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EXTRACTION, PHYTOCHEMICAL EVALUATION AND CHARACTERIZATION OF POLYPHENOLS AND FLAVONOIDS FROM AQUEOUS METHANOLIC LEAF EXTRACTS OF INDIGENOUS PLANT *CLERODENDRUM COLEBROOKIANUM* WALP AND *CENTELLA ASIATICA* LINN OF NORTH EAST INDIA

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ABSTRACT

Extraction of polyphenols and flavanoids were done for two indigenous plants *Clerodendrum colebrookianum* walp and *Centella asiatica*. Extraction was done by both cold maceration and continuous hot percolation. Determination of total phenolic and flavanoid content was carried out. Characterizations was done by TLC study, UV analysis, melting point determination and FTIR study. More total phenolic content found in *Clerodendrum colebrookianum* walp (621.02 ± 0.03 mg of GAE/gm plant extract) and total flavonoid content was found more in *Centella asiatica* L.(596.40 ± 0.53 mg of Quercetin equivalent/gm plant extract). UV and FTIR study revealed the presence of both flavonoids and phenolics in the respective extracts as compared with gallic acid and quercetin. High content of phenolic and flavanoid shows the sign of antioxidant activity of the respective plants. Both the plants showing rich in total polyphenols and total flavonoids**.** Datas were mean ± SD and analyzed by One-way ANOVA, using Graphpad INSTAT. Confidence interval has been considered as 99% and $p < 0.01$ were considered significant. Phenolics have shown to possess an important antioxidant activity toward free radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure. It has been established a highly positive relationship between total phenols and antioxidant activity in many plant species.

KEYWORDS: Polyphenols**,** flavonoids, extraction, indigenous plant, phytochemical evaluation.

INTRODUCTION

Medicinal herbs have a relevant and crucial role to play towards attaining the goal of a proper human healthcare. The effective phyto-components obtained from plants are generally regarded as safe (GRAS) and are ecofriendly. The components used are believed to have better compatibility with human systems. Though effective, herbal medicines are not scientifically exploited, therefore this domain needs proper study in the light of modern science.[1] *Clerodendrun colebrookianum* Walp (Family, Verbenaceae) is one of such important medicinal plants, widely used by the local people of this region as a cardio protective agent and most popularly known as "Nefafu" in Assam, "Phuinum" in Mizoram and "Arun" in Nagaland.[2,3] *Clerodendrum colebrookianum* is distributed widely in the South and South-east Asia.[4] The Mizo people of North east region are claiming that low incidence of hypertensive people among their community member is due to the regular intake of this medicinal plant as vegetables. The plant reported to contain triacontane, amyrin, clerodin, (24s) ethyl cholesta 5, 22, 25 trien 3-ol, clerodolone, clerodendoside, B-sitosterol, clerosterol, daucosterol, colebrin A-E.[5,6,7] The leaves and leaf twigs of this plant are used as home remedy for high blood pressure by the people of North-Eastern regions of India.[5,8] *Clerodendrum colebrookianum* Walp has been considered as the most important medicinal species which is used in the treatment of hypertension by different tribes of north east India.[8] However, the use of the plant for cure or treatment of diseases is based on administration of the leaves or flowers either by boiling or as vegetable.

Centella asiatica (L.) Urban, known as Asian pennywort, guta kola (Indian) and bua-bok (Thai), is a small creeping herb that has long been used in traditional medicine and various purposes. It is a tropical medicinal plant with a long history of therapeutic used for many conditions such as dermal disorder, vascular diseases, microangiopathy, and inflammatory.[9,10] *Centella asiatica* extract exerted potent antioxidative activity as indicated by various assay system.[11] *Centella asiatica* (L.) contains high total phenolic content which contributed by the flavonoids such as quercetin, kaempherol, catechin, rutin, apigenin and naringin[12] and

is said to have a direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in Ayurvedic Pharmacopoeia.[13,14] A flavonoid quercetin found in *Centella asiatica* (L.) was used as positive control in various study since it has been shown to promote relaxation of cardiovascular smooth muscle (antihypertensive effects).[15]

Aim of present study is to extract polyphenols and flavonoids from both the plants and phytochemical evaluation and characterization of the same.

MATERIALS AND METHODS

Collection and authentication of plant materials

The leaves of *Clerodendrum colebrookianum* Walp. and *Centella asiatica* Linn. were collected from Sivasagar district of Assam, India during the month of June-July and January-February. The leaves were washed thoroughly followed by shade drying and were preserved as herbarium sheet. The authentication was carried out in Botanical Survey of India (BSI), Eastern Regional Centre, Shillong, authentication number being DU/PSC/HRB/RJD/1/2016 and DU/PSC/HRB/RJD/2/2016 respectively.

Preparation of Plant Extract

The shade dried leaves were grinded to obtain powder passing through sieve no 60 of ASTM series. About 500 grams of the powdered leaves were taken and extracted in a Soxhlet apparatus with aqueous methanol as a solvent. The extraction was allowed to continue for 72 hours and were concentrated to a dry mass by using a rotary evaporator (BUCHI, Switzerland) followed by lyophilization (IIC, India) into a dried mass by lyophilyzer. Cold maceration was also followed for extraction.

Phytochemical evaluation of plant extracts

Extracted material was subjected to various preliminary phytochemical screening for the detection of various secondary metabolites as shown in Table 1. Detection of alkaloid (Mayer's test, Wagner's test, Hager's test, Dragendorff's test), carbohydrate (Molish's test, Fehling's test, Benedict's test, Barfoed's test), saponins, phenols (Ferric Chloride Test, Gelatin Test), glycosides (Borntrager's test, Lead Acetate Test), flavonoids(Magnesium and Hydrochloric acid reduction/Shinoda test, Alkaline reagent test), proteins and amino acids (Millon's test, Biuret test, Ninhydrin test, Legal's test, Keller-Killiani test), phytosterols (Liebermann- Burchard's test), fixed oils (Spot test, Saponification test), fats and gums and mucilages were performed.[16,17]

Chromatographic technique

Chromatography is the method of separation of a mixture of components into individual components. TLC study was performed to separate out individual components or secondary metabolites from the plant extract using different solvent systems.[16] The extracts was spotted on an activated pre-coated TLC plates (TLC Silica gel 60 F_{254}) and was exposed to various mixtures of organic solvents in different ratios for segregation of the constituents. R_f values were calculated using the following formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance traelleDd by solvent}} \quad \text{(Equation 1)}$$

Analytical methods used for characterization of the plant extracts

UV-Visible spectroscopy

Aqueous methanolic extracts of *C. colebrookianum* Walp and *C. asiatica* Linn, Quercetin and Gallic acid were examined by UV-Spectrophotometer in the range of 300nm to 800nm.

FTIR spectroscopy

Aqueous methanolic extracts of *C. colebrookianum* Walp and *C. asiatica* Linn, Quercetin and Gallic acid were determined by IR absorption spectrophotometer in the range 500 cm^{-1} to 4000 cm^{-1}.

Estimation of total phenolic content

The total phenolic content of the aqueous methanolic extract of *C. colebrookianum* and *C.asiatica* were determined by the method adopted by Ordonez 2006 using Folin-Ciocaltau reagent with slight modifications.[18] The content of phenolics in extracts was expressed in Gallic acid equivalents (GAE). All samples were analyzed in triplicates.

Estimation of total flavonoids content

Total flavonoid content was measured by the aluminum chloride colorimetric assay. The aqueous methanolic extracts of *C. colebrookianum* and *C.asiatica* were dissolved in methanol in the concentration of 1mg/ml and diluted. 1ml of the diluted extract solution was taken in a test tube and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer (UV-1800 Shimadzu, Japan) at 415 nm. The calibration curve was prepared by using quercetin (standard compound) methanolic solutions at different concentrations between (10 – 90) µg/ml. The content of flavonoids in extracts was expressed in terms of Quercetin equivalent (QE).[19] All samples were analyzed in triplicates.

Statistical Analysis

The data were subjected to statistical analysis. All the values are expressed as mean ± SD and data were analyzed by One-way ANOVA, using Graphpad INSTAT. The post-hock analysis was carried out by Dunnet's multiple comparison tests to estimate the significance of difference between individual groups (**P<0.01). Confidence interval has been considered as 99% and $p< 0.01$ were considered significant. IC_{50} value was calculated by plotting a graph with percent inhibition on y-axis and concentration on x-axis.

RESULTS

Phytochemical evaluation of plant extracts

Specific reagents were used to perform the test, the results were observed either in the form of colour or precipitation reaction and additionally TLC profiling was done to suffice the specificity of the class of component. The results observed are given in Table 1, 2 and 3 and Figure 1 and 2.

Table 1: Phytochemical evaluation of plant extracts.

Sl. no	Constituents	*Centella asiatica*	*Clerodendrum colebrookianum*
1.	Alkaloid	+	+
2.	Carbohydrate	+	+
3.	Saponin	-	+
4.	Phenol compounds	+	+
5.	Glycosides	+	+
6.	Flavonoids	+	+
7.	Phytosterols	+	+
8	Protein and Amino acid	-	-

Table 2: R_f value and the solvent used as a mobile phase in *Clerodendrum colebrookianum*.

Constituents	Solvents used	Ratio	$R_{f\ value}$
Flavonoids	Chloroform – Acetone - Formic acid	(7.5:1.7:0.9)	0.85
	Ethylacetate - Chloroform	(4:6)	0.92
	Chloroform	(10)	0.81
	Benzene – Pyridine - Formic acid	(7.2:1.8:1.0)	0.93
	Ethylacetate - Formic acid - Glacial Acetic acid - Water	(6.8:0.7:0.7:1.8)	0.96
	Ethylacetate - Formic acid - Methanol-Water	(5:0.7:0.3:0.1)	0.82
Alkaloids	Toluene - Ethylacetate - Diethylamine	(7:2:1)	0.65
Anthracene Glycoside	Ethylacetate – Methanol – Water	(7.7:1.3:1)	0.73
Carbohydrates	Acetonitrile – Water	(8.5:1.5)	0.78
Saponin	Chloroform – Methanol	(9.9:0.1)	0.90
Protein	n-Butanol – Acetic acid – Water	(6:2:2)	-

Table 3: R_f value and the solvent used as a mobile phase in *Centella asiatica*.

Constituents	Solvents used	Ratio	$R_{f\ value}$
Alkaloids	Methanol: Ammonium hydroxide	17:3	0.71,0.78
Flavanoids	Chloroform: Methanol	18:2	0.16,0.30,0.45,0.60,0.73,0.82,0.85
Saponins	Chloroform:Glacial acetate:methanol:water	6:2:1:1	0.08
Terpenoids	Benzene: Ethyl acetate	1:1	0.36,0.40,0.46,0.73,0.86

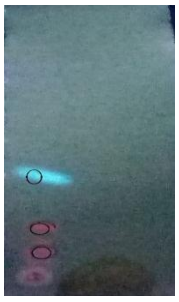

Fig. 1: TLC study of *C.asiatica.*

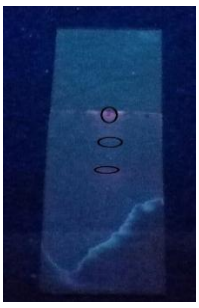

Fig. 2: TLC study of *C.colebrookianum*.

Analytical methods used for characterization of the plant extracts

UV-Visible spectroscopy

Aqueous methanolic extracts of *C. colebrookianum* Walp and *C. asiatica* Linn, Quercetin and Gallic acid were examined by UV-Spectrophotometer in the range of 300nm to 800nm and spectra are reported as Figure 3 to figure 6. Similar peaks were shown by the two extracts with standard flavonoids and phenols.

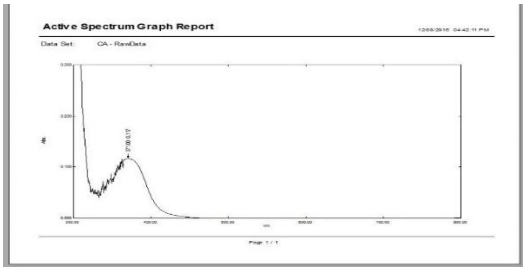

Fig. 3: UV spectrum of *C.colebrookianum*.

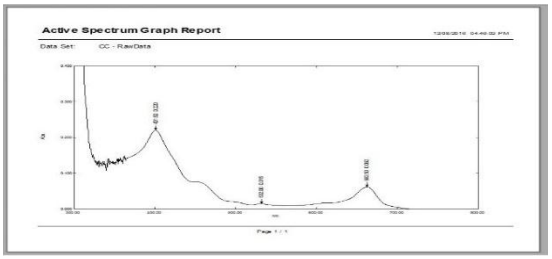

Fig. 4: UV spectrum of *C.asiatica*.

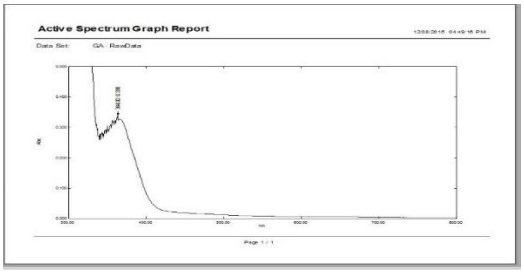

Fig. 5: UV spectrum of Gallic acids.

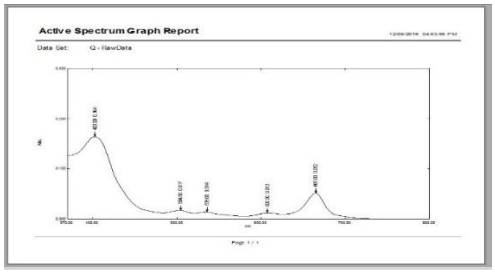

Fig. 6: UV spectrum of Quercetin.

FTIR spectroscopy

Aqueous methanolic extracts of *C. colebrookianum* Walp and *C. asiatica* Linn, Quercetin and Gallic acid were determined by IR absorption spectrophotometer in the range 500cm^{-1} to 4000cm^{-1} spectra are reported as Figure 7 to figure 10.

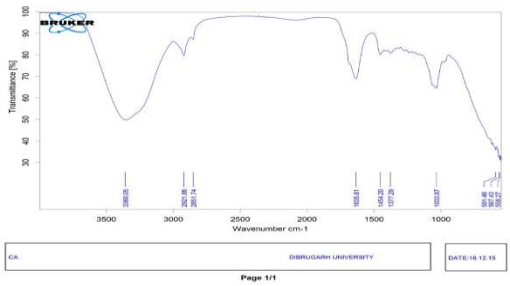

Fig. 7: FTIR Spectra of *C.asiatica*.

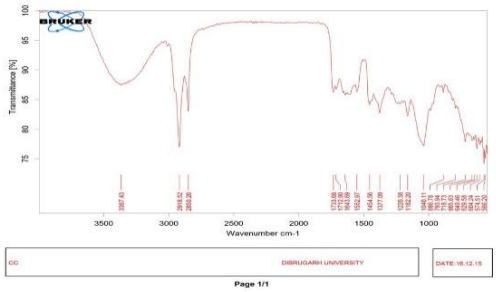

Fig. 8: FTIR Spectra of *C.colebrookianum*.

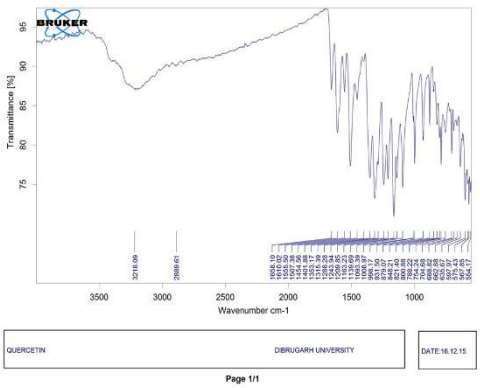

Fig. 9: FTIR Specra of Quercetin.

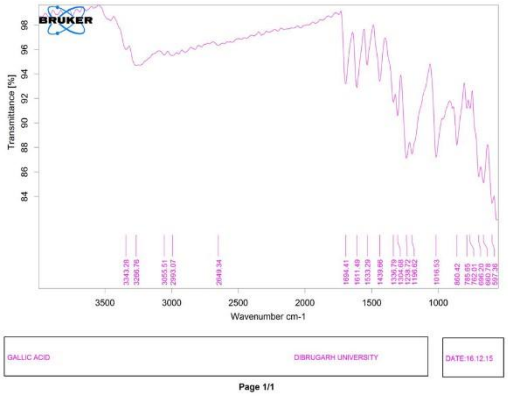

Fig. 10: FTIR Specra of Gallic aids.

Interpretation of FTIR

From the FTIR study some groups were found common with standard flavonoids quercetin and standard phenol gallic acid. Illustrated in table 4.

Table 4: Interpretation of FTIR.

Functional group	Wave number cm^{-1}			
	Quercetin	Gallic acid	*Centella asiatica*	*C. colebrookianum*
Hydrogen bonded alcohol or phenol (OH)	3218.09	3343.28 3266.76 3055.51	3360.05	3367.43
CH_2 (Cycloalkane), hydrogen bonded acids	2889.61	2649.34	2921.86	2918.52 2850.26
C=O (ketone, aldehyde, ester, acid or amide)	1658.10	1694.41	-	1733.68 1712.90
Aromatic rings, alkene	1610.02 1555.50 1507.38	1611.49 1533.29	1635.81	1643.69 1552.97
CH (Alkane)	1454.56 1401.88	1439.66	2851.74 2921.86	1454.56 1377.09
C-O (Alcohols, Ethers)	1353.17 1288.28 1243.94 1209.85	1336.79 1304.68 1238.72	1377.29	1220.38

Estimation of Total phenolic content

The total phenolic content in the examined plant extract using the Folin-Ciocalteu's reagent is expressed in terms of GAE (gallic acid equivalent). The standard curve equation is y = 0.0115x, R^2 = 0.9907. The phenolic content for *C. colebrookianum* extract was found to be 621.02 ± 0.03 mg of GAE/gm plant extract whereas for *C.asiatica* was found to be 234.37 ± 0.54 and were shown in table 4.

Estimation of Total Flavonoid Content

Flavonoids are the most important and diverse phenolic compounds. The content of flavonoids present in the extracts was evaluated using quercetin as a standard. In the procedure the flavonoid present in the extract reacts with the aluminium ion (Al^{3+}) to form the stable flavonoid-Al^{3+} complex, which has a yellow color and whose intensity is proportional to the flavonoid concentration present in it. The content of flavonoids was expressed in terms of quercetin equivalent (the standard curve equation: y = 0.0191x, R^2 = 0.9956), mg of Quercetin/g of extract. The flavonoid content for *C. colebrookianum* extract was found to be 460.07 ± 0.11 mg of Quercetin equivalent/gm plant extract whereas for *C.asiatica* was found to be 596.40 ± 0.53 mg of Quercetin equivalent/gm plant extract and were reported in table 4.

Table 4: Total phenolic content and total flavonoid content.

Plant extract	Total flavonoid content	Total phenolic content
Clerodendrum colebrookianum Walp	460.07 ± 0.11	621.02 ± 0.03
Centella asiatica Linn	596.40 ± 0.53	234.37 ± 0.54

Values are expressed as Mean ± SD.

DISCUSSION

Phytochemical evaluation of plant extracts

Phytochemical test revealed the presence of constituents well known for vivid medicinal and physiological activities. The plant extracts are found to be rich in phenolic compounds as well as flavanoids which are the main components for the anti-hypertensive activity although other classes of compounds also contribute for the blood pressure normalizing activity. From the knowledge of literature, the plants thus can be said to give good results when formulated in a dosage form.[24,25]

Thin Layer Chromatography

When flavonoids are exposed to UV radiation in a UV chamber (254-365nm) it is excited and known to produce yellow, green or blue fluorescent spots.[26] From R_f value data shown we can conveniently conclude that flavonoid is present, so are the other components showing their particular spots except protein.

Total phenolic and flavonoids content study

The conducted preliminary phytochemical studies confirmed the presence of flavonoids in test samples.[23] The total phenolic contents and total flavonoids content were estimated in respect to standard gallic acid and quercetin respectively to understand the phyto–pharmacological relationship. The maximum total phenolic contents of *C.colebrookianum* was found to be 621.02 ± 0.03 mg of GAE/gm plant extract whereas for *C.asiatica* was found to be 234.37 ± 0.54 mg of GAE/gm plant extract and total flavonoids content *C. colebrookianum* extract was found to be 460.07 ± 0.11 mg of Quercetin equivalent/gm plant extract whereas for *C.asiatica* was found to be 596.40 ± 0.53 mg of Quercetin equivalent/gm plant extract. The bioflavonoid

quercetin has demonstrated for its antihypertensive effects when given chronically in the most common rodent models of hypertension including high-sucrose diet induced hypertension[27] which reduced blood pressure and heart rate. Further, Free radical caused damage to G-protein coupled with muscarinic receptors and ascorbic acid, a free radical scavenger, offers protection from reactive oxygen species at the receptor site. Leaf extract of *C. colebrookianum* and *C.asiatica* increases the antioxidant capacity of blood and had an inhibitory effect on the basal level of lipid peroxidation of liver and kidney[28,29], exhibited protection against ischemia–reperfusion injury in rat hearts.[30] *C. colebrookianum* extract also exhibited the reduction in total cholesterol (TC) and low density lipoprotein (LDL) level and enhanced cardioprotective high density lipoprotein (HDL) level.[29] The antioxidant activity of both the extracts were found to be statistically significant when compared with control or standard. The extracts were found to have good antioxidant activity as evidenced from various radical scavenging activity results performed. Hence, it can be concluded that the plants can be used as good source of antioxidant which may be an important factor to efficiently lower the blood pressure, by reducing aldehyde conjugate/AGE formation resulting in reduced oxidative stress and improving insulin resistance and other endothelial functions.[23,31-34]

CONCLUSION

The observations made in the present study revealed that both the plant *C.colebrookianum* and *C.asiatica* possesses significant antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related cardiovascular diseases.

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