

## COMPARATIVE ASSESSMENT OF PHYTOCHEMICALS AND ANTIOXIDANT POTENTIAL OF METHANOLIC AND AQUEOUS EXTRACTS OF *CLERODENDRUM COLEBROOKIANUM* WALP. LEAF FROM NORTH-EAST INDIA

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### ABSTRACT

**Objective:** The present study was undertaken, to evaluate the *in vitro* antioxidant capacity as well as phytochemical quantification of *Clerodendrum colebrookianum*.

**Methods:** A 70% methanolic extract (CCLM) and aqueous extract (CCLA) of *C. colebrookianum* Walp. leaves to evaluate different *in vitro* tests in diversified fields including the total antioxidant activity and the extent of their abilities to scavenge for various reactive oxygen species (ROS), reactive nitrogen species (RNS) and the analysis of various phytochemicals present in both the extracts followed by an attempt to clarify the active compounds through High performance liquid chromatography (HPLC) conferring antioxidant capacity.

**Results:** Results confirmed a better efficacy of CCLM in projecting total antioxidant activity and scavenging of DPPH, hydroxyl, superoxide, nitric oxide and HOCl radicals. CCLM also displayed impressive amounts of phytochemicals like phenolic & flavonoid compounds, carbohydrates, tannins and alkaloids. HPLC chromatogram revealed the presence of compounds that represent the retention peaks of the compared standard phytocompounds-tannic acid, quercetin, catechin, reserpine, ascorbic acid and gallic acid.

**Conclusion:** These results confirm the possible role of the 70% methanolic extract as a promising free radical scavenger and as a potent antioxidant source.

**Keywords:** Free radicals, HPLC, Phytochemical analysis.

### INTRODUCTION

The use of traditional medicine is widespread and plants provide a large source of natural antioxidants that might serve as leads for the development of novel drugs[1]. Normally formed as resultant by-products of a plethora of metabolic processes, the reactive oxygen species (ROS) and reactive nitrogen species (RNS), apart from playing a beneficial role in physiological processes, are more pronounced for their detrimental effects on living systems. The overproduction of ROS, resulting either from mitochondrial electron transport chain or excessive stimulation of NAD(P)H leads to oxidative stress thereby causing damage to cell membranes, lipids, proteins and nucleic acids[2]. These deleterious changes are implicated in the pathogenesis of various human diseases such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation and ageing process. To deal with oxidative stress, antioxidant enzymes including superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) etc., along with other intrinsic repair systems try to establish the redox equilibrium, but again fail occasionally, to entirely prevent the damage. This has encouraged the development of chemical antioxidants in the past few years and many have been clinically accepted, but their wide application has been restricted due to negative side effects and escalating costs[3]. Polyphenols, flavonoids, tannins and other bioactive substances of herbal origin display various biological roles, including antioxidant potential, free radical scavenging ability, anti-inflammatory, anti-diabetic, anticarcinogenic activities[4], and work as potential iron chelators[5]. Thus, many eyes drifted towards evaluating the natural antioxidants of plant origin for their respective efficacies which, on the other hand, showed lesser or no side effects.

The East Indian Glory Bower *Clerodendrum colebrookianum* Walp., one of the most well-known among ~400 species of *Clerodendrum*, is an impressive inclusion in the folklore medicinal practices of North-East India for the treatment of various ailments and disorders since ages. Apart from antihypertensive property[6], its use has been reported for dealing with infant anti-colic pain, diabetes[7], helminthic infections[8], stomach disorder and headache[9] and for

the treatment of cough, dysentery and some skin diseases[10]. Among the *Khasi* and *Jaintia* tribes in Meghalaya where it is commonly known as *Sla Jarem*, consuming the water after boiling the leaves is a traditional practice for the treatment of high blood pressure, malaria and liver troubles and in case of rheumatic pains, application of the warmed leaf - paste on the affected area is a common traditional practice. Experimental evidence on high-fat diet fed Wistar rats show a declining level of total cholesterol, triglycerides and LDL, upon treatment with extracts of *C. colebrookianum*[6]. A dose dependent hypoglycemic effect is also observed with the aqueous extract of *C. colebrookianum*[11]. The present study aims towards the antioxidant and free radical scavenging activities of aqueous and 70% methanolic extracts of *C. colebrookianum* leaves followed by the analysis of the availability and abundance of different phytochemicals.

### MATERIALS AND METHODS

#### Chemicals

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was procured from Roche diagnostics, Mannheim, Germany, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), 2-deoxy-2-ribose, ethylene diamine tetraacetic acid (EDTA), ascorbic acid, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine metho-sulfate (PMS), sodium nitroprusside (SNP), 1,10-phenanthroline, sulphanilamide, naphthyl ethylenediamine dihydrochloride (NED), L-histidine, lipoic acid, sodium pyruvate, quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. HPLC grade acetonitrile, ammonium acetate, hydrogen peroxide, potassium hexacyanoferrate, Folin-ciocalteu reagent, sodium carbonate, mercuric chloride, potassium iodide, anthrone, vanillin, thiourea, 2,4-dinitro-phenylhydrazine, sodium hypochlorite, aluminium chloride, xylene orange, butylated hydroxytoluene (BHT) and N,N-dimethyl-4-nitrosoaniline were taken from Merck, Mumbai, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, (+) catechin and

curcumin were obtained from MP Biomedicals, France. Catalase, reserpine and sodium bicarbonate were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue was purchased from BDH, England. D-glucose was procured from Qualigens Fine Chemicals, Mumbai. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

#### Plant material and preparation of extract

Fresh leaves of *C. colebrookianum* were collected from East Khasi hills district of Meghalaya in the winter season and specimens of the same were submitted and authenticated by the herbarium curator, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India and a voucher no. 6786 was obtained. The leaves were cleaned and dried at room temperature, finely powdered and used for extraction. The leaf powder was mixed with the respective solvents (H<sub>2</sub>O and 70% methanol) in a 1:10 ratio w/v, i.e. 100 g in 1000 ml, using a magnetic stirrer for 15 h and then centrifuged for obtaining the supernatant. The process was repeated by mixing the precipitated pellet with 1000 ml fresh solvent. The supernatants from both the phases were mixed and concentrated under reduced pressure in a rotary evaporator, followed by lyophilisation. The lyophilized extract was stored at -20°C until experimentation. The aqueous and 70% methanol extracts of *C. colebrookianum* leaves were marked as CCLA and CCLM, respectively.

#### HPLC standardization of the extract

The method was carried out as previously described[5]. 10 µg/ml stock solutions are prepared in mobile phase for the sample and standards. The detection is carried out at 254 nm. The injection volume is 20 µl and the sample and standards are analyzed in triplicates with gradient elution for 80 min.

#### Phytochemical analysis:

The analysis of resident phytochemicals like alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, anthraquinones and triterpenoids in both the extracts were carried out using standard qualitative methods as described previously[12,13].

#### Determination of Total Phenolic Content

According to the protocol described earlier[14], Folin-Ciocalteu (FC) reagent was used to determine the total phenolic content of the CCLA and CCLM. Absorbance was measured at 725 nm. All tests were performed six times. The phenolic content was calculated from a gallic acid standard curve.

#### Determination of total flavonoids

Total flavonoid content was determined according to a previously discussed method[14] using quercetin as a standard. The absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve.

#### Quantification of carbohydrate content

Carbohydrate contents of both the extracts were quantified using previously described method[14]. Absorbance of the resultant dark green coloured solution was measured at 630 nm. All tests were performed six times. The carbohydrate content was evaluated from a glucose standard curve.

#### Quantification of alkaloid content

Quantification of alkaloid content for CCLA & CCLM was carried out using previously described method[14]. The absorbance was taken at 500 nm and all tests were performed six times. The alkaloid content was evaluated from the reserpine standard graph.

#### Quantification of ascorbic acid content

This quantification was carried out according to the previously described method[14]. All tests were performed six times. The ascorbic acid content was evaluated from a L-ascorbic acid standard curve.

#### Quantification of Tannin content

This assay was performed as per a previously described method[14]. The absorbance of the resulting magenta-pink colour was measured at 500 nm. All tests were performed six times. The tannin content was evaluated from a catechin standard graph.

#### In vitro antioxidant and free radical scavenging assays

##### Total Antioxidant activity

Antioxidant capacities of both the leaf extracts (0.05–10 mg/ml) were evaluated by ABTS•<sup>+</sup> radical cation decolourisation assay in comparison to trolox standard[15]. Their absorbance was measured at 734 nm.

##### DPPH radical scavenging assay

The complementary study for the antioxidant capacity of the plant extract as well as standard ascorbic acid was confirmed by the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging[14]. The optical density (OD) of the resulting solution was measured at 517 nm.

##### Hydroxyl radical scavenging assay

This assay, as described previously[15] is based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe<sup>3+</sup>-ascorbate-EDTA-H<sub>2</sub>O<sub>2</sub> system (the Fenton reaction). Both the extracts in the dose range (0–200 µg/ml) were quantified for their respective hydroxyl radical scavenging activities spectrophotometrically at 532 nm. Mannitol, a classical OH• scavenger, was used as a positive control.

##### Superoxide radical scavenging assay

The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals that reduce nitro blue tetrazolium (NBT) into a purple-colored formazan. The scavenging activity of the superoxide radical was measured spectrophotometrically at 562 nm, for the leaf extracts (0–20 µg/ml) as described previously[15]. Quercetin was taken as a standard.

##### Nitric oxide radical scavenging assay

Nitric oxide generated from the SNP aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess Illosvoy reaction[15]. A pink coloured chromophore was generated through diazotization of sulphanilamide with nitrite ions and subsequent coupling with NED which was spectrophotometrically measured at 540 nm against the blank sample. Various doses of CCLA and CCLM (0–70 µg/ml) along with the standard curcumin were assessed for their respective activities.

##### Peroxynitrite radical scavenging assay

Peroxynitrite (ONOO<sup>-</sup>) was synthesized 12 h before the experiment[15] and stored at -20°C until use. The percentage of peroxynitrite scavenging was measured spectrophotometrically at 611 nm with Evans blue bleaching and the results were calculated by comparing those of the tests (0–200 µg/ml) and blank samples. Gallic acid was used as reference compound.

##### Singlet oxygen radical scavenging assay

The production of singlet oxygen (<sup>1</sup>O<sub>2</sub>) was determined by monitoring *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported method[15]. Singlet oxygen was generated by a reaction between NaOCl and H<sub>2</sub>O<sub>2</sub> and the bleaching of RNO was read at 440 nm. The scavenging activity of the extracts (0–100 µg/ml) were compared with that of lipoic acid (reference compound).

##### Hypochlorous acid scavenging assay

This assay was carried out as described previously[15]. The scavenging activity of the leaf extracts (0–100 µg/ml) and the standard ascorbic acid was evaluated by measuring the decrease in absorbance of catalase at 404 nm.

### Measurement of reducing power

The Fe<sup>3+</sup> reducing power of the extracts was determined by the method performed earlier[15]. Various concentrations (0–1.0 mg/ml) of the extracts were tested and their absorbance was measured at 700 nm against an appropriate blank.

### Inhibition of lipid peroxidation

The ability of CCLA and CCLM to inhibit lipid peroxidation, was assayed following a method previously described[16]. Various concentrations of plant extracts (2.5–25 µg/ml) were assessed along with a standard Trolox. The absorbance of the reaction mixtures was taken at 532 nm.

### Statistical analysis

All the above tests were performed six times and in each case the percentage of scavenging of the respective radical was calculated using the following equation:

$$\% \text{ scavenging} = [(A_0 - A_1) / A_0] \times 100$$

Where A<sub>0</sub> was the absorbance of the control, and A<sub>1</sub> was the absorbance in the presence of the sample of fruit extracts and standard. All data were reported as the mean ± SD of six measurements. The statistical analysis was performed by KyPlot

version 2.0 beta 15 (32 bit). The IC<sub>50</sub> values were calculated by the formula,

$$Y = 100 \times A_1 / (X + A_1)$$

where A<sub>1</sub> = IC<sub>50</sub>, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC<sub>50</sub> values were compared by paired t test (two-sided). p < 0.05 was considered significant.

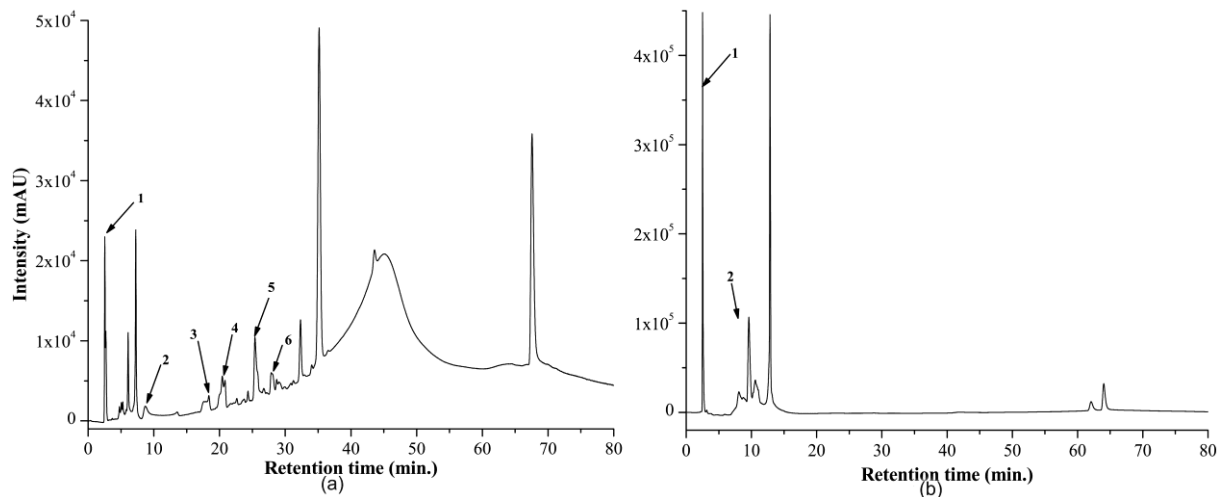
### RESULT

The analysis for the phytochemical contents of the aqueous and 70% methanolic extracts revealed that *C. colebrookianum* leaves showed positive results for phenolic, flavonoid, carbohydrate, and alkaloid compounds, among all the other components tested for (Table 1). In case of tannins, it was only evident in CCLM. The total phenolic content in CCLA was found to be quite low whereas in case of CCLM it was found to be extremely high, i.e., 104.4±0.73 mg gallic acid equivalent per 100 mg extract. Flavonoid contents in CCLA and CCLM appeared as moderate amounts (Table 1) where CCLM showed a better content than CCLA. CCLA & CCLM both exhibited good carbohydrate contents, where again CCLM showed more amounts of carbohydrate as compared to its counterpart. CCLA and CCLM both showed fairly good quantities of alkaloids. In case of ascorbic acid, CCLM displayed a better content than CCLA. Lastly, CCLM showed a tannin content of 1.65±0.04 mg/ 100 mg extract catechin equivalent (Table 1).

**Table 1: Qualitative and quantitative phytochemical analysis of CCLA and CCLM**

Tests	Samples	Phytochemicals									
		Phen	Flav	Carbo	Tan	Alka	Ter	Triter	Anth	Sap	Gly
Qualitative	CCLA	+	+	+	-	+	-	+	-	+	+
	CCLM	+	+	+	+	+	+	+	-	+	+
Quantitative	CCLA	3.99 ± 0.69	10.5 ± 0.27	39.9 ± 0.17	-	13.4 ± 0.29	ND	ND	ND	ND	ND
	CCLM	104.4 ± 0.73	22.1 ± 0.21	56.5 ± 0.21	1.7 ± 0.04	14.08 ± 0.38	ND	ND	ND	ND	ND

Phen- Phenol, Flav- Flavonoid, Carbo- Carbohydrate, Tan.- Tannin, Alka- Alkaloid, Ter- Terpenoids, Triter- Triterpenoids, Anth-Anthraquinones, Sap- Saponin, Gly- Glycoside; Total phenolics (mg/100 mg extract gallic acid equivalent), Total flavonoids (mg/100 mg extract quercetin equivalent), Carbohydrate (mg/100 mg extract glucose equivalent), Tannin (mg/100 mg extract catechin equivalent). "+" Represents presence of the phytoconstituent; "-" represents absence of the phytoconstituent; "ND" represents Not Determined



**Fig. 1: HPLC chromatogram of CCLA (a) CCLM (b).**

Peaks marked 1 (r.t-2.54 min), 2 (9.02 min), 3 (18.37 min), 4 (20.44 min), 5 (25.39 min), 6 (27.83 min) represent the retention peaks of the compared standard phytocompounds Tannic acid, Quercetin, Catechin, Reserpine, Ascorbic acid and Gallic acid respectively.

When matched with the retention time peaks of the standard phytocompounds the HPLC chromatogram of CCLA confirmed the presence of phytocompounds tannic acid, quercetin, catechin, reserpine, ascorbic acid and gallic acid in the extract (Fig. 1a) marked as 1,2,3,4,5,6 respectively. In case of CCLM, tannic acid and quercetin were evident (Fig. 1b).

The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to

determine the trolox equivalent antioxidant capacity (TEAC). To calculate TEAC, gradient of the plot for the sample was divided by the gradient of the plot for trolox. The total antioxidant activities of the leaf extracts (CCLA & CCLM) and trolox, as shown in Fig. 2(a) and (b), respectively were calculated from the spectral data of decolourisation of ABTS•<sup>+</sup>, obtained at 734 nm. The TEAC value of CCLA was found to be 0.15±0.01 and that of CCLM was 0.45±0.01 (Table 2).

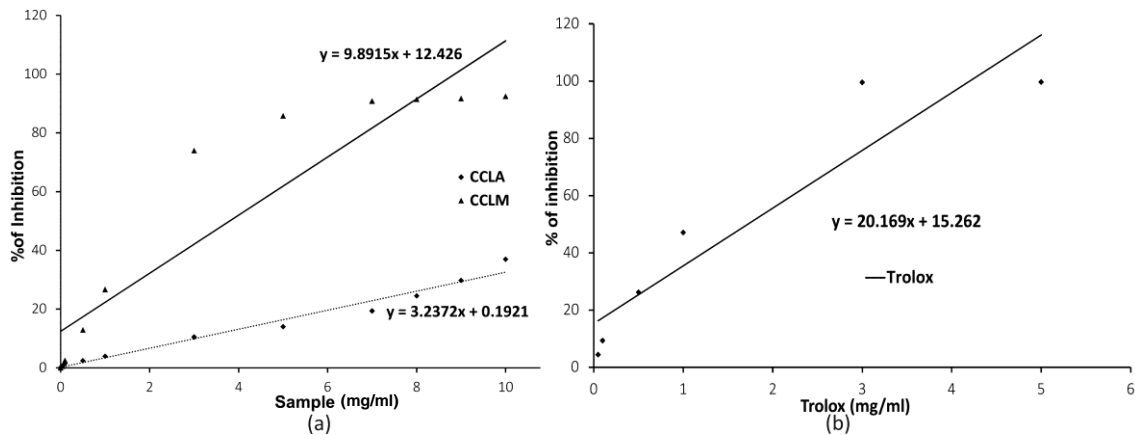


Fig. 2: Total antioxidant activity of CCLA and CCLM (a) trolox standard curve (b)

The TEAC values were determined from and plotted against the concentration of samples. All data are expressed as mean  $\pm$  S.D. (n=6).

The results shown in Fig. 3 is a comparison of the DPPH radical scavenging activities of both CCLA & CCLM and the standard ascorbic acid. CCLM showed a lesser an impressive  $IC_{50}$  value as compared to CCLA (Table 2). The percentage of scavenging of DPPH radical at 100  $\mu$ g/ml for CCLA, CCLM and ascorbic acid is 46.66%, 82.83% and 93.58%, respectively.

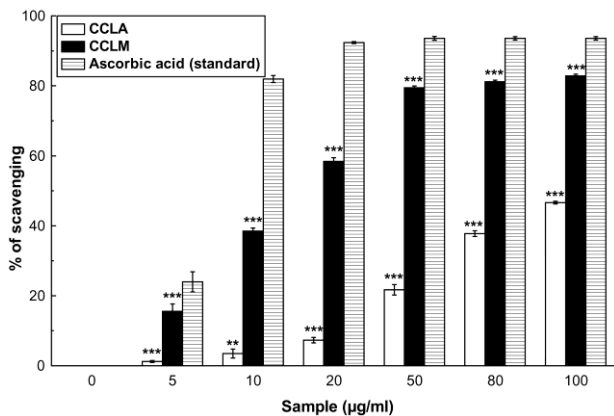


Fig. 3: DPPH Radical Scavenging of the plant extracts and ascorbic acid

The results are mean  $\pm$  S.D. of six parallel measurements. \*\*p < 0.01 and \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.

When analyzed for the abilities of the extracts and standard mannitol to scavenge hydroxyl radical (Fig. 4), the  $IC_{50}$  value (Table 2) for CCLM was found to be very low ( $38.40 \pm 0.27$   $\mu$ g/ml) as compared to that of CCLA and the standard mannitol. The percentages of hydroxyl radical scavenging at the highest dose, 200  $\mu$ g/ml, were found to be 30.27%, 75.08% and 21.9% for CCLA, CCLM and standard mannitol, respectively.

Fig. 5 shows the abilities of the leaf extracts and the reference compound quercetin to quench superoxide radicals in the PMS-NADH reaction mixture. The  $IC_{50}$  values of both CCLA and CCLM were found to be impressive (Table 2) where CCLM showed much better results than even the standard quercetin. The CCLA and CCLM extracts showed percentages of superoxide radical scavenging activity as 64.04% and 62.54%, respectively at the maximum dose 120  $\mu$ g/ml, whereas Quercetin showed 50.67% as the same at the highest dose.

CCLM showed a better nitric oxide radical scavenging activity as compared to CCLA (Fig. 6) which is well evident from their  $IC_{50}$  values (Table 2). CCLA and CCLM showed scavenging percentages of 22.28% and 38.61%, respectively at the highest dose of 70  $\mu$ g/ml. Although throughout the low doses, CCLM showed better scavenging

activity than the standard curcumin, the latter showed 43.91% of scavenging at the highest dose.

When assayed for their possible peroxy nitrite radical scavenging activity (Fig. 7), both CCLA and CCLM showed poor activities of peroxy nitrite radical scavenging (Table 2). CCLA showed a scavenging percentage of 7.90% at the highest dose 200  $\mu$ g/ml, standard gallic acid, 15.44% and CCLM showed a percentage of scavenging of 7.49% at the same dose.

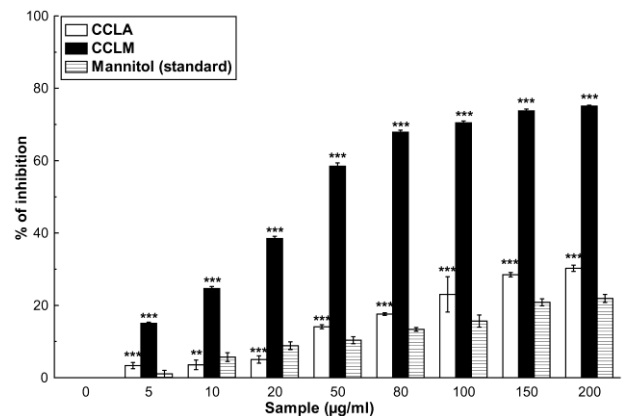


Fig. 4: Hydroxyl radical scavenging activity of CCLA, CCLM and the reference compound mannitol.

All data are expressed as mean  $\pm$  S.D. (n=6). \*\*p < 0.01 and \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.

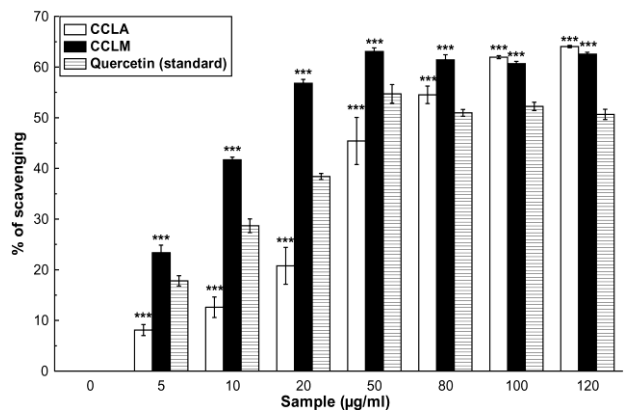
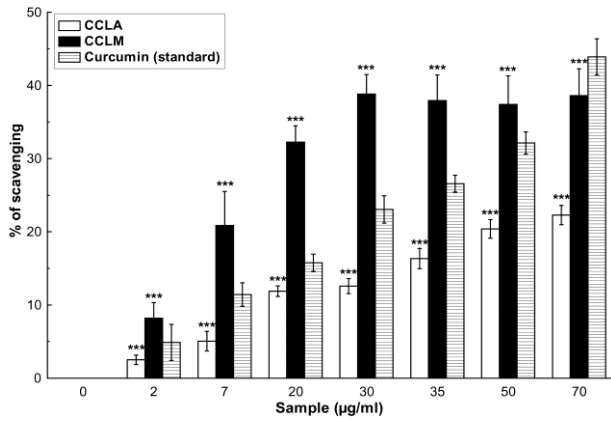


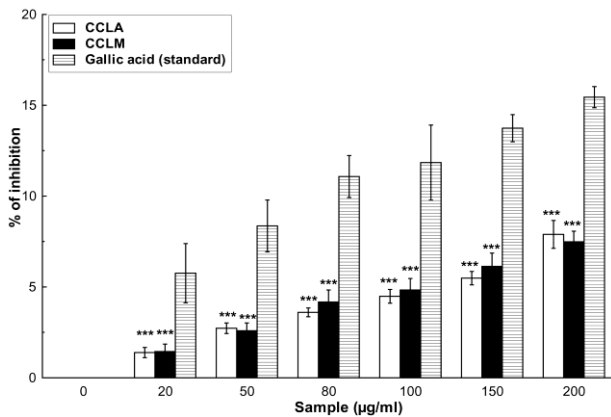
Fig. 5: Superoxide Radical Scavenging of CCLA, CCLM and standard quercetin

All data are expressed as mean  $\pm$  S.D. (n=6). \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.



**Fig. 6: Nitric oxide Radical Scavenging of CCLA, CCLM and standard curcumin**

Each value represents mean  $\pm$  S.D. (n=6). \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.



**Fig. 7: Peroxynitrite Anion Scavenging of CCLA, CCLM and standard gallic acid**

Each value represents mean  $\pm$  S.D. (n=6). \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.

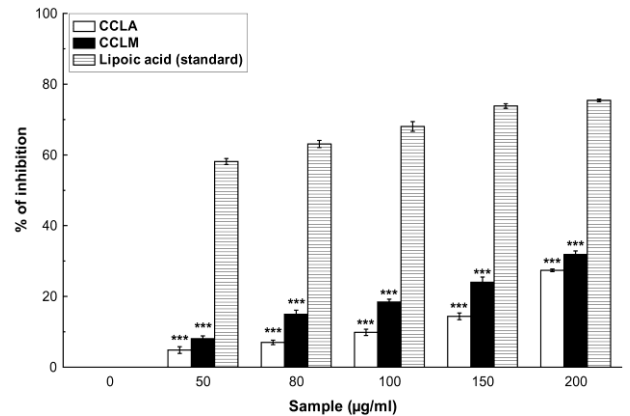
Both extracts showed low but dose-dependent activity as scavengers of singlet oxygen (Fig. 8) in comparison to the standard, lipoic acid (Table 2). The percentages of scavenging of CCLA, CCLM and standard lipoic acid were evaluated as 27.37%, 31.84% and 75.39%, respectively at the highest dose 200  $\mu$ g/ml.

CCLM showed an extremely good dose-dependent HOCl scavenging activity as compared to ascorbic acid, at all doses; CCLA showed lesser activity than CCLM and standard (Fig. 9). The same was evident from their IC<sub>50</sub> values which appeared as 476.31 $\pm$ 71.96  $\mu$ g/ml, 100.16 $\pm$ 8.32  $\mu$ g/ml and 235.96 $\pm$ 5.75  $\mu$ g/ml for aqueous & 70% methanolic extracts and standard ascorbic acid, respectively (Table 2). The percentages of scavenging were evaluated as 15.01%, 47.25% and 35.47% for CCLA & CCLM and standard ascorbic acid, respectively at the highest dose 100  $\mu$ g/ml.

Reducing power of a compound is determined by its ability to facilitate the transformation of Fe<sup>3+</sup> to Fe<sup>2+</sup>. As witnessed by the optical density graphs (Fig. 10), CCLM showed a dose dependent increase in activity. On the other hand CCLA showed a very weak activity at all doses. Ascorbic acid (standard) although, showed a better reducing power than both the leaf extracts.

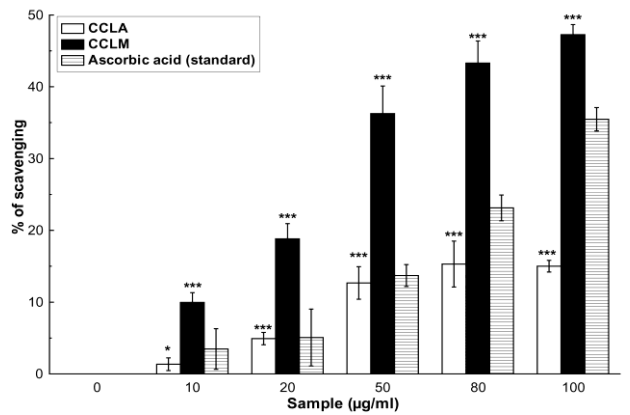
CCLA showed absolutely no effect in inhibiting lipid peroxidation, whereas CCLM showed a modest dose dependent activity (Fig. 11; Table 1) Standard trolox was better in activity than CCLA and CCLM which is evident from their respective percentages of inhibition at

the maximum dose of 25  $\mu$ g/ml, i.e., 78.87%, 24.09% and 0.92%, respectively.



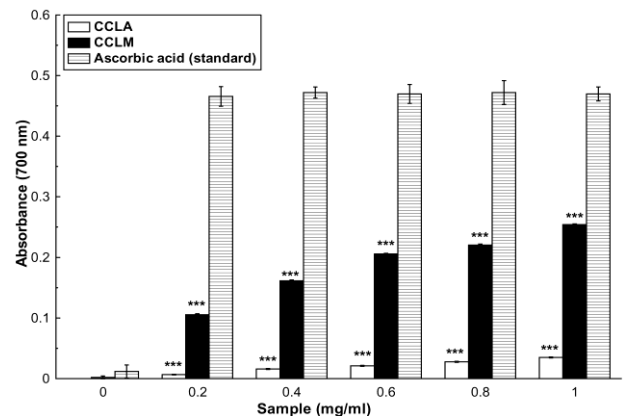
**Fig. 8: Singlet Oxygen Scavenging of CCLA, CCLM and standard lipoic acid**

The results are mean  $\pm$  S.D. of six parallel measurements. \*\*\*p < 0.001 vs. 0  $\mu$ g/ml.



**Fig. 9: HOCl Scavenging of CCLA, CCLM and standard ascorbic acid**

All data are expressed as mean  $\pm$  S.D. (n=6). \*\*\*p < 0.001 vs 0  $\mu$ g/ml.



**Fig. 10: Reducing Power of CCLA, CCLM extracts and standard ascorbic acid**

Each value represents mean  $\pm$  S.D. (n=6). \*\*\*p < 0.001 vs 0 mg/ml.

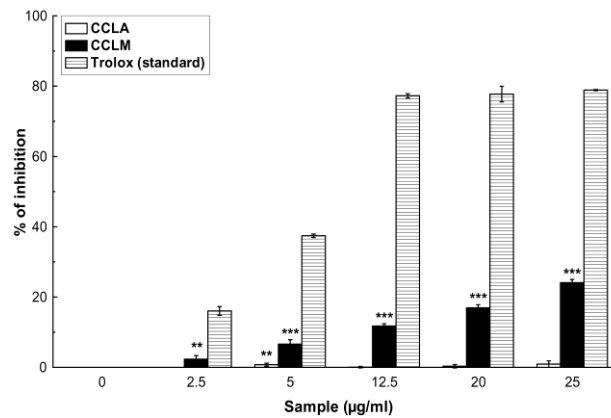


Fig. 11: Inhibition of Lipid Peroxidation of CCLA, CCLM and the standard trolox

Each value represents mean  $\pm$  S.D. (n=6). \*\*p < 0.01 and \*\*\* p < 0.001 vs. 0  $\mu$ g/ml.

Table 2: TEAC and IC<sub>50</sub> values of CCLA & CCLM and standard compounds for ROS scavenging and iron chelating activity

Activity	Extract/Reference	IC <sub>50</sub> (#)
TEAC Values	CCLA	0.45 $\pm$ 0.01
	CCLM	0.15 $\pm$ 0.01
DPPH radical (DPPH <sup>•</sup> ) scavenging	CCLA	141.65 $\pm$ 2.37 ***
	CCLM	16.59 $\pm$ 0.66***
	Ascorbic acid	5.29 $\pm$ 0.28
Hydroxyl radical (OH <sup>•</sup> ) scavenging	CCLA	385.77 $\pm$ 17.35 ***
	CCLM	38.40 $\pm$ 0.27 ***
	Mannitol	571.45 $\pm$ 20.12
Superoxide anion (O <sub>2</sub> <sup>•-</sup> ) scavenging	CCLA	65.27 $\pm$ 5.34
	CCLM	29.23 $\pm$ 0.90 ***
	Quercetin	42.06 $\pm$ 1.35
Nitric oxide radical (NO) scavenging	CCLA	205.13 $\pm$ 14.01 ***
	CCLM	65.42 $\pm$ 8.16 ***
	Curcumin	90.82 $\pm$ 4.75
Peroxynitrite (ONOO <sup>-</sup> ) scavenging	CCLA	2320.07 $\pm$ 136.96***
	CCLM	2264.60 $\pm$ 260.78***
	Gallic acid	876.24 $\pm$ 56.96
Singlet oxygen ( <sup>1</sup> O <sub>2</sub> ) scavenging	CCLA	727.16 $\pm$ 25.96 ***
	CCLM	454.18 $\pm$ 11.95 ***
	Lipoic acid	46.15 $\pm$ 1.16
Hypochlorous acid (HOCl) scavenging	CCLA	476.31 $\pm$ 71.96 ***
	CCLM	100.16 $\pm$ 8.32 ***
	Ascorbic acid	235.96 $\pm$ 5.75
Inhibition of Lipid Peroxidation	CCLA	4729.76 $\pm$ 2716.97**
	CCLM	87.06 $\pm$ 4.47***
	Trolox	6.76 $\pm$ 0.17

# IC<sub>50</sub> values of all activities are determined in  $\mu$ g/ml.

Data expressed as mean  $\pm$  S.D (n=6). Data in parenthesis indicate number of independent assays. EDTA represents Ethylenediamine tetraacetic acid. \*\*p < 0.01, \*\*\*p < 0.001 vs. respective standards.

## DISCUSSION

Kingdom plantae has treasured a wide variety of free-radical scavengers and antioxidants, like flavonoids, carotenoids, anthocyanins, dietary glutathione, vitamins and endogenous metabolites[17] since antiquity. These herbal compounds are affluent antioxidants which have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists in many ways[18]. ROS are generated when electron acceptors, such as molecular oxygen, react easily with free radicals to become radicals themselves and then forming an initiator in the array of damages it is well known for[19]. As mentioned before, due to the stringency in availability of ideal synthetic antioxidants, the fringe is moving towards the herbal natural products for their efficacy as ROS scavengers and safer post-consumption symptoms.

Plant polyphenols (phenolic acids, tannins, flavonoids) are being well documented as potent antioxidants and thus have always been

promising agents as in the vital pathways of tumor-suppression, diabetes and many lethal human disorders and diseases[20]. Phenolic compounds are an important group of secondary metabolites, which are synthesized by plants due to plant adaptation in response to biotic and abiotic stresses (infection, water stress, cold stress, and high visible light)[21]. *C. colebrookianum* leaves have shown extremely good amounts of total phenolic contents in its 70% methanolic extract (Table 1). In addition to that it showed moderate amounts of total flavonoid contents in both, the aqueous and 70% methanolic extracts, the later showing almost double the content of what was displayed in case of CCLA. Moreover almost equal and fairly good amounts of alkaloids were evident in both the extracts, further supporting its folklore medicinal evidence, as mentioned earlier in the text. There were more amounts of carbohydrates in CCLM as compared to CCLA as shown in Table 1, indicating its nutritional value. Ascorbic acid too was found in both the extracts in indicative amounts. In case of tannins some amount was evident only in CCLM.

The above phytochemical data was supplemented by HPLC, where we observed the presence of tannic acid, quercetin, catechin, reserpine, ascorbic acid and gallic acid (Fig. 1). The mentioned compounds are potent antioxidants, as documented in many places and have played a substantial role in the treatment of various diseases and disorders[4,19,20]. In both the crude extracts what we obtained was a speculated synergistic or a probable collective effect of them working in natural concentrations as present in the plants which may show interesting inferences, when worked upon further.

ABTS•<sup>+</sup> is a blue chromophore produced by the reaction between ABTS and potassium persulfate which is reduced to ABTS in a concentration dependant manner upon addition of the 70% methanolic and aqueous extracts. The results are compared with trolox and the TEAC values demonstrate that CCLM was proved to be a potent antioxidant (Table 2). On the other hand, CCLA showed a good total antioxidant activity, although lesser than that of its counterpart. The antioxidant capability of the extract mainly relies on phenolic compounds[21].

The ability to scavenge DPPH radicals further compliments the total antioxidant capacity of a drug. The results here too support the aforementioned fact where both the extracts showed promising activities; CCLM out of both showed a very good tendency towards scavenging the radical, which is evident from the IC<sub>50</sub> value of the same, i.e., 16.59±0.66 µg/ml which is very near to that of the standard ascorbic acid.

Among all the radicals imposing detrimental effects, hydroxyl radical is the most common and versatile of all as it is responsible to lead to enormous amounts of damage to biomolecules and lipid peroxidation[22]. They were produced in this study by incubating ferric-EDTA with ascorbic acid and H<sub>2</sub>O<sub>2</sub> at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH[23]. CCLM showed a tremendously good activity in scavenging these radicals *in vitro* as compared to CCLA and standard.

Superoxide anion is also another harmful reactive oxygen species as it damages cellular components in biological systems. The ability of the leaf extracts and the reference compound quercetin to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance at λ = 560 nm. The results (Fig. 5 & Table 2), indicate the fact that the leaf extracts are more potent scavengers of superoxide radical than the reference compound quercetin; out of which, CCLM showed much better activity of scavenging than the rest, even at low doses.

Direct tissue toxicity and vascular collapse associated with septic shock, may result from a sustained production of the nitric oxide radical; furthermore chronic expression of the radical contributes in many carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis[24]. CCLM showed good NO• scavenging activity which appeared even better than that of the standard, whereas CCLA showed a sound but a dose dependent effect, but not as good as the rest two.

The formation of highly reactive peroxynitrite (ONOO<sup>-</sup>), in inflamed tissues by diffusion-limited reaction of NO• with superoxide anion, in excess amounts, contribute in many pathological conditions[25]. To our unfortunation both the extracts showed poor peroxynitrite scavenging abilities which was evident from their respective IC<sub>50</sub> values.

UVA radiation-generated singlet oxygen reacted with phosphatidylcholine to form lipid hydroperoxides, and the breakdown of these hydroperoxides to thiobarbituric acid-reactive species was dependent on iron[26]. Singlet oxygen induces hyperoxidation, oxygen cytotoxicity and decreases the antioxidant activity[27] Both CCLA & CCLM although showed an attractive dose-dependent activity towards scavenging singlet oxygen, but none could show an IC<sub>50</sub> value as impressive as the standard lipoic acid.

When tested for scavenging ability towards hypochlorous acid CCLM showed excellent results as compared to ascorbic acid (standard) and to its aqueous counterpart. CCLM showed a scavenging

percentage of 47.25% at the highest dose of the experiment which was much higher than that of the rest. Moreover, the IC<sub>50</sub> value of CCLM was lesser than half of what we observed in case of the standard compound. CCLA on the other hand showed a dose dependent but lesser efficiency in scavenging HOCl. HOCl is generated as a result of the oxidation of Cl<sup>-</sup> ions by the neutrophil enzyme myeloperoxidase at the sites of inflammation, and eventually causes breakdown of the heme prosthetic group and inactivates the antioxidant enzyme catalase and thus results in various complications.

The reducing capacity of any compound may serve as a remarkable indicator of its potential antioxidant activity. The reducing power of the leaf extracts was compared with standard ascorbic acid which conferred a decent dose-dependent activity of the 70% methanolic extract, although its net activity, along with that of CCLA, is lesser than that of the standard ascorbic acid (Fig. 10).

Lipid peroxidation is an oxidative amendment of polyunsaturated fatty acids in the cell membranes that result in a huge number of degradation products. The inhibition of FeSO<sub>4</sub>-ascorbic acid induced TBARS formation in the brain homogenate by any compound, indicates its lipid peroxidation inhibitory activities. Although CCLM showed a fair dose dependent efficacy in inhibiting the lipid peroxidation but it failed to work as efficiently as the standard trolox. CCLA on the other hand provided utter disappointing results at all doses by showing no activity. As depicted in Fig. 11, the increase in inhibition of lipid peroxidation with increasing concentration of CCLM reflects its antioxidant property.

All these information leads us to conclude that *C. colebrookianum* Walp. harbours immense qualities and can further prove a pivotal role in the field of antioxidant and anticancer research. The results suggest that among both, the 70% methanolic extract will play a substantially more important role in further experiments as compared to the aqueous extract. There is a need for further research work on the active compounds of the 70% methanolic extract for their individual activities, and understanding the mechanism of action in each case.

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