Antioxidant Activity of *Caesalpinia sappan* Heartwood

Shrishailappa BADAMI,* Sudheer MOORKOTH, Sujay Rammanoharsingh RAI, Elango KANNAN, and Suresh BHOJRAJ

J. S. S. College of Pharmacy; Rocklands, Ootacamund-643 001, Tamilnadu, India.
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Antioxidant activity of *Caesalpinia sappan* heartwood was studied both by *in vitro* and *in vivo* models. The ethyl acetate, methanol and water extracts exhibited strong antioxidant activity as evidenced by the low IC_{50} values in both 1,1-diphenyl-2-picryl hydrazyl (DPPH) and nitric oxide methods. The values were found to be less or comparable to those of ascorbic acid and rutin, the standards used. Administration of the successive methanol and water extracts at 50 and 100 mg/kg body weight given for four days prior to carbon tetrachloride (CCl_4) treatment caused a significant increase in the level of superoxide dismutase (SOD) and catalase and a significant decrease in the level of thiobarbituric acid reactive substances (TBARS), when compared to CCl_4 treated control in both liver and kidney. These changes observed at 100 mg/kg body weight treatment were comparable to those observed for standard vitamin E at 50 mg/kg treatment. The results support significant antioxidant nature of *Caesalpinia sappan* heartwood extracts.

Key words antioxidant; *Caesalpinia sappan*; lipid peroxidation; 1,1-diphenyl-2-picryl hydrazyl (DPPH); nitric oxide; carbon tetrachloride (CCl_4)

In recent years, considerable interest has been evinced by the public and the medical professional regarding the use of indigenous drugs in the treatment of diseases. Several members of the species *Caesalpinia* (family: Caesalpiniaeceae) are being used traditionally for a wide variety of ethnomedical properties.[1] *Caesalpinia sappan* is one among them found in India, Peru, Malaya, *etc.* Greatest medicinal value is ascribed to its heartwood. The wood is orange-red, hard, very heavy, straight grained with a fine and even texture. According to Ayurveda, the heartwood is useful in vitiated conditions of *Pitta*, burning sensation, wounds, ulcers, leprosy, skin diseases, diarrhoea, dysentery, epilepsy, menorrhagia, leucorrhoea, diabetes, *etc.*[1—3] The plant is one of the ingredients of an indigenous drug "Lukol"™ which is administered orally for the treatment of non-specific leucorrhoea (post IUD) and gave encouraging results for bleeding following IUD insertion[1] and several other traditional Ayurvedic formulations. A decoction of the heartwood is commonly used in Kerala, India for its antithirst, blood purifying, antidiabetic, improvement of complexion and for several other properties (personal communication). Its anticonvulsant,[4] anticomplementary,[5] modulation of immune function,[6] hepatoprotective,[7] antiinflammatory,[8] cytotoxic,[9] hypoglycemic[10] and several other biological activities have been reported.[1] Several triterpenoids,[11] flavonoids,[12,13] phenolics,[12,14,15] steroids[11] have been isolated from the heartwood.

Plants containing flavonoids and phenolics are known to possess strong antioxidant properties.[16] Some of the ethnomedical and reported biological activities of this plant may be related to its antioxidant nature. Hence, in the present study the *in vitro* and *in vivo* antioxidant activity of the extracts of *Caesalpinia sappan* heartwood is being investigated.

MATERIALS AND METHODS

Plant Material The heartwood of *Caesalpinia sappan* was collected during October 2001 from the campus of M.G. University, Kottayam, India. Mr. S. Rajan, Medicinal Plants Survey and Collection Unit, Govt. Arts College, Ootacamund, India, authenticated the plant. A voucher specimen is preserved in our laboratory for further reference.

Extraction The shade dried heartwood was powdered and extracted (135 g) successively with 700 ml each of petroleum ether (40—60°), chloroform, ethyl acetate and methanol in a Soxhlet extractor for 18—20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40—50 °C). The petroleum ether extract yielded an oily-yellow residue, weighing 1.89 g (1.4% w/w). The chloroform extract yielded an oily-brown residue, weighing 2.14 g (1.6% w/w). Similarly, the ethyl acetate and methanol extracts yielded reddish brown crystalline solids, weighing 5.05 g (3.8% w/w) and 11.57 g (8.9% w/w), respectively. The powdered heartwood (60 g) was also subjected to a crude extraction with 50% methanol (350 ml) in a Soxhlet extractor for 18—20 h. The extract was concentrated similarly to yield a reddish-brown solid (9.54 g, 15.9% w/w). Similarly a water extract was also prepared by heating heart wood powder (50 g) in a RB flask under reflux for 2 h with 300 ml of distilled water. The mixture was filtered after cooling and the filtrate was concentrated as above to yield a reddish-brown crystalline solid (6.72 g, 13.4% w/w). All the extracts were preserved in a refrigerator till further use.

Chemicals Rutin was obtained from Acros Organics, New Jersey, U.S.A. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals Co., St. Louis, U.S.A. Naphthyl ethylene diamine dihydrochloride was obtained from Roch-Light Limited, Suffolk, England. Petroleum ether (40—60°), chloroform, ethyl acetate, methanol, dimethyl sulfoxide (DMSO), acetic acid and sodium nitroprusside were obtained from Ranbaxy Laboratories Ltd., Mohali, India. Butanol, carbon tetrachloride (CCl_4), sulfanilic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium carbonate and sodium bicarbonate were obtained from E-Merck (India) Ltd., Mumbai, India. Sucrose, sodium carboxy methylcellulose (Sodium CMC), ascorbic acid, sodium lauryl sulfate and vitamin E were obtained from S.D. Fine Chem. Ltd., Biosar, India. Ethylene diamine tetra acetic

* To whom correspondence should be addressed. e-mail: shribadami@rediffmail.com

acid (EDTA), sodium chloride, potassium chloride and 30% hydrogen peroxide were obtained from Qualigens Fine Chemicals, Mumbai, India. Adrenaline bitartarate, thiobarbituric acid and pyridine were obtained from Loba Chemie, Mumbai, India. Phosphate buffer saline (PBS) was obtained from Hi-Media Laboratories, Mumbai, India. Pure arachis oil used was purchased from the local market.

Preparation of the Extracts and Standards For *in vitro* experiments, a weighed quantity of the extract was dissolved in petroleum ether and chloroform, respectively for petroleum ether and chloroform extracts and the other extracts were dissolved in distilled DMSO and used. Solutions of ascorbic acid and rutin used as standards for these studies were prepared in distilled DMSO. All these solutions were serially diluted with respective solvents to get lower dilutions.

For *in vivo* experiments the suspensions of crude water and successive methanol extracts were prepared in sodium CMC (0.3% w/v) using distilled water. Vitamin E standard was dissolved in pure arachis oil and used.

Animals Male Wistar rats (180—220 g) were obtained from the animal house, J. S. S. College of Pharmacy, Ootacamund, India and were maintained under standard environmental conditions (22—28 °C, 60—70% relative humidity, 12 h dark/light cycle) and were fed with standard rat feed (Amrut Rat Feed, Nav Maharastra Chakan Oil Mills Ltd., Pune, India) and water *ad libitum*. The experiments were conducted as per the guidelines of CPCSEA, Chennai, India (approval no: JSSCP/IAEC/Pharma Chemistry/01/2002—2003).

***In-Vitro* Assays** DPPH Method: The antioxidant activity of the plant extract and the standards were assessed on the basis of the radical scavenging effect of the stable DPPH free radical.[17] Ten microliter of each extract or standard (from 21 mg/ml to 21 μg/ml) was added to 200 μl of DPPH in methanol solution (100 μM) in a 96 well microtitre plate. After incubation at 37 °C for 30 min, the absorbance of each solution was determined at 490 nm using ELISA micro plate reader (Bio Rad Laboratories Inc., California, U.S.A., Model 550). The corresponding blank readings were also taken and the remaining DPPH was calculated.[18] IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical.

Nitric Oxide Radical Inhibition Assay: Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction.[19] In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide.[20] The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and extract or standard solution (0.5 ml) was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25 °C. A pink colored chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions in microtitre plate using ELISA reader. IC_{50} value is the concentration of sample required to inhibit 50% of nitric oxide radical.

***In-Vivo* Antioxidant Activity** The water extract is the one being traditionally used and the successive methanol extract showed lowest IC_{50} value in the DPPH method. Hence, both these extracts were selected for the *in vivo* antioxidant studies.

Animals were divided into seven groups comprising of six animals in each group. Group I served as normal and received 1 ml of 0.3% sodium CMC. Group II served as CCl_4 treated control and received 1 ml of 0.3% sodium CMC. Group III received the standard Vitamin E, at 50 mg/kg body weight. Group IV and V animals received the water extract at 100 and 50 mg/kg body weight, respectively. Group VI and VII animals received the successive methanol extract at 100 and 50 mg/kg body weight, respectively. All these treatments were given orally for 4 d. On the fifth day except for group I, all other group animals received 0.5 ml/kg body weight of CCl_4, intraperitoneally. On the seventh day, all the animals were sacrificed by decapitation. The liver and kidney were removed, weighed and homogenized immediately with Elvenjan homogenizer fitted with teflon plunger, in ice chilled 10% KCl solution (10 mg/g of tissue). The suspension was centrifuged at 2000 rpm at 4 °C for 10 min and clear supernatant was used for the following estimations.

Catalase was estimated by following the breakdown of hydrogen peroxide according to the method of Beer and Seizer (1952).[21] Superoxide dismutase (SOD) was assayed according to Misra and Fridevich (1972)[22] based on the inhibition of epinephrine auto-oxidation by the enzyme. Lipid peroxidation was measured in terms of malondialdhyde (MDA) content following the thiobarbituric acid method of Ohkawa *et al.* (1979).[23] Statistical analysis was carried out using the Student's *t*-test and the results were judged significant, if $p<0.05$.

RESULTS AND DISCUSSION

Among the six extracts tested for antioxidant activity using DPPH method the successive ethyl acetate and methanol extracts and the crude 50% methanol and water extracts exhib-

Table 1. *In-Vitro* Antioxidant Activity of *Caesalpinia sappan* Heartwood

Sl. No.	Tested materials	IC_{50} values ± S.E. (μg/ml)[a]	
		DPPH method	Nitric oxide method
	Successive extracts		
1	Petroleum ether extract	>1000	>1000
2	Chloroform extract	>1000	>1000
3	Ethyl acetate extract	1.71±0.15	50.25±4.02
4	Methanol extract	1.44±0.12	234.25±13.32
	Crude extracts		
5	50% Methanol extract	2.31±0.17	39.50±3.32
6	Water extract	4.09±0.31	75.50±4.82
	Standards		
7	Ascorbic acid	2.85±0.20	—
8	Rutin	9.12±0.73	172.65±14.06

a) Average of ten determinations.

ited strong antioxidant activity as evidenced by their low IC_{50} values (Table 1). The values were found to be less or comparable to that of standard ascorbic acid and less than the standard rutin. In the nitric oxide method the successive ethyl acetate and crude 50% methanol and water extracts showed potent antioxidant activity as evidenced by low IC_{50} values lower than the standard rutin. The successive petroleum ether and chloroform extracts did not show any antioxidant activity by both the methods.

The administration of CCl_4 to the control animals caused a significant decrease in the level of catalase and SOD and significant increase in the level of thiobarbituric acid reactive substances (TBARS) in both liver and kidney ($p<0.05$ to $p<0.001$), when compared to normal rats (Tables 2, 3). A significant reversal of these changes towards the normal group was observed by the administration of the crude water and successive methanol extracts at 50 and 100 mg/kg body weight, for four days before CCl_4 treatment, in both liver and kidney ($p<0.05$ to $p<0.001$), when compared to CCl_4 treated control. These changes with both the extracts at 100 mg/kg body weight treatment were comparable to that of standard vitamin E at 50 mg/kg.

The significant decrease observed in the level of TBARS in liver by the successive methanol extract and in kidney by the water extract treatment at 100 mg/kg body weight, respectively, was found to be lower than the normal values ($p<0.05$, when compared to normal). Values higher than the normal group were also obtained in successive methanol extract treatment at 100 mg/kg body weight in the liver catalase and at both the doses in the kidney SOD and for the water extract at 100 mg/kg body weight in the kidney SOD.

Lipid peroxidation is found to be an important pathophysiological event in a variety of diseases including aging, cancer, diabetes, cardiovascular disorders and rheumatoid arthritis.[24] Hence, current interest has focused on the potential role of antioxidants in the treatment and prevention of these diseases. In the present study, the *in vitro* and *in vivo* antioxidant activity of various extracts of *Caesalpinia sappan* heartwood was investigated. A decoction of the heartwood is traditionally being used for the treatment of a variety of ailments in several parts of the world. The *in vitro* antioxidant screening using DPPH and nitric oxide methods showed strong antioxidant activity of all the extracts of heart wood, except the petroleum ether and chloroform extracts. The values were found to be lower or comparable to the standards used.

The lipid peroxidative degradation of the biomembrane is one of the principle causes of toxicity of CCl_4.[25] This is evidenced by the elevation of TBARS and decrease in the activity of free radical scavenging enzymes, *viz.*, SOD and catalase in the CCl_4 treated animals. SOD is the key enzyme in scavenging the superoxide radicals. Catalase is also another key enzyme in the scavenging, which helps in cleaning the H_2O_2 formed during incomplete oxidation. As a whole, these antioxidant enzymes play an important role in the body defense mechanism against the harmful effects of the oxygen free radicals in biological systems.[26] Lipid peroxidation also yield a wide range of cytotoxic products most of which are aldehydes, as exemplified by MDA, which can be measured following the TBA method.[23]

In the present study, administration of the successive methanol and the water extracts at 50 and 100 mg/kg body weight prior to CCl_4 treatment, caused a significant increase in the level of SOD and catalase and a significant decrease in the level of TBARS when compared to CCl_4 treated control in both liver and kidney. The values at 100 mg/kg body weight treatment were comparable to that of vitamin E at 50 mg/kg, used as standard. These results indicate that the

Table 2. Effect of Various Extracts of *Caesalpinia sappan* on Antioxidant Enzymes in Rat Liver

Sl. No.	Treatment	Dose (mg/kg body wt)	Catalase (IU/min/mg of tissue)	SOD (Unit/min/mg of tissue)	TBARS (nM/mg of tissue)
1	Normal	—	1.209±0.100	0.155±0.007	3.065±0.350
2	Control (CCl_4 treated)	0.5 ml	0.963±0.070†	0.044±0.002††	14.899±1.090††
3	Standard (vitamin E+CCl_4)	50	1.276±0.130*	0.183±0.010***	4.599±0.480***
4	Water extract+CCl_4	100	1.188±0.140*	0.101±0.010**	4.484±0.420***
		50	1.131±0.110*	0.087±0.009**	4.869±0.440***
5	Methanol extract+CCl_4	100	1.219±0.100**	0.088±0.008**	1.552±0.140***$^{\#}$
		50	1.127±0.070*	0.075±0.010**	2.985±0.280***

Results are mean±S.E. ($n=6$), $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.001$, when compared to normal. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, when compared with CCl_4 control (Student's *t*-test). $^{\#}p<0.05$, when compared with normal.

Table 3. Effect of Various Extracts of *Caesalpinia sappan* on Antioxidant Enzymes in Rat Kidney

Sl. No.	Treatment	Dose (mg/kg body wt)	Catalase (IU/min/mg of tissue)	SOD (Unit/min/mg of tissue)	TBARS (nM/mg of tissue)
1	Normal	—	1.771±0.068	0.144±0.010	2.829±0.150
2	Control (CCl_4 treated)	0.5 ml	1.273±0.110†	0.066±0.008†	10.609±0.580†
3	Standard (Vitamin E+CCl_4)	50	1.468±0.130*	0.212±0.020***	5.615±0.490***
4	Water extract+CCl_4	100	1.675±0.130*	0.154±0.010***	1.275±0.230***$^{\#}$
		50	1.616±0.140*	0.127±0.008***	8.097±0.070*
5	Methanol extract+CCl_4	100	1.552±0.100**	0.152±0.010***	4.612±0.150***
		50	1.387±0.080*	0.145±0.009***	5.127±0.500***

Results are mean±S.E. ($n=6$), $^{\dagger}p<0.001$, when compared to normal. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, when compared with CCl_4 control (Student's *t*-test). $^{\#}p<0.05$, when compared with normal.

extracts of *Caesalpinia sappan* heartwood protect the tissues from lipid peroxidation.

The antioxidant properties of the heartwood observed in the present study supports its ethnomedical use in Kerala, India and several other parts of the World. Preliminary phytochemical tests indicated the presence of flavonoids, glycosides, triterpenoids and tannins in both methanol and water extracts. The separation of methanol extract by HPTLC using chloroform : methanol (90+10) as solvent system showed the presence of eight compounds. The compounds with *Rf* values 0.25 and 0.32 were found to be the major ones with 28.9 and 42.6 percentages. Similarly, the separation of water extract by HPTLC using chloroform : acetone : glacial acetic acid : water (10+3+4+4) as a solvent system showed the presence of three compounds having *Rf* values 0.30, 0.35 and 0.74, with 22.5, 15.5 and 61.9 percentages, respectively. Hence, the water extract is found to contain more polar compounds compared to the methanol extract.

Several flavonoids and phenols have been isolated from the heartwood.[1,12—15] Brazilin, a phenolic oxygen heterocycle is found to be the major constituent of the heartwood and found to possess immuno stimulant,[6] hypoglycemic,[10] anticomplementary,[5] hepatoprotective[7] and vaso relaxation[27] properties. Free radical mediated processes have been implicated in the pathogenesis of most of these diseases. Thus, the observed antioxidant activity of the heartwood may be due to the presence of brazilin, flavonoids and phenolic compounds present in it. Hence, the plant merits further investigation for identifying antioxidant nature of its constituents.

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