Antioxidant content and in vitro antioxidant activities of extracts from popped Euryale ferox seeds

Kaneez Haleema, Chandan Vinay Srigiripura and *Asna Urooj

Department of Food Science and Nutrition, University of Mysore, Mysore, India

Email: haleemawillreach@gmail.com, knowdiet@gmail.com

*Corresponding author E-mail id: asnau321@gmail.com; Phone/Fax: 0821-2419632

Abstract

Euryale ferox has been widely used in traditional oriental medicine, ayurveda to treat several illnesses and also in Indian cookery. The aim of this study was to analyze the antioxidant content and in vitro antioxidant activity of popped Euryale ferox seeds. Popped seeds were found to contain low quantities of total phenolics (52.7 mg/100g gallic acid equivalents) and flavonoids (1.25 mg/100g rutin equivalents). The ethanol and 80% methanol extracts showed 1,1 Diphenyl-2-picyyl-hydrazyl (DPPH) radical scavenging activity in a dose dependant manner with half maximal inhibitory concentration (IC$_{50}$) values of 109.3 µg/ml, 91 µg/ml respectively. Ferric reducing ability of 80% methanol and ethanol extracts were lower than butylated hydroxy toluene used as a standard and aqueous extract had the least activity. These results suggest that popped seed extracts are relatively lower in antioxidant content and in vitro antioxidant activity in comparison with reported values of extracts from raw seeds and seed shell which can be the outcome of high temperature applied during the processing of seeds.

Keywords: “antioxidant” “DPPH” “Euryale ferox” “FRAP” “makhana” “reducing power”.

Introduction

Euryale ferox Salisbury (nymphaeceae) is popularly known as makhana, fox nut or gorgan nut is a stemless, prickly aquatic herb currently limited to tropical and subtropical regions of South East and East Asia. Makhana is considered as a delicious food item in India. Makhana is either eaten as raw, puff or blended with vegetables, dal (a split pulse based preparation), etc. They are also a part of cookery involving bread, seed flour, salad and desserts. The seeds are processed by traditional methods which includes sun drying, size grading, pre heating, roasting and popping where temperatures ranging from 280-335°C are employed (Shankar et al., 2010). The seeds are edible after being processed and are highly nutritious. There are various medicinal and dietary uses attached to the seeds (Kumari et al., 2014). Euryale ferox has been widely used in oriental medicine and ayurveda in the treatment of kidney problems, diarrhoea, leucorrhea and hypofunctioning of the spleen (Das et al., 2006). There are no known side-effects or drug interactions associated with Euryale ferox. It is classified under CLASS 1 herbs - herbs that can be safely consumed when used appropriately and CLASS A- herbs for which no clinically relevant interactions are expected (Gardner and McGuffin, 2013).

With research consistently unraveling the health benefits of phytochemicals found in food, the interest in such foods and their phytochemicals are of prime interest among researchers in the field of nutrition. The phytochemicals have been attributed to various benefits such as antioxidant, anti-inflammatory, anti-carcinogenic, lowering the blood pressure, antihyperlipidemic etc (Heneman and Karrie, 2008).

Euryale ferox is known to be rich in phenolics, sesquineolignans and flavanone making it a food with potent antioxidant activity (Song et al., 2011). Biological and in vitro studies have indicated significant antioxidant properties of the seed shell/seed coat, raw seed and the
whole plant. The extract from seed and seed shell have significant DPPH radical scavenging activity with percentage inhibition ranging from 83.06 – 89.5% and IC\textsubscript{50} values of seed coat extracts ranging between 1.5-29.4 ug/ml (Zhang, 2014; Lee \textit{et al.}, 2002). The IC\textsubscript{50} values of aqueous and methanol extracts of the dried whole herb is reported to be >2,000 ug/ml and 307.35 ± 1.61 ug/ml respectively (Ho \textit{et al.}, 2012). Improvement in the levels of redox – regulated proteins thioredoxin related protein-32 and Thioredoxin-1 (Trx-1) has been reported in mice treated with \textit{Euryale ferox} seed extract (Das \textit{et al.}, 2006) and improvements in activity of super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase in mice treated with \textit{Euryale ferox} seed coat extracts (Wu \textit{et al.}, 2013).

The lack of studies on the antioxidant profile and \textit{in vitro} antioxidant activity of popped \textit{Euryale ferox} seeds, which is the common form of consumption in India, has driven the present study with an objective to analyze selected antioxidant composition and \textit{in vitro} free radical scavenging potential and ferric reducing ability of popped \textit{Euryale ferox} seed extracts.

Materials and Methods

Materials

L-ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich, India. 2,4,6-triarylidyl-s-triazine (TPTZ) from Hi-media laboratories; Folin-ciocalteau reagent, gallic acid and tannic acid from loba chemie were purchased.

Popped \textit{Euryale ferox} seeds were procured from the local market of Mysore, Karnataka, India. The duration of storage of popped seeds by the retailer was between one to two months at the time of purchase. The popped seeds were powdered finely in a blender, packed in air tight pouches and stored in ambient condition until further analysis.

Preparation of extracts

Powdered seeds (10g) were macerated with 100ml of 80% methanol, ethanol and water separately and kept in a mechanical shaker for 16 hours. The residue was re extracted and filtered. The extract was concentrated by evaporating the solvents using vacuum evaporator under 70\textdegree C. The dried extract was weighed, dissolved in 80% methanol, ethanol and water and stored at -20\textdegree C for further analysis where all the measurements were made in triplicates.

Analysis of selected antioxidants

Ascorbic acid, total phenolics, total flavonoids and reduced glutathione were determined in the sample. The ascorbic acid content was estimated using 2,4 dinitrophenyl hydrazine method (Kapur \textit{et al.}, 2012).

0.23ml of 3% bromine water and 0.13ml of 10% thiourea were added to 4ml of extract. 1ml of 2, 4 dinitrophenyl hydrazine solution was further added and the mixture was incubated at 37\textdegree C for 3hours. The mixture was cooled in an ice bath for 30min followed by slow addition of 5ml of cold sulphuric acid (85%). The resulting red colour was read at 521 nm against reagent blank. Standard curve was prepared using ascorbic acid as standard.

The extract for the estimation of total phenolics and flavonoids was prepared by the method of Jimenez-Escrig \textit{et al.} (2003). The total phenolic content was estimated according to Folin ciocalteau micro method (Slinkard \textit{et al.}, 1977). 20\mu l of the extract was mixed with 4.28 ml of distilled water and 100ul of folin ciocalteau reagent, followed by the addition of 20% sodium carbonate after 1 minute and before 8 minutes. Subsequently the mixture was incubated at 40\textdegree C for 30 minutes and the absorbance was measured at 760nm. Gallic acid was used as reference and total phenolic content was expressed as gallic acid equivalents.

The total flavonoid content of the extracts was estimated using pharmacopeia method (Evans, William Charles, 1986). One ml of extract in methanol (10g/L) was mixed with 1 ml of aluminium trichloride in ethanol (20g/L) and diluted with ethanol to 25ml. The mixture was incubated at 20\textdegree C and the absorbance was measured at 415 nm. Rutin was used as reference and total flavonoid content was expressed as rutin equivalents.

Reduced glutathione (GSH) content was estimated according to the method of Beutler and Kelly (1963) which is based on the reaction of Ellman’s reagent with compounds containing sulphhydryl groups. 1g of the sample powder was homogenized with 8 ml of tris buffer (0.2 M, pH 8.2) and filtered. 1 ml of filtrate was mixed with 2ml of tris buffer (0.4 M, pH 8.2) and 0.1ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and the absorbance was read at 412 nm within 2 minutes using reduced glutathione as a reference.

Analysis of \textit{in vitro} antioxidant activity

\textbf{a. DPPH radical scavenging assay}

This activity of popped \textit{Euryale ferox} seed extracts were measured by the method described by Blois (1958). 1 milliliter of the fraction solutions was made up to 3ml with methanol and 1ml of DPPH solution (0.1mM in ethanol) was added to samples and control. The absorbance of the solution was measured at 517 nm after 30 mins. The activity of each fraction was determined by comparing with 4ml of methanol (blank). L-Ascorbic acid was used as a standard to compare the efficacy of the extracts and half maximal inhibitory concentration (IC\textsubscript{50}) was also calculated. The ability of the extracts to scavenge the DPPH radical was calculated using the following equation:

\[
\text{DPPH scavenging activity (\%)} = \left(\frac{A_0 - A_I}{A_0}\right) \times 100
\]
Where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample.

b. Evaluation of reducing power

The reducing power of popped *Euryale ferox* seeds was analyzed using methods described below, applying the reduction of iron from ferric ferrous form.

i. Ferric Reducing Antioxidant Power (FRAP) Assay

The ability to reduce ferric ions was measured using the method described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM (tripyridyl triazine) TPTZ solution and 20.0 mM ferric chloride hexahydrate solution in a ratio of 10:1:1. Samples at different concentrations were then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance at 593 nm was measured after 4 mins of incubation. Ferrous sulphate was used to obtain a standard curve and the activity of sample was expressed as mmol FeSO$_4$ equivalents per gram of sample (dry weight).

ii. Reducing power assay

Reducing power was determined by the method described by Oyaizu (1986). The sample in 1ml of methanol, methanol-water, ethanol and deionized water at various concentrations were mixed with a phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%), and the mixture was incubated at 50°C for 20 min. 5ml of trichloroactic acid (10%) was added to the reaction mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5ml) and ferric chloride (1 ml, 1%), further the absorbance was measured at 700 nm. A stronger absorbance indicates higher reducing power. Butylated hydroxy toluene was used as a standard to compare the efficacy of the sample.

Results

Antioxidant composition

The selected antioxidant composition of popped *Euryale ferox* seeds is shown in the table 1. Phenolic antioxidants were found to be the most predominant than flavonoid content in the popped seeds. Reduced glutathione was found in minute quantities. Ascorbic acid was absent which can be due the higher processing temperature used.

<table>
<thead>
<tr>
<th>Antioxidant components</th>
<th>Amount/100gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (mg)</td>
<td>0/Nil</td>
</tr>
<tr>
<td>Total Phenolics (GAE* in mg)</td>
<td>52.7 ± 2.83</td>
</tr>
<tr>
<td>Reduced glutathione (µg)</td>
<td>16.92 ± 0.47</td>
</tr>
<tr>
<td>Flavonoids (mg RE**)</td>
<td>1.25±0.35</td>
</tr>
</tbody>
</table>

*GAE-gallic acid equivalents; **RE- rutin equivalents

Analysis of *in-vitro* antioxidant activity

a. DPPH radical scavenging assay

Water extract exhibited zero activity. The DPPH radical scavenging activity of various extracts of popped *Euryale ferox* seeds is shown in table 2. A dose dependant response was observed for ascorbic acid, 80% methanol (EF-M) and ethanol extracts (EF-E). Water extract showed no activity. The $IC_{50}$ values of EF-M, EF-E and ascorbic acid standard were found to be 109.3µg/ml, 91µg/ml and 29.78µg/ml respectively presented in figure no 1. Ascorbic acid had the lowest $IC_{50}$ value followed by ethanol extract and methanol water extract. The $IC_{50}$ values indicate relatively low potential of *Euryale ferox* seed extracts to scavenge the DPPH radical compared to ascorbic acid.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Percentage inhibition of DPPH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid (Std)</td>
</tr>
<tr>
<td>100</td>
<td>96.51</td>
</tr>
<tr>
<td>200</td>
<td>96.80</td>
</tr>
<tr>
<td>300</td>
<td>97.67</td>
</tr>
</tbody>
</table>
Figure 1: DPPH radical scavenging activity - IC50 values of three extracts of popped *Euryale ferox* seeds

<table>
<thead>
<tr>
<th></th>
<th>400</th>
<th>97.38</th>
<th>83.89</th>
<th>88.82</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>97.97</td>
<td>86.73</td>
<td>90.13</td>
<td></td>
</tr>
</tbody>
</table>

**b. Evaluation of reducing power**

**i. FRAP Assay**

Ferric reducing ability of various popped *Euryale ferox* seed extracts are shown in figure 2. A dose dependent increase in the activity was observed in all the extracts except aqueous extracts. 80% methanol extract exhibited the highest ferric reducing activity among the seed extracts followed by ethanol and water extract had the lowest activity. The increase in activity was found to be in a dose dependent manner except for aqueous extract which displayed least activity. Ascorbic acid displayed the highest ferric reducing activity which was predominantly higher than all the popped seed extracts.
ii. Reducing power assay

The reducing powers of various *Euryale ferox* seed extracts are presented in figure 3. When compared with activity of butylated hydroxyl toluene (BHT) standard, all the popped *Euryale ferox* seed extracts had lower reducing power. Water extract had the least activity. The reducing power of the extracts increased with concentration except for water extract.
Discussion

Oxidative stress has been implicated in several diseases as well as in the process of aging. Damage resulting from free radicals to the cells of the body leads to impaired cell function and even cell death resulting to a disease. Membrane lipids, carbohydrates, proteins, deoxyribonucleic acid (DNA) are susceptible to oxidative stress and have been implicated in diseases such as arthritis, cancer, Alzheimer’s etc (Devasagayam et al., 2004). The evaluation of effectiveness of antioxidant action of food in vitro gives an approximate insight into the potential antioxidant actions of foods in vivo.

Total phenolic content in seed shell extracts have been reported to be 113.30 mg/g dry weight (Wu et al., 2013) and 55005 – 74002 mg/100g (Zhang, 2014) which is higher than the popped seed content. The total polyphenol content in the raw seeds have been reported to be 7.7 mg/g dry weight expressed as tannic acid equivalents (Chen et al., 2011). These values are suggestive of prominent reductions in total phenolic content compared to raw seeds is evident and the seed shell appears to have the highest content of total phenolics followed by raw seeds but comparison cannot be made due to differences in the analytical methods used by the researchers. The flavonoid content of popped Euryale ferox seeds appears to be relatively low compared to the total plant extract which is reported to contain 3.28 μg/mg rutin equivalents (Ho et al., 2012). Glutathione is a potent antioxidant found in plant foods and is known to increase serum levels of reduced glutathione thus boosting antioxidant capacity in vivo. There are currently no reports on reduced glutathione content in raw seeds or seed shells of Euryale ferox. Raw seeds appear to be an acceptable source of ascorbic acid indicating complete loss on processing.

The stable DPPH radical undergoes decolourization on receipt of electron or hydrogen atom from a donor in the medium. Higher the antioxidant content of a particular food, higher will be the degree of decolourization which is measured colorimetrically (Kedare and Singh, 2011). The IC$_{50}$ values of popped Euryale ferox seed extracts are lower compared to raw Euryale ferox seed extracts (hexane, dichloromethane, ethyl acetate, butanol and water) which have been shown to exhibit an average IC$_{50}$ values of 5.6 μg/ml (Lee et al., 2002) and an IC$_{50}$ value of 29.4, 28.3 and 27.6 μg/ml have been reported for ethyl acetate, 100% ethanol and 50% ethanol respectively in seed shell extracts (Zhang, 2014) indicating lower potential of the processed seed used in this study in exhibiting antioxidant action.

The FRAP assay is based on the reduction of ferric complex by antioxidants to ferrous form leading to generation of a blue coloured complex which is measured (Benzie and Strain, 1996). The colour development is directly proportional to the antioxidant content in the medium. Although the reducing ability increased with concentration of the popped seed extracts used, the results clearly indicate very low activity of these extracts compared to ascorbic acid used as standard. The presence of antioxidants drives the reduction of ferricyanide to ferrous form. Literature review indicates a higher reducing power of ethyl acetate, 100% ethanol and 50% ethanol extracts of Euryale ferox seed shell (200ug/ml) with optical density between 1.0 to 2.0 (Zhang, 2014) followed by 0.76 ± 0.12 being the optical density expressed by whole plant extract (Ho et al., 2012) which are relatively higher compared to reducing power of popped seed extracts.

Antioxidant potential of the popped seeds appears to be diminished in all the in vitro assays employed in the study compared to raw seeds, seed shell or whole plant. Popped seed extracts exhibit relatively low antioxidant activity in vitro in terms of DPPH radical scavenging activity and ability to reduce ferric ions in comparison with the activity of seed shell and raw seeds reported by earlier studies which may be attributed to the nature of processing of the seeds which is the only distinctive feature in popped seeds. Thermal processing has been known to act differently on types of bioactives and food systems. Thermal processing is also known to alter the phytochemical composition considerably (Kanekanian and Ara, 2010). Polyphenols are known to be unstable at high temperature (Liangli et al., 2012) and loss of polyphenols exposed to processing temperatures above 180°C has been reported (Ross et al., 2011). Losses in flavonoid content of fruits and vegetables on boiling, frying, baking or micro waving also have been reported (Palermo et al., 2014). The temperature used in conventional processing of Euryale ferox seeds in India is between 230-335°C (Shankar et al., 2010), which may be the causative factor for low antioxidant activity of extracts from popped seeds compared to raw seeds or seed coat. This observation necessitates further research for development of a modified processing technique with an aim to preserve the antioxidant composition of popped Euryale ferox seeds.

Conclusion

Popped Euryale ferox seeds certainly exhibit antioxidant activity in vitro but the activity is low when compared to the activity of raw seeds or seed coat. The minute quantities of polyphenols and flavonoids found in the popped Euryale ferox seeds may be attributed to the DPPH radical scavenging activity and ferric reducing power analysed, although the effect of thermal processing on the antioxidant cannot be neglected and necessitates detailed research on behavior of individual bioactive on thermal processing simultaneously creating opportunities for modifications in the processing methods with an aim to preserve the reported high antioxidant potential of Euryale ferox. Overall, the results suggest that popped Euryale ferox seeds are less potent sources of antioxidants and antioxidant activity in vitro in view of the low antioxidant
content and in vitro activity compared to raw seeds and seed shells.

Acknowledgements

Authors are grateful to the University Grants Commission - Special Assistance Program- Phase II from Department of Science & Technology, Ministry of Science 17 Technology, India for funding the current study.

References


