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In vitro antioxidant studies in leaves of *Annona* species

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Antioxidant potential of leaves of three different species of *Annona* was studied by using different *in vitro* models eg., 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothizoline-6-sulphonate) (ABTS), nitric oxide, superoxide, hydroxy radical and lipid peroxidation. The ethanolic extract of *A.muricata* at 500 µg/ml showed maximum scavenging activity (90.05%) of ABTS radical cation followed by the scavenging of hydroxyl radical (85.88%) and nitric oxide (72.60%) at the same concentration. However, the extract showed only moderate lipid peroxidation inhibition activity. In contrast, the extract of *A.reticulata* showed better activity in quenching DPPH (89.37%) and superoxide radical (80.88%) respectively. *A.squamosa* extract exhibited least inhibition in all *in vitro* antioxidant models excepting hydroxyl radical (79.79%). These findings suggest that the extracts of *A. muricata* possess potent *in vitro* antioxidant activity as compared to leaves of *A. squamosa* and *A.reticulata* suggesting its role as an effective free radical scavenger, augmenting its therapeutic value.

Keywords: *Annona* species, Antioxidant, Free radicals, Lipid peroxidation

Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and aging[1]**.** Free radicals induced by peroxidation have gained much importance because of their involvement in several pathological conditions such as atherosclerosis, ischemia, liver disorder, neural disorder, metal toxicity and pesticide toxicity[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 ($OH^{\cdot}$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing injury through covalent binding and lipid peroxidation[4]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease[5]. Foods rich in antioxidants have been shown to play an essential role in the prevention of cardiovascular diseases, cancer, neurodegenerative diseases, inflammation and problems caused by cell and cutaneous aging[6].

Annona species belonging to Custard Apple family is cultivated all over India for its edible fruit. All parts of *Annona* are used in natural medicine in the tropics. It is considered to be good source of natural antioxidants for various diseases. Therefore, attention in recent times has been focused on the isolation, characterization and utilization of natural antioxidants. Of the species, *Annona squamosa* (AS) 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[7].Fruits of *Annona reticulata* (AR) are astringent, sweet and useful in blood complaints. It is also used as anti-dysentric and anti-helminthic[8]. Its leaves and stem constituents include anonaine, roemerine, corydine, isocorydine and many other aporphine alkaloids[8].

Fruits of *Annona muricata* (AM), also known as Graviola in South America are taken internally for worms and parasites, to cool fevers, to increase mother's milk after child birth and as an astringent for diarrhoea and dysentery. Leaves are considered antitumorous[9], sedative, anti-spasmodoic[10] and hypotensive[11].

Preliminary reports have confirmed the antioxidant potential of AS in different *in vitro* models. This has been attributed to the presence of flavonoids like rutin and hyperoside in leaves[12]. In contrast, the leaves of AM are rich in Annonaceous acetogenins responsible for its antitumorous property[9]. However, no reports

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are available to show the antioxidant property of the plant with relation to its anticancer activity. Hence, the present study has been undertaken to investigate the antioxidant activity of the leaves of *Annona muricata* with reference to the two other species of *Annona* i.e. *A. squamosa* and *A. reticulata* using different *in vitro* models.

Materials and Methods

Materials—All chemicals and solvents were of analytical grade and were obtained from HiMedia Chemicals, Mumbai, India. 2,2-azinobis (3-ethylbenzo-thiozoline-6-sulphonate) (ABTS) was obtained from Sigma Chemicals, USA. The other chemicals used were 1,1-diphenyl, 2-picryl hydrazyl (DPPH), sodium nitroprusside, sulphanilamide, o-phosphoric acid, napthyl ethylene diamine dihydrochloride, ferrous sulphate ($FeSO_4$), thiobarbituric acid (TBA), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT) and ethylene diamine tetra acetic acid (EDTA).

Plant material—The leaves of AS, AR and AM were collected during December 2005 from Coimbatore, Tamil Nadu. The plant material was authenticated by the Agricultural Officer, Horticultural Research Station, Coonoor, Tamil Nadu. Voucher specimens (VR 101, 102, 103) of the samples have been deposited in the herbarium of Dr G R Damodaran College of Science, Coimbatore.

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

DPPH radical scavenging activity—DPPH scavenging activity was measured by the spectrophotometric method[13]. To a methanolic solution of DPPH (200μM), 0.05ml of the test compounds dissolved in ethanol were added at different concentration (100 – 500 μg /ml). An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula[14]:

$$\text{Inhibition (\%)} = \frac{(\text{control} - \text{test})}{\text{control}} \times 100$$

ABTS radical cation decolorisation assay[15]—In this improved version, ABTS.-, the oxidant is generated by persulfate oxidation of 2,2,-azinobis (3-ethylbenzoline-6- sulfonic acid) – ($ABTS^{2-}$).

ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12 – 16 hr before use. For the study, different concentration (100 – 500 μg/ml) of 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 and the percentage inhibition calculated.

Scavenging of nitric oxide radical[16,17]—Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction[18,19]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentration (100 – 500 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% napthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthylethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Scavenging of hydroxyl radical—Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao[20] by studying the competition between deoxyribose and test extract for hydroxyl radical generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8 m*M*), $FeCl_3$ (0.1 m*M*), EDTA (0.1 m*M*), H_2O_2 (1 m*M*), ascorbate (0.1 m*M*), KH_2PO_4- KOH buffer (20 m*M*, *p*H 7.4) and various concentrations of the sample extracts in a final volume of 1.0 ml. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reacting substances (TBARS) and the percentage inhibition calculated.

Scavenging of superoxide radical—The scavenging activity towards the superoxide radical ($O_2^{\cdot-}$) was measured in terms of inhibition of generation of $O_2^{\cdot}$ (ref. 21). The reaction mixture consisted of phosphate buffer (50 m*M*, *p*H 7.6), riboflavin

(20 µg/0.2 ml), EDTA (12 mM), NBT (0.1 mg/3ml) and sodium cyanide (3 µg/0.2 ml) Test compounds of various concentrations of 100 -500 µg/ml were added to make a total volume of 3 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 min against a control instead of sample. The percentage inhibition was calculated by using the same formula as given above.

In vitro anti-lipid peroxidation assay—Freshly excised goat liver was processed to get 10% homogenate in cold phosphate buffered saline, *p*H 7.4 using glass teflon homogenizer and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the TBARS by using the standard method[22] with minor modifications[14]. Different concentrations of the extracts (100 – 500 µg/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100 µl of 15 mM ferrous sulphate solution to 3 ml of the tissue homogenate. After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10 % TCA. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50 % acetic acid. The mixture was heated for 30 min in a boiling water bath. The intensity of the pink coloured complex formed was measured at 535 nm. The results were expressed as percentage inhibition using the formula as given above.

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

Results

Several concentrations ranging from 100-500µg/ml of the ethanolic extract of AS, AR and AM 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 in all the models. From the results given in Tables 1-3, it was inferred that, with respect

Table 1— Effect of ethanolic extract of AS on different antioxidant models

[Values are mean of 3 replicates]

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	75.12	10.45	34.69	37.56	10.29	7.64
200	78.88	33.83	40.27	45.60	26.47	11.70
300	86.23	50.25	45.96	52.46	50.00	26.24
400	87.47	72.14	52.60	69.78	66.91	44.52
500	88.77	88.06	68.03	79.79	77.21	50.83
IC_{50}(µg/ml)	65	300	370	300	300	480

Table 2— Effect of ethanolic extract of AR on different antioxidant models

[Values are mean of 3 replicates]

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	63.75	3.98	39.47	40.80	13.97	6.31
200	69.38	35.32	47.44	57.51	34.56	9.97
300	75.00	51.74	55.40	65.03	52.92	10.63
400	81.63	73.63	64.39	69.43	66.18	30.23
500	89.37	89.05	71.10	77.72	80.88	35.54
IC_{50} (µg/ml)	80	260	225	215	285	315

Table 3—Effect of ethanolic extract of AM on different antioxidant models

[Values are mean of 3 replicates

Conc. (µg/ml)	Inhibition (%)					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	72.72	4.48	32.65	44.05	16.18	3.65
200	76.23	27.36	39.02	54.53	33.09	7.30
300	80.74	49.25	43.45	63.73	49.26	12.63
400	83.89	74.00	56.31	69.80	66.18	39.80
500	88.77	90.05	72.60	85.88	78.68	58.47
IC_{50} (µg/ml)	70	305	350	155	300	455

to maximum percentage inhibition in all models viz., ABTS, nitric oxide, hydroxyl radical, lipid peroxidation, the extracts of AM showed 90.05, 72.60, 85.88, 58.47% inhibition respectively at 500µg/ml concentration. In contrast, the extracts of AR showed 89.37 and 80.88 % inhibition in quenching DPPH and superoxide radical respectively at the same concentration. However, the IC_{50} value of AR for ABTS, nitric oxide and hydroxyl radical were shown to be 260, 225 and 215 µg/ml respectively. The extracts of AS showed least inhibition at 500 µg/ml concentration in all the models excepting hydroxyl radical (79.79 %).

Discussion

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place through ferryl-perferryl complex[23] or through ·OH radical by Fenton's reaction[24], thereby initiating a cascade of oxidative reactions. The results obtained in the present study 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 [25].

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals[26]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals.

The 1, 1-diphenyl -2-picryl hydrazyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition.

DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electrons become paired off and solution loses color stochiometrically depending on the number of electrons taken up[27]. DPPH was used to determine the proton radical scavenging action of extracts of AS, AR and AM, because it possesses a proton free radical and shows a characteristic absorbance at 517 nm. From the present results, it may be postulated that AR reduces the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles[21].

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[21]. The results imply that the extracts of AM inhibit or scavenge the $ABTS^{\cdot +}$ radicals since both inhibition and scavenging properties of antioxidant towards $ABTS^{\cdot +}$ radicals have been reported earlier[15, 28].

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological process[29]. Excess concentration of NO is associated with several diseases[30,31]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals[26, 32]. In the present study, the ethanolic extract of AM showed better activity in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions.

The hydroxy radical scavenging activity is measured as the percentage of inhibition of hydroxy radicals generated in the Fenton's reaction mixture[25] by studying the competition between deoxyribose and the extract for hydrogen radicals generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 systems. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation. From the present results, it is observed that the extract of AM have better hydroxyl radical scavenging activity as reflected in terms of percentage inhibition.

Superoxides are produced from molecular oxygen due to oxidative enzymes[32] of body as well *via* non-enzymatic reaction such as autooxidation by catecholamines[33]. The scavenging activity towards the superoxide radical ($O_2^{\cdot -}$) is measured in terms of inhibition of generation of $O_2^{\cdot -}$. In the present study, superoxide radical reduces NBT to a blue colored formosan that is measured at 560 nm[34]. The result shows that extract of AR has a potent scavenging activity with increasing percentage inhibition. The

probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of the extract towards generation of superoxides in the *in vitro* reaction mixture.

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radicals induces lipid peroxidation in polyunsaturated lipid rich areas like brain and liver[35]. Initiation of lipid peroxidation by ferrous sulphate takes place through hydroxy radical by Fenton's reaction[25]. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxy radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. In this study, *in vitro* lipid peroxidation was induced in goat liver by using $FeSO_4$ and ascorbic acid. The present results show that extract of AM shows better dose-dependent prevention towards generation of lipid peroxides.

From the above results, it can be concluded that ethanolic extracts of leaves of AM showed the most potent *in vitro* antioxidant activity with high percentage inhibition as compared to leaves of AS and AR. This may be attributed to the presence of acetogenins, which probably play a role as an effective free radical scavenger and hence an effective antitumorous agent.

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