Butea Monosperma Shows Anti-cancer and Anti-Oxidant Activity: *In-vitro* and *In vivo* Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Breast cancer is one of the predominant cancers in Indian subcontinent and the global scenario is not very different. Treatment of this type of cancer is possible by chemotherapy, surgery, and radiotherapy, which relieve the patient; however, off target activity and severe toxicity of non-cancerous cells remained the major drawbacks of these chemotherapeutics. In order to overcome these issues, modern cancer therapeutics emphasizes the implication of natural bioactive compounds. In this current piece of work, we have studied the anti-cancer potentiality of *Butea monosperma* flower in *in vitro* breast cancer cells. Various extracts of this flower were found to be non-toxic to breast epithelial cells but toxic to breast cancer cells. However, the methanol extract of this flower was most effective amongst all other extracts. Data suggest that *Butea monosperma* flower induce DNA damage mediated apoptosis and inhibit cell proliferation, angiogenesis and metastasis. It also inhibits the angiogenesis and metastasis in xenograft mice model system. Moreover, the anti-cancer phenomenon was found to be induction of inflammatory cytokines mediated oxidative stress in breast cancer. Collectively, *Butea monosperma* flower shows excellent cancer therapeutics in order to induce DNA damage, inhibit angiogenesis and metastasis simultaneously.

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1. INTRODUCTION

Breast cancer is the most common non-skin cancer and primary cause of mortality among women around the world. About 2 million new breast cancer cases were diagnosed in 2020 with almost 60% of deaths occurring in developing countries and it is the most commonly diagnosed cancers worldwide in 2020 [1-4]. It is predicted that one in every eight females in the world will develop breast cancer and only 5-10% of cases of this cancer are associated with genetic disorders, whereas the remaining 90-95% of cases are linked to environmental and lifestyle factors. It is a hormonal malignancy which is influenced by a number of hormones and growth factors [5-8]. Despite the advancements in diagnosis and therapy, relapse and metastasis of breast cancer is commonly seen after treatment. Screening and diagnosis of breast cancer can be done by physical examination by qualified healthcare provider, mammography, ductography, ultrasonography or MRI. Though surgery remains the mainstay of breast cancer treatment [7-9], other frontline therapeutic approaches are radiation therapy, hormone therapy, chemotherapy or monoclonal antibody based targeted therapy [10-17].

Majority of the chemotherapeutic agents are associated with many drawbacks like cytotoxicity. Now a days, natural products are showing promising results in order elucidate cancer and among them *Allium sativum, Vinca rosea, Taxus wallichiana, Viscum album, Panax pseudoginseng, Zingiber officinale, Tinospora cordifolia* are in the front line of cancer therapeutic [18]. In addition, *Butea monosperma* (Palash) flower is found to be natural, non-toxic, bioactive compounds and do not affect the healthy cells in the body. *Butea monosperma* of Leguminosae family is a well-known traditionally used remedy for a numerous liver disease in India. An Ayurvedic medicine have been utilizing bark leaves, flowers, gum, and in many cases the seeds of *Butea monosperma* as herbal remedies. Various parts of Butea monosperma is reported to be used as remedy for anti-diarrhoeal, antiovulatory, anti-implantation, anti-eastrogenic, anthelminthic, fungicidal, bactericidal, and antitumour properties against hepatic carcinoma [19-25].

The methanol extracts collected from powdered *Butea monosperma* is reported to show effects against tumour promotion or carcinogenesis in rat liver. Isobutrin and Butrin are the two major constituents associated with the protective activity of plant. The aqueous extract of flowers of *B. monosperma* can be used as a treatment for hepatic carcinoma. It also inhibits cell proliferation and accumulation of cells in G1 phase. It was followed by initiation of apoptotic cell death. Butein, butrin, isobutrin, and chalcones are some of the principles of *B. monosperma* that has shown a lot of antiproliferative effect on human tumour cells and also breast carcinoma. The plant polyphenol butein is known to hinder testosterone induced augmentation in breast cancer cells expressing aromatase [20-22,26-30].

In this current piece of study, we have performed the *in vitro* evaluation of anti-cancer & anti-oxidant activity of crude extract of *Butea monosperma* flower by using human breast cancer cell line. We have also validated our result by our developed mice xenograft model system.

2. MATERIALS AND METHODS

2.1 Cell Culture and Chemicals

MCF-10A (normal breast epithelial cell line), MCF-7 and MDA-MB-231 breast cancer cell lines were procured from ATCC, Manassas, VA, USA. The cells were maintained in DMEM and RPMI (50:50, v/v) medium supplemented with 10% FBS, 1% antibiotic (100 U/mL of penicillin, 10 mg/mL of streptomycin in 0.9% normal saline) and 0.5 mg/mL of hydrocortisone at 37^0C in a humidified 95% air and 5% CO_2 incubator as described by Chatterjee et al. [31].

All the cell culture chemicals were purchased from GIBCO, ThermoFisher Scientific, India. Antibodies like anti-CD-44 (E-AB-11364), anti-CD133 (E-AB-33462), anti-GAPDH (E-AB-20072), anti-BCL-XL (E-AB-30640), anti-BAX (E-AB-13814), anti-CASPASE-3 (E-AB-13815), antibodies were purchased from Elabscience Biotechnology Inc. Anti-ANG-1 (ab8451), anti-VEGF-A (ab1316) antibodies were procured from Abcum, Cambridge, United Kingdom. Anti-mouse (SC516102), anti-rabbit (SC2357) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti- ANG-2 (#2948), anti-TGF-β (#3711),

MMP-9 (#3852), MMP-2 (#40994) antibodies were bought from Cell Signaling Technology (MA, USA). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) used for this study were purchased from Sigma Chemicals (Sigma, St Louis, MO, USA).

2.2 Cell Proliferation and Viability Assay

The anchorage-dependent cell viability of different plant extract treated cells was measured by performing MTT assay [31]. Briefly, exponentially growing MCF-7 and MDA-MB-231 (8000–10,000 cells/well) were seeded in 96 well plates and allowed to grow for 70–80% confluence. Cells were treated with increasing concentration of various extracts (aqueous, methanol, butanol and ethyl acetate) of *Butea monosperma* flowers (AEBM; MEBM; BEBM and EAEBM) for 24h, 48h and 72 h, respectively. After washing the cells with 1X PBS, 0.05% MTT was added to each well and incubated at 37°C for 5h for the formation of formazan crystals. After 1h incubation in the dark, the crystals were dissolved by the addition of 100µL of 0.2% NP-40 detergent. Then the color intensity was measured spectrophotometrically at 570 nm by using a microplate reader. The data obtained were presented graphically as percentage viability against concentrations of the plant extracts. Each data point was calculated in triplicate and all the assays were performed at least thrice.

2.3 Measurement of DNA Damage by Alkaline Single Cell Gel Electrophoresis (SCGE) or Comet Assay

To measure the DNA damaging potentiality of the plant extract in the breast cancer cells, single-cell gel electrophoresis or comet assay was performed [32]. Approximately, 8000-10,000 MCF-7 cells were seeded per well in a 96-well tissue culture plate. After 24h, cells were treated with MEBM (180µg/ml) for 24h. Then the cells were harvested, washed with 1X PBS, and re-suspended in ice-cold 1X PBS. Next, the samples were stained with SYBR® green dye (Sigma-Aldrich), incubated in the dark for 30 min at room temperature and the migration of DNA was observed using a fluorescence microscope (Nikon, Japan) at a magnification of 20X. Comet lengths were analyzed by TriTek Comet Score™ software (Tritek Corporation, VA, USA).

2.4 γH2AX Immunofluorescence Assay

An immunofluorescence assay was performed to check the expression of γH2AX, a DNA damage marker [32]. MCF-7 cells were grown on 96-well tissue culture plate and treated with the plant extract for indicated time duration. Then the cells were washed with 1X PBS and fixed with acetone: methanol (1:1) for 20 mins at -20°C. Cells were washed twice with 1X PBS and incubated with primary antibody anti–γH2AX (1:500 dilutions in 1X PBS) for 2h at 37°C. Then unbound antibodies were removed by washing twice with 1X PBS and incubated with secondary antibody conjugated with TRITC (1:750 dilutions in 1X PBS) for 1 h at 37°C. Cells were washed thrice with 1X PBS and nuclei were counter-stained with DAPI. Finally, the images were captured using an inverted fluorescence microscope (Nikon, Japan) at 20X magnification.

2.5 DAPI Nuclear Staining

To determine the DNA damage mediated apoptosis of breast cancer cells after treatment with the indicated plant extracts, 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining was carried out [33]. 70–80% confluent cells were treated with the desired plant extract for the indicated time period. The cells were then washed with 1X PBS and fixed with acetone: methanol (1:1) at 20°C in the dark for 15mins. Fixed cells were washed once with 1X PBS and then DAPI solution was added and incubation was continued for 1h at 37°C in the dark. Excess DAPI was removed by washing with 1X PBS and the stained cells were visualized under a fluorescence microscope (Nikon, Japan) at 40X magnification.

2.6 Matrigel Invasion Assay

The matrigel invasion assay was performed using a 24 well transwell plate (#3422, Corning, NY, USA) with a pore size of 8 µM; the inserts were coated with 20 µl of matrigel (356234, BD Biosciences, CA, USA) [32] 3×10^5 cells were resuspended in 100 µl of serum free media and seeded either on uncoated inserts (migration) or on the matrigel coated inserts (invasion). Complete media was added to the lower chamber, incubated for 24 h. Non-invaded cells were removed, and invaded cells were fixed with 4% paraformaldehyde, stained with DAPI and counted at 40X under the fluorescence microscope. The data were expressed as %

invasion against cell types. % invasion was calculated using the following formula:

% Invasion = [Number of cells invaded the matrigel coated inserts towards the complete media / Number of cells migrated the uncoated inserts towards the complete media] × 100.

2.7 Western Blot Analysis

Western blot analysis was performed to check the expressions of several apoptotic, anti-apoptotic and angiogenic markers [32] MCF-7 cells were trypsinized and cultured in 96-well plates at a cell density of 8000-10,000 per well. Then the cells were treated with above-mentioned plant extract for indicated concentrations and time duration. Whole-cell extracts were isolated using RIPA lysis buffer (1% Triton X-100, 20mM Tris–HCl pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM sodium pyrophosphate, 1μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mg/ml PMSF). The extracts were then fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated successively with the primary (1:1000 dilution) and secondary (1:2000 dilution) antibodies and exposed to the chemiluminescence reagent for signal detection. Densiometric analysis was done by using ImageJ software.

ELISA: Expressions of Ki-67 and various other angiogenic markers (like VEGF-A, ANG-1, ANG-2) were measured by ELISA [32]. Protein antigens were coated onto 96 well microplates (3679, Corning, NY USA) with coupling buffer prior to blocking with super cocktail buffer in a triplicate manner. Then, primary (1:5000 dilution) followed by HRP conjugated secondary (1:10000 dilution) antibodies were added. Lastly, absorbance was measured at 405nm using a microplate reader (Berthold, Germany) after addition of 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution.

Annexin V-FITC/PI dual staining for apoptosis measurement: Annexin-V-FITC/PI dual staining was performed to measure the plant extract mediated apoptosis in MCF-7 breast cancer cells [33]. Briefly, 8000-10,000 cells were seeded in each well of a 96-well cell culture plate and incubated for 24h. After incubation, treatment procedure was followed as mentioned earlier. Cells were harvested post-incubation by trypsinization and washed twice with 1X PBS. Then the cells were resuspended in 1X binding buffer and stained with Annexin-V-FITC and PI detection kit (Sigma) according to the protocol provided by the manufacturer. The stained cells were incubated for 10 min and analyzed using a FACS Canto-II flow cytometer (BD, USA).

Determination of oxidative stress, enzymatic antioxidant and catalase activity: After the above-mentioned exposures of MEBMin MCF-7 cells, reduced Glutathione (GSH) level was measured by commercially available ELISA kits according to manufacturer's instructions (Cayman, Ann Arbor, MI). Also, level of Superoxide Dismutase (SOD) was measured according to manufacturer's instructions (Cayman, Ann Arbor, MI) as markers of enzymatic antioxidant activity. In addition to this, catalase and malondialdehyde (MDA) levels were also measured according to the kit method.

Establishment of mice xenograft model system: Basic and translational breast cancer research relies heavily on experimental animal models [34]. Mice xenograft model was developed to check the effectiveness of the plant extracts in *in vivo* model system [35-36]. 6 weeks old female nude mice were housed in a proper light/dark cycle of 12/12 h. 1×10^7 MCF-7 cells in 200μl freshly prepared sterile 1X PBS were injected into the left mammary fat pads of 3 different groups of mice (each group contained 5 mice). First group of mice were used as control and other 2 groups of mice were implanted with MCF-7 cells for tumor development. Tumor growth and mice health were monitored regularly. After 25 days of implantation of cells when a measurable amount of tumor was noticed, then 100μl of MEBM (at their IC_{50} concentrations of *in vitro* system) were administered in 2 groups by oral route. Mice body weight was measured every alternative day for 55 days. Tumor dimensions were measured using slide calipers, and tumor volumes were calculated using the formula: $(W^2 \times L)/2$, where width (W)≤length (L). Then, the mice were euthanized and tumor tissues were collected and processed for H&E staining and immunohistochemistry (IHC) analysis.

Histopathological study (H&E staining) analysis of mice tissue: H&E staining was performed to check the true establishment of mice xenograft model [33]. For this, paraffin-embedded mice breast tissues were cut into a section of 5μm thickness, mounted on slides and heated at 60°C for 30–40 min. Tissue sections were deparaffinized with xylene and rehydrated

with immersing in decreasing concentrations of alcohols (100%, 90%, 70% and 50%, respectively). Then the slides were dipped into hematoxylin followed by eosin stain (H&E) (Fig, No. 7C) and rinsed in distilled water. Then slides were subjected to dehydrate by dipping in increasing concentrations of alcohols (50%, 70%, 90% and 100%) and finally incubated in xylene for 2mins. The sections were mounted by using DPX mountant. Images were captured in bright-field microscope (Leica DM200, USA) at 20X magnification.

Immunohistochemistry (IHC) analysis of xenograft mice tissue: For IHC, tissue specimens were dewaxed with xylene, rehydrated with decreasing concentrations of ethanol (100%, 90%, and 70%, respectively) [33]. Next, the tissue sections were washed with 1X PBS and the antigen was retrieved by citric acid buffer (pH 6). In slides, endogenous peroxide activity and nonspecific sites were blocked by 5% fetal bovine serum (FBS) and hydrogen peroxide, respectively. The sections were incubated with primary antibody at 4°C followed by washing 3 times with 1X PBS. Next, the sections were incubated with HRP-conjugated secondary antibody. The slides were washed with 1X PBS and visualized using 3,3-Diaminobenzidine (DAB) peroxidase substrate kit (SK-4100, Vector Laboratories, CA, USA) followed by hematoxylin counter stain. The sections were then mounted with DPX. The images were captured at 20X magnification using bright-field microscope (Leica DM200, USA).

2.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software, USA. Results represented here were the mean ±standard deviation (SD) of three separate experiments. Data were analyzed using one-way and two-way ANOVA where applicable, followed by Bonferroni's multiple comparison tests. Statistical significance of difference in the central tendencies compared to control groups was designated as '*' ($P < 0.05$), '**' ($P < 0.001$) and '***' ($P < 0.0001$).

3. RESULTS

Various extracts of Butea monosperma inhibits the breast cancer cells viability in time dependant manner: The short-term cell viability effect on normal breast epithelial cells (MCF-10A cell line) after the exposure of various extracts (aqueous, methanol, butanol and ethyl acetate) of *Butea monosperma* flowers (AEBM; MEBM; BEBM and EAEBM) was studied by MTT assay. In our present study, none of the above-mentioned extracts showed cytotoxic action on non-cancerous MCF-10A cells at different time points (24h, 48h and 72h) (Fig. 1).

Then, the cell proliferative study was performed after the exposure of above-mentioned extracts of *Butea monosperma* flowers of on ER +ve breast cancer cell line, MCF-7 and triple negative (ER –ve, PR –ve, HER-2 –ve) human breast cancer cell line MDA-MB-231. The cytotoxicity was found to be dose and time dependent. On MCF-7, the extracts exhibited antiproliferative activity in following order: MEBM> AEBM> BEBM> EAEBM. (Fig. 2A). The IC_{50} values of the extracts are documented in (Table 1). However, when we exposed MDA-MB-231 cell line with MEBM (found to be more toxic in MCF-7 cell line), IC_{50} value was noted to be at 1000 µg/ml. (Fig. 2B).

Table 1. IC_{50} values of various extracts of Butea monosperma flowers; on human breast cancer cell lines (MCF-7 and MDA-MB-231). All values are in µg/ml. "-" represents that IC_{50} value is above 1000 µg/ml

Cell lines	Hours	Extracts of Butea monosperma flowers			
		AEBM	**MEBM**	**BEBM**	**EAEBM**
MCF- 7	24	400	180	690	-
	48	340	100	500	800
	72	300	50	450	500
MDA- MB-231	24	-	-	-	-
	48	-	-	-	-
	72	-	1000	-	-
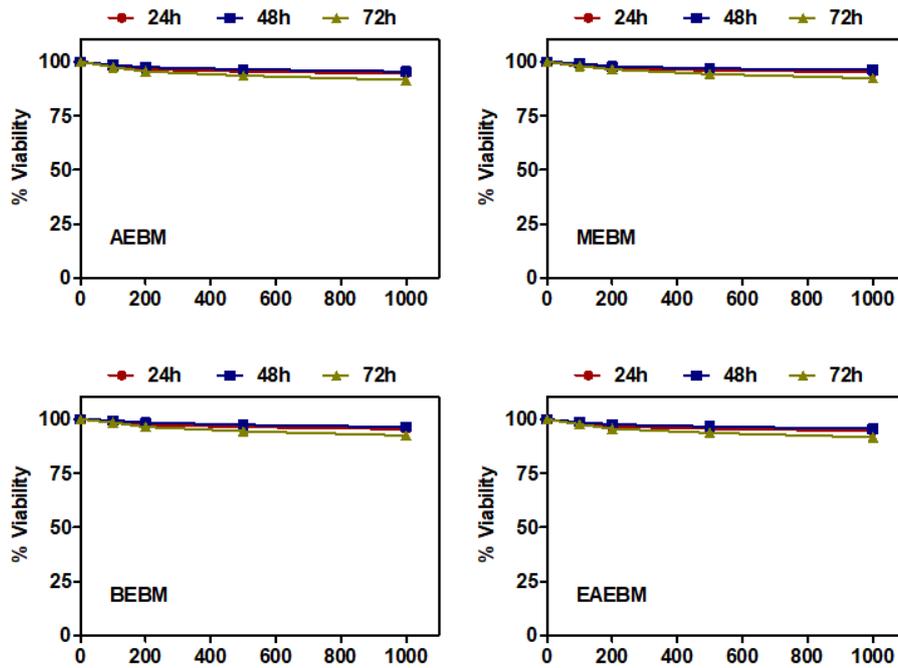
Fig. 1. Effect of various extracts of Butea monosperma flowers on cell viability of MCF-10A cell line at different time points (24h, 48h and 72h). Values were represented as Mean ± SD of 3 independent experiments
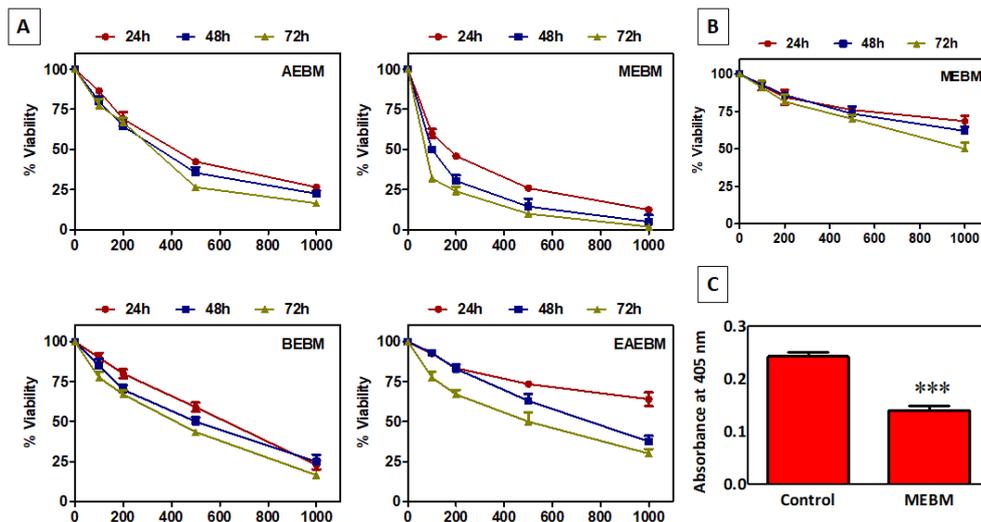
Fig. 2. Effect of various extracts of Butea monosperma flowers on cell viability and cell proliferation of MCF-7 (A) and MDA-MB-231 (B) breast cancer cell line. (C) Expression of Ki-67 in MCF-7 human breast cancer cell line after the exposure of 180 µg/ml MEBM. Values were represented as Mean ± SD of 3 independent experiments

Taking these data in consideration, in the present study, we have used MCF-7 as an *in-vitro* breast cancer model system and we have used MEBM at its IC_{50} values (24h) to evaluate the anti-cancer and anti-oxidant mechanism.

Further, we have checked the anti-cell proliferative abilities of the above-mentioned plant extract by checking the expression of Ki-67 in MCF-7 cell line (Fig. 2C). Approximately, 1.5-fold decrease in Ki-67 expression was

noted upon the 24h exposure of 180µg/ml MEBM (Fig. 2C).

***Butea monosperma* flower (MEBM) caused DNA damage in MCF-7 breast cancer cell line:** From MTT assay, we got the idea that MEBM caused reduce in cell population in MCF-7 cell line. Reduced expression of KI-67 upon MEBM treatment also supported that fact. Further to check if this reduces in cell viability is through DNA damage or not, we have performed alkaline comet assay. (Fig.3A) show the comet tail formation in MEBM treated MCF-7 cell line. Significant comet tails were formed in MEBM treated MCF-7 cells (Fig.3B). To confirm that we have also performed γ-H2AX immunofluorescence assay (Fig.3C). γ-H2AX is a DNA damage marker and statistically higher expression of γ-H2AX was noted in MEBM treated MCF-7 as compared with untreated control (Fig.3C,D).

Butea monosperma flower (MEBM) caused apoptosis mediated cell death of MCF-7 breast *cancer cells:* Above observations appeared the fact that MEBM caused DNA damage in breast cancer cells. To validate this, we have performed DAPI nuclear staining to check the phenotypical microscopic changes of the nucleus upon the exposure of the plant extract. As predicted, exposure of MEBM caused significant toxicity in nucleus (Fig.4A, B).

Further, we wanted to check the apoptotic potentiality of the plant extract and for this we have checked the expressions of some representative apoptotic and anti-apoptotic proteins by western blot analysis. (Fig. 4C) showed the expressions of BCL-XL (anti-apoptotic marker), BAX (apoptotic marker) and CASPASE-3 after the exposure of MEBM in MCF-7 cell line. Higher expressions of BAX and CASPASE-3 and lower expression of BCL-XL were found after the treatment of MEBM (Fig.4C). In MEBM treated cells, approximately 3-fold decrease in expression of BCL-XL, 3.1- and 4.3-fold increase in expression of BAX and CASPASE-3 were noted (Fig. 4C). GAPDH served as loading control.
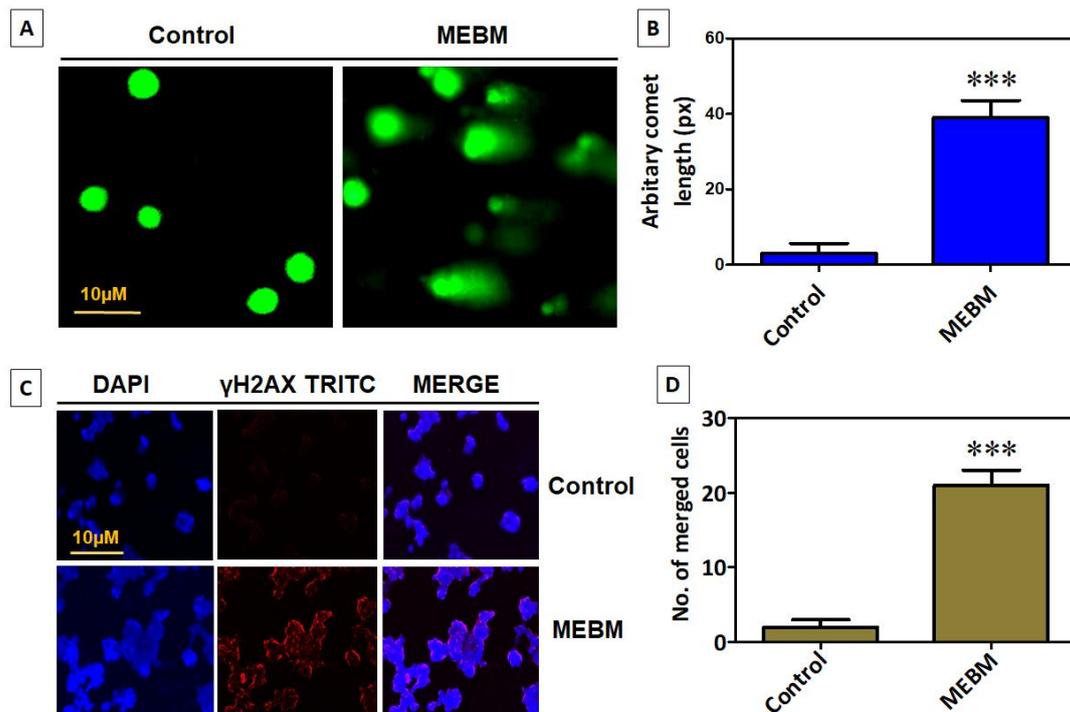
Fig. 3. Methanol extract of Butea monosperma flowers (MEBM) caused DNA damage in MCF-7 cells. (A) Comet assay was performed after the exposure of MEBM in MCF-7 cells. (B) Graphical representation of comet tail length. (C) Expression of γ-H2AX upon the treatment of MEBM. (D) Graphical representation of Figure 4C. Experiments were carried out three times and representative image was provided

Again, to validate this, we have performed Annexin-V-FITC/ PI staining after treating the cells with the plant extract and analyzed by flow cytometry. (Fig. 4D) showed population of cells undergoing early apoptosis, late apoptosis or necrosis in untreated MCF-7 (control) and MEBM treated cells, respectively. Comparatively more cell death [early apoptosis (Q4) + late apoptosis (Q2) + necrosis (Q1)] was noted in MEBM treated cells as compared with untreated control. Approximately, 36% breast cancer cells were undergone cell death upon MEBM treatment (Fig. 4E).

Butea monosperma flower (MEBM) inhibits angiogenesis and metastasis in MCF-7 breast cancer cells: Angiogenesis is the new blood vessel formation and it is one of prime cancerous potentiality and for successful development of cancer therapeutics, the agents ought to have anti-angiogenic potentiality along with their DNA damaging abilities. Hence, we wanted to check if the plant extract has any significant impact on angiogenesis or not.

For this, at first, we have checked the expression of two prime angiogenic marker VEGF-A and TGF-β by western blot analysis. Fig. 5A showed the expression of VEGF-A and TGF-β in MEBM treated MCF-7 cells. Upon exposure of MEBM, approximately, 3.3- and 2.5-fold downregulation of VEGF-A and TGF-β were noted (Fig. 5A).

Degradation of extracellular matrix plays an important role during the extravasation process of angiogenesis and for this, matrix metalloprotease plays prime role. Among these matrix metalloprotease, MMP-9 and MMP-2 play the central role. In our current study, when we checked the expressions of MMP-9 and MMP-2 after the exposure of MEBM in MCF-7 cell line, their expressions were found to be decreased (Fig. 5B). Approximately, 2.5- and 3.3-fold decrease in expressions of MMP-9 and MMP-2 were found in MEBM treated MCF-7 cells (Fig.5B).
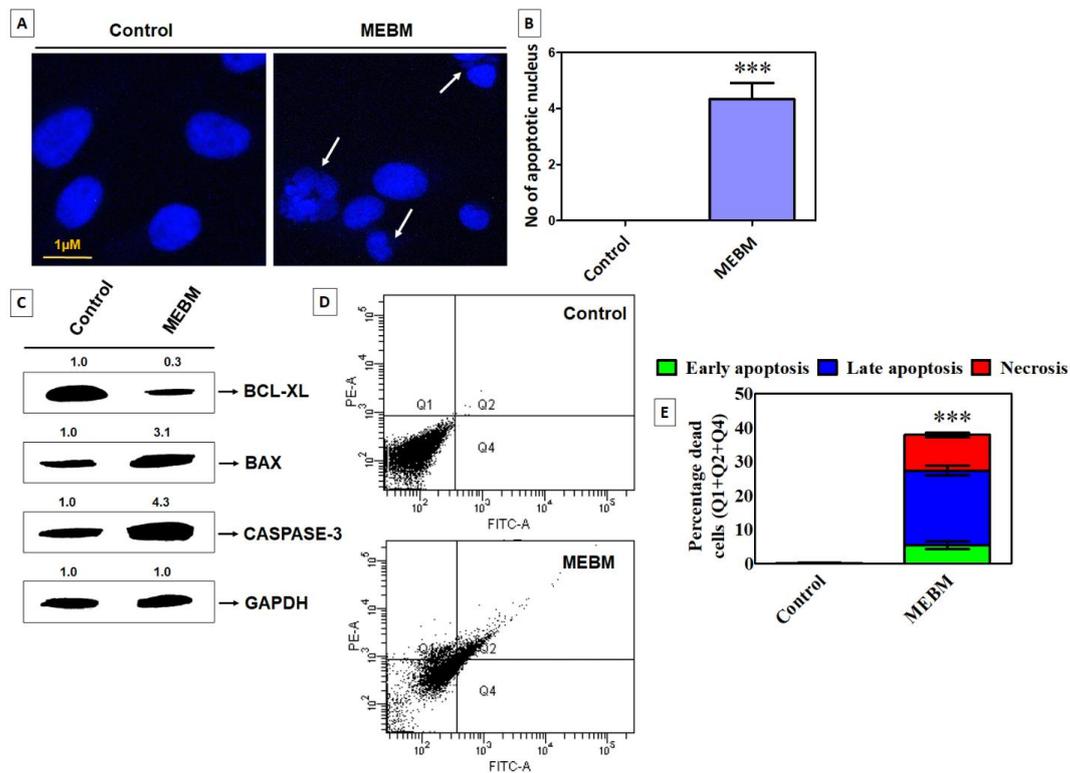
Fig. 4. Methanol extract of Butea monosperma flowers (MEBM) caused apoptosis in MCF-7 cells. (A) DAPI nuclear staining after the exposure of MEBM in MCF-7 cells. (B) Graphical representation of Figure 5A. (C) Expression of BCL-XL, BAX, CASPASE-3 upon the treatment of MEBM. GAPDH served as a loading control. (D) Annexin-V-FITC analysis of cell death population after treating MCF-7 cells with MEBM. (E) Graphical representation of Figure 5D. Experiments were carried out three times and representative data was provided

Further, we have checked the secretory angiogenic markers like Ang-1 (Angiopoetin-1), Ang-2 and VEGF-A in the supernatant of MCF-7 and in the treatment condition of MEBM (Fig.5C, D, E). Here also, decreased expressions of Ang-1, Ang-2 and VEGF-A were observed in MEBM treatment conditions.

Again, we wanted to check the involvement of these plant extracts on metastasis and for this, we have performed matrigel invasion assay and checked the expressions of some prime metastasis marker s (CD-44, CD-133, OCT-4) by Western Blot analysis. Figure 5F shows the percentage of cells invaded in the matrigel, which is indirectly proportional to the anti-invasive potentiality of our plant extracts. From (Fig. 5F) it was found that MEBM was effective in order to inhibit the invasiveness of breast cancer cells. Western Blot analysis of showed the similar observation that down regulation of CD-44, CD-133 and OCT-4 was observed in the treatment condition of MEBM. GAPDH served as a loading control.

Butea monosperma flower (MEBM) mediated anti-cancer activity may be through induction of oxidative stress of breast cancer cells: Further, we wanted to investigate the molecular mechanism behinds the MEBM mediated inhibition of cancer proliferation, angiogenesis or metastasis. For this, the abilities of the plant extract to cause oxidative stress were evaluated by measuring the Superoxide dismutase,

Catalase, MDA and GSH levels in MCF-7 cell line. Literature suggests that induction of oxidative stress was majorly determined by checking the expressions of SOD, Catalase, MDA and GSH.

When we checked the SOD level, upon the treatment of MEBM, SOD level was found to be delineated. (Fig. 6A). Plant extract-induced oxidative stress was further evidenced by depletion of catalase activity in breast cancer cells. Upon the exposure of MEBM, catalase level was found to be down regulated (Fig. 6B). To validate the above observation, we checked the MDA level in different treatment condition of MEBM in breast cancer cells. Elevation of lipid peroxides was noted upon the treatment with plant extract (Fig. 6C). Finally, we have checked the GSH level. Depletion of GSH upon the treatment with MEBM, further supported the plant extract mediated oxidative stress (Fig. 6D).

Further, to illustrate the mechanism behind the MEBM mediated oxidative stress, have checked the expressions of some inflammatory and anti-inflammatory cytokines upon MEBM treatment in MCF-7 cells. Interestingly, inflammatory cytokines levels were found to be uplifted in the treatment condition of MEBM as compared with untreated control. Increase in inflammatory cytokines like TNF-α, IL-1β, IL-6 was noted along with decrease in anti-inflammatory cytokine IL-10 was found in MEBM treatment (Fig. 6E-H).
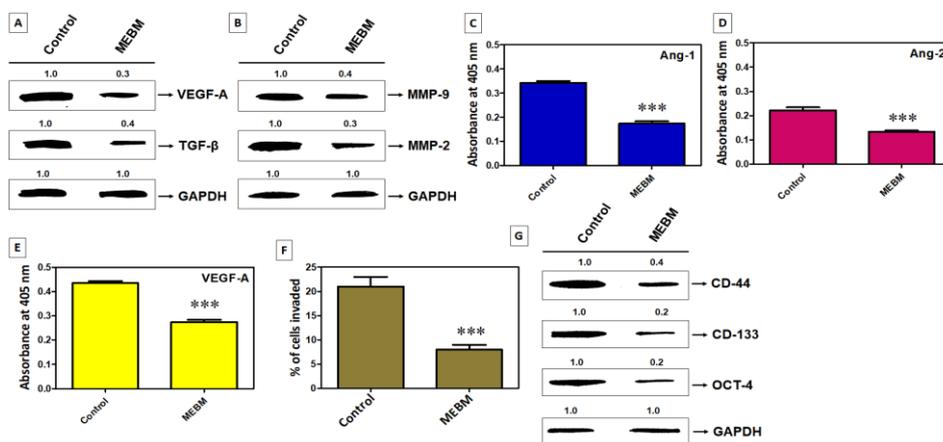
Fig. 5. Methanol extract of Butea monosperma flowers (MEBM) caused inhibition of angiogenesis and metastasis in MCF-7 cells. (A) and (B) Western blot analysis of prime angiogenic proteins. GAPDH served as a loading control. (C), (D and (E) Expression of ANG-1, ANG-2 and VEGF-A in the supernatant of MCF-7 cells upon the treatment of MEBM. (F) Anti-invasiveness potentiality of MEBM in MCF-7 cells. (G) Expressions of prime metastatic markers after the exposure of MEBM in MCF-7 cells. Experiments were carried out three times and representative data was provided
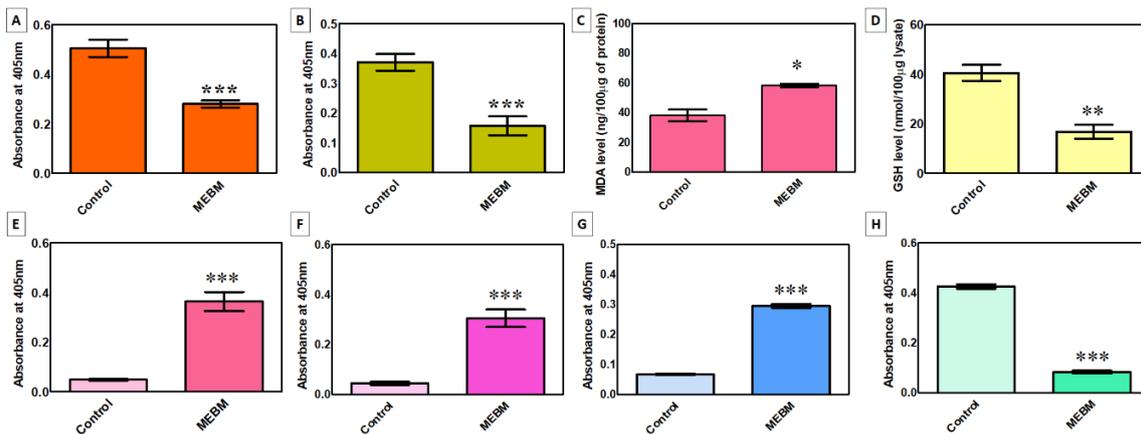
Fig. 6. Methanol extract of Butea monosperma flowers (MEBM) caused induction of oxidative stress in MCF-7 cells. (A)Biochemical detection of Superoxide dismutase, Levels of Catalase (B), lipid peroxides (C) and GSH (D) MCF-7 cells after the treatment of MEBM in MCF-7 breast cancer cells. (E) to (H) Effect of MEBM on pro and anti-inflammatory cytokines in MCF-7 cells. (E-H) are the graphical representation of optical density of cell supernatants at 405 nm for TNF-α, IL-1β, IL-6 and IL-10. Data represents mean ± S.D. of 3 independent experiments

Butea monosperma flower (MEBM) exhibits anti-cancer potentiality in in-vivo mice xenograft model system: Our *in vitro* cell culture-based study revealed the anti-cancer action of above-mentioned different plant extract. To validate the anti-cancer potentiality of the plant extracts, we have developed an *in vivo* mice xenograft model using nude mice. After 10 days implantation of MCF-7 cells, tumor formation was found in the mice. The body weight of the mice was found to be significantly reduced and the tumor volume was elevated day-by-day (Fig. 7A, B). After 25 days of administration of cells, tumor volume was found to be significantly increased and the body weight of mice was noted to be significantly decreased (Fig. 7A, B). Oral administration of the plant extract was given for next 25 days in every alternative day. Interestingly, little recovery from lost body weight was noted and reduction of tumor volume was also noticed at the end of the treatment (Fig. 7A, B).

After 50 days of administration of cells, the mice were euthanized and tumor tissues were analyzed for H&E staining as well as IHC. H&E staining showed enlarged and non-uniform nuclei in tumor bearing mice in comparison to control mice (Fig.7C).

Further, the expressions of representative proteins of metastasis and angiogenesis were analyzed using IHC of tissue section. A higher expression of VEGF-A andOCT-4breast tissues

were noted in tumor bearing mice. But MEBM treatment significantly decreased the above elevated levels of markers in tissue samples (Fig. 7D).

4. DISCUSSION

Breast cancer is the most common reason of cancer mortality for women. It represents 1 in 4 cancers diagnosed among women globally. Early stage of breast cancer is painless and do not cause any symptoms. However, the main problem appeared because it remains undetectable at the very early stage. Once diagnosed, lots of therapeutic approaches are there in order to treat it. Majorly chemotherapeutic drugs are used along with radiation; however, due to off target effects, it causes several side effects. From last couple of decades, cancer therapeutic studies inclined the therapeutic applications of bioactive plant derived compounds in order to apply it as an alternative of toxic chemical compounds. Amongst this, *Butea monosperma* is widely studied and it was reported to be non-toxic. However, detailed anti-cancer potentiality of this compound was not fully established. Hence, a detailed study to illustrate the *Butea monosperma* mediated anti-cancer efficacy is of outmost interest. In this current piece of work, we have systematically studied the anti-cancer potentiality of *Butea monosperma* by using MCF-7 breast cancer cell line.
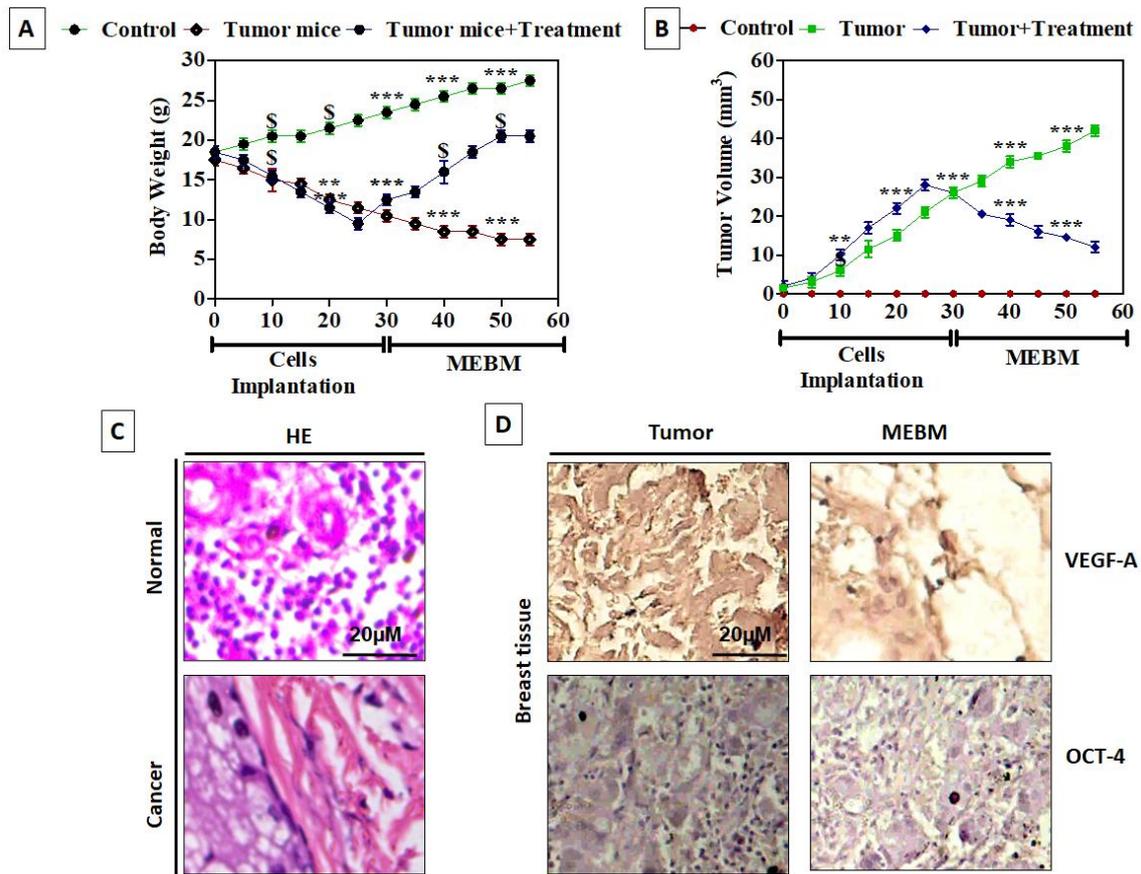
Fig. 7. MEBM decreased the metastasis and angiogenesis markers in xenograft mice model. (A) Average body weight of mice before and after treatment with the MEBM. (B) Average tumor volume of mice. Data was presented as the mean ±SD. (C) H&E staining of the xenograft tissue section. Scale bar 20 μm. (D) Immunohistochemical expressions of VEGF-A and OCT-4 in different tumor tissues. Image used was the representative of three independent experiments

The desired property of any chemotherapeutic agent relies on its minimum off target effect. In order to determine this, we have exposed normal breast epithelial cells (MCF-10A) to different extracts (aqueous, methanol, butanol and ethyl acetate) of *Butea monosperma* flowers (AEBM, MEBM, BEBM and EAEBM). Up to 1000μl treatment concentrations of these extracts were found to be non-cytotoxic to MCF-10A cells even at their 72h of treatment (Fig. 1). This signifies that the extracts of *Butea monosperma* flowers are non-toxic to normal cells. However, when we treated breast cancer cells like MCF-7 or MDA-MB-231 with these extracts, significant reduction in cell proliferation was noted (Fig. 2A, B). Moreover, MEBM was found to be more effective than that of other extracts and it only showed fifty percent reduction in cell proliferation in MDA-MB-231 cells up to the treatment concentration of 1000μg/ml (where IC_{50} values of AEBM, BEBM and EAEBM were not reached).

Hence, we streamlined our experiments by using MEBM and MCF-7 cell line and the IC_{50} value of MEBM (24h) was followed in all the follow-up experiments. Decreased in expression of Ki-67 further suggested that MEBM is effective in order to decrease the breast cancer cell proliferation (Fig. 2C).

Comet and γ-H2AX immunofluorescence data suggested that MEBM caused significant DNA damage in breast cancer cells (Fig. 3A, B). Apoptotic nuclei upon MEBM treatment again supported the nuclear toxicity of MEBM in MCF-7 cells (Fig. 4A). MEBM was also found to be involved in inducing apoptosis in breast cancer cells. Decreased expression of anti-apoptotic protein (BCL-XL) and enhanced expressions of apoptotic proteins (BAX and CASPASE-3) supported the above observation (Fig. 3C). Further, flowcytometric analysis by using Annexin V FITC concluded the MEBM mediated

apoptosis in MCF-7 breast cancer cells (Fig. 3D, E).

Methanol extract of *Butea monosperma* flower was also found to be effective in order to inhibit the angiogenesis and metastasis of breast cancer cells. Reduced expressions of prime angiogenic markers (VEGF-A, TGF-β, MMP-9, MMP-2, Ang-1, Ang-2) upon the treatment of MEBM strengthen the anti-angiogenic potentiality of MEBM (Fig. 4A-E). Data obtained from matrigel invasion assay and western blotting of prime metastatic markers (CD-44, CD-133, OCT-4) give us the conclusive evidence of MEBM dependant inhibition of metastasis of breast cancer cells (Fig. 4F, G).

In order to illustrate the mechanism of anti-cancer potentiality of *Butea monosperma* flower, we checked the MEBM mediated oxidative stress in MCF-7 cells. Reduced expressions of SOD, catalase and GSH and enhanced expression of MDA upon MEBM treatment further give us the experimental evidence of MEBM mediated oxidative stress in breast cancer cells (Fig. 5A-D). Increase in expressions of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and down regulated expression of anti-inflammatory cytokine IL-10 revealed the molecular mechanism of MEBM mediated inhibition of breast cancer properties (Fig. 5E-H).

Again, to validate the anti-cancer efficacy of MEBM, we have developed mice xenograft model system. Increase in decreased body weight and decrease in enhanced tumor volume suggested the truly development of xenograft model (Fig.7A, B) as also studied by Bello E and *et al* 2013. Enlarged and non-uniform nuclei in tumor bearing mice in comparison to control mice further confirmed the genuine formation of tumor (Fig. 7C). Decrease in enhanced expression of VEGF-A and OCT-4 upon MEBM treatment in mice model exclusively concluded the MEBM mediated anti-angiogenic and anti-metastatic potentiality (Fig. 7D).

5. CONCLUSION

The current study suggested the therapeutic implication of *Butea monosperma* flower in breast cancer. The methanol extract of *Butea monosperma* flower was found to be effective in order to inhibit breast cancer without affecting the normal breast epithelial cells. MEBM was found to be effective in inducing DNA damage, apoptosis, reducing cell proliferation, angiogenesis and metastasis in MCF-7 *in vitro* breast cancer model system. MEBM also reduced expression of angiogenic and metastatic markers in our developed mice xenograft model system. Hence, MEBM can be an excellent anti-cancer therapeutic agent in order to target angiogenesis, metastasis and DNA repair in breast cancer.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocol was approved by the institutional animal ethical committee with protocol no. IAEC/CPCSEA/RCPIPER/2019-20.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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