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Free radical scavenging and reducing power of *Lawsonia inermis* L. seeds

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ABSTRACT

Objective: To determine the antioxidant activity, total phenolic and flavonoid content of Petroleum ether extract (PE), Dichloromethane extract (DCM), Ethanol extract (ET) and aqueous extract (AQ) of henna seeds. **Methods:** Total antioxidant assay (phosphomolybenum method), DPPH radical scavenging assay, reducing power assay and lipid peroxidation inhibition assay were used to ascertain the potential of seeds as an antioxidant. **Results:** In all the assays carried out ET showed a greater potential to scavenge DPPH radical, reduce MO (Ⅵ) to MO (Ⅴ) complex and Fe (Ⅲ) to Fe (Ⅱ) and to inhibit lipid peroxidation. The IC_{50} of ET was far greater than that of the standard, ascorbic acid (AS) in the lipid peroxidation assay. The activity of AQ was lesser when compared with that of ET but greater than PE and DCM. The amount of phenolics and flavonoids were present in higher amounts in ET followed by AQ. Trace amounts of phenolics were detected in PE and DCM, but the amount of flavonoids were below the detection level. The study showed that the antioxidant activity and the concentrations of phenolics and flavonoids are proportionate to each other. **Conclusions:** Ethanolic extract of henna seeds are efficient antioxidants, which can be utilized for further isolation of active compounds and pharmaceutical applications.

1. Introduction

A wide variety of sources contribute to the formation of highly reactive free radicals and oxygen species in the biological systems which oxidize nucleic acids, proteins and lipids initiating degenerative diseases and antioxidants play a major role in reducing this risk by trapping the free radicals[1]. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical tissue injury and substituting synthetic antioxidants like butylated hydroxyl toluene (BHT) and propyl gallate (PG) whose safety has been questioned[2,3]. Natural antioxidants are safe, readily acceptable by the consumers and they are identical to the food which people have taken for over a hundred years or have been mixing with food[4].

Some of the studies in our lab include the analgesic activity of *Pleurotus eous (P. eous)*[5], hepatoprotective activity of *Crossandra infundibuliformis (C. infundibuliformis)*[6] and CNS activity of *Lawsonia inermis (L. inermis)*[7]. *L. inermis* is an important medicinal plant of Indian systems of medicine. It is commonly called henna, which grows in warm and arid regions. The dye derived from green leaves of henna is used to decorate the body with intricate designs and the principle coloring matter is lawsone, 2-hydroxy-1,4-naphthoquinone[8]. In our previous study, the seeds were found to have CNS depressant and anticonvulsant property acting via glycine receptors[7]. The leaves showed a significant antioxidant property. All the compounds isolated exhibited a free radical scavenging activity comparable with that of ascorbic acid. This may account for the immunomodulatory effect, anti-sickling activity and hepatoprotective activity of henna leaves[9]. The methanolic extracts of henna seeds were checked for its antioxidant property using ABTS, DPPH and FRAP assays[10]. However the property is only with respect to methanolic extract which lacks clear distinction of the influence of the nature of compounds on the activity. In the present study, solvents with increasing polarity were utilized in order to observe the effect of the extracts and their components on the antioxidant activity.

2. Materials and methods

2.1. Plant material and preparation of extracts

Fresh seeds of *L. inermis* (local name: marudani) were

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obtained from Naturaa Farms, Odugathur, Vellore district, Tamil Nadu. The seeds were shade dried and authenticated by Dr. Annadurai B, Department of Botany, C. Abdul Hakeem College, Melvisharam. The dried seeds were crushed to fine powder using mortar and pestle. About 100 g of the powder was extracted with petroleum ether, dichloromethane and ethanol in a soxhlet apparatus to obtain petroleum ether extract (PE), dichloromethane extract (DCM) and ethanol extract (ET) respectively. The material was then dried and the powder was soaked in 400 mL of water for 24 h and filtered to obtain aqueous extract (AQ). The extracts were concentrated in a rotary evaporator and stored in vacuum desiccators. The total yield of the extracts was 4.02%, 2.32%, 10.6%, and 7.6% for PE, DCM, ET, and AQ respectively.

2.2. Preliminary phytochemical analysis

Preliminary screening for the presence of alkaloids, saponins, phytosterols, phenolic compounds, flavanoids, tannins, oils and fats were carried out using the protocol given by Raaman N[11].

2.3. Determination of total phenolics

The amount of total phenolics in the extracts was determined as described by Javanmardi *et al*[12]. To 50 μL of each sample, 2.5 mL 1/10 dilution of Folin-Ciocalteau's reagent and 2 mL of Na_2CO_3(7.5% w/v) were added and incubated at 45 ℃ for 15 min. The absorbance of all the samples was measured at 765 nm. A calibration curve was prepared using a standard solution of gallic acid (20-1 000 mg/L, r^2=0.966). Results were expressed as mg gallic acid equivalents per gram of extract.

2.4. Determination of total flavonoids

The total flavonoids were determined as described by Liu[13] with some modifications. About 250 μL of the extract was diluted with 1.25 mL of distilled water. Then, 75 μL of 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added and the mixture was allowed to stand for 5 min; 0.5 mL of 1 mol/L NaOH was added and the total mixture was made up to 2.5 mL using distilled water. The solution was mixed well and absorbance was measured immediately against the prepared blank at 510 nm using quercetin as standard. The results were expressed as milligrams of quercetin equivalents per gram extract.

2.5. Total antioxidant assay

Different concentrations of PE, DCM and ET (10-1 000 μg/mL) were prepared by dissolving the extracts in ethanol and water for AQ. The samples were mixed with one milliliter of the reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The tubes were capped with silver foil and incubated at 95 ℃ for 90 min. The tubes were cooled to room temperature and the absorbance was measured at 695 nm against a blank. Ascorbic acid was used as a standard. Total antioxidant capacity was expressed as equivalents of ascorbic acid using the following linear equation:

$$Y = 0.003X + 0.392, \quad r^2 = 0.956$$

Y is the absorbance at 695 nm and X the concentration of ascorbic acid equivalent μg/mL[14].

2.6. DPPH radical scavenging assay

The scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by the extracts is based on the method described by Alothman[15]. 1 mL of the sample solutions containing different concentrations were mixed with 3 mL of 0.1 mmol/L solution of DPPH. The mixture was kept in dark for 30 min. The absorbance was measured after incubation at 517 nm, against a blank of ethanol without DPPH. The control solution consisted of a mixture of 1 mL ethanol and DPPH. In case of aqueous extracts distilled water was used instead of ethanol for blank and control. Ascorbic acid was used as a standard. Results were expressed as percentage of inhibition of the DPPH radical. Percentage of inhibition of the DPPH radical was calculated according to the following equation:

$$\text{Inhibition of DPPH} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100\%$$

IC_{50} values which is the concentration of the extract to cause 50% loss of DPPH activity was calculated from the graph plotted between percentage of inhibition and concentration of the sample.

2.7. Reducing power assay

Plant extracts of various concentrations were well mixed with 0.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 0.5 mL of 1.0% K-ferricyanide. Then, the mixture was incubated at 50 ℃ in a water bath for 20 min. The samples were centrifuged at 1 500 g for 10 min followed by the addition of 0.5 mL of 10% trichloroacetic acid to the supernatant. The solution was well mixed with 1.0 mL distilled water and 0.2 mL of 0.1% $FeCl_3 \cdot 6H_2O$. It was then allowed to stand at room temperature for 10 min. Colour changes were monitored at 700 nm by a spectrophotometer. Ascorbic acid was used to compare the reducing power. The higher the absorbance, the better the reducing power of the sample[16].

2.8. Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay was carried out using the spectrophotometric determination given by Subhasree[17]. Chick liver instead of goat liver was used for the experiment. The liver was washed thoroughly in cold phosphate buffered saline (pH 7.4). The liver was then homogenized in a homogeniser to give a 10% homogenate. The homogenate was filtered using muslin cloth to remove the unwanted debris. The filtrate was centrifuged at 10 000 rpm for 10 min under refrigerated conditions. The supernatant was then used to carry out the assay. To 0.5 mL of the 10% homogenate, 0.5 mL of the extracts at various concentrations was added. To this, 0.05 mL of 0.07 mol/L ferrous sulphate was added. The solution was incubated at room temperature for 30 min. To the incubated solution, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% TBA (in 1.1% SDS) and 0.05 mL

of 20% TCA were added and mixed well. The tubes were then incubated at 100 ℃ for 1 h and cooled to room temperature. About 3 mL of butanol was added to each tube. The solution was mixed well and then centrifuged at 3 000 rpm for 10 min. The upper layer was used to read the absorbance at 532 nm. The percentage inhibition was then calculated using the formula

$$\text{Inhibition rate} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100\%$$

IC_{50} was calculated as explained in the DPPH scavenging assay.

2.9. Statistical analysis

Determination of total antioxidant assay, DPPH radical scavenging assay, ferric reducing assay, lipid peroxidation assay and measurements of total phenolic and total flavonoid contents were done in triplicate and the values reported are the mean of three measurements. Correlation analysis between antioxidant assays and total phenolic and flavonoid contents were done using the Pearson correlation in SPSS 9.05 software (SPSS Inc., Chicago).

3. Results

3.1. Phytochemical studies

Preliminary phytochemical analyses revealed the presence of oils, fats, phytosterols and terpenoids in petroleum ether and dichloromethane extracts, whereas phenolics, tannins, saponins, and flavanoids are present in ethanol and water extracts. The extracts did not show any positive result for alkaloids.

3.2. Total phenolic content (TPC) and total flavonoid content (TFC)

Table 1 shows the amount of phenolics and flavonoids present in each extract. The concentration of phenolics in ET and AQ are higher when compared with that of the other two extracts. Flavonoids were below the detection limit in case of PE and DCM. Phenolics and flavonoids played a major role in most of the activities performed. The concentration of phenolics and flavonoids in the extracts are in the following order: ET > AQ > DCM > PE.

Table 1
Total phenolics and total flavonoid content.

Extract	TPC (mg of Gallic acid equivalents/g extract)	TFC (mg of Quercetin equivalents/g extract)
PE	3.72±0.23	–
DCM	4.60±0.03	–
ET	141.65±0.29	31.59±1.00
AQ	51.46±0.44	6.86±0.23

3.3. Total antioxidant assay

Figure 1 shows the total antioxidant capacity of the extracts. Ethanol extract exhibited a good activity followed by the aqueous extract but that of PE and DCM were negligible.

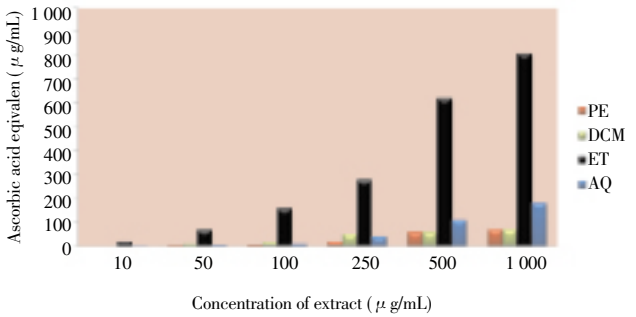

Figure 1. Total antioxidant assay of the extracts at different concentrations.

3.4. Reducing power assay

The reducing power of ET is better than that of the standard from 10–500 μg concentration while it is more or less similar case of AQ and ET–1 000 μg (Figure 2). The activity was far less when PE and DCM are considered.

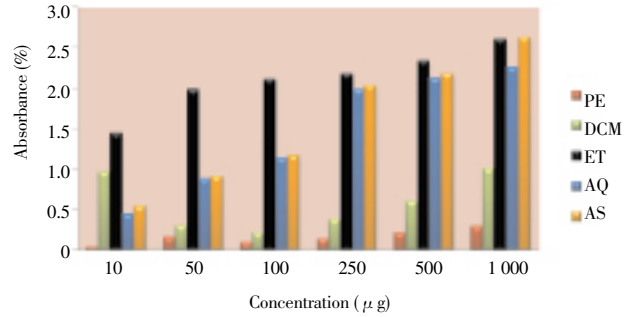

Figure 2. Reducing power assay of different concentration of PE, DCM, ET, AQ and AS.

3.5. DPPH radical scavenging assay

The ability of the extract to scavenge DPPH radicals is represented in Figure 3. On an average all the extracts were able to scavenge the DPPH. But the effect of PE is negligible since its IC_{50} value (Table 2) is comparatively low.

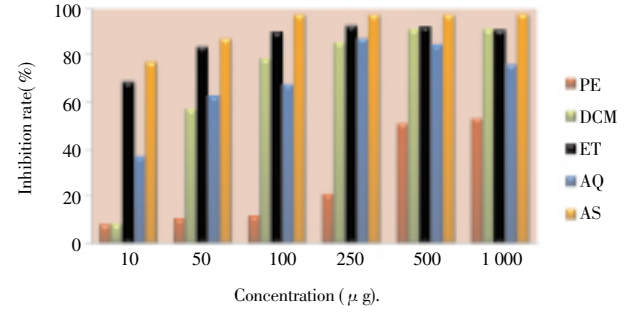

Figure 3. DPPH radical scavenging assay of the extracts.

Table 2
IC_{50} values of different extratcs and standard (μg).

Extract	DPPH radical scavenging assay	Lipid peroxidation assay
PE	254.398	>1 000
DCM	44.509	>1 000
ET	0.083	43.934
AQ	19.186	1 107.058
AS	0.012	82.921

3.6. Lipid peroxidation inhibition assay

Figure 4 shows the inhibition of lipid peroxidation by PE, DCM, ET, AQ and AS. The results show the effective inhibition of lipid peroxidation by ethanol extract. The inhibition by aqueous extract was better than that of the petroleum ether and dichloromethane extracts, all of which had an IC_{50} greater than 1 000 μg. IC_{50} values (Table 3) showed that ET was having the highest lipid peroxidation inhibition potential compared to that of the extracts and standard.

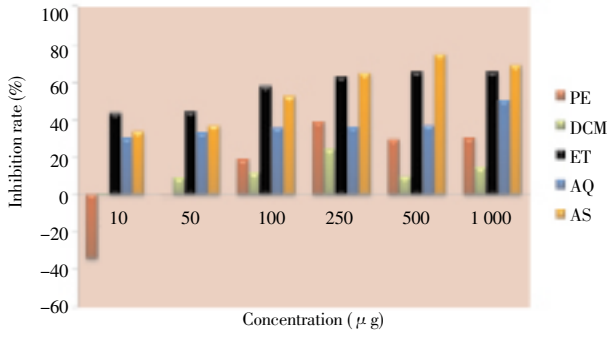

Figure 4. Percentage inhibition of lipid peroxidation by PE, DCM, ET, AQ and AS.

3.7. Correlation

The correlation analysis showed r^2 was 0.991 between TPC and TFC, 0.534 between TPC and DPPH, 0.966 between TPC and TAC, 0.850 between TPC and RPA, 0.939 between TPC and LPA, 0.503 between TFC and DPPH, 0.992 between TFC and TAC, 0.774 between TFC and RPA, 0.928 between TFC and LPA, 0.464 between DPPH and TAC, 0.662 between DPPH and RPA, 0.225 between DPPH and LPA, 0.688 between TAC and RPA, 0.902 between TAC and LPA, and 0.765 between RPA and LPA.

4. Discussion

The concept behind the total antioxidant assay (phosphomolybdenum method) and reducing power assay (RPA) is reduction. While phosphomolyndenum method involves the reduction of molybdenum Ⅵ to a green coloured molybdenum Ⅴ complex[14], RPA involves the reduction of Fe^{3+} to Fe^{2+}[18]. The total antioxidant capacity expressed in ascorbic acid equivalents and ferric reducing ability of the extracts. ET had an excellent reducing ability and its concentrations from 10–500 μg showed even higher ferric reducing ability than the ascorbic acid and it had higher ascorbic acid equivalents. AQ showed good reduction of the Mo(Ⅵ) than PE and DCM and exhibited its ability to reduce Fe(Ⅲ) comparable to that of Ascorbic acid. The activity of PE and DCM in phosphomolybdenum method are negligable since 1 000 μg/mL of both the extracts is equivalent to the activity of ascorbic acid of concentration below 80 μg/mL. The effective reduction by the ethanol extract shows its effeciency in preventing oxidation. Naturally occuring reductants are involved in oxidative defence mechanisms and the reducing capacity may serve as a significant indicator of its potential antioxidants[19]. Thus the antioxidant capacity of the extracts based on its ability to reduce can be given in the following descending order: ET > AQ > DCM > PE.

The DPPH radical scavenging activity of the extracts. On an average all the extracts were able to scavenge DPPH radicals. The IC_{50} of ET was very close to that of the ascorbic acid. The scavenging of DPPH radical by PE and DCM may be due to the presence of trace amounts of phenolics. Phytosterols are triterpenes that are important structural componenets of plasma membranes which may have antioxidant activity[20]. Preliminary phytochemical analysis showed the presence of phytosterols in both the extracts which could have been the responsible compounds for the radical scavenging and also for its ability to reduce and inhibit peroxidation in smaller amounts at higher concentrations. Even the correlation between phenolics, flavonoids and the DPPH assay was not so close which shows the involvement of other compounds such as phytosterols, terpenoids, saponins in addition to phenoilics and flavonoids. Based on the IC_{50} values the ability of the extracts to scavenge the DPPH free radical can be given in the follwing order PE < DCM < AQ < ET.

Lipid peroxidation is a basic membrane damage process which involves free radicals leading to oxidative degradation of polyunsaturated fatty acids and resulting in deleterious effects. In the present study ferrous ions present in the system initiate lipid peroxidation which results in the formation of hydroxyl radicals through Fenton's reaction. The hydroxyl radicals intiate the chain reaction to form malondialdehyde which reacts with thiobarbituric acid to form a pink chromogen. This inhibition could be caused by the absence of ferryl–perferryl complex or by scavenging the hydroxyl radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} ratio or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself[17,21-22]. ET showed a very good ability to inhibit the lipid peroxidation. Moreover it showed 50% inhibition at 43.934 μg which is far better than that of ascorbic acid which showed at 82.921 μg. Cheng[23] reported the multiple mechanisms underlying the reactions between hydroxyl radicals and phenolics. This could explain the effective inhibition by ET. And its lesser concentration in AQ could have been responsible to inhibit at higher concentration (1 107.058 μg–50% inhibition concentration). The inhibition by PE and DCM were negligible since its IC_{50} was far greater than 2 000 μg. The ability of the extracts to inhibit lipid peroxidation would be: ET > AQ > PE > DCM.

The correlation between antioxidant assays and total phenolic, flavonoid contents is given. A close correlation of phenolic content with total antioxidant capacity (0.966), reducing power assay (0.850) and lipid peroxidation assay (0.939) was observed. A good correlation was also observed between total flavonoid content and total antioxidant assay (0.992), reducing power assay (0.774) and lipid peroxidation assay (0.928). But the correlation of phenolic and flavonoid contents with the DPPH assay was not good (0.534 and 0.503, respectively). This may be due the probable involvement of compounds like phytosterols, saponins or terpenoids in addition to phenolics and flavonoids. But the involvement of phenolics, which comprise of a great diversity of compounds, such as flavonoids and several classes of non-flavonoids[24] in most of the antioxidant assays performed supports the fact that they play a role as antioxidants through different mechanisms such as quenching of reactive oxygen

species[25], interaction with biomembranes[26], scavenging of free radiclals[27].

But there is not much information about the phytochemical constituents of henna seeds particularly phenolics and flavonoids which are the major constituents for all the activities we discussed above. Phytochemicals isolated from henna seeds are behenic acid, arachidic acid, palmitic acid, stearic acid, linoleic acid, lawnermis acid and its methyl ester[28,29]. Thus further isolation of compounds from the ethanolic extract could well explain the mechanisms involved for its efficient radical scavenging and reducing power.

The results showed that phenolics and flavonoids played a major role in the antioxidant assays, since the amount of the compounds were proportionate to the activity. The activity shown by PE and DCM were because of the presence of phytosterols and terpenoids as discussed above in addition to trace amounts of phenolics. But higher levels of phenolics and flavonoids in the ethanolic extract resulted in the efficient radical scavenging and reduction. And its lesser amounts in AQ resulted in decrement of the activity. The relation between the constituents of the ethanol extract and the antioxidant activity can be better understood with the individual compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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