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Flavonoids and phenolic compounds in two mangrove species and their antioxidant property

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In the present study the content of phenolic compounds and flavonoids in root extract of *Rhizophora apiculata* and *Acanthus ilicifolius* was determined, their antioxidant property was assayed and the protective effect of R. apiculata root extract on induced-oxidative stress was examined. *R. apiculata* and *A. ilicifolius* root extracts were found to be rich sources of phenolic compounds and flavonoids. DPPH free radical scavenging assay has shown that *R. apiculata* has a better antioxidant activity than *A. illicifolius*. *R. apiculata* root extract had protective effect on sodium nitrite-induced oxidative stress in brain of rats. The extract normalized the sodium nitrite-induced alterations in the levels of glutathione, lipid peroxides and antiperoxidative enzymes like catalase and superoxide dismutase. Mangrove root extract had a beneficial effect in ameliorating oxidative stress in the brain of rats which may be attributed to the presence of flavonoid and polyphenolic compounds.

[Key words: Phenolic compounds, flavonoids, antioxidant property, DPPH free radical].

Introduction

Mangroves produce secondary metabolites. It is estimated that 100,000 to 200,000 secondary metabolites exist[1] and some 20% of the carbon fixed by photosynthesis is channeled into the phenylpropanoid pathway, thus generating the majority of the naturally-occurring phenolics, such as flavonoids[2]. As many studies indicate mangroves may be a rich source of novel compounds along with providing a new source for many already known biologically active compounds[3,4]. Numerous mangrove plants are being used in folklore medicine and extracts from mangroves have proven activity against human, animal and plant pathogens but only limited investigations have been carried out to identify the metabolites responsible for their bioactivities [5, 6,7,8,9].

There is extensive medical research on phenolics and flavonoids. They have been reported to possess many useful properties including antiallergic, anti-inflammatory, antimicrobial, antiviral, antioxidant[10], oestrogenic, enzyme inhibition, vascular and cytotoxic antitumour activity[11]; but the antioxidant activity is, perhaps the most studied property attributed to flavonoids. Plants are good sources of natural antioxidants[12], which has been the basis of numerous studies in the last decade. Perera *et al.*[13] extracted polyphenolic compounds and flavonoids from root and bark of *Rhizophora mangle*, a species closely related to *R. apiculata*. Several investigators have established that mangroves are also rich in polyphenols, among which flavonoids are a significant group[14,15]. Present study had examined the total phenolic and flavonoid content and free radical scavenging capacity in root extracts of two species of mangrove plants *viz.*, *Acanthus ilicifolius* and *Rhizophora apiculata* and further studied the effect of the root extract of *Rhizophora apiculata* on sodium nitrite-induced oxidative stress in brain of rats.

Materials and Methods

Mangrove plants-Rhizophora apiculata and *Acanthus illicifolius* used for the experiments were collected from the Kumbalangi backwaters in Cochin.

Chemicals- All reagents and solvents used in this investigation were of analytical grade. Glutathione, epinephrine, glutathione reductase, NADPH tetraethoxy propane, Folin–Ciocalteu reagent, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and 2-aminoethyl diphenylborinate (NP-reagent) were purchased from Sigma–Aldrich GmbH (Steinheim, Germany); polyethylene glycol 4000 (PEG reagent), (±)-catechin, gallic acid and quercitin from Fluka AG (Buchs, Switzerland). Standards, used were prepared

as 0.05% solutions in methanol. Standards used for thin layer chromatography (TLC) were obtained from Sigma Chemicals *viz*., rutin, quercetin, and gallic acid.

TLC Plates-10×20 cm pre-coated silica gel plates Si 60 F_{254} and 20x20 cm pre-coated silical gel 60 plates from Merck were used for performing TLC.

Sample Preparation

Roots of the mangrove plants were collected from Kumbalangi backwaters in Cochin and dried in a shaded and well-ventilated place at room temperature. Dried plant material (1 g) was reduced to a fine powder and macerated twice with 10 mL of ethanol and refluxed for 30 mins. The extracts were filtered, concentrated under reduced pressure and adjusted to known volume.

Determination of weight of extracts

A 1ml aliquot of resulting extract was transferred into an evaporating dish and left overnight in a stream of air to produce a dry residue, which was later kept in a vacuum-desiccator and accurately weighed. The dry extract was further diluted, if necessary, with the same solvent to make the test-solution.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed[16]. Briefly, samples 10 µl each and standard 5 µl each were applied on the plates. The solvent systems used were ethyl acetate: formic acid: acetic acid: water, 100:11:11:26(V/V) and ethyl acetate: formic acid: water 8:1:1 (V/V). Visualization of the flavonoids and phenolic acids was achieved by spraying with 1% methanolic diphenyl boric acid-ethylaminoester (NP) followed by 5% ethanolic polyethylene glycol-400 (PEG) and examining in UV light at 365 nm.

Biochemical assays

Total polyphenols and flavonoids were determined. DPPH free radical scavenging activity was measured in the extracts as an index of antioxidant capacity. Test-solution (100–1000 µl, final concentration range: 2.7–270 µg/mL) was dispensed to a set of test tubes and the volumes were made up to 4.0 mL by the addition of methanol. Finally, 1 mL of 0.5 mmol/l methanolic DPPH solution was transferred into each test tube. Absorbance was recorded at 517 nm after 30 min incubation at room temperature in the dark, against methanol as a blank. Percent inhibition was calculated against the control solution, containing methanol. IC_{50} value, which denotes the concentration of sample that is required to scavenge 50% DPPH free radicals, was also calculated.

Animal study

For the study of antioxidant effect of the *R. apiculata* root extract, adult male Wistar strain albino rats, weighing 100-120 g were selected. Animals were housed individually in polyurethane cages under hygienic conditions and maintained at ambient temperature. Animals were allowed food and water *ad libitum*. The experiment was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee.

Experimental protocol: Four days after acclimatization, the animals were divided into three groups of 6 rats each. Group I rats were fed commercial diet and taken as control. Group II rats were also fed commercial diet and administered a single dose of sodium nitrite (s.c.) on day 10 of the study. Oxidative stress in the brain of the experimental groups of rats was induced by injecting a single subcutaneous dose of sodium nitrite (75 mg.kg body wt^{-1}. day^{-1})[17]. Group III rats were given *R. apiculata* root extract orally for a period of 10 days. 30 min after giving the last dose of the extract, sodium nitrite was injected. One hour after the injection the rats were sacrificed and brain tissue was excised immediately and washed with chilled isotonic saline. Accurately weighed brain tissue was homogenized in ice-cold 0.1 M Tris-HCl buffer, *p*H 7.2 and centrifuged. The supernatant was used for further biochemical analyses. Total reduced glutathione[18], lipid peroxides[19] and antiperoxidative enzymes, superoxide dismutase[20] (SOD), catalase[21] (CAT) and glutathione peroxidase[22] were assayed. Protein was estimated by the method of Lowry[23]. Results were expressed as mean ± SD, and Student's t-test was used to assess statistical significance.

Results and Discussion

Results of the TLC experiments are presented in Table 1 and Fig 1. TLC of the root extracts showed intense yellow orange and blue bands under UV light (Fig 1). Rfs of the standards and samples and the corresponding colors obtained are given in Table 1. The standard Rfs correspond to Rutin (Rf: 0.566), gallic acid (Rf: 0.8) and Quercitin (Rf: 0.833). Flavonoids (rutin & quercitin) are known to give yellow-orange fluorescence and phenols (gallic acid)

Table1. Rf values of flavonoid standards and *R. apiculata and A. illicifolius* root extracts

S.No	Sample/ Standard	Identified compound	Color	Solute front (cm)	Rf	Solvent front (cm)
1.	Standard	Quercitin	Orange	12.5	0.833	15
		Gallic acid	Blue	12.0	0.8	
		Rutin	Orange	8.5	0.566	
2.	A.*illicifolius*	Quercitin	Orange	12.5	0.833	
		Gallic acid	Blue	12.0	0.8	
		Rutin	Orange	8.5	0.566	
		Compound X	Bright bluish green	7.0	0.466	
3.	R. *apiculata*	Quercitin	Orange	12.5	0.833	
		Gallic acid	Blue	12.0	0.8	
		Rutin	Orange	8.5	0.566	
		Compound X	Bright bluish green	7.0	0.466	

Fig 1. TLC image of the standards and *R. apiculata* extract. Lane 1: rutin, lane 2: quercitin, lane 3: gallic acid, lane 4: *R. apiculata* extract showing the 3 spots corresponding to the standards.

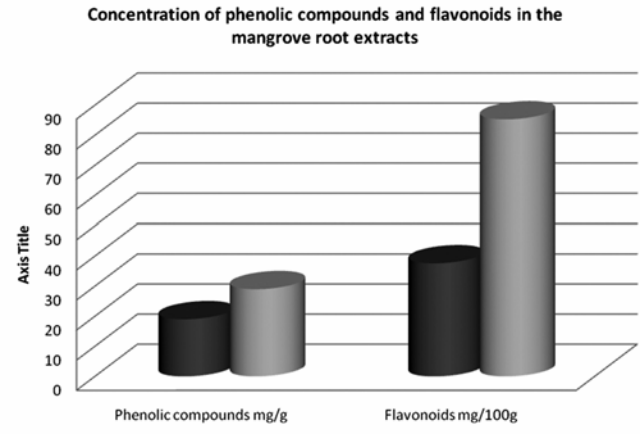

Fig 2. Levels of phenolic compounds and flavonoids in *R. apiculata* (RRE) *and A. illicifolius*(GRE) root extracts.

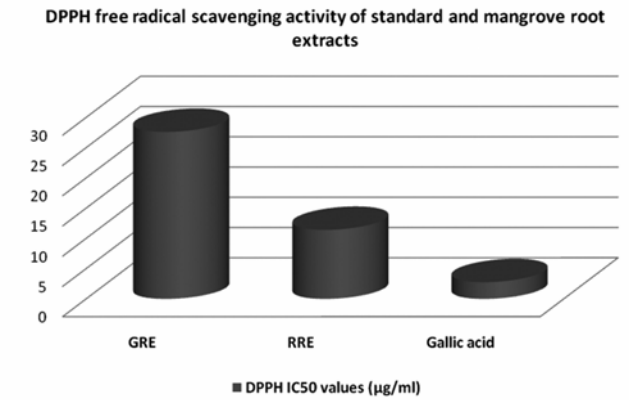

Fig 3. DPPH free radical scavenging activity of standard gallic acid and *R. apiculata* (RRE) *and A. illicifolius* (GRE) root extracts

appear blue[16]. The yields of the root extracts of *R. apiculata* and *A. illicifolius* were 11.65 g/100 g and 7.48 g/100 g respectively. Concentration of phenolic compounds and flavonoids are given in (Fig. 2). Phenolic compounds and flavonoid content are higher in the root of *R. apiculata* than in *A. illicifolius.* As a correlation the DPPH free radical scavenging activity (Fig. 3) was found to be higher in *R. apiculata*. DPPH IC_{50} values for *R. apiculata, A. illicifolius* and standard gallic acid were 11.4, 27.6 and 2.75 ug/ml respectively.

Sodium nitrite combines with haemoglobin in the blood to form methaemoglobin, which has a much higher (up to 20 times) affinity for oxygen and therefore cannot be exchanged with CO_2 in the tissues, thus causing hypoxia and oxidative stress. Brain utilizes glucose as the chief source of energy and in the process generates considerable levels of free radicals. It is uniquely vulnerable to oxidative

injury, due to its high metabolic rate and elevated levels of polyunsaturated lipids, the target of lipid peroxidation[24]. Endogenous oxygen- and nitrogen-centered free radicals are considered to play a decisive role in a variety of neurodegenerative diseases. In the present study oxidative stress in the brain is induced by injection of a single dose of sodium nitrite (s.c.). The levels of lipid peroxides were significantly (p<0.001) elevated (Fig. 4) in sodium nitrite-injected Group II rats when compared to Group I control rats. This is in agreement with other published reports[25]. Sodium nitrite is reported to stimulate free radical generation[17]. It combines with biological amines to form nitrosoamines which in turn react with biomolecules like DNA and enzymes disrupting their structure and function. The content of lipid peroxides was considerably (p<0.001) decreased in mangrove root extract-supplemented Group III (Fig. 4) rats when compared to Group II rats. Several reports support this observation. For instance, Shutenko *et al.*[26] described that flavonoids attenuate free radical-induced peroxidation during ischemia in rat brain. Teselkin *et al.*[27] reported that flavonoids reduce lipid peroxidation in rats following radiation exposure. The results of the present study are also in tune with these observations.

Glutathione is a small non-protein tripeptide (L-glutamyl cysteinyl glycine) thiol antioxidant molecule that plays a significant role in thwarting oxidative damage in all types of cells and organs. Disturbance in GSH status of a biological system is a sign of impaired antioxidant defense system. In the present study, a significant (p<0.001) decrease was observed in the levels of brain GSH content in sodium nitrite-treated Group II rats when compared to Group I normal control rats (Fig. 4). Similar observations were reported by other investigators [28] which establish that GSH depletion occurs following sodium nitrite administration. Depletion in GSH was thought to be due to enhanced consumption in the ROS (reactive oxygen species) ravaged brain cells[29]. In view of the numerous physiological roles that GSH plays in a living cell, a decline in GSH levels would deprive the cells of many of its specific roles exacerbating the oxidative damage. In the present study the brain content of GSH was significantly (p<.0.001) increased in mangrove root extract-treated Group III rats when compared to Group II rats (Fig. 4). Flavonoid rich mangrove root extract was shown to augment GSH and exert cytoprotective effect in experimental conditions that produce oxidative stress. Myhrstad *et al.*[30] had demonstrated that flavonoids increased intracellular GSH levels by activating gamma glutamyl synthetase activity. In a study reported by Ishige *et al*[31], one of the mechanisms by which flavonoids protected neuronal cells from oxidative damage was by elevating the cellular GSH content. The prior administration of flavonoid rich root extract to rats intoxicated with sodium nitrite prevented the decline in GSH content in the brain tissue of Group III rats. The extract strengthens the endogenous antioxidant defenses to fight ROS damage and restore the healthy state of the cell by neutralizing the reactive species.

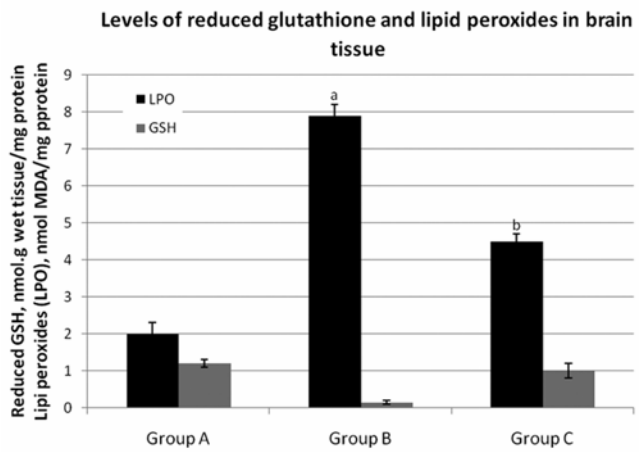

Fig 4: Levels of reduced glutathione and lipid peroxides in brain tissue of control (A), Sodium nitrite administered (B) and Mangrove, *R. apiculata* root extract administered (C) rats. a: p>0.001 significantly different from Group A, b: p>0.01 significantly different from Group B.

A significant (p<0.001) decrease was observed in the activities of the antiperoxidative enzymes SOD and CAT (Fig. 5) in the brain of sodium nitrite-treated Group II rats when compared to normal Group I rats. These results are in concurrence with other previous studies[32]. SOD and CAT along with GPX form the first line of defense against ROS and are referred to as primary antioxidants. Reduction in the activities of these enzymes leads to the accumulation of O^{2-} and H_2O_2, which in turn can form hydroxyl radical ($OH^{\cdot}$) and bring about a number of reactions harmful to the cellular and subcellular membranes[33]. Free radical damage of the active sites of SOD and CAT might be a possible cause of the decline in their activity in sodium nitrite-intoxicated rats[34]. The enzymes have amino acids arginine and histidine in their active sites that have an unpaired electron each and are susceptible to free radical damage. Group III rats that

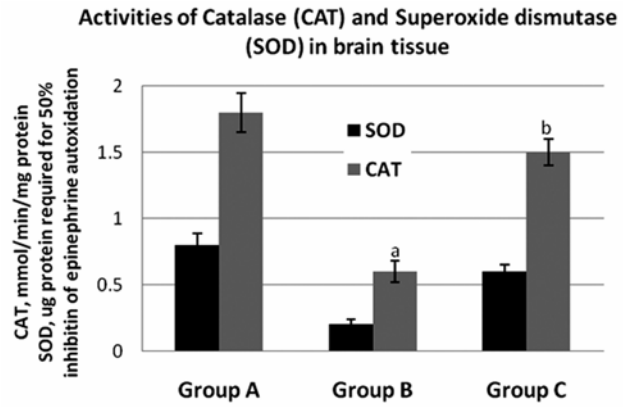

Fig. 5: Activities of Catalase (CAT) and Superoxide dismutase (SOD) in brain tissue of control (A), Sodium nitrite administered (B) and Mangrove *R. apiculata* root extract administered (C) rats. a: $p>0.001$ significantly different from Group A, b: $p>0.01$ significantly different from Group B.

were on mangrove root extract-supplemented diet showed a significant ($p<0.001$) rise in the levels of CAT and SOD (Fig. 5) that implies the protective effect of the extract in oxidative stress in brain. Similar findings were reported by Choi *et al*[35] where they had shown that plant extracts rich in flavonoids reduced antioxidant damage by elevating the levels of antiperoxidative enzymes.

The flavonoid-rich extract supplementation could have reduced lipid peroxidation by directly scavenging the ROS which protected the antiperoxidative enzymes from oxidative damage. Other investigators[36,37,38] have shown the beneficial effects of the ROS-quenching capacity of flavonoids, specifically in relation to attenuation of lipid peroxidation, reduction of membrane damage and permeability, and inhibition of intracellular oxidation in different cells.

Mangroves inhabit a very hostile environment that is characterized by high salinity, low nutrition and high solar radiation during low tide[39]. Exposure to stressful conditions causes the production of ROS in these plants. Interestingly, the concentration and activity of the antioxidative enzymes is high in these species[40] to neutralize the ROS. Also mangroves are generously endowed with polyphenolic compounds[41] that help to fight the oxidative stress by acting as potent antioxidants[42,43]. The antioxidant property of the flavonoids and other polyphenolic compounds is attributed to the free radical scavenging ability mediated by their hydroxyl groups[44]. We conclude that the beneficial effect of the mangrove root extract as an antioxidant in ameliorating oxidative stress in the brain of albino rats in our study is attributed to the presence of flavonoid and polyphenolic compounds.

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