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Evaluation of Antioxidant Activities of *Radermachera xylocarpa* K. Schum

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Abstract: Antioxidant activity of methanolic extract of stem and root bark of *Radermachera xylocarpa* was evaluated using various *in vitro* free radical scavenging assay systems including superoxide, hydroxyl, DPPH and $ABTS^+$ radicals. The inhibition of liver membrane lipid peroxidation in the Fe^{2+}/ascorbate system and hydrogen peroxide mediated RBC hemolysis by the extract was also assessed. Among these, $ABTS^+$ radical could be scavenged most effectively by stem and root bark extracts with IC_{50} values of 28.3 ± 4.04 and 20.3 ± 5.13 µg/ml, respectively. The extract of root bark was effectively protected RBC from lysis and prevented lipid peroxidation with IC_{50} values of 7.8± 1.2 and 216.12 ± 4.0 µg/ml. The study revealed that the root of *R. xylocarpa* is a potential source of natural antioxidants.
Key Words: *Radermachera xylocarpa*; reactive oxygen species; antioxidant; RBC lysis.

Introduction

Reactive oxygen species (ROS) encompasses free radicals including hydroxyl, superoxide, singlet oxygen, lipid peroxides and various non radicals such as hydrogen peroxide, nitric oxide and hypochlorite. Excess generation of these radicals can attack membrane lipids, nucleic acids, proteins, enzymes and other molecules, resulting in cellular damage[1] and play an important role in pathological processes of various diseases such as cancer, atherosclerosis, cataract, inflammation, aging[2], Alzheimer's and neurodegenerative diseases etc[3, 4].

Even though, a well organised endogenous antioxidant system is protecting our body from deleterious effect of free radicals, the consumption of exogenous antioxidants are much beneficial at times when overwhelming production of free radicals. There are several commercially available synthetic antioxidants, but their use is strictly under restriction due to their potential health risks and toxicity. Hence, the search for natural antioxidants with less toxicity, especially from plant origin has increased greatly in recent years. This attraction for herbal medicine with antioxidant properties led to the present investigation on *R. xylocarpa*.

Radermachera xylocarpa K. Schum belonging to the family Bignoniaceae is a medium sized deciduous tree and is mainly distributed in Western Ghats regions of India. Although much phamacological activities are not yet reported with this plant, the antimicrobial activity of lapachol, a naphthaquinone isolated from the stem bark[5] and anti-inflammatory activity of radermachol[6] separated from root was reported. The oil extracted from the wood is used for treating various skin diseases[7]. Several other compounds like dinatine-7-glucuronide (from the

leaves)[8], o-acety-loleanic acid[8] and stismasterol[6] (from the root) were also reported from this plant. Considering the presence of biologically active phytochemicals, present study aims to explore antioxidant properties of *R. xylocarpa*.

Materials And Methods

Radermachera xylocarpa was collected from Thrissur District of Kerala and authenticated by Dr. N Sasidharan, Taxonomist, Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala, India. A voucher specimen was deposited in the herbarium of KFRI for further references.

Preparation of Extracts

Stem and root bark of *R. xylocarpa* were dried in hot air oven at 50°C and powdered using mixer grinder. Approximately, 20 g of powder was successively extracted with 250 ml of petroleum benzene, chloroform, acetone and methanol using a soxhlet extraction system. The solvent of each fraction was evaporated to dryness in a water bath set at 40°C and the residue was dissolved in a minimum volume of dimethyl sulfoxide, appropriately diluted with PBS and used for *in vitro* antioxidant analysis. Vitamin C was used as standard for all antioxidant assays.

Chemicals

All the chemicals and solvents used were of analytical grade. Nitroblue tetrazolium (NBT) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and 2, 2-azobis 3-ethylbenthiozoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picryl hydrazyl (DPPH) were from Sigma Aldrich (St. Louis, USA). Acid-citrate dextrose treated human blood was obtained from blood bank of Amala Institute of Medical Sciences. Vitamin C was purchased from Merck, India.

Analysis of Antioxidant Activity[9-13]

1. Scavenging of Superoxide Radicals

Superoxide radical scavenging activity was determined by the NBT reduction method[9]. Reaction mixture contained 6 µM EDTA, 0.0015% NaCN, 2 µM riboflavin, 50 µM NBT, various concentrations of extract and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 minutes and the optical density was measured at 560 nm before and after illumination. The percentage inhibition of superoxide radical generation was evaluated by comparing the absorbance values of control and experimental tubes.

2. Scavenging of Hydroxyl Radicals

Hydroxyl radicals generated from Fe^{2+}/ascorbate/H_2O_2 system degrades deoxyribose producing thiobarbituric acid reacting substance (TBARS)[10]. The efficacy of the test materials to inhibit TBARS formation was assessed. The reaction mixture contained 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM EDTA, 1 mM H_2O_2, 0.1 mM ascorbic acid, 20 mM KH_2PO_4-KOH (pH 7.4) and various concentrations of extract in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37°C. The TBARS formed was measured by the method of Ohkawa *et al* (1979)[11] and the percentage inhibition was calculated from the optical measurements of control and test tubes.

3. Inhibition of Lipid Peroxidation

The assay system contained 0.1 ml rat liver homogenate (25% w/v) in 20 mM Tris-HCl buffer (pH 7.0), 30 mM KCl, 0.16 mM $FeSO_4$ $(NH_4)_2SO_4 6H_2O$ and 0.06 mM ascorbic acid and various concentrations of extract in a final volume of 0.5 ml and was incubated for 1 h at 37°C. After incubation, 0.1 ml was removed and treated with 0.2 ml 8% SDS, 1.5 ml 0.8% TBA and 1.5 ml 20% acetic acid (pH 3.5). The total volume was made up to 4 ml by adding distilled water and kept in water bath at 95°C for 1 h. After cooling, 1 ml distilled water and 5 ml butanol-pyridine mixture (15:1, v/v) were added. Following vigorous shaking, the tubes were centrifuged at 4,000 rpm for 10 minutes. The organic layer was removed and its absorbance was measured at 532 nm.

Inhibition of lipid peroxidation in the treated samples was determined by comparing their optical density to that of control tubes.

4. Scavenging of DPPH Radicals

Stable radical, 2, 2-diphenyl-1-picryl hydrazyl (DPPH) in methanol was used as substrate to evaluate anti-oxidant activity. This method is based on the reduction of DPPH radical in the presence of hydrogen donating antioxidant that leads to the formation of a non-radical form DPPH-H by the reaction. DPPH in its radical form has an absorption peak at 515 nm which disappeared upon the reduction by antioxidant compounds. Absorbance was measured 20 minutes after the reaction was started.

5. Scavenging of $ABTS^+$ Radicals

$ABTS^+$ (2, 2-azobis -3-ethylbenzthiozoline-6-sulfonic acid) radical scavenging activity of the extract was determined by the method described by Alzoreky and Nakahara (2001)[12]. The principle involves the oxidation of ABTS to its cation radicals by ferryl myoglobin formed in the reaction of H_2O_2 and metmyoglobin. Briefly, the stock solutions of 500 µM ABTS diammonium salt, 400 µM myoglobin (MbIII), 740 µM potassium ferrycyanaide and 450 µM H_2O_2 were prepared in PBS (pH 7.4). Metmyglobin was prepared by mixing equal volumes of myoglobin and potassium ferrycyanide solutions. The reaction mixture (2 ml) contained ABTS (150 µM), MbIII (2.25 µM), varying concentrations of extracts and PBS. The reaction was initiated by adding 75 µM H_2O_2 and oxidation reaction was monitored at 734 nm by spectrophotometer.

6. *In Vitro* Inhibition Assay of Human Erythrocyte Hemolysis

The inhibition of H_2O_2 induced human erythrocyte hemolysis by methanolic extracts of *Radermachera xylocarpa* was evaluated according to the procedure described by Tedesco *et al* (2000)[13] with slight modifications. Hemolysis of erythrocytes was performed with H_2O_2 as free radical initiator. Different concentrations of extract was added to 100 µl of 5% (v/v) suspension of erythrocytes followed by 100 µM H_2O_2, swirled gently and incubated at 37^{o}C for 3 h. The mixture was diluted with 3 ml of PBS and centrifuged at 2,000 g for 10 minutes. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer to determine the hemolysis. The erythrocytes treated with 100 µM H_2O_2 (without inhibitors) was kept to obtain a complete hemolysis and used as control. The IC_{50} (the concentration required for the inhibition of 50% hemolysis) value was calculated from the plot.

Statistical Analysis

The results were presented as the average ± standard deviation of three experiments.

Results

Among the extracts studied, only methanolic extract showed considerable radical scavenging activity. Methanolic extract of stem and root bark showed superoxide radical scavenging activity in a dose dependent manner with IC_{50} values of 74.1 ± 3.4 and 122 ± 3.6 µg/ml, respectively (Table 1). Root bark extract was found to be the significant one for scavenging hydroxyl radicals and the IC_{50} value 75.6 ± 3.51 µg/ml obtained was less, compared to stem bark extract (328.7 ± 2.0 µg/ml) (Table 1). The IC_{50} values of vitamin C, a standard antioxidant in superoxide and hydroxyl radical scavenging activities were higher (483.9 ± 7.65 and 90.5 ± 6.36 µg/ml), compared to root bark extract of *R. xylocarpa*.

The ability of extracts to scavenge free radicals was also assayed with the use of a commercially available free radical compound 2, 2-diphenyl-1- picrylhydrazyl (DPPH). Methanolic extract of both stem and root bark were effective in reducing the stable radical, DPPH by donating hydrogen equivalents to DPPH-H. The IC_{50} values for stem and root bark were 241.3 ± 7.5 and 104.3 ± 4.2 µg/ml, respectively. Both of these extracts also showed greater ability to quench $ABTS^+$ radicals when tested *in vitro* with IC_{50} values of 28.3 ± 4.04 and 20.3 ± 5.13 µg/ml, respectively. Ability of vitamin C to scavenge DPPH and $ABTS^+$ radicals was superior to that of root and stem methanolic extracts (Table 2).

Fe^{2+}- ascorbate system induced significant degree of lipid peroxidation in rat liver homogenate. Addition of extracts was effective in reducing the level of lipid peroxidation as reflected by the level of TBARS. For stem

and root bark extracts, the concentration needed for 50% inhibition was 299.32 ± 6.5 and 216.12 ± 4.0 µg/ml, respectively. The result of hemolysis prevention assay were presented in table 3 shows the efficacy of root extract (IC_{50}; 7.8 µg ± 1.2 µg/ml) in preventing H_2O_2 induced RBC hemolysis. IC_{50} values for vitamin C in these two assays were 126.46 ± 6.0 and 82.3 ± 6.6 µg/ml, respectively.

Table 1. Superoxide and hydroxyl radical scavenging activity of methanolic extract of *R. xylocarpa* stem and root bark (IC_{50} values are in µg/ml)*.

Plant part	Extract	Superoxide radical	Hydroxyl radical
Stem bark	Methanol	74.1 ± 3.4	328.7 ± 2.0
Root bark	Methanol	122 ± 3.6	75.6 ± 3.51
Vitamin C		483.9 ± 7.65	90.5 ± 6.36

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture. Values are mean ± standard deviation of three independent replicates.

Table 2. DPPH and $ABTS^+$ radical scavenging activity of methanolic extract of *R. xylocarpa* stem and root bark (IC_{50} values are in µg/ml)*.

Plant part	Extract	DPPH radicals	$ABTS^+$ radicals
Stem bark	Methanol	241.3 ± 7.5	28.3 ± 4.04
Root bark	Methanol	104.3 ± 4.2	20.3 ± 5.13
Vitamin C		3.71 ± 1.21	11.9 ± 4.06

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture. Values are mean± standard deviation of three independent replicates.

Table 3. Effect of methanolic extract of *R. xylocarpa* stem and root bark on lipid peroxidation and RBC hemolysis (IC_{50} values are in µg/ml)*.

Plant part	Extract	Anti-lipid peroxidation	RBC hemolysis
Stem bark	Methanol	299.32 ± 6.5	72 ± 8.1
Root bark	Methanol	216.12 ± 4.0	7.8 ± 1.2
Vitamin C		126.46 ± 6.0	82.3±6.6

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture. Values are mean ± standard deviation of three independent replicates.

Discussion

Results of the present study demonstrate that the methanolic extract of stem and root bark of *R. xylocarpa* exhibit antioxidant activity in various *in vitro* free radical scavenging assays in a dose dependent manner. According to Chanwitheesuk et al (2005)[14], antioxidants can act as reducing agents, free radical scavengers or singlet oxygen quenchers. In the present study, methanolic extract of *R. xylocarpa* shows pronounced reducing property possibly due to its hydrogen donating efficacy. Compared to standard antioxidant vitamin C, which is a known reducing agent, *R. xylocarpa* showed moderate activity. This might be due to the difference in their antioxidant mechanisms or variations in their ability to scavenge free radicals. Being a pure compound, vitamin C is expected to have a better activity than crude extracts.

In an *in vivo* system, free radicals generated are known to induce peroxidation of membrane lipids. The secondary products thus generated are thought to be involved in the pathophysiology of many degenerative diseases[15]. In the current study, anti-lipid peroxidative effect of root bark extract is reflected by its efficacy in preventing liver tissue lipid peroxidation induced by Fe^{2+} /ascorbate system and hydrogen peroxide mediated RBC lysis. Compared to stem bark extract and standard vitamin C, root bark extract show higher activity in these assays. Radical reducing property of root bark extract might be responsible for the anti-lipid peroxidative effect. It is likely that free radicals responsible for membrane lipid peroxidation generated from the hydrogen peroxide molecule in the assay systems could be reduced by the extract. It is also possible that extract inhibit secondary radical generation (lipid peroxide radicals) that are responsible for further propagation of peroxidative chain reaction.

It has been suggested that the high antioxidant and free radical scavenging activities of *R. xylocarpa* may be partially contributed by the bioactive molecules such as radermachol, lapachol etc present in this plant. Lapachol is a naphthaquinone which is already reported as an antioxidant[16] and exhibit a variety of biological properties such as anticarcinogenic, anti-inflammatory, antitumor, antiviral, bactericidal[17]etc.

The study thus reveals that the methanolic extract of *R. xylocarpa* is a potential source of natural antioxidants. At present, we are not sure whether the bioactive compounds present in the stem and root barks are similar. Further studies are needed for the purification of compounds responsible for the observed antioxidant activity.

Conclusion

Present study concludes that *R. xylocarpa* possess antioxidant activity. Further extensive studies are needed to prove the nature of active compounds responsible for antioxidant activity.

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