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Original Research Article

Identification of Cholinesterase Inhibitors and Antioxidant Molecules from Leaf of *Clerodendrum splendens* G. Don (Verbenaceae) Using GC-MS

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ABSTRACT

More than half of people with dementia globally have Alzheimer's disease (AD). Memory and cognitive impairment is one of the clinical symptoms of AD. Currently there is no cure for AD; hence the need arises to search for new neurotherapeutic agent. The rationale of this research is to identify molecules with cholinesterase inhibition and antioxidant potential from *Clerodendrum splendens* using GC-MS. The powdered leaves of *Clerodendrum splendens* G.Don (Verbenaceae) was extracted by successive method. Cholinesterase inhibitory activities were measured according to Ellman's method. Antioxidant capacity was assessed using standard *in vitro* chemical analysis and bioactive compounds were identified using GC-MS analysis. The ethyl acetate fraction showed the highest acetylcholinesterase inhibitory activity at 1 mg/mL with IC_{50} of 0.42±0.01 mg/mL (81.11±1.7%) in comparism with eserine (IC_{50} of 0.050±0.01 mg/mL). The ethyl acetate fraction at 1 mg/mL also showed good metal chelating activity (IC_{50} of 0.287±0.02 mg/mL) in comparism with EDTA (IC_{50} = 0.086±0.00 mg/mL). The ethyl acetate fraction showed highest nitric oxide scavenging activity (IC_{50} of 0.564±0.1 mg/mL) when compared to ascorbic acid (IC_{50} of 0.064±0.06 mg/mL). The total anthocyanin was higher in ethyl acetate fraction (52.184±2.0 mg cyanidin 3-glucoside/100g of sample). The GC-MS analysis of the ethyl acetate fraction revealed fifty-eight (58) compounds which include 9,12-Octadecadienoic acid (Z,Z)- (RT-15.974, 13.56%), n-Hexadecanoic acid (RT-14.802, 8.88%). Molecules identified by GC-MS from the ethyl acetate fraction could be possible drug lead for the treatment of AD.

Keywords: Cholinesterase inhibition, Antioxidant, GC-MS, *Clerodendrum splendens*, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a neurological disorder clinically characterized by memory and intellectual impairment. It is mainly an age related disease.[1,2] worldwide, 35 million people are currently living with AD and is projected to nearly doubling in every 20 years, reaching 66 million in 2030 and 115 million in 2050.[3] AD has no known cause or cure and these numbers make finding a cure for AD an urgent priority. The etiology of age-related progressive and psychocognitive dysfunction remains largely unknown, despite significant advances in our understanding of neuropsychiatric events.

Acetylcholinesterase (AChE) is a hydrolase of the serine hydrolase class that plays a major role in the hydrolysis of ACh at cholinergic synapses in the autonomic and central nervous systems.[4], Butyrylcholinesterase (BChE), a second type of cholinesterase breaks down acetylcholine more slowly but break choline ester of butyrylcholine faster.[5-6] Drugs that block the cholinesterase enzymes are called anticholinesterase drugs. The unique pharmacological actions of anticholinesterases occur primarily through inhibition of ACh hydrolysis by the AChE enzymes in cholinergic pathway.

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Cholinesterase inhibition induces ACh receptors, resulting in increased ACh at neuronal synapses and neuromuscular junctions.[7] Therefore, inhibition of AChE and BChE is the most effective therapeutic approach to manage AD symptoms.[8,9] The search for new molecules from natural products has attracted a great deal of attention from researchers around the world. As a result, many plants used as memory enhancers in various traditional medicine systems have been tested for anticholinesterase activity.[10-12]

Clerodendrum splendens, usually called flaming glory bower, is native to tropical West Africa. It is an evergreen climbing plant with intertwined woody stems that usually grows 10 to 12 feet in length. The elliptic to ovate leaves (up to 7 inches long) are glossy dark green. Salver morph (thin tube with abrupt corolla) with bright red flowers in dense terminal clusters (up to 4-5 inches long).[13] *C. splendens* root and leaf extracts have been used to treat rheumatism, asthma, and other inflammatory diseases.[14] Gubedema *et al.* [15] also reported the antibacterial and wound-healing effects of *C. splendens* leaves. *Clerodendrum splendens* leaves are used by indigenous peoples as a traditional medicine to treat shingles, infant spleen, asthma, rheumatism, ulcers and malaria.[16]

In recent years, many drug candidates with different pharmacological mechanisms have been proposed and tested in neurobiological AD models. Clinically tested drugs have limited efficacy or toxicity and have yielded negative results when tested in randomized controlled trials.[17] Hence there is need to search for new agents with optimal efficacy and lesser side effect/toxicity profile. The purpose of this research is to identify bioactive molecules from the leaves of *Clerodendrum splendens* with cholinesterase inhibitory and antioxidant potential using Gas Chromatography Mass Spectrometer (GC-MS) analytical technique.

Materials and Methods

Solvents and reagents used

Ethyl acetate, methanol and n-hexane, Acetylthiocholine iodide (ATChI), Butyrylthiocholine chloride (BuChCl), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), Chloroform ($CHCl_3$), Copper (II) Sulphate solution ($CuSO_4$), Fehling's solution A and B, Ferric chloride solution ($FeCl_3$), Hydrochloric acid solution (HCl), Lead acetate solution, N-1-napthylethylenediamine dihydrochloride solution, Potassium chloride solution (KCl), Sodium acetate solution, Sodium hydroxide solution (NaOH), Sodium nitroprusside solution, Sulphuric acid solution (H_2SO_4), Wagner reagent, Ethylenediamine tetraacetic acid (EDTA), obtained from Sigma Aldrich.

Plant collection, identification and preparation

The leaves of *Clerodendrum splendens* G.Don (Verbenaceae) was collected in the month of February, 2020 by the taxonomist Mr Felix Nwafor from Nsukka Local Government Area of Enugu State, Nigeria and voucher specimen deposited at the herbarium of the Department of Pharmacognosy and Environmental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka with authentication number (PCG/UNN/0373).

Extraction of powdered material

To the powdered plant material (560 g) in an air tight container was macerated successively with 2L of n- hexane ensuring that the solvent level was well above the powered material. The container was agitated after every 8 hours. The solution was filtered after 72 hours. The resulting filtrate obtained was stored and then labeled appropriately. The marc was air dried and macerated with 2 L of ethyl acetate and allows standing for about 72 hours with constant agitation every 8 hours. It was also filtered, stored and labeled appropriately. Then, the marc obtained was dried and macerated with 2 L of methanol and also allowed to stand for 72 hours with constant agitation every 8 hours. It was also filtered, stored and labeled appropriately. Each filterate was concentrated under reduced pressure using a rotary evaporator to yield extracts.

Qualitative phytochemical screening

Qualitative phytochemical testing of extracts was performed according to the procedure described by Abulude.[18]

Cholinesterase inhibitory assay

Acetylcholinesterase and butyrylcholinesterase inhibition was determined spectrophotometrically by a modified Ellman method.[19] 20 µl of different concentrations of test samples (0.03125-1 mg/ml), 240 µl of buffer (50 mM Tris-HCl, pH 8.0) and 20 µl of enzyme preparation (0.28 U/ml) were added to a 96-well plate. The reaction mixture was incubated at 37°C for 30 min, then 20 µl of 10 mM DTNB was added. 20 µl of 25 mM substrate was added to initiate the reaction. Spectrophotometrically, the hydrolysis rate of substrate was determined by measuring the change in absorbance per minute (ΔA/min) due to the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm. Buffer was used as a negative control. All assays were performed in triplicate. Eserine was used as standard.

The percentage inhibition (%I) of test sample was obtained using the formula:

$$I(\%) = [(A_o - A_i)/ A_o] * 100$$

Where, A_o = absorbance of control without tested samples and A_i = absorbance of a tested samples.

Metal chelating ability assay

Metal chelation assays were performed using the method of Singh and Rajini. [20] A solution of 2mM $FeCl_2$ $4H_2O$ and 5mM ferrozine was diluted 20 times. Briefly, aliquots (1 mL) of different extract concentrations were mixed with 1 mL $FeCl_2$ $4H_2O$. Ferrozine (1mL) was added to initiate the reaction after 5 minute incubation. Spectrophotometrically at 562 nm, the solution absorbance was measured after the mixture was shaken vigorously and incubated for additional 10 minute period. The % inhibition of ferrozine–Fe^{+2} complex formation was calculated using the below formula:

$$\text{Chelating effect } \% = [(A_o-A_i)/A_o] \times 100$$

Where, A_o = absorbance of control without tested samples and A_i = absorbance of a tested samples.

Nitric oxide radical inhibitory assay

Inhibition of nitric oxide radical activity of extracts was performed according to the method of Green *et al.*[21] as described by Marcocci *et al.*[22] Nitric oxide produced from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrate ions measured by the Griess reaction. Reaction mixtures containing 0.1 mL of various concentrations (0.03125 to 1 mg/mL) of extract and 0.9 mL of sodium nitroprusside (2.5 mM) in phosphate-buffered saline were incubated under illumination for 150 minutes. After incubation, 0.5 ml of 1% sulfanilamide in 5% phosphoric acid was added and incubated in the dark for 10 minutes before adding 0.5 ml of 0.1% NED (N-1-naphthylethylenediamine dihydrochloride). The absorbance of the formed chromophore was measured at 546 nm. Percent inhibition of nitric oxide radical formation was calculated using the following formula:

$$I\% = [(A_{blank}-A_{sample})/A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound.

Total anthocyanin content determination

The total anthocyanin content of the extract was determined by the pH differential method of Fuleki and Francis. [23] A pH 1.0 buffer solution was prepared by dissolving 125 mL of 0.2 N KCl with 385 mL of 0.2 N HCl and 490 mL of distilled water. The pH of the buffer was adjusted to pH 1.0 with 0.2N HCl. The buffer (pH 4.5) was prepared by mixing 440 mL of 1.0 M sodium acetate with 200 mL of 1.0 M HCl and 360 mL of distilled water. The pH of the solution was measured and adjusted to pH 4.5 with 1.0 M HCl. 0.5 mL of extract was diluted to 12.5 mL with pH 1.0 and 4.5 buffers and equilibrated for 2 hours in the dark. The absorbance of the samples at 512 nm (λ512 nm) and 700 nm (λ700 nm) was measured with a UV-Visible spectrophotometer. The difference in absorbance (ΔA) between the anthocyanin extract diluted in pH 1.0 and pH 4.5 buffers was calculated using the equation below:

ΔA = (A512pH1.0-A700nmpH1.0) - (A512nmpH4.5-A700nm pH 4.5).

The A700 nm was employed in the calculation of ΔA to correct for any background absorbance due to turbidity on the extracts. Anthocyanin content was expressed as mg of cyanidin-3-glucoside per 100 g of sample using the molar absorbance coefficient (ε) of 26900$L^{-1}M^{-1}cm^{-1}$) TACY= $\frac{(\Delta A \times MW) \times DF \times 1000}{\varepsilon \times 0.1 \times l}$

where

TACY represent total anthocyanins content

MW represent cyanidin-3-glucoside molecular weight (449.2 g/L)

DF represent dilution factor of the extract expressed on per gram of plant basis

ε represent molar absorbance coefficient of cyanidin-3-glucoside

0.1 represent conversion factor per 1000 grams to 100 grams basis

Gas Chromatography Mass Spectrophotometry analyses

The GC-MS procedure was performed using a GCMS-QP2010SE SHIMADZU JAPAN machine. The analysis of compounds in crude extracts of *Clerodendrum splendens* was programmed at temperature 60.0 °C- 300 °C with hold of time 1.5-4.6 min. The chromatographic conditions are as follows: column flow rate was 3.22 mL/min, injection mode: split and carrier gas was Helium 99.999%, Column Oven Temp (60.0°C), Injection Temp (250.00°C), 144.4 kPa Pressure, 22.6 mL/min Total Flow, 3.22 mL/min Column Flow, 46.3 cm/sec Linear Velocity, 3.0 mL/min Purge Flow, Split Ratio:5.1. Compounds

were identified by their GC-MS spectra and their relative retention indices using bulk library searches (National Institute Standards and Technology (NIST)-based AMDIS software).

Statistical analyses

Graphpad Prism 7 was used for the statistical analyses. All data were expressed as mean ± standard deviation (n=3). One-way ANOVA followed by Dunnett's multiple comparison test at $\alpha_{0.05}$ was performed. Mean differences at the 5% level ($P \leq 0.05$) were considered significant. A standard curve was generated and 50% inhibitory concentration (IC_{50}) values were calculated using Microsoft Excel.

Results and Discussion

Percentage yield and Qualitative phytoconstituents screening

Secondary metabolites are substances that plants produce to be competitive in their own environment. The metabolites were distributed among the three menstrum (n-hexane, ethyl acetate and methanol). The methanol fraction has the highest percentage yield of 3.8% of starting plant material of 560 g followed by n-hexane (3.4%) and ethyl acetate having the least yield (1.178%) as seen in Table 1. These small molecules have various effects on the plants themselves and other organisms. Phytochemical screening of leaves of *Clerodendrum splendens* revealed the presence of phytochemicals such as flavonoids, terpenoids, tannins, steroids, saponins, phenols, alkaloids and carbohydrates (Table 2).

Cholinesterase inhibitory activities

The most appropriate treatment strategy for treating AD and other forms of dementia is to reinstate acetylcholine levels by inhibiting both major forms of the cholinesterase enzyme. In this study, the extracts showed a dose-dependent inhibition of cholinesterase enzyme at 1 mg/mL with the ethyl acetate extract showing the highest activity with IC_{50} of 0.429±0.01 mg/mL (81.11±1.7% inhibition) and 0.296±0.09 mg/mL (79.10±1.4% inhibition) for AChE and BuChE, respectively followed by the methanolic extract having an IC_{50} of 0.729±0.05 mg/mL (58.46±5.3% inhibition) and IC_{50} of 0.354±0.04 mg/mL (78.85±2.1% inhibition) for AChE and BuChE, respectively in comparison with eserine (IC_{50} of 0.050±0.01 mg/mL, 63.64±3.9% inhibition) for AChE and IC_{50} 0.049±0.00 mg/mL (61.33±2.3% inhibition) for BuChE. The n-hexane extract had the least activity (IC_{50} of 1.260±0.1 mg/mL, 41.91±4.4% inhibition) for AChE and IC_{50} of 1.036±0.2 mg/mL (48.92±3.9% inhibition) for BuChE (Figure 1-2). Ethyl acetate fraction of the leaves of *Clerodendrum splendens* indicates its abilities in the management of Alzheimer's disease due to its ability to significantly inhibit AChE. The higher inhibitory activity of this plant could be attributed to the high alkaloid content, as most AChEIs are known to contain nitrogen.[24] Also, Phytol identified in this plant has been reported to show improvement in cognitive function due to their ability to inhibit cholinesterase enzymes in the nerve terminals.[25-26]

Ferrous ion-chelating activity

The brain encompasses comparatively high concentrations of many transition metals such as Fe, Zn and Cu that are involved in intrasynaptic neuronal activity. Comprehending the multifaceted structural and functional interactions between metal ions and various intracellular and extracellular components of the central nervous system, under normal conditions and in neurodegeneration has implications for the development of active therapeutics. Therefore, metal chelation therapy may now be considered as a promising clinical approach to AD treatment.[27] Numerous metal ion chelation therapy in AD have been reported.[28] In this study the metal chelating abilities of fractions of *Clerodendrum splendens* was evaluated. The ethyl acetate fraction at a concentration of 1 mg/mL showed good metal chelating activity by inhibiting ferrozin-Fe^{+2} complex formation (IC_{50} = 0.287 ± 0.02 mg/mL) equated to standard EDTA (IC_{50} = 0.086 ± 0.00 mg/mL). The methanol extract also demonstrated good activity (IC_{50} = 0.481 ± 0.02 mg/mL), while the n-hexane extract showed the lowest activity (IC_{50} = 1.21± 0.14 mg/mL) (Figure 3).

Molecules found in the ethyl acetate fraction of *Clerodendrum splendens* may have the ability to disrupt metal-Aβ interactions by metal chelation therapy, thereby reducing the neurotoxicity of metal-Aβ species and increasing brain activity by the restoration of metal ion homeostasis.

Nitric oxide scavenging activity

Oxidative stress in the brain is mediated by the over expression of inducible nitric oxide synthase (iNOS) and the action of constitutive neuronal NOS (nNOS), which upsurges the making of nitric oxide (NO) and its derivatives may also stimulate additional damage.[29] Therefore, plants that can reduce excessive levels of NO in the system have beneficial effects against diseases such as AD.

Table 1: Percentage yields of extracts of *Clerodendrum splendens* leaf

Fractions of CS	Weight of starting material/sample (g)	Percentage yield
n-hexane	560	3.400
Ethyl acetate	560	1.178
Methanol	560	3.800

Table 2: Qualitative phytochemical screening of *Clerodendrum splendens* leaf

Phytochemicals	Observation
Tannins	+
Glycosides	-
Resins	+
Saponins	+
Phlobatannins	-
Flavonoids	+
Sterols	-
Phenols	+
Carbohydrates	+
Alkaloids	+
Terpenoids	+

Key: (+): present, (-): absent

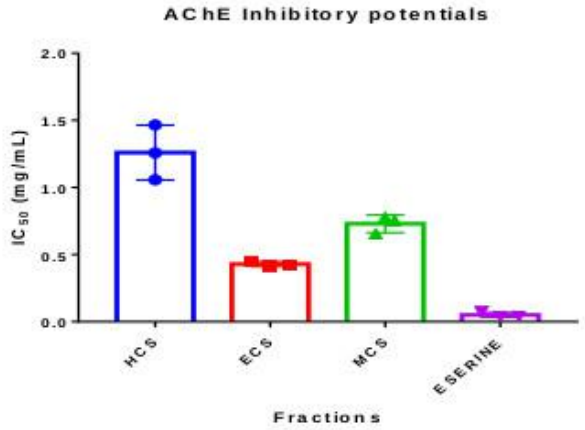

Figure 1: AChE inhibition of *Clerodendrum splendens* leaf extracts at 1 mg/mL.
Where HCS=hexane fraction, ECS =ethyl acetate fraction, MCS= methanol fraction

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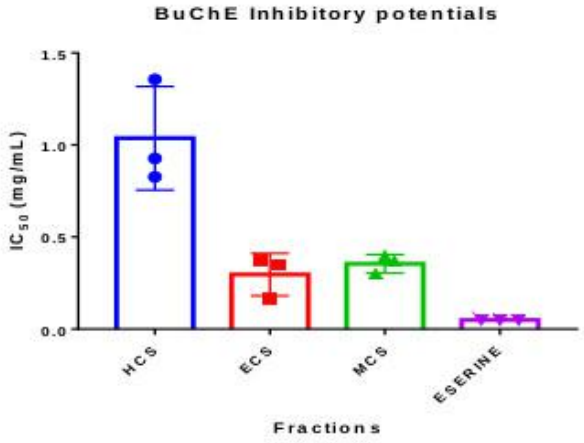

Figure 2: BuChE inhibition of *Clerodendrum splendens* leaf extracts at 1 mg/mL.
Where HCS=hexane fraction, ECS =ethyl acetate fraction, MCS= methanol fraction

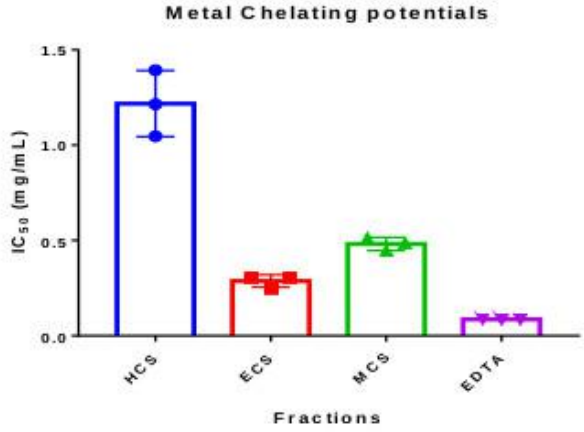

Figure 3: Metal chelating potentials of *Clerodendrum splendens* leaf extracts at 1 mg/mL.
Where HCS=hexane fraction, ECS =ethyl acetate fraction, MCS= methanol fraction

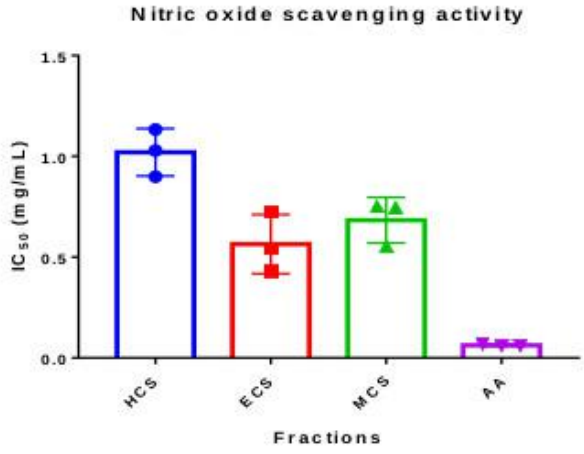

Figure 4: Nitric oxide scavenging activity of *Clerodendrum splendens* leaf extracts at 1 mg/mL.
Where HCS=hexane fraction, ECS =ethyl acetate fraction, MCS= methanol fraction

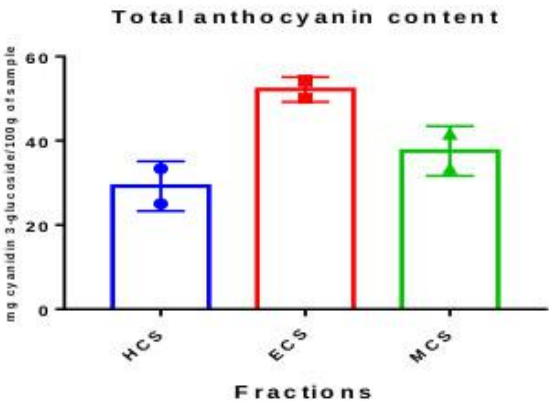

Figure 5: Total anthocyanin content of *Clerodendrum splendens* leaf extracts at 1 mg/mL.
Where HCS=hexane fraction, ECS =ethyl acetate fraction, MCS= methanol fraction

In this study the ethyl acetate extract of *Clerodendrum splendens* showed better NO scavenging activity with the highest percentage inhibition of 64.43±8.8% (IC_{50} of 0.564±0.1 mg/mL) at 1 mg/mL when equated to the methanol and n-hexane fraction which has a percentage inhibition of 55.41±1.1% (IC_{50} of 0.683±0.09 mg/mL) and 48.23±0.9% (IC_{50} of 1.020±0.09 mg/mL), respectively. The standard, ascorbic acid inhibited nitric oxide by 86.86±3.53% (IC_{50} of 0.064±0.00 mg/mL) (Figure 4). The NO radical scavenging activity of this plant may be attributed to the phytochemicals present, such as flavonoids, tannins and phenols. Flavonoids and other polyphenols are highly sensitive due to the hydroxyl groups that give them their redox properties or free radical scavenging capabilities. They play an active role in free radical quenching, which makes them effective antioxidants.[30]Tannins have also been shown to help treat neurodegenerative diseases.[31-32]

Total anthocyanin
Anthocyanin provides health benefits to plants and humans such as antidiabetics, anti-inflammatory and anticancer. Therefore, identification of the accurate quantification is of paramount importance.[33] The higher the test sample concentration, the lower the absorbance capacity and the higher the inhibition rate. The concentration is proportional to the percentage of inhibition of free radicals. In this research, the total anthocyanin capacity was higher in the ethyl acetate fraction with value of 52.18±2.0 mg cyanidin 3-glucoside/100g of sample (R^2 = 0.927) when compared to the methanol and n-hexane fractions which has a value of 37.57±4.1 and 29.22±4.1 mg cyanidin 3-glucoside/100g of sample, respectively (Figure 5). Constituents that can reduce oxidative stress have been proposed as potential drug candidates for therapeutic or preventive therapy in neurological diseases such as Alzheimer's disease.[34] The research have shown that high level of antioxidants in ethyl acetate fraction may help decrease the danger of developing AD due to the presence of phytochemicals (flavonoids, phenols) that can quench ROS by donating electrons or providing hydrogen atom.

Gas Chromatography Mass Spectrometer (GC-MS)
GC-MS is one of the best techniques for identifying components such as volatiles, long chain branched hydrocarbons, alcohols, acids and esters. Fifty-eight (58) components that may contribute to the plant's medicinal properties was revealed by GC-MS analysis of the ethyl acetate fraction of leaves of *Clerodendrum splendens*. Phytochemical identities were confirmed by peak areas, retention times, and molecular formulas.[35] The compounds 9,12-Octadecadienoic acid (Z,Z)- (RT-15.974, 13.56%), n-Hexadecanoic acid (RT-14.802, 8.88%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (RT-14.645, 7.81%), Phytol (RT-15.858, 6.65%), Bis(2-ethylhexyl) phthalate (RT-18.882, 4.29%), 2-methylhexacosane (RT-21.546, 4.05%), cis-9-

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Hexadecenal (RT-16.038, 4.04%), alpha.-Tocopheryl acetate (RT-23.395, 3.19%), 9-Octadecenamide, (Z)- (RT-17.503, 2.41%), 13-Oxabicyclo[9.3.1]pentadecane, 15-chloro-(RT-19.835, 1.94%), [1,1'-Bicyclohexyl]-4-carboxylic acid, 4'-propyl-, 4-fluorophenyl ester (RT-20.941, 1.91%) had the highest areas (Table 3 and Figure 6-7). Compounds like phytol (a diterpene), Octadecanoic acid, n-Hexadecanoic acid (Palmitic acid) are among the fifty-eight (58) compounds identified by the GC-MS analysis of *Clerodendrum splendens* reported to possess anti-oxidant activities.[36] Some of the identified compounds have been reported in other plant species e.g n-hexadenoic acid, octadecanoic acid, phytol and squalene.[37-41]

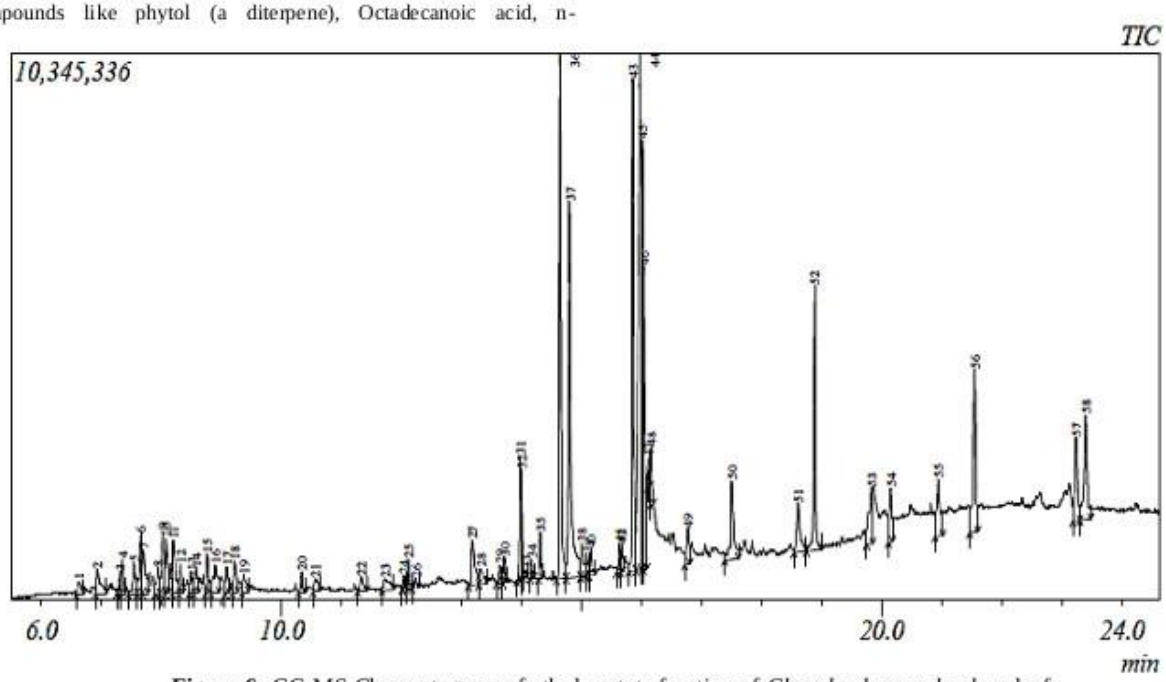

Figure 6: GC-MS Chromatogram of ethyl acetate fraction of *Clerodendrum splendens* leaf

Table 3: Molecules identified by GC-MS from ethyl acetate fraction of *Clerodendrum splendens* leaf

Peak#	R. Time (min)	Area (%)	M.W (g/mol)	Molecular formula	Compound name
1	6.624	0.46	414	$C_{22}H_{26}N_2O_6$	N-carbobenzyloxy-l-tyrosyl-l-valine
2	6.937	0.95	134	$C_{10}H_{14}$	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-
3	7.309	0.35	134	$C_{10}H_{14}$	Benzene, 1,2,4,5-tetramethyl-
4	7.349	0.53	134	$C_{10}H_{14}$	Benzene, 1,2,4,5-tetramethyl-
5	7.545	0.80	132	$C_{10}H_{12}$	Benzene, 1-methyl-2-(2-propenyl)-
6	7.657	1.17	132	$C_{10}H_{12}$	1-Phenyl-1-butene
7	7.702	1.06	164	$C_{11}H_{16}O$	6,7-Dimethyl-3,5,8,8a-tetrahydro-1H-2-benzopyran 1H-2-Benzopyran
8	7.947	0.58	176	$C_{13}H_{20}$	trans-8-tert-Butyl-bicyclo(4,3,0)non-3,7-diene
9	8.016	1.47	128	$C_{10}H_8$	Naphthalene
10	8.085	1.15	146	$C_{11}H_{14}$	1H-Indene, 2,3-dihydro-1,3-dimethyl-
11	8.200	1.43	174	$C_{13}H_{18}$	Benzene, 1-cyclopropylmethyl-4-(1-methylethyl)
12	8.322	0.55	204	$C_{14}H_{20}O$	1-Hexene, 2-(O-anisyl)-4-methyl-
13	8.498	0.50	162	$C_{11}H_{14}O$	Penten-3-ol, 1-phenyl-
14	8.582	0.69	146	$C_{11}H_{14}$	Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethyl-
15	8.760	0.64	146	$C_{11}H_{14}$	1H-Indene, 2,3-dihydro-4,7-dimethyl-
16	8.902	1.08	146	$C_{11}H_{14}$	Benzene, 2-ethenyl-1,3,5-trimethyl-
17	9.094	0.47	148	$C_{10}H_{12}O$	2-Allyl-4-methylphenol
18	9.219	0.85	142	$C_{11}H_{10}$	Naphthalene, 1-methyl-
19	9.369	0.56	142	$C_{11}H_{10}$	1,4-Methanonaphthalene, 1,4-dihydro-
20	10.337	0.32	226	$C_{16}H_{34}$	Hexadecane
21	10.568	0.48	192	$C_{11}H_{12}O_3$	Benzoic acid, 2-(1-oxopropyl)-, methyl ester

22	11.328	0.45	180	$C_{11}H_{16}O_2$	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-
23	11.726	0.65	284	$C_{18}H_{36}O_2$	Octadecanoic acid
24	12.036	0.19	346	$C_{20}H_{39}ClO_2$	3-Chloropropionic acid, heptadecyl ester
25	12.118	0.41	296	$C_{21}H_{44}$	Heptadecane, 2,6,10,15-tetramethyl-
26	12.238	0.24	208	$C_{13}H_{20}O_2$	3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one
27	13.184	1.39	238	$C_{14}H_{22}O_3$	Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester
28	13.315	0.60	284	$C_{18}H_{36}O_2$	Octadecanoic acid
29	13.642	0.24	242	$C_{16}H_{34}O$	1-Hexadecanol
30	13.714	0.30	306	$C_{16}H_{34}O_3S$	Sulfurous acid, 2-propyl tridecyl ester
31	13.976	1.73	278	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
32	13.998	1.00	338	$C_{22}H_{42}O_2$	Phytol, acetate
33	14.067	0.11	212	$C_{11}H_{16}O_4$	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione
34	14.170	0.26	296	$C_{20}H_{40}O$	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
35	14.311	0.67	360	$C_{22}H_{32}O_4$	Phthalic acid, isobutyl trans-dec-3-enyl ester
36	14.645	7.81	334	$C_{20}H_{30}O_4$	1,2-Benzenedicarboxylic acid, butyl octyl ester
37	14.802	8.88	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
38	15.003	0.70	298	$C_{19}H_{38}O_2$	Ethyl 14-methyl-hexadecanoate
39	15.093	0.40	212	$C_{11}H_{16}O_4$	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione
40	15.156	0.48	352	$C_{25}H_{52}$	2-methyltetracosane
41	15.632	0.47	346	$C_{20}H_{39}ClO_2$	3-Chloropropionic acid, heptadecyl ester
42	15.668	0.38	334	$C_{22}H_{38}O_2$	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester
43	15.858	6.65	296	$C_{20}H_{40}O$	Phytol
44	15.974	13.56	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (Z,Z)-
45	16.011	7.49	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (Z,Z)-
46	16.038	4.04	238	$C_{16}H_{30}O$	cis-9-Hexadecenal
47	16.121	0.31	252	$C_{17}H_{32}O$	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
48	16.155	0.75	212	$C_{11}H_{16}O_4$	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione
49	16.769	0.97	238	$C_{16}H_{30}O$	cis-9-Hexadecenal
50	17.503	2.41	281	$C_{18}H_{35}NO$	9-Octadecenamide, (Z)-
51	18.608	1.67	568	$C_{35}H_{68}O_5$	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
52	18.882	4.29	390	$C_{24}H_{38}O_4$	Bis(2-ethylhexyl) phthalate
53	19.835	1.94	244	$C_{14}H_{25}ClO$	13-Oxabicyclo[9.3.1]pentadecane, 15-chloro-
54	20.147	1.12	366	$C_{26}H_{54}$	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
55	20.941	1.91	346	$C_{22}H_{31}FO_2$	[1,1'-Bicyclohexyl]-4-carboxylic acid, 4'-propyl-, 4-fluorophenyl ester
56	21.546	4.05	380	$C_{27}H_{56}$	2-methylhexacosane
57	23.238	2.23	380	$C_{27}H_{56}$	2-methylhexacosane
58	23.395	3.19	472	$C_{31}H_{52}O_3$	alpha.-Tocopheryl acetate

Conclusion

Compounds identified from the most active fractions by GC-MS analysis could be potential drug lead for the treatment of Alzheimer's disease due to their significant AChE inhibition and antioxidant activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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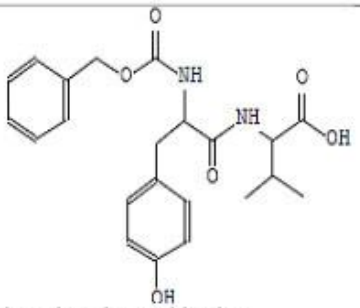
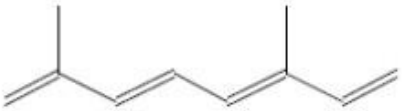
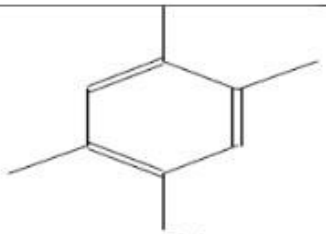
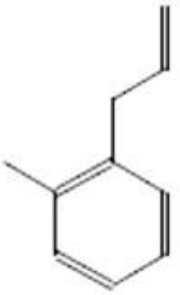
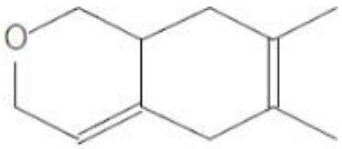
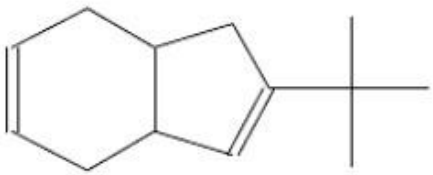
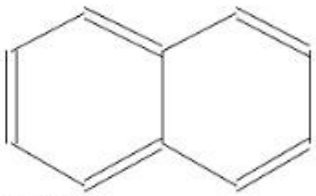
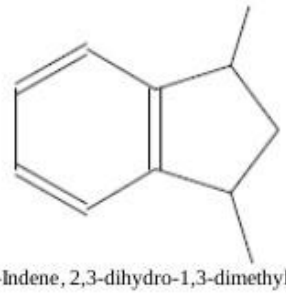
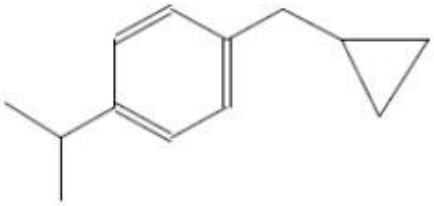
 N-carbobenzyloxy-l-tyrosyl-l-valine	 2,6-Dimethyl-1,3,5,7-octatetraene, E,E
 ***Benzene, 1,2,4,5-tetramethyl***	 Benzene, 1-methyl-2-(2-propenyl)-
 1-Phenyl-1-butene	 6,7-Dimethyl-3,5,8,8a-tetrahydro-1H-2-benzopyran 1H-2-Benzopyran
 trans-8-tert-Butyl-bicyclo(4,3,0)non-3,7-diene	 Naphthalene
 1H-Indene, 2,3-dihydro-1,3-dimethyl-	 Benzene, 1-cyclopropylmethyl-4-(1-methylethyl)

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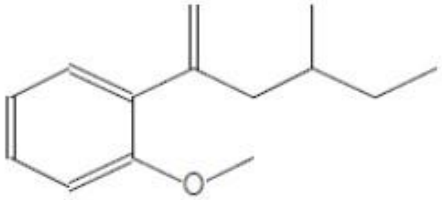
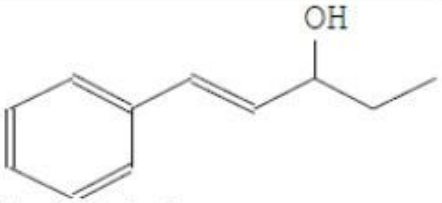
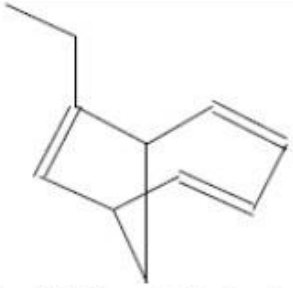
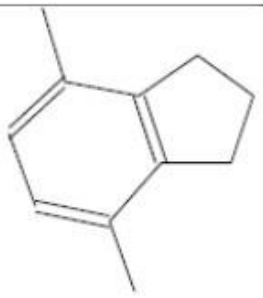
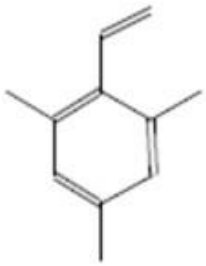
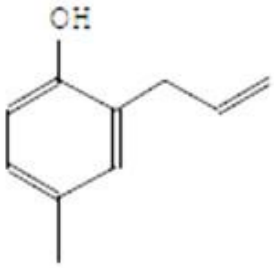
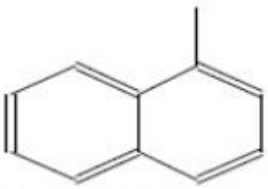
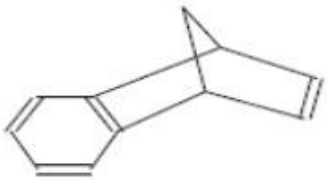
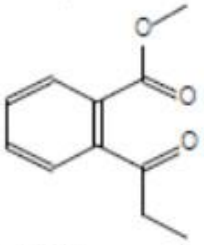
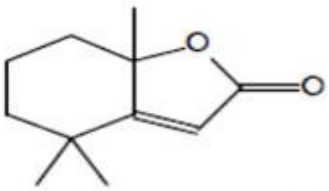
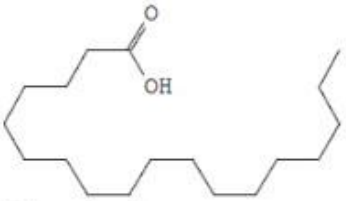
 1-Hexene, 2-(O-anisyl)-4-methyl	 1-Penten-3-ol, 1-phenyl
 Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethyl	 1H-Indene, 2,3-dihydro-4,7-dimethyl
 Benzene, 2-ethenyl-1,3,5-trimethyl-	 2-Allyl-4-methylphenol
 Naphthalene, 1-methyl-  Hexadecane	 1,4-Methanonaphthalene, 1,4-dihydro-  Benzoic acid, 2-(1-oxopropyl)-, methyl ester
 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-	 Octadecanoic acid

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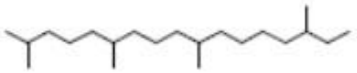
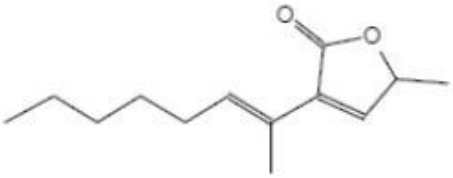
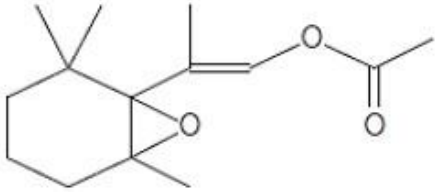
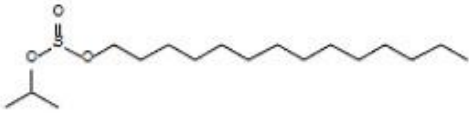
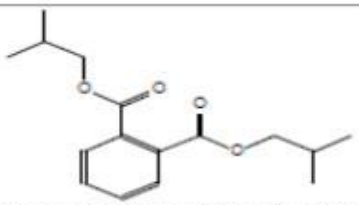
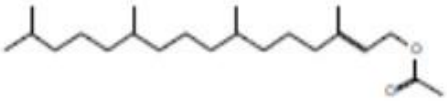
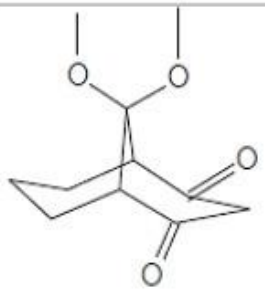
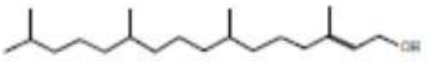
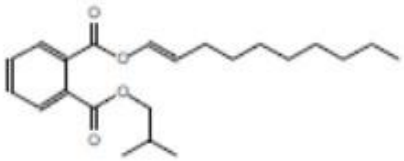
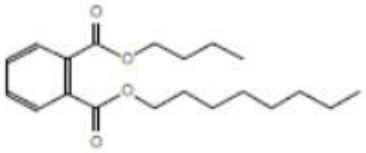
 3-Chloropropionic acid, heptadecyl ester	 Heptadecane, 2,6,10,15-tetramethyl-
 3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one	 Acetic acid, 2-(2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-propenyl ester
 1-Hexadecanol	 Sulfurous acid, 2-propyl tridecyl ester
 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	 Phytol, acetate
 9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	 3,7,11,15-Tetramethyl-2-hexadecen-1-ol
 Phthalic acid, isobutyl trans-dec-3-enyl ester	 1,2-Benzenedicarboxylic acid, butyl octyl ester

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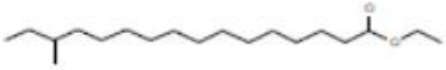
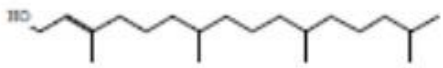
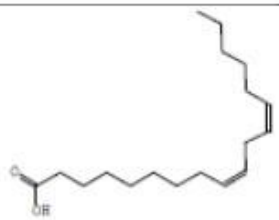
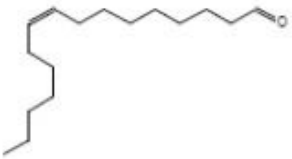
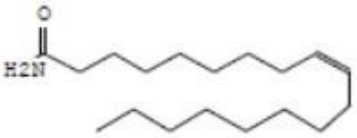
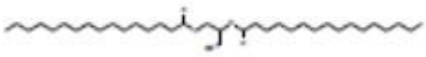
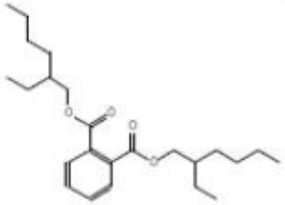
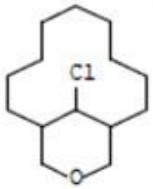
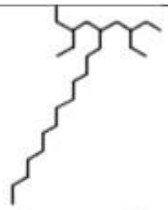
 n-Hexadecanoic acid	 Ethyl 14-methyl-hexadecanoate
 2-methyltetracosane	 3-Chloropropionic acid, heptadecyl ester
 Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester	 Phytol
 9,12-Octadecadienoic acid (Z,Z)-	 cis-9-Hexadecenal
 (R)-(-)-14-Methyl-8-hexadecyn-1-ol	 9-Octadecenamide, (Z)-
 Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	 Bis(2-ethylhexyl) phthalate
 13-Oxabicyclo[9.3.1]pentadecane, 15-chloro-	 Octadecane, 3-ethyl-5-(2-ethylbutyl)-

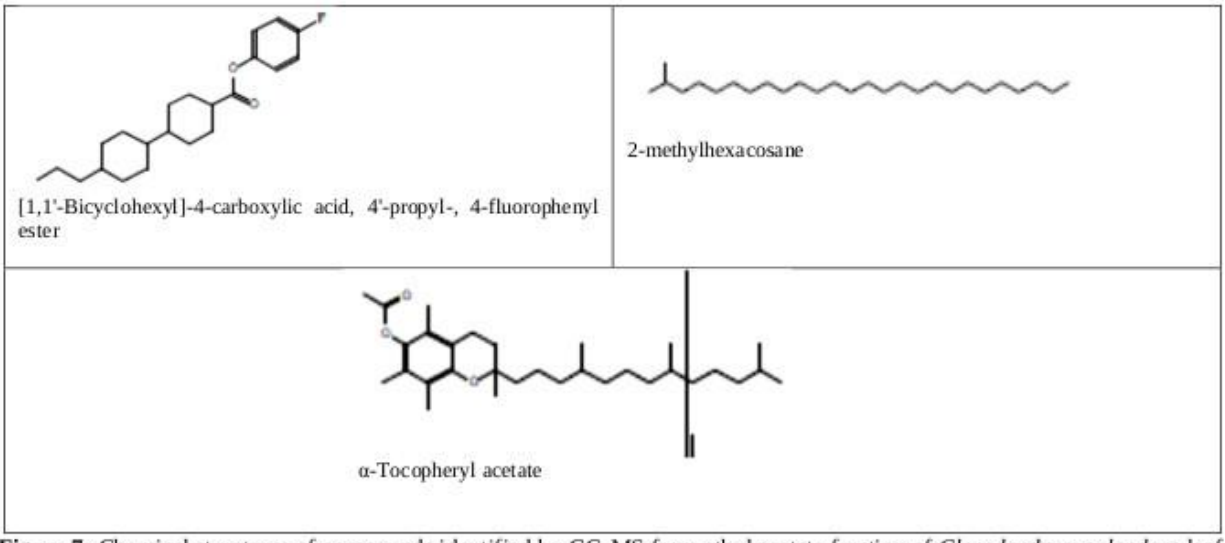


Figure 7: Chemical structures of compounds identified by GC-MS from ethyl acetate fraction of *Clerodendrum splendens* leaf.

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