



PRECLINICAL EVALUATION OF *INVIVO* ANTICANCER ACTIVITY OF *PUNICA GRANTUM* L AND *ZIZIPHUS MAURITIANA* USING EHRlich ASCITES CARCINOMA IN SWISS ALBINO MICE.

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ABSTRACT

OBJECTIVE: Preclinical evaluation of *invivo* anticancer activity of *Punica grantum* L and *Ziziphus mauritiana* using Ehrlich Ascites Carcinoma in Swiss albino mice. **METHODOLOGY:** Experimental tumor was induced by inoculation of 1×10^6 Ehrlich ascites carcinoma cells from the tumor bearing mice aseptically. Group 1 mice (n=12) served as normal control, group 2 mice (n=12) were Ehrlich ascites carcinoma cells control. Group 3 mice (n=12) received standard drug 5- Fluorouracil 20 mg/kg b.w, i.p., group 4, 5 and 6 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of *Punica grantum* L of 200 mg/kg b.w, respectively, for nine days, group 7, 8 and 9 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of *Ziziphus mauritiana* of 200 mg/kg b.w, respectively, for nine days, whereas group 10, 11 and 12 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of combination of both plants of 200 mg/kg b.w, respectively, for nine days. Change in body weight, survival time, ascites fluid volume and packed cell volume were noted. At the end of study, 6 animals from each group were sacrificed, blood samples were collected and WBC, RBC, Hb content were estimated. **RESULTS:** Ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* showed significant reduction in body weight, tumor volume, packed cell volume and percentage increase in life span. Significant increase in RBC, Hb content and reduction in WBC count were observed. **CONCLUSION:** Ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* exhibited significant anticancer activity ($r < 0.001$).

KEYWORDS: *Punica grantum* L, *Ziziphus mauritiana*, Ehrlich ascites carcinoma.

1. INTRODUCTION

Cancer is one of the most common devastating disease affecting millions of people per year. Cancer has been estimated as the second leading cause of death in humans. The plant kingdom has provided a variety of medicines for cancer treatment, currently over 60% of the drugs are derived in one or other way from natural source including plant, marine organism and micro-organism. There are worldwide efforts to discover anticancer agents from plants.^[1] Plants have proved to be an important natural source of anti-cancer therapy for several years.^[2] The first agents to advance into clinical use were the so-called vinca alkaloids, vinblastine (VLB) and vincristine (VCR) as active agents, isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae). These compounds reported potential activity against lymphocytic leukemia in mice. The mechanism of action of Vinca alkaloids is that they inhibit the cell proliferation by affecting the microtubular dynamics during mitosis, and this causes a characteristic block during mitosis leading to apoptosis.^[1] Podophyllotoxin is obtained from the roots of Podophyllum species, namely, *Podophyllum peltatum*

Linnaeus and *Podophyllum emodi* Wallich. Epipodophyllotoxin is an isomer of podophyllotoxin. The two clinically important semi-synthetic analogs generated from Epipodophyllotoxin are Etoposide and Teniposide which were found very potential in treating lymphomas, bronchial and testicular cancers.^[2] Paclitaxel (Taxol) is obtained from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae). Another species, *Taxus baccata*, an Indian Ayurvedic medicine have also been in use for cancer therapy. Research is still undergoing in the area of anti-cancer therapy.^[2] Curcumin (diferuloylmethane), a polyphenolic compound is isolated from the Indian plant spices, *Curcuma longa* (commonly known as turmeric), now finds its application as potential anti-cancer compound. Curcumin is involved in modulating the cell cycle pathway and induces apoptosis of various cancer cells.^[2] A plant alkaloid, Ellipticine (5, 11-dimethyl-6H-pyrido [4,3-b] carbazole) and its derivatives were isolated from Apocynaceae plant species (eg. *Ochrosia borbonica*, *Excavatia coccinea*, *Ochrosia elliptica*). They exhibit significant anti-tumor properties. The primary function of this drug is that it intercalates with DNA and

also causes inhibition of Topoisomerase II activity. It is also reported that this drug, inhibits cell growth and causes apoptosis of human hepatocellular carcinoma HepG2 cells.^[2] Major research programs are being undertaken in a number of laboratories in various parts of the world, screening plant extracts for anticancer activity.^[3] Therefore, the present study taken up for screening the anticancer potential of *Punica grantum* L and *Ziziphus mauritiana*. The recent studies focused on the potential role of the leaves extraction of *Ziziphus mauritiana* as prevention or regression agent affecting the growth of certain tumors.^[4] The extracts from fruits^[5], leaves^[6-7] of *Ziziphus mauritiana* have been reported to exhibit antioxidant activity, whereas bark^[8-9] and pulp^[10] are reported to possess cytotoxicity against different cancer cell lines. *Ziziphus mauritiana* belongs to the family *Rhamnaceae*. It contains flavonoids, oleic and linoleic acid, triterpene oligoglycosides^[11] *Ziziphus mauritiana* is used for many of their ailments such as burns, wounds, conjunctivitis, cold, cough, mouth ulcer, fever, hypertension, diuretic, anthelmintic, antiemetic, etc.^[12-15] Several studies have indicated immunomodulatory activity^[16], hepatoprotective activity^[6-7], antioxidant and free radical scavenging activity^[17-18], antiulcer activity^[19], antidiarrhoeal activity^[20], antimicrobial activity^[21-22], antiplasmodial and antimycobacterial activity^[23], antihyperlipidaemic activity.^[24] Recent studies have shown that pomegranate is a potent anticancer agent that causes the induction of apoptosis and cell cycle arrest in cancer cells, inhibition of multiple signaling pathways in cancer cells, inhibition of tumorigenesis in animal models of various carcinomas.^[25-28] *Punica grantum* L belongs to family *Punicaceae* commonly called Pomegranate, recently described as nature's power fruit.^[29] It contains antocyanins, glucose, ascorbic acid, punicic acid, sterols, polyphenol and flavonols.^[30-31] Traditionally *Punica grantum* is used as anti-inflammatory^[32], antidiabetic^[33], and neuroprotective.^[34] It has been reported for treatment against influenza virus^[35], diarrhea and dysentery^[36], anti-atherosclerotic^[37] and antioxidant activity.^[38] Keeping above in view, the present study was taken up to predict potential antitumor effect of aqueous, ethanol and chloroform seed extracts of *Ziziphus mauritiana* and *Punica grantum* L in combination to evaluate the synergic effect of both plants in EAC tumor bearing mice as there is no previous anticancer activity reported for the combination of the two plants. This research will enable us to suggest, increase the frequency of the more potent extract of these plants in diet for prophylactic/curative purpose.

2. MATERIALS AND METHODS

2.1. Plant material and preparation of extracts

The authenticated aqueous, ethanol and chloroform extracts of *Punica grantum* L and *Ziziphus mauritiana* were obtained from "Green Chem", Bangalore-560071. The seeds of *Punica grantum* L and *Ziziphus mauritiana* were obtained from local markets. The seeds were washed with water for the removal of adhering

material and sun dried. Seeds were powdered with a mechanical grinder, passing through sieve # 40 and stored in airtight container. The seed powder (1kg) was extracted in a soxhlet with hexane (4000ml) for 6h for the removal of fatty matters. The hexane extract was discarded and residues were successively extracted with distilled water, ethanol and chloroform (3200ml each) for 8h each. The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) to get concentrated extracts (60g), which was dried in vacuum oven and stored in a desiccator.

2.2. Animals

Healthy male adult Swiss albino mice weighing 25±5 g were obtained from the Drug Control Laboratory (DCL), Bangalore, India, housed in well ventilated cage under standard laboratorial conditions, i.e. room temperature of 25 ± 3 °C, relative humidity 45-55 % and natural day and night cycle. The animals were allowed free access to standard laboratory cube pellets and drinking water *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee. (Registration No: 152/1999, renewed in 2012).

2.3. Tumor Cells

EAC cells were obtained by Amala Cancer Research Center, Thrissur, Kerala, India and were maintained by weekly intraperitoneal (i.p) inoculation of 10⁶ cells/mouse in the laboratory. Ehrlich Ascites Carcinoma (EAC) cells maintained in the peritoneal cavity of Swiss albino mice were collected from an animal having 7 days old ascitic tumor by aspirating the ascitic fluid in sterile isotonic saline. The viable EAC cells were counted (trypan blue indicator) under microscope. A fixed number of viable cells 10⁶ cells were inoculated into the peritoneal cavity of each recipient mouse.

2.4. Grouping and treatment

Experimental tumor was induced by inoculation of 1x10⁶ Ehrlich ascites carcinoma (EAC) cells from the tumor bearing mice aseptically. Healthy, adult Swiss albino mice were divided into 12 groups of 12 animals each and following treatment was given: Group 1 mice (n=12) served as normal control, group 2 mice (n=12) was EAC control. Group 3 mice (n=12) received standard drug 5-Flourouracil 20 mg/kg b.w, i.p., group 4, 5 and 6 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of *Punica grantum* L of 200 mg/kg b.w, respectively, for nine days, group 7, 8 and 9 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of *Ziziphus mauritiana* of 200 mg/kg b.w, respectively, for nine days, whereas group 10, 11 and 12 (n=12) mice were administered, orally, aqueous, ethanol, chloroform extract of combination of both plants (ZP) of 200 mg/kg (100 mg from each plant) b.w, respectively, for nine days.

2.5. Tumor growth response

The anticancer effect of plant extracts were assessed by change in tumor volume, packed cell volume, median

survival time (MST) and percentage increase in life span (%ILS). The tumor volume was measured by taking the ascetic fluid in a graduated centrifuge tube following its collection from the peritoneal cavity; the total volume of fluid was centrifuged at 2000 rpm for 20 minutes for determining the packed cell volume. MST and %ILS were determined using following formulae.^[39]

$$MST = \frac{\text{day of first death} + \text{day of last death}}{2}$$

$$\% ILS = \frac{(\text{MST of treated group} - \text{MST of control group})}{\text{MST of the control group}} \times 100$$

2.6. Hematological parameters

At the end of experimental period, on day 10, after an overnight fasting, blood was collected from freely flowing tail vein and used for the estimation of the hemoglobin (Hb) content, red blood cells (RBC) count and white blood cell (WBC) count by standard procedures.

2.7. Statistical analysis

The data were expressed as mean ± S.E.M (n=6). The statistical analysis was performed by means analysis of variance (ANOVA) followed by Dunnett's post hoc test where the difference was considered significant if p < 0.05.

3. RESULTS

3.1. Survival Time

Table 1 shows the effect of different extracts of *Punica grantum* L and *Ziziphus mauritiana* on mean survival time and % ILS against EAC. In the EAC control group, the mean survival time was 14 days and it increased significantly to 23 and 21 days with ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* L ZP (E) and ethanol extract *Punica grantum* L P(E) of treated groups respectively. The mean survival time in 5-Flurouracil treated mice found to be 26 days (r < 0.001). The %ILS was found to be 63.38 %, 52.11%, 84.51% in EAC induced animals treated with ZP (E), P (E) and 5-Flurouracil respectively when compared with vehicle treated cancerous animals.

Table1: Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on mean survival time and percentage increase in life span in EAC tumor bearing mice.

TREATMENT GROUP	MST (Days)	ILS%
EAC CONTROL	14.20 ± 0.58	-
5-FLUOROURACIL	26.20 ± 0.73 ^r	84.51
P-E	21.60 ± 0.51 ^{rz}	52.11
P-Aq	19.80 ± 0.37 ^{rz}	39.44
P-Ch	19.00 ± 0.71 ^{rz}	33.80
Z-E	20.20 ± 0.58 ^{rz}	42.25
Z-Aq	18.60 ± 0.93 ^{rz}	30.99
Z-Ch	17.60 ± 0.40 ^{rz}	23.94
ZP-E	23.20 ± 0.86 ^{rx}	63.38
ZP-Aq	20.20 ± 0.86 ^{rz}	42.25
ZP-Ch	19.40 ± 0.74 ^{rz}	36.62

n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. P values: q < 0.01, r < 0.001, as compared with EAC control; x < 0.05, z < 0.001, as compared with 5-Flurouracil treated group

3.2. Effect of drugs on body weight and tumor growth response

Figure 1 show the percentage decrease in the body weight after treatment with different extracts of *Punica grantum* L and *Ziziphus mauritiana* on day 10. The percentage decrease was found to be 41.46%, 50.47% and 64.77% in EAC induced animals treated with P (E), ZP(E) and 5-Flurouracil respectively when compared with vehicle treated cancerous animals. ZP (E) significantly (r<0.001) decreased the tumor volume and packed cell volume as compared to that of the EAC control group. [Figure 2,3].

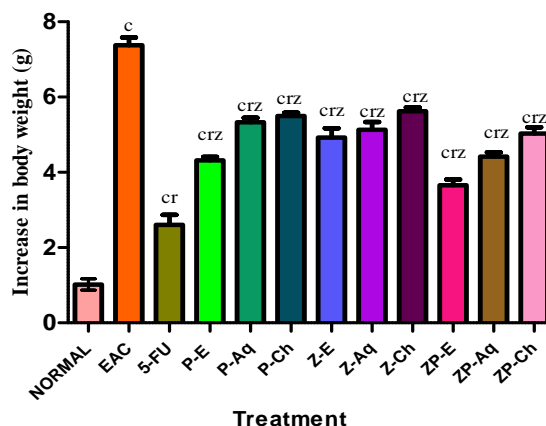


Fig 1: Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on body weight in EAC tumor bearing mice. n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnett's multiple comparison test. P values: c < 0.001, as compared with normal group; r < 0.001, as compared with EAC control; z < 0.001, as compared with 5-Flurouracil treated group.

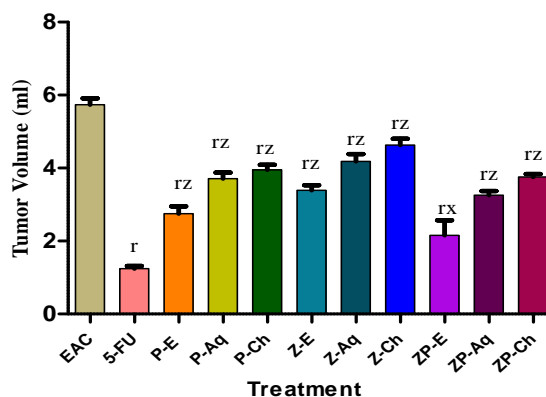


Fig 2: Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on tumor volume in EAC tumor bearing mice. n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnett's

multiple comparison test. P values: $r < 0.001$, as compared with EAC control; $z < 0.001$, $x < 0.05$, as compared with 5-Fluorouracil treated group.

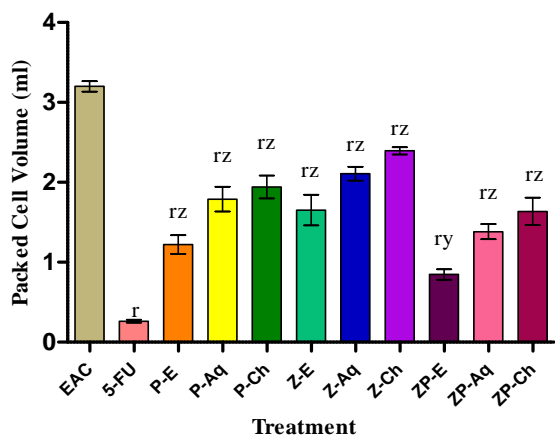


Fig 3: Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on packed cell volume in EAC tumor bearing mice. n = 6, Values are mean \pm S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. P values: $r < 0.001$, as compared with EAC control; $z < 0.001$, $y < 0.01$ as compared with 5-fluorouracil treated group.

Table 2: Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on hematological parameters in EAC tumor bearing mice.

TREATMENT GROUP	RBC COUNT ($\times 10^6/\text{mL}$)	WBC COUNT ($\times 10^3/\text{mL}$)	Hb (g%)
NORMAL	5.68 ± 0.11	6.96 ± 0.18	15.99 ± 0.39
EAC	2.17 ± 0.1^c	18.58 ± 0.55^c	7.07 ± 0.42^c
5-FU	4.65 ± 0.25^f	6.50 ± 0.34^f	12.29 ± 0.56^{br}
P-E	3.95 ± 0.34^{bq}	9.94 ± 0.70^{ary}	9.74 ± 0.76^{cp}
P-Aq	3.61 ± 0.32^{cp}	11.30 ± 0.52^{crz}	8.66 ± 0.57^{cy}
P-Ch	3.07 ± 0.33^{cy}	11.85 ± 0.67^{crz}	8.26 ± 0.43^{cy}
Z-E	3.29 ± 0.25^{cx}	10.95 ± 0.38^{crz}	9.42 ± 1.00^{cx}
Z-Aq	3.07 ± 0.30^{cy}	12.22 ± 0.82^{crz}	8.93 ± 0.30^{cy}
Z-Ch	2.79 ± 0.37^{cy}	13.58 ± 0.80^{crz}	8.09 ± 0.68^{cz}
ZP-E	4.26 ± 0.31^{af}	8.06 ± 0.78^f	10.89 ± 1.00^{cq}
ZP-Aq	3.89 ± 0.32^{cq}	10.36 ± 0.69^{brz}	9.70 ± 0.81^c
ZP-Ch	3.66 ± 0.40^{cq}	11.70 ± 0.72^{crz}	9.27 ± 0.28^{cx}

n = 6, Values are mean \pm S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. p values: $a < 0.05$, $b < 0.01$, $c < 0.001$, compared to the normal group; $p < 0.05$, $q < 0.01$, $r < 0.001$, as compared with EAC control; $x < 0.05$, $y < 0.01$, $z < 0.001$, as compared with 5-Fluorouracil treated group.

4. DISCUSSION

Search for a selective and less toxic molecule for cancer treatment is an ongoing process. Plants have played an important role as a source of effective anticancer agents and about 60% of the currently available anticancer drugs are derived from plant sources.^[1] The global trend is also towards natural bioactive substances due to their low toxicity and cost. The exploration of medicinal plants for their therapeutic efficacy still holds the hope for the treatment and prevention of cancer. The present study was designed to explore the possible *in vivo* anticancer activity of plant extracts of *Ziziphus*

3.3. Hematological parameters

Table 2 reveals the effect of different extracts of *Punica grantum* L and *Ziziphus mauritiana* on hematological parameters against EAC induced animals estimated on 10th day of treatment. The total WBC count found significantly increased in the EAC control group when compared with the normal group ($c < 0.001$). Ethanol and aqueous extracts of combination of *Ziziphus mauritiana* and *Punica grantum* L (ZP) as well as ethanol extract of *Punica grantum* L P(E), showed activity at par with 5-Fluorouracil as standard and these differences were statistically non-significant for ethanol extract of ZP and $y < 0.01$ for both aqueous of ZP and P(E). Ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* L (ZP) when administered to EAC bearing mice significantly decreased the WBC count as compared with EAC control. RBC count and Hb content in the EAC groups were significantly ($c < 0.001$) decreased as compared to normal group. Treatment with ZP(E) 200 mg/kg showed significant ($r < 0.001$) increase in the RBC count and Hb content when compared to the EAC control group, and restored these values towards normal [Table 2].

mauritiana and *Punica grantum* L (ZP). The ‘‘appropriate’’ transplantable mouse tumors models still have their place in the drug development programs and are used to investigate the antineoplastic effects of plant extracts. Hence in the present study the *in vivo* antitumor efficacy of plant extract were assessed in EAC cell lines in mice respectively. The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma and is able to grow in almost all mice strains.^[1] It has reported that Ehrlich ascetic tumor implantation induces a local inflammatory reaction, with increasing vascular

permeability, which results in an intense edema formation, cellular migration, and a progressive ascetic fluid formation.^[1] The ascetic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells.^[19] In this ascites tumor model, a substantial increase in body weight of the animals was observed in EAC-bearing control mice owing to the rapid and progressive accumulation of ascites tumor cells. The reliable criterion for judging the value of any anticancer drug is the prolongation of life span of animal.^[40] The present study showed that ethanol extract of *Ziziphus mauritiana* and *Punica grantum* L (ZP) 200 mg/kg administered to EAC mice significantly increased the life span than that of EAC control. Furthermore the reduced volume of tumor and increased survival time of mice suggest the delaying impact of ethanol extract of ZP on cell division.^[41] In the present study both ethanol extract of *Ziziphus mauritiana* and *Punica grantum* L (ZP) and ethanol extract of *Punica grantum* L showed the significant decrease in body weight, tumor volume ($r < 0.001$), and packed cell volume ($r < 0.001$) when compared with the EAC control group. Since the prolongation of life span is a reliable criterion for judging the anticancer efficacy of any compound.^[1] An enhancement of life span of group treated by ethanol extract of ZP by 63.38 % more over that of EAC control was considered as effective antitumor response. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or Hb content, this occurs either due to iron deficiency or due to hemolytic or myelopathic conditions.^[42] Treatment with ethanol extract of *Punica grantum* L P(E) at 200 mg/kg dose significantly increased the RBC level ($q < 0.01$) and Hb content ($p < 0.05$) when compared to the EAC control. Ethanol extracts of combination of *Ziziphus mauritiana* and *Punica grantum* L (ZP) at 200 mg/kg dose showed significant effect than the ethanol extract of *Punica grantum* L P(E) for RBC level ($r < 0.001$) and Hb content ($q < 0.01$). The WBC count has been decreased significantly ($p < 0.001$) when compared with the EAC bearing mice and restored more towards the normal level in the group which is treated with ethanol extract of ZP at 200 mg/kg. Ethanol extract of ZP at 200 mg/kg dose showed non-significantly decrease in WBC level when compared to 5-FU at 20 mg/kg thus, increase in life span, and decrease in body weight, tumor volume and packed cell volume and the restoration of hematological parameter as a result of treatment and in the group treated by ethanol extract of *Ziziphus mauritiana* and *Punica grantum* L (ZP) at 200mg/kg may be attributed to antioxidant as well as anticancer activity. The previous phytochemical analysis of *Punica grantum* L with *Ziziphus mauritiana* has also revealed the presence of phenolic, alkaloid and flavonoids, which could also be expected to be responsible for apoptosis as well as anticancer activity.^[43]

5. CONCLUSION: Based on these data, ethanol extract of combination of *Punica grantum* L with *Ziziphus*

mauritiana exhibited significant anticancer activity ($r < 0.001$).

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