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Plant-derived Tetranortriterpenoid, Methyl Angolensate Activates Apoptosis and Prevents Ehrlich Ascites Carcinoma Induced Tumorigenesis in Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KKC, BC and SCR conceived and designed the experiments. Authors KKC, MN, VG, SRR and SKT performed the experiments. Authors KKC, MN, BC and SCR analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Cancer is a leading health problem throughout the world. For decades, natural plant products have been playing promising roles as anticancer agents.
Objective: The present study aims to investigate the chemotherapeutic potential of methyl angolensate (MA), purified from *Soymida febrifuga* in mice bearing carcinoma and examines the molecular basis for its anticancer actions.
Study Design: The inhibitory effects of MA treatment on the survival of mice bearing Carcinoma and adverse side effects of MA treatment in mice were analyzed.
Methods: Tumor volume, life span, histopathology, immunohistochemical (IHC) analysis, estimation of liver enzyme, alkaline phosphatase and metabolites, creatinine and urea.
Results: Oral administration of MA in mice with Ehrlich Ascites Carcinoma showed significant

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inhibition of tumor growth compared to untreated mice. We observed a significant increase in the life span (~4-fold) of tumor bearing animals following treatment with MA. MA affected tumor cell proliferation by activating intrinsic pathway of apoptosis without imparting any side effect on normal cells. MA treatment in mice showed no major side effects.

Conclusion: MA treatment showed significant inhibition of tumor growth by inducing apoptosis as well increased life span of mice, with no adverse side effects to normal cells. Altogether, the present *in vivo* study provides new insights of MA serving as a cancer chemotherapeutic agent.

Keywords: Methyl angolensate; apoptosis; tumor; carcinoma; chemotherapy; natural products.

ABBREVIATIONS

MA-Methyl Angolensate, EAC- Ehrlich Ascites Carcinoma, IHC- Immunohistochemistry, b.wt.- Body weight, ALP- Alkaline phosphatase, p53BP1-p53 binding protein.

1. INTRODUCTION

Cancer continues to be a leading health problem throughout the world, despite momentous research efforts towards development of therapeutics. Significant advancement in preventive and therapeutic approaches is crucial for reducing cancer associated mortality. For decades, plant products have been playing promising roles as anticancer agents. One such group of phytochemicals, the triterpenoids have demonstrated chemopreventive and therapeutic effects [1-5]. In this regard, the recently reported natural tetranortriterpenoid, methyl angolensate (MA) (Fig. 1A), is of immense interest. MA is known to be chemopreventive, antimalarial [6], anti-inflammatory [7], antiallergic [8], antifungal [9] antiulcer [10], spasmolytic [11], insect antifeedant [12] and antibacterial in action.

Previously, utilizing cell based assays, we reported that MA exhibited time- and dose-dependent cytotoxicity to human T-cell leukemia, chronic myelogenous leukemia, Burkitt's lymphoma and breast cancer cells [13-16]. The observed cytotoxicity in normal lymphoblastoid cell lines was minimal suggesting that its mode of action was specific to cancer cells. Besides, treatment of MA led to activation of NHEJ machinery in Burkitt's lymphoma cells suggesting activation of DNA repair pathway [16]. Strikingly, MA treatment also resulted in phosphorylation of signaling molecules such as ERK1/2, JNK and MEK1/2 in cancer cells implying their role in apoptotic signal transduction. Although the action of MA against various types of cancer cell lines suggested its potential as an anticancer agent, its effects in primary tumor cells or tumors *in vivo* remained unexplored.

Here, we show that MA impedes tumor progression in mice similar to a positive control, SCR7, a small molecule inhibitor that possess anticancer properties. Histopathological and immunohistochemistry studies demonstrate that MA treatment affects only tumor cell proliferation, sparing normal cells. Further, our data indicates that MA activates intrinsic pathway of apoptosis. Finally, we show that treatment with MA results in significant increase in life span of mice with no adverse effects.

2. MATERIALS AND METHODS

2.1 Methyl Angolensate (MA)

MA used in the present study was dissolved in 0.5% methyl cellulose and used for animal experiments.

2.2 Ethical Statement

Mice were maintained as per the principles and guidelines of the ethical committee for animal care at Indian Institute of Science, which was in accordance with Indian National Law on animal care and use. The experimental design of the present study was approved by Institutional Animal Ethics Committee (Ref. CAF/Ethics/125/2007/560), Indian Institute of Science, Bangalore, India.

2.3 Evaluation of Anticancer Effect of MA on Solid Tumor Progression

Swiss albino mice, 8-10 weeks old, weighing 18-24 g were used for the present study. For the pilot experiments to determine the optimum dose of MA required for tumor regression, 6 Swiss albino mice were injected with EAC cells (1×10^6 cells/animal) into left thigh for the development of solid tumor. The mice were divided randomly into three groups of two animals each, on the 15th

day of tumor induction. Group I animals served as tumor control and received equivalent volume of 0.5% methylcellulose (vehicle control). Group II and III were orally administered 10 and 30 mg/kg b. wt. MA (6 doses), respectively, every alternate day. Increase in tumor volume was measured using vernier calipers every alternate day.

MA used for further experiments was 30 mg/kg b. wt. 30 mice were used per batch, which was divided randomly into three groups of 10 animals each. Group I animals served as untreated control and received no treatment. Group II and III were injected with EAC cells (1×10^6 cells/animal) into left thigh for the development of solid tumor. Group II animals served as tumor control and received equivalent volume of 0.5% methyl cellulose. Group III animals received oral administration of MA (30 mg/kg b.wt., dissolved in 0.5% methyl cellulose, 9 doses) at an interval of two days using intra gastric gavage from 12^{th} day of tumor development.

Tumor volume was measured every alternate day during the entire life span and was calculated using the formula $V = 0.5 \times a \times b^2$, where 'a' and 'b' indicates the major and minor diameters, respectively [17-20]. At the end of 25^{th} and 45^{th} day, one animal from each group was sacrificed and organs of interest were collected from untreated control, tumor control and MA treated animals and further processed for histological evaluation.

2.4 Determination of Effect of MA on Survival of Tumor Bearing Mice

The percentage increase in life span was calculated by using the formula $((T - C)/C) \times 100$, where 'T' indicates the number of days the treated animals survived and 'C' indicates the number of days the control tumor animals survived [17-21]. Animals were maintained till their natural death. However, few animals which were high in tumor burden were sacrificed before their natural death.

2.5 Determination of the Side Effects of Administration of MA in Mice

Serum was collected from blood of control (n=4) and MA administered (n=4; 30 mg/kg b. wt., 9 doses, every alternate day). Body weights of mice from both the groups were recorded 20 days after treatment. Liver and kidney function tests were performed as described, to check the toxicity induced by MA treatment [17,22]. Levels of alkaline phosphatase activity served as an index of liver functionality. Kidney function was determined by checking urea and creatinine levels in the serum. Blood collected from mice in both the groups was used for obtaining mean values of total red blood (RBC) and white blood cells (WBC) counts using a haemocytometer. Values are presented as mean ± SEM for the control and MA administered mice.

2.6 Histopathological Evaluation Immuno-histochemical (IHC) Analysis

The tissues and organs of interest (tumor and liver) collected from the animals were processed as per standard protocol and embedded in paraffin. Microtome sectioning was done at 3–5 µm in a rotary microtome (Leica Biosystems, Germany) and stained with hematoxylin and eosin [17,18,22,23]. Each section was evaluated using light microscopy and images were captured (Carl Zeiss, Germany).

2.7 Immunohistochemical (IHC) Analysis

Immunostaining of tumor and MA treated tissue was performed on formalin fixed, paraffin embedded tissues and sectioned at 3-5 µm thickness as described [18,22]. Rabbit polyclonal antibodies Ki67, BID and p53BP1 (Santa Cruz Biotechnology) and biotinylated anti-rabbit IgG were used. Multiple images from control and treated groups were quantified using ImageJ.

2.8 Statistical Analysis

Values were expressed as mean ± SEM for control and experimental samples and statistical analysis was performed by one-way ANOVA followed by Turkey-Kramer Multiple Comparison test using GraphPad prism 5.1. Values were considered statistically significant, if the p-value was less than 0.05.

3. RESULTS

3.1 MA Prevents Tumor Progression in Mice

In order to evaluate the potential of MA as an anticancer agent, mice harboring EAC induced tumor were utilized. EAC is a murine breast adenocarcinoma extensively used for analyzing effects of small molecule inhibitors on tumor progression. A pilot study was performed using

EAC induced Swiss albino mice for determining the dose of MA needed for tumor regression (Fig. 1B). The tumor induced mice were treated with two doses of MA (10 or 30 mg/kg b.wt) or an equivalent volume of vehicle control. Interestingly, we found that, while tumor volume increased exponentially in the untreated group, in both the treated groups the tumor progression was reduced (Fig. 1B). The tumor regression was pronounced in the group treated with 30 mg/kg b.wt. MA, while it was minimal when the lower dose was used (Fig. 1B). Importantly, the mice showed no observable difference in appearance or body weight (data not shown) implying that MA did not adversely affect its health.

Based on the above results, therapeutic potential of MA was extensively assayed in three independent batches of experiments (in each experiment, 10 each untreated normal control, untreated tumor control and tumor mice treated with MA) at a dose of 30 mg/kg b.wt. (Fig. 1C). Following the development of tumor, 12^{th} day post EAC injection, the mice were orally fed with MA on every alternate day (9 doses). Fig. 1C showed remarkable reduction in tumor size upon treatment with MA as compared to untreated animals. Fig. 1D shows average tumor volume in untreated animals was 14.78, 13.84, 18.94, 23.89 and 38.76 cm^3 on 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} day respectively, whereas it was only 6.56, 5.22, 6.16, 1.57 and 0.0 cm^3 respectively, upon MA treatment (Fig. 1C). We found that 83% (25/30) of the mice with tumor were dead by 50^{th} day of tumor development when left untreated. Interestingly, 70% (21/30) of the mice survived following treatment with MA up to at least 300 days (Fig. 1D).

SCR7 is an inhibitor of nonhomologous DNA end joining (NHEJ), and shown to possess antitumor activity in mice [18,24-26]. Therefore, we have used SCR7 as a positive control for tumor regression. Upon treatment with SCR7 in mice bearing EAC induced tumors we found significant tumor regression in tumor volume, while the growth was exponential in the untreated group (Fig. 1E).

3.2 MA Treatment Significantly Increased the Survival of Tumor Bearing Mice

We observed lifespan was significantly increased in MA treated tumor mice compared to that of untreated tumor animals (Fig. 1D). While control animals survived maximum of only 68 days after tumor development, 50% of the MA treated mice survived upto >200 days and 25% of the mice survived upto 300 days indicating significant increase in life span (Fig. 1D). Therefore, our results suggest that MA treatment inhibited tumor progression and improved the survival of mice, significantly.

Next, we performed histopathological examination of tumor and liver tissues of MA treated mice on 25^{th} and 45^{th} days, for analyzing cellular level changes and toxicity caused by MA. Hematoxylin-eosin stained sections of 25^{th} and 45^{th} day untreated tumor showed cellular damage, angiogenesis and tumor cell proliferation (Fig. 2A (b, e), B (b, e)) as compared to normal untreated mice thigh tissue (Fig. 2A (a, d), B (a,d)). However, tumor tissues showed very high tumor cell proliferation and complete destruction of cellular architecture. Interestingly, tumor tissue from MA treated mice showed only mild cell architecture damage, while proliferation of tumour cells was reduced (Fig. 2A (c, f), B (c, f)). When similar tissue sections were examined after 45 days of tumor development, the effect was very prominent following MA treatment and it could be seen that the tissues were restored to normal cellular morphology (Fig. 2B (c, f)).

Histopathological sections of liver from normal mouse showed proper arrangement of hepatocytes with no infiltration (Fig. 2C (a, d), D (a, d)), while liver from tumor bearing mouse after completion of 25 days of treatment showed infiltration of hepatocytes, cellular damage and dilation of central veins (Fig. 2C (b, e), D (b, e)). In contrast, liver from MA treated mouse showed dilated sinusoids with minimum infiltration of hepatocytes and nearly normal rearrangement of hepatic cell chords (Fig. 2C (c, f)). However, the effect was more pronounced when tissues were analysed following 45 days of MA treatment (Fig. 2D (c, f)). Thus, our results showed that MA treatment significantly reduced the tumor burden.

3.3 MA Abrogates Tumor Progression with no Side Effects

Next we evaluated the side effects of MA treatment for serum levels were analysed for liver enzyme such as Alkaline phosphatase (ALP), and metabolites like creatinine and urea. Results showed no significant difference in the serum levels of ALP, creatinine and urea between untreated control and MA treated mice groups suggesting that there were no adverse effects of

MA on liver and kidney functions (Fig. 3A). Further, we did not observe any effect of MA on the total RBC and WBC counts suggesting that it did not affect hematopoiesis. Analysis for toxicity on 0th day after oral administration of MA showed that there was no significant change in the body weight among the control and treated groups (Fig. 3B). Thus, our data indicates that MA treatment does not result in any apparent side effects in normal mice.

3.4 MA Induces Cell Death through Activating Apoptosis

Previously studies on cancer cell lines suggested that MA induced apoptosis by activating

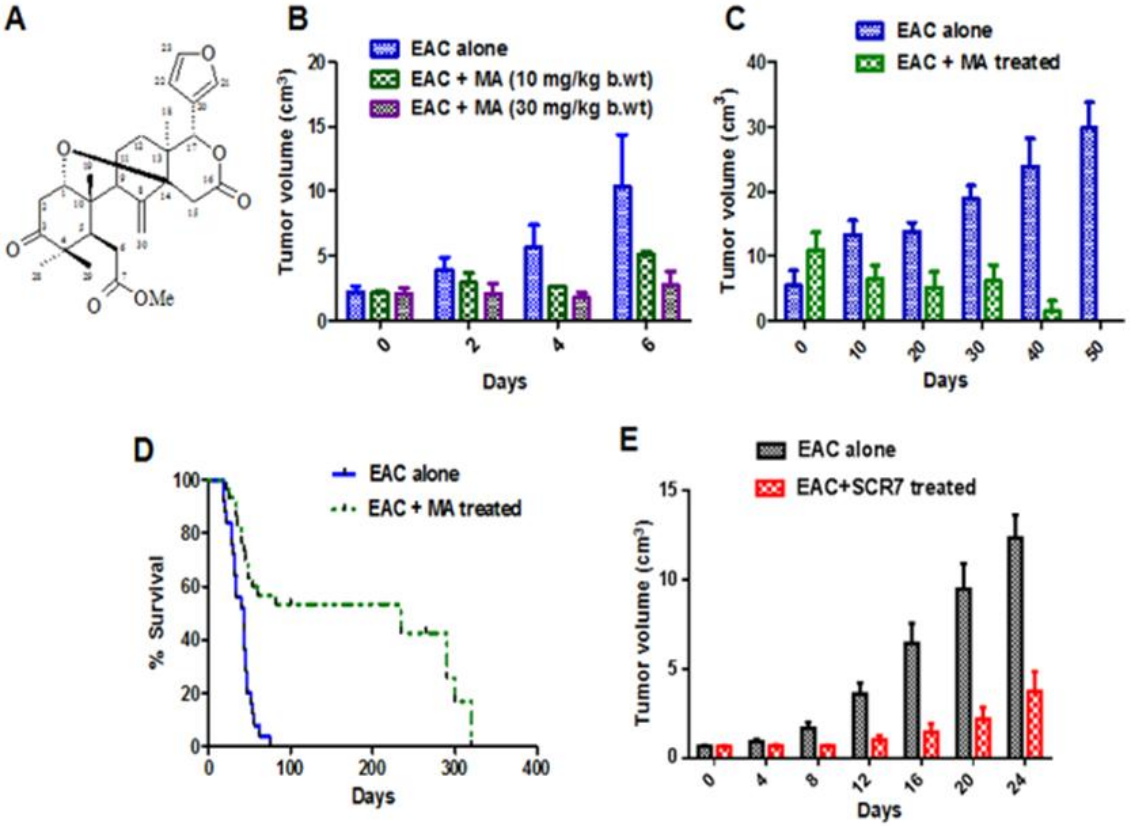

Fig. 1A. Structure of methyl angolensate. B. Pilot experiment to evaluate the effect of MA on mice bearing tumor. Solid tumor was induced intramuscularly in Swiss albino mice by injecting EAC cells (1x10^6 cells/animal). From 15th day of EAC injection, MA (10 or 30 mg/kg b. wt.) was orally administered (6 doses) every alternate day. The tumor volume was measured and plotted against the day of measurement. Two mice per group were used for the preliminary study. C-E. Effect of MA on tumor progression and life span of EAC induced mice. C. Solid tumor was induced as described above. Tumor volume in control and MA treated tumor-bearing mice at different time intervals. From 12th day of EAC injection, MA (30 mg/kg b. wt.) was orally administered (9 doses) at an interval of two days and the tumor volume was calculated. D. Evaluation of effect of MA treatment on life span in tumor bearing mice. Kaplan–Meier survival curves for control and MA treated mice. ''EAC alone'' refers to control mice injected with EAC cells while ''EAC+MA'' indicates the mice-bearing tumor treated with MA. Error bars indicate SD from independent experiment. Data shown is obtained from three independent batches of experiments containing 10 animals per group. E. Effect of SCR7, a positive control on tumor progression and life span of EAC induced mice. EAC induced mice were treated with vehicle control or SCR7 (10 mg/kg b. wt.) intraperitonially (6 doses) every alternate day. The tumor volume was measured and plotted against as above. Each group consisted of six mice and the error bar is plotted

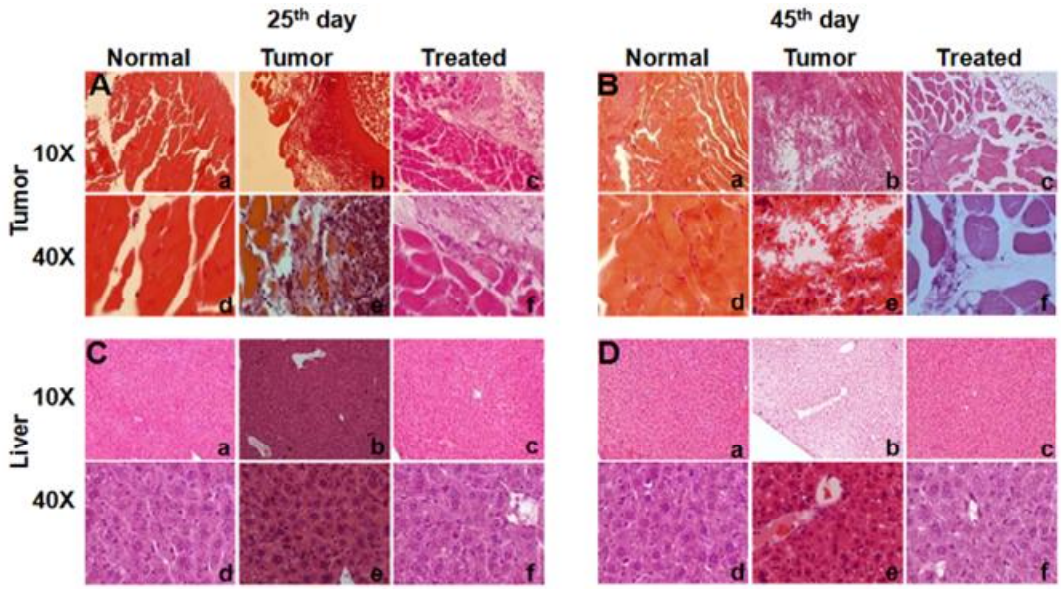

Fig. 2. Histopathology of tumor and liver following MA treatment

Histopathological sectioning of tumor and liver tissues of mice following MA treatment after 25 and 45 days of treatment with MA. A-B. Microscopic images of histological sections of thigh tissues on 25th and 45th days, respectively. C-D. Histological sections of liver tissues on 25th and 45th days, respectively. In all panels, "Normal" refers to tumor mice, untreated with MA (a, d), "Tumor" indicates mice injected with EAC cells with no MA treatment (b, e). "Treated" refers to the mice bearing tumor treated orally with MA (c, f). In all the cases, a, b, c indicates images shown at 10X magnification, while d, e, f at 40X magnification

A

20th Day Serum Test	Control	MA
Alkaline Phosphatase (IU/L)	27.18 ± 4.41	23.91 ± 1.43
Creatinine (mg/dL)	0.04 ± 0.02	0.07 ± 0.03
Urea (mg/dL)	29.18 ± 2.88	32.70 ± 2.34
RBC (10^6/µl)	8.92 ± 0.16	8.96 ± 0.63
WBC (10^3/µl)	2.92 ± 0.11	2.83 ± 0.25

B

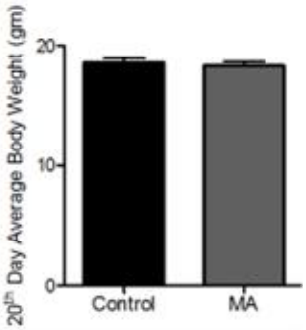

Fig. 3. Evaluation of side effects of MA administration in Swiss albino mice on 20th day

Swiss albino mice, treated with MA (30 mg/kg b. wt, 9 doses) and untreated mice, were tested for various toxicological parameters after 20 days of treatment. A. Blood was collected and serum levels were checked for liver and kidney enzymes such as alkaline phosphatase (ALP), creatinine and urea. Variation in the counts of RBC and WBC were also compared following MA treatment. B. Body weight (g) was measured to assess the effect of MA following 20 days of oral administration. In all the cases, error bars indicate mean ± standard error mean

mitochondrial pathways. In order to investigate whether the same holds true in tumor tissues, immunohistochemical analysis was performed using antibodies against protein markers of cell proliferation and apoptosis in histological sections of mouse tumor tissues following MA treatment (30 mg/ kg, 9 doses, 25th day) (Fig. 4). We observed efficient Ki67 nuclear staining in untreated tumor sections (Fig. 4A (a, g)). while number of Ki67 positive cells were substantially less in MA treated tumors, although there was no significant difference in staining pattern between control and treated groups (Fig. 4A (b, h)). Besides we observed that the expression of proapoptotic protein, BID was significantly high following MA treatment compared to untreated

tumor tissues (Fig. 4A). This indicates the activation of intrinsic pathway of apoptosis in MA treated tumor tissues, which is consistent with our earlier studies that shows that MA induces intrinsic pathway of apoptosis [13,15,16]. Further, we also tested the expression of DNA repair protein, p53 binding protein, p53BP1. The expression of p53BP1 was significantly high in MA treated tissues compared to that in tumor tissues (Fig. 4B). Hence, our results suggest that MA treatment resulted in the abrogation of tumor cell proliferation by activating the apoptotic pathway in mice induced with carcinoma.

4. DISCUSSION

Compounds purified from medicinal plants have been considered as valuable sources for development of anticancer drugs. Differential effects of these natural products on tumor cells could be due to their difference in the efficacy to induce apoptotic pathways and this also might play a significant role in their chemopreventive potential. Among various plant derived molecules, limonoids are triterpenoids, which are biologically important and possess tumor preventive properties as shown by several *in vitro* and *in vivo* studies. Our previous studies, utilizing various cell line based assays, identified that MA possesses anticancer properties [13,15,16]. However, this is the first study to test the anticancer properties of MA in primary tumor tissues using an *in vivo* model system.

Here, we have uncovered that MA has the potential to cause tumor regression in mice bearing breast adenocarcinoma. Further, this study, in conjunction with previous *ex vivo* studies show that MA activates intrinsic pathway of apoptosis to inhibit tumor growth [13,15,16].

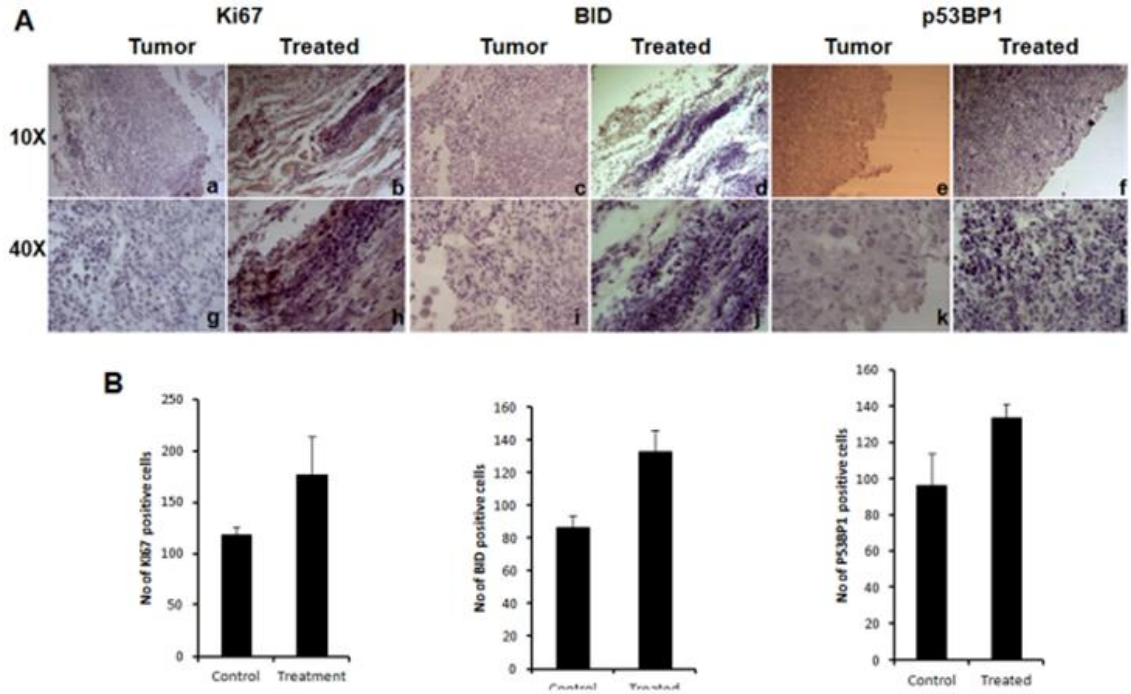

Fig. 4. Immunohistochemical evaluation for cell proliferation, apoptosis and DNA damage markers following administration of MA

Immunostaining studies in tumor and MA treated tissues on 25th day. One representative animal from the treated and untreated tumor mice were used for the evaluation. A. Antibody staining for Ki67, BID and 53BP1. Although cell proliferation marker, Ki67 showed comparable staining in both control (a, g) and MA treated tissues (b, h), note that the number of surviving tumor cells were much less following MA treatment. Low BID expression in tumor tissue (c, i) versus high BID expression in MA treated tissue (d, j) is also noted. Low expression levels of 53BP1 in tumor tissues (e, k) versus high expression levels in tumor bearing mice treated with MA (f, l) was also observed. Magnification of the images shown in upper panel is 10X, while lower panel is 40X. B. The signals were quantified with the help of "ImageJ" software. Multiple images from control and treated groups were used for the quantification and the results were plotted and presented with error bars

Previous studies using tetranortriterpenoids have also shown their antiproliferative effects, particularly inhibition of carcinomas by inducing apoptosis [4,27-29]. Breast adenocarcinoma cells possess malignant features of cancer and are commonly used for inducing tumor in Swiss albino mice, for evaluating anticancer activity of small molecules *in vivo.*

Several lines of evidence support that MA abrogated the tumor progression in EAC induced mice without adverse side effects. Our studies show more than 4-fold increase in lifespan following MA treatment compared to untreated tumour mice. Besides, there were no significant alterations in hepatic or renal functions and the whole blood cell counts following MA treatment. Histological evaluation of tumor and MA treated tumor tissues further indicates that effect of MA treatment was restricted to tumor cells and does not affect other organs. The present observation that oral administration of MA did not lead to any side effects was consistent with our previous findings of MA displaying no cytotoxicity in noncancerous cell lines [13,16]. We also tested the therapeutic potential of MA (30 mg/kg b. wt., 9 doses) in the Dalton's lymphoma mouse model. Our results showed no reduction of tumor in MA treated animals (data not shown). These results suggest that MA was effective against tumor induced by breast adenocarcinoma cells, while it was insensitive to tumors generated by Dalton lymphoma cells.

Apoptosis is one of the important mechanisms, which determines the efficacy of anticancer drugs. Our previous findings showed that, upon treatment with MA, intrinsic pathway of apoptosis leading to cell death was activated in both leukemia and lymphoma cells. During apoptosis, proapoptotic proteins promote the release of mitochondrial Cytochrome C into cytoplasm, while antiapoptotic proteins block the Cytochrome C release by preserving the integrity of mitochondrial membrane. Previously, it has been shown that MA treatment resulted in increased BAD and decreased BCL2 expressions leading to an altered ratio of antiapoptotic/proapoptotic proteins [13]. The increase in the BAD/BCL2 ratio resulted in disruption of mitochondrial membrane potential and Cytochrome C release, which in turn activated Caspase 9 and Caspase 3 culminating in cell death, indicating that MA displayed activation of the intrinsic pathway of apoptosis [13].

In the present study, we measured expression of one of the proapoptotic proteins, (BID) in tumor tissues. An increase in BID expression in MA treated tumor tissues as compared to that in untreated tissues suggest that MA increased the levels of BID, causing its translocation from the cytosol into mitochondria. Thus, our findings in conjunction with previous studies suggested the activation of mitochondrial pathway of apoptosis following treatment with MA in tumor tissues. Similar activation of the intrinsic pathway has also reported in the case of other terpenoids [30-32]. DNA damage is a critical event preceding cellular apoptosis. Accumulation of DNA double strand breaks leads to activation of apoptosis in cancer cells. The observed elevated expression of 53BP1 in tumor tissues was also consistent with this. Overall, DNA strand breakage following MA treatment in tumor cells supports the notion of activation of apoptosis in cancer cells and recapitulates DNA fragmentation induced by MA in cancer cell lines during apoptosis.

5. CONCLUSIONS

Taken together, our data suggests that MA abrogated tumor progression blocking cell proliferation by inducing intrinsic pathway of apoptosis in EAC induced mice as well increased life span of mice, with no adverse side effects to normal cells. Therefore, our current study in conjunction with previous reports reveals the potential of plant-derived MA as a potential cancer chemotherapeutic agent.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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