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Antiproliferative Activity of *Bombax ceiba* Flower Extract against Mammary Gland Carcinoma in Rats

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ABSTRACT

Breast cancer is the leading cause of cancer-related deaths among women. The present study evaluates the potential therapeutic effects of a methanol extract of *Bombax ceiba* flowers on cancerous changes in the mammary glands of rats. Mammary gland tumors were induced in female rats with 7,12 dimethylbenz (a) anthracene, with phorbol used as a promotor. PI3K/AKT, Ki-67, TGFβ, and Bcl2 as markers for tumor-cell proliferation and progression were assessed by an enzyme-linked immunosorbent assay in serum and tissues. Mammary gland tissue samples were collected for histopathology and immune staining for Ki-67. All markers increased significantly in the rat-breast cancer model. The extract minimized cancer-cell proliferation, as evidenced by a substantial reduction in the levels of all markers in both serum and tissues and supported by histopathological findings and immunexpression of Ki-67.*B.Ceiba.* The flower extract ameliorate the action against exaggerated cell proliferation associated with mammary gland tumors, suggesting a possible use of it as a complementary anticancer therapy.

Keywords: Breast cancer - *Bombax ceiba* flower methanol extract - Proliferation markers- Histopathology

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INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women, affecting 2.1 million women annually and causing the greatest number of malignant neoplastic disease-related deaths. In 2018, an estimated 627,000 women died from breast cancer. Breast cancer rates are the highest in developed regions, but rates are increasing in nearly every region globally[WHO, 2013]. To emulate human breast carcinogenesis, rodents have long been used as experimental models. Mammary tumors can be initiated in susceptible rat strains with a single dose of a carcinogen such as 7,12 dimethylbenz (a) anthracene (DMBA) or nitrosomethylurea. Tumors were generated morphologically and histologically mimic those observed in human estrogen-dependent breast cancer[Abba *et al*. 2016]. These carcinogens disrupt the tissue redox balance and cause biochemical and pathophysiological disturbances due to oxidative stress [Lai and Singh, 2006]. Phorbol (12-O-tetradecanoyl-phorbol-13- acetate) was also used to promote cancer [Shellabarger *et al*.,1979]. Phytochemical-rich diets can reportedly decrease cancer risks by 20% [Bradford and Awad, 2007]. Epidemiological data indicate that the phytosterol content of a diet is associated with its ability to decrease the incidence of tumors, including those of breast cancer. Data from previous studies using various tumor models indicated that phytosterols could modify host systems, improve immune recognition of tumor cells, and affect the growth of hormone-dependent tumors [Bradford and Awad, 2007]. Induction of apoptosis (programmed cell death) was thought to be the mode of action of chemotherapeutic agents in general[Soengas and Lowe, 2003].

Aalcohol extract of Egyptian *Bombax ceiba* flowers (BCFE) contains a variety of constituents with therapeutic effects. BCFE (80%) contains 3 xanthones: isomangiferin, 4-O-p-hydroxybenzoyl, and mangiferin, along with 9 flavonoids, including apigenin, vitexin, vicinine-2, quercetin, quercetin 3-O-β-arabinoside, isorhamnetin 3-O-β-glucuronide, and rutin [El-Toumy *et al*., 2013]. The n-hexane and methanol of BCFE contains the β-D-glucoside of β-sitosterol, hentriacontane, and hentriacontanol [Rani *et al*., 2016]. Treatment of Ehrlich ascites carcinoma cells in mice with BCFE resulted in a modest decrease in the number of tumor cells and tumor size and prolonged the lifespans of tumor-bearing mice [El-Toumy *et al*., 2013].

The present study was designed to evaluate the potential therapeutic effects of BCFE on DMBA-induced mammary gland tumors in rats by estimating the expression levels of the biomarkers related to tumor-cell proliferation and progression in both serum and tissue and evaluating the associated histopathological alterations.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used in the experiments were of analytical grade. DMBA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phorbol was obtained from Theodor Schuchardt GmbH and Co., (Munich, Germany). ELISA kits were used for the quantitative determination of AKT, Ki-67, and TGFβ for quantitative determination.

Plant material

B. ceiba flowers were collected in January, 2018 from the Orman botanical garden in Giza, Egypt. The flowers were authenticated by a botanist at the National Research Centre. A voucher specimen (No. 3241) was deposited in the herbarium of National Research Centre, Doki, Giza, Egypt.

Preparation of aqueous methanol extract of *Bombax ceiba* flowers

Shade-dried *B. ceiba* flowers (1 kg) were immersed in aqueous methanol (70% v/v) for three days. The extracted solutions were combined, filtered, and reduced by a rotary evaporator under reduced pressure to eliminate the methanol solvent. The selection was then subjected to lyophilization to remove excess water, yielding 100 g of a

crude dark-brown, sticky BCFE and stored at −20° for later use.

Phytochemical screening, total phenolic assay, total flavonoid assay, and high-performance liquid chromatography of flavonoids and phenolic compounds of the same plant (No. 3241 in the herbarium of National Research Centre, Dokki) were previousely described by Abd-Elhakim *et al*., 2019.

Animals

Forty adult female Wistar rats weighing 180 ± 5 g were obtained from the animal house of the National Research Centre Dokki, Giza. All rats were kept in clean cages under normal laboratory conditions (22 ± 5 °, for 12 h and light/dark cycle). They were supplied with access to a standard pellet diet and a loose approach to water. All animal protocols were performed with the approval of the Animal Care and Experimental Committee, National Research Centre, Cairo, Egypt (Approval No: 18163).

Induction of breast cancer

Twenty Wistar female albino rats each received 50 mg of DMBA per kg of body weight in sesame oil by oral gavage [Hakkak *et al*., 2002] . One week after DMBA treatment, 0.5 mL of phorbol (8 mg/L acetone) was injected intraperitoneally twice weekly for 10 weeks [Armuthand Berenblum, 1974]. All rats were palpated twice weekly to detect mammary tumors. After 6 weeks, the rats were given a booster dose of DMBA (25 mg/kg).

Experimental design

Rats were allocated into 4 groups of 10 animals each. Group 1, the control negative, were given only a vehicle. Group 2 animals were fed BCFE by oral gavage (150 mg/kg). Group 3 received DMBA and phorbol. Group 4 received DMBA with oral gavage BCFE twice weekly for 4 weeks.

At the end of the treatment period (28 ± 10 days after starting the experiment), blood samples were drawn from the animals by puncturing the retro-orbital venous plexus with a thin sterilized capillary tube under light anesthesia; using isoflurane as an inhaled anesthetic in a drop jar at a concentration of 4-5% then 2% for maintenance. Blood specimens were centrifuged to separate serum and stored at −80° till needed. Rats were then sacrificed by decapitation and their breasts were removed. Part of each breast tissue sample was rinsed and homogenized in phosphate buffer saline (pH 7.4) and centrifuged at 4000 rpm and 4 ℃ for 15 min. The supernatants were used for biochemical assays. Another part of the dissected mammary gland tissue was kept at 10% neutral buffered formalin.

Biochemical analysis

***In-vitro* studies:**

Cell cycle time determination

Cell cycle checkpoints are G1 (Start or restriction checkpoint), S (metaphase), and G2/M [MacLachlan *et al*.,1995]. The principal function of the anticancer agents is to cessat the cell division at these checkpoints**.** The cells were stained with Annexin V/PI and examined using flow cytometry procedure [Amin *et al*., 2018; Anwar *et al*., 2019].

Apoptotic assay

The flow cytometry technique was carried out using propidium iodide (PI) and annexin-V-FITC in MCF-7 cells to evaluate the apoptotic effect**.**

Quantification of AKT

AKT was quantitative determined in rat serum ELISA kit from Sinogeneclon Co., Ltd. (Catalog Nos. SG- 20692) according to the manufacturer's instructions.

Quantification of Ki-67

Ki-67 was measured in serum and tissues using a commercially available rat AKT ELISA kit (Glory Science, Del Rio, TX, USA) according to the manufacturer's instructions. The kit uses a double antibody sandwich enzyme linked immunosorbent assay to measure the level of Ki-67.

Quantification of TGF-β1

TGF-β1 was determined using a double-antibody sandwich enzyme-linked immunosorbent assay in serum and tissues using (Rat (TGF-β1) ELISA kit) from Sinogeneclon Co., Ltd. (Catalog No. 20060).

Quantification of BcL2

BcL2 was determined in serum using ELISA kit . A BcL2 kit was purchased from Fine Test (Catalog No. ER0762) for quantitative determination.

Histopathological examination

After fixation the tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological evaluation and Masson's trichrome (MTC) stain to differentiate myoepithelial and fibrous connective tissue according a previously described protocol[Bancroft, 2013]. Tissue slides were examined using an Olympus BX43 light microscope and photographed with an Olympus DP27 camera linked to CellSens dimension software (Olympus). Elston / Nottingham modification of Bloom-Richardson histologic grading system[Elstonand Ellis, 1991] was applied; three main characters were evaluated including (a) tumor tubule formation, (b) number of mitotic figures in most active areas and (c) nuclear pleomorphism. Tumor tubule formation was considered if the tumor cells are forming tubules with clear center lumina :1 point: > 75% of tumor, 2 points: 10 - 75% of tumor and 3 points: < 10% of tumor. Number of mitotic figures was counted in 10 high power microscopic fields and scored as 1 point if 0 to 11 figures were counted per field, 2 points if 12 to 22 figures were seen and 3 points were given if more than 23 figures were observed in the most active areas of tumor. For the nuclear pleomorphism I point was given to the minimal variations in size and shape of cells, 2 points for the moderate and 3 points to the marked cellular pleomorphism. Finally, a total score was calculated; from 3-5 points considered well-differentiated carcinoma (grade I), 6-7 points for moderately differentiated carcinoma (grade II) and 8-9 points for the poorly differentiated carcinoma (grade III).

Immunohistochemistry studies

To demonstrate Ki-67 expression in mammary gland tissue, sections were blocked for endogenous peroxidase and placed in an antigen-retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min. Mouse anti-rat Ki-67 (clone MIB-5; DAKO) was applied to the sections and incubated. A secondary detection system was used to visualize antibody binding with avidin-conjugated horseradish peroxidase (ExtrAvidin Peroxidase Staining Kit, Extra-2; Sigma) and staining was developed with a diaminobenzidine (DAB; Sigma) substrate. Finally, the sections were counterstained with Meyers' hematoxylin, dehydrated, and mounted. For negative control slides, primary antibodies were deleted[Muskhelishvili *et al*., 2003].

Statistical analysis

Data are presented as mean ± SE. The data are analyzed using one way analysis of variance, followed by Duncan's multiple range test for post hoc comparison of group means. Results with a probability of $p<0.05$ are considered statistically significant.

RESULTS

***In-vitro* analysis:**

In this study, MCF-7 cells were incubated with BCFE at two concentration values; 10 and 50 µg /ml for 24 h to analyze its effect on the cell cycle profile and apoptosis induction. The resultant data investigated that there was cell accumulation of percentages 15.61 % and 27.38 % due to the two concentration values of the extract at pre G1 phase comparing to 1.49 % of the untreated MCF-7 cells. Furthermore, cell accumulation was also detected at G2/M phases in MCF-7 cells of percentages 19.03 and 29.1 % due to treatment with BCFE by 10 and 50 µg / ml respectively, compared to 10.75% of the untreated MCF-7 cells. This result represents that there was a cell cycle arrest at G2/M phase and partialy cessation of mitotic cycle (Table1).

Table I. Determination of cell cycle inhibition of MCF-7 cancer cells by BCFE.

Sample data	***DNA content***				
code	%G0-G1	%S	%G2/M	%Pre-G1	Comment
BCFE/mcf7_10ug/ml	47.36	33.61	19.03	15.61	Cell cycle arrest@G2/M
BCFE/mcf7_50ug/ml	41.52	29.38	29.1	27.38	Cell cycle arrest@G2/M
control_MCF7	52.44	36.81	10.75	1.49	---

Apoptotic assay

There was an increase in the late apoptosis produced by the BCFE at a concentration of 10 µg / ml of 0.15 % (DMSO control) to 4.81 % and an increase in the early apoptosis from 0.28 % (DMSO control) to 7.29 % with necrosis percent 3.51% *vs* 1.06 % produced in the untreated MCF-7. On the other hand, treatment of MCF-7 with the extract at a concentration of 50 µg / ml exhibited a significant increase in the late apoptosis reaching 12.76 % and an increase in the early apoptosis of 5.41 % with necrosis percent 9.21 % compared to the untreated MCF-7 **(**Table 2) (Fig.2). It could be noted that the late apoptosis percentages were higher than that of the early phase, which makes it more challenging to recover the apoptotic cells to innate ones.

Table II. Percentage of apoptosis and necrosis in MCF-7 cancer cells after treatment with two doses of *B. ceiba* flower extract

Code	**Apoptosis**			
	Total	Early	Late	Necrosis
BCFE/mcf7_10ug/ml	15.61	7.29	4.81	3.51
BCFE/mcf7_50ug/ml	27.38	5.41	12.76	9.21
control_MCF7	1.49	0.28	0.15	1.06

***In-Vivo* analysis:**

Effect of *B. ceiba* extract on breast cancer markers in female rat serum

Data in Fig. 3 shows that the treatment of rats with DMBA significantly increased PI3K/AKT signaling, Ki-67, TGFβ, and Bcl2 ($p \leq 0.05$) in serum by 68.22%, 111.59%, 110.52% and 229.22%, respectively which represents nearly 1.7-, 2-, 2-, and 3-fold increases compared with the control group. On the other hand, BCFE inhibited abnormal tumor cells proliferation by decreasing the PI3K/AKT signaling, Ki-67, TGFβ, and Bcl2 in serum significantly ($p \leq 0.05$) compared with the DMBA non-treated group.

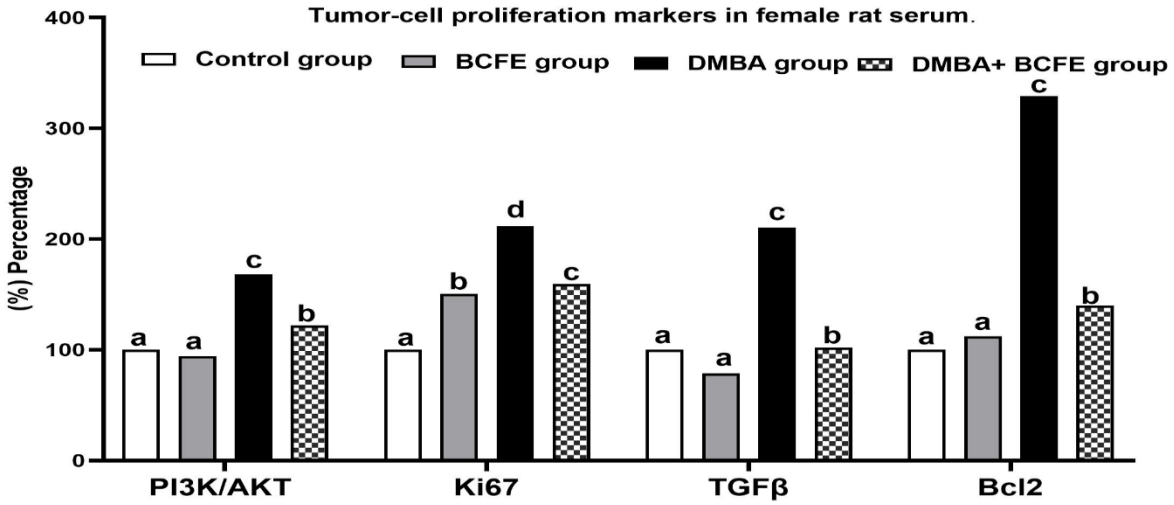

Figure 1. Effect of *B. ceiba* flower extract on tumor-cell proliferation markers in female rat serum.

Effect of *B. ceiba* extract on breast cancer marker in female rat tissue

Figure 4 shows that administration of DMBA resulted in significant increases ($p \leq 0.05$) in Ki-67and TGFβ expression in tissue by 200% and 60% respectively, compared with the control group. PI3K/AKT exhibited only statistically insignificant increases ($p \leq 0.05$) compared with the control group. Administration of BCFE

ameliorated proliferation and progression of tumor cells by decreasing all measured parameters significantly ($p \leq 0.05$).

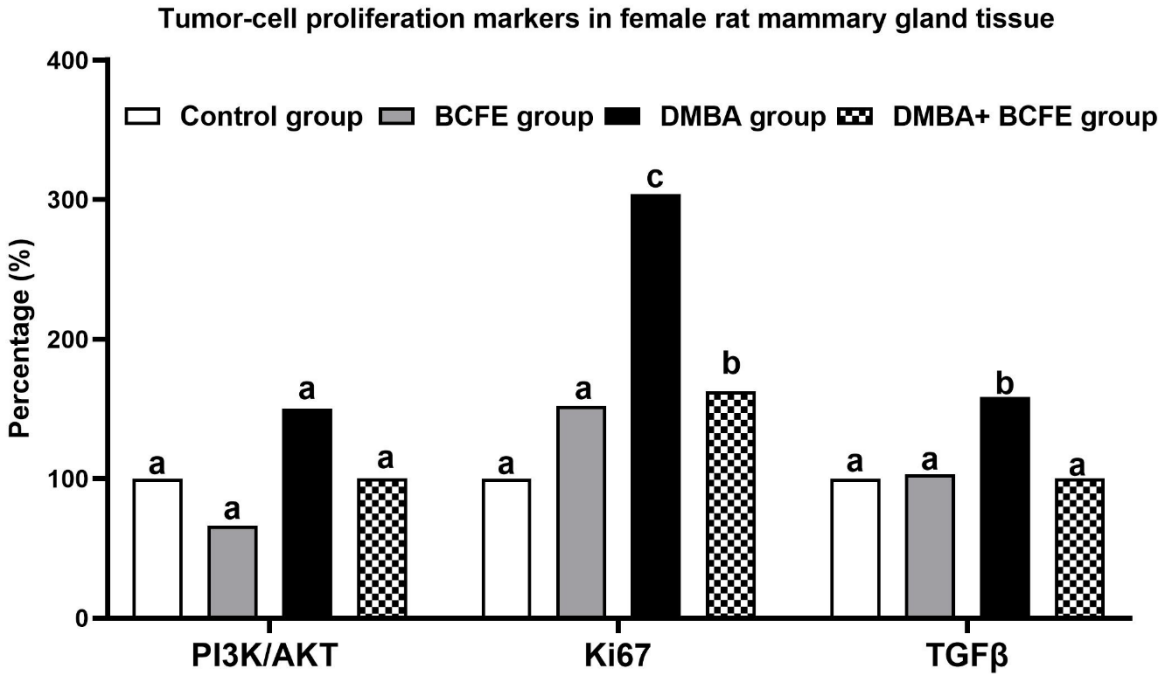

Figure 2. Effect of *B. ceiba* flower extract on tumor-cell proliferation markers in female rat tissue.

Histopathology

Histopathological examination of mammary gland tissue from Group 1 (controls) revealed normal histological architecture of murine mammary glands (Fig. 3a and b), which appeared to be composed of branching ducts surrounded by connective tissues, few acini, and adipose tissue. Similar to the normal group, the mammary glands of female rats that received only BCFE (150 mg/kg) (Group 2) showed apparently normal duct and glandular acini structures (Fig. 3c). These structures appeared to be surrounded by normal, but scanty amounts of connective tissues (Fig. 3d).

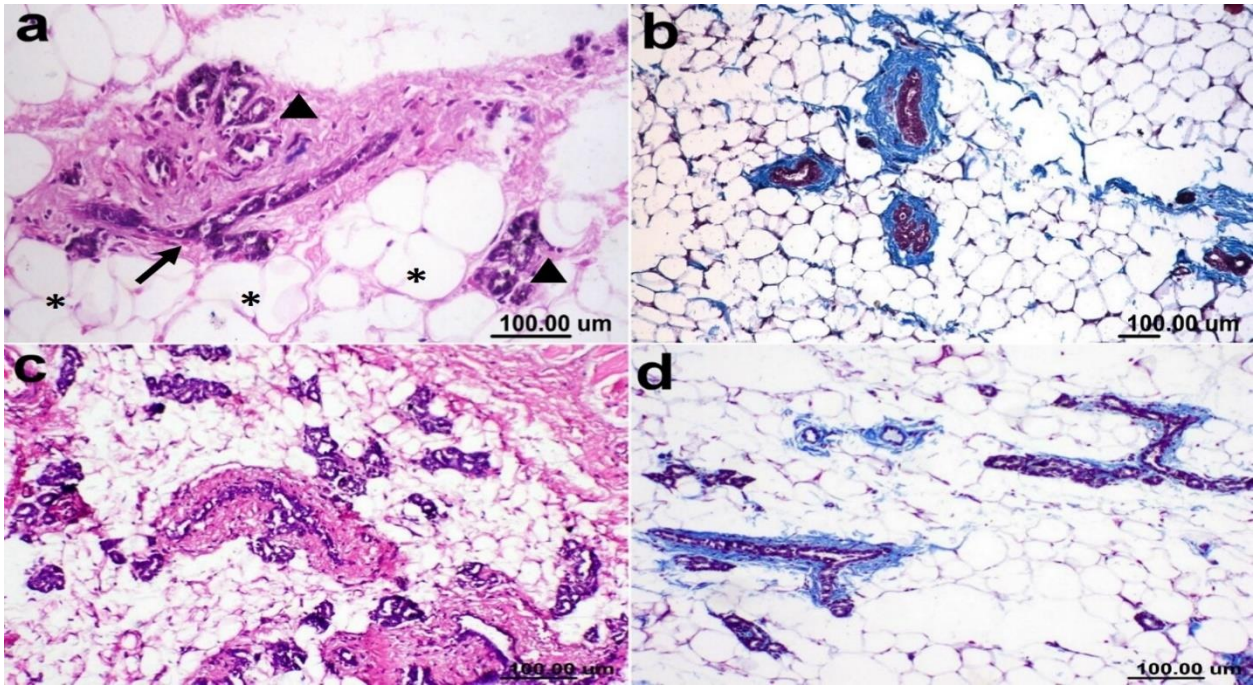

Figure 3. Photomicrograph of a rat mammary gland showing normal histological structures of a mammary gland (**a**) from a normal control female rat, branching duct (arrow), few acini (arrow head) and adipose tissue (asterisk) (via H&E staining) and (**b**) from a normal control female rat, blue-stained fibrous connective tissue within the normal mammary gland (MTC).. (**c**) The group that received *B. ceiba* extract showed normal histological structures of ducts and acini (H&E) and (**d**) scanty blue-stained fibrous tissue surrounding a mammary gland structure (MTC).

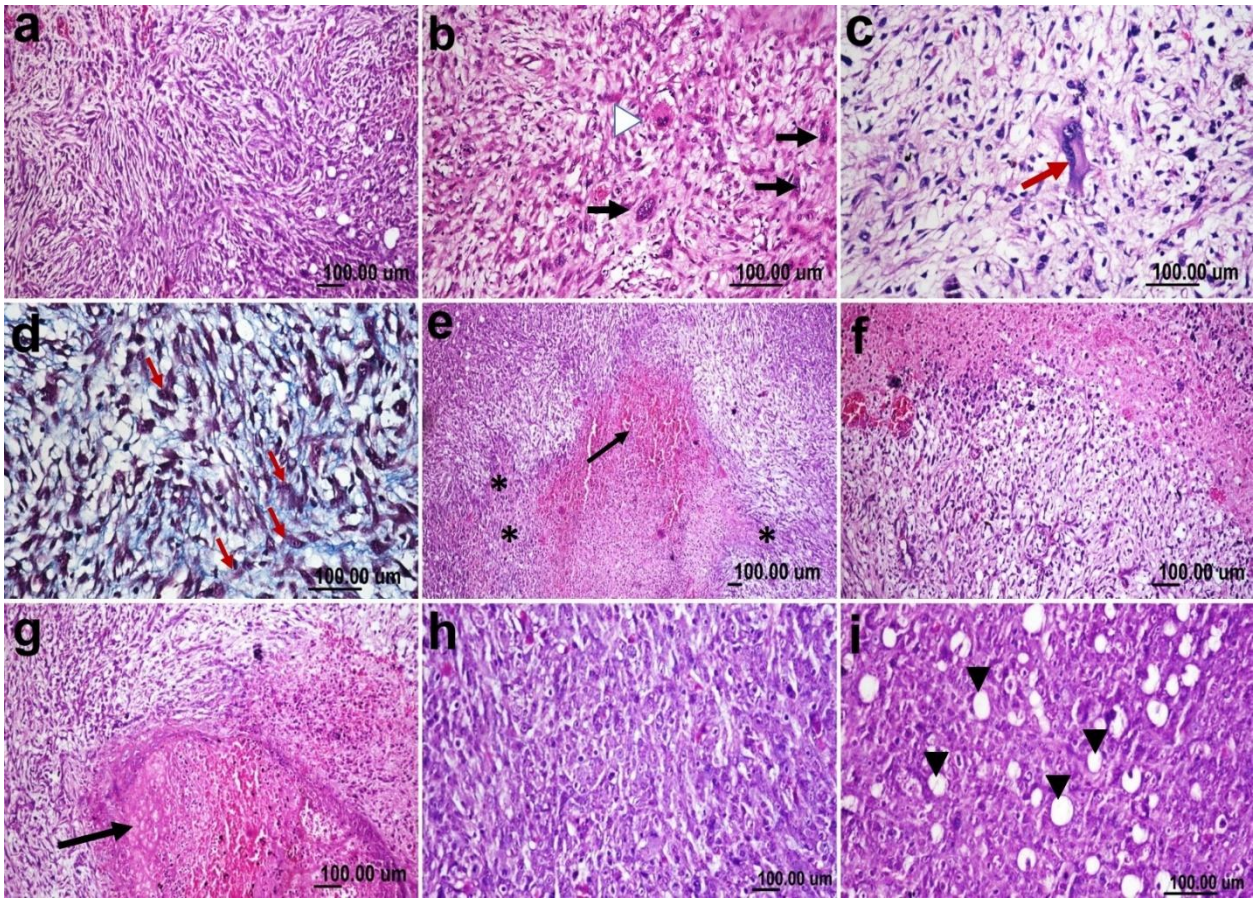

Figure 4. Photomicrograph of a rat mammary gland from the control DMBA group: (**a**) Malignant myoepithelial cells arranged in a characteristic storiform pattern (via H&E staining). (**b**) Plump spindle malignant myoepithelial cells (arrows) displaying criteria for malignancy (karyomegaly and pleomorphism) in the presence of a multinucleated cell (arrow head) (H&E). (**c**) Multinucleated tumor giant cell (red arrow); note the marked pleomorphism of tumor cells (H&E), (**d**) Red-stained myoepithelial cells (red arrows) (MTC). (**e**) Severe hemorrhage (arrow) and inflammatory reaction (asterisk) within a tumor stroma (H&E). (**f**) Necrosis, congestion, and hemorrhaging within a tumor stroma (higher magnification) (H&E). (**g**) Thrombosis (arrow) in a blood vessel with perivascular inflammatory-cell infiltration and hemorrhage (H&E). (**h**) A solid carcinoma; malignant cells are arranged in sheets or clusters (H&E). (**i**) Frequent apoptosis (arrow heads) within carcinoma cells (H&E).

However, histopathological examinations of mammary gland sections from control DMBA rats (Group 3) revealed complete destruction of the mammary gland architecture, while the normal surrounding adipose tissue were poorly detected and occupied by intense inflammatory reactions with marked fibrosis. In addition, the glandular tissue was completely replaced by dense populations of malignant tumor cells bearing two distinct criteria. First, some malignant tumor cells were identified as malignant myoepithelioma arranged in a characteristic storiform pattern (Fig. 4a). The cells appeared as plump spindle cells with ovoid nuclei and eosinophilic cytoplasm, and tumor cells showed characteristic signs of malignancy, including hyperchromatic, pleomorphism, and frequent atypical mitosis with numerous mitotic figures (Fig. 4b). Multinucleated tumor giant cells were frequently detected (Fig. 4c). MTC- stained sections showed red-stained spindle myoepithelial cells and a few blue-stained fibrous tissue stroma (Fig. 4d). The supporting stroma showed signs of inflammatory reactions, hemorrhaging, necrosis (Fig. 4e & f), and thrombosis (Fig. 6g). Second, tumor-cell population recognized was defined as a solid mammary gland carcinoma, in which the tumor cells were predominantly arranged in solid sheets and clusters (Fig. 4h), with no tendency for acinar formation (highly malignant). Necrosis and apoptosis (Fig. 4i) were frequently observed within the tumor cells.

The first histological characteristics of mammary gland tissue to be detected in the DMBA group treated with BCFE was the decreased load of malignant myoepithelial cells per microscopic field with progressive necrosis accompanied by hyalinization in many sections (Fig. 5a and b). Although the tumor cells displayed criteria of malignancy, mitosis were infrequent, and no giant cells formed. The glandular carcinoma was represented by tumor cells showing a tendency for acini formation (Fig. 5c), indicting a lower grade of malignancy.The ductal carcinoma (Fig. 5d) was characterized by a typical cell growing within ducts with a weak tendency for invasion. Severe inflammatory reactions were detected, especially within the lumen of the ducts (Fig. 5e). In some sections, the associated adipose tissues appeared normal, with little sign of perivascular inflammatory-cell infiltration and no evidence of tumor-cell invasion ig. 5f).

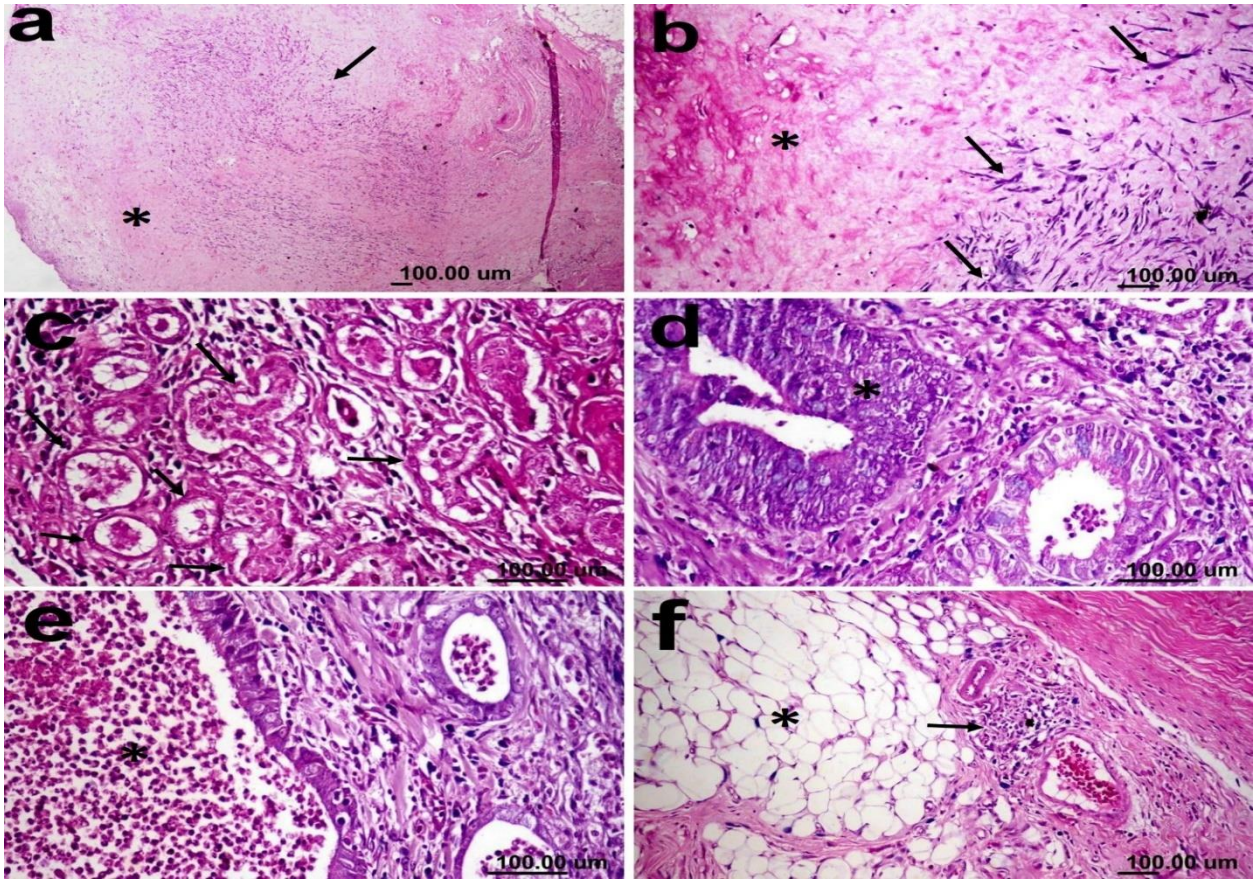

Figure 5. Photomicrograph of a rat mammary gland from the DMBA group treated with BCFE showing (**a**) fewer tumor myoepithelial cells (arrow) with necrosis and hyalinization (asterisk) (via H&E staining). (**b**) Higher magnification of necrosis and hyalinization (asterisk) within the tumor cells (arrows) (H&E). (**c**) An adenocarcinoma, in which tumor cells are arranged into acini (arrows) (H&E). (**d**) A ductal carcinoma (asterisk) (H&E). (**e**) Inflammatory-cell infiltration (mainly neutrophils) (asterisk) within a ductal lumen (H&E). (**f**) Apparently normal adipose tissue (asterisk) with mild perivascular inflammatory-cell infiltration (arrow) (H&E).

The total histologic score is illustrated in (Fig. 6). The detected mammary gland carcinoma in DMBA group was graded as moderately differentiated (grade II) carcinoma characterized by poor tubular formation, increased mitotic figures per microscopic field and marked nuclear and cellular pleomorphism, On the other hand DMBA group treated with BCFE exhibited significant regression of the tumor grade it was scored as well- differentiated (grade I) carcinoma.

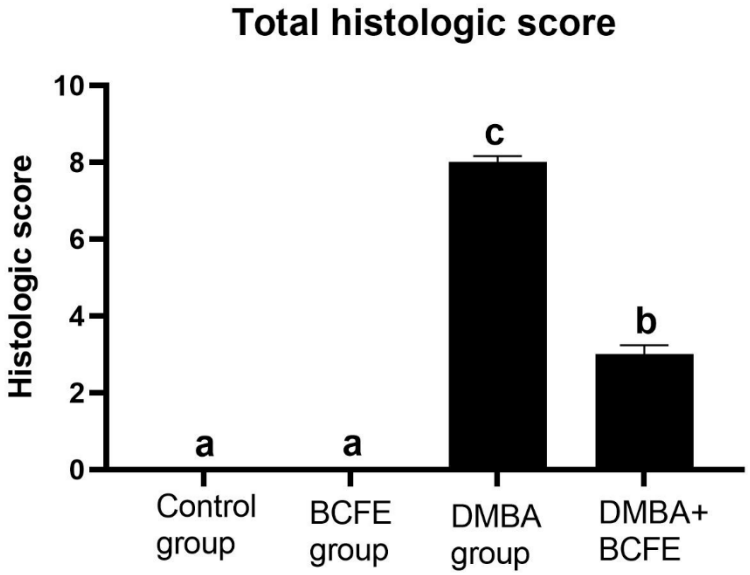

Figure 6. Percent of total histologic score.

Immunohistochemistry

The normal control group and the group that received BCFE showed normal expression of Ki-67 in few acinar and ductal cells (Fig. 7a and b). The DMBA control group exhibited a marked increase in Ki-67–positive cells in the malignant myoepithelial cells as well as the ductal carcinoma cells (Fig. 7c) indicating a high cell proliferation rate, while the DMBA group treated with the extract showed mild expression of this marker in proliferating ductal and carcinoma cells (Fig. 7d and e).

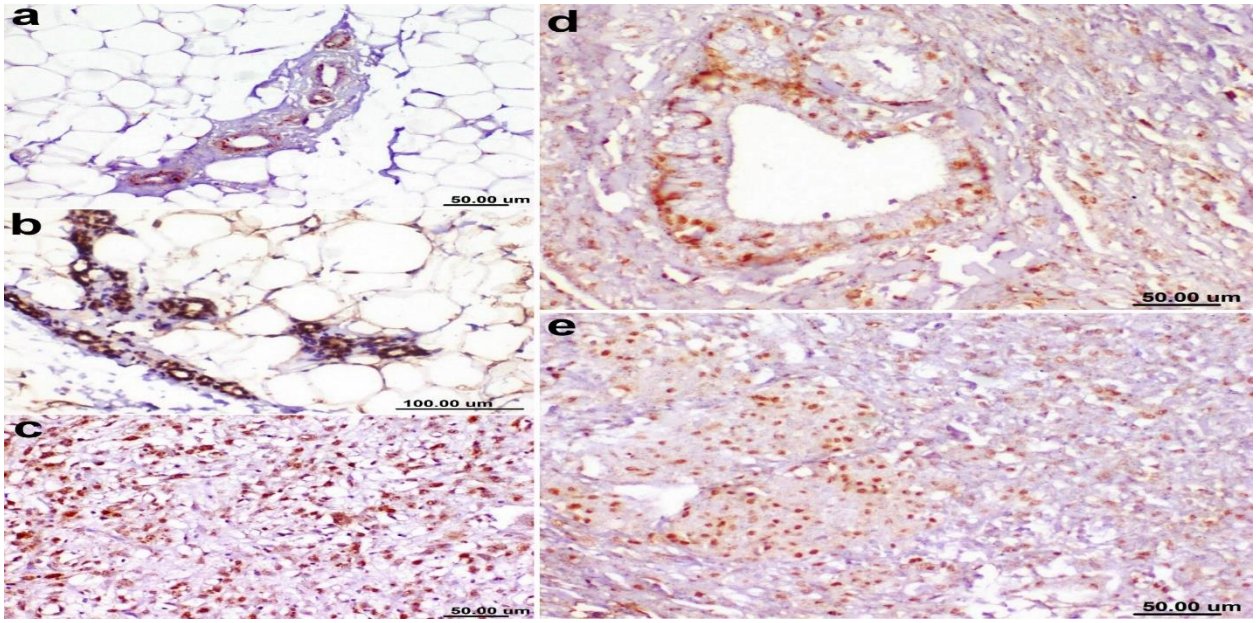

Figure 7. Photomicrograph of a mammary gland of rat with immunostaining: (**a**) Normal control group, showing normal expression of a few positive Ki-67 cells in the lining of the epithelium of ducts and acini. (**b**) The group that received *B. ceiba* extract displayed normal mild expression of ki-67 antigens in ductal and acinar cells. (**c**) The DMBA control group displayed marked expression of Ki-67–positive cells in malignant myoepithelial cells. (**d**) The DMBA group treated with *B. ceiba* extract displayed mild expression of Ki-67–positive cells in the ductal epithelium. (**e**) The DMBA group treated with *B. ceiba* extract displayed mild expression of Ki-67–positive nuclei in solid carcinoma cells.

Quantified expression of Ki 67 is shown in (Fig. 8), DMBA group exhibited marked increase in expression of Ki-67 compared to the control group. Co-administration of BCFE resulted in significant decrease in Ki 67 within the tumor cells.

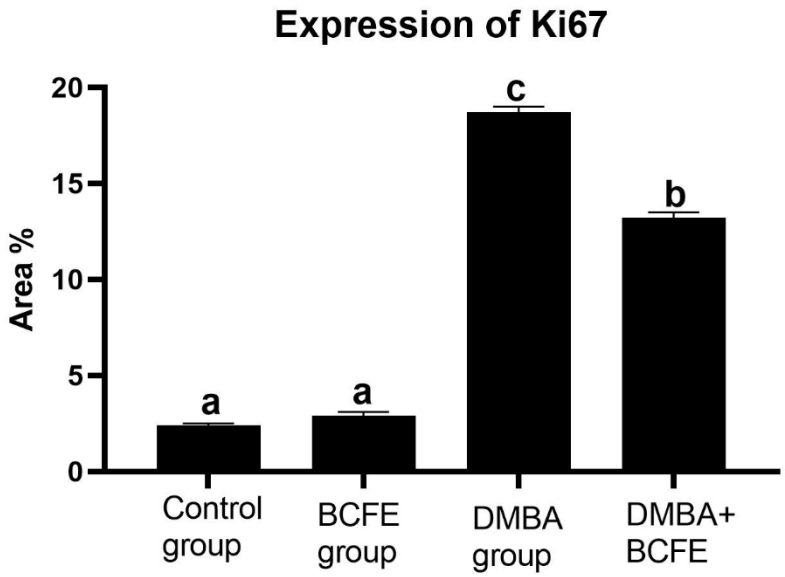

Figure 8. Area percent of Ki67 expression.

DISCUSSION

Malignant neoplastic change is a complex process characterized by increased cell proliferation, dysplasia (dysregulation of cellular differentiation), and infrequent apoptosis [Wang *et al*., 2017]. Induction of apoptosis is considered as one of the most important tools that confirm the effectiveness of cancer treatment [Liu *et al*., 2013]. The present study evaluated the potential therapeutic effects

of BCFE on cancerogenic disorders observed in rats treated with DMBA.This was achieved through analysis of expression levels of some markers related to proliferation and progression of tumor cells.

In the in-vitro studies, the data investigated that there was cell accumulation due to using BCFE by two concentration values at pre G1 phase and G2/M phases in MCF-7 cells in comparison to untreated MCF-7 cells. Thess results represented that there was cell cycle arrest at G2/M phase and cessation of mitotic cycle due to the treatment of cancer cells with BCFE (table 1). In addition, there was an increase in the apoptotic percent produced by the examined extract at the two used concentrations in the early and late apoptosis with necrosis percent 9.21 % compared to the untreated MCF-7 (table 2). It could be noted that the late apoptosis percentages were higher than that of the early phase which makes it more challenging to recover the apoptotic cells to safe ones. The resultant data showed that the apoptotic effect was increased by increasing the concentration of the used extract.

The process of carcinogenesis is activated by different markers such as PI3K/AKT. PI3K is activated through the binding of a ligand or growth factors to its compatible receptor tyrosine kinases (RTKs) [Bland *et al*., 2009]. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates protein kinase B (AKT) [Thapa *et al*., 2015]. Cell growth and protein synthesis is triggered by phosphorylation of AKT via activating rapamycin (mTOR)[Schulz, 2005] which often occurs in various types of cancer cells [Bhaskar and Hay, 2007] and acts as an apoptotic inhibitor.

Mammary gland tissue of DMBA-treated rats showed that excessive proliferation of the epithelium of acini and ductulus was ameliorated by BCFE. The increase in PI3K/AKT signaling affected cell proliferation was found in both tumor initiation and oncogenic phenotypes of mammary gland tumors [Sinn *et al*., 2010]. Our results are in agreement with previous observations which represented that phosphorylated AKT can inhibit cytochrome C and apoptotic factors, thereby inhibiting apoptosis and promoting the proliferation of cancer cells [Sinn *et al*., 2010; Krajarng *et al*., 2017].

Because AKT signaling is fundamental to the growth of various types of cancer cells, including breast carcinoma cells [Tang *et al*., 2014], It is assumed that hypophosphorylation of AKT is responsible for the decrease in proliferation of cancer cells. In the current study, BCFE inhibited the AKT phosphorylation in the breast cancer cells. This effect could be related to the presence of apigenin and xanthone in BCFE [El Toumy *et al*., 2013; Harrison *et al*., 2014].

Despite the proven effects of AKT in promoting tumor growth and progression, AKT inhibitors have shown only limited effect in breast cancer treatment in many trials [Carmona *et al*., 2016]. The inhibition of both AKT and Ki-67 may have a synergistic effect in limiting breast cancer-cell proliferation. Ki-67 expression appeared through the cell cycle, and was found at various points during the G1, G2/M, and S phases but it was not appeared during the G0 phase. So, AKTcould be considered as an effective biomarker for evaluation of growth and proliferation of cancer cells [Pan *et al*., 2017].

Many studies have demonstrated the role of Ki-67 as a prognostic factor for breast cancer [De Azambuja *et al*., 2007; Yerushalmi *et al*., 2010]. The aforementioned marker was decreased in serum and tissues of rats treated with BCFE, indicating limitations in tumor growth and repair of tissue damage.

In cancer cells, TGFβ was considered a tumor-derived immunosuppressor, tumor-mitogen initiator, invasion inducer, and pro-metastatic cytokine stimulator. Blocking of TGFβ in breast cancer prevented primary or metastatic tumors from metastasizing further. In the present study TGFβ expression was decreased in the group treated with BCFE, indicating decreased tumor mutagens and reduced tumor size [Gonzalez-Junca *et al*., 2019].

The present study targeted the anti-apoptotic factors affecting cancer cells. Bcl-2 is a major protein that blocks programmed cell destruction, which prevents the apoptosis of different cells, including cancer cells, and inhibits the release of cytochrome C. The synthesis of Bcl-2 proteins is related primarily to the incidence and progression of breast cancer [Zhang *et al*., 2005]. In our results, serum mammary tumors in the DMBA group showed extensive Bcl-2 expression, which decreased after treating the animals with BCFE (Fig. 1) indicating a role of BCFE in stimulating pro-apoptotic mechanism. The extract prevented breast cancer progression through changes in the mitochondrial cell membrane, where phosphatidyl serine changes its position from inside to outside the membrane and enhanced the macrophages to engulf the tumor cells [Krajarng *et al*., 2017].

BCFE has several modes of biological activity. It inhibits fatty-acid synthesis, which represses cancer-cell proliferation and induces cancer-cell apoptosis without affecting nonmalignant fibroblasts [Alli *et al*., 2005]. Inhibition of carcinogenesis and angiogenesis are other modes of action of flavonoids in cancer treatment. The extract, which is composed of flavonoids, does not induce cytotoxicity in normal Vero cells, but can decrease cell viability of mouse melanoma B16 cells and induce nuclear condensation [Krajarng *et al*., 2017]. Moreover, flavonoids can change the mitochondrial membrane potential, which is an early marker of apoptosis and can reduce mitochondrial membrane integrity, inhibit the Bcl-2 expression, stimulate caspase-3, and induce apoptosis. However, antiproliferative activity of several flavonoids, including kaempherol and quercetin, has been reported against the proliferation of B16 cells [Iwashita *et al*., 2000]. In addition, other flavonoids have been associated with nuclear DNA fragmentation and induction of apoptosis by increasing Bax and suppression of Bcl-2.

Another route through which BCFE can control breast cancer proliferation involves its antioxidant activity. Sitosterol, one of BCFE's components, can act as an anti-inflammatory agent by inhibiting the cycloxygenase (COX) enzyme [Khedir *et al*., 2016]. Ergosterol, another BCFE component, triggers the antioxidant defense system and protects against tissue damage [Krishnamoorthy and Sankaran, 2016] exerted by different cancer models [Li *et al*., 2015; Nadumane *et al*., 2016].

Results of a histopathological analysis showed that a DMBA-induced tumor was malignant and of epithelial origin. The histological pattern of solid tumors without a cribriform appearance in the DMBA group confirmed a high grade of malignancy as previously reported [Costa *et al*., 2002]. Cribriform and well-differentiated patterns of adenocarcinoma observed in BCFE–treated groups were previously reported as a sign of low-grade malignancy[Pugalendhi and Manoharan, 2010]. Given the intense inflammatory reaction observed in the stroma of mammary gland tumors in the DMBA group [Costa *et al*., 2002], these infiltrations can be associated with the

histologic grade of the tumor. Members of the BCFE–treated group displayed weaker inflammatory reactions in the stroma, indicating a lesser histological grade of tumor. Tumors were necrotic in the BCFE–treated group, suggesting a role of our extract as an antitumor agent in inducing local death of cancer cells. The extract appears to act as a mild inhibitor of tumor volume by decreasing tumor-cell counts and increasing the life span of the tumor-bearing mice in Ehrlich ascites carcinoma model [El Toumy *et al*., 2013]. A limited appearance of Ki-67 antigens expression in the BCFE–treated group in our study compared with that in DMBA group, can be considered a mark of decreased tumor size and activity, as reported elsewhere [Pula *et al*., 2013]

We found that the protective efficacy of BCFE depended on its capacity to either reduce harmful effects of DMBA or maintain normal homeostasis of cells and tissues.

CONCLUSION

The remarkable changes in PI3K/AKT, Ki-67, TGFβ and Bcl-2 associated with reactive oxygen species produced by DMBA administration resulted in defects in membrane integrity and other pathological changes in mammary tissues. Our findings addressed one of the mechanisms through which BCFE exerts a therapeutic role in modulating the proliferation of mammary gland carcinoma in vivo. BCFE can therefore be considered a probable anticancer agent through the induction of cancer-cell apoptosis and decrease proliferation.

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