

Anti-Proliferative and Antimetastatic Activity of Methanolic Extract of *Butea monosperma* Stem Bark in MDA- MB 231 Cell Line

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ABSTRACT: *Buteamonosperma* is an ethnomedicinal tree of remedial value in the treatment of diabetes, bone fractures, and liver and neurological disorders. However, the information available on the anti-proliferative and antimetastatic potential of the bark of this tree is scarce. In the present study, the methanolic extract prepared from bark of *Buteamonosperma* was evaluated for anti-proliferative, anti-inflammatory, and antimetastatic effects on Triple-Negative Breast cancer cell line MDA MB 231, using *in vitro* assays like MTT assay, cell cycle analysis and apoptotic assays, Transwell migration and invasion assays. Genes involved in apoptosis and metastasis were studied by Real-Time PCR analysis. The anti-inflammatory effect was assessed by measuring IL 6 level in cell culture supernatant as well as by measuring the expression of NF kB at the mRNA level. The extract inhibited the growth of MDA MB 231 cell line at an IC₅₀ value of 25µg/ml, induced cell cycle arrest at S phase and apoptosis. The extract inhibited the migration and invasion potential of MDA MB 231 cell line and reduced the expression of inflammatory markers NF kB and IL 6. The inhibitory potential of the extract is possibly due to the anti-inflammatory effect of polyphenols enriched in the *Butea monosperma* bark extract.

KEYWORDS: TNBC, Apoptosis, Metastasis, Anti-inflammation, *Butea monosperma* Bark extract.

I. INTRODUCTION

Breast cancer is the most frequent cancer among women worldwide, impacting 2.1 million women each year and considered as the leading cause of cancer-related death among women. Triple-negative breast cancer (TNBC) is a subtype of breast cancer which is defined by the absence of estrogen receptor (ER), and progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) overexpression by immunohistochemistry (IHC). TNBC represents approximately 10–15% of all diagnosed breast cancers and is associated with earlier age of onset, aggressive clinical course, and dismal prognosis with a high rate of recurrence compared to hormone receptor- and HER2-positive breast carcinomas (1). Chemotherapy is the primary established systemic treatment for TNBC patients in both the early and advanced stages of the disease. There are no approved targeted treatments available other than chemotherapy. The dose-limiting toxicity and development of drug resistance hamper the treatment with chemotherapeutics. Currently, there is growing interest in the identification of new anticancer natural products that overcome the limitations of cell toxicity and adverse reactions that affect the patient's quality of life. Traditional medicine such as Ayurveda, make use of plant-derived products in cancer treatment, which may reduce adverse side effects. Several medicinal plant species and their phytochemicals inhibit the progression and development of cancer. *Butea monospermous* is a medicinal plant commonly known as Flame of the forest, belonging to the family Fabaceae. The plant is reported to have anti-inflammatory and antioxidant potential. Considering the medicinal importance of the plant the present study was

designed to investigate the antiproliferative and anti-metastatic effect of methanolic extract of *Butea monosperma* stem bark in TNBC cell line

II. LITERATURE SURVEY/RELATED WORK

Butea monosperma (Palasa) is a medium-sized deciduous tree that belongs to family Fabacea and sub-family Papilionaceae. This tree is also called 'Flame of the Forest' and 'Bastard Teak'. All parts of the plant have been used as a crude drug for the treatment of tumors, piles, skin diseases, wounds, and ulcers. *Butea monosperma* is extensively used in Ayurveda, Unani, and Homeopathic medicine and has become a treasure of modern medicine (2). Various parts of the plant have been explored for antioxidant, antidiarrheal, anti-diabetic, anti-stress, anti-convulsive, anti-hepatotoxic, anti-estrogenic, and anti-helminthic activities (3). The hydroalcoholic and alcoholic extracts of the flower and the alcoholic extract of the stem proved to have antioxidant, anti-glycation, and anti-inflammatory activities (4). The of the plant are rich in polyphenols like Butein, Butrin, Isobutrin, Plastron, Coreipsin, and Isocoreipsin and are widely used in the treatment of hepatic disorders, viral hepatitis, diarrhea, depurative and tonic (5). The stem bark of the plant is reported to possess antitumor, antiulcer, antifungal, and antidiarrheal activities. Hepatoprotective potentials of *Buteamonospermastem* bark extract against carbon tetrachloride-induced hepatotoxicity has been reported in albino rats (6). The stem bark of *Buteamonosperma* displays antifungal activity which is due to the presence of an active constituent medicarpin (7). The seed oil of *Buteamonosperma* shows significant bactericidal and fungicidal effects in in-vitro testing (8). The aqueous and ethanol extracts of *Buteamonosperma* leaves extracts have potential antioxidant, antigenotoxic, and chemopreventive activity which could be used as an adjuvant in chemotherapeutic applications (9). Intraperitoneal administration of the aqueous extract of *Butea monosperma* flower maintained liver architecture, nuclear morphometry, and down-regulated the serum VEGF level in the X15-myc oncomice (10). The n-butanol fractions of *Buteamonosperma* floral extract inhibited aberrant crypt foci (ACF) formation in the colon of azoxymethane-induced Wister albino rats (11). Methanolic extract of *Buteamonospermastem* bark produced anti-inflammatory and analgesic activity in carrageenan - induced paw edema model (12). The ethanolic and the acetone extract of *Buteamonospermastem* bark showed significant wound healing activity in excision, incision, and deep space wound healing model (13). Methanolic extract of *Butea monosperma* flowers exerts selective cytotoxic activity in MCF-7 estrogen-positive breast cancer cells and inhibits the growth of mammary carcinoma in-vivo (14). *Butea monosperma* bark fraction exerts anti-oxidant and anti-proliferative effect against MCF 7 cell line via Reactive Oxygen Species mediated pathway (15). *Butea monosperma* is a plant rich in phytochemicals and chemopreventive ability of plant is due to the inhibitory potential of the phytochemicals on cancer growth due to their pleiotropic actions on multiple target sites. So in the present study, we evaluated the anticancer potential of methanolic extract of *Butea monosperma* stem bark in the TNBC cell line.

III. METHODOLOGY

PREPARATION OF EXTRACT: The plant materials used for the present study were collected from the Kollam district of Kerala, India. The pulverized, shade dried powder of the stem bark of *Buteamonosperma* was extracted with 5 ml of methanol for 5 days with intermittent shaking at room temperature.

CELL CULTURE: Triple Negative breast cancer cell line MDA MB 231 was used as the experimental model. Cell lines were purchased from NCCS (National Center for Cell Science), Pune. MDA MB231 cell line was maintained in DMEM with 10% FBS (Gibco) at 37°C in the CO₂ incubator.

MTT ASSAY: The cells were seeded at optimal density into sterile 96-well plates and incubated overnight for cell adhesion. The stock solution of methanolic extracts of *Butea monosperma* was serially diluted and added to the cells at concentrations ranging from 10-80 µg/ml. After 48 hours, cytotoxicity screening was performed using

MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 200µl of MTT reagent was added to each well and the plate was incubated for 2 hours at 37°C in CO₂ incubator. Then 100µl of MTT lysis buffer was added to the wells and kept in dark for 3 hours at room temperature and absorbance was measured at 570nm. Each measurement was performed in triplicates. The half-maximal inhibitory concentration (IC₅₀) value was determined from the dose-response curve.

CELL CYCLE ANALYSIS: Cell cycle status was determined by measuring cellular DNA content after staining with Propidium Iodide (PI). Briefly, after the treatment cells were harvested and washed in PBS, then fixed in cold 70% ethanol. Ethanol was added dropwise to the pellet while vortexing, which should ensure the fixation of all cells and minimize clumping. The ethanol- fixed cells were spun and washed twice with ice-cold PBS. Then the cell pellet was stained with PI (50µg/ml) in PBS containing RNase A (100µg/ml) in dark for 15minutes before analyzing on a flow cytometer. The percentage of cells at the different phases of the cell cycle was quantified.

APOPTOSIS ASSAY: FITC Annexin V was used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. Cells were seeded at a density of 2×10^5 cells per well in 6-well plates. Briefly, cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. 100µl of the solution (1×10^5 cells) was transferred into a 5ml culture tube. 5µl of FITC Annexin V and 5µl PI was added. Gently vortexed the cells and incubated for 15min at RT (25°C) in the dark. To each tube 400µl of 1X Binding Buffer was added and analyzed by flow cytometry.

CELL MIGRATION AND INVASION ASSAY: Cell migration was determined using 24-well transwell chamber (Corning) with 8 µm pore polyethylene terephthalate (PET) membrane while the matrigel coated transwell chamber was used for invasion assay (BD Biosciences). After the treatment, cells were seeded into the upper compartment at a concentration of 2×10^5 cells/ml in a volume of 100 µl serum-free medium/well. The lower compartment contained 500 µl of medium supplemented with 10% FBS as a chemoattractant. After incubation for 48 hours at 37°C, cells that had migrated and invaded through the matrigel were fixed with 100% methanol and stained with 0.5% crystal violet. The cells were then imaged under a light microscope and counted.

ANALYSIS OF SECRETORY IL 6: IL 6 level was measured using the Quantikine ELISA kit from R& D systems. The assay was carried out using the manufacturer's protocol. Briefly, the cells were seeded in 6-well plates (2×10^5 cells/well) and treated with extracts in serum-free medium for 48 h. The conditioned medium was centrifuged at 4 °C and the supernatant was collected to carry out the assay. To 100µl assay diluent, 100µl of IL6 standard solution/sample was added and incubated for 2h at room temperature. After washing 4 times, 200µl of IL 6 conjugate was added to each well and incubated for 2h at room temperature. After washing, 200µl substrate solution was added and incubated for 20minutes in dark. 50µl stop solution was added to each well and absorbance was measured at 450nm.

REAL-TIME REVERSE TRANSCRIPTION PCR:RNA was extracted from MDA-MB-231 cells using TRI reagent (Sigma) following the manufacturer's instructions. DNase treated RNA samples were reverse transcribed using the Reverse Transcriptase Core kit (Eurogentec). RT PCR was performed using SYBR green dye on the Applied Biosystems Step One Real-Time PCR system. RT-PCR was performed in a total volume of 20µl, containing 50 ng cDNA, 1µmol of each primer (forward and reverse), and 10µl of TakyonRox SYBR Master Mix. The PCR condition comprised incubation at 95°C for 10 minutes (for denaturation) followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1-minute and was followed by melt curve analysis The primers used are described in [Table 1](#). *β actin* was used as the reference genes for the mRNA levels. Fold-expression was calculated according to the $\Delta\Delta C_t$ method (16).



Gene	Primer sequence
Bax Forward	5'TTTCATCCAGGATCGAGCAG3'
Bax Reverse	5'AAAGTAGAAAAGGGCGACAA3'
Bcl- 2 forward	5'CTGCACCTGACGCCCTTACC3'
Bcl- 2 reverse	5'CACATGACCCACCGAACTCAAAGA3'
MMP- 2 Forward	5'CTCATCGCAGATGCCTGAA3'
MMP- 2 Reverse	5'TTCAGGTAATAGGCACCCTTGAAGA3'
MMP 9 Forward	5'ACGCACGACGTCTTCCAGTA3'
MMP 9 Reverse	5'CCACCTGGTTCAACTCACTCC3'
NF kB p65 Forward	5'GCACAGATACCACCAAGACC3'
NF kBp65 Reverse	5'TCAGCCTCATAGAAGCCATC3'
β -actin Forward	5'GCCAACACAGTGCTGTCTGG 3'
β -actin Reverse	5'GCTCAGGAGGCAATGATCTTG3'

TABLE 1: List of primer sequence used for Real Time PCR analysis

STATISTICAL ANALYSIS: Data were analyzed by software GraphPad Prism (6.0). Results are presented as mean± SD. We have considered $p < 0.05$ as statistically significant.

IV. RESULTS AND DISCUSSION

CYTOTOXICITY SCREENING: MTT assay was performed to determine the cytotoxic potential of the extract and the result has shown that the methanolic extract of *Butea monosperma* was toxic to MDA MB 231 cell line. The half-maximal inhibitory concentration (IC_{50}) value was determined from the dose-response curve and was used for subsequent experiments. *Butea monosperma* had an IC_{50} of 25 μ g/ml against TNBC cells (Figure 1).

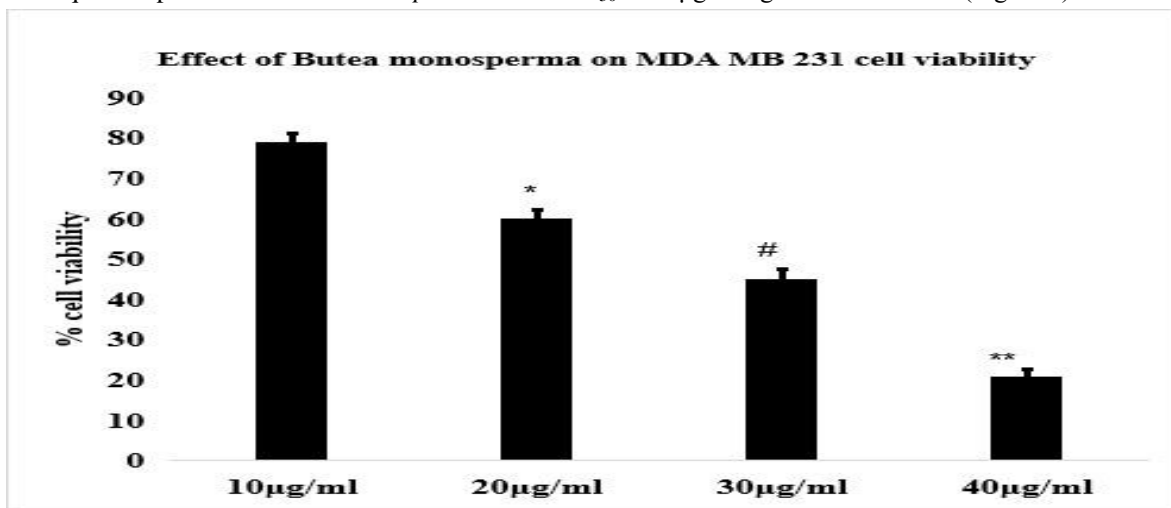


Figure 1: Effect of methanolic extract of *Butea monosperma* stem bark on MDA MB 231 cell viability after 48 hours of treatment measured by MTT assay. Data are presented as the mean±SD. * $p < 0.05$

CELL CYCLE ANALYSIS: MDA MB231 cells were treated with BM extract with IC_{50} values for 48 hours and were analyzed by flow cytometry. Extracts exhibited cell cycle arrest at S phase in MDA MB 231 cell line (Figure 2). This is possibly due to interference with DNA synthesis, thereby halting the progression of cells at S phase and leading to apoptosis.

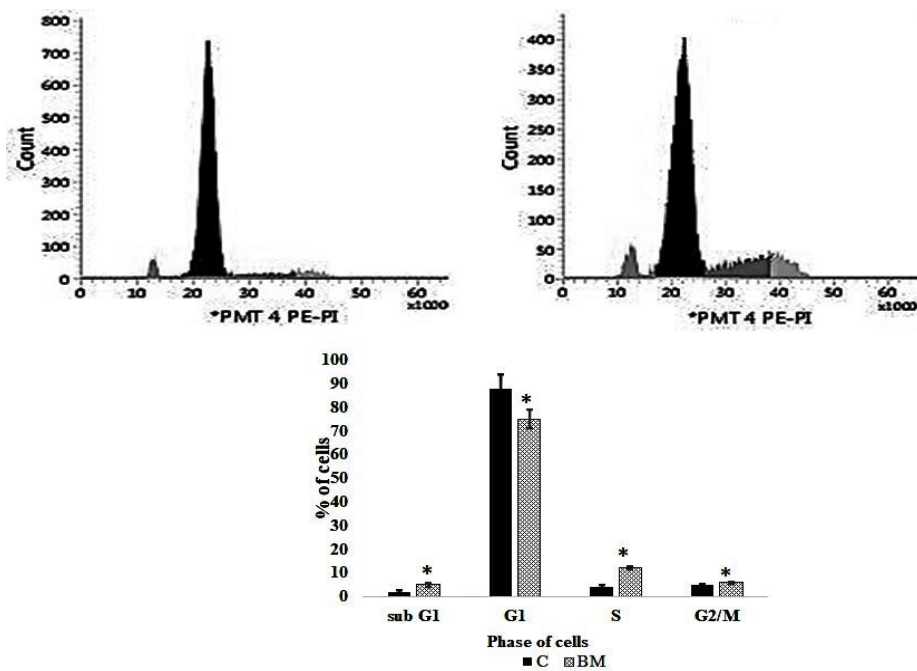


Figure2:Effectof methanolic extract of Butea monosperma on cell cycle phase distribution of MDA MB 231cell lines after 48 hours of treatment. Data are presented as the mean±SD. *p<0.05 vs C.

APOPTOTIC EFFECT: The apoptotic effect of the extract was analyzed in MDA MB 231 cell line by Annexin/PI staining. Expression of pro-apoptotic Bax gene and antiapoptotic Bcl-2 expression was measured by Real-Time PCR analysis. BM extract treatment significantly enhanced the percentage of early and late apoptotic cells (Figure 3). Extract significantly increased Bax levels, and decreased Bcl-2 level resulting in a significant increase in Bax/Bcl-2 ratio (Figure 5). Bax promotes cell death and inhibits the protective effect conferred by Bcl-2, which in turn prevents the activation of caspases in cultured cells (17). The balance of proapoptotic Bax and anti-apoptotic Bcl-2 was also associated with apoptosis. An increase of the Bax/Bcl-2 ratio observed in response to the treatments may amplify the mitochondrial apoptotic pathway to release caspase-9 and downstream effector caspase-3 to induce apoptosis. A large body of evidence indicates that polyphenols trigger apoptosis by modulating key elements involved in the apoptotic signalling pathway and studies have confirmed *Butea monosperma* bark extract is rich in polyphenols.

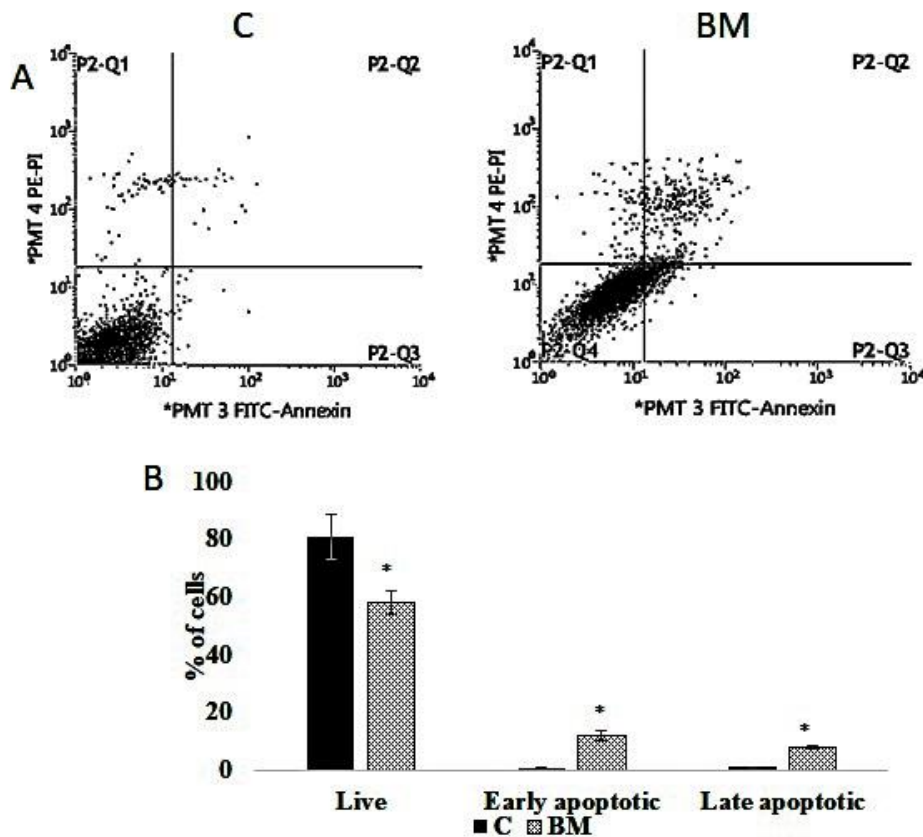


Figure3:(A)Effect of methanolic extract of Butea monosperma bark extract on apoptosis induction in MDA MB231 cell lines assessed by Annexin/PI staining followed by Flow cytometry and(B) the graphical representation of the percentage of cells at different phases of apoptosis after 48 hours of treatment. Data are presented as the mean±SD.

*p<0.05 vs C.

ANTIMETASTATIC EFFECT: Metastasis is the major cause of mortality in patients with cancer and is a multifaceted process that results from coordinated events, including cancer cell invasion, migration, and adhesion. To determine the inhibitory effect of the extract on migration and invasion of MDA-MB-231 cells Transwell migration and invasion assays were employed. A significant reduction in the number of migrated cells was observed in the extract-treated samples in transwell migration assay, confirming the anti-migratory effect of the extracts (Figure 4A). Invasion is a canonical feature of cancer cells, and it is a prerequisite for cancer metastasis. We examined the ability of cell invasion using Matrigel-coated Transwell assay. The results showed that treatment with extracts significantly decreased cell invasion (Figure 4B). Matrix metalloproteinase MMP -2 and MMP-9 are cancer-associated endopeptidases, which cause proteolytic degradation of the ECM and then regulate signaling pathways promoting the mobility of cancer cells, angiogenesis, and eventually leading to metastases (18). The extracts down-regulated the expression of MMP2 and MMP9. (Figure 5)

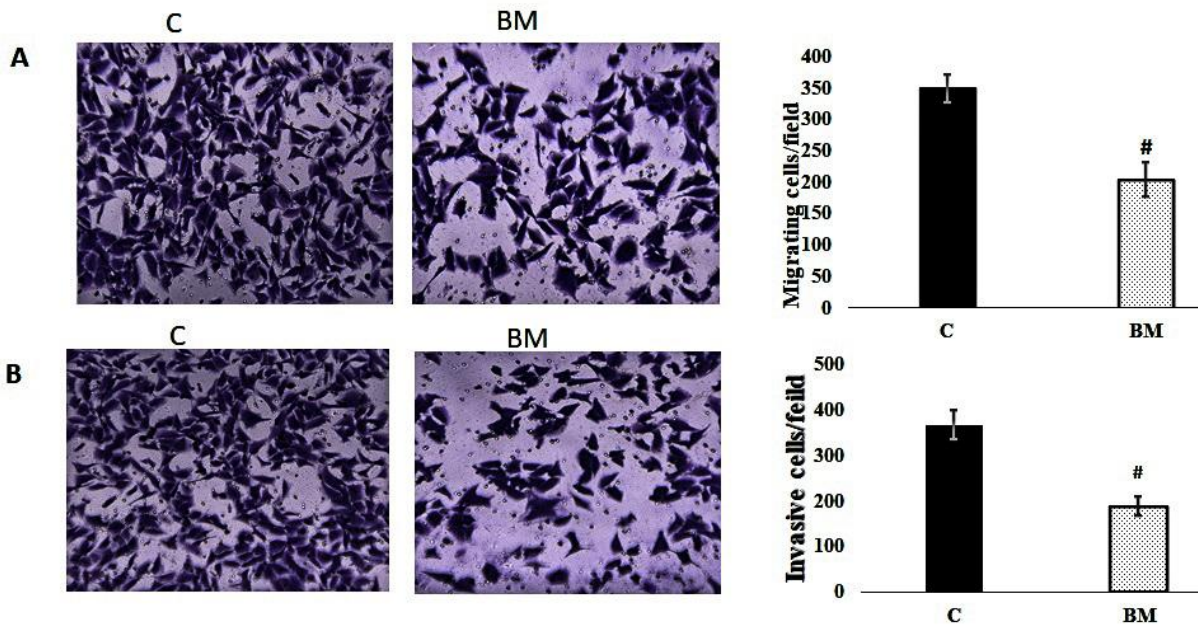


Figure 4:Effect of Butea monosperma extracts on migration(A) and cell invasion(B) after 48 hours of extract treatment. (magnification 10x).Migrated and invaded cells from the five photographed fields in each group were counted. Results are presented as means ± SD of three independent experiments.#p<0.01 vs C.

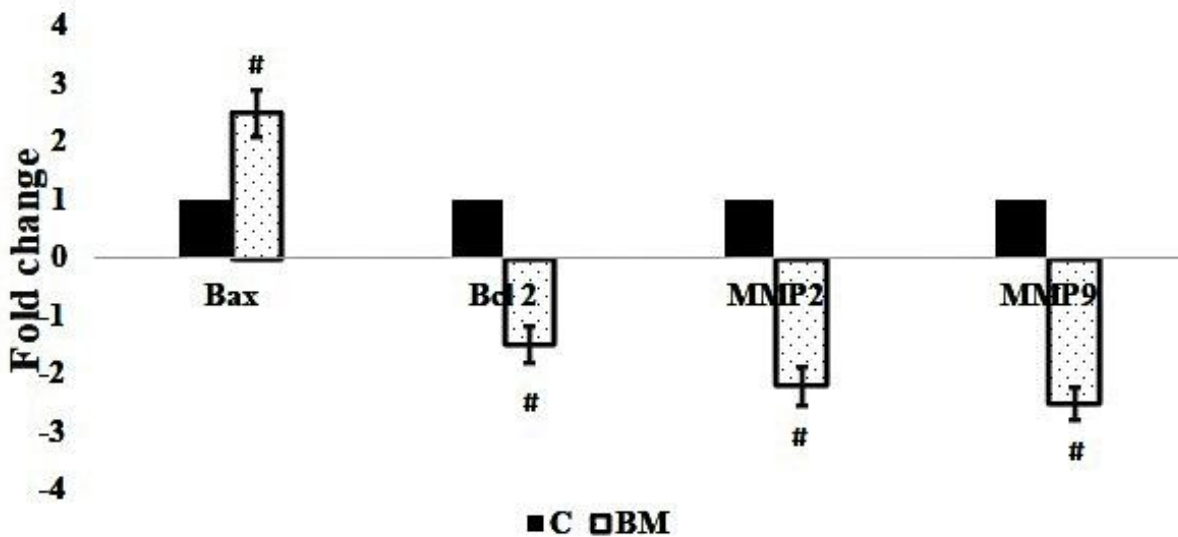


Figure5:Real-time PCR analysis of Bax, Bcl-2,MMP 2 and MMP9 mRNA levels in MDA MB 231cell lines treated with methanolic extracts of Butea monosperma Bark extract. The values have been normalized to beta-actin. The graph represents three independent experiments with each bar as mean ±SD. #p<0.01 vs C.

ANTI-INFLAMMATORY EFFECT: The anti-inflammatory effect of the extracts was studied by measuring the expression of IL 6 in cell culture supernatant by ELISA kit as well as by measuring the expression of NF kB at the mRNA level. The result has shown that extract treatment for 48 hours significantly reduced inflammatory response. Inflammation is associated with the development and malignant progression of most cancers.NF kB is a key regulator of the inflammatory response associated with cancer. The herbal extracts inhibited the expression of NF kB in the MDA



MB 231 cell line (Figure 6). IL 6 is a biomarker associated with systemic inflammation linked to poor prognosis among patients with TNBC. Studies have shown that gallic acid and its derivatives have a potential anti-inflammatory effect and decreased the expression of NF kB and IL6 in primary endometriotic culture cells (19). The higher content of gallic acid in BM may be the reason for the enhanced inhibition of NF kB and IL6 in AR treated cells (Fig 6). Plant polyphenols can inhibit NF kB which involves the redox cycling property of phenolic antioxidants (20). Reports have shown that butrin, isobutrin and butein isolated from *Butea monosperma* selectively inhibit NF kB and IL 6 in activated human mast cells (21).

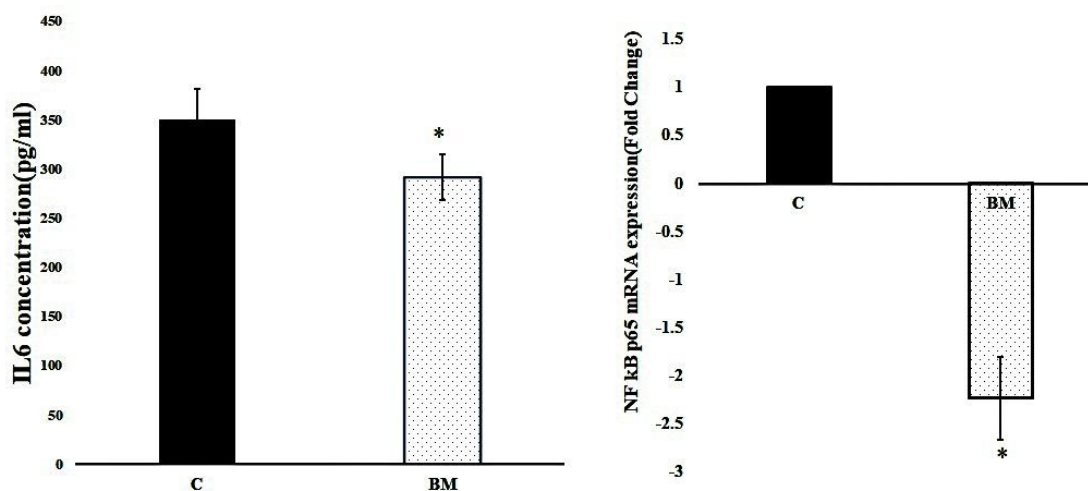


Figure 6: Anti-inflammatory effect of the methanolic extracts of *Butea monosperma* Bark extract. Extract treatment down regulated the mRNA expression of NF kB and serum level of IL 6 in MDA MB 231 cells. Data are presented as the mean±SD. *p<0.05 vs C.

V. CONCLUSION

Triple-negative breast cancer is considered to be an aggressive breast cancer subtype with a poorer prognosis than other types of breast cancer, mainly because there are fewer targeted medicines that treat triple-negative breast cancer. Chemotherapy is often accompanied by debilitating side effects. Plants are reservoirs of hitherto unknown chemical entities and provide a promising line for research on cancer. The quest for pharmaceutical drug leads from Ayurvedic medicinal plants has been rapidly growing due to cost-effectiveness, reliability, and least side effects. The present study has shown that the methanolic extract of *Butea monosperma* stem bark induced antiproliferative and antimetastatic effect in the TNBC cell line. Scientific studies have reported that BM bark extract is rich in polyphenols like gallic acid, epicatechin, and kaempferol. The inhibitory potential of the extract against the growth and metastasis of MDA MB 231 cells, possibly due to its anti-inflammatory effects as evident from the reduced expression level of IL 6 and NF kB, can be attributed to the presence of polyphenols. The observations of the study envisage the development of a novel formulation for effective treatment of TNBC

ACKNOWLEDGMENTS: The study was supported by a grant from the Kerala State Council for Science Technology and Environment.

REFERENCES

1. Al-Mahmood S, Sapiezynski J, Garbuzenko OB, Minko T. Metastatic, and triple-negative breast cancer: challenges and treatment options. *Drug delivery and translational research*. 2018 Oct;8(5):1483-507.

2. Fageria D, Rao D. A review on *Butea monosperma* (Lam.) Kuntze (sic): A great therapeutic valuable leguminous plant. *International Journal of Scientific and Research Publications*. 2015;5(6):1-7.
3. Burlia DA, Khadeb AB. Phcog Rev.: Plant Review A Comprehensive review on *Butea monosperma* (Lam.) Kuntze. *Pharmacognosy Reviews*. 2007 Jul;1(2).
4. Vaidya A, Pandita N. Comparative pharmacognostic and phytochemical studies of flower, leaf, and stem extracts of *Butea monosperma*. *Asian Journal of Biomedical and Pharmaceutical Sciences*. 2017;7(63):10-8.
5. Mishra A, Verma S, Mishra AP. A plant review: *Butea monosperma* (Lam.) Kuntze. *Res. J. Pharm. Biol. Chem. Sci*. 2012;3(1):700-14.
6. Sharma N, Shukla S. Hepatoprotective potential of aqueous extract of *Butea monosperma* against CCl₄ induced damage in rats. *Experimental and Toxicologic Pathology*. 2011 Nov 1;63(7-8):671-6.
7. Bandara BR, Kumar NS, Samaranyake KS. An antifungal constituent from the stem bark of *Butea monosperma*. *Journal of ethnopharmacology*. 1989 Feb 1;25(1):73-5.
8. Sharma AK, Deshwal N. An overview: On phytochemical and pharmacological studies of *Butea monosperma*. *Int. J. of PharmTech Research*. 2011 Apr;3:864-71.
9. Singh A, Kaur M, Choudhary A, Kumar B. Effect of *Butea monosperma* leaf extracts on cyclophosphamide-induced clastogenicity and oxidative stress in mice. *Pharmacognosy research*. 2015 Jan;7(1):85.
10. Mathan G, Fatima G, Saxena AK, Chandan BK, Jaggi BS, Gupta BD, Qazi GN, Balasundaram C, Anand Rajan KD, Kumar VL, Kumar V. Chemoprevention with aqueous extract of *Butea monosperma* flowers results in normalization of nuclear morphometry and inhibition of a proliferation marker in liver tumors. *Phytotherapy research*. 2011 Mar;25(3):324-8.
11. Subramaniyan B, Polachi N, Mathan G. Isocoreopsin: An active constituent of n-butanol extract of *Butea monosperma* flowers against colorectal cancer (CRC). *Journal of pharmaceutical analysis*. 2016 Oct 1;6(5):318-25.
12. William CM, Mohan KG. Anti-inflammatory and analgesic activity of *Butea monosperma* (Lam) stem bark in experimental animals. *Pharmacologyonline*. 2007; 2:88-94.
13. Muralidhar A, Babu KS, Sankar TR, Reddanna P, Latha J. Evaluation of Wound Healing Properties of Bioactive Fractions from the Extract of *Butea monosperma* (Lam) Stem Bark. *International Journal of Phytomedicine*. 2011 Jan 1; 3(1):41.
14. Karia P, Patel KV, Rathod SS. Breast cancer amelioration by *Butea monosperma* in-vitro and in-vivo. *Journal of ethnopharmacology*. 2018 May 10; 217:54-62.
15. Kaur V, Kumar M, Kumar A, Kaur S. *Butea monosperma* (Lam.) Taub. Bark fractions protect against free radicals and induce apoptosis in MCF-7 breast cancer cells via cell-cycle arrest and ROS-mediated pathway. *Drug and chemical toxicology*. 2018 Sep 21:1-1.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ ΔΔCT method. *Methods*. 2001 Dec 1; 25(4):402-8.
17. Morales-Cano D, Calviño E, Rubio V, Herráez A, Sancho P, Tejedor MC, Díez JC. Apoptosis induced by paclitaxel via Bcl-2, Bax and caspases 3 and 9 activation in NB4 human leukemia cells is not modulated by ERK inhibition. *Experimental and toxicologic pathology*. 2013 Nov 1; 65(7-8):1101-8.
18. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*. 2010 Apr 2; 141(1):52-67.
19. Bustami A, Sopia P, Muharam R, Wibowo H. Effects of Gallic Acid and Its Derivates on Inflammatory Regulation of Endometriotic Primary Cultures: Study on NF-κB mRNA Expression and IL-6 Secretion. *Biomedical and Pharmacology Journal*. 2018 Sep 21; 11(3):1479-84.
20. Rasheed Z, Akhtar N, Khan A, Khan KA, Haqqi TM. Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor-κB in activated human mast cells: Suppression of tumor necrosis factor-α, interleukin (IL)-6, and IL-8. *Journal of Pharmacology and Experimental Therapeutics*. 2010 May 1; 333(2):354-63.
21. Yahfoufi N, Alsadi N, Jambi M, Matar C. The immunomodulatory and anti-inflammatory role of polyphenols. *Nutrients*. 2018 Nov; 10(11):1618.