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Der Pharma Chemica, 2010, 2(1): 261-272
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X

GC-MS Evaluation of Bioactive Compounds and Antibacterial Activity of the Oil Fraction from the Leaves of *Alstonia boonei* De Wild

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Abstract

The ethanolic extract of the leaves of *Alstonia boonei* yielded yellow oil (2.38g). The oil was subjected to GC-MS studies. Fifteen phyto-constituents were identified with Ascorbic acid 2,6-hexadecanoate (17.27%) constituting the bulk of the oil, followed by 9-Octadecenoic acid, 1,2,3-propanetriyl ester (12.89%). Other monoterpenoids, oxygenated compounds and fatty acids identified include Octadecanoic acid (10.31%), Octadecanoic acid methyl ester (9.02%), Benzoic acid thio (7.73%), 9-Octadecene (E) (6.44%), 2,3-Diazabicyclo [2.2.1] hept-2-ene, 4-methyl-1-(Pent-4-en-1-yl) (5.15%), 1,2-Oxathiane, 6-Dodecyl-2,2-dioxide (3.87%) while 1-Undecene-9-methyl and 1-Heptanol-6-methyl respectively contained 2.58%. n-Decanoic acid contained 1.55% while 1-Hexanol, 5-methyl and Bicyclo [3.1.1] heptane 2,6,6-trimethyl constitutes 1.29% of the oil respectively. The volatile oil showed antibacterial activity against *Escherichia coli*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Proteus mirabilis*. These results lend credence for the use of oil extract from the leaves of *Alstonia boonei* in the treatment of asthma, coughs and wounds in herbal medicine in Nigeria.

Keywords: *Alstonia boonei*, Bioactive compounds, essential oils, Antibacterial activity, herbal medicine.

Introduction

Nigeria is richly endowed with plants which are used as both food and medicine. These plants have various effects on living systems. They are sedative, analgesic, cardio-tonic, anti-inflammatory, oxytocic, anti-spasmodic and immune modulators [1]. The utilization of plants against diseases such as cancer, jaundice, typhoid, fibroid, malaria, gonorrhea, syphilis, hypertension and tumor growth are thought to be derived from the presence of the chemicals available in the plants [2]. These plant chemicals are classified as primary or secondary metabolites. The primary metabolites include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids and chlorophyll. Secondary metabolites are the

remaining plant chemicals which are produced from the primary metabolites. These include alkaloids (derived from amino acids), terpenoids (a group of lipids), phenolics (derived from carbohydrates), tannins, steroids and volatile oils [3].

One of these plants with medicinal values is *Alstonia boonei* De Wild (Devil tree) Apocynaceae a medicinal plant that is widely used in Africa for the treatment of various ailments. Numerous therapeutic properties have been attributed to *A. boonei*, like antifungal, antibacterial, antiviral, anti-thrombosis, anti-tumor, anti-inflammatory, analgesic, antioxidant and antipyretic activities [4,5]. The stem bark is commonly used in treating malaria, toothache and rheumatism [6,7]. The stem bark is used as anti-venom for snake bites [5]. The latex or exudates from the plant is used in treating coughs, throat sores and fever [1]. Infusion of the roots and stem barks is taken as a remedy for asthma. Extracts from the stem barks and leaves is drunk to treat impotence in women. The extracts are also administered to women after child delivery, to help in expelling the placenta [5]. It is applied topically to reduce oedema and to clear sores, wounds and exposed fractures [5]. The antibacterial activities of some other members of the same family have been reported [8]. Other members such as *Alstonia scholaris* and *Razia stricta* are used in traditional medicine for the treatment of diabetes mellitus, skin infections and stomach disorders. The chloroform and methanol extracts from the roots showed activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* [8]. However, very little is known about the chemical constituents of *A. boonei* and so far there have been few previous phytochemical investigations which reported the isolation and structural elucidation of alkaloids such as echitamine [6]. The plant contains poisonous alkaloids comprising ditamine. echitamine and echitamidine [8].

As part of our chemical studies on Nigeria medicinal plants we describe herein the chemical constituents of the volatile oil and lipid content of *A. boonei* and consequently evaluate the antibacterial activity of the oil against some pathogenic bacteria for possible development of new drugs for the prevention and treatment of infections.

Results and Discussion

The yellow oil (2.38 g) obtained from ethanol extract of the leaves of *A. boonei* showed fifteen peaks from the chromatogram of the oil (Figure **1**). These peaks indicate the presence of fifteen compounds (**1-15**) in the oil (Figure **2**). The molecular formula, percentage constituents and molecular mass of the compounds is shown in Table **1**. These compounds comprise mainly hydrocarbons, fatty acids, alcohols, thiols, esters and alkaloids. The composition of the oil comprises; esters (54.64%), fatty acids (14.44%), thios and sulphones (11.60%), hydrocarbons (10.31%), alkaloids (5.15%) and alcohol (3.87%).

Compound **1** was identified as thiobenzoic acid and has molecular formula of C_7H_6OS (m/z 138) with base peak at m/z 105. It comprises 7.73% of the oil. The base peak m/z 105 occur due to alpha cleavage of SH group from the compound. Compound **2** contains 1.29% of the oil with molecular formula $C_7H_{16}O$ (m/z 116) and base peak at m/z 43 which occurred due to the detachment of a propyl fragment C_3H_7 (m/z 43) from the compound. Compound **3** has molecular formula $C_8H_{18}O$ (m/z 130) and base peak at m/z 55. The compound was identified as 6-Methyl-1-heptanol comprising 2.58% of the oil. Compound **4** is a hydrocarbon 9-Octadecene (E) with molecular formula $C_{18}H_{36}$ (m/z 252). The constituent was 6.44% of the oil. Compound **5** was identified as n-Decanoic acid with molecular formula $C_{10}H_{20}O_2$ (m/z 172) and base peak at m/z 73. The base peak occurred as a result of the detachment of

$C_3H_5O_2$ (m/z 73) fragment from the compound. It comprises 1.55% of the oil. Compound **6** is 9-Methyl-1-undecene with molecular formula $C_{12}H_{24}$ (m/z 168) and comprises 2.58% of the oil. Compound **7** was identified as Bicyclo-[3.1.1]-heptane-2,6,6-trimethyl and with molecular formula $C_{10}H_{18}$ (m/z 138). It comprises 1.29% of the oil. Compound **8** was identified as 1-Methyl-4-(4-pentenyl)-2,3-diazobicyclo-[2.2.1]-hept-2-ene has molecular formula $C_{11}H_{18}N_2$ (m/z 178) and it comprises 5.15% of the oil. Compound **9** has molecular formula $C_{38}H_{68}O_8$ (m/z 652) and comprises 17.27% of the oil. The base peak occurred at $C_3H_5O_2$ (m/z 73). This peak occurred due to McLafferty re-arrangement. Other prominent peaks observed on the compound occurred at m/z 43 ($C_3H_7^+$) and m/z 41 (C_3H_5). These peaks occurred due to proton migration and rearrangement. Compound **9** was identified as L-(+)-Ascorbic acid- 2,6-dihexadecanoate. Compound **10** was identified as methyl (7E)-7-Octadecenoate with molecular formula $C_{19}H_{36}O_2$ (m/z 296). It comprises 12.89% of the oil. Compound **11** has a molecular formula of $C_{19}H_{38}O_2$ (m/z 298). It was identified as methyl n-Octadecanoate and it comprises 9.02% of the oil. Compound **12** was identified as 2,3-Bis-(9E)-9-octadeconoate with molecular formula $C_{57}H_{104}O_6$ (m/z 884) and it comprises 15.46% of the oil. Compound **13** has molecular formula $C_{18}H_{36}O_2$ (m/z 284) and comprises 10.31% of the oil. It was identified as Octadecanoic acid. Compound **14** has molecular formula $C_{16}H_{30}O_4$ (m/z 286) and it was identified as Hexadecane-1,16-dioc acid. It comprises 2.58% of the oil. Compound **15** has molecular formula $C_{16}H_{32}O_3$ (m/z 304) and was identified as Delta-hexadecane sultone. It comprises 3.87% of the oil.

Organosulphur compounds (OSCs) prevent or slow down the carcinogenic process induced by a variety of chemical carcinogens [10]. OSCs offer protection against cancer. These include inhibition of the carcinogens, dermatitis and other minor wounds [11]. The occurrence of thiobenzoic acid and L-(+)-Ascorbic acid 2,6-dihexadecanoate in the leaves of *A. boonei* may be the reason behind the use of the extracts in the treatment of wounds in herbal medicine in Nigeria [5]. Natural ascorbic acid is crucial for the body performance. It possesses anti-scorbutic activity. Ascorbic acid in the body helps in absorption from the intestine [5]. It is required for connective metabolism especially the tissue, bones and teeth [5]. It is necessary as anti-stress and protects against colds, chills and dumps. It prevents muscle fatigue and scurvy which is characterized by the hemorrhages, bleeding gums, fragile bones, anemia and pains in the joints and defects in skeletal calcification. This function of ascorbic acid also accounts for its requirement for normal wound healing [12]. This also supported the use of *A. boonei* in treating wounds by the natives in Nigeria. Ascorbic acid and OSCs act as antioxidant in the skin by scavenging and quenching free radicals generated by ultraviolet (UV) radiation stabilization. Ascorbic acid is an important antioxidant. It act as an electron donor for eight important enzymes in humans [5]. Ascorbic acid may protect against the oxidative damage of light in the eye [13] and may also play an important role in sperm maturation [14]. It helps in stabilizing various plasma components and has been shown to be an effective scavenger of super oxide radical anion (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($O^{\cdot}$) and reactive nitrogen oxide (NO) [15].

Fatty acids always occur in plants. The presence of fatty acids, aromatics and alkaloids in *A. boonei* shows the pharmacological properties of the plant. Fatty acids and alcohols in the plant undergo esterification reaction to form the esters. One or both of the oxygen atoms of carboxylic acid can be replaced by sulphur giving a thio acid or dithio acid respectively. Thio acids react readily with alcohols to form thio esters. Thio esters play an important part in the break down and synthesis of lipids and steroids in living tissues. Carboxylic acids are transferred from one enzyme reaction to another as thio-esters of the complex thiol, Co enzyme A (CoA-SH). The thio-ester of benzoic acid with Co-enzyme A is the form in which

acetate enters the sequence of enzyme catalyzed reactions which results in the synthesis of fatty acids and glycerides[16]. Insecticidal and germicidal activities of the leaves of *A. boonei* may be due to the synergetic effect of these chemical constituents or any single chemical compound may have toxic effect. The dominant component of the essential oil of this plant L-(+)-Ascorbic acid 2,6-dihexadecanoate has been reported to have antioxidant, anti-inflammatory and anti-nociceptive properties [5,12]. Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agent for its analgesic, antispasmodic and antibacterial properties [17]. Alkaloids exhibit marked physiological activity when administered to animals. Most of the plants used in the cure of diseases have been reported to contain traces of alkaloids. For instance *Azadiracheta indica* (Meliaceae) is employed in the treatment of malaria and fever in Nigeria folk medicine. The plant contains gedunin which has anti-malaria properties [18,19]. The roots of *Eurycoma longifola* Jack (Simaroubaceae) contain canthin-6-one alkaloid, a quassinoid eurycoma-anone and 7-Methyl-b-carboline-1-proprionic acid. However, only the last two showed anti-malarial activity [18]. *Cinchona* bark consists of various species, races and hybrids of *Cinchona*. These plants produce quinine-type alkaloid. Quinine is used for the treatment of malaria [19]. The presence of alkaloid 1-Methyl-4-(4-pentenyl)-2,3-diazabicyclo [2.2.1] hept-2-ene from the leaves of *A. boonei* investigated suggest that the plant has medicinal properties. This lends credence for the use of the leaves of *A. boonei* for the treatment of malaria by the natives in Nigeria [4].

The oil from the leaves of *A. boonei* successfully inhibited *E. coli, S. pneumoniae, P. mirabilis* and *S. aureus* (Table 2). Many of these organisms are natural flora of the skin and also known etiologic agent of several skin and mucous membranes infections of man [20,24]. The result obtained from this study show that the oils from the leaves of *A. boonei* showed inhibition towards pathogenic bacteria (*E. coli, S. aureus* and *S. pneumoniae*). These findings confirm the traditional therapeutic claims for the use of this plant for the treatment of cough, asthma, sores and wounds [5]. Wounds and boils provide environment conducive for the growth of microbial organisms. Usually, microbial contaminants of wounds and boils involve a variety of organisms such as *P. aeruginosa*, *S. aureus* and *E. coli* [21,24]. Evaluation of the effects of the oils from the leaves of *A. boonei* on these clinically isolated microbial contaminants of wounds and boils showed varying levels of inhibitory activity against these pathogens (Table 2). Microbial infection of wounds delays healing which can lead to further tissue injury and damage [21,24]. The antimicrobial activity of the oils on these wound pathogens may contribute to wound healing, eliminate infections, thereby resulting to cell proliferation [21,24]. *P. aeruginosa, E. coli* and *S. aureus* are common human commensals and have been incriminated in the infection of wounds [22]. These findings also justify the application of *A. boonei* oils in dermatological creams and soaps and indicate that effective skin protection could be achieved at very low concentrations. The leaves of *A. boonei* possess phyto-constituents capable of inhibiting the growth of microbial wound contaminants; accelerate wound healing and consequently resulting to cell proliferation.

These findings justifies the use of *A. boonei* in the treatment of skin infections such as boils, carbuncles, breast abscess, sores and wound treatment in herbal medicine in Nigeria.

Materials and Methods

General experimental procedure

GC analysis were carried out in SHIMADZU JAPAN gas chromatography 5890-11 with a fused GC column (OV-101) coated with polymethyl silicon (0.25nm x 50m) and the conditions were as follows: Temp programming from 80–200^{o}C held at 80^{o}C for 1 min, rate

5°C/min and at 200°C for 20 min. FID temp 300°C, injection temp 250°C, carrier gas nitrogen at a flow rate of 1 ml/min, split ratio 1:75. GC–MS [Gas Chromatography Mass Spectrum] analysis was conducted using GCMS – QP 2010 PLUS SHIMADZU JAPAN with injector temperature of 230°C and carrier gas pressure of 100 kpa. The column length was 30 m with a diameter of 0.25 mm and the flow rate of 50 ml/min. The elutes were automatically passed into a mass spectrometer with a dictator voltage set at 1.5kv and sampling rate of 0.2 sec. The mass spectrum was also equipped with a computer fed mass spectra data bank. HERMLE Z 233 M-Z centrifuge Germany was used. Reagents and solvents like ethanol, chloroform, diethyl ether, hexane, were all of analytical grade and were procured from Merck, Germany. The nutrient agar was purchased from Scharian chemie (APHA) Spain.

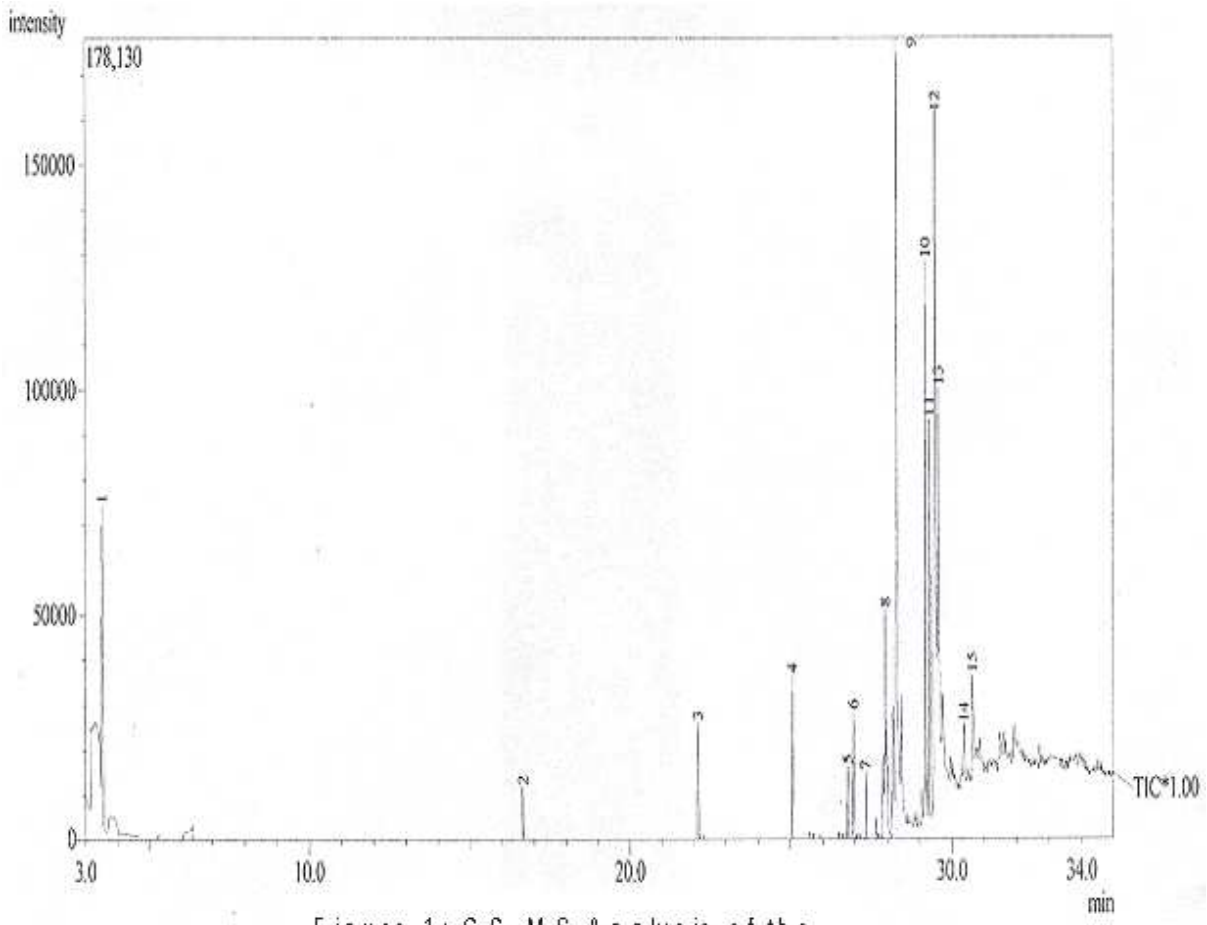


Figure 1: GC-MS Analysis of the oil from *A. Boonei*

Plant Materials

Fresh leaves and stem barks of *A. boonei* were collected on 20th January 2008, from Umudike, Abia State, Nigeria. Plant materials (stems and leaves) were identified by Dr. A Nmeregini of Taxonomy Section, Forestry Department, Michael Okpara University of Agriculture, Umudike, Nigeria. Voucher specimen No AS/250 has been deposited at the Forestry Department of the University.

Extraction of Plant Materials

The leaves (1kg) of *A. boonei* were cleaned with pure water and dried on the laboratory bench for 10 days. The dry sample was milled and ground into powder (800 g) using a

Thomas Wiley Machine (Model 5 USA). The powdered plant sample (300 g) was successively extracted with 2 L of benzene (8 hours/3 times/80^{o}C) followed by 2 L of ethanol (8 hours/3 times/65^{o}C). The extracts were concentrated under reduced pressure and the supernatant yellow oil was decanted (2.38 g) after complete removal of the solvent. The oil was centrifuged at 10,000 rpm for 20 minutes and the clear supernatant oil (1 µl) was subjected to systematic GC and GC-MS analysis.

Component Identification

Oil components were identified by matching the peaks with Computer Wiley MS libraries and confirmed by comparing mass spectra of the peaks and those from literature [11,23-24].

Bioassay

The *in vitro* antibacterial activity of the oil was carried out for 24h culture of four selected bacteria. The bacteria organisms used were *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae* and *Proteus mirabilis*. All the test organisms are clinical isolates of human pathogens obtained from the Federal Medical Centre (FMC) Umuahia, Nigeria. Cultures were brought to laboratory conditions by resuscitating the organism in buffered peptone broth (Scharian chemie) and incubated at 37^{o}C for 24 hours. The antibacterial activity was performed by filter paper disc diffusion technique. The medium (7 g nutrient agar in 250 ml distilled water, autoclave at 115^{o}C for 15 mins) was cooled to 50^{o}C. 20 ml of the medium was poured into a sterile Petri-dish and allowed to solidify. It was allowed to stay for 8 hours and observed for contamination. The oil (1 g) was dissolved in 1 ml of absolute ethanol and made up to 10 ml with distilled water to a concentration of 100 mg/ml (10% dilution), 50mg/ml. 25mg/ml, 12.5mg/ml 6.5mg/ml respectively. A colony of each test organism was sub-cultured on nutrient broth which contained peptone (5g/l) and meat extract (3 g/l) and incubated at 37^{o}C for 8 hours. 30 ml of the nutrient broth was used to flood the agar plates. A sterilized Whatman No. 1 filter paper disc soaked in the oil (0.02 ml) was used to test for the sensitivity or antimicrobial effect of the oil. The plates were incubated at 37^{o}C for 24 hours. After incubation plates were observed for zones of inhibition (in mm diameter). The minimum inhibitory concentration was determined by comparing the different concentrations of the oil.

Statistical Analysis

All measurements were replicated three times and standard deviation determined. The student's t-test at $P<0.05$ was applied to assess the difference between the means [9].

Table 2: Inhibitory effects of the essential oils from the leaves of *Alstonia boonei*

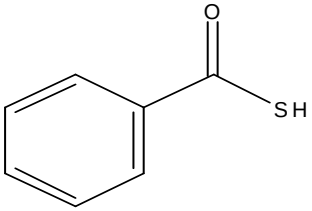
Pathogens	Concentration of essential oil 1 mg/ml				Mic mg/ml
	50	25	12.5	6.5	
Staphylococcus aureus	12	8	3	-	12.5
Escherichia coli	12	8	6	-	12.5
Streptococcus pneumoniae	16	10	6	-	12.5
Proteus mirabilis	10	8	5	-	12.5

Data are means of triplicate determinations
- No inhibition

Table 1: GC-MS Analysis and Mass Spectral Data of Ethanol fractions from the leaves of *Alstonia boonei,* showing the fragment ion peaks and retention time.

Peak	Compound	Molecular Formula	Molecular Weight	Retention Time	Percentage Content	Fragment Peaks (m/z)
1	Benzenecarbothioic acid Benzoic acid, thio Benzoyl thiol Monothiobenzoic acid Thiobenzoic acid	C_7H_6OS	138	3.533	7.73	27(10%), 51(20%), 77(70%), 105(100%)
2	1-Hexanol, 5-methyl 5-methyl-1-hexanol	$C_7H_{16}O$	116	16.642	1.29	27(20%), 41(60%), 43(100%), 56(70%), 70(80%).
3	1-Hexanol, 6-methyl 6-methyl-1-heptanol	$C_8H_{18}O$	130	22.133	2.58	27(20%), 41(80%), 55(100%), 69(90%), 70(60%), 84(50%), 98(10%)
4	9-Octadecene (E), (9E)-9-Octadecene	$C_{18}H_{36}$	252	25.067	6.44	27(10%), 41(70%), 55(100%), 83(80%), 97(50%), 111(40%), 125(10%), 252(5%)
5	n-Decanoic acid Decanoic acid n-capric acid Capric acid	$C_{10}H_{20}O_2$	172	26.775	1.55	26(10%), 27(30%), 41(60%), 60(90%), 73.05(100%), 87(20%) 101(10%), 115(20%), 129(50%), 143(10%), 172(10%).
6	1-Undecene, 9-methyl 9-methyl-1-undecene	$C_{12}H_{24}$	168	26.942	2.58	27(20%), 41(60%), 55(80%), 57(60%), 70(100%), 97(30%), 98(20%), 139(10%)
7	Bicyclo[3.1.1] heptane, 2,6,6-trimethyl Pinane Dihydropinene 2,6,6-Tribicyclo (3.1.1) heptane	$C_{10}H_{18}$	138	27.325	1.29	27(20%), 41(70%), 55(90%), 67(70%), 82(60%), 95.15(100%), 109(10%), 123(30%)
8	2,3 Diazabicyclo [2.2.1] hept-2-ene, 4-methyl-1-(pent-4-en-1-yl 1-methyl-4-(4-pentenyl)-2,3-diazabicyclo [2.2.1] hept-2-ene	$C_{11}H_{18}N_2$	178	27.933	5.15	38(10%), 41(205), 55(10%), 81.10(100%), 93(10%), 107(20%)
9	L-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652	28.275	17.27	27(20%), 41(50%), 43(80%), 57(90%), 73.05(100%), 85(50%), 98(40%), 115(40%), 129(50%), 143(20%), 157(30%), 171(30%), 185(30%), 256(40%)
10	7-Octadecenoic acid methyl ester methyl (7E)-7-Octadecenoate	$C_{19}H_{36}O_2$	296	29.175	12.89	27(20%), 41(90%), 55(100%), 69(60%), 74(70%), 84(50%), 98(40%), 123(20%), 222(20%)
11	Octadecanoate acid methyl ester	$C_{19}H_{38}O_2$	298	29.308	9.02	27(10%), 41(30%), 43(40%), 57(30%), 74.05(100%),

	Stearic acid methyl ester, n-Octadecanoic acid methyl ester Methyl n-octadecanoate					101(10%), 115(5%), 255(10%)
12	9-Octadecenoic acid, 1,2,3-Propanetriyl ester (E,E,E) 2,3-Bis (9E)-9-Octadecenoyloxyl (9E)-9-Octadecenoate	$C_{57}H_{104}O_6$	884	29.467	15.46	27(20%), 41(50%), 55(100%), 69(80%), 83(70%), 97(60%), 98(50%), 264(20%), 339(10%), 393(10%)
13	Octadecanoic acid Stearic acid n-Octadecanoic acid Humko industrene	$C_{18}H_{36}O_2$	284	29.573	10.31	27(20%), 41(70%), 43(90%), 73.05(100%), 85(40%), 98(30%), 115(20%), 129(50%), 143(10%), 171(10%), 185(30%), 227(10%), 241(30%), 284(40%)
14	Hexadecanedioc acid n-Tetradecane-omega, omega di-carboxylic acid Hexadecane-1, 16-dioc acid	$C_{16}H_{30}O_4$	286	30.367	2.58	27(10%), 41(20%), 55(40%), 69(20%), 73(20%), 84(50%), 98(100%), 112(40%), 126(20%), 209(10%), 242(10%)
15	1,2-Oxathiane, 6-dodecyl-2,2-dioxide delta-Hexadecyl 1,4-Sultone Hexadecene-1-Sulfonic acid 4-hydrox-del	$C_{16}H_{32}O_3S$	304	30.64	3.87	18(10%), 27(20%), 41(80%), 55(100%), 69(90%), 83(80%), 98(50%), 112(40%), 126(20%), 154(10%), 304(10%).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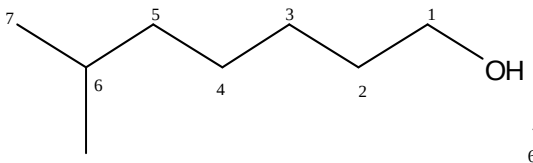
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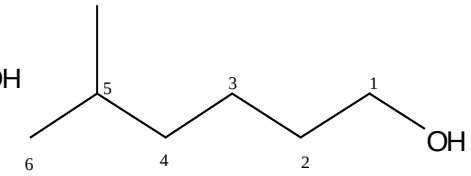
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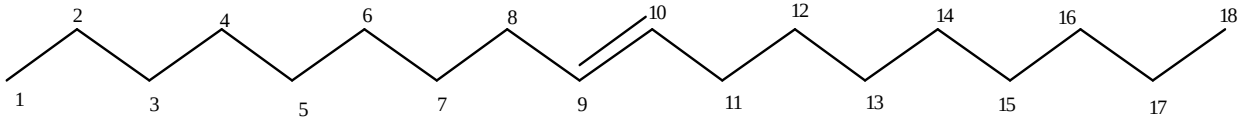
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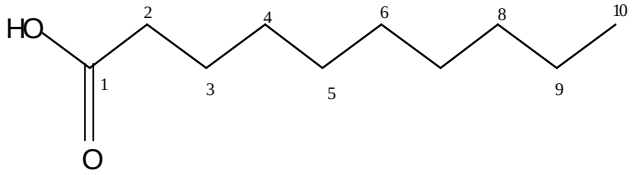
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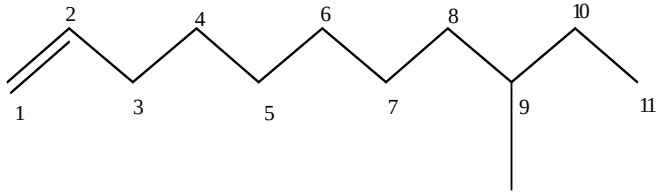
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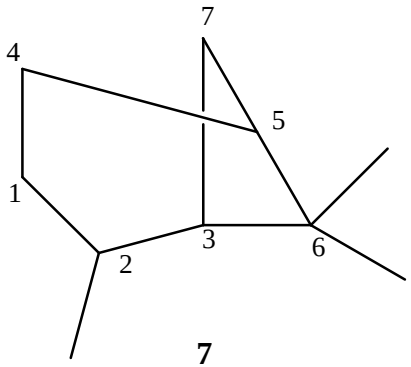
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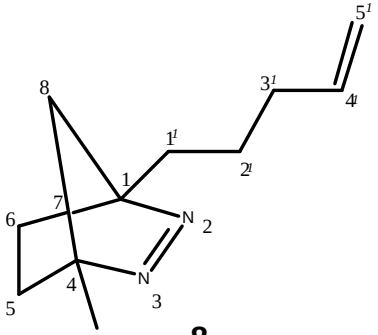

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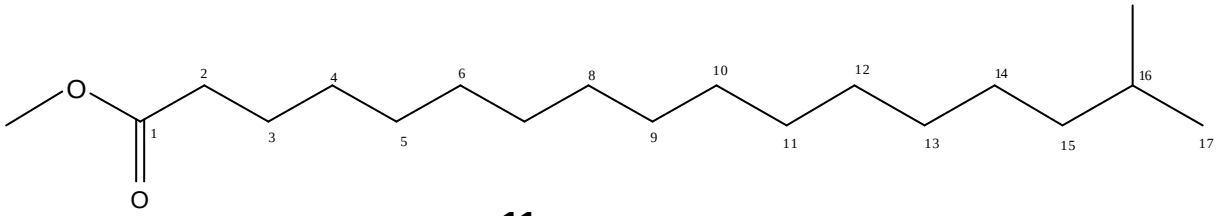

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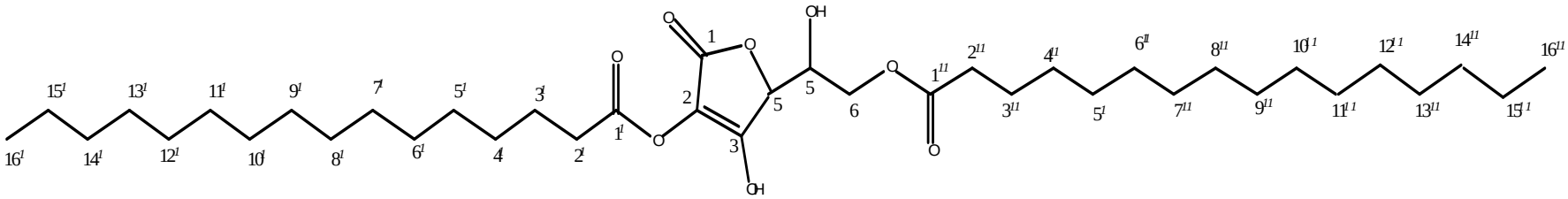

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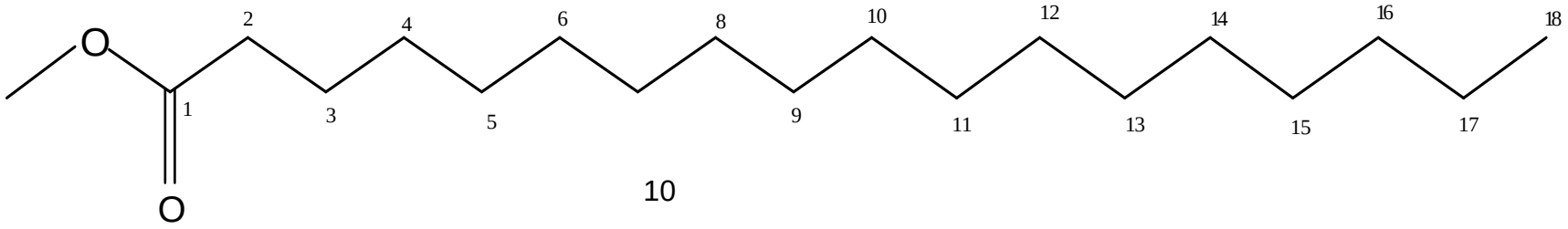

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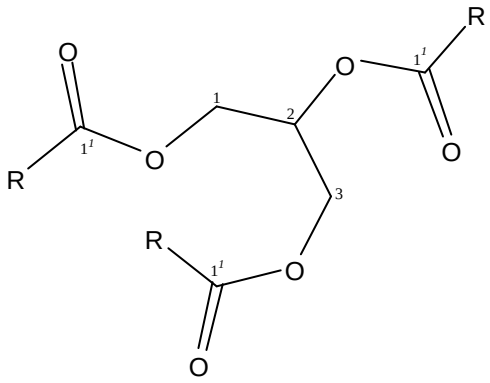

12

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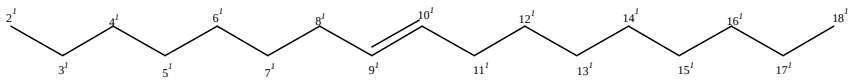

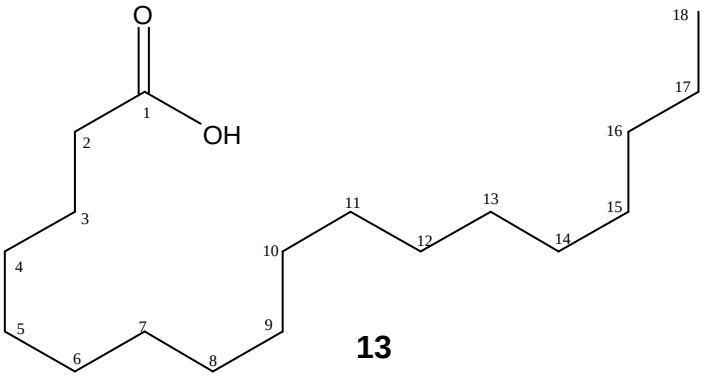

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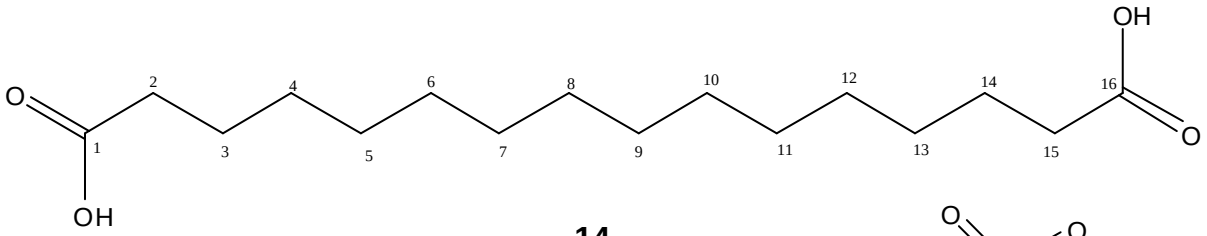

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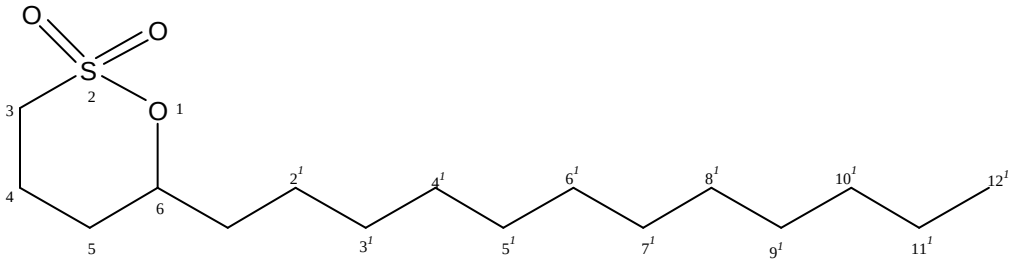

15

Figure 2: Structures of the compounds from GC-MS Analysis of the oil from the leaves of *Alstonia boonei*

Acknowledgements

We are grateful to Dr. N.O. Eddy of Ahamdu Bello University Zaria Nigeria and Mr Ime Ekanem of Akwa-Ibom State Polytechnic for their assistant in carrying out the GC-MS analysis. We are also grateful to Dr. A. Nmeregini of Forestry Department, Michael Okpara University of Agriculture, Umudike Nigeria who has been kind in authenticating our plant samples.

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