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IN VITRO FREE RADICAL SCAVENGING ACTIVITY OF RAW LEAVES OF HENNA (LAWSONIA INERMIS)

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ABSTRACT

The aim of this research was to investigate the antioxidant activity of henna leaves. The content of total phenols, flavonoid, the level of antioxidant potential by DPPH, OH radical, reducing power, chelation, FRAP and total antioxidant in water extract of Lawsonia inermis were determined. In all the methods the extract showed good scavenging activity. The EC50 values of raw water extract on DPPH radical, reducing power, ferrous ion chelation, FRAP and hydroxyl radical were found to be 69.25,0.159,0.0183,83.49,0.1887,0.01075,86.86,0.0245 and 0.8436 mg/ml, respectively. Substantial amount of phenol and flavonoids was noticed. The free radical scavenging and antioxidant characteristics of the extract may be due to the presence of polyphenols in the leaf extract. This study showed the potential of using fresh leaves to develop functional foods with high antioxidant activity

KEYWORDS: Henn leaves, Total phenols, Flavonoids, Antioxidant, Scavenging activity.

INTRODUCTION

The importance of free radicals and reactive oxygen species (ROC) has attracted increasing attention. Reactive oxygen species (ROS) were produced by normal cell metabolism, both in animals and plants. Oxidative stress occurred due to excess of ROS, resulting in oxidative DNA damage which is implicated in the pathogenesis of many diseases, e.g., atherosclerosis, cataractogenesis, reperfusion injury, cardiovascular, rheumatoid arthritis, inflammatory disorders and cancer.[1,2]

The use of synthetic antioxidants such butylated hydroxyanisole (BHA), butylated

hydroxytoluene (BHT) and propyl gallate (PG) as must be under strict regulation due to potential health hazards.[3] The search for natural antioxidants as alternatives is therefore of great interest among researchers.

The consumption of a diet rich in fresh fruits and vegetables has been associated with a number of health benefits including the prevention of chronic diseases. This beneficial effect is believed to be due, at least partially, to the action of antioxidant compounds, which reduce oxidative damage in the body.[4]

The henna leaves (Lowsonia inermis) which is an fresh leaves, is also known as smooth assonia, AL-henna, Henne, Al-Khanna. Although it is native to northen Africa, asia, and it is also grown in Australia. It contais a alkaloids, Quinones, tannins, flavonoids ideal for a number of metabolic and antioxidant reaction.[5]

In the present study, the water extract of raw henna leaves was determined for its antioxidant activities by in vitro methods, including DPPH, ABTS, OH radical scavenging assay, reducing power, chelating activity, FRAP and total molybdenum assay and for its total phenolic and flavonoid contents.

MATERIAL AND METHODS

Collection and Identification of plant materials

The leaves of Lawsonia inermis L. was collected in the month of feb 2016, from Kalasalingam university campus, Krishnankoil, Tamil nadu, India. The plant material was taxonomically identified by a botanist. The fresh leaves collected were washed with distilled water to remove dust and was shade dried, pulverized by a mechanical grinder and stored in airtight containers for further use.

Sample extraction

The leaves were cleaned and cut into small pieces before being dried in a hot air-blowing oven at 50°C. All samples, after drying, had water contents below 10%. They were ground to a fine powder in a mechanical blender and kept at room temperature prior to extraction. 10 g of the sample were extracted by using a Soxhlet extractor for 3h with 100 ml of water under reflux conditions. The extract was then rotary evaporated at 40°C to dryness. The extract was stored at 4°C for further use. Analyses were carried out in triplicate.

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH) and rutin were obtained from Sigma Co. (St. Louis, MO, USA). ferrozine, gallic acid, 2, 4, 6-tripyridyl-s-triazine (TPTZ) and ascorbic acid were obtained from Himedia, Mumbai. Potassium ferricyanide, ferric chloride, trichloroacetic acid, aluminium chloride, potassium persulphate, ammonium persulphate, ferrous suphate, sodium salicylate, ammonium molybdate, sodium carbonate, aluminium chloride, sodium nitrate, sodium hydroxide, Folin-Ciocalteu's phenol reagent, ferrous chloride, sodium hydroxide and solvents from Ranbaxy chemicals, Mumbai.

Phytochemicals

Estimation of total flavonoid content

Total flavonoid content was determined as described by Jia Z. 0.25 ml of various extracts was diluted with 1.25 ml of distilled water. 75 µl of a 5% $NaNO_2$ solution were added and after 6 min 150 µl of a 10% $AlCl_3.H_2O$ were added and mixed. After 5 min, 0.5 ml of 1 M NaOH was added. The absorbance was measured immediately against the prepared blank at 510 nm. Rutin was used as a standard and the results were expressed as mg of rutin equivalents (RE) per g of dry extract.

Determination of total phenolic content

Total phenol content was determined by the method adapted from Singleton VL, with some modifications using the Folin-Ciocalteu reagent. 1 ml of the extract was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 3min, 1ml of saturated Na_2CO_3 (35%) was added to the mixture and it was made up to 10 ml by adding deionised distilled water. The mixture was kept for 90 min at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per gram of dry extract.

Antioxidant capacity

Phosphomolybdenum assay

The antioxidant capacity of water extract of Henna was evaluated by the method of Prieto and colleagues.[8] A 0.1 ml aliquot of sample solution (equivalent to 100 µg) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The blank was 0.1 ml of methanol used in place of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 90 minutes. After the samples

were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm. The antioxidant capacity was expressed as an equivalent of ascorbic acid (mg of ascorbic acid/g of dried extract).

DPPH radical scavenging activity

DPPH-radical scavenging activity was determined according to the technique outlined by Shikmmada.[9] A 0.002% of DPPH in methanol was prepared and 1 ml of this solution was added to 1 ml of different concentrations of extracts (10µg/ml to 100µg/ml and standard (Ascorbic acid), allowed to stand for 30 min at room temperature. All tests were performed in triplicate. The change in color from purple to yellow was measured at 517nm in a spectrophotometer (SYSTRONICS 2201). Methanol with extract served as the blank and DPPH in methanol without the extracts served as the positive control. The percentage of radical scavenging activity was calculated using the following formula:

% Antioxidant scavenging activity = $[(A_0 – A_1) / A_0] \times 100$

Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

Determination of reducing power

The reducing power of the extracts was assessed by the method proposed by Oyaizu.[10] A 0.75 ml of various concentrations of the extracts (10 µg/ml - 100µg/ml) was mixed with 0.75 ml of phosphate buffer (0.2 M pH 6.6) and 0.75 ml of potassium ferricyanide (1% v/v). Incubated at 50°C for 20min. The reaction was stopped by adding 0.75 ml of 10% trichloroacetic acid, centrifuged at 800 rpm for 10 minutes. 1.5 ml of supernatant was mixed with 1.5 ml distilled water and 0.1 ml ferric chloride (0.1%). Incubated at room temperature for 10 minutes and the absorbance at 700 nm were measured with Double beam UV-visible Spectrophotometer (SYSTRONICS 2201). All tests were performed in triplicate. Higher absorbance of reaction mixture indicates the greater reducing power as compared to ascorbic acid (Standard). The percentage of radical scavenging activity was calculated using the following formula:

% Antioxidant scavenging activity = $[(A_0 – A_1) / A_0] \times 100$ Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

Chelating effects on ferrous ions

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis and colleagues.[11] To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 seconds, 0.1 ml ferrozine (5 mM) was added.

Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 minutes at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as:

$$\text{Chelating rate (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Ferric reducing antioxidant power (FRAP) assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu.[13] The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 minutes. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 minutes. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 minutes. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Hydroxyl radical scavenging assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration.[14] Aliquot of 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H_2SO_4 and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM Sodium thiosulphate (NaS_2O_3) until yellow colour disappeared. Percentage of scavenging of hydrogen peroxide was calculated as:

$$\text{\% Inhibition} = [(V_0 - V_1)/V_0 \times 100],$$

Where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of the extracts.

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± SD. The data were subjected to one way analysis of variance (ANOVA) and the differences between various concentrations were determined by DMRT test using SPSS software. The P values of

< 0.05 were considered significant.

RESULTS AND DISCUSSION

The extraction yield, total phenolic content, total flavonoid content and total antioxidant activity of Henna leaf extract is presented in Table 1. Percent yield of water extract of henna leaf was found to be 12.36%.

It was known that plant phenolic compounds are responsible for effective free radical scavenging and antioxidant activities.[15] The total phenol and flavonoid content of Henna extract were found to be 23.34 mg GAE/g dry weight, 65.22 mg RE/g dry weight respectively.

The phosphomolybdenum method is based on the reduction of molybdenum by the antioxidants and the formation of a green molybdenum (V) complex, which has absorption at 695 nm. The total antioxidant capacity observed in the ripe ethyl acetate extract of henna was 0.08436 nM GAE/g respectively (Table 1) The antioxidant properties of Henna leaf were evaluated by different in vitro antioxidant assays such as reducing power, DPPH / OH / radical scavenging activity, FRAP and chelation activity.

DPPH radical scavenging activity

Being a stable free radical, DPPH is frequently used to determine radical scavenging activity of natural compounds. In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form, DPPH–H.[16] Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution. Figure 1 shows free radical scavenging activity of the hena extract at different concentrations. The radical scavenging activity of henna extract increased with increasing concentrations, with 69.25%, 75.01%, 98.98%, and scavenging activity for 83.49, 86.86, 98.98, mg/ml extract, respectively (Table 1). The IC50 values was found to be 0.113 mg/ml. These results indicated that henna water extract exhibited the ability to quench the DPPH radical, which indicated that extract was good antioxidant with radical scavenging activity.

Reducing power

The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow

color of the test solution changes to green and blue depending on the reducing power of test specimen. Greater absorbance at 700 nm indicated greater reducing power. The table 1 presents the reductive capabilities of water extract of Henna leaves. In the concentration range investigated, water extracts of henna demonstrated reducing power that increased linearly with concentration. At 0.159, 0.1709, 0.1902, mg/ml, reducing power of henna extract were found to be 0.1887, 0.26625, 0.4240, respectively. The IC50 values was found to be 0.113 mg/ml. The reducing power of water extract of henna might be due to their hydrogen-donating ability. Possibly, henna leaf contain high amounts of reduction one, which could react with radicals to stabilize and terminate radical chain reactions.

Metal chelating activity

Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms, since it reduces the concentration of the catalysing transition metal in LPO.[17] The chelating effects of henna extract on ferrous ions increased with increasing concentrations (Table 1). At concentrations of 10 and 50 mg/ml, the henna extract exhibited chelating effects of % and %, respectively (Table 1). The IC50 values was found to be mg/ml. The results of the present study suggest that an ethyl acetate extract of henna leaf exhibits good chelating activity on ferrous ions.

Ferric reducing antioxidant power (FRAP)

FRAP assay is based on the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}TPTZ complex with an absorption maximum at 593 nm. The absorbance decrease is proportional to the antioxidant content.[13] The trend for ferric ions reducing activities of henna extract at different concentrations are shown in Table 1. The IC50 values was found to be 1.51 mg/ml. Our results showed significant ferric reducing power which indicated the hydrogen donating ability of the extract.

OH radical scavenging activity

The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage in adjacent bio molecules.[19] The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins.[20] The •OH scavenging` activity of mushroom extracts was assessed by its ability to compete with salicylic acid for •OH radicals in the •OH

generating/detecting system. In the present study, the hydroxyl radical-scavenging effect of the water extract of henna, in a concentration of 0.2 mg/ml, was found to be 46.46% and in a concentration of 1.0 mg/ml, was found to be 89.63%. The IC50 value was found to be 0.23 mg/ml. Hence, the henna water extract can be considered as a good scavenger of hydroxyl radicals.

Table 1: Free radical scavenging activity and antioxidant activity of water extract of henna.

Name of drug	Concentration (µg/ml)	DDPH radical scavenging activity % inhibition	Reducing assay Absorbance± SEM	Phosphodenum reduction assay Absorbance± SEM	FRAP assay	OH radical scavenging activity % of inhibition
Standard drug (Gallic acid)	1	69.25±0.26	0.159±0.002	0.0183±0.0001		
	1.5	75.01±1.06	0.1709±0.002	0.0282±0.02		
	5	98.98±1.08	0.1902±0.005	0.0498±0.08		
Ic50 (µg/100ml)		0.113				
Test	50	83.49±0.24	0.1887±0.002	0.01075±0.0001	624.17±12.5	32.154±0.120
	100	86.86±0.32	0.2662±0.003	0.0245±0.008	325.86±18.09	56.12±0.161
	200	96.98±0.03	0.4240±0.004	0.8436±0.0001	19397.212±296.20	62.11±0.121

CONCLUSION

In present study, antioxidant activities of the ethyl acetate extract obtained from henna were investigated. The extracts were found to possess radical scavenging and antioxidant activities, as determined by scavenging effect on the DPPH, ABTS, OH radical, reducing power, chelating effect on ferrous ions, FRAP and total antioxidant activity. Generally, EC50 values of lower than 10 mg/ml indicated that the extracts were effective in antioxidant properties. In the present study it is found that the water extract of henna contains substantial amount of phenolics and flavonoids and it is the extent of phenolics present in this extract being responsible for its marked antioxidant activity as assayed through various in vitro models. Thus, it can be concluded that ethyl acetate extract of henna leaf can be used as an accessible source of natural antioxidants with consequent health benefits.

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