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IN VITRO ANTIOXIDATIVE ROLE OF *HELICTERES ISORA* (L)

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Abstract: Free radicals are implicated for more than 80 diseases including diabetes mellitus, arthritis, cancer, ageing, etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance and research is now directed towards finding naturally occurring antioxidant of plant origin. In a step in this direction we have evaluated antioxidant potency of a plant extract *Helicteres isora* of four different solvent extracts. But only hexane extract was investigated for its free radical scavenging action towards 1, 1-Diphenyl–2picryl hydrazyl, nitric oxide and superoxides and Reducing assay, FRAP, Total phenolic content etc and found that the hexane extract is having promising free radical scavenging activity in dose dependent manner. This antioxidant potency may be related to the presence of antioxidant vitamins and other compounds present in the extract. These results clearly indicate that it is effective against free radical mediated diseases.

Keywords: *Helicteres isora,* Oxidative Stress, Free Radical Scavenging activity

INTRODUCTION

High levels of free radicals or active oxygen species create oxidative stress, which leads to a variety of biochemical and physiological lesions and often results in metabolic impairment and cell death (3). Epidemiological evidence indicates that the consumption of food stuffs containing antioxidant phychemicals (notably flavonoids and other polyphenols) is advantageous for our health (8; 10; 28), since they can protect the human body from free radicals and retard the progress of many chronic diseases. Moreover, many biological functions, such as antimutagenicity, anticarcinogenicity and antiaging, among others, originate from this property (9; 30; 38; 39). A number of synthetic antioxidant such as 2-3-ter-butyl-4-methoxyphenol (i.e., BHA), 2, 6-di-ter-butyl-4-methylphenol (i.e., BHT) and ter-butylhydroquinone (TBHQ) have been added to food stuffs but, because of their toxicity, their use has been questioned (36). For this reason, the development and isolation of natural antioxidants from plant species are in progress.

The many number of medicinal plants are used in the cellular and metabolic disease treatment such as diabetes, obesity and cancer etc. There are some speculations that the generation of free radicals inside the body in some physiological conditions is resulted in the cellular changes and development of cancer etc. and this could be neutralized by the antioxidants from different medicinal plants. Several studies have shown that plant derived antioxidant neutraceuticals scavenge free radicals and modulate oxidative stress-related degenerative effects (2; 18). Free radicals have been implicated in many diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and aging (16; 40).

Previous research reports suggest that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly cardiovascular diseases and cancer (35).

Antioxidants are important in the prevention of human diseases. Naturally occurring antioxidants in leafy vegetables and seeds such as ascorbic acid, vitamin E and phenolic compounds possess the ability to reduce the oxidative damage associated with many diseases, including cancer, cardiovascular disease, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (28). Antioxidant compound may function as free radical scavengers, complexes of pro-oxidants metals, reducing agents and quenchers of singlet oxygen formation (22).

They are often used in oils and fatty foods to retard their autoxidation. Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have restricted use in foods as they are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants greatly increased in the recent years. The researchers have focused on natural antioxidants and numerous crude extracts and pure natural compounds have been reported to possess antioxidant properties (17).

Helicteres isora (L). Is a large arborescent shrub of the family Sterculiaceae (12). It is used as an antigastrospasmodic, anthelmintic, antispasmodic, antipyretic, antidiarrheal, antidysenteric (1) and as a tonic after childbirth (17). Stems of this plant are used as anthelmintic, colic and aphtha, while fruits are used as colic, anticonvulsant, and abdominalgia (13).

Traditionally, the root juice is claimed to be useful in diabetes, emphysema, and snakebite (20), (32). From the roots, betulic acid, daucosterol, sitosterol and isorin (29) were isolated. Cucurbitacin B and isocucurbitacin B were isolated and reported to possess cytotoxic activity (4). In addition, Hattori and Co-workers reported an inhibitory activity of the water extract of fruits of *H. isora* against reverse transcriptase from avian myeloblastosis virus (21) and anti-HIV-1 activity (25). Six neolignans, the helicterins A-F were isolated from aqueous extract of the fruits (34), plant also contains flavonoid glucosides (19). This species is also known as Indian screw tree occurs often gregariously, through India, South East Asia, the southern part of China and in dry deciduous forests, up to 1500 m on the hill slopes (Wealth of India). The present study was undertaken to verify the claim and evaluate the antioxidant property of the fruits of *H. isora.*

MATERIALS AND METHODS

Chemicals and reagents:

All solvents used were of analytical grade and obtained from Merck and the chemicals for *In-vitro* antioxidant were purchased from Sigma–Aldrich India Ltd.

Plant material Collection:

Wild populations of *Helicteres isora* were collected during September to March in Tamil Nadu. Voucher specimens were deposited in the Herbarium of Department of Botany, Annamalai University. Thorny Fruits parts of *Helicteres isora* were taken for the present study.

Preparation of *H. isora* fruit extracts:

About 6 (Kg) of shade dried fruits of *Helicteres isora* were grinded into fine powder using fine grinder. The finely powdered seeds were then weighed and extracted with different solvents like Acetone, Hexane, Isopropanol and distilled water (aqueous) using a soxhlet device. Each extract is concentrated using a rotary evaporator and final yield is assessed for the entire *In vitro* studies.

DPPH Scavenging Assay:

DPPH scavenging potential of each extract was measured, based on the scavenging ability of stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The ability of extracts to scavenge DPPH radicals was determined by the method of (6). 1 ml of 1 mM methanolic solution of DPPH was mixed with 1 ml of extract solution (containing 100, 300, 500, µg ml^{-1} of the extract). The mixture was then vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging activity relative to the control, using the following equation:

% Radical scavenging activity = [Control – Sample / Control] x 100

Ferrous ion chelating activity:

Based on the methodology of (11), the ferrous ion chelating effects of Helicteres isora seed extraction were done. Each extracts were added to a solution of 2mM$FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After a while absorbance of the solution was quantified spectrophotometrically at 562 nm. A control run was performed which contains $FeCl_2$ and ferrozine alone. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was given below formula:

% Inhibition = $[(A_0 – A_1) / A_0]$ x 100

Where, Ao was the absorbance of the control and A1 was the absorbance in the presence of the *Helicteres isora* seed extract sample and standard.

Superoxide anion radical scavenging activity:

Superoxide radical scavenging effect was assessed by following the methodology of (23) with slight modification. It is based on the capacity of the *Helicteres isora* to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT) in the riboflavin system. 1ml of each extract was mixed with the reaction mixture containing 50mM sodium phosphate buffer, pH 7.8, 13mM methionine, 2uM riboflavin, 100uM EDTA, and NBT (75uM). The reaction mixture was monitored for the absorbance at 560nm after 10 min illumination from a fluorescence lamp for the formation of blue coloured formazan. Reaction mixture alone was served as a control blank. The percentage inhibition was determined by comparing the results of control and test samples.

% Inhibition = ((A0-A1) / A0 × 100)

Where, Ao was the absorbance of the control (blank, without extract) and A1 was the absorbance in the presence of the extract.

Reductive assay:

Based on the methodology of (26) the reductive ability of different concentrations of *Helicteres isora* was quantified. Increasing gradient concentration of *H. isora* fruit extract (100, 300, 500ug/ml) in 1ml of distilled water was mixed with phosphate buffer [2.5ml, 0.2M (pH 6.6)] and 2.5ml of potassium ferricyanide [K_3Fe (CN) 6] (1%). The reaction mixture was incubated at 50 °C for 20 min. A proportion of (2.5ml) of tricholro acetic acid (10%) was added to the reaction mixture and then it is subjected to centrifugation for 10 min at 1000g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer.

Nitric oxide scavenging activity;

Nitric oxide when reacts with sodium nitroprusside leads to produce nitric ions by the interaction of oxygen molecules. This reaction was quantified using Griess reagent. Nitric oxide scavenging assay was performed on the basis of (33) with slight modifications. Different concentrations of the sample extract (100, 300,500µg/ml) was added to the reaction mixture of 3ml containing 10mM sodium nitroprusside in phosphate buffered saline and incubated at 25°C for 150 min. 0.5ml of aliquot of the incubated sample was removed at 30 min intervals and 0.5ml Griess reagent was added. The colour absorbance was measured at 546nm. Curcumin was used a reference drug.

Ferric Reducing Ability of Plasma (FRAP) assay:

The FRAP assay was done according to (5) with some modifications. The stock solutions included 300mM acetate buffer (3.1 g $C_2H_3NaO_2$ _ $3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mM HCl, and 20mM FeCl3.6H2O solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5mL TPTZ solution, and 2.5mL $FeCl_3$ - $6H_2O$ solution and then warmed at 37 °C before using. Fruit extracts (150µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was linear between 25 and 800 M Trolox. Results are expressed in µM TE/g fresh mass. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

RESULTS AND DISCUSSION

DPPH radical scavenging effect:

It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate or propagate further oxidation. 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances. In this model it was observed that the test extract (in dose of 100µg/ml, 300µg/ml, 500µg/ml) significantly scavenged 0.57%, 0.60%, and 0.60% DPPH free radical respectively in a dose dependent manner which can be comparable to the standards (Fig. 1).

Extracts were subjected for the evaluation of antioxidant activity by using various in vitro model systems. DPPH radical scavenging activity was observed in all the extracts, the curcuma extract.

The DPPH radical scavenging assay is regularly used for the relatively rapid evaluation of the antioxidant activity. DPPH is a stable free radical, even at room temperature, and shows strong absorbance at 517 nm. The DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule with a different colour. Thus, the degree of its discolouration from purple to yellow is attributed to the hydrogen donating ability of the added compound, which is indicative of its radical scavenging potential (15). In the present study, the DPPH radical scavenging ability of *H. isora* was calculated in a dose-dependent manner (Fig. 1). *Helicteres isora* proved to be an effective scavenger of DPPH radicals. At concentrations ranging from 100µg/ml to 500µg/ml the DPPH radical scavenging ability of *H. isora* was measured at 517nm with respect to that of Gallic acid (the reference compound used in this test). The maximum scavenging ability was recorded in at 500µg/ml than the rest of concentration.

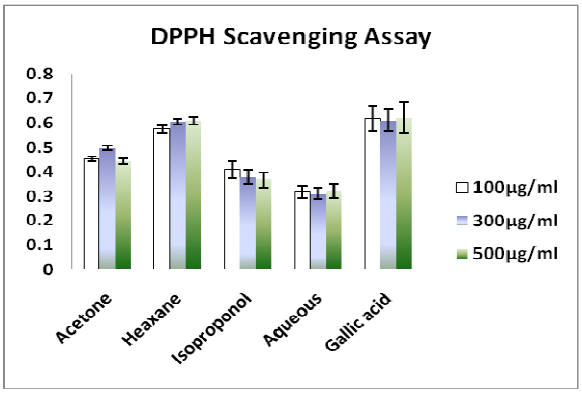

Fig. 1. Showing DPPH scavenging assay

Ferrous Ion chelating activity:

The hydroxyl radicals are the most reactive species of ROS and are formed by the Fenton reaction of H_2O_2 with ferrous iron or the Haber–Weiss reaction of the superoxide anion with H_2O_2. Ferrous ions play an important role in the catalysis reaction of hydroxyl radical formation (11). Several studies have demonstrated that the scavenging of hydroxyl radicals by antioxidants were effective mainly via metal ion chelation. Therefore, the estimation of metal ion chelating ability is important for appraising the free radical scavenging activity of natural antioxidants (16). In the present study, the ferrous ion chelating ability was determined by the reduction of absorbance at 562 nm; this red colour is quantitatively formed by the reaction of ferrozine with ferrous ions. Fig. 2 shows the ferrous chelating abilities of *H. isora* and is compared with that of citric acid as reference compounds. At concentrations ranging from 100µg/ml to 500µg/ml, the ferrous chelating ability of *Helicteres isora* was measured at 562nm. The *H. isora* exhibited significant metal chelating activity when compared to citric acid which was found to have a more and strong chelating ability for ferrous ions at the same concentration range. These results suggest that *Helicteres isora* has a beneficial effect on ferrous chelating ability and may thus exert protection against oxidative damage.

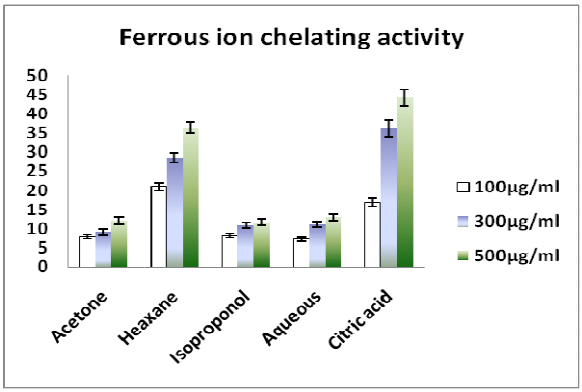

Fig. 2. Showing Ferrous Ion chelating activity

Superoxide anion radical scavenging activity;

Superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 3 shows the percentage inhibition of superoxide radical generation by 100µg/ml, 300µg/ml and 500µg/ml of hexane extracts of *H. isora* extract and comparison with same concentration of Quercetin. Hexane extracts of *H. isora* have strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity compared with that of Quercetin. The results were found statistically very significant ($p < 0.01$). However, Isoproponol, Acetone as well as Aqueous extracts of *Helicteres isora* have relatively less inhibition of superoxide radical generation, statistically.

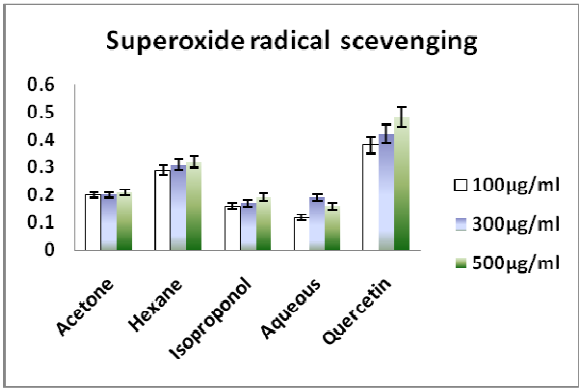

Fig.3: Showing Superoxide anion radical scavenging activity

Reductive assay:

Fig. 4 shows the reductive capabilities of *H. isora* extracts compared to BHA. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (41). Like the antioxidant activity, the reductive potential of *H. isora* increased with increasing concentration. All of concentration of *H. isora* fruit extracts showed higher activities than control and these differences were statistically very significant ($p < 001$). Hexane extract exhibited stronger reductive potential which was found to be significant statistically ($p < 0.01$) and it is relatively very close to that of standard drug Butylated hydroxyanisole (BHA) which reduces almost 90%.

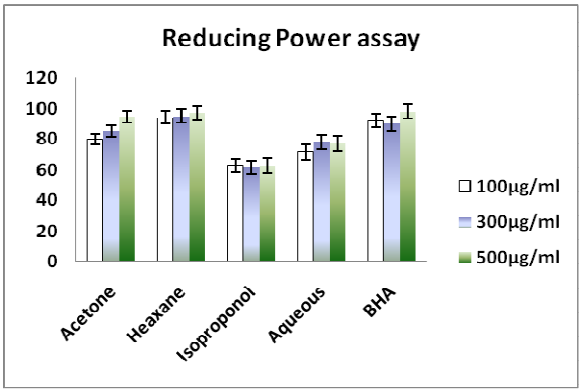

Fig. 4: Showing Reductive assay

NO scavenging assay:

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (42). The scavenging of NO by the extracts was increased in dose dependent manner. Fig.5 illustrates a significant decrease in the NO radical due to the scavenging ability of extracts and ascorbic acid. The hexane extracts showed maximum activity of 58.7% at 500µg /ml; whereas ascorbic acid at the same concentration exhibited 60.9% inhibition.

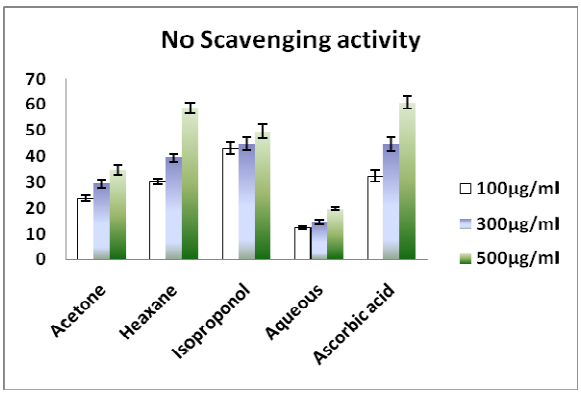

Fig.5: Showing NO scavenging assay

Ferric reducing antioxidant potential assay (FRAP):

The FRAP assay was carried out according to the method of Benzie and Strain (1996) with few modifications. The FRAP reagent was prepared from sodium acetate buffer (300mmol/L, pH 3.6), 10 mmol/L

2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ) solution in proportions of 10:1:1 v/v), respectively. The FRAP reagent was freshly prepared. Antioxidants potential was determined by adding 651µl sodium acetate buffer solution, 279 µl of FRAP reagent and 100µl/ml to 500µl/ml of each plant extract was explored. The absorbance reading was taken at 593 nm exactly 4 min after initial mixing. The plant extracts were first adequately diluted to fit within the linear dynamic range. Solvent blanks were also prepared and the absorbance reading taken. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as mmol Trolox equivalent (TEAC) per100g of plant material.

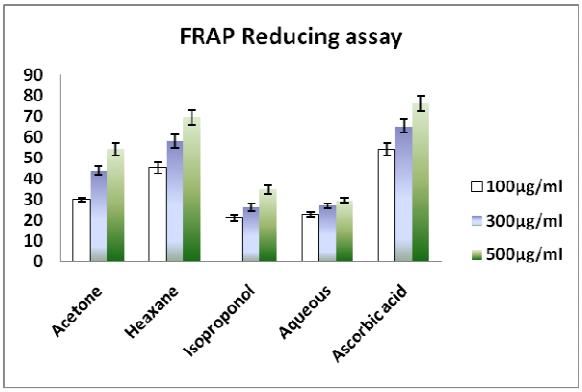

Fig.6: Showing ferric reducing antioxidant potential assay (FRAP)

Total phenolic content:

Phenolics constitute one of the major groups of compounds which act as primary antioxidants (24). They inhibit autoxidation of unsaturated lipids, thus preventing the formation of oxidized low-density lipoprotein (LDL), which is considered to induce cardiovascular diseases. The assay used for total phenol determination detects phenolic acids, flavonoids, tannins, anthocyanins, lignans and coumarins. The content of phenolic compounds is expressed as milligrams gallic acid equiva lence (mg GAE) was calculated by the following formula:

$$C = c.\ V/m'$$

Where: C- Total content of phenolic compounds, mg/g plant ext racts (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 hexane extracts (g).

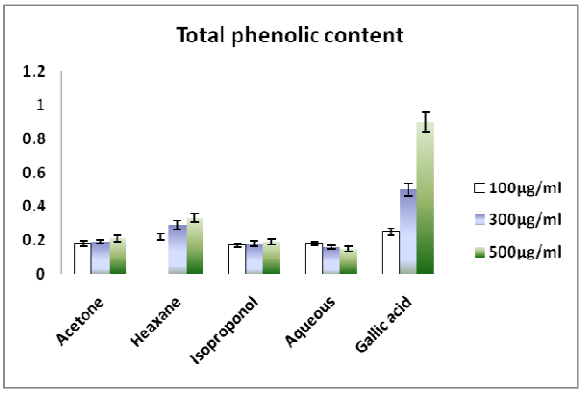

Fig.6: Showing total phenolic content

The amount of total phenolics of the plant extracts are shown in Fig. 6. The total phenolic content was very high in Hexane extract among other extracts like acetone, Isopropanol and water.

Statistical analysis:

Values are expressed as mean ± S.D. The data were statistically analyzed using ANOVA. The values were considered statistically significant if the p-value was less than >0.05.

CONCLUSION

In order to characterize antioxidant activity of a plant extract, it is desirable to subject it or the tests that evaluate the range of activities such as scavenging of the reactive oxygen species, inhibition of membrane LPO and metal ion chelation. Antioxidant-rich plant extracts serves as sources of nutraceuticals that alleviate the oxidative stress and therefore prevent or slow down the degenerative diseases. An effort has been made to explore the antioxidant properties of *Helicteres isora* a medicinal herb. This indicates the potential of the extracts as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

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