***Original Research Article***

# Antioxidant activity, Total Phenols, Flavonoids and LCMS Pprofile of *Chamaecrista hildebrandtii* (Vatke) Lock ~~(Fabaceae-Caesalpinioideae)~~ and *Clerodendrum rotundifolium* ~~(Verbanaceae)~~


## ABSTRACT

**Aims:** The purpose of the study was to determine the antioxidant activity, quantify total phenols and total flavonoids and characterize the secondary metabolites present in methanolic extracts of *Chamaecrista hildebrandtii* and *Clerodendrum rotundifolium* using liquid chromatography coupled to mass spectrometry (LC-MS).
**Methodology:** The total ~~phenolic~~ phenol and flavonoid contents were determined spectrophotometrically while the antioxidant activity was evaluated using the ~~DPPH~~ 2, 2-Diphenyl-1-Picrylhydrazyl free radical scavenging method. The secondary metabolites present in the methanolic leaves extracts were evaluated using ~~liquid chromatography coupled to mass spectrometer~~ LC-MS
**Results:** The extracts of *C. hildebrandtii* showed a significantly higher antioxidant activity ($IC_{50}$ = 8.7 mg/mL) compared to *C. rotundifolium* ($IC_{50}$= 28.5 mg/mL). Both methanolic extracts of *C. hildebrandtii* and *C. rotundifolium* had common and different types of flavonoids such as quercetin, rutin, (+)-catechin 3-O-gallate and luteolin 6-C-glucoside among others that could be responsible for the observed antioxidant activity. The total phenolic content of *C. hildebrandtii* (1.33±0.07 mg/g tannic acid equivalents ~~TAE~~) was significantly higher than that of *C. rotundifolium* (0.25±0.00 mg/g tannic acid equivalents ~~TAE~~). However, there was no statistically significant difference ($p>0.05$) in total flavonoid content of *C. hildebrandtii* (2.69±0.33 mg/g catechin equivalents ~~CE~~) and *C. rotundifolium* (2.36±0.16 mg/g catechin equivalents ~~CE~~).
**Conclusion:** The results of the present study suggested~~s~~ that the good antioxidant activity exhibited by *C. hildebrandtii* may probably have been brought about by various secondary metabolites functioning in synergy.



*Keywords: Antioxidant, Reactive Oxygen Species, LC-MS, phenolic, Flavonoids*


## 1. INTRODUCTION

Biological functions such as protection from carcinogenesis, inflammation and aging are as a result of oxidative effects of free radicals produced during oxidation reactions. Free radicals are essential for production of energy required for biological processes in most living organisms. ~~However~~ Thus, excessive production of free radicals such as sup and hydroxyl radicals, hydrogen peroxide and nitric oxide are associated with several health-related issues [1]. The radicals initiate cancer progression through binding via electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage, including mutation, coupled with lipid peroxidation [2]. Free radicals and peroxidative damage are implicated in many human and animal pathological disorders

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including inflammatory ailments and microbial infections [3], neurodegenerative diseases, cancer, cardiovascular disease, hypertension, ischemia/reperfusion injury, atherosclerosis, diabetes mellitus, rheumatoid arthritis, immunosuppression, ageing and hair loss [4,5,6,7,8]. Consequently, there is an urgent need to search for new strategies to fight such health disorders including the use of natural antioxidants available in plants such as vitamins C and E, terpenoids, phenolic acids, tannins, flavonoids, quinones, lignins, stilbenes, coumarins, alkaloids, betalains, selenium, ß-carotene, lycopene, lutein and other carotenoids [9,10,11,12,13]. Antioxidants are substances that prevent damage to cells caused by free radicals by supplying electron to these free radicals [14], thus stabilizing the molecules and preventing damage to other cells. Antioxidants also turn free radicals into waste by-products which are then eliminated from the body. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plant phenolics act as reducing agents, metal chelators and singlet oxygen quenchers and many studies have shown that phenolics are useful in preventing the onset and/or progression of many human diseases [15,16]. Therefore, a number of medicinal plants have been extensively investigated for the presence and activity of polyphenols and other secondary metabolites with antioxidant properties [17,18].

The 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical scavenging method is a standard method for screening free radical-scavenging ability of compounds. The method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolorizes the DPPH solution. The degree of decolorization is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound or extract under investigation [19].

*Chamaecrista hildebrandtii* is a perennial herb with thick woody rootstock and prostrate or ascending stems. The leaves are glabrous and densely hairy~~;~~ and the midrib is generally distinctly eccentric but ~~giving~~ gives off lateral nerves towards both margins. The leaflets are often sessile, usually in 4 to 13(/14) pairs, oblong or lanceolate oblong with uppermost often somewhat ~~obvate~~ obviate, straight or slightly falcate. Plants belonging to this family have found use in wound healing, use as antioxidants, treatment of microbial infections, diabetes and skin diseases among others [20,21,22]. *Clerodendrum rotundifolium* is an erect shrub, 0.75 to 3 m tall or sometimes climbing and even reported to be a small tree. The stems have dense short spreading pubescence, later glabrescent and lenticellate while the leaves are opposite or in whorls of 3 or rarely alternate, ovate to round. The flowers are usually fragrant, terminal and not very dense but aggregated in one inflorescence. The fruits are usually red, drying black and shiny. Leaves and roots of *Clerodendrum rotundifolium* have been used during induction of labour in childbirth and in treatment of diabetes, stomachache, malaria, microbial infections and intestinal parasites [23].

## 2. MATERIAL AND METHODS

### 2.1 Sample collection, preparation, and extraction

Leaves samples of *Chamaecrista hildebrandtii* and *Clerodendrum rotundifolium* obtained from Bondo Sub-county of Siaya County, Kenya were botanically identified and authenticated at the University of Nairobi herbarium, where voucher specimens were also deposited (RSO/2016/003 and RSO/2016/004). The plant samples were washed thoroughly in water, air dried for two weeks, pulverized in an electric grinder and exhaustively extracted using methanol. The extracts were concentrated using a rotary evaporator, dried and stored at 4°C until required for analysis.

### 2.2 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) antioxidant assay

The method of Liyana-Pathirana and Shahidi [24] was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of methanol extract ranging from 3.9-500 µg/mL. Ascorbic acid was used as the standard at concentrations of 1.5-100 µg/mL. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The Scavenging of DPPH radicals by the extract was calculated using the following formula:

Inhibition (%)= $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where $Abs_{control}$ is the absorbance of DPPH and $Abs_{sample}$ is the absorbance of the DPPH radical + sample extract/standard.

The half maximal inhibitory concentration ($IC_{50}$) values denoting the concentrations of sample required to scavenge 50 % of DPPH free radicals were obtained by interpolation from linear regression analysis.

## 2.3 Total phenolic and flavonoid contents

### *2.3.1 Total flavonoid content*

The total flavonoid content of each extract was estimated by the method described by Odhiambo *et al*., [25] with some modifications. 1.0 mL of each extract was mixed with 4 mL distilled water and subsequently with 0.30 mL of 10 % $NaNO_2$ solution. After 5 min, 0.30 mL of 10 % $AlCl_3$ solution followed by 2.0 mL of 1 % NaOH solution were added to the mixture. Immediately, the mixture was thoroughly mixed and absorbance determined at 510 nm versus the blank. A standard curve of catechin was prepared (0-1.25 mg/mL) and the flavonoid concentration expressed as catechin equivalents (mg catechin/g dried sample).

### 2.3.2 Total phenolic content

Total phenol content was determined using Folin-Ciocalteu Reagent as described by Odhiambo *et al*., [25] with some modifications. 200 µL of the plant extract was mixed with 1000 µL of 1 N Folin-Ciocalteau reagent and kept at 30 °C for 4 min, then 800 µL of 7 % sodium carbonate solution added. The reaction mixture was then incubated at 30°C for 30 min in the dark and the absorbance measured at 725 nm. The concentration of total phenols in mg/g tannic acid equivalents (TAE) was calculated from the calibration curve established using tannic acid as standard. The samples were prepared in triplicate for each analysis and the mean value of absorbance obtained.

## 2.4 Liquid Chromatography-Mass Spectrometry (LC MS) analysis of the extracts

1 mg of each of the extract was weighed (in triplicates) and dissolved in 1 mL methanol. The samples were vortexed for 10s, ultra-sonicated for 1 hr, centrifuged at 14 000 rpm and the supernatant filtered and analyzed by LC-Qtof-MS (MeOH) under the following conditions: UPLC (Waters ACQUITY I-class system); UPLC column (Waters ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7-µm particle size Waters Corporation, Dublin, Ireland); Column temperature of 25 °C; mobile phase of water (A) and methanol (B), each with 0.01 % formic acid; flow rate of 0.3 mL/min, gradient from 95% A to 100 % B and back to starting solvent proportion., with the run time being 25 min. The Q-tof ion mode was positive, with a nitrogen desolvation flow rate of 500 l/h and an accuracy of < 5 ppm. The quantitative analysis of the secondary metabolites present was based on a standard curve of apigenin (y= 10288x – 11117; $R^2$=0.999).

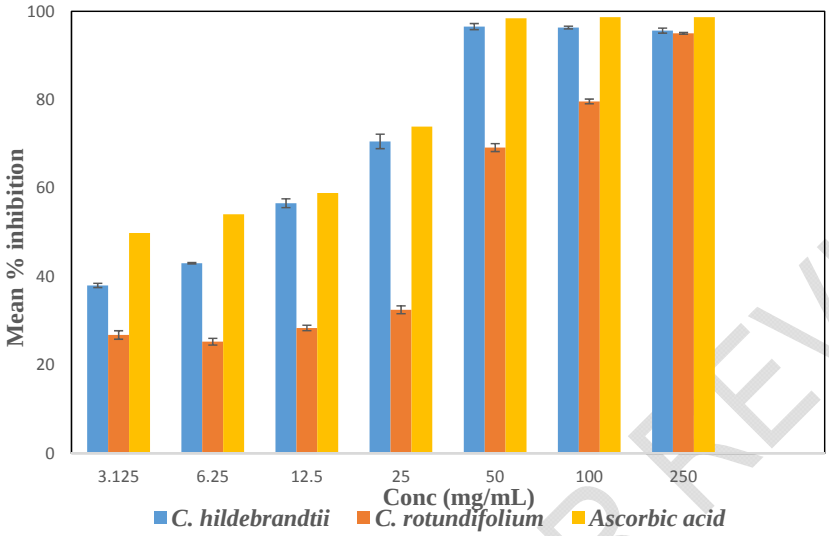
# 3. RESULTS AND DISCUSSION

## 3.1 Results

Globally, the results are poorly commented. More detailed comments of the results should be made with eventually emphasis on statistical significances or not of the differences obtained

### 3.1.1 Antioxidant assay of methanolic extracts

The findings for the antioxidant activity of the methanolic extracts and standard ascorbic acid are shown in Figure 1. The difference in antioxidant activity of both ascorbic acid and methanolic extract of *C. hildebrandtii* at the highest concentration of 250 $mgmL^{-1}$ were not statistically significant (*P*>.05).




The bars must bear letters which indicate the statistical significance or not

**Figure 1 ~~Fig. 1~~: Antioxidant activity of methanolic extracts and ascorbic acid**

In general, there was no statistically significant difference in antioxidant activity between *C. hildebrandtii* ($IC_{50}$ = 8.7 mg/mL) and ascorbic acid ($IC_{50}$ = 2.3 mg/mL) which was used as a positive control (*P*>.05) (Table 1). However, the antioxidant activity of *C. hildebrandtii* leaves extract was significantly higher than that of *C. rotundifolium* leaves extract ($IC_{50}$= 28.5 mg/mL) (*P*<.05). Leaves methanolic extract of *C. rotundifolium* had significantly lower antioxidant activity than both *C. hildebrandtii* and the standard ascorbic acid (*P*<0.05). These findings imply that the methanolic extract of *C. hildebrandtii* have compounds with stronger hydrogen-donating capacity capable of efficiently scavenging DPPH radicals.

**Table 1 showing $IC_{50}$ values of methanolic extracts and ascorbic acid**

**$IC_{50}$ Values must be followed by ± standard deviations. They must also be followed by** letters which indicate the statistical significance or not

| Sample | $IC_{50}$ value (mg/ml) | Lower boundary | Upper boundary |
|---|---|---|---|
| ***Chamaecrista hildebrandtii* leaves** | 8.7 | 3.6 | 15.0 |
| ***Clerodendrum rotundifolium* leaves** | 28.5 | 14.8 | 51.2 |
| Ascorbic acid | 2.3 | 0.08 | 5.4 |

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### 3.1.2 Quantity of total phenols and flavonoids

Phenolic compounds have been implicated in antioxidant activity exhibited by various plant extracts and citrus fruits (references). In this regard, ~~the study sort to quantify the amounts~~ levels of total phenols and flavonoids ~~present~~ in the methanolic leaves extracts of both *C. hildebrandtii* and *C. rotundifolium* were determined in the present study.

**Table 2: Total phenolic and flavonoid contents of the extracts**

**Values must be followed by ± standard deviations. They must also be followed by** letters which indicate the statistical significance or not

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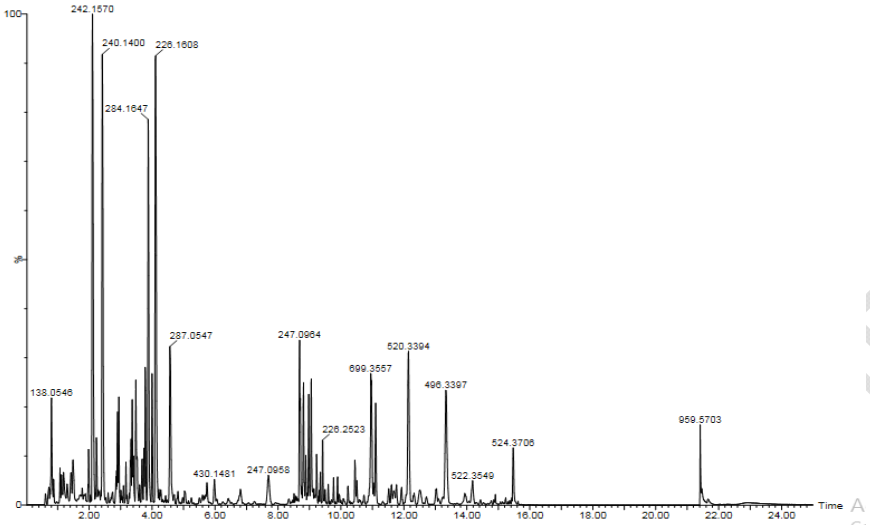
| | Quantity of Phytochemicals | |
|---|---|---|
| **Phytochemicals** | **Flavonoids (mg/g catechin equivalents)** | **Total phenols (mg/g tannic acid equivalents** |
| *C. hildebrandtii* | 2.69±0.33 | 1.33±0.07 |
| *C. rotundifolium* | 2.36±0.16 | 0.25±0.00 |

The flavonoid and total phenolic contents were higher in *C. hildebrandtii* compared to *C. rotundifolium* extract (Table 2). However, there was no statistically significant difference in flavonoid content between the two plant extracts ($P$>.05).

### 3.1.3 LCMS profiles of the methanolic plant extracts and concentrations of identified metabolites

LCMS is a reliable hyphenated technique useful in the qualitative identification and quantification of polar compounds based on fragmentation patterns. For this study, the quantities of the identified compounds was based on standard curve of apigenin (y=10288x-11117; $R^2$=0.999). The total ion chromatogram (TIC) and the identities together with quantities of compounds in methanolic extract of *C. hildebrandtii* are as shown in Figure 2 and Table 3 respectively.




The serial peak numbers of the first column of table 3 should be indicated on tops of peaks. In others words, the existing numbers should be replaced by the peak numbers according to the first column of table 3.

**Figure 2~~Fig. 2~~: TIC for *C. hildebrandtii* methanolic extract (LC-MS base peak in the positive ion mode)**

**Table 3: LC-MS Profile of *Chamaecrista hildebrandtii***

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| ~~S~~/NO | RT | $[M+H]^+$ | Compound name | Average concentration (µ~~u~~g/mg) ±SD |
|---|---|---|---|---|
| 1 | 2.903 | 443.1538 | (+)-Catechin 3-O-gallate | 2.85±0.0097 |
| 2 | 3.997 | 434.2017 | Pelargonidin 3-O-galactoside | 31.03±0.0097 |
| 3 | 4.598 | 449.1071 | Luteolin 6-C-glucoside | 18.07±0.0097 |
| 4 | 5.600 | 355.1718 | Isoxanthohumol | 1.73±0.0097 |
| 5 | 7.717 | 593.1827 | Stigmastanol ferulate | 6.11±0.0097 |
| 6 | 8.976 | 405.1174 | Oleoside 11-methylester | 9.46±0.0097 |
| 7 | 9.049 | 405.1174 | 5-Heneicosylresorcinol | 10.59±0.0097 |
| 8 | 9.348 | 389.1225 | Medioresinol | 1.45±0.0097 |
| 9 | 9.411 | 226.2523 | 2-methyl-6-nonylpiperidine | 4.86±0.0097 |
| 10 | 10.959 | 699.3557 | Asteriidoside L | 15.07±0.0097 |
| 11 | 12.147 | 520.3393 | Pelargonidin 3-O-(6"-malonyl- | 19.56±0.0097 |

| | | | | |
|---|---|---|---|---|
| ~~11~~ | | | glucoside) | |
| 12 | 13.944 | 480.3784 | Petunidin 3-O-galactoside | 2.45±0.0097 |
| 13 | 14.162 | 522.3547 | Petunidin 3-O-(6"-acetyl-galactoside) | 3.40±0.0097 |
| 14 | 15.473 | 508.4101 | Delphinidin 3-O-(6"-acetyl-galactoside) | 1.66±0.0097 |

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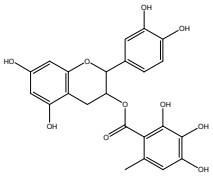
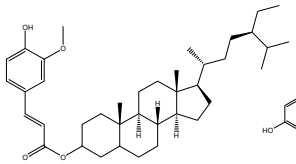
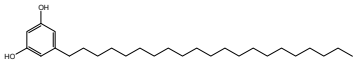
The structures of the identified compounds from *C. hildebrandtii* are shown in Figure 3.



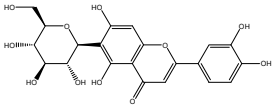
(+)-Catechin 3-O-gallate



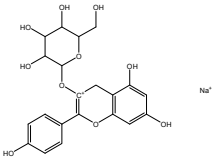
Stigmastanol ferulate



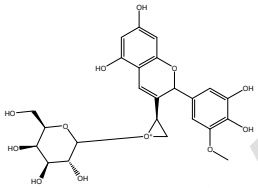
5-Heneicosylresorcinol



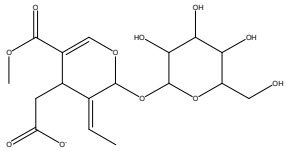
Luteolin 6-C-glucoside



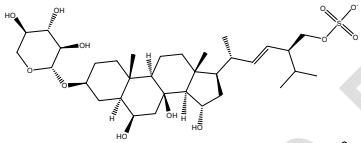
Pelargonidin 3-O-galactoside



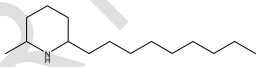
Petunidin 3-O-galactoside



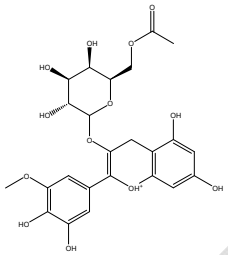
Oleoside 11-methylester



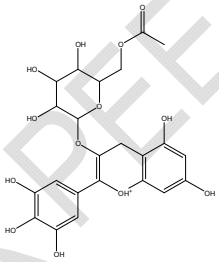
Asteriidoside L



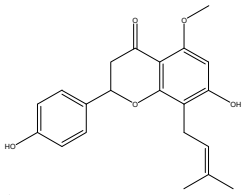
2-methyl-6-nonylpiperidine



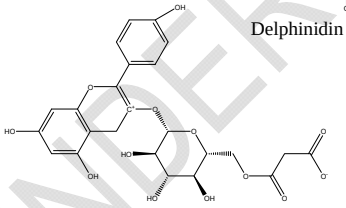
Petunidin 3-O-(6"-acetyl-galactoside)



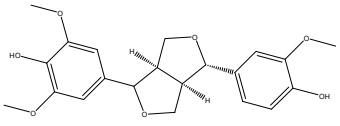
Delphinidin 3-O-(6"-acetyl-galactoside)



Isoxanthohumol



Pelargonidin 3-O-(6"-malonyl-glucoside)



Medioresinol



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158 **The name of compounds should be accompanied by their LCMS peak numbers according to the first column of**
159 **table 3**

160 **Figure 3 ~~Fig. 3~~: Structures of compounds identified from methanolic extract of *C. hildebrandtii***

161 The TIC and the identities together with quantities of compounds in methanolic extract of *C. rotundifolium* are as shown in
162 Figure 4 and Table 4 respectively.

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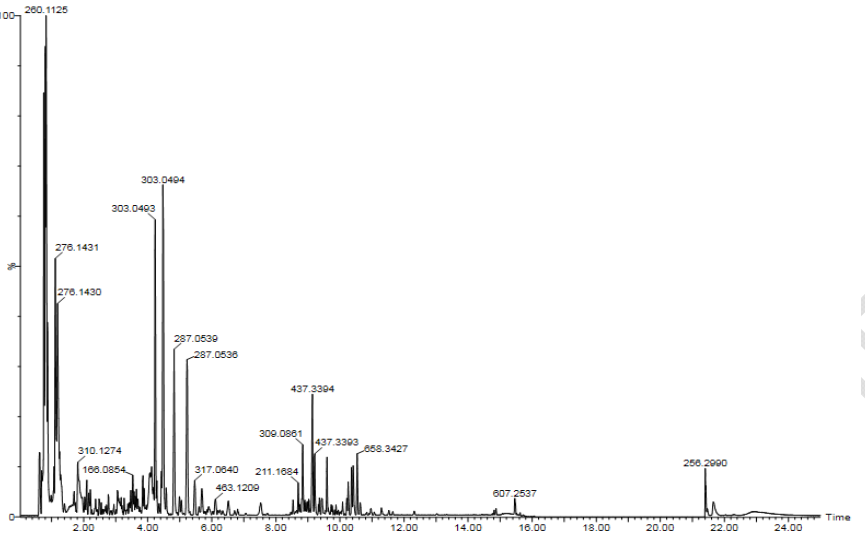



The serial peak numbers of the first column of table 4 should be indicated on tops of peaks. In others words, the existing numbers should be replaced by the peak numbers according to the first column of table 4.

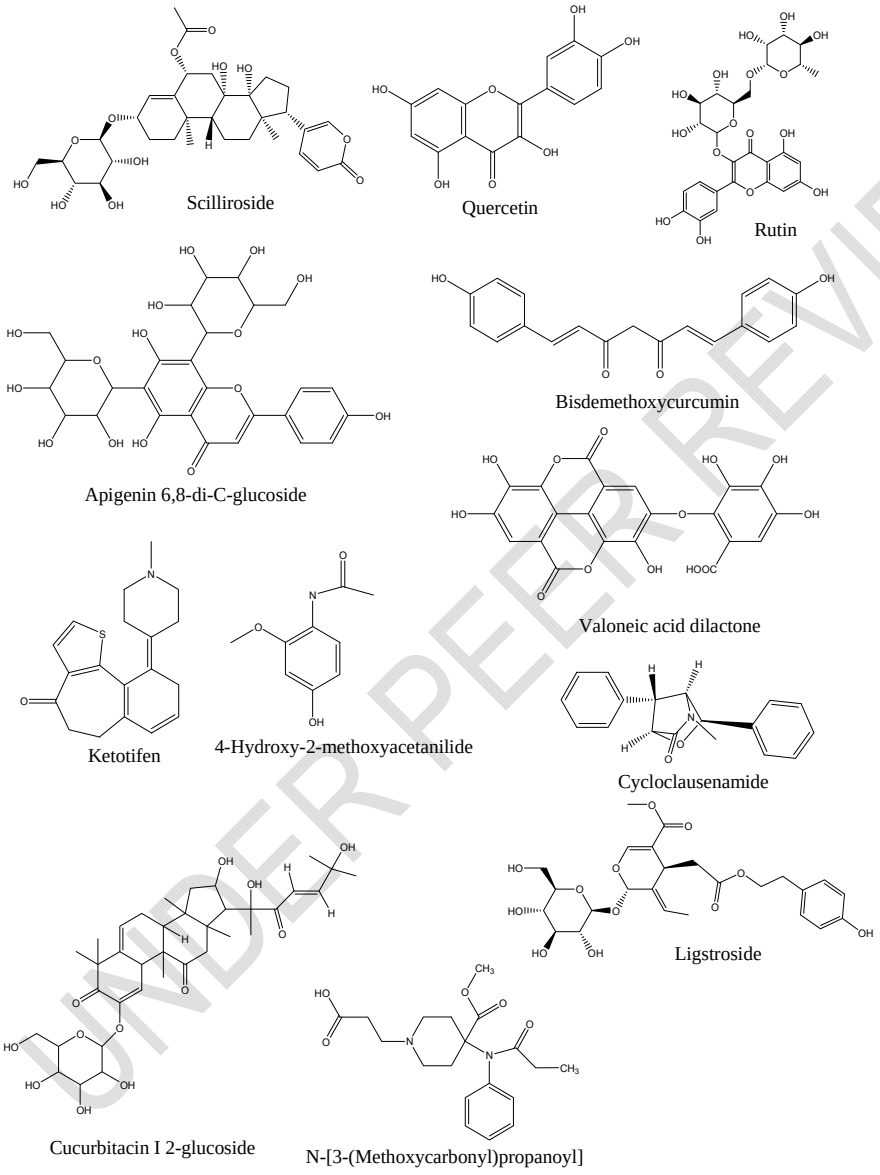
Figure 4 ~~Fig.~~ **4: TIC for *C. rotundifolium* methanolic extract (LC-MS base peak in the positive ion mode)**

**Table 4: LC-MS Profile of *Clerodendrum rotundifolium***

| ~~S~~/NO | RT | $[M+H]^+$ | Compound name | Average concentration (µg/mg) ±SD |
|---|---|---|---|---|
| 1 | 0.621 | 182.9617 | 4-Hydroxy-2-methoxyacetanilide | 3.53±0.0097 |
| 2 | 0.754 | 266.1224 | N-[3-(Methoxycarbonyl)propanoyl] | 9.93±0.0097 |
| 3 | 0.878 | 280.1372 | Cycloclausenamide | 12.55±0.0097 |
| 4 | 1.809 | 310.1274 | Ketotifen | 12.05±0.0097 |
| 5 | 4.235 | 611.1605 | Rutin | 9.50±0.0097 |
| 6 | 4.492 | 303.0495 | Quercetin | 12.63±0.0097 |
| 7 | 4.822 | 595.1647 | Apigenin 6,8-di-C-glucoside | 6.52±0.0097 |
| 8 | 5.237 | 471.0884 | Valoneic acid dilactone | 7.46±0.0097 |
| 9 | 8.831 | 309.0861 | Bisdemethoxycurcumin | 6.61±0.0097 |
| 10 | 9.14 | 525.3018 | Ligstroside | 2.94±0.0097 |
| 11 | 10.422 | 605.2924 | Scilliroside | 2.59±0.0097 |

| | | | | |
|---|---|---|---|---|
| 12 | 10.958 | 676.3459 | Cucurbitacin I 2-glucoside | 1.47±0.0097 |

The structures of the identified compounds from *C. rotundifolium* are shown in Figure 5.






The name of compounds should be accompanied by their LCMS peak numbers according to the first column of table 4

**Figure 5 ~~Fig. 5~~: Structures of compounds identified from methanolic extract of *C. rotundifolium***

## 3.2 Discussion

Research on natural antioxidants with preventive interventions for free-radical mediated diseases is very vital for improvement of human health. Polyphenols, including phenolic acids, flavonoids, tannins and lignans among others, widespread in plants, may function as potent free radical scavengers, reducing agents, quenchers of reactive oxygen species (ROS), and protectors against lipid peroxidation [26]. These compounds also exhibit cytotoxic activity and could have potential as lead compounds in the development of new anti-cancer drugs and drugs against other degenerative diseases. *C. hildebrandtii,* containing these compounds, may also serve as a potential source of bioactive compounds for the prevention and cure of free-radical associated disorders. Both methanolic extracts of *C. hildebrandtii* and *C. rotundifolium* had different types of flavonoids that could be responsible for the exhibited antioxidant activity. The flavonoids present in *C. hildebrandtii* extract included quercetin, rutin and apigenin 6,8-di-C-glucoside. Alkaloids, including N-[3-(Methoxycarbonyl)propanoyl and cycloclausenamide together with terpenoids (scilliroside and cucurbitacin I 2-glucoside) and valoneic acid dilactone, ligstroside, 4-hydroxy-2-methoxyacetanilide and ketotifen were also present in *C. hildebrandtii* extract*. C. rotundifolium* extract had several flavonoids which included (+)-catechin 3-O-gallate, luteolin 6-C-glucoside, pelargonidin 3-O-galactoside, pelargonidin 3-O-(6”-acetyl-galactoside), petunidin 3-O-galactoside, petunidin 3-O-(6”-acetyl-galactoside), isoxanthohumol and delphinidin 3-O-(6”-acetyl-galactoside). Besides, the extract also had triterpenes such as stigmastanol ferulate and asteriidoside L; 5-heneicosylresorcinol, medioresinol and oleoside 11-methylester.

Antioxidant activity of phenolic compounds has been correlated to their chemical structures [27]. In general, free radical scavenging and antioxidant activity of phenolics such as flavonoids and phenolic acids mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, but is also affected by other factors, such as glycosylation of aglycones, and other Hydrogen-donating groups (-NH, -SH). For example, flavonol aglycones such as quercetin, myricetin, and kaemperol containing multiple hydroxyl groups have been shown to have higher antioxidant activity than their glycosides such as rutin. On the other hand, the glycosylation of flavonoids has been shown to reduce their activity [9,27,28,]. Both *C. hildebrandtii* and *C. rotundifolium* had flavonoids with different levels of hydroxylation and glycosylation. However, the better activity exhibited by methanolic extract of *C. hildebrandtii* compared to *C. rotundifolium* could be explained by the possible presence of great synergy among the compounds present which included flavonoids, alkaloids, chalcone, ketotifen and valoneic acid dilactone. On the other hand, in as much as *C. rotundifolium* had more flavonoids than *C. hildebrandtii*, the lower antioxidant activity observed *C. rotundifolium* leaves extracts signifies less synergistic effects or more antagonistic effects of the various classes of compounds present in the extract including flavonoids and terpenoids.

Flavonoids are natural antioxidants with a characteristic $C_6$-$C_3$-$C_6$ carbon skeleton structure. Flavonoids show a wide range of pharmacological activities including being antiallergic, anti-inflammatory, antimicrobial, anti-cancer, antidiarrheal and antiulcer. Due to the presence of a hydroxyl group(s) in their aromatic ring(s), they possess antioxidant activity. Rutin and quercetin isolated from *Piper umbellatum* L. showed antiulcer effect by exerting antioxidant, anti-secretory, anti-inflammatory and mucosa regenerative activities [29]. Garcinol, a flavonoid from *Garcinia indica,* has been established to suppress superoxide anion, hydroxyl radical and methyl radical in rats with acute ulceration stress induced by indomethacin and water immersion [30,31]. The beneficial action of grapefruit seed extract has been attributed to the antioxidative activity of citrus flavonoids such as naringenin whose potent antibacterial activity against *Helicobacter pylori* has been established *in vitro* and has also been implicated in cytoprotection against injury induced by algal toxins in isolated hepatocytes. Naringenin, has also shown gastroprotective activity due to increased expression of prostaglandin biosynthesis and anti-cancer activity against human breast cancer cell lines [32]. Derivatives of Naringenin, such as luteolin 6-C-glucoside, isoxanthohumol, quercetin and rutin, present in the extract under investigation may, perhaps, also exhibit similar activity as naringenin, hence conferring antioxidative properties on these extracts. Phenolics such as gallic acid, catechin and epicatechin are known to be good hydrogen donors and reducing agents hence exert scavenging of ROS [32,33]. Phenolics may also prevent cancer through antioxidant action and/ or modulation of several protein functions. Furthermore, they are also capable of inhibiting carcinogenesis by affecting the initiation, promotion and progression stages [27,34].

## 4. CONCLUSION

From the current study, it can be concluded that *C. hildebrandtii* methanolic extract had significant antioxidant activity as confirmed by the DPPH scavenging assay. This radical scavenging activity could be attributed to the presence of phenolic compounds such as flavonoids working in synergy with other secondary metabolites such as alkaloids and dilactones. However, the study recommends further research on complete isolation and characterization of the compounds responsible for the observed activity.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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