In vitro studies on antioxidant and free radical scavenging activities of aqueous extract of Acorus calamus L.

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Abstract

Acorus calamus is a plant, rich in alkaloids, phenolics and flavonoids as a potential source of compounds possessing beneficial biological activities. The present study is directed to investigate the antioxidant property of the plants. The antioxidant activity of aqueous extract of Acorus calamus was determined by the following radical scavenging assays namely DPPH radical scavenging assay, nitric oxide scavenging assay, superoxide radical scavenging assay, ferrous chelation assay, reducing power assay and phosphomolybdenum assay. In DPPH assay, the percentage of inhibition was found to be 86.45±0.38 at 300 µg/mL. In nitric oxide radical scavenging activity, the percentage of inhibition was found to be 57.1±0.14 at 700 µg/ml. In superoxide assay, the percentage of inhibition was found to be 91.92±0.45 at 300 µg/ml. In ferrous chelation assay, the percentage of inhibition was found to be 78.4±2.15. The reducing power was found to be 0.25±0.003 at 400 µg/ml. In phosphomolybdenum assay, the concentration range from 25-400 µg/mL, the aqueous extract showed strong dose dependent reducing activity. The results showed that Acorus calamus exhibits free radical scavenging, reducing power and metal chelating property.

Keywords: Acorus calamus, antioxidant, aqueous extract, free radical

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Introduction

An enormous variety of medicinal plants are used worldwide by about 80% of the world population, although in most cases no scientific studies have been done to prove the efficacy of these medicinal plants. Considering that most of the present day western medicines are based on the traditional medicinal plants of European, Mediterranean, and Arabic origin, the variety of plants in use around the world may very well represent an enormous treasure for drug development (Verpoorte, 2000). The immune system is vulnerable to oxidative stress. During certain diseased state, as well as during aging, there is a need to boost the antioxidant abilities, thereby potentiating the immune mechanism (Devasagaya and Sainis, 2002). The antioxidants preserve and stimulate the function of immune cells against homeostatic disturbances (De La Fuente and Victor, 2000). Metabolic activation of carcinogen is a free radical-dependent reaction. DNA damage, which is mediated by free radicals, play a critical role in carcinogenesis (Guyton and Kensler, 1993; Feig, et al., 1994). The potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in fruits and vegetables. These fruits and vegetables can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Waladkhani and Clemens, 1998). Our present aim is focussed on the studies on aqueous extracts of Acorus calamus L. a plant rich in secondary metabolites. These assays showed that the plant had antioxidant potential and further studies could be directed to exploit its medicinal and cosmetic value.

Materials and methods

Collection and authentication of plant materials

Rhizomes of Acorus calamus were collected from the herbal product market, during August 2009. A specimen was deposited at the Herbal Garden, Centre for Advanced Studies in Botany, University of Madras,
Chennai. The identification was made to be *Acorus calamus* with the help of flora of presidency of madras (Gamble, 1967) and the flora of the Tamil Nadu and carnatic (Matthew, 1983). The rhizomes were washed with water, air-dried (30°C), pulverized and stored in a sterile air-tight container for further use.

**Preparation of crude aqueous extract**

The powdered plant material (100 g) was extracted in distilled water (1000 ml) and boiled at 50°C for 1 hr. The extract was filtered using filter paper. The filtrate was condensed to give a yield of 14.3 g of extract. The resulting extract was reconstituted with sterile distilled water to give different concentrations, ranging from 25 to 300 µg/ml.

**Antioxidant assays**

The antioxidant activity of plant material was assayed by employing the following methods.

**DPPH radical scavenging assay**

DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available stable free radical, which is purple in colour. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH (Baumann, 1979) and convert it into Di-phenyl hydrazine, which is yellow in colour. The degree of discoloration of purple to yellow was measured at 520 nm, which is a measure of scavenging potential of plant extracts. 10 µL of plant extract was added to 100 µL of DPPH solution (0.2 mM DPPH in methanol) in a microtitre plate. The reaction mixture was incubated at 25°C for 5 minutes, after that the absorbance was measured at 520 nm. The DPPH with corresponding solvents (without plant material) served as the control. The methanol with respective plant extracts served as blank. The DPPH radical scavenging activity of the plant extract was calculated as the percentage inhibition.

\[
\text{Percentage of inhibition} = \frac{(\text{Control} - \text{Test})}{(\text{control})} \times 100
\]

**Nitric oxide generation and assay of nitric oxide scavenging**

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction as described previously. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (Green et al., 1982; Marcocci et al., 1994a, b), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci et al., 1994a, b). Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with different concentrations of the drugs dissolved in the suitable solvent systems and incubated at 25°C for 150 min. The samples from the above were reacted with greiss reagent (1% sulphanilamide, 2% H₃PO₄ and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite treated in the same way with greiss reagent.

**Assay of superoxide radical scavenging activity**

The method used by Martinez et al. (2001) for determination of the superoxide dismutase was followed with modification (Dasgupta and De, 2004) in the riboflavin-light-nitrobluetetrazolium (NBT) system (Beauchamp and Fridovich, 1971). Each 3 ml of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 ml of 1M riboflavin, 100 ml of 1M EDTA, NBT (75 lM) and 1 ml of sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination from a fluorescent lamp.

**Fe²⁺ chelation assay**

The ability of the sample extracts to chelate Fe²⁺ was determined using a modified method of Minotti and Aust (1987) with a slight modification (Puntel et al., 2005). Briefly 150 µL of freshly prepared 500 µM FeSO₄ was added to a reaction mixture containing 168 µL of 0.1 M Tris-HCl (pH 7.4), 218 µl saline and the methanolic leaf extracts (0 - 500 µL). The reaction mixture was incubated for 5 min, before the addition of 13 µL of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in the spectrophotometer.
Reducing power assay

The reducing power was determined according to the method previously described by Oyaizu (Oyaizu, 1986). Different concentrations of extract (100-1000 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean ± standard deviation.

Phosphomolybdenum reducing power assay

The antioxidant power of the extracts has been assessed with the phosphomolybdenum Reduction assay according to Prieto et al. (1999). The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (0.6 M) mixed with the extracts diluted in 50% ethanol solution at the concentration of 5 mg/mL. The samples were incubated for 90 min at 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. For reference, the appropriate solutions (0.2-2 mM) of ascorbic acid have been used. The reducing capacity of the extracts has been expressed as the ascorbic acid equivalents (milligrams per gram extract).

Results and discussion

The antioxidant potential of aqueous extract of *Acorus calamus* L. was studied by the DPPH assay, and superoxide anion assay (Table 1) at different concentrations, ranging from 25 to 300 µg/mL.

The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The percentage of inhibition was found to be 86.45±0.38 at 300 µg/mL. The IC50 value of the aqueous extract of *Acorus calamus* was found to be 77.48 µg/mL. The superoxide scavenging activity showed a marked increase with the increase in concentrations of aqueous extracts of *Acorus calamus*. The percentage of inhibition was found to be 91.92±0.450 at 300 µg/mL. The IC50 of aqueous extract of *Acorus calamus* was found to be at 53.93 µg/mL. These results showed that aqueous extracts of *Acorus calamus* has a potent superoxide radical scavenging effects.

<table>
<thead>
<tr>
<th>Concentration of aqueous extract of Acorus calamus (µg/mL)</th>
<th>% of inhibition of DPPH radical scavenging assay</th>
<th>% of inhibition of superoxide anion radical scavenging assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>17.3±10.20</td>
<td>27.4±10.778</td>
</tr>
<tr>
<td>50</td>
<td>39.2±10.28</td>
<td>46.3±70.829</td>
</tr>
<tr>
<td>100</td>
<td>57.1±0.20</td>
<td>75.0±00.473</td>
</tr>
<tr>
<td>150</td>
<td>76.6±0.33</td>
<td>82.5±0.689</td>
</tr>
<tr>
<td>200</td>
<td>77.6±0.48</td>
<td>86.6±90.631</td>
</tr>
<tr>
<td>250</td>
<td>80.27±0.35</td>
<td>89.11±0.454</td>
</tr>
<tr>
<td>300</td>
<td>86.45±0.38</td>
<td>91.93±0.450</td>
</tr>
</tbody>
</table>

In nitric oxide radical scavenging activity, the percentage of inhibition was found to be 57.1±0.140 at 700 µg/ml with the IC50 being at 58.2 µg/mL. The result indicated that the extract might contain compounds able to inhibit Nitric oxide activity and offers scientific evidence for the use of the rhizomes in indigenous system in inflammatory condition. The compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in plants (Cook and Samman, 1996). In Ferrous chelation assay, the percentage...
of inhibition was found to be 57.84±2.15. The formation of Fe2+ complex is interrupted in the aqueous extracts of *Acorus calamus*, indicating its chelating activity with an IC50 of 54.62 µg/mL (Table 2). The reducing power was found to be 0.85±0.005 at 300 µg/mL. This result showed that the aqueous extract of aqueous extract of *Acorus calamus* showed excellent reducing power activity. In the concentration range from 25-400 µg/mL, the aqueous extract showed strong dose dependent reducing activity. The reducing power was found to be 0.25±0.003 at 400 µg/ml. In phosphomolybdenum assay, the concentration range from 25-400 µg/mL, the aqueous extract showed strong dose dependent reducing activity. The result obtained was confirmed by the high potency of the aqueous extract towards the transition metal ions. The reducing power was found to be 0.25±0.003 at 400 µg/ml.

Table 3. Reducing power assay and phosphomolybdenum assay

<table>
<thead>
<tr>
<th>Concentration of aqueous extract of <em>Acorus calamus</em> (µg/mL)</th>
<th>Reducing power assay absorbance at 700 nm</th>
<th>Phosphomolybdenum assay absorbance at 700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.287 ± 0.00</td>
<td>0.025±0.001</td>
</tr>
<tr>
<td>25</td>
<td>0.370± 0.00</td>
<td>0.050±0.002</td>
</tr>
<tr>
<td>50</td>
<td>0.38±2 0.05</td>
<td>0.078±0.004</td>
</tr>
<tr>
<td>100</td>
<td>0.540±0.01</td>
<td>0.093±0.002</td>
</tr>
<tr>
<td>150</td>
<td>0.598±0.00</td>
<td>0.13±0.004</td>
</tr>
<tr>
<td>200</td>
<td>0.653±0.04</td>
<td>0.147±0.005</td>
</tr>
<tr>
<td>250</td>
<td>0.690±0.03</td>
<td>0.168±0.003</td>
</tr>
<tr>
<td>300</td>
<td>0.847±0.00</td>
<td>0.179±0.003</td>
</tr>
<tr>
<td>350</td>
<td>0.890±0.00</td>
<td>0.183±0.001</td>
</tr>
<tr>
<td>400</td>
<td>0.900±0.03</td>
<td>0.250±0.003</td>
</tr>
</tbody>
</table>

Conclusions

In this study it was concluded that the radical scavenging activity of aqueous extracts of tested plant rhizome is mainly due to the presence of phenolics and flavonoids content and may be used for scavenging the free radicals. The free radical scavenging activity of various assay showed that the aqueous extract is a better radical scavenger (Dorman et al., 2003). The results obtained in the present study, indicate the aqueous extract of *Acorus calamus* extract exhibits free radical scavenging, reducing power, metal chelating activity. Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy (Prior, 2003) the plants may be considered as a good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms (Hasan et al., 2006). Further investigation on the isolation and identification of antioxidant components in the plant may lead to chemical entities with potential for chemical use.

Acknowledgements

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