

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 IJABR 2026; 10(5): 635-646
www.biochemjournal.com
 Received: 23-02-2026
 Accepted: 26-03-2026

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Prophylactic effects of piperine and berberine on DMBA-induced mammary gland tumor model in rats

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i5h.8469>

Abstract

The present study was carried out to evaluate the prophylactic efficacy of piperine and berberine against 7,12-dimethylbenz [a]anthracene-induced mammary gland tumors in female Sprague-Dawley rats. Forty rats were randomly divided into five groups comprising eight animals each. Group I served as the normal control and received vehicle only, whereas mammary gland tumors were induced in Groups II, III, IV and V by single intragastric administration of 7,12-dimethylbenz [a]anthracene at 80 mg/kg body weight in 0.5 ml corn oil at 8 weeks of age. Group II served as the tumor control. Groups III and IV received piperine and berberine, respectively, at 50 mg/kg body weight orally once daily, while Group V received their combination at the same dose. Treatments were administered from day 56 to day 140. Clinical signs, feed consumption, body weight and tumor volume were monitored. Blood and serum samples were analysed for haematological, biochemical, hormonal, inflammatory and oxidative stress markers. Mammary tissue was examined for histopathological and ultrasonographic changes, while quantitative real-time polymerase chain reaction was used to assess the expression of *TP53*, *Caspase-3*, *Cyclin D1* and matrix metalloproteinase-9 genes, with *GAPDH* used as the housekeeping gene. Tumor control rats showed reduced feed intake and body weight gain, increased tumor volume, haematological alterations, altered liver and kidney function markers, increased estradiol, elevated pro-inflammatory cytokines and higher malondialdehyde concentration. Tumor development was also associated with downregulated *TP53* and *Caspase-3* expression and upregulated *Cyclin D1* and matrix metalloproteinase-9 expression, along with marked neoplastic changes in mammary tissue. Prophylactic administration of piperine and berberine improved general health status, reduced tumor burden, corrected several altered biomarkers, lowered oxidative stress and favourably modulated tumor-related gene expression. The combined treatment produced the most consistent protective response, suggesting that piperine and berberine together may offer promising prophylactic benefit against chemically induced mammary gland tumors.

Keywords: Berberine, 7,12-dimethylbenz [a]anthracene, mammary gland tumor, gene expression

Introduction

Cancer is a complex and heterogeneous disease characterized by uncontrolled proliferation of abnormal cells, with the potential to invade surrounding tissues and metastasize to distant organs. It continues to be a major cause of morbidity and mortality in both humans and dogs, thereby emphasizing its comparative biomedical and clinical relevance [1]. Experimental animal models are essential for understanding the mechanisms of carcinogenesis and for evaluating potential preventive and therapeutic agents. Among these models, rats are widely used in mammary Tumor research because of their well-characterized biology, reproducible Tumor development and suitability for controlled experimental studies [2]. The chemical carcinogen 7,12-dimethylbenz [a]anthracene (DMBA) is commonly used to induce mammary Tumors in female Sprague-Dawley rats. Following metabolic activation, DMBA contributes to mammary carcinogenesis through aryl hydrocarbon receptor-mediated pathways, induction of cytochrome P450 enzymes such as CYP1A1 and CYP1B1, generation of reactive intermediates, formation of DNA adducts and subsequent malignant transformation [3].

Although several treatment modalities, including chemotherapy, hormone therapy, radiotherapy, immunotherapy and targeted therapy, are currently used for cancer management, their clinical efficacy is often limited by poor tumor selectivity, toxicity to

normal tissues, tumor recurrence and inadequate bioavailability [4]. Chemotherapeutic agents are generally classified according to their mechanism of action and chemical structure, including alkylating agents, antimetabolites, topoisomerase inhibitors and mitotic spindle inhibitors [5]. Among these, doxorubicin is a widely used antineoplastic agent for the treatment of mammary gland tumors and other solid tumors. Its anticancer activity is mainly attributed to DNA intercalation, inhibition of topoisomerase II, mitochondrial dysfunction and induction of oxidative stress; however, its precise mechanism of action remains complex and not completely understood [6]. Despite its therapeutic efficacy, the clinical use of doxorubicin is restricted by dose-dependent toxicity to normal tissues, particularly myelosuppression, nephrotoxicity and cardiotoxicity [7].

Cancer incidence is increasing globally and continues to account for a large number of deaths each year, underscoring the need for effective preventive and control strategies. Therefore, greater emphasis on suppressing or delaying carcinogenesis may offer a rational approach, rather than focusing only on the treatment of established or advanced-stage cancer [8]. In this regard, chemoprevention may serve as a valuable strategy by employing natural, synthetic or biological agents to suppress, delay or reverse carcinogenic progression and thereby reduce the risk of invasive cancer development [9]. Numerous studies using animal models have demonstrated the potential efficacy of phytochemicals in both cancer prevention and treatment, with encouraging outcomes. Bioactive compounds derived from medicinal plants have been shown to suppress cell proliferation, induce apoptosis, delay metastatic progression and inhibit angiogenesis [10]. Medicinal plants are important sources of bioactive compounds with potential anticancer properties [11]. Piperine, the major alkaloid of black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.), has been reported to exert cytotoxic and pro-apoptotic effects in different cancer models by modulating signalling pathways associated with cell proliferation, apoptosis and survival [12]. Similarly, berberine, an isoquinoline alkaloid obtained from medicinal plants such as *Coptis chinensis* and *Coptis japonica*, has demonstrated antiproliferative activity through interaction with key molecular targets and disruption of cell-cycle progression [13]. Based on the reported anticancer properties of piperine and berberine, the present study was undertaken to investigate their prophylactic efficacy against DMBA-induced mammary gland tumors in Sprague-Dawley rats, focusing on tumor response, clinical health status, haemato-biochemical changes, histopathological alterations and molecular markers involved in mammary carcinogenesis.

Materials and Methods

Animals

The present study was conducted on forty healthy female Sprague-Dawley rats (*Rattus norvegicus*), aged 8 weeks and weighing 103-127 g. The animals were procured from Zydus Research Centre, Ahmedabad, Gujarat, India. The experiment was carried out at the Small Animal House Facility and the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, India. Female Sprague-Dawley rats were procured before the experimental start and acclimatized for 2 weeks. During the study period,

the animals were maintained under standard laboratory conditions with free access to a standard pelleted diet and drinking water. They were housed under controlled environmental conditions, including temperature of 21 ± 1 °C, relative humidity of $55\pm10\%$ and a 12 h light/12 h dark cycle. The experimental protocol was approved by the Institutional Animal Ethics Committee of the College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, India, vide Approval No. 462/VPT/2025. All experimental procedures were performed in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India.

Study Design and Protocol

7,12-Dimethylbenz [a]anthracene (DMBA) (Cayman Chemical, Ann Arbor, MI, USA) was used for the induction of mammary gland tumors. The compound was stored at -20 °C until use. Before administration, DMBA was weighed according to the calculated dose and dissolved in corn oil with continuous magnetic stirring until a homogeneous solution was obtained. Piperine and berberine were procured from Sigma-Aldrich (St. Louis, MO, USA). Piperine with purity $\geq 98\%$ and berberine with purity $\geq 97\%$ were used for the prophylactic intervention. For the prophylactic study, forty female Sprague-Dawley rats were randomly assigned into five groups, with each group comprising eight animals. Group I served as the normal control and received vehicle only. Mammary gland tumors were induced in Groups II, III, IV and V by a single intragastric administration of DMBA at a dose of 80 mg/kg body weight, dissolved in 0.5 ml corn oil, at 8 weeks of age (8-20 weeks of age). Group II served as the DMBA-induced tumor control. Group III received piperine at 50 mg/kg body weight orally once daily, while Group IV received berberine at 50 mg/kg body weight orally once daily. Group V received a combination of piperine and berberine, each at 50 mg/kg body weight orally once daily. The prophylactic treatments were started on the day of DMBA administration and continued up to day 140 of age (8-20 weeks of age). During the experimental period, animals were monitored for general clinical signs, feed consumption, body weight changes and tumor development. Tumor growth was assessed by measuring tumor volume and further evaluation was performed through haematological, serum biochemical, hormonal, inflammatory, oxidative stress, gene expression studies, ultrasonographic examination and histopathological studies at the end of the prophylactic period.

Feed Consumption and Body Weight

During the prophylactic study, feed consumption was recorded weekly by measuring the quantity of feed offered to the animals in each cage and subtracting the leftover feed. Body weight of individual rats was recorded at the beginning of the experiment at 8 weeks of age (day 56), when DMBA was administered to the tumor induction groups and subsequently monitored at weekly intervals up to day 140 of age (8-20 weeks of age).

Measurement of Mammary Tumor Volume

Mammary tumors in rats were measured using a vernier calliper by recording the largest diameter (L) and smallest diameter (B) of each tumor. Tumor volume was calculated using the following formula:

Tumor volume (cm^3) = $(L \times B^2)/2$

Where

L represents the largest diameter and B represents the smallest diameter of the tumor.

Blood and Serum Analysis

At the completion of the prophylactic study, blood samples were collected from all rats on day 140 by retro-orbital plexus puncture under isoflurane anaesthesia. Approximately 1 ml of blood was transferred into K3EDTA vacutainers for haematological analysis. Additional blood samples were collected separately in sterile anticoagulant-free glass tubes for serum separation. The samples were allowed to clot and then centrifuged at 3000 rpm for 15 min at 4 °C. The separated serum was stored at -20 °C until further analysis of biochemical, hormonal, oxidative stress and pro-inflammatory cytokine parameters. Haematological parameters, including haemoglobin, total erythrocyte count, total leukocyte count and differential leukocyte count, were determined using an automated whole blood analyser (BC-2800 Vet, Mindray). Serum biochemical parameters were estimated using commercially available diagnostic kits procured from Q-line Biotech Private Limited with an AutoChem Ingenious® 1.0 Semi-Auto Chemistry Analyser. The parameters assessed included alanine aminotransferase (ALT0250PS), aspartate aminotransferase (ASTG0250PS), blood urea nitrogen (UREG0250PS) and creatinine (CRTG0250PS), according to the manufacturer's instructions. Serum malondialdehyde concentration was estimated as a marker of lipid peroxidation using a malondialdehyde colorimetric assay kit based on the thiobarbituric acid method (PG-520K-BC-M). Serum estradiol and progesterone concentrations were measured using Rat Estradiol Rapid ELISA Kit (EELR015) and Rat Progesterone Rapid ELISA Kit (EELR018), respectively, procured from Thermo Fisher Scientific, Invitrogen BioServices India Pvt. Ltd. Serum levels of pro-inflammatory cytokines were determined using rat-specific ELISA kits procured from Geno Biosciences Pvt. Ltd., including TNF- α (ITRPF009), IL-1 β (PG-2100R) and IL-6 (PG-5100R). All biochemical and ELISA-based assays were carried out as per the respective manufacturer's protocols.

Real-time Quantitative PCR Analysis

Total RNA was isolated from mammary gland tissue samples using QIAzol lysis reagent and the RNeasy Mini Kit, according to the manufacturer's instructions. On-column DNase treatment was performed to eliminate possible genomic DNA contamination. The concentration and purity of isolated RNA were assessed using a NanoDrop 1000 spectrophotometer, and RNA integrity was confirmed by agarose gel electrophoresis. Purified RNA was reverse-transcribed into complementary DNA using a first-strand cDNA synthesis kit (Thermo Scientific), and the synthesized cDNA was subsequently used for quantitative real-time PCR analysis. The gene-specific primer sequences used for quantitative real-time PCR analysis are presented in Table 1. *GAPDH* was used as the endogenous reference gene. Quantitative real-time PCR was performed using the QuantStudio™ 5 Real-Time PCR System with KAPA SYBR FAST qPCR Master Mix (2X). Each 20 μl reaction mixture consisted of 10 μl of 2X SYBR Green PCR Master Mix, 0.5 μl each of forward and reverse primers, 0.3 μl of

cDNA template, 0.6 μl of ROX dye and 8.1 μl of nuclease-free water. The amplification protocol included initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing at 58 °C for 20 s. All reactions were performed in duplicate, and no-template controls were included to check contamination or non-specific amplification. Melt curve analysis was performed after amplification to confirm the specificity of PCR products. Relative mRNA expression levels of *TP53*, *Caspase-3*, *Cyclin D1* and *MMP-9* were calculated using the comparative $2^{-\Delta\Delta C_q}$ method after normalization with *GAPDH*.

Ultrasonographic Examination

Mammary gland ultrasonography was performed on day 140 (20 weeks of age) to evaluate tumor development at the end of the prophylactic study. Real-time B-mode imaging was carried out using an Esaote MyLab™ 40 VET ultrasound system (Esaote Europe B.V., Maastricht, Netherlands) equipped with a 3.5-12 MHz linear transducer. Tumor size, margins, echotexture and surrounding mammary tissue architecture were assessed.

Gross and Histopathological Examination

At the end of the prophylactic study on day 140, rats were euthanized using an overdose of sodium pentothal. Mammary gland tissues were collected and examined for gross lesions. Representative tissue samples were fixed in 10% neutral buffered formalin, processed by routine paraffin embedding, sectioned at 5-6 μm using an automatic microtome (Leica, Germany) and stained with haematoxylin and eosin. The stained sections were examined microscopically and histopathological alterations were recorded.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA) under a completely randomized design with IBM SPSS Statistics software, version 31.0. Significant differences among experimental groups were analysed using Duncan's multiple range test at $p < 0.05$. All data were presented as mean $\pm$ standard error (SE).

Results

Feed Consumption Measurement

Feed consumption during the prophylactic study is presented in Table 2a and 2b. Mean feed consumption was reduced in the DMBA control group (Group II) compared with the normal control group (Group I) during most weeks of the study. A significant decrease was observed in Group II during Week 1 and the later phase of the study from Week 10 to Week 12, indicating the adverse effect of DMBA administration on appetite and general health status. During the intermediate weeks, feed consumption in the DMBA control group remained numerically lower than the normal control group, although the differences were not significant. Compared with the DMBA control group, rats treated prophylactically with piperine, berberine and their combination showed variable improvement in feed intake. The combined treatment group (Group V; piperine + berberine) generally showed better restoration of feed consumption than the individual treatment groups, particularly during the later weeks of the study. However, the improvement observed in the treated groups was mostly

non-significant when compared with the DMBA control group. Overall, DMBA administration reduced feed consumption in mammary tumor-bearing rats, while prophylactic treatment with piperine and berberine, especially in combination, partially improved feed intake during the experimental period.

Body Weight Measurement

Body weight changes during the prophylactic study are presented in Table 3a and 3b. Initial body weight at 8 weeks of age (day 56) did not differ significantly among the groups, indicating comparable baseline body weight before DMBA administration. During the study period, the DMBA control group (Group II) showed lower body weight gain than the normal control group (Group I), with significant reduction observed during several weeks, particularly in the later phase of the experiment. Among the treated groups, the combination treatment group (Group V; PIP + BER) showed comparatively better improvement in body weight, with significantly higher values than Group II during Weeks 2-5. Piperine and berberine-treated groups showed variable but mostly non-significant changes compared with Group II. Overall, DMBA administration reduced body weight gain, whereas prophylactic treatment with piperine and berberine, especially in combination, partially improved body weight status in mammary tumor-bearing rats.

Measurement of Mammary Tumor Volume

Tumor volume in the prophylactic groups is presented in Table 4. The DMBA control group (Group II) showed the highest mean tumor volume. Compared with Group II, tumor volume was significantly reduced in Group III (DMBA + piperine), Group IV (DMBA + berberine) and Group V (DMBA + piperine + berberine). Overall, these results suggest that prophylactic administration of piperine and berberine, particularly berberine, effectively reduced tumor burden in DMBA-induced mammary tumor-bearing rats.

Haematological Analysis

Haematological parameters of day 140 are presented in Table 5. Compared with the normal control group (Group I), the DMBA-induced mammary tumor control group (Group II) showed significantly lower haemoglobin concentration, total erythrocyte count and lymphocyte percentage, together with significantly higher total leucocyte count, monocyte percentage and granulocyte percentage. Overall, piperine and berberine improved haemoglobin concentration and lymphocyte percentage, while the piperine + berberine combination showed the most evident reduction in total leucocyte count.

Serum Biochemical Parameters

Serum biochemical parameters are presented in Table 6. The DMBA control group showed significant increases in serum AST, ALT and BUN compared with the normal control group, indicating hepatic and renal biochemical alterations associated with DMBA-induced mammary tumor development. Prophylactic administration of piperine and berberine consistently attenuated DMBA-associated increases in serum transaminase activities, while their effects on renal biochemical markers were selective and treatment dependent.

Serum Oxidative Stress and Inflammatory Biomarkers

Serum oxidative stress and inflammatory biomarkers are presented in Table 7. The DMBA control group showed a significant increase in MDA, TNF- α , IL-1 β and IL-6 concentrations compared with the normal control group, indicating enhanced lipid peroxidation and inflammatory activation. Overall, prophylactic administration of piperine and berberine significantly attenuated DMBA-associated oxidative stress and pro-inflammatory cytokine elevation, although the most favourable response varied according to the biomarker assessed.

Serum Hormonal Profile

Serum hormonal changes following prophylactic treatment are presented in Table 8. Serum estradiol concentration was significantly increased in the DMBA control group compared with the normal control group, indicating DMBA-associated estrogenic alteration. Findings indicated that DMBA-induced mammary tumor development was mainly associated with increased serum estradiol concentration, whereas progesterone concentration remained largely unaffected by tumor induction or prophylactic intervention.

Gene Expression Analysis

Relative mRNA expression profiles of *Cyclin D1*, *MMP-9*, *TP53* and *Caspase-3* are shown in Fig. 1. Overall, prophylactic administration of piperine and berberine favourably modulated DMBA-associated molecular alterations. The combined treatment showed the most evident response by producing significant upregulation of *Caspase-3* and numerically lowering *Cyclin D1* and *MMP-9* expression, while *TP53* changes remained non-significant.

Ultrasonographic Evaluation of Mammary Gland Tumor

Ultrasonographic observations of mammary glands from different prophylactic groups on day 140 are shown in Fig. 2. The normal control group showed a uniform mammary echotexture with no detectable mass or structural abnormality. In contrast, the DMBA control group revealed irregular hyperechoic mammary lesions with heterogeneous internal echotexture and distortion of the surrounding mammary tissue, confirming tumor development. The groups receiving prophylactic treatment with piperine, berberine and their combination showed comparatively smaller lesions, improved echotexture, more defined margins and reduced disruption of mammary architecture than the DMBA control group. These findings suggest that prophylactic treatment delayed tumor development and reduced the severity of DMBA-induced mammary gland alterations.

Histological Examination of Mammary Gland

Representative histopathological changes in mammary gland tissues from control and experimental rats are presented in Fig. 3. The normal control group exhibited normal mammary architecture, with well-organized ducts and acini lined by uniform epithelial cells, along with intact basement membrane and normal stromal and adipose tissue components. The DMBA control group showed pronounced neoplastic changes, including loss of normal glandular arrangement, densely packed irregular glandular structures, increased cellularity, epithelial atypia, hyperchromatic nuclei, multiple luminal formations and stromal invasion,

confirming successful induction of mammary gland tumors. The piperine-treated group showed milder proliferative changes with better preservation of glandular architecture, minimal nuclear atypia and mild hyperchromasia. The berberine-treated group demonstrated moderate proliferative alterations, with partial disturbance of glandular organization and mild to moderate cellular atypia; however, these changes were less severe than those observed in the DMBA control group. The piperine + berberine-treated group showed comparatively greater improvement, characterized by reduced neoplastic severity, improved glandular organization, decreased cellular atypia and partial restoration of mammary tissue architecture. Overall, prophylactic administration of piperine and berberine reduced DMBA-induced mammary neoplastic changes, with the combined treatment showing better histological protection.

Discussion

DMBA-induced mammary carcinogenesis is strongly associated with oxidative damage, inflammatory responses and systemic toxic effects. After metabolic activation through cytochrome P450 enzymes, DMBA produces reactive carcinogenic intermediates that promote reactive oxygen species generation, DNA damage and subsequent mammary tumor development^[14].

Feed intake, body weight and general body condition are useful indicators of systemic toxicity, tumor burden and overall physiological status during chemically induced carcinogenesis^[15]. In the present prophylactic study, rats in the DMBA control group showed reduced feed consumption and lower body weight gain, indicating systemic stress, altered nutrient utilization and increased metabolic demand associated with mammary tumor development. Similar reductions in feed intake and body weight have also been reported in different DMBA-exposed experimental models^[16, 17].

Prophylactic administration of piperine and berberine, either alone or in combination, improved feed intake and body weight status compared with the DMBA control group, suggesting partial protection against DMBA-induced systemic deterioration. The beneficial effect of piperine may be attributed to its digestive stimulant, bioavailability-enhancing, antioxidant and anticancer properties^[18]. Similarly, the protective effect of berberine may be related to its antioxidant, anti-inflammatory, metabolic regulatory and anticancer activities^[19]. Overall, these findings indicate that piperine and berberine may help attenuate DMBA-associated systemic toxicity and support better nutritional and body weight status during mammary carcinogenesis. Progressive increase in mammary tumor volume in DMBA-administered rats indicates active tumor growth and increased proliferation of mammary epithelial cells. In the present prophylactic study, the DMBA control group showed higher mammary tumor volume, which may be attributed to oxidative stress, DNA damage and enhanced epithelial cell proliferation during carcinogenesis, as described in previous DMBA-induced mammary tumor studies^[20]. Prophylactic administration of berberine reduced tumor volume, suggesting suppression of tumor development, which is consistent with its reported protective effect against DMBA-induced mammary carcinogenesis^[21]. Similarly, the reduction in tumor volume observed in piperine-treated rats may be associated with inhibition of

tumor cell proliferation, invasion and angiogenic signalling. Lai *et al.* (2012)^[22] reported that piperine suppressed tumor growth and metastatic spread in a 4T1 mammary carcinoma model, while Zare *et al.* (2020)^[23] demonstrated that piperine reduced MMP-9 and VEGF expression in breast cancer cells, indicating anti-invasive and anti-angiogenic potential. Its ability to induce apoptosis and interfere with cell-cycle progression further supports the tumor-suppressive effect observed in the present study^[24].

The haematological profile of DMBA-induced mammary tumor-bearing rats reflected anaemia and immune disturbance, as indicated by decreased Hb and TEC along with alterations in TLC and DLC. These changes may be associated with DMBA-induced oxidative injury, erythrocyte damage, impaired haematopoiesis and tumor-related inflammatory stress. Similar haematological disturbances have been reported in DMBA-exposed rats by Selamoglu *et al.* (2007)^[25] and in DMBA-induced mammary carcinoma-bearing Sprague-Dawley rats by Razmi *et al.* (1999)^[26]. Prophylactic treatment with piperine and berberine improved selected haematological parameters compared with the DMBA control group, suggesting partial restoration of erythropoietic and immune status. This improvement may be linked to their antioxidant, anti-inflammatory and immunomodulatory properties. Piperine has been reported to restore altered haematological parameters and modulate immune responses^[27], while berberine has been shown to improve Hb, RBC and WBC profiles under systemic toxicity conditions^[28].

Serum biochemical alterations in DMBA-induced mammary tumor-bearing rats indicated hepatic and renal functional stress during mammary carcinogenesis. In the present study, the DMBA control group showed increased serum AST, ALT and BUN levels, suggesting hepatocellular injury and renal biochemical alteration. The elevation of AST and ALT may be attributed to hepatic bioactivation of DMBA into reactive carcinogenic metabolites, which generate reactive oxygen species, induce oxidative cellular damage and cause hepatocellular membrane disruption, leading to leakage of intracellular enzymes into circulation^[29]. Similarly, increased BUN may reflect renal functional stress associated with systemic oxidative and toxic effects during tumor development. Comparable DMBA-associated increases in hepatic and renal injury markers have been reported in experimental mammary tumor models^[30]. In contrast, prophylactic administration of piperine and berberine improved these biochemical indices, suggesting attenuation of DMBA-associated organ toxicity. The hepatoprotective potential of piperine has been reported in experimental liver injury models, while its nephroprotective effects have also been demonstrated under renal toxic and ischemic conditions^[31]. Similarly, berberine has been shown to reduce hepatic and renal injury through antioxidant and anti-inflammatory mechanisms in experimental toxicity models^[32].

The inflammatory profile of DMBA-induced mammary tumor-bearing rats was characterized by elevated serum TNF- α , IL-1 β and IL-6 concentrations. This increase may be attributed to DMBA-induced oxidative stress and activation of tumor-promoting inflammatory pathways, as these cytokines are known to support neoplastic cell survival, proliferation, angiogenesis and invasiveness through signalling cascades such as NF- κ B, MAPK and IL-6/STAT3. Similar increases in pro-inflammatory cytokines

have been reported in DMBA-induced mammary carcinoma, supporting the role of inflammation in chemically induced mammary carcinogenesis [21]. In the present prophylactic study, administration of piperine, berberine and their combination significantly reduced serum TNF- α , IL-1 β and IL-6 levels compared with the DMBA control group, indicating attenuation of DMBA-associated inflammatory activation. The anti-inflammatory effect of piperine may be related to suppression of inflammatory mediator production, as earlier studies reported reduced TNF- α , IL-1 β and IL-6 levels following piperine treatment in different inflammatory models [33, 34, 35]. Piperine has also been shown to inhibit NF- κ B and MAPK signalling pathways, thereby reducing TNF- α , IL-1 β and IL-6 expression [36]. Similarly, berberine has been reported to reduce inflammatory cytokine production in DMBA-induced mammary carcinoma and other inflammatory models through modulation of ER stress-mediated ERK1/2 signalling [21]. Thus, the reduction in circulating pro-inflammatory cytokines in the treated groups suggests that piperine and berberine may suppress DMBA-induced inflammatory signalling and reduce the tumor-supportive inflammatory microenvironment.

Oxidative stress was evident in DMBA-induced mammary tumor-bearing rats, as shown by increased serum MDA levels, indicating enhanced lipid peroxidation during tumor development. This may be due to metabolic activation of DMBA into reactive intermediates, leading to ROS generation and membrane lipid damage. Similar findings have been reported in DMBA-induced mammary tumor models [37]. Prophylactic treatment with piperine and berberine reduced MDA levels, suggesting attenuation of oxidative damage and improvement in redox balance, which may be related to their reported antioxidant and lipid peroxidation-inhibitory activities [38]. The elevated estradiol level in DMBA-induced rats may reflect estrogen-associated mammary tumor progression, whereas its reduction after treatment, particularly with berberine, suggests modulation of hormone-related tumor promotion [39]. Progesterone levels remained largely unchanged, indicating limited involvement of progesterone under the present experimental conditions, consistent with Akhouri *et al.* (2020) [37].

At the molecular level, DMBA-induced mammary tumors showed a tumor-promoting expression pattern, characterized by reduced *TP53* and *Caspase-3* expression together with increased *Cyclin D1* and *MMP-9* expression. This indicates suppression of tumor-suppressive and apoptotic signalling, along with activation of proliferative and invasion-associated pathways. DMBA-derived reactive metabolites are known to induce genotoxic stress and oxidative DNA damage, which may impair p53-mediated responses such as DNA repair, cell-cycle arrest, apoptosis and senescence [40]. Therefore, the reduced *TP53* and *Caspase-3* expression observed in the DMBA control group suggests weakening of DNA-damage response and apoptotic elimination of damaged cells during mammary carcinogenesis. The increased *Cyclin D1* expression in the DMBA control group indicates enhanced cell-cycle progression and abnormal proliferative signalling, which is consistent with previous reports of *Cyclin D1* upregulation in DMBA-induced mammary tumors [41]. Similarly, elevated *MMP-9* expression

reflects extracellular matrix degradation and increased invasive potential, supporting its role in tumor progression and metastasis-associated changes in DMBA-induced mammary tumor models [42, 43]. Prophylactic administration of piperine and berberine partially modulated these molecular alterations. The combination treatment showed significant upregulation of *Caspase-3* and numerically reduced *Cyclin D1* and *MMP-9* expression, suggesting enhanced apoptotic activity with partial suppression of proliferative and invasion-related signalling. These effects may be related to the reported pro-apoptotic, anti-proliferative and anti-invasive properties of piperine and berberine [24, 44]. Overall, these findings suggest that prophylactic treatment with piperine and berberine may attenuate DMBA-induced mammary tumor progression by favourably modulating apoptosis-, proliferation-and invasion-related molecular pathways.

Histopathological observations supported the biochemical and molecular findings of DMBA-induced mammary carcinogenesis. The DMBA control group showed marked disruption of mammary gland architecture, with irregular ductal and acinar structures, epithelial proliferation, cellular atypia, hyperchromatic nuclei, stromal invasion and inflammatory changes. These lesions confirmed active neoplastic transformation and were comparable with earlier reports in DMBA-induced mammary tumor models, where excessive ductular and acinar epithelial proliferation, stromal fibrosis and inflammatory infiltration were observed [45, 46]. The histological alterations were also consistent with increased tumor volume, oxidative stress, inflammatory biomarkers and altered gene expression observed in the present study, indicating that DMBA promotes mammary tumor development through epithelial proliferation, oxidative injury, inflammatory activation and tissue remodelling. Prophylactic treatment with piperine, berberine and their combination reduced the severity of mammary lesions compared with the DMBA control group. Treated rats showed better preservation of glandular organization, reduced epithelial proliferation, lower cellular atypia and less invasive change. The protective effect of berberine is supported by Karnam *et al.* (2017) [21], who reported improved mammary gland histology in DMBA-induced mammary tumor-bearing Sprague-Dawley rats. Similarly, the response observed with piperine may be related to its reported antitumor, anti-invasive and pro-apoptotic properties [18]. Overall, the histological improvement in the treated groups suggests that piperine and berberine may attenuate DMBA-induced mammary tumor progression through combined antiproliferative, antioxidant, anti-inflammatory and apoptosis-modulating mechanisms.

Ultrasonographic findings further supported the protective response. The DMBA control group showed irregular hyperechoic mammary masses with heterogeneous echotexture and distortion of surrounding tissue architecture, features commonly associated with malignant mammary lesions [47]. In contrast, piperine, berberine and combination-treated groups showed relatively smaller lesions, improved echotextural uniformity, better defined margins and reduced architectural distortion, indicating partial preservation of mammary gland architecture following prophylactic treatment.

Table 1: Primer sequences used for quantitative real-time PCR analysis

Gene	Primer		Accession No.	Product Size
Caspase-3	F	GAAATTC AAGGGACGGGTCA	>NM_012922.2	160
	R	TTCTTTGCATGGAAAGTGGC		
MMP-9	F	AGCCGGGAACGTATCTGGA	>NM_031055.2	173
	R	TGGAAACTCACACGCCAGAAG		
TP 53	F	GCAGAGTTGTTAGAAGGC	>NM_030989.4	138
	R	TTGAGAAGGGACGGAAGA		
Cyclin D1	F	GCGAGCCATGCTTAAGACTGA	>XM_008760168	65
	R	TCTGCACGCACTTGAAGTAAGAA		
GAPDH	F	ACCACGAGAAATATGACAACCTCCC	>XM_063261286	100
	R	CCAAAGTTGTCATGGATGACC		

Table 2a: Feed consumption (g/day) in rats under prophylactic treatment groups (n = 8)

Groups	Treatment	FC1 (9 th WK)	FC2 (10 th WK)	FC3 (11 th WK)	FC4 (12 th WK)	FC5 (13 th WK)
I	Normal control	70.67±5.71 ^b	73.24±1.81	77.43±0.10	87.24±1.90	88.29±1.81 ^b
II	DMBA control	45.52±0.95 ^a	53.33±9.90	59.14±8.10	56.48±14.57	63.81±4.57 ^{ab}
III	DMBA + PIP	45.84±3.50 ^a	51.81±6.86	53.33±11.24	57.05±14.19	55.62±11.43 ^a
IV	DMBA + BER	46.14±1.66 ^a	62.38±9.43	66.50±22.69	71.43±7.62	65.81±12.10 ^{ab}
V	DMBA + PIP + BER	51.71±0.29 ^a	71.24±3.24	69.52±1.71	76.76±2.29	79.58±2.81 ^{ab}

Table 2b: Feed consumption (g/day) in rats under prophylactic treatment groups (n = 8)

Groups	FC6 (14 th WK)	FC7 (15 th WK)	FC8 (16 th WK)	FC9 (17 th WK)	FC10 (18 th WK)	FC11 (19 th WK)	FC12 (20 th WK)
I	88.00±0.57 ^b	99.81±4.19 ^b	104.76±4.00	106.44±0.45	111.17±9.02 ^b	113.57±10.71 ^b	120.93±8.05 ^b
II	65.05±9.24 ^{ab}	80.62±12.00 ^{ab}	84.54±0.11	84.76±4.54	86.17±5.06 ^a	79.01±3.33 ^a	78.64±8.49 ^a
III	58.67±12.95 ^a	71.90±14.76 ^a	75.90±16.29	76.01±10.23	81.05±6.57 ^a	81.65±3.47 ^a	87.16±11.02 ^a
IV	66.76±4.10 ^{ab}	67.14±4.48 ^a	81.52±10.29	82.77±14.48	82.57±7.90 ^a	73.59±4.61 ^a	81.23±5.00 ^a
V	84.12±3.17 ^{ab}	90.25±6.13 ^{ab}	97.89±3.63	96.18±2.05	92.35±0.93 ^{ab}	91.41±0.73 ^a	93.51±2.25 ^{ab}

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$). DMBA: 7,12-dimethylbenz [a]anthracene, 80 mg/kg b.wt., intragastric, single dose; PIP: piperine, 50 mg/kg b.wt., p.o.; BER: berberine, 50 mg/kg b.wt., p.o. Treatments were administered from day 56 to day 140

Table 3a: Body weight (g) in rats under prophylactic group (n = 8)

Group	Treatment	BW0 (8 th WK)	BW1 (9 th WK)	BW2 (10 th WK)	BW3 (11 th WK)	BW4 (12 th WK)	BW5 (13 th WK)
I	Normal control	133.25±2.83	156.75±4.04 ^{bc}	185.13±3.24 ^{ab}	213.75±2.82 ^{ab}	237.38±2.54 ^b	257.25±4.67 ^c
II	DMBA control	127.75±3.12	139.00±5.10 ^a	160.38±5.71 ^a	184.00±7.65 ^a	203.13±8.97 ^a	217.25±7.96 ^a
III	DMBA + PIP	134.50±6.44	150.88±5.93 ^{ab}	172.88±6.41 ^a	191.88±6.67 ^a	208.88±6.36 ^{ab}	221.38±5.37 ^{ab}
IV	DMBA + BER	124.75±2.34	138.38±5.48 ^a	164.75±9.66 ^a	183.25±11.56 ^a	198.38±9.70 ^a	213.00±9.33 ^a
V	DMBA + PIP + BER	136.13±2.90	168.75±7.62 ^c	205.63±12.84 ^b	223.50±16.51 ^b	236.00±16.01 ^b	247.75±15.89 ^{bc}

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$)

Table 3b: Body weight (g) in rats under prophylactic group (n = 8)

Group	BW6 (14 th WK)	BW7 (15 th WK)	BW8 (16 th WK)	BW9 (17 th WK)	BW10 (18 th WK)	BW11 (19 th WK)	BW12 (20 th WK)
I	272.00±6.69 ^c	288.25±7.85 ^{bc}	299.00±10.70 ^c	302.88±11.36 ^c	318.88±12.41 ^c	317.38±12.75 ^b	324.00±12.40 ^c
II	234.00±6.22 ^{ab}	247.50±5.92 ^{ab}	250.75±6.37 ^{ab}	256.38±5.52 ^{ab}	277.13±6.63 ^{ab}	267.63±9.78 ^a	282.88±5.10 ^{ab}
III	230.38±4.17 ^a	242.88±4.81 ^{ab}	250.25±4.34 ^{ab}	258.25±5.17 ^{ab}	269.63±3.96 ^{ab}	274.75±6.53 ^a	280.63±6.70 ^{ab}
IV	221.38±7.83 ^a	234.75±6.74 ^a	244.63±6.55 ^a	248.25±7.37 ^a	266.13±9.66 ^a	268.25±7.71 ^a	272.75±8.88 ^a
V	258.38±15.41 ^{bc}	269.75±16.46 ^{bc}	275.88±16.43 ^{bc}	285.00±15.90 ^{bc}	298.63±15.28 ^{bc}	296.63±14.13 ^{ab}	306.25±14.74 ^{bc}

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$)

Table 4: Tumor volume (cm³) in rats under prophylactic groups

Group	Treatment	Tumor Volume (cm ³)
II	DMBA control	3.50±0.18 ^b
III	DMBA + PIP	1.68±0.48 ^a
IV	DMBA + BER	1.47±0.34 ^a
V	DMBA + PIP + BER	2.40±0.36 ^a

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$)

Table 5: Haematological parameters in rats under prophylactic groups

Group	Treatment	Hb (g/dl)	TEC (10 ⁶ /μl)	TLC (10 ³ /μl)	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)
I	Normal control	14.99±0.41 ^c	7.20±0.20 ^b	9.68±1.18 ^a	72.49±1.37 ^b	4.60±1.37 ^a	22.91±3.92 ^{ab}
II	DMBA control	9.53±0.49 ^a	5.86±0.35 ^a	19.12±1.84 ^c	52.75±1.23 ^a	5.24±1.23 ^b	34.57±2.33 ^c
III	DMBA + PIP	13.77±0.27 ^{bc}	6.33±0.12 ^a	14.12±1.40 ^b	78.52±0.22 ^b	1.93±0.22 ^a	19.85±2.74 ^a
IV	DMBA + BER	14.66±0.49 ^{bc}	6.11±0.14 ^a	12.92±0.72 ^{ab}	77.14±0.59 ^b	3.54±0.59 ^{ab}	21.79±1.28 ^{ab}
V	DMBA + PIP + BER	13.33±0.57 ^b	6.07±0.19 ^a	11.41±0.49 ^{ab}	70.39±0.17 ^b	2.21±0.17 ^{ab}	27.54±0.53 ^{bc}

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$). Hb: Hemoglobin; TEC: Total erythrocyte count; TLC: Total leukocyte count

Table 6: Serum biochemical in rats under prophylactic groups (n = 8)

Group	Treatment	AST (U/L)	ALT (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
I	Normal control	111.73±1.87 ^a	26.51±0.27 ^a	19.56±0.89 ^a	0.68±0.03
II	DMBA control	206.82±6.76 ^d	51.80±0.73 ^c	27.62±1.57 ^b	0.63±0.04
III	DMBA + PIP	175.27±5.76 ^c	32.62±0.65 ^b	17.49±1.50 ^a	0.73±0.06
IV	DMBA + BER	160.93±4.44 ^b	33.36±0.73 ^b	26.20±2.67 ^b	0.76±0.07
V	DMBA + PIP + BER	155.34±3.54 ^b	34.30±0.51 ^b	16.85±1.72 ^a	0.62±0.02

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$). BUN: Blood urea nitrogen; AST (SGOT): Aspartate aminotransferase; ALT (SGPT): Alanine aminotransferase

Table 7: Serum pro-inflammatory cytokine levels (TNF- α , IL-1 β and IL-6) in rats under prophylactic groups

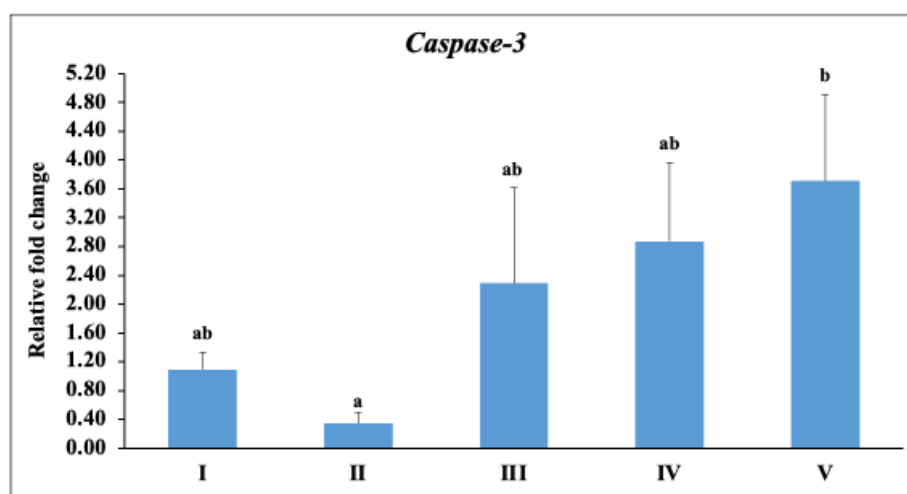
Group	Treatment	MDA (μmol/L)	TNF- α (pg/mL)	IL-1 β (pg/mL)	IL-6 (pg/mL)
I	Normal control	4.09±0.29 ^a	33.63±3.56 ^a	27.82±2.18 ^a	14.44±2.45 ^a
II	DMBA control	9.64±1.24 ^c	80.91±5.60 ^b	61.46±1.62 ^c	53.68±5.49 ^c
III	DMBA + PIP	6.82±0.93 ^b	45.97±5.45 ^a	38.72±3.13 ^b	25.06±2.51 ^b
IV	DMBA + BER	4.64±0.33 ^a	42.73±4.11 ^a	38.97±3.65 ^b	22.83±3.25 ^{ab}
V	DMBA + PIP + BER	5.07±0.69 ^a	40.94±6.07 ^a	42.24±0.57 ^b	17.68±1.72 ^{ab}

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$). MDA: Malondialdehyde. TNF- α : Tumor necrosis factor- α ; IL-1 β : Interleukin-1 beta; IL-6: Interleukin-6

Table 8: Serum hormonal profile (estradiol and progesterone) in rats under prophylactic groups (n = 6)

Group	Treatment	Estradiol (pg/mL)	Progesterone (ng/mL)
I	Normal control	16.14±1.65 ^a	7.165±0.032
II	DMBA control	32.44±0.24 ^d	7.201±0.026
III	DMBA + PIP	27.73±0.75 ^c	7.190±0.010
IV	DMBA + BER	17.66±1.85 ^{ab}	7.122±0.030
V	DMBA + PIP + BER	20.85±2.04 ^b	7.145±0.038

Mean values with dissimilar superscripts in a column vary significantly ($p < 0.05$)



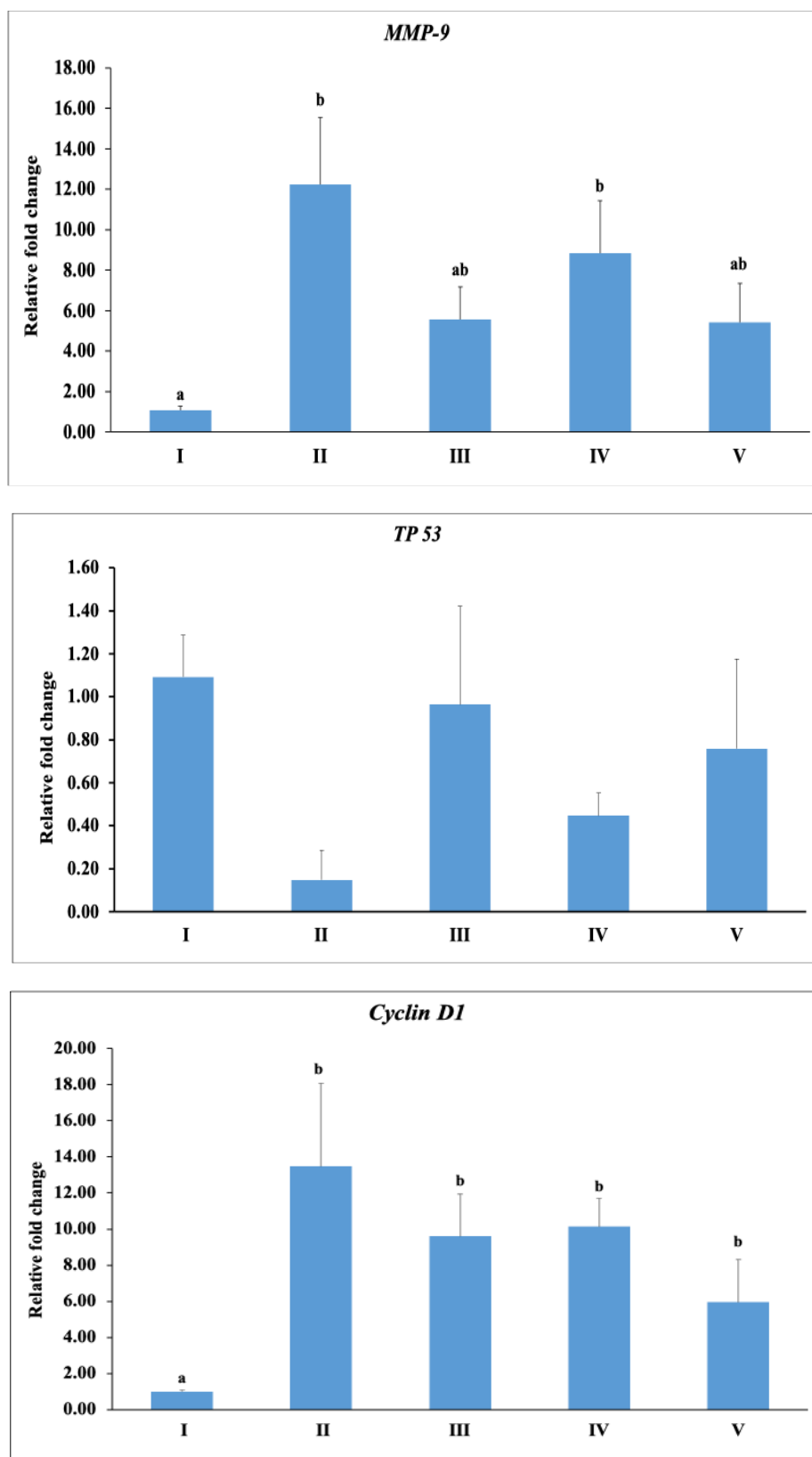


Fig 1: Relative mRNA expression of (A) *Caspase-3*, (B) *MMP-9*, (C) *TP53* and (D) *Cyclin D1* in DMBA-induced mammary tumor-bearing rats following prophylactic treatment (n = 6). Bars bearing different superscript letters differ significantly ($p < 0.05$). Group I: normal control; Group II: DMBA control; Group III: DMBA + PIP; Group IV: DMBA + BER; Group V: DMBA + PIP + BER. DMBA: 7,12-dimethylbenz [a]anthracene; PIP: piperine; BER: berberine

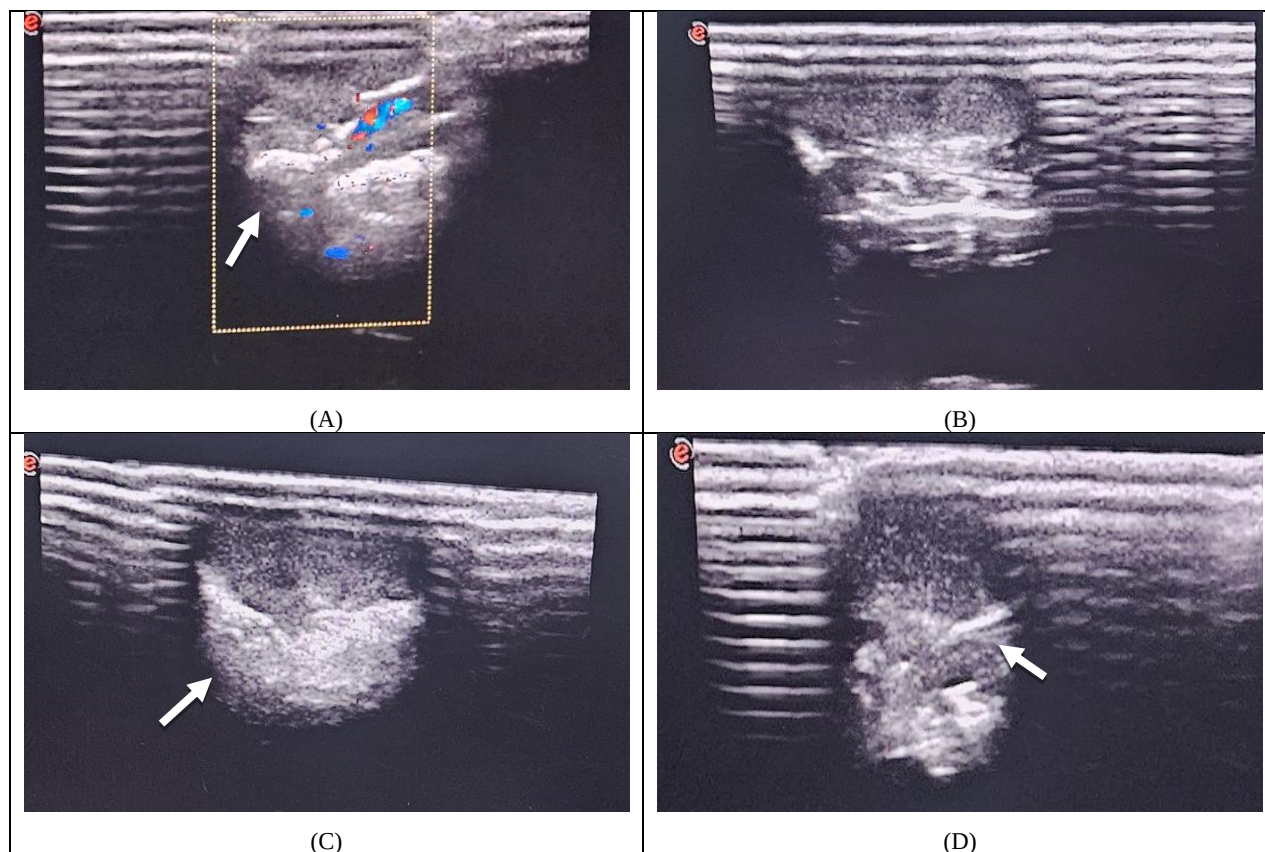


Fig 2: Representative ultrasonographic images of mammary tumors in DMBA-induced mammary tumor-bearing rats following prophylactic treatment. Arrowheads indicate the tumor mass. Panels A-D represent the respective experimental groups: A: DMBA control; B: DMBA + PIP; C: DMBA + BER; D: DMBA + PIP + BER. DMBA: 7,12-dimethylbenz [a]anthracene; PIP: piperine; BER: berberine

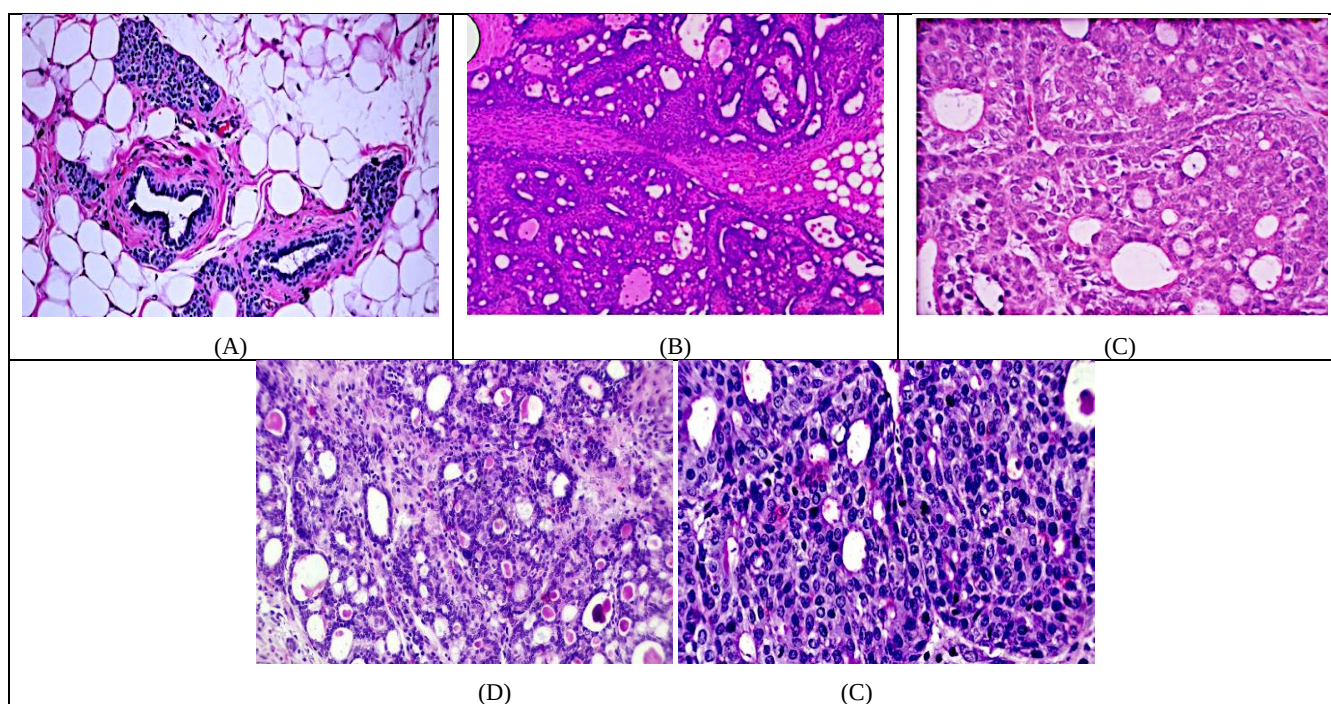


Fig 3: Representative H&E stained mammary gland sections from control and prophylactic experimental groups (120X). A: normal control; B: DMBA control; C: DMBA + PIP; D: DMBA + BER; E: DMBA + PIP + BER. DMBA: 7,12-dimethylbenz [a]anthracene; PIP: piperine; BER: berberine

Conclusion

Prophylactic administration of piperine and berberine reduced DMBA-induced mammary gland tumor development in female Sprague-Dawley rats. DMBA caused increased tumor volume, reduced feed intake and body weight gain, haemato-biochemical and hormonal alterations,

elevated inflammatory cytokines, increased oxidative stress and marked neoplastic changes in mammary tissue. It also altered tumor-related gene expression by increasing *Cyclin D1* and *MMP-9* and reducing *TP53* and *Caspase-3* expression. Treatment with piperine and berberine improved general health status, reduced tumor burden, lowered

oxidative and inflammatory stress, improved mammary gland architecture and favourably modulated molecular markers. Among the prophylactic regimens, berberine showed marked reduction in tumor volume and MDA level, while the piperine + berberine combination produced the most consistent overall protective response. These findings suggest that piperine and berberine, particularly in combination, may have promising chemopreventive potential against DMBA-induced mammary gland tumors.

Acknowledgements

The authors are grateful to the Dean and Principal, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, for providing laboratory facilities and institutional support. The authors also thank the Veterinary Physiology and Biochemistry and Veterinary Surgery and Radiology for their support in haemato-biochemical analysis and ultrasonography studies, respectively.

Conflict of Interest

The authors declare that they have no conflict of interest.

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