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CHEMOPREVENTIVE EFFICACY OF ZINGERONE ON MASTOCYTE INFLAMMATION, CELL PROLIFERATION AND APOPTOSIS AGAINST DMBA-INCITED MAMMARY CARCINOGENESIS IN FEMALE SPRAGUE-DAWLEY RATS

Ayyanar Rajagopal, Ravi Sriragavi and Namasivayam Nalini *

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar - 608002, Tamil Nadu, India.

Keywords:

Apoptosis; Dimethylbenz (a) anthracene; Estrogen Receptor (ER); Lipid per oxidation; Progesterone Receptor (PR) Proliferation; Zingerone

Correspondence to Author:
Dr. N. Nalini

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar - 608002, Tamil Nadu, India.

E-mail: nalininam@yahoo.com

ABSTRACT: Zingerone is an active phenolic acid and the least pungent component of *Zingiber officinale*. It is known to exhibit different pharmacological properties which include anti-inflammatory, antioxidant, anticancer and antimicrobial activities. But, so far there has been no information available on the role of zingerone in experimental breast carcinogenesis. The objective of this experiment is to untwine the anticancer qualities of zingerone against dimethylbenz (a) anthracene (DMBA)-incited mammary carcinogenesis. To achieve this goal, female Sprague-Dawley rats were arbitrarily classified into six groups. Group 1 was notified as the control, groups 2-5 were given a single dose of DMBA (25 mg/kg b.wt, subcutaneously) in the 4th week. Along with DMBA, groups 3 (initium), 4 (post-initium) and 5 (entire period) rats received zingerone (20mg/kg b.wt. p.o) every day in different time periods during the experimental period of 16 weeks. Group 6 rats received 20 mg/kg b.wt. of zingerone alone. Cancer-bearing animal mammary tissue when evaluated by western blot showed increased immune expression patterns of ER, PR, HER2/ neu, cyclin D1 and also antiapoptotic protein (Bcl-2) and decreased expression of the proapoptotic proteins (P53, P21, Bax, caspases 3 and 9. When zingerone (20 mg/kg b. wt.) supplementation was continued for the entire duration of the experiment to DMBA- treated rats, the expression patterns of various tumor and apoptotic markers including the pathological modifications were switched back to essentially normal. Therefore, we conclude that zingerone could be considered as a powerful chemo preventive agent to counter DMBA incited breast carcinogenesis.

INTRODUCTION: Breast cancer is viewed as the widely recognized tumor in India especially in women and it was recorded to be 14% as compared to all other types of cancers that affects women throughout the world [1, 2]. Globocan 2018 data: New cases registered: 1,62,468 Deaths: 87,090.

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The occurrence rate in India begins to increase during early thirties and peaks between the age of 50-64 [National Cancer Registry Programme]. Overall, 1 out of every 28 women is probably at a risk of developing breast cancer during her life span. In urban areas, 1 out of every 22 women and in rural areas 1 out of every 60 women is prone to breast cancer in the course of her life span. Thus, compared to rural areas, women in cities are highly prone to breast cancer [3]. DMBA is an engineered and polycyclic aromatic hydrocarbon; in following its organization, the concentration of DMBA in the entire mammary gland is 110-times higher than that which is acquired from collagenase-dissociated

mammary epithelial cells. Accordingly, it appears that the mammary gland acts as a store for the continual release of the pro carcinogen into the parenchymal tissues. This phenomenon clarifies the high vulnerability of the mammary epithelial cells to carcinogenesis induced by DMBA [4]. It has been clearly reported that a single portion (dose) of DMBA brings good results and it may be due to the dynamic proliferation of the terminal glands in its presence [5]. Increased exposure to exogenous or endogenous estrogens is usually risk factors in the development of breast cancer.

Particular genes such as BRCA1 and BRCA2, HER-2/neu and P53 are sensitive to the exposure and the growth of breast cancer. Over expression of HER-2 was reported both in mammary carcinogenesis as well as its metastasis. The status of ER, PR and HER-2/neu have been utilized as routine markers for prognosis and diagnosis; moreover, they react to the treatment of breast cancer [6]. Proliferating cell nuclear antigen (PCNA), a non-histone nuclear acidic protein as shown in the nuclei of proliferating cells in G1 and S phase [7, 8] is used as an index of proliferation in a wide range of cells [5]. Cyclin D1, a proto-oncogene is significant regulator of G1 to the progression of various kinds of cells in S phase.

Besides cyclin D1 works as a transcriptional modulator in managing the actions of transcription factors such as, NF-κB[9] connected with vitalizing PCNA. The pro-apoptotic Bcl-2 relatives like Bax starts apoptosis discharge and initiates the caspase sequence, although the anti-apoptotic protein Bcl-2 avoids permeability of mitochondrial membrane and represses apoptosis, mainly due to the endurance of the host cell [10]. In spite of advancement in science and technology in the management and the treatment of cancer, no solid or definite medicine has been found. Hence, the discovery of new powerful anticancer drugs has always been the main stream of cancer research. Ginger, the rhizome of *Zingiber officinale*, is commonly used in various food preparations, in almost all parts of the world. Ginger is not an allergenic food, and it is used to treat different kinds of disease across the world. Countries like India, China and also other Eastern countries use ginger for the treatment of cerebral pains, nausea, rheumatism, cold and loose bowels. Zingerone, a vital component of ginger (approximately 9.25%) is known for its powerful pharmacological properties. Though zingerone is extensively present in dry ginger, recooking and drying additionally changes gingerol into zingerone by the retro aldol reaction. Zingerone is a phenolic alkanone that contains vanilloid (3-methoxy-4-hydroxy benzene) group like structure.

It is considered as the pungent element of ginger, which consists of 3% of essential oil with gingerol and Sogaol [11] gingerol includes 6-gingerol, 8-gingerol and 10-gingerol. These are present in low levels in fresh ginger, but when ginger is roasted and dried, the levels of zingerone largely increases. Zingerone has numerous beneficial effects like Antioxidant, [12, 13] anti-inflammatory, antimicrobial [14] and anti-radiation impacts [15]. Although the recent scientific studies have demonstrated that zingerone has anticancer properties, its exact mechanism remains to be elucidated. Our previous study stressed the inhibitory effects of zingerone applies inhibitory impacts of dose-dependently on DMBA-incited mammary carcinogenesis [16]. Presently, our goal is to examine and affirm the chemo preventive adequacy of zingerone on mastocyte inflammation, histochemistry, cell proliferation and apoptosis in DMBA-incited mammary carcinogenesis in female Sprague-Dawley rats.

Required Materials and Procedure:

Chemical Compounds: Acrylamide, zingerone, 2-mercaptoethanol, bovine serum albumin (BSA), bromophenol blue, ethidium bromide, sodium dodecyl sulphate (SDS), diemthylbenz (a) anthracene (DMBA) and N,N,N,N`- tetramethyl ethylenediamine (TEMED) were brought from Sigma Aldrich, Mumbai, India. Antibodies for ER, PR, Her2, cyclin D1, PCNA, Bcl-2, Bax, P53,P21, caspase-3 and caspase-9 were brought from Santa Cruz Biotechnology, USA. Reagents and every additional chemical compounds were of systematic degree, brought from Hi-Media Pvt. Ltd. (Mumbai, Maharashtra, India).

Ethical Statement, Animals and Dietary Supplement: Five week old female Sprague-Dawley rats (130-150 g weight) were focused and kept for experimental analysis at the Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University, Annamalainagar,

Tamil Nadu, India. The animals (rats) were humanely taken care of in accordance to the rules and principles of the Ethical Committee for Animal Care of Annamalai University by the Indian National Law on animal care and use (Reg. No. AU-IAEC/ 1214/4/18). The rats were domiciliated in sterile polypropylene closets with wirework at the top in a special-pathogen-free room under well-organized environment of 12 h light/ 12 h dark cycle, with a temperature of 22 ± 1°C and relative humidity of 50 ± 10% till the end of the experiment. In the entire duration of the experiment, modified pellet diet [16] and water were provided *ad libitum*.

Schedule of the Treatment: Group 1: Rats were provided modified pellet food and considered as control. Group 2: Rats were administered a modified pellet diet a single dose of DMBA (25 mg/kg b.w.) as a subcutaneous injection during the fourth week of the experiment. Group 3: Rats were given a modified pellet diet and received carcinogen (like group 2) and zingerone (20 mg/kg b.w.) right from the very first day and proceeded till the administration of a single DMBA injection in the fourth (4^{th}) week. Group 4: Rats were given a modified pellet diet and received carcinogen (like group 3) and was started zingerone (20 mg/kg b.w.) after two days of carcinogen injection and continued till the end of the experiment. Group 5: Rats were given a modified pellet diet and received carcinogen (same as in group 3) and zingerone (20 mg/kg b.w.) for the whole experimental period. Group 6: Rats were given a modified pellet diet and administered zingerone (20 mg/kg b.w.) every day for 16 weeks. The design of the experiment is schematically portrayed in **Fig. 1.**

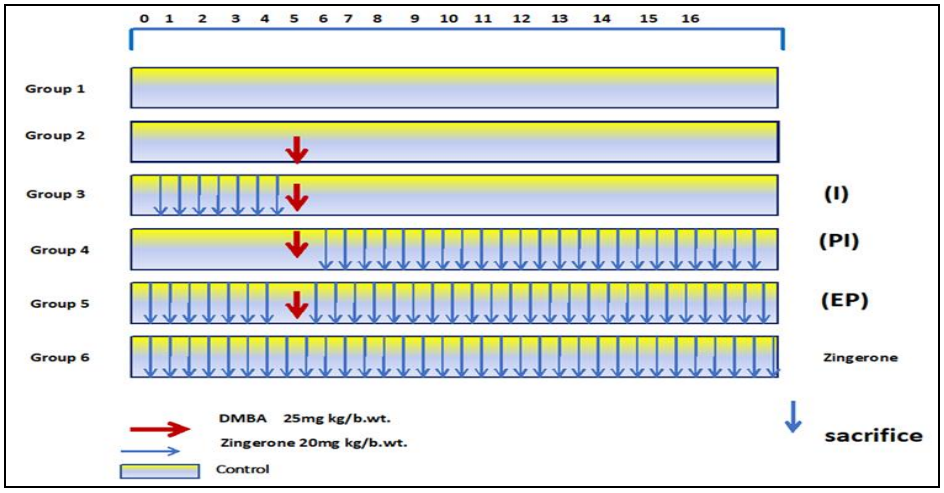


FIG. 1: SIGNIFICANTLY AT P<0.05 VERSUS CONTROL, F P<0.05 VERSUS DMBA, B P<0.05 VERSUS DMBA. INITIUM: I, POST INITIUM: IP, ENTIRE PERIOD: EP

Administration and Preparation of Zingerone and Carcinogen: Zingerone was mixed and dissolved in 2% dimethyl sulfoxide (DMSO) [17], then it was orally administered. The dose of zingerone 20 mg/kg b.w. was given as provided in the experimental design. 25 mg of DMBA was suspended in 1 ml of physiological saline and sunflower oil [18] to ensure the stability of the carcinogen. Then, it was infused subcutaneously in the right thigh once every week for 15 weeks. The process began in the 4th week of the experiment.

Changes in Growth Rate and Body Weight: The experimental and control rats' growth rate and body weight were monitored and surveyed during the whole experimental period of 32 weeks. The weight of rats was measured before the start of the experiment, then weighed once every week and finally weighed before necropsy.

Histopathological Analysis: Histological testing was performed to observe the occurrence of any pathological alterations keenly. A tiny part of mammary tissue and liver were taken from the various groups and then they were fixed in 10% neutral buffered formalin for seven days at normal room temperature. Specimens were dehydrated under equal portions of ethanol, cleared in xylene, and inserted in paraffin wax. Then tissue blocks were segmented (5 µm) and handled by usual histological techniques, which were followed by hematoxylin and eosin (H & E) staining.

At that point, histological evaluation was completed by a pathologist in a blinded way with the assistance of a light microscope (40X) (Carl Zeiss. Germany).

Toluidine Blue Staining for Mastocytes: The tissue segments (4-5 cm) were rehydrated, dewaxed and later they were treated with 0.5% solution of toluidine blue dissolved in 0.5 M HCl (pH 0.5) for the duration of 5 to 7 days and separating the solution on other substitute days. After that, the tissue segments were cleansed with refined water (distilled) covered with 5% ammonium molybdate for 5 min, cleaned for 10 min in running tap water and counterstained by 1% eosin.

Again, the segments were cleaned in running tap water, placed in n-butane for 5 min, cleared in xylene, air-dried, and mounted using DPX. The granules of mammary cells were stained dark blue against a plain back drop, those granules were photographed at the magnification of 40 X using a light microscope. (Axio Scope A1, Carl Zeiss, Jena, Germany).

Immunohistochemistry: The paraffin-embedded (5µm) tissue segments were rehydrated, deparaffinised, and exposed to recover antigen. They were dipped in a solution for blocking the proteins and subsequently incubated with primary antibodies such as ER, PR, Her2, Bcl2, PCNA, and cyclin D1 for three hours at 30ºC. The identification of bound primary antibody (immune) was carried out using a secondary antibody, which was inturn conjugated with horseradish peroxidase polymer for 30 min at room temperature. The immune precipitant was envisioned by treating with 3, 3`- diaminobenzidine (DAB), counterstaining with hematoxylin, and photographed using 40X magnification (AxioScope A1, Carl Zeiss, Jena, Germany) [19].

Immunoblot Analysis: In order to obtain protein samples, the mammary tissues were homogenized in protein buffer, which contained 5% sucrose, 10% Tris-HCl, 10% Triton X-100, 1% EDTA, 0.0125% NaF, 1% EGTA, 4% Sigma protease inhibitor mixed drink (cocktail), 1% sodium orthovanadate and 0.07% β-mercaptoethanol. The lysed mammary cells were homogenized by a tissue homogenizer. Spinned using a centrifuge at 15,000 × g for 20 min at 4 °C, and the supernatant was aliquoted and preserved at -80 °C for further analysis.

SDS-PAGE was performed by utilizing identical protein extracts (55 µg) from every sample, and the resolved proteins were electrophoretically moved on to polyvinylidene difluoride membranes. Subsequently, the smudges were incubated for 2 hours with 1 × PBS containing 5% skimmed dry milk to block unclear binding sites. Then the membranes were incubated with 1:1000 dilution of primary antibody at 4 °C overnight. After cleansing, the membranes were incubated with 1:2000 dilution of horse radish per oxidase-conjugated secondary antibody for two hours at 30 ºC. After satisfactory cleaning, the immune-reactive proteins were envisioned by improved chemiluminescence detection reagents (Sigma) and quantified by Image J (1.51 f) (NIH, Bethesda, MD) [19].

The impact of zingerone and DMBA on the expressions of cyclin D1, Bax, Bcl-2, caspases 3 and 9, PCNA, p^{21} and wild-type p^{53} genes were assessed by immune blotting. To study the sub-cellular fractionation, nuclear proteins and cytoplasm were isolated from the mammary tissue utilizing a compartmental protein extraction pack (Millipore). Cytoplasmic and nuclear protein was isolated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were moved on to polyvinylidene difluoride (PVDF) membranes, as portrayed by Towbin *et al.* [10].

RESULTS:

General Observations on the Changes that Occurred in the Growth Rate and Body Weight of Rats: The changes in the growth rate and body weight of rats in every group of rats are delineated in **Fig. 2.** During the experiment, varying levels of changes in the growth rate and body weight of animals (rats) in different groups were noticed.

DMBA-alone exposed rats (group 2) gained weight (35.01) and growth rate (0.28) significantly slowly when compared with the control and zingerone groups (1^{st} and 6^{th} groups). The growth rate and weight were evidently higher ($P< 0.05$) with the supplementation of zingerone to DMBA-exposed

rats (groups 3-5). The growth rate and weight gain noticed in group 3 was 50.1 and 0.40 and in group 4 was 65.01 and 0.53 separately. It is vital to note that when zingerone was supplemented throughout the experimental period (Group 5), improvement in weight gain (96.10) and growth rate (0.78) could be equated to the other two groups (3 and 4), which underlined the protective effects of zingerone against DMBA-incited mammary carcinogenesis. The growth ratio was compared and contrasted between the last and earlier body weight. Further, the final body weight was divided by the number of total days i.e., 122. There were no important variations in the growth and body weight between the rats in groups 1 and 6.

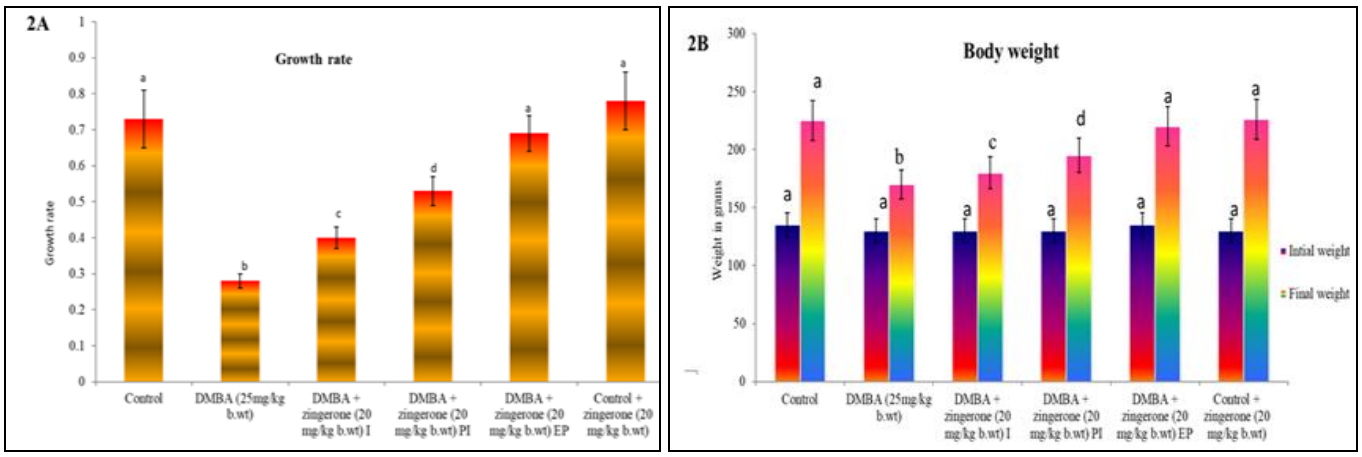


FIG. 2 DIMETHYLBENZ (A) ANTHRACENE: DMBA, INITIUM: I, POST INITIUM: PI, ENTIRE PERIOD: EP. DATA ARE DISPLAYED AS THE MEAN ± SD OF SIX RATS IN EVERY GROUP (n=6). VALUES NOT SHARING TYPICAL SUPERSCRIPT LETTER (a-d) VARY ESSENTIALLY AT P<0.05 (DMRT)

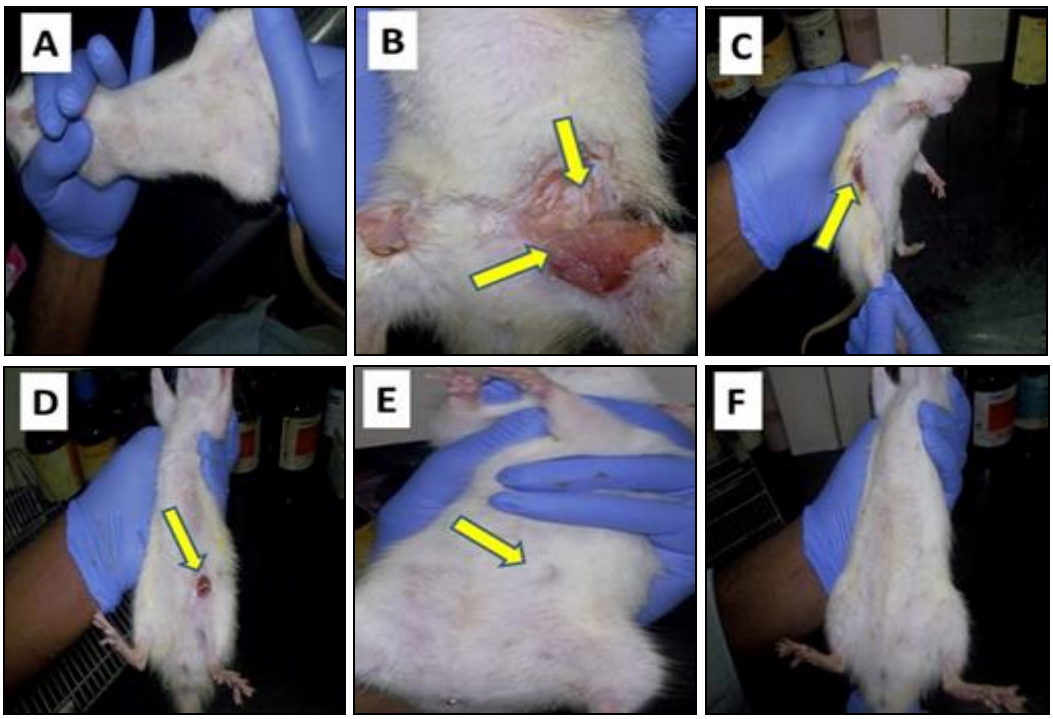


FIG. 3: THE EFFECT OF ZINGERONE AND DMBA ON MAMMARY TUMOR MORPHOLOGY (A-F) IN CONTROL AND EXPERIMENTAL RATS. GROUP 2 AND GROUP 3 ARE CANCER-BEARING RATS AND GROUPS 4 AND 5 ZINGERONE-TREATED RATS. GROUP 1 IS CONTROL AND GROUP 6 ZINGERONE-ALONE-TREATED RATS

Effect of Zingerone and DMBA on Mammary Tumor Morphology: Morphological examination was done to analyze the rats for DMBA-incited tumors in the mammary glands and in other organs. None among the rats in the control or zingerone group was found to have any trace of tumors in

their mammary glands; simultaneously, rats of the DMBA treatment groups had a builtup thick adenomatous skin and a distinct type of tumor that were promptly identified. Rarely in some cases, a huge centrally placed, poorly characterized mass was recognized; based on its general qualities, this gave an impression of being penetrating adenocarcinoma **Fig. 3.**

The growth of tumour was related with the discharge from the nipple, along with the presence of large, delicate and bulky lumps ulcerated via the skin in group 2 (B). Meanwhile, zingerone treatment decreased the impact of DMBA, demonstrating especially the reduced number of tumors in group 5 (E), ulceration and few tumors in group 4 (D) and increased number, but reduced size of tumors in group 3 (C) as equated to DMBA alone treated rats. Control rats and zingerone alone rats 1 & 6 (A&F) showed normal appearing skin with no tumors.

Effect of Zingerone on DMBA Incited Histo-pathological Alterations: Fig. 4. indicates the photomicrographs of hematoxylin and eosin (H&E) stained mammary tissue segments of the experimental and control rats.

Absence of usual mammary epithelial integrity and architecture with extreme dysplasia were recorded in the rats treated with DMBA-alone (2nd group).

Groups 4 and 5 rats appeared to have crumbled mammary architecture with gentle dysplasia. However, control + zingerone-managed rats (groups 6 and 1) indicated usual architecture with intact mammary tissue.

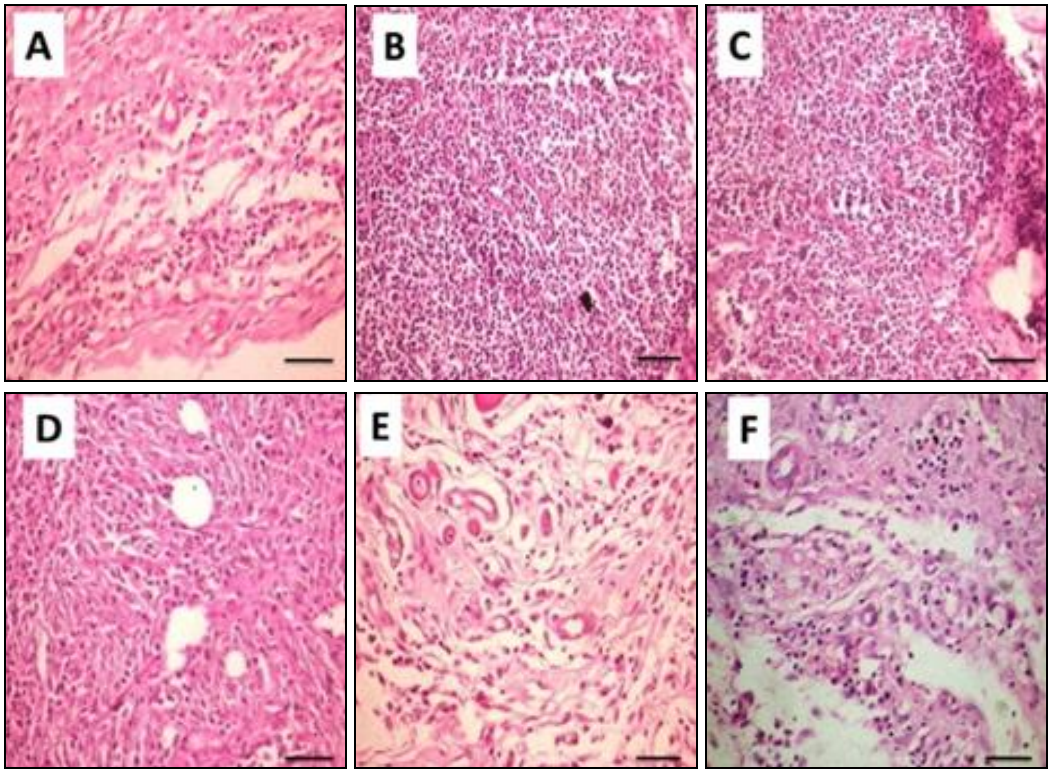


FIG. 4: PHOTOMICROGRAPHS INDICATING THE EFFECT OF ZINGERONE ON THE HISTOPATHOLOGICAL EVALUATION OF H&E STAINING (A-F) IN MAMMARY TISSUES OF CONTROL AND EXPERIMENTAL RATS. GROUP 1 CONTROL AND GROUP 6 ZINGERONE ALONE-TREATED RATS SHOWED USUAL ARCHITECTURE OF MAMMARY TISSUES. INTERESTINGLY, GROUPS 2 AND 3 DMBA-INCITED CANCER-BEARING RATS INDICATED LOSS OF ARCHITECTURE WITH PERMEATING HARMFUL TUMOR. GROUPS 4 AND 5 ZINGERONE-TREATED RATS INDICATED ALMOST NORMAL MAMMARY ARCHITECTURE WITH INCREASED FIBROTIC AREA (40 X MAGNIFICATIONS)

Effect of Zingerone on DMBA Incited Mastocyte Inflammation: His to chemical staining with toluidine blue for mastocytes in the mammary tissue of experimental and control rats is exemplified in **Fig. 5.** DMBA alone-exposed rats (groups 2 and 3) pictured extreme accretion of mastocytes in the mammary tissue (termed as mastocyte density), while zingerone feeding to

DMBA-exposed rats at various intervals of time (groups 3-5) evidently decreased the count of mastocytes. Zingerone supplemented during the time of post-initium (group 4), indicated lower number of mastocytes compared to group 3 rats (initium). A good impact was noticed progressively in the carcinogen-treated rats fed zingerone as a supplement (group 5). Relatively low number of mastocytes were recorded in control + zingerone-fed rats (groups 6 and 1).

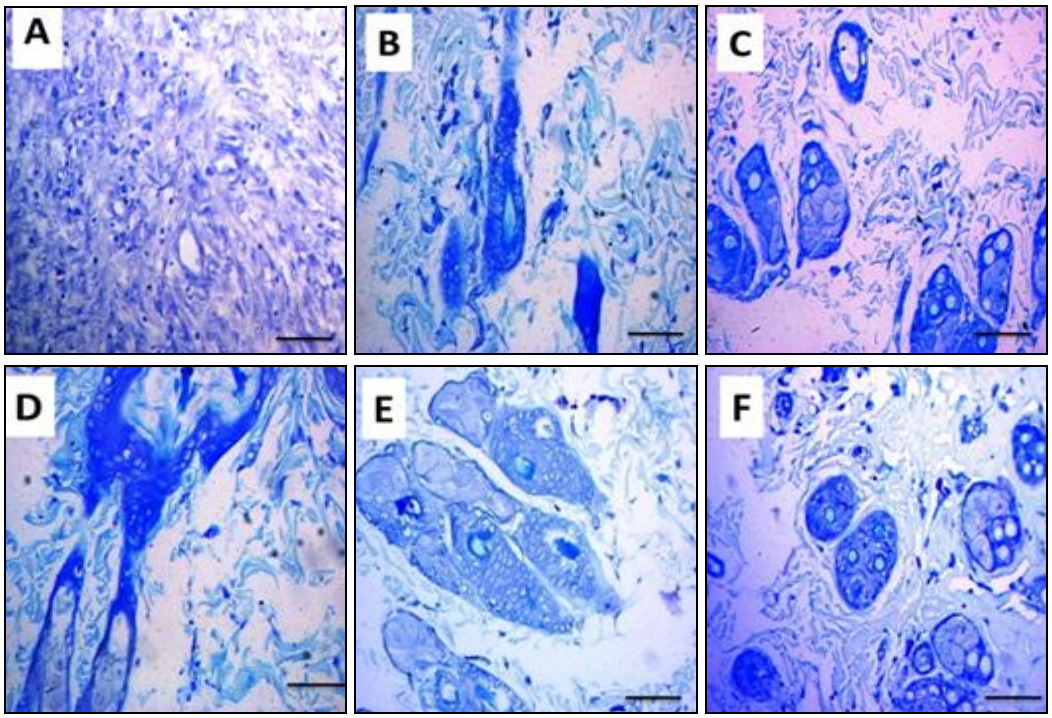
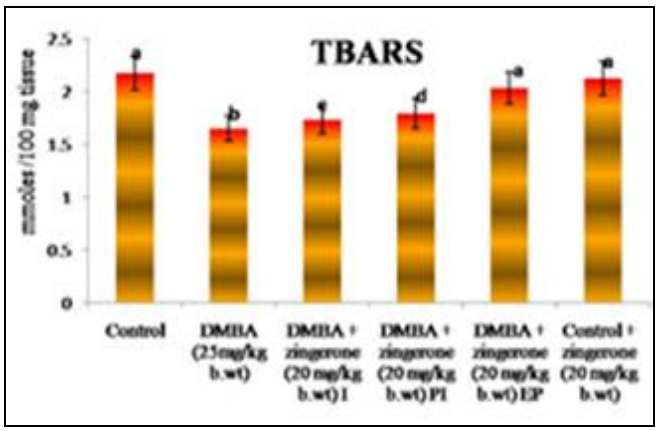


FIG. 5: DELEGATE IMAGES INDICATING THE EFFECT OF ZINGERONE ON THE HISTOPATHOLOGICAL TESTING USING TOLUIDINE BLUE STAINING (A-F) IN MAMMARY TISSUES OF THE RATS FROM THE CONTROL AND EXPERIMENTAL GROUP. GROUP 2 AND GROUP 3 CANCER-BEARING RATS INDICATED CLEARLY THE INCREASED NUMBER OF MASTOCYTE POPULATION; HOWEVER, THEY WERE MODIFIED IN GROUPS 4 AND 5 ZINGERONE-TREATED RATS. THE GROUP 1 CONTROL AND GROUP 6 ZINGERONE-ALONE-TREATED RATS DISPLAYED EXTREMELY LOW ACCRETION OF MASTOCYTES (40 X MAGNIFICATION)

Effect of Zingerone on DMBA Induced TBARS and Antioxidant Status: The levels of antioxidants (CAT, SOD, GSH and GPx) and TBARS in the mammary gland of experimental and control rats in every group are shown in **Fig. 6.** TBARS, CAT and SOD levels activities were lowered. However, GPx and GSH levels/activities were raised in tumor-bearing rats than the control rats. Zingerone alone treated experimental animals revealed no major variations in TBARS and antioxidant levels when compared to control rats. Zingerone offered protection to the mammary gland revealing near-normal range of TBARS and antioxidants in rats treated with DMBA.



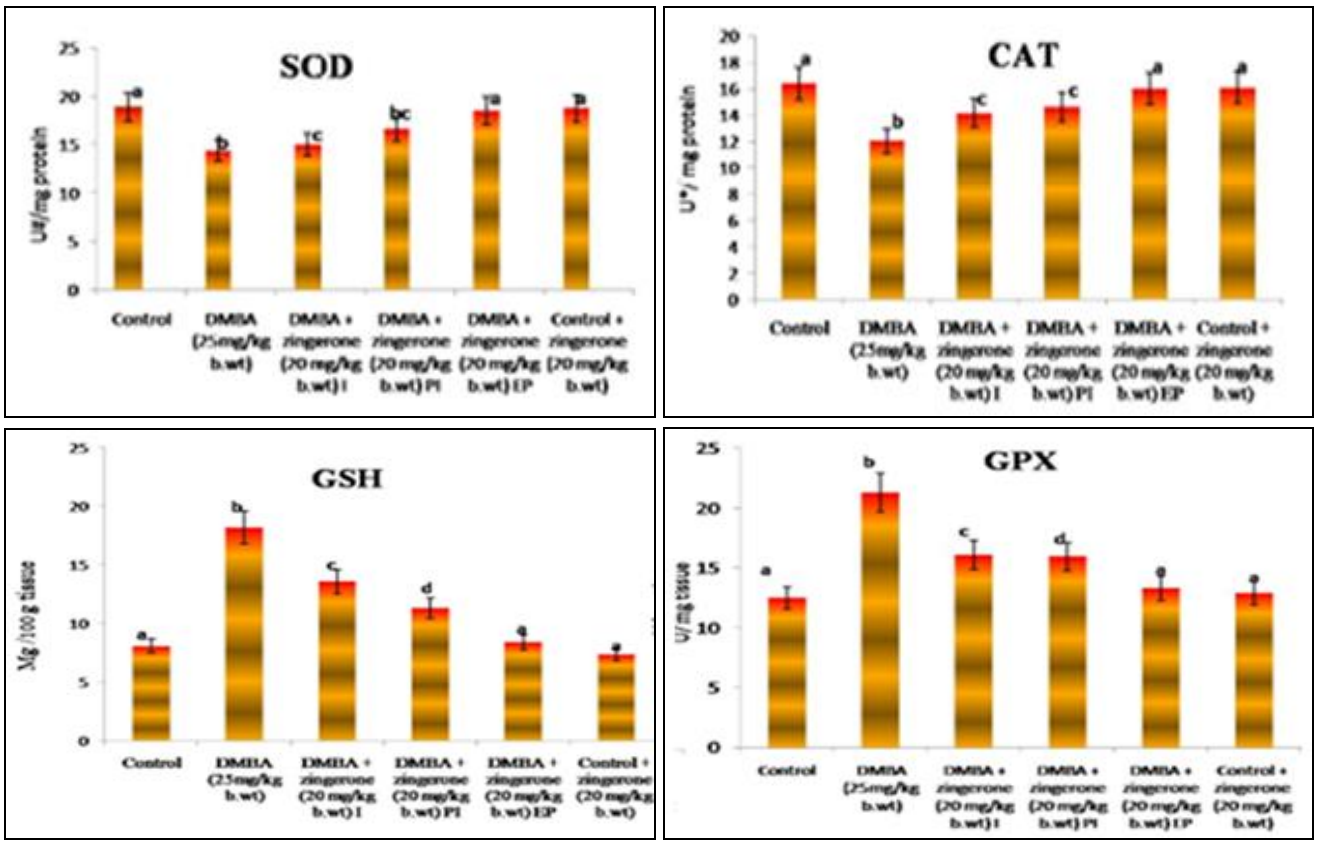


FIG. 6: THE STATUS OF MAMMARY TISSUE TBARS AND ANTIOXIDANTS IN CONTROL AND EXPERIMENTAL RATS IN EACH GROUP (n=6). VALUES ARE GIVEN AS MEAN ± STANDARD DEVIATION (S.D.) FOR SIX RATS IN EVERY GROUP. VALUES NOT SHARING A TYPICAL SUPERSCRIPT BETWEEN THE TWO GROUPS VARY FUNDAMENTALLY AT P<0.05 (DMRT). # -THE AMOUNT OF ENZYME REQUIRED TO HINDER 50% NBT REDUCTION*-MICROMOLES OF HYDROGEN PEROXIDE USED/SEC/MG PROTEIN-MICROMOLES OF GLUTATHIONE UTILIZED/MIN/MG PROTEIN**

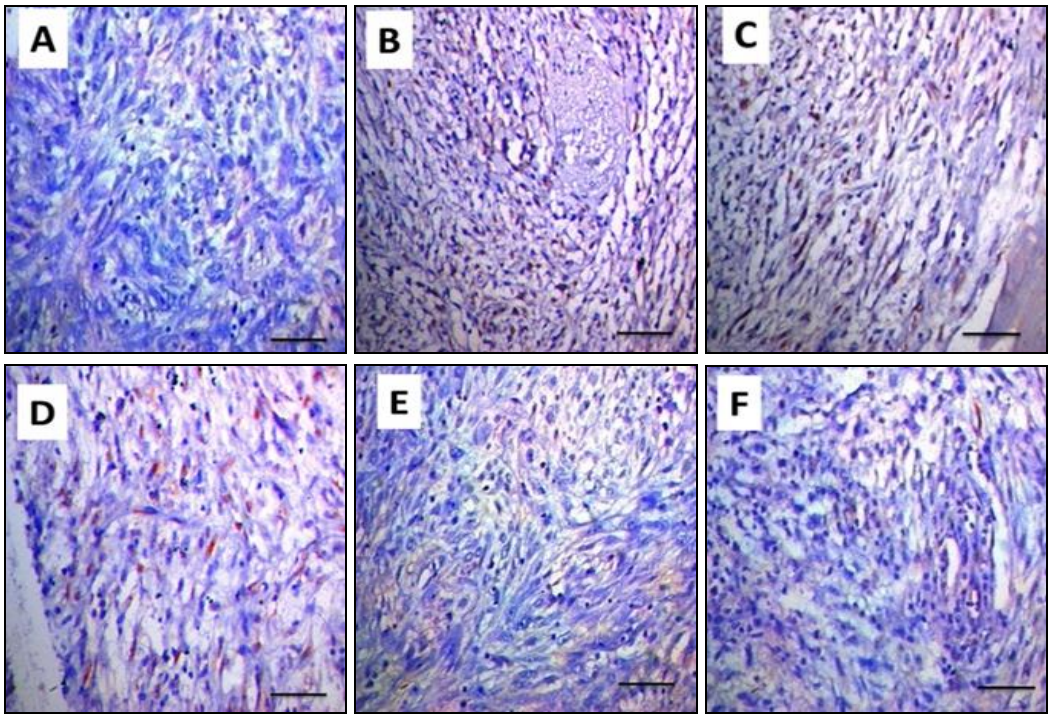


FIG. 7: DEPICTS IMMUNOEXPRESSION OF ER IN (A) CONTROL RAT (MILD EXPRESSION), (B) DMBA ALONE TREATED RAT (OVER EXPRESSION), (C, D AND E) DMBA + ZINGERONE TREATED RATS (DOWN REGULATE EXPRESSION) AND (F) ZINGERONE ALONE TREATED RAT (MILD EXPRESSION)

Zingerone and DMBA Incited Alterations In The Immunoexpression of ER, PR and HER2/Neu: The immune expression pattern and intensity of positively stained cells for ER, PR and HER2/neu receptors of experimental and control rats in every group are illustrated neatly in **Fig. 7.** (A-F), 8 (A-F) and 9 (A-F) separately. The analysis demonstrated positive staining for ER, PR and HER2/neu receptors in tumor tissues (group 2), which are most prominent as compared to normal tissues. We observed increased nuclear expression of ER and PR receptors and membrane staining for HER2/neu receptors in the mammary tissues of DMBA alone –treated rats. Orally administered zingerone (group 3, 4 and 5) to the rats treated with DMBA showed considerably regulated the expressions of ER, PR and ER2/neu receptors. Rats treated with zingerone alone (group 6) showed no major variations in the expression patterns of ER, PR, and HER2/neu receptors when compared with the control rats (referred as group1).

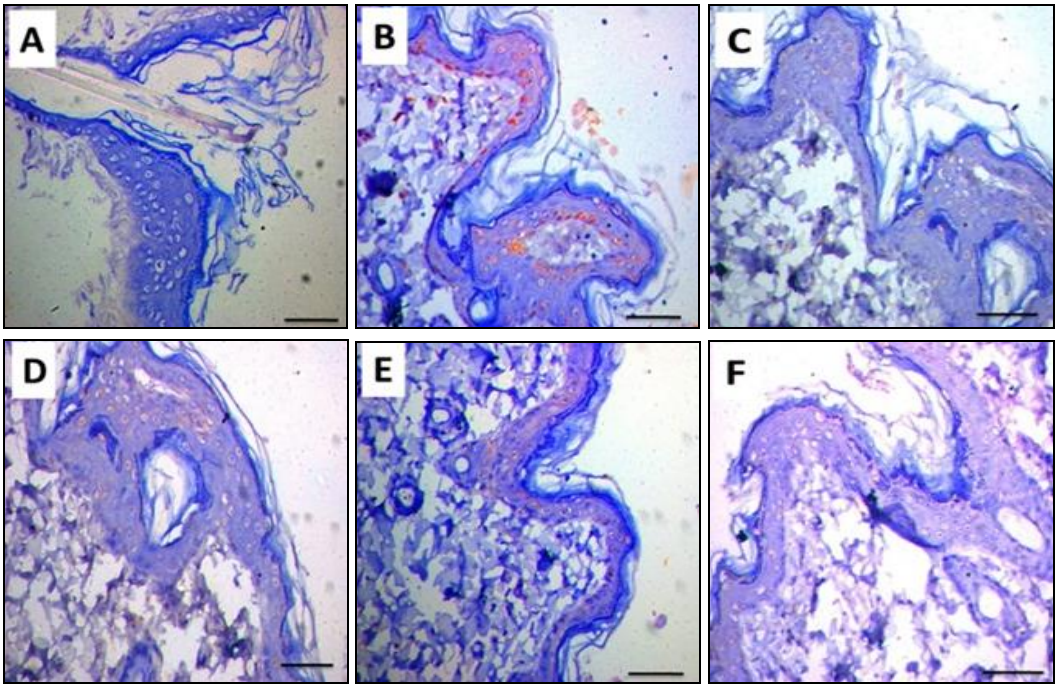


FIG. 8: IMMUNE EXPRESSION OF PR IN (A) CONTROL RAT (MILD EXPRESSION), (B) DMBA ALONE TREATED RAT (OVEREXPRESSION), (C, D AND E) DMBA + ZINGERONE TREATED RATS (DOWN-REGULATED EXPRESSION) AND (F) ZINGERONE ALONE TREATED RAT (MILD EXPRESSION)

Effect of Zingerone on DMBA Induced Cell Proliferation: Fig. 10 and **11.** showed the immunehistochemical staining for cyclin D1 and PCNA separately in the mammary tissues of experimental and control rats. Rats treated with DMBA alone (group 2) demonstrated deep nuclear staining for PCNA, besides cyclin D1 as compared to the control rats. Zingerone feeding at various periods of time (groups 3-5) to DMBA exposed rats indicated low staining for cyclin D1 as well as for PCNA. With additional investigation, in order to unravel the systematic process by which zingerone hinders cell proliferation, we decided to perform western blotting to assess the proteins, cyclin D1 and PCNA. We discovered that rats treated with DMBA altogether increased the protein expressions which could be analyzed by comparing with control. Synchronous supplementation with zingerone at different periods of time to the rats treated with DMBA, evidently decreased the protein expression of cyclin D1 and PCNA **Fig. a** and **b,** underlining its anti-proliferative effect. On the whole, our results uncovered that zingerone feeding during the course of the post-initium (group 4) lessened the expression of cyclin D1 and PCNA when compared to group 3 rats.

However, when zingerone was administered throughout the experimental period (group 5) to rats lowered the expressions of cyclin D1 and PCNA in the mammary tissues as compared to rats in groups 3 and 4. Statistically, no authentic difference was recorded between the rats fed onlyzingerone **Fig. 12.**

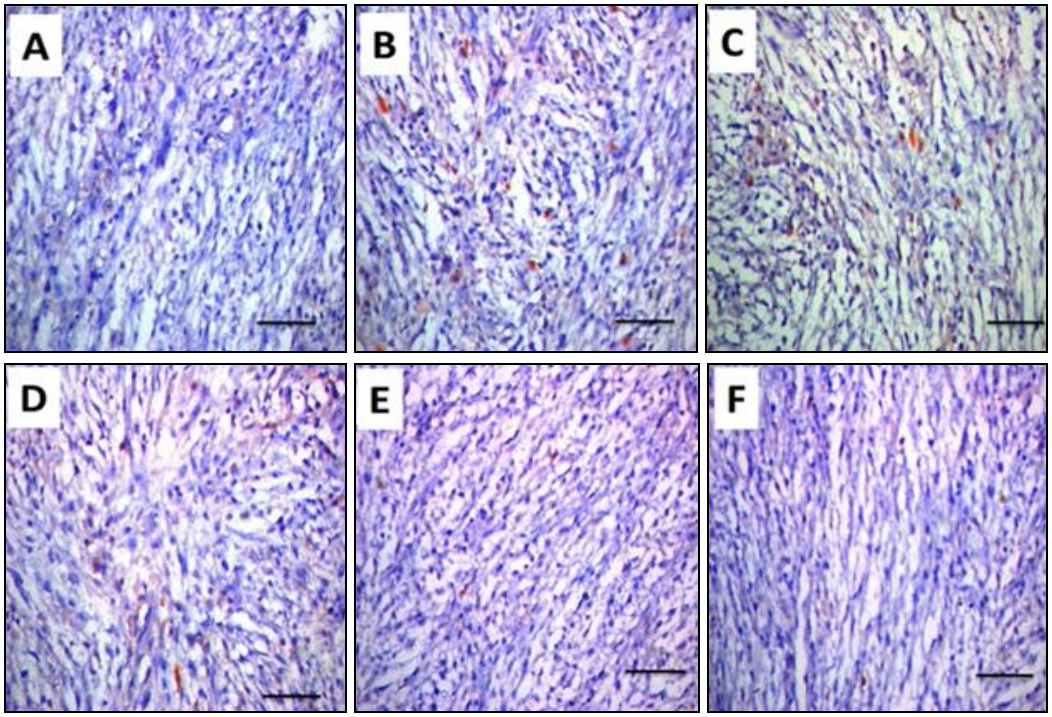
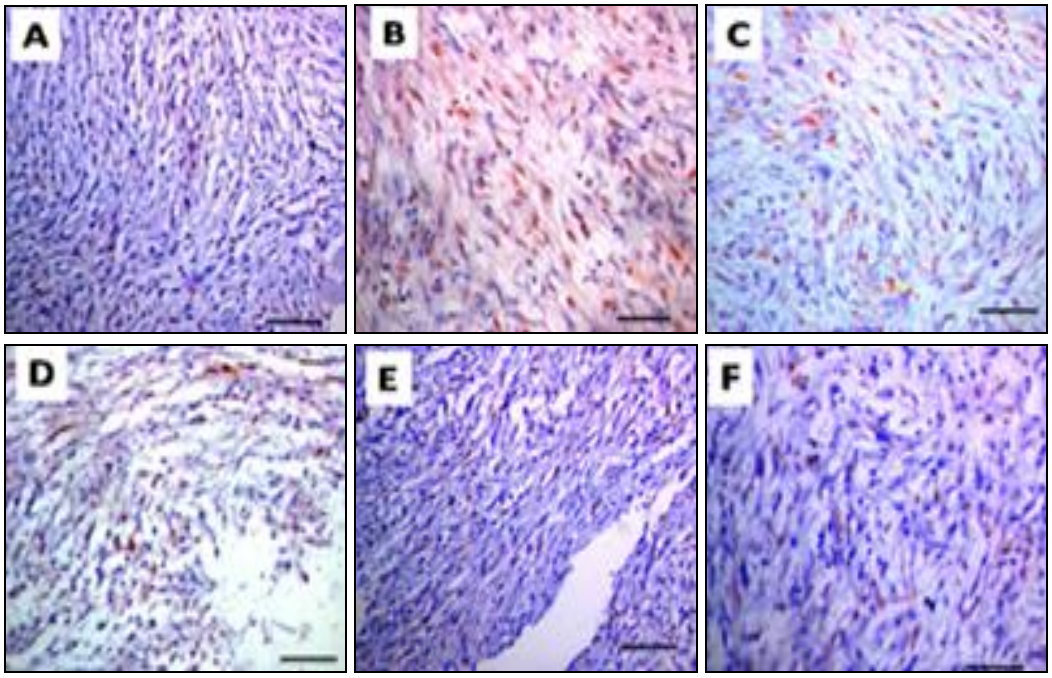


FIG. 9: IMMUNE EXPRESSION OF HER2/NEU IN (A) CONTROL RAT (MILD EXPRESSION), (F) DMBA ALONE TREATED RAT (OVEREXPRESSION), (C, D AND E) DMBA + ZINGERONE TREATED RATS (DOWN-REGULATE EXPRESSION) AND (F) ZINGERONE ALONE TREATED RAT (MILD EXPRESSION)



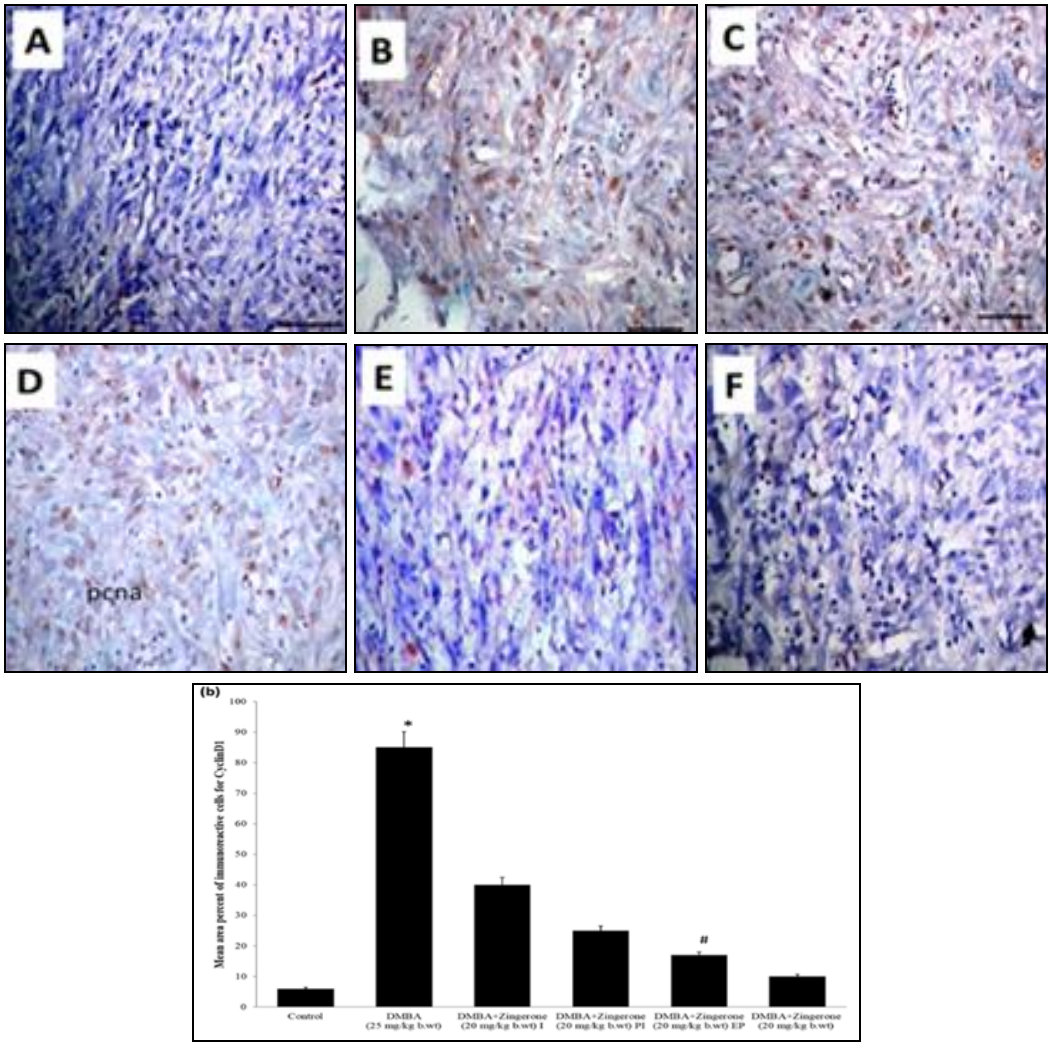


FIG. 10 AND 11: PROTEIN EXPRESSIONS OF CYCLIN D1 AND PCNA IN THE MAMMARY TISSUES OF EXPERIMENTAL AND CONTROL RATS. A: REPRESENTATIVE PHOTOMICROGRAPHS OF THE IMMUNOHISTOCHEMICAL STAINING OF CYCLIN D1 (× 40). THE SCALE BAR IS 100 µm. GROUP 2 (B) SHOWED DEEP NUCLEAR STAINING WHEN COMPARED WITH GROUPS 6 AND 1 (F AND A). OBSERVED REDUCED STAINING IN GROUPS, 3-5 (C-E)

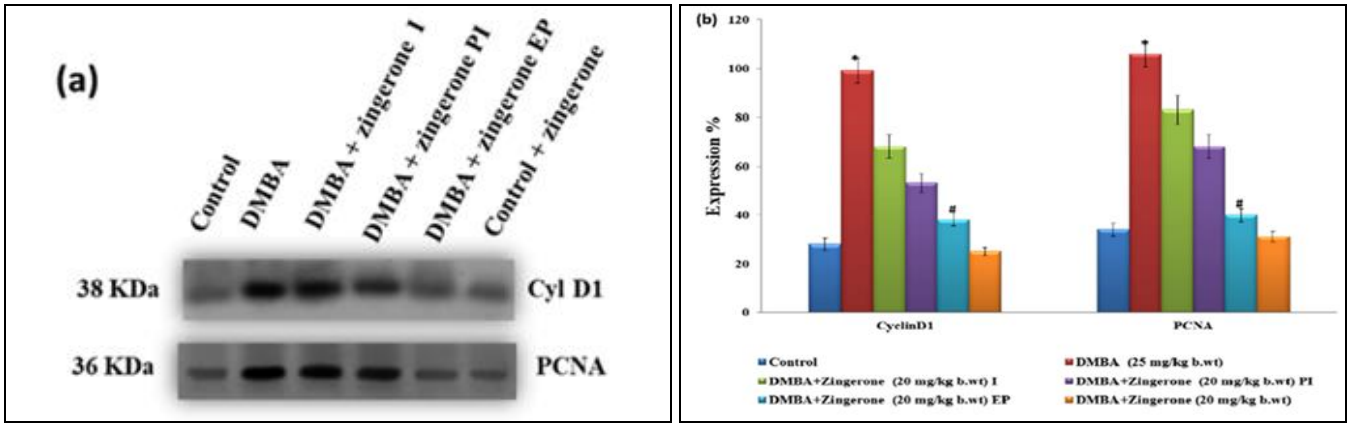


FIG. 12: PROTEIN EXPRESSIONS IN THE MAMMARY TISSUES OF EXPERIMENTAL AND CONTROL RATS. A: IMMUNOBLOT ANALYSIS OF MAMMARY TISSUE CYCLIN D1 AND PCNA EXPRESSIONS. B: EACH LANE WAS ANALYZED BY DENSITOMETRY AND THE EXPRESSION IN THE CONTROL WAS REGARDED AS 100%. THE COLUMN HEIGHTS ARE THE MEAN ± SD OF THREE DETERMINANTS (n=6).

Effect of Zingerone on Dmba Induced Apoptosis: To explore if the inhibitory impacts of zingerone was related to its proapoptotic potential, next we planned to examine the expressions of

Bax, Bcl-2, p53, p21, caspase-3 and caspase-9 by immune blot investigation. Our findings depict that zingerone treatment fundamentally stifled the expression of Bcl-2 with a simultaneous increase in the expressions of Bax, caspases-3 and -9 as compared with rats administered only with DMBA (group 2). Additionally, to confirm the obtained results and we had analyzed the protein expressions of Bcl-2 by immune his to chemical analysis **Fig. 15.** Our study showed that zingerone administration remarkably subdued the Bcl-2 expression with an increase in Bax, p53 and p21 expressions as compared with group 2 rats.

Furthermore, the decrease in expression of Bcl-2 and increase in Bax, caspase-3 and -9 was highly recognizable, when zingerone was fed during the post-initium time period (group 4) in contrast to feeding zingerone during the of initium period (group 3 rats). Collectively, highly pertinent results were displayed when zingerone was fed daily for the entitle period of the experiment (group 5) when compared with the rats of groups 3 and 4. There were statistically no important difference between the rats in control group and zingerone alone group **Fig. 13 and 14.**

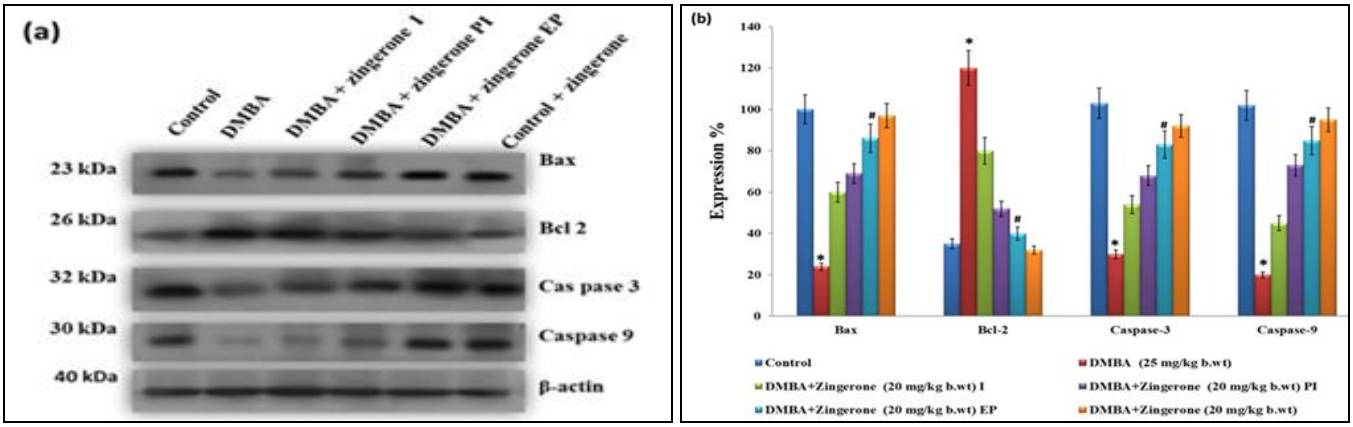


FIG. 13: PROTEIN EXPRESSIONS OF BAX, BCL-2, CASPASE-3 AND CASPASE-9 IN THE MAMMARY TISSUE OF EXPERIMENTAL AND CONTROL RATS. A: IMMUNOBLOT ANALYSIS OF MAMMARY BAX, BCL-2, CASPASE-3 AND CAS.PASE-9 EXPRESSIONS. PROTEIN SAMPLES (100 µg /LANE) RESOLVED ON SDS-PAGE WAS EXAMINED WITH SAME ANTIBODIES. µg β-ACTIN WAS UTILIZED AS A LOADING CONTROL. B. EACH LANE WAS ANALYZED BY DENSITOMETRY AND THE EXPRESSION IN THE CONTROL WAS REGARDED AS 100%. THE COLUMN HEIGHTS ARE THE MEAN ± SD OF THREE DETERMINANTS (n=6). SIGNIFICANCE WAS DENOTED AS A FOR P<0.05 VERSUS CONTROL AND B FOR P<0.05 VERSUS DMBA: PHOTOMICROGRAPHS OF THE IMMUNOHISTOCHEMICAL STAINING OF BAX AND BCL-2 (×40). THE SCALE BAR IS 100 µM. INITIUM: I, POST INITIUM: IP, ENTIRE PERIOD: EP.

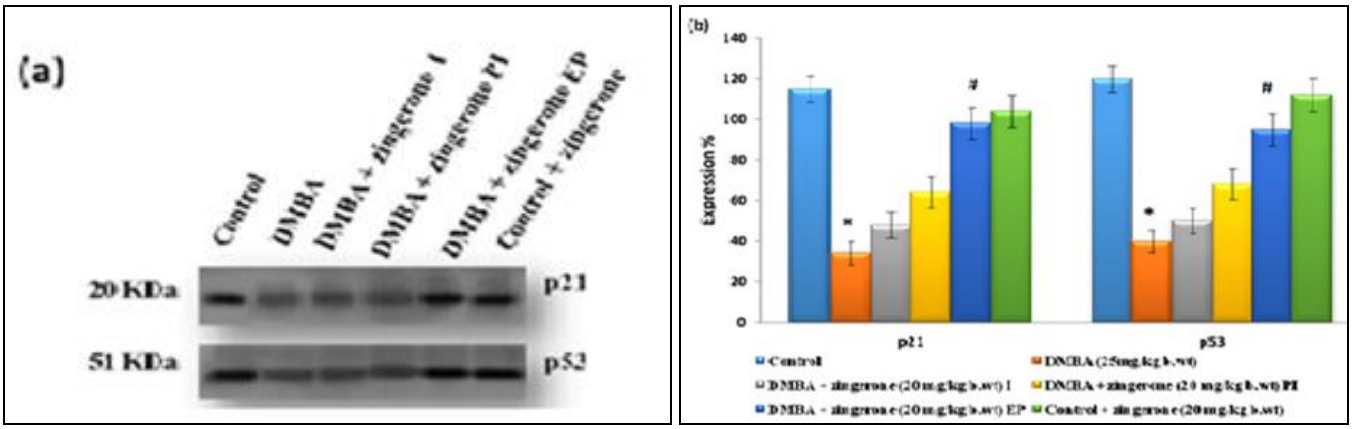


FIG. 14: PROTEIN EXPRESSIONS OF P21 AND P53 IN THE MAMMARY TISSUES OF EXPERIMENTAL AND CONTROL RATS. THE MAMMARY TISSUES P21 AND P53 EXPRESSION WERE EXAMINED BY IMMUNOBLOT ANALYSIS. B: EACH LANE WAS ANALYZED BY DENSITOMETRY AND THE EXPRESSION IN THE CONTROL WAS REGARDED AS 100%. THE COLUMN HEIGHTS ARE THE MEAN ± SD OF THREE DETERMINANTS (n=6). EVIDENTLY, AT P< 0.05 VERSUS CONTROL, F P<0.05 VERSUS DMBA, B P<0.05 VERSUS DMBA INITIUM: I, POST INITIUM: IP, ENTIRE PERIOD: EP

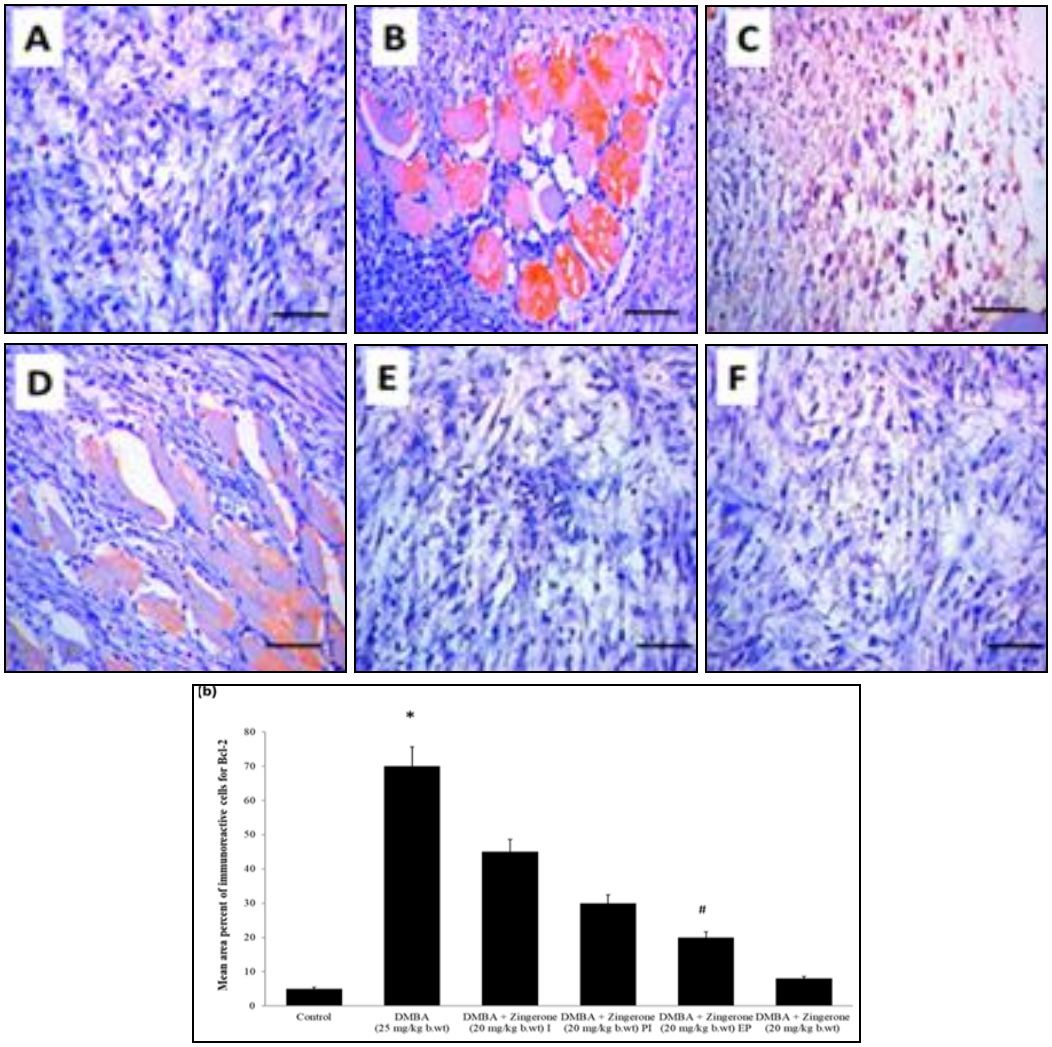


FIG. 15: THE LABORATORY AND CONTROL RATS (40X MAGNIFICATION) HAVE IMMUNOHISTOCHEMICAL STAINING OF MAMMARY TISSUE BCL-2. A AND F: MAMMARY TISSUE OF ZINGERONE-TREATED RATS ALONE AND CONTROL RATS DISPLAYED NORMAL ARCHITECTURE WITH A DELICATE BCL-2 SIGNAL. B: RATS TREATED WITH DMBA ALONE EXHIBIT DECREASED BCL-2 PROTEIN EXPRESSION. C, D and E: DIET WITH ZINGERONE FEEDING TO DMBA INCITED RATS SHOW ELEVATED EXPRESSION OF BCL-2. VALUES ARE PRESENTED AS MEANS ± S.D. FOR SIX RATS IN EVERY GROUP. GROUPS ARE NOT SHARING THE USUAL SUPERSCRIPT LETTER VARY SIGNIFICANTLY AT P<0.05. DUNCAN'S MULTIPLE RANGE TEST (DMRT)

DISCUSSION: The mortality rate of breast cancer is always high; however, enormous advancements are being made in its diagnosis and treatment to eradicate cancer cells. New molecules are being recognized from organic and medicinal plants, which are generally less toxic and have produced good results. It has been demonstrated from extensive *in-vitro* and *in-vivo* studies that phytochemicals display intense anticancer activities. In this manuscript, we have record our findings on the chemopreventive effect of zingerone in DMBA-incited mammary carcinogenicity in rats. In the present analysis, the remarkable increase in the body weight observed on zingerone administration suggests the defensive effects of this phytochemical against mammary tumorigenesis. Experimental animals injected only with DMBA displayed large tumors after 15 weeks of study. However the noteworthy decrease in the volume of tumour observed on feeding on zingerone clearly shows its inhibitory effects against DMBA-induced mammary cancer. In this context, a study reported that moringa, gravila, ginger, cress and artemisinin plant extracts exhibited chemopreventive potential against breast cancer. Zingerone is one such phenolic compound isolated from ginger. A study by Rahmani *et al.*, showed that ginger based nonvolatile pungent active principles had a wide range of health benefits including anticarcinogenic properties.

Further, results acquired from his pathological assessment of the mammary gland segments are in accordance with those observed morphologically the tumor number and its volume. Carcinoma observed in DMBA animals showed that DMBA modifies the normal process of separation of terminal ducts to alveoli and lobules in the mammary glands. However, Zingerone feeding at the dose of 20 mg/kg b.wt. protected the mammary glands from DMBA-incited modifications. Metabolism of DMBA induces ROS production that is capable of producing free radicals and depleting antioxidants, leading to lipid per-oxidation, degeneration and/or tissue injury [20]. In mammary cells the TBARS levels on feeding zingerone at the dose of 20 mg/kg b.wt. wt was highly effective than the other two doses. All three dose ranges of zingerone used were effective ($P>0.05$) and were comparable to the control value, indicating that maximum recovery was achieved by zingerone. This proves that zingerone completely neutralizes the toxic effects of DMBA on the mammary cells and thus can serve as a treatment strategy for cancer cells [21].

Antioxidants have been proposed as a helpful indicator in assessing the danger of oxidation incited carcinogenesis. The enzymic antioxidant system encompasses glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) which counter lipid peroxidation and ROS. SOD is considered as an important enzyme that neutralizes superoxide radicals and is available in almost all cells, including erythrocytes [22]. SOD and catalase are broadly disseminated in the entire tissue and catalyses the hydrogen peroxide breakdown.

Again, the reservoir of hydrogen peroxide is based on SOD-mediated dismutation of superoxide radicals, which is produced by different enzymic systems just like the nonezymic systems. Various types of harmful cancer cells are under heterogeneous levels of oxidative pressure, related with different expression levels of SOD and several other antioxidant enzymes. Decreased amount of SOD activity has been reported in different carcinogenic conditions [23, 24]. Some of the reports have suggested to decreased activities of SOD and catalase in other types of carcinogenic conditions [25]. However, in the present observation, we noted a decrease in SOD and catalase activities, which might increase in spreading out and intense oxidizing radical fit for transiting membranes causing malicious effects of catalase discovered in the dangerous condition, which is because of weariness of these enzymes in catalyzing the overstimulation of hydrogen peroxide by the destructive tumor cells. Our data indicated decreased lipid peroxidation and increased antioxidants conditions in the mammary gland of DMBA induced animals. Conversely, zingerone treatment significantly decreased the antioxidants and increased the lipid peroxidation levels, perhaps this might be one of the mechanisms by which zingerone exhibits its anticancer activity.

The ER/PR pathways act as an integral part of breast cancer pathophysiology in human beings. The mutation or overexpression of HER2 directly leads to tumorigenesis as well as metastasis. ER, PR, and HER2/neu levels have been used as regular markers for the diagnosis, prognosis, and to assess the reaction to breast cancer treatment. DMBA, an effective carcinogen, and a site-specific polycyclic aromatic hydrocarbon is mostly used to study hormone-dependent mammary carcinogenesis in experimental models. Assessment of ER, PR and HER2/neu levels revealed that zingerone treatment remarkably reduced the levels of these receptors, which clearly indicates that zingerone inhibits tumor development by targeting hormone receptors.

In general, phytochemicals are known to inhibit the expression of ER, PR and HER2/neu receptors as evident by a study showing the protective effect of Sophytoestrogen mixture consumption instead of a single compound against DMBA incited mammary carcinogenesis. Similarly, another study, showed that genistein and daidzein in combination inhibited mammary carcinoma by modulating the expressions of ER, PR and HER2/neu receptors [26, 27]. Further, to receive more clarityon the mechanism by which zingerone abrogated mammary tumorigenesis in rats, we proposed to figure out the expression of molecules involved in cellular proliferation. Cell proliferation studies are regularly used in clinical conditions for the evaluation of tumor prognosis as well as to assess the response of harmful cells to anticancer therapy. We noticed an increase in the expression of cyclin D1 and PCNA in DMBA treated tumor-bearing

mammals. Several pieces of evidence have suggested a correlation between cyclin D1 and PCNA expression and the degree of malignancy [28, 29]. However, zingerone treatment inhibited the expression of cyclin D1 and PCNA, which strongly indicates its antiproliferative potential. In this context [30] reported that zingerone can effectively suppress the expression of cell proliferation markers in chemically induced colon cancer models [31] also reported that zingerone administration significantly decreased cyclin D1 in mammary tissues. The development of carcinogenesis evades apoptosis; we aimed to establish the apoptosis-inducing capacity as the main criteria in the chemo preventive potential of zingerone. Tumor cells can resist apoptosis by activation of antiapoptotic proteins such as Bcl-2 or by the downregulation of proapoptotic proteins such as Bax, which is regulated by the p53 gene. In this study, DMBA induced animals showed overexpression of Bcl-2 and decreased expressions of Bax, caspase-3 and 9. However, zingerone feeding activated Bax, caspase-3, caspase-9 and notably decreased Bcl-2 expression in DMBA induced animals [32]. Similarly, at this juncture we can recall the studies by [31, 33, 34] who observed that zingerone increased the expressions of p21 and p53. Therefore, we can stress that the chemopreventive potential of zingerone in DMBA incited mammary gland cancer is associated with apoptosis induction.

CONCLUSION: In conclusion, the present study explicitly proves that zingerone feeding exhibits a remarkable chemopreventive effect against DMBA incited breast cancer. The underlying protective effect of zingerone is due to its ability to resist the growth and development of tumors in mammary glands, and its ability to overcome his to pathological alterations. The constraint on abnormal cell proliferation and initiation of apoptosis illustrate the fundamental cellular mechanisms of tumor growth inhibition by zingerone. Overall, since zingerone inhibits the hallmark coordinates of cancer, it may be considered a promising option for cancer prevention and therapy.

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