Comparative Evaluation of Antioxidant Activity of Methanolic Extract of *Saraca asoca* and its Commonly Used Substitute Plants

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**ABSTRACT**

**Objective**- This study was carried out to compare antioxidant activity of methanolic extract of *Saraca asoca* and its commonly used substitute plants *Kingiodendron pinnatum*, and *Cynometra travancorica*.

**Method**- Chopped bark of each plant where blundered and extracted with methanol, the antioxidant activity were evaluated using Swiss Albino mice.

**Results**- The antioxidant enzymes and glutathione in blood, serum and tissue was evaluated. Enzyme activity was found to be significantly increased in extracts treated with 200 and 400 mg/kg. The percentage increase of catalase enzyme in each extract was, *Saraca asoca* (19.97 and 33.17%), *Kingiodendron pinnatum* (10.09 and 36.87%) and *Cynometra travancorica* (15.92 and 49.19%) respectively. The activity of superoxide dismutase was 16.89 and 31.33%, 24.43 and 39.05%, 27.91 and 48.58%. The percentage increase of glutathione was found to be 25.98 and 51.69%, 19.21 and 43.76%, 9.75 and 56.36% respectively.

**Conclusion**- The data obtained from the study indicate that the extract prepared with *Cynometra travancorica* and *Kingiodendron pinnatum* shown significant increase of antioxidant enzymes. So *Saraca asoca* can be substituted with these two plants.

**Key words**, *Saraca asoca*, *Kingiodendron pinnatum*, *Cynometra travancorica*, antioxidant enzymes.

**INTRODUCTION**

*Saraca asoca* is used in large quantities in Ayurvedic medicines. These are the preferred species in Ayurveda as ‘Asokam’. [1] Asoka is one of the foremost plant utilized from antiquity till to date. The bark of *Saraca asoca* is the useful parts and an important raw drug in ‘Asokarishtam’ and several other medicinal preparations. According to Ayurveda, it is a sacred tree of India, famous for its use in treating gynaecological disorders and is especially relied upon as an astringent to treat menorrhagia. [2] The tannins contained in the bark provide the main astringent action for halting excessive menstrual bleeding, and also for bleeding haemorrhoids, bleeding ulcers, and haemorrhagic dysentery. ‘Asokarishtam’, the fermented formulation of ‘Asokam’ is used as a tonic for menorrhagia. The inhibitory activity of prostaglandin H2 synthetase was reported for the methanolic extract of Asoka. [2] Moreover, oxytocic efficacy of a phenolic glycoside isolated from this plant has also been reported. [3] Some of the compounds such as tannin, catechol, ketosterol and organic calcium have been isolated from the bark. [4,5] Due to the wide spectrum properties of *Saraca asoca*, the plant becomes over exploited and the size of natural populations has been dwindling over the years in the country. The pharmaceutical industry in India requires about 5,300 tones bark annually. The annual consumption of ‘Asokam’ in the Ayurvedic drug industry in Kerala is about 105 tones/year. [6] Due to the wide use of this tree, it has been almost
depleted from its natural habitat. International Union for Conservation of Nature and Natural Resources (IUCN) has listed this species under the threat category, ‘Globally Vulnerable’. [7] The scarcity of this tree has led to substitution with the bark of other related or unrelated trees. The drug is widely adulterated with the bark of *Polyalthia longifolia*. Other Caesalpiniiaceae members particularly *Kingiodendron pinnatum* and *Cynometra travancorica* are commonly used as substitutes. The medicinal properties of these species are not well known. Hence, the work is carried out to assess the antioxidant property of *Saraca asoca* and its substitutes, *Kingiodendron pinnatum*, and *Cynometra travancorica*.

According to modern medicine, most of the diseases are caused by the over production of free radicals. The free radicals damage the biological macromolecules including oxidation of sulfhydryl containing enzymes, modification of amino acids, loss of function and degradation of proteins, damage of polysaccharide and extensive DNA strand breaks. [8] To protect the action of highly reactive oxygen, the body has several safeguard mechanisms. They include enzymes such as superoxide dismutase, glutathione peroxidase, catalase, etc. Many antioxidants in the body such as vitamin E and ascorbic acid (vitamin C) also inhibit the generation of oxygen free radicals. Sometimes these protective mechanisms were not found to be sufficient to the free radicals produced by excess stress. So, the increased level of ROS (reactive oxygen species) cannot be neutralized by enzymes, this will leads to the damaging of cells and the condition is known as oxidative stress. Plants are rich in free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which have more antioxidant activity. [9, 10] Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, anti-mutagenic, anticarcinogenic, antibacterial and antiviral activities. [11, 12, 10] The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing. [13] In these circumstances supplementation of non-toxic antioxidants may have a chemo-protective role in the body. [14] Most of the plant related compounds also have the ability to activate free radical scavenging enzymes. Hence, in the present study analysed the antioxidant properties of plants and its preparations.

**MATERIALS AND METHODS**

**Materials**

**Collection of plant samples**

The bark of *Saraca asoca*, *Kingiodendron pinnatum* and *Cynometra travancorica* were obtained from Kerala Forest Research Institute, Peechi, Kerala.

**Animals**

Swiss albino mice (aged 7 days) were purchased from Small Animal Breeding Station, College of Veterinary, Agricultural University, Thrissur, Mannuthy, Kerala. The animals were maintained under standardized environmental conditions (22-28°C, 60-70% relative humidity, 12 hrs dark/light cycle) and fed with standard mice feed (Sai Durga Feeds and Foods) and water *ad libitum*. All the animal experiments were carried out in Al Shifa College of Pharmacy by the prior permission of Institutional Animal Ethics Committee (IAEC).

**Methods**

**Preparation of plant sample**

The barks of each plant is freshly collected, chopped into small pieces and washed thoroughly with distilled water, shade and air dried at room temperature for 7 days. The dried samples then pulverized using an electric blender and the powdered sample of 150g of each plant was extracted using 500ml of methanol in Soxhlet apparatus for 48 hours at 50°C. The apparatus was intermittently shaken and the extracts were filtered. The solid mass obtained after evaporation and dryness of
the solvent, stored in desiccator for further use.

**Preparation of tissue homogenate**

Tissues (liver and kidney) were homogenized in appropriate buffer, made up to 10 or 25%, centrifuged at 12000 rpm for 20 min at 4°C and the supernatant was used for the assay.

**Determination of superoxide dismutase (SOD) activity in the blood**

SOD activity was determined according to the method of McCord and Fridovich (1969). The photo-illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to O$_2^{-}$, which is allowed to react with a detector molecule NBT. Upon reaction NBT is reduced to a formazan blue. The SOD in the sample inhibits the formazan production. For the determination of SOD, the heparinised blood was centrifuged at 2500 rpm to remove the plasma. To the packed RBCs, normal saline was added, centrifuged and supernatant was removed. The remaining packed RBCs were used for the experiment. The packed RBCs (100 μL) was haemolysed by 900 μL of cold water. The haemolysate was then treated with 250 μL of chloroform and 500 μL of ethanol with vigorous mixing to remove the haemoglobin. The mixture was then centrifuged at 15000 rpm for 60 min at 40°C. Clear supernatant (100 μL) was mixed with 200 μL of 0.1 M EDTA (containing 0.0015% NaCN), 100 μL of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.95 mL. After adding 0.05 mL of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. The tubes were then uniformly illuminated with an incandescent lamp for 15 minutes and absorbance was taken again at 560 nm. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). In the case of tissue homogenate also the volume used was 100 μL and methodology followed was same as given above. The volume of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/g Hb in the case of blood.

**Determination of catalase activity in the blood and in the tissue homogenate**

Catalase activity in the blood was determined by the method of Aebi (1974) and tissue catalase was determined according to the method of Beer and Sizer (1952). The catalase activity was assayed by measuring the decomposition of H$_2$O$_2$. The H$_2$O$_2$ has absorption maxima at 240 nm and absorption decreases with the decomposition of H$_2$O$_2$. The difference in extinction per unit time is a measure of the catalase activity. For the determination of catalase activity, the lysate of packed RBCs was prepared in ice cold water which containing approximately 5g Hb/dL. A 1:500 dilution of this concentrated haemolysate with sodium-potassium phosphate buffer (0.05 M, pH 7) was prepared immediately before the assay. Reference cuvette contained 1 mL of buffer and 2 mL of haemolysate and test cuvette contained 2 mL diluted haemolysate. The reaction was started by addition of 1 mL of H$_2$O$_2$ (30 mM in the buffer, fresh every time) to the test cuvette, mixed well and the decrease in extinction was measured at 240 nm for 1 minute with an interval of 15 sec. Catalase activity was calculated using the formula and expressed as K/g Hb,

Where k is a rate constant of 1st order reaction.

\[
2.303 \times (\log E1 - \log E2) \times \text{dil. Factor} \\
\text{Catalase (K/g Hb)} = \frac{15 \times g \text{ Hb/mL of blood}}{15 \times g \text{ Hb/mL of blood}} \\
E1 \text{ is E240 at } t=0 \text{ and } E2 \text{ is E240 at } t=15 \text{ sec.}
\]
Catalase in the tissue
Tissue homogenate (0.1 mL, approximately 0.1 mg protein) was mixed with 1.9 mL of phosphate buffer. After adding 1 mL of H$_2$O$_2$ solution in buffer, decrease in extinction was measured at 240 nm, at 1 min interval for 3 min. A sample control was placed in the reference cuvette containing 0.1 mL of tissue homogenate and 2.9 mL of the buffer. Activity of catalase was calculated using the molar extinction coefficient of 43.6. Specific activity at 25°C was defined in terms of mmols of H$_2$O$_2$ consumed/min/mg of protein sample.

$$\text{mmoles of H}_2\text{O}_2 \text{ decomposed/min/} \text{Mg protein or (U/mg protein)} = \frac{\Delta \text{ A/min x 1000 x 3}}{43.6 \times \text{mg protein in sample}}$$

Determination of reduced glutathione (GSH) content in the blood and in the tissue homogenate
Reduced glutathione in blood and tissue was determined according to the method of Moron et al. (1979). Reduced glutathione forms a yellow coloured complex with DTNB with an absorbance at 412 nm. For the determination of reduced glutathione, haemolysate of heparinised blood was prepared in distilled water. Haemolysate of 500 µL (500 µL of the tissue homogenate) was mixed with 125 µL of 25% TCA and cooled on ice for 5 minutes followed by further dilution of the mixture with 600 µL of 5% TCA and these were then subjected to centrifugation at 3000 g for 5 min to settle down the precipitate. Supernatant of 150 µL was mixed with 350 µL of sodium phosphate buffer (0.2M, pH 8.0) and 1.0 mL of DTNB (0.6mM in 0.2M, pH 8.0 phosphate buffer). The yellow colour obtained was measured at 412 nm against a blank which contained 5%TCA in place of the supernatant. A standard graph was prepared using different concentrations (10-50 nmoles) of GSH. The GSH content of the sample was calculated from the standard graph and expressed as nmol/mL of blood and for tissue it was expressed as nmol/mg protein.

$$\text{GSH content (nmol/mL or mg protein)} = \frac{\text{O. D of blank - O. D of sample x 1}}{\text{The GPx activity}} \times 1000 \times \frac{1}{0.001 \times \text{g Hb or mg protein x 10}}$$
One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

RESULTS

Results of the present study demonstrated that the enhanced activity of anti-oxidant enzymes such as superoxide dismutase, catalase, glutathione and glutathione peroxidase by the administration of extracts prepared with Saraca asoca and its substitutes Kingiodendron pinnatum and Cynometra travancorica in both blood, serum and tissue is shown in Table 1 and Table 2. Catalase activity was found to be significantly increased in mice treated with 200 and 400 mg/Kg, and the percentage increase of catalase activity of Saraca asoca was 19.97 and 33.17 (P < 0.005), of Kingiodendron pinnatum was 10.09 and 36.87 (P < 0.001) and of Cynometra travancorica was found to be 15.92 and 49.18 (P < 0.001), respectively. The activity of superoxide dismutase was increased by 16.89 and 31.33% in 200 and 400 mg/Kg body weight of extract prepared with Saraca asoca treated groups (P <0.001).

Kingiodendron pinnatum treated group showed 24.43 and 39.05% of increase (P <0.001) and Cynometra travancorica showed 27.91 and 48.58% of superoxide dismutase activity (P <0.001) when compared with untreated control groups. Glutathione was found to be significantly elevated in 200 (25.98, P <0.005) and 400 mg/Kg. body weight (51.69%, P < 0.001) of Saraca asoca treated groups. In this case, Kingiodendron pinnatum showed 19.21 and 43.76 (P <0.001) and Cynometra travancorica showed 9.75 and 56.36% (P < 0.001) increase.

**Table 1** Effect of prepared extracts on antioxidant enzymes in the blood and serum.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catalase (K/g Hb)</th>
<th>Superoxide Dismutase (U/g Hb)</th>
<th>Glutathione (nmols/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>80.75 ± 16.73</td>
<td>910.52 ± 52.2</td>
<td>25.13 ± 2.25</td>
</tr>
<tr>
<td>Saraca asoca 200 mg/kg</td>
<td>100.9 ± 15.41*  (+19.97)</td>
<td>1095.55 ± 82.61***</td>
<td>31.29 ± 2.63*</td>
</tr>
<tr>
<td>Saraca asoca 400 mg/kg</td>
<td>120.83 ± 15.76* (+33.17)</td>
<td>1326.02 ± 58.09***</td>
<td>47.88 ± 7.04***</td>
</tr>
<tr>
<td>Kingiodendron Pinnatum 200 mg/kg</td>
<td>89.81 ± 28.72** (+10.09%)</td>
<td>1204.93 ± 33.64***</td>
<td>28.63 ± 4.75**</td>
</tr>
<tr>
<td>Kingiodendron Pinnatum 400mg/kg</td>
<td>127.91 ± 26.58*** (+39.05)</td>
<td>1493.78 ± 55.74***</td>
<td>41.13 ± 3.54***</td>
</tr>
<tr>
<td>Cynometra travancorica 200mg/kg</td>
<td>96.04 ± 15.51*** (+15.92%)</td>
<td>1262.32 ± 66.05***</td>
<td>25.63 ± 1.65**</td>
</tr>
<tr>
<td>Cynometra travancorica 400 mg/kg</td>
<td>158.88 ± 19.34*** (+49.18)</td>
<td>1770.61 ± 88.43***</td>
<td>53 ± 1.68***</td>
</tr>
</tbody>
</table>

(Values are mean ± SD from 6 animals in each group. Figures in parenthesis are % increase over control values)

**Table 2** Effect of prepared extracts on antioxidant enzymes in the tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catalase (K/g Hb)</th>
<th>Glutathione Peroxidase (U/g Hb)</th>
<th>Glutathione (nmols/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11.2 ± 4.6</td>
<td>16.82 ± 2.6</td>
<td>6.7 ± 2.34</td>
</tr>
<tr>
<td>Saraca asoca 200 mg/kg</td>
<td>13.41 ± 3.35*** (+16.48)</td>
<td>20.94 ± 3.85*** (+19.67)</td>
<td>7.1 ± 1.78*** (+8.23%)</td>
</tr>
<tr>
<td>Saraca asoca 400 mg/kg</td>
<td>14.01 ± 2.07* (+20.06)</td>
<td>23.56 ± 3.85* (+26.81)</td>
<td>9.9 ± 1.43* (+32.32)</td>
</tr>
<tr>
<td>Kingiodendron Pinnatum 200 mg/kg</td>
<td>11.75 ± 2.02* (+4.68)</td>
<td>21.71 ± 4.93* (+22.52)</td>
<td>6.9 ± 1.3* (+6.94%)</td>
</tr>
<tr>
<td>Kingiodendron Pinnatum 400 mg/kg</td>
<td>13.46 ± 2.18* (+16.79)</td>
<td>23.08 ± 4.85* (+27.12)</td>
<td>7.5 ± 1.77* (+10.66)</td>
</tr>
<tr>
<td>Cayanomatra travancorica 200 mg/kg</td>
<td>12.79 ± 1.14* (+12.43)</td>
<td>23.6 ± 2.37* (+28.71)</td>
<td>7.4 ± 1.26* (+14.15)</td>
</tr>
<tr>
<td>Cayanomatra travancorica 400 mg/kg</td>
<td>13.36 ± 2.54* (+16.17)</td>
<td>25.99 ± 4.57* (+35.28)</td>
<td>9.7 ± 0.68* (+30.93)</td>
</tr>
</tbody>
</table>

(Values are mean ± SD from 6 animals in each group. Figures in parenthesis are % increase over control values)

k = the measure of catalase activity (the difference in extinction at 240 nm per 15 seconds)
The effect of each extract on the antioxidant enzymes in mice liver and kidney after treatment for thirty days is given in Table 2. The activity of catalase was increased by 16.48 and 20.06% (P < 0.005) in 200 and 400 mg/Kg in extract of Saraca asoca treated groups when compared with untreated control groups. The activity increase was 4.68 and 16.79% in Kingiodendron pinnatum and 2.43 and 16.17% in Cynometra travancorica treated groups, respectively. Glutathione peroxidase was also increased by 19.67 and 28.61% in 200 and 400 mg/Kg of Saraca asoca treated groups. In this experiment, Kingiodendron pinnatum showed 22.52 and 27.12% and Cynometra travancorica shows 28.71 and 35.28% (P <0.01) of increase, respectively. Glutathione was increased by 8.23 and 32.32% (P <0.005) in Saraca asoca, 6.94 and 10.66% in Kingiodendron pinnatum and 14.1 and 30.93% (P <0.005) in Cynometra travancorica treated group when compared with untreated control groups.

**DISCUSSION**

The results obtained here indicate that the Saraca asoca and its substitutes Kingiodendron pinnatum and Cynometra travancorica show significant antioxidant properties. Among the substitutes, the extract prepared with Cynometra travancorica was most effective in scavenging superoxide radicals. Under various pathological conditions, the balance between the formation of reactive oxygen species and their inactivation by anti-oxidant enzymes can be deregulated, resulting in the increased accumulation of reactive oxygen species (ROS). They have damaging effects on the biological molecules due to the oxidation of DNA, lipids or proteins. These conditions which are described as oxidative stress are often the result of tissue injuries. The antioxidant enzymes like superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (Gpx) convert the free radicals to water molecules. One of the major sources for reactive oxygen is the leakage of activated oxygen like superoxide (O2–) which is produced via the respiratory chain in mitochondria. Also a variety of enzymes like xanthine oxidase, P450 oxidases, FADH2 oxidase, or NADPH oxidase can produce superoxide. A pathophysiologically relevant role for reactive oxygen species has been suggested in various neurodegenerative diseases and stroke. Oxidative stress has also been linked to the development of atherosclerosis which requires early stage oxidation of LDL particles for the formation of atherosclerotic plaques. However, under normal conditions most cells maintain a reducing environment due to the activity of various antioxidant mechanisms. Several enzymes such as superoxide dismutase, glutathione peroxidase, or catalase can convert superoxide to hydrogen peroxide (H2O2) and finally to water (H2O). Anti-oxidants have been shown to be able to treat and prevent various degenerative diseases.

**CONCLUSION**

The data obtained from the present study indicate that extract prepared with Saraca asoca and its substitute plants Kingiodendron pinnatum and Cynometra travancorica showed significant antioxidant. The level of antioxidant enzymes such as superoxide dismutase, glutathione, glutathione peroxidase and catalase were found to be increased in the extract prepared with Cynometra travancorica and Kingiodendron pinnatum treated groups.

In conclusion, the indiscriminate use and unscientific extraction of Asoka bark has led to acute scarcity of the genuine raw drug and this in turn has led to cost escalation and wide spread adulteration of the drug. The data generated from the study thus provides scientific basis for the use of substitute plants and in therapeutic preparations of Saraca asoca. Nowadays almost all Ayurvedic preparations have been adulterated. These adulterations lead to a wide variation in quality control. The result is that, Ayurveda has not been able to capitalize its wealth by promoting its use.
Thus the quality assurance is the integral part of all systems of medicine to ensure quality medicine today.

REFERENCES