

EVALUATION OF ANTI-CANCER PROPERTIES OF HEARTWOOD OF *Caesalpinia sappan*

¹SUDHEER MOORKOTH* AND ²NASEER M

¹Department of Pharmaceutical Quality Assurance, MCOPS, Manipal University, Manipal, 576104, India, ²Department of Pharmacology, MCOPS, Manipal University, Manipal, 576104, India. Email: sudheeremoorkoth@gmail.com

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ABSTRACT

Objectives: To evaluate the anti-cancer potential of the heartwood of *Caesalpinia sappan*. **Methods:** The extraction of the plant material was done using ethyl acetate, methanol and water. Cytotoxicity evaluated using MTT assay. Apoptosis potential was evaluated by cell cycle analysis and Hoechst staining techniques. *In vivo* anticancer evaluation performed by the Ehrlich's ascites carcinoma model and anti-inflammatory study performed using Carrageenan induced rat paw edema method. **Results:** Methanol and water extracts exhibited marked cytotoxic activity against human cancer cell lines such as HeLa, MDA MB, A 549, and HCT-15 in the MTT assay. An evaluation of the apoptotic potential of the water extract on HeLa cells also was performed using flow cytometry and Hoechst staining techniques. The DNA histograms obtained from the cell cycle analysis were apoptotic in nature and the fluorescent microscopic picture revealed the condensation and fragmentation of the nuclei in the treated cells. The *in vivo* study in albino mice using Ehrlich ascites carcinoma model resulted in an increase in the life span. **Conclusion:** The water extract obtained from the heartwood of *Caesalpinia sappan* has shown promising cytotoxic and apoptotic potential. The *in vivo* anticancer and anti-inflammatory activities of the extract were also promising.

Keywords: *Caesalpinia sappan*, cytotoxic activity, Ehrlich ascites carcinoma, Anti-inflammatory activity

INTRODUCTION

Caesalpinia sappan is a species of flowering tree in the family; Caesalpinaceae. It is native to southeast Asia and the Malay. Common names include Sappanwood, *Patanga-Chekke*Sappanga (Kannada name), *Pathimukham* (Malayalam name), *Bakam* or *Patang* (Hindi name) etc. Natives of Kerala add small chips or shavings of the heartwood of this plant to drinking water due to its blood purifying and antioxidant properties. It is propagated from seed and grows as a shrub up to 10m in height. It grows in the tropical region and cultivated for its medicinal and dye yielding properties. The heartwood is orange red and is hard. Branches are rufous-pubescent and have prickles. Leaves have pinnate arrangement (9-14 in pairs). Flowers are yellow and occur as inflorescence. Seeds are black and seen in pods usually 3-4[1]. The aerial parts of the plant are shown in the figure 1.

It is reported that the dye obtained from this plant mordanted with alum displays good fastness towards washing, and is also an ingredient of facials which are resistant to light, heat and water and are non-irritating[2,3]. Another traditional use of this plant is in the treatment of non-specific leucorrhoea to stop bleeding following an IUD insertion [4]. As per the Ayurveda, it is used in vitiated conditions of pitta, burning sensation, wounds, ulcers, leprosy, skin diseases, diarrhea, dysentery, epilepsy, convulsions, menorrhagia, leucorrhoea, diabetes, haemoptysis, haemorrhages and odontopathy[4,5]. The cytotoxic and anticancer activity, especially the topoisomerase inhibitory activity is well established[6]. It is reported that the essential oil and ethanol and water extracts of the wood showed antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and

Escherichia coli[7,8]. The hot water extract of the dried bark of the plant exhibited good antiviral activity[9,10]. It is reported that the methanol extract of the heartwood show anti-inflammatory activity[11]. It is also reported to possess immunostimulant[12], hypoglycaemic[13], anticomplementary[14] and hepatoprotective[15] properties. Reported chemical constituents present in the heartwood and seeds of this plant include triterpenoids, flavonoids, oxygen heterocycles, lipids, steroids, and aminoacids. Brazilin, the main constituent oxidises to produce brazilin[16].

EXPERIMENTAL

Collection

The heartwood of *Caesalpinia sappan* was collected during October 2008 from the campus of M.G. University, Kottayam, India and was authenticated by Dr. Bindu K., Department of Pharmacognosy, CPS, Cheruvandoor, Kottayam, Kerala.

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. Similarly a water extract was also prepared by heating heart wood powder (50 g) in a round bottomed 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).



Figure 1: Aerial parts of *Caesalpinia sappan*.

Biological activity

Ethyl acetate, methanolic and water extracts were evaluated for their *in vitro* cytotoxic activity. *In vitro* cytotoxicity was done by the MTT assay[17]. HeLa, MDA MB, A 549, and HCT-15 cell lines were used for the study. Water extract that showed an IC_{50} value less than 20 μ g/ml against all cell lines were studied for their apoptotic potential by Hoechst staining analysis[18] and flow cytometric cell cycle analysis[19]. *In vivo* anticancer studies were conducted on healthy adult Swiss albino mice weighing 20-25 g were procured from the central animal facility of the Manipal University. They were housed in polypropylene cages in a controlled environment (temperature 25 \pm 2°C, humidity (50 \pm 5 % and 12 hr dark and light cycle) with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance and following the ethical guidelines specified.

Study of the effect of the selected compounds on mice bearing Ehrlich's Ascites Carcinoma (EAC) cells

Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior. It is able to grow in almost all mice strains[20]. The Ehrlich's ascites tumor derived from a spontaneous murine mammary adenocarcinoma, is maintained in the ascitic form by passaging in Swiss mice, by i.p.route[21]. Ehrlich's Ascites Carcinoma (EAC) cells were obtained through the courtesy of Amala cancer research center, Thrissur. They were maintained by weekly intra-peritoneal inoculation of 1 \times 10⁶ cells/ mouse. The study was carried out as per the reported procedure[22]. Albino mice were divided into twenty three groups of 6 animals in each group. All the groups were inoculated with EAC cells (2 \times 10⁶ cells/mouse). Group I served as the tumor control. This group received only 2% acacia suspension intra-peritoneally. Group II served as a positive control and was treated with the standard drug Cisplatin at 3.5 mg/kg/day on alternative days on day 1, day 3 and day 5 for three days. Groups III was treated with the water extract at a dose of 100 mg and 50 mg respectively. All the treatments were given 24 hr after tumor inoculation. The treatments were given once daily for nine days. The mice were weighed every day till the 15th day. The median survival time of each group, consisting of 6 mice was noted. The antitumor efficacies of the compounds (average life span, % increase in life span, and % increase in body weight) were compared with that of the standard drug Cisplatin.

In vivo anti-inflammatory studies

The *In vivo* anti-inflammatory activity of the water extract was studied on Wistar rats by Carrageenan induced rat paw oedema method[23]. The rats were divided into three groups containing six rats in each group. Acute inflammation was induced according to the above reported method. 0.1 ml of 1.0% of carrageenan in normal saline (0.9 % w/v NaCl) was injected to the sub-plantar region of left hind paw. The water extract was suspended in 0.2% carboxy methylcellulose (CMC) and administered to the rats 1 hr before carrageenan injection. Different groups were treated as follows: Group 1: Carrageenan + 0.2% CMC (3.0 ml/kg) (Control), Group 2: Carrageenan + Water extract 100 mg/kg, p.o. (Test), Group 3: Carrageenan + Ibuprofen 100 mg/kg, p.o.

RESULTS AND DISCUSSION

Cytotoxic activity: The preliminary cytotoxic analysis was done by the MTT assay on all the extracts. A mitochondrial enzyme in the living cells namely succinate dehydrogenase, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation. The results were read on a multi-well scanning spectrophotometer (ELISA reader) which showed a high degree of precision. No washing steps were used in the assay. The main advantages of the colorimetric assay are its rapidity and precision. The results of cytotoxic activity are shown in the Table 1. The ethyl acetate extract did not show any cytotoxic response and the IC_{50} values were more than 100 μ g/mL. The water and methanol extracts were showing some promising activity against all the selected cell lines. Among the extracts, water extract was the most active with an IC_{50} value of 7.8 μ g/mL against the HCT 15 colon cancer cell lines.

Table 1: IC_{50} value (μ g/mL) obtained for the MTT Assay

Extract	HeLa	MDA MB	A549	HCT 15
Ethyl acetate	>100	>100	>100	>100
Methanol	34.86	27.71	32.19	23.68
Water	19.34	14.79	17.56	7.8
Doxorubicin	0.69	0.77	0.98	1.42

Hoechst staining for morphological assessment of apoptotic cells

The water extract was selected and studied for their potential for apoptosis at a concentration equal to their IC_{50} values. HeLa cells treated with the compounds were stained with Hoechst 33342 and the appearance of chromatin condensation and fragmentation of nuclei was monitored. Morphological observation of HeLa cells showed that control cells had regular and round-shaped nuclei revealed by nuclear staining with Hoechst 33342. By contrast, condensation and fragmentation of nuclei characteristic of apoptotic cells were evident in HeLa cells treated with Doxorubicin and with the selected compounds. The percentage of apoptotic cells increased significantly in cells exposed to the newly synthesized compounds. As can be seen from the results obtained with the Hoechst staining technique, the cells showed DNA fragmentation, condensed nucleus (highly fluorescent), and membrane blebbing, which are the characteristic features of apoptosis. These morphological changes in the nuclei of the apoptotic cells were visualized by fluorescence microscopy. The results are shown in Figure 2.



Figure 2: Results of the Hoechst staining analysis.

Cell cycle analysis

DNA fragmentation is considered to be the key biochemical event of apoptosis. One of the methods for identifying the DNA fragmentation is by the DNA content analysis by flow cytometry. The technique is based on the accumulation of ethanol-fixed apoptotic cells in the sub-G0/G1 peak of the DNA content histogram. The fluorescence-activated cell sorting (FACS) analyses were performed to study the effect of the synthesized compounds on the cell cycle progression. The results of water extract tested against HeLa cell lines at their 50% inhibitory concentration is shown in Figure 3, as DNA histograms. As seen in the above mentioned figure, the water extract showed the DNA histogram characteristic of apoptosis.

In vivo anticancer studies

The water extract was subjected to *in vivo* studies, to determine the mean survival time, % increase in life span (%ILS), and % increase in body weight in comparison with that of the standard

drug and control. Safe dose was selected from the data obtained from the acute toxicity studies. Accordingly, a dose of 100 mg/kg body weight per day was selected as the safe dose.

The mean survival time and percentage increase in life span were studied on EAC (Ehrlich's ascites tumor) induced mice in two doses of 100 mg/kg/day and 50 mg/kg/day. The treated mice showed an increase in the survival time (mean) from 17 days (for the untreated control) to 27.5 days (Table 2).

In vivo anti-inflammatory studies

Carrageenan induced rat hind paw edema is the standard experimental model of acute inflammation. It was observed that, the increase in paw volume following carrageenan administration in the control (0.46±0.01 ml) and ibuprofen treated group (0.15±0.01ml) corresponds with the findings of previous workers[24]. The water extract showed anti-inflammatory activity with a reduction of paw volume from 0.46 ml (untreated control) to as low as 0.22ml. The results are provided in the Table 3.

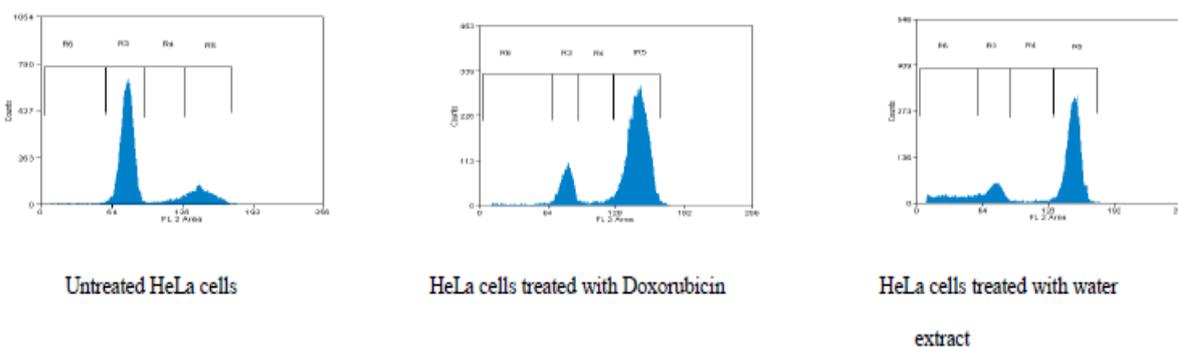


Figure 3: Results of cell cycle analysis

Table 2: Results of *in vivo* anticancer studies

Compound code	Dose (mg/kg/day)	Mean life span		Median life span	
		Mean survival time (days)	Mean % ILS	Median survival time (days)	Median % ILS
- ve control	-	17.33± 0.21	-	17	-
Cisplatin	3.5	34±0.63 ^a	96.19	34	100
Water Extract	100	27.5±0.42 ^a	58.68	27.5	61.76
	50	27.17±0.30 ^a	56.76	27	58.82

N=6 animals in each group, ^a p<0.01 Vs control as per Dunnet Multiple Comparisons Test for one way analysis of variance. Days of treatment =9, values are expressed as mean ±SEM.

Table 3: Results of *in vivo* anti-inflammatory studies

Compound code	Dose (mg/kg, p.o)	Mean Rat Paw Edema Volume after 3h (ml±SEM ^a) (n=6)	Percentage Inhibition±SEM
Control	CMC 2%	0.46±0.01	-
Water extract	100	0.22±0.01 ^a	52.45±3.1
Ibuprofen	50	0.15±0.01 ^a	67.14±2.5

One-way ANOVA followed by Post hoc Scheffe's test a = p<0.05 Vs Control group

CONCLUSIONS

The water extract has shown promising cytotoxic activity against all selected cell lines. Flow cytometry and Hoechst stain analysis confirmed the apoptotic nature of the water extract. Percentage increase in life span was comparable to the standard drug Cisplatin. The promising anti-inflammatory activity seen in our study supports the literature and the hypothesis that there is a relationship between inflammation and cancer. Molecular level mechanism of action can be explored in a future study.

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