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In vitro antioxidant activity of *Diospyros malabarica* Kostel bark

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Antioxidant activity of defatted methanol extract of *D. malabarica* bark was studied for its free radical scavenging property on different *in vitro* models e.g. 1,1-diphenyl-2-picryl hydrazyl (DPPH), nitric oxide, superoxide, hydroxyl radical and lipid peroxide radical model. The extract showed good dose-dependent free radical scavenging property in all the models except in hydroxyl radical inhibition assay. IC_{50} values were found to be 9.16, 13.21, 25.27 and 17.33 µg/ml respectively in DPPH, nitric oxide, superoxide and lipid peroxidation inhibition assays. In hydroxyl radical inhibition assay 1000 µg/ml extract showed only 10% inhibition with respect to the control. Measurement of total phenolic compounds by Folin-Ciocalteu's phenol reagent indicated that 1mg of the extract contained 120.7µg equivalent of pyrocatechol. The results indicate that the antioxidant property of the extract may be due to the high content of phenolic compounds. However, the underlying mechanism may not involve hydroxyl radical scavenging property.

Keywords: Antioxidant, *Diospyros malabarica,* Free radicals, Phenolic compound, Reductive ability

Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals[1,2] like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second[3]. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates[4-6] and this leads to a number of physiological disorders. Free radicals are involved in the development of degenerative diseases[6]. They have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders, and in the process of aging[7]. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins etc., and thus can be utilized to scavenge the excess free radicals from human body[8].

Diospyros malabarica Kostel (Synonym: *D. embryopteris, D. peregrina;* Ebenaceae) is a medium size evergreen plant found in India. In West Bengal, it is popular as Gab or Tinduk. The plant is traditionally used for the treatment of dysentery and menstrual problems. Its stem bark is used for the treatment of intermittent fever and fruit juice for healing of wound and ulcer[9,10]. The plant also possesses antifertility activity[11]. Stem bark of the plant is reported to have hepatoprotective[12] and hypoglycemic[13] activity. Some plants are known to possess hepatoprotective activity due to their antioxidant property[12,14,15]. It is also established that diabetes is associated with low level of antioxidants and many plants show hypoglycemic property due to their antioxidant potential[16-18]. As the plant under consideration possesses both hepatoprotective and hypoglycemic activity it may have good antioxidant property. Thus, the present study has been directed to investigate the antioxidant activity of *Diospyros malabarica* bark in different *in vitro* models.

Materials and Methods

All chemicals used were of analytical grade. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Sodium nitroprusside, sulphanilamide, *O*-phosphoric acid, napthyl ethylene diamine dihydrochloride, nitroblue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), 2-Deoxy-D-ribose, hydrogen peroxide, ascorbic acid, ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium chloride(KCl), potassium ferricyanide [$K_3Fe(CN)_6$], ethylene diamine tetra acetic acid

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(EDTA), tris hydrochloride buffer, Folin-Ciocalteu's phenol reagent (FCR) and all solvents were obtained from SISCO Research Laboratories Pvt. Ltd, Mumbai, India.

Animals—Swiss albino female mice (20±2g) and Wistar albino male rats (130–170g) were used for the present studies. They were housed in clean polypropylene cages (38×23×10 cm) with not more than six animals per cage and maintained standard laboratory condition (temperature 25°±2°C) with dark and light cycle (12/12 hr). They were allowed free access to standard pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*.

Plant material—The bark of *D.malabarica* was collected during February 2004 from Bangaon, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (PG - 211) was retained in our laboratory for further reference.

Plant extract—The bark was dried under shade and then powdered in a mechanical grinder. The powdered material was extracted successively with petroleum ether (60°–80°C) 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 9.1% w/w with respect to dried powder. Preliminary qualitative analysis of the methanol extract showed the presence of alkaloid, tannin, C-anthracene glycoside, saponins, reducing sugar and triterpenes. The methanol extract of *D. malabarica* (MEDM) was used for the antioxidant studies.

In vitro antioxidant study—MEDM was tested for its free radical scavenging property using different *in vitro* models. All experiments were performed thrice and the results averaged. The University Ethical Committee approved the use of animals for the lipid peroxidation assay (Ethical clearance number: 367001//C/CPCACA).

DPPH radical scavenging activity—DPPH radical scavenging activity was measured according to the method of Cotelle *et al.*[19] with some modification. In brief, 3 ml reaction mixture containing 200µl of DPPH (100 µ*M* in methanol) and 2.8 ml of MEDM (at various concentrations; 3-110 µg/ml) in methanol was incubated at 37° 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 formula[20]

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control-Absorbance of test})}{\text{Absorbance of control}} \times 100 \quad \ldots(1)$$

Nitric oxide radical scavenging activity[21,22]—Nitric oxide (NO) radicals were generated from sodium nitroprusside solution at physiological *p*H. Sodium nitroprusside (1ml of 10 m*M*) was mixed with 1 ml MEDM of different concentrations (3-110µg/ml) in phosphate buffer (*p*H 7.4). The mixture was incubated at 25°C for 150 min. To 1ml of the incubated solution, 1ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546nm and percentage inhibition was calculated using the formula (1).

Superoxide radical scavenging activity—Superoxide anion scavenging activity of MEDM was measured according to the method of Robak *et al*[23] with some modification. All the solutions were prepared in 100 mM phosphate buffer (*p*H 7.4). 1ml of NBT (156 µ*M*), 1ml of NADH (468 µ*M*) and 3ml of MEDM (to produce final concentrations of 3-110µg/ml) were mixed. The reaction was started by adding 100 µl of phenazine methosulphate (PMS) (60µ*M*) and the mixture then incubated at 25°C for 5 min followed by measurement of absorbance at 560 nm. The percentage inhibition was calculated from the formula (1).

Inhibition of hydroxyl radical—Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao[24], by studying the competition between deoxyribose and test extract for the hydroxyl radical generated by Fenton's reaction. 1ml of reaction mixture containing 500 µl of MEDM solution of different concentrations (3-110 µg/ml), and 100 µl of each of 2- Deoxy-D-ribose (28 m*M*), EDTA (1.04 m*M*), $FeCl_3$ (0.2 m*M*) and ascorbic acid (1 m*M*) were incubated at 37°C for 1hr. The damage imposed on deoxyribose due to the free radicals was determined colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa *et al*[25]. Percentage inhibition was calculated using the formula (1).

Inhibition of lipid peroxidation[26]—Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Liver was collected immediately

after the sacrifice of the animals by cervical dislocation under ether anesthesia. The liver was homogenized with 40 m*M* tris–HCl buffer (*p*H 7) and centrifuged at 3000 rpm for 10 min to get a clear supernatant. Reaction mixture (4ml) containing 0.5 ml of supernatant, MEDM solution of different concentrations (3-110µg/ml) and 100µl of each of 0.15 *M* KCl, 15 mM $FeSO_4$ and 6m*M* ascorbic acid was incubated at 37°C for 1 hr. TCA (1 ml; 10%) was added to the mixture and the samples centrifuged at 3000 rpm for 20 min at 4°C to remove insoluble proteins. Supernatant was removed and 1ml TBA (0.8%) was added to this fraction followed by heating at 90°C for 20 min in a water bath. After cooling the coloured TBA-MDA complex was extracted with organic solvent (2ml butanol) and absorbance was measured at 532 nm. Percentage inhibition was calculated by formula (1).

Reductive ability[27]—Reducing power of MEDM was determined based on the ability of antioxidants to form coloured complex with potassium ferricyanide, TCA and $FeCl_3$. 1ml of different concentrations of MEDM (to produce final concentration 100–600µg/ml) were mixed with 2.5 ml potassium ferricyanide (1%) and 2.5 ml of phosphate buffer (*p*H 6.6). The mixture was incubated at 50°C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 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.

Determination of total phenolic compounds[28,29]—The content of total phenolic compounds in MEDM was determined by using Folin-Ciocalteu's phenol reagent (FCR) and determining absorbance at 760 nm according to the method of Slinkard and Singleton[28]. The content was expressed as equivalent of pyrocatechol (µg) by using the following equation, which was obtained from a standard pyrocatechol graph.

$$\text{Absorbance} = 0.001 \times \text{pyrocatechol (µg)} + 0.0033$$

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

Results

The extract showed good antioxidant activity in all *in vitro* free radical scavenging models except in hydroxyl radical inhibition assay (Fig. 1a and b) where only 10% inhibition was noted with 1000 µg/ml of MEDM. Thus, IC_{50} value could not be determined for hydroxyl radical inhibition assay. In all other models, MEDM showed dose dependent results. The percentage inhibition in various models *viz.* DPPH, nitric oxide, superoxide radical and lipid peroxidation is shown in Fig. 1(a). IC_{50} values were found to be 9.16, 13.21, 25.27 and 17.33 µg/ml respectively. The reducing power of MEDM was also dose dependent and shown in Table 1. Determination of total phenolic compounds showed that 1mg of MEDM contains 120.7µg equivalent of pyrocatechol.

Table 1—Reductive ability of methanol extract of *Diospyros malabarica* (MEDM)
[Values are mean ± SEM of 3 replicates]

Concentration of MEDM (µg/ml)	Absorbance at 700 nm
100	0.040 ± 0.007
200	0.191 ± 0.021
300	0.310 ± 0.036
400	0.433 ± 0.044
500	0.505 ± 0.060
600	0.545 ±0.068

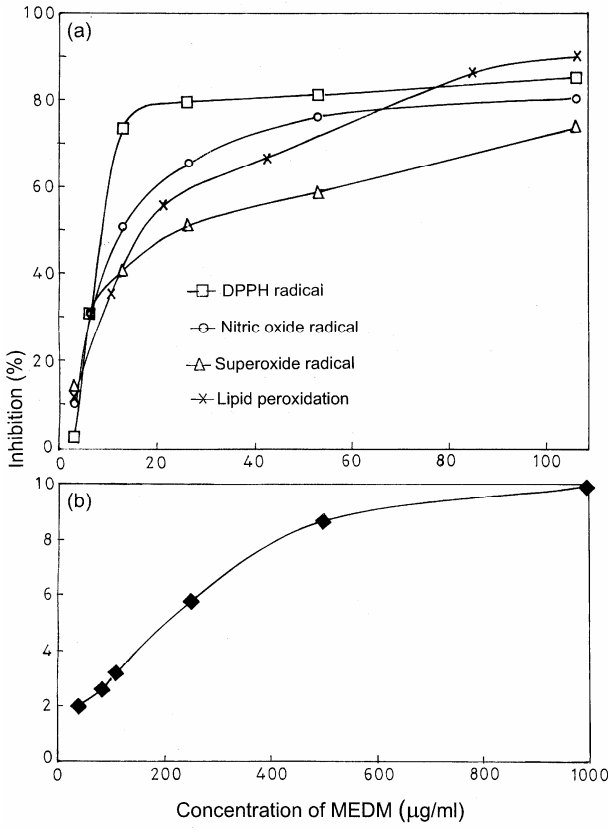

Fig. 1— Effect of MEDM on (a) different *in vitro* free radical models, (b) hydroxyl radical model.

Discussion

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals[4,5]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation etc.

DPPH is a relatively stable free radical and the assay determines the ability of MEDM 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. 1a) indicates that MEDM causes reduction of DPPH radical in a stoichometric manner[30-32].

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes[3]. Excess concentration of NO is associated with several diseases[33,34]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals[4,35]. In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits the generation of the anions.

Superoxides are produced from molecular oxygen due to oxidative enzymes[35] of body as well as *via* non-enzymatic reaction such as autoxidation by catecholamines[36]. In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560nm[37]. The effect of MEDM in this regard is shown in Fig. 1a. The probable mechanism of scavenging the super oxide anions may be due to the inhibitory effect of MEDM towards generation of superoxides in the *in vitro* reaction mixture.

Ferrous salts can react with hydrogen peroxide and form hydroxyl radical *via* Fenton's reaction. The iron required for this reaction is obtained either from the pool of iron or the heme-containing-proteins[4]. The hydroxyl radical ($OH^{\bullet}$) thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA strand breakage[38]. Although MEDM concentration range of 3-110µg/ml is found to be sufficient to calculate IC_{50} values for different free radical inhibition models (Fig.1a), it is not so for hydroxyl radical model and thus, in this regard concentration is increased up to 1000µg/ml[17,20]. However, the result of the present study does not show any promising hydroxyl radical scavenging property (Fig. 1b), although MEDM showed very good effect against other radicals like DPPH, superoxide, etc. These differences can be explained by understanding the nature and generation of radicals as well as studying the differences in physical and chemical properties of the naturally occurring antioxidants[37,39]. The stable radicals like DPPH react stoichometrically with antioxidants which are good hydrogen donors[39-41]. But antioxidants which are effective chelators of transition metal ions may contribute differently to the antioxidant response in hydroxyl radical inhibition assay compared to the assays involving stable radicals, as Fe^{2+} / Fe^{3+} is the active redox couple in Fenton's reaction[39]. The differences in antioxidant activity using different stable radicals are related to different redox potentials and steric properties of the radicals[41].

Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver[42]. In this study, *in vitro* lipid peroxidation was induced to rat liver by using $FeSO_4$ and ascorbic acid. Lipid peroxidation occurs either through ferryl-perferryl complex or through $OH^{\bullet}$ radical by Fenton's reaction[43]. Whatever may be the process of lipid peroxidation, MEDM shows dose dependent prevention towards generation of lipid peroxides (Fig. 1).

A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom[27,37]. The reducing property of MEDM (Table 1) implies that it is capable of donating hydrogen atom in a dose dependent manner. The high content of phenolic compounds in the extract may 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[44,45].

Preliminary phytochemical analysis indicates the presence of tannins in MEDM. Polyphenols, particularly flavonoids and tannins are well known natural antioxidants[8,44-46]. Thus, the antioxidant potential of methanol extract of *D. malabarica* Kostel bark may be due to the presence of polyphenolic compounds, which needs further analysis.

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