QUANTITATIVE DETERMINATION OF POLYPHENOLS AND STUDY OF ANTIOXIDANT ACTIVITY OF A TRADITIONALLY IMPORTANT MEDICINAL PLANT: *HELICTERES ISORA* LINN.

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Abstract: In the present study *in vitro* anti-oxidative potential and free radical scavenging activity were analyzed for hydroethanolic extract of *Helicteres isora* Linn. (HEHI) stem bark by chemical assays including DPPH (2, 2-diphenyl-1-picrylhydrazyl), superoxide anion radical scavenging, ferric ion reducing antioxidant power (FRAP) metal chelating activity. These assays were compared with standard antioxidants such as ascorbic acid, BHT, rutin, EDTA. Total phenolic and flavonoid content were also determined spectrophotometrically. IC_{50} value of HEHI obtained in DPPH, superoxide anion radical scavenging and metal chelating activity were 97.53 ± 0.281, 5.40 ± 0.032, 165.7 ± 0.45 respectively. The results obtained revealed that *Helicteres isora* L. can be used as a potential source of natural antioxidants.

Keywords: Antioxidant, DPPH, Free radical, Phenols, *Helicteres isora*

INTRODUCTION

The biological reactivity of free radicals and their contribution in causing oxidative stress has gained considerable attention in past decade. Due to oxidative damage many cellular activities get affected. In addition biological molecules including RNA, DNA and protein enzymes become susceptible to damage causing disorders like cancer, cardiovascular diseases, diabetes, atherosclerosis (Cerutti, 1985; Moskovitz, 2002).

The generation of free radicals in human body can be blocked by antioxidant substances, inhibiting the initiation of chain reaction by trapping the free radicals and thus preventing the biological damage (Velioglu *et al*., 1998). Dietary antioxidants mainly derived from medicinal plants have gained lot of importance for nullifying the cumulative effects of the oxidatively damaged molecules (Halliwell, 1996).

Helicteres isora Linn. commonly named as East Indian screw tree in English and also known as Avartani in Ayurveda belongs to family Sterculiaceae (Yoganarasimhan, 1996). In different parts of India *Helicteres isora* (HI) has been used by a large number of tribal people/ethnic groups for the treatment of various elements such as gastrointestinal disorder, diabetes, scabies, eczema, sore ear and snake bite (Mohan, 2008; Arjariya, 2009; Meena, 2009; Babu, 2010; Dhare, 2010; Sankaranarayanan, 2010; Basha, 2011; Sharma and Shori, 2013). In view of its significant ethno medicinal importance the objective of the study conducted was to investigate the *in vitro* antioxidant and free radical scavenging activity of hydroethanolic extract of the stem bark of HI compared to various standards such as ascorbic acid, BHT, rutin, EDTA etc.

MATERIAL AND METHOD

Chemicals

Sodium nitrite, Aluminium chloride, Nitroblue tetrazolium (NBT), Ammonium thiocyanate, ferrozine, Phenazine methosulphate (PMS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ethylene diamine tetra acetic acid (EDTA), Rutin, ascorbic acid, gallic acid, potassium ferricyanide, tricholroacetic acid (TCA), folin-ciocalteau's reagent, Na_2CO_3, TPTZ (2,4,6-tripyridyl-s-triazine), BHT (Butylated hydroxytoluene), NADH (Nicotinamide adenine dinucleotide), quercetin were obtained from HI-Media, CDH and Merck.

Preparation of extract

The brown colored powder of stem bark (70 g) was extracted with various solvents of increasing polarity beginning with petroleum ether, chloroform, ethyl-acetate, methanol and then mixture of ethanol: water (7:3) using Soxhlet apparatus. With each solvent, extraction was carried out for 24 hrs. Extracted solvents were then filtered to remove any residue. Solvents were then evaporated using distillation assembly and when around 10 to 15 ml solvent was left in receiver then it was evaporated on water bath. Later, extracts were kept in vacuum dessicator for 72 hrs and then weight was taken for each extract.

Phytochemical screening

Dried hydroethanolic extract of *Helicteres isora* (HEHI) was used for preliminary screening of various secondary metabolites (Kokate, 1994).

Quantitative estimation

Determination of total phenolic content

Total phenolic content in *Helicteres isora* stem bark was determined using Folin-Ciocalteau colorimetric method (Singleton and Rossi, 1996). To 1 ml of plant

extracts was added 5ml of Folin- Ciocalteau reagent (diluted to tenfold). After incubating for 5 min it was followed by the addition of 4ml (75mg/ml) of sodium carbonate for neutralization. Samples were kept in dark for 120 min with intermittent shaking. All the samples of varying concentrations (10-220µg/ml) were then centrifuged as precipitation was observed in them. Absorbance of supernatant was taken at 760nm to measure total phenolic content. Standard curve was plotted using methanolic solution of gallic acid. The results were calculated as gallic acid equivalents (GAE) mg/g of the dry extract using the following formula:

$C = c.\ V/m'$

Where: C- Total content of phenolic compounds, mg/g plant extract (GAE),

c-The concentration of gallic acid established from the

Calibration curve (mg/ml), V- The volume of extract (ml),

m- The weight of pure plant extract (g).

Determination of total flavonoids

Total flavonoid content in the extract was determined according to the method of Sakanaka, 2005. To 500µl of sample of varying concentrations (10 - 220µg/ml) were mixed with 2.5 ml of deionized water in a glass vial, to this was added 150µl of sodium nitrite solution of concentration 0.05g/ml, which was followed by the addition of 300µl of 10% aluminium chloride. The mixture was then allowed to stand for 5 min after which 1 ml of 1M NaOH was added giving light pink color to the mixture. Absorbance of the reaction mixture was taken immediately at 510nm. The results were expressed as rutin equivalent (mg RE/g dry extract) and calculated using the following formula:

$X = (A.\ m_0)/ (A_0.\ m)$

Where, X- The total flavonoid content, mg/mg plant extract in RE,

A- The absorption of plant extract solution,

A_0- The absorption of standard rutin solution,

m- The weight of plant extract (mg)

m_0- The weight of rutin in the solution, (mg)

Antioxidant assays

DPPH radical scavenging activity

The free radical scavenging activity was determined using 1,1-diphenyl-2-picryl-hydrazyl assay. The assay was performed as described by Blois, 1958. In this method DPPH radical was prepared by dissolving 3.94 mg of methanol. This solution (600µl) was added to sample solution (200µl) of various concentrations (10µg/ml, 30µg/ml, 50µg/ml, 70µg/ml, 90µg/ml, 110µg/ml, and 220µg/ml). The mixture was incubated in dark for 30 min; the absorbance of mixture was taken at 517nm and compared with the standard ascorbic acid and BHT. To 600µl of DPPH solution mixed with 200µl of solvent (ethanol: water) were taken as control. The level of percentage inhibition of DPPH radical by the different extracts was calculated according to the following formula.

% Radical Scavenging= $[Abs_{control} - Abs_{sample}]/Abs_{control} \times 100$

Superoxide radical scavenging activity

Superoxide radical scavenging activity of each extract was determined by the method of Robak, 1988 with a slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (150µM in 100mM phosphate buffer, pH 7.4), 1ml of NADH solution (468µM in 100mM phosphate buffer, pH 7.4) and 500µl of test solution (10-220µg/ml) were mixed. To this solution 1 ml of PMS (phenazine methosulfate) solution (60µM, PMS in 100mM phosphate buffer pH 7.4) was added. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560nm was measured. The percentage inhibition of superoxide radical by HEHI was calculated using the formula

% Radical Scavenging= $[Abs_{control} - Abs_{sample}]/Abs_{control} \times 100$

Ferrous ions (Fe^{2+}) chelating activity

The assay was done by the method of Dinis *et al*., 1994. In brief, 500µl of extract was mixed with 1.6ml of demonized water followed by 2mM $FeCl_2.4H_2O$ (5µl). The reaction was initiated by the addition of 5mM ferrozine (100µl). The reaction mixture was vortexed and left at room temperature for 10 minutes. Fe^{2+} chelating ability of HEHI was measured by the absorbance of the ferrous iron- ferrozine complex at 562nm. EDTA was used as positive control and sample without extract or EDTA was used as negative control. The percentage of inhibition of ferrozine-Fe^{2+} complex was calculated using the formula.

Chelating activity % = $[Abs_{control} - Abs_{sample}]/Abs_{control} \times 100$

Ferric ion reducing antioxidant power (FRAP) assay

The ferric ions reducing power of the extract was determined by modified method of Benzie and Strain, 1996. In brief, 150µl of different concentrations (10-220µg/ml) of HEHI were reacted with 2.85ml of the FRAP solution which were then incubated for 30 min at room temperature. FRAP solution was prepared by mixing 25ml of 300mM acetate buffer (3.1g $C_2H_3NaO_2.3H_2O$ and 16ml $C_2H_2O_2$), with pH 3.6, 2.5ml of 10mM TPTZ (in 40mM HCL) and 2.5ml of $FeCl_3.6H_2O$. The solution was kept at 37°C for 30 min before use. Readings of an intense blue color product (ferrous tripyridyl-triazine complex) was taken at 593 nm.

Statistical analysis

The experimental results were performed in triplicates. The data were recorded as mean ±

standard deviation and analysed by ANOVA. Significant differences between means were determined by Bonferroni's multiple comparison test using SPSS (Version 16.6).

RESULTS AND DISCUSSION

Phytochemical Screening

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites. Thus the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development (Mallikharjuna *et al*., 2007). Preliminary phytochemical screening of hydroethanolic extract of *Helicteres isora* stem bark revealed the presence of phenols, tannins, steroids, saponnins, flavonoids, carbohydrates (Table 1), but it showed negative test to alkaloids. The phenolic compounds are known to possess various anti-bacterial actions (Fernández, 1996) this may support the ethno-medicinal use of HI for treating stomach disorders (Arjariya, 2009; Dhare, 2010). Flavonoids, tannins and plant phenolics are known to possess antioxidant or free radical scavenging capability (Polterait, 1997; He and Liu, 2008). The importance of saponins and tannins in various antibiotics used in treating common pathogenic strains has been reported by Kubmarawa *et al*. 2007. This suggests that *Helicteres isora* may also reveal significant pharmacological activities due to the presence of major secondary metabolities.

Quantitative estimation

Phenols are the widely spread class of compounds amongst the plethora of medicinal plants. They are well considered to possess significant antioxidant potential by scavenging free radicals, metal chelation and by reducing ferric ion reducing activity (Rice-Evans, 1995; Zeng, 2012). Total phenolic content (Figure 1) of hydroethanolic extract of *Helicteres isora* stem bark was determined from regression equation of calibration curve ($y= 0.313x + 0.123$, $R^2= 0.842$) and expressed in gallic acid equivalents (GAE). TPC was found to be 1263.6 mg/g of plant extract in stem bark of *Helicteres isora*. The efficiency of flavonoids (Figure 2) to act as antioxidants *in vitro* has been the subject of several studies in the past year. Interest has been shown to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000). Total flavonoid content of HEHI was found to be 0.339 mg/g expressed as rutin equivalent. Results obtained in the present study revealed that the level of phenol and flavonoid in the extract were significantly ($p<0.001$) lower as compared to the reference compound used in the study.

Determination of antioxidant assays

The unpaired electrons readily form free radical molecules which are chemically reactive and highly unstable. Since the electrons have a strong tendency to exist in a paired state, free radicals take electrons from other atoms which in turn converts those other atoms into free radicals. Thus setting up a chain of free radicals, these free radicals contribute to various disorders in humans (Ozawa, 1999; Shori *et al*., 2011). Cell walls and other biological molecules are also susceptible to oxidative damage including RNA, DNA and protein enzymes. Antioxidants are used to terminate the free radical chain reaction and thus preventing biological damage. Based on previous reports the secondary metabolites present in medicinal plants are known to possess significant antioxidant activity (Pracheta *et al*., 2011a; 2011b; Singh *et al*., 2012). Various chemical assays are used for measuring the free radical scavenging activity of plant extracts. In the present work DPPH and superoxide anion scavenging methods were used.

DPPH is a stable free radical which gives rise to deep violet color, characterized by absorption at about 517nm. When the methanolic solution of DPPH is mixed with test solution or standards like ascorbic acid, BHT which can donate hydrogen atom to free radical of DPPH then this gives rise to reduced form by changing the violet color to yellow or colorless. With an increase in concentration of the plant extract the ability of the antioxidants to quench the free radicals increases thus decrease in absorbance was observed. Figure 3 shows that with increasing concentration (10-220 µg/ml) of plant extract the free radical scavenging ability also increases. The DPPH radical scavenging activity of HEHI was compared to standards ascorbic acid and BHT and the order of % inhibition recorded at highest concentration was in the order ascorbic acid (98.3%)> BHT (96.7%) > HEHI (94%). Fig 3 illustrates that HEHI, at the concentration 220 µg/ml put forward the strong scavenging activity although it was less than the standards taken. IC_{50} value of the HEHI, ascorbic acid and BHT were (97.53 ± 0.281, 1.72 ± 0.078 and 2.40 ± 1.011 respectively.

Superoxide anions are also harmful reactive oxygen species, acting as precursor for generating other ROS which initiate lipid peroxidation (Halliwell and Chirico, 1993). In the chemical assay, superoxide anion were generated in a PMS-NADH system and assayed by the reduction of nitro blue tetrazolium (NBT) (Kanatt *et al*., 2007). In the protocol used, superoxide anion reduces the yellow color to produce the blue formazan which is measured spectrophotometrically at 560 nm. With the increase in concentration (10-220 µg/ml) of plant extract or rutin the formation of blue NBT is inhibited and thus decrease in absorbance is recorded. The HEHI exhibits significant superoxide anion radical scavenging activity at all the concentrations compared to the rutin. IC_{50} value of HEHI and rutin

were 5.40 ± 0.032 and 7.77 ± 0.018 respectively. According to the results, (Figure 4) HEHI showed 76.6 % inhibition of superoxide radical scavenging activity which was higher than rutin (73.3%). The study reveals that HEHI significantly reduced the free radicals generated in DPPH and superoxide anion radical scavenging assays, thus it may be a potential source of natural antioxidants.

Ferrous ions (Fe^{2+}) chelation may impart prominent anti-oxidative effects by inhibiting metal-catalyzed oxidation (Gulcin *et al*., 2012). In this assay ferrozine formed magenta colored complex with Fe^{2+}. In the presence of antioxidants, which act as chelating agents the complex formation is distorted, resulting in decrease in the color of the complex. Thus metal chelating activity of plant extract can be estimated by measuring the absorbance of the complex formed. With the increase in concentration of the plant extract and the control (10-220 µg/ml) lower absorbance value was observed indicating higher metal chelating activity. Figure 5 illustrates that metal chelating effect of HEHI increases with increase in concentration showing 64.3% of chelation of Fe^{2+} ions whereas, the standard EDTA showed only 52.5% of chelating capacity. The IC_{50} value of HEHI was noted as 165.78 ± 0.450 which was lesser than IC_{50} value of EDTA (217.63 ± 1.091).

In Fe^{3+} reducing method, antioxidant substances in the plant extract and control causes the reduction of the Fe^{3+} to Fe^{2+}. The absorbance of blue colored Fe^{2+}- tripyridyl-triazine (TPTZ) compound was noted at 593 nm. The reducing capacity of the compound increased with the increasing concentration of the plant extract. Figure 6 illustrates that HEHI had moderate reducing power when compared to BHT. Thus it can be reported that HEHI may act as a free radical scavenger as it demonstrates the electron donating property by terminating the chain of free radicals that may cause oxidative damage.

CONCLUSION

The results of the study indicate that HEHI exhibit potent free radical scavenging, metal chelating and reducing power activity. The antioxidant effect of hydroethanolic extract of *Helicteres isora* stem bark may be due to the presence of various secondary metabolites found in it by phytochemical screening. Based on the discussion above, *Helicteres isora* presents a promising source of antioxidants, which also explains its ethno-medicinal use among tribal people. *Helicteres isora* Linn. needs further exploration so that we may completely utilize its beneficial health effects for treating oxidative damage related diseases.

Table 1. Phytochemical screening of hydro-ethanolic extract of *Helicteres isora*.

Class of compounds	HEHI
Alkaloids	-
Tannins	+
Steroids	+
Flavonids	+
Phenols	+
Saponins	+
Carbohydrates	+

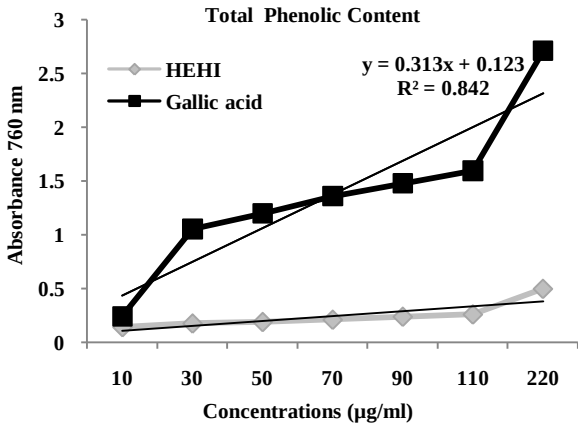

Figure 1: Total Phenolic content in HEHI

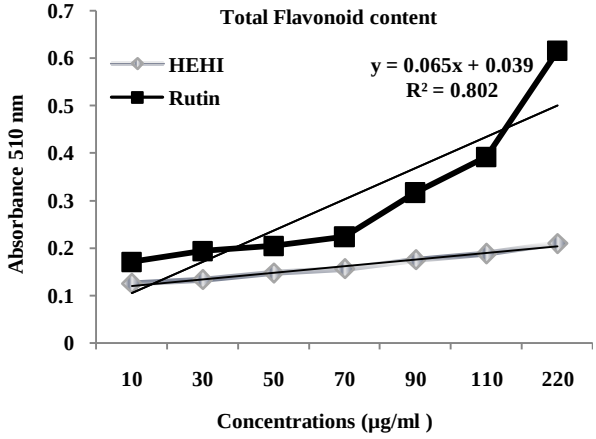

Figure 2: Total Flavonoid content in HEHI

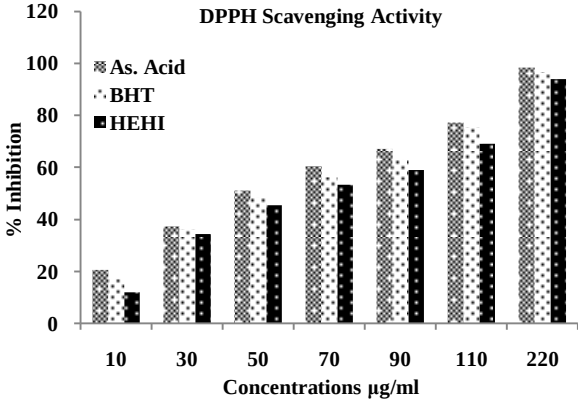

Figure 3: DPPH free radical scavenging activity of HEHI, ascorbic acid and BHT at different concentrations (10-220 µg/ml).

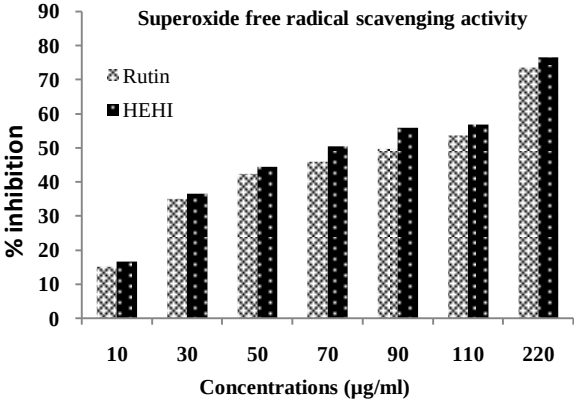

Figure 4: Superoxide anion radical scavenging activity of HEHI and rutin at different concentrations (10-220 µg/ml).

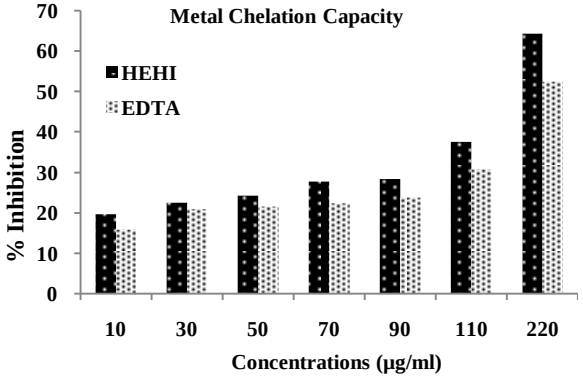

Figure 5: Metal chelation activity of HEHI and EDTA at different concentrations (10-220µg/ml).

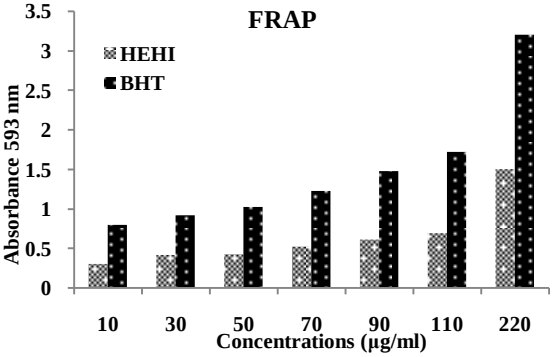

Figure 6: Reductive potential of HEHI and BHT at different concentrations (10-220µg/ml).

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