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ISOLATION OF SQUALENE FROM DICHLOROMETHANE EXTRACT OF *CROTON NIGRITANUS* LEAF

***[1]Abdullahi M. Abdullahi, [2]Olime M. Nkemakonam, [3]Usman Garba, [4]Muhammad A. Mustapha, [5]Muhammad I. Nazifi, [4]Muhammad B. Usman and [4]Umar M. Ibrahim**

[1]Department of Chemistry, Directorate of Science, Remedial and General Studies, Federal University of Health Sciences Azare, Bauchi State, Nigeria.
[2]Sheda Science and Technology Complex Federal Capital Territory, Abuja, Nigeria.
[3]Department of Mathematics/Computer, Gombe State College of Education Billiri, Gombe State, Nigeria.
[4]Department of Chemistry, Faculty of Sciences, Gombe State University, Gombe State, Nigeria
[5]Department of Science Laboratory Technology, Federal Polytechnic Kaltungo Gombe State, Nigeria.

*Corresponding authors' email: abdullahi.muhammadjika@fuhsa.edu.ng

ABSTRACT
A precursor of steroids with a variety of biological activities is squalene. The purpose of the study was to identify and describe the industrial/medical chemical found in Croton nigritanus. Using the solvents such as; dichloromethane, ethyl acetate, and n-hexane, the procedure entails a liquid-liquid extraction stage using the partition method and a solid-liquid extraction step using the maceration method. For separation and purification, gravity column chromatography (GCC) and thin-layer chromatography were employed. The isolated chemical's identity was determined using FTIR, ^{1}H NMR and GC-MS spectroscopy. The inquiry successfully extracted and analyzed the major chemical compound which was identified as Squalene.

Keywords: Isolation, Terpenes, *Croton*, Extraction, Spectroscopy

INTRODUCTION

Croton nigritanus is a shrub known for its beautiful leaves, it belongs to Euphorbiaceae family, consisting of six different species of broadleaf perennials, shrubs, and small trees. The species are widely distributed in tropical regions of Asia, Africa, and the western Pacific (Wen *et al.,* 2018). In tropical landscaping, smaller plants like *Croton nigritanus* are widely being used. In its natural habitat, the plant develops into a bushy shrub with multiple branches that reach a height of around 10 feet. However, the domesticated varieties that are sold as houseplants are mostly smaller than their natural ones. Large, leathery leaves on *Croton nigritanus* range in different sizes (Yue *et al.,* 2022).. They can be linear or spherical in some cases, and can be of different sizes. *Croton nigritanus* leaves contain patterns in green, white, or purple in addition to other colours. When grown inside, it hardly ever blooms, according to Wen et al., (2018), Squalene is a triterpene with molecular formula $C_{30}H_{50}$, in which there are six isoprene units. Squalene was first identified in shark liver extract, and later found in the other tissues of shark and vegetable oils such as olive oil (Yue *et al.,* 2022). Due to the high market demand for squalene, investigation to find other sources of squalene is very essential (Ardhyni *et al.,* 2022).

Isolation of Compounds from Croton Plant

According to Meresa et al., (2019), the following compounds are among those produced from *Croton macrostachyus*: crotepoxide, lupeol and betulin, cis- clerodane, crotomacrine, 3- Acetoxy tetraxer-14-en-28-oic acid, trachylina-19-oic acid, and trachylina-18-oic acid. Sonia *et al.,* 2022, (6) Insulated novel composites from Croton hirtus, 5,14-dihydroxyrotundone-9-(2)-methybut-2-enoate) and (-) 5,14-dihydroxyrotundane-9-benzoate.. Squalene was extracted from DD of rice husk oil by CO_2 supercritical fluid (CSF) chromatography. In another study, squalene was separated from DD of palm oil by CSF extraction in the counter-current packed column (Fatemeh *et al.,* 2018).

Phytochemistry of Croton Species

Phytochemical screening test on ethanol extract of leaves of *C. macrostachyus* indicated the presence of alkaloids, terpenes, flavonoids and saponins while anthraquinones and tannins were not detected (Abeye *et al.,* 2016). Ermias et al., (2022) discovered that *C. megalocarpus* has antiviral substances in addition to other physiologically active molecules. According to Ermias et al., (2022), members of this plant family can provide the active components for novel antivirals that can treat HIV. Phytochemicals derived from *Croton* species include alkaloids, diterpenoids, essential oils, flavonoids, furanoditerpenoids, and triterpenoids (Alfred, 2019). Preliminary qualitative phytochemical testing on crude extracts from various plant parts identified the presence of phenolic substances, tannins, terpenoids, alkaloids, saponins, free anthraquinones, phytosterols, and polyphenols (Meresa *et al.,* 2019). According to Emmanuel et al., (2018), the leaf extracts of *C. gratissimus* in acetone and ethanol showed that it can be employed as a new source of medicinal compounds. Chemicals known as diterpenes, which come in a variety of kinds including clerodanes, cembranoids, halimanes, are frequently found in *Croton*. Several *Croton* species are aromatic because they contain volatile oils (Abdulrahman *et al.,* 2022).

MATERIALS AND METHODS

Plant Materials

The plant component (leaf) was collected in July 2023 from Savannah Woodland, located at 11.0855 ºN and 7.7199 ºE in Sakaru Kaduna State Nigeria. The authenticity of the plant sample was assured by Dr. U.S. Gallah from the Department of Botany at Ahmadu Bello University Zaria, Nigeria ABU/H/13715, a voucher specimen, was kept in the university's botany department's herbarium. *Croton nigritanus* leaves were sent to the Chemistry Research Laboratory of North Eastern University Gombe, Nigeria. in poly-ethene bags, before being reduced to minuscule pieces using pestle and mortar, the leaf segments were air-dried for one week at room temperature. (Andi *et al.,* 2019) and (Ferdinand *et al.,* 2023).

Extraction of Plant Material
According to Nnamdi et al., (2022), 2.5 Liters of CH_2Cl_2, the maceration technique was employed to separate the dried and powdered plant material from the leaves using (500 g). The resulting extracts were dried at 50 ^{0}C in a rotary evaporator to produce crude extracts.

Phytochemical analysis
An initial screening was performed on a dichloromethane extract of *C. nigritanus* leaves to hunt for common secondary metabolites. For this screening, the methods stated earlier in the analytical procedures were applied (Abeje *et al.,* 2016).

Test for terpenes
250 mg of ethanol, 2 ml of dichloromethane extract of *C. nigritanus*, and 30 ml of concentrated H_2SO_4 were combined to produce a layer (Abeje *et al.,* 2016). There was a reddish-brown stain on the interface.

Test for flavonoids
250 mg dichloromethane extract of *C. nigritanus* was dissolved in a small amount of diluted NaOH, and 3 ml of HCl was then added. The resulting golden solution was visible but did not turn colourless upon closer observation (Nnamdi *et al.*, 2022).

Test for Tannins
The dichloromethane extract of *C. nigritanus* was diluted with water, then it was heated in a water bath. A dark-green solution was not observed when a few grams of solid $FeCl_3$ were added to the filtrate. (Wen *et al.,* 2018).

Test for alkaloids
2 cm^3 of strong HCl was added to 250 mg of extract of *C. nigritanus*. The extract was filtered, blended with a small amount of amyl alcohol at ambient temperature. The final combination was used to determine the alcohol layer's colouring indicating the presence of alkaloids (Nnamdi *et al.,* 2022).

Test for saponins
250 mg of dichloromethane extract of *C. nigritanus* and 5 ml of distilled water were shaken for 30 mins. When the mixture boiled, it was checked to see if a creamy mixture with few bubbles was present (Abeje *et al.,* 2016).

Test for anthraquinones
After boiling in strong HCl for a brief period in a water bath, 500 mg of the dichloromethane extract of *C. nigritanus* was filtered. The same quantity of $CHCl_3$ was added after cooling the filtrate. A few drops of ammonia was added to the mixture before it was boiled in a water bath. It was seen to take on a rose-pink colour (Dorine *et al.,* 2021).

Column Chromatography Packing of Crude Extract
The glass column was meticulously cleaned before being secured upright to a retort stand. A layer of cotton wool was placed at the base of the glass. The prepared slurry was gently poured into the column while being lightly tapped to ensure appropriate packing. The column was then treated with the prescribed amount of the crude extract. 100% CH_2Cl_2 was the starting point for the elution, which then included n-hexane (3:1), (4:1), (14:6), (3:2), and (5:5), ethyl acetate (3:2), (14:6), (4:1), and (3:1), and 100% n-hexane. The column was washed with 200 mL of several solvent solutions with increasing polarity. The last fractions were collected, dried in a chamber, and then put into 10 mL vials. Additionally, TLC was carried out on the eluents using a solvent solution that contained only n-hexane. In this step, each eluent's purity, component count, and, if applicable, compositional analysis were all assessed (Akpotu *et al.,* 2017).

Thin Layer Chromatography
The method was used to separate the active polar CH_2Cl_2 fraction from the crude extract using silica gel plates as the stationary phase. These chromatoplates were then subjected to 100% CH_2Cl_2 and 100% n-hexane after being placed in a developing chamber and permitted to progress in a CH_2Cl_2: n-hexane (9:2, 8:2, 14:6, 12:8, 5:5) mobile phase system. After being developed, the TLC plate was taken out, air-dried, and inspected using a spray reagent (10% sulphuric acid in water), which was then heated with a hairdryer (Josephat, 2021).

Isolation and Purification
Eluents that shared comparable components required extra purification, as shown by their Rf values acquired using thin-layer chromatographic techniques in experimental conditions. For column chromatography purification, various column diameters were utilized, packed with silica gel in certain solvent systems were utilized. During the purification procedure, the fractions with equal Rf values were generated, mixed, and showed a white crystals. The isolate was labelled as CNDL-4.

Proton NMR Analysis
The structure was located via spectroscopic analysis. The separated compound was delivered to King Fahad University of Petroleum Saudi Arabia for proton NMR research. The NMR studies used a 400 MHz Bruker Advance III NMR spectrometer. When spectra were collected in $CDCl_3$, the center line at about 7.26 in the ^{1}H NMR spectrum was occasionally utilized as a reference. The processed spectra were done using a program named Delta NMR software.

GCMS Analysis
The GC-MS analysis was carried out using (GC-6890N/5975B MSD) at King Fahad University of Petroleum Saudi Arabia. The investigation of the isolate was performed on an Inert MSD-597CM.

RESULTS AND DISCUSSION

Table 1*:* Phyto-chemical Test Results of CH_2Cl_2 Extract of Croton nigritanus Scott elliot

Plant constituent	Observation
Alkaloids	+
Tannins	-
Terpenes	+
Anthraquinones	+
Saponins	-
Flavonoids	+

(+) detected (-) not detected

From Table 1 Some of the most important secondary metabolites/phytochemicals possessing different industrial/medicinal uses being analyzed such as alkaloids, flavonoids, Terpenes, anthraquinones are present while tannins and saponins were absent.

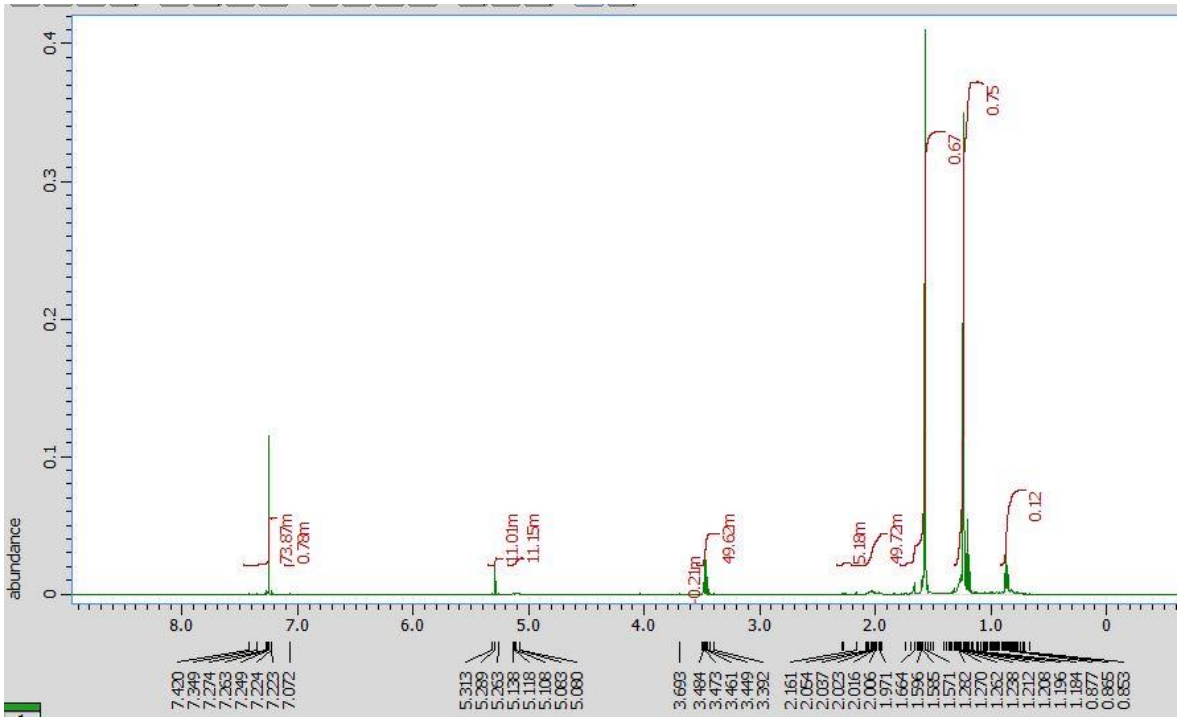

Figure 1: Proton NMR spectrum of CNDL-4

Table 2: Proton NMR result of CNDL-4, 1H NMR (400 MHz; $CDCl_3$)

Signals	**^{1}HNMR(δ ppm)**
1,6,8,9,12,13,17,18,21,22	1.5 -CH_2
2,4,11,14,19,23	2.0 -C=CH
7,15,26,27,28, 29,30,35	1.0 -CH_3

The compound was isolated as a white crystalline compound from the dichloromethane extract of the leaves of *Croton nigritanus*. The GC-MS spectrum of the compound gave a molecular ion peak at m/z 410, which indicated a molecular formula of $C_{30}H_{50}$, corresponding to six degrees of unsaturation. The FTIR spectrum of the compound displayed an absorption band at 1744.84 cm^{-1} that was ascribed to an alkene stretch and absorption band at 3007.92 cm^{-1} that was attributed to sp^2 C-H stretches. Presence of alkene double bonds and alkene protons signifies the present of squalene. The ^{1}H-NMR spectrum displayed proton resonances at δH (7.2, 5.3, 3.5, 2.0, 1.7, 0.8 Hz) that corresponds to squalene with small quantity of impurities. Therefore, the prominent compound was identified as squalene.

Table 3: The result of the GC-MS analysis of CNDL-4

RT	**Area Pct**	**Library/ID reference**	**CAS**	**Quality**	**Molecular formulae**	**Molecular mass ($gmol^{-1}$)**
40.7581	5.0008	Squalene/ 114269	007683-64-9	94	$C_{30}H_{50}$	410

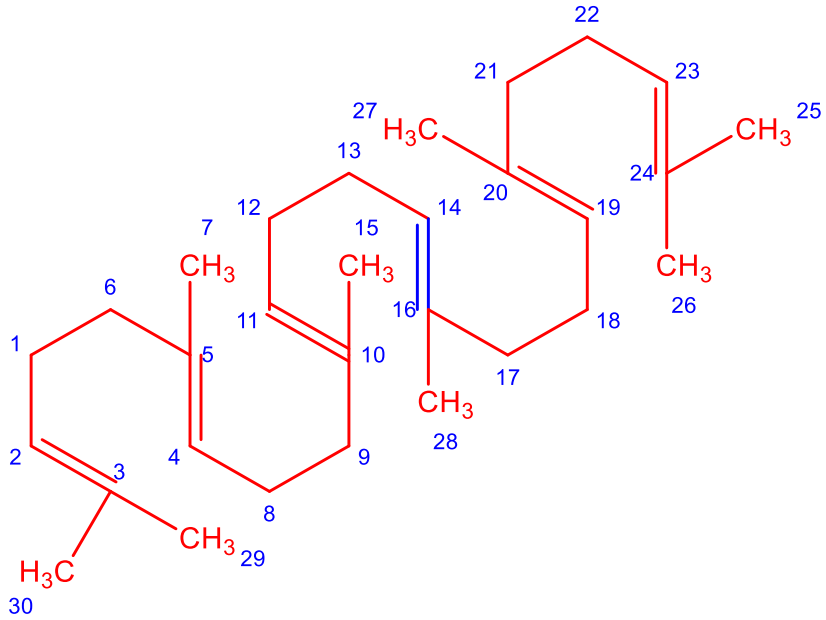

Figure 2: The structure of Squalene.

The suggested structure of the compound as represented in fig.1 above was concluded based on both proton NMR results in Fig. 1, Table 2. and GC-MS results in Table. 3.

CONCLUSION

Dichloromethane leaf extract of *Croton nigritanus* indicated the signs/presents of some secondary metabolites such as; alkaloids, terpenes, and anthraquinones in a preliminary phytochemical study. Spectroscopic examination also revealed the presence of squalene as major compound using FTIR, ^{1}H NMR and GC-MS results. Squalene is currently being used as an adjunctive therapy in a variety of cancers, diabetes, ulcers, malaria and constipation. More research needs to be carried out on the isolation of squalene from different sources due to its lower availability in pharmaceutical industries.

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