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GC-MS Analysis and Phytochemical Screening of n-Hexane Fraction Constituents from the Leaf of *Clerodendrum volubile* P. Beauv.

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ABSTRACT:
The study investigated the bioactive principles from n-hexane fraction of the leaf of *Clerodendrum volubile* using phytochemical screening test and GC-MS techniques. The leaves of *C. volubile* plant material were harvested, air-dried for 72 h. The dried leaves were milled into powdered form using electric blender. Powdered plant material (60 g) was suspended in 3 L of absolute methanol for 48 h at 25^{o}C. The suspension was filtered through a double layer cheese-cloth. The extraction process was repeated five times until the extract became clear. The filtrates were pooled and concentrated under reduced pressure at 35ºC to give dark green residue. The total crude methanolic extract was stored in the desiccator until it was required for further processing. In GC-MS analysis, identification of the constituents was carried out by comparing the mass spectrum fragmentation pattern of each of the constituents with those stored in the data base of National Institute Standard and Technology 11 (NIST11.L) library. The results of the phytochemical screening gave a positive reaction for the presence of flavonoids, alkaloids, resins, tannins and phenols while glycosides, saponins, phlobatannins, sterols, carbohydrates and terpenoids were absent. Also, the GC-MS analysis of the leaf of the plant revealed the presence of six (6) bioactive compounds out of which three compounds were prominent. The prominent constituents of the plant were bicyclo [3.1.1] heptane, 2, 6, 6 –trimethyl-, 1-Hexadecyne and dodeca -1, 6 – dien – 12 – ol, 6, 10 – dimethyl. The study therefore concluded that the bioactive compounds identified in the leaf of *C. volubile* may be responsible for the ethnomedical or traditional use of this plant in the treatment and management of various diseases and also, it could be a plausible explanation for the reported biological activities exhibited by this plant. Hence, *Clerodendrum volubile* leaves could serve as a source for these bioactive principles which may be employed as ingredients for the formulation of novel drug.

KEYWORDS: GC-MS analysis, Phytochemical Screening, *Clerodendrum volubile,* Bioactive Principles.

INTRODUCTION

The role of medicinal plants cannot be overemphasized in the treatment and management of various diseases such as cancer, diabetes, tuberculosis, hypertention, hypercholesterolemia and inflammatory disorders. Medicinal plants contained various bioactive compounds that may act singly, additively or synergistically to impove health [1]. A plant may contain constituents characterized with bitter taste which can enhance digestion, It may also made up of anti-inflammatory, anti-oxidant, anti-bacterial and anti-fungi bioactive principles that can ameliorate swelling and pains, scavenge free radicals, inhibit the growth of bacteria and fungi respectively [1].

Report had shown that plant-based drugs are cheap, readily available and possess little or no side effect compare to synthetic drugs or modern medicines [2]; [3] and [4]. Examples of such plants includes: *Carica papaya, Hydnocarpus gaertn, Cedrus species (cedar), Cupressus sempervirens (cypress), Glycyrrhiza glabra (licorice)* and *Clerodendrum volubile* [5]; [1] and [6].

Clerodendrum volubile belongs to the Family *Verbenaceae*. It is a climbing shrub of about 3m high. The flowers are numerous (1½cm long) and greenish-white. It is found in deciduous forest and secondary jungle. It spans from Senegal to Fernando Po [7]. The macroscopic study showed that *C. volubile* has a simple leaf with a reticulate venation, a cuminate apex and a cureate base. The leaves are green with a blond odour [8]. *Clerodendrum volubile* plant is commonly known as White butterfly, *Marigbo, Obenetete, Ebenote, Nruchinta and Furudugu* in English, Yoruba, Urhobo and Itsekiri, Edo, Igbo and Sierra Leone respectively.

Ethnomedically, the leaves had been reported to be used in treating life-threatening diseases such as arthritis, rheumatism, dropsy and swellings [9]. The leaves are also widely used as anti-abortifacients, analgesics, general body healing and sedatives [7]. Locally, the leaves are often dried and used as spices in cooking. Ogunwa *et al.* [10] evaluated the nutritional value of *Clerodendrum volubile* (Marugbo) leaves. The antiproliferative effect of the fatty acid components of *Clerodendrum volubile* leaves as well as its antioxidant effect on MCF-7 and MDA-MB-231 human breast cancer cell lines were investigated by Erukainure *et al.* [11], Stephen and Ganiyu [12] characterized the interaction of phenolic (free and bound) extracts from white butterfly (*Clerodendrum volubile* P. Beauv) leaves

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with key enzymes relevant to non-insulin dependent diabetes mellitus and hypertension and their antioxidant properties *in vitro*. Akinpelu *et al*. [6] investigated the effect of ethanolic leaf extract of *Clerodendrum volubile* on lipid profile of hyperlipidemic Wistar rats Hence, the present study investigated the bioactive principles in the leaf of *Clerodendrum volubile* using phytochemical screening test and GC-MS techniques.

MATERIALS AND METHODS

PLANT MATERIALS

Fresh leaves of *Clerodendrum volubile* were collected from Fashina Village, Ife-Central Local Government, Ile-Ife, Osun State. The plant material was identified and authenticated at IFE HERBARIUM by Mr. Ademoriyo, Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The specimen sample was deposited at IFE herbarium with specimen identification number IFE 17506.

PREPARATION OF THE EXTRACT

The leaves of *C. volubile* plant were harvested and air-dried for 72 hours. The dried leaves were milled into powdered form using electric blender. Powdered plant material (60 g) was suspended in 3 L of absolute methanol for 48 h at room temperature. The suspension was filtered through a double layer cheese-cloth. The extraction process was repeated five times until the extract became clear. The filtrates were combined and concentrated under reduced pressure on Edwards High Vacuum Pump Rotatory Evaporator (Edward Vacuum Co-operation, Crawley, England) at 35^{o}C to give dark green residue. The total crude methanolic extract was stored in the dessicator until required for further processing.

PARTITIONING OF CRUDE EXTRACT

The methanol extract was partitioned based on the procedure of Adeoye and Oyedapo [13]. The extract (2.0 g) was dissolved in distilled water (150 ml) and partitioned with n-hexane (200 ml x 3). The n-hexane fraction obtained was concentrated in vacuo on rotary evaporator (Edwards Vacuum Components, Crawley England) at 35^{o}C to obtain n-hexane fraction which was used for the GC-MS analyses.

PHYTOCHEMICAL SCREENING OF N-HEXANE FRACTION

Phytochemical screening of n-Hexane fraction of the leaf of *C. volubile* was carried out based on procedure of Oyedapo *et al.* [14], Trease and Evans [15] and Sofowora [16].

TEST FOR ALKALOID

Acidic solution of the extract was prepared by dissolving 50 mg of the extract into 10 ml of 10 % (v/v) HCl, heated and filtered. To 1.0 ml of the filtrate in separate test tubes was added, 1.0 ml of Mayer's reagent, Dragendorff reagent and Wagner's reagent. The mixtures were examined for colour change, turbidity or formation of precipitate. Equal volume of 10 % (v/v) HCl was used as parallel control.

TEST FOR FLAVONOIDS

The extract (0.05 g) was dissolved in 5.0 ml of distilled water and filtered. To 1.0 ml of the filtrate, few drops (2-3) of ethanolic potassium hydroxide solution were added. The formation of suspension, cloudiness or precipitate was taken as the evidence of flavonoids.

TEST FOR TANNINS

The extract (0.05 g) was dissolved in 20 ml of distilled water in separate test tubes and filtered. To 1.0 ml of the filtrate was added few drops (2-3) of 0.1% ferric chloride ($FeCl_3$) in glacial acetic acid solution. The mixture was examined for the formation of blue, brownish green or blue-black precipitate.

TEST FOR CARDIAC GLYCOSIDE

C. volubile extract (0.5 g) was extracted with 2 ml chloroform and filtered into a clean test tube. Concentrated sulphuric acid was carefully added to form a lower layer. A reddish brown colour ring at the chloroform/sulphuric acid interphase indicated the presence of a steroidal ring or glycine of the cardiac glycosides.

TEST FOR TRITERPENOIDS

The extract (20 mg) was suspended in chloroform (10 ml), slightly warmed in water bath and filtered. To the chloroform layer was added concentrated sulphuric acid and mixed properly. The appearance of red colour indicates the presence of triterpenes.

TEST FOR SAPONINS (FROTHING TEST)

The extract (0.1 g) was suspended in water in a test tube, shaken vigorously and checked for froth. It

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was warmed gently at 50°C for 10 min and shaken vigorously again. Frothing which persisted on warming was a preliminary evidence of the presence of saponins.

TEST FOR STEROIDS
Acetic anhydride (2 ml) and 2 ml of H_2SO_4 was added to 0.5 g extract of *C. volubile*. The mixture was shaken vigorously. Colour changes from violet to blue or green indicates the presence of steroids.

TEST FOR RESINS
Two and a half of Copper (II) Sulphate solution was added to 2.5 ml of the extract. The resultant mixture was shaking vigorously and allowed to settle. A green colour indicated positive test.

TEST FOR PHLOBATANNINS
Five (5) ml of distilled water was added to 5 ml of extract solution and boiled with 1%HCl for two minutes. A deep green colour indicates a positive test.

TEST FOR PHENOLS
Equal volumes of the extract solution and $FeCl_3$ were mixed. A deep bluish green solution confirmed the presence of phenols.

TEST FOR CARBOHYDRATE (FEHLING TEST)
Five (5) ml of the mixtures of equal volume Fehling solution A and B were added to 2 ml of the extract in a test tube. The resultant mixtures were boiled for two minutes. A brick red precipitate of copper oxide indicates a positive test.

GC-MS ANALYSIS
The gas chromatography coupled with mass spectrometer (GC-MS) was carried out on a Hewlett Packard Agilent gas chromatography (Model19091J-413:3516.156884, USA) fitted with flame ionization detector and Hewlett Packard mass spectrophotometer 5975C series injector, MS transfer line temperature of 300°C. The GC was coupled with a capillary column-Agilent J HP-5MS (length; 30 m x 250µm; film thickness 0.25µm) treated with phenyl methyl silox. Helium gas was used as a carrier gas (99.999% purity) at a constant flow rate of 1.504 ml/min. Samples was dissolved in acetone and 1µl (split ratio of 30:1; split flow of 45.12 ml/min) injected automatically into the column with the injector temperature set at 300°C. The ion source temperature was 230°C. The gas chromatography (GC) oven was programmed from 45°C (isothermal for 2 min.), with an increase of 0.5°C/min., to 58°C, then 1.5°C/min. to 160°C ending with a 5minutes isothermal temperature at 260°C. The mass spectrum of compounds was taken by ionization energy at 70 eV; and its detector was operated in scan mode from 50 – 600amu (atomic mass unit). A scan interval of 5 minutes and fragment from 50 – 600 Da was maintained. The test was run in triplicate. The total running time was 96 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

IDENTIFICATION OF THE COMPONENTS
The interpretation of each of the mass spectra from GC-MS analysis was carried out using the database of National Institute Standard and Technology 11 (NIST11. L) Library; an online library having more than 62000 patterns. The spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST11 library. The name, molecular weight, molecular formula and the structure of the test materials were ascertained.

RESULTS AND DISCUSSION
Medicinal plants are important source of synthetic and herbal drugs. Virtually all medicinal plants have bioactive principles which are responsible for most of the biological activities they exhibit. These bioactive principles are commonly referred to as phytochemicals. Phytochemicals, also known as plant chemicals are bioactive substances synthesized by plant which are actively associated with protection of human health against chronic degenerative diseases [17]. They are present in fruits, beans, vegetables, grains and extract of plants. Examples of these plant chemicals are tannins, cardiac glycosides, resins, saponins, flavonoids, phenols, carbohydrates, steroids, phlabotannins, alkaloids and terpenoids. Tannins are polyphenolic compounds of plant origin which could be hydrolysable or condensed tannins [18]. Anti-nutritive effects of tannins had been reported to be associated with dietary proteins, polymers (such as cellulose, pectin and hemicelluloses) and minerals thus retarding their digestion. They can also impair digestive processes by forming complex with enzymes and endogenous proteins [18].

Glycosides are tetracyclic steroid with an attached unsaturated lactone ring that may have 5 or 6 members. It possesses a sugar moiety which is usually linked via the C-3 hydroxyl group or the aglycone. Glycosides have been used as drugs for the treatment of cardiac insufficiency [19]. Moreover, flavonoids are hydrophilic (water soluble) molecules containing 15 carbon atoms which can be visualized as two benzene rings which are linked together with a short three carbon chain. Flavonoids are

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involved in scavenging of oxygen derived free radical [20]. It has been identified as potent hypolipidemic agents in a number of studies [21]; [22]. It has also been reported that flavonoids from medicinal plants or extract of plants exhibit a high antioxidant potential which could be attributed to their hydroxyl group and protect more efficiently against free radical related diseases like arteriosclerosis and cardiovascular diseases [23]; [24]. In addition to this, flavonoids enhance vaso-relaxant process [25] and prevent platelet-related thrombosis [26].

Furthermore, alkaloids are a group of complex nitrogen-containing compounds derived from a variety of sources, including microbes, marine organisms and plants, via complex biosynthetic pathways [27]. Alkaloids are used in a wide range of medicinal complaints, for example as anticancer agent, analgesics, and antimalarial and in the treatment of hypertension, Parkinsonism and central nervous system disorders [28]. Also, phenols are very important phytochemicals because of their ability to scavenge free radicals owing to their redox properties which permit them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [29]. Resins are liposoluble substances that are secreted by plants which consist essentially a volatile compound called essential oil and a non-volatile, long-chain terpenoids. Resins find its use since time immemorial as constituents of cosmetics, varnishes, adhesives and as incense in ritual ceremonies in temples and churches [30]; [31]; [32]. However, in this study, the phytochemical screening of n-hexane fraction of the leaf of *Clerodendrum volubile* revealed the presence of tannins, resins, flavonoids, phenols and alkaloids, while glycosides, saponins, Phlobatannins, steroids, carbohydrate and terpenoids were absent (Table 1).

GC-MS is an analytical tool in which the building block consists of gas chromatography and mass spectrometer to detect different substances within a test sample [33]. It had been employed in drug detection, fire and explosive investigation, airport security to detect hidden substances in luggage, baggage or in human beings [33]. Moreso, this technique is a vital tool in the hand of forensic toxicologist in the detection of drugs or poisons in the biological specimens of suspects, victims, or deceased [33]; [34]. In addition, gas chromatography interfaced with mass spectrometer (GC-MS) is an established technique for adequate, effective and reliable identification of bioactive principles inherent in medicinal plants or extract of plants including pesticides, alcohol, aldehydes, esters, terpenes and fattyl acid [35];[36]. However, in this study, the GC-MS analysis of n-hexane fraction of the leaf of *C. volubile* P.
Beauv. was carried out. The chromatogram of the GC-MS analysis of the plant fraction indicating total ion concentration (TIC) is shown in Figure 1.0.

The compounds detected from each of the mass spectra fragmentation patterns are highlighted in Table 2.0. The hexane fraction of the leaf of *C. volubile* plant essentially contains alcohols, aromatic bicyclics, hydrocarbon and organoheterocyclic compounds.
A total of six (6) peaks were shown by the GC-MS chromatogram of the hexane fraction indicating the presence of 6 bioactive compounds (Table 2.0) which consist of three prominent compounds (Table 3.0) and three (3) minor phytoconstituents. The three prominent compounds constitute 83.75% of the plant hexane fraction. The 3 major compounds with their pencentage abundance are Bicyclo [3.1.1] heptane, 2, 6, 6-trimethyl (56.27%), 1-Hexadecyne (16.91%) and Dodeca-1, 6-dien-12-ol, 6, 10-dimethyl- (10.57%). These major compounds were indicated by peaks 4, 6 and 5 with retention times of 92.086, 94,825 and 93.731 respectively. Moreso, Bicyclo [3.1.1] heptane, 2, 6, 6-trimethyl which is synonymous to Dihydropinene and Pinane appeared to be the most prominent compound among the three prominent compounds (Table 3.0).

Furthermore, the compound name, compound nature and reported biological activity of phytocompounds identified in the hexane fraction of the leaf of *C. volubile* plant were explored (Table 4.0). The Bicyclo [3.1.1] heptane, 2, 6, 6-trimethyl had been reported to exhibit antimicrobial activities such as antifungal and antibacterial [37]. Also, Dodeca-1, 6-dien-12-ol, 6, 10-dimethyl- had been documented to possess antioxidative potential [38]. In addition to this, hexadecane which is one of the minor compounds identified in the hexane fraction of this plant with concentration (% peak area)-6.84 possess various biological activities (Table 4.0).

This compound had been well documented to exhibit antifungi, antibacterial, antioxidant, antitumor and anaphylactic activities [42]. Reports had also revealed that hexadecane could be actively involved in cognition disorder treatment [39]. Moreso, hexadecane could also serve as nicotinic alpha 6 beta 3 beta 4 alpha 5 receptor antagonist, albendazole monooxygenase inhibitor, alpha 2 beta 2 receptor, ovulation inhibitor, platelet aggregation stimulant, kidney function stimulant, PFA-M 1 amino peptidase inhibitor, leucopoiesis inhibitor, CYP2A8 substrate, chloride peroxidase inhibitor, taurine-2-oxoglutarate transaminase inhibitor,

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hydroxytryptamine uptake stimulant and (S)-6-hydroxynicotine oxidase inhibitor. However, the biological activity of 2 (4H)- Benzofuranone,5,6,7, 7a-tetrahydro-4, 4, 7a-trimethyl-, (R)- and Tridecane, 7-propyl- have not been reported.

Table 1.0: Phytochemical Constituents of n-Hexane Fraction of the Leaf of *C. volubile* P. Beauv.

Phytochemical	Results
Tannins	+ +
Glycosides	-
Resins	+ +
Saponins	-
Flavonoids	+ +
Phlobatannins	-
Sterols	-
Phenols	+
Carbohydrates	-
Alkaloids	+ +
Terpenoids	-

Key: (+) = moderately present, (+ +) = highly present, (-) = absent

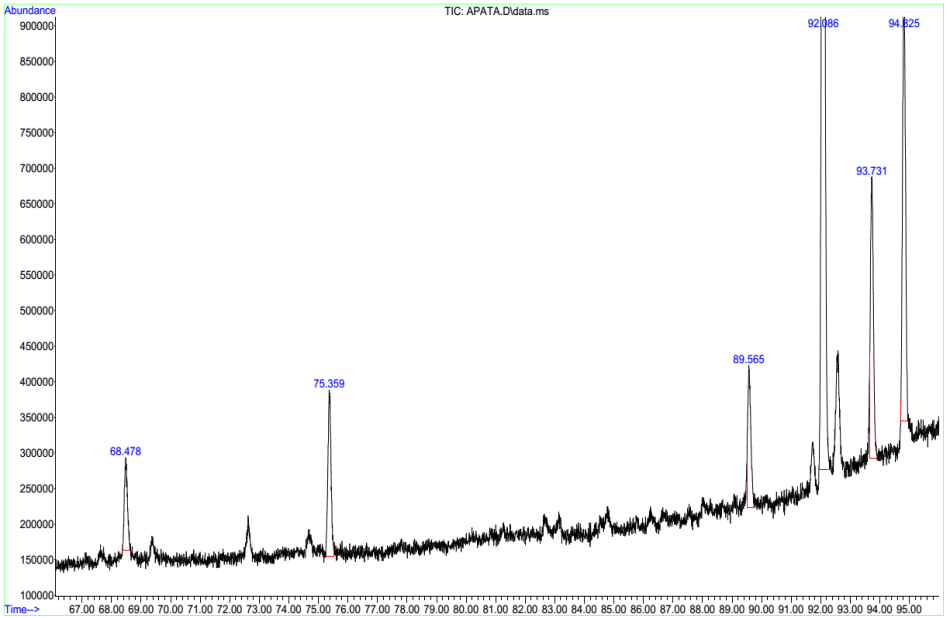

Figure 1.0: GC-MS Chromatogram of n-Hexane Fraction of the Leaf of *Clerodendrum volubile* P. Beauv.

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Table 2.0: Bioactive Compounds Detected in Hexane Fraction from the Leaf of *Clerodendrum volubile* P. Beauv.

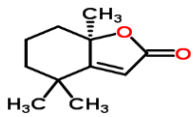
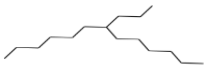
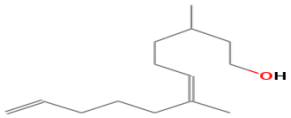
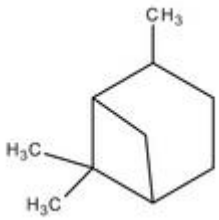
/No	Retention Time	Peak Area (%)	NIST Matching (%)	Compound Name	Molecular Formular	Molecular Weight	Molecular Structure
1	68.478	3.66	87	2 (4H)-Benzofuranone,5,6,7, 7a-tetrahydro-4, 4, 7a-trimethyl-, (R) **Synonyms:** Dihydroactinidiolide	$C_{11}H_{16}O_2$	180.2435	
2	89.565	5.75	87	Tridecane, 7-propyl-	$C_{16}H_{34}$	226.4412	
3	75.359	6.84	91	Hexadecane	$C_{16}H_{34}$	226.448	
4	93.731	10.57	49	Dodeca-1, 6-dien-12-ol, 6,10-dimethyl-	$C_{14}H_{26}O$	210.3556	
5	94.825	16.91	70	1-Hexadecyne	$C_{16}H_{30}$	222.416	
6	92.086	56.27	64	Bicyclo [3.1.1] heptane, 2, 6, 6- trimethyl- **Synonyms:** Dihydropinene, Pinane	$C_{10}H_{18}$	138.25	

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Table 3.0: The Three Prominent Bioactive Compounds Identified in the Hexane Fraction from the Leaf of *Clerodendrum volubile* P. Beauv.

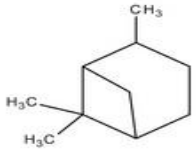
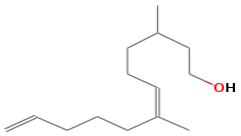
PK/No	Retention Time	Peak Area (%)	NIST Matching (%)	Compound Name	Molecular Formular	Molecular Weight	Molecular Structure
4	92.086	56.27	64	Bicyclo [3.1.1] heptane, 2, 6, 6- trimethyl- **Synonyms:**Dihydropinene, Pinane	$C_{10}H_{18}$	138.25	
6	94.825	16.91	70	1-Hexadecyne	$C_{16}H_{30}$	222.416	
5	93.731	10.57	49	Dodeca-1, 6-dien-12-ol, 6,10-dimethyl-	$C_{14}H_{26}O$	210.3556	

Table 4.0: Bioactive Compounds Identified in Hexane Fraction of the Leaf of *Clerodendrum volubile* and their Reported Biological Activities

S/No	Compound Name	Nature of Compound	Reported Biological Activity	References
1	2 (4H)- Benzofuranone,5,6,7, 7a-tetrahydro-4, 4, 7a-trimethyl-, (R)- Synonyms: Dihydroactinidiolide	Organoheterocyclic compound	No bioactivity reported	
2	Tridecane, 7-propyl-		No bioactivity reported	
3	Hexadecane	Alkane hydrocarbon	Cognition disorders treatment, Nicotinic alpha6beta3beta4alpha5 receptor antagonist, Albendazole monooxygenase inhibitor, alpha2beta2 receptor antagonist, Leukopoiesis inhibitor, CYP2A8 substrate, Chloride peroxidase inhibitor, Taurine-2-oxoglutarate transaminase inhibitor, PfA-M1 aminopeptidase inhibitor, Ovulation inhibitor, Hydroxytryptamine uptake stimulant, Platelet aggregation stimulant, (S)-6-hydroxynicotine oxidase inhibitor, Kidney function stimulant, Antifungi, Antibacterial and Antioxidant activities, Antitumor, Anaphylactic activity.	[39], [40], [41], [42]
4	Dodeca-1, 6-dien-12-ol, 6,10-dimethyl-	Alcoholic compound	Antioxidative potential	[38]
5	1-Hexadecyne (Tetradecylacetylene)	Acetylenic hydrocarbon	Antibacterial	[37]
6	Bicyclo [3.1.1] heptane, 2, 6, 6- trimethyl- **Synonyms:**Dihydropinene, Pinane, Terpene	Aromatic byclics	Antimicrobial	[43]

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CONCLUSION

The preliminary phytochemical screening gave a positive reaction for the presence of phytochemical constituents which exhibit anti-oxidant, anti-inflammatory, anti-malarial and analgesic activity. The GC-MS analysis of n-hexane fraction of the leaf of *C. volubile* afforded three prominent compounds with Bicyclo [3.1.1] heptane, 2, 6, 6-trimethyl- appearing to be the most prominent compound, and three minor compounds including hexadecane. These compounds had been reported to exhibit various biological activities; hence hexane fraction of the leaf of *C. volubile* could serve as a source for these bioactive principles which might be employed as ingredients for formulation of novel drug. Also, the bioactive compounds identified in the leaf of *C. volubile* could be responsible for the ethnomedical or traditional use of this plant in the management and treatment of various disease and their attendant complications.

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