

Liquid Chromatography Mass Spectrometer (Lc/Ms) Profile Revealed Flavonoids And Terpenoids With Antioxidant Potential In Aqueous Fraction Of *Combretum Micranthum* Leaf Extract

Aminu Ibrahim¹ and Musa Bashir^{2*}

¹Department of Biochemistry,
Faculty of Basic Medical Sciences,
Bayero University, Kano-Nigeria.

²Centre for Dryland Agriculture,
Bayero University,
Kano-Nigeria.

Email: mbashir.cda@buk.edu.ng

Abstract

Combretum micranthum (CM) is commonly used for its ethno-medicinal potentials without much or no scientific basis. Thus, the aim of this research is to evaluate aqueous fraction of *C. micranthum* leaf extract for possible antioxidant compounds using liquid chromatography mass spectrometer technique. In vitro antioxidant was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Power (FRAP) assay methods and the results indicated the free radical scavenging activity of 80.351 ± 0.732 and 0.800 ± 0.00 at 2.50 mg/ml concentrations which is significantly different when compared to reference standard of 84.718 ± 0.605 and 0.426 ± 0.000 (ascorbic acid) respectively, with concentration or dose-dependent. LC-MS profile revealed the presence of medicinally important compounds like Myricetin (Flavone), Epioxylubimin (Sesquiterpenoids), Furocoumaric acid (Phenolic glycoside) among others. The findings of this study revealed that *C. micranthum* is rich in potential metabolites and was reflected as its antioxidant potential and could be used for drug and other oxidative stress related management.

Key Word: *Combretum micranthum*, Antioxidant, Metabolite, LCMS

INTRODUCTION

Combretum micranthum, (CM), Combretaceae is an undomesticated shrub species majorly found in western part of Africa and is one of the most used traditional bush teas most especially in Senegal. Traditionally the herbal beverage is used for weight loss, digestion, mild antibiotic, and for other pharmacological benefit. *Combretum micranthum*, specifically used as a general panacea that the name kinkéliba has become a word synonymous with “medicine” in some languages (Eloff, *et al.*, 2008). The use of natural resources emerging in general as one of the most important and suitable means to explore in the search for new and more effective antibacterial

*Author for Correspondence

drugs and particularly medicinal plants (Yoda *et al.*, 2020). Recently many literature revealed the presence of important bioactive metabolites with many biological activities (Yoda *et al.*, 2020). The survey on phytochemical and biological activity of *C. micranthum* has re-confirmed the presence of these metabolites. Hydroalcoholic extract of *C. micranthum* (CM extract) showed a strong antioxidant activity by scavenging AAPH, DPPH, nitric oxide, hydrogen commonly used for pharmaceutical benefit throughout West Africa. (Kpemissi *et al.*, 2019).

Additionally, *C. micranthum* was mentioned as one of the 50 most important African medicinal plants by Association for African Medicinal Plant Standards. Burkhill (1985) has reported many benefits of species found in combretaceae including, but not limited to, anthelmintic, colitis, constipation, diuretic, expectorant, gingivitis, guinea worm, parasites, malaria, nausea, purgative, thrush, tuberculosis and yellow fever. It is clear, from this report that Combretaceae species play an important part in Africa's traditional medicines.

Modern scientific research indicates that leaf extracts exhibit a number of pharmacological activities. For example, a kinkeliba decoction was shown to inhibit both strains of *Plasmodium falciparum in vitro* (chloroquine-sensitive and chloroquine-resistant), indicating its uses for the treatment of malaria infection (Benoit *et al.*, 1996). In many literatures this plant was reported to be medicinally important most especially for antimicrobial and anti-inflammatory activities against both strains of bacteria (Olajide *et al.*, 2003; Udoh *et al.*, 2012). Therefore continuous research to investigate the bark, roots and branches of the plant, may lead to elucidate, develop the mode of therapeutic action that is utilized in the tea and ultimately employed in foods, beverages and, if therapeutically valid, it can be useful in modern health care or medicine.

In a normal cellular condition the ratio between appropriate oxidant and antioxidant is balanced. However, any shift of this balance may cause the increased production of oxygen species or decreased antioxidant level and consequently causes oxidative stress. Similarly this would result in damaging the biopolymers such as nucleic acids, proteins, poly-unsaturated fatty acids and carbohydrates. It also causes serious cell damage leading to a variety of health conditions such as Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders among others. Many literatures reported that plant extracts is rich in phenol acids exhibit strong antioxidant and antiradical potentials *in vitro* (Mary *et al.*, 2003) and *in vivo* (Rajlakshmi *et al.*, 2003). However, the use of synthetic antioxidants have been restricted due to their health risks and toxicity (Linderschmidt *et al.*, 1986). The need to evaluate or identify phytochemical from natural sources with antioxidant potential or with pharmacological benefit will not be overemphasis. Therefore this study is aimed to determine the antioxidant potential of *C. micranthum* aqueous fraction and identify the possible metabolites responsible using LCMS technique.

MATERIALS AND METHODS

Chemical/Reagent. All chemicals and reagents used are of analytical grade and purchased from sigma Aldrich.

Sample Collection and Extraction

The leaves of *C. micranthum* were collected from Filin Shagari Area, Bauchi State, Nigeria. It was taken to herbarium unit of biological science department, Bayero University Kano for identification and voucher specimen no. BUKHAN0349 was deposited. The leaves were shade

dried and ground into powder using a clean metal mortar and pestle and were extracted using maceration method (Ibrahim *et al.* (2017).

DPPH Assay

This assay was carried out as described by Shen *et al.* (2010) with slight modification. About 0.1mM solution of DPPH in methanol was prepared and 2cm³ of this solution was added to 1cm³ of various concentration of the extract fraction (0.16, 0.3125, 0.62, 1.25 and 2.50 mg/cm³).The mixtures were shaken vigorously and incubated at room temperature for 30 minutes. Methanol was used as a blank and DPPH in methanol without the plant extract was used as positive control. Then the absorbance was measured at 517 nm using a UV-VIS Spectrophotometer. Ascorbic acid was used as the reference. The capability of scavenging the DPPH radical was calculated by using the following formula; DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1) / A_0\} * 100$.

Where, A₀ is the absorbance of the control reaction, and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicate. The IC₅₀ value was determine as the half-maximum concentration of the extract (in mg/mL) that scavenge 50% of DPPH.

Ferric Reducing Power Assay

The reducing power of aqueous fraction *C. micranthum* was measured using the method described by Ferreira *et al.* (2007) with slight modification. About 2.5 cm³ of extracts fractions at different concentrations (0.1625, 0.625, 0.3125, 1.25 1.25 and 2.50 mg/cm³) were mixed with 2.5 cm³ phosphate buffer (0.2 M, pH 6.6) and 2.5 cm³ of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 minute, and allowed to cooled, mixed with 2.5 ml of 10% trichloroactic acid and centrifuged at 6500 rpm for 10 minute. The supernatant (2.5 cm³) for each were mixed with distilled water (2.5 cm³) and ferric chloride (0.5 cm³, 0.1%) then finally allowed to stand for 10 min. The absorbance was read at 700 nm using UV-Vis spectrometer. Ascorbic acid was used as standard against reagent in distilled water as blank.

Liquid Chromatography Mass Spectrometer (LC-MS) Analysis

The LCMS profile was carried out according to the method (Piovesana *et al.* (2018) with some modifications. The extract samples were dissolved in Methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. Then, the filtrate (10.0 µl) was injected into the LC system and allowed to separate on Sunfire C18 (5.0µm 4.6mm x 150 mm) Column. The run was carried out at a flow rate of 1.0 mL/min, Sample and Column temperature at 25°C. The mobile phase consists of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in Acetonitrile) with a gradient as shown in table 1.

Table 1: Liquid Chromatography Mass Spectrometer (LC-MS) Solvent gradient (%)

Time	%A	%B
0	95	5
1	95	5
13	5	95
15	5	95
17	95	5
19	95	5
20	95	5

NOTE: Solvent A (0.1% formic acid in water), Solvent B (0.1% formic acid in Acetonitrile) A ratio of A/B 95:5 was used from the beginning and hold for further 1 min, then A/B 5:95 for 13min and hold to 15min. then A/B 95:5 to 17min, 19min and finally 20min. the PDA detector was set at 210-400nm with resolution of 1.2nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 - 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative); probe temperature 600°C; flow rate 10 mL/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The data was processed with Empower 3. The compounds were identified on the basis of the following information, elution order, and retention time (Rt), fragmentation pattern, and Base m/z.

Statistical Analysis

Data were analyzed using SPSS Minitab package. ANOVA was used to identify the cause of differences. The results were expressed as mean \pm standard deviation (SD) of triplicates determination and IC₅₀ for percentage inhibition was calculate.

RESULTS

DPPH and FRAP Radical Scavenging Activities of *Combretum micranthum* Leaf Extract in aqueous Fraction.

In vitro antioxidant studies were carried out using DPPH and Ferric Reducing Power (FRAP) assay methods as presented in Table 2 and 3 respectively. The findings revealed the free radical scavenging activity of 80.351 \pm 0.732 and 0.800 \pm 0.00 at 2.50mg/ml concentrations which is significantly different when compared to reference standard of 84.718 \pm 0.605 and 0.426 \pm 0.000 (ascorbic acid) respectively. And concentration or dose-dependent, with IC₅₀ 1.491 \pm 0.716.

Liquid Chromatography Mass Spectrometer (LC-MS) Analysis

The LCMS profile of aqueous fraction of *C. micranthum* leaf was conducted as presented in Table 4. However the results revealed the presence of many medicinally important compounds like Myricetin (Flavone), Epioxylubimin (Sesquiterpenoids), and Furocoumaric acid (Phenolic glycoside) among others at positive mode, which tentatively identified using data base by comparing their molecular fragmentations pattern.

TABLE 2: DPPH scavenging activity of *C. micranthum* leaf extract aqueous fractions using ascorbic acid as standard.

Results are expressed in mean \pm standard deviation

Concentration of the extract (Mg/ml).	Antioxidant inhibition (%)	Antioxidant inhibition (%)
	Ascorbic acid	Aqueous
2.5	84.718 \pm 0.605	80.351 \pm 0.732
1.25	82.481 \pm 1.359	80.032 \pm 0.889
0.625	80.405 \pm 2.567	78.594 \pm 1.365
0.3125	78.754 \pm 3.503	76.358 \pm 0.319
0.15625	69.861 \pm 14.279	68.424 \pm 2.272
IC 50	1.438 \pm 0.688	1.491 \pm 0.716

TABLE 3: FRAP scavenging activity of *C. micranthum* leaf extract aqueous fractions using ascorbic acid as standard.

Concentration of the extract (Mg/ml).	Antioxidant inhibition (%)	Antioxidant inhibition (%)
	Ascorbic acid	Aqueous
2,5	0.426 \pm 0.000	0.80 0 \pm 0.002
1,25	0.425 \pm 0.001	0.359 \pm 0.001
0,625	0.364 \pm 0.045	0.35 1 \pm 0.001
0,3125	0.281 \pm 0.001	0.31 3 \pm 0.001
0,15625	0.167 \pm 0.001	0.204 \pm 0.001

Results are expressed in mean \pm standard deviation

TABLE 4: LC-MS analysis of aqueous fraction of leaf extract of *C. micranthum*.

PEAK	COMPOUNDS NAME	MW	M/Z
1	Furocoumarinic acid (phenolic glycoside)	366	367.809
2	12-tricosanol	340	341.666
3	Fenarimol	331	332.132
4	Myricetin (Flavonol)	318	319.062
5	Epioxylubimin (sesquiterpenoid)	269	270.268

MZ - Mass to charge ratio, MW - Molecular Weight

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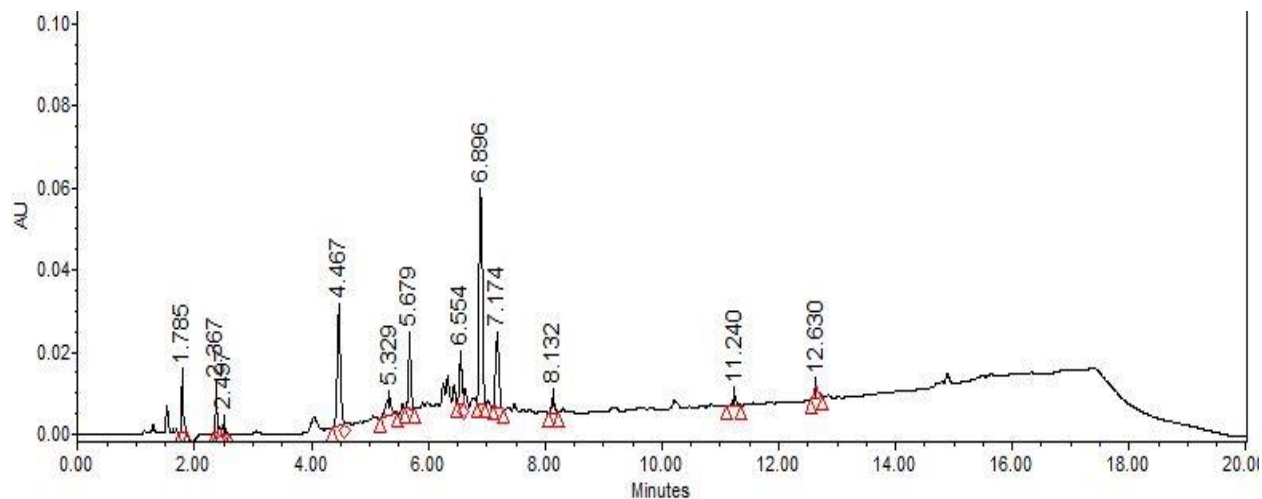


Figure 1: Total chromatogram of aqueous fraction of *C. micranthum* leaf

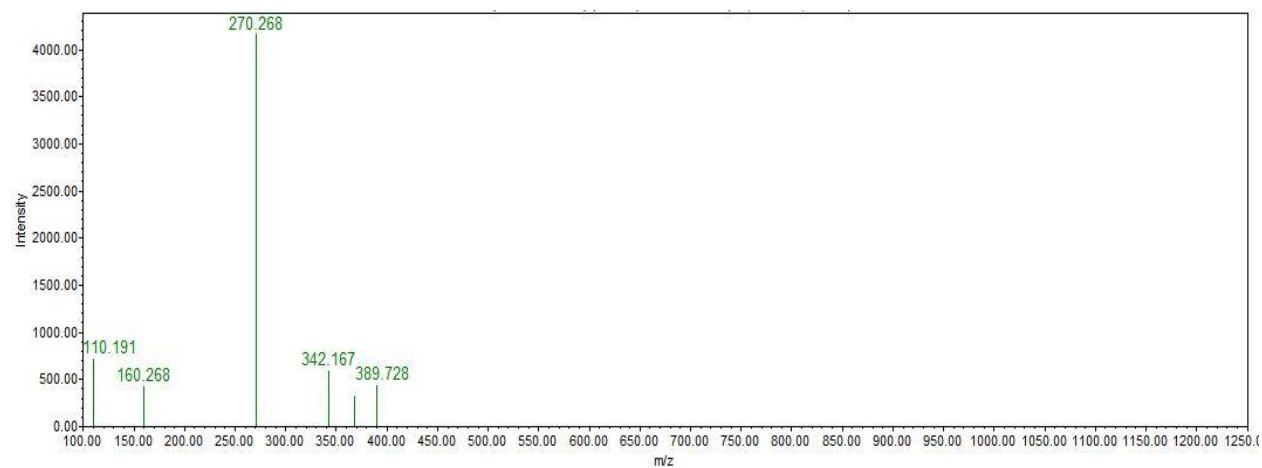


Figure 2: Molecular fragmentation of Epoxylubimin (Sesquiterpenoid) 270.268 m/z

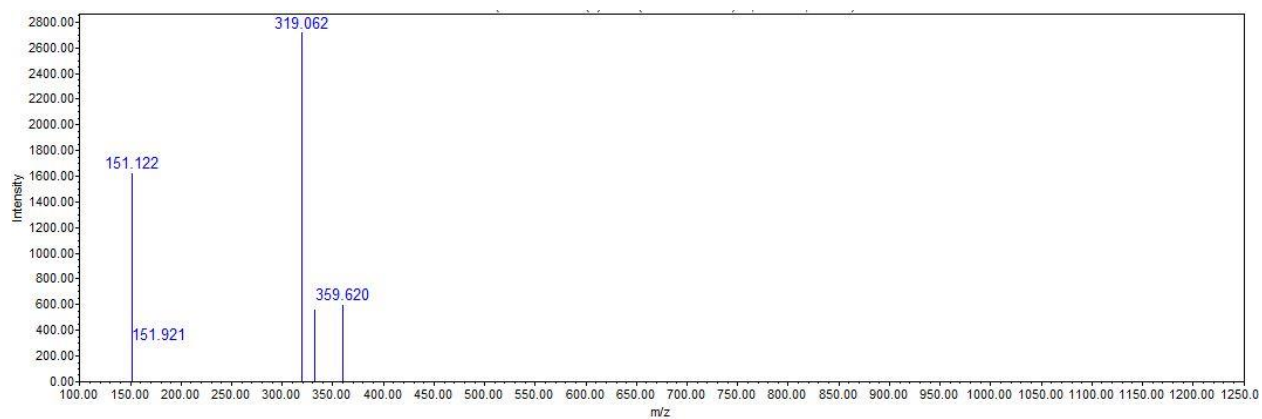


Figure 3 Molecular fragmentation of Myricetin (flavonol) 319.062 m/z

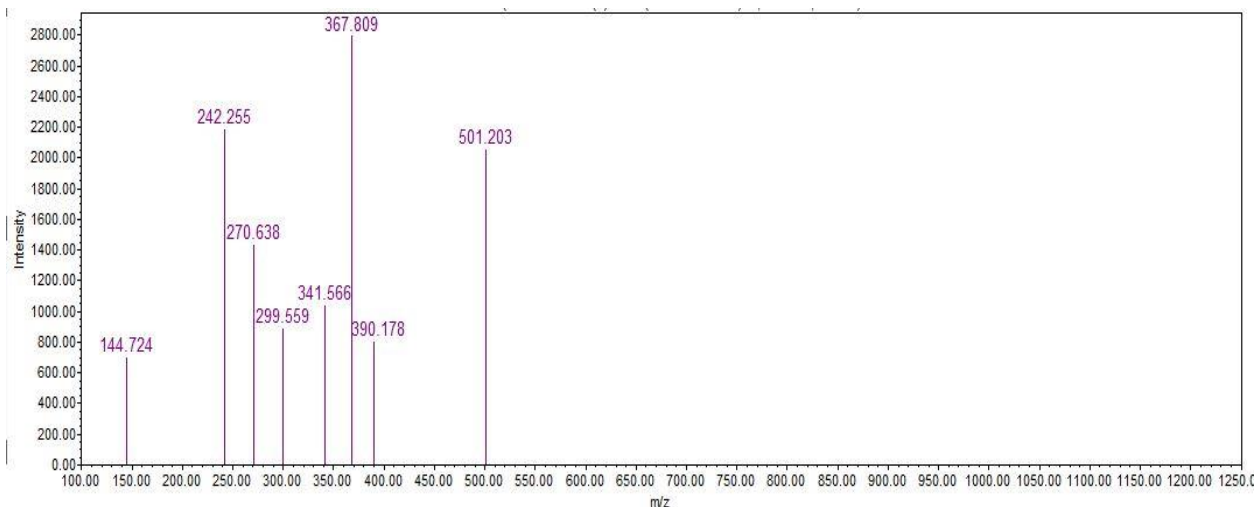


Figure 4: Molecular fragmentation of Furocoumarinic acid (phenolic glycoside) 367.809 m/z

DISCUSSION

In the present study, aqueous leaf extract of *C. micranthum* exhibited DPPH radical scavenging activity of 80.351 ± 0.732 at 2.5 mg/ml concentration which is significantly different with reference standard of 84.718 ± 0.605 (Ascorbic acid), and concentration or dose-dependent. The antioxidant capacity was also expressed as 50% inhibitory concentration (IC_{50}) and it shows a value of 1.491 ± 0.716 , however the ascorbic acid used as a reference standard has higher antioxidant potential than the extract used with IC_{50} value of 1.039 ± 0.006 . Similarly, for the FRAP, the result indicated that aqueous leaf extract of *C. micranthum* revealed appreciable antioxidant activity with 0.800 ± 0.00 at 2.5mg/ml of the extract as compared to 0.426 ± 0.000 for ascorbic acid as reference standard, however the results shows concentration or dose-dependency.

Previous studies revealed there was positive and potential effect on cardiac performance as a results of flavonoid intake (Banjarnahor and Artanti 2014). Recently, *in vivo* studies conducted by Liu, *et al.* (2014) using experimental rat found that production of antioxidant enzyme activity increases by quercetin consumption, such as glutathione-peroxidase (GSH-Px), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT), similarly it helps to preserve membrane integrity and subsequently prevents lipid peroxidation. Therefore based on these findings, the extract could be a good supplement that will help in prevention of lipid peroxidation or oxidative stress and subsequently organ damage.

The result of the LC-MS analyses aqueous leaf extract of *C. micranthum* revealed the presence of medicinally important compounds like Myricetin (Flavone), Epioxylubimin (Sesquiterpenoids), Furocoumaric acid (Phenolic glycoside) among others. Myricetin (Flavone), one of the most important flavonols, has revealed its protective effect on HIT-T15 pancreatic beta cells from oxidative damage (Banjarnahor and Artanti 2014) due to its antioxidant properties. Therefore, the activity observed in this study could be as a result of these metabolites. In similar studies, some of these compounds were shown to be good scavengers of ROS produced by glucose toxicity in type 2 diabetes (Banjarnahor and Artanti 2014). Therefore the ability to directly scavenge the activity of ROS, should be the best way to described antioxidant activity of flavonoids. Flavonoids are able to chelate free radicals immediately by donating a hydrogen

atom or by single-electron transfer, another possible mechanism of action of flavonoids is through the chelation of transition metal elements. Flavonoids possess chelating potential, which enabled them to chelate, or binds to metal ions in human body to prevent them being accessible for oxidation. Certain flavonoids have potential capacity to chelate trace metal ions such as Fe²⁺ and Cu²⁺ that play a vital role in oxygen metabolism and free radical formation (Banjarnahor and Artanti 2014). The anti-inflammatory, antioxidant, antimicrobial and antidiabetics properties of most of the identified metabolites from this extract were reported in different literature. Flavonoids have cardiovascular protective effect which resembles their antioxidant activity. Other studies showed cumulative evidence that strongly linked the oxidative stress to cardiovascular diseases, such as myocardial infarction, myocardial ischemia or reperfusion, and atherosclerosis as well as hypertension and heart failure (Banjarnahor and Artanti 2014).

A model on the antioxidant properties of terpenoids constituents in 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, was proposed by Wojtunik *et al.* (2014). The role of terpenoids as chemopreventive and therapeutic agent against some health conditions was reported by Thoppil *et al.* (2011). In similar study conducted by Zwenger and Basu (2008), the roles and future potentials of plant terpenoids was also evaluated. The therapeutic uses with special emphasis on antidiabetic potential of diterpenes, triterpenoids from *Salvia macrochlamys* were compared (Topçu *et al.*, 2007; Nagarajan and Brindha 2012). Kasote *et al.* (2015) proved remarkable nature of polyphenolics in terms of antioxidant potential to cure many lifestyle diseases, as they contain one or many aromatic rings with hydroxyl groups. Generally, the antioxidant capacity of the phenolic is directly related with the number of free hydroxyls and conjugation of side chains with the aromatic rings. (Mohandas and Kumaraswamy 2018) Flavonoids and phenolic acids of plant phenolics, are metabolically originated from the acetate via shikimate pathways, as well as the shikimate pathway from phenylalanine or tyrosine (Mohandas and Kumaraswamy 2018). Phytochemicals of this group are excellent antioxidants in both *in vitro* and *in vivo* studies. In addition, they are proven to interplay with other natural antioxidants like ascorbate or tocopherol and to synergistically enhance their biological potentials (Mohandas and Kumaraswamy 2018).

CONCLUSION

The present study shows the potentiality of the aqueous leaf extract of *C. micranthum* based on the *in vitro* assay using DPPH and Ferric Reducing Power methods. The LCMS profile conducted identify prominent metabolites (flavonols and Terpenoids) that were reported to be good antioxidant. Therefore the activities observed from this study could be as a results of the metabolites identified. Also the extract could be used against oxidative stress related diseases and or for other pharmacological benefits.

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