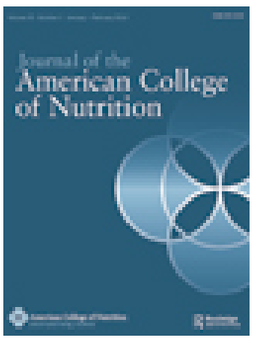

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Aqueous Extract of *Moringa oleifera* Exhibit Potential Anticancer Activity and can be Used as a Possible Cancer Therapeutic Agent: A Study Involving *In Vitro* and *In Vivo* Approach

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ABSTRACT

Objectives: New cases of cancers are increasing at an alarming rate globally. It has been hypothesized that modern cancer treatment is associated with lots of side effects and thus evoking the need to develop safer treatment measures. Thus, the present study was undertaken to evaluate the anti-carcinogenic potential of a highly nutricious plant "*Moringa oleifera*" (MO) *in vitro* and *in vivo*.

Methods: GC-MS analysis of aqueous extract of *Moringa oleifera* (AEMO) was employed to identify the bioactive compound present. Anti-tumor activity of AEMO was assessed in EAC (Ehrlich acites carcinoma) induced solid tumor bearing mice by analyzing tumor weight (TW) and Tumor volume (TV). To assess AEMO induced cytotoxicity, EAC and HEp-2 (Human laryngeal carcinoma) cells were treated with AEMO (0.05, 0.1, 0.25, 0.5 and 1 mg/ml) for both 48 h and 72 h and trypan blue, MTT and LDH released assay was done. Further, cell cycle assay and apoptosis assay was done in EAC cells to understand the mechanism of AEMO induced tumor regression.

Results: GC-MS analysis revealed the presence of quinic acid, octadecanoic acid, hexadecanoic acid (palmitic acid), α-tocopherol (Vitamin-E) and ɤ-sitosterol as major bioactive compounds. AEMO administration reduced the TV and TW of tumor-bearing mice and increases the life span. Side effect analysis showed that AEMO treatment did not induce significant alterations of liver and kidney function and hematological parameters. Further, *in vitro* cytotoxicity assays revealed that AEMO treatment induced dose and time-dependent toxicity in both the cell lines tested. Flow cytometric analysis confirmed significant induction of apoptotic cells by changing the mitochondrial membrane potential in EAC cell line.

Conclusion: The results of the present study suggest that AEMO has immense potential to inhibit the tumor progression without affecting the normal physiology and functioning of the body and thus can be used as a cancer therapeutic agent.

Abbreviations: EAC: Ehrlich ascites carcinoma; AEMO: Aqueous extract of *Moringa oleifera*; TV: Tumor volume; TW: Tumor weight; BW: Body weight; MO: *Moringa oleifera*; MMC: Mitomycin C; MEM: Minimum essential medium; FBS: Fetal bovine serum; PBS: Phosphate buffer saline; DMSO: Dimethyl sulphoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; DMS: Degree minute second; LDH: Lactate dehydrogenase; FITC: Fluorescein isothiocyanate; PI: Propidium iodide, JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide; ILS: Increase in life span; AST: Aspertate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cells; RBC: Red blood cells; PS: Phosphotidyle serine; GC-MS: Gas chromatography mass spectrophotometry; Apaf-1: Apoptotic protease activating factor 1; TRAIL: TNF-related apoptosis-inducing ligand; TRAMP: Transgenic adenocarcinoma of the mouse prostate

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Moringa oleifera; cytotoxicity; apoptosis; solid tumor; Ehrlich ascites carcinoma

Introduction

Cancer is a cluster of disease characterized by uncontrolled cellular proliferation with the potential to spread/invade in different parts of the body. And it is considered as the most leading cause of morbidity and mortality in many high-income countries as well as in developing nations (1). According to the most recent report using GLOBOCAN 2018 estimates, 18.1 million new cancer cases and 9.6 million deaths were reported in 2018 (2). Chemotherapy, surgery, radiation therapy, targeted therapy, adjuvant therapy, and immunotherapy are modern ways of treating cancer (3, 4). But these interventions failed to show anticipated results and produce adverse side effects like nausea, vomiting, fever, tumor site pain etc. (5, 6). Therefore, search for new and effective novel phytochemicals with lower toxicity and side effects are in constant demand.

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The efficacy of plants toward cancer treatment is attributed to the bioactive compounds present in plants. Scientific reports indicate that bioactive compounds have immense potential to block or suppress cancer (7). *Moringa oleifera* (MO) is one of the most cultivated angiospermic plants belonging to the family Moringaceae. The plant has nutritional values as it contains high amount of proteins, vitamins and minerals. Different parts of *Moringa oleifera* have been traditionally used by Indians and Africans in the treatment of more than 300 ailments including cancer (8). The plant possess various bioactive compounds like flavonoids, phenolics, vitamin C (ascorbic acid), α-tocopherol, β-carotenoids, etc. (9–13). The antioxidant, antidiabetic or anti-inflammatory potential of *Moringa oleifera* can be attributed to the bioactive compounds present in it (14–17).

We observed that AEMO administration significantly reduced the growth of EAC induced solid tumor as compared to tumor control and Mitomycin C (MMC). MMC, in our study, is considered as a positive control. It binds to DNA molecule by alkylation and thus inhibiting DNA synthesis. However, other modes of action like redox cycling and rRNA inhibition that may also contribute to the biological action of the drug. Apart from its anti-tumor activity, MMC has lots of side effects like myelosuppression, thrombocytopenia, mild to infrequent anorexia, vomiting and diarrhea, nausea etc. Thus, screening of plants for its anticancer activity is very much beneficial as they contain excellent bioactive components with less side effects.

Studies on the anticancer properties of *Moringa oleifera* are inadequate and mostly carried out in cell lines (18, 19). In the present study, we have also reported that AEMO causes cytotoxicity in HEp-2 and EAC cells. HEp-2 is a human laryngeal carcinoma cells and EAC is an undifferentiated mouse breast adenocarcinoma. EAC cells have high transplantable ability, rapid proliferation rate and 100% malignancy (20). To report a novel drug or compound for its anticancer potential, its testing in cell lines only is insufficient and should surmount preclinical and clinical trials. Therefore, the present study was carried out to evaluate the anticancer activity of *Moringa oleifera* in EAC and HEp-2 cells *in vitro* and in mice allograft model *in vivo*. Based on the above studies, it is expected that *Moringa oleifera* should induce cytotoxicity, apoptosis in EAC and HEp-2 and therefore act as a potent antitumor agent in mice test system."

Materials and methods

Chemicals and reagents

All the chemicals used in the present study were of analytical grade. MTT (CAS No. 298-93-1) was purchased from E. Merck (India) Limited. Mitomycin C (MMC), Minimum essential medium (MEM), Fetal bovine serum (FBS), Trypan blue, Hematoxylin, Eosin (2% w/v), Phosphate buffer saline (PBS), Dimethyl sulphoxide (DMSO), Formaldehyde solution and Triton X-100 were purchased from Himedia Laboratories Pvt. Ltd.

Collection and identification of plant specimen

The fresh healthy leaves of *Moringa oleifera* Lam. were collected directly from the plants of Southern Assam, India. Geographically, the latitude of the plant collection area is 24°41′29N and longitude is 92°44′39E on degree minute second (DMS) unit. The herbarium was prepared and the plant species was identified by the taxonomist from Botanical Survey of India, Shillong, Meghalaya, India having the reference number BSI/ERC/Tech/2017/616.

Preparation of aqueous extracts of Moringa oleifera (AEMO)

The collected plant materials were washed two times with double distilled water, air-dried and ground into a coarse powder. The chemical ingredients were extracted using a Soxhlet apparatus (BOROSIL, India) at 37 ± 1°C. Briefly, 30 g of ground powder was taken in an extractor with 200 ml of double distilled water. The extraction process was continued up to 6–8 h. The soluble extract was filtered using Whatman filter paper No. 1 until clear and lyophilized. The lyophilized AEMO extract was dissolved in doubled distilled water prior to use. The extract thus obtained was stored in a refrigerator at −20°C and were used within one month. The percentage of yield of AEMO was 17.8%.

Gas chromatography and mass spectrometry (GC-MS) analysis

GC-MS analysis of AEMO was employed to identify the chemical composition of AEMO. The extraction process of AEMO for GC-MS analysis was same as described earlier. The GC-MS analysis was performed using GC-MS QP2010 at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi. The GC-MS ultra system attached with an Elite 5MS (5% diphenyl in diethyl polysiloxane) fused to a capillary column. The inert gas helium (99.99%) was used as a carrier gas at the constant flow rate of 1.21 ml/min in split mode. The injector and detector temperature were adjusted to 260°C and 270°C respectively. The column oven temperature was fixed to 50°C for 2 min. Thereafter, the column oven temperature was raised from 50°C to 250°C at a constant rate of 7°C min^{-1} with hold time 2 min and then from 250°C to 280°C at a constant rate of 15°C min^{-1} with hold time 16 min. The total run time was 46.56 min. The spectra obtained from the extracts in GC-MS analyses were interpreted using the database of National Institute of Standards and Technology (NIST, USA).

In vivo experiments

Animals

Balb/c animals (*Mus musculus*), weighing 25–30 g were purchased from Pasteur Institute, Shillong, Meghalaya, India and maintained in the animal house at the department. The experimental animals were kept in polypropylene cages

(TARSONS, India) provided with standard food pellets (Mice Maintenance Feed, LA-061119; Hindustan Animal Feeds, Gujarat, India) and water *ad libitum*. The mice were housed with a controlled temperature of 25 ± 5°C and photoperiod of 12 h light and 12 h dark cycles.

Ethics statement

Experimental animals were maintained in the animal house of the department as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The present study has the approval of Institutional Ethics Committee (IEC) of Assam University, Silchar having reference number: IEC/AUS/2015-030 dt-4/9/15.

Preparation of breast adenocarcinoma model in mice test system

A fixed number of viable EAC cells (1 × 106 cells) were inoculated in the peritoneal cavity of mice and allowed to multiply. After a successful growth, the tumor cells were collected, diluted in PBS and counted. Thereafter, the experimental animals were re-injected with tumor cells (1 × 106 cells/animal) in the right thigh to develop a solid tumor as per procedure of Sharma et al. (2013) (21).

Experimental design

To evaluate the antitumor activity of AEMO in the mice test system, 50 numbers of Balb/c mice (6–8 weeks; 25–30 g body weight) of both sexes were used in which 40 animals were inoculated with EAC cells to develop solid tumors. 10 animals (5 male and 5 female) without treatment were kept for control (Group 1). In the 12th day of EAC inoculation, solid tumor was observed and the EAC treated animals were randomly divided into subsequent treatment groups (n = 10). Group 2 animals received no treatment and termed as tumor control. Group 3 animals were received MMC (2 mg/kg body wt.). Experimental animals of Group 4 and 5 were received oral feeding of AEMO at a dose of 200 mg/kg body and 400 mg/kg body respectively.

Dose and treatment of MMC and AEMO for in vivo study

In a study by Ali et al. (2015), exposure to MMC with 0.5, 1 and 2 mg/kg BW per week showed significant reduction of tumor weight in EAC induced solid tumor bearing mice (22). For our study, we have selected 2 mg/kg BW per week as it showed maximum reduction of tumor weight. The dose of AEMO for *in vivo* study is selected based on the previous report of Awodele et al. (2012) (23). In their study, treatment of aqueous extract of leaves of *Moringa oleifera* upto a dose of 6400 mg/kg BW did not elicit any mortality in male wistar mice. Moreover, treatment with 250, 500 and 1500 mg/kg BW of *Moringa oleifera* extract did not showed any toxicity in mice. Thus, in the present study, we have selected 200 and 400 mg/kg BW of AEMO to evaluate its anti-tumor activity.

Evaluation of the antitumor activity of AEMO in mice

Antitumor activity of AEMO was evaluated by measuring the change in tumor volume (TV), tumor weight (TW), and body weight (BW).

Measurement of tumor volume (TV) and tumor weight (TW)

The size of developing tumor of the experimental animals except control group was measured in every alternative day by using a vernier caliper (Mitutoyo 530 Series Vernier Caliper). TV was calculated employing the equation, $V = 0.5\,ab^2$, where 'V' is the volume and 'a' and 'b' is the major and minor diameter respectively (24).

Body weight (BW) measurements

The changes in BW of the experimental animals were measured in every alternative day up to 50th day. The change of body weight was examined at 30th and 50th day for initial, final and net body weight. The net body weight and change in body weight were calculated using the equation given below-

$$\text{Net body weight (g)} = \text{Final body weight} - \text{Tumor weight}$$

$$\text{Change in body weight (g)} = \text{Net body weight} - \text{Initial weight}$$

Assessment of life span in tumor-bearing mice

To assess the effect of AEMO on survival, 40 animals of both sexes were inoculated with EAC cells left for tumor development. After 12th day of EAC inoculation, solid tumors were formed in mice. The solid tumor bearing mice were randomly divided into four groups (n = 10, 5 male and 5 female). Group 1 animals received no treatment and considered as tumor control (EAC). Group 2 animals received MMC (2 mg/kg). Group 3 (EAC + AEMO 200) and Group 4 (EAC + AEMO 400) animals received oral feeding of AEMO at a dose of 200 mg/kg BW and 400 mg/kg BW respectively till 50th day of tumor development. After completion of AEMO treatment, the survival was monitored for an entire lifetime and the death pattern was recorded. The percentage of increase in life span (ILS) as compared to the tumor control group was calculated by using the following formula,

$$\%\ \text{ILS} = \frac{\text{Mean survival time of treated animals}}{\text{Mean survival time of tumor control animals}} \times 100$$

Histological evaluation of thigh and liver tissues

Thigh and liver tissues from control, tumor control, MMC (2 mg/kg) and AEMO (200 mg·kg and 400 mg/kg) treated animals of both 30th and 50th days of experimental periods were fixed in 10% formalin and processed for histological evaluation. Briefly, fixed tissues were washed with water, dehydrated, embedded in paraffin wax and cut in 5 µm thicknesses. The sectioned tissues were rehydrated, stained

with hematoxylin and counterstained with eosin. Each stained slides were studied under a light microscope- Leica DMLS (Leica, Wetzer, Germany) at different magnification and images were captured.

Side effect analysis of AEMO in normal animals

A total of twenty Balb/c mice of both sexes having a BW of 25–30 g were randomly divided into two groups (n = 10, consisting of 5 male and 5 female in each group). Group 1 animals were considered as control and Group 2 animals were orally fed with AEMO (400 mg/kg) for a period of 14 days. To evaluate the effect of AEMO on physiological function in mice, liver, and kidney function test was performed. Levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Urea and Uric acid in the blood plasma were measured biochemically. Moreover, hematological parameters like hemoglobin content, white blood cell (WBC) and red blood cell (RBC) count was done.

In vitro experiments

Cell culture

Ehrlich ascites carcinoma (EAC) and human laryngeal carcinoma (HEp-2) cells were procured from National Center for Cell Sciences (NCCS, Pune, India) and was cultured in minimum essential medium (MEM) containing 10% Fetal bovine serum (FBS), 100 U of antibiotic-antimycotic solution (Himedia). The culture was maintained in humidified atmosphere within a CO_2 incubator at 37°C with the constant supplement of 5% CO_2.

Trypan blue dye exclusion assay

To assess the effect of AEMO on cell viability, trypan blue dye exclusion assay was performed in EAC and HEp-2 cells. Briefly, EAC cells (1×10^4 cells/ml) were seeded in six-well culture plates. After attaining approximately 70% confluence, the cultured cells were treated with increasing concentrations of AEMO (0.05, 0.1, 0.25, 0.5 and 1 mg/ml) in triplicates (n = 3) to determine IC_{50} (50% inhibitory concentration) value at 48 h and 72 h. Cells without treatment was considered as control. After treatment, cells were harvested and the numbers of the viable cell were counted at least three times per treatment using trypan blue dye under a light microscope.

MTT assay

To validate the data of trypan blue dye exclusion assay, MTT [3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide] assay was done. EAC cells (1×10^4 cells/well) were seeded in 96 well culture plates. After 60–70% confluence, increasing doses of AEMO (0.05, 0.1, 0.25, 0.5 and 1 mg/ml) was added to the culture medium in triplicates. Cells without treatment were used as control. After 48 h and 72 h exposure, the media was aspirated and fresh media was given. 20 µl of MTT (5 mg/ml) was added to each well and incubated for an additional 3 h. After that, the media was aspirated carefully and formed formazan crystals were dissolved in DMSO. The optical density (OD) was recorded at a wavelength of 570 nm using a microplate reader.

Lactate dehydrogenase (LDH) release assay

The LDH release assay was done as per the standard protocol of Korzeniewski and Callewaert (1983) (25) with modifications of Kavitha et al. (2009) (26). Briefly, 1×10^4 cells/well were seeded in 96 well culture plates. AEMO treatment was given in increasing concentration (0.05, 0.1, 0.25, 0.5 and 1 mg/ml) for 48 h and 72 h. Cells without treatment was considered as control. The level of LDH released in both culture media and cell lysate was measured at 490 nm using a microplate reader. The percentage of LDH released was calculated as LDH released in culture media/(LDH released in culture media + LDH released in cell lysate) × 100. The experiment was performed in triplicates (n = 3).

Cell cycle assay

For cell cycle assay, EAC cells (1×10^4 cells/ml) were seeded in 6 well culture plates. As the cells were attains ~70% confluence, cells were exposed to AEMO (0.05, 0.1 and 0.2 mg/ml) for 24 h and 48 h. After treatment, the cells were treated with 0.1% hypotonic citrate buffer containing 0.3 µl/ml NP40. Thereafter, the cells were stained with PI/RNase solution (BD Biosciences, Product code: 550825) for 20 min in the dark followed by flow cytometric detection of cellular DNA content (Accuri, BD Biosciences, USA). The experiment was done in triplicates (n = 3) and 15000 cells per sample was acquired.

Annexin V-FITC/PI staining

Annexin V-FITC/PI dual staining method to detect apoptosis was followed as per manufacturer instruction (BD Biosciences, USA) using FITC Annexin V Apoptosis Detection Kit (Product code: 556547). Briefly, EAC cells (1×10^4 cells/ml) were seeded in 6 well culture plates. After, ~70% confluence the cells were treated with AEMO (0.05, 0.1 and 0.2 mg/ml) for 24 h and 48 h in triplicates (n = 3). After treatment, the harvested cells were washed with cold PBS, stained with 5 µl of annexin V-FITC/PI conjugate (BD Biosciences) per 100 µl cell suspension and left for incubation (15 mins) at room temperature. After incubation 400 µl of binding buffer was added to each sample and 10,000 cells per sample were analyzed.

Mitochondrial membrane potential ($\Delta\Psi m$)

The mitochondrial membrane potential was assessed as per manufacturer instructions (BD Biosciences, USA) using BD MitoScreen (JC-1) kit (Product code: 551302). Briefly, EAC cells (1×10^4 cells/ml) were seeded in 6 well plates. After, ~70% confluence the cells were treated with AEMO (0.05, 0.1 and 0.2 mg/ml) in triplicates (n = 3) for 24 h and 48 h. After treatment, the harvested cells were centrifuged at 1500 rpm for 5 minutes. The cells were gently re-suspended and stained with JC-1 dye (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-

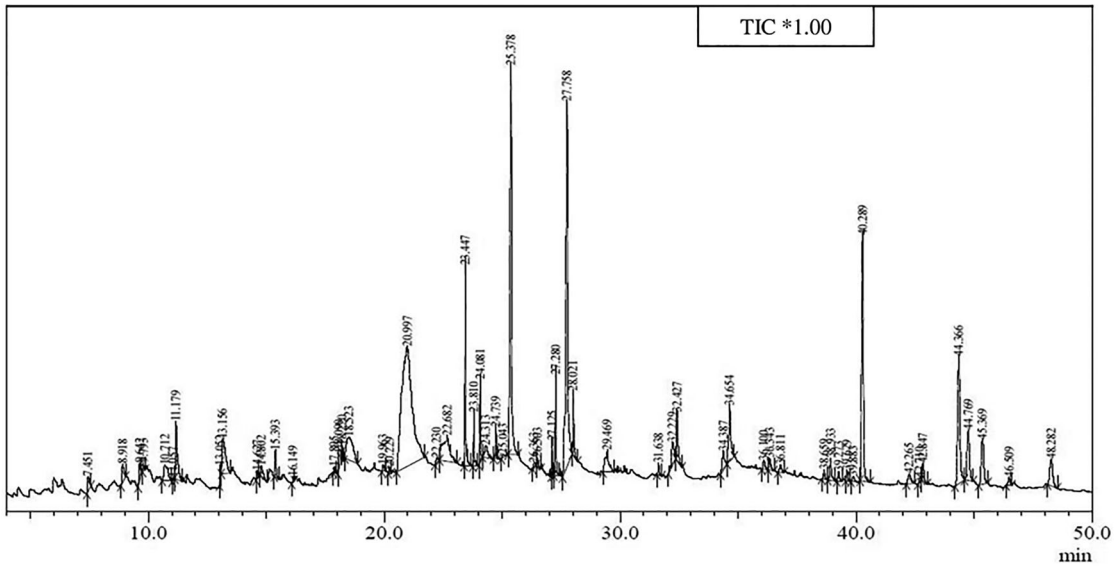


Figure 1. GC-MS chromatogram showing the presence of bioactive compounds in AEMO.

tetraethyl-benzimidazolylcarbocyanine iodide, BD, Biosciences) and incubated at 37˚C for 10–15 min. After staining, the cells were washed with 1X assay buffer (BD, Biosciences) for 2–3 times and finally 0.5 ml of 1X assay buffer was added and the cells were analyzed in a flow cytometer. At least 10,000 cells per sample were analyzed and depolarized cells were quantified.

Statistical analysis

One way analysis of variance (ANOVA) followed by Tukey post hoc analysis was performed to test the levels of significant among the groups. Values were expressed in means ± SD for control and experimental groups. Values were considered significantly different at $p < 0.05$. All the analyses were done in Graph Pad Prism software version 4.0 (San Diego California USA). The analysis of survival data was done using IBM SPSS software for Windows, version 21, Armonk, NY: IBM Corp.

Results

Gas chromatography and mass spectrometry (GC-MS) analysis revealed the presence of numerous bioactive compounds

The GC-MS analysis of AEMO showed the presence of a number of bioactive chemical compounds in the chromatogram as shown in Figure 1 and listed in Supplementary Table S1. Extracts of *Moringa oleifera* leaves mainly consisted of phenolics, hydrocarbons, fatty acids, and vitamins. Interestingly, we did not found the presence of flavonoids in the leaves of *Moringa oleifera*. Chemically, Quinic acid (1,3,4,5-Tetrahydroxy-cyclohexane carboxylic acid); 9,12,15-Octadecatrienoic acid, (Z, Z, Z)-; n-Hexadecanoic acid; α-Tocopherol and γ-Sitosterol are the major bioactive compounds present in the leaves of *Moringa oleifera*.

AEMO treatment effects on the body weight of tumor-bearing mice

Body weight of experimental animals was recorded in every alternative day and was assessed on 30th and 50th day tumor development. In the present study, at 30th day, we found a body weight gain of 13.56% (Table 1A) in the control group, not inoculated with EAC cells. The tumor-bearing animals had a body weight loss of 9.45%. Treatment of MMC and AEMO had a considerable effect on BW. We observed a BW gain of 5.91% in MMC treated animals. The BW gain in animals treated with 200 mg/kg and 400 mg/kg of AEMO was found to be 8.82% and 11.23% respectively (Table 1A). Moreover, we observed a time dependent gain in BW in tumor bearing mice treated with AEMO. At 50th day, we noticed a BW gain of 10.41% and 16.62% when the tumor-bearing mice were treated with 200 mg/kg and 400 mg/kg of AEMO respectively (Table 1B).

AEMO inhibits tumor growth in solid tumor-bearing mice

In the present study, we observed a dose and time-dependent decrease of TV in mice bearing a solid tumor and maximum reduction in TV was observed in animals treated with 400 mg/kg AEMO (Figure 2A). By the 50th day of AEMO treatment with low dose (200 mg/kg) showed 36.97% decrease ($p < 0.001$) in TV as compared to tumor control (EAC), whereas at a high dose (400 mg/kg), the reduction was 78.69% ($p < 0.001$) (Figure 2A).

AEMO increases the survival time of tumor-bearing mice

To assess the affect of AEMO on life span, the survival time of tumor bearing animals was recorded and it was calculated from the day of AEMO administration. We observed that the tumor control group survived only for a maximum of 59 days. MMC treatment had detectable effect on life span

Table 1A. Assessment of body weight (BW) and tumor weight (TW) at 30th day of AEMO administration in Balb/c mice bearing solid tumor.

	At 30th day of treatment				
Parameters	Control	Tumor control	MMC (2 mg/kg)	AEMO treated (200 mg/kg)	AEMO treated (400 mg/kg)
Initial body wt. (g)	25.81 ± 0.62	25.50 ± 0.36	25.84 ± 0.82	26.13 ± 0.74	26.19 ± 1.00
Final body wt. (g)	29.31 ± 0.86	38.60 ± 1.49***	35.72 ± 0.74***	36.31 ± 1.22***	35.57 ± 1.54***
Tumor wt. (g)	–	15.51 ± 1.31[a,b,c]	8.26 ± 0.60[a]	7.78 ± 0.32[b]	6.44 ± 0.68[c]
Net body wt. (g)	29.31 ± 0.86	23.09 ± 0.63***	27.46 ± 0.95**	28.53 ± 1.23	29.13 ± 1.72
Body wt. change (g)	+3.50	−2.41	+1.62	+2.40	+2.94
% of change of body wt. from initial wt.	+13.56%	−9.45%	+6.26%	+9.18%	+11.23%

MMC, Mitomycin C. Each value indicates the mean values of 10 experimental animals (n = 10) and expressed as mean ± SD.
**$p < 0.01$ vs control.
***$p < 0.001$ vs control.
[a,b,c]$p < 0.001$ vs tumor control. Negative and positive values of BW change indicate loss and gain of BW respectively. Body wt. change (g) and % of change of body wt. from initial wt. was calculated by taking the average value. Body wt. change (g) = Net body wt. (g) – Initial body wt. (g).

Table 1B. Assessment of body weight (BW) and tumor weight (TW) at 50th day of AEMO administration in Balb/c mice bearing solid tumor.

	At 50th day of treatment				
Parameters	Control	Tumor control	MMC (2 mg/kg)	AEMO treated (200 mg/kg)	AEMO treated (400 mg/kg)
Initial body wt. (g)	26.30 ± 0.99	27.53 ± 1.25	26.89 ± 0.82	27.68 ± 1.28	27.14 ± 1.30
Final body wt. (g)	32.26 ± 0.92	52.35 ± 2.74***	43.60 ± 1.64***	42.85 ± 1.92***	40.08 ± 2.12***
Tumor wt. (g)	–	27.91 ± 1.50[a,b,c]	14.42 ± 1.09[a]	12.45 ± 1.20[b]	8.43 ± 0.49[c]
Net body wt. (g)	32.26 ± 0.92	24.44 ± 1.51***	29.18 ± 0.77***	30.40 ± 1.07*	31.65 ± 1.77
Body wt. change (g)	+5.96	−3.09	+2.29	+2.72	+4.51
% of change of body wt. from initial wt.	+22.67%	−11.22%	+8.52%	+9.83%	+16.62%

MMC, Mitomycin C. Each value indicates the mean values of 10 experimental animals (n = 10) and expressed as mean ± SD.
***$p < 0.001$ and
*$p < 0.01$ vs control.
[a,b,c]$p < 0.001$ vs tumor control. Negative and positive values of BW change indicate loss and gain of BW respectively. Body wt. change (g) and % of change of body wt. from initial wt. was calculated by taking the average value. Body wt. change (g) = Net body wt. (g) – Initial body wt. (g).

of tumor bearing animals and MMC treated animals were survived for a maximum of 115 days. Treatment with AEMO at a dose of 200 mg/kg increased the life span of tumor-bearing mice by 259.54% and at least 40% mice were found to have survived for a maximum of 138 days. We observed a dose-dependent effect of AEMO in ILS of tumor-bearing mice. AEMO treatment (400 mg/kg) increases the survival time of tumor-bearing mice by 423.22% and 40% of animals treated with AEMO at this dose were survived up to 235 days (Figure 2B).

AEMO treatment reduced tumor weight (TW) in tumor-bearing mice

On the 30th day, the TW was found to be decreased significantly when the tumor-bearing mice were treated with 200 mg/kg and 400 mg/kg of AEMO and it was found to be 7.80 ± 0.32 ($p < 0.001$) and 6.44 ± 0.68 ($p < 0.001$) respectively as compared to tumor control group (15.51 ± 1.31) (Figure 2C). The TW of MMC treated animals was 8.26 ± 0.60 ($p < 0.001$) as compared to tumor control. Similarly, on the 50th day, TW of MMC treated animals was significantly reduced to 14.42 ± 1.09 ($p < 0.001$) as compared to tumor control. AEMO treatment with 200 mg/kg and 400 mg/kg reduced the TW in experimental animals and it was found to be 12.45 ± 1.20 ($p < 0.001$) and 8.43 ± 0.49 ($p < 0.001$) (Figure 2D) respectively.

The gross anatomical appearance of thigh, liver, and spleen of normal control and tumor-bearing mice at 30th and 50th days was represented in Figure 2E, F. A dose-dependent decrease of tumor growth (right thigh of experimental mice) was observed in AEMO treated animals. Treatment with a high dose of AEMO (400 mg/kg), especially in the 50th day showed a contrasting variation with respect to tumor development as compared to tumor control (Figure 2F). The morphological appearance of mice and its organs itself is an indicator of tumor growth reduction in AEMO treated mice, indicating the potential of AEMO toward tumor regression.

AEMO treatment led to the restoration of tissue architecture in tumor-bearing mice

Histopathological evaluation of thigh muscles and liver tissues was performed in two different time points (30th day and 50th day) in mice inoculated with or without EAC cells. Tissue architecture of control mice showed the normal structure of skeletal muscle fiber that contains multinucleated cells whose nuclei are visible in the periphery of the cell (Figure 3A (a–c), B (a–c)). The experimental animals of the positive control group showed altered tissue architecture with high nuclear staining that may resulted due to the proliferation and growth of EAC cells forming tumor masses that infiltrates to the muscle fibers (Figure 3A (d–e), B (d–f)). Treatment with MMC and AEMO showed reestablishment of abolished tissue architecture of tumor bearing animals. At 30th day, AEMO treatment (200 mg/kg body wt.) did not show marked restoration of tissue architecture (Figure 3A (j–l)). However, treatment of AEMO (400 mg/kg BW) at the 30th day showed reestablishment of tissue organization (Figure 3A (m–o)). On the other hand, when the tumor-bearing mice were treated with the same doses for a relatively longer time period, more prominent restoration of tissue

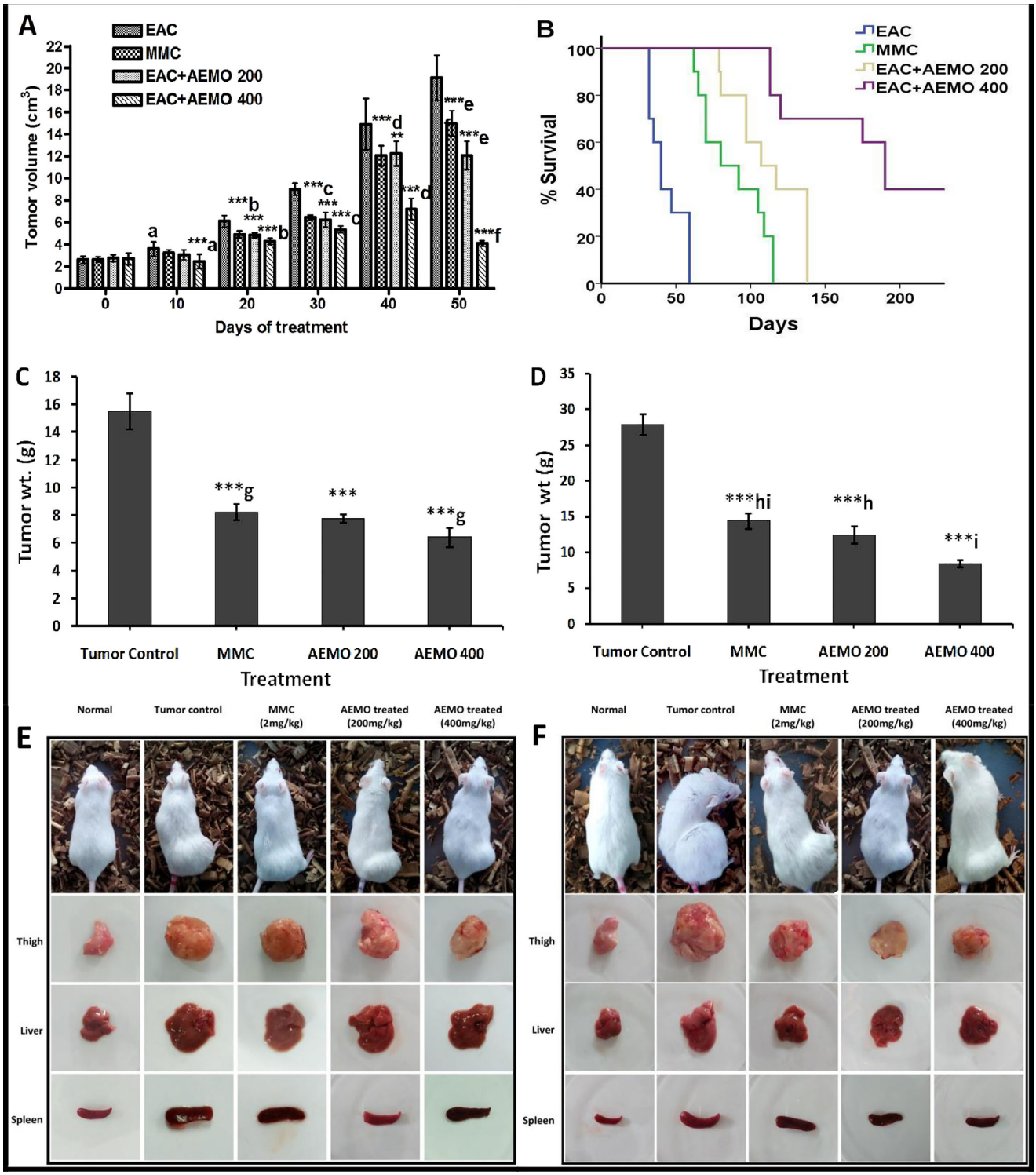


Figure 2. Effect of AEMO on tumor volume, life span and tumor weight in solid tumor-bearing mice.

architecture was observed. The tumor-bearing animals were treated with AEMO (200 mg/kg body wt.) for a period of 50th day, we observed a gradual reduction of tumor cell masses along with degenerated muscles indicating the inhibition of tumor growth (Figure 3B (j–l)). The maximum restoration of tissue architecture and reduction in tumor masses with infiltrated cells was observed in tumor-bearing animals treated with 400 mg/kg BW of AEMO for a period of 50 days (Figure 3B (m–o)). The histological evaluation was also done in liver tissues following AEMO treatment to analyze the adverse effect of AEMO administration on other organs. The liver sections of the tumor control group showed altered tissue structure consisting of highly nuclear-stained cells (Figure 4A (d), B (d)). However, liver sections in AEMO (200 mg/kg and 400 mg/kg) treated groups showed restored tissue architecture containing large round nuclei (Figure 4A (j–l), B (j–l)), similar to normal control animals (Figure 4A (a–c), B (a–c)).

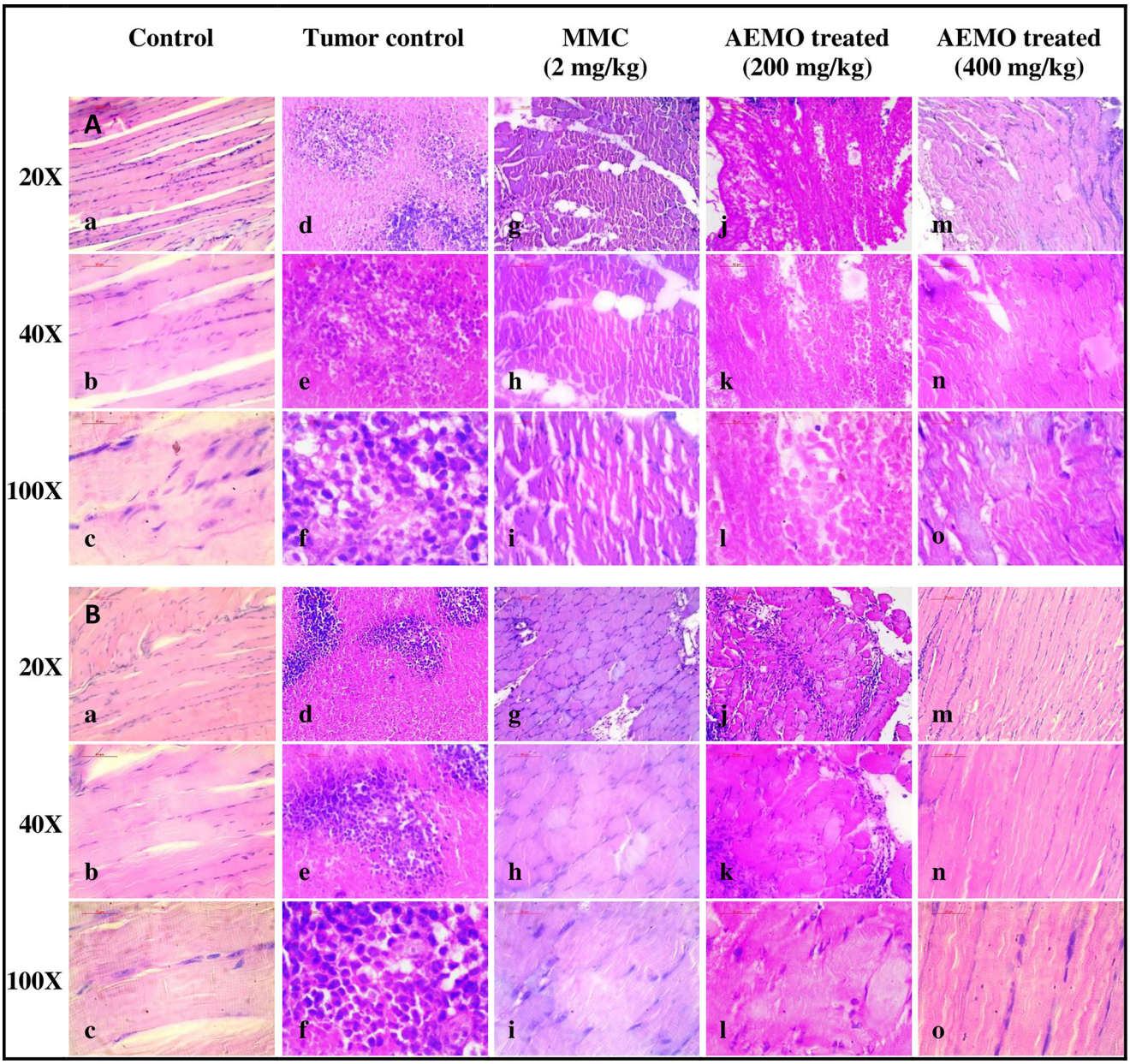


Figure 3. Histological evaluation of thigh tissues of control and treated tumor-bearing mice.

Administration of AEMO does not alter the physiology of normal mice

Our findings showed similar levels of AST, ALT, urea and uric acid in control and AEMO treated animals (Figure 5B). Further, hemoglobin (Hb) content and WBC and RBC count did not show any significant differences between control and AEMO treated animals (Figure 5B). Besides, we did not observe significant change in the body weight of control and AEMO treated animals (Figure 5A) indicating that AEMO administration of 400 mg/kg does not have any side effect.

AEMO causes dose-dependent cytotoxicity in EAC and HEp-2 cells

Data on trypan blue dye exclusion assay indicate a dose and time-dependent effect of AEMO on EAC cells (Figure 6A). In MTT assay, similar results were observed. A dose-dependent decrease in viable cells upon AEMO treatment was more prominent in MTT assay for both 48 h and 72 h (Figure 6B). In our study, the highest decrease in live cells was observed at 1 mg/ml AEMO at 72 h and only 1.30% of cells were found to be survived. Treatment of AEMO on HEp-2 cells also resulted a significant decrease of viable cells and showed dose and time dependent cytotoxicity (Figure 6D, E). The IC_{50} values of AEMO on EAC and HEp-2 cells for 48 h and 72 h was calculated by probit analysis (Table 2).

LDH release assay was done to determine the cellular integrity following AEMO (0.05, 0.1. 0.25, 0.5 and 1 mg/ml) treatment. Results of LDH release assay showed a dose and time dependent release of LDH in both EAC and HEp-2 cells (Figure 6C, F).

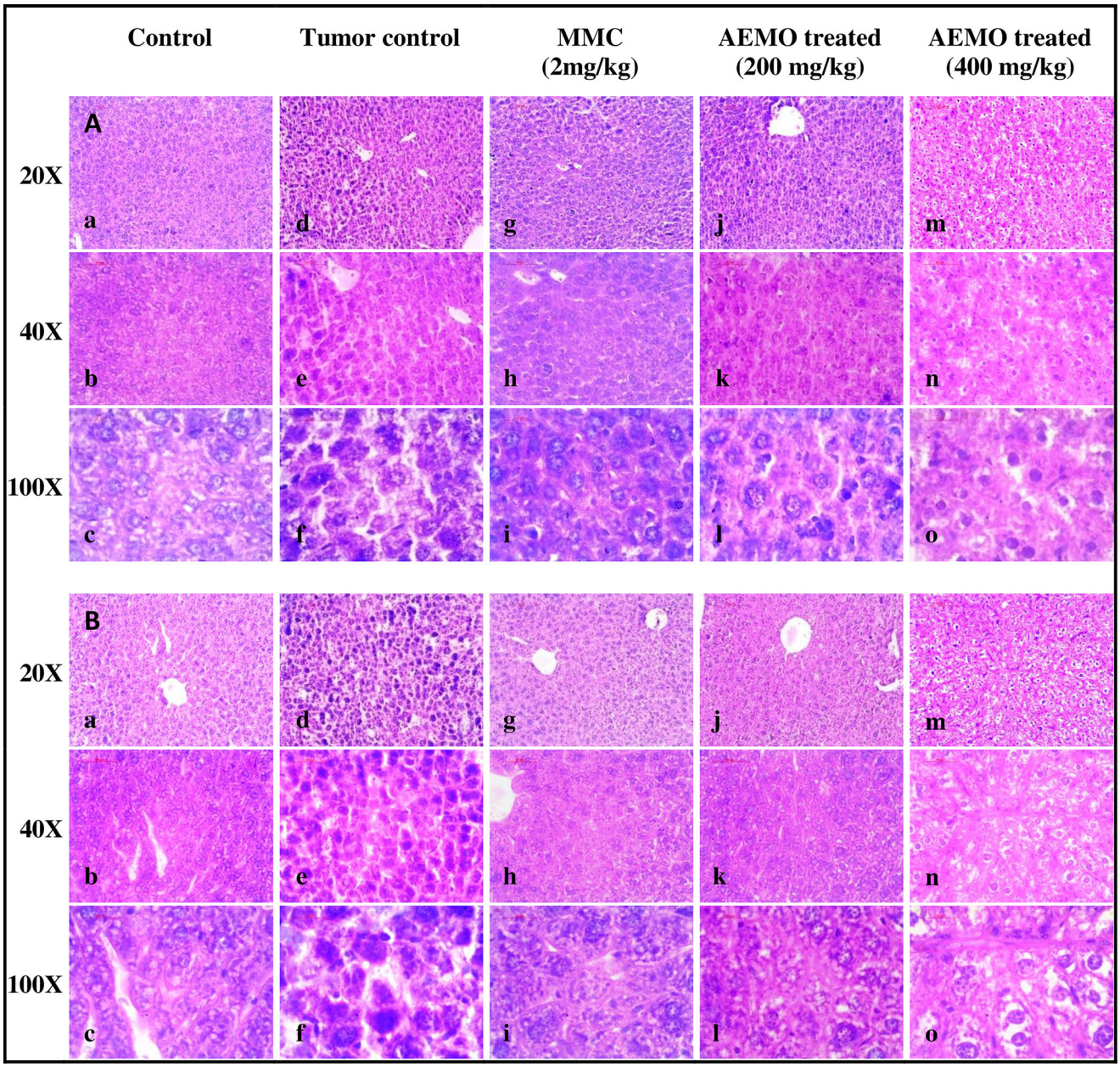


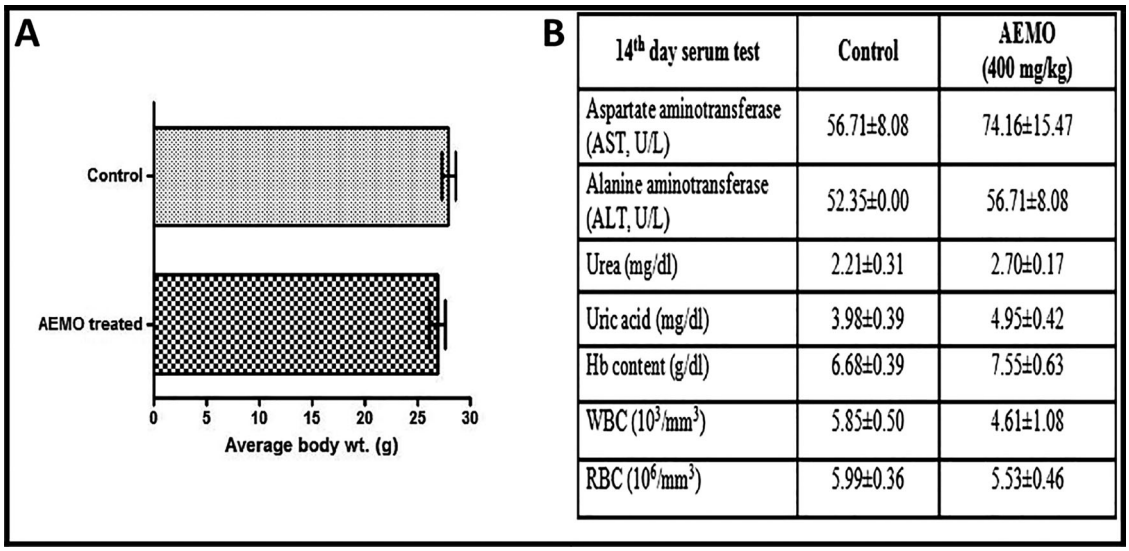
Figure 4. Histological evaluation of liver tissues of control, tumor control and AEMO treated tumor-bearing mice.

AEMO exposure induces cell death in EAC cells without arresting cell cycle

EAC cells were treated with AEMO (0.05. 0.1 and 0.2 mg/ml) for 24 h and 48 h. No significant change in cell cycle distribution following AEMO exposure was observed. However, a dose and time-dependent accumulation of cells in the sub-G1 phase were observed (Figure 7B, C).

AEMO treatment leads to apoptosis in EAC cells

In order to validate whether AEMO induced cytotoxicity is indeed due to apoptosis, annexin V-FITC/PI dual staining following AEMO treatment for 24 h and 48 h were performed. Annexin V-FITC has high binding affinity to phosphatidylserine which is a phospholipid and considered as a biomarker of apoptosis. Phosphatidylserine is found to be present in the cytoplasm of normal cells but it translocates out from the cell cytoplasm in apoptotic cells and in cells that undergo apoptosis. The percentage of the total apoptotic cell was calculated by combining the early and late apoptotic populations. In the present study, we observed that AEMO treatment significantly increases the percent of apoptosis in EAC cells. Upon treatment with 0.2 mg/ml AEMO for 24 h, an approximately two-fold increase in the percentage of apoptotic cells ($p < 0.001$) from 9.37% to 18.43% as compared to control (Figure 8B) was observed. On the other hand, more than 5 fold increase of apoptotic population, from 7.30% to 40.97% ($p < 0.001$, Figure 8B), was observed upon 0.2 mg/ml AEMO treatment for 48 h.



14th day serum test	Control	AEMO (400 mg/kg)
Aspartate aminotransferase (AST, U/L)	56.71±8.08	74.16±15.47
Alanine aminotransferase (ALT, U/L)	52.35±0.00	56.71±8.08
Urea (mg/dl)	2.21±0.31	2.70±0.17
Uric acid (mg/dl)	3.98±0.39	4.95±0.42
Hb content (g/dl)	6.68±0.39	7.55±0.63
WBC (10^3/mm^3)	5.85±0.50	4.61±1.08
RBC (10^6/mm^3)	5.99±0.36	5.53±0.46

Figure 5. Side effect analyses of AEMO in normal mice and identification of potential bioactive compounds.

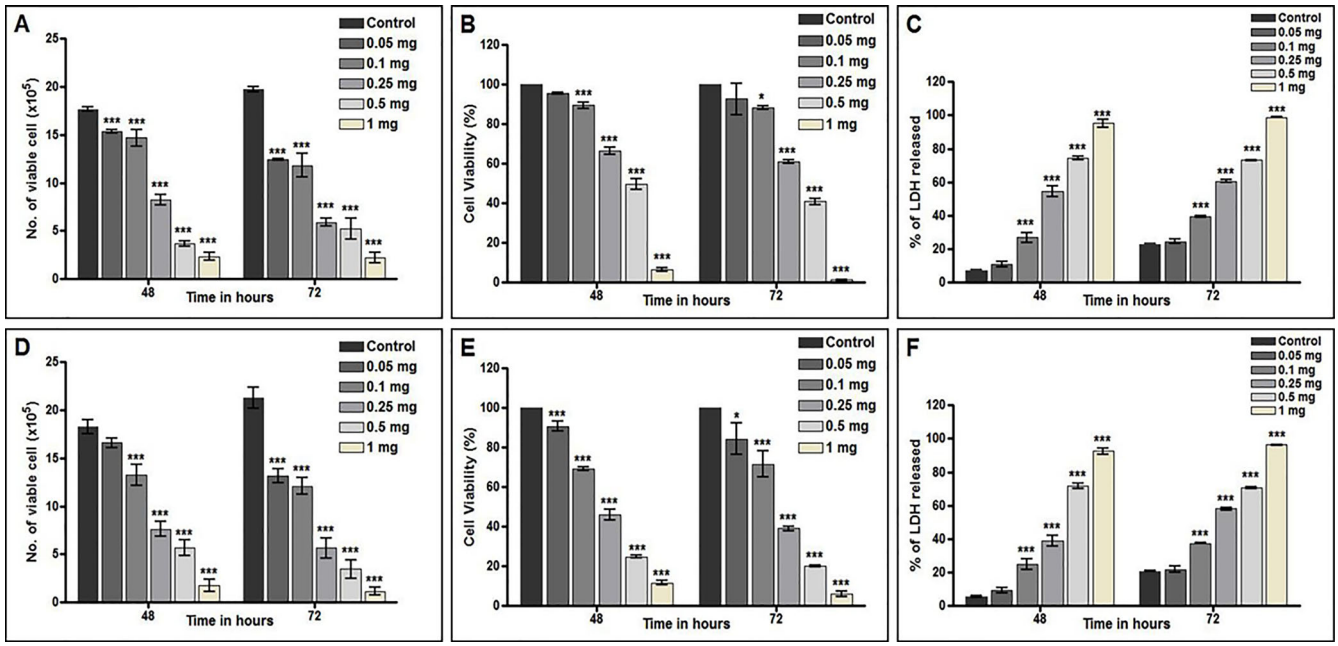


Figure 6. Histograms showing AEMO induced cytotoxicity in EAC and HEp-2 cell lines.

Table 2. IC_{50} value of AEMO in Ehrlich ascites carcinoma (EAC) and human laryngeal carcinoma (HEp-2) cells at different exposure time.

	IC_{50} (mg/ml), 48 h		IC_{50} (mg/ml), 72 h	
Cell lines	Trypan blue	MTT	Trypan Blue	MTT
EAC	0.23 ± 0.01	0.33 ± 0.01	0.12 ± 0.01	0.21 ± 0.07
HEp-2	0.23 ± 0.01	0.22 ± 0.02	0.10 ± 0.01	0.18 ± 0.03

EAC and HEp-2 cells were treated with different doses of AEMO (0.05, 0.1, 0.25, 0.5 and 1 mg) for 48 and 72 h and cell viability (n = 3) was determined by trypan blue dye exclusion and MTT assays. IC_{50} value was calculated by probit analysis for both 48 and 72 h. each value indicates mean ± SD.

AEMO treatment results in depolarization of mitochondrial membrane potential (ΔΨm) in EAC cells

Mitochondrial membrane potential was assessed using JC-1 dye staining method of treated and untreated EAC cells. This assay was performed using a flow cytometer to test whether the apoptosis is attributable to depolarization of cellular mitochondrial membrane potential. The depolarization of cells occurs due to the release of cytochrome c and thus it leads to a shift from red to green fluorescence. At 24 h of exposure, AEMO showed a significant increase of depolarized cells ($p < 0.01$) as compared to control (Figure 9B). At 48 h of exposure, a dose-dependent increase of depolarized cells was observed and even at a low dose (0.05 mg/ml), there is a drastic increase of depolarized cells as compared to control (Figure 9B). Upon treatment with 0.2 mg/ml AEMO, more than the five-fold increase ($p < 0.001$) of depolarized cells from 6.75% to 36.53% was observed as compared to control suggesting that apoptosis has occurred due to change in mitochondrial membrane potential.

Discussion

The inverse association between the higher uptake of vegetables and fruits with decreased risk of developing cancers

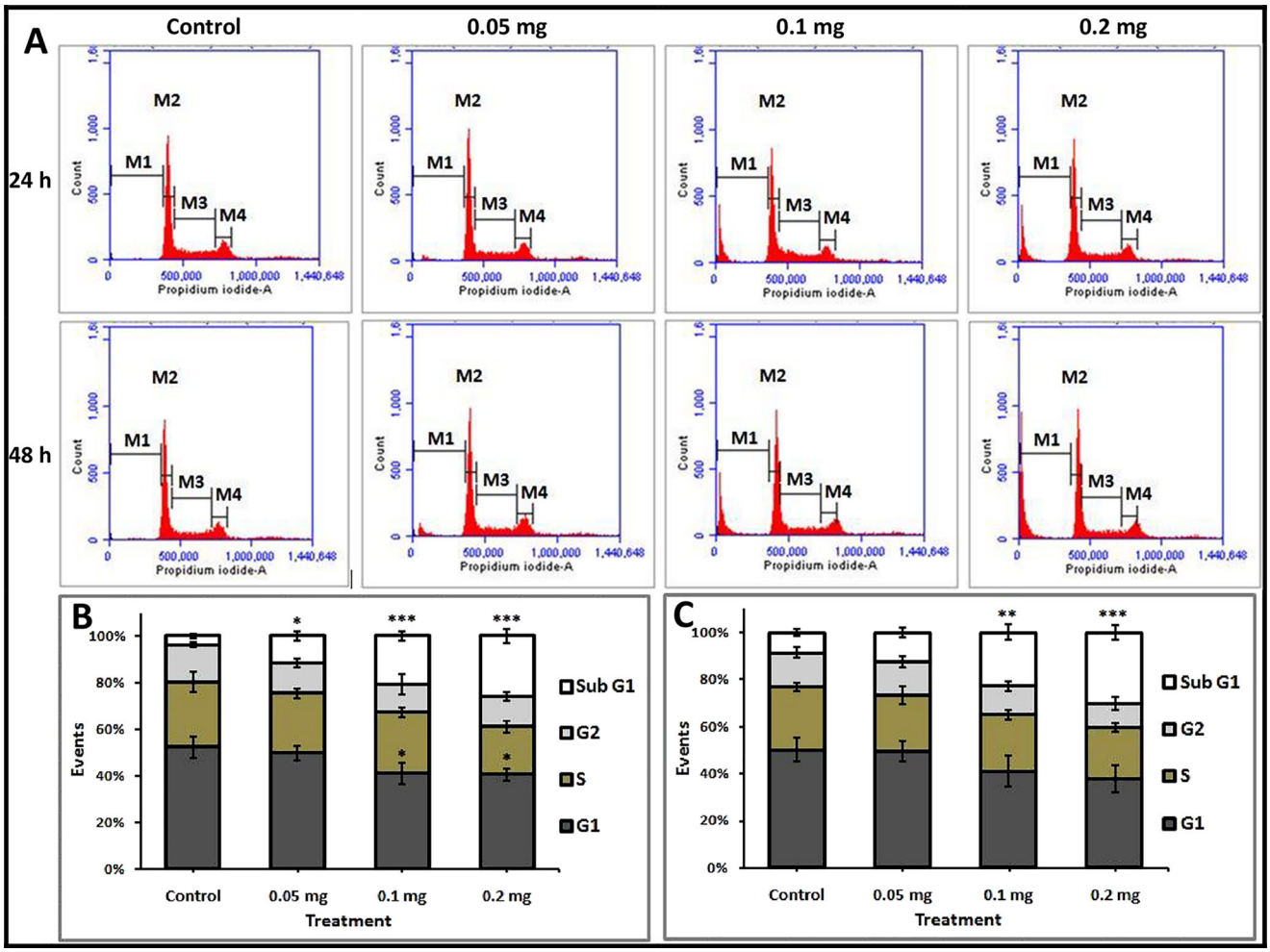


Figure 7. Histograms showing cell cycle stages in EAC cells following AEMO exposure.

could be attributed to the antioxidant content present in plants (27). GC-MS analysis of AEMO showed the presence of numerous phytoconstituents in the chromatogram (Figure 1) of which quinic acid (1, 3, 4, 5-Tetrahydroxy-cyclohexane carboxylic acid), octadecanoic acid, hexadecanoic acid (palmitic acid), α-tocopherol (Vitamin-E) and ɤ-sitosterol are the major bioactive compounds. Most of these compounds possess anticancer activity in cell lines and/or *in vivo*. There is evidence that quinic acid has anti-inflammatory, anti-mutagenic, DNA damage inhibitory and anti-oxidant property (28, 29). Recently published work showed that quinic acid attenuates the proliferative potentials of oral cancer cells by down-regulating the expression of cyclin D1 and Akt signaling (30). Besides, there is evidence that quinic acid and its derivatives display potent anticancer activity against HT-29 human colon cancer and prostate cancer (31, 32). 9, 12-Octadecadienoic acid (Z, Z)- and hexadecanoic acid possess anti-inflammatory activity (33, 34). There are numerous reports that palmitic acid showed anticancer activity in HCT-116 cell lines (35), human leukemic cell lines and *in vivo* mice model too (36). α-tocopherol and γ-sitosterol have antioxidant and immunomodulatory activity (37). It has been reported that α-tocopherol showed anti-tumor activity in oral squamous cell carcinoma (ORL-48) cell lines (38) and chemopreventive activity against tobacco-induced carcinogenesis (39). Thus, in the present study, the AEMO induced tumor regression in tumor-bearing mice could be attributed to the presence of above mentioned bioactive compounds.

Researchers across the world reported the anticancer potential of *M. oleifera* using *in vitro* assays but studies on animal model is inadequate (18, 40–43). In this study, we have shown the oncostatic potential of AEMO in solid tumor-bearing mice. Our study revealed a dose and time-dependent attenuation of TV and improved survival time of tumor-bearing mice following AEMO treatment. Similar studies on mice model were carried out by other workers (21, 44–46) by taking different plant extracts or isolated compounds or synthetic chemicals. Recently, Srivastava et al. (2016) reported quercetin mediated reduction of TV in solid tumor-bearing mice (45). In the same year, Thomas et al. (2016) showed inhibition of tumor growth in the mice test system exerted by extracts of *Vernonia condensata* (46). In the year 2008, Noaman et al. also observed a remarkable and progressive reduction of TW in arabinoxylan rice bran, MGN-3/biobran treated solid tumor mice (47), which is in concordance with our results, where a dose and time-dependent reduction of TW and TV was observed in solid tumor-bearing mice.

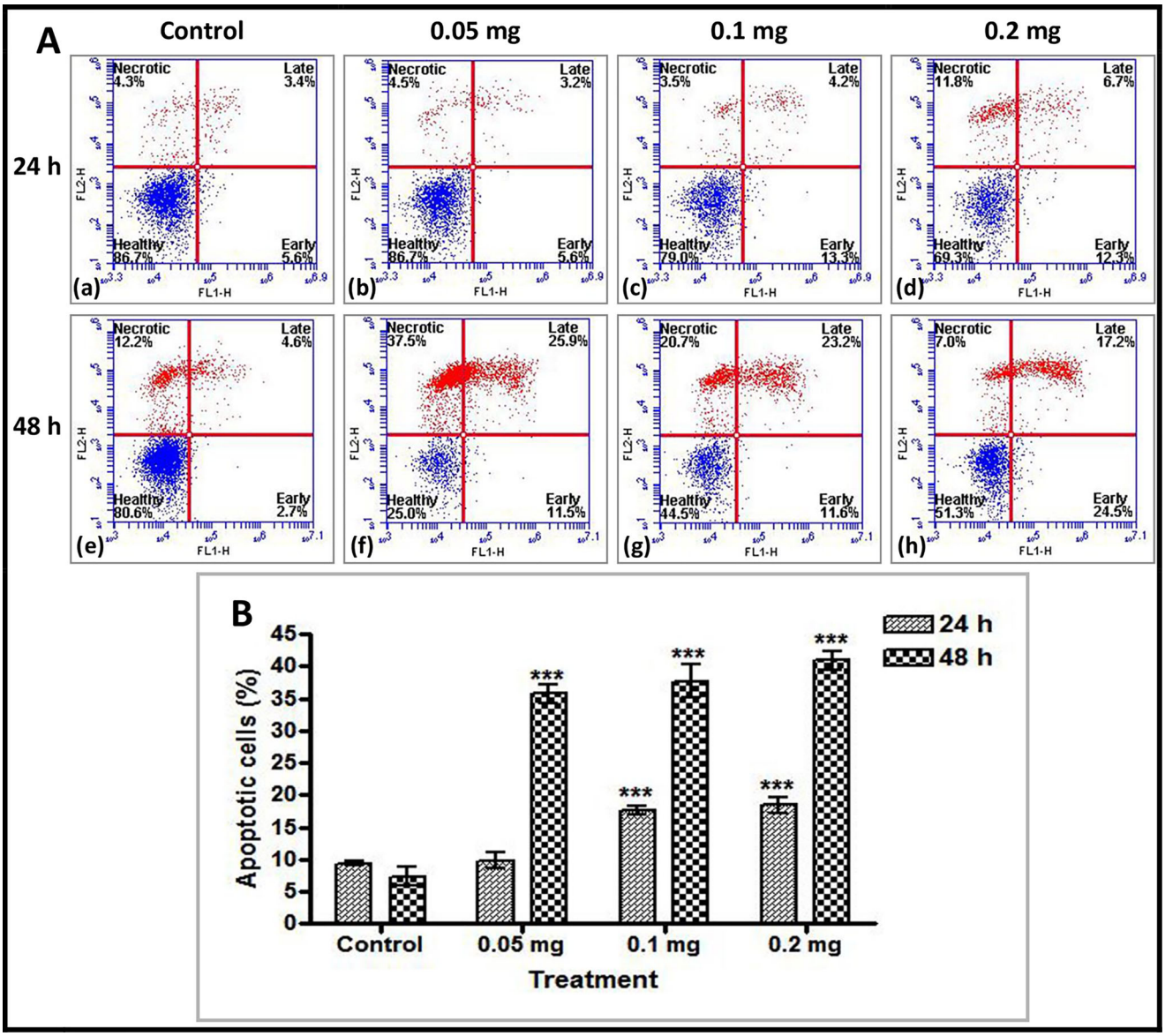


Figure 8. Histogram showing induction of apoptosis in EAC cells following AEMO treatment.

In the present study, we observed that AEMO administration induced a ~4 fold and ~2 fold increase in survival time as compared to the untreated tumor control and MMC treated group respectively. From our study, it is apparent that at least 40% of tumor-bearing mice were survived up to 235 days after treatment with 400 mg/kg AEMO. In contrast to this, all the untreated tumor control mice died within 59 days. Interestingly, histological evaluation of liver showed that EAC solid tumor not only affects the tumor tissues but also other organs. Histology of liver tissues showed altered tissue morphology in solid tumor bearing mice. Our results are in concordance with the earlier reports (48–50) where they observed several changes in liver tissue architecture like sinusoidal infiltration of highly stained carcinoma cells, hypertrophy and hydrophic degeration of hepatocytes upon EAC inoculated solid tumor in mice. Other studies also suggests that EAC tumors metastases in to other organs such as lung, kidney, spleen, adrenal gland and liver and effects the tissue morphology (51) by which it may affects the organs of the body. However, administration AEMO showed restoration of liver and thigh tissue morphology as compared to untreated tumor control. Thus, our study clearly indicates that AEMO is cytotoxic to cancer cells and reduces the proliferative potential of tumor cells, thereby increasing the survival time of tumor-bearing mice.

The analysis of biomarkers for liver and kidney function showed no significant changes in the levels of AST, ALT, urea and uric acid in AEMO treated animals as compared to normal mice (Figure 5B). Further, analysis of hematological parameters in AEMO treated and untreated animals did not show any significant differences (Figure 5B). It indicates that *M. oleifera* is a good and safe candidate for the development of a new therapeutic drug that can inhibit tumor progression without affecting the normal body function.

There are few studies that *M. oleifera* exhibit cytotoxic potential in human KB cells (52); human multiple myeloma cultured cells (53); human non-small cell lung cancer, A549 and hepatocellular carcinoma, HepG2 cells (42); African

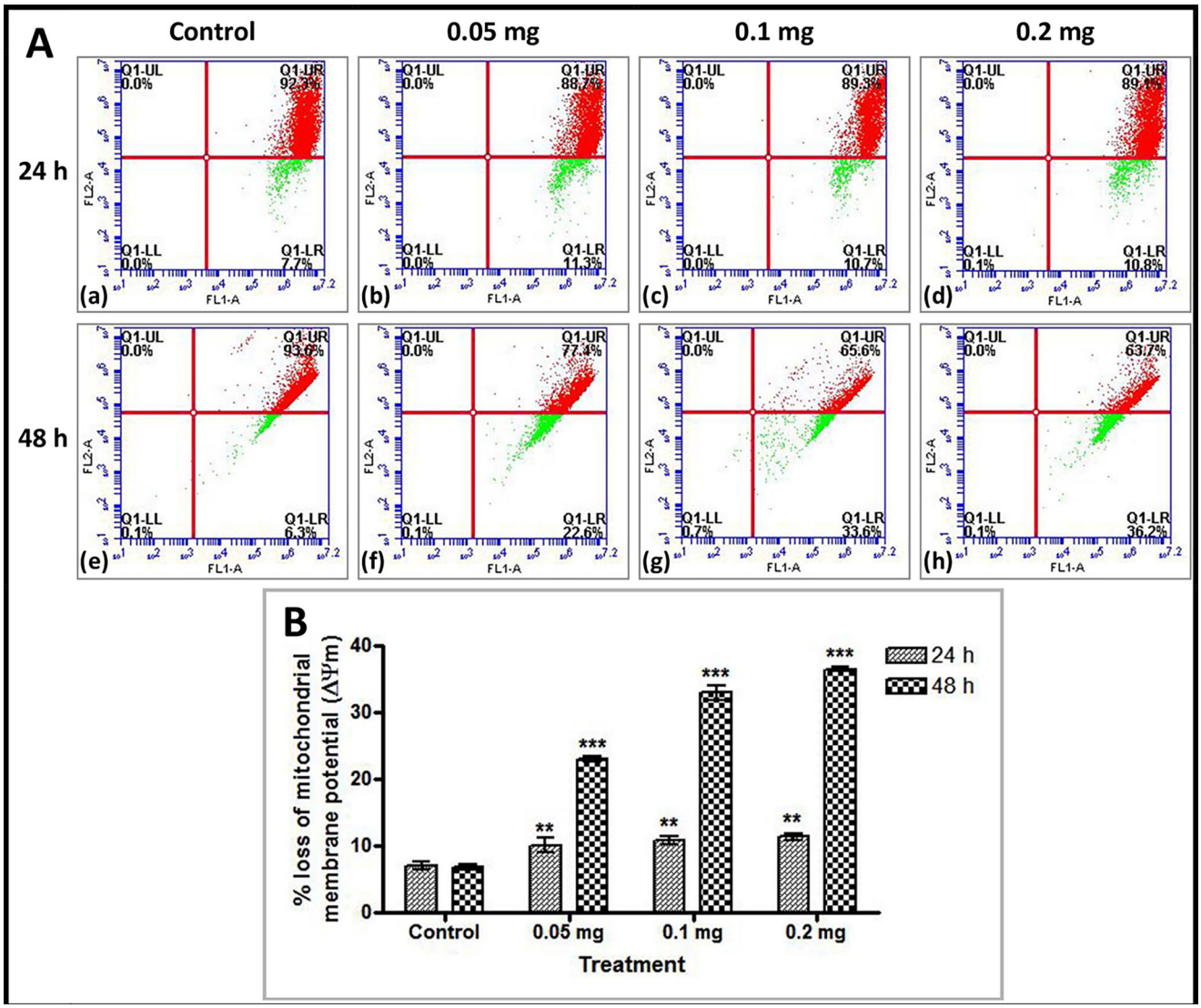


Figure 9. Assessment of mitochondrial membrane potential (ΔΨm) induced by AEMO in EAC cells.

green monkey kidney, COS-7 cells (41); and human pancreatic cancer, COLO-357, Panc-1 and p34 cells (18). There is also a report that *M. oleifera* treatment in MDA-MB-231 and HCT- 8 cells significantly reduced the number of viable cells (19) which is comparable to our findings where AEMO treatment induced cytotoxicity in EAC and HEp cells in a dose and time-dependent manner.

In the present study, we have performed the cell cycle assay to assess cell cycle progression following AEMO treatment. Interestingly, no cell cycle arrest was observed in our study. However, a dose and time-dependent accumulation of cells at sub-G1 was noted. This suggests that AEMO treatment causes cell death without causing cell cycle arrest. Further, to understand the mechanism of AEMO induced tumor reduction, we have performed apoptosis assay and it was established that the anticancer potential of AEMO is due to its strong induction of apoptosis. Apoptosis assay was done by staining the cells with annexin V FITC-PI. Annexin V FITC is a Ca^{2+} dependent phospholipid binding protein having high affinity to PS that translocated out from the cytoplasm in apoptotic cells and/or in cells undergoing apoptosis. In our study, we observed a time-dependent increase of apoptotic cells following AEMO treatment. AEMO treatment for a period of 48 h drastically increases the percent of total apoptotic cells even at a low dose (0.05 mg/ml) and the effect was almost steady on further increment in the doses (Figure 8B). At 24 h, we observed relatively more early apoptotic cells (annexin V positive and PI negative cells) in treated groups as compared to late apoptotic (annexin V-PI positive) cells. In contrast to this, with the increase of exposure time, 48 h, we noted more late apoptotic cells as compared to early apoptotic cells, which indicates the activation of apoptotic processes that might be a cause behind the sudden increase of total apoptotic cells at 48 h exposure. Our findings of apoptosis assay are in agreement with the previous reports of Badmus et al. (2015) (54) where they observed a dose and time-dependent increase of apoptotic cells in a variety of cell lines. Further, we evaluated the mitochondrial membrane potential (ΔΨm) to analyze the probable mechanism behind apoptosis. It has been reported that change of membrane potential results in cytochrome c release from the mitochondria that activates further downstream processes of apoptosis (55). In the presence of cytochrome c, apoptotic protease activating

factor 1 (Apaf 1) forms an apoptosome complex (56, 57); to execute apoptosis. Reports available that apoptosome complex activates the caspase 9 which in turn activates a downstream cascade of caspase-like caspase 3 to stimulate apoptosis (58). We found a dose-dependent increase of cells with the depolarized mitochondrial membrane at 48 h exposure (Figure 9B). The increased percentage of apoptotic cells in our study might be due to the activation of the intrinsic pathways of apoptotic signaling that ultimately results in cell death.

Conclusion

From the present findings, we can conclude that AEMO treatment has immense potential to induce apoptosis in cancer cells by changing the mitochondrial membrane potential. The results of *in vivo* assays showed that AEMO treatment on mice bearing solid tumor resulted in the reduction of tumor growth and increases the life span of tumor bearing allograft mice. However, this study lacks the translational study. Characterization of mitochondrial proteins in tumor tissues responsible for mitochondrial mediated apoptotic pathway is the future direction that will provide a more clear image. Histological evaluation of tumor sections showed restoration of the disrupted architecture of thigh muscles following AEMO administration. Further, AEMO administration to normal mice did not show alteration of physiological function of the body. Thus, our study clearly indicates that *Moringa oleifera* could be an excellent and safe candidate for the development of new therapeutics against cancer.

Author's contribution

Dharmeswar Barhoi and Sarbani Giri conceived and designed the experiments. Dharmeswar Barhoi performed all the experiments. Dharmeswar Barhoi and Anirudha Giri jointly performed the experiments on mice tumor model. Dharmeswar Barhoi and Sarbani Giri analyzed the data and wrote the manuscript. Puja Upadhaya and Sweety Nath Barbhuiya equally contributed during experiments and data collection. All the authors critically reviewed and approved the manuscript.

Disclosure statement

The authors declare is no conflict of interest.

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