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ESTIMATION OF FLAVONOID, PHENOL CONTENT AND ANTIOXIDANT POTENTIAL OF INDIAN SCREW TREE (*HELICTERES ISORA* L.)

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ABBREVIATIONS:

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH: 2,2-diphenyl-1-picrylhidrazyl
FRAP: Ferric Reducing Antioxidant Power
GAE: Gallic Acid Equivalent
RE: Rutin Equivalent
RP-HPLC: Reverse Phase – High Performance Liquid Chromatography
TEAC: Trolox Equivalent Antioxidant Capacity
TFC: Total Flavonoid Content
TPC: Total Phenolic Content

ABSTRACT: *Helicteres isora* (L.), a South-Asian plant, is a rich source of medicinal and antioxidant compounds and has been widely used in traditional medicine. Antioxidants are important to neutralize damaging free radicals in the body, especially when the systemic defence mechanisms prove insufficient. The objective of this study was to determine the antioxidant potential of its fresh and dry plant parts in various solvent systems. Plant material was collected from Karnala forest of Maharashtra. Extracts of leaves, bark, root and fruits (fresh and dry) were prepared using four different solvents viz. Distilled water, Ethanol, Methanol and Acetone. Each extract was tested for total phenolic content, total flavonoid content, and antioxidant activity by – FRAP, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays while phenolic compounds like Gallic acid, Cathechol, Vanillin, Caffic acid, p-Coumaric acid and Ferulic acid were detected using RP-HPLC. Antioxidant potential was significantly high in dried plant parts than the fresh ones. Leaves showed highest phenolic and flavonoid content (11.47 ± 0.50 mg GAE/g) and (54.16 ± 1.22 mg RE/g) with 90.21 ± 0.57% DPPH$^{\bullet}$ radical scavenging activity.The FRAP and ABTS$^{\bullet+}$ radical scavenging activity was found considerably high in leaves as well as in roots and fruits. Hence, the dried plant material like leaves, root and fruit when prepared in distilled water and methanol showed best antioxidant activity and holds potential for development into herbal formulation to be used as medicine, though further study is necessary to confirm its activity and suitability within the body.

INTRODUCTION: *Helicteres isora* L. (Family: Sterculiaceae) distributed widely in forests throughout India and commonly known as East Indian screw tree, is a medicinally important sub-deciduous shrub or a small tree [1].

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The different plant parts reportedly contain an array of important compounds like phytosterols, saponins, sugars, lignin, alkaloids, triterpenoids and their acetates, cucurbitacin B, isocucurbitacin B, flavonoids, neolignans, rosmarinic acid derivatives, betulic acid, daucosterol, tannnins, anthoquinones, sterols, lupeol, β-sitosterol, α and β amyrin, taraxerone and volatile oil [2]. It has been used in traditional medicine for treatment of various diseases and disorders like skin problems, dermatitis, eczema, acne, gastropasm. It is used as an antispasmodic, antipyretic, anti-diarreoheal,

anti-dysentric, anti-helmintic for tapeworms and as a tonic after childbirth. The root juice is claimed to be useful in treating cough, asthma, diabetes, emphyenma, intestinal infection, snake bites and a cure for scabies when applied topically [3, 4]. The root extracts are also found to have hypoglycaemic, hypolipidemic and anti-nonciceptive activity in mice. The fruits are astringent, refrigerant, useful in griping bowels and used in treatment of paralysis in some parts of India [2]. The fruit extract has also been reported to be potent inhibitor of HIV type-1 [5]. Aqueous extract of *H. isora* improves the level of plasma insulin, decrease glucose levels and reverses the changes in the levels of the carbohydrate moieties of glycoproteins and protein marker enzymes. It can reinstate brain antioxidant enzymes, heart antioxidant enzymes, and hepatic enzymes [6].

Medicinal plants have multiple biological effects on human system including antioxidant activity[7] due to presence of various compounds such as flavonoids, phenolic acids, tannins, coumarins, lignans and lignins[8,9,10] in different parts of plant. The free radicals produced during metabolism, when not sufficiently neutralized by indigenous antioxidants, cause serious damage to cells leading to various diseases like atherosclerosis, arthritis, ischemia, gastritis, cancer, AIDS etc [11].

Hence there is need of antioxidant supplements from natural sources, especially plant derived phenolics, which have recently received a lot of interest since synthetic antioxidants suffer from several side effects [12]. It has been proved with epidemiological studies that consumption of plant food containing antioxidants are beneficial to health as it controls many degenerative processes and can reduce the incidence of cancer and cardio-vascular diseases [13].

Unfortunately, information about phytochemical content and related antioxidant activity in *H. isora* is very limited. Some reports focusing only on antioxidant activity of fruit are available. Fruits of *H. isora* are seasonal and come annually once; this limits its quantity and availability throughout the year. There is constant pressure on fruit explants to meet the demand of pharmacy sector. Therefore, it is a need of the hour to explore the properties of the other parts *H. isora*.

Therefore, this work aims to study the phytochemical content and antioxidant activity of *H. isora* to determine the best plant part (in fresh or dry state) as well as solvent for extraction.

MATERIALS AND METHODS:

Plant Material: Leaves, bark, root and fruits of *H. isora* were collected from Karnala, Raigad District, Maharashtra, India. The plant was authenticated by Dr. Milind Sardesai, Botany Department, (Dr. B.R. Ambedkar Marathwara University), Maharashtra, India. The plant parts were separated and half of each explant was left to air dry for around two weeks (for dry plant extracts), while the rest was used for fresh extracts.

Chemicals: Methanol, Acetone, Folin-Ciocalteu reagent, Sodium Acetate, Sodium Carbonate were purchased from SRL Pvt. Ltd. (Mumbai, India); Trolox, Rutin, TPTZ, DPPH, ABTS and Potassium Persulfate were obtained from Sigma-Aldrich Chemical Co. (St. Lois, MO, USA); Aluminuium chloride, Gallic Acid, Acetic Acid were acquired from Molychem (Mumbai, India); Ferric Chloride was from HiMedia Laboratories (Mumbai India); Ethanol, HPLC grade water, Methanol and Acetic acid were procured from Merck (Darmstadt, Germany);

Extract preparation [14]: The fresh and dry plant part extracts were made using Distilled water, Ethanol, Methanol and Acetone and stored at -20^{0}C refrigerator until further use [14].

Phytochemical Assays: All methods described below are spectrophotometric methods and absorbance at specific wavelengths was taken using a UV spectrophotometer (Shimadzu – Model UV-1700 Pharma spec). For the assays, the extracts were diluted to 1% in their respective solvents and these dilutions were used. All readings were taken in triplicates and the activities/concentration of all three was averaged to give final value.

Total Phenolic Content (TPC) [15]: The TPC was determined by a Spectrophotometric method using Folin-Ciocalteu (FC) reagent. The Phenolic content of each sample had been determined from the standard curve of Gallic Acid using the calibration equation $y = 3.1368x - 0.0023$ ($R^2 = 0.9903$) where 'x' is the GAE in mg and 'y' is the absorbance at

760 nm and the content had been expressed as milli gram Gallic Acid Equivalent per Gram of Sample (mg GAE/g).

Total Flavonoid Content (TFC) [16]: The total flavonoid content had been estimated by a spectrophotometric method, which detects the amount of coloured complex formed between the flavonoids and aluminium ions. The flavonoid content had been estimated from standard curve of Rutin using the calibration equation of $y = 6.3771x - 0.0084$ ($R^2 = 0.9964$) where 'x' is the mg Rutin Equivalent and 'y' is the absorbance at 368 nm, and the content had been expressed as milli gram Rutin Equivalent per Gram of sample (mg RE/g).

DPPH Radical Scavenging Activity Assay [16]: This spectrophotometric assay, measures the radical scavenging ability of antioxidants present in the sample, towards the stable DPPH (2,2-diphenyl-1-picrylhidrazyl) radical rendering it colourless. The absorbance of the DPPH reagent was recorded as the control and the radical scavenging activity of the samples were calculated by the given formula:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Ferric Reducing Antioxidant Power (FRAP) assay [17]: This assay quantitates the amount of antioxidants in the sample, based on its ability to reduce Fe^{3+} to Fe^{2+}. The antioxidant content based on ferric ion reducing ability, had been calculated using a standard curve of Trolox and the calibration equation had been $y = 1.32x - 0.0044$ ($R^2 = 0.9717$) where 'x' is the mg Trolox Equivalent and 'y' is the absorbance at 595 nm and the content had been expressed as milli gram Trolox Equivalent Antioxidant Capacity per Gram of sample (mg TEAC/g).

ABTS Radical Scavenging Assay[18]: The antioxidants in a sample were quantified by a de-colorizing reaction with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radicals $ABTS^{\bullet+}$, generated during the reagent preparation. Absorbance of the ABTS reagent was recorded as the control and the ABTS radical scavenging activity of the sample was calculated by the given formula:

$$\text{ABTS radical Scavenging activity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Detection and quantification of Phenolic compounds using RP-HPLC: Detection and quantification of types of phenolic compounds present in each solvent extract of each fresh or dry plant part were done using reverse-phase HPLC (High Performance Liquid Chromatography). Waters HPLC (Model 2487) instrument with a UV spectrophotometer as detector was used. The column used was a 15 cm hypersil C18 reverse phase column with 5µ particle packing and while the mobile phase (Composition – 20% Methanol, 1% Acetic acid and 79% water) passed through the column at the rate of 1ml/min.

A linear gradient elution scheme was used and detection was done at 280 nm. The phenolic compounds were identified based on their retention time as identified in a standard chromatogram of a mixture of the pure phenolic compounds obtained beforehand, while the concentration of individual species was estimated from the peak area measurements and the output is given in the units of ppm. The results were converted from ppm and given in the units of µg/mg. All the solvents and chemicals used were HPLC purity grade.

Data analysis: The mean and ± standard deviation of the triplicate readings of each sample for each assay was determined and the mean values were analysed using Microsoft excel and SPSS version 19.0 software. Significant differences between samples were analysed using analysis of variance (ANOVA), Bonferroni test at $P<0.01$. Pearson correlation coefficient (R) and Coefficient of Determination (R^2) between different assays i.e. phenolic, flavonoid content and the antioxidant assays was carried out using SPSS software version 19.0.

RESULTS AND DISCUSSION: Plant extracts rich in polyphenols are important for preparation of medicines as polyphenols are easily obtained from natural sources. Though medicinal plants of India constitute about 20% of total plant species [19], but the medicinal properties of most of them are not completely explored.

There are very few studies available where commercially viable formulations are being prepared, therefore, it is imperative to promote their studies for their application in curing diseases. The present investigation evaluates the phenolic and flavonoid content as well as antioxidant activity of one such scantily explored but potentially useful plant species *H. isora* (L.)

Solvent extraction is most frequently used technique for isolation of plant antioxidant compounds with varied characteristics and polarities that may or may not be soluble in a particular solvent. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix [13]. The selection of an appropriate solvent is one of the most relevant previous steps in estimating phytochemical activities. The yield of antioxidant compounds from plant parts is influenced mainly by the conditions under which the process of liquid-solid extraction is achieved, the type of solvent used to separate the soluble fraction from the permeable solid, the degree of polymerization of phenolics and their interaction with the other components[20].

In present investigation, four types of extracts with water, ethanol, methanol, and acetone were prepared from different plant parts and used to check TPC, TFC and their antioxidant potential.

Total Phenolic Content: Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Phenolic compounds mainly contribute to antioxidant potential of plant due to their redox properties. The mechanism of phenolic compounds for antioxidant activity are neutralizing free radicals and preventing decomposition of hydroperoxides into free radicals [21, 22].

The TPC of different plant parts of *H.isora* ranged from 0.73±0.0 to 11.47±0.5 mg GAE/g in distilled water, ethanol, methanol and acetone (Table 1). Dried plant part showed maximum amount of phenolic compound in comparison to fresh plant parts. Dried leaves showed the highest (11.47±0.5 mg GAE/g) phenolic content when prepared in ethanol, while it was minimum in fresh roots (0.73±0.0mg/g GAE/g) (**Table 1**). Similar studies with leaf explants of different medicinal plant species when prepared in ethanol showed highest extraction of TPC [23, 24]. While dried root and fruit extracts, in water showed maximum phenolic content (7.21±0.2 mg GAE/g and 2.33 ±0.32 mg GAE/g). Strong *in vitro* polyphenol content in aqueous extracts of fruits was reported [25]. Dried leaves showed maximum TPC extractability in ethanol followed by water and the levels of significance between solvent and explants was studied by Bonferroni ANOVA and it was found highly significant at P<0.01significance level (**tables 2 & 3**).

Total Flavonoid Content: Flavonoids are one of the most common and universally occurring group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. These compounds contain hydroxyls which are responsible for the radical scavenging effects of most plants through scavenging or chelating process [26]. TFC of various plant parts in different solvents was shown in Table 1. Among different plant parts, fresh and dried leaves showed higher content (55.31±0.09 and 54.16±0.22 mg RE/g) in acetone and ethanol where as in other plant part, flavonoid content ranged from 2.76±0.06 to 15.52±0.04 mg RE/g in different solvents (Table 1).

When analysed statistically highest flavonoid content was found in acetone and ethanol, while within explants, dry leaves were found to be good source for extracting maximum amount of flavonoid. Fruit and bark [25] showed same kind of results. In some medicinal plants, the flavonoid content was better in methanol than ethanol [13] while in present study Acetone > Ethanol > Methanol > Water order was observed (tables 2 & 3).

2, 2-diphenyl-1-picrylhidrazyl (DPPH˙) radical scavenging assay: The measurement of the scavenging activity of DPPH˙ radical allows a substance to donate electrons or hydrogen ion to reactive species in a homogenous system. The method is based on the reduction of methanolic-DPPH˙ solution because substances having hydrogen donating groups (RH) such as phenolic and flavonoid compounds produce reduced DPPH-H form[27]. Due to this reaction, free radicals produced that control the number of molecules of DPPH˙ reduced by one molecule of the reductant.

The % DPPH˙ activity of different plant parts in various solvents is shown in (Table 1).

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TABLE 1: TOTAL PHENOLIC CONTENT (TPC), TOTAL FLAVONOID CONTENT (TFC), DPPH RADICAL SCAVENGING ACTIVITY, FERRIC REDUCING ANTIOXIDANT POWER (FRAP) AND ABTS RADICAL SCAVENGING ACTIVITY OF VARIOUS PLANT PART EXTRACTS OF *HELICTERES ISORA* (L.) IN VARIOUS SOLVENTS

Plant Part	Solvent	TPC (mg GAE/g)		TFC Assay (mg RE/g)		FRAP (mg TEAC/g)		DPPH Scavenging Activity (%)		ABTS Scavenging Activity %)	
		Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Leaves	D/W	1.29 ± 0.09	4.65 ± 0.03	20.23 ± 0.47	23.89 ± 0.71	1.69 ± 0	23.13 ± 1.14	81.09 ± 2.08	86.48 ± 0.28	59.29 ± 1.01	80.98 ± 1.47
	Ethanol	1.39 ± 0.04	11.47 ± 0.50	47.36 ± 4.25	54.16 ± 1.22	1.69 ± 0.13	7.05 ± 0.24	83.23 ± 0.75	73.55 ± 1.08	65.66 ± 1.03	73.22 ± 1.19
	Methanol	1.22 ± 0.06	7.25 ± 0.98	45.85 ± 4.75	51.33 ± 0.15	1.57 ± 0.11	44.77 ± 3.78	90.21 ± 0.57	85.64± 0.62	64.70 ± 0.16	100 ± 0
	Acetone	1.28 ± 0.01	6.68 ± 0.24	55.31 ± 0.09	52.28 ± 1.6	1.59 ± 0.24	9.27 ± 1.51	82.41 ± 0.91	81.88 ± 5.06	100 ± 0	100 ± 0
Bark	D/W	1.04 ± 0.03	4.28 ± 1.2	2.76 ± 0.06	2.45 ± 0.58	0.33 ± 0	0.33 ± 0	60.51 ± 5.25	62.72 ± 5.6	64.99 ± 1.47	60.24 ± 1.43
	Ethanol	0.97 ± 0.01	7.78 ± 0.35	6.91 ± 0.65	5.49 ± 0.01	0.33 ± 0	0.33 ± 0	57.71 ± 1.67	62.90 ± 1.34	67.28 ± 1.19	66.66 ± 0.51
	Methanol	1.00 ± 0.03	2.71 ± 0.06	5.42 ± 0.38	4.34 ± 0.01	0.33 ± 0	0.73 ± 0.08	83.36 ± 4.86	63.77 ± 0.43	68.53 ± 0	65.46 ± 0.86
	Acetone	1.13 ± 0.01	5.28 ± 0.04	6.08 ± 0.24	5.26 ± 0.01	0.86 ± 2.6	0.33 ± 0	78.35 ± 4.71	62.90 ± 0.82	99.76 ± 0	100 ± 0
Root	D/W	0.76 ± 0.03	7.21 ± 0.2	4.05 ± 0.04	15.52 ± 0.04	1.82 ± 0.61	64.97 ± 2.58	50.04 ± 0.03	73.39 ± 0.22	54.41 ± 3.51	98.36 ± 0.83
	Ethanol	0.73 ± 0	2.04 ± 0.08	6.95 ± 0.05	6.57 ± 0.12	1.34 ± 0.04	14.87 ± 0.59	51.23 ± 0	54.75 ± 0.08	54.46 ± 1.61	78.18 ± 1.85
	Methanol	0.82 ± 0.08	3.27 ± 0.01	6.57 ± 0.05	6.69 ± 0.09	1.64 ± 0.11	37.42 ± 1.22	53.84 ± 0	70.45 ± 0.03	57.74 ± 1.97	99.68 ± 0.07
	Acetone	0.97 ± 0.15	1.39 ± 0.1	7.74 ± 0.31	5.92 ± 0.26	1.11 ± 0.24	10.15 ± 0.37	57.61 ± 0.08	51.06 ± 0.12	96.35 ± 0.75	99.90 ± 0.07
Fruits	D/W	2.25 ± 0.01	2.33 ± 0.32	5.05 ± 0.06	4.95 ± 0.01	13.23 ± 0.81	20.88 ± 0.21	54.69 ± 0.05	51.87 ± 0.22	79.32 ± 0.43	91.57 ± 0.15
	Ethanol	1.02 ± 0.03	0.86 ± 0.03	5.56 ± 0.05	7.01 ± 0.15	14.25 ± 0.41	3.36 ± 0.07	50.05 ± 0.26	61.78 ± 0.08	81.14 ± 0.36	63.06 ± 0.15
	Methanol	2.52 ± 0.12	1.50 ± 0.03	6.23 ± 0.08	6.17 ± 0.18	25.58 ± 1.39	11.36 ± 0.15	55.56 ± 0.85	59.36 ± 0.08	93.98 ± 1.89	80.37 ± 0.31
	Acetone	1.47 ± 0.14	1.34 ± 0.23	6.36 ± 0.1	9.32 ± 0.4	10.53 ± 0.67	8.26 ± 0.04	51.64 ± 0.46	57.61 ± 0.08	99.77 ± 0.07	99.77 ± 0.15

Total Phenol Content (TPC), expressed in milligrams of Gallic Acid Equivalent per gram of sample.

Total Flavonoid Content (TFC), expressed in milligrams of Rutin Equivalent per gram of sample.

Ferric Reducing Antioxidant power (FRAP), expressed in milligrams of Trolox Equivalent Antioxidant Capacity per gram of sample.

$DPPH^{\cdot}$ radical scavenging activity, expressed in terms of % inhibition of $DPPH^{\cdot}$ radicals.

$ABTS^{\cdot+}$ radical scavenging activity, expressed in terms of % inhibition of $ABTS^{\cdot+}$ radicals.

It was ranging from 50.69% in fresh fruit to 90.21% in fresh leaves. Maximum DPPH˙ scavenging activity was observed in methanol and aqueous extracts. Fresh and dry leaves showed highest DPPH˙ radical scavenging activity (90.21% and 85.64 %) amongst various plant parts.

Similar reports of high antioxidant activity in methanolic extracts of *H. isora* have been reported [6, 13, 28]. It has been found statistically (**Table 2, 3**) that leaves are good source of antioxidants and methanol and water are the best solvents to extract the content.

TABLE 2: ANOVA FOR TOTAL PHENOLIC CONTENT (TPC), TOTAL FLAVONOID CONTENT (TFC) AND ANTIOXIDANT ACTIVITY ASSAYS (FRAP, DPPH, ABTS) AMONG DIFFERENT PLANT PARTS OF *H. isora* (L.)

Pairwise Comparisons						
		Mean Difference (I-J)				
(I) Plant Part	(J) Plant Part	TPC	TFC	FRAP	DPPH	ABTS
Fresh leaves	dry leaves	-6.220770*	-3.228*	-19.419*	2.349	-16.140*
	fresh bark	.257759	36.895*	1.174	14.253*	-2.730*
	dry bark	-3.714923*	37.802*	1.206	21.162*	-.682
	fresh root	.473002	35.860*	.158	31.056*	6.669*
	dry root	-2.181654*	33.513*	-30.221*	21.823*	-21.621*
	fresh fruit	-.520833*	36.390*	-14.331*	31.248*	-16.145*
	dry fruit	-.215242	35.326*	-11.578*	26.580*	-11.284*
Dry leaves	fresh leaves	6.220770*	3.228*	19.419*	-2.349	16.140*
	fresh bark	6.478529*	40.123*	20.593*	11.904*	13.410*
	dry bark	2.505846*	41.030*	20.625*	18.814*	15.457*
	fresh root	6.693771*	39.088*	19.577*	28.707*	22.809*
	dry root	4.039116*	36.741*	-10.802*	19.474*	-5.481*
	fresh fruit	5.699936*	39.617*	5.088*	28.899*	-.005
	dry fruit	6.005527*	38.554*	7.841*	24.231*	4.856*
Fresh bark	fresh leaves	-.257759	-36.895*	-1.174	-14.253*	2.730*
	dry leaves	-6.478529*	-40.123*	-20.593*	-11.904*	-13.410*
	dry bark	-3.972683*	.907	.032	6.909*	2.047*
	fresh root	.215242	-1.035	-1.016	16.802*	9.399*
	dry root	-2.439413*	-3.382*	-31.395*	7.569*	-18.891*
	fresh fruit	-.778593*	-.506	-15.505*	16.995*	-13.415*
	dry fruit	-.473002	-1.569	-12.753*	12.327*	-8.554*
Dry bark	fresh leaves	3.714923*	-37.802*	-1.206	-21.162*	.682
	dry leaves	-2.505846*	-41.030*	-20.625*	-18.814*	-15.457*
	fresh bark	3.972683*	-.907	-.032	-6.909*	-2.047*
	fresh root	4.187925*	-1.942*	-1.048	9.893*	7.352*
	dry root	1.533270*	-4.289*	-31.427*	.660	-20.938*
	fresh fruit	3.194090*	-1.413	-15.537*	10.086*	-15.462*
	dry fruit	3.499681*	-2.476*	-12.784*	5.418*	-10.601*
Fresh root	fresh leaves	-.473002	-35.860*	-.158	-31.056*	-6.669*
	dry leaves	-6.693771*	-39.088*	-19.577*	-28.707*	-22.809*
	fresh bark	-.215242	1.035	1.016	-16.802*	-9.399*
	dry bark	-4.187925*	1.942*	1.048	-9.893*	-7.352*
	dry root	-2.654656*	-2.347*	-30.379*	-9.233*	-28.290*
	fresh fruit	-.993835*	.529	-14.489*	.193	-22.814*
	dry fruit	-.688244*	-.534	-11.736*	-4.475*	-17.953*

Dry root	fresh leaves	2.181654*	-33.513*	30.221*	-21.823*	21.621*
	dry leaves	-4.039116*	-36.741*	10.802*	-19.474*	5.481*
	fresh bark	2.439413*	3.382*	31.395*	-7.569*	18.891*
	dry bark	-1.533270*	4.289*	31.427*	-.660	20.938*
	fresh root	2.654656*	2.347*	30.379*	9.233*	28.290*
	fresh fruit	1.660821*	2.876*	15.890*	9.426*	5.476*
	dry fruit	1.966412*	1.812	18.643*	4.758*	10.337*
Fresh fruit	fresh leaves	.520833*	-36.390*	14.331*	-31.248*	16.145*
	dry leaves	-5.699936*	-39.617*	-5.088*	-28.899*	.005
	fresh bark	.778593*	.506	15.505*	-16.995*	13.415*
	dry bark	-3.194090*	1.413	15.537*	-10.086*	15.462*
	fresh root	.993835*	-.529	14.489*	-.193	22.814*
	dry root	-1.660821*	-2.876*	-15.890*	-9.426*	-5.476*
	dry fruit	.305591	-1.064	2.753*	-4.668*	4.861*
dry fruit	fresh leaves	.215242	-35.326*	11.578*	-26.580*	11.284*
	dry leaves	-6.005527*	-38.554*	-7.841*	-24.231*	-4.856*
	fresh bark	.473002	1.569	12.753*	-12.327*	8.554*
	dry bark	-3.499681*	2.476*	12.784*	-5.418*	10.601*
	fresh root	.688244*	.534	11.736*	4.475*	17.953*
	dry root	-1.966412*	-1.812	-18.643*	-4.758*	-10.337*
	fresh fruit	-.305591	1.064	-2.753*	4.668*	-4.861*

Based on estimated marginal means (Bonferroni test SPSS version 19.0). * The mean difference is significant at the 0.01 level.

TABLE 3: ANOVA FOR TOTAL PHENOLIC CONTENT (TPC), TOTAL FLAVONOID CONTENT (TFC) AND ANTIOXIDANT ACTIVITY ASSAYS (FRAP, DPPH, ABTS) AMONG DIFFERENT SOLVENT EXTRACTS OF *H. ISORA* (L.)

Pairwise Comparisons						
(I) Solvent	**(J) Solvent**	**Mean Difference (I-J)**				
		TPC	**TFC**	**FRAP**	**DPPH**	**ABTS**
Distilled water	Ethanol	-.304*	-7.640*	10.363*	3.199*	4.936*
	Methanol	.438*	-6.715*	-.751	-5.176*	-5.162*
	Acetone	.533*	-8.672*	10.537*	-.334	-25.797*
Ethanol	Distilled water	.304*	7.640*	-10.363*	-3.199*	-4.936*
	Methanol	.743*	.925	-11.114*	-8.374*	-10.098*
	Acetone	.837*	-1.032	.174	-3.533*	-30.732*
Methanol	Distilled water	-.438*	6.715*	.751	5.176*	5.162*
	Ethanol	-.743*	-.925	11.114*	8.374*	10.098*
	Acetone	.094	-1.957*	11.288*	4.841*	-20.635*
Acetone	Distilled water	-.533*	8.672*	-10.537*	.334	25.797*
	Ethanol	-.837*	1.032	-.174	3.533*	30.732*
	Methanol	-.094	1.957*	-11.288*	-4.841*	20.635*

Based on estimated marginal means (Bonferroni test SPSS version 19.0). * The mean difference is significant at the 0.01 level.

The DPPH˙ free radical is known to react with numerous antioxidants (like tocopherols, carotenoids, flavonoids, phenolics) and give an estimate of the total antioxidant activity of the sample [29] which is also supported by the strong correlation found in the present investigation between DPPH˙ radical scavenging activity, TPC and TFC of dry plant parts (**Table 4**).

TABLE 4: CORRELATIONS (R AND R^2) BETWEEN DIFFERENT ANTIOXIDANT CAPACITY PARAMETERS (BY ABTS, DPPH AND FRAP ASSAYS), TOTAL PHENOLIC CONTENT (TPC) AND TOTAL FLAVONOID CONTENT (TFC) OF *H. ISORA* FRESH AND DRY PLANT PARTS.

Fresh Plant Parts				
R(R^2)	**TPC**	**TFC**	**DPPH**	**FRAP**
TFC	0.058 (0.003)			
DPPH	0.006 (0)	0.721** (0.519)		
FRAP	0.790** (0.623)	-0.244 (0.06)	-0.418 (0.175)	
ABTS	0.439 (0.193)	0.051 (0.003)	0.11 (0)	0.414 (0.172)
Dry Plant Parts				
R(R^2)	**TPC**	**TFC**	**DPPH**	**FRAP**
TFC	0.704** (0.496)			
DPPH	0.62* (0.384)	0.754** (0.569)		
FRAP	0.184 (0.34)	0.216 (0.47)	0.416 (0.173)	
ABTS	0.002 (0.0)	0.238 (0.056)	0.164 (0.027)	0.516* (0.266)

R = Correlation coefficient; R^2 = Coefficient of Determination. The values in parenthesis represent the R^2 value. ** Significance at P<0.01. * Significance at P<0.05

Ferric Reducing Antioxidant Power (FRAP) assay: FRAP assay showed a wide range of variation among the plant parts and solvents used (Table 1). Dried roots (64.97±2.58) and leaves (44.77±3.78) showed the highest FRAP activity while fresh bark showed the lowest FRAP value (Table 1). Upon drying, FRAP activity increased in all the plant parts except in fruits. Fresh fruit showed 25.58±1.39 mg TEAC/g whereas in dry fruit it significantly lowered at 11.36±0.15 mg TEAC/g. Maximum FRAP activity was found in dried leaves when extracts prepared in methanol (44.77±3.78). In a study of *H. isora* fruit explants similar results were reported [30, 31].

It has been observed that dried fruit exhibited significant metal chelating ability in hexane solvent and none in acetone, isopropanol and aqueous extracts [32]. Though the fruit of the plant is used extensively in herbal formulations but there are no reports on analysis of leaves, root and bark for their antioxidant activity. In present investigation, it was observed that leaves and roots have maximum antioxidant potential in methanol (Table 2 and 3).

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) radical scavenging assay: The scavenging capacities of the different extracts for the $ABTS^{\bullet+}$ radical were measured and compared (Table 1). The $ABTS^{\bullet+}$ scavenging activity was higher in dried tissues and ranged from 52.1% to 100%. Maximum activity was observed in acetone extract in all fresh and dry plant parts, followed by methanol. The differences in the $ABTS^{\bullet+}$ scavenging activity exhibited by various extracts indicated that the extracting solvent influenced the antioxidant content of the extracts. The significant difference was observed between dried root prepared in acetone and methanol at 0.01 levels.

Correlation analysis between TPC, TFC and Antioxidant activity ($DPPH^{\bullet}$, FRAP and $ABTS^{\bullet+}$ assays): To establish the suitability, reliability and relationship amongst TPC, TFC and the total antioxidant capacity analysed through different assays, linear regression and correlation analysis was performed. The Pearson correlation coefficients (R) and coefficients of determination R^2 for fresh and dry plant parts are given in Table 4. All R values were found significant at P<0.01 significance level and in case of dried tissue the values of antioxidant capacities observed by three assay and TPC and TFC are correlated.

The TPC in fresh plant parts showed high correlation with FRAP (R=0.79**) and moderate with $ABTS^{\bullet+}$ (R= 0.439) whereas TFC has good correlate on with $DPPH^{\bullet}$ (R= 0.721**). Similarly, antioxidant potential observed in dried plant parts showed significant correlation amongst themselves indicating dried tissue had more potential than fresh ones. TPC was highly correlated with TFC (R= 0.704**) and $DPPH^{\bullet}$ (R= 0.620*) and TFC also showed significant linear correlation with $DPPH^{\bullet}$ (R= 0.754**). This suggests that flavonoids (a group of polyphenols) are the main compounds responsible for the antioxidant activity detected by the $DPPH^{\bullet}$ assay. This was also explained by the fact that flavonoids are low molecular weight, polar polyphenol [33] and $DPPH^{\bullet}$ radicals react preferentially with low molecular weight phenolic compounds [27].

Amongst the three antioxidant assays, FRAP with $ABTS^{\bullet+}$ have moderate relation in fresh tissues (R=0.414) and good correlation in dry tissues (R=0.516) but there was no relation found between either of these two with $DPPH^{\bullet}$ assay, indicating some congruency between $ABTS^{\bullet+}$ and FRAP assay and their incompatibility with $DPPH^{\bullet}$ assay, as also found in other studies [34]. This suggests that single assay is not sufficient for the accurate estimation of the total antioxidant capacity and thus a combination of assays should be performed to get better estimate.

HPLC analysis for Phenolic compounds: Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids [35]. Individual phenolic acids like Gallic acid, Cathechol, Vanillin, Caffic acid, p-Coumaric acid and Ferulic acid in *H. isora* were analysed and quantified using RP-HPLC (**Fig. 1**) for all the plant parts (fresh and dried) prepared in different solvents (**Table 5**).

TABLE 5: QUANTIFICATION OF PHENOLIC COMPOUNDS IN DIFFERENT EXTRACTS OF *HELICTERES ISORA* (L.) IN VARIOUS SOLVENTS BY RP-HPLC

Plant Part	Solvent	Gallic acid (µg/g)	Catechol (µg/g)	Vanillin (µg/g)	Caffeic acid (µg/g)	p-Coumaric acid (µg/g)	Ferullic acid (µg/g)
Fresh Leaf	D/W	2.21	4.16	5.64	5.14	4.48	4.9
	Ethanol	0.33	-	-	0.18	-	-
	Methanol		-	-	0.42	-	-
Dry leaf	D/W	21	-	18.6	-	-	-
	Ethanol	0.49	-	6.97	0.13	-	-
	Methanol	5.8	1.65	42.4	5.13	1.1	4.1
Fresh Bark	D/W	-	-	2.1	0.8	1	-
	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	2.25	-	-
Dry Bark	D/W	-	-	0.11	-	-	-
	Ethanol	2.7	-	-	0.4	0.5	-
	Methanol	-	-	-	0.14	0.38	-
Fresh Root	D/W	-	0.39	0.28	-	-	-
	Ethanol	0.38	-	-	-	-	-
	Methanol	2.72	-	-	0.3	-	-
Dry Root	D/W	44.8	9.17	-	-	-	-
	Ethanol		10.32	-	-	-	-
	Methanol	42.1	-	0.35	0.41	-	-
Fresh Fruit	D/W	-	4	-	-	4.43	-
	Ethanol	-	-	-	-	-	-
	Methanol	-	1.56	1.47	0.67	-	-
Dry Fruit	D/W	34	71	25.1	10.1	-	-
	Ethanol	23.5	-	-	-	-	-
	Methanol	5.9	-	2.4	-	-	-

The content of phenolic acids in fresh and dried plant part measured by HPLC showed slightly different results to those determined by the Folin-Ciocalteu method. The highest concentration of total phenolic acid (140.2 µg/g) was found in aqueous extracts of dry fruit of *H. isora.* In case of dried leaves, methanol showed all six types of studied phenolic compounds and Vanillin was found in maximum concentration (Table 4). Dried plant parts proved to be much better in terms of containing phenolic compounds in comparison to fresh ones.

The results indicated that Gallic acid is the major free phenolic compound in distilled water (44.8 µg/g in dry root, 34.0 µg/g in dry fruits and 21.0 µg/g in dry leaf) extracts. Aqueous extracts showed the highest extraction capacity for Gallic acid (44.8 µg/mg), Cathechol (71 µg/g), Vanillin (18.6 µg/g), Caffeic acid (10.1 µg/g), p-Coumaric acid (4.4 µg/g) and Ferulic acid (4.9 µg/g) in various explants followed by methanol. Dried plant parts leaves, root and fruit of *H. isora* contains high amount of phenolics compared to bark tissue in different solvents.

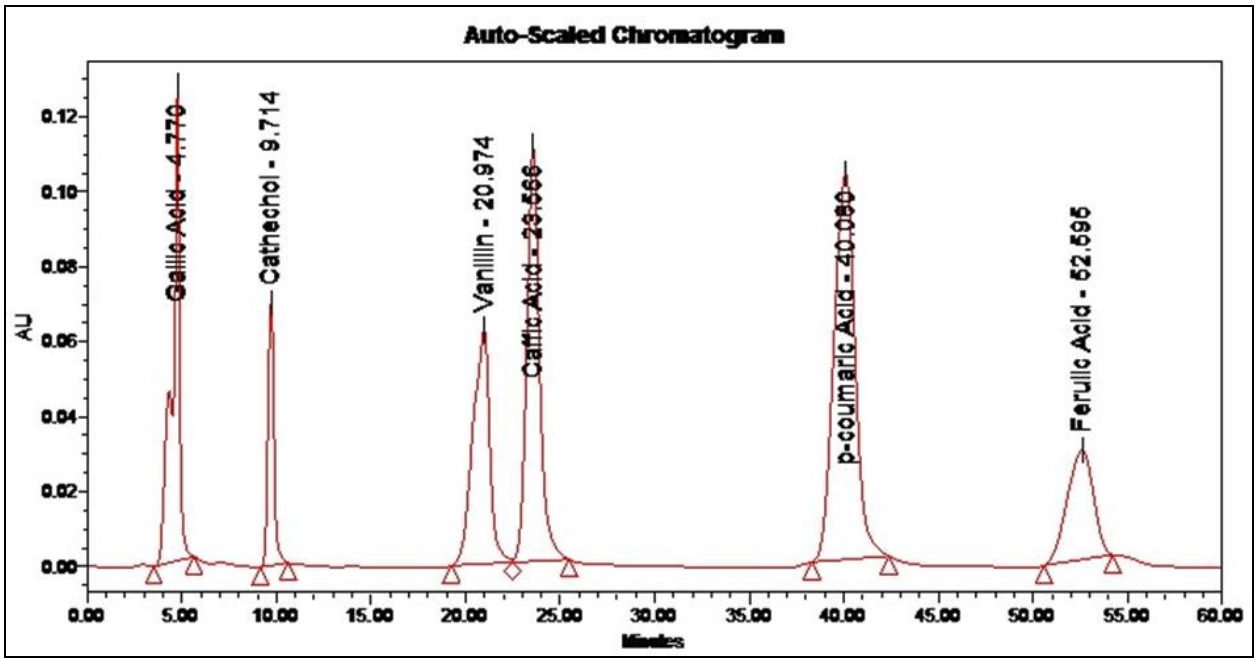

FIGURE 1: HPLC FINGERPRINTING: HPLC CHROMATOGRAM OF STANDARD, SEPARATED ON C18 COLUMN (REVERSE PHASE COLUMN 15 CM; PARTICLE SIZE 5 µM FROM HYPERSIL, USA) USING GRADIENT ELUTION – ACETIC ACID, METHANOL, AND WATER AT FLOW RATE OF 1ML/MIN. The chromatograms of absorbance at 280 nm were analyzed and compared.

The leaves showed maximum amount of flavonoids and antioxidant potential as supported through different assays of present investigation.

CONCLUSION: *H. isora* is a potential source of natural antioxidants for preparation of crude extracts or further isolation and purification of antioxidant components. Especially, if the crude extract is non-toxic after the toxicological assessment, further isolation and purification of antioxidant components is not necessary because health benefits of the extract might be from additive and synergistic effects of phytochemicals in the extract [36].

In this study, it was demonstrated for the first time that extracts from different plant parts in various solvents of *Helicteres isora* exhibited good antioxidant activity as measured by various antioxidant assays. From the correlation and regression analysis, it can be inferred that DPPH activity is highly dependent on the flavonoid and phenolic content of the tissue. The DPPH assay showed leaves are better source of antioxidant activity while the FRAP and ABTS assays shows roots and fruits.

On generalizing the results for all the five assays, it can be concluded that dried leaves, roots and fruit are good antioxidant sources.

Methanol proved to be the best solvent for extraction of flavonoid and antioxidant compounds whereas in case of total phenol content assay distilled water was found to be better. The RP-HPLC results further confirmed these findings. This study substantiates the potency of the crude extract of dry leaf >dry root>fresh fruit>dry bark in methanol as significant source of natural antioxidants. Further research in identifying individual components and forming antioxidative system will aid in developing their application for herbal medicines.

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