Anti Tumor Potential of *Premna integrifolia* Linn Against Ehrlich Ascites Carcinoma Cell Lines

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Summary

The present study is aimed at to investigate the antitumor and antioxidant potentials of the ethanolic extract of ***Premna integrifolia*** **Linn. (EEPI)** on Ehrlich Ascites Carcinoma cell lines. Tumor was induced intraperitoneally using Ehrlich Ascites Carcinoma cells ($1X10^6$cells/mouse). Ethanolic extract of ***Premna integrifolia*** **Linn** was administered at the dose level of 100 & 200 mg/kg.bw./day for 14 days to the experimental animals after 24h of tumor inoculation. The antitumor effect of EEPI was accessed by studying the parameters such as Tumor volume, PCV, viable and non-viable cell counts, life span, Hematological and Glycoprotein profiles. Administration of EEPI decreased the Ascites fluid volume, PCV and Viable cell counts and increased the mean survival time of tumor bearing animals. The EEPI brought back the altered levels of hematological parameters, and glycoprotein levels in dose dependent manner in EAC bearing mice. The results obtained were comparable with the standard drug 5-Flurouracil (20mg/kg.bw.). The data of the results of the present study depicted that altered levels of Glycoprotein content and hematological parameters due to EAC induction were brought back to near normal values after oral administration of ethanolic extract of ***Premna integrifolia*** **Linn.**

Key words: Ehrlich Ascites Carcinoma(EAC), Ethanolic extract of ***Premna integrifolia*** **Linn.*(EEPI),*** Mean survival time (MST), Tumor growth response, Hexsose, Hexsoamine, Sialicacid, and Fucose

The alternative systems of medicines like Ayurvedha, Siddha, Unnani and other tribal folklore medicines which are herbal based have significantly contributed to the health care of the population of India. Today, these systems are not only complementary but also competitive in the treatment of various diseases (**1**). Herbs and herbal based compounds have always been an important source of medicines for various diseases and have received considerable attention in recent years due to their diverse pharmacological actions including anticancer potentials. According to World Health Organization, more than 10 million new cases of cancer are diagnosed every year, and the statistical trends indicate that this number would double by 2020 **(2)**. Cancer is the uncontrolled growth and spread of abnormal cells, associated with dysregulation of apoptosis, a programmed cell death. Most of the current anticancer drugs are derived from plant sources, which act through different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity. This prompted us to evaluated the anticancer potentials of plant sources available in Tamilnadu. A common ***Verbenaceae*** member botanically equated as ***Premna integrifolia Linn*.** was selected and its ethanol extract was subjected to preliminary anticancer screening.

Premna integrifolia Linn. is a large shrub with greyish-white smooth bark, cordate or ovate leaves with dirty yellow small flowers in paniculate corymbs.**(3)**.

Materials and Methods

Plant collection and extraction

The aerial parts of ***Premna integrifolia*** **Linn**. were collected from in and around Chennai in the month of December 2009 and identified with the help of Flora of Presidency of Madras **(4)** and authenticated with the specimens deposited at RAPINAT Herbarium, Department of Botany, St. Joseph's college, Trichy. The plant material was shade dried and pulverized. About 500gms of plant material was soaked in ethanol for 48hrs. The solvent was distilled off under reduced pressure at 50°C and dried in vacuum (yield: 5.5% w/w) and dissolved in isotonic normal saline and used for the studies.

Animals:

Male Swiss albino mice (25+ 2g) were procured from the Tamil Nadu Vetinary University Chennai, and were housed in microlon boxes in a controlled environment (temperature 25+2°C and 12h dark/light cycle). They were fed with standard laboratory diet and were given sterilized water *ad libitum.* The animals were maintained in standard Animal house (Approval no:790/03/ac/CPCSEA). The study was conducted after obtaining the necessary clearance from Institutional Animal Ethical Committee.

Cells:

EAC cells obtained through the courtesy of Amla Cancer Research Centre, Thrissur, were used in the present work **(5)**

Experimental protocol :

Male Swiss albino mice were divided into 6groups (n=6). All the groups except normal control (Group I) were injected with EAC cells ($1X10^6$ cells/mouse) intraperitoneally.

GROUP I - Normal control
GROUP II - Ehrlich Ascites Carcinoma cells ($1X10^6$cell mouse)
GROUP III - Ehrlich Ascites Carcinoma cells ($1X10^6$cells) treated with 100mg /kg bw of the ethanolic extract of ***Premna integrifolia*** **Linn**
GROUP IV - Ehrlich Ascites Carcinoma cells ($1X10^6$cells) treated with 200mg /kg bw of the ethanolic extract of ***Premna integrifolia*** **Linn**
GROUP V - Ehrlich Ascites Carcinoma cells ($1X10^6$cells) treated with 5 **-** Fluorouracil (5-FU)(20mg/kgbw.) (Standard Drug)

Treatment started after 24hrs of EAC inoculation. The plant extract was administered orally for 14 days. After 14days of treatment animals from each group were sacrificed by cervical decapitation method to evaluate the antitumor activity.

Life Span

The percentage of increase in life span was calculated as follows

ILS (%) = [(Mean survival time of treated group/
Mean survival time of control group)-1] X100

MST = (Day of first death + day of last death)/2

Tumor volume:

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min.

Tumor cell count:

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares were counted.

Viable/non-viable tumor cell count:

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were treated as viable and those that took up the stain were treated as nonviable. Thus viable and nonviable cells were counted.

$$\text{Cell count} = \frac{\text{No of cells X dilution}}{\text{Area X Thickness of liquid film}}$$

Hematological studies:

Blood was collected and used for the estimation of Hemoglobin (Hb) levels, red blood cell counts (RBC) **(6)** and white blood cell counts (WBC) (**7**) . WBC differential counts were determined using Leishman stained blood smear method (**8**)

Assay of Glycoprotein in Liver tissue and Serum

To the weighed amount of defatted tissues and serum, 2ml of 4N HCl was added and the mixture was refluxed at 100 ◦C for 4 h in a test tube with suitable marble lids. The hydrolysate was neutralised with sodium hydroxide. Aliquots of the neutralised samples were taken for the analysis of hexose and hexosamine. For estimation of

sialic acid, the tissues and serum were added with 2.0 ml of 0.1N sulphuric acid and hydrolysed for 1h at 80◦C. The level of hexose was estimated by the method of (**9**). Hexosamine was estimated by the method of **(10)**. Sialicacid and fucose levels were estimated **(11,12).**

Statistical Analysis:

Data of the results obtained were subjected to statistical analysis. Values were recorded as mean ± S.E.M., student *t*-test was performed followed by ANOVA. $P<0.05$ is considered as statistically significant value.

Results

Survival Time:

Life span of the tumor bearing animal increased upto 27.60% and 53.64% respectively in drug treated group compared to Mean survival time of Tumor bearing animals (**Table 1**).

Tumor response

Treatment of EEPI at two different dose levels effectively reduced the elevated levels of Tumor volume, Packed cell Volume, and Viable cell counts in tumor bearing animals (**Table 2**) which further provided supporting scientific evidences for the antitumor potentials of the test drug (EEPI)

Hematological profiles

Hematological parameters (**Table 3**) of tumor animals such as levels of Hemoglobin and counts of RBC and WBC reverted back to near normal and Differential counts of Lymphocytes, Neutrophils and Monocytes, which were also altered reverted back to normal value after the test drug administration.

Glycoprotein Content

Table 4,5 depicts the Tissue and Serum glycoprotein levels. The inoculation of Ehrlich Ascites carcinoma at the dose levels of $1X10^6$ cells/mouse resulted in the elevation of both serum and tissue glycoproteins such as Hexose, Hexosamine. Sialic acid and Fucose. Oral administration of EEPI at various concentration for 14 days to tumor bearing animals resulted in a significant ($P<0.05$) decrease in both tissue and serum glycoproteins.

Table 1: Effect of EEPI on Survival time

Particulars	**Mean survival Time MST (days)**	**Increase in Life span(ILS) (%)**
Group 1	19.2 ± 1.1	**---**
Group 2	24.5 ± 1.3	27.60
Group 3	29.5 ± 2.71*	53.64*
Group 4	35.3±0.10*	83.85*

Values are mean ±S.E.M., n=6
*p<0.05 statistically significant when compared to EAC control group

Table 2. Effect of EEPT on Tumor growth

Group	**Tumor volume(ml)**	**Packed cell volume (ml)**	**Viable cells**	**Non-viable cells**
Group II	1.5 ± 0.32	2.1±0.02	7.9±0.19	0.32±0.04
Group III	1.1 ± 0.41	1.5±0.02*	5.3±0.37*	0.90±0.07*
Group IV	0.7 ± 0.27	1.1±0.04*	4.0±0.51*	1.4±0.18*
Group V	0.8±0.11*	0.3±0.14*	2.2±0.11*	1.99±0.57*

Values are mean± S.E.M., n = 6
*p<0.05 statistically significant when compared to EAC group
Viable cells : Not Stained with Trypan blue
Non-viable cells: Stained with Trypan blue

Table 3 Effect of EEPI on Hematological parameters

Particulars	Hb (g/dl)	RBC count 10^6cells/mm^3	WBC count 10^3 cells/mm^3	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
Group I	13.3 ± 1.4	4.9 ± 0.3	6.7 ± 0.8	59±0.14	21±0.97	1.3±1.5
Group II	9.1 ± 1.3 [a]	3.3 ± 0.5 [a]	9.3 ± 0.5 [a]	26±0.24 [a]	68±0.12 [a]	2.1±0.5 [a]
Group III	10.3 ± 0.57	3.8 ± 0.3	8.6 ± 0.3	37±1.1	47±0.62	1.8±0.84
Group IV	12.3 ± 0.48 [b]	4.3 ± 0.6 [b]	7.3 ± 0.7 [b]	46±1.5 [b]	31±0.47 [b]	1.6±0.39 [b]
Group V	12.9 ± 0.35 [b]	4.7 ± 0.2 [b]	6.7 ± 0.5 [b]	69±1.5 [b]	30±0.1 [b]	1.7±0.8 [b]

Values are mean ±S.E.M. n=6
[a] $p<0.05$ statistically significant when compared with normal group
[b] $p<0.01$ statistically significant when compared with EAC group

Table 4. Effect of EEPI on Liver Glycoprotein levels

Groups	Hexose (mg/g Defatted tissue)	Hexosamine (mg/g Defatted tissue)	Sialicacid (mg/g Defatted tissue)	Fucose (mg/g Defatted tissue)
Group I	4.50 ± 0.24	3.92 ± 0.22	2.45 ± 0.25	3.07 ± 0.04
Group II	9.33 ± 0.87*	9.45 ± 0.56*	8.18 ± 0.23*	7.63 ± 0.14*
Group III	8.43 ± 0.36	7.63 ± 0.18	5.92 ± 0.14	5.74± 0.17
Group VI	6.21 ± 0.12	5.91 ± 0.0.19	4.38 ± 0.74	4.46 ± 0.14
Group V	4.93 ± 0.16**	4.03 ± 0.10**	2.67 ± 0.14**	3.63 ± 0.11**

Values are mean ±S.E.M. n=6
* $p<0.05$ statistically significant when compared with normal group
** $p<0.01$ statistically significant when compared with EAC group

Table 5 Effect of EEPI on Serum Glycoprotein levels

Groups	Hexsose (mg/dl)	Hexsoamine (mg/dl)	Sialicacid (mg/dl)	Fucose (mg/dl)
Group I	138.74 ± 7.66	32.98 ± 1.95	68.98 ± 3.54	9.21 ± 1.16
Group II	239.79 ± 7.12*	79.78 ± 2.46*	133.94 ± 3.68*	18.90 ± 1.58*
Group III	174.07 ± 2.78	56.86 ± 2.45	106.93 ± 2.07	15.42 ± 0.48
Group VI	155.24 ± 2.73	41.18 ± 1.48	89.58 ± 2.67	11.30 ± 0.61
Group V	134.50 ± 4.01**	35.82 ± 1.36**	71.83 ± 2.77**	9.80 ± 0.56**

Values are mean ±S.E.M. n=6

* $p<0.05$ statistically significant when compared with normal group

** $p<0.01$ statistically significant when compared with EAC group

Discussion

An extremely promising strategy for cancer prevention today is chemoprevention which is defined as use of synthetic and natural agents (alone or combined) to block the development of cancer **(13)**.During the last decade the search for new anticancer dugs has taken many different approaches. The need to find a safe and highly effective cure for neoplastic diseases remains a major challenge for modern scientists. Recently a greater emphasis has been given towards the alternative medicine that deals the cancer management through various plants. In the present study ***Premna indigrifolia*** **Linn.** a traditional drug source identified was screened for its anticancer potentials against Ehrlich Ascites Carcinoma cell lines with a view to develop an ecofriendly Herbal drug molecule to combat this serious killer disease.

One of the reliable criteria for judging the merit of any anticancer drug are prolongation of life span of the tumor bearing animals **(14)**. In the present study it is observed that EEPI (the drug under study) increased the life span of tumor bearing animal upto 53.64%. The increase in the life span of the animals suggested the tumor growth inhibiting property of EEPI in a dose-dependent manner.

Tumor bearing mice possessed increased peritoneal cell counts and ascites fluid. Ascites fluid is the direct nutritional source for tumor cells and fluid volume will directly represent the tumor growth **(15)**. In the present study it is noticed that treatment with EEPI at various dose levels decreased the Ascites fluid volume as well as peritoneal cell counts.

Usually, in cancer chemotherapy the major problems that are often encountered are myelosupression and anemia The anemia encountered in cancer animals are mainly due to reduction in RBC and Hemoglobin content which may be due to iron deficiency or hemolysis **(16)**. Treatment with EEPI brought back the Hemoglobin, RBC and WBC contents more or less to near normal. This suggested that EELN has a protective action on the hemopoietic system.

Membrane associated carbohydrate is exclusively in the form of oligosaccharides covalently attached to proteins forming glycoprotein. Glycoproteins on cell surface are important for communication between cells for maintaining cell structure. Glycoproteins plays a vital role in cell to cell recognition,

intracellular processing of proteins, cell activation and ability of cancer cell to metastasize **(17)**. The elevated levels of hexose in serum and cancerous tissue are useful indicators of carcinogenic process and these change altered the structure, rigitidy, amd function of cell membrane **(18)**. Sialic acid is an aceylated derivative of nuraminic acid and exist as amino acid terminal compound of non-reducing end of carbohydrate chains of glycoprotein in mammals. Sialic acid acts a tumor marker which should be examined from the perspective of aberrant glycosylation occuring in cancer cells membranes owing to activation of new glycosyl transferase. The high sensitive Sialic acid as a tumor marker has reported in variety of cancers conditions **(19)**.

In the present study it noticed that levels of Hexoses, Hexosamine, Sialic acid and Fucose levels in serum and liver tissue of EAC bearing mice were found to increased. This may be due to leakage of the distributed membrane compounds from either disintegrating or dying neoplastic cell or as a consequence shedding of plasma membrane and neoplastic transformation. Treatment with various dose levels of EEPI glycoprtoein components levels were restored (Table 4 and 5). This reduction in the glycoprotein components indicates that the test drug has cytostabilising property, ability to suppress malignancy of modulating cells transformation, decreasing the degree of metastases, inhibiting of tumor growth and thus controlling the cancer cell proliferation and differentiation.

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