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ORIGINAL ARTICLE

Chemopreventive Effect of Tomentosin against 7,12-Dimethylbenz[a] anthracene-Induced Breast Cancer Progression and Inhibits the Cell Proliferation in MCF-7 Cells via Downregulation of PI3K/AKT Signaling Pathway

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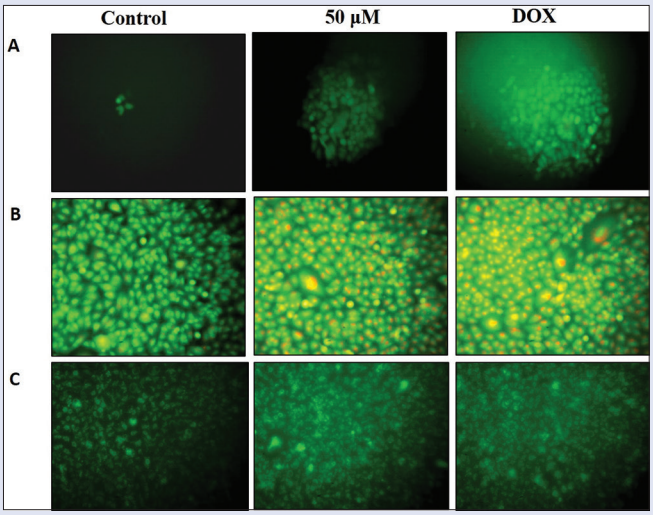
ABSTRACT

Background: Breast cancer is the most common cancer which disturbs not only the older population but also women under 35 years old. It also ranks to be the first in cancer-connected deaths of women. **Objectives:** Hence, we intended the study to investigate the efficacy of tomentosin as an anticancer agent against breast cancer *in vitro* and *in vivo* conditions. **Materials and Methods:** Breast cancer was persuaded to the rats by DMBA administration and then treated with the tomentosin for 28 days. The levels of estrogen receptor α (ER-α), oxidative stress markers, biotransformation enzymes, carcinoembryonic antigen, and cytokines were assessed. Histopathological analysis was done to check the anticancer effect of tomentosin. *In vitro* studies were finished with MCF-7 cells and the cells were exposed to cell viability assay and different fluorescent staining assays such as H2DCFDA, JC-1, and AO/EtBr dual staining to inspect the tomentosin effects. The expression of PI3K/AKT signaling molecules was considered by quantitative polymerase chain reaction (qPCR) analysis. **Results:** Our *in vivo* consequences recommend tomentosin knowingly reduced the levels of ER-α, CYP450, CYT-b5, carcinoembryonic antigen, and cytokines and augmented the levels of CAT, GST, and GR enzymes. Histological results authorize anticancer effects of tomentosin. The qPCR results exhibited that tomentosin significantly lessened the expression of PI3K/AKT and augmented the apoptosis-related p38/JNK1 expression in MCF-7 cells. **Conclusion:** Overall, our results settle tomentosin possibly inhibit the DMBA-convinced breast cancer induction in rats and they also induce apoptosis in estrogen-dependent breast cancer MCF-7 cell lines.

Key words: Apoptosis, breast cancer, MCF-7 cell line, PI3K/AKT pathway, tomentosin

SUMMARY

- Tomentosin treatment effectually abridged the levels of estrogen alpha receptors, cytokine levels, oxidative stress markers levels in breast cancer induced rats.
- The induction of apoptosis by tomentosin was established with H2DCFDA, JC-1, AO/EtBr staining.
- It also diminished the expression of genes PI3K, ERK involved in cell proliferation and augmented the apoptosis inducing genes AKT, p38, JNK in MCF-7 cells



Abbreviations used: MDA-malondialdehyde; SOD-super oxide dismutase; ROS -reactive oxygen species;CEA- carcino embryonic antigen

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INTRODUCTION

Breast cancer is the most common type of cancer which touches not only the older population but also women under the age of 35 years. It also ranks to be the first in cancer-associated deaths of women. According to the approximation of 2018, about 2.1 million new breast cancer patients were identified and about 6.25 lakh women were died due to breast cancer.[1,2] Compared to under established and developing countries, the prevalence of breast cancer is more among settled countries. Modern lifestyle, age, high hormonal levels, diet, race, and family histories are some of the recognized risk factors for breast cancer occurrence.[3,4] The

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existence rate of breast cancer patients is more in patients identified with early stage, whereas it is much dangerous for the patients who are spotted at a later stage. Even though a lot of treatments such as chemotherapy, surgery, and radiation were there to treat breast cancer, they afford hilarious side effects and the relapse rate is more. The major compression for usage of chemotherapy drugs and radiation in younger patients is it disturbs their fertility. Hence, it is necessity of today to learn a drug which hypothetically treats breast cancer and does not yield any side effects.

Tomentosin, sesquiterpene lactone, existing in the plants fits to the family of Asteraceae. In ancient Chinese treatment, tomentosin is employed as a drug to treat numerous diseases.[5,6] It is naturally found that sesquiterpene lactone overturns the proliferation of various cancer cell lines.[7] Tomentosin declines the reactive oxygen species (ROS) and induces the apoptosis in cancer cells and arrests the cancer cell proliferation.[8] Effect of tomentosin was verified *in vivo* DMBA persuaded breast cancer rat model and compared. Molecular targeted therapy which targets transmembrane and intracellular molecules is presently in use to arrest the progression of breast cancer. Tomentosin persuades the apoptotic pathway by blocking inflammatory mediatory in cancer cell lines. Hence, we intended the study to examine the efficacy of tomentosin as an anticancer drug in *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

Chemicals

Tomentosin, DMBA, and other chemicals were acquired from Sigma Aldrich, USA. All the ELISA assay kits were obtained from Abcam, USA, MyBioSource, USA, and Thermo Fisher Scientific, USA, respectively. Other chemicals were conquered from HiMedia, USA.

Drug preparation

Tomentosin was found from Sigma Aldrich, USA. 40 mM stock solution of tomentosin was prepared by dissolving tomentosin drug in DMSO. The stock solution was deposited in 20°C until further usage.

Animal and treatment

Female adult Sprague Dawley rats weighing about 250g were adapted in laboratory conditions for a week. Acclimatization, the rats were assembled into five groups (each group contains of 20 rats). Group I continued untreated and kept as control. Group II, III, IV, and V were injected with DMBA (Sigma Aldrich) intraperitoneally each with a single dose of 20 mg/kg body weight. The incidence of tumor was evaluated every week and followed up to 3 months since DMBA administration. Group II endure kept as breast cancer control, Group III and IV administered with 25 and 50 mg/kg orally with tomentosin for 28 days. Group V was treated with the standard drug tamoxifen 20 mg/kg orally for 14 days. The body weight of rats was restrained both during the initiation of experiment and before euthanizing the rats. At the end of 130 days, the rats were killed by cervical dislocation.[9] Breast tissues were dismembered and subjected to further analysis.

Estimation of estrogen receptor α level

The levels of estrogen receptor α in the breast cancer-persuaded rats and concurrently tomentosin-treated rats were enumerated using the ELISA technique. The experiments were reiterated thrice and completed according to the instructions given (MyBioSource, USA).

Estimation of oxidant/antioxidant status

The oxidant/antioxidant status in breast cancer-induced rats and tomentosin-treated rats was dignified. Lipid peroxidation (LPO) was leisurely according to the protocol of Devasagayam and Tarachand,[10] the levels of malondialdehyde (MDA) were measured at 532 nm since the synthesis of malondialdehyde is unswervingly proportional to LPO. The levels of superoxide dismutase (SOD) were quantified according to the method of Marklund and Marklund.,[11] Catalase activity was unrushed according to the protocol of Sinha[12] and the levels of glutathione were quantified using the protocol of Aykaç *et al.*[13]

Estimation of biotransformation enzymes

The biotransformation enzymes such as CYP-450, CYT-b5, glutathione-S-transferase, and glutathione reductase were measured with commercially available kits. CYP-450 kit (Abcam), GST (Merck), and GR (Abcam) were dogged using the colorimetric detection method. CYT-b5 was projected using ELISA kit procured from MyBioSource, USA. The assay was achieved according to the manufacturer's protocol.

Quantification of carcinoembryonic antigen and cytokine levels

Carcinoembryonic antigen (CEA), cytokines interleukin (IL)-1β, and tumor necrosis factor (TNF)-α levels were assessed in the serum of breast cancer-induced rats and breast cancer-induced tomentosin-treated rats. The tests were done using a commercially obtainable ELISA kit (MyBioSource, USA). The assay was accomplished according to the instructions delivered in the kit and the test was performed in triplicates.

Histopathological analysis

Histopathological analysis of control and treated group rat's breast tissue was achieved using hematoxylin and eosin staining. The tissue was fixed with 4% paraformaldehyde for 24 h at RT and then exposed dehydration with a sequence of ethanol. The dehydrated tissue was entrenched with paraffin wax and then sectioned into 5 µ thickness using tissue microtome. The sections were deparaffinized and located on albumin-coated glass slides. The sections were then exposed to alcohol, xylene dehydration and alcohol-free slides were stained with hematoxylin and eosin stain, viewed, and photographed under a light microscope (Olympus, USA).

Culturing of breast cancer cells

MCF 7 cells were grown up in DMEM at 37°C under 5% CO_2 in a humidified incubator. Upon attaining 80% confluence, the cells were trypsinized and the cells were exploited for further investigation.

Cytotoxicity assay

Tomentosin in various snowballing concentrations was added to MCF 7 cells and incubated for 24 h. 25 µL of the MTT (5 mg/mL) solution was added to each of the 96 wells and incubated at 37°C for 4 h. After incubation period the supernatant was removed and 200 µL of DMSO was added to dissolve the formazan crystals. The OD value of each well was quantified at 575 nm using a microplate reader.

H2DCFDA staining

2',7'-dichlorodihydrofluorescein diacetate staining technique was completed to quantify the amount of ROS generated in MCF-7 cells by tomentosin. The cells were treated with 30 µM concentration of tomentosin and incubated for 24 h. After the incubation period, the cells were stained with 5 mM 2',7'-dichlorodihydrofluorescein diacetate for 15 min and the stained cells were watched under a fluorescent microscope.

JC1 staining

The mitochondrial membrane potential of control and tomentosin-treated breast carcinoma MCF-7 cells was evaluated using JC-1 staining. The cells were treated with 30 µM concentration of tomentosin and

incubated for 24 h. After incubation period, the cells were stained 2 μM JC-1 dye and the cells were incubated at 37°C for 30 min. The stained cells were observed and photographed under a fluorescent microscope.

AO/EtBr staining

MCF-7 cells treated with tomentosin were dual stained with ethidium bromide and acridine orange (1:1) for 15 min at 37°C. The cells were then eroded thrice with phosphate-buffered saline and observed under the fluorescent microscope; the images were photographed and investigated with ImageJ software.

Quantitative polymerase chain reaction analysis

The gene expression of PI3K/AKT signaling proteins was assessed in breast carcinoma cell line MCF-7 treated with tomentosin using quantitative polymerase chain reaction (qPCR) analysis. Cell lysates of each group were exposed to RNA isolation with TriZol reagent. The RNA was then quantified with a NanoDrop Spectrophotometer and imperiled to cDNA conversion with Cells-to-cDNA™ II Kit, Thermo Fisher Scientific, USA. The cDNA was then exposed to qPCR analysis with individual primers using Light Cycler Fast Start DNA master mix for SYBR Green I (Roche Diagnostic).

Statistical analysis

The data obtained were statistically analyzed with GraphPad Prism software, one-way ANOVA and Dunnett *post hoc* test were achieved to examine the statistical significance between the groups. $P < 0.05$ was measured to statistically significant.

RESULTS

Effect of tomentosin on body weight of breast cancer-induced rats

Figure 1 portrays the body weight of the rats in each group that was itemized and monitored for every week. Compared to control rats, the breast cancer-persuaded untreated rats have shown a drastic diminution in their body weight. There was no much difference gotten in the body weight of tomentosin- and tamoxifen-treated rats.

Effect of tomentosin on estrogen receptor α levels in breast cancer-induced rats

Estrogen receptors (ERs) shows key role in breast cancer induction. In the current study DMBA treatment augmented the levels of estrogen, whereas both the doses of tomentosin treatment declined the estrogen alpha receptor levels [Figure 2].

Effect of tomentosin on oxidative stress markers in breast cancer-induced rats

The oxidative stress markers were reduced in tomentosin- and tamoxifen-treated rats when compared to breast cancer-tempted rats.

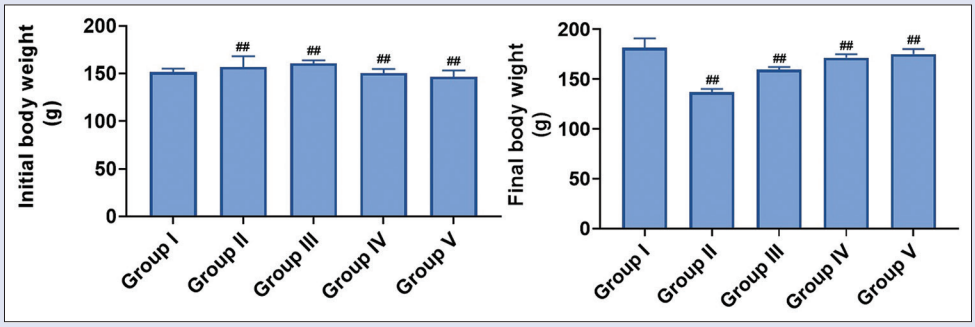

Figure 1: Effect of tomentosin on body weight of cancer-induced rats. Rats were divided as control, breast cancer-induced, low-dose tomentosin-treated cancer-induced, high-dose tomentosin-treated cancer-induced, and tamoxifen-treated cancer-induced rats. The weight of rats was monitored every week and the final weights were tabulated. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

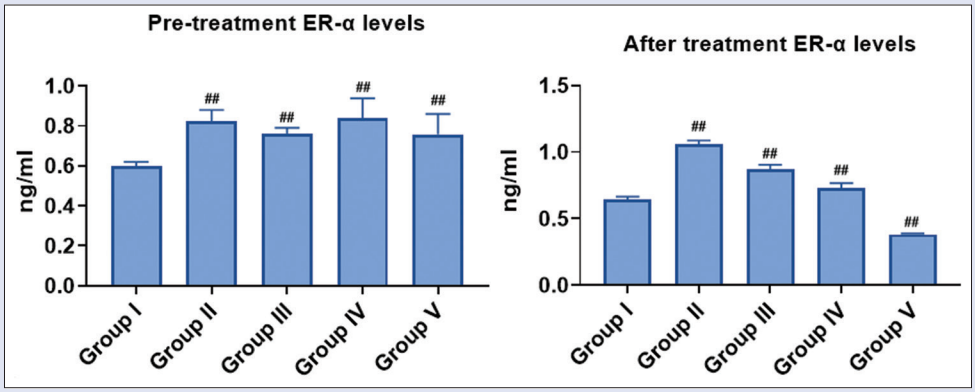

Figure 2: Effect of tomentosin on estrogen receptor α levels in breast cancer-induced rats. The levels of estrogen receptor α were estimated using commercially available ELSIA kit. Control, breast cancer-induced, low-dose tomentosin-treated cancer-induced, high-dose tomentosin-treated cancer-induced, and tamoxifen-treated cancer-induced rats. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

LPO level was diminished in tomentosin treated compared with breast cancer-persuaded untreated group. The level of reduced glutathione level was augmented in all treated groups except untreated breast cancer-induced rats. There was no exhaustion of glutathione, CAT, and SOD in all tomentosin-treated groups [Figure 3].

Effect of tomentosin on biotransformation enzymes in breast cancer-induced rats

Figure 4 shows the levels of biotransformation enzymes such as CYP-450, CYT-b5, glutathione-S-transferase, and glutathione reductase in the breast cancer-induced untreated and tomentosin-treated rats. The breast cancer-tempted rats have shown augmented levels of CYP450 and CYT-b5 and reduced levels of GST and GR enzymes, whereas tomentosin treatment pointedly decreased the levels of CYP450 and CYT-b5 and enlarged the levels of GST and GR enzymes.

Effect of tomentosin on carcinoembryonic antigen and cytokine levels in breast cancer-induced rats

Figure 5 exemplifies the levels of CEA, cytokine IL 1β, and TNF-α in the breast tissue of breast cancer-induced untreated and tomentosin-treated rats. The levels CEA, cytokine IL 1β, and TNF α were meaningfully improved breast cancer-induced rats, whereas the tomentosin and tamoxifen treatment knowingly dwindled the levels of cytokines in breast cancer-induced rats.

Effect of tomentosin on breast tissue histomorphology of breast cancer-induced rats

Figure 6 portrays the histomorphometric changes in the breast tissue of breast cancer-induced untreated, tomentosin-treated, and tamoxifen-treated rats which were stained with H and E staining and images were measured using ImageJ software. The number of inflammatory cell infiltration, neoplastic cells, and epithelial hyperplasia was augmented in breast cancer-induced rats compared to the control rats. Tomentosin treatment ominously diminished the number inflammatory cell infiltration, neoplastic cells, and epithelial hyperplasia in breast cancer-induced rats.

Cytotoxic effect of tomentosin on MCF-7 breast carcinoma cells

The cytotoxic effect of tomentosin on MCF-7 breast carcinoma cells was evaluated using MTT assay and the results are shown in Figure 7. The cell viability was considerably condensed to 80%, 65%, and 50% for the concentrations of 25, 50, 100, and 200 µg/mL of tomentosin-treated and MCF-7-treated cells, respectively ($P < 0.05$).

Effect of tomentosin on apoptosis induction in MCF-7 breast carcinoma cell lines

The apoptotic effect of tomentosin on breast carcinoma MCF-7 cells was evaluated using H2DCFDA, JC-1, and AO/EtBr dual staining cells [Figure 8]. Untreated control cells have shown uniform green fluorescence in H2DCFDA, JC-1 staining, whereas the tomentosin-treated cells have shown an augmented number of bright green-stained nucleus representing apoptotic cells. AO/EtBr-stained tomentosin-treated cells exposed yellowish-orange cells demonstrating the cells in later apoptotic stages.

Effect of tomentosin on PI3K/AKT signaling pathway in MCF-7 cell line

The gene expression of PI3K/AKT signaling proteins in untreated breast carcinoma cells and tomentosin-treated breast carcinoma MCF-7 cells

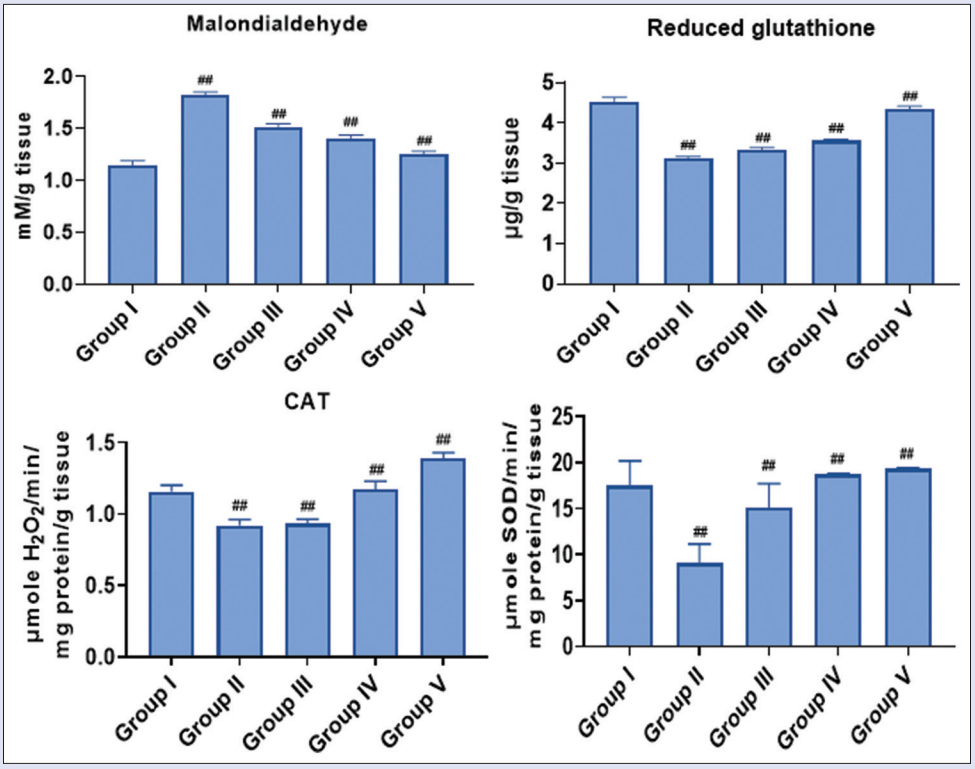

Figure 3: Effect of tomentosin on oxidative stress markers in breast cancer-induced rats. The levels of malondialdehyde, glutathione, SOD, and CAT were assessed in control, breast cancer-induced, low-dose tomentosin-treated cancer-induced, high-dose tomentosin-treated cancer-induced, and tamoxifen-treated cancer-induced rats. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

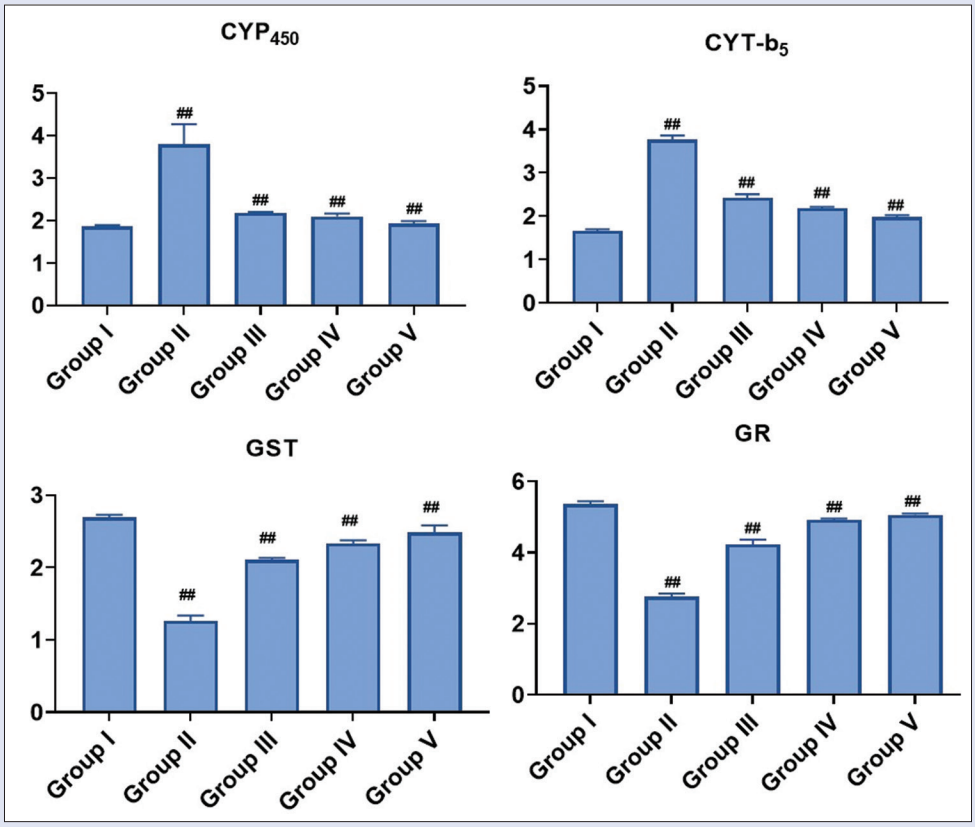

Figure 4: Effect of tomentosin on biotransformation enzymes in breast cancer-induced rats. The levels of biotransformation enzymes CYP-450, CYT-b5, glutathione-S-transferase, and glutathione reductase were estimated using commercially available kits. The data were statistically analyzed with one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

was enumerated using qPCR analysis and the consequences are portrayed in Figure 9. Untreated breast carcinoma MCF-7 cells have shown augmented expression of cell proliferating p-PI3K1/2, PI3K1/2, p-ERK1/2, and ERK1/2 genes and decreased expression apoptotic inducing genes AKT, p-AKT, p-p38, p38, p-JNK1/2, and JNK 1/2, whereas tomentosin and tamoxifen treatment meaningfully diminished the expression of genes p-PI3K1/2, PI3K1/2, p-ERK1/2, and ERK1/2 and enlarged AKT, p-AKT, p-p38, p38, p-JNK1/2, and JNK 1/2 gene expression in breast carcinoma MCF-7 cells.

DISCUSSION

Breast cancer, a worldwide threat, is the greatest predominant type of cancer with a rapid upsurge in the occurrence rate each year. The frequency rate of breast cancer in US women is about 12.5% and death befalls one in every 35 breast cancer exaggerated individual.[14] Various treatments were arranged to treat breast cancer based on the stage of disease and patient condition. The breast-conserving surgery or mastectomy is usually agreed, but it causes pain, arm morbidity, and discomfort in patients. Mastectomy is followed by radiation therapy that is operative in treating breast cancer patients, but the usage of high-energy radiation disrupts the normal cellular DNA along with destructing the cancer cells. Apart from these systemic therapies such as chemotherapy drugs, hormone therapy is also agreed, but these drugs also reduce hilarious side effects.[15] Natural compounds play a crucial role in treating cancer and numerous phytomedicines were recommended to treat cancer. One such drug with immense pharmacological properties is tomentosin, it is confirmed to have antiproliferative, antibacterial, and anti-inflammatory properties.[16] It renders anticarcinogenic effect against cervical cancer cells,[8] melanoma cells,[17] and osteosarcoma cells,[18] etc., Hence, we examined the anticancer potency of tomentosin on breast cancer-persuaded rats and also in estrogen-dependent breast cancer MCF-7 cell line.

Well-known risk factors for breast cancer are old age, female sex, white race, obesity, and high exposure of hormones. Before menopause, women's ovaries make most of the body's estrogen and fat cells make a very minor amount. After menopause, all the estrogen comes from fat tissues due to sojourn of ovaries production of estrogen. It is a high risk of estrogen. Thus, we evaluated the role of tomentosin in changeable the estrogen levels in breast cancer-induced rats. Tomentosin treatment efficiently diminished the levels of ERs in breast cancer-induced rats.

Oxidative stress shows a crucial role in cancer induction via the unregulated generation of ROS which leads to cancer cell proliferation and growth.[19] Phytochemicals are supposed to condense anticarcinogenic effect via suppressing the reactive oxygen generation in cancer cells.[20] This finding associates with the current study tomentosin treatment has effectively reduced the MDA levels and increased the levels of reduced glutathione levels. Tomentosin treatment does not display any alteration in the levels of SOD and catalase.

CEAs are cell-surface glycoproteins which are employed as diagnostic tumor markers for various tumors such as breast, lung, colorectal, and gastrointestinal.[21] Augmented levels of CEA were stated in most of the breast cancer patients and patients with growing levels of CEA which was described to be a sign of resistance to cancer drugs or recurrence of cancer.[22] We projected the potency of tomentosin to inhibit CEA levels, tomentosin treatment meaningfully diminished the levels of CEA in breast cancer-induced rats. Tomentosin treatment meritoriously dwindled the levels of cytokines which is a crucial factor that controls the

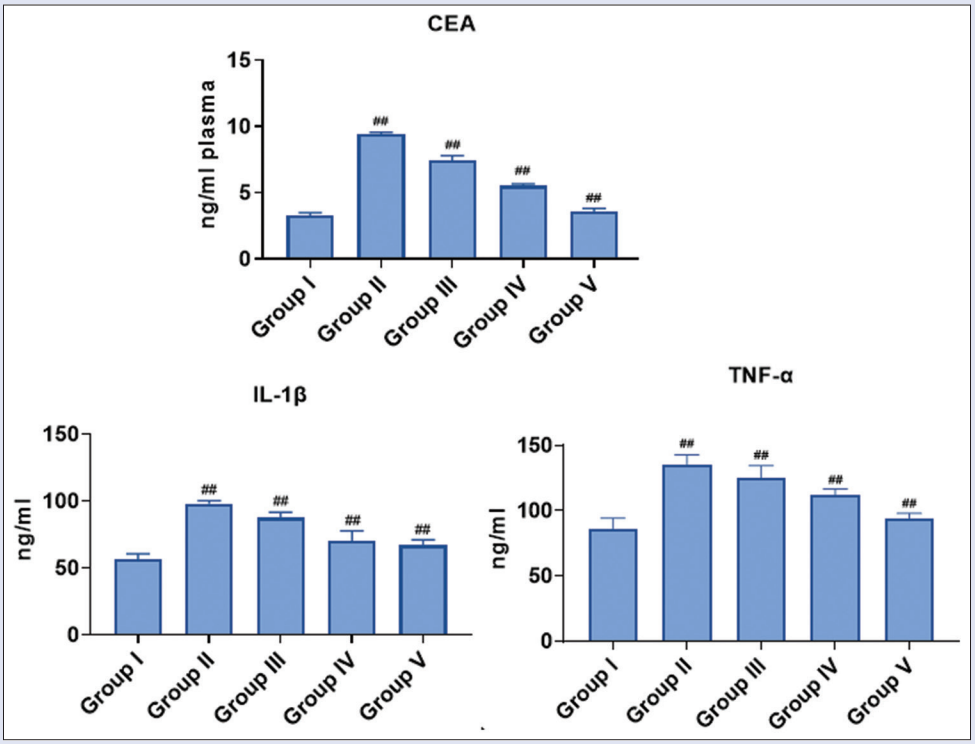

Figure 5: Effect of tomentosin on carcinoembryonic antigen and cytokine levels in breast cancer-induced rats. The levels of carcinoembryonic antigen, cytokines interleukin-1β, and tumor necrosis factor-alpha were estimated using commercially available ELISA kits. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

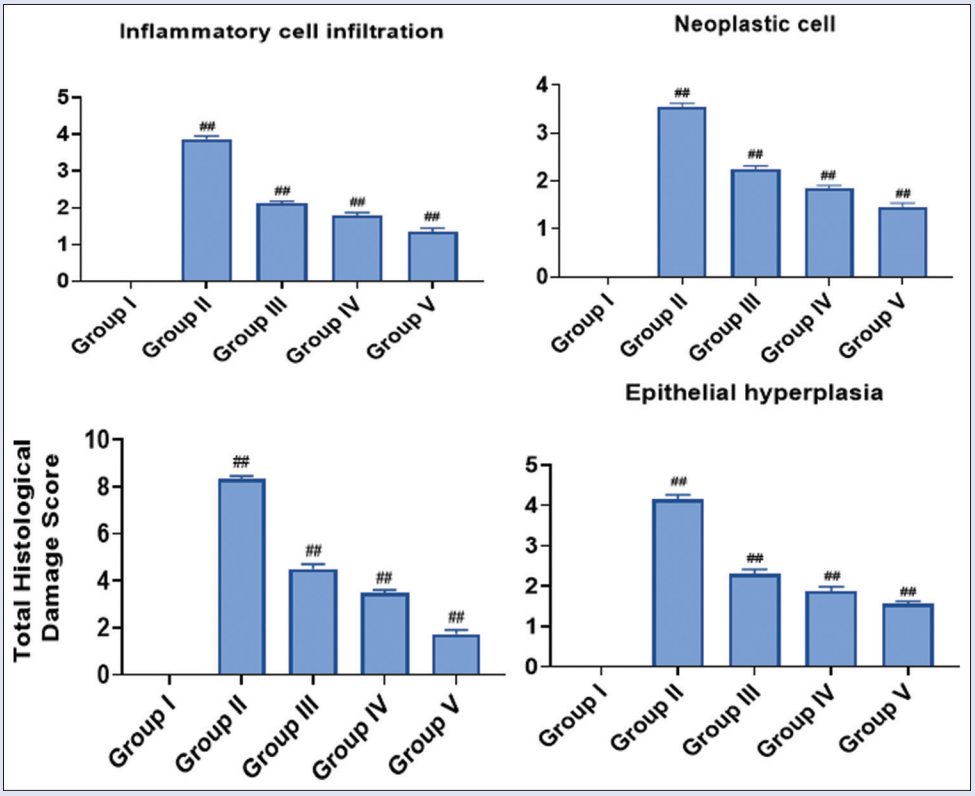

Figure 6: Effect of tomentosin on breast tissue histomorphology of breast cancer-induced rats. Histological damage in breast tissue of control, breast cancer-induced, low-dose tomentosin-treated cancer-induced, high-dose tomentosin-treated cancer-induced, and tamoxifen-treated cancer-induced rats. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

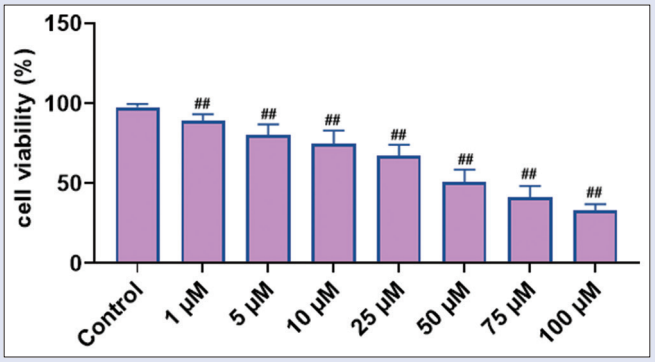

Figure 7: Cytotoxic effect of tomentosin on MCF-7 breast carcinoma cells. The cytotoxic effect of tomentosin on MCF-7 breast carcinoma cells was assessed using an MTT assay. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

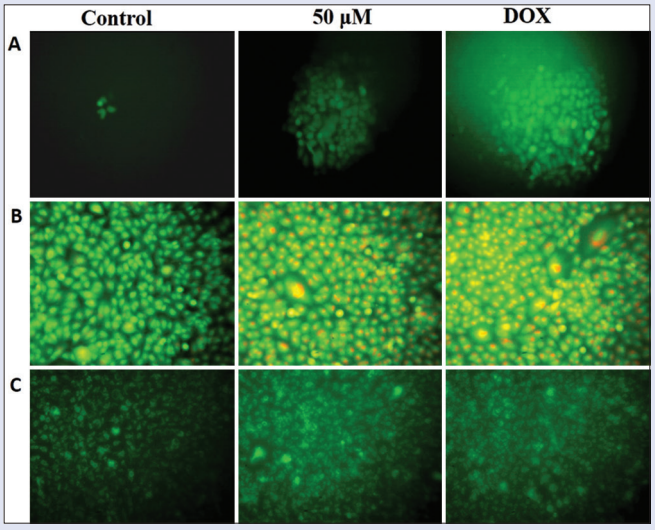

Figure 8: Effect of tomentosin on apoptosis induction in MCF-7 breast carcinoma cell lines. The apoptotic induction of tomentosin in MCF-7 breast carcinoma cell lines was assessed with (a) H2DCFDA Staining, (b) AO-EtBr staining, and (c) JC-1 staining. The images were viewed and photographed under a fluorescent microscope

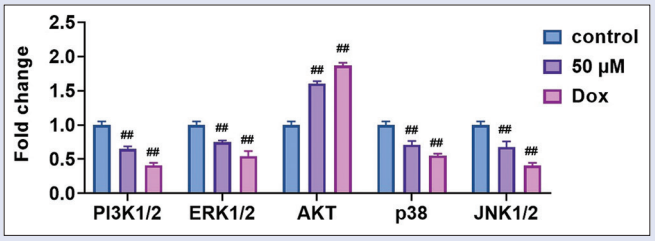

Figure 9: Effect of tomentosin on PI3K/AKT signaling pathway in MCF-7 cell line. MCF-7 cell lines were treated with tomentosin and cell lysates were subjected to quantitative polymerase chain reaction analysis. The gene expression of P-PI3K1/2, ERK1/2, AKT, p-AKT, p-p38, p38, p-JNK1/2, and JNK 1/2 was estimated using quantitative polymerase chain reaction analysis and the fold changes obtained between control and treated cells are represented in the figure. The data were statistically analyzed using one-way ANOVA followed by Dunnett *post hoc* test and the statistical significance was considered to be $^{\#\#}P < 0.05$

induction and progression of cancer. The protective action of tomentosin against DMBA carcinogen was obviously verified with histopathological analysis. Tomentosin treatment meaningfully prohibited damage to breast tissue induced by DMBA carcinogen treatment.

Further to check the anticancer potency of tomentosin, we measured the cytotoxic effect of tomentosin on MCF-7 cells. Tomentosin treatment knowingly abridged the viability of cells in dose-dependent manner. Apoptosis is the vital and decisive mechanism that controls the equilibrium between the survival and death in cells to evade the progression of cancers and other related diseases. The triggering of apoptosis in cancer cells and prevent parallel cell death of normal cells is the main objective of cancer therapy.[23] Deregulation of apoptotic signaling is one of the foremost causes for cancer induction.[24] Tomentosin suggestively augmented the levels of ROS, declined mitochondrial membrane potential, and thereby improved apoptosis in MCF-7 cells.

PI3K/AKT signaling pathway is an important signaling pathway changed in most of the cancer cells. The deregulation of PI3K/AKT signaling leads to augmented cell proliferation and growth and survival.[25] It was formerly showed that the chemodrug tamoxifen induces apoptosis in numerous cancer cells such as MDA-MB-231, MDA-MB-468, MDA-MB-453, and SK-BR-3 via inhibiting the phosphorylation of AKT.[26] In the current study, the tomentosin treatment significantly diminished the expression of genes p-PI3K1/2, PI3K1/2, p-ERK1/2, and ERK1/2 and increased AKT, p-AKT, p-p38, p38, p-JNK1/2, and JNK 1/2 gene expression in breast carcinoma MCF-7 cells. This associates with an earlier study, which stated that phytochemical hesperidin induces apoptosis in MCF-7 cells via the activation of ASK1/JNK signaling pathway.[20] Overall, our *in vivo* and *in vitro* results show that tomentosin is a potent drug which effectively inhibits the breast cancer cell growth, survival, and proliferation via the inhibition of PI3K/AKT pathway.

CONCLUSION

To accomplish, tomentosin treatment effectually abridged the levels of estrogen alpha receptors, cytokine levels, and oxidative stress markers levels in breast cancer-induced rats. Our histological analysis of breast tissue authorizes that tomentosin convincingly prevents DMBA-induced injury in breast tissue. *In vitro* results also rope the data of *in vivo* results, tomentosin induced cytotoxic effect on MCF-7 carcinoma cells in a dose-dependent manner. The induction of apoptosis by tomentosin was established with H2DCFDA, JC-1, AO/EtBr staining. It also diminished the expression of genes such as PI3K and ERK involved in cell proliferation and augmented the apoptosis-inducing genes such as AKT, p38, and JNK in MCF-7 cells. Overall, our results validly prove that tomentosin is a strong anticancer drug which induces apoptosis via varying PI3K/AKT signaling pathway.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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