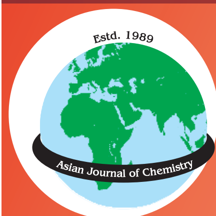

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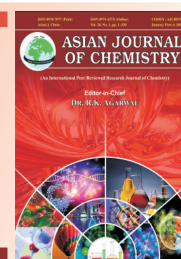

Studies on Antiproliferative and Antioxidant Efficacy of *Caesalpinia sappan* L. Heartwood†

C. David Raj, M.G. Dhinesh, R. Lavanya and P. Brindha*

Centre for Advanced Research in Indian system of Medicine, SASTRA University, Thanjavur-613 401, India

*Corresponding author: E-mail: brindha@carism.sastra.edu

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In the present study, cytotoxic and antioxidant potentials of *Caesalpinia sappan* heart wood were evaluated. The heartwood of *C. sappan* was collected, authenticated, coarsely powdered and subjected to solvent extraction using 70 % ethanol. Preliminary phyto-chemical screening was carried out for the hydro-alcoholic extract thus obtained. Antioxidant potential of the extract was evaluated employing *in vitro* methods such as DPPH assay and reducing capacity. *In vitro* antiinflammatory studies were also performed. The data of the results of antioxidant and antiinflammatory studies were encouraging. Besides cytotoxicity efficacy of different concentrations of *C. sappan* heartwood extract was studied against HEp-2, HT-29, HeLa, MCF-7, T47D, HepG2 and Vero cell lines using 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dose which showed highest cytotoxic effect was subjected to further studies to understand the Apoptosis mechanism involved. The present study depicted the anticancer and antioxidant potentials of hydro-alcoholic extract of heartwood of *C. sappan*.

Keywords: *Caesalpinia sappan*, DPPH Assay, MTT Assay, Cytotoxicity, Antioxidant.

INTRODUCTION

In consonance with World Health Organization (WHO), globally cancer is the second leading deadly disease accounting for nearly 13 % casualties[1]. Worldwide, deaths due to cancer are predicted to increase many folds by 2030[2]. In 2010, more than 71.1 % cancer deaths occurred in people aged between 30-69 years. The most common type of fatal cancer in men and women were oral (23 %) and cervical (17 %) cancer, respectively[3]. Chemoprevention, by definition, is controlling cancer by which the disease occurrence can be entirely prevented, slowed down, or reversed by the administration of one or more naturally occurring and/or synthetic agents[4]. A successful anticancer moiety should destroy or damage cancer cells without inducing severe damage to normal cells. The search for anticancer drugs from natural sources initiated in 1950s, resulted in the exploration of vincristine and podophyllotoxins[5]. The most important of these bioactive compounds from plants are alkaloids, flavanoids, tannins and phenols[6]. These compounds perform their anticancer action through mechanisms such as inhibiting genotoxic effects, increasing antioxidant potentials, promoting antiinflammatory activity, preventing proteases and cell proliferation, finally protecting intracellular communications to modulate apoptosis and signal transduction pathways[7].

Caesalpinia sappan belonging to Caesalpiniaceace is widely distributed in India. According to Ayurveda, the heart wood is used for treating wounds, ulcers, leprosy, skin diseases, diarrhea, diabetes, epilepsy and other diseases[8]. The heart wood decoction is commonly used in Kerala state for its antithirst and antidiabetic potentials. The methanol extract of *C. sappan* has been reported for its antioxidant[9], anticonvulsant[10] and antiinflammatory[11] activities. In the present study, antiproliferative, antiinflammatory and antioxidant potentials of hydro-alcoholic extract of *Caesalpinia sappan* are evaluated employing *in vitro* methods.

EXPERIMENTAL

The heart wood of *Caesalpinia sappan* was collected from Kuzhanthupuzha, Kerala. The wood specimens were identified and authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India (Ref. BSI./SC/5/23/07-08/Tech.1226, Dt.: 28-11-2007.). The herbarium of the plant has been preserved at CARISM, SASTRA University, Thanjavur, Tamilnadu state, India. (Voucher No. C0058).

Preparation of plant extracts: The heart wood was collected, shade dried and coarsely powdered using mechanical pulverizers. The coarsely powdered plant material was subjected to cold maceration using 70 % ethanol for 3 days.

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This process was repeated thrice and the extract thus obtained was filtered. The filtered extract was dried under reduced pressure, yielding dark reddish brown solid. The obtained extract was finally powdered and stored in refrigerator for further use. The percentage yield of hydro-alcoholic extract was found to be 18.74 % w/w.

RPMI-1640 and Fetal bovine serum (FBS) were purchased from GIBCO/BRL Invitrogen (Gaithershurg, MD). Trypsin, methylthiazolyldiphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide were purchased from (SISCO, Mumbai). All other chemicals and reagents were obtained from Sigma Aldrich, USA.

Preliminary phytochemical analysis: The preliminary phytochemical screening of test extract was carried out to detect the presence of phytoconstituents according to the method described by Harborne[14] and Trease and Evans[15].

Total phenolic and tannin content: Total phenolic content of hydro-alcoholic extract of *C. sappan* was determined using Folin Ciocalteu method[16]. The Folin-Ciocalteu reagent, diluted 10 times (1 mL) was mixed with 1 mL of saturated sodium carbonate (75 g/L) and 1 mL of extract (1 mg/mL). The mixture was vortexed for 10 seconds and incubated at 45 ºC for 15 min. The absorbance was measured at 765 nm after cooling at room temperature. The absorbance of test sample was compared with the standard curve of gallic acid (1000-1 µg/mL). The total phenolic content of the extract was expressed in mg/g of extract, gallic acid equivalent (GAE). The total tannin content was estimated by the above mentioned procedure and finally compared with standard curve of tannic acid. The total tannin content of the extract was expressed in mg/g of extract, tannic acid equivalent (TAE).

Total flavonoids content: Total flavonoid content of the plant extract was estimated by using aluminium chloride method[17]. Different concentrations of plant extracts (1000 to 1 µg/mL) dissolved in methanol were mixed with 2 % aluminium trichloride in methanol (1 mL). The resultant solution was mixed well and kept at room temperature for 10 min. Finally the absorbance was read at 415 nm. Blank sample consist of a 1 mL of plant extract with 1 mL of methanol without aluminium trichloride. Quercetin was considered as standard and expressed as mg/g quercetin equivalent.

HPTLC analysis: HPTLC analysis was performed using Silica Gel 60 F_{254} as stationary phase and the mobile phase as toluene:ethyl acetate: acetic acid: methanol in the ratio of 2.5:7:0.25:0.25. The standard and test solutions of volume 5 µL were spotted on a pre-coated silica gel 60 F_{254} HPTLC plate (E. Merck) of uniform thickness 0.2 mm using Linomat5 sample applicator. The plate was developed in the solvent system to a distance of 8 cm and was scanned densitometrically at 254 nm using TLC Scanner 3. Finally, the plate was observed under UV light at 254 and 366 nm using CAMAG Reprostar 3.

LC-MS/MS analysis: LC-MS/MS analysis was performed using MicrOTOF Q II UHPLC-ESI- TOF (Bruker, Germany). The instrument settings were optimized as follows: LC condition: UV at 330 nm, 0.2 mL/min flow rate, gradient mobile system start with 5 % acetonitrile and 95 % water (1 % acetic acid) to 20 % acetonitrile in 5 min, this was brought to 30 % acetonitrile in 5 min to 35 % acetonitrile in next 5 min to 45 % acetonitrile in next 5 min to 75 % acetonitrile in next 5 min to 95 % acetonitrile untill run ends. The chromatographic separation was accomplished using a Dionex C_{18} RP Acclaim 120 and the MS conditions were optimized as follows: MS condition: ESI, Negative mode, Nebulizer 30.5 psi with 6.0 L/min N_2 flow, m/z range: 50-1000 m/z, Capillary voltage 4500 V, dry heater temperature at 280 ºC. The flow rate was maintained as 0.2 mL/min and the injection volume was 10 µL.

In vitro antioxidant assays

Ferric reducing capacity of extract: The ferric reducing capacity of hydro-alcoholic extract of *C. sappan* was evaluated as reported by Oyaizu[18]. Different concentrations of the extracts (1000-1 µg/mL) in deionized water (1 mL) were mixed with phosphate buffer (1 mL) and potassium ferricyanide (1 mL). The mixture was incubated at 50º C for 20 min. Aliquots of TCA (1 mL) were added to the mixture, vortexed and centrifuged at 3000 rpm for 10 min. The supernatant (1 mL) was diluted with 1 mL of distilled water and 100 µL of freshly prepared ferric chloride solution was added. The absorbance was measured at 700 nm. Vitamin C at various concentrations (100 to 1 µg/mL) was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

DPPH assay: DPPH assay was carried out as described by Brand-Williams *et al*. with slight modifications[19]. The hydro-alcoholic extract of *Caesalpinia sappan* was dissolved in methanol and the various concentrations (1000 to 1 µg/mL) of each extract were prepared. Hydro-alcoholic extract dissolved in methanol (500 µL) mixed with 125 µL of 1 mM of freshly prepared DPPH solution and 375 µL of solvent (methanol). The contents were mixed vigorously in a vortex mixer for 10 s and incubated at room temperature in the dark (wrapped with aluminum foil) for 0.5 h. The absorbance was read at 517 nm using a spectrophotometer. All experiments were carried out in triplicates. Vitamin C was used as a standard and free radical scavenging activity of test samples corresponded to the intensity developed due to quenching DPPH. The results were expressed as percentage inhibition and their IC_{50} values were calculated and tabulated.

In vitro Antiinflammatory assays

Protein denaturation inhibition assay: Protein denaturation inhibition assay was performed according to Saso*et al*.[20]. The reaction mixture (0.5 mL) consisted of 450 µL 5 % aqueous bovine serum albumin and 50 µL of hydro-alcoholic extract of *C. sappan* (50, 100, 250, 500 and 1000 µg/mL). The pH of the reaction mixture was maintained to 6.3 using 1 N HCl. The test samples were incubated at 37 ºC for 20 min followed by heating at 57 ºC for 3 min. This mixture was then immediately brought to room temperature and finally phosphate buffer saline (1 mL, pH 6.3) was added. The absorbance of the resulting solution was measured at 660 nm. The percentage inhibition of protein denaturation was calculated and tabulated.

RBC membrane stabilization assay: RBC membrane stabilization assay was performed as per Shinde *et al*.[21]. Briefly, 300 µL hypotonic saline (0.25 % NaCl) was diluted with 100 µL of 0.15 M phosphate buffer (pH 7.4). To the resulting solution, 100 µL test solution (50, 100, 250, 500 and 1000 µg/mL) in normal saline and 500 µL of 10 % human RBC in normal saline were added. The reaction mixtures were incubated at

56 ºC for 0.5 h and the test tubes were cooled under running tap water for 20 min followed by centrifugation at 1500 rpm for 10 min. Absorbance of the supernatant was read at 560 nm. The percentage membrane stabilizing activity was calculated and tabulated.

Proteinase inhibitory assay: Antiproteinase assay was performed according to Oyedepo *et al.*[22]. Briefly, the reaction mixture (1.3 mL) contained 0.06 mg trypsin, 500 µL of 25 mM *Tris*-HCl buffer (pH 7.4) and 100 µL of hydro-alcoholic extract of *C. sappan* (50, 100, 250, 500 and 1000 µg/mL). The reaction mixture was incubated at 37 ºC for 5 min followed by the addition of 500 µL 0.8 % (w/v) casein. This was then incubated for 20 min followed by addition of 200 µL 70 % (v/v) perchloric acid, to terminate the reaction. The cloudy suspension was centrifuged and the absorbance was read at 280 nm against buffer as blank. The percentage of inhibition was calculated and tabulated.

***in vitro* assay for cytotoxicity activity by MTT assay:** The cell lines such as human laryngeal cancer cell lines (HEp2), human cervical cancer cell lines (HeLa), human breast cancer cell lines (MCF 7), human liver adenocarcinoma (HEP G2), African green monkey kidney cell lines (VERO) and human ductal breast cancer cell lines (T47D) were obtained from National Centre for cell sciences, Pune (NCCS) and were maintained in RPMI-1640 supplemented with 10 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified atmosphere of 5 % CO_2 at 37 ºC. The cytotoxicity of hydro-alcoholic extract of *C. sappan* on these cell lines were determined by employing MTT assay[23]. Cells (1×10^5/well) were plated in 96 well plates along with 100 µL of medium. The cells were incubated in the presence of various concentrations of the samples (1000-1 µg/mL) in 0.1 % DMSO for 48 h at 37 ºC. After removal of the sample solution, the wells were washed with phosphate-buffered saline and 20 µL of 0.5 % w/v MTT solution was added. After 4 h incubation, 0.04 M HCl/ isopropanol were added and the absorbance was read at 570 nm using microplate reader (Bio-Rad, Richmond, CA). The cytotoxic effect of the samples on the proliferation of individual cancer cells were expressed as the % cell viability, using the following formula:

$$\text{Cell viability (\%)} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100\ \%$$

Statistical analysis: All the experiments were carried out in triplicates and represented as mean ± SD. The IC_{50} (µg/mL) values were calculated. The statistical analyses were performed using the computer software Graph Pad Prism 6 for Windows (GraphPad Software, USA).

RESULTS AND DISCUSSION

The preliminary phytochemical analysis revealed the presence of phyto-constituents such as saponin, tannins, phenol, cardiac glycosides, flavonoids, steroids and terpenoids. The total phenolic content in hydro-alcoholic extract of *C. sappan* was found to be 49.71 ± 0.49 µg/mL equivalent to gallic acid. Similarly the total tannin and flavonoid contents were found to be 55.76 ± 0.53 and 27.13 ±0 .88 µg/mL equivalent to tannic acid and quercertin, respectively. The *in vitro* antiinflammatory assays were performed using three methods such as inhibition of protein denaturation, Membrane stabilization and proteinase inhibition assays and their results were tabulated and presented (Table-1).

Inflammation is a complex process and is associated with increase in vascular permeability and protein denaturation. Agents that prevent the above mentioned mechanism might be used as antiinflammatory drugs. Protein denaturation is one of the important causes of inflammation and arthritis. The synthesis of auto antigens in arthritic conditions may be due to protein denaturation[12]. In the present study, the inhibition of protein denaturation bioassay was selected to screen anti-inflammatory potential of hydroalcoholic extract of *C. sappan* and the IC_{50} value was determined as 239.9 µg/mL.

The membrane stabilization activity using Human RBC is considered as good *in vitro* model to screen antiinflammatory activity of the test drugs. The stabilization of lysosomal membrane will prevent the release of lysosomal content and thus prevent the tissue from inflammation. RBC membrane used in the present assay is comparable with lysosomal membrane[13]. The IC_{50} value for hydro-alcoholic extract of *C. sappan* using human RBC membrane stabilization assay was determined as 154 µg/mL.

Neutrophils released during inflammatory condition are rich in serine proteinase and thus develops tissue damages during inflammation. Any agents that prevent the release of proteinase enzyme will prevent inflammation hence considered as proteinase inhibitors. Hydro-alcoholic extract of *C. sappan* exhibited significant antiproteinase activity at different concentrations. The data obtained were recorded, calculated and the IC_{50} value was determined as 208.9 µg/mL.

The DPPH assay was performed for hydro-alcoholic extract of *C. sappan* and vitamin C and the IC_{50} value was found to be 5.43 and 4.40 µg/mL, respectively. The data obtained on reducing capacity of hydro-alcoholic extract of *C. sappan* and vitamin C were recorded and compared (Fig. 1).

HPTLC analysis was performed for *C.sappan* extract and compared with standard quercertin. The chromatogram was observed under both 254 and 366 nm. The HPTLC finger-

TABLE-1
in vitro ANTIINFLAMMATORY ACTIVITY OF *C. sappan*

Treatment	Concentrations (µg/mL)	Inhibition of protein denaturation (%)	Membrane stabilization (%)	Proteinase inhibition (%)
Hydro-alcoholic extract of *Caesalpinia sappan*	50	10.6 ± 1.6	13.0 ± 1.9	14.7 ± 0.6
	100	24.0 ± 3.5	41.1 ± 2.5	35.7 ± 3.1
	250	47.7 ± 2.7	65.0 ± 3.1	54.5 ± 1.9
	500	65.5 ± 2.6	74.8 ± 3.0	67.2 ± 1.1
	1000	80.7 ± 2.0	86.4 ± 2.4	84.0 ± 2.7
	IC_{50}	239.9	154.0	208.9

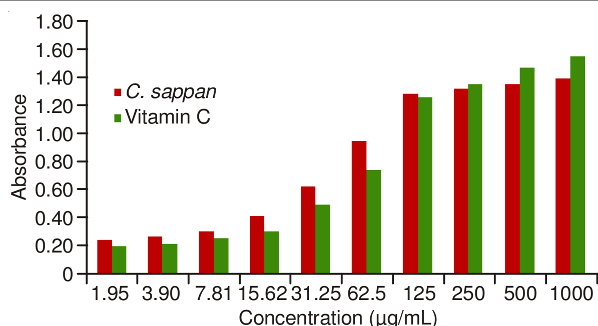

Fig. 1. *in vitro* ferric reducing capacity assay of *C. sappan* heartwood

printing of standard quercetin showed R_f at 0.72, whereas *C. sappan* extract also showed band with similar R_f 0.72. This confirms the presence of quercetin or its derivatives in the extract (Fig. 2). In addition, LC-MS/MS analysis confirmed the presence of quercetin derivative (dihydroquercetin) in the selected plant extract (Fig. 3).

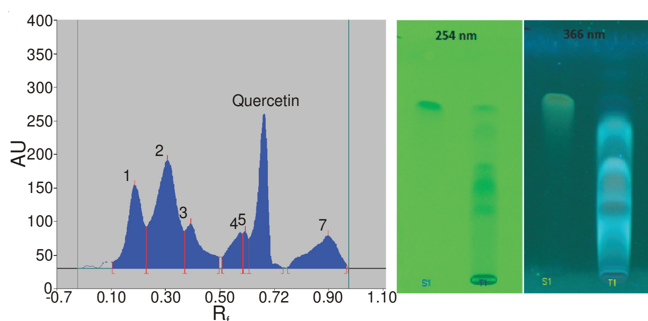

Fig. 2. HPTLC-profiles of *Caesalpinia sappan* hydro alcoholic extract

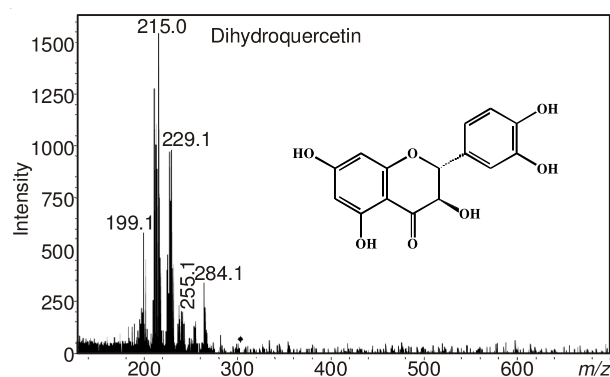

Fig. 3. LC-MS/MS fragmentation pattern of dihydroquercetin

In vitro cytotoxicity employing MTT assay was performed using different human cancer cell lines. The hydro-alcoholic extract of *C. sappan* showed good cytotoxicity against Human cervical cancer cell lines (HELA) as compared to other cell line studied and the IC_{50} value was found to be 180.75 µg/mL (Table-2).

TABLE-2
in vitro ANTICANCER EFFICACY OF *C. sappan* HEARTWOOD EXTRACT ON DIFFERENT CANCER CELL LINES

Human cancer cell line	IC_{50} (µg/mL)	Human cancer cell line	IC_{50} (µg/mL)
HEp2	231.37	T47D	340.13
HELA	180.75	HEPG2	283.39
MCF7	211.34	VERO	572.99

Conclusion

Data of the results obtained in the present study depicted that hydro-alcoholic extract of *Caesalpinia sappan* heart wood possess significant antioxidant, antiinflammatory and cytotoxicity potentials. The presence of quercetin-derivatives dihydroquercetin a bioactive flavonoid was also detected through LC-MS/MS analysis in hydro-alcoholic extract of *Caesalpinia sappan* heart wood.

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