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Research Article

# Evaluation of anti-tumor activity of *Momordica cymbalaria* fenzl

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## ABSTRACT

**Background:** The aim of the current study is to evaluate the anti-tumor activity of saponins isolated from the roots of *Momordica cymbalaria* (MC) against dimethylbenz[a]anthracene (DMBA) induced rats.
**Methods:** A steroidal saponin MC (SMC) was isolated from MC fenzl and purified by preparative high-performance liquid chromatography. Breast cancer was induced in 50-day-old female Sprague-Dawley rats by injecting DMBA (6 mg/kg intravenous) in three doses on day 50, 54, and 57. The rats were randomized into four groups; control, DMBA, SMC (100 mg/kg), and tamoxifen (6.6 mg/kg) to DMBA breast cancer rats. The tumor size, volume, hormonal, antioxidant, and whole mount parameters were estimated.
**Results:** Mean tumor size and volume, luteinizing hormone, and progesterone with superoxide dismutases, catalase, and glutathione levels increased significantly ($p<0.001$); serum estradiol, follicle stimulating hormone with lipid peroxidation decreased significantly ($p<0.001$) in DMBA-induced breast cancer and *vice versa* in SMC and tamoxifen. Terminal end buds, terminal ducts, alveolar buds, and lobules decreased significantly ($p<0.001$) in DMBA-induced breast cancer whereas increased significantly in SMC and tamoxifen. Histological necrosis and hemorrhage along with focal desmoplastic reaction in DMBA-induced breast cancer; ductile elongation and hyperplasia of both ducts and alveoli were prominent, with increased secretory activity in SMC group. The results confirmed the chemopreventive effect of SMC and tamoxifen in DMBA-induced breast cancer.
**Conclusions:** The SMC exhibited anti-tumor activity against mammary cancer, which may be due to its anti-estrogenic, antioxidant activity.

**Keywords:** Antiestrogen , Antioxidant, Breast tissue, Dimethylbenz[a]anthracene, Hormonal parameters, Mammary cancer, *Momordica cymbalaria*, Saponins, Whole mount parameter

## INTRODUCTION

Cancer is the leading cause of death and second most common type in developing countries.[1] From which the most leading and diagnosed is breast cancer in females accounting 23% total cases and 14% of deaths.[1] Most of the chemotherapy is less effective in treatment with estrogen (ER–) breast carcinoma when compared to estrogen (ER+) breast carcinoma. Therefore, there is an urgent need for the discovery and development of agents efficacious against ER– breast cancer to close or eliminate the breast cancer difference gap. *Momordica chiranta* strongly inhibited the growth of cancer and shown anticancer activity against breast cancer[2] whereas, *Momordica cymbalaria* (MC) is used as antidiabetic,[3,4] abortifacient and anti-ovulatory,[5] anti-hyperglycemic, anti-diarrheal, and anti-implantation activities[6] effectively. Saponins of MC (SMC) are increasingly emerging as a very strong entrant for breast cancer treatment.

The importance of carcinogenicity experiment in cancer research studies with chemicals such as dimethylbenz[a] anthracene (DMBA) to induce the mammary carcinoma in rat models is well-established.[7] Herbal medicines such as resveratrol, black tea polyphenols, green tea extracts, rosemary, operculum turpethum, and lycopene have reported having beneficial effects in DMBA-induced mammary cancer.[8] *M. chiranta* (cucurbitaceae) is reported to have chemopreventive effect in skin carcinogenesis.[9] Hence, this study is to evaluate the antitumor activity of steroidal of SMC.

## METHODS

Tamoxifen was purchased from Astra-Zenica Ind. Pvt. Ltd; dimethyl sulfoxide (DMSO), Ellman's reagent (2,4-dithionitrobenzene [DTNB]) nitro blue tetrazolium chloride, and 5,5 dithiobis(2 nitrobenzoic acid) were

purchased from Himedia India. Also purchased DMBA, caramine alum stain distyrene plasticizer xylene (DPX) mountant.

### *Animals*

Female Sprague-Dawley (SD) rats were purchased from Indian Institute of Sciences, Bangalore. They were housed in propylene cage under standard laboratory conditions at room temperature (RT) (25°C± 2°C) with 12 hrs light/dark cycle. The animals were provided with pellet chow and water ad libitum, except during experimentation was followed under the norms of CPCSEA guidelines. Ethical clearance was obtained from Institutional Animal Ethical Committee of Karnataka College of Pharmacy, Bangalore with 1564/PO/a/11/CPCSEA for further reference.

### *Plant collection, extraction, and isolation*

The fresh roots of MC were collected from Gadag district, South India, identified and authenticated. The dried powdered roots of MC were soxhlet extracted with 95% v/v methanol. Methanoloic extract of MC was hydrolyzed with 0.5 N potassium hydroxide (KOH) for 1-2 hrs. After hydrolysis, free fatty acids were separated. Unsaponified fraction of free fatty acid was then extracted with 100 mL diethyl ether and allowed to stand for a few minutes, and collected the upper layer for complete separation of sterols. After the repeated separation, finally the ethereal layer was combined and washed with distilled water and KOH and pH adjusted to alkalinity. Ether is removed by distillation and added 6 mL of acetone to get a precipitate. Dry off the residue not above 80° temperature to get unsaponified matter. With the help of chemical, tests confirmed the presence of triterpenoid saponins.[10]

### *Phytochemistry*

In MC, cucurbitaceae family has been found as triterpenoids of saponinis as main chemical constituents in it with single spot total leukocytes count (TLC) and infrared (IR), mass, nuclear, and magnetic resonance (NMR) spectra.

### *Experimental design*

#### *Tumor induction*

Female SD rats weighing l30-140 g were used for the study. Cancer was induced in rats (l50-170 g) at the age of 50-57 days by intravenous injection of 40 mg/kg of DMBA dissolved in 0.05 ml of DMSO on the day 50, 54 and 57.[11]

#### *7, 12 DMBA-induced mammary tumors*

The SD rats were divided into four groups consisting of six rats each:

Group 1: DMSO (0.05 ml): normal control,
Group 2: DMBA control,
Group 3: Rats bearing tumors, treated with SMC (100 mg/kg; po; day/30 days),
Group 4: Tamoxifen (6.6 mg/kg; po; day/30 days).[12]

At the end of the experiment, the rats were sacrificed by cervical decapitation. Breast tissues were removed from the animals. The breast tissue was taken for observing whole mount preparation and histopathological analysis. The breast tissue homogenate was analyzed for oxidative stress parameters - superoxide dismulase (SOD), catalase (CAT), lipid peroxidation (LPO), glutathione (GSH), and serum was collected for measuring hormonal parameters.[13]

### *Mean tumor diameter*

The tumor sizes of the rats were measured using vernier calipers.[14] First, hold the rat by grasping the whole body with the palm over the back, with forefinger behind the head and the thumb and second, finger under the opposite axilla. Turn the rat, so it is lying on its posterior, and palpate to detect any mammary tumors; palpable tumors should feel like a "lump." Next use a caliper to measure the width, length, and height of the tumor. Calculate the tumor volume using the ellipsoid formula,

Volume=1/6πabc,

Where "a"=width, "b"=length, and "c"=height.

### *Removal of the abdominal mammary gland and preparing a whole mount*

The mammary gland was isolated as per the procedure described by Sonia de Assis.[15] The animals were killed, and the mammary gland was incised with a Y marked incision from the midline toward the hind legs.

Dissect the mammary gland free from the skin either using sharp scissors and/or a scalpel, starting from the proximal area close to the nipple and working toward the distal end of the gland toward the spine of the animal.

Immediately spread the detached gland onto an appropriately labeled glass slide for few minutes and do not dry it. Put the slide into a jar containing Carnoy's fixative (75% glacial acetic acid, 25% absolute ethanol (EtOH), let it get fixed at RT in the fume hood for 2 days or longer. Slides can also be left in the fixative for a longer period. Wash the slides in 70% EtOH for 1 hr at RT then slowly rinse in distilled water for 30 mins at RT.

Stain in carmine alum stain - place 1 g carmine and 2.5 g aluminum potassium sulfate in 500 ml distilled water and boil for 20 mins. Adjust final volume to 500 ml with water. Filter and refrigerate. The solution can be used for several months.

Discard when color becomes weak. For 2 days or longer (until you see that the lymph nodes have stained through; look at the back side of the slide). Wash in increasing series of EtOH, 1 hr in each: 70% → 95% → 100%. After the last wash in absolute EtOH, put the glands in xylene. Let sit in the fume hood in RT at least for 2 days.

This last step is clearing of the mammary gland meaning de-lipidation of the mammary fat pad, and subsequent increase in transparency. The fattier the gland the longer clearing time is required.

Mount with cover-slips using a mounting media, such as DPX mountant. Let the slides dry well (several days) before observing under a stereo microscope to observe epithelial growth, number of terminal end buds and differentiation of alveolar buds score.

### *Anti-oxidant enzyme estimation*

*Estimation of SOD*

To 2.78 mL sodium carbonate buffer (0.05 mM, pH 10.2), 100 µL of 1 mM ethylenediaminetetraacetic acid (1 mM, 0.0037 g in 10 mL distilled water) and 20 µL tissue supernatant/sucrose were added and incubated at 30°C for 45 mins. The reaction was initiated by adding 100 µL of adrenaline. The change in the absorbance was recorded at 480 nm for 3 mins.[16] Sucrose was used as a blank. The values were expressed as units/milligram of protein.

### *Estimation of CAT*

To 1.9 mL phosphate buffered saline (pH 7.0), 100 µL of supernatant was added. To this, 1 mL of $H_2O_2$ was added and the change in the absorbance was recorded at 240 nm for 3 mins.[17] The values were expressed as units/milligram of protein.

### *LPO*

Based on the Liu and Ng[18] method, the presence of markers of LPO was determined. 0.5 mL supernatant, 0.1 mL of 10 mM $FeSO_4$, and 0.1 mL of 0.1 mM ascorbic acid were incubated at 37°C for 1 hr. The reaction was stopped by addition of 0.75 mL of 28% (w/v) trichloroacetic acid (TCA) and 0.5 mL of 1% (w/v) thiobarbituric acid (TBA), successively. The mixture was then heated at 100°C for 45 mins. After centrifugation, all precipitated proteins were removed, and the color of the malondialdehyde (MDA) TBA complex in the supernatant was detected at 532 nm.[18] The values of MDA are expressed as nmol/mg of protein.

### *Estimation of GSH*

To an equal volume of 10%, TCA and tissue homogenate were taken and centrifuged at 5000 rpm for 10 mins. To 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB reagent and 1.9 ml of 0.2 M phosphate buffer (pH 9.0) were added. Absorbance was measured at 412 nm against a blank containing TCA instead of supernatant. A series of standard treated in a similar way also run to determine the GSH content. Standard graph with reduced GSH at concentrations of 0, 20, 40, 60, 80, and 100 µg were plotted, and the test OD was compared with standard graph. The amount of GSH is expressed as µg/g wet tissue.[19]

### *Statistical analysis*

The results are expressed as mean ± standard deviation from n=5 rats in each group, using one-way analysis of variance, and two-way analysis of variance followed by Tukey's multiple comparison tests was used to determine statistical significance.

## RESULTS

### *Phytochemical analysis of SMC*

Cucurbitaceae family, SMC was analyzed qualitatively. Positive triterpeniod by Liebermann Burchard Test, single spot TLC, Interpretation of IR, MASS, NMR compound is pentacyclic triterpenoid saponin (Figure 1) and its IUPAC name is methyl 11-cyano-2, 2, 6a, 9, 9, 12a-hexamethyl-10, 14-dioxo, 2, 3, 4, 4a, 5, 6, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-icosahydropicene-4a-carboxylate monohydrate.[20]

### *Tumor measurement*

The tumor sizes of the rats were measured using vernier calipers.[14] The tumor size and tumor volume of control and experimental animals in each group are shown in Table 1. We have observed 75% tumor formation with mean tumor size in DMBA treated animals. Oral administration of SMC 100 mg/kg and tamoxifen 6.6 mg/kg body weight prevented the tumor size and tumor volume up to 80% in Group III and 90% in Group IV (Figures 2 and 3). No tumors were

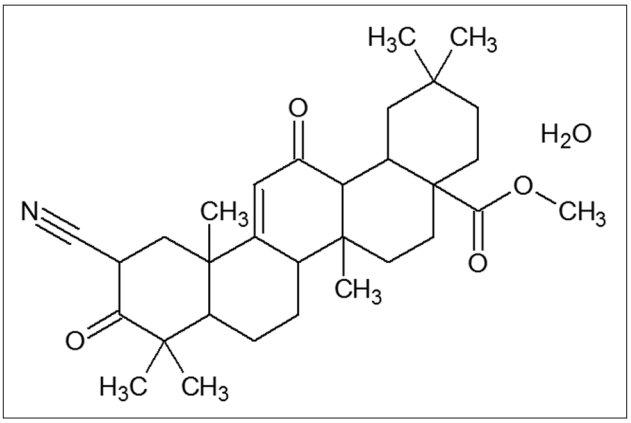


**Figure 1: Triterpinoidal saponin.**

**Table 1: Terminal ductal structures in mammary glands of female rats exposed to SMC or tamoxifen.**

| Treatment | Terminal ducts | Terminal end buds | Lobules |
|---|---|---|---|
| Normal control | 39.33±0.51 | 31±0.7303 | 50.5±1.432 |
| DMBA control | 20.67±0.760*** | 16.67±0.988*** | 21.83±0.833 |
| SMC | 35±0.632### | 26.17±0.972### | 43.83±0.792 |
| Tamoxifen | 31.33±0.843### | 21.5±0.991## | 35.67±0.614 |

***p<0.001 when compared to normal control; ###p<0.001 when compared to DMBA control, DMBA: Dimethylbenz[a] anthracene, SMC: Saponin of *Momordica cymbalaria*

observed significantly in Group I control animals treated with DMSO alone 0.05 ml.[21]

### *Whole mount preparation and histopathological parameters*

The whole mount preparation and histopathological features observed in breast tissue of control and experimental animals in each group (Figures 4 and 5).

The myoepithelial layer appears intact with collagen and contrast to MC, had limited effects on ductal elongation and epithelial hyperplasia in Group IV (Table 1 and Figures 6-10).

### *Hormonal parameters*

A significant reduction in serum estradiol, prolactin level and consequent increase in progesterone (PR), luteinizing hormone (LH), and follicle stimulating hormone (FSH) when compared to DMBA control group. Estradiol may exert its negative feedback mechanism on FSH, LH, and PR when compared to DMBA control to decrease mammary cancer incidence in DMBA-treated rats (Table 2).

### *Antioxidants*

The enzymatic and non-enzymatic antioxidants activity and breast tissue of control and experimental groups were analyzed (Table 3).

## DISCUSSION

Herbal and natural products represent one of the most popular alternative treatments. Many of the natural products had hormonal activity and used to prevent and treat diseases including cancers and might act as a good for the development of anti-cancer drugs. Several natural plant products, such as phenolics, indoles and flavonoids, saponins, and steroids have been shown to alter the initiation phase of carcinogenesis.

Haque et al.; showed that data cucurbitane type of triterpenoids are the main source, found in *M. chiranta*, whereas, in MC, cucurbitaceae family has been found as triterpenoids of saponins as main chemical constituents in it.[20]

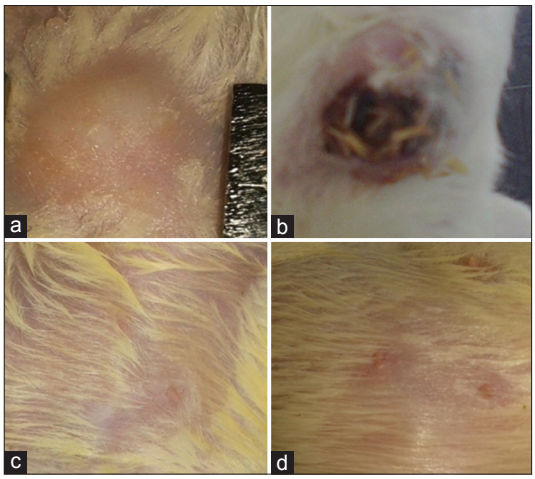


**Figure 2: Effect of saponin of *Momordica cymbalaria* (SMC) and tamoxifen on dimethylbenz[a]anthracene (DMBA) induced mean breast tumor volume, (a) Mammary gland in normal control, (b) Mammary gland in DMBA control, (c) Mammary gland of SMC (100 mg/kg; po; day/30 days) treated group, (d) Mammary gland of tamoxifen (6.6 mg/kg; po; day/30 days) treated group.**

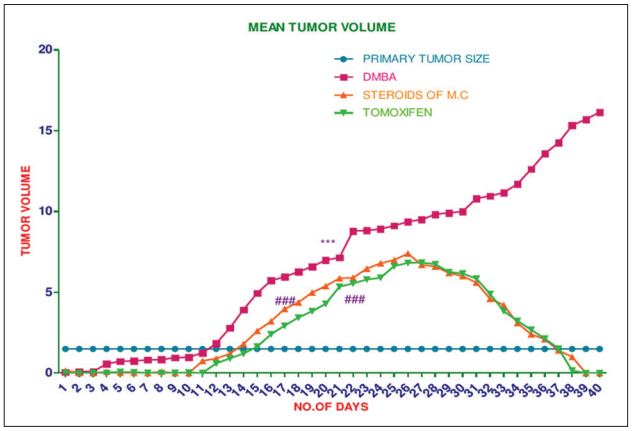


**Figure 3: Effect of saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days) on mean tumor volume, values are expressed as mean ± standard mean of error, n=6, ***p<0.001 when compared to normal control; ###p<0.001 when compared to dimethylbenz[a]anthracene control.**

In the present study, there has been significantly decreased the mean tumor diameter of mammary tumor compared to

**Table 2: Effect of SMC (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days) on hormonal changes.**

| Group | FSH (mIU/mL) | LH (mIU/mL) | Estradiol (ng/mL) | Progesterone (ng/mL) | Prolactin (ng/mL) |
|---|---|---|---|---|---|
| Normal control | 4.037±0.011 | 18.67±0.0136 | 59.05±0.014 | 36.15±0.0061 | 3.05±0.0073 |
| DMBA control | 5.91±0.018 | 9.96±0.0137 | 75.64±0.0042 | 15.34±0.0114 | 3.15±0.0067 |
| 100 mg SMC | 7.853±0.016 | 13.18±0.0093 | 50.37±0.0089 | 26.56±0.013 | 3.103±0.0066 |
| Tamoxifen | 7.278±0.0094 | 15.67±0.00138 | 46.23±0.010 | 24.14±0.0098 | 1.76±0.0315 |

***p<0.001 when compared to normal control; ###p<0.001 when compared to DMBA control, DMBA: Dimethylbenz[a] anthracene, LH: Luteinizing hormone, SMC: Saponin of *Momordica cymbalaria*, FSH: Follicle stimulating hormone

**Table 3: Effect of SMC and tamoxifen on oxidative stress DMBA induced mammary cancer.**

| Treatment | Oxidative stress parameters | | | |
|---|---|---|---|---|
| | SOD (units/mg protein) | CAT (µ moles of $H_2O_2$ metabolized/mg protein/mins) | GSH (µg/g wet tissue) | LPO (nmol/g wet tissue) |
| Normal control | 4.472±0.1160 | 8.087±0.05270 | 71.75±0.6455 | 26.45±0.4045 |
| DMBA control | 1.997±0.04551*** | 2.198±0.02545*** | 15.58±0.5151*** | 81.70±0.3618*** |
| SMC | 3.032±0.03587### | 6.978±0.04028### | 58.60±1.999### | 20.31±0.3005### |
| Tamoxifen | 2.647±0.1046### | 5.578±0.1052### | 51.54±1.552### | 13.87±0.3177### |

***p<0.001 when compared to normal control; ##p<0.001 when compared to DMBA control, DMBA control, DMBA: Dimethylbenz[a] anthracene, SMC: Saponin of *Momordica cymbalaria*, SOD: superoxide dismutases, CAT: Catalase, LPO: Lipid peroxidation, GSH: Glutathione

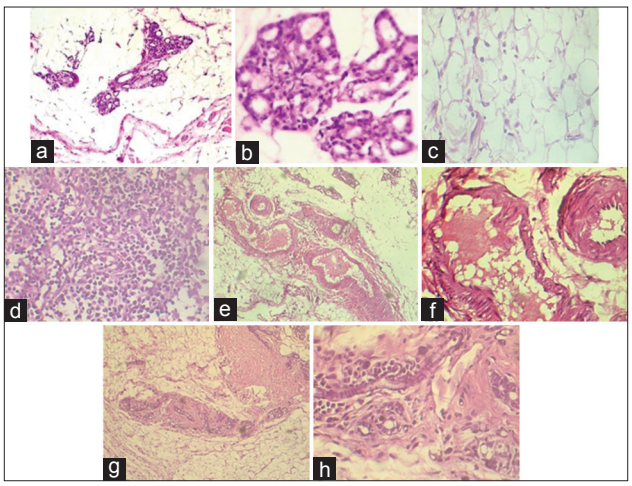


**Figure 4: Effect of saponin of *Momordica cymbalaria* (SMC) (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days) on histological pictures of dimethylbenz[a]anthracene (DMBA) induced mammary cancer, (a) Light microscopy of breast sections of normal control (H and E, ×100), (b) Light microscopy of breast sections of normal control (H and E, ×400), (c) Light microscopy of breast sections of DMBA (6 mg/each animal intravenous [IV]) control (H and E, ×100), (d) Light microscopy of breast sections of DMBA (6 mg/each animal IV) control (H and E, ×400), (e) Light microscopy of breast sections of SMC 100 mg/kg po treated group (H and E, ×400), (f) Light microscopy of breast sections of SMC mg/kg po treated group (H and E, ×100), (g) Light 100 microscopy of breast sections of tamoxifen 6.6 mg/kg po (H and E, ×100), (h) Light microscopy of breast sections of tamoxifen 6.6 mg/kg po (H and E, ×400)**

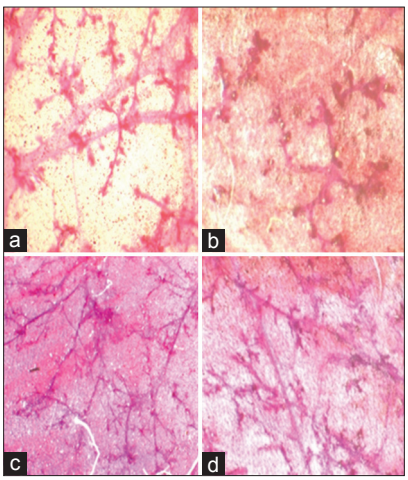


**Figure 5: Effect of saponin of *Momordica cymbalaria* (SMC) (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days) on morphology of whole mount preparation after staining, (a) Mammary gland in normal control, (b) Mammary gland in dimethylbenz[a]anthracene control, (c) Mammary gland of SMC (100 mg/kg; po; day/30 days) treated group, (d) Mammary gland of tamoxifen (6.6 mg/kg; po; day/30 days) treated group.**

DMBA control. Tamoxifen has an antitumor effect of similar magnitude in DMBA-induced rat mammary carcinoma in terms of tumor regression, inhibition of tumor growth, and new tumor development. In the present study, tamoxifen has significantly decreased mean tumor diameter of mammary tumor compared to DMBA control. Morphologically, a significant decrease in tumor size has been observed to tamoxifen treatment was reported earlier in the DMBA-tumor model.[21]

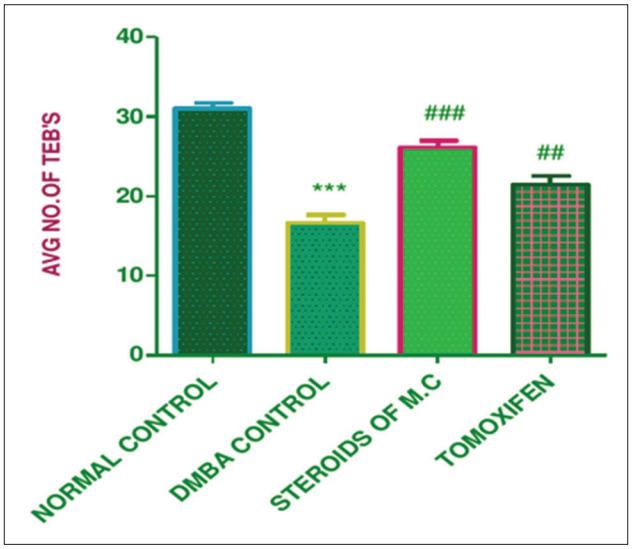


**Figure 6: Terminal end buds in mammary gland of female rat exposed to saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days).**

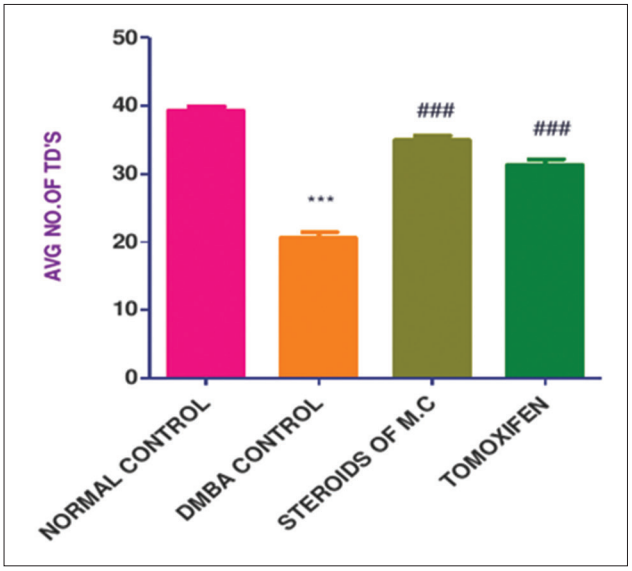


**Figure 7: Terminal ducts in mammary gland of female rat exposed to saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days).**

Hypotheses have been proposed to explain the inhibitory effects that include modulation of the reproductive neuro-endocrine axis (down-regulation of the gonadotropic axis and decrease of estrogen and prolactin levels).[22]

Studies reported that consistent with the result of a number of animal studies that have demonstrated a direct pituitary site of estrogen negative feedback. Although both isoforms of the estrogen receptor (ERα [also known as ESR1] and ERβ [also known as ESR2]) are present in the pituitary, receptor models which imply that ERα is the predominant receptor mediating estrogen negative feedback.[23]

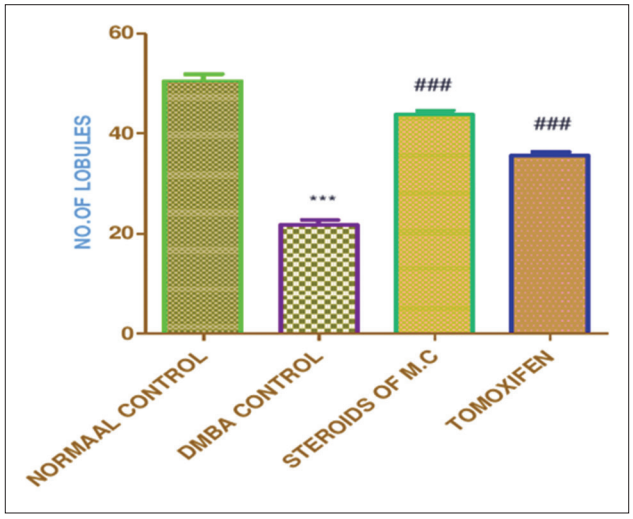


**Figure 8: Number of lobules in mammary gland of female rat exposed to saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tamoxifen (6.6 mg/kg; po; day/30 days).**

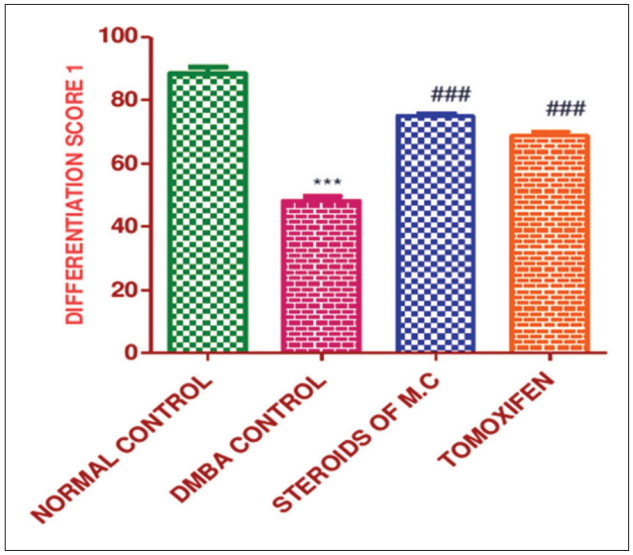


**Figure 9: Effect of saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tomoxifen (6.6 mg/kg; po; day/30 days) on differentiation score 1.**

Tamoxifen-treated tumors reduced its estrogen receptor value to after treatment, as would be expected because of the binding of tamoxifen to the estrogen receptor as in reported earlier. In the present study, tamoxifen is the standard endocrine (anti-estrogen) therapy for hormone-positive early breast cancer. Tamoxifen inhibited the stimulatory action of the hormones.

The Breast tissue from DMBA-treated animals shown severe malignant ductal cells infiltrating into the surrounding stroma that have a prominent nucleoli with abundant necrosis and hemorrhage indicate rapid proliferation of focal desmoplastic reaction and decreased epithelial growth, with small proliferative lesions in Group II. In drug-treated animal's ducts with fibrous stroma and adipose tissue having

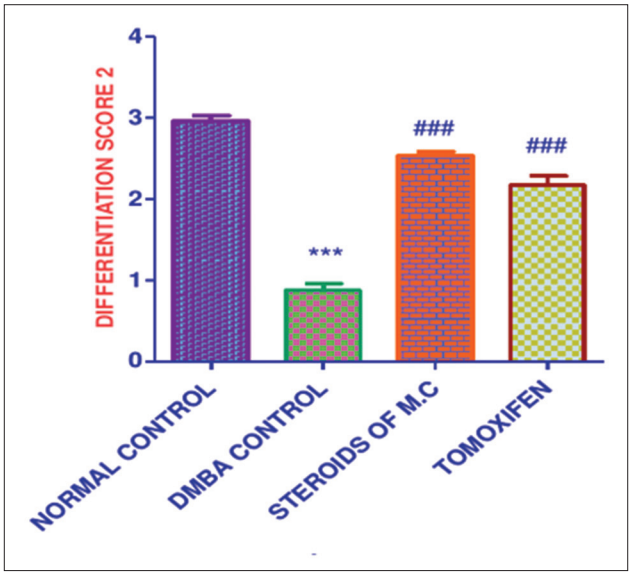


**Figure 10: Effect of saponin of *Momordica cymbalaria* (100 mg/kg; po; day/30 days) and tomoxifen (6.6 mg/kg; po; day/30 days) on differentiation score 2.**

scattered mononuclear inflammatory cells within the stroma which shows absence necrosis and presence of more collagen is observed as a part of repair mechanism of drug and the mammary tubule alveolar acini were increased in number and enlarged in volume.[24]

The ducts became more elongated; the ductal walls were lined with increased layers of epithelial cells. The alveoli were enlarged and increased, with lumens visible in Group III whereas prominent nucleoli and moderate cytoplasm indicating that cells are in in-situ stage. No areas of necrosis are seen.

The SOD, CAT, and GSH were significantly decreased, and LPO significantly increased in (Group II) DMBA treated animals. In tamoxifen-treated animals (Groups III and IV) the SOD, CAT, and GSH activities were significantly increased whereas, LPO significantly decreased when compared to DMBA cancer animals (Group II).

Steroidal SMC has been reported for its antioxidant activity[25] and the studies on leutolein,[26] taxol,[27] and spirulina were reported protection against DMBA-induced mammary tumors by their antioxidant activity.

## CONCLUSION

The findings of the current study evince that SMC reveals the significant preventive effect of anti-tumor activity against mammary cancer, which may be due to its anti-estrogenic, hormonal level is decreasing by the estrogen level and by increasing the LH, FSH level in DMBA control and by its antioxidant activity with equal reduction of the epithelial and the stromal components was unexpected, and seems to stress the importance of the epithelial-stromal and elongation of ductal as well as alveolar changes noted the interactions in the regression of these neoplasms. Further research is needed.

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