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RESEARCH ARTICLE

EVALUATION OF ANTI TUMOUR EFFECT OF *Caeselpinia sappan* ON EHRLICH ASCITES CARCINOMA IN MICE

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ABSTRACT

To investigate the anti tumor potential of 50% hydro ethanolic extract of *Caeselpinia sappan* (EECS) on Ehrlich ascites carcinoma in mice. The anti tumor potential was carried out both by *in vitro* (MTT assay in Hep G 2 cell line) and *in vivo* methods. The cytotoxic effect (Ic 50 value) on Hep G 2 cell line and Vero cell line was found to be 1.25mg/ml and 2.50mg/ml. Various hematological parameters (RBC, WBC, Hb and PCV) and marker enzymes (Cathepsin-D, β-D-glucuronidase, Acid phosphatase, 5'-nucleotidase and lactate dehydrogenase) were analyzed. The extract was administered at the dose of 400mg/kg of body weight and with the 5-FU (20 mg/kg.*ip*) as the standard drug. Tumor was induced in mice by intraperitoneal injection of EAC cells ($1x10^6$cells/mouse). Significant changes were observed in biochemical parameters of ascitic carcinoma mice when compared to *C.Sappan* treated mice. The anti tumor potential activity was mainly due to the presence of poly phenolics and flavonoids present in the plant extract. Thus, the present study confirms the anti tumor potential of the ethanolic extract of *C.Sappan*.

INTRODUCTION

Traditional medicine has a long history of serving people all over the World. The Ethano botany and ubiquitous plants provide a rich resource for natural drug research and development (Garg *et al*., 2007). Medicinal plants are used for antidiabetic, anti tumor, central nervous system activity, insecticidal and anti microbial agents. Plant derived compounds have played an important role in the development of several clinically useful anti cancer agents (Cragg *et al*., 2005). *Caeselpinia sappan* is a tropical tree distributed in Tamil Nadu, Kerala, Karnataka and West Bengal. The heart wood is reported to contain a glycoside containing β-amyrin and 2-deoxy ribose. The heart wood contains several compounds such as branzilin, caeselpin, sappanol, episappanol and quercetin (Guha bakshi *et al*., 1999). The plant contains secondary metabolites such as glycosides, flavonoids, phenols and tannins (Varies *et al*., 1994). The present investigation was carried out to evaluate the anti tumor activity of the ethanolic extract of *C. sappan* heart wood against EAC induced mice.

MATERIALS AND METHODS

Chemicals

5-flurouracil, nicotiamide adenine dinucleotide, deoxyribonucleic acid, and ribonucleic acid were purchased from Biochem Ltd, India. All the other chemicals used were of analytical grade.

Collection of plant and extract preparation

The heart wood of *C.sappan* was procured from a local market in Coimbatore, Tamilnadu, India. The plant was authenticated by a botanist in Botanical Survey of India, Coimbatore. Freshly collected material was chopped, shade dried, and coarsely powdered in a mechanical grinder. 100 g of dried powder was extracted with 150 ml of ethanol–water (1:1) several times at room temperature. After 3 days, the suspension was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature (<40° C) under reduced pressure in a rotary evaporator. The yield of the plant extract was noted to be 12%.

MTT assay

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT) was used to determine cell viability in assays of cell proliferation and cytotoxicity. The study was carried out with different concentrations (0.078-10 mg) of plant extract against Vero cell line and Hep G 2 cell line (Thabrew *et al*., 2005)

Animals

Male Swiss albino mice weighing 20 ± 2 g were procured from the animal house of PSG Institute of Medical Sciences and Research, Coimbatore. They were housed in the standard microbon boxes and given standard mouse pellet and water ad libit. The clearance of the ethical committee for experimentation on animals was obtained before the start of the experiment (No: 158/99/CPCSEA).

Tumor cells

EAC cells were obtained through the courtesy of Amala Cancer Research Center, Kerala (Thrissur), India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal (*i.p.)* transplantation of 1×10^6 cells/mouse after every 10 days.

Treatment schedule

The mice were categorized into five groups (n=9). Except group I (normal) and group V (plant control), all the other groups were

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injected with EAC cells (1 × 10^6 cells/mouse) *i.p.* This was taken as day zero. On the first day, normal saline was administered to group I and group II. *C. sappan* extract at a dose of (400mg/kg.bw.po) was administered to group III and group V for 14 days. The standard drug 5-FU (20mg/kg.bw.ip) was administered to group IV for 14 days. After the last dose and 18-hr fasting, six mice from each group were sacrificed for various hematological and cancer marker enzyme parameters.

Measurement of biochemical parameters

The blood collected after killing was anticoagulated by adding EDTA and used for determining various hematological parameters such as RBC, Hb, total WBC count and PCV (Mukherjee, 1998). One gram of liver tissue was taken and homogenized with 10 ml of 0.1 M cold Tris-buffer, pH 7.4. Cancer marker enzymes, namely Cathepsin-D (Sapolsky, 1973) β-D-glucuronidase (Kawai and Anno, 1971) Acid phosphatase (Fiske and Subbarow, 1925) 5′-nucleotidase (Heppel and Hilmoe, 1951) and Lactate dehydrogenase (King J, 1965), were estimated in ther Liver homogenate.

Statistical analysis

All results were expressed as mean ± SD. The significance of the *in vivo* data was analyzed by the one-way analysis of variance (ANOVA), followed by the *post hoc* LSD comparison test using SPSS version 10.0. $P < 0.05$ was considered as statistically significant.

RESULTS

***In vitro* cytotoxicity assay**

The extract at a concentration 0.078 mg/ml showed 95% cell viability in MTT assay and 90% reduction was observed for 10mg/ml concentration. IC_{50} value was calculated after 48 hrs of exposure and was found to be 1.25 mg/ml for Vero cell line and 2.50 mg/ml Hep G 2 cell line. The maximum percentage of cytotoxic inhibition was reported for 10 mg/ml concentration and the minimum percentage of inhibition was for 0.07mg/ml concentration. The cytotoxic activity decreases gradually from 10mg/ml concentration to 0.07mg/ml concentration from 90 to 5% respectively (Figure 1 and Figure 2).

***In vivo* parameters**

Hematological parameter of EAC infected mice on day 14 were found to be remarkably altered when compared to normal mice. The WBC count was incremented but the Hb and RBC count were markedly declined. Among the various white blood cells analyzed, neutrophills were found to be elevated. Supplementation of EECS and 5-flurouracil to EAC infected mice has restored the above alterations to a significant extent (Table 1).

Cancer marker enzymes

Cancer marker enzyme in lysosomes like cathepsin-D, β-D-glucuronidase and acid phosphatase was incremented in EAC-bearing mice. The Cathepsin-D was elevated twice (37.72±2.55) than the normal mice (18.18±1.65). β-D-glucuronidase and acid phosphatase were increased in EAC control when compared to normal mice. The EECS extract on EAC bearing mice was found to control the leakage of lysosomes and retrieve the normal function. (Table 2). Liver Marker enzymes such as lactate dehydrogenase and 5′-nucleotidase were found to significantly increased when compared to the control animals. In EAC control mice 5′-NT was elevated thrice (5.51± 0.96) when compared to normal mice (1.72± 0.80). LDH activity in the liver of EAC-transplanted mice (0.55± 0.36) was markedly declined when compared to the normal mice (1.57 ±0.75). The activities of these enzymes were significantly brought back to near normal level by the administration of EECS. (Table 3).

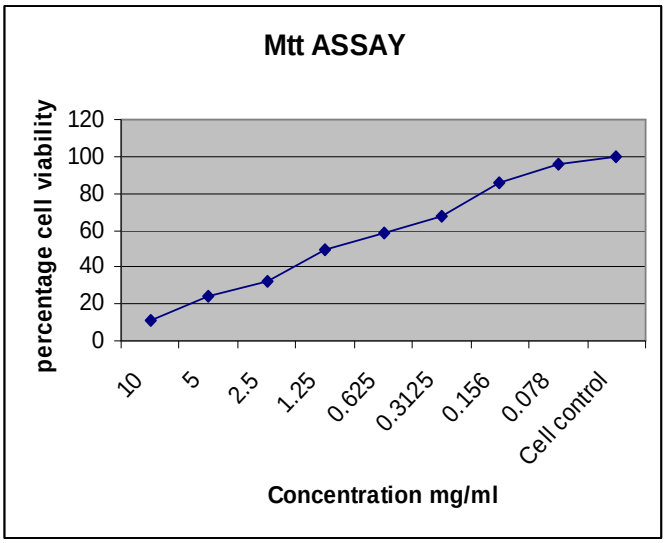

Figure 1. *C.sappan* extract Against Vero cell line

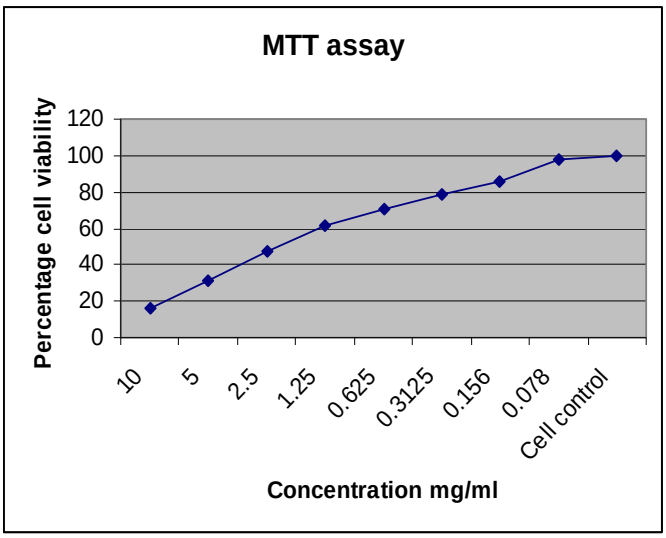

Figure 2. *C.sappan* extract against Hep G2 cell line

Table 1. Antitumor activity of ethanolic extract of *C.sappan* on haematological parameters in normal and experimental group of mice

Groups	RBC (millions/cu.mm)	Hb (g/dl)	WBC (10^3 / cu.mm)
Normal	4.51 ± 0.82	11.90 ± 0.11	9.22 ± 1.36
EAC-control (1X10^6 cells/mouse)	2.41± 0.98 [a]	6.84 ± 0.28 [a]	14.88 ± 0.31 [a]
EAC-control + *C.sappan* (400 mg/kg.bw.*p.o*)	3.35 ± 0.63 [a,b]	10.34 ± 0.54 [a, b]	11.03 ± 0.77 [a, b]
EAC-control+5-fluorouracil (20 mg/kg.bw.*i.p*)	4.22± 0.91 [b]	11.11 ± 0.51 [b]	10.73 ± 0.52 [a b]
Plant control (400 mg/kg.bw.*p.o*)	4.29 ± 1.03	11.42 ± 0.63	9.50± 1.77

Values are Mean ± SD (n=6)
*P< 0.05 statistically significant when compared with EAC control group

Table 2. Effect of ethanolic extract of *C.sappan* on the activities of Lysosomal marker enzymes in liver of normal and experimental group of animals

Groups	Cathepsin-D (µmoles of tyrosine liberated/hr/mg protein)	β-D-glucuronidase (µmoles of *p*-nitrophenol formed/ min/mg protein)	Acid phosphatase (µmoles of *Pi* liberated/ min/mg protein)
Normal	18.18 ± 1.65	20.93 ± 1.17	3.22 ± 1.24
EACcontrol (1X10^{6}cells/mouse)	37.72 ± 2.55 ^{a}	37.19± 1.56 ^{a}	11.34 ± 1.52 ^{a}
EAC-control + *C.sappan* (400 mg/kg.bw.*p.o*)	20.5 ± 2.22 ^{a,b}	25.45 ± 3.06 ^{a,b}	5.65 ± 1.41 $^{a, b}$
EAC-control+5-fluorouracil (20mg/kg.bw.*i.p*)	19.87 ± 1.45 ^{b}	23.73 ± 3.09 ^{a,b}	4.46 ± 0.83 ^{b}
Plantcontrol (400mg/kg.bw.*p.o*)	19.20 ± 1.85	22.88 ± 2.1	3.71 ± 1.01

Values are Mean ± SD (n=6)
**P*< 0.05 statistically significant when compared with EAC control group

Table 3. Effect of ethanolic extract of *C.sappan* on the activities of liver marker enzymes in normal and experimental group of animals

Groups	5'-Nucleotidase (units/mg protein)	Lactate dehydrogenase (units/mg protein)
Normal	1.72 ± 0.80	1.57 ±0.75
EAC-control (1X10^6 cells/mouse)	5.51± 0.96 ^{a}	0.55± 0.36^a
EAC-control + *C.sappan* (400 mg/kg.bw.*p.o*)	3.65± 0.78 ^{a,b}	1.10 ± 0.30^b
EAC-control + 5-fluorouracil (20 mg/kg.bw.*i.p*)	3.56 ± 0.82 $^{a, b}$	1.12 ± 0.20 ^{b}
Plant control (400 mg/kg.bw.*p.o*)	2.45 ± 0.85	1.27 ± 0.52

Values are Mean ± SD (n=6)
**P*< 0.05 statistically significant when compared with EAC control group

DISCUSSION

Ehrlisch ascites carcinoma is one of the experimental breast tumor model. MTT assay is a well-established *In vitro* method for cytotoxicity against cancer cell lines and non-cancer cell lines (Abu-Dahab and Afifi, 2007). When the Ic 50 value lower than 30 g/ml, they can be used to discover and develop several potential anticancer natural compounds (Riss and Moravec, 2004). Cytotoxicity screening models provide important preliminary data to help in selecting plant extracts with potential antineoplastic properties for future work (Baskar *et al*., 2010). Several plant species rich in flavanoids are reported in preventing diseases and therapeutic properties. Cytotoxic activity recorded in this study flavonoids in *C. sappan* with promising activity. The cytotoxic activities of this active plant are probably due to the presence of several secondary metabolites. It is of interest that the extracts of the plants showed cytotoxicity against cancer cell line, and if this also occur *in vivo*, the use of these plants by traditional healer for the treatment of cancer patients would have some scientific support (Kanadaswami *et al*., 2005). The reliable criteria for judging the value of any anticancer drug are prolongation of life span, inhibition of gain in average body weight and decrease of WBC from blood (Clarkson and Burchanal, 1965). The ascites fluid is the direct nutritional source to tumor cells and the rapid increase in ascites fluid with tumor growth could possibly by a means to meet nutritional requirements of tumor cells and packed cell volume.

The body weight was incremented in EAC-mice due to ascitic tumor volume (Price and Greenfield, 1958). It is mainly due to the actively proliferating peritoneal cells (Fecchio *et al*., 1990). The decrease in life span is by low level of Hb in EAC-mice (Prasad and Giri, 1994). The changes in the body weight and life span in EAC mice with EECS group exhibited the inhibitory property of tumor present in the plant extract. The major problems encountered are hematopoiesis, immune response, myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to the reduction in RBC and hemoglobin percentage, deficiency of iron or due to the hemolytic or myelopathic conditions in EAC mice (Feninger and Mider, 1954). Lysosomes are membrane bounded cytoplasmic organelles contain hydrolytic enzymes. The enormous production of free radicals in the cancerous condition leads to the abnormal fragility of the lysosomes results in the elevated levels of lysosomal enzymes (Geetha, 1993). Oral feeding of *C.sappan* lowered the leakage of lysosomal marker enzymes most likely via stabilizing the membrane architecture. This could be attributed to the presence of flavonoids in the extract that have an inhibiting property on lysosomal membranes (Niebes and Ponard, 1975). Degradation of basement membrane and extracellular membrane plays a crucial role in tumor invasion and metastasis. Extra cellular matrix degradation by proteases in involved not only in local invasion, but also in several stages of metastastais cascade, including angiogenesis, intravasation and extravasations (Pellentier and Novikoff, 1972). Cathepsin-D (CD) plays a proteolytic role in the digestion of ECM components. In most breast tumors, CD is over expressed from 2 to 50-fold compared to its concentration in other cell types (Capony *et al*., 1989). β-D-glucuronidase was shown to be a sensitive marker of lysosomal integrity (Kalra *et al*., 1990). Increased serum glycoside levels found in cancerous conditions may thus be associated with structural changes in the enzymes which make the liver cells unable to recognize them, thereby preventing their clearance from the blood. The levels glycan moieties and the activities of glycosides can be used as diagnostic marker to assess the site of cancer and can be used as prognostic markers during therapy (Bhuvarahamurthy *et al*., 1995).

Acid phosphatase is also a cytoplasmic enzyme that has been considered to be associated with the lysosomes which catalyses the hydrolysis of organic phosphate. 5'-nucleotidase is a glycoprotein hydrolysis of nucleotides to nucleoside inorganic phosphate was found to be elevated in the sera of 90% breast cancer patients before treatment. This elevation of the marker enzymes may be correlated with the progression of the malignancy. Dao *et al*., 1980 have reported that the increased activity of 5'-nucleotidase seems to have originated from the proliferating breast cells. This elevation of the marker enzyme may be correlated with the progression of the malignancy (Dao *et al*., 1980). That LDH is a tetrameric enzyme recognized as a marker with potential role in assessing the progression of the proliferating malignant cells. The elevation will further release of the isoenzymes from the destroyed tissues (Helmey and Ei-moneim, 1998). This may due to the higher glycolysis in the cancerous condition, which is the only producing pathway for the uncontrolled cells (Karunairatnam *et al*., 1949). All these marker enzymes were brought back to normal suggesting that the presence of flavonoids in the plant extract, exerting anti proliferative action in the cancer cells.

Conclusion

On the basis of the results obtained in the present study, it is concluded that a 50% ethanolic extract of *Caeselpinia sappan* heart wood , capable of suppressing tumor cells both in *in vitro* and *in vivo*. This anti proliferative activity of the plant extract contains large

amounts of flavonoid and phenolic compounds which exhibit high antioxidant and free radical scavenging activities. Thus, the plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

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