Anticancer potential of aqueous ethanol seed extract of *Ziziphus mauritiana* against

cancer cell lines and Ehrlich ascites carcinoma

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Abstract

Ziziphus mauritiana (Lamk.) is a fruit tree that has folkloric implications against many ailments and diseases. In the present study, anticancer potential of seed extract of *Ziziphus mauritiana in vitro* against different cell lines (HL-60, Molt-4, HeLa and normal cell line HGF) by MTT assay as well as *in vivo* against Ehrich ascites carcinoma bearing Swiss albino mice was investigated. The extract was found to markedly inhibit the proliferation of HL-60 cells. Annexin and PI binding of treated HL-60 cells indicated apoptosis induction by extract in a dose dependent manner. The cell cycle analysis revealed a prominent increase in sub Go population at concentration of 20 µg/ml and above. Agarose gel electrophoresis confirmed DNA fragmentation in HL-60 cells after 3 h incubation with extract. The extract also exhibited potent anticancer potential *in vivo*. Treatment of Ehrlich ascites carcinoma bearing Swiss albino mice with varied doses (100-800 mg/kg b.wt.) of plant extract significantly reduced tumor volume and viable tumor cell count, and improved haemoglobin content, RBC count, mean survival time, tumor inhibition and percentage life span. The enhanced antioxidant status in extract treated animals was evident from decline in levels of lipid peroxidation and increased levels of glutathione, catalase and superoxide dismutase.

Key Words: *Ziziphus mauritiana*, anticancer, HL-60, apoptosis, Ehrlich ascites carcinoma

Introduction

The plant derived compounds have always been an important source of medicines for various diseases, and have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (1). During the last few years, novel chemopreventive agents of natural origin have been targeted with fruits and vegetables being a key interest due to high content of bioactive compounds (2). Cancer is the second leading cause of death all over the world (3). According to World health organization, more than 10 million new cases of cancer are diagnosed every year, and the statistical trends indicate that this number would double by 2020 (4). Cancer is the uncontrolled growth and spread of abnormal cells, associated with dysregulation of apoptosis, a programmed cell death. Most of the current anti-cancer drugs are derived from plant sources, which act through different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity.

Ziziphus mauritiana commonly known as Indian jujube is a fruit tree belonging to family Rhamnaceae. Traditionally, the fruit has been used as anodyne, sedative, tonic anticancer, potent wound healer, applied on cuts and ulcers and has also been used against asthma (5, 6). The extracts from fruits (7), leaves (8, 9) and seeds (10) of *Ziziphus mauritiana* have been reported to exhibit antioxidant activity, whereas bark (11, 12) and pulp (13) are reported to possess cytotoxicity against different cancer cell lines. Keeping above in view, the present study was aimed at investigating the effect of aqueous-ethanolic seed extract of *Ziziphus mauritiana* against different cancer cell lines *in vitro* and against Ehrlich ascites carcinoma *in vivo*.

Materials and Methods

Chemicals and Reagents

3-(4,5-dimethylthiazole-2-yl)-2,5–diphenyltetrazolium bromide (MTT), RPMI-1640, L-glutamine, penicillin, streptomycin, HEPES, 2-mercaptoethanol, propidium iodine (PI), DNase free RNase, proteinase K, 2-Deoxy-D-ribose, ascorbic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma chemical Co, St. Louis, USA. Fetal

Bovine serum (FBS) was purchased from GIBCO Invitrogen Corporation, USA. Annexin V-FITC apoptosis detection kit and Cycle test[TM] were purchased from BD Bioscience USA. Electrophoresis apparatus and reagents were procured from Bio-rad, USA. Tri-chloro acetic acid (TCA), Thiobarbituric acid (TBA) and pyragallol were purchased from Merck, USA. All other chemicals used were of analytical grade, available locally.

Plant source, extract preparation and standardization (ZMS)

Fruits of *Ziziphus mauritiana* (Lamk.), variety Umran were collected from Botanical Gardens of Punjabi University, Patiala, Punjab, India and authenticated by Prof. R.C. Gupta, Botany Department, Punjabi University, Patiala, Punjab, India. Plant sample has been kept in Voucher specimen DOB (305) PUP at Punjabi University, Patiala. Aqueous-ethanolic seed extract of *Ziziphus mauritiana* (ZMS) was prepared and standardized as described earlier (14).

Anticancer activity of ZMS *in vitro*

Cell lines, growth conditions and treatment

Human promyelocytic leukemia cells (HL-60), acute lymphoblastic leukemia cells (Molt-4), human cervical cancer cells (HeLa) cells and normal human gingival fibroblast cells (HGF) were obtained from National Cancer Institute (NCI), Bethesda, USA.

The cells were grown in RPMI-1640 medium, supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES 15 mM, L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), 2-mercaptoethanol (50 μM), NaHCO$_3$ (0.37%) and incubated at 37°C in an atmosphere of 95% air and 5% CO$_2$, with 98% humidity. ZMS was dissolved in 30% dimethyl sulfoxide (DMSO) and delivered to cell culture in complete medium while the controls received only DMSO (<0.2 %, v/v).

Cell proliferation assay

Cell proliferation was assessed according to the method of Bhushan *et al.* (15). Briefly, 200 μl of medium containing 2.5 x 10^4 cells obtained from different cell lines (HL-60, Molt-4, HeLa cells and HGF) were seeded in each well of 96-well microtiter plates. Cells were incubated with different concentrations of ZMS for 48 hrs at 37°C, 5% CO$_2$ with 98%

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humidity. The medium was replaced with fresh medium containing 100 μg/ml of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hrs. The supernatant was aspirated and MTT-formazan crystals were dissolved in 100 µl DMSO. Absorbance was measured at 570 nm. Cell growth was calculated by comparing the absorbance of treated and untreated cells. Cell line showing maximum sensitivity was subjected to further study.

Flow cytometric analysis of apoptosis and necrosis

Flow cytometric analysis of apoptosis and necrosis was done by method of Del Bino *et al*. (16). HL-60 cells treated with ZMS were washed twice with phosphate buffered saline (PBS) and then resuspended in 100 µl of binding buffer provided with apoptosis detection kit (BD Pharmingin). Cells were stained with annexin-V FITC antibody and PI as per instructions provided by the manufacturer and scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fractions of cell population in different quadrants were analyzed using quadrant statistics. Cells in the lower right quadrant represented apoptosis and in the upper right represented post-apoptotic necrosis.

DNA content and cell cycle phase distribution

Cell cycle phase distribution was done by the method of Del Bino *et al*. (16). Cells were treated with different concentrations of ZMS for 24 hrs and collected at 160 × g for 5 min in 5 ml polystyrene tubes. Cells were washed once with PBS and fixed in 70% ethanol overnight at 4°C. Cells were again washed with PBS, resuspended in 250 µl PBS, incubated with RNase at 37°C and stained with propidium iodine using procedures and reagents as described in the instruction manual. The preparations were analyzed for DNA content using BD-LSR flow cytometer. Data was collected in list mode on 10,000 events for FL2-A versus FL2-W.

DNA fragmentation

DNA fragmentation was assessed by electrophoresis of extracted genomic DNA from HL-60 cells as described by Muller *et al.* (17) with slight modifications. Briefly, 2×10^6 cells were incubated with different concentrations of ZMS for 24 hrs and washed with PBS containing 10 mM EDTA. The pellet was lysed in 250 µl of lysis buffer (100 mM NaCl,

5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 5% Triton X-100, 0.25% SDS), containing 400 µg/ml DNase-free RNase and incubated at 37°C for 90 min followed by 1 hr incubation with proteinase-K (200 µg/ml) at 50°C. DNA was extracted with 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) for 1 min and centrifuged at 13000 × g for 3 min. The aqueous phase was further extracted with chloroform and centrifuged. DNA was precipitated from aqueous phase with 3 volumes of chilled alcohol containing 0.3 M sodium acetate at 4°C overnight. The precipitate was centrifuged at 13000 × g for 10 min. DNA pellet was washed with 80% alcohol, dried, dissolved in 50 µl TE buffer and electrophoresed in 1.8% agarose gel at 50 V, stained with ethidium bromide and visualized in Bio-rad Gel Documentation System.

For the time dependent study, 2×10^6 cells were incubated with specified concentrations of ZMS for 24 hrs and studied at different time intervals.

Anticancer activity of ZMS *in vivo*

Animals

Male Swiss albino mice (18-22 g) maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India), having free access to tap water, were employed in the present study. The animals were housed in the departmental animal house and exposed to 12 hr cycle of light and dark. Experimental protocol was approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No- 107/ 1999/ CPCSEA).

Acute toxicity

The acute toxicity of *Ziziphus mauritiana* seed extract was assessed as described earlier (18).

Experimental design

Ehrlich Ascites Carcinoma (EAC) cells maintained in the peritoneal cavity of Swiss albino mice (male) were collected from an animal having 8-10 days old ascitic tumor by aspirating the ascitic fluid in sterile isotonic saline. The viable EAC cells were counted

(trypan blue indicator) under microscope. Male Swiss albino mice weighing 18-22 g were injected with 1×10^7 EAC cells intraperitoneally on day 0. A day of incubation was allowed for multiplication of the cells. On day 1, the animals were randomized and divided in seven groups (n=10), viz.: Group I, vehicle (Distilled water, i.p); Group II, Control 5-fluorouracil (20 mg/kg b.wt.); Group III, extract (100 mg/kg b.wt.); Group IV, extract (200 mg/kg b.wt.); Group V, extract (400 mg/kg b.wt.); Group VI, extract (800 mg/kg b.wt.); Group VII, Control $(1 \times 10^7$ EAC cells). Test groups were treated with plant extract prepared in distilled water intraperitoneally at the interval of 24 hrs from day 1-9. Positive control group was treated with 5-fluorouracil (commercial anticancer drug) from day 1-9, parallel control group was treated with distilled water only. The animals were sacrificed on day 13 by cervical dislocation and tumor volume, tumor cell count, hematological parameters and antioxidant status were assessed. The second set of animals was continued with the similar design so as to observe their life span.

Tumor growth response

The antitumor effect of ZMS was assessed by change in body weight, ascites tumor volume, viable tumor cell count, percentage tumor inhibition, mean survival time and percentage increased life span (19, 20).

The tumor growth inhibition was calculated as follows:

% tumor inhibition= 1-Av. No. of cells in test group/ Av. No. of cells in control group × 100

The effect of ZMS on tumor growth was monitored by recording the mortality for the period of 40 days and percentage increase in life span was calculated as:

% ILS= [(Mean survival time of treated group/Mean survival time of control group)-1] × 100

Mean Survival = (Day of 1st death + Day of last death)/2

Body weight of the animals was recorded both in treated and control groups at the beginning of the experiment and subsequently on every 5th day.

Hematological studies

Blood was collected from retero oribital plexus of animals and used for the estimation of hemoglobin (Hb) content, red blood cell count (RBC) and white blood cell count (WBC) (21) and animals were sacrificed by cervical dislocation.

Biochemical assays

The liver of animals was excised, rinsed in ice-cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% (w/v) homogenates was prepared in 0.15 M Tris-HCl buffer and was used for the estimation of lipid peroxidation (LPO) (22) and glutathione content (GSH) (23). Rest of the homogenate was centrifuged at 2500 × g for 15 min at 4°C and superoxide dismutase (SOD) (24), catalase (CAT) (25) and total protein (26) was estimated in the supernatant.

Statistical analysis

All the results of *in vivo* experiment were expressed as Mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA followed by Tukey's multiple range test, applied for *post-hoc* analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

Results

Inhibition of cell proliferation in various cell lines by ZMS

In order to determine the anticancer potency of extract *in vitro*, ZMS was tested for cell growth inhibition in different cancer cell lines at various concentrations for 48 hrs. The extract did not show any significant inhibitory effect against normal cell line HGF (Normal human gingival fibroblast cell line). Inhibitory effect against HL-60, Hela and Molt-4 was observed with IC50 value at 20 μg/ml, 40 μg/ml and 40 μg/ml, respectively. Of the tested cell lines, maximum growth inhibition was observed in HL-60 cells as shown in Fig. 1, and thus HL-60 was selected for further study.

Flow cytometric analysis of apoptosis/necrosis induced by ZMS in HL-60 cells

In order to determine whether ZMS-induced cytotoxicity is due to apoptosis or necrosis, HL-60 cells were incubated with different concentrations of ZMS for 12 hrs, and the percentage of cells undergoing apoptosis or necrosis was determined by staining with annexin

V-FITC and PI (Fig. 2). Cells in the lower right quadrant indicated apoptosis while in the upper right quadrant represented post-apoptotic necrotic population. ZMS at 5 µg/ml and 80 µg/ml induced about 18.8% and 61.2% apoptosis. The increase in annexin V-FITC/PI positive cell population suggests that ZMS is a potent inducer of apoptosis and triggers events leading to apoptotic cell death.

Cell cycle analysis by ZMS treatment

For the confirmation of apoptotic cell death induced by ZMS in HL-60 cells, the cells were treated with ZMS for 24 hrs. The cells exhibited concentration dependent increase in hypo diploid sub-Go/G1 DNA fraction (<2nDNA) (Fig. 3). The sub-Go/G1 fraction was <1% in control cells (untreated HL-60 cells) which increased with increasing concentration of ZMS and reached to 64% at concentration of 80 µg/ml.

DNA fragmentation

DNA fragmentation, a characteristic feature of apoptosis, was assessed by ladder formation on agrose gel electrophoresis. HL-60 cells treated with extract showed apoptosis in a concentration dependent manner as evidenced by the formation of internucleosomal DNA fragments (Fig. 4 a). Apoptosis induction in cells started at 20 µg/ml and this concentration was further selected for time dependent study. The extract was found to induce apoptosis in cells after 3 hrs incubation as shown in Fig. 4 b.

Survival time and tumor growth response

Antitumor activity of ZMS against EAC tumor bearing mice is shown in Table 1. The tumor volume and viable cell count significantly increased in EAC control animals. Administration of ZMS (100, 200, 400 and 800 mg/kg b.wt.) to tumor bearing mice significantly ($p<0.001$) decreased tumor volume and viable cell count and enhanced the mean survival time in a dose dependent manner. Tumor growth inhibition upto $75.42 \pm 0.96\%$ was observed at 800 mg/kg b.wt. with 70% increase in life span of test animals.

EAC bearing mice showed rise in body weight, while the extract treatment inhibited the rise in body weight in the pattern similar to the standard drug (Fig. 5).

Hematological parameters

Hematological parameters of tumor bearing animals on day 13 were found to be significantly altered as compared to normal group (Table 1). Hemoglobin content and RBC count in EAC control group decreased while WBC count increased as compared to normal group. Treatment with ZMS at various doses increased hemoglobin content and RBC count and reduced the WBC count towards normalcy.

Lipid peroxidation and glutathione

Increase in malondialdehyde (MDA) levels is a measure of lipid peroxidation. Fig. 6 depicts the level of lipid peroxidation in terms of MDA in liver tissue of experimental animals. It was observed that MDA levels increased by 60.6% in EAC control group as compared to normal control group ($p<0.001$). After administration of ZMS at different doses (100, 200, 400 and 800 mg/kg) to EAC bearing mice, the level of lipid peroxidation reduced by 12.22%, 21.83%, 31.44% and 43.23% respectively, as compared to EAC control group ($p<0.05$).

Fig. 7 illustrates the effect of ZMS on reduced glutathione in EAC bearing mice. Tumor induction by inoculation of EAC drastically decreased the GSH content by 70% ($p<0.001$) in control group. The administration of ZMS at 100, 200, 400 and 800 mg/kg to EAC bearing animals increased the GSH content by 21.97%, 30.39%, 37.71% and 41.32% respectively, as compared to untreated EAC control group ($p<0.05$).

Antioxidant enzymes

ZMS significantly restored the antioxidant status of tumor bearing mice. The effect of administration of ZMS to EAC bearing animals at 100, 200, 400 and 800 mg/kg efficiently enhanced the superoxide dismutase activity by 11.01%, 24.62%, 31.52% and 47.12% respectively, as compared to EAC control group (Fig. 8).

Catalase activity was evaluated in terms of H_2O_2 reduced/min/mg of protein. The catalase level in EAC bearing mice was found to decrease by 57.80% as compared to normal control group ($p<0.001$). However, treatment of EAC bearing mice with different doses of ZMS enhanced the catalase activity in a dose dependent manner (Fig. 9.) At the highest dose

of 800 mg/kg, rise in catalase activity was found to be 44.31% as compared to EAC control group (p<0.05).

Discussion

One of the goals of anticancer potential of any drug/extract is the induction of apoptosis in cancer cells (27). Apoptosis or programmed cell death is one of the most important targets for cancer treatment comprising chemotherapy as well as chemoprevention. It is characterized by membrane blebbing, cytoplasmic condensation, formation of apoptotic bodies, DNA fragmentation, alteration in membrane symmetry, activation of cascade of caspases and loss of mitochondrial membrane potential (28). In the present study, cytotoxic potential of ZMS was assessed by MTT assay against cancer cell lines (HL-60, Molt-4, HeLa). Assay is based upon reduction of yellow tetrazolium salt (MTT) by metabolically active cells to a dark blue formazan (29), has been employed by many workers to measure cytotoxicity to cells (30, 31). ZMS showed significant cytotoxic effect against different cell lines with minimum IC50 value of 20 μg/ml against HL-60 cells and no significant cytotoxicity to normal cell line was observed. The criteria of cytotoxicity activity for crude extracts established by American National Cancer Institute is an IC50 value of < 30 μg/ml in preliminary assay (32), which was observed only against HL-60 cells and hence further investigations were carried out using HL-60 cell line. To investigate the mechanism of cell death induced by ZMS in HL-60 cells, flow cytometric analysis was done by PI and annexin V-FITC labelling to confirm apoptosis/necrosis as a marker to assess apoptosis (33). During apoptosis, a number of changes occur in cell surface markers that show affinity for PI and annexin V-FITC labelling (34). The results of the present study indicated an increase in apoptotic population induced by the extract in a concentration dependent manner. Earlier Malik *et al*. (35) reported withaferin A as apoptosis inducer utilizing flow cytometric analysis. Another important characteristic of apoptosis induction is DNA fragmentation and measurement of DNA content makes it possible to identify apoptotic cells. To recognize the cell cycle phase specificity and to quantify apoptosis, propidium iodide (PI) dye binds to DNA in cells at all stages of the cell cycle, and the intensity with which a cell nucleus emits

fluorescent light is directly proportional to its DNA content. The increase in hypo diploid sub Go population in our results indicate the induction of apoptosis, as sub Go peak is reported to be a quantitative indicator of apoptosis (16). Uvaretin, Isouvaretin and Diuvaretin are known triterpenoid established as apoptosis inducer using the same parameters (36). DNA fragmentation was confirmed by agarose gel electrophoresis. The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments, which is regarded as the hallmark of apoptosis (37).

Plant extract at various doses was tested *in vivo* against Ehrlich ascite carcinoma induced tumor in Swiss albino mice. Ascite fluid is the direct nutritional source for tumor cells and a rapid increase in ascite fluid with tumor growth would be a mean to meet the nutritional requirement of tumor cells (38). Treatment with ZMS inhibited tumor volume and viable cell count and also increased the life span of tumor bearing mice. Prolongation of life span of animal has been well documented as criteria for judging the drug activity (39). Myelosuppression and anaemia are the major problems of cancer chemotherapy (40). Treatment with ZMS reverted back the Hb content, RBC and WBC count towards normalcy showing its protective action on hematopoetic system. Our results are in corroboration with the findings of Gupta *et al*. (41) who reported a similar effect of *Indigofera aspalathoides* against Ehrlich ascites carcinoma.

Excessive production of free radicals results into oxidative stress which leads to damage of macromolecules like lipids and induces lipid peroxidation (42, 43). Malondialdehyde (end product of lipid peroxidation) has been reported to increase in carcinomous tissues than non-diseased organs (44). Glutathione, a potent inhibitor of neoplastic process is found particularly in liver and known to exhibit protective function against free radicals (42). In the present study, ZMS treatment reduced lipid peroxidation and enhanced glutathione content in tumor bearing animals. Superoxide dismutase and catalase are free radical scavenging enzymes present in all oxygen metabolizing cells and provide defence against potentially damaging entities of superoxide and hydrogen peroxide. Inhibition of both the enzymes as a result of tumor growth observed in the present study is in

corroboration with earlier reports (45). Administration of ZMS at different doses increased the SOD and CAT levels in a dose dependent manner indicating the protective function of ZMS. In our previous study (14), phytochemical analysis of the extract showed the presence of betulinic acid which is a known anticancer agent (11) and the antitumor and apoptosis inducing property of extract may be in part due to the presence of betulinic acid. It is well established that betulinic acid activates apoptosis independent of the death receptors (CD95/Fas), but induces the successive activation of caspase 9 and caspase 3 (46, 47). The phytochemical analysis of the extract in our previous study has also revealed the presence of alkaloids and flavonoids, which could also be expected to be responsible for its bioactivity. Although at this stage the exact mechanism underlying apoptosis induction by extract is difficult to predict, but it could be due to activation of mitochondrial pathway or its effect on proteosome or other gene and transcription factors. The hypothetical mechanism is outlined in Fig. 10.

In conclusion, the present study highlights the antitumor and cytotoxic potential of seed extract of *Ziziphus mauritiana*. Further studies to characterize the active principles and elucidate the mechanism of action are in progress.

Acknowledgement

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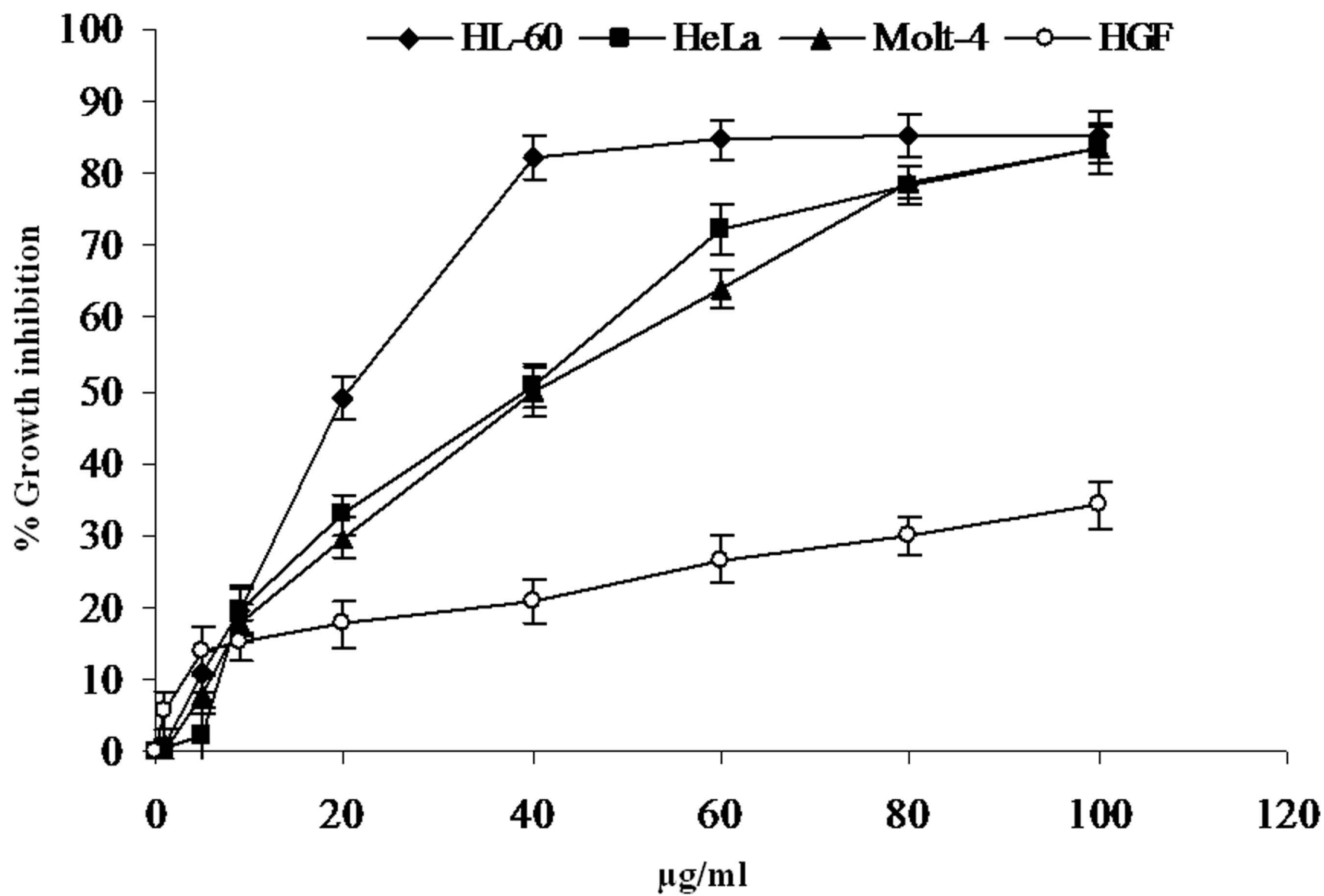
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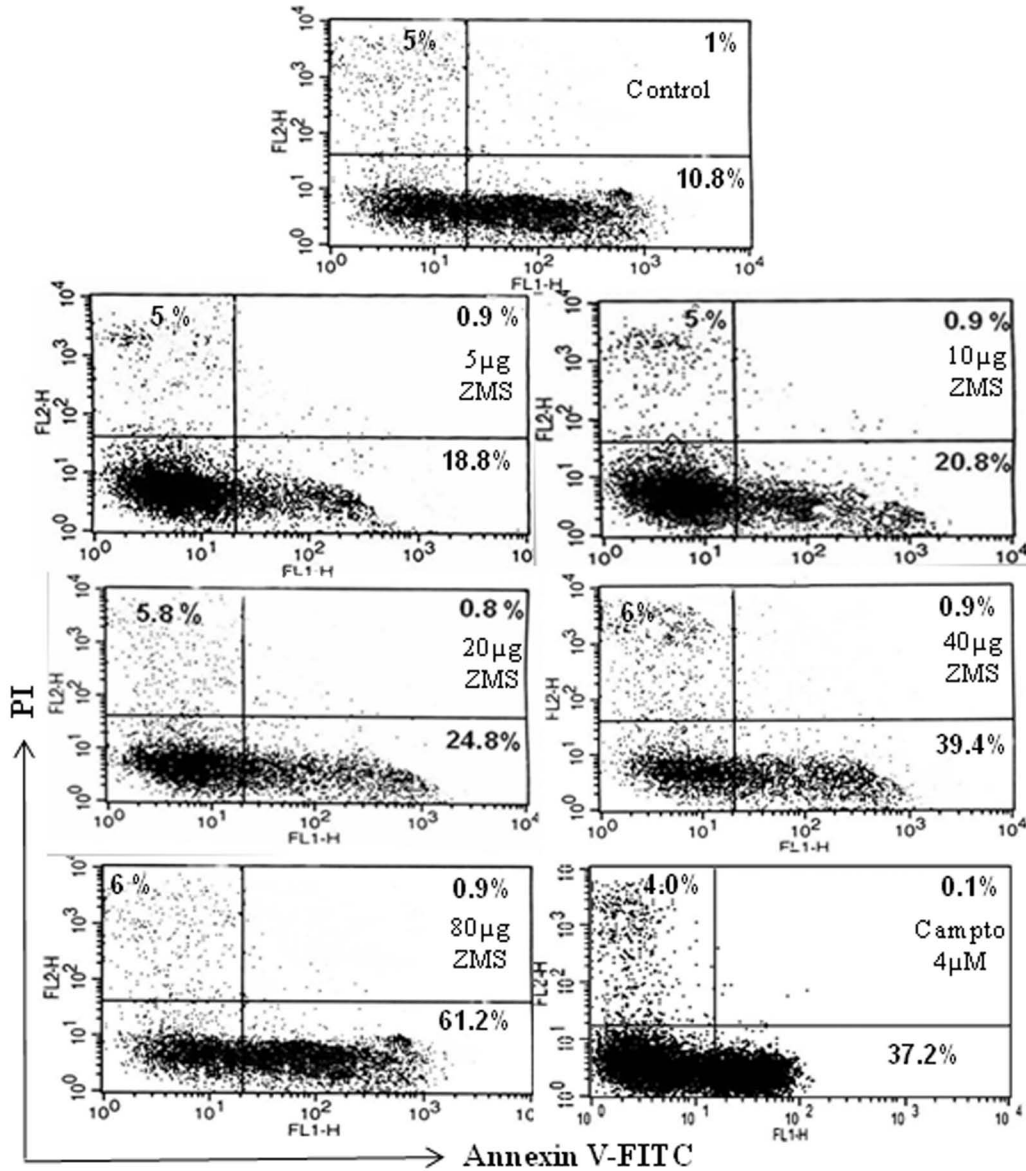
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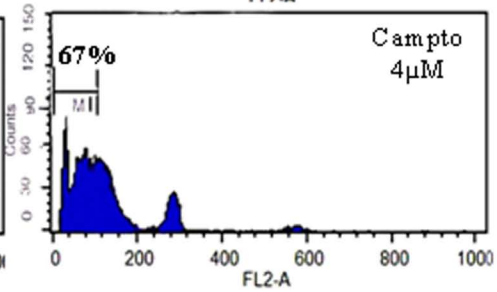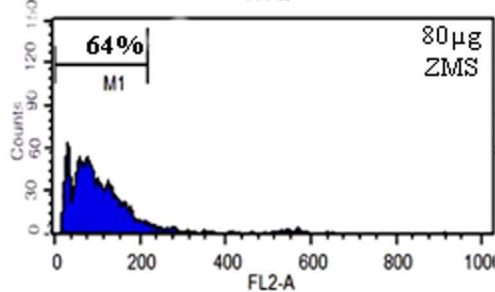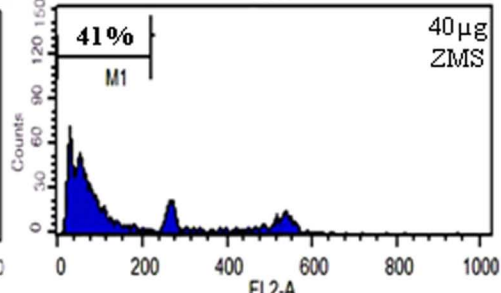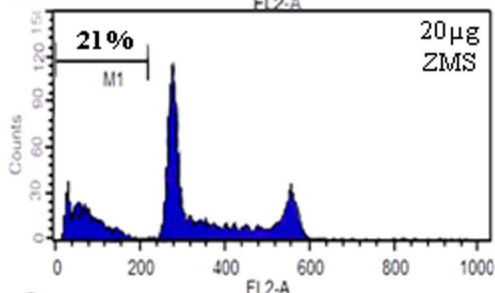
Table 1. Effect of ZMS treatment on EAC bearing mice.

Parameters	Control	EAC	EAC + 5FU	EAC + ZMS	EAC + ZMS	EAC + ZMS	EAC + ZMS
			Treatment of different groups				
		Dose (mg/kg b.wt.)	**20**	**100**	**200**	**400**	**800**
Hb content (mg/dl)	13 ± 0.5	9.5 ± 0.73^a	13.1 ± 0.4^b	11.24 ± 0.67^b	11.59 ± 0.6^b	12.4 ± 0.81^b	12.2 ± 0.45^b
WBC (cells/ml $\times 10^6$)	7.17 ± 0.11	15.6 ± 0.64^a	7.73 ± 0.68^b	12.1 ± 0.75^b	9.4 ± 0.83^b	9.2 ± 1.26^b	8.5 ± 0.40^b
RBC (cells/ml $\times 10^6$)	5.33 ± 0.45	3.5 ± 0.41^a	5.2 ± 0.11^b	4.2 ± 0.55^b	4.3 ± 0.35^b	4.9 ± 0.15^b	5.0 ± 0.12^b
Tumor Volume (ml)	----	8.3 ± 0.79	0.7 ± 0.20^b	7.6 ± 0.94^b	5.4 ± 0.96^b	4.5 ± 1.04^b	2.1 ± 0.51^b
Viable tumor Cell Count ($\times 10^7$)	---	283.02 ± 1.63	11.17 ± 0.96^b	186.12 ± 1.57^b	146.27 ± 1.43^b	109.57 ± 1.08^b	69.59 ± 1.62^b
Mean Survival time (Days)	----	20 ± 1.41	39 ± 0.81^b	23 ± 1.53^b	26 ± 1.73^b	30 ± 0.81^b	34 ± 1.63
Tumor inhibition (%)	---		96.11 ± 0.73	25.67 ± 1.09	39.78 ± 2.55	57.54 ± 1.03	75.42 ± 0.96
Increase in life span (%)	---		95	15	30	50	70

The results are presented as Mean ± S.E.M. (n=10). $^a p<0.001$ in comparison to normal group; $^b p<0.05$ in comparison to EAC control group
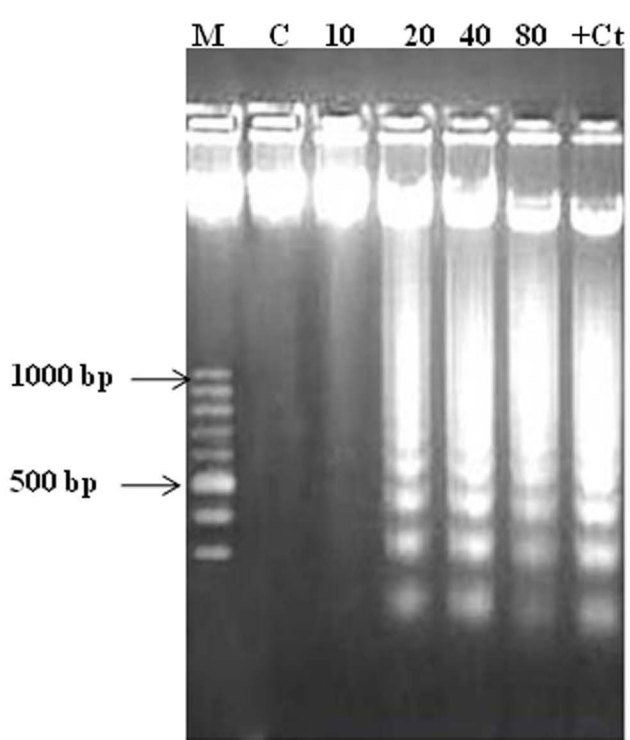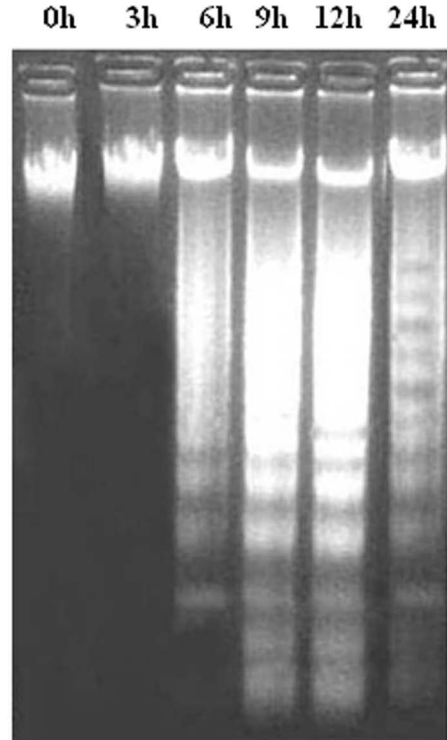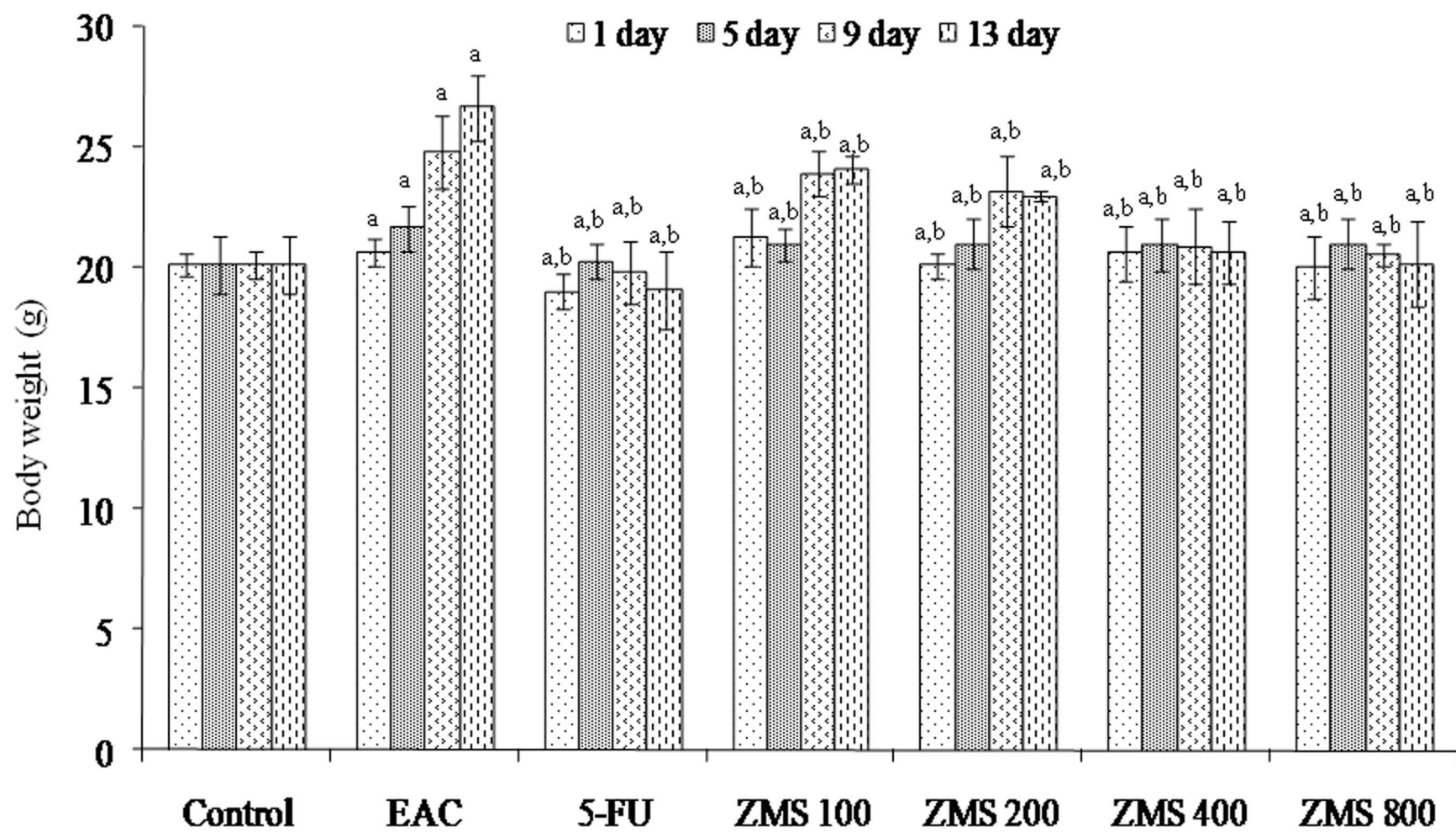
Control

5%   1%
            Control
            10.8%

5%   0.9%
            5μg
            ZMS
            18.8%

5%   0.9%
            10μg
            ZMS
            20.8%

5.8%   0.8%
            20μg
            ZMS
            24.8%

6%   0.9%
            40μg
            ZMS
            39.4%

6%   0.9%
            80μg
            ZMS
            61.2%

4.0%   0.1%
            Campto
            4μM
            37.2%

Annexin V-FITC

Cell Count (vertical axis label)

DNA Content (horizontal axis label)

(a)   (b)
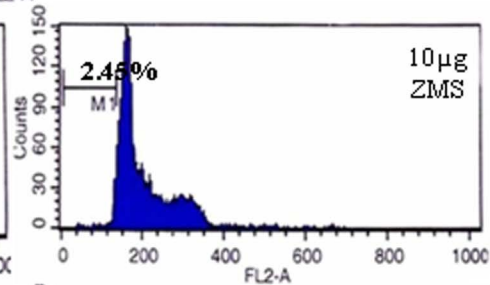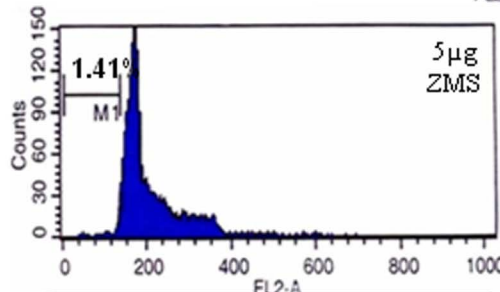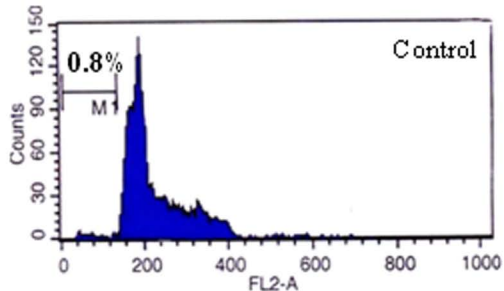
μmoles H₂O₂ reduced/min/mg protein

Legend: □ Control □ EAC □ 5-FU □ ZMS 100 □ ZMS 200 □ ZMS 400 □ ZMS 800