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In vitro antioxidant studies of *Annona squamosa* Linn. Leaves

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The free radical scavenging potential of the leaves of *A. squamosa* was studied by using different antioxidant models of screening. The ethanolic extract at 1000 µg/ ml showed maximum scavenging of the radical cation, 2,2- azinobis- (3-ethylbenzothiazoline- 6- sulphonate) (ABTS) observed upto 99.07% followed by the scavenging of the stable radical 1,1-diphenyl, 2- picryl hydrazyl (DPPH) (89.77 %) and nitric oxide radical (73.64 %) at the same concentration. However, the extract showed only moderate scavenging activity of superoxide radicals and antilipid peroxidation potential, which was performed using rat- brain homogenate. The findings justify the therapeutic applications of the plant in the indigenous system of medicine, augmenting its therapeutic value.

Keywords: *Annona squamosa*, Antioxidant, Free radicals

There is extensive evidence to implicate free radicals in the development of degenerative diseases[1]. Free radicals have been implicated in causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity etc[2]. Together with other derivatives of oxygen they are inevitable byproducts of biological redox reactions[3]. Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical (.OH) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation[4], and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered to be a universal feature of stress conditions. Plants and other organisms have evolved a wide range of mechanisms to contend with this problem, with a variety of antioxidant molecules and enzymes.

Annona squamosa Linn[5]. (Annonaceae), popularly known as custard apple is cultivated all over India for its edible fruit. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, fainting, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors, ulcers and also as an abortifacient[6, 7]. Several tribal populations in northern India use the leaves of the plant to cure diabetes[8, 9]. A detailed review of literature afforded no information on the antioxidant potential of the plant. It was therefore thought worthwhile to investigate the antioxidant potential of A*nnona squamosa* (AS).

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Materials and Methods

All chemicals and solvents were of analytical grade and were obtained from Ranbaxy Fine Chemicals, Mumbai, India. 1,1-diphenyl, 2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. The other chemicals used were 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulphonate) (ABTS), sodium nitroprusside, sulphanilamide, potassium superoxide, O-phosphoric acid, napthyl ethylene diamine dihydrochloride, potassium chloride (KCl), ferrous sulphate ($FeSO_4$), thio barbituric acid (TBA), trichloro acetic acid (TCA), butyrated hydroxy toluene (BHT), nitroblue tetrazolium (NBT), dimethyl sulphoxide (DMSO), ethylene diamine tetra acetic acid (EDTA) and sodium hydroxide (NaOH).

Plant material—The leaves of AS were collected during July 2002 from Painkulam village in Kanyakumari district, Tamil Nadu. The plant material was authenticated by Dr. Stephen Julius, botanist, Nesamony Memorial Christian College, Marthandam, Tamil Nadu. A voucher specimen (PP 519) has been submitted in the herbarium of the college.

Plant extract—About 350 g of the shade dried, powdered leaves of AS were exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The residue was filtered and concentrated *in vacuo* to a syrupy consistency (yield 60 g). The extract was then stored in a dessicator until further use.

Preparation of rat brain homogenate[10]—Adult Wistar rats of either sex and of approximately the same age weighing about 200-250 g were used. The rats were fed with standard chow diet (Pranav Agro Industries Ltd., Sangli, Maharashtra) and water *ad libitum*. They were housed in polypropylene cages maintained under standard conditions (12: 12 hr L: D cycle; 25°±3°C; 35-60% RH). The experimental protocols were subjected to the scrutinization of the Institutional Animal Ethics Committee and were cleared by the same (No: IAEC/ KMC/ 01/ 2002-2003). Randomly selected rats were fasted overnight. They were sacrificed by cervical dislocation, dissected and the whole brain except cerebellum was removed quickly. It was further processed to get 10% homogenate in 0.15 *M* KCl[11] using a teflon homogeniser. The homogenate was filtered to get a clear solution and used as a source of polyunsaturated fatty aids for determining the extent of lipid peroxidation.

In vitro antilipid peroxidation assay—The extent of lipid peroxidation in rat brain homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS). Different concentrations of the extract (2-1000 μg/ml) were made up with ethanol. The ethanolic extract was expressed in terms of dry weight (mg/ml) in ethanol. These samples were individually added to the brain homogenate (0.5 ml). This mixture was incubated with 0.15 *M* KCl (100 μl). Lipid peroxidation was initiated by adding 100 μl of 15 m*M* $FeSO_4$ solution. The reaction mixture was incubated at 37°C for 30 min. An equal volume of TBA : TCA (1:1, 1 ml) was added to the above solution followed by the addition of 1 ml BHT. This final mixture was heated on a water bath for 20 min at 80°C and cooled, centrifuged and absorbance read at 532 nm[12] using a spectrophotometer (Shimadzu 160 IPC). The percentage inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the extract as per the formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

ABTS radical cation decolorization assay[13]—ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS solution (7 m*M*) with 2.45 m*M* ammonium persulfate and the mixture were allowed to stand in dark at room temperature for 12-16 hr before use. For the study, different concentrations (2-1000 μg/ml) of the ethanolic extract (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1 ml. The absorbance was read at 745 nm[10] and the percentage inhibition calculated by using the same formula as given above.

DPPH radical scavenging activity—DPPH scavenging activity was measured by the spectrophotometric method[14]. To an ethanolic solution of DPPH (200 μ*M*), 0.05 ml of test compounds dissolved in ethanol were added at different concentrations (2-1000 μg/ml). An equal amount of ethanol was added to the control. After 20 min the decrease in absorbance of test mixtures (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated[12].

Scavenging of nitric oxide radical[15,16]—Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction as described previously[17-18]. Sodium nitroprusside (5 m*M*) in standard phosphate buffer solution was incubated with different concentrations (2-1000 μg/ml) of the ethanol extract dissolved in phosphate buffer (0.025 *M*, *pH* : 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess' reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Scavenging of superoxide radical (potassium superoxide assay)—The scavenging activity towards the superoxide radical ($O_2^{\cdot -}$) was measured in terms of inhibition of generation of $O_2^{\cdot -}$ [19]. The method was performed by using alkaline DMSO method[20]. Potassium superoxide and dry DMSO were allowed to stand in contact for 24 hr and the solution was filtered immediately before use. Filtrate (200 μl) was added to 2.8 ml of an aqueous solution containing NBT (56 μ*M*), EDTA (10 μ*M*) and potassium phosphate buffer (10 m*M*). Test compounds (1 ml) at various concentrations (2-1000 μg/ml) were added and the absorbance was recorded at 560 nm against a control in which pure DMSO was added instead of alkaline DMSO.

Statistical analysis—Linear regression analysis was used to calculate the IC_{50} values.

Results

Several concentrations, ranging from 2 - 1000 μg/ml of the ethanolic extract of AS were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner upto the given concentration in all the models (Table1). The maximum percentage inhibition in all the models *viz*, ABTS, DPPH, nitric oxide, lipid peroxidation and superoxide dismutase were found to be 99.07, 89.77, 73.64, 51.61 and 51.71% respectively at 1 mg/ml concentration. On a comparative basis the extract showed better activity in quenching ABTS with an IC_{50} value of 40 μg/ml and DPPH radicals with an IC_{50} value of 110 μg/ml (Table 1). However, the extract showed encouraging response in quenching nitric oxide radicals (IC_{50} value –60 μg/ml). The activity was moderate in the remaining antioxidant models.

Discussion

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[2]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent disease[21].

Table 1—Effect of alcoholic extract of AS on different antioxidant models
[Values are mean of 3 replicates]

Conc. (μg/ml)	Inhibition (%)				
	DPPH	ABTS	Superoxide dismutase	Nitric oxide	Lipid peroxidation
1000	89. 78	99. 08	51. 71	73. 65	51. 61
500	88. 18	97. 47	51. 42	72. 81	46. 71
250	77. 66	97. 47	51. 17	69. 20	44. 76
125	76. 89	87.79	51. 17	51. 66	44. 44
63	34. 79	74. 88	37. 18	51. 60	43. 09
32	17. 20	47. 00	32. 35	41. 46	42. 77
16	02.33	32. 03	21. 82	40. 65	38. 99
8	01.76	26. 04	15. 63	40. 54	34. 41
4	01.18	14. 98	15. 54	38. 16	34. 17
2	0. 58	09. 45	15. 40	35. 50	33. 49
IC_{50} (μg/ml)	110	40	115	60	955

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl- perferryl complex[22] or through · OH radical by Fenton reaction[23] thereby initiating a cascade of oxidative reactions. The results obtained in the present studies may be attributed to several reasons *viz*, the inhibition of ferryl- perferryl complex formation; scavenging of OH or superoxide radical or by changing the ratio of Fe^{3+}/ Fe^{2+}; reducing the rate of conversions of ferrous to ferric or by chelation of the iron itself[24]. The moderate activity of the extract may probably be due to the rapid and extensive degradation of the antioxidant principles in an *ex vivo* state thereby corroborating the finding that was observed in a study carried out in Australia with a group of human volunteers[25]. It is also known that the · OH radical which initiates lipid peroxidation has a very short life time (10^{-9} s at 37° C) and is hence very difficult to investigate by conventional methods[26].

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long wavelength absorption spectrum[19]. The results obtained imply the activity of the extract either by inhibiting or scavenging the $ABTS^{\cdot+}$ radicals since both inhibition and scavenging properties of antioxidants towards $ABTS^{\cdot+}$ radicals have been reported earlier[13,27].

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases[28,29]. In the present study the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the ethanolic extract of AS. This may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide[30] thereby inhibiting the generation of nitrite.

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical $DPPH^{\cdot}$. From the present results it may be postulated that AS reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles[19]. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stoichometrically depending on the number of electrons taken up[31].

Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide[32]. Superoxide anion is the first reduction product of oxygen[33] which is measured in terms of inhibition of generation of $O_2^{\cdot-}$.

Leaves of AS are rich in flavonoids like rutin and hyperoside[6]. Flavonoids are natural products, which have been shown to possess various biological properties related to antioxidant mechanisms[34-39]. Thus, the antioxidant potential of AS observed in the present study may be attributed to the presence of flavonoids therein.

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