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Research Article

EVALUATION OF ANTIOXIDANT ACTIVITY OF TLC FRACTIONS OF *CLERODENDRON COLEBROOKIANUM* LEAF EXTRACT CONTAINING FLAVONOIDS

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ABSTRACT

In this study, we are presenting the results of the antioxidant activity of different TLC fractions isolated from the extract of *Clerodendron colebrookianum* leaves. Two different extracts (extract-1 and-2) of the leaves were prepared and their total flavonoid content was determined. Among the extracts, the one with higher total flavonoid content was selected for further separation using thin layer chromatography (TLC). Each TLC fractions including the crude extract-2 were individually assessed for total antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, hydroxyl radical scavenging activity. Their UV spectral analysis was also performed. As per the result, since the extract-2 was showing higher total flavonoid content than the extract-1, therefore, extract-2 was selected for further separation using TLC. Following TLC analysis of extract-2, three fractions were obtained with Rf values of fraction-1: 0.32, fraction-2: 0.53 and fraction-3: 0.82 respectively. The TLC fractions were shown to possess antioxidant activities. Also, the UV spectral analysis indicated that the TLC fractions may contain flavonoid component as they individually absorbed UV radiation in two different ranges between λ240~280 nm (band II) and λ300~550 nm (band I) as the flavonoids. Further studies will be conducted to identify the flavonoids present in each TLC fractions.

Keywords: Antioxidant activity, *Clerodendron colebrookianum*, Flavonoids

INTRODUCTION

Flavonoids are widely distributed phenolic compounds in the plant kingdom and they occur in all parts of plants as complex mixtures of different components. Flavonoid consists of different subgroups where flavones, flavonols and flavanones are considered to be important sub-groups of flavonoid due to the recognition of their *in vitro* antioxidant properties [1-4]. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Flavonoids possess strong anti-oxidative activity [5] as well as other potential beneficial effect including anti-atherosclerotic [6], anti-inflammatory [7], anti-cancer [1, 8] and anti-diabetic [9] activity.

A large number of medicinal plants and their purified constituents have been shown to exhibit antioxidant activity [10-14]. Northeast region of India is full of natural resources especially in medicinal and aromatic plants, which are extensively used by the traditional user from time immemorial. *Clerodendron 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 "jarem" in khasi, "Nefafu" in Assam, "Phuinum" in Mizoram and "Arun" in Nagaland [15-17]. *C. colebrookianum* is distributed widely in the South and South-east Asia [18]. The Mizo people of this 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. Antioxidant, hypolipidemic, anti-peroxidative activities of *C. colebrookianum* leaf extract have already been reported [19, 20].

In the present study, two different extracts (extract-1 and extract-2) of *C. colebrookianum* leaf was investigated. The aim of this study was to optimize extraction methods in order to maximize the recovery of flavonoids in the crude extracts of *C. colebrookianum*. This was accomplished by examining the influence of two different extraction solvents on the total flavonoid content. The extract with higher flavonoid content was selected and further separated by TLC and the antioxidant activity of each scraped TLC fractions was determined. Antioxidant activity was examined using different antioxidant assays such as total antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and hydroxyl radical scavenging activity which was measured spectrophotometrically.

MATERIALS AND METHODS

Chemicals

Aluminium chloride, ascorbic acid, boric acid, deoxyribose, ferric chloride, hydrogen peroxide, oxalic acid, silica gel GF_{254}, sodium nitrite, trichloroacetic acid were purchased from Sisco research laboratories (Mumbai, India). Ammonium molybdate was purchased from Merck Limited, Mumbai. Ethylenediaminetetraacetic acid (EDTA), DPPH, 2, 4-dinitrophenyl hydrazine (2,4-DNPH) was purchased from HiMedia Laboratories Private, Mumbai. Thiobarbituric acid was procured from Central Drug House Private Limited, New Delhi. All the chemicals used including the solvents, were of analytical grade.

Plant collection

C. Colebrookianum leaves were collected twice a year from Ri-Bhoi District (Latitude 25°46' N, Longitude 91°46' E, Elevation 525 m), Meghalaya, India. The specimens were submitted and identified by herbarium curator, Department of Botany, North Eastern Hill University, Meghalaya, India (voucher no. 6786 of Department of Botany Herbarium, NEHU). The leaves were thoroughly washed with water and dried at 40°C [21] and were finely powdered using grinder and the powder was stored at 4°C. Two different extraction methods were used to prepare the leaf extract of the plant for further investigation.

Test for flavonoid

A portion of crude powder was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and yellow coloration confirms the presence of flavonoid [22].

Preparation of extracts

There are different methods of flavonoid extraction from plants. Below are the two methods used for obtaining flavonoids:

(i) Extract-1: The dried leaf powder was immersed in the solution containing methanol and water with the proportion of 9 to 1 by volume. The total volume applied was 10 times that of the dry weight of the sample. The samples were minced into nearly homogeneity and left to stand for 4 h. The extracts were later filtered through white linen and the methanol was eliminated through the evaporator at 95°C. The samples were then extracted 3 times with chloroform 1/1 by v/v. The aqueous solution from the chloroform extraction was then evaporated until the sample volume was reduced to one third [23].

(ii) Extract-2: 2.5 g of the dried leaf powder was kept overnight in 30 ml of 70% ethanol in a shaker at room temperature and filtered thereafter. The residue was again extracted twice with 5 ml of 70 % ethanol [24].

Total flavonoid content determination

(i) Aluminum Chloride Colorimetric Method

An aliquot (1ml) of extract-1 (1mg/ml) or extract-2 (1mg/ml) or standard solution of quercetin (QE) (0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml) was added to 10 ml volumetric flask containing 4 ml double distilled water. To the flask 0.3 ml of 5% sodium nitrite was added. After 5 min, 0.3 ml of aluminium chloride solution followed by 2 ml of sodium chloride solution was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorption was measured against prepared reagent blank at λ510 nm. Total flavonoid content was expressed as mg QE equivalents /g dry plant material. Samples were analyzed in triplicates [25].

(ii) 2,4-DNPH Colorimetric Method

Exactly 40 µl of the extract-1 or extract-2 (20 mg/ml) was combined with 80 µl of a 50.5 mM 2,4-DNPH solution (100 mg dissolved in 10 ml methanol with 200 µl sulfuric acid in a 5 ml screw test tube). The contents were heated for 50 min at 50˚C on a water bath then allowed to cool down to room temperature. Exactly 280 µl of a 10% potassium hydroxide (w/v) in methanol was added. For calibration curve, methanolic standards of naringenin (NE) at (0, 0.25, 0.50, 0.75, 1.0 and 2.0 mg/ml) were added instead of the sample. Absorption was measured at λ495 nm. Results are expressed as mg NE equivalent/g of dry material [26].

Thin-Layer Chromatography (TLC)

Extract-2 (1mg/ml) dissolved in 70% ethanol was further separated using TLC (silica gel GF_{254} on a glass of 7.5 x 2.5 cm) with a solvent system consisting of ethyl acetate-formic acid-water (8:1:1) and revealing reagents for detecting flavonoid: Boric acid- Oxalic acid in the ratio of 3:1 (3g of boric acid in 100 ml of distilled water and 1g of oxalic acid in 100 ml of distilled water) followed by heating at 60˚C and fractions were visualized under UV light [27]. Rf was calculated for every constituent. Individual TLC fractions were scraped off the TLC plates and extracted with 70% ethanol. Individual TLC fractions were analyzed for their antioxidant activity.

Total Antioxidant Capacity

An aliquot of 0.1 ml of extract-2 or individual TLC fractions or standard was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a tube. The tubes were capped, incubated in a water bath at 95˚C for 90 min, cooled to room temperature and the absorbance measured at λ695 nm against a blank solution. The total antioxidant capacity was expressed as the ascorbic acid equivalent/g of the extract [28].

DPPH Free Radical Scavenging Activity

The DPPH solution (1.0 mM, 1 ml) was incubated with 3 ml of extract-2 (1 mg/ml) or individual TLC fractions (1 mg/ml). Incubation was carried out at room temperature for 30 min. At the end of the incubation period, the optical density (OD) of each sample was determined at λ517 nm. The blank used was methanol. The readings were noted and recorded [29]. The percentage of scavenging of hydrogen peroxide was calculated as:

$$\text{Free radical Scavenged (\%)} = \frac{A_{cont} - A_{test}}{A_{cont}} X100$$

Where, Acont was the absorbance read in the presence of methanol (without extract),

Atest was the absorbance read in the presence of extract.

Hydroxyl Radical Scavenging Activity

To the reaction mixture containing deoxyribose (3mM, 0.2 ml), ferric chloride (0.1 mM,0.2 ml), EDTA (0.1 mM, 0.2ml), ascorbic acid (0.1 mM, 0.2ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH 7.4, 20 mM), 0.2 ml of extract-2 (1 mg/ml) or individual TLC fractions (1 mg/ml) to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 30˚C. After incubation, ice-cold trichloroacetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v) in 0.25 N hydrochloric acid were added. The reaction mixture was kept in boiling water bath for 30 min and then cooled, and the absorbance was measured at λ532 nm [28]. The percentage of scavenging of hydrogen peroxide was calculated as:

$$\text{Hydroxyl radical Scavenged (\%)} = \frac{A_{cont} - A_{test}}{A_{cont}} X100$$

Where, Acont was the absorbance read in the presence of methanol (without extract)

Atest was the absorbance read in the presence of extract.

UV Spectra Analysis

Flavonoids are normally characterized by their specific chromatographic behavior and distinctive UV-VIS spectra [30]. To determine the wavelength for flavonoid analysis the extract-2 and TLC fraction-1,-2,-3 were scanned in the wavelength range of λ200–500 nm using UV-VIS Thermo Scientific spectrophotometer.

Statistical analysis

Statistical analysis was done using students *t*-test. All the values represent mean of triplicates and are expressed as Mean ± SEM.

RESULTS AND DISCUSSION

Test for flavonoids

The yellow coloration confirmed the presence of flavonoids in a crude leaf powder of *C. colebrookianum* [22, 30]. This test gave an indication that flavonoids can be extracted from the leaves of the studied plant and two different extraction methods were used for this purpose.

Extraction yield and Total flavonoid content

Two extraction methods were employed in order to obtain the biologically active components of *C. colebrookianum* leaf. The percentage (%) yield for the prepared extracts (extract-1 and extract-2) from *C. colebrookianum* leaf is shown in **Fig 1**. It is obvious that the percentage yield of extract-2 was higher than the extract-1 for *C. colebrookianum* leaf. Total flavonoid content of extract-1 and extract-2 was also determined by adding up the values obtained from two methods: $AlCl_3$ and DNPH as the flavones, flavonols and isoflavones formed complexes only with $AlCl_3$, while flavanones strongly react only with 2,4-dinitrophenylhydrazine [25]. Results indicated that, among the two extracts (extract-1 and extract-2) of *C. colebrookianum* leaf investigated, extract-2 contained higher total flavonoid content (164 ± 3.75 mg/g dry material) than the extract-1 with total flavonoid content of 145 ± 2.55 mg/g dry material (**Table 1**). This suggests that the total flavonoids content in ethanolic extract is more than aqueous extract which may be due to their solubility in ethanol more than in aqueous solution.

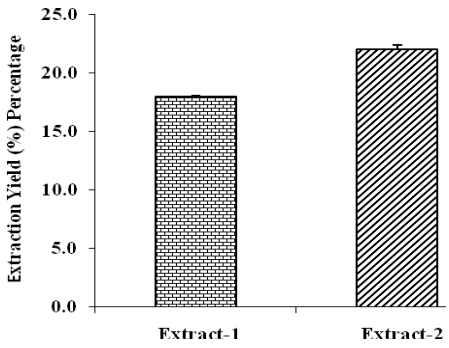

Fig. 1: Extraction yield (%) percentage for extract-1 and extract-2 of *C. colebrookianum* leaf.

Table 1: The total flavonoid content of different leaf extracts of *C. colebrookianum.*

Extracts of *C. colebrookianum* leaf	Flavonoid content		
	2,4-DNPH	$AlCl_3$	Total
Extract-1	95±1.08	50±1.47	145±2.55
Extract-2	99±2.67	65±1.08	164±3.75

TLC Analysis

As extract-2 contained more flavonoid than extract-1, hence, extract-2 was selected for further separation using TLC. TLC analysis of extract-2 using ethyl acetate: formic acid: water (8:1:1) as the solvent system with boric acid: oxalic acid (3:1) as a spraying reagent resulted in three fractions which were observed as fluorescent bluish-green color under UV light and were marked as fraction-1, fraction-2 and fraction-3 with Rf value (based on their distance from the solvent front) of 0.32, 0.53 and 0.82 respectively. Each TLC fractions were scraped and tested for total antioxidant activity, DPPH free radical scavenging and hydroxyl radical scavenging activity.

Table 2: The total antioxidant activity, DPPH free radical scavenging and hydroxyl radical scavenging activity.

Sample	Total Antioxidant activity(mg ascorbic acid equivalent/g of dry material)	DPPH Free radical Scavenging (%)	Hydroxyl radical scavenging (%)
Extract-2	6.9 ± 0.05	66 ± 0.75	88.0 ± 2.64
TLC Fraction-1	1.9 ± 0.07	59 ± 2.21	57.8 ± 1.10
TLC Fraction-2	2.5 ± 0.15	67 ± 0.71	41.0 ± 1.22
TLCFraction-3	1.9 ± 0.05	70 ± 1.20	68.7 ± 1.67

Total Antioxidant Capacity

Total antioxidant capacity of the extract-2 or TLC fraction-1, -2 and -3 was calculated using phosphomolybdate assay which is based upon the color measurement of a green colored phosphomolybdenum (V) complex formed through reduction of Mo (VI) and shows a maximum absorbance at λ695 nm. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid [28]. The extract-2 of *C. colebrookianum* exhibited total antioxidant capacity of 6.9 ± 0.05 mg ascorbic acid equiv./ g of dry material which was higher than all the three TLC fractions. The results obtained for all the three TLC fractions were in the range of fraction-1: 1.9 ± 0.07; fraction-2: 2.5 ± 0.15; fraction-3: 1.9 ± 0.05 mg ascorbic acid equivalent/g of dry material (**Table 2**).

DPPH Free Radical Scavenging Activity

The method with DPPH as a stable free radical to measure radical-scavenging activity has been widely used. Antioxidants react with DPPH, which is a stable free radical, and convert it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine. The stable radical DPPH has been used widely for the determination of primary antioxidant activity [29, 31, 32]. The degree of discoloration indicates the scavenging potentials of the antioxidant compounds. All the three TLC fractions for free radicals of DPPH showed scavenging activities in the range of fraction-1: 59 ± 2.21; fraction-2: 67 ± 0.71; fraction-3: 70 ± 1.20 (%) which was similar to the scavenging activity shown by extract-2 of *C. colebrookianum* with an activity of 66 ± 0.75 (%) (**Table 2**).

Hydroxyl Radical Scavenging Activity

The •OH radical is an extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells [33]. These radical combines with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical (•OH) scavenging capacity of an extract is directly related to its antioxidant activity [34]. The extract-2 of *C. colebrookianum* exhibited hydroxyl radical scavenging activity of 88 ± 2.64 (%) which was higher than all the three TLC fractions. The results obtained for hydroxyl radical scavenging activity of fraction-1, fraction-2 and fraction-3 were 57.8 ± 1.10, 41.0 ± 1.22 and 68.7 ± 1.67 (%) respectively (**Table 2**).

Ultraviolet (UV) spectra

The application of standardized UV (or UV–Vis) spectroscopy has for years been used in analyses of flavonoids. The various flavonoid classes can be recognized by their UV spectra and UV-spectral characteristics of individual flavonoids including the effects of the number of aglycone hydroxyl groups, glycosidic substitution pattern, and nature of aromatic acyl groups have been reviewed [23]. All the flavonoids contain at least one aromatic ring and consequently absorb UV light [35]. The typical UV-Vis spectra of flavonoids include two absorbance bands maxima in the ranges of λ240~280 nm (band II) and λ300~550 nm (band I) [23]. Flavonoids are composed of three rings structure (A, B, and C) with various substitutions. Changes in the substitution of the A-ring tend to be reflected in the band II absorption while alterations in the substitution of the B- and C-rings tend to be more apparent from band I absorption [23]. Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band to the longer wavelengths. Based on the UV-visible absorbance spectra, the flavonoid class can be predicted for each chromatographic peak separated.

The extract-2 and the TLC fraction-1, -2 and -3 were spectrophotometrically scanned in a wavelength range of λ200–500 nm and the graph showing peaks at different wavelength were observed (**Fig 2-5)**. The extract-2 showed seven peaks at wavelengths 260 nm, λ280 nm, 380 nm, 410 nm, 440 nm, 460 nm and 480 nm (**Fig 2**). The TLC fraction-1 showed four peaks at wavelengths 220 nm, 260 nm, 450 nm and 490 nm (**Fig 3**). The fraction-2 showed four peaks at wavelengths 260 nm, 280 nm, 450 nm and 480 nm (**Fig 4**) and fraction-3 showed three peaks at 260 nm, 280 nm and 440 nm (**Fig 5**).

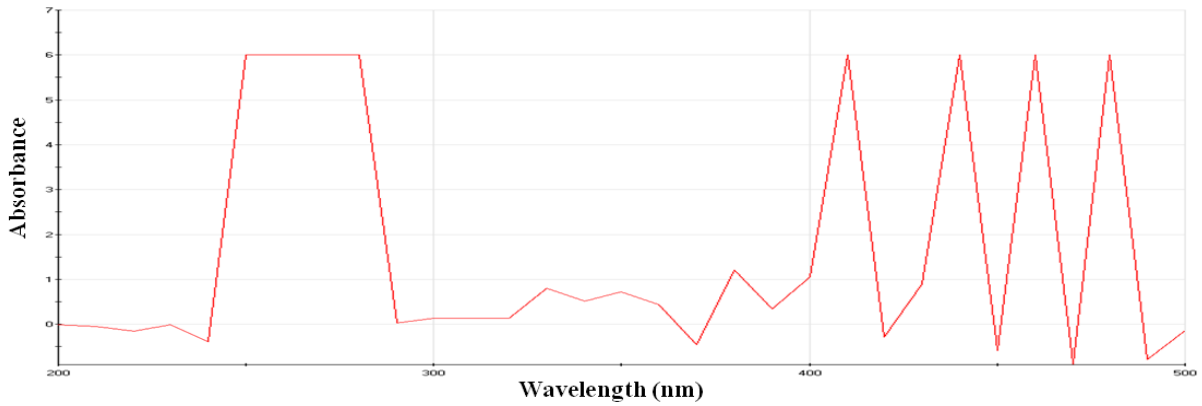

Fig. 2: Scanning spectra of extract-2 of *C. colebrookianum leaf*.

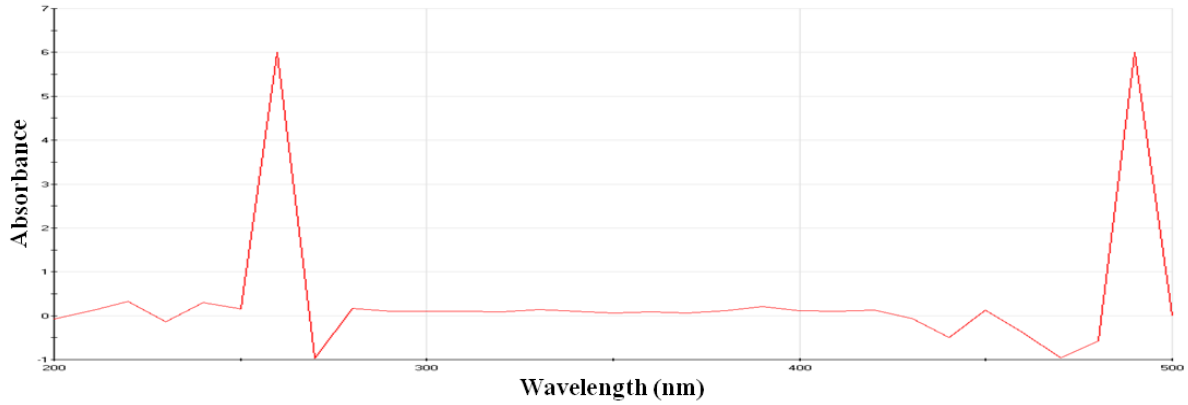

Fig. 3: Scanning spectra of TLC fraction-1.

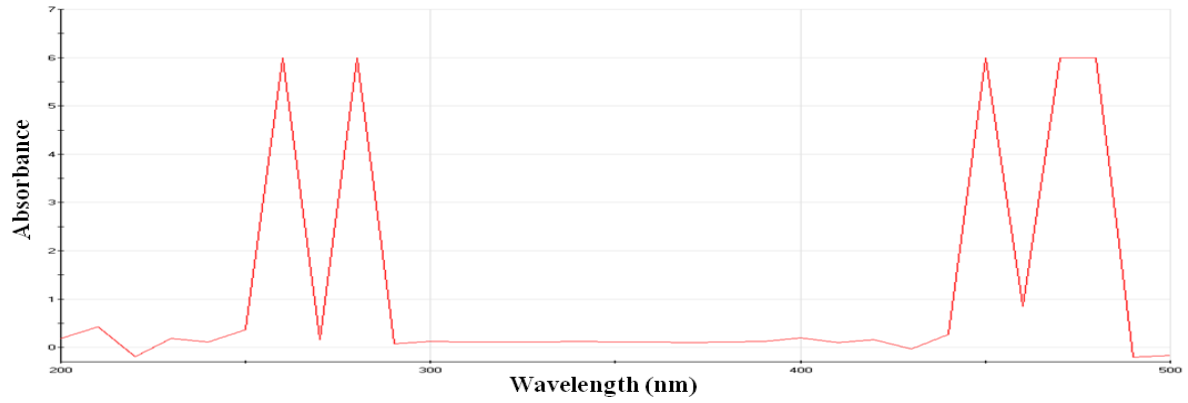

Fig. 4: Scanning spectra of TLC fraction-2.

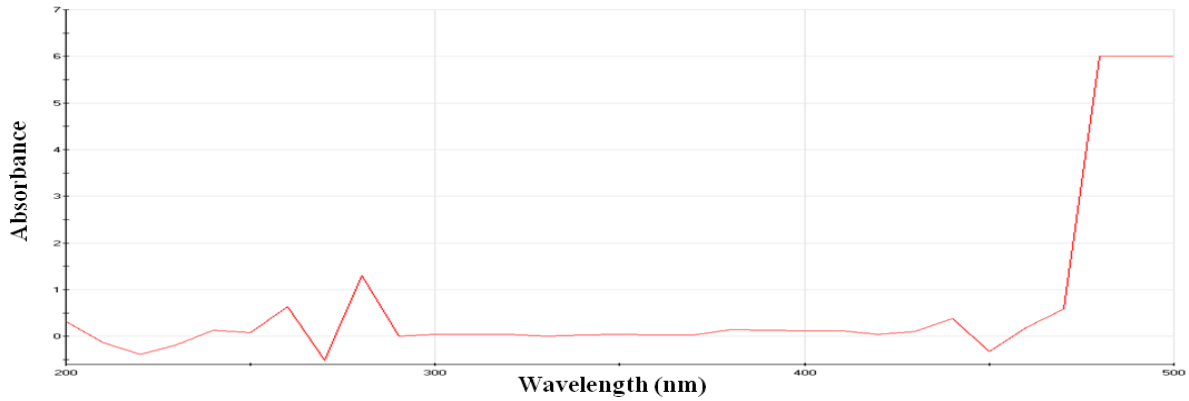

Fig. 5: Scanning spectra of TLC fraction-3.

From the scanning results shown in **Fig 2-5**, it indicates that the extract-2 of *C. colebrookianum* and the TLC fraction-1,-2, and -3 may contain flavonoid component as they individually absorbed UV radiation in two different range between λ240~280 nm (band II) and λ300~550 nm as the flavonoids [23]. In extract-2, the number of peaks was more than the peaks shown by individual TLC fractions could be the extract-2 being a crude extract it contained more compounds than the isolated TLC fractions. TLC fractions were also shown to contain more than one peak showing that the TLC fractions need to be further purified into individual component using other methods like high-performance liquid chromatography (HPLC) [36].

CONCLUSION

Medicinal plants have complex anti-oxidative machinery that protects them against oxidative damage. Sources of these natural antioxidants are primarily phenolics and flavonoids that may occur in all products and parts of a plant. *C. colebrookianum* leaf is a valuable model species for flavonoid research. With various biological activities, flavonoids are the principal components in evaluating the quality as well as taxonomy of various taxa of *C. colebrookianum* leaf. On the basis of the considerations obtained in the present study, it is concluded that the TLC fractions extracted from the leaves of *C. colebrookianum* may contain flavonoid component which exhibit antioxidant activities. Further studies will be conducted to identify the flavonoids present in each TLC fractions isolated from the leaves of *C. colebrookianum* ethanolic extract (extract-2).

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