Antioxidant and anti-inflammatory activity of extract obtained from *Aspergillus candidus* MTCC 2202 broth filtrate

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Received 20 September 2005; revised 13 March 2006

Antioxidant potential of *Aspergillus candidus* MTCC 2202 broth filtrate extract was studied using different antioxidant models, whereas anti-inflammatory potential was studied using carrageenan-induced rat paw oedema model. The ethyl acetate extract at 1000 µg/ml showed maximum scavenging activity of the stable radical 1,1-diphenyl,2-picryl hydrazyl up to 96.65% (IC50=430.36 µg/ml) and scavenging of the radical cation, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) up to 92.25% (IC50=606.29 µg/ml) at the same concentration. The extract had good reducing power, however showed moderate inhibition for conjugated dienes and thiobarbituric acid reactive acid substances (59.56 and 51.45%). The total phenolic content of various extracts of *A. candidus* broth filtrate was measured and a correlation between radical scavenging activities of extracts with total phenolic content was observed. The ethyl acetate extract (125 mg/kg ip) showed significant anti-inflammatory activity in carrageenan-induced rat paw oedema model. The exhibited antioxidant activity of ethyl acetate extract of *A. candidus* broth filtrate was comparable with BHA and ascorbic acid, while anti-inflammatory activity was comparable with standard diclofenac sodium.

**Keywords:** Anti-inflammatory activity, Antioxidant activity, *Aspergillus candidus*, Phenolic compound, Reducing power

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular disease, inflammatory conditions, cancer and ageing. Almost all organisms possess antioxidant defenses that protect against oxidative damage and numerous damage removal and repair enzymes to remove or repair damaged molecules. However, the natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds become important. Although synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) have been widely applied in food processing, they have been reassessed for their possible toxic and carcinogenic components formed during their degradation. In addition, it has been suggested that there is an inverse relationship between dietary intake of antioxidant-rich foods and incidence of a number of human diseases. Therefore, search into the determination of natural antioxidant sources is important.

In recent years, researchers have reported that metabolites produced by microorganisms may serve as antioxidants, lipoxygenase inhibitors, synergists and/or metal chelating agent. In addition, these metabolites can decompose lipid peroxides and use them as nutrients. It is also demonstrated that Penicillium and Aspergillus could produce antioxidants as metabolites. Thus, the present study was aimed at evaluating antioxidant and anti-inflammatory potential of *Aspergillus candidus* broth filtrate extract.

**Materials and Methods**

*Source of chemicals—* Ammonium persulphate, potassium ferricyanide, trichloroacetic acid, Folin-Ciocalteu reagent, catechin, linoleic acid emulsion, Tween-20, thiobarbituric acid (TBA), diclofenac sodium, butylated hydroxyl anisole (BHA), sucrose, yeast extract, potassium chloride (KCl), ferrous sulphate (FeSO₄·7H₂O), sodium nitrate, dipotassium hydrogen phosphate (K₂HPO₄), magnesium sulphate (MgSO₄·7H₂O), and ascorbic acid used were of analytical grade and obtained from E-Merck, India. 1,1-diphenyl,2-picryl hydrazyl (DPPH), Carrageenan and 2,2-azinobis-(3-...
ethylbenzothiazoline-6-sulphonate) (ABTS) were obtained from Sigma Chemicals, USA.

Microorganism— Aspergillus candidus MTCC 2202 was obtained from the MTCC (Microbial Type Culture Collection, Chandigarh, India). The stock culture of A. candidus was grown on potato dextrose agar medium and maintained at 25°C.

Medium and culture conditions: — The medium used for cultivation of molds in this study consisted of (%): 3, sucrose; 0.1, yeast extract; 0.1, polypeptone; 0.1, K₂HPO₄; 0.05, MgSO₄.7H₂O; 0.05, KCl; and 0.001, FeSO₄. 7H₂O. From a petri plate culture, two agar blocks (5 mm in diam) of A. candidus was inoculated into 500 ml Hilton flask containing 100 ml of medium, and the flask was incubated at 25°C for 6 days on a rotary shaker set at 150 rpm.

Preparation of extract— The culture filtrate was separated from the mycelium by filtration through Whatman no.1 filter paper under vacuum and mixed with equal volume of ethyl acetate. The mixture was then shaken in a separating funnel and the phase separated ethyl acetate layer was collected. The ethyl acetate extracts were dehydrated with anhydrous sodium sulphate (1 % w/v). The solvent was removed in vacuo using a rotary evaporator below 30°C. The residue obtained was then tested for antioxidant and anti-inflammatory properties.

Animals— Healthy Wistar albino rats of either sex (Haffkin Biopharmaceutical Corporation Ltd., Parel, Mumbai) and of approximately the same age, weighing about 150-200 g, were used for the study. They were fed with standard pellet diet (Amrut Ind Ltd., Pune, Maharashtra) and water ad libitum. They were housed in polypropylene cages maintained under standard condition (12 h light/dark cycle; 25°C ±3°C, RH 35-60%).

Experimental protocols were approved by our institutional ethics committee, which follow guidelines of CPCSEA (Committee for the purpose of control and supervision of experiments on animals) that complies with international norms of INSA.

DPPH radical scavenging assay— DPPH (1,1-diphenyl-2-picryl hydrazyl) scavenging activity was measured by the spectrophotometric method. To an ethanolic solution of DPPH (200 µM), 0.05 ml of ethyl acetate extract dissolved in ethanol were added at different concentrations (50-2000 µg/ml). An equal amount of ethanol was added to the control. After 20 min the decrease in absorbance of test solutions (due to quenching of DPPH free radicals) was read at 517 nm and percentage inhibition calculated.

ABTS radical cation decolorization assay— ABTS radical cation (ABTS⁺) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulphate and the mixture was allowed to stand in dark at room temperature for 12-16 h before use. For the study, different concentrations (50-2000 µg/ml) of the ethanolic solution and the final volume were made up with ethanol to make 1 ml. The absorbance was read at 745 nm and the percentage inhibition calculated.

Reducing power—The reducing power of the extract was determined according to the method of Oyaizu. Different concentrations of extract (50-2000 µg/ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 ml of 10 %) was added to it, the mixture was mixed and centrifuged at 650 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of ferric chloride (1%), and the absorbance was measured at 700 nm. Higher absorbance indicated higher reducing power.

Determination of antioxidant activity using linoleic acid emulsion system— Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween-20 as emulsifier and 50 ml of phosphate buffer (pH 7.2). The mixture was homogenized for 5 min, and the antioxidant was added at the final concentrations of 50-2000 µg/ml of extract. The mixture was incubated in an oven at 37°C for 24 hr and the course of oxidation was monitored by measuring conjugated dienes formation and thiobarbituric acid reactive substances (TBARS).

Estimation of conjugated dienes (CD) formation— Aliquots of 20 µl were taken every hour from the emulsion during 24 hr of incubation. To each aliquot, 2 ml of methanol in deionized water (60 %) were added, and the absorbance of the mixture was measured at 233 nm (Jasco UV-Visible spectrophotometer).

Estimation of thiobarbituric acid reactive substances— Sample (100 µl) was taken every hour from the emulsion and the followings were sequentially added - 100 µl, BHA (3.6 %) and 2 ml of TBA-TCA solution [20 mM TBA in 15% trichloroacetic acid (TCA)]. The mixture was heated in a water bath at 90°C for 15 min and cooled at room
temperature. Two ml of chloroform was added to it, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and absorbance of the supernatant was measured at 532 nm (Jasco UV-Visible spectrophotometer) against a blank containing 0.1 ml of double distilled water and 2 ml of TBA-TCA solution. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content.

**Total phenolic content**— The total phenolic content of various extracts of *A. candidus* broth filtrate was determined using Folin-Ciocalteu reagent. 

**Evaluation of anti-inflammatory activity**— The ethyl acetate extract was evaluated for its anti-inflammatory activity by carrageenan-induced rat paw oedema model. The animals were divided into three groups of six animals each. The first group served as vehicle control (saline with a drop of Tween-20) and the second group received 125 mg/kg ip body weight of EAEAC broth filtrate. The third group served as positive control and received diclofenac 9 mg/kg body weight. Food was withdrawn overnight, but adequate supply of water was given to the rats before the experiment. The drugs were given intraperitoneally. After half an hour, a sub-plantar injection of 0.1 ml of 1% carrageenan was administered in the left hind paw to all the three groups. The paw volume was measured plethysmographically at 0, 1, 3, and 5 h. The average paw of swelling in a group of extract treated rats was compared with control group (treated with vehicle) and the positive control (diclofenac).

**Statistical analysis**— The data was subjected to unpaired Student’s *t* test. Values have been expressed as mean ± SD. *P*<0.05 were considered statistically significant.

**Results**

**Antioxidant activity**— Several concentrations, ranging from 50-2000 µg/ml of ethyl acetate extract of *A. candidus* broth filtrate was tested for their antioxidant activity in different *in vitro* models. It has been observed that antioxidant activity exerted by ethyl acetate extract of *A. candidus* (EAEAC) broth filtrate increased with increasing concentrations in all models. Maximum percentage inhibition in all the models *viz*, DPPH, ABTS, conjugated dienes and thiobarbituric acid reactive substances were found to be 96.65, 92.25, 59.56 and 51.45%, respectively at 1 mg/ml concentration.

On a comparative basis, the extract showed better activity in quenching DPPH radical with an IC50 value of 430.36 µg/ml than ABTS radicals with an IC50 value of 606.29 µg/ml (Table 1). Reducing power of extract was good and increased with increasing concentration, and they showed moderate to good reducing ability in comparison with BHA and ascorbic acid; however, the activity was moderate in the remaining antioxidant models.

The content of phenolic compounds (µg/ml) in ethyl acetate, diethyl ether and hexane extract of *A. candidus* broth filtrate was determined from regression equation of standard curve for catechin (*y* = 0.0009X-0.0111, *R*2 = 0.9967) and expressed in catechin equivalents (CE), varied between 4.01 and 34.56 (Table 2). It was also observed that the content of phenolics in the extract correlates with their antiradical activity (e.g. correlation coefficient

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**Table 1**— Effect of EAEAC broth filtrate on different antioxidant models

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>DPPH</th>
<th>ABTS</th>
<th>Conjugated dienes</th>
<th>TBARS</th>
<th>Reducing power</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>95.09 ± 2.54</td>
<td>92.00 ± 2.6</td>
<td>55.05 ± 2.52</td>
<td>49.06 ± 0.98</td>
<td>0.5689 ± 0.036</td>
</tr>
<tr>
<td>1500</td>
<td>95.58 ± 3.57</td>
<td>92.30 ± 4.21</td>
<td>58.26 ± 4.82</td>
<td>49.25 ± 2.93</td>
<td>0.603 ± 0.025</td>
</tr>
<tr>
<td>1000</td>
<td>96.65 ± 2.76</td>
<td>92.25 ± 3.26</td>
<td>59.56 ± 4.59</td>
<td>51.45 ± 2.58</td>
<td>0.6354 ± 0.031</td>
</tr>
<tr>
<td>750</td>
<td>88.82 ± 1.09</td>
<td>76.54 ± 3.89</td>
<td>48.50 ± 2.7</td>
<td>44.05 ± 3.07</td>
<td>0.5524 ± 0.029</td>
</tr>
<tr>
<td>500</td>
<td>76.25 ± 2.62</td>
<td>60.46 ± 1.89</td>
<td>40.63 ± 3.29</td>
<td>42.25 ± 1.25</td>
<td>0.4586 ± 0.019</td>
</tr>
<tr>
<td>250</td>
<td>38.90 ± 1.67</td>
<td>29.18 ± 2.25</td>
<td>36.51 ± 3.87</td>
<td>35.55 ± 1.6</td>
<td>0.3045 ± 0.025</td>
</tr>
<tr>
<td>100</td>
<td>17.59 ± 2.1</td>
<td>11.58 ± 1.25</td>
<td>28.59 ± 1.45</td>
<td>31.89 ± 1.04</td>
<td>0.2578 ± 0.014</td>
</tr>
<tr>
<td>50</td>
<td>09.22 ± 1.11</td>
<td>05.52 ± 1.14</td>
<td>19.89 ± 2.45</td>
<td>30.21 ± 0.85</td>
<td>0.2014 ± 0.013</td>
</tr>
<tr>
<td>IC50</td>
<td>430.36</td>
<td>606.29</td>
<td>1144.72</td>
<td>1578.43</td>
<td>-</td>
</tr>
<tr>
<td>BHA (1 mg/ml)</td>
<td>98.50 ± 4.43</td>
<td>95.40 ± 4.59</td>
<td>85.17 ± 1.99</td>
<td>78.50 ± 1.58</td>
<td>0.8856 ± 0.015</td>
</tr>
<tr>
<td>Ascorbic acid (1 mg/ml)</td>
<td>82.56 ± 3.67</td>
<td>78.89 ± 3.99</td>
<td>66.05 ± 2.89</td>
<td>57.54 ± 1.98</td>
<td>0.7125 ± 0.035</td>
</tr>
</tbody>
</table>
between data of DPPH assay and total phenolic compounds is 0.991).

**Anti-inflammatory activity** — The EAEAC (125 mg/kg i.p) broth filtrate showed significant inhibition of oedema by 40.42 and 66.66 % at the end of 3 and 5 h, respectively, which was similar to the standard drug, diclofenac (Table 3).

**Discussion**

Lipid peroxidation causes oxidative damage not only in food system but also in human body. Peroxyl radical is a key step in lipid peroxidation and is an important cause of tissue damage and thus, oxidative stress. Antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and/or some other mechanisms.

DPPH is a relatively stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as antioxidants, the radical functions as scavenger and the absorbance is reduced. From the present results it may be postulated that ethyl acetate extract of *Aspergillus candidus* broth filtrate reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles.

DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up.

ABTS assay, another *in vitro* antioxidant screening method, applicable for both lipophilic and hydrophilic antioxidants. The assay is based on inhibition of the absorbance of radical cation, ABTS⁺ which has a characteristic long wavelength absorption spectrum. The results obtained implied the activity of the extract either by scavenging or inhibiting ABTS⁺ radicals, since both inhibition and scavenging properties of antioxidants towards ABTS⁺ radicals have been reported earlier.

A particular antioxidant can promote the formation of hydroperoxides at the early stage of oxidation and the same antioxidant may inhibit the formation of secondary oxidation products (aldehydes, ketones, alcohols and hydrocarbon) at later stages. Therefore, this extract was evaluated for its antioxidant properties using different lipid oxidation indicators (conjugated dienes hydroperoxides and TBARS) in an emulsion system model. It was observed that the inhibition of CD formation was higher than TBARS by extract, however, the control (BHA) and ascorbic acid showed more inhibition for CD and TBARS at the same concentration. This difference in extent of inhibition could be ascribed to several factors, including the indicators of the different steps of lipid oxidation, temperature, polarity of polyphenolic compounds present in the extract and the antioxidative mechanisms exhibited by the compounds. Last but not the least, synergistic effects of the different compounds involved could contribute to observed results.

Reducing power of the extract was strong and increased with increasing concentration, and it showed moderate to good reducing ability in comparison with BHA and ascorbic acid. Antioxidant activity has been reported to be concomitant with development of reducing power. This shows that extract might contain reductones formed during fermentation, which could react with free radicals to stabilize and terminate radical chain reaction.

High content of phenolic compounds was found in ethyl acetate extract, indicating that phenolic compounds in *A. candidus* were mainly soluble in ethyl acetate. By comparing the results of radical scavenging assay (DPPH) and total phenolic content of various extracts of *A. candidus* broth filtrate, good correlation between them can be observed confirming that phenolic compounds are likely to contribute to the radical scavenging activity of the extract.
EAEAC broth filtrate produced marked inhibition of carrageenan-induced rat paw inflammation, a test that has a significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation\textsuperscript{29}. Carrageenan induces an inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 2 h after injection of carrageenan, has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability\textsuperscript{30}. Inflammation volume reaches its maximum approximately 3 h post treatment after which it begins to decline\textsuperscript{31}. The late phase, which is also a complement-dependent reaction, has been shown to be due to overproduction of prostaglandin in tissues\textsuperscript{32}. EAEAC broth filtrate inhibited the inflammation by acting on late phases, which suggested that anti-inflammatory activity of extract mediated by inhibiting the overproduction of prostaglandin.

\textit{A. candidus} is known that produces flavonoid metabolites\textsuperscript{33}. Flavonoids are natural products, which have been shown to possess various biological properties related to antioxidant and anti-inflammatory mechanisms\textsuperscript{34,35}. Thus, the antioxidant and anti-inflammatory potential of EAEAC broth filtrate may be attributed to the presence of flavonoids therein.

**Acknowledgement**

The authors are thankful to the Department of Biotechnology, New Delhi for providing financial assistance and Institute of Microbial Technology, Chandigarh, India, for providing the microbial strain for the study.

**References**