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CORRELATION ANALYSIS OF PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITIES OF *HELIOTROPIUM INDICUM* L. LEAVES EXTRACTS

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Keywords:

Heliotropium indicum L., Phytochemicals, Antioxidants, Oxidative stress, Soxhlet extraction, Rotary vacuum evaporator

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ABSTRACT: The present study aimed to investigate the correlation between the total phenolic and flavonoid contents and *in-vitro* antioxidant activity of the Soxhlet-extracted leaves extracts (hexane, chloroform, ethyl acetate, methanol and aqueous) of *Heliotropium indicum* L, a medicinal weed. The preliminary phytochemical analysis was carried out in all five solvents using the standard methods of Harbonne. The phenols were quantified using a spectrophotometer by using Folin-Ciocalteau method by Singleton and Rossi and used catechol as standard. Flavonoids were quantified by using the aluminium chloride colorimetric method by Woisky and Salatino and used quercetin as standard. Soxhlet extracts of plant leave to scavenge DPPH radicals using Mensor *et al.*, method and reducing power was studied using Gow-Chin et al. method. The qualitative phytochemical studies of the crude extracts showed the presence of alkaloids, flavonoids, phenols, saponins, terpenoids, glycosides, proteins, fats and oils, and carbohydrates. The aqueous extract had the highest total phenol contents (165.6 mgGAE/g), and the ethyl acetate extract had the highest flavonoid contents (28.01 mgQE/g) at a concentration of 500 µl. Aqueous extract exhibited higher radical scavenging activity, with an IC_{50} value of 198.34µg/ml, and the reference standard, ascorbic acid with an IC_{50} value of 12.51 µg/ml. High correlations were observed between antioxidant activity and total phenols, and total flavonoid contents. *H. indicum* L. aqueous leaves extract exhibited significant DPPH radical scavenging and reducing power abilities. The present study concluded that the *H. indicum* L. aqueous leaves extract contained high amounts of total phenolic contents and exhibited significant antioxidant activity.

INTRODUCTION: Medicinal plants have been traditionally used in the treatment of various diseases in almost all cultures. The global interest has recently increased in plant-derived drugs that are found to be relatively safe and even free from serious side effects.

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According to the WHO estimates, about three-quarters of the world population currently relies chiefly on herbs and other traditional local medicinal plants as their therapeutic aid for mitigating ailments of various diseases [1].

Plant products have been a part of phytomedicines since time immemorial. The various parts of the plants including barks, leaves, flowers, roots, fruits, seeds, possess therapeutic value, which produces a definite physiological action on the human body. Plant-derived bioactive compounds have a protective role in combating oxidative stress.

A large number of plant's crude extract contained high oxidative potential and a significant amount of total phenolic compounds. Unbalanced generation of free radicals creates abnormal physiological conditions that lead to oxidative damage to cells by degrading lipids, proteins, and nucleic acids biomolecule, and in consequence, overexpression of oncogenes, mutagens formation, induction of atherogenic activity, or inflammation occurs. Indeed, many studies have been demonstrated that phytochemical constituents in plants like flavonoids, polyphenols, tannins, carotenoids, and phenolic terpenes exert antioxidant activities by quenching free radical production in the body. The plant-derived drugs are relatively non-toxic, safe, and even free from serious side effects in medical practice [2]. Oxidative stress leads to the overproduction of reactive oxygen species (ROS), including free radicals, and strongly implicated in the pathophysiology of diseases, such as cancer, rheumatoid arthritis, alzheimer's disease, parkinson's disease, neurodegeneration, aging, cirrhosis, arteriosclerosis, etc. Antioxidants could neutralize free radicals by donating electrons and prevent cell and tissue damage. Many studies have been reported that phytochemical constituents in plants like flavonoids, polyphenols, tannins, carotenoids, and phenolic terpenes exert antioxidant activities by quenching free radicals in the body when antioxidant protection become unbalanced [3].

Heliotropium indicum Linn. (Boraginaceae) is an annual herbaceous weed commonly known as the Indian heliotrope and is native to India. It is very common in India and grows in tropical, subtropical, and warm temperate regions. Most of the plants belonging to Boraginaceae are herbs. The majority of the heliotropes are popular garden plants, and some others occur as a weed. Many plants that are considered as weeds are also grown in gardens and other cultivated areas; these are known as beneficial weeds [4]. The study showed that the leaf extract of this plant showed potential antioxidant activity, which could be attributed to its total phenolic and flavonoid contents.

This study aimed to evaluate the correlation between antioxidant activities and total phenol and flavonoid contents in *H. indicum* L. leaves extract, as it is essential for scientific support to be made available for the evaluation of pharmacological properties of the plant. Nowadays, phytomedicine has high prospects worldwide, and many ethnomedicinal plants are considered contributors to drug discovery. Therefore, the total phenolics and flavonoids which contribute to the antioxidant activities and their correlation are evaluated in this study.

MATERIALS AND METHODS:

Leaf Sample Collection: The plant sample of *Heliotropium indicum* L. was collected from a barren paddy field in Cherthala, Kerala, India, during the month of May 2018. This plant was authenticated at the Botanical Survey of India, Coimbatore, India, as *Heliotropium indicum* L. (Boraginaceae), and the herbarium is stored at BSI, Coimbatore (BSI/SRC/5/23/2018/Tech/412). The leaves of the plant were washed in water and shade dried for two weeks.

The dried leaves were then powdered and kept in airtight container for analysis. The *in-vitro* experimental analysis was performed at the Department of Biotechnology and Research, KVM College of Science and Technology, Cherthala, Kerala, India.

Preparation of Plant Extract: The powdered leaves were subjected to hot extraction using Soxhlet apparatus for 24 h. 100 g of sample was extracted successively with 500 ml of hexane, chloroform, ethyl acetate, methanol, and water.

The solvents from these extracts were removed under reduced pressure using a rotary vacuum evaporator (Heidolph, Germany) and stored in sterile pre-weighed screw-capped container's at 4 ^{o}C until used. The sample weight of different solvent extracts is recorded in **Table 1**.

Preliminary Phytochemical Analysis: The phytochemical screening of leaves extracts of *Heliotropium indicum* L. was performed using standard methods by 5.

All the extracts of *H. indicum* L. prepared were subjected to a preliminary phytochemical screening for the presence of carbohydrates, alkaloids, flavonoids, phenols, tannins, glycosides, saponins, fats and oils, proteins and terpenoids.

Quantification of Total Phenols and Flavonoids:

Total Phenolic Content (TPC): Total natural phenolics present in the extracts were determined using Folin-Ciocalteu reagent using the method of 6. An aliquot of 0.5ml of the extract was treated with 2.5 ml of Folin-Ciocalteu reagent (1:10 diluted with water). Mixed thoroughly and after 5 min, 2ml of 7.5% sodium carbonate (Na_2CO_3) solution was added. The reaction mixture was kept for incubation at 45 ºC for 15 min. The blue colour developed was read at 765 nm. Gallic acid was used as the reference standard, and the TPC was estimated from the calibration graph of gallic acid. The total phenolic content (TPC) in the extracts was calculated using the equation

$$T = C \times (V/M)$$

Where, T is the total phenolic content (mg/g) of the extracts, C is the concentration of gallic acid (mg/ml) obtained from the calibration graph, Vis the volume of the extract taken (ml), and Mis the weight of the extract (g). The results were expressed as milligram gallic acid equivalents (mg GAE/g) of sample dry weight.

Total Flavonoid Content (TFC): Total flavonoids in the extracts were evaluated by aluminium chloride colourimetric method according to the method of7. To 0.5 ml of extract, 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$ (diluted in methanol), 0.1 ml of 1M potassium acetate (CH_3COOK), and 2.8 ml of distilled water were added.

The mixture was kept for incubation at room temperature (35 ºC) for 30 min. The absorbance was measured at 415 nm. In the blank $AlCl_3$ was substituted by the same amount of methanol. Quercetin was kept as the standard. TFC was calculated from the calibration graph plotted for quercetin using the formula

$$T = C \times (V/M)$$

Where T is the total flavonoid content (mg/g) of the extracts, C is the concentration of quercetin (mg/ml) obtained from the calibration graph, V is the volume of the extract taken (ml), and M is the weight of the extract (g). TFC is expressed as milligram quercetin equivalent (mg QE)/g sample dry weight.

Estimation of Antioxidant Activity:

***In-vitro* Antioxidant activity by DPPH Method:** Quantitative measurement of radical scavenging property was carried out according to the method of 8. A methanolic solution of 1 ml of DPPH (0.1 mM) was added to 1 mL of different concentrations (50-1000 µg/ml) of the extract and allowed to react at room temperature for 30 min in the dark. Absorbance was measured at 517 nm. Methanol served as the blank. DPPH in methanol without the extract served as the control, and ascorbic acid was taken as the reference standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

The capacity to scavenge the DPPH radical was calculated using the following equation:

% radical scavenging activity = (Abs. of control – Abs. of the sample) × 100/ Abs. of control

A graph was plotted with the percentage of inhibition against concentration. The IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression graph. The smaller the IC_{50} higher will be the antioxidant activity of the extract. The experiment was done in six replicates (n = 6), and the average of six radical scavenging assay (RSA) values at each concentration was calculated. The plot of averaged percentage RSA against extract concentrations was used to produce a linear regression line to determine the half-maximal inhibitory concentration (IC_{50}) value, which is the concentration of the extract that caused a reduction of DPPH initial concentration by 50%.

***In-vitro* Antioxidant Activity by Reducing Power Assay:** Reducing power assay was evaluated according to the procedure of9. Various concentrations of extract (500 µl) were mixed with 1.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min, followed by the addition of 10% trichloroacetic acid, which was then centrifuged at 6000 rpm for 5 min at 4 ºC. The upper layer of the solution (1.5 ml) was mixed with 1.5 ml of distilled water and 300 µl of ferric chloride (0.1%), and absorbance was read at 700 nm. Ascorbic acid was used as standard. Increased absorbance of the reaction mixture indicated increased reducing power. Concentration at 0.5

absorbances was marked as the reference to know the scavenging potential of each extract. The experiment was done in six replicates (n = 6).

Statistical Analysis: The experimental data were reported as the mean ± standard deviation (SD) having six replicates (n = 6). Linear regression coefficient (R^2) for phenolic and flavonoid content with antioxidant activity was analyzed by Graph Pad Prism for Windows, Version 7 (Graph Pad Software, San Diego, CA, USA). A p-value < 0.05 was considered significant.

RESULTS:

Extraction Yields: Soxhlet extraction of *H. indicum* L. hexane, chloroform ethyl acetate, methanol, and aqueous crude extracts yielded 2.64%, 5.43%, 4.2%, 7.16%, and 5.36% of extractable components, respectively, relative to the weight of dried leaves used.

The sample weight of different solvent extracts and percentage extractive of the crude matter is recorded in **Table 1**.

TABLE 1: PERCENTAGE EXTRACTIVE OF CRUDE EXTRACTS

Solvent	Appearance	Total weight (g) from 250 g	% extractive of crude matter
Hexane	Black-brown colour, semi-solid paste	6.6	2.64
Chloroform	Black colour, semi-solid paste	13.57	5.43
Ethyl Acetate	Green-brown colour, semi-solid paste	10.49	4.2
Methanol	Pale Green-yellow colour, semi-solid paste	17.91	7.16
Aqueous	Black colour, semi-solid paste	13.41	5.36

Preliminary Phytochemical Analysis: Preliminary qualitative phytochemical tests revealed that *H. indicum* L. hexane leaves extract showed the presence of flavonoids, phenols, and terpenoids and absence of carbohydrates, tannins, glycosides, saponins, fats and oils, and proteins. The chloroform extract showed the presence of flavonoids, phenols, tannins, saponins, and terpenoids and the absence of carbohydrates, alkaloids, glycosides, fats and oils, and proteins. The ethyl acetate extract showed the presence of flavonoids, phenols, saponins, and terpenoids and the absence of carbohydrates, alkaloids, glycosides, fats and oils, and proteins. The methanolic extract showed the presence of alkaloids, flavonoids, phenols, tannins, saponins, and proteins and the absence of carbohydrates, glycosides, fats and oils, and terpenoids. The aqueous extract showed the presence of carbohydrates, alkaloids, flavonoids, phenols, tannins, glycosides, saponins, and proteins and the absence of fats and oils, and terpenoids. Phytochemical analysis of *H. indicum* L. leaves extracts are summarized in **Table 2**.

TABLE 2: PHYTOCHEMICAL ANALYSIS OF *H. INDICUM* L. LEAVES CRUDE EXTRACTS

S. no:	Tests	Method	Strongly present (++), Weakly present (+), Absent (-)				
			Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous
1	Carbohydrate	Molisch's	-	-	-	-	++
		Benedict's	-	-	-	-	++
		Barfoed's	-	-	-	-	++
2	Alkaloids	Wagner's	-	-	-	+	+
		Meyer's	-	-	-	+	+
		Dragendorff"s	-	-	-	+	+
3	Flavonoids	Alkaline reagent	+	-	+	++	++
		Shinoda	+	+	+	+	++
4	Phenols	5% Ferric chloride	-	++	++	+	-
		Libermann	++	++	++	++	++
5	Tannins		-	+	++	++	-
6	Glycosides	Keller killiani	-	-	-	-	++
		Legal's test	-	-	-	-	-
		Borntrager's test	-	-	-	-	-
7	Saponins	Foam test	-	+	+	+	+
8	Fats and oils	Spot test	-	-	-	-	-
9	Proteins	Biuret	-	-	-	++	++
		Millon's	-	-	-	++	++
		Ninhydrin	-	-	+	++	++
10	Terpenoids	Salkowski's test	+	+	+	-	-

Quantification of Total Phenols and Flavonoids:

Determination of Total Phenolic Content (TPC): Total phenolic content (TPC) of *H. indicum* L. leaves extract was estimated using Folin-Ciocalteau reagent. The TPC was expressed as mg of gallic acid/g dried extracts. From the obtained standard graph and evaluation, it was found that the TPC of *H. indicum* L. leaves extract in hexane, chloroform, ethyl acetate, methanol, and aqueous extracts was found to be 40 mg GAE/g, 70.3 mg GAE/g, 151.9 mg GAE/g, 5.4 mg GAE/g, 165.6 mg GAE/g respectively at a concentration of 500 µg/ml. The aqueous extract (165.6 mg GAE/g) contained the highest total phenolic content when compared to other extracts. **Fig. 1** shows the TPC of *H. indicum* L. leaves extracts.

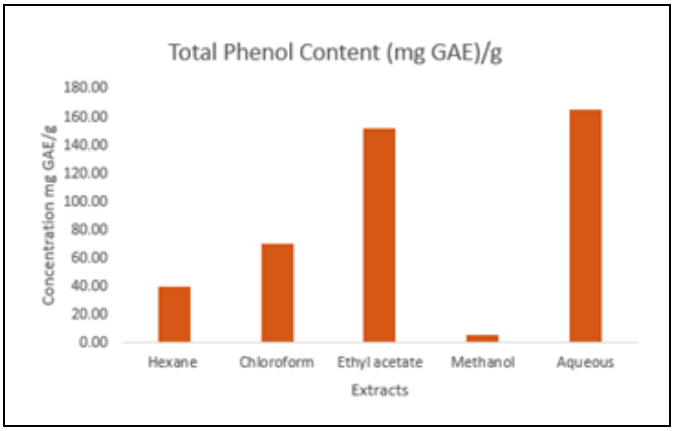

FIG. 1: DETERMINATION OF TOTAL PHENOL CONTENT (TPC)

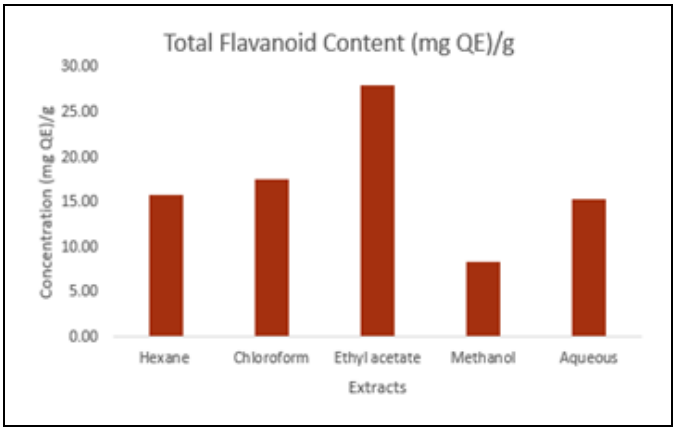

FIG. 2: DETERMINATION OF TOTAL FLAVONOID CONTENT (TFC)

Determination of Total Flavonoid Content (TFC): Total flavonoid content (TFC) of *H. indicum* L. leaves extract was estimated using the $AlCl_3$ method. The TFC was expressed as mg of Quercetin /g dried extract. The TFC of *H. indicum* L. leaves extract in hexane, chloroform, ethyl acetate, methanol, and aqueous was found to be 15.8 mg QE/g, 17.5 mg QE/g, 28.01 mg QE/g, 8.44 mg QE/g, and 15.27 mg QE/g, respectively, at a concentration of 500 µg/ml. The ethyl acetate extract contained (28.01 mg QE/g) the highest total flavonoid contents when compared to other extracts. **Fig. 2** shows the TFC of *H. indicum* L. leaves extracts.

***In-vitro* Antioxidant Activity by DPPH Method:** The DPPH radical scavenging activity was evaluated using different concentrations (50, 100, 250, 500, 1000 µg/ml) of *H. indicum* L. leaves extract and also ascorbic acid as standard for comparison. The DPPH radical scavenging ability of *H. indicum* L. leaves in hexane, chloroform, ethyl acetate, methanol, and aqueous extracts was found to be 51.6%, 54.07%, 67.15%, 66.67%, and 81.4% at 1000 µg/ml. The IC_{50} values of hexane, chloroform ethyl acetate, methanol, and aqueous was determined as 940.33 µg/ml, 898.5 µg/ml, 395.44 µg/ml, 415.72 µg/ml and 198.34 µg/ml, respectively. Higher antioxidant activity is indicated by having a lower IC_{50} value. The aqueous extract showed the highest radical scavenging ability when compared to other extracts, which indicated that the aqueous extract was more in DPPH radical scavenging when compared to the other extracts. The standard ascorbic acid had IC_{50} value of 12.51 µg/ml. **Fig. 3**. shows the antioxidant activities of *H. indicum* L. leaves extracts at different concentrations. Each value represents the mean ± SD of six replicates.

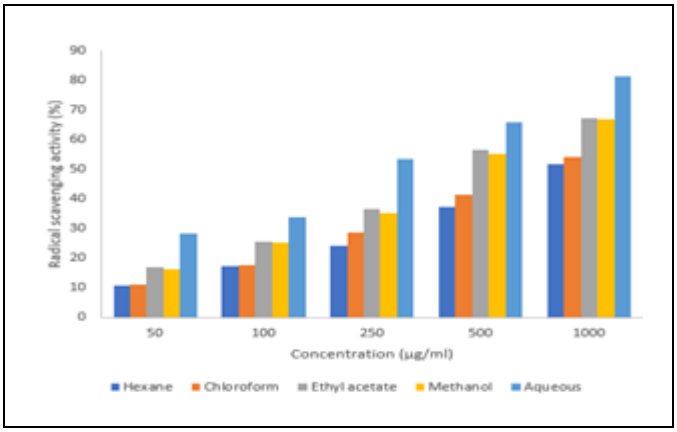

FIG. 3: DPPH RADICAL SCAVENGING ACTIVITIES OF *H. INDICUM* L. LEAVES CRUDE EXTRACTS AT VARIOUS CONCENTRATIONS

***In-vitro* Antioxidant Activity by Reducing Power Assay:** The reducing power of hexane, chloroform,

ethyl acetate, methanol, and aqueous crude leaves extracts of *H. indicum* L. at 0.5 Abs was found to be 350 µg/ml, 334 µg/ml, 165 µg/ml, 167 µg/ml and 70 µg/ml respectively. The aqueous extract showed the highest reducing ability (70 µg/ml) when compared with the other extracts. The radical scavenging activity of standard ascorbic acid was found to be 1.6 µg/ml at 0.5 Abs. **Fig. 4**. shows the reducing power activities of *H. indicum* L. leaves extract. Each value represents the mean ± SD of six replicates.

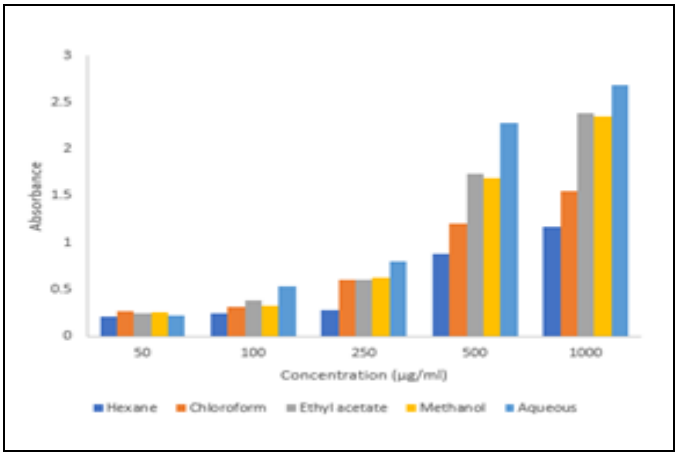

FIG. 4: REDUCING POWER ACTIVITIES OF *H. INDICUM* L. LEAVES CRUDE EXTRACTS AT VARIOUS CONCENTRATIONS

Statistical Analysis: High correlation was observed between antioxidant capacity and total phenols (DPPH, R^2 = 0.88; Reducing power, R^2 = 0.73) and total flavonoids (DPPH, R^2 = 0.62; Reducing power, R^2 = 0.68). A significant correlation was also observed between values of total phenolic and flavonoid contents (R2 = 0.81). A significant positive correlation of IC_{50} values of DPPH was established with the standard ascorbic acid (R^2 = 0.92, $p<0.05$). A significant positive correlation was also established with IC_{50} values of reducing power at 0.5 Abs and the standard ascorbic acid (R^2 = 0.78, $p<0.05$). The p-value<0.05 showed the significant presence of phenols and flavonoids in the plant extracts.

DISCUSSION: The extraction yield for hexane, chloroform, ethyl acetate, methanol, and aqueous leaves extract of *H. indicum* L. showed that methanolic extract exhibited a higher percentage yield (7.16%w/v). These results are similar to those of 10, which generated a 7.2% w/v percentage yield for methanolic extract. The selection of solvents for extraction plays a major role in the quantity and quality of extracts. The type and polarity of solvent can affect the extraction quality, quantity, extraction velocity, inhibitory compounds, toxicity, other biological activities, and biosafety [11]. Preliminary qualitative phytochemical analysis of leaves extracts of *H. indicum* L. revealed carbohydrates, alkaloids, flavonoids, phenols, tannins, glycosides, saponins, proteins, and absence of fats and oils. These results are consistent with those of reports of 12. Various secondary metabolites were isolated and identified from *Heliotropium* species such as pyrrolizidine alkaloids, flavonoids, triterpenes, and benzofurans [13].

The phytochemicals such as phenols and flavonoids were present in all the solvents and were quantified. Out of the ten secondary metabolites studied in *H. indicum* L. leaves extracts, the aqueous extract showed the presence of eight metabolites, and total phenols were also found to be rich in aqueous extract. However, total flavonoids were present in higher amounts in ethyl acetate extracts. The study revealed a highly significant correlation between the values of total phenolic and flavonoid contents (R^2 = 0.81). The quality, quantity, and biological activities of the phytoconstituents depend on the plant developmental stage, plant parts, and the solvents used for the extraction and isolation [11].

Antioxidants are molecules that neutralize free radicals by accepting or donating an electron, and it becomes a free radical and converting a free radical molecule to a non-free radical molecule. The concentration of antioxidant compounds is needed to decrease the DPPH radicals by IC50. DPPH is a stable, nitrogen-centered free radical which produces deep purple colour in methanol solution. The principle of DPPH assay is based on the reduction of purple coloured methanolic DPPH solution in the presence of hydrogen donating antioxidants by the formation of yellow coloured diphenyl-picryl hydrazine. In DPPH assay, as the absorbance decreases, the antioxidant activity of the extract is found more efficient. The antioxidants present in the extract is determined by DPPH reduction and are measured spectrophotometrically at 517 nm. The results of the study suggested that different concentrations have different radical scavenging activities, and maximum activity

(81.4%) was observed at 1000 µg/mL concentration. The aqueous leaves extract of *H. indicum* L. exhibited significant DPPH radical scavenging activity when compared with the standard, ascorbic acid. High correlation between IC_{50} values of DPPH was established with the IC_{50} values of the standard, ascorbic acid (R^2 = 0.92, $p<0.05$). This might be due to the presence of higher amounts of phenols in the extract. The p-value<0.05 showed the significant presence of polyphenols in plant extracts. The findings of 14in ethyl acetate extract support the present investigation as well, demonstrating a strong correlation between DPPH activity and total polyphenols, and total flavonoid content. Effective antioxidant activity was reported by 12 & 15 in ethanolic extracts of *H. indicum,* and 16 reported good antioxidant activity in ethyl acetate extract. The total secondary metabolites and their anti-oxidant activity depend mainly on the solvent and plant part used for extraction [11]. Reports on many biological activities of extracts and isolated compounds from Heliotropium plants were documented like antimicrobial, wound healing, antioxidant, antineoplastic, cytotoxic activities, and anti-inflammatory activity [13].

The reducing power of the extracts is a significant indicator to determine the antioxidant activity. In reducing power assay, the yellow colour of the test solution changes to shades of green and blue, depending on the reducing power of the test sample. It is seen that the reducing power increased with the increase in the extract concentrations.

A significant positive correlation was established with IC_{50} values of reducing power at 0.5 Abs and the standard ascorbic acid (R^2 = 0.78, $p<0.05$). In the present study, aqueous leaves extract of *H. indicum* L. exhibited significant reducing power ability. The aqueous extract may have a good amount of reductones and hence the antioxidant activity. Literature reports showed that reducing power and antioxidant activities of flavonoids are positively correlated to each other [17].

The study evaluated the phytochemical constituents, total phenolic contents, total flavonoid contents, and antioxidant activities in *H. indicum* L. leaves crude extracts. The study revealed a significant correlation between antioxidant activity and total phenolic and flavonoid contents.

Phenolic compounds from medicinal plants have several biological properties, such as anti-inflammatory, antioxidant and can be used in the prevention of many diseases [18]. This plant has been used in traditional medicine in many countries and warrants further scientific research in order to identify the phenolics responsible for the anti-oxidant activity.

CONCLUSION: The phytochemical constituents in *Heliotropium indicum* L. extracts were evaluated and found the aqueous leaves extract possessed the majority of secondary metabolites. The aqueous extract contained high amounts of phenol, and the ethyl acetate extract contained high amounts of flavonoid contents. The antioxidant activities were determined by DPPH radical scavenging and reducing power assays. The correlation analysis revealed that there was a significant correlation of total phenolic and flavonoid content with anti-oxidant activity, and this plant could be a potential source of antioxidants.

The study evaluated the phytochemical constituents, total phenolic contents, total flavonoid contents, and antioxidant activities in *H. indicum* L. leaves crude extracts. The study revealed a significant correlation between antioxidant activity and total phenolic and flavonoid contents. The important antioxidant components in plants, *viz.* phenols and flavonoids, could deactivate the free radicals as they have ideal structural characteristics for free radical scavenging. This plant has been used in traditional medicine in many countries, and the present study accounts for its antioxidant activities. Future research warrants identifying the phenolics responsible for its antioxidant activity.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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