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Antioxidant Activity, Total Phenols, Flavonoids and LCMS Profile of *Chamaecrista hildebrandtii* (Vatke) Lock and *Clerodendrum rotundifolium* (Oliv.)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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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 phenol and flavonoid contents were determined spectrophotometrically while the antioxidant activity was evaluated using the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical scavenging method. The secondary metabolites present in the methanolic leaves extracts were evaluated using LC-MS.

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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) was significantly higher than that of *C. rotundifolium* (0.25±0.00 mg/g tannic acid equivalents). 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) and *C. rotundifolium* (2.36±0.16 mg/g catechin equivalents).
Conclusion: The results of the present study suggested 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 some biological processes in living organisms. Thus, excessive production of free radicals such as superoxide and hydroxyl radicals, hydrogen peroxide and nitric oxide are associated with several health-related issues [1]. These free 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 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]. Based on the foregoing, the aim of this 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.

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 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, 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 [19,20,21]. *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 [22].

2. MATERIALS 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 and then pulverized in an electric grinder. 1 kg of powdered plant samples of *C. hildebrandtii* and *C. rotundifolium* were separately weighed and extracted by maceration with analytical grade methanol (3 L) at room temperature. After 24 hours, the mixtures were filtered through Whatman filter papers and the marc re-extracted two more times using fresh methanol solvent. After the third extraction, similar filtrates were pooled and vacuum-dried using rotary evaporator at 40°C. The concentrates were then transferred to pre-weighed sample bottles, kept in a desiccator and weight of the dry extract recorded and stored at 4°C until required for bioassay. The extract yield for *C. hildebrandtii* and *C. rotundifolium* were 7.8% and 6.7% respectively.

2.2 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Antioxidant Assay

The method of Liyana-Pathirana and Shahidi [23] 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:

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

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 Phenol 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. [24] 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 phenol content

Total phenol content was determined using Folin-Ciocalteu Reagent as described by Odhiambo et al. [24] 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

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 Fig. 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).

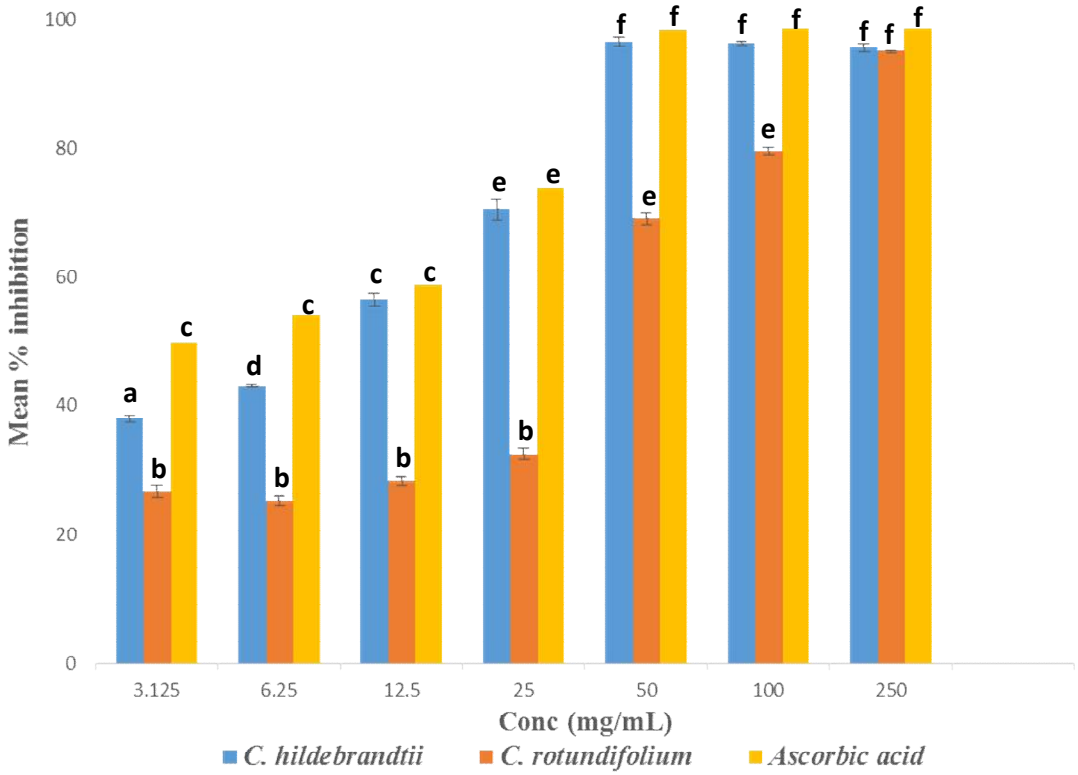

Fig. 1. Antioxidant activity of methanolic extracts and ascorbic acid

Note: Values are the mean ± SD (n = 3); Bars for a particular concentration with different letters on top have statistically significant different mean percent inhibition (p<0.05)

Table 1. Showing IC_{50} values of methanolic extracts and ascorbic acid

Sample	IC_{50} value (mg/mL)	Lower boundary	Upper boundary
Chamaecrista hildebrandtii leaves	8.7^a	3.6	15.0
Clerodendrum rotundifolium leaves	28.5^b	14.8	51.2
Ascorbic acid	2.3^a	0.08	5.4

Note: Values are the mean ± SD (n = 3)

Values in the column followed by a different letter superscript are significantly different (p<0.05)

Table 2. Total phenolic and flavonoid contents of the extracts

Plant extracts	Quantity of phytochemicals	
	Flavonoids (mg/g CE)	Total phenols (mg/g TAE)
C. hildebrandtii	2.69±0.33[a]	1.33±0.07[a]
C. rotundifolium	2.36±0.16[a]	0.25±0.00[b]

Note: Values are the mean ± SD (n = 3); CE: Catechin equivalents; TAE: Tannic acid equivalent
Values in the column followed by a different letter superscript are significantly different (p<0.05)

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.

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 [9]. In this regard, levels of total phenols and flavonoids in the methanolic leaves extracts of both *C. hildebrandtii* and *C. rotundifolium* were determined in the present study.

The flavonoid and total phenolic contents were significantly 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 Fig. 2 and Table 3 respectively.

The TIC and the identities together with quantities of compounds in methanolic extract of *C. rotundifolium* are as shown in Fig. 3 and Table 4 respectively.

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 [25]. 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), isoxantho-humol 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 [26].

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 [9,26,27]. 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 in *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.

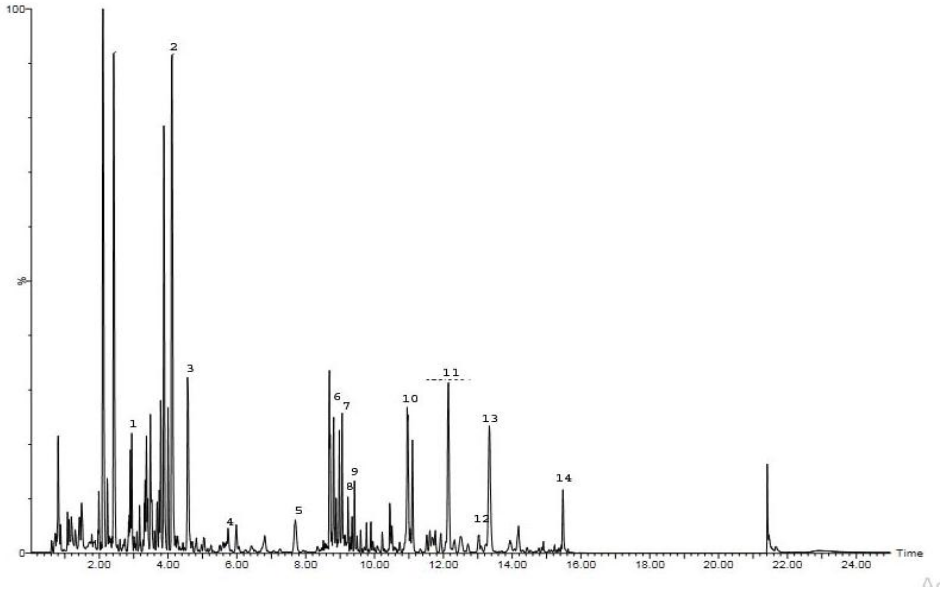

Fig. 2. TIC for *C. hildebrandtii* methanolic extract (LC-MS base peak in the positive ion mode)

Table 3. LC-MS profile of *Chamaecrista hildebrandtii*

NO	RT	[M+H]+	Compound name	Average concentration (µg/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-glucoside)	19.56±0.0097
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

Note: Values are the mean ± SD (n = 3)

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 [28]. 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 [29,30]. 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 [31]. 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 [31,32]. 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 [26,33].

Other scientific investigations have also confirmed the antioxidant potential of plants in the same families as *C. hildebrandtii* and *C. rotundifolium.* In most instances, the antioxidant activity has always been attributed to presence of phenolic compounds, including flavonoids. The antioxidant activity observed in this study could partly be attributed to the various phenolic compounds such as flavonoids present as identified by LC-MS. According to Ilavarasan [34], the aqueous and methanolic extracts of *Cassia fistula* bark exhibited free radical scavenging effect of DPPH in a concentration dependent manner and its methanolic extract showed better free radical scavenging activity (IC_{50}=213 µg/mL) than the aqueous extract (IC_{50}= 233 µg/mL). A study by Singh et al. [35], attributed the antioxidant activity of *Cassia occidentalis* to polyphenolic compounds such as flavonoids present in its seed methanolic extract. Also a study by Akter et al. [36] on antimicrobial and antioxidant activity and chemical characterization of *Erythrina stricta* Roxb (Fabaceae) stem bark, led to the isolation of seven flavonoids derrivatives, with erynone exhibiting the greatest DPPH free radical scavenging activity. Other studies on plant extracts belonging to the family Fabaceae have also led to similar conclusions regarding the role of phenolic compounds on antioxidant activity of plant extracts [37,38]. Other studies have also reported antioxidant activity and secondary metabolites from plant species belonging to the genus Clerodendrum. Several classes of compounds such as steroids, terpenoids and

Table 4. LC-MS profile of *Clerodendrum rotundifolium*

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

Note: Values are the mean ± SD (n = 3)

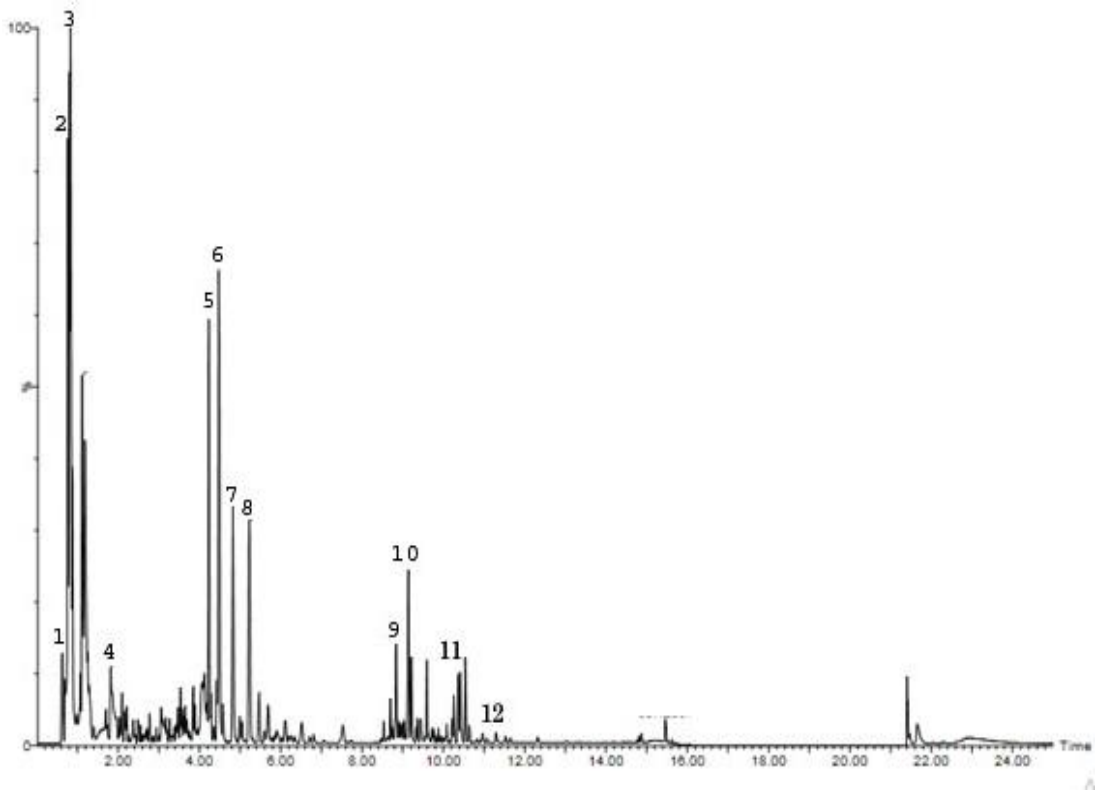

Fig. 3. TIC for *C. rotundifolium* methanolic extract (LC-MS base peak in the positive ion mode)

flavonoids have been isolated from species such as *Clerodendrum indicum, C. phlomoides* and *C. inerme* [39]. Phenolic compounds whose antioxidant activity have been established and have been isolated from members of this genus include Hispidulin, Luteolin, Scutellarein, Apigenin and Quercetin derivatives [40,41,42]. Ethanolic extracts of both aerial parts of *C. serratum* and *C. infortunatum* showed good antioxidant activity. Leaves methanolic extracts of *C. inerme* have also been reported to exhibit higher antioxidant activity [43,44,45,46].

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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