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Evaluation of *In Vitro* Cytotoxicity and Antioxidant Activity of *Punica Grantum* L And *Ziziphus Mauritiana* Extracts For Anticancer Screening

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ABSTRACT

This survey was designed to investigate the evaluation of synergic anticancer activity of *Punica grantum* L and *Ziziphus mauritiana* extracts by using *invitro* cytotoxicity assay, *invitro* and *invivo* antioxidant estimation. The *invitro* antioxidant activity of ethanol, aqueous and chloroform extracts of *Punica grantum* L and *Ziziphus mauritiana* and combination of them was estimated by reducing power, diphenyl picryl hydrazyl scavenging and hydrogen peroxide scavenging assays using standard procedure, the *invivo* antioxidant activity was also performed by estimation of Catalase and Glutathione in liver homogenate. The extracts were screened for their *invitro* cytotoxicity by trypan blue exclusion assay and *Allium cepa* root tips. The results displayed the 100µg/ml of both ethanol and aqueous extracts i.e. *Punica grantum* L and it's combination with *Ziziphus mauritiana* having the maximum *invitro* antioxidant activity. Ethanol and aqueous extract of combination of *Punica grantum* L and *Ziziphus* and ethanol extract of *Punica grantum* L has produced the significant increase in the glutathione and catalase levels. The cytotoxic effect of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana* significantly increased in Ehrlich ascites tumour cells with an increase in concentration by trypan blue cytotoxic assay and also significant root growth inhibition in *Allium cepa* after 48h. The percentage decrease in mitotic index was found to be 27.28 % in 800 µg/ml of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana*. Accordingly, the synergic anticancer activity of illustrated extracts was evinced.

Key words: Antioxidant, cytotoxic assay, *Punica grantum* L, *Zizipus mauritiana*

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INTRODUCTION

The chemoprevention of cancer is delineated as the use of natural agents or synthetic (alone or combination) to impede of cancer. Surgery, radiotherapy and chemotherapy for miscellaneous cancers are pricey, having severe side effects. The novel chemopreventive agents of natural origin have been aimed with fruits and vegetables being a key interest due to high content of bioactive compounds [1]. The evidence advocates that a number of plants are known to be the source of useful drugs in modern medicine [2] and have been achieved currently as one of the main source of cancer chemoprevention drug discovery and development [3] due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [4]. There are a lot of pathological factors, inclusive of reactive oxygen species (ROS) involved in the process of cancer initiation, promotion and progression [5]. First, an oxidative stress can motivate DNA damages that accelerate genomic instability and stimulate cancer progression [6]. Second, elevated ROS levels are responsible for activation of transcription factors [7]. Consequently, the exploration for antioxidants as cancer chemopreventive agents is a persisted process. A number of studies signified that plant derived natural products, such as polyphenols, possess diverse pharmacological properties, among which antioxidant activity [8]. The recent studies aim attention at the potential role of the leaves extraction of *Ziziphus mauritiana* as impediment or regression agent affecting the growth of certain tumors [9]. The extracts from fruits [10], leaves [11,12] of *Ziziphus mauritiana* have been denoted to display antioxidant activity, whereas bark [13,14] and pulp [15] are remarked to possess cytotoxicity against different cancer cell lines. *Ziziphus mauritiana* belongs to the family *Rhamnaceae*, It consists of flavonoids, oleic and triterpene oligoglycosides [16]. *Ziziphus* is used for many of their illnesses such as burns, wounds, conjunctivitis, cough,hypertension, diuretic, anthelmintic, antiemetic, etc [17-20]. Several researches have displayed immunomodulatory [21], hepatoprotective activity [11,12], free radical scavenging activity [22,23], antiulcer activity [24],antidiarrhoeal activity [25], antimicrobial activity [26,27] antimycobacterial activity [28], antihyperlipidaemic activity [29]. Current studies have revealed that pomegranate is a potent anticancer agent that instigates the induction of apoptosis and cell cycle arrest in cancer cells, prohibition of multiple signaling pathways in cancer cells, inhibition of tumor genesis in animal models of various carcinomas [30-33]. *Punica grantum* L belongs to family *Punicaceae* commonly [34]. It consists of antocyanins, ascorbic acid, punicic acid, sterols, polyphenol and flavonols [35,36]. Traditionally *Punica grantums* was used as anti-inflammatory [37], antidiabetic [38], and neuroprotective [39]. It has been delineated for remedy against influenza virus [40], diarrhea and dysentery [41], anti-atherosclerotic [42] and antioxidant activity [43]. Hold

above opinions, the present investigation was taken up to anticipate potential anticancer effect of aqueous, ethanol and chloroform seed extracts of *Ziziphus mauritiana* and *Punica grantum* L in combination to assess the synergic effect of both plants in EAC tumor bearing mice as there is no preceding reported for their anticancer activity.

MATERIALS AND METHOD

Plant material and Preparation of extracts

The seeds of *Punica grantum* L (*Punicaceae*) and *Ziziphus mauritiana* (*Rhamnaceae)* were collected from Udupi District, Bangalore, Karnataka State, India and authenticated by Green Chem of India, Bangalore, Karnataka, India, a voucher specimens (NRI-COP-204; NRI-SOP-309) for *Punica grantum* L and *Ziziphus mauritiana* respectively were preserved for future references. 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 vaccum (Buchi, Switzerland) to get concentrated extracts (60g), which was dried in vaccum oven and stored in a desiccator.

Chemicals

Hydrogen peroxide was purchased from BioVision company. DPPH (1,1 Diphenyl-2-picryls-hydrazyl) was acquired from Ultra Lab company. All other chemicals were analytical up grade.

Tumor cells line for Hydrogen peroxide assay

The Hela cell line (Cervical cancer) was obtained from National Center for Cell Science, Pune, India. Cells were grown and maintained in RPMI-1640 medium, PH 7.4. The media was supplemented with 10% fetal calf serum, glutamine (2mM), penicillin (100units/ml) and streptomycin (100µg/ml). The cell culture was grown in carbon dioxide incubator at 37^{0C} with 90% humidity and 5% CO_2.

Tumor cell line for *in vitro* cytotoxic test by Trypan blue method and *invivo* antioxidant activity

EAC cells were obtained by Amala Cancer Research Center, Thrissur, Kerala, India and were maintained by weekly intraperitonial (i.p) inoculation of 10^6 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 10^6 cells were inoculated into the peritoneal cavity of each recipient mouse.

Animals

Healthy male adult Swiss albino mice weighing 25±5 g was obtained from the Drug Control Laboratory (DCL), Bangalore, were housed in well ventilated cage and animals had natural day and night cycle with temperature between 25±3^{o}C. The animals were housed in large spacious hygienic cages during the course of the experimental period. 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 (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore. (Registration No: 152/1999, renewed in 2012).

Reducing power assay

The reducing power was determined according to the method of Oyaizu [44]. About 2.5 ml of sample/std solution (10, 25, 50. 75 and 100 µg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, PH 6.6) and Potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50^o C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. A blank was prepared without adding standard or test compound. Increased absorbance of the reaction mixture indicates increase in reducing power.

The percentage increase in reducing power was calculated using the following equation:

$$\text{Increase in reducing power (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of sample/standard.

Free radical scavenging activity using DPPH assay

The DPPH scavenging was determined according to the method of Sabir and Rocha [45]. A portion (1ml) of various concentration of extract sample/standard (10, 25, 50, 75, 100 µg/ml) was mixed with 1ml DPPH solution (24 mg of DPPH was dissolved in 100 ml methanol and then stored at -20^o C until needed. The working solution was obtained by mixing 10 ml of stock solution with 45 ml methanol.)The mixture contains 1ml of methanol and 1ml of DPPH solution was used as control. After incubation for 30 min in dark, absorbance was recorded at 517nm.

The % scavenging was calculated using this formula:

$$\% \text{ Scavenging} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of sample/standard.

Hydrogen peroxide scavenging assay

The Hydrogen peroxide scavenging was determined according to the method of Vijayabhaskaran [46]. HeLa cells were cultured in 24 flasks (0.1×10^6 cells per wells) then trypsinzed, subcultured and stabilized the cells during 14 days. Drugs/std (10, 25, 50, 75 and 100 µg/ml) were added and kept it in contact with cells for 24 hours. Cell culture supernatant was collected and proteins were removed by PCA precipitation. 30µl of sample were added to each well and made up the volume to 50µl with assay buffer. Added 50 µl reaction mixture to each well and optical density was measured at 545 nm in a microplate reader.

Treatment schedule

Experimental tumor was induced by inoculation of $1x10^6$ Ehrlich ascites carcinoma (EAC) cells from the tumor bearing mice aseptically. Group 1 mice (n=6) served as normal control, group 2 mice (n=6) were EAC control. Group 3 mice (n=6) received standard drug 5- Flourouracil 20 mg/kg b.w, i.p., group 4, 5 and 6 (n=6) 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=6) 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=6) mice were administered, orally, aqueous, ethanol, chloroform extract of combination of both plants (ZP) of 200 mg/kg b.w, respectively, for nine days. Animals from each group were sacrificed, liver homogenate was collected and antioxidant activity was estimated by following parameters.

Biochemical parameters

The mice were sacrificed. Then the liver was excised, rinsed in ice cold normal saline followed by ice cold 10% KCl solution, blotted, dried and weighed. A 10% w/v hemoginate was prepared in ice cold KCl solution and centrifuged at 1500 rpm for 15 min at 4˚C. the supernatant thus obtained were used for the estimation of glutathione (GSH) [47], catalase (CAT) [48].

***In-vitro* Cytotoxicity:**

Trypan blue dye exclusion method

Trypan blue dye exclusion method was carried out according to the method of Saluja [49]. Ethanol extract of combination *Punica grantum* L and *Ziziphus mauritiana* was used for the preparation of the stock solution (1mg/ml). Then different concentrations of drug (10, 25, 50, 75 and 100 µg/ml) were added to each tube which contains 10^6 cells/ml. A control had tumor cells with solvent. All

the tubes were incubated at 37^{0C} for 3 hours then 0.1ml of 1% trypan blue was added to each tube. The number of viable (unstained) and dead (stained) cells were counted using haemocytometer. Results were expressed as percentage mortality (% cytotoxicity) using this formula :

$$\% \text{ cytotoxicity} = \frac{(\text{Total cells counted} - \text{total viable cells})}{\text{Total cells counted}} \times 100$$

***Allium cepa* root model**

Allium cepa root tip meristems were determined according to method of Namita and Sonia [50]. Healthy and equal sized bulbs of common onion (*Allium cepa*) were chosen and the loose outer scales of bulbs and old roots were removed with the help of sharp and pointed forceps so as to expose the root primodia series of bulbs were grown in different concentration of ethanol extract of combination of *Ziziphus mauritiana* and *Punica grantum* L (200, 400, 600 and 800 µg/ml) in beakers at 27 ± 2 $^{\circ}C$ in a dark cupboard. At end of exposure periods (48 hours), root lengths were measured in cm with ruler .The onions were grown on distilled water (without any extract) serve as the control. Colchicine was employed as standard (1mg/ml). Roots were excised at 48 hrs and suspended in fixative liquid (ethanol: acetic acid (3:1 v/v)) for 24 hrs. They were washed with distilled water, hydrolyzed at 60^{oC} in 1N HCl for 1 min and transferred to watch glass containing aceto-orcien and 1N HCl (9:1). They were then heated intermittently for 5-10 min, covered and kept aside for 20-30 min. The tip of root was cut with sharp blade and placed on a glass slide in a drop of 45% glacial acetic acid and covered with coverslip. The root tip was squashed by tapping with match stick and sealed with DPX (Distyrene a plasticizer and xylene). The cells were scored under high power objective in the compound microscope for mitotic index (% MI).

$$\% \text{ MI} = \frac{\text{Total number of cells in mitosis}}{\text{Total number of cells counted}} \times 100$$

Statistical analysis

The data were expressed as mean ± S.E.M (n=3 for *in-vitro* study and n=6 for *in-vivo* study). The statistical analysis involving five groups was performed by means analysis of variance (ANOVA) followed by Dunnett's post hoc test where the difference was considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Reactive oxygen species (ROS) attacks and motivates oxidative damage to numerous biomolecules and are implied in several human disease. It is utterly impressive to denote that plants have favorable antioxidants activity which can be attributed to the various mechanisms like prevention of chain initiation, binding at transition metal ion catalysis, decomposition of peroxides, reductive capacity and radical scavenging activity [51]. There are abundant antioxidant methods for assessment

of antioxidant activity out of which the present study has used three antioxidant assays for evaluation of antioxidant activity. Evaluation of reducing power is based on the principle of increase in absorbance of the reaction mixture by the plant extract. Increase in the absorbance indicates increase in the reductive power. For the measurements of reductive ability, Fe^{3+}-Fe^{2+} transformation in the presence of sample was selected using the method of Oyaizu [44]. The reducing capacity of a compound may aid as a significant indicator of its potential antioxidant activity [45]. Table 1 shows significant percentage increase of reducing power at 100µg of ethanol extracts of both *Punica grantum* L and it's combination with *Ziziphus mauritiana*. Free radicals involved in the process of lipid peroxidation are contemplated to play a fundamental role in various chronic pathologies such as cancer and cardiovascular disease and are implicated in aging process. Therefore, the extracts were assessed against scavenging of various free radicals like hydrogen peroxide radical and DPPH. DPPH radical serves as the oxidative substrate, which can be reduced by an antioxidant compound to its hydrazine derivative via hydrogen donation and as the reaction indicator molecule [45]. DPPH radical is considered to be lipophilic. It is stable nitrogen centered free radical that easily receives an electron or hydrogen radical then convert to a stable diamagnetic molecule. DPPH radical react with suitable reducing agents, as a result of which the electrons become paired off forming the corresponding hydrazine, hence, the solution loses colour stoichiometrically depending on the number of electrons taken up. The decrease in the absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecule and DPPH radical and results in the scavenging of the radical by hydrogen donation. It is visually perceptible as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to figure out antioxidant activity. The 100µg of both ethanol and aqueous extracts i.e. *Punica grantum* L and it's combination with *Ziziphus mauritiana* has shown maximum DPPH scavenging (Table 2).Result also indicates that the DPPH scavenging activity is concentration dependent. Hydrogen peroxide is immensely cardinal because of its ability to pass through the biological membrane. Hydrogen peroxide is formed spontaneously or by catalytic dismutation of O_2^-. Hydrogen peroxide itself is not very reactive but it can be toxic to the cell because it may generate the hydroxyl radical in the cells. Hydrogen peroxide gives rise to the cellular damages in the presence of ferrous ions resulting in the formation of hydroxyl radical. Therefore it is important to remove the hydrogen peroxide. Table 3 shows the percentage increase of Hydrogen peroxide scavenging of ethanol extract of combination of *Punica grantum* L with *Zizipus mauritiana* as well as ethanol extract of *Zizipus mauritiana* at par with ascorbic acid as standard and these differences were statistically non-significant. The Ehrlich tumor growth produces an inhibition of catalase and

glutathione enzymes, which are crucial in the withdrawal of free radicals as hydrogen peroxide. The prohibition of CAT and GSH activities as a result of tumor growth was also denoted. The ethanol and aqueous extract of combination of *Punica grantum* L and *Ziziphus* has produced the significant rise in the glutathione levels (Figure 1). Increased in CAT level was found in EAC induced animals treated with aqueous extract of combination of *Punica grantum* L and *Ziziphus mauritiana* ZP(aq) ,ethanol extract of *Punica grantum* L P(E) , ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* ZP(E) when compared with vehicle treated cancerous animal.(Figure 2). The results of the *invitro* cytotoxicity assay revealed that ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana*, ZP (E) is toxic to the EAC cells as there was an increase in the number of cells stained with trypan blue dye and the percentage of death of Ehrlich ascites tumour cells *invitro* increases with the concentration of ehanol extracts of *Punica grantum* L and *Ziziphus mauritiana* (Figure 3).

Table 1: Percentage increase of reducing power of different extracts of *Punica grantum* L and *Ziziphus mauritiana*.

Conc. µg/ml	% Increase of Reducing Power									
	ASC	P (E)	P (Aq)	P (Ch)	Z (E)	Z (Aq)	Z (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
10	67.11 ±0.51	42.38± 0.03^{C}	35.33± 0.35^{C}	24.91± 0.16^{C}	41.00± 0.44^{C}	31.24± 0.56^{C}	21.90± 0.28^{C}	42.39± 0.33^{C}	36.86± 0.39^{C}	26.53± 0.06^{C}
25	80.32 ±0.51	53.62± 0.71^{C}	46.40± 1.10^{C}	33.51± 0.86^{C}	50.65± 0.45^{C}	43.15± 0.37^{C}	27.26± 0.68^{C}	54.16± 0.42^{C}	47.43± 0.11^{C}	35.33± 0.46^{C}
50	89.91 ±0.19	61.38± 0.26^{C}	53.53± 0.19^{C}	38.44± 0.25^{C}	57.38± 0.37^{C}	51.25± 0.36^{C}	32.67± 0.22^{C}	65.45± 0.26^{C}	55.32± 0.17^{C}	39.71± 0.41^{c}
75	93.41 ±0.12	70.56± 0.27^{C}	57.65± 0.38^{C}	40.43± 0.47^{C}	65.81± 0.11^{C}	53.91± 0.47^{C}	39.05± 0.19^{C}	72.31± 0.24^{C}	59.02± 0.29^{C}	41.82± 0.17^{c}
100	95.52 ±0.18	73.74± 0.73^{C}	60.35± 0.05^{C}	43.79± 0.42^{C}	68.77± 0.45^{C}	56.04± 0.38^{C}	41.22± 0.12^{C}	74.49± 0.10^{C}	61.37± 0.21^{C}	44.75± 0.43^{c}

n = 3, values are mean ± S.E.M, one way ANOVA followed by Dunnet's multiple test.P values: a < 0.05, b < 0.01 and c < 0.001 as compared to ascorbic acid treated group as standard. ASC: Ascorbic acid, P: *Punica gratum* L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

Table 2: Percentage inhibition of DPPH free radical activity of different extracts of *Punica grantum* L and *Ziziphus mauritiana.*

Conc.	% Inhibition									
µg/ml	ASC	P (E)	P (Aq)	P (Ch)	Z (E)	Z (Aq)	Z (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
10	90.27±0.13	88.74±0.43^{a}	87.49±0.28^{c}	73.21±0.12^{c}	50.17±0.6 ^{c}	46.28±0.14^{c}	45.49±0.29^{c}	90.24±0.35^{c}	88.2±0.70	66.93±0.63^{c}
25	94.79±0.42	93.74±0.17	92.52±0.14^{b}	77.51±0.15^{c}	52.32±0.19^{c}	47.03±0.23^{c}	46.3±0.39^{c}	93.85±0.35	93.67±0.78	71.11±0.35^{c}
50	95.83±0.29	94.42±0.28	94.34±0.96^{c}	82.71±0.05^{c}	55.23±0.06^{c}	47.71±0.23^{c}	47.07±0.1^{c}	95.09±0.11	94.95±0.06	75.87±0.32^{c}
75	97.95±0.02	95.55±0.32^{c}	95.49±0.12^{c}	87.03±0.49^{c}	59.41±0.23^{c}	49.1±0.48^{c}	48.45±0.26^{c}	96.40±0.18^{b}	96.19±0.05^{b}	78.48±0.03^{c}
100	99.07±0.08	97.67±0.21	97.59±0.23	89.96±0.37^{c}	61.21±0.12^{c}	50.00±.15^{c}	49.54±0.26^{c}	98.59±0.29	98.34±0.2	81.75±0.36^{c}

n = 3, values are mean ± S.E.M, one way ANOVA followed by Dunnet's multiple test.P values: a < 0.05, b < 0.01 and c < 0.001 as compared to ascorbic acid treated group as standard.ASC: Ascorbic acid, P: *Punica gratum* L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

Table 3: Percentage of hydrogen peroxide scavenging activity of different extracts of *Punica grantum* L and *Ziziphus mauritiana.*

Conc.	% Scavenging									
µg/ml	ASC	P (E)	P (Aq)	P (Ch)	Z (E)	Z (Aq)	Z (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
10	35.37±0.13	25.64±0.35^{c}	15.94±0.6^{c}	6.35±0.08^{c}	34.55±0.28	27.36±0.01^{c}	16.05±0.03^{c}	35.43±0.06	29.10±0.41^{c}	26.19±0.18^{c}
25	37.98±0.02	28.56±0.07^{c}	19.00±0.05^{c}	9.33±0.01^{c}	37.15±0.43^{a}	30.25±0.13^{c}	18.75±0.14^{c}	37.63±0.17	30.78±0.13^{c}	28.58±0.24^{c}
50	39.00±0.05	29.14±0.06^{c}	21.46±0.27^{c}	10.13±0.05^{c}	38.16±0.48	31.44±0.12^{c}	19.09±0.05^{c}	38.61±0.57	31.86±0.37^{c}	29.22±0.13^{c}
75	40.02±0.33	30.21±0.21^{c}	22.85±0.37^{c}	10.85±0.04^{c}	39.16±0.42	32.09±0.08^{c}	19.90±0.41^{c}	39.64±0.20	32.67±0.36^{c}	30.25±0.10^{c}
100	42.17±0.64	31.24±0.32^{c}	24.75±0.43^{c}	11.60±0.34^{c}	40.13±0.51^{a}	32.71±0.42^{c}	20.83±0.47^{c}	40.72±0.39	33.39±0.19^{c}	31.01±0.57^{c}

n = 3, values are mean ± S.E.M, one way ANOVA followed by Dunnet's multiple test.P values: a < 0.05, b < 0.01 and c < 0.001 as compared to ascorbic acid treated group as standards.ASC: Ascorbic acid, P: *Punica gratum*L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

Table 4: Effect of ethanol extracts of combination of *Punica grantum* L and *Ziziphus mauritiana* on root length and mitotic index of *Allium cepas.*

Name of the extract	48 h Root length (in cms)	% MI
Control (Distilled Water)	1.98 ± 0.27	76.08
Colchicine 1mg/ml	0.74 ± 0.32[b]	21.55
ZP-E 200 µg/ml	1.90 ± 0.32[y]	71.3
ZP-E 400 µg/ml	1.64 ± 0.19[x]	64.2
ZP-E 600 µg/ml	1.45 ± 0.11	45.8
ZP-E 800 µg/ml	1.05 ± 0.23[a]	27.28

n=3, Values are mean ± 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 control group; x< 0.05, y<0.01, z< 0.001, as compared with colchicine treated group.

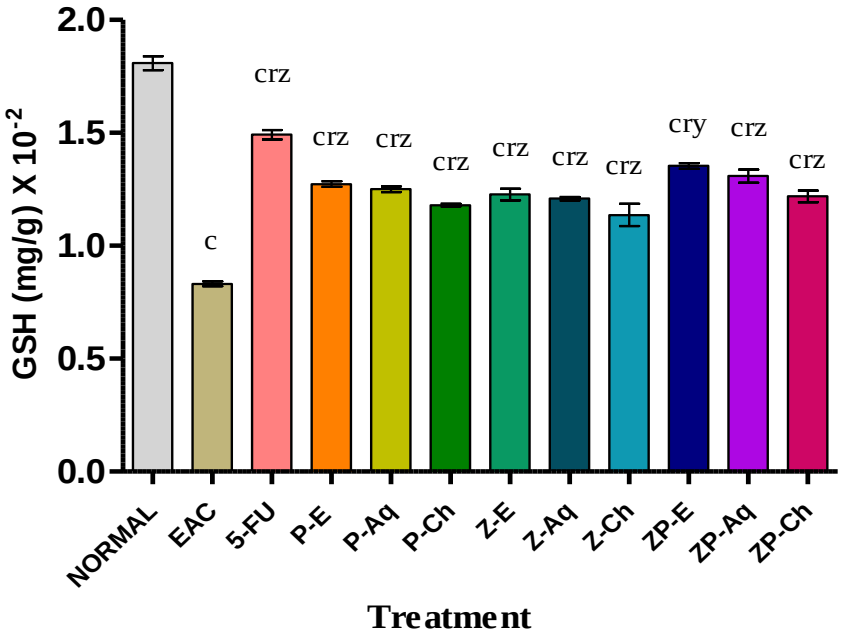

Figure 1: Effect of different extracts of *Punica grantum* L and *Ziziphus mauritiana* on glutathione level in EAC tumor bearing mice. n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. p values: c< 0.001, compared to the normal group; r< 0.001, as compared with EAC control; y < 0.01, z< 0.001, as compared with 5-Fluorouracil treated group. 5-FU: 5-Fluorouracil, P: *Punica gratum*L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

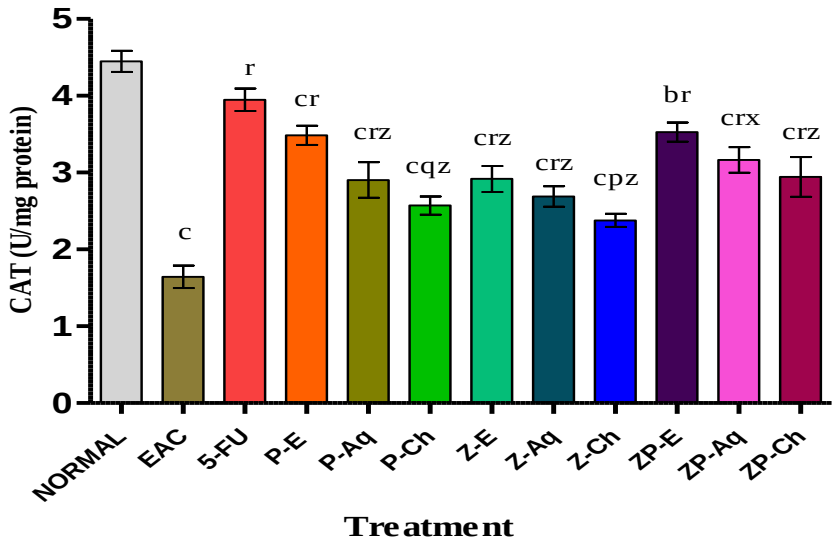

Figure 2: Effect of different extracts of *Punica grantum* L and *Ziziphus mauritiana* on catalase level in EAC tumor bearing mice. n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. p values: a< 0.05, b< 0.01, c< 0.001, compared to the normal group; p< 0.05, 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. 5-FU: 5-Fluorouracil, P: *Punica gratum*L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

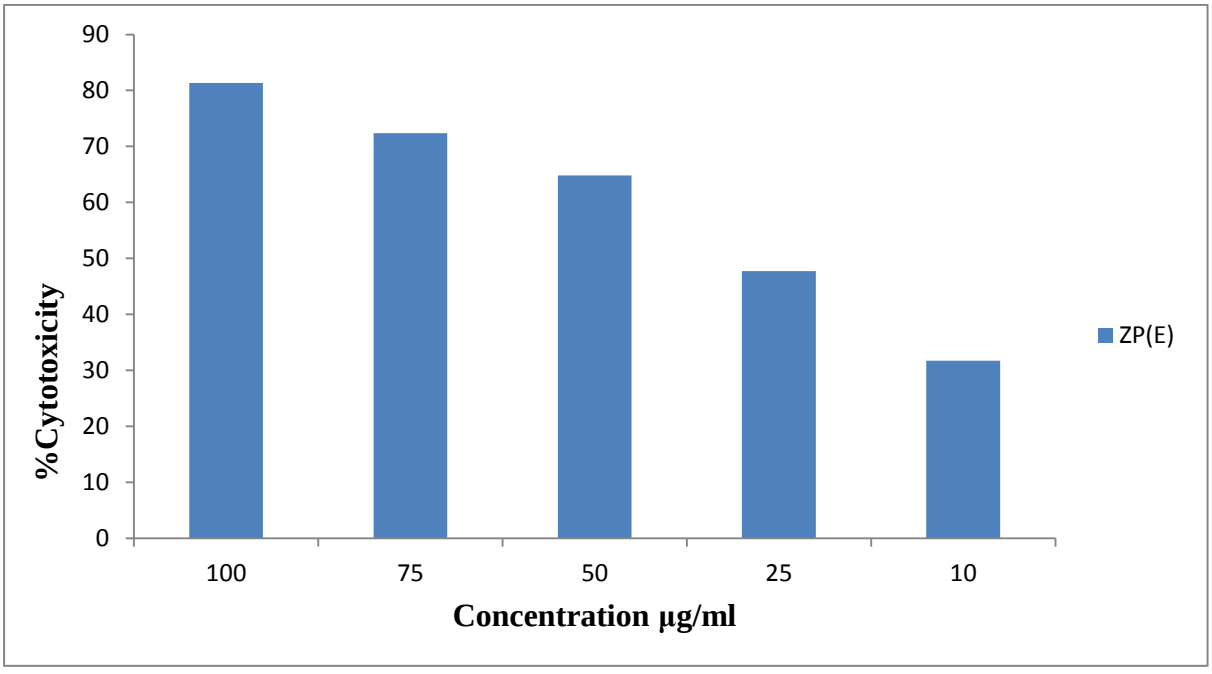

Figure 3: *In-vitro* cytotoxic effect of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* on Ehrlich ascetic carcinoma cells.

This cytotoxic activity might be due to mechanisms other than direct catalytic effects such as directly on the tumor cells and cause their lysis and/or indirectly by destroying the microenvironment i.e., the ascites fluid produced by the tumor cells. Table 4 shows effect of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana* ZP (E) on root length and mitotic index of *Allium cepa*. The ZP (E) samples and standard (Colchicine treated group) showed significant root growth inhibition in *A. cepa*. ZP (E) at concentrations of 200, 400, 600, 800 µg/ml induced root growth inhibitions of 1.90, 1.64, 1.45, and 1.05 cm respectively at 48h. The percentage decrease in mitotic index was found to be 27.28 % in 800 µg/ml of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana*. The previous phytochemical analysis [52,53] of *Punica grantum* L with *Zizipus mauritiana* has also disclosed the presence of alkaloids and phenolic compounds such as flavonoids, tanin which could also be expected to be responsible for antioxidant as well as anticancer activity. It has been suggested that the antioxidant properties of phenolic compounds can be interceded by free radical scavengers and metal chelators [54]. The inhibitory effect of natural phenolics in carcinogenesis and tumor growth may be done by two mechanisms: (1) modifying the redox status and, (2) interfering with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis) [55].

CONCLUSION

The results from the present investigation are coherent with the earlier reports, which confirm the antioxidant and cytotoxic potential of *Punica grantum* L and Ziziphus mauritiana. Moreover, the synergic anticancer activity of ethanol extract of *Punica grantum* L and *Ziziphus mauritiana* was verified, so it can be considered beneficial for cancer therapy.

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