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Induction of Apoptosis and Antiproliferative Activity of *Alangium salviifolium* against Non-melanoma and Melanoma Cancer Cells

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ABSTRACT

Ayurveda is a traditional medicine of system in India which includes plants and plant-based products for treatment and cure of diseases. *Alangium salviifolium* is an ethnomedicinal plant and has been reported to be useful against skin cancer. In the present study, we used thee concentrations of methanolic bark extract of *A. salviifolium* (MBEAS) to evaluate its anticancer activity against epidermoid carcinoma A431cells and melanoma B16F10 cells. Assays performed were trypan blue dye exculsion to assess the cytotoxicity, colony formation for cell growth and AO/EtBr staining to check the apoptotic/necrotic effect of *A. salviifolium* on skin cancer cells. Exposure of cells to *A. salviifolium* for 24 h and 48 h strongly reduced the cell number of A431 and B16F10 cells, inhibited the colony formation ability and induced apoptosis in a concentration-dependent manner. The results from the study suggest the antiproliferative activity of *A. salviifolium* against both melanoma and non-melanoma skin cancer cells. Overall, it provides the needed scientific validity to its traditional use against skin cancer.

Keywords: Anticancer, ethnomedicine, antiproliferative, non-melanoma, melanoma.

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Abbreviations: MBEAS; Methanolic bark extract of *Alangium salviifolium,* DMSO; Dimethyl sulfoxide, DMEM; Dulbecco's modified Eagles medium, AO; Acridine orange, EtBr; Ethidium bromide.

1 INTRODUCTION

Cancer incidences are rising around the globe. An increase in new cancer cases to 19.3 million per year by 2025 has been estimated on the basis of GLOBOCAN 2012 estimates (Ferlay et al., 2015). According to GLOBOCAN 2018 statistics the global cancer burden has increased to 18.1 million cases and cancer deaths to 9.6 million. According to this report, Asia is contributing to half of the all new cancer cases and cancer deaths. In these estimates, Asia and Africa have higher percentage of cancer deaths 7.3% and 57.3% respectively, as compared to global incidence of 5.8% and 48.4% respectively. According to the International Agency for Research on Cancer (IARC), this is because of the cancer types with poorer prognosis and also delay in diagnosis and lack of access to the treatment (Bray et al., 2018). Thus to increase the access of cancer treatment, economic and efficient treatment options are needed to be explored. In this regard, plant and plant-based products provide a good option because of its easy availability and relatively lesser side effects (Atanasov et al., 2015).

Ethnomedicinal plants are getting much importance and many of them are getting evaluated for their claimed activities to provide with the much needed scientific evidence. Phytochemical and pharmacological studies of these plants are useful and support their traditional uses and provide new source for evaluation and development of drugs. *A. salviifolium* belongs to genus *Alangium* and family Alangiaceae and in local languages known as Ankora, Ankol, Ankolam, Ankola, etc. It is found as shrub or as small to medium-sized tree that has thorns and rough bark surface with reddish-brown fruits. In India, it is commonly found in Western Ghats (Tanwer, 2010). Many of the traditional use of *A. salviifolium* are as vasodilators, antidote, and purgative and to cure diarrhoea, asthma, eye diseases, back pain, blood disorders, and skin diseases (Plants, 1995; Kritikar and Basu, 1918; Nandakarni, 1996; Pullaiah, 2003; Panara et al 2016). It is used by many tribes of Chhattisgarh and Rajasthan to treat cancer (Jain and Jain, 2010; Tanwer and Vijayvergia, 2014).

Different parts of the plant have been explored for the phytochemical constituents that revealed the presence of primary metabolites such as lipids, starch, phenols, ascorbic acid, soluble sugars etc and secondary metabolite such as steroids, flavonoids, alkaloids, tannins, terpenoids and

glycosides (Sreekanth et al, 2011; Murugan et al 2000; Jubie, 2008). Some of the medicinal properties of stem bark of *A. salviifolium* have been evaluated previously. Stem bark of *A. salviifolium* has been reported to have antimicrobial (Katyayani et al, 2002), anti-arthitic (Jubie, 2008), antidiabetic (Rajkumar and Kumar, 2011), anticonvulsant (Parida et al, 2010) and androgenic activity (Murugan et al, 2000).

Currently, there is no study reporting the anticancer activity of bark extract of *A. salviifolium* against skin cancer. Therefore in the present study, we evaluated the anticancer activity of *A. salviifolium* against melanoma and non-melanoma skin cancer cells.

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Chemicals used for the study were 0.4% trypan blue from Himedia laboratories, crystal violet, acridine orange (AO) from Thermo Fisher Scientific, and ethidium bromide (EtBr), dimethyl sulfoxide (DMSO) from Sigma (Saint Louis, MO, USA), Dulbecco's modified Eagles medium (DMEM; Sigma, Saint Louis, MO, USA), fetal bovine serum (Gibco), penicillin-streptomycin (Himedia, India). Other chemicals and reagents used were of analytical grade.

2.2. Plant material

The stem bark of *A. salviifolium* was freshly collected from District Balod, Chhattisgarh, India. The plant material was authenticated by Dr. Mohammad Irfan Qureshi (Jamia Millia Islamia, New Delhi), and a voucher specimen (Acc. No. 20150218) was deposited at the Herbarium of PBL, Department of Biotechnology, Jamia Millia Islamia, New Delhi, India.

2.3 Cell culture

Cell lines used for the study are squamous cell carcinoma A431 cells, and melanoma B16F10 cells. It was obtained from National Centre for Cell Sciences (NCCS), Pune, India. These cells were cultured and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin-amphotericin B cocktail at 37° C temperature with 5% CO_2 in a humidified cell culture incubator.

2.4 Extract preparation

For preparation of methanol extract of bark of *A. salviifolium*, it was air dried and grounded and then subjected to Soxhlet extractor. The extract was then concentrated in rotary vacuum evaporator at 45°C and freeze lyophilized to get the powder form.

MBEAS in the dried powder form were weighed and dissolved in dimethyl sulfoxide (DMSO) for making the stock solution. For the use in experiments, it was diluted in culture medium at required concentrations, in which DMSO concentration doesn't exceed the final concentration of 0.1% in all the treatment groups. The stock was stored at -20°C and used for future experiments.

2.5 Trypan blue assay

To evaluate the cytotoxic effect of MBEAS, trypan blue exclusion assay was performed. A431 and B16F10 cells were seeded at the density of $1x10^5$ cells/well in 60 mm plates. Cancer cells were treated with *A. salviifolium* bark extract at the concentration of 0.05, 0.1 and 0.5 µg/ml for 24 h and 48 h. DMSO was present in all the groups with the final concentration not exceeding 0.1%, v/v. After each treatment time points, cells were collected and mixed with trypan blue and counted using hemocytometer under the phase contrast microscope at 100X magnification, (Bhat, Nambiar, Pal, Agarwal, & Singh, 2012). Counting was done in duplicate and its average value was taken and the experimental groups were in triplicate. The experiment was repeated twice.

Total cells were calculated using the following formula:

Total cells = average number of cells counted in each chamber X dilution factor X 10^4 cells/ ml.

2.6 Clonogenic assay

To assess the cell survival and proliferation ability, colony assay was performed. Seeding density was 500 cells per well in 6 well culture plate, and the cells were allowed to attach for 24 h. After that, cells were treated with MBEAS (0.05, 0.1 and 0.5 µg/ml) for 24 h. Post treatment time; media was replaced with fresh complete culture media and incubated in CO_2 incubator. Experiment was terminated on 7th day. Plates were gently washed with PBS, cells were fixed with 2 ml of fixative (12.5% acetic acid and 30% methanol in DW) for 5 mins at RT. Colonies were stained with 2 ml of crystal violet (0.5%) for 15-20 mins at RT. Colonies were observed and counted under phase contrast microscope at 100X magnification >50 cells were included in the colony (Nambiar et al., 2015). Experiment was performed in triplicates and repeated twice.

Colony formation rate (%) was calculated using the formula;

Colony formation rate (%) = (number of colonies formed/ total number of cell seeded) X 100

2.7 AO/EtBr assay

After the desired treatment time point of 24 h cells were collected by trypsinization and centrifuged. Cell pellet was washed with PBS (1X) and then suspended in 100 µl of PBS. Then from this, 10µl of cell suspension was incubated with 2µl of AO: EtBr (1:1 of 100 µg/ml stock solution) for 2 minutes. Stained suspension was placed on microscopic slide and covered with a glass cover slip, such that there is no air bubble trapped. Cells were examined under the fluorescence microscope at 100 X magnifications (Kasibhatla et al., 2006). A minimum of 200 were counted for analysis of the live, early apoptotic, late apoptotic, and necrotic population in the control and treated groups. Total apoptotic cells included both early and late apoptotic populations in each sample.

2.8 Statistical analysis

Statistical analysis was done using GraphPad Prism5 software. Difference between groups was analyzed by Student's t-test. All the data are represented as the mean ± standard error. A value of $p \leq 0.05$ indicates a significant difference between the groups.

3 RESULTS

3.1 MBEAS decreases the proliferation of A431 and B16F10 cells

In vitro cytotoxicity of the extract was determined by trypan blue dye exclusion assay on A431 and B16F10 cells. On treatment of A431 cells with MBEAS for 24 h, the total cell number decreased by 40 %, 60 % ($p< 0.001$) at lower concentrations of 0.5 and 0.1 µg/ml and approximately 66 % ($p<0.001$) decrease at the concentration of 0.5 µg/ml. With increased time period of exposure of cells to MBEAS, total cell number decreased significantly by around 80 to 90% ($p<0.001$) at all the three concentrations (0.05. 0.1, 0.5 µg/ml) after 48 h of treatment (Figure 1a and 1b). In case of B16F10 cells, MBEAS was highly cytotoxic with around 70 % ($p<0.01$) decrease in total cell number after 24 h of

treatment and at higher concentration after 48 h of treatment, cells were barely left with around 90 % ($p<0.01$) decrease in total cell number (Figure 1c and 1d). Hence, MBEAS was found to be cytotoxic for both the cancer cell lines at all the three concentrations (0.05, 0.1, 0.5 µg/ml) after 24 and 48 h of treatments.

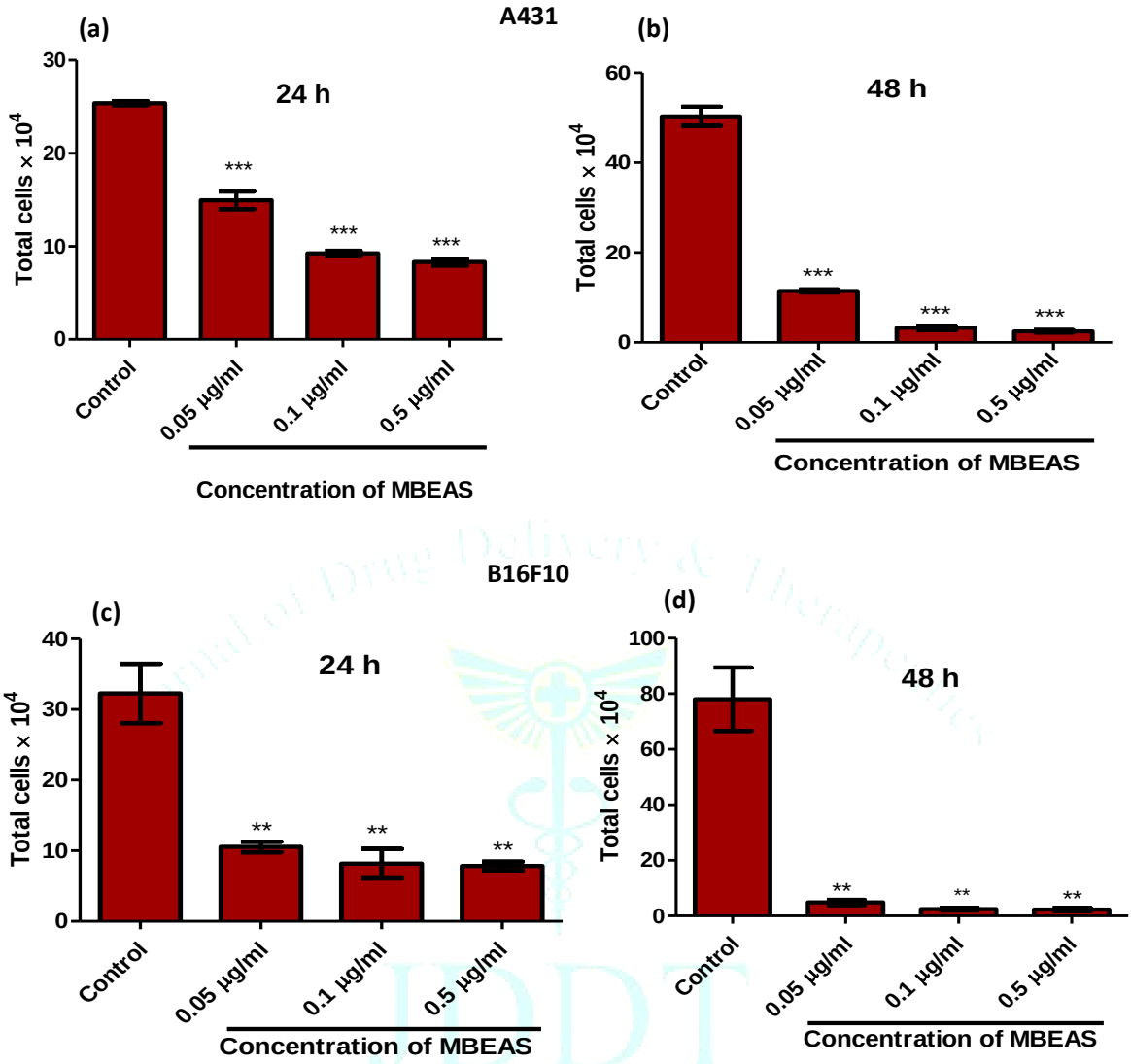

Figure 1: Effect of MBEAS on cell viability of skin cancer cells. Quantitative data of trypan blue exclusion assay performed on A431 and B16F10 cells after 24 h and 48 h treatment with MBEAS. Data are represented as total cell number as percent of control in A431 cells at (a) 24 h, (b) 48 h and B16F10 cells at (c) 24 h, (d) 48 h with treatments of MBEAS. Counting was done in duplicate and the experimental repeat was in triplicate. Data are represented as mean ± SEM, **p< 0.01 and ***p<0.001 is the significance difference compared to control.

3.2 Decrease in the clonogenicity of skin cancer cells by MBEAS

On treatment with MBEAS for 24 h resulted in decrease in colony numbers as well as the colony forming rate in both A431 and B16F10 cells. The colony having >50 cells were counted; with increase in concentration of treatment the size of the colony was also highly reduced. In A431 cells, treatment with 0.05 µg/ml concentration of MBEAS showed 80 % decrease ($p< 0.01$) in number of colonies and colony forming rate (Figure 2b and 2c) as compared to control. In B16F10 cells the reduction was almost 90% ($p<0.05$) as compared to control. With higher concentration of MBEAS colony size was too small (Figure 2d and 2e). The results indicate that MBEAS causes a strong decrease in the proliferation ability of skin cancer cells.

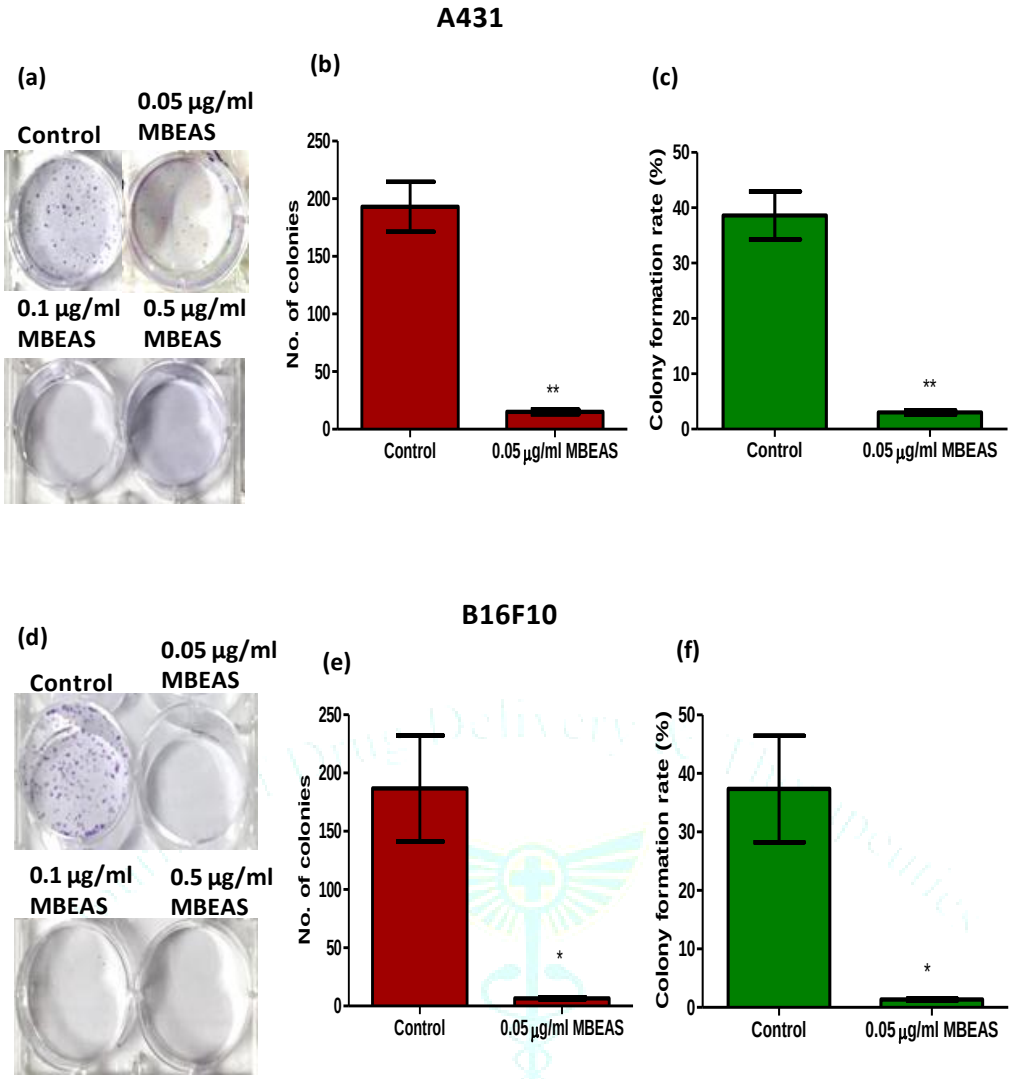

Figure 2: Effect of MBEAS on clonogenicity of skin cancer cells. A431 and B16F10 cells were allowed to form colony post 24 h treatment with MBEAS, the colony having >50 cells were counted and evaluated. Representative data of colony formation assay (a) and (d). Quantitative data are represented as number of colony and colony formation rate compared to control of A431 (b), (c) and B16F10 cells (e) and (f). The data are represented as mean ± SEM, *p<0.05, **p<0.01 represents the significance difference as compared to control.

3.3 MBEAS treatment induces apoptosis in A431 and B16F10 cells

Further to study the cell death effect of MBEAS, AO/EtBr staining assay was performed. Acridine orange and ethidium bromide dual staining method are used to stain necrotic and apoptotic cells. Microscopy was performed and cells in different fields were counted, population of necrotic cells was not found to be significant. After 24 h treatment of A431 cells with MBEAS showed an increase in apoptotic cells percentage in a concentration-dependent manner. The increase was around 10%, 12% ($p<0.1$) and 15% ($p<0.1$) for 0.05, 0.1 and 0.5 µg/ml concentrations respectively, as compared to control (Figure 3). Similarly, there was also an increase in percentage of apoptotic cells in case of B16F10 cells. The increase was concentration-dependent with around 4%, 6% ($p<0.05$) and 8% for 0.05, 0.1 and 0.5 µg/ml concentration respectively as compared to control (Figure 3). This observation indicated the apoptosis-inducing effect of MBEAS in skin cancer cells.

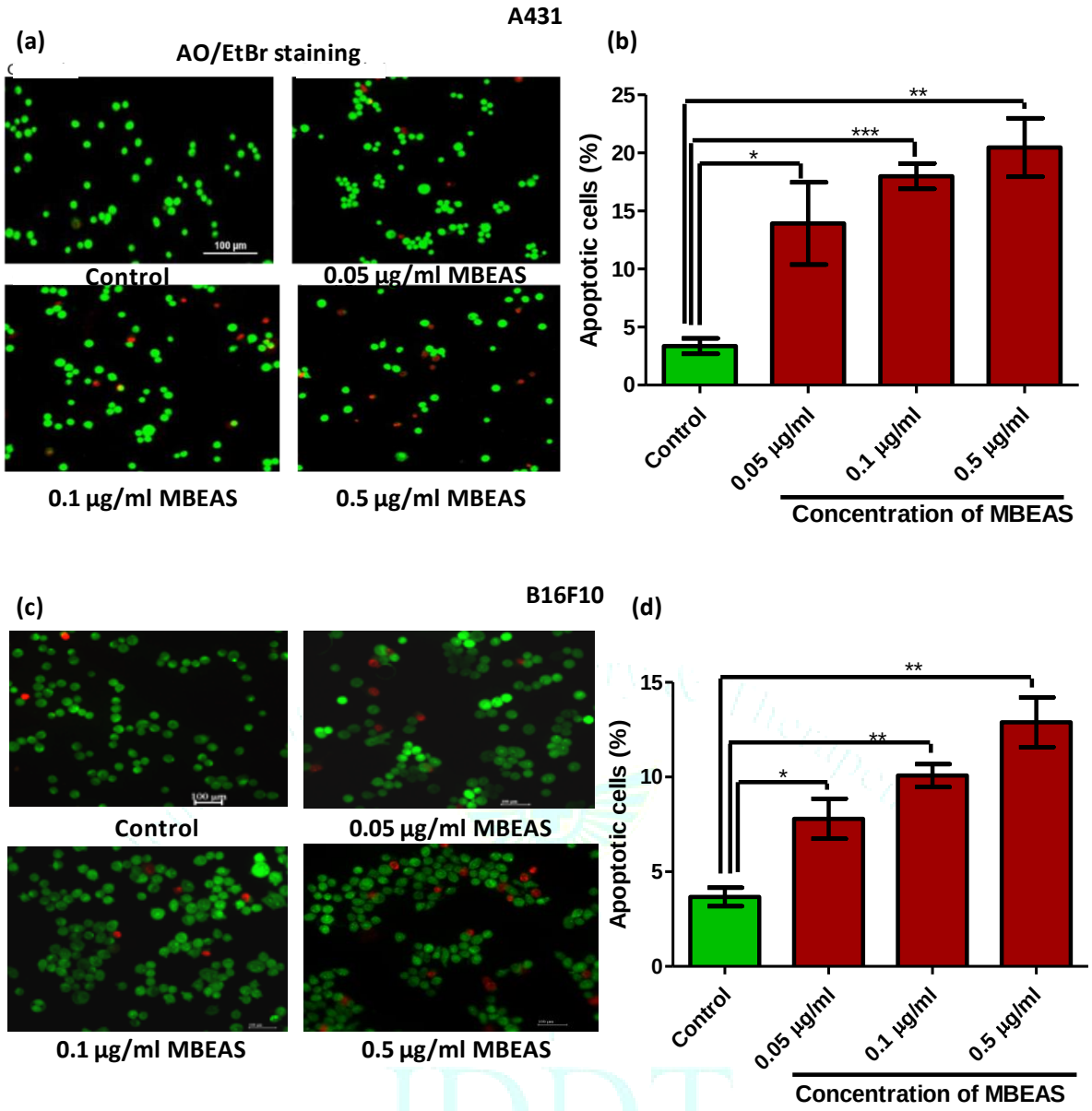

Figure 3: Effect of MBEAS on apoptosis in skin cancer cells. AO/EtBr dual staining assay was performed on A431 and B16F10 cells to detect the necrotic and apoptotic cells after 24 h treatment with MBEAS. Representative images (a) and (c) and quantitative data (b) and (d) of A431 and B16F10 cells showing apoptotic cells. Total 200 cells were counted to quantitate the apoptotic population in the respective treatment groups. Experiment was performed in triplicate and data are represented as mean ± SEM. *p<0.05 signifies the significant difference compared to control.

4 DISCUSSION

Studies on cancer cell lines have shown the anticancer activity of test compounds is exhibited by inhibiting the cell proliferation as well as by inducing apoptosis. The end result of apoptosis may be brought about by causing DNA damage and arresting the cell cycle (Ahamad et al., 2014; Huang et al., 2013; Ji Hye Kim, 2015; Pieme et al., 2014). Results from the present study also suggest the antiproliferative activity of MBEAS accompanied with induction of apoptosis in A431 and B16F10 cells.

The literature available about *A. salviifolium* have described it as a medicinal plant. The phytochemical analysis of bark of *A. salviifolium* has shown the presence of flavonoids, phenols, steroids and saponins (Tanwer and Vijayvergia, 2014). The flavonoids and phenolic compounds present in plants contribute to the antioxidant and therefore the cancer preventive activity which has been observed in many plants studied. Methanolic leave and stem extract of *A. salviifolium* showed maximum antioxidant activity as compared to other solvent extracts and thus it might contribute to the anticancer activity of *A. salviifolium*. Many medicinal plants have been extensively evaluated for their anticancer activities and has received great attention recently. Camptothecin is an alkaloid that has exhibited anticancer activities both *in vitro* and *in vivo*. It is isolated from the plant, *Mappia foetida* and has shown inhibitory activity against cancer cells. Clinical trials have been underway for camptothecins and its analogues (Wall and Wani, 1977; Potmesil, 1994). *Boswellia serrata* has been evaluated for its medicinal properties and many of its pharmacological properties have been documented. Triterpenediol isolated from it is cytotoxic against many cancer cells (Syrovets et al,

2005; Hostanska et al, 2002; Liu et al, 1998). It inhibits DNA synthesis, topoisomerase induces apoptosis (Shao et al, 1998; Lee et al 1966).

There have been previous reports that showed the anticancer activity of *A. salviifolium* against various *in vitro* and *in vivo* models of cancer. It inhibits the growth of Daltons ascetic lymphoma murine cells (Venkateshwarlu et al, 2012). Flowers of *A. salviifolium* showed anticancer potential against Ehrlich ascites carcinoma model in mice and substantially increased the life span of tumor-bearing mice (Haque, 2011; Nahar et al, 2012). Some of the alkaloids isolated from the stem of *A. salviifolium* exhibited antioxidant activity and selectively inhibited the growth of HepG2 cancer cells (Phanruethai et al, 2011).

Currently, no study has been carried out using the bark extract of *A. salviifolium* against skin cancer cells to evaluate its anticancer potential. So, the present study was carried out and we explored the anticancer activity of MBEAS on skin cancer A431 and B16F10 cells. Bark extract of *A. salviifolium* efficiently decreased the cell viability, cell proliferation and clonogenicity of epidermoid carcinoma A431cells and melanoma B16F10 cells at its very low concentrations of 0.05, 0.1, 0.5 µg/ml. For cytotoxicity study trypan blue exclusion assay was performed, live cells do not take up the dye due to membrane integrity hence it only stains the dead cells. There was significant decrease in cell number in MBEAS treatment groups compared to control suggesting that the increased cell death in addition to inhibition of cell proliferation in these treated groups. Further to check the effect on cell growth, colony formation assay was performed that indicated the decreased ability of cells to form colony in MBEAS treated groups as compared to control. The colony size was remarkably reduced at higher concentration of MBEAS.

Further our results from AO/EtBr assay indicated that MBEAS induced apoptosis in A431 and B16F10 cells at all the thee tested concentrations (0.05, 0.1, 0.5 µg/ml) after 24 h of treatment. Acridine orange (AO) stains all the cells, live cells as well as cells undergoing cell death. Live cells are stained evenly with AO but EtBr is only taken up by the cells undergoing death as the membrane integrity is lost and it is permeable to cells, where it goes and intercalates within DNA. Necrotic cells get evenly stained and the cell size increases where as in the apoptotic cells, chomatin condensation and fragmentation is visible.

This is a novel study reporting the cytotoxic effect of bark extract of *A. salviifolium* against human as well as mouse skin cancer cells. The results from the present study on skin cancer cell lines provide a first line evidence for the anticancer activity of *A. salviifolium* bark.

5 CONCLUSION

In summary, the results from the study report the antiproliferative activity of MBEAS against both melanoma and non-melanoma cancer cells. It inhibited the growth of A431 and B16F10 cells, showed cytotoxicity by inducing apoptosis in both the cell lines. Hence it suggests that *A. salviifolium* has potential to be developed as an anticancer agent against skin cancer but for that further detailed study including *in vivo* studies will be required to be carried out to better understand the efficacy and implications of *A. salviifolium* in cancer management.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest for this article.

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