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Original Article

Synergistic Suppression of DMBA-Induced Breast Cancer in Rats: Exploring the Preventive Potential of Diclofenac Sodium and *D*-Limonene Combination through mTOR Inhibition

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ABSTRACT

Background: To examine the combination of Diclofenac sodium (DCF) and *D*-Limonene's (LIM) preventative effects against DMBA-induced breast cancer in a rat model. **Materials and Methods:** 7,12-Dimethylbenz[a]Anthracene (DMBA) was administered subcutaneously once to rats to trigger mammary cancer. DCF+LIM (DL) supplementation improved the level of antioxidants in mammary tissues, levels of lipids in serum, along with serum cytokines. The pathological alterations brought on by DL treatment were identified by a histological analysis of breast tissue, both malignant as well as normal. It was also investigated how DL impacted the mTOR gene as well as the build-up of the associated gene product. **Results:** PI3K/Akt/mTOR plays a critical role in cell metabolism and proliferation in both malignant and normal cell populations. Rats given DL treatment had lower levels of mTOR expression in their breast tissues, according to transcriptional and immunohistochemical investigations. Lastly, the results suggest that by blocking the mTOR pathway, DL can reduce tumor development in rats with breast cancer generated by DMBA. **Conclusion:** DMBA-induced breast cancer in rats showed a significant improvement in their preclinical chemoprevention capability when treated with a combination of diclofenac sodium and *D*-limonene, which inhibited the mTOR pathway.

Keywords: DMBA, Breast cancer, Diclofenac sodium, *D*-limonene, mTOR, Immunohistochemistry.

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INTRODUCTION

Breast cancer is the frequent type of malignancy that mainly affects premenopausal women and is also the second leading contributing factor to cancer deaths worldwide.[1,2] Breast cancer must be prevented from developing due to the cumulative death and mortality rates. Tamoxifen is a synthetic estrogen receptor antagonist which is commonly utilized as hormonal treatment for the management of breast cancer in women. A longer consumption of this medicine, however, might result in significant consequences which include stroke,[3] embolism of the lungs,[4] along with obstruction of veins in the retina. Drug resistance and the toxic effects of the drug to healthy cells are the major downsides of the various chemotherapeutic medicines available nowadays. For the treatment of different disorders, several medications are being repurposed. Anti-inflammatory, vasodilator, vasoconstrictor, a pain reliever along with antipyretic characteristics are shared by a family of substances known as NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). They work by preventing the enzymes cyclooxygenase and lysyl oxidase from producing prostaglandins.[5] Prostaglandins, thromboxanes, leukotrienes, lipoxins, resolvins, along with eoxins are eicosanoids which are generated from the compound arachidonic acid by the enzymes cyclooxygenase and lysyl oxidase.[6] A number of studies have demonstrated a link between NSAID use and the development of a variety of cancers, notably mammary,[7,8] lung,[9,10] and gastric.[11] The results of these studies have prompted research into the possible anti-cancer properties of NSAIDs.

According to research by Nina Myorek, mice implanted with PANC02 cells had a diclofenac therapy (30 mg/kg BW for 11 days), which resulted in a 60% reduction in tumor weight and an increase in tumor cell death. They hypothesized that diclofenac therapy involves additional mediators present *in vivo* since this effect was not found *in vitro* on cultivated $PANCO_2$ cells. Diclofenac, in fact, significantly reduced tumor blood vessel development by inhibiting VEGF in tumor as well as abdominal cavity fluid.[12] Okamoto investigated the effects of diclofenac or celecoxib on cisplatin induced nephrotoxicity as well as the anticancer impact

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of cisplatin in a xenograft mice model implanted with A549/DDP cells.[13] Despite the fact that cisplatin did not diminish size of the tumor or weight, when combined with diclofenac, these attributes decreased substantially when compared to the control group. On the other hand, cisplatin's anticancer impact was only slightly decreased by celecoxib. Treatment of grafted neuroblastoma transplants in nude rats with the dual COX-1/COX-2 inhibitor diclofenac or the celecoxib, a specific COX-2 inhibitor significantly reduced the tumor progression.[14]

D-limonene is the most important bioactive food component in citrus peel oil. In animal and cell culture models, *D*-limonene might suppress or delay the progression of numerous cancers, notably lymphomas,[15] breast,[16,17] stomach,[18] and liver.[19] It possesses additional qualities that are comparable to several chemopreventive drugs. Considering the complexity of the processes driving the formation and advancement of various cancers. *D*-limonene is likely to affect multiple anti-cancer pathways.[20] Drugs for both preventive and curative purposes of breast cancer must develop this characteristic in order to proceed. Chemically non-polar, highly lipophilic and sensitive to lodging in tissues with fat cells after oral consumption, particularly in the breast. The *in vitro* studies for the combination DCF and LIM were also performed in MCF7 breast cancer cell lines and are reported to have enhanced anti-cancer activity.[17]

Earlier studies claimed that *D*-limonene treatment resulted in an overexpression of Bax and a small a decrease in the expression of Bcl-2, resulting in a significant rise in the ratio of Bax to Bcl-2, which promoted death in LS174T human colon cancer cells, according to research done by Jia *et al.*[21] According to Yu *et al.*[22] *D*-limonene upregulated Bax as well as cleaved PARP right through the treatment, demonstrating the possibility that the mitochondria-mediated intrinsic death pathway is possibly vital for the eradication of cells associated with lung cancer and this is consistent with certain aspects of its relevance in the curative efforts of numerous different malignant tumors. Due to diclofenac's induction of PTEN, PDK and Akt dephosphorylation, the PI3K/Akt survival axis was suppressed in colon cancer cells (HCT 116).[23] Despite this, no research has been conducted on the combination anti-cancer capabilities of *D*-limonene and diclofenac sodium. We expected that combining these compounds could ultimately have an enhanced impact, lowering the dose as well as the adverse effects of diclofenac, such as ulcers. In this study, we looked at the effects of diclofenac sodium, *D*-limonene and their combination on a breast cancer *in vivo* rat model. The mTOR pathway is a complex biochemical mechanism that contributes to growth of cells and tumor development and plays an essential role in the development of endocrine resistance in breast cancer. Clinical trials are currently underway for a number of compounds that target this pathway.[24] In this study, we show that the combination of Diclofenac sodium and *D*-limonene prevents DMBA-induced breast cancer in rats via inhibiting mTOR.

MATERIALS AND METHODS

Chemicals

HIMEDIA supplied D- Limonene (Mol. Wt: 136.23). The diclofenac sodium (Voveran® solution for Intramuscular injection 75 mg/mL) was provided by a local drugstore to test the commercially available medicine, which people use, rather than the pure molecule. The solutions were diluted impromptu. All the chemicals and reagents were used directly from their original forms, without any extra purification. *D*-Limonene (LIM) and Diclofenac Sodium (DCF) were mixed with 1% carboxymethyl cellulose sodium and used.

In vivo Experimental Design

Animal ethical approval was acquired in accordance with the Institutional Animal Ethical Committee (IAEC) procedure (VIT/IAEC/21/Sep22/13). Experimentation on animals that were alive was carried out as directed. The experiment was carried out on Wistar albino rats-female that weighs 100-120 g as well as aged 5 to 6 weeks. The animals used in this experiment received from the animal house at the Vellore Institute of Technology in Vellore, India. The experimental rats were kept in separate boxes made from polypropylene in a controlled experimentation room that maintained a regulated humidity level of 55±5%, a temperature of 27 to 2% and a 12 hr light/dark cycle. A pellet meal that had 4.1% fat, 22.2% of the protein content and 4% carbohydrate was given on a regular basis along with water.

Three milliliters of sterile air were injected beneath the fat area in the breast, so as to form a hollow pouch concerning the mammary glands.[25] 24 hr after the air injection, the drug 7,12-Dimethylbenz[a]anthracene (25 mg/kg BW) mixed in 0.5 mL sesame oil was subcutaneously injected into the air pouch in order to promote mammary carcinogenesis.[26] Rats were divided into six groups, each with 6 rats: a control group, a vehicle control group, a group injected with DMBA, a group administered with DCF (10 mg/kg BW), a group administered with DMBA and LIM (50 mg/kg BW) and a group administered with DMBA and combination DL-1:5 (30 mg/kg BW). Following the 16-week duration of therapy, rats were put down to fasting, followed by cervical dislocation. The mammary tissue samples of the rats were stored at 10% formalin for histopathological, immunohistochemical analysis and in TRIZOL reagent for the isolation of total RNA.

Estimation of Biochemical parameters and Pro-Inflammatory Cytokines

The isolated tissues from the breast were thoroughly homogenized using a mixture comprising 0.25 M sucrose, 10 mM HEPES-NaOH, with 1 mM EDTA after having been rinsed with ice-cold saline solution. A transparent supernatant was obtained after centrifuging the ensuing mixture at 10,000 *g* for a period of 20 min at 4ºC,[27] and it was then applied to biochemical research.

Malondialdehyde, also known as MDA, concentration was analyzed to assess the amount of Thiobarbituric Acid Reactive compounds (TBARS). Spectrophotometric measurements were used to quantify Superoxide Dismutase (SOD) as well as Glutathione Peroxidase (GPx).[28,29] Serum was separated for biochemical analysis (AST and ALT) by centrifuging blood taken from the rats for 20 min at 2,000 g. The kits were supplied by ARKRAY®, India and the blood concentrations of HDL, triglycerides and total cholesterol were measured. A rat ELISA kit (RayBio®, USA) was used to estimate the Tumor Necrosis Factor-alpha (TNF- α) and interleukin-6 (IL-6) expressions.

Gene expression analysis using Quantitative Real-Time PCR

Total RNA was isolated from the breast tissues utilizing TRIZOL reagent (Applied Biosystems, USA). Thermoscientific's NanoDrop UV-Visible spectrophotometer was used to measure the amount of RNA extracted. Takara Bio kit technique for reverse transcription of RNA into cDNA was also used. Furthermore, qRT-PCR was carried out in a Bio-Rad RT PCR system with SYBR® Premix Ex TaqTM II (Takara Bio, Japan) using the cDNA isolated from the sample as a template. Rat mTOR primers were used for amplification and GAPDH was used as the reference gene to measure the relative abundance of each gene in breast tissue. The graph depicts the fold change between the treatment group and the control group.

Histopathology and Immunohistochemical Analysis

All animals were quickly sacrificed and their mammary tissues were removed. The removed mammary tissues were fixed with 10% formalin solution and finally, they were embedded in paraffin after being dehydrated using ethanol concentrations ranging from 50% to 100%. Hematoxylin and eosin staining and light microscopy examination of sections with a thickness of 3-5 µm were performed. The receptors for Estrogen and Progesterone (ER, PR), the ki-67 (marker for proliferating cells) and the mTOR protein in the breast tissues were examined using Immunohistochemistry (IHC) on paraffin-embedded tissues.[30]

Statistical analysis

Graph pad prism 8 software was used for evaluating the statistically significant disparity among the samples using one-way ANOVA followed by Dunnett's test. p values <0.05 were deemed significant.

RESULTS

Primary observation of breast cancer in rats

Following the induction of tumor, the DMBA administered group had the greatest incidence of tumors (6/6). Despite the decrease in tumor incidence (3/6), the DMBA+DCF group rats appeared less energetic. In comparison to DMBA and DMBA+DCF, the DMBA+LIM group responded and demonstrated a significantly reduced prevalence of tumors (2/6). In comparison to each of the other tumor-induced groups, the DMBA+DCF+LIM group showed a decreased incidence of tumors (1/6).

Effect of the drug combination DL on antioxidant levels of mammary tissues

Following a 16-week experiment, the rats were sacrificed, then the mammary tissues were obtained to determine their antioxidant levels. By using TBARS, the traditional diagnostic biomarker for assessing oxidative stress, the quantity of MDA was determined. MDA was substantially higher in the DMBA group and significantly lower in the DMBA+DL treatment group as when compared with the untreated control group. SOD and GPx serve as the body's preliminary line of defense against free radicals (Figure 1 A-C). A higher concentration of these indicators suggests oxidative stress-induced cell damage. In comparison to the control, the DMBA group had significantly lower levels of the antioxidant enzymes, but the DMBA+DL group that received treatment had substantially higher levels of the antioxidant enzymes.

Determining the levels of Serum parameters

Serum lipids are one of the indicators for breast cancer and total cholesterol is one of these lipids that is important to consider since it is the essential precursor for estrogen. Elevated blood cholesterol, triglycerides and lower HDL levels, as seen in Figure 2 A-C, all contribute to an increased risk of developing breast cancer. Serum cholesterol and triglyceride levels were found to be higher in the DMBA group, whereas HDL cholesterol levels were shown to be lower. The DMBA+DL group improved blood lipid levels by lowering cholesterol, lowering triglycerides and increasing HDL levels.

Due to their great potential for being utilized in determining the prognosis and detection of various types of cancer and their stages, cytokines associated with inflammation have drawn a lot of focus on it. TNF-α along with IL-6 are 2 of the most important cytokines that promote inflammation. In comparison to the untreated control group, the DMBA group's pro-inflammatory cytokine levels were considerably greater (Figure 3 A, B). The amount of IL-6 and TNF-α level appear to have been reduced in the DMBA+DL administered group and it agrees with the delayed metastasis observed in this group. The liver function test ALT and AST levels showed significant increase in the DMBA induced rats, whereas the rats that received DMBA+DL showed decrease in the ALT and AST levels (Figure 4 A, B). The body weight of the rats was also measured and it showed decreased body weight in DMBA induced rats, whereas the rats which received the DMBA+DL treatment showed body weight similar to the weight of the control (Figure 5).

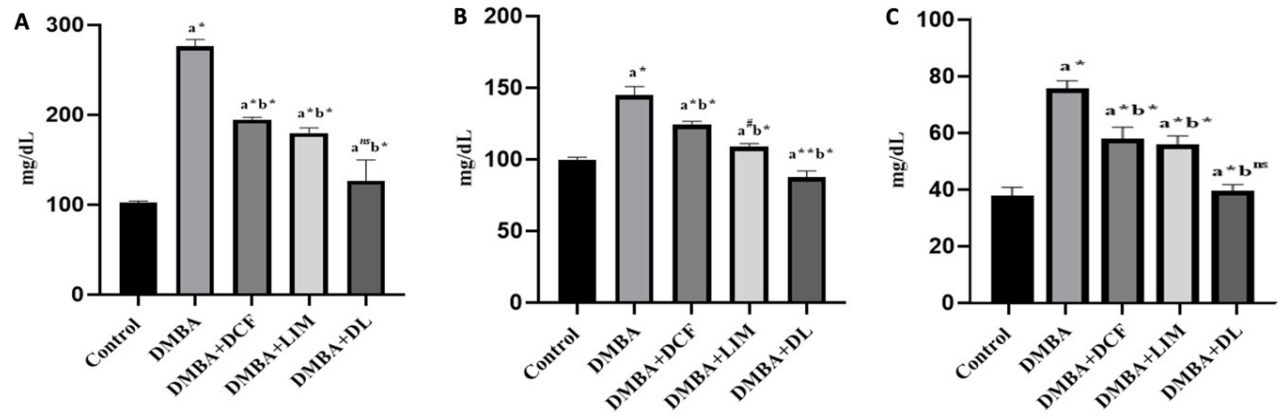

Figure 1: Antioxidant levels in mammary tissue from the rats subjected to experiments. A represents the amount of TBARS produced, B represents the amount of SOD generated, whereas C represents the amount of GPx produced. #p<0.05 **p<0.002 and *p<0.001; a=the control group is compared with other groups, b=the DMBA group is compared with other groups.

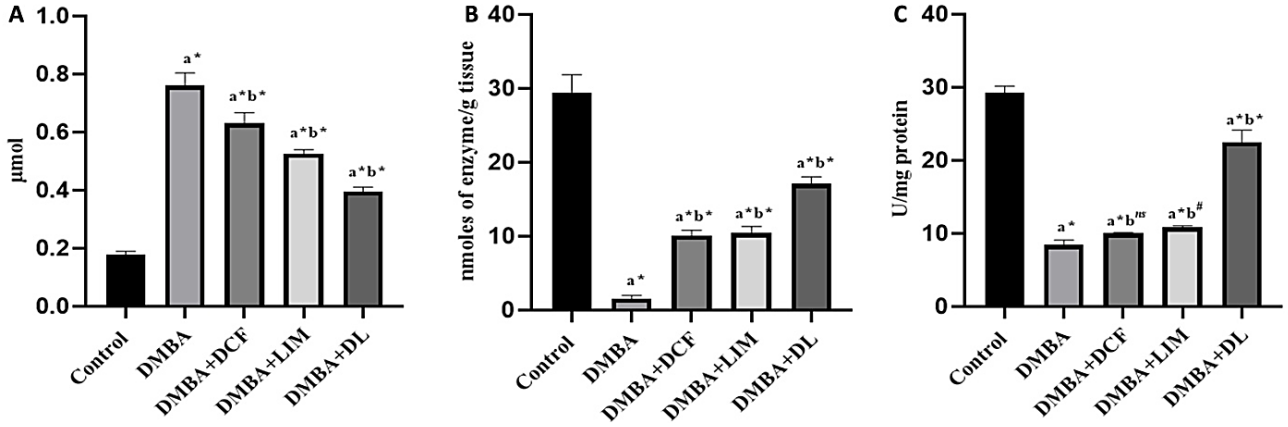

Figure 2: Lipid concentrations in the serum of the rats subjected to experiments. A, B and C stand for total cholesterol, triglyceride and HDL-C, respectively. #p<0.05 **p<0.002 and *p<0.001; a=the control group is compared with other groups, b=the DMBA group is compared with other groups.

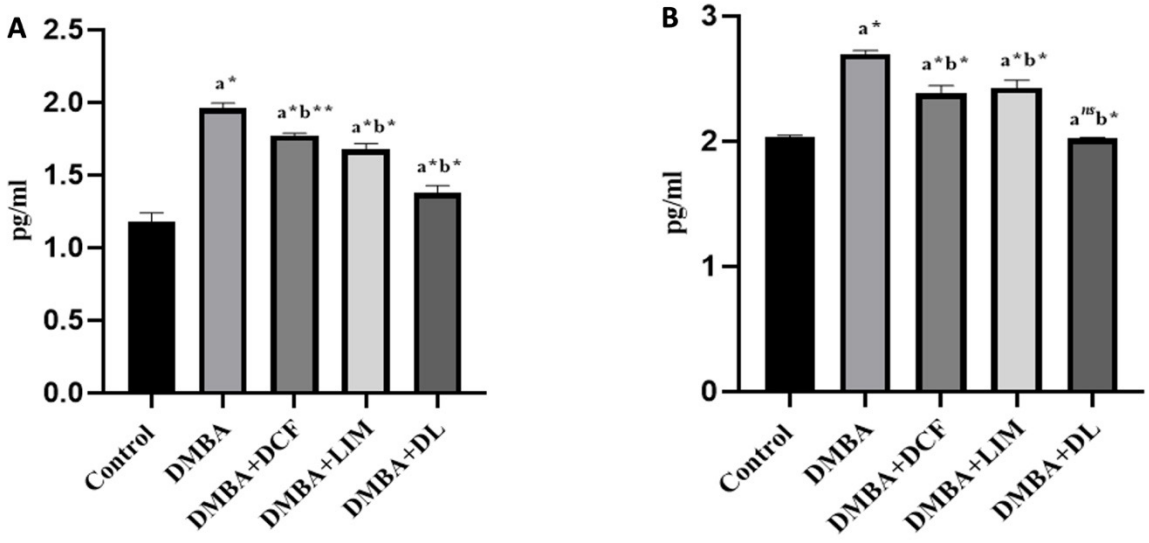

Figure 3: Levels of pro-inflammatory cytokines. A and B denotes the level of IL-6, TNF- α levels, respectively. #p<0.05 **p<0.002 and *p<0.001; a=the control group is compared with other groups, b=the DMBA group is compared with other groups.

Histopathological analysis of Mammary Tissue

H and E staining was used to investigate the influence of DL on the pathophysiology concerning the tissues of the breast (Figure 6). The Terminal Duct Lobule (TDL) as well as fatty tissues (F) of the control group's mammary morphology was typical. Invading tumor cells were seen in the group treated with DMBA alone, which was then followed by underlying membrane disruption along with permeation into connective tissues. In contrast to the DMBA+DCF group, which displayed aberrant proliferative cells that developed cribriform, the DMBA+LIM group displayed a normal tumor morphology. Despite a disorganized structure of proliferating cells in the DMBA+DL group, ductal cells of epithelium as well as fatty tissues seemed normal.

ER, PR, ki-67 and mTOR levels following DL Treatment

IHC analysis of endocrine receptors such as the hormone receptor for Estrogen (ER) and Progesterone (PR), ki-67 (marker for the proliferating cells), as well as mTOR protein in breast cancers was performed in order to correlate the acquired mTOR protein levels with the mTOR levels of gene expression (Figure 7 a). The DMBA induced group showed higher degrees of ER, PR and ki-67 expression than the DMBA+DCF and DMBA+LIM groups, while these markers were more sparsely expressed in the DMBA+DL group. The DMBA induced group exhibited greater levels of mTOR protein expression, whereas the DMBA+DL group had extremely low levels of mTOR protein expression.

DL (DCF+LIM) treatment downregulates the mTOR pathway

The mTOR is believed to be associated with several biological activities, particularly progression of cell cycle as well as cancer metastasis. As a result, we pondered if the diclofenac sodium and *D*-limonene combination treatment acted on the mTOR pathway to decrease tumor growth. DMBA+DCF administered group reduced the mTOR gene expression, when compared to the DMBA+LIM group (Figure 7 b). DMBA+DL group significantly downregulated the mTOR expression compared to the groups in which both the drugs were administered alone.

DISCUSSION

Cancer recurrence, treatment resistance and detrimental impacts affecting normal tissues are the most common side effects of the most commonly prescribed chemotherapeutic drugs and these limitations may restrict their usage and drastically reduce patients' lives.[31] To address the difficulties with existing treatment, researchers are looking for potentially effective anticancer medicine with greater effectiveness and fewer adverse reactions. Generally, combination chemopreventive approaches are favored over single-agent chemoprevention. A combined technique employs different chemopreventive medications at low doses to provide maximum chemopreventive efficiency with minimal toxicity.[32] Multiple epidemiological and preclinical studies have persuadingly suggested that a number of dietary elements have a role in both the prevention of cancer and its therapeutic management. Many clinical investigations involving the chemopreventive capabilities of the aforementioned natural substances are now underway. In experimental models of lymphomas, breast, gastric, liver and lung cancers, *D*-limonene as well as structural analogues have shown significant chemopreventive benefits.[15,33] The preliminary evidence is the strongest evidence of mammary carcinogenesis and points to a possible chemopreventive effect. Non-steroidal anti-inflammatory drug diclofenac is a common prescription drug. Actinic keratosis, which is typically classified as pre-cancerous lesions, is treated topically with it and has a widely recognized role in oncological treatment. Numerous malignancies, including neuroblastoma,[14] ovarian cancer,[34] pancreatic cancer,[12] melanoma,[10] liver cancer,[11] and prostate cancer,[35] have been extensively examined to determine the anti-proliferative effects of diclofenac. Results from our previous

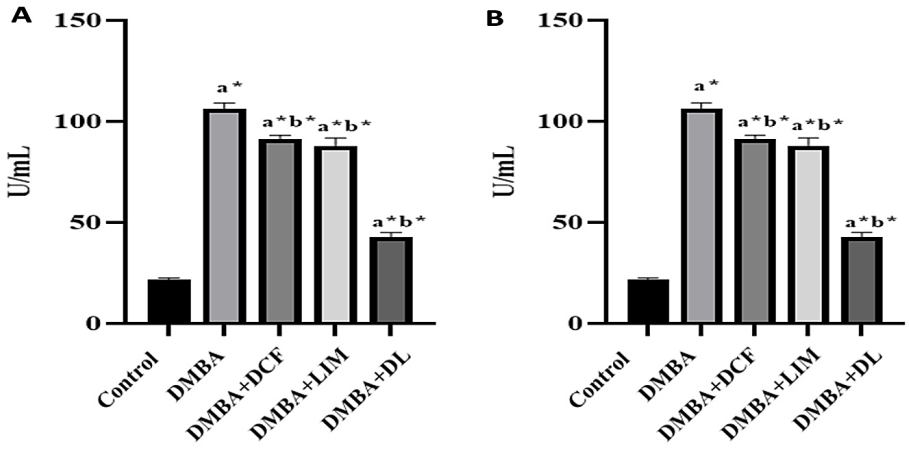

Figure 4: Levels of AST and ALP. A and B denotes the AST and ALP levels, respectively. #$p<0.05$ **$p<0.002$ and *$p<0.001$; a=the control group is compared with other groups, b=the DMBA group is compared with other groups.

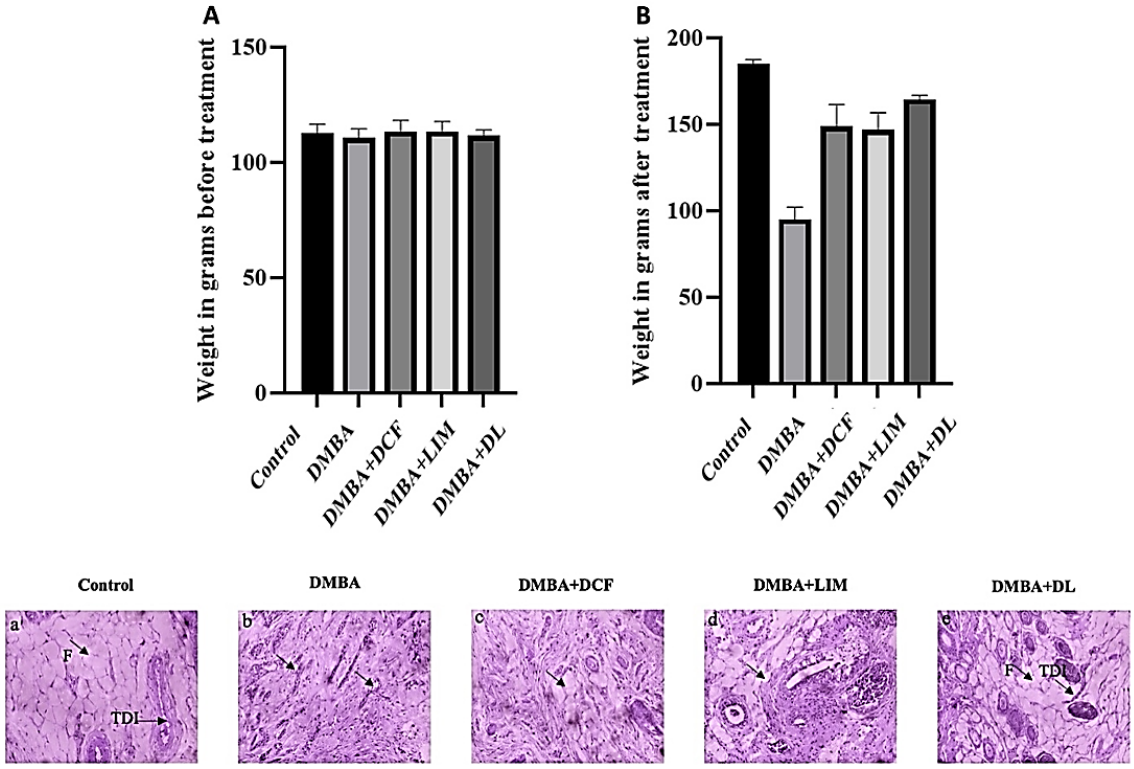

Figure 5: Breast tissue stained with H and E (20X magnification). a: Normal breast morphology with Terminal Duct Lobule (TDL) along with fatty tissues (F); b: DMBA group: Invasive tumor cells (invasive ductal carcinoma) followed by basement membrane damage; c: DMBA+DCF group: Solid tumor pattern; d: DMBA+LIM group: Atypical proliferative cells that formed cribriform; e: DMBA+DL group: Healthy ductal cells of the epithelium along with fatty tissues exhibiting a disorganized arrangement of proliferative cells.

study shows diclofenac sodium and *D*-limonene in combination displayed growth inhibitory effects on MCF-7 breast cancer cell line.[17]

The influence of DL on preventing breast cancer by downregulating mTOR in rat models is being reported for the first time in the current investigation. DMBA causes oxidative damage at the site where it is injected.[36] A single administration of 25 mg/kg BW of DMBA was utilized to induce breast cancer, which was confirmed by the development of tumor. In DMBA-induced rats, DL (30 mg/kg BW) treatment displayed preventative benefits as demonstrated by decreased tumor formation, enhanced level of antioxidants, as well as substantially decreased breast cancer associated biomarker levels. Breast cancer patients generally have higher triglycerides, higher total cholesterol and lower HDL cholesterol levels,[37] and the rats given DMBA showed an identical pattern, but those treated with DL showed improved lipid levels. Furthermore, DL-treated rats substantially transformed the morphology of mammary tissues towards more normal forms. Histopathological investigation provided significant support for the reported result.

Tumor development and metastasis are facilitated by cytokines which are pro-inflammatory. TNF-α primarily drives inflammation by interfering with cellular signaling pathways, whereas IL-6 plays a role in cancer metastasis. Individuals with elevated levels of the aforementioned cytokines had a greater death rate than individuals with low levels of IL-6 as well as TNF- α.[38] Reactive Oxygen Species (ROS) production along with increased levels of glucose in cells with cancer might be both indirectly influenced by elevated levels of inflammatory cytokines. Glycolysis and oxidative stress are integrated metabolically by ROS, which act as key mediators.[39] DMBA-treated rats had elevated levels of cytokines, which was linked to the cancer metastasis. The DL therapy dramatically enhanced levels of antioxidants in mammary tissues while lowering serum cytokine levels which results in delaying metastasis. During the advancement of tumors, mTOR controls the metabolism of cells along with the proliferation.[40] Inhibiting autophagy and promoting the growth of cancer cells, overexpression of mTOR promotes the spread of cancer. IHC for the mammary tissues of the rats was also performed which showed correlation between the mTOR gene expression with mTOR protein expression. In breast cancer, the majority of genetic alterations take place upstream of mTOR and cause an excessive stimulation of the signaling protein mTOR.[41] DMBA+DL therapy significantly lowered the mRNA and protein levels of mTOR in the animals. Inhibiting the mTOR pathway, DL therefore likely prevents the spread of DMBA-induced tumor development.

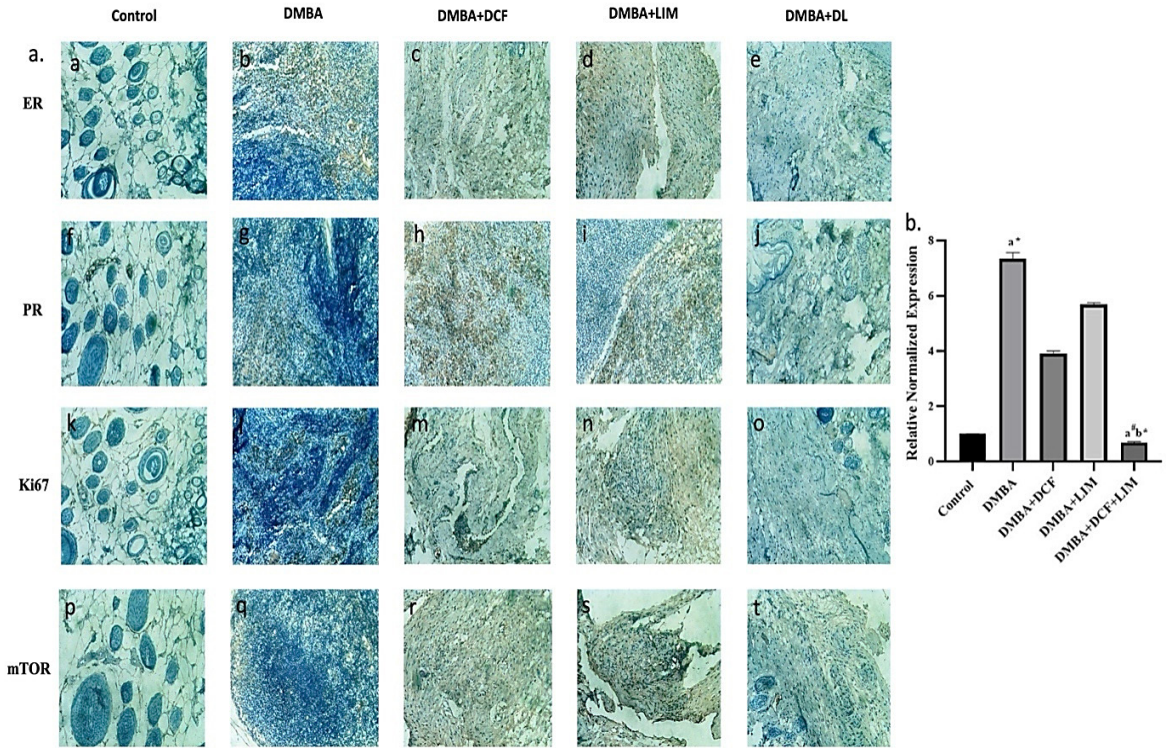

Figure 6: The breast tissues of the experimental rats were immunohistochemically positive for ER, PR, Ki-67 and mTOR (40X magnification). ER: a) The control group showed negligible expression; b) the DMBA-induced group showed upregulated expression; c) the DMBA+DCF group showed modest expression; d) the DMBA+LIM group showed overexpression; and e) the DMBA+DL group showed downregulation. PR: f) There was absence of expression in the control group; g) unregulated expression in the group that received DMBA; h and i) overexpression in the groups that received DMBA+DCF and DMBA+LIM; j) downregulation in the group that received DMBA+DL treatment. Ki67: k) No expression was observed in the control group; l) overexpression in the DMBA induced group; m and n) moderate expression in the DMBA+DCF and DMBA+LIM group; o) Downregulation was observed in the group treated with DMBA+DL. mTOR: p) No expression was observed in the control group; q) overexpression in the DMBA induced group; r and s) moderate expression in the DMBA+DCF and DMBA+LIM group; t) Downregulation was observed in the group treated with DMBA+DL. b. The quantitative real-time PCR investigation of the gene mTOR, #$p<0.05$ **$p<0.002$ and *$p<0.001$; a=the control group is compared with other groups, b=the DMBA group is compared with other groups.

CONCLUSION

It has been observed that the phytochemicals, both individually and in combinations with other anti-cancer medications, have improved anti-cancer effects without causing considerable harm. The current study highlights the preclinical chemoprevention potential of diclofenac sodium and *D*-limonene by downregulating the mTOR pathway.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Bax: Bcl-2-associated X protein; **Bcl2:** B-cell lymphoma-2; **PKB:** Protein Kinase B; **mTOR:** Mammalian target of rapamycin; **PI3K:** Phosphoinositide 3-kinase; **GAPDH:** Glyceraldehyde-3-Phosphate Dehydrogenase; **TNF-α:** Tumour Necrosis Factor alpha; **IL-6:** Interleukin-6; **DMBA:** 7,12-Dimethylbenz[a]anthracene; **DMEM:** Dulbecco's Modified Eagle Medium.

AUTHORS' CONTRIBUTION

SS contributes to the conception and design, acquisition of data, drafting of the article and final approval of the manuscript. KMG contributes to the analysis of data, critically revised the manuscript content and final approval of the manuscript.

ETHICAL APPROVAL

Animal ethical approval was acquired in accordance with the Institutional Animal Ethical Committee (IAEC) procedure (VIT/IAEC/21/Sep22/13). Experimentation on animals that were alive was carried out as directed.

SUMMARY

Combining diclofenac sodium with *D*-limonene improved the preclinical chemoprevention efficacy of rats with DMBA-induced breast cancer and improved serum parameters. Rats treated with the DL combination showed reduced levels of TNF-α and IL-6, which helps to postpone metastases. Despite a disorganized structure of proliferating cells in DMBA, DCF and DL groups, the ductal cells of the epithelium and the fatty tissues in the DMBA+DL group seemed normal. While the DMBA+DL group showed very low levels of mTOR protein expression, the DMBA-induced group showed higher levels of mTOR protein expression. Therefore, from this study we conclude that Diclofenac sodium and *D*-limonene combination, inhibits the proliferation of cancer cells, by downregulating the mTOR pathway.

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