OPEM
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Oriental Pharmacy and Experimental Medicine 2008 **8(3)**, 260-265
DOI 10.3742/OPEM.2008.8.3.260

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Free radical scavenging property of *Annona reticulata* leaves

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Received for publication March 30, 2007; accepted December 07, 2007

SUMMARY

Annona reticulata belonging to the family Annonaceae is traditionally used as anthelmintic and it is reported to have anticancer activity. Free radicals are known to be involved in various types of diseases like cancer, diabetes, neurological disorders etc. The present study was aimed to evaluate free radical scavenging property of methanol extract of the leaves of *Annona reticulata* in some *in vitro* models e.g. DPPH, nitric oxide, superoxide and lipid peroxide radical model. The extract showed good dose-dependent free radical scavenging property in all the models. IC_{50} values were found to be 3.22, 170.01, 25.12 and 140.12 μg/ml respectively in DPPH, nitric oxide, superoxide and lipid peroxidation inhibition assays. Reductive ability of the extract was also tested based on potassium ferricyanide reduction where dose dependant reducing capability was observed. Measurement of total phenolic compounds by Folin-Ciocalteu's phenol reagent indicated that 1mg of the extract contained 146.20 μg equivalent of pyrocatechol. The findings ascertain promising free radical scavenging property of the extract and the antioxidant property of the extract may be due to the high content of phenolic compounds.

Key words: *Annona reticulata*; Free radicals; Reductive ability; Phenolic compound

INTRODUCTION

Oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals are collectively termed as reactive oxygen species (ROS) and have been implicated in the pathogenesis of various diseases (Yu, 1994; Halliwell, 1997; Lata and Ahuja, 2003). When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders (Yu, 1992; Campbell and Abdulla, 1995; Cotran *et al.*, 1999). The oxidative stress has also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders, as well as in the process of aging (Marx, 1987). Many plants contain substantial amounts of antioxidants and can be utilized to scavenge the excess free radicals from human body (Pratt, 1992).

Annona reticulata belonging to the family Annonaceae is a medium size plant found in countries with tropical climate like India (Hooker, 1985). Its fruit is popular as Custard apple and bullock's heart. The unripe fruit is considered to be

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an anthelmintic whereas the bark, a powerful astringent. The leaves and seeds possess insecticidal properties (Maeda *et al.,* 1993). The plant contains a number of acetogenins which are known to bear anticancer property via Bax-, Bad- and caspase-3 related pathways (Yuan *et al*., 2003, 2006). The methanol extract of the leaves showed cytotoxicity against CaCO2 and Hep G2 cell lines (Mondal *et al.,* 2007). This extract also showed inhibition of human recombinant caspases (Mondal *et al.,* 2007). Different phytochemicals isolated from bark include reticulatacin, reticulacinone, bullatacin, liriodenine, kaurane diterpene (Etse *et al.,* 1987; Saad *et al.*, 1991; Hisham *et al.,* 1994) and that from fruit include squamocin (Duval *et al.,* 2005) and annonacin (Chang *et al.,* 1993). However there is no phytochemical report on leaves.

The present study was directed to evaluate the antioxidant activity of *Annona reticulata* leaves by investigating free radical scavenging property in different *in vitro* models.

MATERIALS AND METHODS

Plant material

The leaves of *Annona reticulata* was collected during its fruiting season from Bangaon, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (PG - 212) was retained in our laboratory for further reference.

Plant extract

The leaves were dried under shade and powdered in a mechanical grinder. The powdered material was extracted successively with petroleum ether (60 - 80 °C), chloroform and methanol using soxhlet apparatus. The methanol extract was concentrated in *vacuo* and kept in a vacuum dessicator for complete removal of solvent. The yield was 7.5% w/w with respect to dried powder. The methanol extract of *Annona reticulata* (MEAR) was used for the present study.

Experimental procedure

All the following experiments (i-vi) were performed thrice and the results averaged.

i) DPPH radical scavenging activity

1,1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity was measured according to the method of Cotelle *et al.* (1996) with some modification as done by Mondal *et al.* (2006). In brief, 3 ml of reaction mixture containing 200 µl of DPPH (100 µM in methanol) and MEAR (at various concentrations; 1 - 32 µg/ml in methanol) was incubated at 37 °C for 30 min and absorbance of the test mixture was read at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the following formula (Mondal *et al.,* 2006).

Percentage inhibition = (Absorbance of control - Absorbance of test) × 100/Absorbance of control

ii) Nitric oxide radical scavenging activity

Nitric oxide (NO) radicals were generated from sodium nitroprusside solution in buffer saline and measured by Griess reagent (Marcocci *et al.,* 1994; Sreejayan and Rao 1997). 1 ml of 10 mM sodium nitroprusside was mixed with 1 ml of MEAR of different concentrations in phosphate buffer (pH 7.4). The mixture was incubated at 25 °C for 150 min. To 1 ml of the incubated solution, 1 ml of Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% Napthyl ethylene diamine dihydrochloride) was added (Mondal *et al.,* 2006). Absorbance was read at 546 nm and percentage inhibition was calculated using the formula shown above.

iii) Superoxide radical scavenging activity

Superoxide scavenging activity of MEAR was measured according to the method of Robak and Gryglewski (1998) with some modification. All the solutions were prepared in 100 mM phosphate

buffer (pH 7.4). 1ml of nitroblue tetrazolium (NBT, 156 μM), 1 ml of reduced nicotinamide adenine dinucleotide (NADH, 468 μM) and 3 ml of MEAR (to produce final concentrations of 10 - 320 μg/ml) were mixed. The reaction was started by adding 100 μl of phenazine methosulphate (PMS, 60 μM). The reaction mixture was incubated at 25 °C for 5 min followed by measurement of absorbance at 560 nm. The percentage inhibition was calculated from the above formula.

iv) Inhibition of lipid peroxidation

Rat liver served as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation (Prasanth *et al.,* 2000; Mondal *et al.,* 2006). Wistar albino male rat (130 - 170 g body weight) was sacrificed under ether anesthesia and liver was collected immediately. The liver was homogenized with M/150 phosphate buffer (pH 7.4) and centrifuged at 3,000 rpm for 10 min to get a clear supernatant. 4 ml of reaction mixture containing 0.5 ml of supernatant, MEAR solution of different concentrations (10 - 215 μg/ml) and 100 μl of each of 0.15 M potassium chloride, 15 mM ferrous sulphate ($FeSO_4$) and 6 mM ascorbic acid was incubated at 37 °C for 1 h. 1 ml of trichloroacetic acid (TCA, 10%w/v) was added to the mixture and the samples were centrifuged at 3,000 rpm for 20 min at 4 °C to remove insoluble proteins. 2 ml of the supernatant was removed and 1 ml thiobarbituric acid (TBA, 0.8% w/v) was added to this and heated on water bath for 20 min. Further 20 min was allowed for cooling. The coloured TBA-MDA complex was extracted with 2 ml butanol and absorbance was measured at 532 nm. Percentage inhibition was calculated as above.

v) Reductive ability

Reducing power of MEAR was determined based on the ability of antioxidants to form colored complex with potassium ferricyanide, Trichloro acetic acid (TCA) and ferric chloride ($FeCl_3$), which is measured at 700 nm (Jayprakash *et al.,* 2001). 10 - 50 μg/ml of MEAR was mixed with 2.5 ml potassium ferricyanide (1%) and 2.5 ml of phosphate buffer (pH 6.5). The mixture was incubated at 50 °C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3,000 rpm for 10 min. 2.5 ml of the supernatant was taken. 2.5 ml of water and 0.5 ml of $FeCl_3$ (0.1%) were added to it. Absorbance was measured at 700 nm.

vi) Determination of total phenolic compounds

The content of total phenolic compounds in MEAR was determined by using Folin-Ciocalteu's phenol reagent and determining absorbance at 760 nm according to the method of Slinkard and Singleton (1977). The content was expressed as equivalent of pyrocatechol (μg) by using the following equation, which was obtained from a standard pyrocatechol graph (Mondal *et al.,* 2006).

Absorbance = 0.001 × pyrocatechol (μg) + 0.0033

vii) Preliminary phytochemical screening

MEAR was subjected to various preliminary phytochemical tests (Ravishankara *et al.,* 2002) for the presence or absence of various classes of compounds.

Statistical analysis

Linear regression analysis was used to calculate IC_{50} values where ever needed. All the results are shown as Average ± S.E.M.

RESULTS

The extract showed dose dependant free radical scavenging activity in all the *in vitro* models. The percentage inhibition in various models *viz.* DPPH, NO, super oxide radical and lipid peroxidation is shown in Figs. 1 and 2. IC_{50} values were found to be 3.22, 170.01, 25.12 and 140.12 μg/ml respectively. Reductive ability of the extract was also dose dependent which was indicated from the increase in absorbance with the increase in concentration of

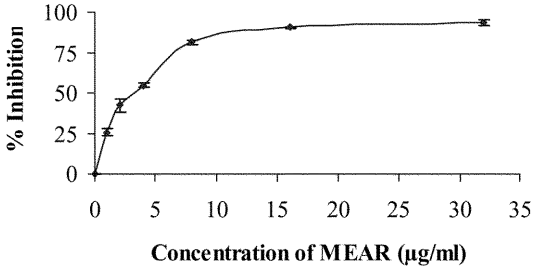

Fig. 1. Inhibition of DPPH radical by MEAR. Each data point is average of three replicates ± S.E.M.

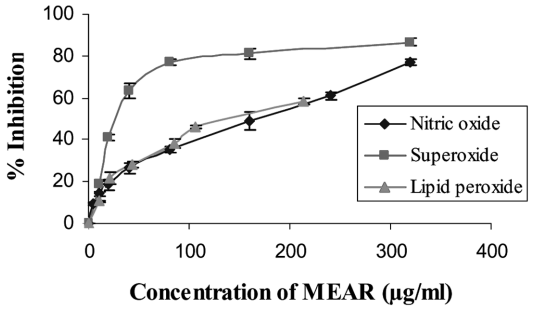

Fig. 2. Inhibition of NO, superoxide and lipid peroxide radicals by MEAR. Each data point is average of three replicates ± S.E.M.

MEAR. In this reductive ability experiment MEAR at 10, 20, 30, 40 and 50µg/ml showed absorbance of 0.237 ± 0.017, 0.447 ± 0.012, 0.511 ± 0.018, 0.598 ± 0.043 and 0.761 ± 0.012 respectively. Determination of total phenolic compounds showed that 1 mg of MEAR contains 146.20 ± 3.95 µg equivalent of pyrocatechol. Preliminary phytochemical analysis of the methanol extract showed the presence of alkaloid, flavonoids, tannin, gum, saponins and triterpenes.

DISCUSSION

During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets reduced to oxygen derived free radicals which are capable of attacking lipids, proteins and DNA leading to several diseases (Yu, 1994; Halliwell, 1997). Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation etc.

As DPPH is known to abstract the labile hydrogen atom from the antioxidants, it is widely used for screening of antioxidant activity of plant drugs (Ratty *et al.*, 1988; Vani *et al.*, 1997; Sanchez *et al.*, 1999, 2002). The assay determines the ability of MEAR to reduce DPPH radical to the corresponding hydrazine by converting the unpaired electrons to paired ones. Antioxidants can act by converting the unpaired electrons to paired ones. The dose dependent inhibition of DPPH radical (Fig. 1) indicates that MEAR causes reduction of DPPH radical in a stoichometric manner.

NO, because of its unpaired electron, is classified as free radical and displays reactivities with proteins and other free radicals (Khanam *et al.*, 2004). Generation of excessive NO is associated with several diseases (Ialenti *et al.*, 1993; Ross, 1993). Oxygen reacts with the excess NO to generate nitrite and peroxynitrite anions, which act as free radicals (Sainani *et al.*, 1997; Cotran *et al.*, 1999). In the present study the extract competes with oxygen to react with NO and thus inhibits the generation of the anions (Fig. 2).

Superoxides are produced from molecular oxygen due to oxidative enzymes (Sainani *et al.*, 1997) of our body as well as from non-enzymatic reaction such as autoxidation by catecholamines (Hemmani and Parihar, 1998). In the present study superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm (Khanam *et al.*, 2004). The dose dependent effect of MEAR in this regard is shown in Fig. 2 which signifies that probably MEAR renders inhibitory effect towards generation of super oxide anions in the *in vitro* reaction mixture.

Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver (Coyle and Puttfarcken, 1993). In this study *in vitro* lipid peroxidation was induced to rat liver by using $FeSO_4$ and ascorbic acid. MEAR shows dose dependent prevention towards generation of lipid peroxides (Fig. 2).

An antioxidant may reduce ROS by donating

hydrogen atom (Jayprakash *et al.,* 2001; Khanam *et al.,* 2004). The reducing property of MEAR implies that it is capable of donating hydrogen atom in a dose dependent manner. The high content of phenolic compounds in the extract may also be a contributing factor towards antioxidant activity because the phenolic compounds are known to have direct antioxidant property due to the presence of hydroxyl groups which can function as hydrogen donor (Duh *et al.,* 1999; Dreosti, 2000).

Preliminary phytochemical analysis of MEAR indicates the presence of flavonoids and tannins. These types of polyphenols are well known natural antioxidants due to their electron donating property which either scavenge the principal propagating radicals or halt the radical chain (Bors *et al.,* 1990; Pratt, 1992; Duh *et al.,* 1999; Sugihara *et al.,* 1999; Dreosti, 2000). Thus the antioxidant potential of methanol extract of *Annona reticulata* leaves may be due to the presence of polyphenolic compounds.

ACKNOWLEDGEMENTS

The help and cooperation of Mr. Sumit Kumar Shai and Ms. Monali Chakraborty to perform the experiments is gratefully acknowledged.

REFERENCES

Bors W, Heller W, Michel C, Saran M. (1990) Flavonoids as antioxidants: determination of radical scavenging efficiencies. *Meth. Enzymol.* **186**, 343-355.

Campbell IC, Abdulla EM. (1995) Strategic approaches to in vitro eurotoxicology, In: Approaches and Methods: Neurotoxicology, p. 495, Academic Press, London.

Chang FR, Wu YC, Duh CY, Wang SK. (1993) Studies on the acetogenins from the Formosan annonaceous plants. II. Cytotoxic acetogenins from Annona reticulata. *J. Nat. Prod.* **56**, 1688-1694.

Cotelle A, Bernier JL, Catteau JP, Pommery J, Wallet JC, Gaydou EM. (1996).

Antioxidant properties of hydroxy-flavones. *Free Radic. Biol. Med.* **20**, 35-43.

Cotran RS, Kumar V, Collins T. (1999) Robbin's Pathological basis of diseases, pp. 1, Thomson Press (I) Ltd., Noida, India.

Coyle JT, Puttfarcken P. (1993) Oxidative stress, glutamate and neurodegenerative diseases. *Science* **219**, 1184-1190.

Dreosti IE. (2000) Antioxidant polyphenols in tea, cocoa and wine. *Nutrition* **16**, 692-694.

Duh PD, Tu YY, Yen GC. (1999) Antioxidant activity of aqueous extract of harn jyur (Chrysanthemum morifolium Ramat*). Lebensm. Wiss. Technol.* **32**, 269-277.

Duval RA, Duret P, Lewin G, Peris E, Hocquemiller. (2005) Semisysnthesis and biological activity of aminoacyl triesters of squamocin, an annonaceous acetogenin. *Bioorg. Med. Chem.* **13**, 3773-3781.

Etse JT, Gray AI, Waterman PG. (1987) Chemistry in the annonaceae, XXIV. Kaurane and Kaur-16-ene diterpenes from the stem bark of Annona Reticulata. *J. Nat. Prod.* **50**, 979-983.

Halliwell B. (1997) Antioxidants and human disease: a general introduction. *Nutr. Rev.* **55**, S44-S52.

Hisham A, Sunitha C, Sreekala U, Pieters L, Bruyne D, Heuvel HVD, Claeys M. (1994) Reticulacinone, an acetogenin from Annona reticulata. *Phytochemistry* **l35**, 1325-1329.

Hemmani T, Parihar MS. (1998) Reactive oxygen species and oxidative DNA Damage. *Indian J. Physiol. Pharmacol.* **42**, 440-452.

Hooker JD. (1985) The Flora of British India, Vol 1, pp. 85, L Reeves, India.

Ialenti A, Moncada S, Di Rosa M. (1993) Modulation of adjuvant arthritis by endogenous nitric oxide. *Br. J. Pharmacol.* **110**, 701-706.

Jayprakash GK, Singh RP, Sakariah KK. (2001) Antioxidant activity of grape seed extracts on peroxidation models in vitro. *J. Agric. Food Chem.* **55**, 1018-1022.

Khanam S, Shivprasad HN, Kshama D. (2004) In vitro antioxidant screening models: a review. *Indian J. Pharm. Educ.* **38**, 180-183.

Lata H, Ahuja GK. (2003) Role of free radicals in health and disease. *Ind. J. Physio. & Allied Sci*. **57**, 124-132.

Maeda U, Hara N, Fujimoto Y, Srivastava A, Gupta YK, Sahai M. (1993) N-Fatty acyl tryptamines from *Annona reticulata. Phytochemistry.* **34**, 1633-1635.

Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. (1994) The nitric oxide scavenging properties of *Ginkgo biloba* extract EGB 761. *Biochem. Biophys. Res. Commun.* **201**, 748-755.

Marx JL. (1987) Oxygen free radicals linked to many diseases. *Science* **235**, 529-531.

Mondal SK, Chakraborty G, Gupta M, Mazumder UK. (2006) In vitro antioxidant activity of *Diospyros malabarica* Kostel bark. *Indian J. Exp. Biol.* **44**, 39-44.

Mondal SK, Mondal NB, Mazumder UK. (2007) *In vitro* cytotoxic and human recombinant caspase inhibitory effect of *Annona reticulate* leaves. *Indian J. Pharmacol.* **39**, 253-254.

Prasanth Kumar V, Sashidhara S, Kumar MM, Shidhara BY. (2000) Effect of Luffa echinata on lipid peroxidation and free radical scavenging Activity. *J. Pharm. Pharmacol.* **52**, 891-894.

Pratt DE. (1992) Natural antioxidants from plant material. In: Phenolic compounds in food and their effects on health II: Antioxidants and Cancer prevention (ACS Symposium Series 507), edited by Hang M, Ho C, Lee C p. 54-71, American Chemical Society, Washington DC.

Ratty AK, Sunamoto J, Das NP. (1988) Interaction of flavonoids with 1,1-Diphenyl picrylhydrazyl free radical scavenging activity, some structural aspects. *J. Pharm. Pharmacol.* **43**, 486-491.

Ravishankara MN, Shrivastava N, Padh H, Rajani M. (2002) Evaluation of antioxidant properties of root bark of *Hemidesmus indicus* R. Br. (Anantmul). *Phytomedicine* **9**, 153-160.

Robak J, Gryglewski RJ. (1998) Flavonoids are scavengers of superoxide Anions. *Biochem. Pharmacol.* **37**, 837-841.

Ross R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* **362**, 801-809.

Saad JM, Hui Y, Rupprecht K, Anderson JE, Kozlowski JF, Zhao G, Wood KV, McLaughlin JL. (1991) Reticulatacin: A new bioactive acetogenin from *Annona reticulata* (Annonaceae). *Tetrahedron* **47**, 2751-2756.

Sainani GS, Manika JS, Sainani RG. (1997) Oxidative stress: a key factor in pathogenesis of chronic diseases. *Med. Update.* **1**, 1.

Sanchez-Moreno C, Larrauri J, Saura-Calixto F. (1999) Free radical scavenging capacity of selected red and white wine. *J. Sci. Food Agric.* **79**, 1301-1304.

Sanchez Moreno C. (2002) Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Tech. Int.* **8**, 121-137.

Slinkard K, Singleton VL. (1977) Total phenol analysis; automation and comparison with manual methods. *Am. J. Enol. Vitic.* **28**, 49-55.

Sreejayan N, Rao MNA. (1997) Nitric oxide scavenging by curcuminoids. *J. Pharm. Pharmacol.* **49**, 105-107.

Sugihara N, Arakawa T, Ohnishi M, Furunko K. (1999) Anti- and pro-oxidative effects of flavonoids as meta induced lipid hydroperoxide- dependent lipid peroxidation in cultured hepatocytes loaded with alpha- linoleic acid. *Free Radic. Biol. Med.* **27**, 1313-1323.

Vani T, Rajani M, Sarkar S, Shishoo CJ. (1997) Antioxidant properties of ayurvedic formulation triphala and its constituents. *Int. J. Pharmacognosy.* **35**, 313-317.

Yu BP, Suescun EA, Yang SY. (1992) Effect of age–related lipid peroxidation on membrane fluidity and phospholipase A_2: modulation by dietary restriction. *Mech. Ageing Dev.* **65**, 17-33.

Yu BP. (1994) Cellular defenses against damage from reactive oxygen species. *Physiol. Rev.* **74**, 139-162.

Yuan SSF, Chang HL, Chen HW, Yeh YT, Kao YH, Lin KH, Wu YC, Su JH. (2003) Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax- and caspase-3-related pathway. *Life Sci.* **72**, 2853-2861.

Yuan SSF, Chang HL, Chen HW, Kuo FC, Liaw CC, Su JH, Wu YC. (2006) Selective cytotoxicity of squamocin on T24 bladder cancer cells at the S-phase via Bax-, Bad- and caspase-3-related pathways. *Life Sci.* **78**, 869-874.