Synergic Anticancer Activity of Different Extracts of Ziziphus Mauritiana and Punica Grantum L on the Inhibition of Hela Cell Proliferation and Tumor Growth in EAC Bearing Mice

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Abstract

In consideration of the synthetic chemicals chemotherapy has induced obnoxious hazards, there is a ubiquitous tend towards the natural resources, which are remedially potent and affordable for the poor people. Development of phenolic compounds or extracts has become a major area medical research due to their cancer preventive effects through induction apoptosis and prevention of oxidative stress associated cancer. The present scrutiny was designed to investigate the synergic anticancer effect of Punica grantum L and Ziziphus maritiana. The ethanol extract of combination of both plants were found to be the most effective in BSL and trypan blue assay, also stimulated caspase-8 and-9 protease by fold increase in Ac-IETD-pNA. The expression of P53 in cytoplasm and nuclei and the increase of TdT in the nuclei of carcinoma cells in mice treated while Bcl2 expression was declined. Consequently, the synergic anticancer activity of ethanol extract of combination of both plants was proved.

Index terms— anticancer activity, apoptosis, carcinoma cells, Punica grantum L, Ziziphus maritiana.

1 I. Introduction

ancer is a multi-process disease integrating environmental, chemical, physical, metabolic, and genetic factors which play a direct and/or indirect role in the induction and retrogression of cancers. Strong and consistent epidemiology declaration implies a diet with high consumption of antioxidant rich fruits and vegetables significantly reduces the risk of many cancers, suggesting that certain dietary antioxidants could be effective agents for the precaution of cancer incidence and mortality. These agents present in the diet are a very promising group of compounds because of their safety, low toxicity, and general acceptance (Fresco et al., 2006). Accordingly, in the last few years, the identification and development of such agents has become a major area of experimental cancer research. Phenolic compounds constitute one of the most numerous and ubiquitous group of plant metabolites, and are an intrinsic part of the human diet. It was found that besides their primary antioxidant activity, this group of compounds exhibit a wide variety of biological functions which are mainly related to modulation of carcinogenesis. Various in vitro and in vivo systems have been exerted to determine the anticarcinogenic and anticancer potential of these natural phenolic compounds or extracts. For example, invitro study of berry extracts and isolated polyphenols from strawberry including anthocyanins, kaempferol, quercetin, esters of coumaric acid and ellagic acid, were exhibited to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT-116) and prostate (LNCaP, DU-145) tumor cell lines in a dosedependent manner with different sensitivity between cell lines (Zhang et al., 2008;Seeram et al., 2006). Similar results have also been recited in several cell system with wine extracts and isolated polyphenols (resveratrol, quercetin, catechin, and epicatechin) (Damianaki et al., 2000;Kampa et al., 2000), tea extract and major green tea polyphenols (epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-gallate) (Zhang et al., 1999;Weisburg et al., 2004;Nichenametla et al., 2006), despite the effective concentrations depend on the system and the

tested substances. Other phenolic extracts or compounds profoundly studies are from olives, legumes, citrus, apples, and also curcumin from spice turmeric. Moreover, in vitro studies on cancer cell lines, abundant in vivo experiments have also been executed to certify the antitumor efficacy of plant foodderived phenolic extracts or compounds with tumor prevalence and multiplicity (e.g., number of tumors per animal) as endpoints (Yang et al., 2002;Lambert and Yang, 2003;Gerhauser, 2008;Thomasset et al., 2009). The animal models frequently employed are either chemically, genetically, or ultraviolet light-induced tumor, as well as xenograft models, including colon, lung, breast, liver, prostate, stomach, esophagus, small intestine, pancreas mammary gland and skin tumors. **??**Lalaet al., 2006) investigated the chemoprotective activity of anthocyanin-rich extracts (AREs) from bilberry, chokeberry, and grape in Fischer 344 male rats treated with a colon carcinogen, azoxymethane (AOM). After 14 weeks, rats on ARE diets had significantly fewer colonic aberrant crypt foci (ACF) when compared with the control group. Moreover, rats fed bilberry ARE had 70% fewer large ACF compared with rats fed the control diet, indicating significant chemoprevention. Chokeberry-fed rats had a 59% reduction in large ACF, whereas the reduction was only 27% in rats fed grape ARE. The authors wound up that AREs from bilberry, chokeberry, and grape notably prohibited ACF formation induced by AOM. One more investigation by (Ding et al., 2006), cyanidin-3-glucoside (C3G), the major anthocyanin in blackberry, was surveyed for the potential ability to inhibit 7,12-dimethylbenz[a]anthracene (DMBA)-12-Otetradecanolyphorbol-13-acetate (TPA)-induced skin papillomas in animal skin model. The results revealed that treatment of the animals with C3G (3.5 ?M, topical application, twice/week) decreased the number of tumors per mouse at all exposure times. After 20 weeks of TPA promotion, a greater than 53% inhibition of papillomagenesis by C3G was observed. 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 (which contain phenolic componds) 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.

2 II. Materials and Methods

3 a) 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 rinsed with water for the evacuation of adhering material and sun dried. Seed powders were prepared mechanically, passing through sieve # 40 and stowed in sealed container. The seed powder (1kg) was extracted in a soxhlet with hexane (4000ml) for 6h for the segregation of fatty compounds. 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.

4 b) Chemicals

Apotarget caspase colorimetric protease assay kit was purchased from Invitrogen Company. (TUNEL) assay kit and biochemical assay kits were bought from Roche Molecular Biochemicals Company and Sigma-Aldrich Company respectively. Trypan blue was obtained from Bio-tech Pvt Ltd (India), All other chemicals were analytical grade.

5 c) Tumor cells line for caspase 8 and 9 assay

The Hela cell line (Cervical cancer) was obtained from National Center for Cell Science, Pune, India. Cells were grown and maintained in DMEM medium which contained 10% fetal calf serum, penicillin (100units/ml) and streptomycin (100µg/ml)., PH 7.4. The cell culture was maintained in carbon dioxide incubator at 37 0C with 90% humidity and 5% CO 2 .

6 d) Tumor cell line for in vitro cytotoxic test and invivo

study 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 ascetic 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.

7 e) Animals

Healthy male adult Swiss albino mice: BALB/cJ, with age of 3 months and weighing 25±5 g was obtained from the Drug Control Laboratory (DCL), Bangalore. that 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.

8 f) Brine shrimp lethality (BSL) bioassay

The brine shrimp (Artemiasalina) eggs were bought from Brine Shrimp Direct, Ogaden, UT, USA. The assay was accomplished for the investigation of cytotoxicity of the selected extracts. The procedure and method was pursued as illustrated by (Meyer et al., 1982).The chamber was divided into two equal parts. Aeration was given in both the compartments. One part was lighted up with a bulb (60 W), while the other was darkened. Brine shrimp eggs were scattered in the dark side and incubated at room temperature for 48h. As the hatching occurred, the nauplii were swum towards the illuminated side, where they were compiled by a Pasteur pipette. Samples of the extracts were prepared by dissolving 5mg of extract in 5ml of DMSO to get 500 ppm stock solution and further diluted with sea water to get the required concentration (10, 50, 100 and 150 µg/ml). 5-Flourouracil (5-FU) was used as standard (1, 10, 25 and 50 µg/ml). Dried vials were taken and ten shrimps were transferred in each vial and then volume was made up to 5ml with sea water. A drop of dry yeast suspension (3mg in 5ml sea water) was added to each vial as food for shrimps. For each concentration test was done in triplicate. Control vials were prepared by adding equal volumes of distilled water. The vials were sustained under illumination. After 24h survivors were counted, by using 3× magnifying glass and the percentage of deaths (% Mortality) and IC 50 value were calculated by using Finney Computer program.

9 g) In-vitro Cytotoxicity (Trypan blue dye exclusion method)

In-vitro cytotoxic activity was accomplished using the Trypan Blue cytotoxic assay. Concisely, ethanol extract of combination of Punica grantum L and Ziziphus mauritiana was used for the preparation of the stock solution (1000 ?g/ ml) in Phosphate buffered saline. Serial dilutions (200, 20, 2 and 0.2 ?g/ml of sample solution) were prepared in PBS. Sample solutions (200?l) were placed in tubes. The volume in all the tubes was made up to 800 ?l with PBS (Phosphate buffered saline). 100?l of EAC with a concentration of 10 6 cells/ml of Phosphate buffered Saline was added to the tubes. A control having solvent alone was also prepared. Incubate at 37 0C for 3 hours and add 100 ?l of trypan blue to all test tubes. Ascitic tumour cell counts are done in a Cell Counting machine (Cedex, Roche) using the trypan blue dye exclusion method. Results were expressed as percentage cell viability (Saluja et al., 2011).

10 h) Caspase assay

Caspase activity was governed by using Apotarget caspase colorimetric protease assay sampler kit (Catalog number: KHZ1001; Invitrogen, USA). HeLa cells were treated or without ethanol extract of ZP, 5-FU for 24 h. The cells were then accumulated, rinsed in PBS and lysed in 50 µl of lysis buffer on ice for 10 min. After centrifugation, the supernatant containing 150 µg proteins were incubated with 200 µM Caspase-8 (AC-IETD-pNA) and Caspase 9 (Ac-LEHD-pNA) substrates in reaction buffer at 37 0C for 1h. The levels of released pNA were measured with microplate reader (Fluostar optima, BMG Labtech, Germany) at 405 nmwavelength. The fold-increase in caspase 8 and caspase 9 activities was determined by direct comparison to the level of the untreated control, which was considered as 1 (Kumar et al., 2014).

11 i) DNA isolation from Ehrlich Ascites Carcinoma Cells

The EAC cells assembled from treated and untreated animals were used for DNA fragmentation assay using the modified method of (Jun-yaet al., 2002). Cells were rinsed twice in 800 ?L of PBS and pelleted.

Pelleted cells were lysed in 600 ?L ofLysis buffer (10 mMTris-HCl buffer, pH 8.0, 10mM EDTA and 0.2% Triton X-100) for 10 minuteson ice. The lysate was centrifuged at 6000 rpm for20 mins. Then, the supernatant was extracted with1000 ?L of PCIAA (Phenol -chloroform -Isoamyl alcohol solution, 25:24:1). The mixturewas then centrifuged at 6000rpm for 20 mins andthe upper layer decanted off and precipitated with50 ?L of 3M NaCl and 1000 ?L of cold ethanol at -200C overnight. After drying, the isolated DNAwas dissolved in TE buffer. Contamination byRNA was removed by incubation with 40 units of RNase at 370C for 30 minutes.

12 j) DNA fragmentation assay on 2% agarose gel

Loading buffer was added, and (fragmented) DNA electrophoresed on 2% agarose gel in TBE(40 mMTris, 20 mM Boric acid, 1mM EDTA) at100 V for 45 minutes and visualized by EtBrstaining.

13 k) 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., whereas group 4, 5 and 6 (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 Biochemical parameters were estimated.

14 l) Biochemical parameters

Abundant laboratory surveys have been suggested in the appraisal of liver dysfunction. For assessment of drug induced hepatotoxicity, liver function tests were carried out and values obtained for treated groups were correlated with the normal value. From among the host of liver function tests, the following battery of blood tests namely bilirubin, SGOT, SGPT, ALP and protein levels were done. (Pain et al., 2003)

15 m) Histological of plasma and immunohistochemical examination

The immunohistochemical investigations were done by staining with haematoxylin and eosin. Sections mounted onto positive charged slides were applied to distinguish the Bcl-2 and p53 reactivity or apoptotic cells by using the TUNEL assay (Gao and Zhou, 2005). Streptavidinbiotin or avidin-biotin peroxidase (ABC/ HRP) was used and bound antibody complex was visualized by the reaction of 3, 3'-diaminobenzidine (DAB) substrate and counter stained with haematoxylin.

16 n) Statistical analysis

The data were expressed as mean ± S.E.M (n=3 for invitro study n=6 for invivo). 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 $p < 0.05$.

17 III. Results

18 a) Brine shrimp lethality bioassay

Ethanol extract of combination of Punica grantum L and Ziziphus Mauritiana was found the most effective with lesslethal concentration LC 50 . 5-FU showed IC 50 : 1.98 µg/ml as standard drug.

19 ZP (E): Ethanol extracts of combination of

20 d) DNA fragmentation

Figure 4 revealed the fragmentation of DNA extracted from the ethanol extract of combination of Punica grantum L and Ziziphus mauritiana treated cells in agarose gel electrophoresis. The DNA from the control was perceived to be complete and unbroken although the fragmentation pattern of 5-Fluorouraciltreated cells was more intense that compared to the pattern of ZP (E)-treated cells.

21 e) Biochemical parameters

There were significant increase in the level of ALP (Alkaline phosphatase) and also in level of both transaminases SGPT (Serum glutamic pyruvic transaminase) and SGOT (Serum glutamic oxaloacetic transaminase) in EAC untreated group while their values stayed in the normal range in ZP (E), (100mg/kg) treated group, redolent of its efficiency with less toxic effect compared with the standard as seen in Figes 5-7 respectively. The levels of protein andbilirubin didn't differ significantly from normal in ZP (E) treated group which were depicted in Figs 8 and 9 accordingly.

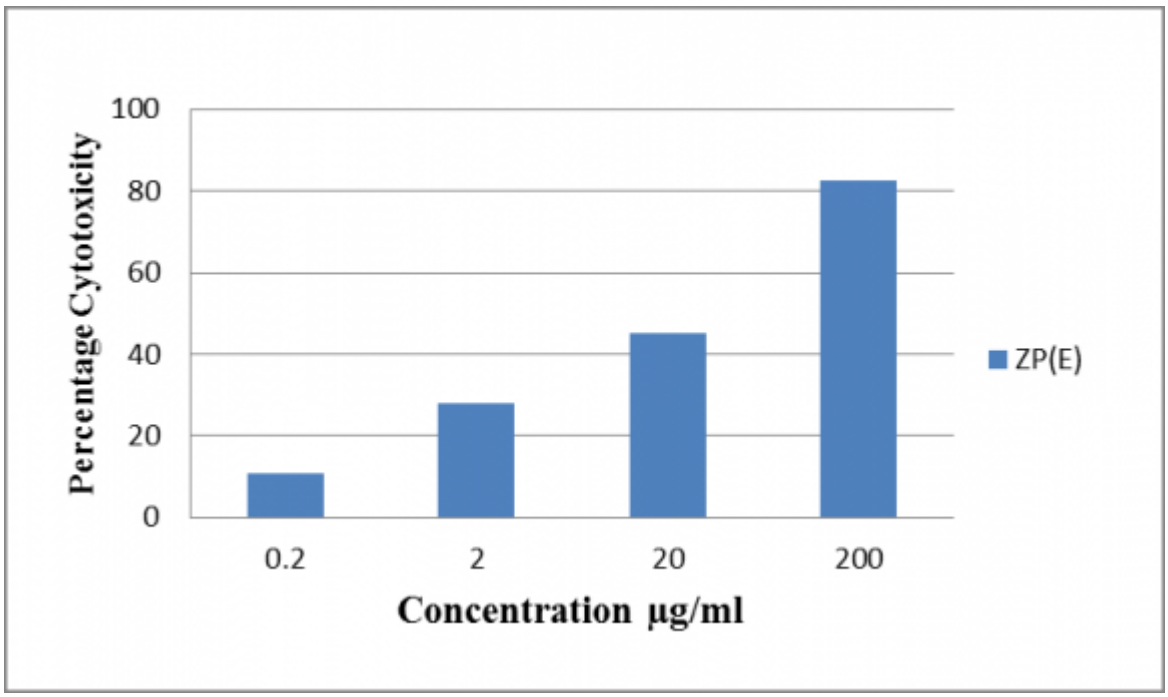
22 f) Histological and immunohistochemical effects in EAC bearing mice

Figure 10 (A, C and E) showed the highly proliferated rate, abundant basophilic and dark stained cytoplasm and moderate sized nuclei of EAC cells of control mice. Many dividing cells are depicted in (Figure 10 E). The number of EAC cells were decreased and also some cells were degraded due to the treatment with ethanol extract of combination of Punica grantum L and Ziziphus mauritiana at dose of 100mg/kg (Figure 10D). Figure 10 (B, D, F) illustrated phenotypic apoptotic signs including irregular shape, cell shrinkage, plasma membrane blebbing, cytoplasmic azurophic lytic vesicles, fragmenting nuclei and apoptotic bodies. Figure 11 A signified the decreasing in density of Bcl-2 in cytoplasm of EAC cells treated with ZP (E) extract as compared with control. EAC treated with ZP (E) revealed considerably upgrading of p53 in cytoplasm and nuclei and TdT in nuclei compared with untreated control mice (Figure **??**2

23 IV. Discussion

The advanced stage of cancer can be controlled by synthetic compounds, but almost all of these compounds display normal tissue toxicity with unenviable side effects. Consequently, the research for safe compounds that

may have anticancer efficiencies should be evaluated. Different extract of combination of Punica grantum L and Ziziphus Mauritiana as natural product was tried out to assess biochemical parameters as consequence of getting better result from ethanol extract of combination of both plant, this extract was surveyed for caspase activity, cytotoxic activity and histological and immunohistochemical investigation. There are significant ascents in the level of ALP, SGPT and SGOT in case of liver disease in cancer. The ethanol extracts of combination of Punicagrantum L and Ziziphus Mauritiana (100mg/kg) remained the level of these parameters in the normal range. Total plasma proteins provide most convenient data in chronic liver diseases. Liver is the site of ?, ? globulins and albumin synthesis. Advanced liver diseases are characterised by decline in level of albumin and rising in level of globulin. Serum bilirubin is also exhibitive of hepatotoxicity. In the case of ethanol extracts of combination of both plants treated mice, the protein levels didn't alter significantly. The total bilirubin levels were also in normal range. In preliminary cytotoxic screening, considerable cell death was observed in the brine shrimp lethality bioassay particularly, ethanol extract of combination of both plants ZP (E) which showed promising activity. Brine shrimp lethality bioassay gave preliminary information about toxic nature of compound in rapidly multiplying cells and supported its cytotoxic nature. (Silva et al., 2007;Genupur et al., 2006). To confirm it's cytotoxic activity, study was further carried out in Ehrlich Ascites Carcinoma (EAC) cells. Ethanol extract of combination of PunicagrantumL and Ziziphus mauritiana caused significant cytotoxicity in vitro EAC cell. The results of the in-vitro cytotoxicity assay revealed that ethanol extract of combination of Punica grantum L and Ziziphus mauritiana is toxic to the EAC cells as there was an increase in the number of cells stained with trypan blue dye, with an increase in the concentration of ZP (E). This cytotoxic activity might be due to mechanisms other than direct catalytic affects 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 tumour cells (Berenbaum, 1970). In the present study, revealed that after treatment with ethanol extract of combination of both plants ZP (E), the caspase-8 and 9 activity were increased and also histological study of stained EAC cells section from EAC bearing mice showed evidence of apoptosis such as shrinkage, plasma membrane blebbing, apoptotic bodies, fragmentation of nuclei and cytoplasmic azurophilic lytic vesicles. To prove this clarification based on morphologic, the mediator such as p53, Bcl-2 and TdT which involved in the molecular mechanism of apoptosis were detected by immunohistochemical methods. Presently, it well perceived that apoptosis is a form of cell death characterized by active suicide of cells. Hence, the study of this process is a profitable in cancer therapy (Sobenin et al., 1998) by elimination of seriously damaged cells or tumor cells by chemoperventive or chemotherapeutic agents (Galati et al., 2000;Thompson, 1995). The cells have undergone apoptosis which have frequently shown chromatin condensation and DNA fragmentation. They are immediately recognized by macrophages before cell lysis and then can be evacuated without inflammation. Hence, apoptosis inducing compounds are anticipated to be ideal anticancer drugs. Phenolic compound (phenolics acids, flavonoids, tannins) 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, 2007).Pervious phytochemical analysis have revealed presence of bioactive phenolic compounds in both plants (Agata et al., 2009;Sharrif and Hamed, 2012). Apoptosis relies on the balance between pro-apoptotic protein for example P21, P53, Bax, Bid, Back, etc., and antiapoptotic protein such as Bcl-2, Bcl-xl and survivin. Change in ratio of proapoptotic protein/antiapoptotic protein stimulates apoptosis process, so increase in level of pro-apoptotic proteins or decrease in level of anti-apoptotic proteins which lead to initiate the apoptosis process. (Bhattacharyya et al., 2003;Chinni et al., 2001;Giannakakou et al., 2001;Ahmed et al., 2009). There are two distinct but interconnected pathways in the regulation of apoptosis process, viz., death receptor pathway (Extrinsic) and mitochondriamediated pathway (Intrinsic). Caspase-8 is frequently related with the inception of the death receptor pathways which are independent to mitochondria pathways whereas caspase-9 is associated in the intrinsic pathways (Chen and wang, 2002; Hengartner, 2000). Apoptosis can be induced through the activation of death receptors including: Fas, TNF? R, DR3, DR4 and DR5 by their ligands such as FasL, TNF?, APO-3L/ TWEAK and APO-2L/TRAIL respectively. Activated caspase-8 can stimulate apoptosis via two parallel cascades. Firstly, it can directly cleave and activate caspase -3 which induce apoptosis. Secondly, it can cleave pro-apoptotic proteins such as P53 and translocate its fraction to mitochondria, releasing cytochrome-c which sequentially activates caspase-9 and caspase-3 and initiates apoptosis certainly (Fuchs and Steller, 2000). The key element of the intrinsic pathways is the releasing of cytochrome-c from A cell undergoing apoptosis would illustrate the following morphological changes: cell shrinkage, membrane blebbing, chromatin condensation and finally DNA fragmentation. DNA fragmentation assay showed that the ethanol extract of combination of both plants ZP (E) induced ladder-like DNA fragmentation which is characteristic of DNA damage. Most anticancer drugs of plant origin or synthetic have been known to cause DNA damage or suppress its replication, not necessarily killing the cells directly but promoting apoptosis. During apoptosis, a specific nuclease (now known as caspase-activated DNase or CAD and pre-existed in living cells as an inactive complex) breaks the genomic DNA between nucleosomes and generates DNA fragments. This ladder has been used extensively as a marker in studies on apoptotic cell death (Wyllie, 1980;Nagata, 2000). Consequently, based on the present data, the



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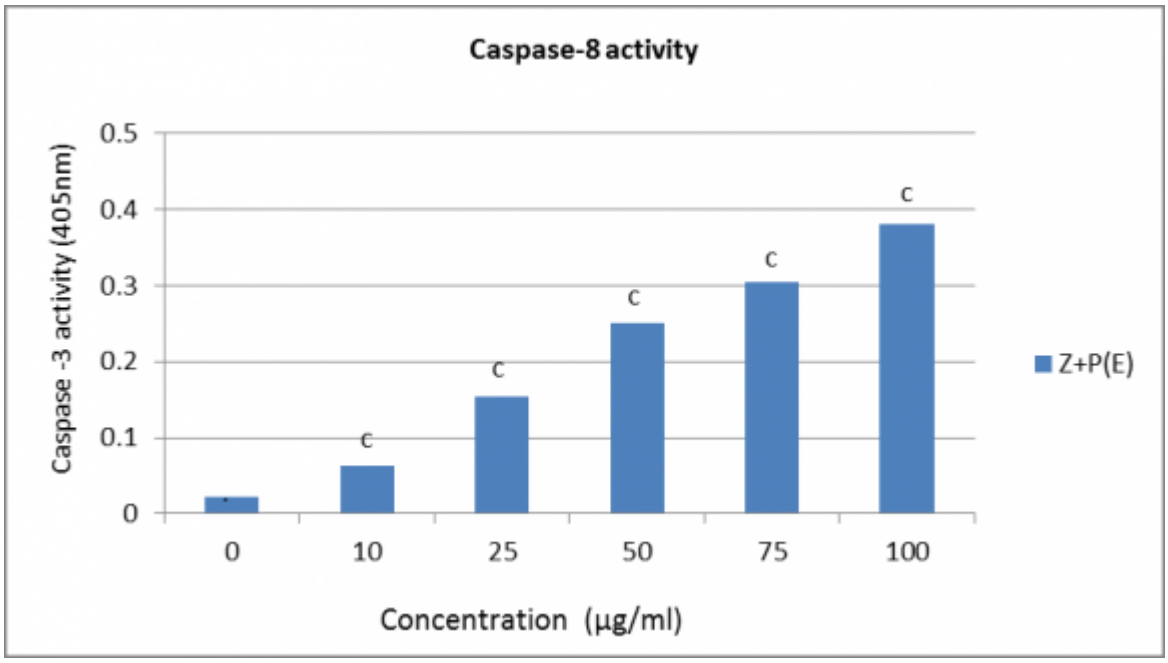

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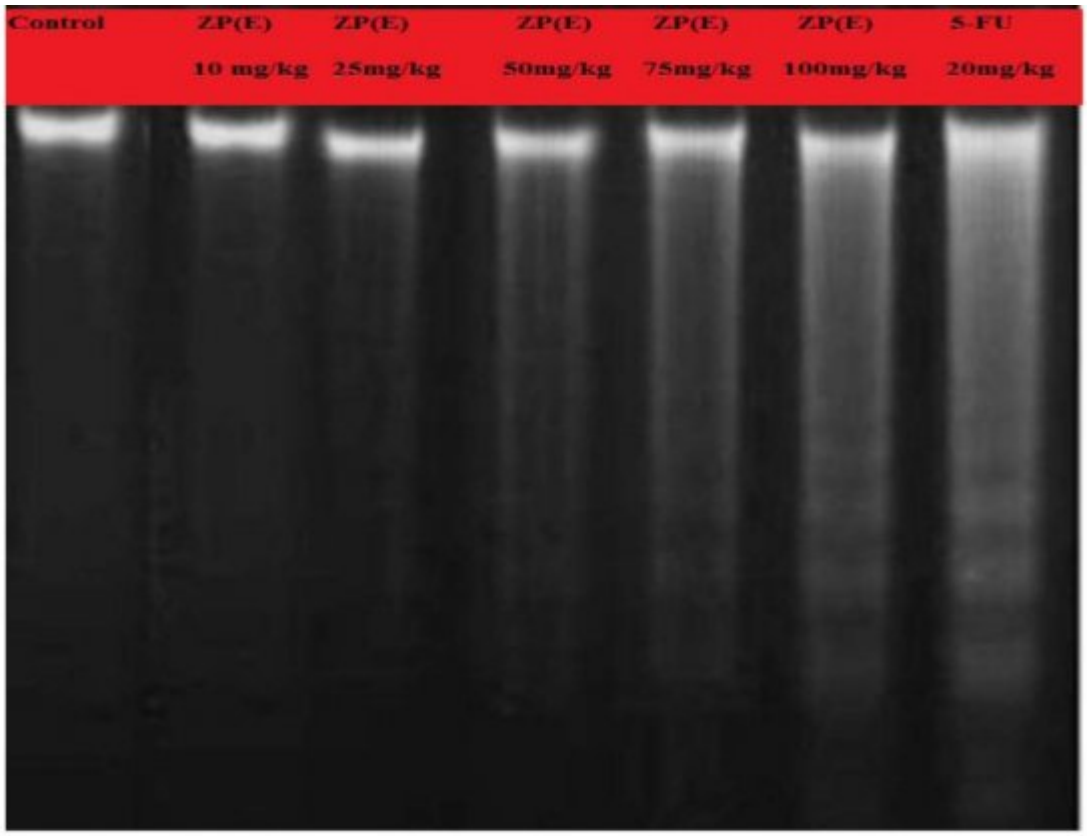
Control
ZP(E)
10 mg/kg
ZP(E)
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75mg/kg
ZP(E)
100mg/kg
5-FU
20mg/kg

Figure 3:

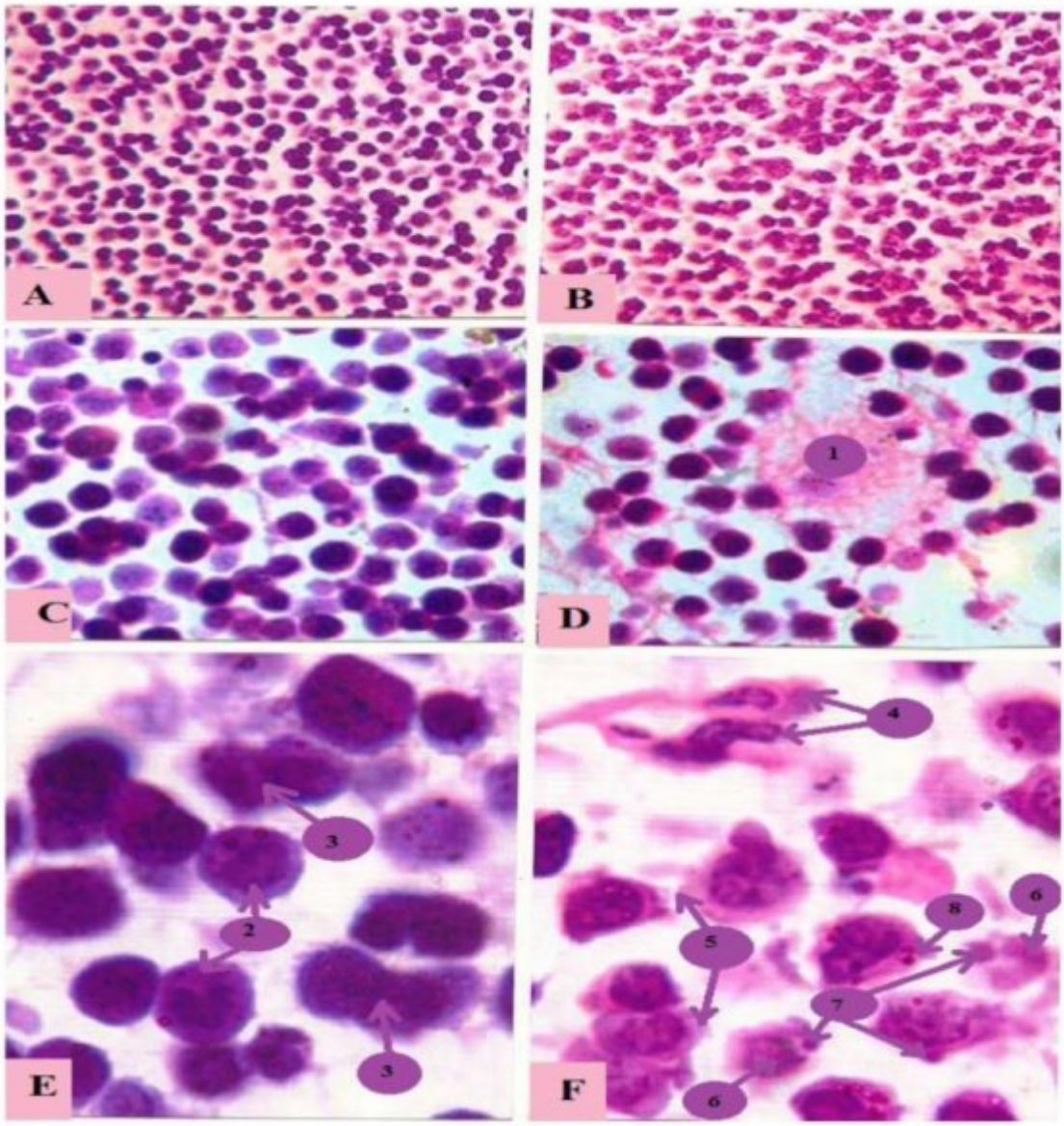

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Figure 4: Figure 1 :Figure 2 :Figure 3 :

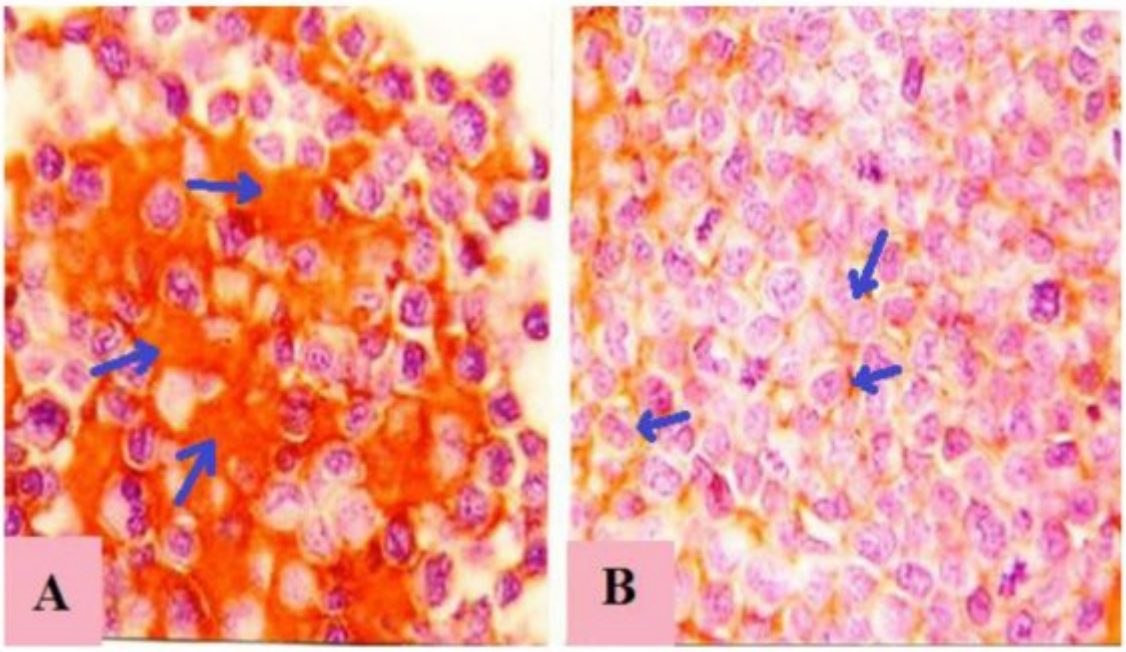

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Figure 5: Figure 4 :

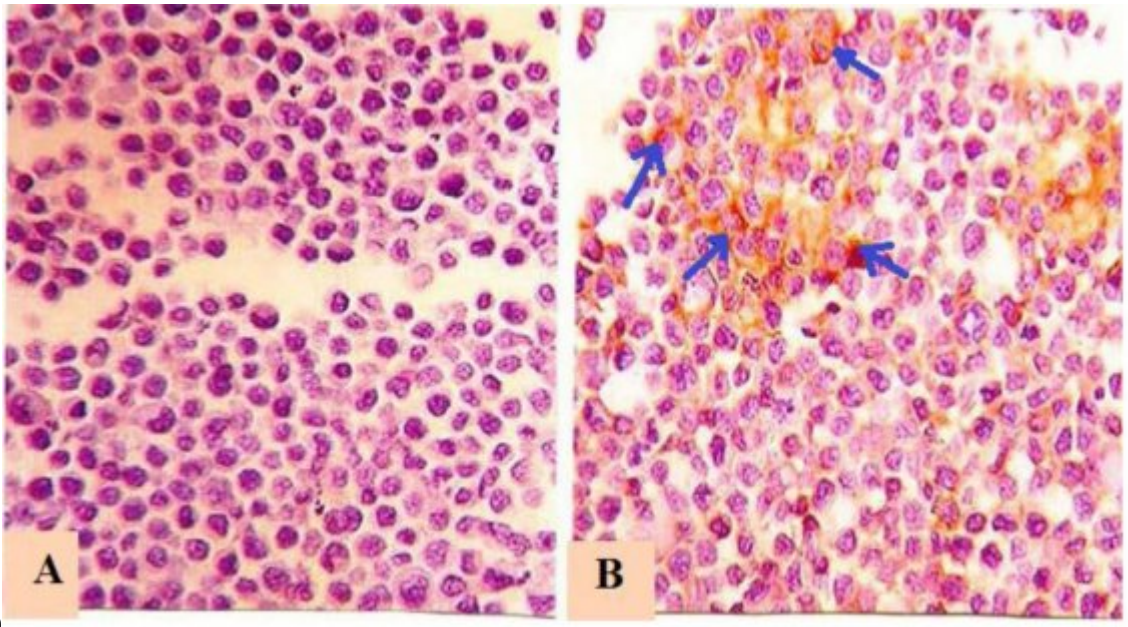

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Figure 6: Figure 8 :Figure 9 :

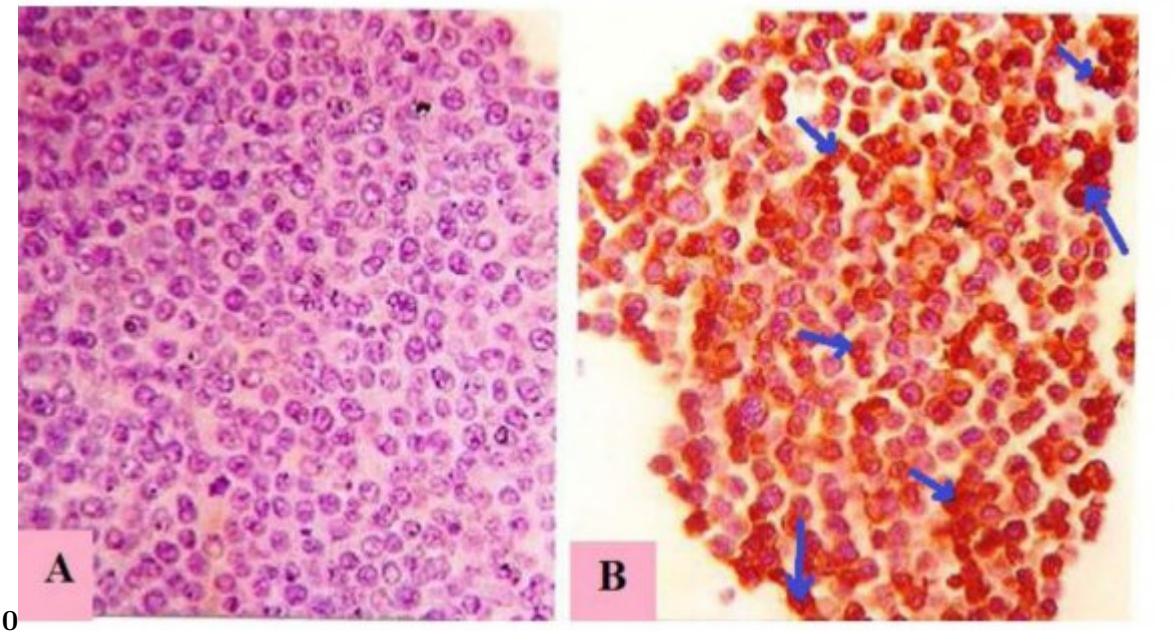

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Figure 7: Figure 10 :

1

EXTRACT	10 (µg/ml)	% Mortality after 24 h 50 (µg/ml)	100 (µg/ml)	150 (µg/ml)	LC 50 (µg/ml)
Control	0.00	0.00	0.00	0.00	–
ZP (E)	80.65	92.21	98.66	99.83	2.03
ZP (Aq)	64.08	83.34	97.32	98.22	7.83
ZP (Ch)	12.32	26.00	35.32	45.98	378.43

Figure 8: Table 1 :

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Figure 9: Figure 5 :

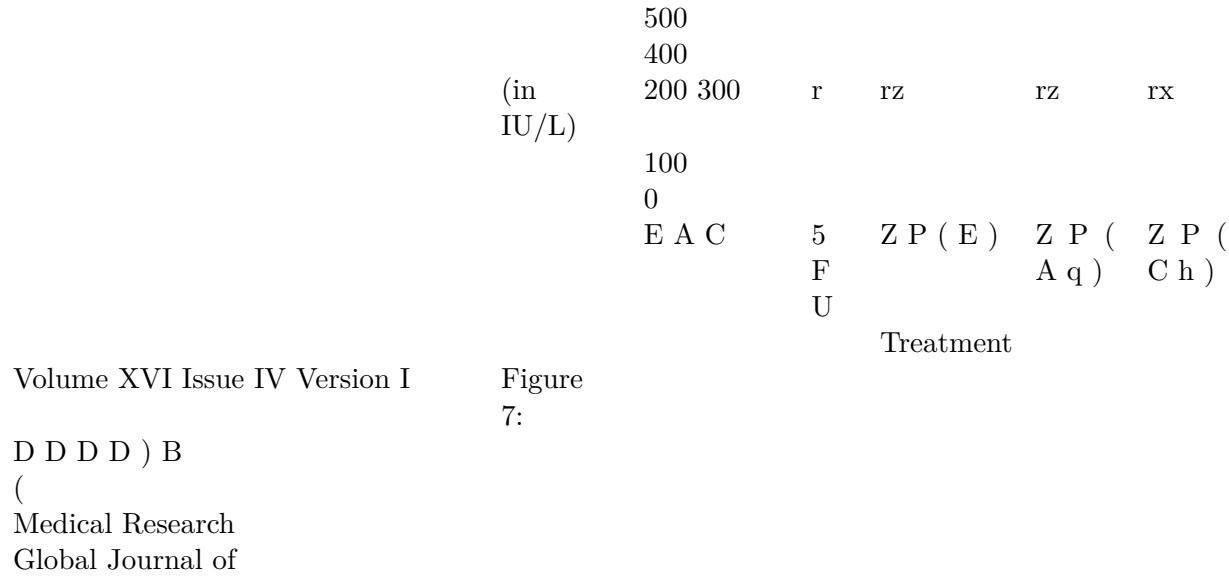

Figure 7:

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Figure 10: Figure 6 :

synergic anticancer activity of ethanol extract of Punica grantum L and Ziziphus mauritiana was verified, so it can be considered beneficial for cancer therapy and further medicinal investigation. [1]

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