

IN VITRO ANTIOXIDANT ACTIVITY OF VARIOUS LEAF EXTRACTS OF *CANTHIUM COROMANDELICUM* (BURM.F.) ALSTON

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ABSTRACT

Objectives: The objective of this study was to estimate the total phenolic and flavonoid contents and to explore the antioxidant potential of various leaf extracts (chloroform, ethyl acetate, and ethanol) of *Canthium coromandelicum*, which is considered traditionally as an important medicinal plant.

Methods: Antioxidant properties of the extracts were assessed using 1, 1-diphenyl-2-picrylhydrazyl and hydrogen peroxide radical scavenging assays for ascorbic acid equivalents. The total phenolic and flavonoid contents were also investigated to determine their correlation with the antioxidant activity of the leaf extracts and expressed in Gallic acid and quercetin equivalents, respectively.

Results: The results showed that the content of total phenols and flavonoids was found to be high in ethyl acetate extract which was recorded as 61.02±1.30 mg Gallic acid equivalent (GAE/g) and 81.72±0.61 QE/g, respectively. Compared to other extracts, ethyl acetate leaf extract was found to possess high antioxidant activity at p<0.05 level, with high percentage of inhibition at 100 µg/ml concentration (82.70%) toward hydrogen peroxide radical scavenging with IC₅₀ value 62.94 µg/ml. Statistically, two-tailed Pearson's correlation showed strong positive correlations between hydrogen peroxide radical scavenging activity and total phenolic contents (TPC) (r=1.000) at p<0.05 level.

Conclusion: The results obtained in this study clearly signifies that the ethyl acetate leaf extract of *C. coromandelicum* has high content of total phenols which are correlated to its antioxidant activity and thus has the potential to use as a source of natural antioxidants and can be explored as a therapeutic agent in free radical induced diseases.

Keywords: *Canthium coromandelicum*, In-vitro antioxidant activity, Ethyl acetate extract, Pearson's correlation, 1, 1-diphenyl-2-picrylhydrazyl, Hydrogen peroxide, Nitric oxide, Superoxide.

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INTRODUCTION

Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes, approximately 5% oxygen gets univalently reduced to oxygen-derived free radicals such as superoxide, hydrogen peroxide, hydroxyl, and nitric oxide radicals. Excessive generation of these radicals disrupts the antioxidant defense system of the body which may lead to oxidative stress [1]. All these radicals known as reactive oxygen species (ROS) exert oxidative stress toward the cells of human body rendering each cell to face about 10,000 oxidative hits per second [2,3]. Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular, and neurodegenerative diseases [4]. The antioxidant enzymes and free radical scavengers may provide a defensive mechanism against the deleterious acting on ROS.

Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent generation of ROS [5]. The two most commonly used synthetic antioxidants butylated hydroxyanisole and butylated hydroxytoluene have begun to be restricted because of their toxicity and DNA damage induction [6]. Significant antioxidant properties have been recorded with phytochemicals that are necessary for the reduction in the occurrence of many diseases. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins and thus can be utilized to scavenge the excess free radicals from human body [7]. Therefore in recent years, considerable attention

has been directed toward the identification of plants with antioxidant activity [8].

Natural antioxidants tend to be safer, and they also possess antiviral, anti-inflammatory, anticancer, antitumor, and hepatoprotective properties [9,10]. Therefore, the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals. In this respect, polyphenolic compounds, such as flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity [11-13].

Canthium coromandelicum (Burnm.f.) Alston. (Syn. *Canthium parviflorum*) of family Rubiaceae is a bushy thorny suffruticose herb, native of India found mainly in *Coromandelicum* region. The plant is popularly recorded under the local name, i.e., in Odisha "Tutidi saga" (Odia language). *Canthium coromandelicum* is native to India, Sri Lanka, and tropical East Africa [14]. *Canthium* genus was named by Jean Baptiste Lamarck in 1785 in Encyclopedie Methodique (Lamarck J-B 1785). The name is a latinisation of "Kantankar," a Malayalam name from Kerala for *C. coromandelicum*. Kantan means "shining" and kara means "a spiny shrub" [15]. The biological type for the genus consists of specimens originally described by Jean - Baptiste Lamarck as *C. parviflorum* (*Canthium* In: Index Nomenum Genericorum), but this species is included in *C. coromandelicum* [14]. Traditionally, the leaves and roots were found to be effective in the treatment of kapha, diarrhea, strangury, fever, leukorrhea, intestinal worms, and general debility [16]. The present work was aimed to estimate the estimate the total phenolic and flavonoid contents and to compare the

in vitro free radical scavenging activity between the leaf extracts of *C. coromandelicum*.

METHODS

Plant material (*C. coromandelicum* Burm. f.) alston

C. coromandelicum (Burm. f.) Alston. plant was collected from Unnamalaikadai region of Kanyakumari District, Tamil Nadu, India, in the month of September 2013. The plant sample was identified and authenticated by Dr. A.G. Pandurangan, Director and Head, Plant Systematic and Evolutionary Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, Kerala, India (Collection number:76878). The voucher specimens (JNTBGRI/PS/173/2016) have been deposited at TBGT, JNTBGRI, Palode, Thiruvananthapuram, Kerala, India, for future reference.

Preparation of powder

After authentication, *C. coromandelicum* plant material was collected and the leaf was separated carefully, washed thoroughly in running tap water and then with distilled water to remove any adhering material like soil and any other material, and dried under shade. After drying, the plant part materials were ground well using mechanical blender into a coarse power. About 500 g of leaf was obtained by mechanical grinding and stored in airtight containers for further use.

Extraction of the plant material

Air dried coarsely powdered leaf (500 g), of plant *C. coromandelicum* were successively extracted with chloroform, ethyl acetate and ethanol separately by continuous hot percolation using soxhlet apparatus for 48 hrs. The temperature was maintained (25-100°C) on an electric heating mantle with thermostat control. The extracts were then concentrated by evaporating the solvents under reduced pressure. Preliminary phytochemical studies were carried out in the different extract to access the presence of various phytoconstituents.

Quantitative phytochemical screening

The quantitative estimation of phytochemicals in different leaf extracts of *C. coromandelicum* according to the standard protocols.

Determination of TPC

The TPC in the ethyl acetate leaf extract of *C. coromandelicum* was determined by spectrophotometric method using Folin-Ciocalteu reagent method of Singleton *et al.* [17] with some modifications. Ethyl acetate leaf extract in the concentration of 1 mg/ml was used in the analysis. Briefly, 0.5 ml of ethyl acetate extract was mixed with 1.5 ml (1:10 v/v diluted with distilled water) Folin-Ciocalteu's reagent and allowed to stand for 22°C for 5 minutes. Then, 2 ml of sodium carbonate (Na₂CO₃, 7.5%, w/v) was added, and the mixture were allowed stand for another 90 minutes and kept in the dark with intermittent shaking. Then, the absorbance of the blue color that developed was measured at 725 nm using spectrophotometer (HITACHI U-1900 spectrophotometer 200V). Blank was concomitantly prepared with 0.5 ml ethyl acetate instead of extract solution. The sample was performed in triplicates, and the results were averaged. Gallic acid was used for constructing the standard curve (20-100 mg/ml). Based on the measured absorbance, the concentration of total phenolic compounds in ethyl acetate leaf extract was determined (mg/ml) using an equation that was obtained from the standard Gallic acid graph (20-100 mg/ml), and the total phenolic compounds concentration in the leaf extract was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (mg GAE/g) of extract.

Determination of total flavonoids contents

The flavonoid content in the extract was determined spectrophotometrically by the method of Zhishen *et al.* [18] 1 ml (1 mg/ml) of extract was added to a 10 ml volumetric flask, containing 4 ml of distilled water. Subsequently, 0.3 ml of 5% sodium nitrate was added to the flask. After 5 minutes, 0.3 ml of 10% aluminum chloride was added. At the 6th minute, 2 ml of 1 M sodium hydroxide was added

and the total volume was made up to 10 ml with distilled water. Then, the absorbance of the reaction mixture was measured at 512 nm, with a UV scanning spectrophotometer (Unico® 1200, m Alexandria, Egypt), along with the standard, quercetin, and blank reagent. All tests were performed 3 times and averaged. The total flavonoids content was determined as microgram, quercetin equivalent by using the standard, quercetin graph, obtained by comparing the calibration curve prepared from a reference solution containing quercetin (20-100 mg/ml). The data of the total flavonoid content (TFC) of *C. coromandelicum* ethyl acetate extract were expressed as milligrams of quercetin equivalents per gram of plant extract (mg quercetin equivalents/g extract).

In vitro antioxidant activity

1, 1-diphenyl -2- picrylhydrazyl (DPPH) radical scavenging assay

The scavenging ability of ethyl acetate extract on DPPH free radicals was estimated according to the method of Madaan *et al.* [19] Solution of DPPH (0.1 mm) in methanol was prepared by dissolving 1.9 mg of DPPH in methanol and volume was made up to 100 ml with methanol. The solution was kept in darkness for 30 minutes to complete the reaction. 1 ml of DPPH solution was added to 1 ml of different (20, 40, 60, 80 and 100 mg/ml) concentrations of extracts and allowed to stand at room temperature for 30 minutes. The mixture was measured spectrophotometrically (UV-1800, UV-VIS spectrophotometer, Shimadzu) at 517 nm. The experiments were repeated in triplicate. The free radical scavenging activity was calculated as using following formula,

$$\% \text{ DPPH radical scavenging activity} = (A_0 - A_1) / A_0 \times 100$$

Where, A₀ is the absorbance of the control and A₁ is the absorbance of the sample.

A standard of ascorbic acid was used using same concentrations as that of extracts [19,20]. From the absorbance obtained, percentage inhibition and regression curves were made, and the linear equation was applied to calculate the IC₅₀ value [21].

Hydrogen peroxide radical scavenging (H₂O₂) assay

About 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide solutions were prepared as per the Indian Pharmacopoeia 1996 standards. 50 ml potassium dihydrogen phosphate solution was placed in a 200 ml volumetric flask and 39.1 ml of 0.2 M sodium hydroxide solution was added, and finally, volume was made up to 200 ml with distilled water to prepare phosphate buffer (pH-7.4). 50 ml of phosphate buffer solution was added to equal amount of hydrogen peroxide to generate the free radicals and solution was kept aside at room temperature for 5 minutes to complete the reaction. Extracts (1 ml) in distilled water were added to 0.6 ml hydrogen peroxide solution, and the absorbance was measured at 230 nm in a spectrophotometer (UV-1800, UV-VIS spectrophotometer, Shimadzu) against a blank solution containing phosphate buffer solution without hydrogen peroxide [22,23]. Concentrations selected for extract were ranging from 20 to 100 mg/ml. Ascorbic acid was used as standard. The experiments were repeated in triplicate. The percentage of scavenging of H₂O₂ of extract was measured using the following equation:

$$\% \text{ NO radical scavenging activity} = (A_0 - A_1) / A_0 \times 100$$

Where, A₀ is the absorbance of the control and A₁ is the absorbance of the sample.

Statistical analysis

All statistical analysis was performed using standard software IBM SPSS (Ver. 21). Data were expressed as mean ± standard deviation and its difference was compared using one-way analysis of variance. Duncan's multiple range test (p<0.05) was also used to find out the significant difference in values. Data were evaluated using two-tailed Pearson's correlation using SPSS to identify the inter-relationships among TPC, TFC, and antioxidant activity.

RESULTS AND DISCUSSION

Quantitative phytochemical screening

Chloroform, ethyl acetate and ethanolic leaf extracts of *C. coromandelicum* were prepared to examine the TFC and TPC.

Estimation of total flavonoids content

The TFC for various leaf extracts was measured with aluminum chloride spectrophotometric method using quercetin as standard. The flavonoid contents were expressed in terms of mg/g quercetin equivalent using the standard curve equation: $Y=0.0106X - 0.01$, with a regression coefficient $R^2=0.9989$. The plot has a slope (m) = 0.0106 and intercept = 0.01 (Fig. 1). The calibration curve of quercetin has exhibited linearity. Using the calibration curve, the TFC was calculated and it was found that ethyl acetate extract showed very high quantity of flavonoid as 81.72 ± 0.61 QE/g. Ethanol and chloroform extracts possess satisfactory quantity of flavonoid and the lowest flavonoid concentration 13.89 ± 1.32 was measured in hexane extract.

Estimation of TPC

The amount of TPC was determined with Foiln-cioalteu reagent. Gallic acid was used as a standard compound, and the TPC of the extracts was calculated from the regression equation of calibration curve: $Y=0.013X - 0.178$; $R^2=0.9984$ and expressed as mg GE/g of sample in dry weight (mg/g) (Fig. 2). The TPC in various leaf extracts ranged from 25.10 ± 0.43 to 61.02 ± 1.30 mg GAE/g. The highest amount of phenolic contents was detected in ethyl acetate extract 61.02 ± 1.30 mg GAE/g sample when compared to other extracts. The concentration of phenolic content in hexane extract 30.64 ± 1.01 mg GAE/g was very similar to the value of chloroform extract 34.79 ± 0.84 mg GAE/g. Ethanol extract contains considerable concentration of phenolic, whereas lowest phenolic concentration was measured in aqueous extract.

Antioxidant activity

Chloroform, ethyl acetate, and ethanolic extracts were subjected to screening for their possible antioxidant activity using DPPH free radical scavenging assay, hydrogen peroxide radical scavenging assay, superoxide radical scavenging activity, total antioxidant activity, and reducing power activity.

DPPH radical scavenging assay

The DPPH assay has been largely used as a quick, accurate, reliable and sensitive method for testing preliminary radical scavenging activity of the plant extracts [24]. DPPH is usually used as a substance to determine the antioxidant activity [25]. DPPH is a stable free radical which accepts an electron to become stable and cause discoloration from purple to yellow which shows maximum ultraviolet and visible (UV-VIS) absorbance at 517 nm [26,27]. In this study, the percentage of scavenging effect on the DPPH radical was concomitantly increased with the increase in the concentration of chloroform, ethyl acetate and ethanolic leaf extracts of *C. coromandelicum* from 20 to 100 μ g/ml.

The DPPH scavenging activity of ethyl acetate leaf extract was well pronounced at higher concentrations of 100 μ g/ml with a mean percentage of 75.10 ± 1.01 while at the same concentration, ascorbic acid scavenged $92.70\pm 0.52\%$ of DPPH radicals. The IC_{50} values of the extracts and ascorbic acid were obtained using the linear regression equation. The results of DPPH radical scavenging activity of *C. coromandelicum* leaf extracts along with the reference standard ascorbic acid were shown in Table 1 and Fig. 3. The concentration of the sample necessary to decrease the initial concentration of DPPH by 50% (IC_{50}) under the experimental condition was determined. The lower value of IC_{50} indicates a higher antioxidant activity. Ethyl acetate extract (IC_{50} 59.11 μ g/ml) showed maximum DPPH radical scavenging activity which is comparable to the reference standard ascorbic acid (IC_{50} 19.66 μ g/ml). The Duncan test analysis showed that there is a significant difference in the DPPH radical scavenging activity among the different extract of test sample and standard ascorbic acid. A study revealed that the DPPH radical scavenging activity of methanol leaf

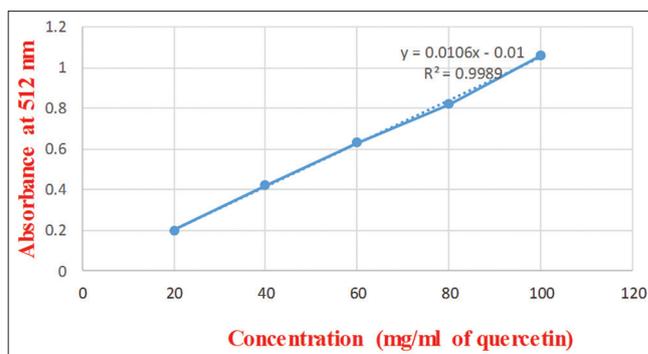


Fig. 1: Standard curve for determination of quercetin equivalents for total flavonoid contents

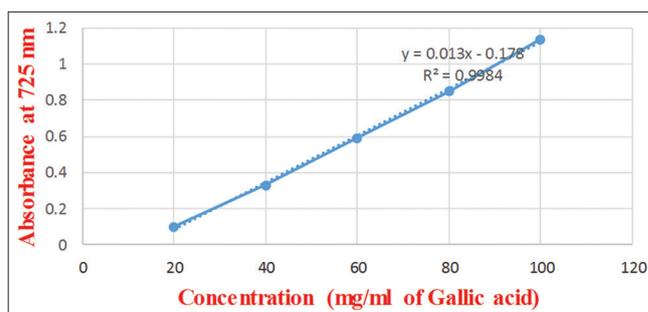


Fig. 2: Standard curve for determination of gallic acid equivalents for total phenolic contents

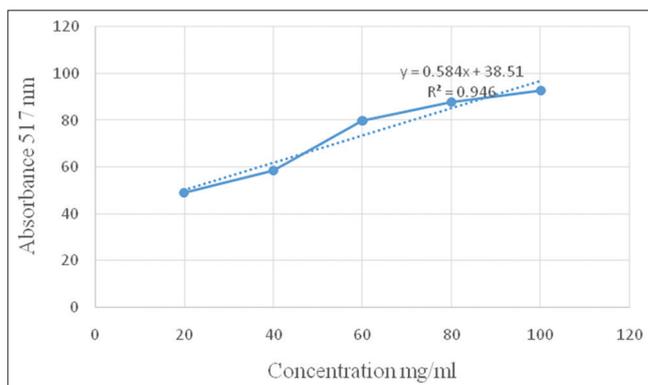


Fig. 3: The IC_{50} value obtained by linear regression equation for standard ascorbic acid of 1, 1-diphenyl-2-picrylhydrazyl

extract of *Hypericum hookerianum* was recorded as 90.24% with the IC_{50} value of 3.03 μ g/ml and showed highest antioxidant activity as well as phenolic content (128 mg/g) [28].

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, hydrogen peroxide can probably react with Fe^{2+} , and possibly Cu^{2+} to form hydroxyl radical and this may be the origin of many of its toxic effects [29]. The scavenging of hydrogen peroxide by the extract may be attributed to active secondary metabolites, phenolics which neutralize hydrogen peroxide by donating electrons thereby neutralizing it to water [30].

All the three leaf extracts of *C. coromandelicum* with different concentrations (20, 40, 60, 80, and 100 μ g/ml) were screened for

Table 1: DPPH radical scavenging activity of various leaf extracts of *C. coromandelicum*

S. No.	Concentration $\mu\text{g/ml}$	% of inhibition			Standard
		Chloroform	Ethyl acetate	Ethanol	
1.	20	09.70 \pm 1.51 ^a	22.83 \pm 0.47 ^a	19.06 \pm 1.05 ^a	49.03 \pm 0.37 ^a
2.	40	15.53 \pm 1.00 ^b	31.30 \pm 1.12 ^b	21.53 \pm 0.85 ^b	58.40 \pm 0.91 ^b
3.	60	26.60 \pm 0.81 ^c	55.16 \pm 1.25 ^c	39.53 \pm 1.44 ^c	79.80 \pm 1.11 ^c
4.	80	32.70 \pm 1.95 ^d	68.76 \pm 0.58 ^d	61.86 \pm 1.96 ^d	87.90 \pm 0.70 ^d
5.	100	43.80 \pm 1.15 ^e	75.10 \pm 1.01 ^e	69.03 \pm 1.72 ^e	92.70 \pm 0.52 ^e
6.	IC50	116.99	59.11	71.11	19.66

C. coromandelicum: *Canthium coromandelicum*, DPPH: (1, 1-diphenyl-2-picrylhydrazyl), ascorbic acid was used as reference standard, values were performed in triplicates and represented as mean \pm SD, mean values followed by different superscript in a column are significantly different ($p < 0.05$), a,b,c,d,e: Values followed by different letters are significantly different ($a > b > c > d > e$, $p < 0.05$)

Table 2: Hydrogen peroxide radical scavenging activity of various leaf extracts of *C. coromandelicum*

S. No.	Concentration $\mu\text{g/ml}$	% of inhibition			Standard
		Chloroform	Ethyl acetate	Ethanol	
1.	20	11.16 \pm 1.04 ^a	21.43 \pm 0.97 ^a	15.36 \pm 1.12 ^a	50.00 \pm 0.10 ^a
2.	40	16.73 \pm 1.53 ^b	30.00 \pm 1.70 ^b	28.80 \pm 1.30 ^b	61.90 \pm 0.72 ^b
3.	60	22.66 \pm 0.90 ^c	39.50 \pm 0.78 ^c	31.40 \pm 1.25 ^c	72.63 \pm 0.75 ^c
4.	80	33.16 \pm 1.23 ^d	64.80 \pm 1.08 ^d	43.96 \pm 1.00 ^d	84.53 \pm 1.00 ^d
5.	100	41.60 \pm 0.85 ^e	82.70 \pm 1.01 ^e	54.80 \pm 0.91 ^e	91.66 \pm 0.76 ^e
6.	IC50	124.49	62.94	92.19	18.20

C. coromandelicum: *Canthium coromandelicum*, ascorbic acid was used as reference standard, values were performed in triplicates and represented as mean \pm SD, mean values followed by different superscript in a column are significantly different ($p < 0.05$), a,b,c,d,e: Values followed by different letters are significantly different ($a > b > c > d > e$, $p < 0.05$)

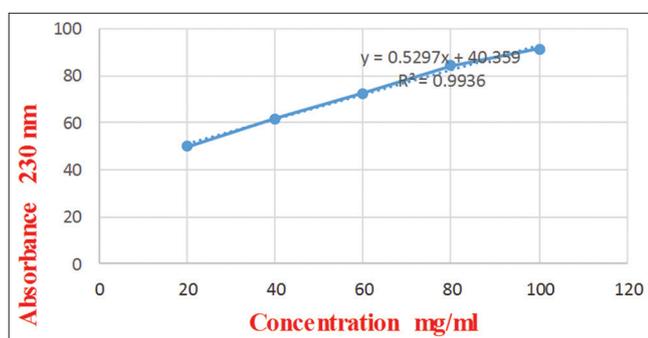


Fig. 4: The IC₅₀ value obtained by linear regression equation for standard ascorbic acid of H₂O₂

Hydrogen peroxide scavenging activity, and the results were shown in Table 2 and Fig 4. The absorbance was found to decrease with an increase in the dose of extracts used. It indicates that ethyl acetate leaf extract of *C. coromandelicum* (100 $\mu\text{g/ml}$) exhibited the maximum hydrogen peroxide scavenging activity of 82.7 \pm 1.01% which is comparable to the standard effect is 91.66 \pm 0.76%. The ethyl acetate extract exhibited potent hydrogen peroxide radical scavenging activity in concentration dependent manner. The IC₅₀ values of chloroform extract (IC₅₀ 124.49 $\mu\text{g/ml}$) followed by ethyl acetate and ethanol extract (IC₅₀ 62.94 $\mu\text{g/ml}$) and (IC₅₀ 92.19 $\mu\text{g/ml}$), respectively, using linear regression equation. The Duncan test analysis showed that there is a significant difference in the hydrogen peroxide radical scavenging activity among the different extract of test sample and standard ascorbic acid with IC₅₀ (18.20 $\mu\text{g/ml}$). In the present investigation, the ethyl acetate leaf extract of *C. coromandelicum* exhibited strong hydrogen peroxide radical scavenging activity in a dose-dependent manner. The ethanol extract showed lower level of activity, while the chloroform extract revealed moderate activity. A significant ($p < 0.05$) dose-dependent response was found in the hydrogen peroxide scavenging activity in methanolic leaf extract of *Emblca officinalis*. Maximum scavenging activity (84.15%) was observed at 100 $\mu\text{g/ml}$ concentration, and the IC50 value of *E. officinalis* leaf extract and ascorbic acid were found to be 42.87 $\mu\text{g/ml}$ and 34.51 $\mu\text{g/ml}$, respectively [31].

Correlation analysis between TPC, TFC and antioxidant assays

The two-tailed Pearson's correlation coefficient between antioxidant assays with TPC and TFC of *C. coromandelicum* ethyl acetate leaf extract showed positive correlations and is shown in Table 3. Strong and high positive correlation was found between hydrogen peroxide and TPC ($r = 1.000$) at $p < 0.05$ level in a two-tailed Pearson's correlation. Furthermore, the correlation ($r = 0.997$, $p < 0.01$) for TFC indicate that it has significant positive relationship with total phenolic content (TPC). Hence, it was clear that the TPC of the extract showed a satisfactory and significant correlation with hydrogen peroxide and thus suggesting that the phenolic compounds may be responsible for the antioxidant activity of the ethyl acetate leaf extract of *C. coromandelicum*. Similar correlation analysis was done in *Mitragyana parvifolia* leaves and bark extracts, where the total phenol content showed positive correlation with antioxidant assays [32]. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [33].

CONCLUSION

Today, antioxidative properties of the plants have become a great interest due to their possible uses as natural additives to replace synthetic ones. The result of this study showed that, among the three extracts, ethyl acetate leaf extract of *C. coromandelicum* exhibited higher potency of free radical scavenging activity. Phytochemical analysis revealed the presence of phenolic and flavonoid compounds in the extract, indicating that the free radical scavenging activity of ethyl acetate leaf extract of *C. coromandelicum* is highly related to the presence of these compounds. Thus, the data suggest that *C. coromandelicum* leaf could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing or slowing the oxidative stress-related degenerative diseases. Further isolation of bioactive compounds was done to identify the unknown compounds and to establish their pharmacological properties.

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Table 3: Correlation between TPC, total flavonoid content and antioxidant assays

	TPC	TFC	DPPH	H ₂ O ₂
TPC				
Pearson correlation	1	0.997*	0.862	1.000**
Significant (two-tailed)		0.046	0.338	0.008
N	3	3	3	3
TFC				
Pearson correlation	0.997*	1	0.897	0.996
Significant (two-tailed)	0.046		0.292	0.054
N	3	3	3	3
DPPH				
Pearson correlation	0.862	0.897	1	0.856
Significant (two-tailed)	0.338	0.292		0.346
N	3	3	3	3
H₂O₂				
Pearson correlation	1.000**	0.996	0.856	1
Significant (two-tailed)	0.008	0.054	0.346	
N	3	3	3	3

TPC: Total phenolic content, TFC: Total flavonoid content, DPPH: 1, 1-diphenyl-2-picrylhydrazyl, H2O2: Hydrogen peroxide, *Correlation is significant at the 0.05 level (two-tailed), **Correlation is significant at the 0.01 level (two-tailed)

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