K through the blood cell [3].

Table 5: Experimental chemical shift of CHDS12 compared to literature

Proton	Experimental Chemical shift δ and Multiplicity	SDBS Literature
CH=CH9,10,12,13	5.28 (m)	5.35
H-C-O-2	5.19 (t)	5.27
CH_2O-1^1	4.22 (dd)	4.295
CH_2O-1^2	4.07 (dd)	4.147
CH_2-11	2.72 (dq)	2.769
CH_2-2	2.24 (td)	2.315
CH_2-8,14	1.99 (m)	2.049
CH_2-3	1.54 (d)	1.61
CH_2--7,15	1.21(m)	1.36
CH_2-4,5,6,16,17	1.21 (m)	1.30
CH_3-18	0.81 (td)	0.889

Experimental NMR data showing the protons and chemical shifts in ppm.

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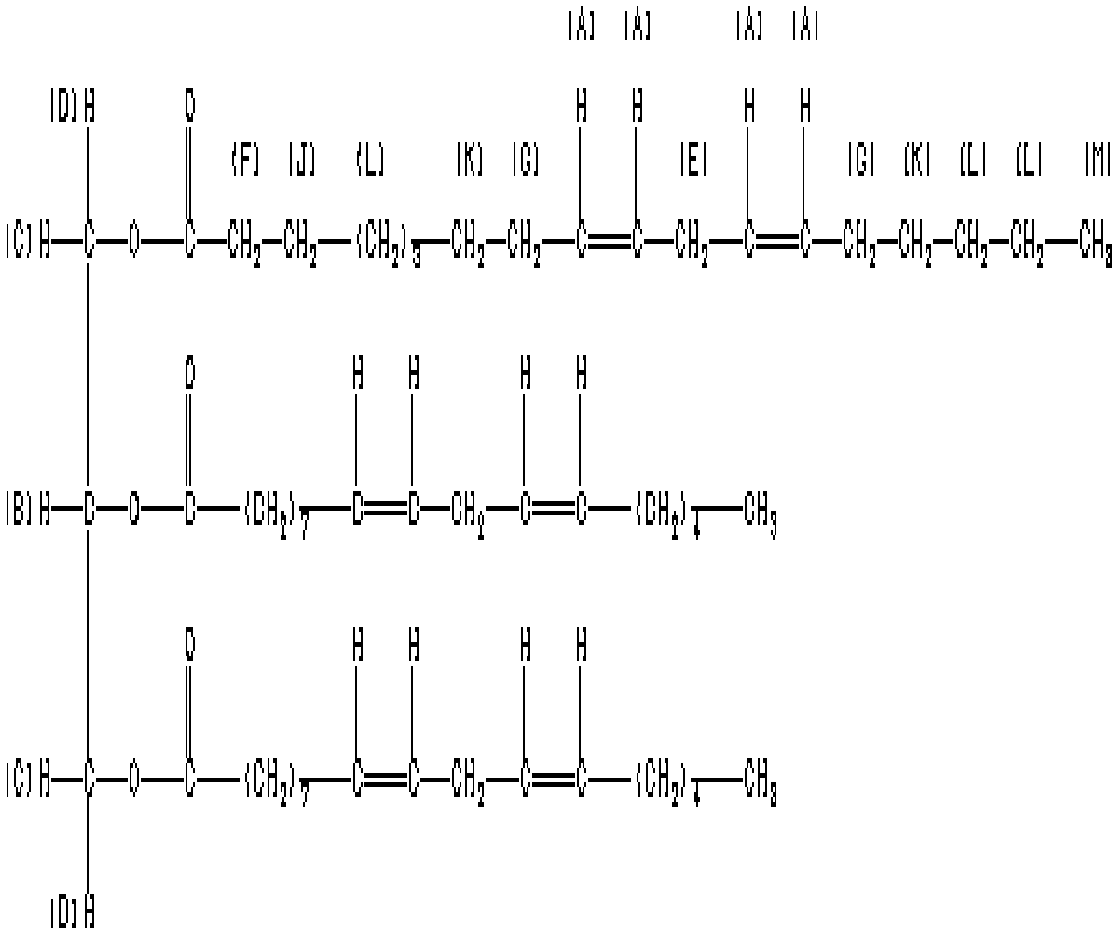

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Figure 2: 1,2,3-propanetriyltris(cis,cis-9,12-octadecadienoate)

^{1}HNMR SPECTRUM OF1,2,3-propanetriyltris(cis,cis-9,12-octadecadienoate)

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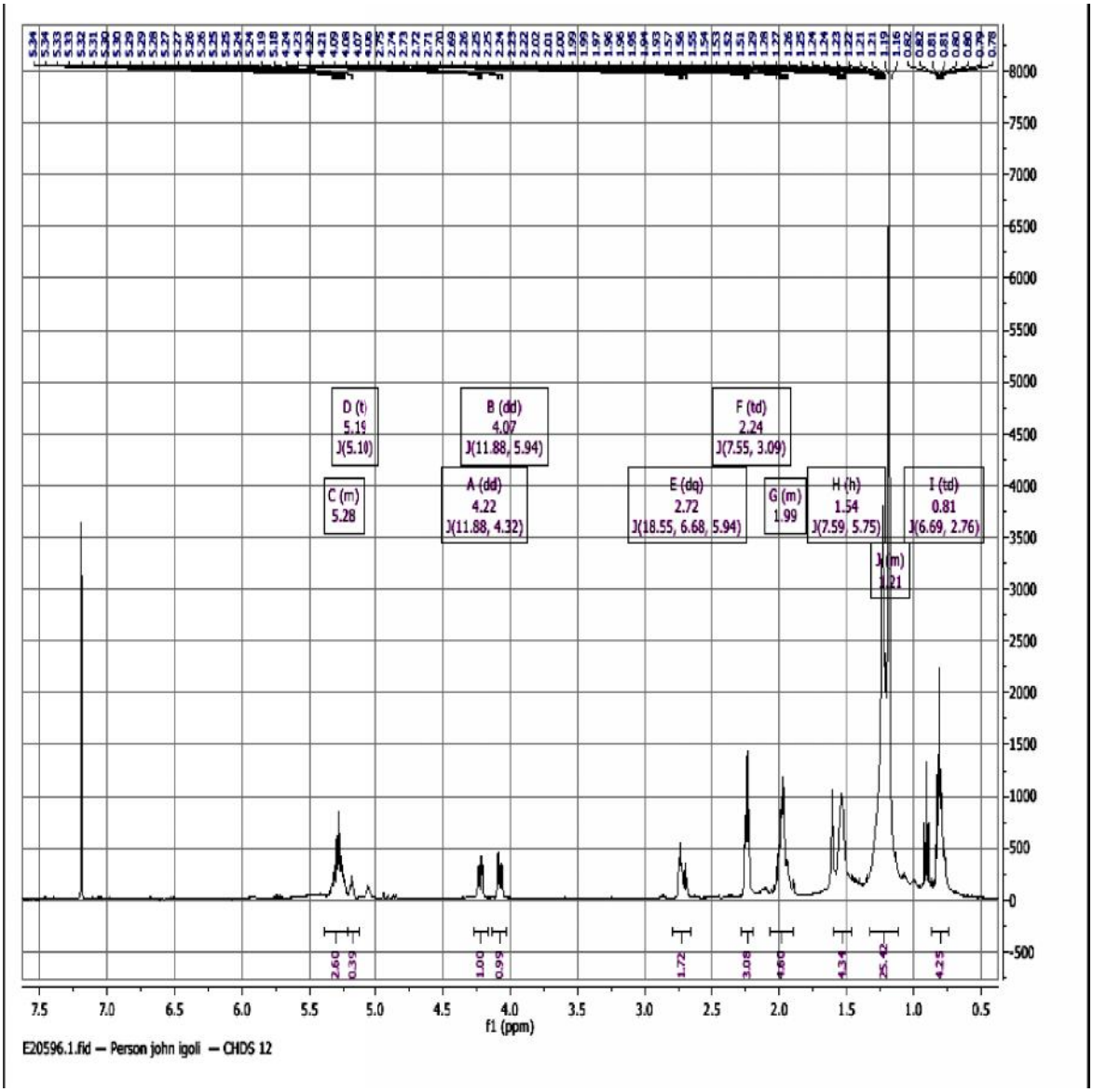

CONCLUSIONS

The phytochemical analysis carried out showed the presence of steroids, glycosides, alkaloids, saponins, in n-hexane leaves crude extract, steroids, and saponins in the crude ethylacetate extract and steroids, saponins, alkaloids. The n-hexane crude extract was fractionated and two pure samples were obtained and were labeled CHDS11, CHDS12.

The ^{1}H NMR spectral revealed that the sample labeled CHDS11 contained cis,cis,-9,12-octadecadienoic acid, CHDS12 contained 1,2,3-propanetryltris(cis,cis-9,12-octadecadienoate). The presence of these compounds has shown that the traditional use of the plant could be correct.

UNDER PEER REVIEW

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UNDER PEER REVIEW

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