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RESEARCH ARTICLE

ANTI PROLIFERATIVE AND APOPTOTIC EFFECTS OF *PUNICA GRANTUM* LAND *ZIZIPHUS MAURITIANA* AGAINST CARCINOMA CELLS: *INVITRO* AND *INVIVO.*

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This study was designed to investigate the anticancer effects of *Punica grantum* Land *Ziziphus mauritiana* against carcinoma cells *invitro* and *invivo*. *Invitro* antioxidant activity of ethanol, aqueous and chloroform seed extracts of *Punica grantum* L and *Ziziphus mauritiana* showed that the ethanol extract of combination of both plants having significant Super oxide, Nitric oxide and ABTS scavenging activity with IC_{50} value of 32.79,31.95,78.85 µg/ml respectively. The MTT assay indicated that the percentage decrease of HeLa cells survival was found to be 18.68% in 100 µg of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* and it also stimulated caspase-3 protease by 17.9-fold increase in DEVD-pNA cleavage.*Invivo* antioxidant displayed theethanol and aqueous extract of combination of both plants and ethanol extract of *Punica grantum* L has produced the significant increase in the super oxide dismutase and decrease in lipid peroxidation ($c<0.001$).The ethanol extracts of combination of both plants induced chromosomal aberrations such as spindle disturbance, bridge chromosome, sticky chromosomes, and polar deviations in root tips of onions and also showed root growth inhibition activity at part with colchicine as standard and these differences were statistically nonsignificant after 72h.Consequently, the synergic anticancer activity of ethanol extract of *Punica grantum* L and *Ziziphus mauritiana* was proved.

Introduction:-

Cancer is a major public health burden in both developed and developing countries. World Health Organization (WHO) reported that there are 7.6 million deaths in 2008 and it is estimated up to 13.1 million deaths in 2030 **(Kimman et al., 2012).**Cancer cell growth is different from normal cell growth. Instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells has capability to invade other tissues. The cells become cancerous because of damage done to DNA. In a normal cell, when DNA gets damaged, the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired but the cell does not die. Preferably, this cell goes on making new cells that the body does not need **(Collin, l. and Workman, P., 2006)**and it is associated with deregulation of apoptosis, a programmed cell death. Since the increase in the use of synthetic chemicals in cancer has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources, which are therapeutically effective, culturally acceptable and economically within the reach of the poor people **(Fauziah et al., 2005).**Thus, the search for new anticarcinogenic agents from plants is a main goal of research by many scientists. Generation of free radicals or reactive oxygen species (ROS) during metabolism or as a result of exposure to gamma radiation beyond the antioxidant capacity of a biological system gives rise to oxidative stress, which leads to a decrease in mitochondrial ATP synthesis, causing liver and muscle dysfunction and cell death. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process **(Sian, B.A., 2003)**.ROS are tightly controlled by antioxidant defence systems, including non-enzymatic radical scavengers as reduced glutathione and enzymes that can either directly detoxify ROS or indirectly regulate their

levels **(Alasalvar et al., 2005)**. Dietary antioxidants oppose the oxidative damage and lower risk of degenerative disease and thus arises a necessity to extract these antioxidants from the plant matrices. These plant-based dietary antioxidants are believed to have an important role in the maintenance of human health because the endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species **(Fridovich, I., 1998)**. In addition, most of the current anticancer drugs are derived from plant sources, which act throught different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity (**Tulika, M., 2011)**.The recent studies focused on the potential role of the leaves extraction of *Ziziphus mauritiana* asprevention or regression agent affecting the growth of certain tumors **(Kunwar et al., 2009)**. The extracts from fruits **(Ndhala et al., 2006)**, leaves **(Dahiru et al., 2005; Dahiru, D. and Obidoa ,O., 2007)**of *Ziziphus mauritiana* have been reported to exhibit antioxidant activity, whereas bark **(Pisha et al., 1995; Ramadoss et al., 2000)**and pulp **(Vahedi et al., 2008)** are reported to possess cytotoxicity against different cancer cell lines. *Ziziphus mauritiana* belongs to the family *Rhamnaceae*, It contains flavonoids, oleic and triterpene oligoglycosides **(Yoshikawa et al., 1997)**. *Ziziphus* is used for many of their ailments such as burns, wounds, conjunctivitis, cough,hypertension, diuretic, anthelmintic, antiemetic, etc **(Chopda, M.Z. and Mahajan, R.T., 2009; Jyotsana et al., 2010; Ashok et al., 2010; Jain et al., 2010).**Several studies have indicated immunomodulatory **(Wadekar, R. and Patil, K.S., 2008)**, hepatoprotective activity **(Dahiru et al., 2005; Dahiru, D. and Obidoa ,O., 2007)**,free radical scavenging activity **(Kalidose, A. and Krishnamoorty, P.,2011; Abalaka et al., 2011)**, antiulcer activity **(Panchal et al., 2010)**,antidiarrhoeal activity **(Dahiru et al., 2006)**, antimicrobial activity **(Abalak et al., 2010; Mahesh, B. and Satish, S., 2008)**, antimycobacterial activity **(Panomwan et al., 2011)**, antihyperlipidaemic activity **(Dahiru, D. and Obidoa, O., 2009).** 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 tumor genesis in animal models of various carcinomas **(Sartor, O. and Pal, S.K., 2013; Ryan et al., 2013; Yakes et al., 2011; Smith et al., 2013).***Punica grantum* L belongs to family *Punicaceae* commonly **(Jurenka, J., 2008).** It contains antocyanins, ascorbic acid, punicic acid, sterols, polyphenol and flavonols **(Aviram et al., 2000; Sharaf, A. and Nigm, S.A., 1964).** Traditionally *Punica grantums* is used as anti-inflammatory **(Ahmed et al., 2005)**, antidiabetic **(Huang et al., 2005)**, and neuroprotective **(Loren et al., 2005).** It has been reported for treatment against influenza virus **(Haidari et al., 2009)**,diarrhea and dysentery **(Ismail et al., 2012**), anti-atherosclerotic **(Cam et al., 2009)**and antioxidant activity **(Chidambara et al., 2002)**. 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 plantsin Hela cells for *invitro* study and EAC tumor bearing mice for *invivo* study as there is no previous anticancer activity reported for the combination of the two plants.

Materials & methods:-

Plant material and preparation of extracts:-

The authenticated aqueous, ethanol and chloroform extracts of *Punica grantum* Land *Ziziphus mauritiana* were obtained from "Green Chem", Bangalore-560071.The seeds of *Punica grantum* Land *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 vaccum (Buchi, Switzerland) to get concentrated extracts (60g), which was dried in vaccum oven and stored in a desiccator.

Tumor cells line for *invitro* antioxidant activity, MTT assay and Caspase -3/ CPP32 colorimetric 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 *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 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).

***Invitro* antioxidant activity:-**
Nitric oxide scavenging activity:-
The Nitric oxide scavenging was determined according to **(Sreejayan, N. and Rao, M.N.A., 1997; Marcocci et al., 1994).**Nitric oxide can interact with oxygen to produce nitrite ions which can be measured using the Griess reagents. Optical density was measured at 54o nm in a microplate reader.

Superoxide scavenging activity:-
The superoxide anion scavenging was determined according to **(Gulcin et al., 2002).**The superoxide ions generated from the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxide (XOD) converts tetrazolium salt to formazan dye. Formazan dye absorbed light and measured at 460nm.
ABTS assay (2,2-azino bis (3-ethylbenz-thiazoline-6-sulphonic acid) diazonium salt):-
ABTS assay was determined according to **(Thaipong et al., 2006).**The blue green chromogen $ABTS^+$ radical cation in the presence of antioxidant reductant, the coloured radical is converted into colourless ABTS which measured at at 734nm.

Caspase -3/ CPP32 colorimetric assay:-
The Caspase 3 activity was determined according to **(Lavrik et al., 2005).**Activation of caspases initiates apoptosis in mammalian cells. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labelled substrate DEVD- pNA.The pNA light emission which was quantified using microtiter plate reader at 405 nm.

Cytotoxicity assay:-
The *invitro* cytotoxic evaluationwas determined using MTT assayand Allium Cepa root tips.

MTT assay:-
The MTT assay was determined according to **(Gothoskar, S.V. and Ranadive, K.J., 1971).**The reduction of tetrazolium MTT salts is widely accepted as a reliable way to examine cell proliferation to generate MTT fromazan which was measured using microtiter plate reader at 570 nm.

***Allium cepa* root model:-**
Allium cepa root tip meristems was determined according to **(Namita, K. and Sonia, S., 2013)**. At end of exposure periods (72 hours), root lengths was measured in cm with ruler.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).

***Invivo* antioxidant activity:-**
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 lipid peroxidation (LPO) **(Ohkawa et al., 1979)**,superoxide dismutase (SOD) **(Sathish, R., 2009)**.

Histological of plasma and immunohistochemical examination:-
The fixed samples were transferred to the Department of Pathology, Health Care Global Cancer Institute for processing, blocking, sectioning and staining with haematoxylin and eosin or mounting on +ve slides for immunhistochemical investigations. Sections mounted onto positive charged slides were used to detect the Bcl-2 and p53 reactivity or apoptotic cells using the TUNEL assay according to **(Gao, H. and Zhou, Y.W., 2005)**. Streptavidinbiotin or avidin-biotin peroxidase (ABC/ HRP) was applied and bound antibody complex was visualized by the reaction of 3, 3`-diaminobenzidine (DAB) substrate and counter stained with haematoxylin.

Phytochemical screening:-
Theaqueous, ethanol and chloroform seed extracts of *Punica grantum* Land *Ziziphus mauritiana* were subjected to phytochemical screening according to the phytochemical methods described by **(Raman, N., 2006; Harbone, J.B., 2005)**.

Statistical analysis:-
The data were expressed as mean ± S.E.M (n=6). 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:-

***Invitro* antioxidant activity:-**

Nitric oxide scavenging activity:-
Table.1.1 shows the percentage increase of nitric oxide scavenging activity of different extracts of *Punica grantum* L and *Ziziphus mauritiana*. As the nitric oxide scavenging activity is recorded in terms of percentageinhibition, it is observed that all the extract have demonstrated dose dependent increase in the nitric oxide scavenging activity. The 100µg of Ascorbic acid (as standard) has 77.64% nitric oxide scavenging property. The 100µg/ml of both ethanol and aqueous extracts i.e. *Punica grantum* L and its combination with *Ziziphus mauritiana* has shown maximum Nitric oxide scavenging 65.19%, 57.66%,65.71% and 58.33% respectively. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* showed good antioxidant activity with IC_{50} value of 31.95µg/ml (Table.1.2).

(Table.1.1): Percentage of nitric oxide scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Conc µg/ml	% Scavenging									
	ASC	Z (E)	Z (Aq)	Z (Ch)	P (E)	P(Aq)	P(Ch)	ZP(E)	ZP(Aq)	ZP(Ch)
10	45.41±0.13	35.8±0.34^{c}	25.63±0.71^{c}	16.54±0.16^{c}	44.91±0.39^{a}	37.49±0.16^{c}	26.13±0.06^{c}	45.52±0.03	38.98±0.39^{c}	35.98±0.33^{c}
25	59.23±0.24	39.59±0.14^{c}	30.19±0.47^{c}	20.32±0.13^{c}	48.52±0.21^{c}	41.31±0.17^{c}	29.84±0.51^{c}	48.51±0.21^{c}	41.69±0.33^{c}	39.51±0.25^{c}
50	64.65±0.27	44.64±0.21^{c}	36.61±0.27^{c}	25.48±0.16^{c}	53.21±0.36^{c}	46.61±0.21^{c}	34.43±0.22^{c}	53.36±0.38^{c}	45.73±0.44^{c}	44.65±0.22^{c}
75	70.01±0.34	50.33±0.35^{c}	42.91±0.41^{c}	33.89±3.21^{c}	59.21±0.41^{c}	52.51±0.41^{c}	39.81±0.37^{c}	59.41±0.09^{c}	52.86±0.31^{c}	47.23±0.11^{c}
100	77.64±0.66	56.23±0.44^{c}	49.65±0.33^{c}	36.74±0.36^{c}	65.19±0.21^{c}	57.66±0.34^{c}	50.78±0.48^{c}	65.71±0.59^{c}	58.33±0.15^{c}	51.02±0.68^{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.1.2): IC_{50} values for nitric oxide scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Extracts	ASC	Z (E)	Z (Aq)	Z (Ch)	P (E)	P(Aq)	P(Ch)	ZP(E)	ZP(Aq)	ZP(Ch)
IC_{50} µg/ml	13.45	72.85	101.3	150.12	33.15	64.63	99.49	31.95	63.25	90.05

Superoxide scavenging activity:-

Table.2.1 shows the percentage increase of superoxide scavenging of different extracts of *Punica grantum* L and *Ziziphus mauritiana*.It is observed that all the extract have demonstrated dose dependent increase in the super oxide scavenging activity. The 100µg/ml of ascorbic acid has shown maximum super oxide scavenging (77.17%). Ethanol extract of combination of *Punica grantum* Lwith *Zizipus mauritiana* as well as ethanol extract of*Punica grantum* L, showed activities at par with ascorbic acid as standard and these differences were statistically b < 0.01 and c < 0.001 respectively. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* showed good super oxide scavenging activity with IC_{50} value of 32.79 µg/ml (Table.2.2).

(Table.2.1): Percentage increase of superoxide scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Conc .µµg/ml	% Scavenging									
	ASC	Z (E)	Z (Aq)	Z (Ch)	P (E)	P (Aq)	P (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
10	42.31±0.31	27.38±0.04^{c}	17.46±0.74^{c}	12.96±0.31^{c}	36.16±0.19^{c}	29.35±0.61^{c}	18.48±0.22^{c}	37.09±0.41^{a}	30.99±0.31^{c}	23.22±0.18^{c}
25	57.77±0.26	38.02±0.31^{c}	28.91±0.29^{c}	19.31±0.04^{c}	47.08±0.51^{c}	39.93±0.25^{c}	29.09±0.13^{c}	47.48±0.18^{c}	40.49±0.26^{c}	38.72±0.43^{c}
50	68.73±0.23	49.31±0.04^{c}	41.61±0.37^{c}	26.12±0.49^{c}	56.13±0.41^{c}	51.43±0.17^{c}	39.00±0.37^{c}	58.31±0.61^{c}	51.75±0.51^{c}	44.13±0.25^{c}
75	72.46±0.32	52.23±0.35^{c}	54.83±0.31^{c}	27.91±0.23^{c}	60.62±0.23^{c}	54.39±0.17^{c}	41.67±0.21^{c}	67.64±0.23^{b}	54.66±0.28^{c}	47.25±0.17^{c}
100	77.17±0.64	56.45±0.14^{c}	58.52±0.16^{c}	30.56±0.39^{c}	65.12±0.65^{c}	57.24±0.15^{c}	43.46±0.19^{c}	72.89±0.32^{b}	56.36±0.037^{c}	51.32±0.05^{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. 2.2): IC_{50} values for superoxide scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Extracts	ASC	Z (E)	Z (Aq)	Z (Ch)	P (E)	P (Aq)	P (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
IC_{50} µ g/ml	18.84	67.53	72.12	180.85	42.85	62.37	102.24	32.79	45.57	79.41

ABTS assay (2,2-azino bis (3-ethylbenz-thiazoline-6-sulphonic acid) diazonium salt):-

Table.3.1 shows thepercentage increase of ABTS radical scaveging of different extracts of *Punica grantum* L and *Ziziphus mauritiana*. All extracts significantly (c<0.001) increase in scavenging activity as compared to the ascorbic acid as standard. The 100µg/ml of Ascorbic acid (as standard) has 74.17% ABTS scavenging property. The percentage increase in scavenging activity was found to be 52.13% and 62.72% in 100µg/ml of ethanol extracts of both *Punica grantum* L and its combination with *Ziziphus mauritiana* respectively. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* showed good ABTS scavenging activity with IC_{50} value of 78.85 µg/ml compared to IC_{50} value of ascorbic acid as standard 10.4µg/ml (Table.3.2).

(Table.3.1): Percentage increase of ABTS scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Conc µg/ml	% Scavenging									
	ASC	ZM (E)	Z (Aq)	Z (Ch)	P (E)	P (Aq)	P (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
10	49.61± 0.21	30.64±0. 35^{c}	20.94±0. 61^{c}	11.35±0. 08^{c}	39.55±0. 28^{c}	32.36±0. 02^{c}	21.05±0.0 3^{c}	40.43±0.0 6^{c}	33.1±0.4 1^{c}	31.19±0. 18^{c}
25	62.98±0. 02	33.56±0. 35^{c}	24.00±0. 05^{c}	14.33±0. 01^{c}	42.15±0. 43^{c}	35.25±0. 13^{c}	23.75±0.1 4^{c}	53.63±0.1 7^{c}	34.45±1. 41^{c}	33.58±0. 24^{c}
50	66.00±0. 06	36.14±0. 06^{c}	28.79±0. 58^{c}	17.13±0. 05^{c}	45.16±0. 48^{c}	38.44±0. 12^{c}	26.09±0.0 5^{c}	55.61±0.5 7^{c}	40.86±0. 37^{c}	36.22±0. 13^{c}
75	70.00±0. 33	40.21±0. 21^{c}	32.85±0. 37^{c}	20.85±0. 04^{c}	49.16±0. 42^{c}	42.12±0. 11^{c}	29.9±0.41^{c}	59.64±0.2 1^{c}	45.67±0. 36^{c}	40.25±0. 11^{c}
100	74.17±0. 64	43.24±0. 32^{c}	36.75±0. 43^{c}	23.61±0. 34^{c}	52.13±0. 51^{c}	44.71±0. 42^{c}	32.83±0.4 7^{c}	62.72±0.3 9^{c}	50.39±0. 19^{c}	43.01±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 standard. ASC: Ascorbic acid, P: *Punica gratum* L, Z: *Zizipus mauritiana*, E: Ethanol extract, Aq: Aqueous extract, Ch: Chloroform extract.

(Table.3.2): IC_{50} values for ABTS scavenging activity of different extracts of *Punica grantum* L and *Zizipus maritiana*

Extracts	ASC	Z (E)	Z (Aq)	Z (Ch)	P (E)	P (Aq)	P (Ch)	ZP (E)	ZP (Aq)	ZP (Ch)
IC_{50} µg/ml	10.4	147.1	173.3	293.15	83.3	134.8	231.8	78.85	97.15	151.55

Caspase 3 activity of ethanol extracts of combination of *Punica grantum* L and *Ziziphus mauritiana*:-

Table 4 shows caspase-3 protease activity of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana*.It is observed thatethanol extracts of combination of*Punica grantum* L and *Ziziphus mauritiana* significantly stimulated caspase-3 protease activity. Cells showed a 17.9-fold increase in DEVD-pNA cleavage at dose of 100µg/ml.

(Table 4): Caspase-3 activity of ethanol extract of combination of *Punica grantum* L and *Zizipus maritiana*

Conc µg/ml	absorbance	Fold increase
0	0.0213±0.0007	-
10	0.0629±0.0013^{C}	2.4
25	0.1534±0.0027^{C}	7.2
50	0.2501±0.003^{C}	11.7
75	0.3036±0.0023^{C}	14.2
100	0.3812±0.0042^{C}	17.9

n = 3, values are mean ± S.E.M, one way ANOVA followed by Dunnet's multiple test. P values: c < 0.001 as compared to untreated group as standard.

Cytotoxicity assay:-

Cells survival of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* by using MTT assay:-

Table.5.1 shows the percentage decrease of cells survival of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana*. It is observed that extract has demonstrated dose dependent decrease in amount of cells survival. The 100 µg of Ascorbic acid has shown maximum cells growth inhibition by reducing the cell proliferation up to 8.63% after 48 h. The percentage decrease of cells survival was found to be 18.68% in 100 µg of ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana*. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* showed good cell growth inhibition with IC_{50} value of 30.89 µg/ml (Table.5.2).

(Table.5.1): Effect of ethanol extract of combination of *Punica grantum* Land *Zizipus mauritiana* on cell survival in HeLa cells

Conc (µg/ml)	% Cells Survival	
	Asc	ZP(E)
0	100	100
10	57.99 ± 1.23^{c}	68.66 ± 1.13^{c}
25	29.17 ± 0.83^{c}	52.57 ± 1.63^{c}
50	18.10 ± 0.25^{c}	35.17 ± 1.01^{c}
75	10.73 ± 0.11^{c}	27.66 ± 0.49^{c}
100	8.63 ± 0.03^{c}	18.68 ± 0.21^{c}

n = 3, Values are mean ±S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. P values; c< 0.001, compared to the untreated group.

(Table 5.2):IC_{50} values for ethanol extracst of combination of *Punica grantum* Land *Zizipus mauritiana* on cell survival in HeLa cells

Extracts	ASC	ZP (E)
IC_{50} (µg/ml)	11.01	30.89

***Allium cepa* root model:-**

Table 6 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 2.40, 2.07 , 1.72, and1.09 cm respectively at 72h. 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 ZP(E) showed root growth inhibition activity at part with colchicine as standard and these difference were statistically nonsignificant after 72h. The 800 µg/ml of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana* indicated 20.85% reduction in mitotic index after 72h.The figure 10 show macroscopic effects on *Allium cepa* root after 72h to distilled water, colchicine, 200, 400, 600,800 µg/ml of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana* ZP(E). The figure 11 show aberrations observed in *Allium cepa* root tip cells exposed to ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana*ZP(E). The ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana* induced chromosomal aberrations in root tips of onions at all tested concentrations compared to the control. The aberrations include; spindle disturbance, bridge chromosome, sticky chromosomes, and polar deviations.

(Table 6): Effect of ethanol extracts of combination of *Punica grantum* Land *Ziziphus mauritiana* on root length and mitotic index of *Allium cepas.*

Name of the extract	72 h	
	Root length (in cms)	% MI
Control (Distilled Water)	3.04 ± 0.06	64.08
Colchicine 1mg/ml	0.80 ± 0.34^{c}	15.5
ZP-E 200 µg/ml	2.40 ± 0.29^{az}	63.78
ZP-E 400 µg/ml	2.07 ± 0.13^{cz}	56.7
ZP-E 600 µg/ml	1.72 ± 0.16^{cz}	35.4
ZP-E 800 µg/ml	1.09 ± 0.28^{c}	20.85

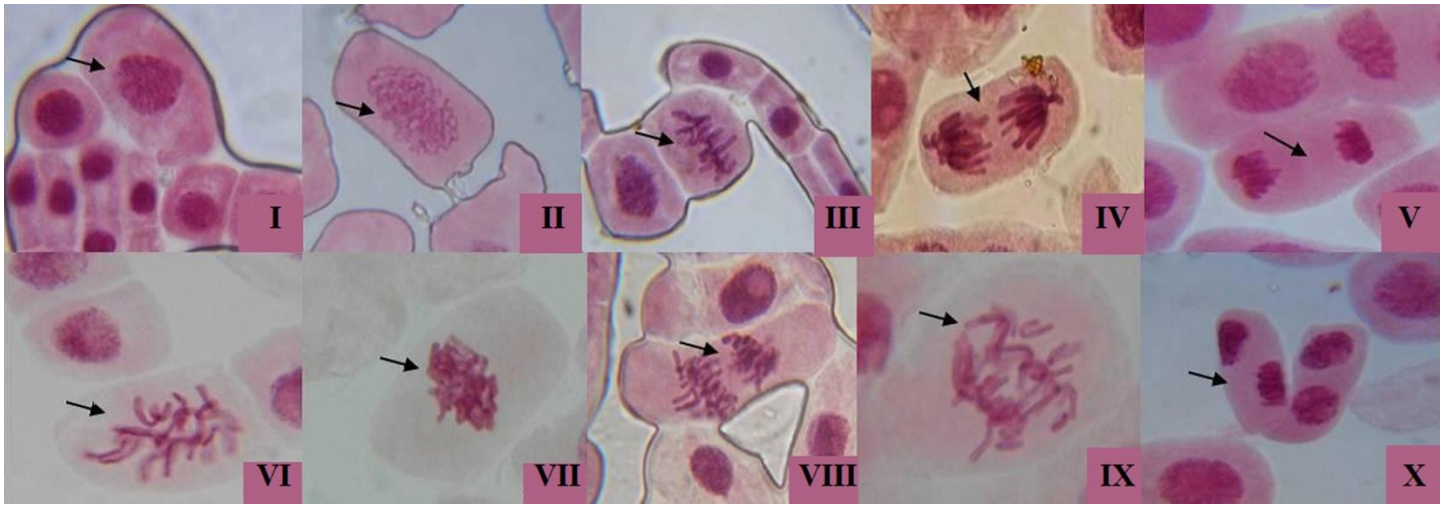
n=3, Values are mean ± S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. P values: a< 0.05, b<0.01, c< 0.001, compared to the control group; x< 0.05, y<0.01, z< 0.001, as compared with colchicine treated group.

Control (distilled water)

Colchicine1mg/ml

ZP(E)-200µg/ml	ZP(E)-400µg/ml	ZP(E)-600µg/ml	ZP(E)-800µg/ml

(Fig 1): **macroscopic effects on *Allium cepa* root after 72h to distilled water, colchicine, 200, 400, 600 and 800 µg/ml of ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana***

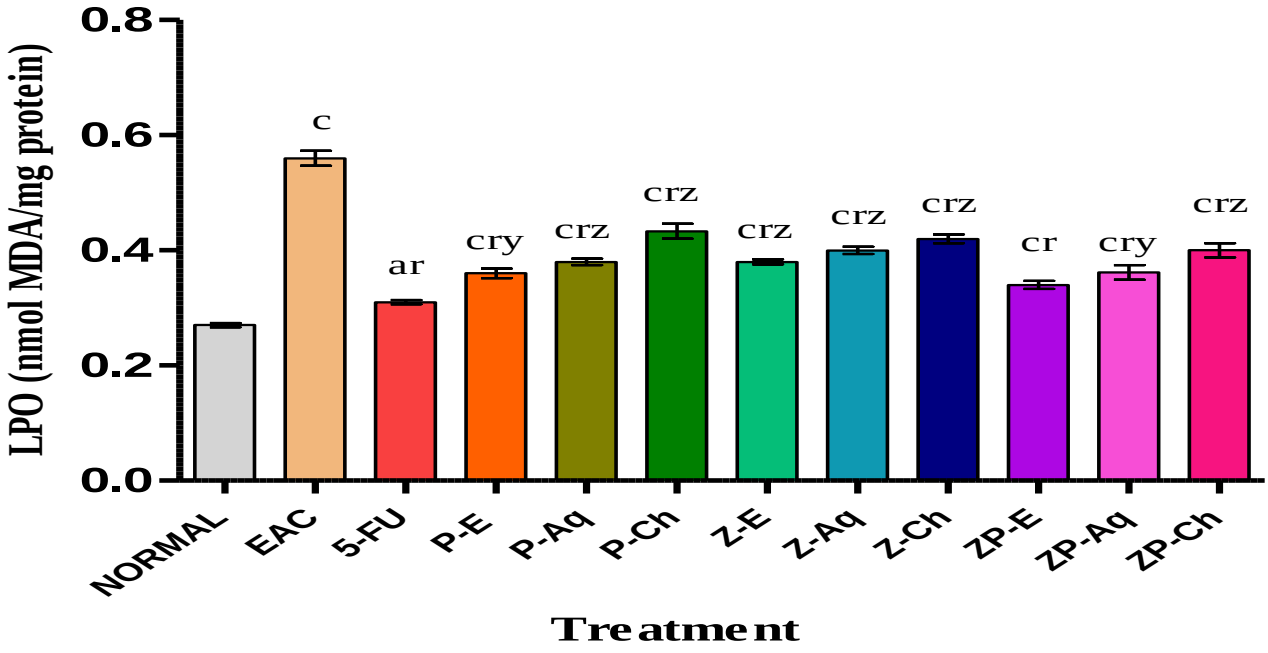


(Fig 2): Aberrations observed in *Allium cepa* root tip cells exposed to ethanol extracts of *Punica grantum* L and *Ziziphus mauritiana*.(I–V) Normal cells at: Interphase (I), prophase (II), metaphase (III), anaphase (IV) and telophase (V); (VI) Spindle disturbance at metaphase; (VII,VIII) stickiness at metaphase (VII) and anaphase (VIII); (IX) Bridges and non-disjunction at anaphase; (X) polar deviations at telophase.

***Invivo* antioxidant activity:-**

Lipid Peroxidation:-

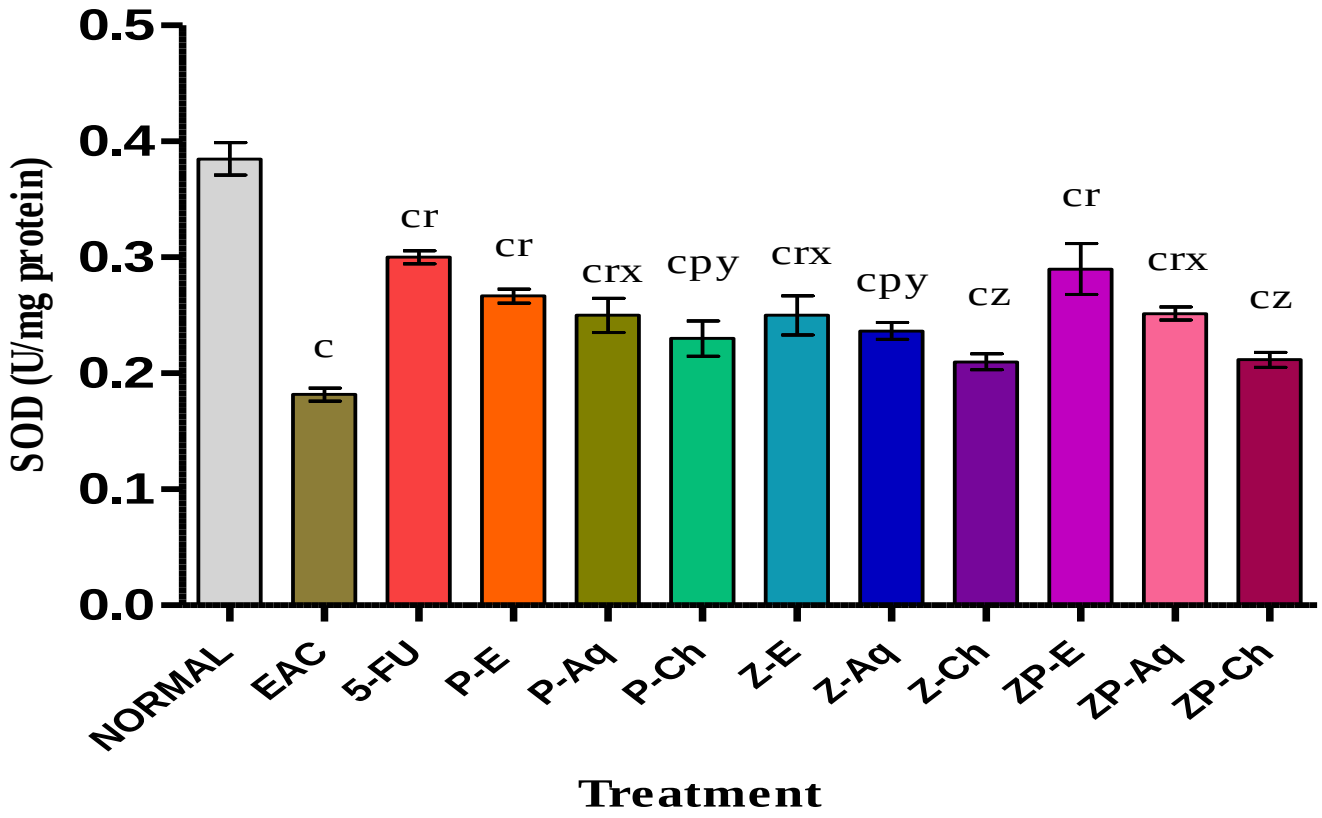
Figure 3 showsLPO levels in various experimental groups. EAC control group has showed significant increase in the LPO level (0.56 ± 0.03 nmolMDA/mg protein) when compared to the normal mice (0.27 ± 0.01 nmolMDA/mg protein). Treatment with 5-Flurouracil has restored the LPO(0.31 ± 0.01 nmolMDA/mg protein) towards normal level whereas ethanol extract *Punica grantum* L P(E) (0.36 ± 0.02 nmolMDA/mg protein) and ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* ZP (E) (0.34 ± 0.02 nmolMDA/mg protein) (c<0.001) has shown significant activity compared to the normal mice.



(Fig 3):Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on lipid peroxidation 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, 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.

Superoxide Dismutase:-

Figure 4 shows the SOD levels in various experimental groups. EAC control group (0.18 ± 0.01 U/mg protein) has showed significant decrease in the SOD level when compared to the normal mice (0.39 ± 0.03U/mg protein). The entire treated group has shown significant increase in the SOD level as compared to the normal group. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* ZP(E) treated group (0.29 ± 0.05 U/mg protein) has showed significant (c<0.001) activity by restoring SOD level towards normal than other treated groups.



(Fig 4): Effect of different extracts of *Punica grantum* L and *Zizipus mauritiana* on superoxide dismutase 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; p< 0.05, r< 0.001, as compared with EAC control; x< 0.05, y< 0.01, z< 0.001, as compared with 5-fluorouracil treated group.

Qualitative phytochemical screening of different extract of *Punica grantum* L and *Ziziphus mauritiana*:-

Results (Table 7) show phytochemical compounds which are present in aqueous, ethanol and chloroform of *Punica grantum* L and *Ziziphus mauritiana*. Ethanol extract of *Punica grantum* L contained triterpenoids, steroids, glycosides, alkaloids, flavanoids, tannins, proteins, carbohydrates, vitamin C and fat and oil. Aqueous extract of of *Punica grantum* Lcontained triterpenoids, flavanoids, tannins, proteins, carbohydrates, vitamin C and fat and oil. Chloroform extract of of *Punica grantum* L contained glycosides, flavanoids, tannins, proteins and vitamin C. Ethanol extract of *Ziziphus mauritiana* contained triterpenoids, alkaloids, flavanoids, proteins, carbohydrates, vitamin C and fat and oil. Aqueous extract of *Ziziphus mauritiana* contained flavanoids, proteins, carbohydrates, vitamin C and fat and oil. Chloroform extract of *Ziziphus mauritiana* contained flavanoids, proteins and Vitamin C.Pervious analyses of spectroscopy methods have revealedthe bioactive phytoconstituents in *Ziziphus mauritiana* (ZM) and *Punica grantum* L (PG) **(Agata et al., 2009; Sharrif, M.M. and Hamed, H.K., 2012; Borochov et al., 2011).***Ziziphus mauritiana* contains bioactive alkaloids such as cyclic peptide alkaloid zizyphine E,D,F,G, franguloine, and three peptide alkaloid; sanjoinine B, D and G_2, bioactive flavonoid for instance swertish, spinosin, puerarin, 6-feruloylspinosin, apigenin-6-C-b-Dglucopyranoside, 6-feruloylisospinosin, isospino, isovitexin-2-O-b-D glucopyranoside, 1-quercetin 3-O-robinobioside, 2-quercetin 3-Qrutinoside, 5-quercetin 3-O-a-L-arabinosyl-(1→2)-a-L-rhamnoside, 6-quercetin 3-O-b-D-xylosyl-(1→2)-a-L-rhamnoside, 12-quercetin 3-O-b-D-xylosyl-(1→2)-a-L-rhamnoside-40-O-a-L-rhamnoside.*Punica grantum* L contains bioactive alkaloid such as Pseudopelletierine, pelletierine, isopelletierine,bioactive flavonoid containsvitamin P which is called as flavonoids or bioflavonoids particularly Quercetol and anthocyanin. The major anthocyanins are mono- and diglucosylated delphinidins and

cyanidins and pelargonidins is a minor components. The bioactive tanins in*Punica grantum* L are punicacortein A,B,C,D, granatin A, granatin B with punicalin and punicalagin.

(Table 7): Qualitative phytochemical screening of different extract of *Punica grantum* L and *Ziziphus mauritiana*:

Chemical tests	***Punica grantum***			***Ziziphus mauritiana***		
	Ethanol	Aqueous	Chloroform	Ethanol	Aqueous	Chloroform
I. Test for Triterpenoids /Steroids						
Liebermann Burchard Test	+/+	+/-	-/-	+/-	-/-	-/-
II. Test for Glycosides						
Keller Killiani Test	+	-	+	-	-	-
Bromine water	+	-	+	-	-	-
III. Test for Saponins						
Foam test	-	-	-	-	-	-
IV. Test for Alkaloids						
Hager's Test	+	-	-	+	-	-
V. Test for Flavanoids						
Ferric Chloride test	+	+	+	+	+	+
Alkaline reagent test	+	+	+	+	+	-
Lead Acetate Solution test	+	+	-	+	+	-
VI. Test for Tannins						
Gelatin Test	+	+	+	-	-	-
5% $FeCl_3$	+	+	-	-	-	-
VII. Test for Proteins						
Biuret test	+	+	+	+	+	+
VIII. Test for Free amino acids						
Ninhydrin Test	-	-	-	-	-	-
IX. Test for Carbohydrates						
Benedict's Test	+	+	-	+	+	-
X. Test for Vitamin C						
DNPH test	+	+	+	+	+	+
XI. Test for fat and oil						
Saponification test	+	+	-	+	+	-

Discussion:-

Humans have always relied on nature for survival since ancient times**(Arora et al., 2003; Sandhya et al., 2006)**.Natural products have been regarded as important sources that could produce potential chemotherapeutic agents**(Kim, J. and Park, E., 2002).**Plant-derived compounds, in particular have a special place in anticancer therapy and some of the new chemotherapeutic agents currently available for use in a clinical setting include paclitaxel, vincristine, podophyllotoxin and camptothecin, a natural product precursor for water-soluble derivatives. 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**(Newman et al., 2003)**.The global trend is also towards natural bioactive substances due to their low toxicity and cost. Nowadays they are used to inhibit cancer cell growth through cell cycle regulation including the G2/M accumulation**(Gerzon, K.,1980; Douglas, K.A and Manuel, F., 1993; Tanaka el al., 2004).**Antioxidants are substances which retard or prevent damage or destruction caused by oxidation. During the last few years research has confirmed that many of common disease and aliment of the 21st century cardiovascular diseases, cataract, diabetes, hepatic damage, rheumatoid arthritis , Alzheimer's disease, several type cancer and even mental illness are associated with tissue deficiency or dietary levels of antioxidants compounds. They act by radical scavenging, suppression of radical formation and repair of damage membranes. Fortunately, the human body has an army of antioxidants for delaying or inhibition of oxidation. A family of antioxidant enzymes are provide in the healthy tissue such as SOD, CAT and GSH which defense the body**(Bhalodia et al., 2011).** There are numerous methods for evaluation of antioxidant activity, out of which the present study has used three antioxidant assays for evaluation of antioxidant activity. Evaluation of Nitric oxide scavenging is based on generation of nitric oxide, which interact with oxygen to produce nitrite ions, which can be measured using a modified **Griess-illosvoy** method. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ion**(Govindarajan, R., 2012).** From the result it is evident that *Punica grantum* Lethanol extract and ethanolic extract of combination of *Punica grantum* Lwith *Zizipus mauritiana*has potential nitric oxide scavenging activity. Aqueous extracts of the *Punica grantum* Landaqueous extract of the combination of both has partially scavenging activity, thus they are able to scavenge the nitric oxide. Result also indicates that the Nitric oxide scavenging activity is concentration dependent. ABTS assay is based on the inhibition of the absorbance of $ABTS^{+}$ radical which has characteristic wavelength at 734nm using the method of **(Thaipong et al., 2006).**In this assay, the ABTS radical which is a blue green chromogen is generated in a stable form using potassium persulphate. When antioxidant react with $ABTS^{+}$ radical then colored radical is converted to colorless ABTS**(Sreejayan, M., 2013).**All of the amounts of extracts showed activities less than ascorbic acid as standard and these differences were statistically significant ($P<0.001$). The superoxide ions generated from the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxide (XOD) converts tetrazolium salt to formazan dye. Formazan dye can absorb light at 460nm. SODs reduce superoxide ion and thereby the rate of formazan dye formation. The extent of reduction in absorbance shows the increase in SOD activity. Ethanol extract of combination of *Punica grantum* Lwith *Zizipus mauritiana* as well as ethanol extract of *Punica grantum* L,showed activities at par with ascorbic acid. The activation of caspase 3 is a central mechanism of apoptosis in the death receptor pathway. Therefore, caspases are considered to be the executioner in this cell death process. In the present study, the caspase 3 activity was increased by 17 fold ($P<0.001$) in ethanol extract of combination of *Punica grantum* Lwith *Zizipus mauritiana* –treated Hela cells at dose of 100µg/ml compared with control cells. The pervious phytochemical analysis of *Zizipus mauritiana* showed the presence of betulinic acid (Pentacyclic triterpenoid) which is known as anticancer agent and apoptosis inducing property of *Zizipus mauritiana* may be in part due to the presence of betulinic acid. It is well established that betulinic acid activates apoptosis independent of the death receptor (CD_{95}/ Fas) but induces the successive activation of caspase 9 and caspase 3**(Pokrovskii et al., 2006)**.In addition; anthocyanin of *Punica grantum* L has the ability to induce apoptosis due to activation of mitochondrial pathway. The antioxidant properties of anthocyanins involves (1) scavenging radical species such as ROS/RNS (Reactive Oxygen Species/ Reactive Nitrogen Species); (2) suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production**(Cotelle, N., 2001).**Cytotoxicity of anticancer drugs to the normal cells are major problems in cancer therapy and engender the risk of inducing secondary malignancy**(Little, M.P., 2001).**A dose of anticancer drug sufficient to kill tumor cells is often toxic to the normal tissue and leads to many side effects, which in turn, limits its treatment efficacy. There has been a concerted search in the recent years for the discovery of novel selective antitumor agents, devoid of many of the unpleasant side effects of conventional antitumor agents. Therefore, in the present study the cytotoxicity of the ethanol extract of ZP on HeLa cells was determined to check the selectivity of the extract. Ethanol extract of combination of *Punica grantum* L and *Ziziphus mauritiana* showed significantly cytotoxic effect against HeLa cell line with IC_{50} value of 30.89 µg/ml. The results of the present study indicated an increase in apoptotic population induced by extract in a concentration-dependent

manner. To evaluate the cytotoxic of known and unknown drug, more than 200 short term assays have been developed. Among those assay, *Allium cepa* root model was selected for the present study.Consistent with many reports, *Allium cepa* assay , which is established by the international programmed on chemical safety and the World Health Organizattion (WHO), one of the most effective and sensitive methods for mutagenic testing**(Kwankua, W., 2010).**The cytotoxic potential of ethanol extract of combination of *Punica grantum* L and *Ziziphus*ZP(E) was screened by using *Allium cepa* root meristem assay. The inhibitory effect of ZP(E) extract was evaluated on the growth of *Allium cepa* root meristem. Root lengths and % mitotic index were calculated in control group and treated group at 72h. Root growth decrease indicates the presence of toxic nature of substances**(Robinson, B.H., 2009)** having sub lethal effects on plants. Significant reduction in MI, noted in the present study may be due to the inhibition of DNA synthesis or the blocking in the G2 phase of the cell cycle**(Dimitrova et al., 1994).** Chromosomal abnormalities are characterized by change in either total number of chromosomes or in chromosomal structure which occur as a result of the exposure of chemical treatment. To evaluate the different chromosomal abnormalities (CA), several types of CAs are considered in different stages of cell cycle (Prophase, metaphase, anaphase and telophase). CAs were grouped into 2 types, clastogenic and physiological aberrations. Clastogenic aberrations include chromatin bridge,chromosomal break and ring chromosome/s whereas physiological aberrations include c-mitosis, vagrant, stickiness, delayed anaphase and laggard. Lagging chromosomes resulted due to failure of the chromosomes to get attached to the spindle fibre and to move to either of the two poles. Stickiness of chromosomes has resulted from increased chromosomal contraction and condensation or might from the depolymerization of DNA and partial dissolution of nucleoproteins. Chromosome stickiness reflects toxic effects, usually of an irreversible type and probably leading to cell death. Same results are in line with the results of many research groups that examined the effects of different chemicals on different materials**(Leme, D.M. and Marin, M.A., 2009; Ozkan et al., 2011).**In vagrant chromosome/s, a chromosome moves ahead of from its chromosomal group toward poles and leads to the unequal separation of number of chromosomes in the daughter cells. Vagrant chromosomes have been observed by many workers in different studies**(Gupta et al., 2001).** Ring chromosomes are the result of loss of chromosomes from the telomeric side. Chromatin bridges could happen during the translocation of the unequal chromatid exchange and cause structural chromosome mutation. The spindle disturbance, bridge chromosome, sticky chromosomes, and polar deviations are type of anomaly which were observed in the mitosis of *Allium cepa* after treatments with *Punica grantum* L and *Ziziphus*ZP(E). The cytotoxic activity raised with increases in concentration and the time of exposure to the test solutions. Oxidative stress as a result of overproduction of reactive oxygen species (ROS) has been implicated in the pathogenesis of a number of human diseases including cancer**(Benkovic et al., 2007).** Implantation of EAC cells into mice exerted significant ($p < 0.001$) increased in lipid peroxidations (LPx) represented by malondialdehyde (MDA) content in blood and liver as compared to normal group. Lipid peroxidation induced by reactive oxygen species might be involved in tumor progression and promotion of carcinogenesis**(Schumann et al., 2002).**Increased level of lipid peroxidation, including 4-hydroxynonenal and MDA were found in colon, liver, breast and kidney carcinogenesis**(Schumann et al., 2002).**Many observations indicate a direct correlation between 8-oxo-7,8-dihydroguanine formation and carcinogenesis**(Bergmeyer et al., 1977).**Treatment with ethanol extract *Punica grantum* L, P (E) and ethanol extract of combination of *Punica grantum* L and *Ziziphus* ZP (E) at 200mg/kg dose reduced the elevated levels of lipid peroxidation ($c<0.001$). Results of lipid peroxidation inhibiting activity indicate that ZP (E) was significantly effective to inhibit the peroxidation compared to the EAC control and all treated group. SOD eliminates the superoxide by catalyzing dismutation of superoxide into oxygen and hydrogen peroxide; inhibition of SOD activity has been reported in the cancer condition. Similar finding were observed in the present study with EAC-bearing mice. Plant-derived extracts containing antioxidant principle showed cytotoxicity towards tumor cells**(Reddy et al., 2003)**and antitumor activity in experimental animals**(Spiridon, K.E., 2006).** The antitumor activity of ethanol extract of *Punica grantum* LP(E) ,aqueous extract of combination of *Punica grantum* L and *Ziziphus* ZP(aq) and ethanol extract of combination of *Punica grantum* L and *Ziziphus* ZP(E) was accompanied with the increase of antioxidant status. The data revealed that the concentration of reduced SOD levels was significantly ($c<0.001$) increased when EAC bearing mice were pretreated with ethanol extract of combination of *Punica grantum* L and *Ziziphus* ZP(E). The phytochemical screening in the present study, has revealed the presence of triterpenoids, steroids,glycosides, flavonoids, tannins, alkaloid,carbohydrate and vitamin C in different extract of *Punica grantum* L and *Ziziphus mauritiana*. Phenolic compounds such as flavonoids,tannins and alkaloid are a major group of compounds that act as primary antioxidants or free radical scavengers. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Plant phenolics include phenolics acids, flavonoids, tannins and the less common stilbenes and lignans. Plant phenolics

are generally involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to plants' colors. They are ubiquitous in all plant organs and are therefore an integral part of the human diet**(Jin, D. and Russell, J.M., 2010).**Antioxidant properties of phenolic compounds: Antioxidants are defined as compounds that can delay, inhibit, or prevent the oxidation ofoxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and/or nitrogen species (ROS/RNS,e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite) overcome endogenous antioxidant capacity, leading to oxidation of a varieties of biomacromolecules, such as enzymes, proteins, DNA and lipids. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer and aging**(Ames et al., 1993)**. Recently, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids**(Evans et al., 1995; Evans et al., 1996)**.The inverse relationship between fruit and vegetable intake and the risk of oxidative stress associated diseases such as cardiovascular diseases, cancer or osteoporosis has been partially ascribed to phenolics**(Scalbert et al., 2005; Hollman et al., 1999).**It has been proposed that the antioxidant properties of phenolic compounds can be mediated byfree radical scavengers and metal chelators**(Cotelle, N., 2001).**Free radical scavengers and metal Chelators:Phenolic compounds (POH) act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (R):

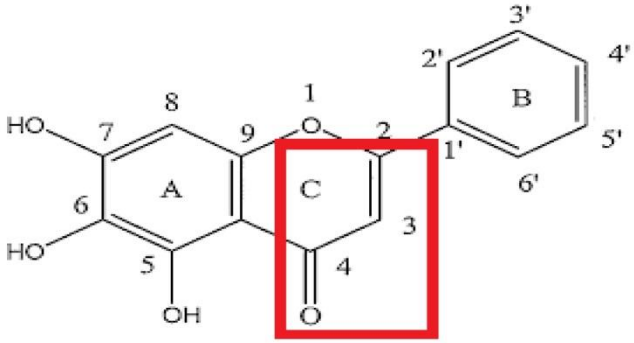
$$R + POH \rightarrow RH + PO^{\cdot}$$

The phenoxy radical intermediates ($PO^{\cdot}$) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals:

$$PO^{\cdot} + R^{\cdot} \rightarrow POR$$

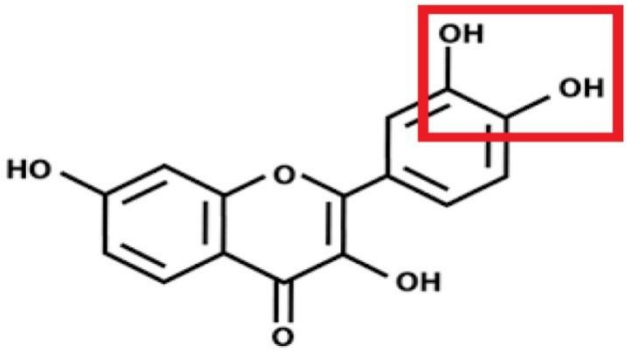
Phenolic compounds possess ideal structure chemistry for free radical scavenging activities because they have: (1) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; (2) extended conjugated aromatic system to delocalize an unpaired electron. Several relationships between structure and reduction potential have been established as follows:(1) For phenolic acids and their esters, the reduction activity depends on the number of freehydroxyl groups in the molecule, which would be strengthened by steric hindrance**(Dziedzic, S.Z. and Hudson, B.J., 1983).**Hydroxycinnamic acids were found to be more effective than their hydroxyl benzoic acid counterparts, possibly due to the aryloxy-radical stabilizing effect of the –CH=CH–COOH linked to the phenyl ring by resonance**(Evans et al., 1996)** (2) For flavonoids, the major factors that determine the radical-scavenging capabilityare**(Shahidi, F. and Wanasundara, P.K.,1992; Bors, W. and Michel, C., 2002).**

(i) The 2,3-double bond a 4 –oxo functionof quercetin which is responsible for electrondelocalization from the B ring.



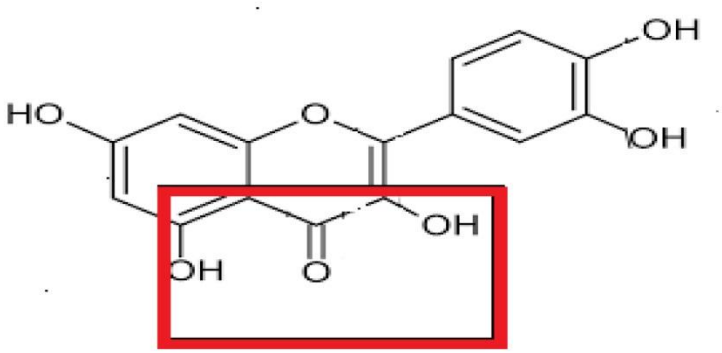
(Fig 5): The 2,3-double bond with a 4-oxo function in the C ring

(ii) The B ring of quercetin at the ortho-dihydroxy position, which has the best electron-donating properties and confers higher stability to the radical form and participates in electron delocalization.



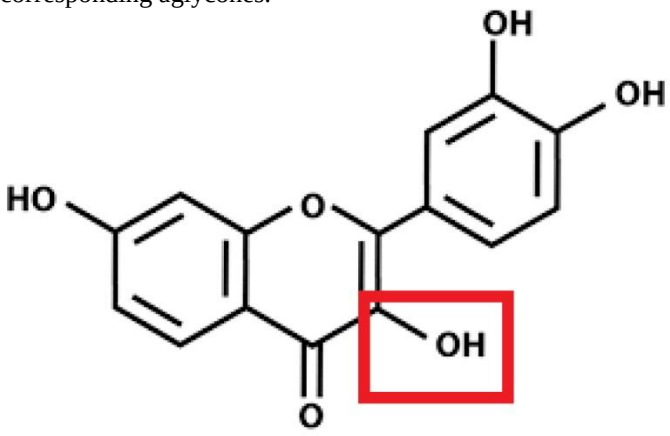
(Fig 6): The ortho-dihydroxy structure on the B ring

(iii) The 3- and 5-hydroxyl groups with the 4-oxo function of quercetin, which are essential for maximum radical scavenging potential.



(Fig 7): The 3- and 5-hydroxyl groups with the 4-oxo function in A and C rings

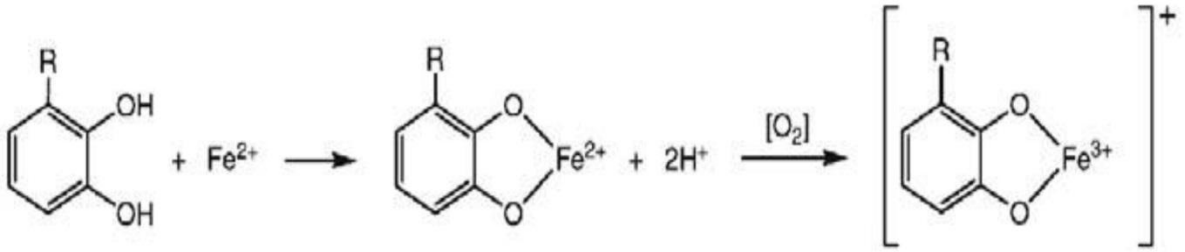
(iv) The 3-hydroxyl group is quercetin. Here, the 3-glycosylation reduces their activity when compared with corresponding aglycones.



(Fig 8):The 3-hydroxyl group is important for antioxidant activity

Quercetin is a flavonol that possess all of the factors described in (2). Anthocyanins are particularly reactive toward ROS/RNS because of their peculiar chemical structure of electron deficiency. As an alternative antioxidant property, some phenolic compounds with dihydroxy groups can conjugate transition metals, preventing metal-induced free radical formation. The redox active metal ions such as Cu^{+} or Fe^{2+} interact with hydrogen peroxide (H_2O_2) through Fenton chemistry (as shown in below reaction) to form hydroxyl radicals (·OH), which is the most reactive ROS known, being able to initiate free radical chain reactions by abstracting hydrogen from almost any molecule. Phenolic compounds with catecholate and gallate groups can inhibit metal-induced oxygen radical formation either by coordination with Fe^{2+} and enhancing autoxidation of Fe^{2+}(as shown in below reaction), or the formation of inactive complex with Cu^{2+}, Fe^{2+}, or Cu^{+} with relatively weaker interaction**(Yoshino. M. and Murakami, K., 1998; Perron, N.R. and Brumaghim, J.L., 2009).**The attachment of metal ions to the flavonoid molecule can be 3',4'-*o*-diphenolic groups in the B ring,3,4 or 3,5-*o*-diphenolic groups, and the ketol structures 4-*keto*,3-hydroxy or 4-*keto*,5-hydroxy groups in the C ring**(Hudson, B.J. and Lewis, J.I., 1983; Evans et al., 1997)**.It was also proposed that optimum metal-binding and antioxidant activity is associated with the structures which contain hydroxy-keto group (a 3-OH or 5-OH plus a 4-C = O), as well as a large number of catechol/gallol groups**(Hudson, B.J. and Lewis, J.I., 1983;Khokhar, S. and Apenten, R.K., 2003).**

H2O2 + Cu+ or Fe2+ → Cu2+ or Fe3+ + OH + ·OH-



(Fig 9): Phenolic compounds with catechol groups

Theoretically, these two antioxidant actions can cause a reduction of the steady state concentrations of free radicals and oxidant species. As a result, the subsequent oxidation of target molecules such as lipids, proteins and nucleic acids is diminished.The inhibitory effect of natural phenolics in carcinogenesis and tumor growth may be through two main mechanisms: 1) modifying the redox status and, 2) interfering with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis)**(Kampa et al., 2007).**

1. Modifying the redox status:

ROS/RNS are constantly produced during normal cellular metabolism or by other exogenous means including the metabolism of environmental toxins or carcinogens, by ionizing radiation and by phagocytic cells involved in the inflammatory response. When the cellular concentration of oxidant species is increased to an extent that overcome the endogenous antioxidant defense system, oxidative stress occurs, leading to lipid, protein, and DNA damage. In addition, ROS, particularly H_2O_2, are potent regulators of cell replication and play an important role in signal transduction**(Khan, A.U. and Wilson, T., 1995).**Hence, oxidative damage is considered a main factor contributing to carcinogenesis and evolution of cancer. Due to their ability to scavenge and reduce the production of free radicals and act as transition metal chelators, natural phenolic compounds can exert a major chemopreventive activity**(Kampa et al., 2007).**Indeed, it has been shown that natural polyphenols can inhibit carcinogen/toxin-induced cellular oxidative damage. For example, in nicotine-treated rat peripheral blood lymphocytes, ellagic acid effectively restored the antioxidant status and reduced DNA damage as well as lipid peroxidation**(Sudheer et al., 2007)**.A phenolic apple juice extract as well as its reconstituted polyphenol mixture (rutin, phloridzin, chlorogenic acid, caffeic acid and epicatechin) were shown to effectively reduce menadione-induced oxidative DNA damage and increasing of cellular ROS level**(Schaefer et al., 2006).**Tea polyphenols**(Higdon, J.V. and Frei, B., 2003)** and other extensively studied polyphenols such as resveratrol**(Damianaki et al., 2000; Burkhardt et al., 2001)**quercetin**(Alia et al., 2006; Aherne, S.A. and Brien, N.M., 1999; Johnson, M.K. and Loo, G., 2000)**were also showed to exert protective effects against cellular oxidative damage in different human cell lines.

2) interfering with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis)(Kampa et al., 2007).

Natural phenolics can affect basic cell functions that related cancer development by many different mechanisms.

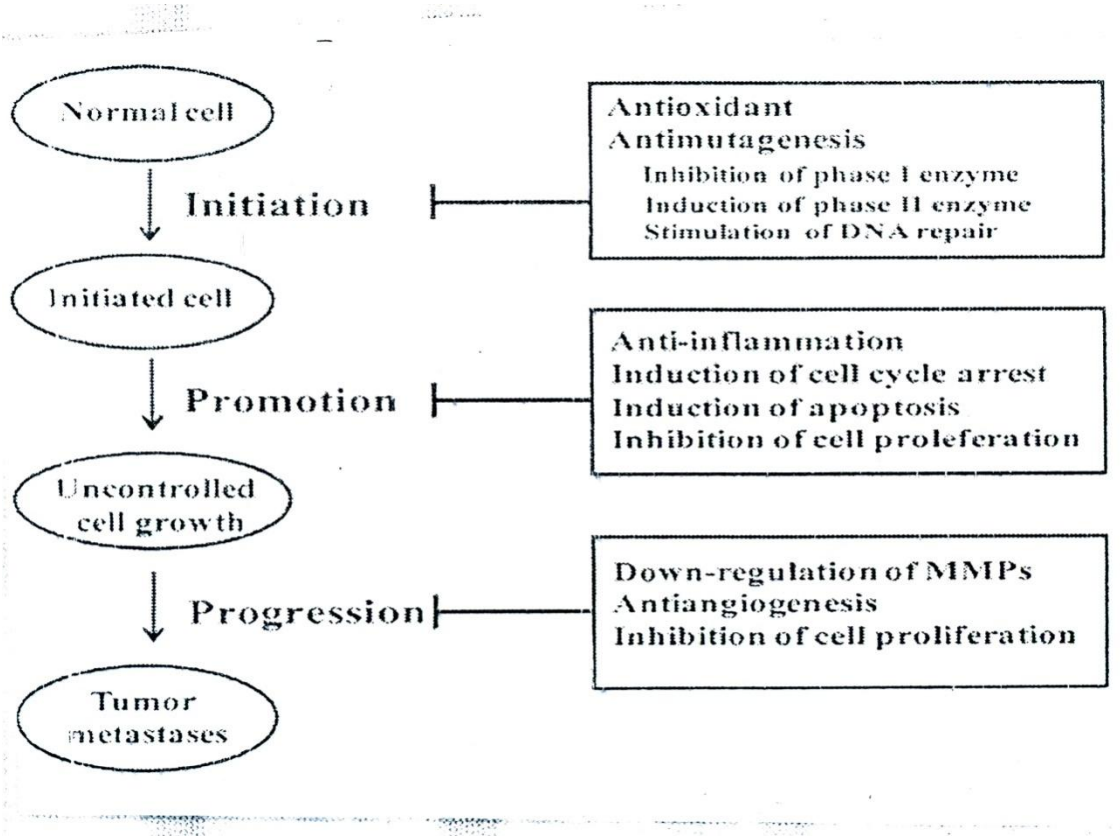


Fig 10):Potential anticancer mechanisms of plant phenolics during cancer development.

Firstly, in the initiation stage, phenolics may inhibit activation of procarcinogens by inhibiting phase I metabolizing enzymes, such as cytochrome P_{450}**(Hodek et al., 2002)** and also facilitate detoxifying and elimination of the carcinogens by induction of phase II metabolizing enzymes such as glutathione S-transferase (GST), NAD(P)H quinine oxidoreductase (NQO), and UDP-glucuronyltransferase (UGT)**(Galati et al., 2000)**. They may also limit the formation of the initiated cells by stimulating DNA repair**(Webster et al., 1996; Imanishi et al., 1991).**Secondly, phenolics may inhibit the formation and growth of tumors by induction of cell cycle arrest and apoptosis. Malignant cells are characterized by excessive proliferation, inability to terminally differentiate or perform apoptosis under normal conditions, and an extended or immortalized life span. The regulation of cell cycle is altered in these cells. Thus, any perturbation of cell cycle specific proteins by phenolics can potentially affect and/or block the continuous proliferation of these tumorigenic cells. Natural phenolics have been reported induce cell cycle arrest at different cell phases: G1, S, S-G2, and G2 by directly down-regulating cyclins and cyclins-dependent kinases (CDKs) or indirectly inducing the expression of P_{21}, P_{27} and P_{53} genes**(Fresco et al., 2006; Ramos, S., 2008).**Moreover, some studies have shown that natural phenolics exhibit differential effect in cancer *versus* normal cells. For example, anthocyanin-rich extract from chokeberry was found to induce cell cycle block at G_1/G_0 and G_2/M phases in colon cancer HT-29 cells but not in NCW460 normal colonic cells**(Malik et al., 2003).** Apoptosis has been reported to play an important role in elimination of seriously damaged cells or tumor cells by chemopreventive or chemotherapeutic agents**(Galati et al., 2000; Thompson, C.B., 1995).**The cells that have undergone apoptosis have typically shown chromatin condensation and DNA fragmentation. They are rapidly recognized by macrophages before cell lysis, and then can be removed without inducing inflammation. Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Polyphenols have been found to affect cancer cell growth by inducing apoptosis in many cell lines such as the hepatoma ($HepG_2$), the colon (SW620, HT-29, CaCo-2, and HCT-116), the prostate (DU-145 and LNCaP), the lung (A549), the breast (MCF-7), the melanoma (SK-MEL-28 and SK-MEL-1), the neuroblastoma (SH-SY5Y) and the HL-60 leukemia cells**(Ramos et al., 2005; Ramos, S., 2007).**In many cases, apoptosis induced by polyphenols was caspase-3-dependent. The induction of apoptosis and/or inhibition of proliferation/survival by polyphenols has been reported to result from a number of mechanisms including inducing cell cycle arrest; blocking the extracellular regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and P_{38} mitogen-activated protein kinase (MAPK) pathway; inhibition of the activation of transcription factors, NF-κB and activator protein-1 (AP1); suppression of protein kinase C (PKC); suppression of growth factor-mediated pathways**(Fresco et**

al., 2006; Ramos, S., 2008). For example, **(Afaq et al., 2005)**showed that pomegranate fruit extract, rich in anthocyanins and hydrolysable tannins, protected against the adverse effect of both UV radiation in normal human epidermal keratinocytes *in vitro*and 12-*O*-tetradecanoylphorbol-13- acetate (TPA) in CD-1 mouse skin *in vivo***(Afaq et al., 2005; Sai et al., 2000)**, by inhibiting the activation of NF-κB and MAPK pathway. One important aspect of carcinogenesis is recognized to be the involvement of inflammation. For instance, prostaglandins are mediators of inflammation and chronic inflammation predisposes to carcinogenesis. The over-expression of inducible cyclooxygenases (COX-2), the enzyme which catalyzes a critical step in the conversion of arachidonic acid to prostaglandins and is induced by pro-inflammatory stimuli, including mitogens, cytokines and bacterial lipopolysaccharide (LPS), is believed to be associated with colon, lung, breast and prostate carcinogenesis. Natural phenolics have been reported to inhibit transcription factors closely linked to inflammation (e.g., NF-κB)**(Karlsen et al., 2007; Tsoyi et al., 2008)**, pro-inflammatory cytokines release**(Karlsen et al., 2007; Gauliard et al., 2008)**and enzymes such as COX-2**(Hou et al., 2007; Hou et al., 2005)**, lipoxygenases (LOX)**(Hong et al., 2001)**, inducible nitric oxide synthase (iNOS)**(Pergola et al., 2006)** that mediate inflammatory processes, both *in vitro* and *in vivo***(Jin et al., 2006)**. In many cases, polyphenols exhibit anti-inflammatory properties through blocking MAPK-mediated pathway. Another study by Herath *et al.* suggested that the double bond between carbon 2 and 3 and the ketone group at position 4 of flavonoids are necessary for potent inhibitory effects on LPS-induced tumor necrosis factor-alpha (TNF-α) production in mouse macrophages (J774.1)**(Herath et al., 2003)**.Finally,natural phenolics such as hydrolysable tannins and anthocyanins, were found to suppress malignant cell migration, invasion and metastasis *in vitro* and *in vivo*.The inhibition effect has been shown to be related to their ability to down-regulate the matrix metalloproteases (MMPs), namely, MMP-2 and MMP-9, as well as urokinase-plasminogen activator (uPA) and uPA receptor (uPAR) expression. In addition, phenolic compounds possess antiangiogenesis effects**(Mojzis et al., 2008**), which is an important aspect in the inhibition of tumor growth, invasion and metastasis. It has been reported that phenolic compounds such as ellagic acids, EGCG, genistein and anthocyanin-rich berry extracts inhibit tumor angiogenesis through down-regulation of vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR-2), platelet-derived growth factor (PDGF), PDGF receptor (PDGFR), hypoxia-inducible factor 1α (HIF-1α) and MMPs, as well as inhibition of phosphorylation of EGFR, VEGFR and PDGFR**(Ramos, S., 2008).**Alkaloids have been found to be potent inhibitors of singlet oxygen species (O_2), superoxide radical scavenger O_2^- and lipid peroxidation inhibitors. Alkaloid have been shown to OH^- scavenger and inhibitor for 5-lipogenase**(Nabilah et al., 2011).**The alkaloid exhibited metal chelating properties effectively reduced Fe^{3+}-induced LPO and blocked the process at its initial stage**(Neganova et al., 2010).**

Conclusion:-

Ethanol extract of combination of *Punica grantum* Lwith *Zizipus mauritiana* exhibited significant strong *invitro* antioxidant activity, Nitric oxide, ABTS radical and super oxide scavenging activities when compared with ascorbic acid as standard also revealed significant convincing *invivo* antioxidant activity, Lipid peroxidation (LPO), Super oxide dismutase (SOD), when compared with 5-Flurouracil as standard. The results of this study show that this combination can be of use as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. In addition, present study showed that ethanol extract of combination of *Punica grantum* Lwith *Zizipus mauritiana* , ZP (E) induces the cell cytotoxicity on Hela cancer cells, likely undergo through an apoptotic pathway on the basis of increase of caspase-3 activity. The ZP(E) extract manifested significant inhibitory effect on the growth of *Allium cepa* root meristem. The cytotoxic activity raised with increases in concentration. The presence of phytoconstituents make the plant useful for treating different ailments and have a potential of providing useful drugs of human use. The present study indicates that most of the biologically active phytochemicals such as flavonoids, tannins, phenolic compounds and alkaloid were present in the ethanolic extract of *Punica grantum* L with *Zizipus mauritiana*. Since the ethanolic extract of both plants contains more constituents it can be considered beneficial for cancer therapy and further medicinal investigation.

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