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Research

Evaluation of in-vitro and in-vivo anticancer activity of leaf extracts of pisonia alba

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Abstract

Pisonia alba leaves have been traditionally used for many ailments including cancer. In the present study, anti-cancer activity of ethanolic leaf extract of *Pisonia alba* (EEPA) was evaluated using both in vitro and in vivo methods. EEPA was subjected to preliminary qualitative phytochemical investigations by using standard procedures. In vitro antitumor activity of EEPA was evaluated by the MTT assay method using The human cervical cancer cell line (HeLa). Then the extract subjected to in vivo anti cancer activity using Dalton's Lymphoma Ascites (DLA) tumor model. The activity was assessed Increase in life span, average increase in body weight, changes in food intake, tumor volume, tumor weight, viable cell count, non viable cell count, PCV, Total cell count and hematological studies. The potency of the extract was compared with standard 5-flurouracil (20 mg/kg i.p.).In in vitro anti cancer activity EEPA exhibited significant cytotoxic activity against both cell lines even at different concentrations. Oral administration of EEPA at the dose of 200 and 400 mg/Kg, significantly ($p < 0.001$) increased the survival time, non viable cell count and decreased the average body weight and food intake, viable cell count of the tumor bearing mice. After 14 days of inoculation, EEPA was able to reverse the changes in the hematological parameters, protein and PCV consequent to tumor inoculation.The results indicate that EEPA possess significant antitumor activity on dose dependent manner.

Keywords: Pisonia alba, HeLa, DLA, tumor, non-viable

INTRODUCTION

Cancer is one of the most severe health problems in both developing and developed countries, worldwide. Among the most common (lung, stomach, colorectal, liver, breast) types of cancers, lung cancer has continued to be the most common cancer diagnosed in men and breast cancer is the most common cancer diagnosed in women. An estimated 12.7 million people were diagnosed with cancer across the world in 2008, and 7.6 million people died from the cancer during the same year [1]. Lung cancer, breast cancer, colorectal cancer and stomach cancer accounted for two-fifths of the total cases of cancers diagnosed worldwide [1]. More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths due to cancer are projected to continuously increase and it has been estimated that there will be 11.5 million deaths in the year 2030 [1] and 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world by 2050 [2]. According to Canadian cancer statistics,

issued by the Canadian Cancer Society, it is estimated that 186,400 new cases of cancer (excluding 81,300 non-melanoma skin cancers) and 75,700 deaths from cancer will occur in Canada in 2012 [1]. The lowest number of incidences and mortality rate is recorded in British Columbia. Both incidence and mortality rates are higher in Atlantic Canada and Quebec [3].

Pisonia alba also known as *Pisonia alba spanoghe, pisonia umbellifera*, belongs to the family of Nyctaginaceae. *P. alba* is a large evergreen shrub. It is originally from the beach forests of Andaman Islands. Leaves: Long, bounty, and fresh green in color. If planted in good sunlight, the leaves may acquire a light yellow color. Flowers: The tree rarely flowers in India. The flowers are small, green, and inconspicuous. Uses: The leaves are edible. Young leaves are used as a vegetable. Leaves make good cattle feed too and are mostly used to treat rheumatism or arthritis. In traditional Indian medicine, they are used as an anti-diabetic; Leaves , of course, are used by natives as cattle feed; They are cooked and eaten for arthritis; The leaves are also carminative; Leaves are an antidote for snake bites; Researches have revealed that favonoids, steroids and phenolic compounds are present in the leaf. [4,5] The aim of the present study was to explore the anti-cancer potential of ethanolic extract of leaves of *Pisonia alba.*

MATERIALS AND METHODS

Plant Collection & Extraction

The plant *Pisonia alba* was collected from Institute of Kanchi Mamunivar Govt. Institute for Postgraduate Studies and Research, Pondicherry. The plant material was identified and authenticated by Dr. M. KUMARESAN, Assistant Professor of Botany, Kanchi Mamunivar Govt. Institute for Postgraduate Studies and Research, Pondicherry. The plant was shade dried at room temperature and was subjected to sizereduction to a coarse powder by using dry grinder. 60grams of this coarse powder was packed in to soxhlet apparatus and was subjected to extraction sequentially with 500ml of petroleum ether followed by ethyl acetate and ethanol. The extraction was continued until the colour of the solvent in the siphon tube become colourless.

***In-vitro* anticancer activity**

The anticancer activity of *Pisonia alba* was evaluated by MTTassay against Human cervical cancer cell line (HeLa).

Cell line

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passages weekly and the culture medium waschanged twice a week.

MTT assay

It is otherwise called as tetrazolium salt assay or Microculturetetrazolium test. MTT assay is an *in-vitro* method for screening, which has been internationally accepted. 3-[4,5- dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

The cytotoxicity of samples on Chang Liver cells was determined by the MTT assay (*Mosmann et al.,*1983). Cells (1×10^5/well) were plated in 5ml ofmedium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 hoursincubation the cell reaches the confluence. Then, cells were incubated in the presence samples for 24 - 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 1ml/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-- tetrazolium bromide cells(MTT) phosphate- buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol was added. [6-11]

Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) wasdetermined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of Chang Liver cells was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%

Linear regression graph was plotted between % cell viability and Log concentration. The IC_{50} was determined by using graphical method.

***In- vivo* anticancer activity**

The anticancer activity of *Pisonia alba* was evaluated in Swiss albino mice. Tumor was induced by intra-

peritoneal injection of ($1x10^6$) Dalton's Lymphoma Ascites (DLA) cells. The anticancer effect of plant extract was compared with standard drug 5- fluorouracil at 20mg/kg body weight.

Experimental procedure

Selection, Grouping and Acclimatization of Laboratory Animal

Male Swiss albino mice (20-25gm) were produced from animal xperimental laboratory and used throughout the study .They were housed in micro nylon boxes in a control environment (temp 25+2ºC) and 12 hr dark/light cycle with standard laboratory diet and water *ad libitum*. All animal procedures were performed after approval from the Institutional ethical committee. The experimental protocol has been approved by Institutional ethical committee,

Evaluation of anticancer activity

Induction of cancer using DLA cells

Dalton's Lymphoma Ascitic (DLA) cells were supplied by Amala cancer research centre, Trissur, Kerala, India. The cells are maintained in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animalthe DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilutions were made so that total cell should be 1×10^6, this dilution was given intraperitoneally. Let the tumor grown in the mice for minimum seven days before starting treatments.

Treatment Protocol

Swiss Albino mice were divided into five group of six each. All the animals in four groups were injected with DLA cells (1×10^6cells per mouse) intraperitoneally and the remaining one group is normal control group. The five groups were treated as follows. Group – 1 Served as the normal control, Group – 2 Served as the tumor control. Group 1 and 2 receives normaldiet and water. Group –3 Served as the positive control, was treated with injection of5-fluorouracil at 20mg/kg body weight, intraperitoneally. Group –4 Served as treatment control received (200mg/kg.bw) EEPAadministered through orally. Group – 5 Served as treatment control received (400mg/kg.bw) EEPAadministered through orally. Drug treatment was given after the 24hr of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital puncture blood collecting method. From the collected blood haematological parameters were checked, the remaining blood was centrifuged and serum was used for the estimation of biochemical parameters.

Determination of percentage Increase of Life Span (ILS)

In this study, drug treatment was given after the 24hr of inoculation, once daily for 14 days. The animals were monitored daily twice for 30 days. Antitumor effect of *Pisonia alba* was determined by monitoring the death pattern of animals due to tumor burden and calculating the percentage increase in life span (%ILS). The percentage increase in life span was calculated using the following formula:

$$\text{ILS (\%)} = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100$$

Determination of Average Body weight changes

The body weight (BW) of the control and treated group animals were measured from 0th day to 30th day interval up to 30 days.

Cancer cell count

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8ml of ice cold normal saline or sterile Phosphatebuffer Solution and 0.1ml of tryphan blue (0.1mg/ml) and total numbers of the living cells were counted using haemocytometer.

Cell count = No. of cells Dilution/Area × Thickness of liquid film

Histopathological analysis

A small portion of liver was taken and fixed in to 5% formaldehyde, after several treatments for dehydration in alcohol, sections having 4µm thickness were cut and strained with haematoxylin and eosin and histopathological analysis was carried out for the treated as well as control group of mice. [6-11]

Statistical analysis

Values are expressed as mean (±SEM). The statistical analysis was performed using one-way analysis of

variance (ANOVA) followed by Dunnett test using Graph padIn Stat version 3.0, Graph Pad Software, San Diego, California< USA. P-Values (i.e., *p<0.05, **P<0.01) were considered statistically significant compared to DLA tumor control.

RESULTS AND DISCUSSIONS

MTT assay

Dried leaf part of *Pisonia alba* were extracted with solvents like Petroleum ether, Ethyl acetate and Ethanol. *In-vitro* cytotoxic activity was carried out in Human Cervical Cancer cell line (HeLa) with extracts of Petroleum ether, Ethyl acetate and Ethanol. Test for cytotoxicity was carried out by MTT assay. Among the three extracts evaluated, the effective extract was found to be Ethanol extract with IC_{50} valueof 15.6µg/ml followed by ethyl acetate and petroleum ether with IC_{50} value of 62.5µg/ml and 15.6µg/ml respectively. Linearity was expressed with help of graph plotted in Microsoft excel. By carrying out MTT assay ethanol extract was found to be more effective of all three extracts and further studies carried out with ethanol extract. Acute toxicity studies were carried out by using Swiss albino mice at dose level up to 2000mg/kg. Figure 1-3 & 4-6. [19-21]

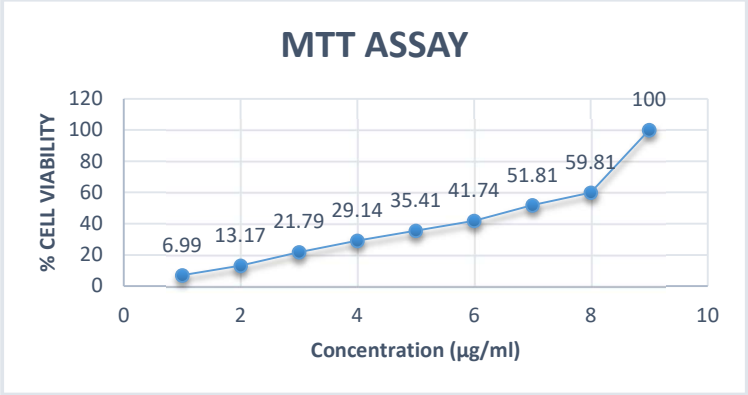


Fig 1: % Cell viability Vs Concentration in µg/ml of ethanolic extract of *Pisonia alba*

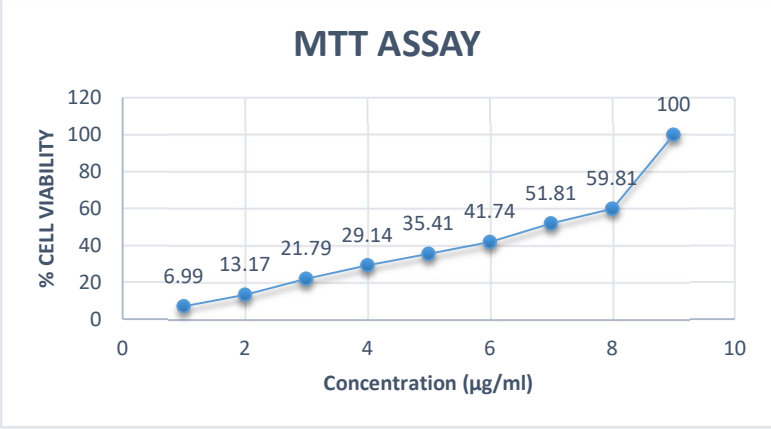


Fig 2: % Cell viability Vs Concentration in µg/ml of Ethyl acetate extracts of *Pisonia alba*

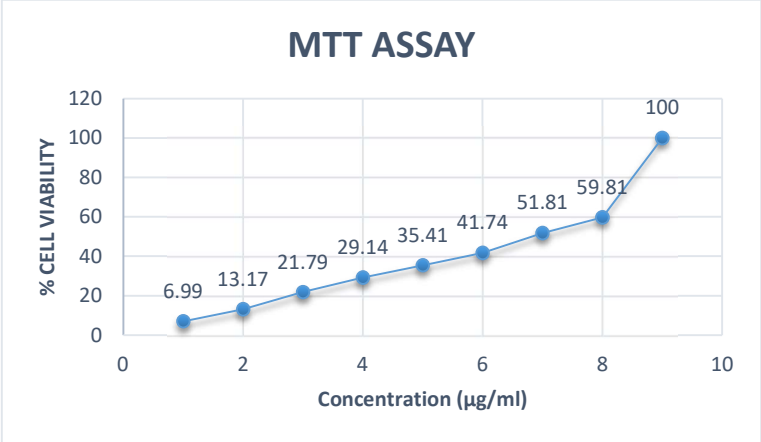


Fig 3: % Cell viability Vs Concentration in µg/ml of Petroleum ether extract of *Pisonia alba*

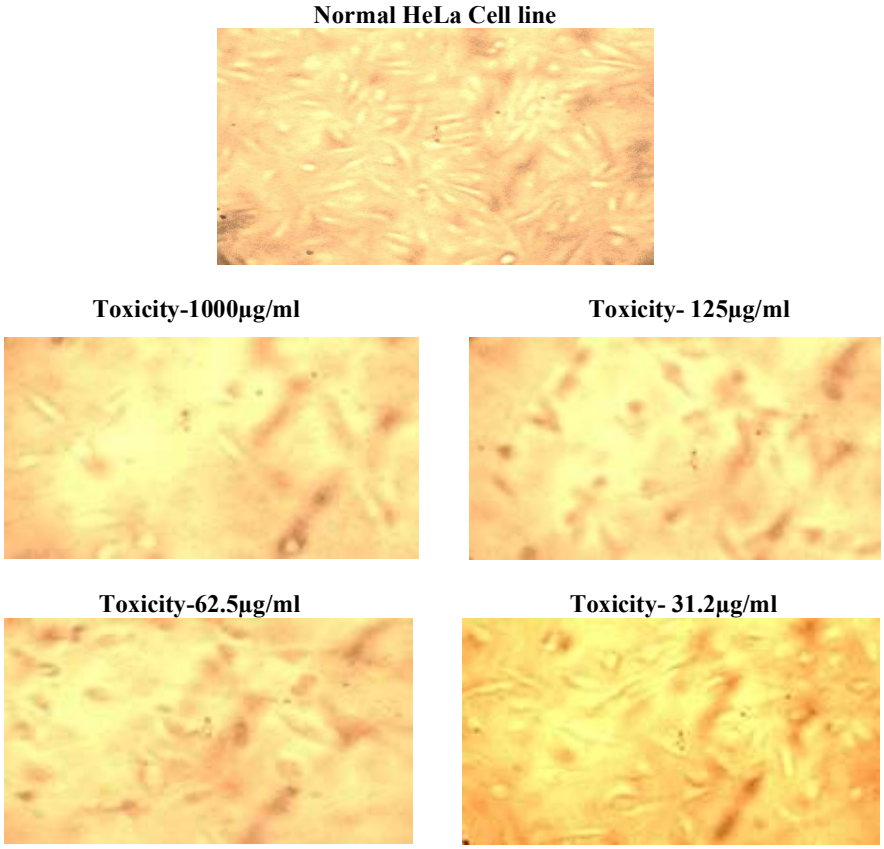
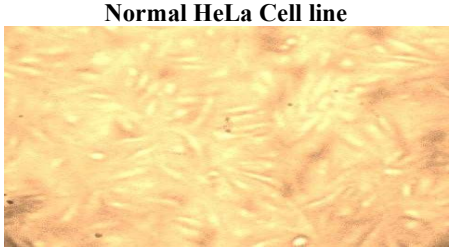


Fig 4: Anticancer effect of Ethanol extract on *HeLa* Cell line



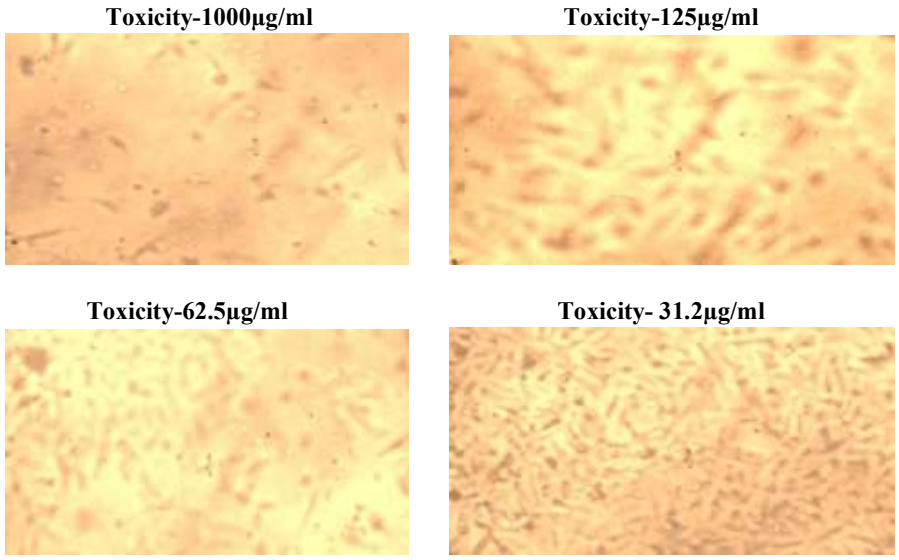


Fig 5: Anticancer effect of Ethyl acetate extract on *HeLa* Cell line

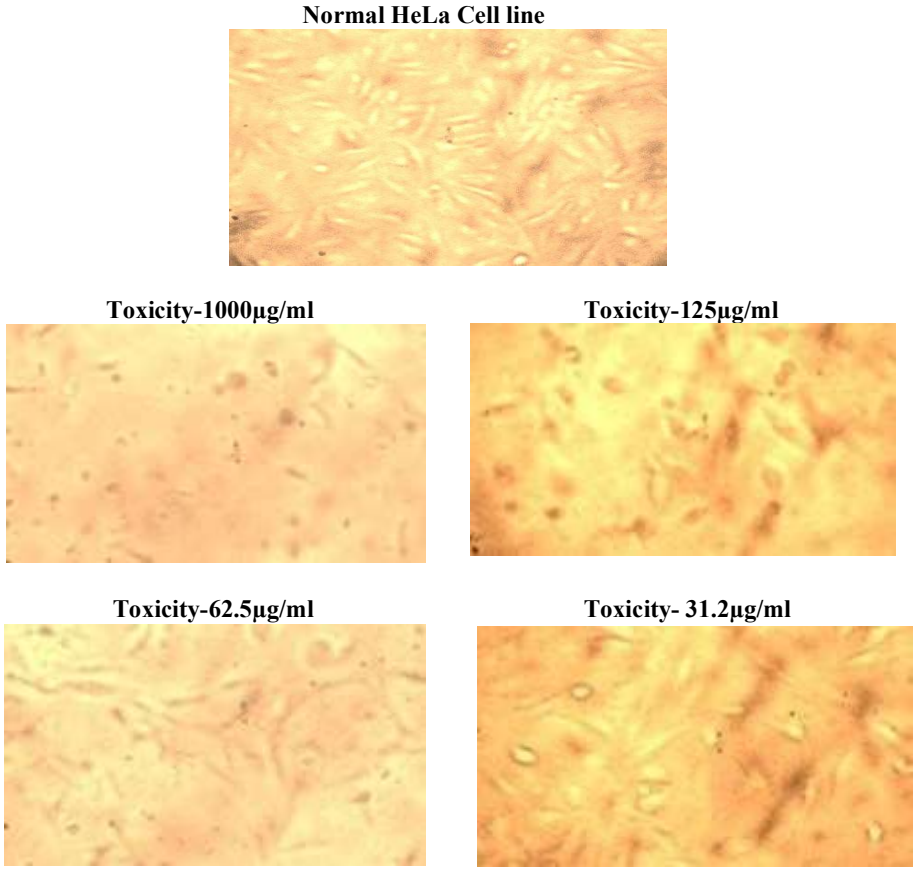


Fig 6: Anticancer effect of Petroleum Ether extract on *HeLa* Cell line

***In- vivo* anticancer activity**

Evaluation of Hematological parameters

As shown in (Table: 1) RBC, Hb, Platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with ethanolic extract of *Pisonia*

*alba*at a dose of 200 and 400 mg/kg significantly increases the Hb content, RBC, Platelets and significantly decreased the WBC count to normal level. All these results suggested the anticancer nature of the ethanolic extract of *Pisonia alba*at a dose of 200 and 400 mg/kg. However, the standard 5FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Table 1: Effect of EEPA on Hematological parameters

Treatment	Total WBC Cells /mlx10^3	RBC Count Mill/cum	Hb Gm/dl	PCV %	Platelets Lakhs/cum
G1	9.20 ±0.25	3.22±0.80	11.25 ±1.40	13.10±2.30	2.28±0.65
G2	15.10 ±11.97^{a**}	1.15±0.40^{a**}	5.05 ±0.80^{a**}	35.20±3.20^{a**}	0.99±0.45^{a**}
G3	12.15 ±0.94^{b**}	2.85±1.30^{b**}	10.17±1.40^{b**}	15.35±1.35^{b**}	1.88±0.82^{b**}
G4	13.20 ±0.81^{b**}	3.01±0.52^{b**}	11.05±1.25^{b**}	19.52±2.25^{b**}	2.08 ±0.56^{b**}
G5	13.10±1.00^{b**}	3.12±0.25^{b**}	11.15±1.55^{b**}	17.60±2.20^{b**}	2.38±0.65^{b**}

*G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatmentcontrol (200mg/kg) G5 – Treatment control (400mg/kg). All values are expressed as mean ± SEM for 6 animals in each group. **a – Values are significantly different from normal control (G1) at P < 0.001. **b – Values are significantly different from cancer control (G2) at P < 0.01*

Serum enzyme and lipid profile

Abnormal blood lipid profile has been associated with cancer. In Hodgkinlymphoma, high cholesterol level and low triglyceride level has been reported. Abnormal liver function seen in patient with Hodgkin lymphoma that these liver enzyme levels markedly increase in tumor bearing mice. ALP is an enzyme mainlyderived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes. An increase in ALP levels in the serum is frequently associated with the variety of disease. ALP comprises a group of enzyme that catalyzes the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate. Markedly elevated serum ALP, hyperalakline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis. [Table 2] [22-26].

Table 2: Effect of EEPA on serum Enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT(U/L)	ALP (U/L)
G_1	109.15±2.35	133.71±2.50	37.34 ±0.62	32.29 ±1.39	129.29 ±1.34
G_2	141.81±3.15^{a**}	227.21±4.45^{a**}	73.3±2.60^{a**}	59.29±2.64^{a**}	257.34±3.29^{a**}
G_3	127.21±2.70^{b**}	161.21±2.62^{b**}	45.34 ±1.60^{b**}	31.49±1.64^{b**}	174.39±1.35^{b**}
G_4	113.14±1.50^{b**}	169.14±2.50^{b**}	47.39±1.85^{b**}	37.37 ±1.69^{b**}	193.34±1.48^{b**}
G_5	111.11±2.22^{b**}	165.51±2.70^{b**}	39.49 ±2.09^{b**}	34.21±1.94^{b**}	195.31±1.11^{b**}

*G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatmentcontrol (200mg/kg) G5 – Treatment control (400mg/kg). All values are expressed as mean ± SEM for 6 animals in each group. **a – Values are significantly different from normal control (G1) at P < 0.001. **b – Values are significantly different from cancer control (G2) at P < 0.01*

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Derived parameters

In the DLA tumor control group, the average life span of animal was found to be 48% whereas ethanolic extract of *Pisonia alba*at a dose of 200mg/kg and 400mg/kg body weight increase the life span to 79%, and 86% respectively. These valueswere significant. However the average life span of 5-FU treatment was found to be 94%, indicating its potent antitumor nature. The antitumor nature of ethanolic extract of *Pisonia alba*at a dose of 200 and 400 mg/kgwas evidenced by the significant reduction in percent increase in body weight of animal treated with ethanolic extract of *Amaranthus spinous* . at a dose of 200 and 400 mg/kg body weight when compared to DLA tumor bearing mice. [Table 3]. [26-30]

Table 3: Effect of EEPA on the life span, body weight and cancer cell count oftumor induced mice

Treatment	Number of animals	% ILS Lifespan	Increase in Body weight grams	Cancer cellcount ml X 10^6
G_1	**6**	**>>30 days**	**2.19±0.44**	**-**
G_2	**6**	**41%**	**9.59±0.84^{a**}**	**2.69±0.39^{a**}**
G_3	**6**	**93%**	**5.84±0.41^{b**}**	**1.14±0.34^{b**}**
G_4	**6**	**78%**	**6.39±0.61^{b**}**	**1.49±0.46^{b**}**

G_5	**6**	**85%**	**5.94±0.77^{b**}**	**1.41±0.27^{b*}**

*G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatmentcontrol (200mg/kg) G5 – Treatment control (400mg/kg). All values are expressed as mean ± SEM for 6 animals in each group. **a – Values are significantly different from normal control (G1) at P < 0.001. **b – Values are significantly different from cancer control (G2) at P < 0.01.*

Histopathological studies

Histology of liver sections of normal control animals (Group I) showed normal liver architecture with were brought out central vein, were preserved cytoplasm and prominent nucleus and nucleolus (Fig: 7). The liver sections of DAL treated animals (Group II) showed hepatic cells with serum toxicity characterized by inflammatory cell collection, scattered inflammation across liver parenchyma, focal necrosis and swelling up of vascular endothelial cells (Fig no: 8). 5FU (Group-III) exhibited protection from DAL cell line induced changes in the liver (Fig no: 9). EEPA pretreatment at a dose of 200mg and 400mg/kg (group IV and V) appeared to significantly prevent the DAL toxicity as revealed by the hepatic cells with were preserved cytoplasm. EEPA pretreatment also caused marked decrease ininflammatory cells (Fig no: 10 and 11).

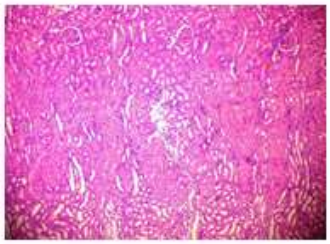

Fig 7: Normal control group treated with 10ml/kg ofnormal saline

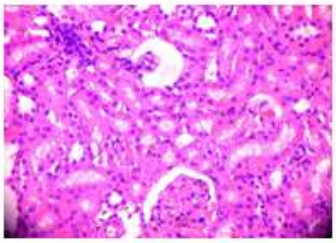

Fig 8: Cancer control group (Tumor control)

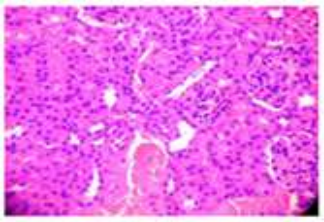

Fig 9: Positive control (5-fluorouracil)

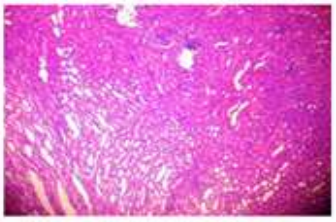

Fig 10: Treatment control received 200mg/kg of EEPA

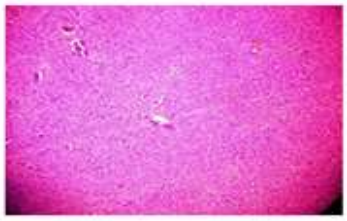

Fig 11: Treatment control 400mg/kg of EEPA

CONCLUSION

In-vitro studies by MTT assay, the ethanol extract of *Pisonia alba* L. wasfound to be effective, which shows viability 50% of cell growth at concentration in 15.6µg/ml. The ethanolic extract of *Pisonia alba* L. (200mg/kg and 400mg/kg) exhibited significant reduction in packed cell volume and viable Tumor cell countand also increase the life span of cells in DLA induced tumor mice. *In- vivo* studies of *Pisonia alba* L. ethanolic extract revealed by enhancing the survivability of the tumor bearing mice. Thus it was concluded thatthe ethanol extract of *Pisonia alba* L. has antitumor activity. On the basis of the above result it was suggested that, the *In-vitro* and *In-vivo* anticancer activity of ethanolic extract of *Pisonia alba* leaves possessed significant anticancer effect. This may probably due to the presence of phytochemicals such as phenols, terpenoids and flavonoids. Further isolation and purification of bioactivecompound from *Pisonia alba* may reveal the presence of potent novel anticancer agent and also to unveil the molecular mechanism behind its therapeutic action.

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