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ANTIOXIDANT POTENTIAL OF MEDICINALLY IMPORTANT PLANTS *EHRETIA MICROPHYLLA*, *DIPTERACANTHUS PATULUS* AND *HYDNOCARPUS LAURIFOLIA*

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ABSTRACT

Ehretia microphylla, *Dipteracanthus patulus* and *Hydnocarpus laurifolia* is a main constituent of ethnomedicinal formulations used in Indian traditional medicine, there is very minimal systematic investigation done on this plant, it is not sufficient to support its traditional claim. The current study was attempted to assess the phytochemical and antioxidant activity of the *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia*. The ABTS radical scavenging assay, DPPH radical scavenging assay, FRAP assay and Phosphomolybdate assay were performed for the confirmation of antioxidant activity. The Petroleum ether, Chloroform, Ehyl acetate and Methanolic extracts of these three plants were evaluated for the preliminary Phytochemical study which confirms the presence of various phytochemicals such as Alkaloids, Flavonoids, Tannins, Saponins, Glycosides, and Phytosterols. Petroleum ether extract of all the three plants do not have these secondary metabolites, so that excluding this petroleum ether extract for quantification of flavonoids

and phenolic contents using spectrophotometer. The ethyl acetate and chloroform extracts of all the three plants having higher flavonoid content since further antioxidant activity was evaluated in both ethyl acetate and chloroform extracts of three plants. All the plant extracts shows good antioxidant activity and from the results of the current study the ethyl acetate and chloroform extracts of *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia* were shows potent antioxidant activity which is similar to the standard. This study concluded that the flavonoids present in *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia* may be the reason for antioxidant activity.

Keywords: Flavonoid, DPPH, ABTS, Quercetin, Gallic acid

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical (OH), and hydrogen peroxide have close association with oxidative stress. Over production of ROS can worsen the quantum of oxidative stress leading to combining mechanism of injury associated with liver damage. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play a significant role in the prevention of tissue damage by oxidative stress through inhibition of free radicals. Herbal medicines are in great importance in planet due to their efficacy, safety and lesser side effects **[1, 2]**.

Herbal drugs are most widely used than allopathic drugs as antioxidants because they are inexpensive, have better cultural acceptability, better compatibility with the human body and minimal side effects **[3]**. *Ehretia microphylla* is classified under the family Boraginaceae. It is known locally in the Philippines as tsaanggubat. It is an erect, much branched shrub that grows 1-4 m in height. It is found all over the Philippines, specifically in thickets and secondary forests of low and medium altitude **[4]**. *Dipteracanthus patulus* belongs to Acanthaceace family and very important indigenous medicinal plant, which present in remedy for ear disease and believed to be anti-cancer against the nasopharynx region, slightly hypoglycemic, anti-inflammatory and anti-microbial and also the leaves are eaten as vegetable **[5]**. *Hydnocarpus laurifolia*, belonging to the family of Flacourtiaceae is distributed in the tropical forests along the Western Ghats (South India). Seeds and oil of this plant are acrid, bitter, thermogenic, purgative, vermifuge, haematinic and tonic. They are used in the treatment of leprosy, skin diseases, leucoderma, dermatitis, bronchopathy, eczema, tubercular laryngitis, verminosis, wounds, ulcers and also as a fish poison **[6, 7]**.

Even though *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia* is a main constituent of ethnomedicinal formulations used in Indian traditional medicine, there is very minimal systematic investigation done on this plant, it is not sufficient to support its traditional claim. The current study was attempted to assess the phytochemical and antioxidant activity of the *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia*.

MATERIALS AND METHODS

Chemicals and reagents

For the entire research work, Borosil glass wares were used. They were soaked in chromic acid for 3 days, washed with tap water and rinsed with distilled water and dried over a hot air oven. Analytical grade chemicals supplied by SD Fine chemicals, SRL, Hi Media, Merck India and Sigma Aldrich Chemicals were used for this research.

Collection of herbal plants

The aerial parts of *Ehretia microphylla* and entire parts of *Dipteracanthus patulus*, the seeds of *Hydnocarpus lorifolia*, were collected locally Sattankulam at Thoothukudi District in Tamil Nadu state in India. After collecting the Leaves, Aerial parts, seeds, were washed and shade dried and powdered using a micropulverizer and the powder passed through sieve no. 40# **[8]**. The collected herbal plants were authenticated by Dr. V. Chelladurai, Survey of Medicinal and Aromatic plants Unit Siddha, CCRAS (Central Council for Research in Ayurvedic Sciences), Palayamkottai, Tamil Nadu, India, and voucher specimen was deposited in the Department of School of Biotechnology, NITC, Calicut, Kerala for future reference.

Extraction of herbal plants

Powdered plant materials were subjected to continuous hot extraction method by using soxhlet apparatus. Increasing orders of polarity solvents (petroleum ether, chloroform, ethyl acetate and methanol) were used. 1 kg of finely powdered plant materials were extracted with 5 L of petroleum ether in a soxhlet apparatus for 72 h, after the successive extraction, the obtained marc was further extracted with solvents of increasing order of polarity (Chloroform, Ethyl acetate and Methanol) **[9]**. After extraction the extracts were separately concentrated under vacuum using rotary vacuum evaporator at 40^0C until get viscous solid mass. The obtained crude extracts were weighed and stored at 4^0C for the further analysis.

Preliminary phytochemical analysis

The obtained extracts were subjected to phytochemical evaluation and identified the various plant constituents present in the test sample qualitatively **[10, 11]**.

Quantification of total phenolics and flavonoids

Estimation of total phenolics

The total phenolic content of extracts was determined by folin-ciocalteu assay method **[12-14]**. To an aliquot 100 µl of extract (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100 µg/ml) added 50 µl of folin-ciocalteu reagent followed by 860 µl of distilled water and the mixture is incubated for 5 min at room temperature. 100 µl of 20% sodium carbonate and 890 µl of distilled water were added to make the final solution to 2 ml. It was incubated for 30 min in dark to complete the reaction. The absorbance of the mixture was measured at 765 nm against blank. The total phenolic content was found out from the calibration curve of Gallic acid and it was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract.

Estimation of total flavonoids

The total flavonoid content of extracts was determined using Aluminium chloride colorimetric method. To an aliquot of 100µl of the extract or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) ethanol was added separately to make up the solution up to 2 ml 13, 14. The resulting mixture was treated with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8ml of distilled water. Mixed and allowed to stay at room temperature for 30 minutes. The absorbance was measured at 415 nm against blank. The total flavonoid content was determined from the standard Quercetin calibration curve and it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract.

***In vitro* antioxidant activity**

ABTS radical scavenging assay

To determine ABTS radical scavenging assay, the method of Re *et al*., **[15]** was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. One ml of plant extracts (20-100 µg/ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer **[16]**. ABTS radical scavenging activity (%) = (Absorbance of control - Absorbance of sample)/ (Absorbance of control)×100

DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of

Liyana- Pathiranan and Shahidi **[17]**. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm **[18]**. Quercetin was used as standard. DPPH radical scavenging activity (%) = (Absorbance of control - Absorbance of sample)/(Absorbance of control)×100

Reducing ability (FRAP assay)

The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mMHCl, and 20 mM $FeCl_3$. $6H_2O$. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3$. $6H_2O$. The temperature of the solution was raised to 37^0C before use. Plant extracts (1 mg/ml) 150 µl were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product were taken at 593 nm. The results were reported as µg of ascorbic acid equivalents (AAE) per ml **[19]**.

Phosphomolybdate assay for total antioxidant capacity

Plant extract (1 mg/ml) 300 µl was mixed with 3 ml phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tube was covered with aluminum foil and incubated at 95^0C for 90 min. The mixture was then allowed to reach room temperature when its absorbance was recorded at 765 nm. Blank was run using the same procedure but containing an equal volume of methanol in place of the plant sample. Ascorbic acid was used as a standard. A stock solution of ascorbic acid (20 - 100 µg/ml) was prepared with distilled water, to obtain a standard calibration curve. The antioxidant capacity was reported as µg of ascorbic acid equivalents (AAE) per ml **[20]**.

RESULTS AND DISCUSSION

The percentage yield of different extracts of *Ehretia microphylla*, *Dipteracanthus patulus* and *Hydnocarpus laurifolia* are shown in **Table 1**. From the results ethyl acetate and methanol extracts of EM & DP shows higher yield of extract when compared with other extracts. For HL the petroleum ether and chloroform extracts showed more percentage yield.

The preliminary phytochemical studies of different extracts are shown in **Table 2**. All the extracts except PEEM, PEDP, PEHL contains essential phyto constituent such as flavonoids present in CEM, EAEM, MEM, CDP, EADP, MDP, CHL, EAHL, and MHL. Phenolic compounds present in CEM, EAEM, MEM, CDP, EADP, MDP, CHL and EAHL. Glycosides present in EAEM,

MEM, MDP, PEHL, CHL, EAHL and MHL. Phyto sterols present in all the extracts except PEEM, PEDP, CDP and CHL. Alkaloids was absent in all the plant extracts.

Quantification of total phenolic content and total flavonoid content was estimated by using standard calibration curve of Gallic acid and Quercetin. The results for the quantitative estimation of total phenolic content and total flavonoid content, in chloroform and ethyl acetate extracts of three medicinal plants are shown in **Table 3 & 4** respectively.

From the results of total phenolic and total flavonoid content CEM has higher amount of phenolic content and flavonoid content and EAHL has less amount of phenolic and flavonoid content when compared with all the other extracts. From the results of total phenolic and flavonoid content Ethyl acetate and chloroform extracts shows higher phenolic and flavonoid content so that Ethyl acetate and chloroform extracts of all plants were undergoes the *in vitro* antioxidant studies.

ABTS radical scavenging assay IC_{50} values of extracts and standard Quercetin are shown in **Table 5**. From the results of ABTS assay CDP was found to be 7.6 µg/ml has higher scavenging activity when compared with other extracts.

DPPH radical scavenging assay IC_{50} values of extracts and standard Quercetinare shown in **Table 6**. From the results of DPPH assay CDP has higher scavenging activity 15.60 µg/ml when compared with other extracts.

Ferric ions are reduced to ferrous ions in the presence of an antioxidant. Ferric reducing antioxidant potential (FRAP) values of extracts in terms of Ascorbic acid equivalent is shown in **Table 7**. EAEM was found to be 30.77µg/ml has higher activity when compared with other extracts.

Molybdenum (VI) is reduced to molybdenum (V) in the presence of a reducing agent (antioxidant), forming a green phosphomolybdate (V) complex. Total antioxidant capacity of extracts according to phosphomolybdate assay, expressed as µg/mL of Ascorbic acid equivalents. EAEM was found to be 25.28µg/ml has higher activity when compared with other extracts. From the results of the current research the ethyl acetate and chloroform extracts of *Ehretia microphylla, Dipteracanthus patulus* and *Hydnocarpus laurifolia* were shows potent antioxidant activity which is similar to the standard.

Table 1: Percentage yield of plant extracts

Plants	Solvents	Amount of extract obtained from 10 kg of plant material (g)	Percentage Yield (% w/w)
Ehretia microphylla	Petroleum ether (PEEM)	82	0.82
	Chloroform (CEM)	236	2.36
	Ethyl acetate (EAEM)	264	2.64
	Methanol (MEM)	625	6.25
Dipteracanthus patulus	Petroleum ether (PEDP)	78	0.78
	Chloroform (CDP)	296	2.96
	Ethyl acetate (EADP)	348	3.48
	Methanol (MDP)	542	5.42
Hydnocarpus laurifolia	Petroleum ether (PEHL)	345	3.45
	Chloroform (CHL)	236	2.36
	Ethyl acetate (EAHL)	195	1.95
	Methanol (MHL)	162	1.62

Table 2: Preliminary phytochemical investigations of different extracts of *Ehretia microphylla, Dipteracanthus patulus* and *Hydnocarpus laurifolia*

	PEEM	CEM	EAEM	MEM	PEDP	CDP	EADP	MDP	PEHL	CHL	EAHL	MHL
Flavonoids	-	+	+	+	-	+	+	+	-	+	+	+
Phenolic compound	-	+	+	+	-	+	+	+	-	+	+	+
Glycosides	-	-	+	+	-	-	-	+	+	+	+	+
Phytosterols	-	+	+	+	-	-	+	+	+	-	+	+
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-

Table 3: Total phenol content in chloroform and ethyl acetate extracts of *Ehretia microphylla, Dipteracanthus patulus* and *Hydnocarpus laurifolia*

S. No	Extract	Total phenol content (mg/kg/g of Gallic acid equivalent)
1	CEM	3.62±0.65
2	EAEM	2.93±0.08
3	MEM	0.63±0.03
4	CDP	2.32±0.021
5	EADP	1.52±0.28
6	MDP	0.57±0.4
7	CHL	1.02±0.11
8	EAHL	0.98±0.32
9	MHL	0.74±0.12

Table 4: Total flavonoids content in chloroform and ethyl acetate extracts of *Ehretia microphylla, Dipteracanthus patulus* and *Hydnocarpus laurifolia*

S. No	Extract	Total flavonoids content (mg/kg/g of Quercetin equivalent)
1	CEM	2.36±0.16
2	EAEM	2.19±0.26
3	MEM	0.81±0.43
4	CDP	2.09±0.48
5	EADP	1.52±0.45
6	MDP	0.67±0.31
7	CHL	1.49±0.67
8	EAHL	0.55±0.74
9	MHL	0.41±o.23

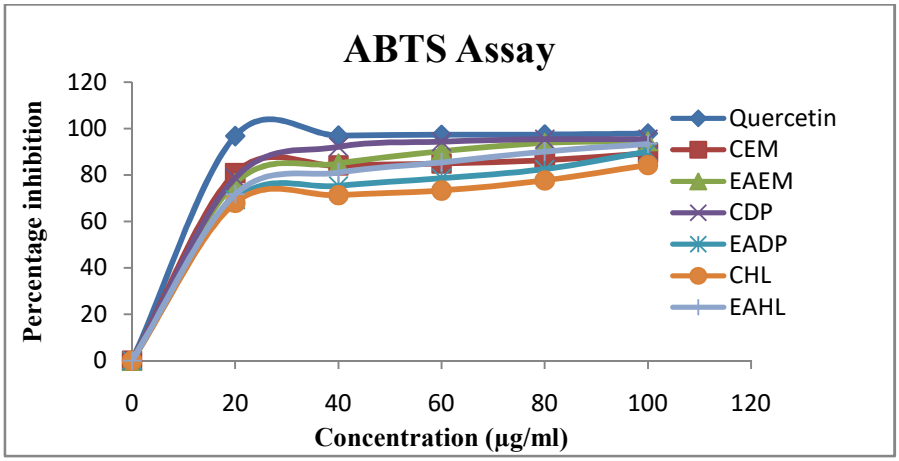

Figure 1: ABTS scavenging activities of different extracts and standard Quercetin

Table 5: ABTS radical scavenging assay IC_{50} values of extracts and standard Quercetin

S. No	Extracts	ABTS assay IC_{50} value of extract (µg/ml)
1	CEM	9.04
2	EAEM	14.69
3	CDP	7.66
4	EADP	19.33
5	CHL	17.96
6	EAHL	15.51
7	Quercetin	6.50

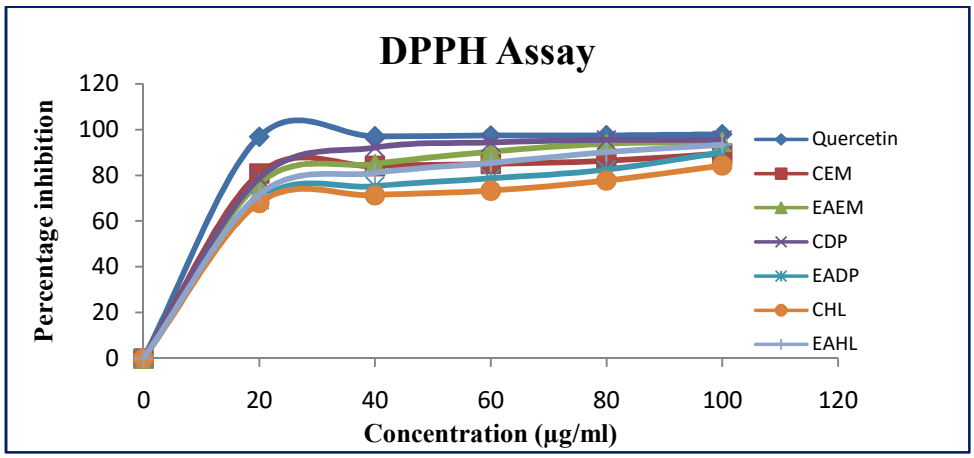

Figure 2: DPPH scavenging activities of different extracts and standard Quercetin

Table 6: DPPH radical scavenging assay IC_{50} values of extracts and standard Quercetin

S. No	Extracts	DPPH assay IC_{50}value of extract (µg/ml)
1	CEM	18.46
2	EAEM	19.23
3	CDP	15.60
4	EADP	27.31
5	CHL	30.78
6	EAHL	23.03
7	Quercetin	5.74

Table 7: FRAP and Phosphomolybdate assay values of extracts in terms of Ascorbic Acid Equivalents (AAE)

S. No	Extracts	FRAP assay values in terms of AAE (µg/ml)	Phosphomolybdate assay values in terms of AAE (µg/ml)
1	CEM	29.61	14.50
2	EAEM	30.77	25.28
3	CDP	28.13	13.81
4	EADP	28.24	12.21
5	CHL	20.16	13.39
6	EAHL	29.32	15.02

CONCLUSION

This study concluded that, the flavonoids present in *Ehretia microphylla, Dipteracanthus patulus* and *Hydnocarpus laurifolia* may produce the antioxidant activity. The future direction of this research is to conduct the *in vivo* studies and find out the exact mechanism of antioxidant activity.

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