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Protective effect of *Rhizophora mangle* bark on lipid peroxidation and erythrocyte hemolysis

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Abstract

Excessive peroxidation of biomembranes is thought to contribute to the initiation and progression of numerous degenerative diseases. The present study examined the effects of *Rhizophora mangle* bark aqueous extract and its polyphenol fraction on the peroxidation of rat-brain lipids and human erythrocyte hemolysis. The lipid peroxidation was assessed by incubating with Fe^{3+} / ascorbic acid and erythrocyte hemolysis was induced from 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH). The extract and its polyphenol fraction exhibited dose-dependent protection against peroxidation of rat-brain lipids and human erythrocyte heamolysis. The correlation coefficients for percentage of lipid peroxidation inhibition versus the tannins concentration were, r=0.999 ($p<0.05$) for extract and r= 0.868 ($p<0.05$) for polyphenol fraction. In the free radicals-induced hemolysis assay the values of IC_{50} were 113,99 μg/ml and 204,02 μg/ml for the extract and its polyphenol fraction respectively. In both case, the extract was more efficient than its polyphenol fraction in protecting rat-brain lipids from oxidation and human erythrocyte from heamolysis, it suggests a synergic effect of all components of the extract which increase in its antioxidant activity, although polyphenol fraction plays an important role in these effects. These results show that *Rhizophora mangle* extract and its polyphenol fraction can provide membrane protective effects.

Key Words: *Rhizophora mangle L.*, polyphenols, lipid peroxidation, membrane oxidation, erythrocytes.

Introduction

Excessive oxidative damage to cellular membranes is thought to contribute to the initiation and progression of numerous degenerative diseases, including certain cancers and cardiovascular disease (1, 2, 3, 4). Erythrocytes are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals (5, 6). Reactive oxygen species (ROS) generated in the aqueous or lipid phase can attack erythrocyte membranes and can induce the oxidation of lipids and proteins, triggering disruptions in the membrane and heamolysis (7, 8). Numerous investigations have used erythrocytes as model systems for studying biomembrane oxidative damage (5, 7, 8). In many of these studies, free radical initiators such as 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) have been used to generate free radicals in the aqueous phase that can attack the erythrocyte membrane and propagate lipid peroxidation, leading to hemolysis (7, 8). The erythrocyte has several membrane systems to protect itself against oxidative damage and hemolysis; these systems include superoxide dismutase, glutathione peroxidase, and catalase. In addition, water-soluble chain-breaking antioxidants such as ascorbic and uric acids can scavenge oxygen radicals residing in the aqueous phase, whereas lipid-soluble scavengers such as α-tocopherol can scavenge radicals within the lipid region of erythrocyte membranes (7). Although the erythrocyte oxidative defense system is robust, deficiencies in the above systems, a large oxidant insult, or certain disorders such as ß-thalassaemia, sickle cell anemia, and glucose-6-phosphate dehydrogenase deficiency can increase the susceptibility of the erythrocyte to peroxidation (5).

Rhizophora mangle L is a medicinal plant that grows spontaneously in the coasts of America, Occidental Africa and some Pacific islands (9). The bark of this specie is reported to possess antibacterial activity (10), gastric antiulcer properties (11), antifungal activity (9) and efficacy in wound healing (12). Previous studies on *Rhizophora mangle* L bark aqueous extract concerned its chemical characterization, revealed the presence of several polyphenols, carbohydrates, fatty acids and

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sterols (13). However, there is no available information related to the antioxidant properties of this specie.

Given previous reports have demonstrated antioxidant properties of polyphenols (14, 15, 16, 17, 18, 19), we hypothesized that the *Rhizophora mangle* extract and its polyphenol fraction have antioxidant properties. To test this hypothesis we evaluated the effect of *Rhizophora mangle* extract and its polyphenol fraction to inhibit lipid peroxidation in rat brain and reduce the risk of free radical-induced heamolysis.

Materials and Methods

Reagents

Ferric chloride (III), ascorbic acid, BHT, trichloroacetic acid, thiobarbituric acid and NaCl were purchased from Sigma (St.. Louis, MO, USA). Sodium dihydrogen phosphate and sodium hydrogen phosphate were purchased from Merck (Germany). Trolox C and AAPH were obtained from Aldrich Chemical Co.

Tested material

Rhizophora mangle L. bark was dried at room temperature. The extract was obtained by aqueous decoction of 1 kg of plant material in 1000 mL of boiling distilled water for 15 min. The mixture was then filtered. The obtained extract was freeze-dried (SuperModulyo 4K Freeze Dryer Edwards). The yield of the lyophilized extract was 30-40 %. The dry residue of extract was 30-40 mg/mL, with Tannins content above 12 mg/mL (13). Tannin content in the extract and polyphenol fraction was estimated as previously described by (20). Briefly, this method measures the amount of tannins precipitated by a standard protein, bovine serum albumin (BSA). The reaction mixture contained: *Rhizophora mangle* extract, its polyphenol fraction or tannic acid as standard (1ml) and BSA (1mg/ml), which was mixed in vortex and put to sit for 15 min at room temperature. To follow it was centrifuged at 3000 x g for 15 min and then the supernatant was poured off. The pellet was dissolved in 15 ml of lauryl sodium sulphate (1%) and a 1 ml of it was added 3 ml of a solution that contain: lauryl sodium sulphate (1 %) and triethanolamine (7 %) and a 1 ml $FeCl_3$ (0.01 M in HCl 0.1 N). Immediately the mixture was shaken in vortex. After 15 min. the absorbance was read at 510 nm and the tannin concentration in the *Rhizophora mangle* extract or its polyphenol fraction was calculated using a patron curve with tannic acid as standard. The polyphenol fraction of the *Rhizophora mangle* aqueous extract was obtained by precipitation with NaCl (13).

Effect on Lipid Peroxidation

The ability of the *Rhizophora mangle* extract and its polyphenol fraction to inhibit membrane lipid peroxidation at pH 7.4 was tested using rat-brain homogenate as described by (21). Rat brain homogenate was obtained from male Sprague-Dawley rats (180–220 g). The animal protocol used was in accordance with the Guide for the Care and Use of Laboratory Animals, and was approved by the CENSA Animal Use and Care Committee. Briefly, the rats were sacrificing by cervical dislocation, after which brain was extracted by surgery. The brains were homogenized at 10 000 rpm for 10 min in ice-cold PBS (pH 7.0, 50 mM), for each 3.2 g of brain was added 12.8 ml of PBS. To follow it was centrifugated (2 120 x g) at 4 °C, the supernatant was used as phospholipid source in the experiment. Reaction mixtures contained, in a final volume of 1.05 ml: brain homogenate in PBS (900 µl), $FeCl_3$ (50 µM), *Rhizophora mangle* extract or its polyphenol fraction (0.2, 1.0 and 5.0 mg dry weight/ml) and ascorbic acid (100 µM). Trolox C (0.05, 0.15 x 6 mg/ml) was used as comparable standard antioxidant. Then the mixture was incubated at 37 °C for 1 h and after malondialdehyde production was determined (22). Briefly, a solution of 0.25 N HCl containing 15 % trichloroacetic acid (w/v) and 0.5 % thiobarbituric acid (w/v) was added to incubated mixture, followed by 1% butylated hydroxytoluene solution. Then the mixture was heated in a water bath at 100 °C for 15 min. The absorbance of the resulting solution was measured at 535 nm. The malondialdehyde production in presence of all components of reaction mixture mentioned above, except the extract and its polyphenol fraction, was considered as 100 % lipid peroxidation. The malondialdehyde concentration (MDA) was determined using its molar extintion coefficient (epsilon) at 535 nm (ε_{535}= 1.56 x 10^5 M^{-1} cm^{-1}) by the following equation:.

$MDA = A * \varepsilon_{535} * b$

Where *A* is the absorbance of the sample at 535 nm containing or not the *Rhizophora mangle* extract or its polyphenol fraction and *b* is the cuvette width in cm.

The percentage of inhibition of lipid peroxidation was calculated by the following equation:

$$\% \text{ Inhibition} = [(MDA_{reaction\ mixture} - MDA_{extract\ or\ fraction})/ MDA_{reaction\ mixture}] * 100$$

Where $MDA_{extract\ or\ fraction}$ is the malondialdehyde concentration in the reaction medium containing either the *Rhizophora mangle* extract or its polyphenol fraction, and $MDA_{reaction\ mixture}$ is the malondialdehyde concentration in the reaction medium containing neither *Rhizophora mangle* extract nor its polyphenol fraction. Three replicates were performed for each concentration the extract or its polyphenol fraction, and the whole experiment was repeated at least three times on different days.

Effect on Hemolysis by Radicals initiated from AAPH

Normal blood was collected from healthy subjects. They were all volunteers in this study. Erythrocytes were separated from plasma, and were washed three times with 5 vol of NaCl (0.75 M). During the three washes, the erythrocytes were centrifuged at 3000 x *g* for 10 min to obtain a packed cell preparation. The packed

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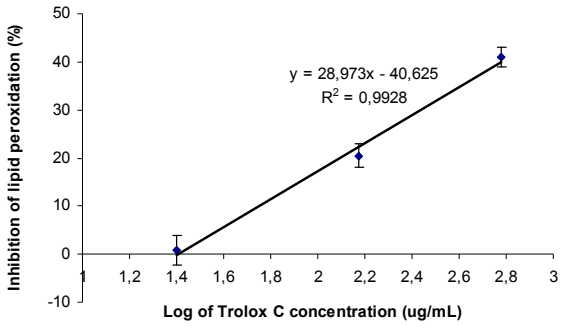
erythrocytes were then suspended in 2 vol of NaCl (0.75 M) solution.



In the present study, the method described by Miki *et al*. (7) was used to determine erythrocyte hemolysis mediated by AAPH. The erythrocyte suspension (250 µL) was mixed with 500 µL of NaCl (0.75 M) solution containing varying amounts of *Rhizophora mangle* extract (100–200 µg dry weight/ml) or its polyphenol fraction (100–700 µg dry weight/ml). 250 µL of 100 m*M* AAPH in NaCl (0.75 M) was then added to the mixture. The reaction mixture was shaken while being incubated at 37 °C for 2.5 h. After incubation, the reaction mixture was centrifuged at 750 X g for 10 min. The degree of hemolysis (%) was determined from absorbance (*A*) of hemoglobin at 540 nm in the supernatant. The value of 100 % hemolysis was determined from AAPH (100 mM) induced hemolysis. The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [(A_{AAPH} - A_{\text{extract or fraction}}) / A_{AAPH}] \cdot 100$$

Where $A_{\text{extract or fraction}}$ is the absorbance of the sample containing either the *Rhizophora mangle* extract or or its polyphenol fraction, and A_{AAPH} is the absorbance of the sample containing neither *Rhizophora mangle* extract nor its polyphenol fraction. Three replicates were performed for each concentration the extract or its polyphenol fraction, and the whole experiment was repeated at least three times on different days.

Statistical analyses

One-way ANOVA parametric comparison test followed by a DUNCAN test in order to compare between groups was used for statistical analysis. Probability values less than 0.05 were considered to be statistically significant. Regression analysis was done by linear and logarithmic regressions.

Results and discussion

Fig.1 shows respectively the regression analysis of the lipid peroxidation inhibition in a rat brain homogenate, as a Trolox C concentration function (Fig 1.A), and as a tannins concentration function in the *Rhizophora mangle* extract and in its polyphenol fraction (Fig 1.C). The extract and its polyphenol fraction significantly decreased lipid peroxidation as measured by malondialdehyde production (Fig 1.B). The Trolox C, a positive control, resulted almost twice more effective than polyphenol fraction and almost twice less effective than extract in reduced rat brain lipids damage (Fig 1.A and Fig 1.C).

Fig 1.C showed that the antioxidant activity of extract and its polyphenol fraction positively correlate to the tannin concentration. The correlation coefficients for percentage of inhibition versus the tannins concentration were, r=0.999 ($p<0.05$) for extract and r= 0.868 ($p<0.05$) for polyphenol fraction. The difference of the slopes indicate that the extract was more efficient than its polyphenol fraction in protecting the lipids from oxidative injury, however, the polyphenol fraction is a responsible component, at least in part, of the protective effect against lipid oxidation showed for the extract. Similar antioxidant effects have been obtained from green tea polyphenols like epigallocatechin gallate (EGCG), it was the most potent antioxidant in inhibiting H_2O_2 or ferric ion-induced lipid peroxidation in the gerbil brain homogenates (18) and it reduced MDA production, the formation of postischemic brain edema and infarct volume caused by cerebral ischemia in gerbils (23).

The reasons for these differential effects between extract and its fraction deserve further study; they most likely include chemical properties of compounds presents in it. Previous studies reported the effects of various external factors on the affinity of tea catechins for lipid bilayers by using liposomes as model membranes. These researchers found that the number of hydroxyl groups on the B-ring, the presence of the galloyl moiety, the stereochemical structure of each catechin, salt concentration in aqueous medium, electric charge of the membrane and the presence of others catechins govern their affinity for lipid bilayers (24, 25). The specific mode of inhibition of lipid peroxidation by polyphenols is not clear; but *Rhizophora mangle* polyphenols may protect lipids from damage oxidative by chelating transition metals to inhibit the decomposition of lipid hydroperoxide (LOOH), or by scavenging chain-propagating peroxyl radicals, because the reduction potential of catechin radicals was reported to be lower than those of alkylperoxyl and hydroperoxyl (26).

Fig 2 show AAPH-induced human erythrocyte hemolysis as a function of tannin concentration in the *Rhizophora mangle* extract and in its polyphenol fraction. The addition of AAPH to the suspension of erythrocytes induces the membrane damage, resulting in hemolysis. The extract and its polyphenol fraction provided a protective effect on red blood cells lysis. The AAPH-induced hemolysis inhibition was dependent of the tannins concentration in the extract and in its polyphenol fraction. The values of IC_{50} were 113.99 µg/ml and 204.02 µg/ml for the extract and its fraction respectively. These results suggest a synergic effect of all components of the extract which increase its antioxidant activity, although polyphenol fraction play

an important role in this effect. In agreement with ours results, other researchers have reported that resveratrol and quercetin, well known red wine antioxidants, showed lower antioxidant properties compared with red wine extract, they suggest that interaction between constituents may bring about effects that are not necessarily properties of the singular components (14). Also the antioxidant activity had been reported for others non-polyphenol components presents in *Rhizophora mangle* extract, such as: essential oils and phytosterols. Various articles show antioxidant properties in the essential oil of differents plants, such as: *Nigella sativa L.* (27), *Achillea millefolium* (28), Basil *(Ocimum basilicum*), in this case the authors tell that in all basils, the essential oil contribution to the total antioxidant activity was low, varying from 0.05% to 5.9%, these results strongly suggest that the main antioxidant activity from these plants does not arise from their essential oils, but rather from other phenolics such as flavonoids in green basils and anthocyanins in purple basils (29). The antioxidant properties of phytosterol and its components, beta-sitosterol, stigmasterol, and campesterol, against lipid peroxidation, besides have been reported (30, 31).

The excessive peroxidation of biomembranes is a processes by which tissues can be damaged during ischemia/reperfusion, inflammation and aging (5, 7). The peroxidation of erythrocyte membranes and hemolysis induced by various agents such as hydrogen peroxide, dialuric acid, xanthine oxidase, organic hydroperoxides, and AAPH (7, 32) has been extensively studied as a model for membrane-peroxidative damage. In the present study, we investigated the antioxidative activity *of Rhizophora mangle* extract and its polyphenol fraction. A strong dose-dependent protection was demonstrated toward the hemolysis of red blood cells *in vitro*. Similar effects have been obtained from green tea polyphenols like epigallocatechin gallate (EGCG), a

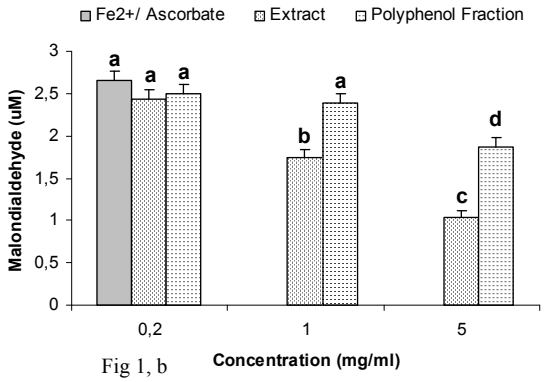


Fig 1, b

powerful antioxidant that is capable of protecting erythrocyte membrane against oxidative stress (33). Others authors report that red wine polyphenols prevent hemolysis, methemoglobin production and lipid peroxidation in human erythrocytes challenged with H_2O_2 (14).

Potential mechanisms by which *Rhizophora mangle* polyphenols may protect erythrocytes from hemolysis include the following: (i) The polyphenols may function as primary antioxidants by directly reducing the formation of free radicals mediated by AAPH; (ii) they may spare, maintain, or regenerate α-tocopherol (34) and other antioxidants by donating a hydrogen; and (iii) they may function as chelators and bind redox active metals, including $Fe^{2+}$and Cu^{2+}, involved in the

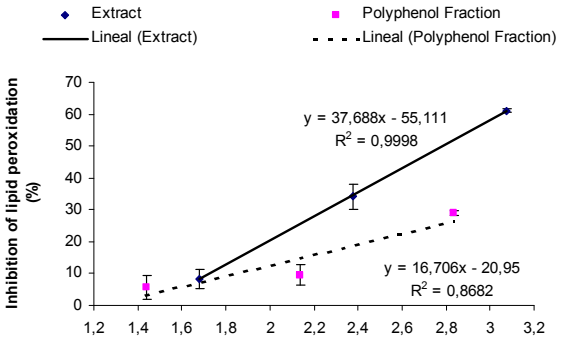


Fig 1, c

propagation of oxidative stress.

In conclusion, considering the obtained results, aqueous extract of *Rhizohpora mangle* bark and its polyphenol fraction can provide membrane protective effects under in vitro conditions, whether a similar effect is evident under in vivo conditions has to be investigated.

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