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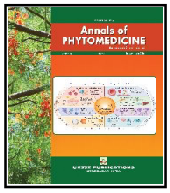

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Therapeutic efficacy of piperine, berberine and doxorubicin alone and their combinations against 7, 12-dimethylbenz[a]anthracene-induced mammary gland tumors in female Sprague-Dawley rats

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Abstract

The present study was designed to evaluate the therapeutic efficacy of piperine and berberine, alone and in combination with doxorubicin, against 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary gland tumors in female Sprague-Dawley rats. A total of nine groups were formed randomly, comprising normal control, DMBA-induced tumor control, doxorubicin-treated, piperine-treated, berberine-treated and their respective combination-treated groups. Mammary gland tumors were induced by a single intragastric administration of DMBA at 80 mg/kg body weight in female rats at 8 weeks of age (day 56) and tumor development was confirmed on day 140. After tumor confirmation, therapeutic interventions were continued for 28 days, from day 140 to day 168. Tumor-bearing rats received doxorubicin at 2.5 mg/kg intraperitoneally once weekly, piperine at 50 mg/kg orally once daily, berberine at 50 mg/kg orally once daily, or their combinations. DMBA-induced tumor-bearing rats showed reduced feed intake and body weight, increased tumor volume, haematological alterations, altered serum biochemical and hormonal profiles, increased pro-inflammatory cytokine levels and elevated serum malondialdehyde level. These changes were associated with downregulation of TP53 and Caspase-3 and upregulation of Cyclin D1 and MMP-9gene expression, indicating impaired apoptosis, enhanced proliferation and invasive tumor behaviour. Treatment with piperine, berberine and doxorubicin, particularly in combination, improved feed intake and body weight, reduced tumor volume, ameliorated haematological and serum biochemical alterations, modulated hormonal and inflammatory profiles, attenuated oxidative stress, upregulated TP53 and Caspase-3 gene expression and downregulated Cyclin D1 and MMP-9 gene expression. Histopathological and ultrasonographic observations further supported reduced tumor burden and partial restoration of mammary gland architecture. Among the therapeutic regimens, the combination of piperine, berberine and doxorubicin showed broad improvement across multiple pathological and molecular parameters. The findings suggest that piperine and berberine may enhance the therapeutic efficacy of doxorubicin and may serve as promising phytogenic adjuvants against chemically induced mammary gland tumors.

1. Introduction

Cancer is a heterogeneous disease characterised by uncontrolled proliferation of abnormal cells with the capacity to invade surrounding tissues and metastasise to distant sites and it remains a major cause of morbidity and mortality in both humans and dogs, highlighting its comparative clinical importance (Sung *et al*., 2021).Animal models are valuable for investigating disease mechanisms and evaluating therapeutic approaches. The rat model is widely used in mammary tumor research due to its well-characterised biology, reproducibility and experimental suitability (Tulotta *et al*., 2019). Among experimental models, 7,12-dimethylbenz[a]anthracene (DMBA) is widely used to induce mammary tumors in female Sprague-Dawley rats, where it promotes carcinogenesis through metabolic activation involving the aryl hydrocarbon receptor pathway, induction of CYP1A1 and CYP1B1, formation of reactive intermediates and DNA adducts and subsequent malignant transformation (Alhoshani *et al*., 2021)

Although, current cancer treatment includes chemotherapy, hormone therapy, radiotherapy, immunotherapy and targeted therapy, these modalities remain limited by inadequate tumor selectivity, toxicity to normal tissues, recurrence and poor bioavailability (Eisenmann *et al*., 2022; Amini *et al*., 2023).Chemotherapeutic agents are broadly classified according to their mechanism of action and chemical structure into groups such as alkylating agents, antimetabolites, topoisomerase inhibitors and mitotic spindle inhibitors (Mohseni *et al*., 2016). Doxorubicin is widely used for mammary gland and other solid tumours, exerting anticancer activity mainly through DNA intercalation, topoisomerase II inhibition, mitochondrial dysfunction and oxidative stress induction (Tacar *et al*., 2013). Although, doxorubicin is an effective antineoplastic agent, its use is limited by toxicity to normal tissues, including myelosuppression, nephrotoxicity and cardiotoxicity (Kciuk *et al*., 2023). Plants are recognised as

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important sources of bioactive compounds with potential anticancer activity (Olawale *et al*., 2022). Piperine, the principal alkaloid of black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.), has shown cytotoxic and pro-apoptotic effects in cancer models through modulation of signalling pathways involved in cell proliferation and survival (Xia *et al*., 2015).Likewise, berberine, an isoquinoline alkaloid isolated from medicinal plants such as *Coptis chinensis* and *Coptis japonica*, exhibits antiproliferative activity through interactions with key cellular targets and disruption of cell-cycle progression (Li *et al*., 2019). In view of the growing evidence supporting the anticancer potential of these phytochemicals, the present study was undertaken to evaluate the therapeutic efficacy of piperine and berberine, alone and in combination with doxorubicin, against DMBA-induced mammary gland tumors in Sprague-Dawley rats, with emphasis on tumor response, clinical status, biochemical and pathological alterations and molecular changes associated with mammary carcinogenesis.

2. Materials and Methods

2.1 Animals

The study was performed in seventy-two healthy female Sprague-Dawley rats (*Rattus norvegicus*), aged 8 weeks and weighing 103-127 g, procured from Zydus Research Centre, Ahmedabad, Gujarat, India. The experiment was conducted 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. Following procurement, the animals were acclimatised for 2 weeks and maintained under standard laboratory conditions with *ad libitum* access to a standard pelleted diet and water throughout the study. The animals were housed under controlled environmental conditions at 21 ± 1°C, relative humidity of 55 ± 10% 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 (Approval No. 462/VPT/2025) and all procedures were carried out in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA).

2.2 Study design and protocol

7,12-dimethylbenz[a]anthracene (DMBA) (Cayman Chemical, Ann Arbor, MI, USA) was used to induce 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). Berberine (B3251; purity-98%) and piperine (W290904; purity < 95%) were procured from Sigma-Aldrich and used in their standard chemical forms for the therapeutic interventions. DOX (Duxocin 50) was obtained from Zydus Lifesciences Limited (Ahmedabad, India). For the therapeutic study, rats were randomly allocated into nine groups (n = 8/group), as detailed in Table 1. Group I served as the normal control, while mammary gland tumours were induced in Groups II-IX by a single intragastric administration of DMBA at 80 mg/kg body weight in 0.5 ml corn oil on day 56. Tumour development was allowed for 12 weeks and confirmed on day 140 by ultrasonography and tumour volume measurement. After confirmation, therapeutic interventions were administered from day 140 to day 168. DMBA was used at 80 mg/kg intragastrically to reliably induce mammary tumors in female Sprague-Dawley rats, while berberine was selected at 50 mg/kg based on its reported protection in the same model (Karnam *et al*., 2017). Piperine was used at 50 mg/kg based on reported safety and biological activity in rodents (Sankar *et al*., 2023). Doxorubicin was administered at 2.5 mg/kg intraperitoneally once weekly, as similar low-dose regimens have been used in rat mammary tumour models (Bose *et al*., 2018).

Table 1: Experimental design and treatment group allocation

Group	Experimental groups (n = 8)	Intervention
I	Normal control	Vehicle only
II	DMBA control	DMBA only, no treatment
III	DMBA + doxorubicin	Doxorubicin (2.5 mg/kg, i.p.), once weekly from day 140 to 168
IV	DMBA + piperine	Piperine (50 mg/kg/day, p.o.) from day 140 to 168
V	DMBA + berberine	Berberine (50 mg/kg/day, p.o.) from day 140 to 168
VI	DMBA + piperine + berberine	Piperine (50 mg/kg/day, p.o.) + berberine (50 mg/kg/day, p.o.) from day 140 to 168
VII	DMBA + piperine + doxorubicin	Piperine (50 mg/kg/day, p.o.) + doxorubicin (2.5 mg/kg, i.p.), once weekly from day 140 to 168
VIII	DMBA + berberine + doxorubicin	Berberine (50 mg/kg/day, p.o.) + doxorubicin (2.5 mg/kg, i.p.), once weekly from day 140 to 168
IX	DMBA + piperine + berberine + doxorubicin	Piperine (50 mg/kg/day, p.o.) + berberine (50 mg/kg/day, p.o.) + doxorubicin (2.5 mg/kg, i.p.), once weekly from day 140 to 168

DMBA: 7,12-dimethylbenz[a]anthracene; i.p.: intraperitoneal; p.o.: per oral.

2.3 Feed consumption and body weight

During the therapeutic study, feed consumptionwas recorded weekly by measuring the quantity of feed offered to the animals in each cage and subtracting the leftover feed. The 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. For assessment of therapeutic response, body weight changes were

evaluated during the treatment phase from day 140 to day 168 (20th to 24th week of age).

2.4 Measurements of mammary tumorvolume

Mammary tumors in rats were measured using a vernier calliper, recording the large diameter (L) and small diameter (B). Tumor volume was calculated using the formula:

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

2.5 Blood and serum analyses

At the end of the therapeutic study, blood samples were collected from all rats on day 168 through retro-orbital plexus puncture under isoflurane anaesthesia. Approximately 1 ml of blood was collected in K_3EDTA vacutainers for haematological evaluation.Separate blood samples were collected in anticoagulant-free sterile glass tubes for serum separation. After clotting, the samples were centrifuged at 3000 rpm for 15 min at 4°C, and the separated serum was stored at -20°C until biochemical, hormonal, oxidative stress and pro-inflammatory cytokine analyses were carried out. Haematological parameters, including haemoglobin, total erythrocyte count, total leukocyte count and differential leukocyte count, were analysed 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 analysed parameters included alanine aminotransferase (ALT0250PS), aspartate aminotransferase (ASTG0250PS), blood urea nitrogen (UREG0250PS) and creatinine (CRTG0250PS), following the manufacturer's instructions. Serum malondialdehyde concentration, used as an indicator of lipid peroxidation, was estimated using a malondialdehyde colorimetric assay kit based on the thiobarbituric acid method (PG-520K-BC-M). Serum estradiol and progesterone concentrations were quantified 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 pro-inflammatory cytokines were measured using rat-specific ELISA kits procured from Geno Biosciences Pvt. Ltd., including TNF-α (ITRPF009), IL-1β (PG-2100R) and IL-6 (PG-5100R). All biochemical and ELISA-based assays were performed according to the respective manufacturer's protocols.

2.6 Real-time quantitative PCR analysis

Total RNA was extracted from mammary gland tumor tissues using QIAzol lysis reagent and the RNeasy Mini Kit according to the manufacturer's protocol, with on-column DNase treatment to remove possible genomic DNA contamination. The concentration and purity of RNA were assessed using a NanoDrop 1000 spectrophotometer and RNA integrity was verified by agarose gel electrophoresis. Purified RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Thermo Scientific) and the synthesised cDNA was used for quantitative real-time PCR analysis. The gene-specific primer sequences used for quantitative real-time PCR analysis are presented in Table 2. GAPDH was used as the endogenous reference gene. Real-time PCR was performed using the QuantStudio™ 5 Real-Time PCR System withKAPA SYBR FAST qPCR Master Mix (2X). Each 20 µl reaction contained 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 programme consisted of 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 monitor contamination or non-specific amplification. Melt curve analysis was carried out after amplification to confirm product specificity. Relative mRNA expression levels of *TP53*, *Caspase-3*, *Cyclin D1* and *MMP-9*were calculated using the comparative 2{ ÄÄCq method after normalisation with GAPDH (Livak and Schmittgen, 2001)

Table 2: Primer sequences used for quantitative real-time PCR analysis

Gene	Primer		Accession No.	Product size
Caspase-3	F	GAAATTCAAGGGACGGGTCA	>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	ACCACGAGAAATATGACAACTCCC	>XM_063261286	100
	R	CCAAAGTTGTCATGGATGACC		

2.7 Ultrasonographic examination

Mammary gland ultrasonography was performed on day 140 to confirm tumor development and on day 168 to evaluate therapeutic response. Real-time B-mode imaging was carried out using an EsaoteMyLab™ 40 VET ultrasound system (Esaote Europe B.V., Maastricht, Netherlands) with a 3.5-12 MHz linear transducer. Tumor size, margins, echotexture and surrounding mammary tissue architecture were assessed.

2.8 Histopathological examination

At the end of the therapeutic study on day 168, rats were euthanised 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.

2.9 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 (DMRT) at $p<0.05$. All data were presented as mean ± standard error (SE).

3. Results

3.1 Feed consumption measurement

Feed consumption during the therapeutic period is presented in Table 3. Mean feed consumption was significantly lower in the DMBA control group (Group II) than in the normal control group (Group I) from Week 20 to Week 24. Compared with Group II, the treated groups showed variable improvement in feed consumption during the therapeutic period. Group IX (PIP + BER + DOX- treated) consistently showed the greatest improvement, whereas Groups III and VI also demonstrated marked increases in feed intake. Overall, therapeutic treatment partially restored feed consumption in DMBA-induced mammary tumor-bearing rats.

Table 3: Feed consumption (g/day) in rats under therapeutic groups (n = 8)

Group andtreatment	Week 20	Week 21	Week 22	Week 23	Week 24
I (Normal Control)	125.24 ± 0.67^{f}	125.81 ± 2.38^{e}	128.57 ± 1.71^{d}	126.76 ± 1.43^{e}	128.95 ± 0.76^{f}
II (DMBA Control)	77.14 ± 1.33^{a}	76.57 ± 0.57^{a}	76.29 ± 2.19^{a}	75.14 ± 1.62^{a}	77.14 ± 0.76^{a}
III (DMBA + DOX)	91.38 ± 0.43^{d}	94.95 ± 1.05^{c}	96.95 ± 1.14^{c}	103.14 ± 0.76^{c}	110.36 ± 0.50^{d}
IV (DMBA + PIP)	72.57 ± 0.95^{a}	78.86 ± 1.52^{c}	83.62 ± 0.95^{b}	86.29 ± 3.81^{b}	84.10 ± 2.76^{b}
V (DMBA + BER)	85.71 ± 1.33^{bc}	91.52 ± 0.29^{c}	93.71 ± 0.95^{c}	96.00 ± 3.81^{c}	95.62 ± 2.29^{c}
VI (DMBA + PIP + BER)	84.95 ± 2.10^{b}	90.76 ± 0.48^{bc}	95.24 ± 0.76^{c}	100.19 ± 2.67^{c}	106.95 ± 2.57^{d}
VII (DMBA + PIP + DOX)	84.38 ± 2.10^{b}	85.05 ± 1.62^{b}	86.86 ± 2.10^{b}	84.57 ± 0.95^{b}	84.38 ± 2.48^{b}
VIII (DMBA + BER + DOX)	90.40 ± 2.93^{cd}	92.86 ± 4.29^{c}	96.17 ± 4.21^{c}	97.29 ± 4.14^{c}	100.00 ± 2.38^{c}
IX (DMBA + PIP + BER + DOX)	104.19 ± 0.76^{e}	109.24 ± 1.05^{d}	113.05 ± 0.86^{d}	115.14 ± 1.05^{d}	117.43 ± 1.62^{e}

Mean values with dissimilar superscripts in a column vary significantly ($p<0.05$). DMBA: 7,12-dimethylbenz[a]anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine.

3.2 Body weight measurement

Initial body weight recorded at 8 weeks of age (day 56) showed no significant changes among the experimental groups, suggesting that all groups were comparable at the start of the study before tumor induction. Body weight during the therapeutic period is presented in Table 4. Mean body weight was significantly lower in the DMBA control group (Group II) than in the normal control group (Group I) throughout the therapeutic period. Group I showed a gradual increase in body weight from Week 20 to Week 24, whereas Group II showed a declining trend. The treated groups remained largely similar to Group II up to Week 23. At Week 24, Groups IV, V and VI showed significantly higher body weight than Group II, while Groups III, VII, VIII and IX showed intermediate values. These findings suggest that selected treatments partially mitigated DMBA-associated body weight reduction in mammary tumor-bearing rats.

Table 4: Body weight (g) in rats under therapeutic groups (n = 8)

Group andtreatment	Week 8	Week 20	Week 21	Week 22	Week 23	Week 24
I (Normal Control)	127.10 ± 2.10	327.30 ± 10.29^{b}	332.90 ± 10.11^{b}	341.90 ± 10.94^{b}	347.20 ± 10.05^{b}	361.80 ± 9.48^{c}
II (DMBA Control)	114.10 ± 3.56	282.00 ± 8.41^{a}	280.70 ± 9.44^{a}	276.00 ± 10.81^{a}	270.00 ± 11.26^{a}	265.80 ± 10.70^{a}
III (DMBA + DOX)	112.30 ± 2.21	271.70 ± 6.15^{a}	272.70 ± 9.79^{a}	273.50 ± 5.70^{a}	273.70 ± 5.45^{a}	281.40 ± 6.50^{ab}
IV (DMBA + PIP)	113.60 ± 9.14	281.50 ± 6.20^{a}	281.90 ± 5.81^{a}	291.70 ± 6.36^{a}	291.00 ± 5.83^{a}	294.30 ± 6.68^{b}
V (DMBA + BER)	103.50 ± 8.58	281.10 ± 10.34^{a}	278.30 ± 9.07^{a}	282.60 ± 10.47^{a}	282.80 ± 8.48^{a}	296.30 ± 8.96^{b}
VI (DMBA + PIP + BER)	113.60 ± 2.34	272.50 ± 8.83^{a}	276.20 ± 7.91^{a}	279.90 ± 9.04^{a}	290.10 ± 8.84^{a}	294.80 ± 8.83^{b}
VII (DMBA + PIP + DOX)	123.80 ± 3.37	290.90 ± 11.14^{a}	280.00 ± 9.18^{a}	278.00 ± 10.68^{a}	272.10 ± 9.95^{a}	280.70 ± 9.29^{ab}
VIII (DMBA + BER + DOX)	104.70 ± 6.15	297.80 ± 6.50^{a}	281.70 ± 5.91^{a}	278.40 ± 7.94^{a}	274.70 ± 5.73^{a}	286.50 ± 6.31^{ab}
IX (DMBA + PIP + BER + DOX)	116.50 ± 15.19	274.90 ± 9.81^{a}	285.50 ± 9.43^{a}	289.10 ± 7.66^{a}	296.60 ± 8.01^{a}	291.60 ± 7.03^{ab}

Mean values with dissimilar superscripts in a column vary significantly ($p<0.05$). DMBA: 7,12-dimethylbenz[a]anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine.

3.3 Measurement of mammary tumorvolume

Tumor volume in the therapeutic groups are presented in Table 5. The DMBA control group (Group II) showed the highest mean tumor volume (2.83 ± 0.36 cm^3). Compared with Group II, tumor volume was significantly reduced in Group III (DMBA + doxorubicin), Group V (DMBA + berberine) and Group VI (DMBA + piperine + berberine).

Table 5: Tumor volume (cm^3) in rats under therapeutic groups (n = 8)

Group	Treatment	Tumor volume (cm^3)
II	DMBA Control	**2.83 ± 0.36^{b}**
III	DMBA + DOX	**1.33 ± 0.22^{a}**
IV	DMBA + PIP	**1.81 ± 0.40^{ab}**
V	DMBA + BER	**1.38 ± 0.20^{a}**
VI	DMBA + PIP + BER	**1.45 ± 0.28^{a}**
VII	DMBA + PIP + DOX	**2.01 ± 0.49^{ab}**
VIII	DMBA + BER + DOX	**2.00 ± 0.31^{ab}**
IX	DMBA + PIP + BER + DOX	**1.85 ± 0.39^{ab}**

Mean values with dissimilar superscripts in a column vary significantly (p<0.05). DMBA: 7,12-dimethylbenz[a]anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine.

3.4 Haematological analysis

Haematological parameters of day 168 are presented in Table 6. Compared with the normal control group (Group I), the DMBA-induced mammary tumor control group (Group II) showed significantly lower haemoglobin concentration and total erythrocyte count, together with significantly higher total leucocyte count, lower lymphocyte percentage and higher granulocyte percentage. Monocyte percentage did not differ significantly among the groups. Haemoglobin concentration was significantly increased in all treated groups compared with Group II, indicating a general improvement in DMBA-associated anaemia. Among these, the piperine-treated group (Group IV) and the berberine-treated group (Group V) showed haemoglobin values closest to the normal control group. Total erythrocyte count was not significantly improved by any treatment. Total leucocyte count was significantly reduced in the doxorubicin-treated group (Group III), piperine-treated group (Group IV), piperine + berberine-treated group (Group VI), piperine + doxorubicin-treated group (Group VII), berberine + doxorubicin-treated group (Group VIII) and piperine + berberine + doxorubicin-treated group (Group IX), whereas the berberine-treated group (Group V) showed no significant change compared with Group II. Among the differential leucocyte parameters, lymphocyte and monocyte percentages showed no significant change in any treated group compared with Group II. Granulocyte percentage was significantly reduced only in the piperine + berberine-treated group (Group VI), indicating that the combination produced a clearer reduction in granulocyte-related inflammatory changes. Overall, piperine and berberine alone showed better restoration of haemoglobin, whereas the piperine + berberine combination showed the broadest haematological response by improving haemoglobin, reducing total leucocyte count and lowering granulocyte percentage.

Table 6: Haematological parameters in rats under therapeutic groups (n = 8)

Group	Hb (g/dl)	TEC (10^6 /µl)	TLC (10^3/µl)	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)
I	14.58 ± 0.16^{c}	6.88 ± 0.07^{c}	9.73 ± 0.62^{a}	75.18 ± 1.08^{c}	2.56 ± 0.37^{a}	22.86 ±1.06^{a}
II	10.09 ± 0.65^{a}	5.91 ± 0.32^{ab}	23.40 ± 3.96^{c}	64.13 ± 4.07^{ab}	5.24 ± 0.97^{bc}	33.63 ± 4.00^{bc}
III	12.18 ± 0.28^{b}	5.58 ± 0.11^{a}	10.59 ± 1.61^{ab}	58.92 ± 2.67^{a}	2.91 ± 0.25^{ab}	38.65 ±2.75^{c}
IV	13.44 ± 0.63^{bc}	5.93 ± 0.28^{ab}	15.88 ± 3.18^{ab}	72.46 ± 4.85^{bc}	2.54 ± 0.40^{a}	24.98 ± 4.78^{ab}
V	13.38±0.47^{bc}	6.32 ± 0.27^{bc}	17.65 ± 1.87^{bc}	70.26 ± 3.55^{bc}	3.59 ± 0.76^{ab}	26.15 ± 3.39^{ab}
VI	12.71 ± 0.51^{b}	5.85 ± 0.24^{ab}	15.01 ± 2.50^{ab}	73.78 ± 2.65^{bc}	4.29 ± 1.01^{ab}	22.06 ±1.82^{a}
VII	12.51 ± 0.27^{b}	5.88 ± 0.12^{ab}	14.08 ± 1.05^{ab}	55.44 ± 2.63^{a}	7.20 ± 0.97^{c}	36.86 ±2.19^{c}
VIII	12.74 ± 0.24^{b}	6.01 ± 0.12^{ab}	9.88 ± 1.71^{a}	63.16 ± 3.79^{ab}	5.44 ± 0.90^{bc}	19.70 ± 3.32^{abc}
IX	12.90 ± 0.16^{b}	5.98 ± 0.07^{ab}	13.19 ± 2.22^{ab}	59.08 ± 4.47^{a}	5.41 ± 1.07^{bc}	33.61 ± 2.51^{bc}

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

3.5 Serum biochemical analysis

Serum biochemical parameters are presented in Table 7. The DMBA control group showed significant increases in serum AST, ALT, BUN and creatinine compared with the normal control group, indicating hepatic and renal biochemical alterations associated with DMBA-induced mammary tumor development. All therapeutic treatments significantly reduced AST activity compared with the DMBA control group. All treated groups showed significant reductions in ALT activity compared with the DMBA control group. The numerically lowest ALT activity was recorded in the piperine + berberine + doxorubicin group (32.50 ± 1.04 U/l), followed by the piperine + berberine group (34.51 ± 1.20 U/l), suggesting better restoration of hepatic biochemical status.Overall, treatments consistently attenuated DMBA-associated increases in serum transaminase activities, while their effects on renal biochemical markers were selective and treatment dependent.

Table 7: Serum biochemical parameters in rats under therapeutic groups (n = 8)

Group	Treatment	AST (U/l)	ALT (U/l)	BUN (mg/dl)	Creatinine (mg/dl)
I	Normal Control	112.41 ± 4.06^{a}	27.79 ± 0.41^{a}	17.20 ± 1.35^{ab}	0.43 ± 0.02^{ab}
II	DMBA Control	224.00 ± 5.17^{e}	53.77 ± 1.29^{e}	27.62 ± 2.30^{d}	0.53 ± 0.01^{cd}
III	DMBA + DOX	164.17 ± 5.68^{b}	40.54 ± 1.65^{d}	17.49 ± 1.96^{abcd}	0.53 ± 0.03^{cd}
IV	DMBA + PIP	171.70 ± 3.63^{b}	35.80 ± 0.53^{bc}	21.06 ± 2.72^{abcd}	0.50 ± 0.03^{bc}
V	DMBA + BER	195.39 ± 3.47^{cd}	36.41 ± 0.73^{c}	16.45 ± 1.59^{a}	0.60 ± 0.03^{d}
VI	DMBA + PIP + BER	201.50 ± 5.17^{d}	34.51 ± 1.20^{bc}	25.21 ± 2.36^{cd}	0.43 ± 0.02^{ab}
VII	DMBA + PIP + DOX	186.34 ± 6.37^{c}	40.27 ± 1.26^{d}	19.67 ± 2.13^{abc}	0.36 ± 0.03^{a}
VIII	DMBA + BER + DOX	195.89 ± 2.26^{cd}	36.48 ± 1.80^{c}	23.93 ± 1.24^{cd}	0.50 ± 0.03^{bc}
IX	DMBA + PIP + BER + DOX	190.83 ± 4.28^{cd}	32.50 ± 1.04^{b}	20.69 ± 1.05^{bcd}	0.41 ± 0.03^{a}

Mean values with dissimilar superscripts in a column vary significantly (p<0.05). DMBA: 7,12-dimethylbenz[a]anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BUN: Blood urea nitrogen.

3.6 Serum oxidative stress and inflammatory biomarkers

Serum oxidative stress and inflammatory biomarkers are presented in Table 8. The DMBA control group showed a significant increase in MDA, TNF-α, IL-1β and IL-6 concentrations compared with the normal control group, indicating enhanced lipid peroxidation and inflammatory activation.

The DMBA control group also showed significant elevation of TNF-α, IL-1β and IL-6 concentrations, reaching 85.64 ± 3.27, 70.69 ± 1.64 and 44.18 ± 2.37 pg/ml, respectively, compared with 28.91 ± 0.92, 36.64 ± 2.45 and 16.41 ± 2.66 pg/ml in normal rats. All therapeutic interventions produced a significant reduction in TNF-α, IL-1β and IL-6 concentrations compared with the DMBA control group. Overall, therapeutic interventions significantly attenuated DMBA-associated oxidative stress and pro-inflammatory cytokine elevation, although the most favourable response varied according to the biomarker assessed.

Table 8: Serum oxidative stress and inflammatory biomarkers in rats under therapeutic groups (n=8)

Group	Treatment	MDA (µmol/l)	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
I	Normal Control	4.79 ± 0.25^{a}	28.91 ± 0.921^{a}	36.64 ± 2.45^{a}	16.41 ± 2.66^{a}
II	DMBA Control	11.01 ± 0.89^{b}	85.64 ± 3.27^{d}	70.69 ± 1.64^{f}	44.18 ± 2.37^{c}
III	DMBA + DOX	9.40 ± 0.98^{b}	35.47 ± 2.23^{b}	49.72 ± 2.03^{bc}	32.53 ± 1.58^{b}
IV	DMBA + PIP	4.30 ± 0.56^{a}	49.94 ± 1.62^{c}	42.82 ± 1.78^{ab}	36.80 ± 1.75^{b}
V	DMBA + BER	3.87 ± 0.49^{a}	54.11 ± 2.13^{c}	60.85 ± 3.71^{e}	34.19 ± 2.34^{b}
VI	DMBA + PIP + BER	4.53 ± 0.77^{a}	51.36 ± 3.28^{c}	52.41 ± 3.75^{cd}	31.82 ± 1.59^{b}
VII	DMBA + PIP + DOX	8.84 ± 0.71^{b}	39.24 ± 1.94^{b}	51.87 ± 2.90^{c}	36.14 ± 2.19^{b}
VIII	DMBA + BER + DOX	10.03 ±1.87^{b}	39.03 ± 1.84^{b}	52.47 ± 2.18^{cd}	34.84 ± 2.76^{b}
IX	DMBA + PIP + BER + DOX	3.36 ± 0.60^{a}	42.40 ± 2.35^{b}	60.10 ± 1.77^{de}	33.52 ± 1.81^{b}

Mean values with dissimilar superscripts in a column vary significantly (p<0.05). DMBA: 7,12-dimethylbenz[a]anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6.

3.7 Serum hormonal profile

Serum hormonal changes following therapeutic treatment are presented in Table 9. Findings indicate that DMBA-induced mammary tumor development was primarily associated with increased serum estradiol concentration, whereas progesterone concentration remained largely unaffected by tumor induction or therapeutic intervention.

3.8 Gene expression analysis

Relative mRNA expression profiles of TP53, Caspase-3, Cyclin D1 and MMP-9 are shown in Figure 1. Overall, therapeutic interventions favourably modulated DMBA-associated molecular alterations, with the clearest significant effect observed for restoration of TP53 expression by the triple-combination regimen, while changes in Caspase-3, Cyclin D1 and MMP-9 were mainly non-significant or treatment-dependent.

3.9 Ultrasonographic evaluation of mammary gland tumor

Ultrasonographic findings of mammary glands from different therapeutic groups on day 168 are presented in Figure 2. Ultrasonographic examination on day 168 showed uniform mammary echotexture without detectable lesions in the normal control group. The DMBA control group exhibited irregular hyperechoic mammary masses with heterogeneous echotexture and distortion of surrounding tissue architecture. In contrast, therapeutic groups treated with piperine, berberine, doxorubicin and their combinations showed comparatively reduced lesion size, decreased echogenicity, more

homogeneous echotexture and less irregular margins than the DMBA control group. These findings indicated attenuation of DMBA-induced mammary tumor progression following therapeutic treatment.

3.10 Histological examination of the mammary gland

Representative histopathological features of mammary gland tissue sections from control and experimental rats are shown in Figure 3. The normal control group exhibited preserved mammary architecture, with well-arranged ducts and acini lined by uniform epithelial cells, along with normal stromal and adipose components. In contrast, the DMBA control group showed clear neoplastic alterations, including disruption of normal glandular architecture, densely packed and irregular glandular structures, increased cellularity, epithelial atypia, hyperchromatic nuclei and stromal invasion, confirming successful induction of mammary tumors.

Table 9: Serum estradiol and progesterone concentrations in rats under therapeutic groups (n=6)

Group	Treatment	Estradiol (pg/ml)	Progesterone (ng/ml)
I	Normal Control	22.42 ± 0.98^{a}	7.098 ± 0.0092
II	DMBA Control	34.13 ± 0.68^{e}	7.091 ± 0.0359
III	DMBA + DOX	32.51 ± 1.99^{de}	7.097 ± 0.0251
IV	DMBA + PIP	30.40 ± 1.25^{cde}	7.055 ± 0.0244
V	DMBA + BER	25.66 ± 1.43^{abc}	7.050 ± 0.0170
VI	DMBA + PIP + BER	30.51 ± 0.61^{cde}	7.023 ± 0.0101
VII	DMBA + PIP + DOX	31.60 ± 0.53^{de}	7.067 ± 0.0316
VIII	DMBA + BER + DOX	29.54 ± 1.97^{bcd}	7.021 ± 0.0212
IX	DMBA + PIP + BER + DOX	25.36 ± 2.13^{ab}	7.074 ± 0.0265

Mean values with dissimilar superscripts in a column vary significantly (p<0.05). DMBA: 7,12-dimethylbenz [a] anthracene; DOX: doxorubicin; PIP: piperine; BER: berberine.

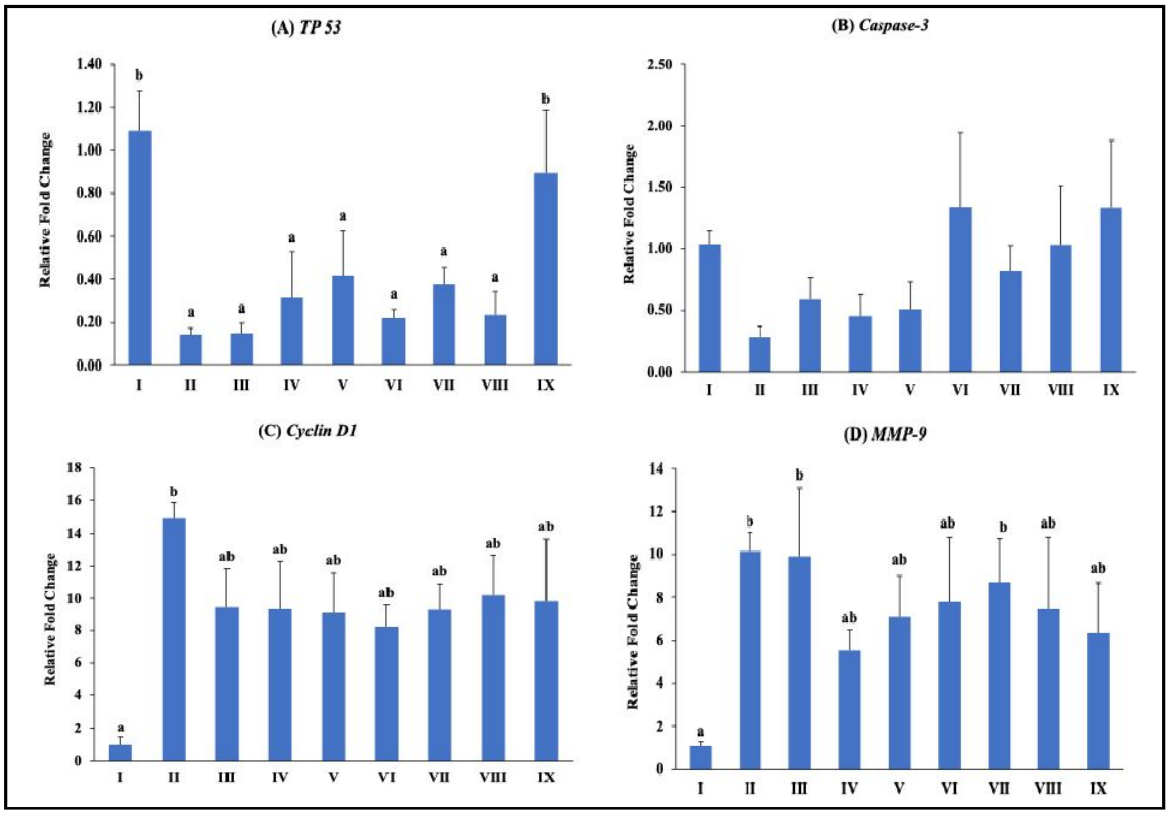

Figure 1: Relative mRNA expression of (A) *TP53*, (B) *Caspase-3*, (C) *Cyclin D1* and (D) *MMP-9* in DMBA-induced mammary tumor-bearing rats following therapeutic treatment(n=6). Bars bearing different superscript letters differ significantly (p<0.05).Group I: normal control; Group II: DMBA control;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

Rats treated with piperine or berberine also showed milder tumor-associated histological changes, reflected by reduced cellular atypia and partial preservation of ductal and glandular structures. The combination-treated groups showed more evident histological improvement than the individual treatment groups, particularly through reduced cellular proliferation and better tissue organization. Among all therapeutic regimens, the piperine + berberine + doxorubicin group showed the most favourable microscopic appearance, with improved preservation of mammary architecture, reduced atypical changes and lower proliferative activity. Overall, these findings indicate that therapeutic treatments attenuated DMBA-induced mammary neoplastic changes, with the strongest qualitative improvement observed in the triple-combination group.

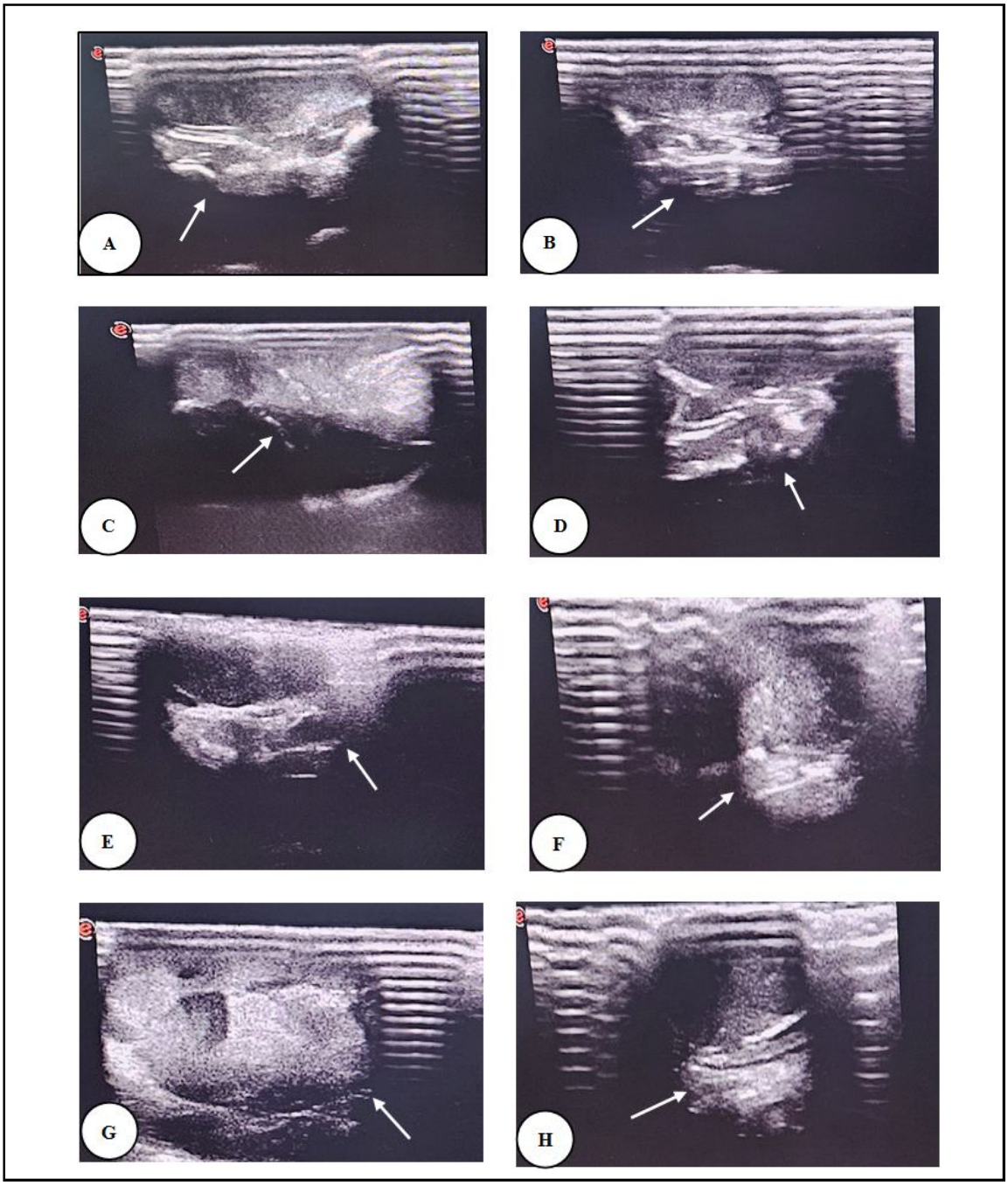

Figure 2: Representative ultrasonographic images of mammary tumors in DMBA-induced mammary tumor-bearing rats following therapeutic treatment. Arrowheads indicate the tumor mass. Panels A-H represent the respective experimental groups: A: DMBA control; B: DMBA + DOX; C: DMBA + PIP; D: DMBA + BER; E: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

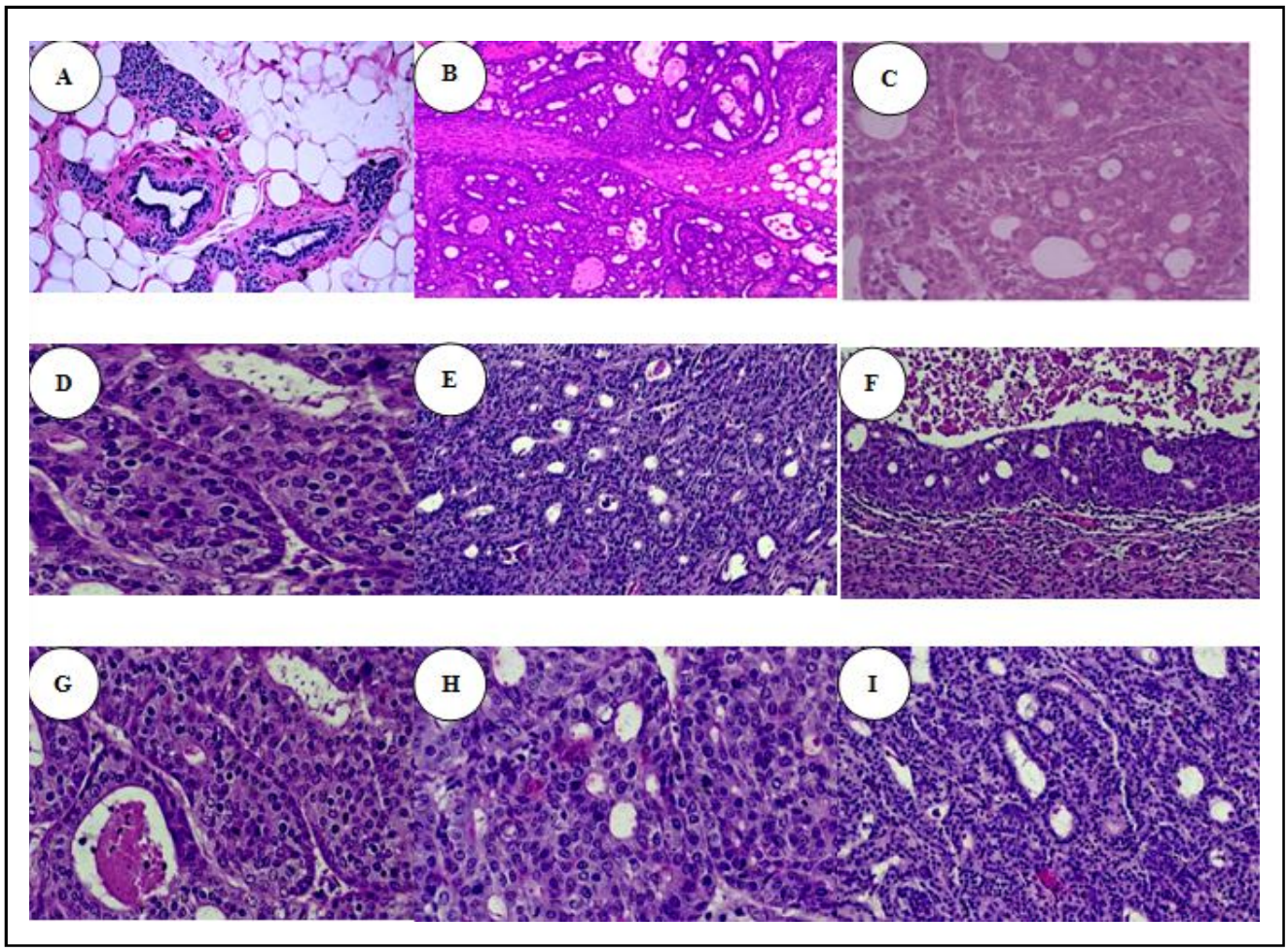

Figure 3: Representative H&E stained mammary gland sections from control and therapeutic experimental groups (120X). A, normal control; B, DMBA control; C, DMBA + DOX; D, DMBA + PIP; E, DMBA + BER; F, DMBA + PIP + BER; G, DMBA + PIP + DOX; H, DMBA + BER + DOX; I, DMBA + PIP + BER + DOX. DMBA: 7,12-dimethylbenz[a]anthracene;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

4. Discussion

DMBA-induced mammary carcinogenesis is closely associated with oxidative stress, inflammatory activation and systemic toxicity. Following metabolic activation by cytochrome P450 enzymes, DMBA forms carcinogenic metabolites that generate ROS and promote mammary tumor development (Krishnamoorthy and Sankaran, 2016). Body weight, body condition and feed intake are practical indicators of systemic toxicity, tumor burden and overall physiological status during chemically induced carcinogenesis (Marinkovic *et al*., 2018). In the present study, DMBA-induced tumor-bearing rats showed reduced feed intake and a significant decrease in body weight gain, suggesting tumor-associated systemic stress, impaired nutrient utilisation and altered metabolic demand during mammary carcinogenesis. Similar reductions in food consumption and body weight have been reported in DMBA-exposed experimental models (Rojas-Armas *et al*., 2020; Suleiman *et al*., 2024). In contrast, piperineand berberine-treated tumor-bearing rats showed improved feed intake and body weight gain compared with the DMBA control group, indicating partial recovery of general physiological condition and attenuation of disease-associated wasting. The beneficial response observed with piperine may be related to its digestivesupportive, bioavailability-enhancing, antioxidant and anticancer activities (Mad-adam *et al*., 2023). Likewise, the improvement observed with berberine may be associated with its antioxidant, anti-inflammatory, metabolicregulatory and anticancer properties (Xiong *et al*., 2022). Collectively, these findings suggest that piperine and berberine may help counteract DMBA-induced systemic deterioration, thereby supporting better nutritional and body weight status during tumor progression.

Progressive enlargement of mammary tumor volume in DMBA-treated rats reflects active tumor development and enhanced mammary epithelial proliferation. DMBA-induced rats exhibited increased mammary tumor volume, which may be associated with oxidative stress, DNA damage and enhanced proliferation of mammary epithelial cells during carcinogenesis, as reported in earlier DMBA-based mammary tumor studies (Kontogeorgi *et al*., 2025). Treatment with berberine reduced tumor volume, suggesting inhibition of tumor progression, consistent with its reported protective effect against DMBA-induced mammary carcinogenesis (Karnam *et al*., 2017). Similarly, the lower tumor volume observed in piperine-treated rats

may reflect suppression of tumor cell proliferation, invasion and angiogenic signalling. Lai *et al*. (2012) reported that piperine inhibited tumor growth and metastatic spread in a 4T1 mammary carcinoma model, while Zare *et al*. (2020) showed that piperine reduced MMP-9 and VEGF expression in breast cancer cells, indicating anti-invasive and anti-angiogenic activity. Its reported effects on apoptosis induction and cell-cycle arrest further support the tumor-suppressive response observed in the present study (Qiu *et al*., 2019).

The haematological profile of DMBA-induced tumor-bearing rats indicated anaemia and immune disturbance, as evidenced by reduced Hb and TEC together with altered TLC and DLC. These changes may be attributed to DMBA-induced oxidative stress, erythrocyte damage, impaired haematopoiesis and tumor-associated inflammatory stress. Similar haematological alterations have been reported in DMBA-exposed rats by Selamoglu *et al*. (2007) and in DMBA-induced mammary carcinoma-bearing Sprague Dawley rats by Razmi *et al*. (1999). Treatment with piperine and berberine improved the haematological profile compared with the DMBA control group, suggesting partial restoration of erythropoietic and immune status. This improvement may be related to their antioxidant, anti-inflammatory and immunomodulatory activities, as piperine has been reported to reverse altered haematological parameters and modulate immune responses (Divakar *et al*., 2015), while berberine has been shown to ameliorate reductions in Hb, RBC and WBC counts under systemic toxicity conditions (Labib *et al*., 2025).

Serum biochemical changes in DMBA-induced tumor-bearing rats indicated hepatic and renal stress during mammary carcinogenesis. Elevated serum hepatic biomarkers such as SGPT, SGOT and bilirubin in DMBA-treated animals indicate hepatic injury, which may be attributed to hepatic bioactivation of DMBA into reactive carcinogenic metabolites that generate ROS, induce oxidative cellular damage and contribute to carcinogenesis and tissue injury (Rodgers *et al*., 2018).The increase in AST and ALT may reflect hepatocellular membrane disruption and leakage of intracellular enzymes into circulation, whereas elevated BUN and creatinine suggest impaired renal function due to systemic oxidative and toxic stress. Similar DMBA-associated increases in hepatic and renal injury markers have been reported in experimental mammary tumor models (Akhouri *et al*., 2020). In contrast, piperine and berberine-treated rats showed improvement in these biochemical indices, suggesting attenuation of DMBA-associated organ toxicity. The hepatoprotective potential of piperine has been supported in experimental liver injury models, while its nephroprotective effects have also been reported under renal toxic and ischemic conditions (Morsy *et al*., 2020). Similarly, berberine has been shown to reduce hepatic and renal injury through antioxidant and anti-inflammatory mechanisms in experimental toxicity models (Mohammadi *et al*., 2020).

The inflammatory status of DMBA-induced tumor-bearing rats was characterized by increased circulating TNF-á, IL-1â and IL-6. This response may be linked to DMBA-induced oxidative stress and activation of tumor-promoting inflammatory pathways, since these cytokines can support neoplastic cell survival, proliferation, angiogenesis and invasiveness through signalling networks such as NF-κB, MAPK and IL-6/STAT3. Similar elevation of pro-inflammatory cytokines has been reported in DMBA-induced mammary carcinoma, supporting the inflammatory basis of chemically induced mammary carcinogenesis (Karnam *et al*., 2017). Treatment with piperine, berberine and their combination reduced TNF-α, IL-1β and IL-6 levels, with the combined treatment showing the greatest anti-inflammatory response. The effect of piperine may be associated with suppression of inflammatory mediator production, as previous studies showed that piperine reduced TNF-α and IL-1β in a 6-OHDA-induced rat model, decreased IL-1β and IL-6 in ovalbumin-induced allergic rhinitis and inhibited IL-6/MMP-13 expression in inflammatory models (Aswar *et al*., 2015). Piperine has also been shown to suppress TNF-α, IL-1β and IL-6 expression through inhibition of NF-κB and MAPK signalling (Zhai *et al*., 2016). Similarly, berberine reduced IL-1β, IL-6 and TNF-α in DMBA-induced mammary carcinoma-bearing rats and inhibited *Pseudomonas aeruginosa* lipopolysaccharide-induced inflammatory cytokine expression through modulation of ER-stressmediated ERK1/2 activation in macrophages and hepatocytes (Wang *et al*., 2020). Thus, the reduction in serum pro-inflammatory cytokines in treated groups may indicate attenuation of DMBA-associated inflammatory signalling and suppression of a tumor-supportive microenvironment.

Oxidative imbalance was evident in DMBA-induced mammary tumor-bearing rats, as shown by increased MDA levels, indicating enhanced lipid peroxidation during tumor progression. This may result from metabolic activation of DMBA into reactive intermediates that generate ROS and damage membrane lipids. Similar oxidative alterations have been reported in DMBA-induced mammary tumor models (Akhouri *et al*., 2020). The reduction in MDA levels after piperine and berberine treatment suggests attenuation of oxidative membrane injury and partial restoration of redox balance. This effect is supported by the reported antioxidant and lipid peroxidation-inhibitory activities of berberine (Chang *et al*., 2016) and piperine (Morsy *et al*., 2020). The elevated estradiol level in DMBA-induced rats may reflect estrogen-linked mammary tumor progression, whereas its reduction after treatment suggests partial modulation of hormone-associated tumor promotion, particularly with berberine (Roheel *et al*., 2024). Progesterone levels remained largely unchanged, consistent with Akhouri*et al*. (2020), indicating limited progesterone involvement under the present experimental conditions.

At the molecular level, DMBA-induced mammary tumors showed a pro-tumorigenic expression pattern, characterised by reduced *TP53* and *Caspase-3* expression together with increased *Cyclin D1* and MMP-9 expression.This pattern suggests impaired tumor-suppressive and apoptotic signalling, accompanied by enhanced cell-cycle progression and invasion-associated activity. DMBA-derived reactive metabolites are known to induce genotoxic stress and oxidative DNA damage, which can disrupt p53-mediated cellular responses. The p53 pathway normally regulates DNA repair, cell-cycle arrest, apoptosis and senescence in damaged cells (Zender *et al*., 2010), whereas persistent exposure to polycyclic aromatic hydrocarbons such as DMBA may promote p53 mutation or functional inactivation (Munoz and Albores, 2011). Therefore, the down regulated *TP53* and Caspase-3 expression observed in the DMBA control group may indicate weakening of the DNA-damage response and apoptosis during mammary carcinogenesis. In parallel, increased *Cyclin D1* expression supports abnormal proliferative signalling and cell-cycle advancement, consistent with previous reports of *Cyclin D1* upregulation and oncogenic pathway activation in DMBA-induced mammary tumors (Currier *et al*., 2005). The elevation of *MMP-9* further suggests extracellular matrix remodelling and increased invasive potential, as MMP-9 facilitates matrix degradation and tumor

dissemination, and has been associated with progression-related changes in DMBA-induced mammary tumor models (Dong *et al*., 2018). Overall, these gene-expression changes indicate that DMBA-induced mammary tumor progression involves coordinated suppression of apoptotic control with activation of proliferative and invasion-related pathways.

Treatment with piperine and berberine partly corrected the gene-expression alterations associated with DMBA-induced mammary carcinogenesis, suggesting improvement in apoptosis-related signalling together with suppression of proliferative and invasion-linked pathways. The increased expression of TP53 and Caspase-3 genes in treated groups may indicate reactivation of DNA-damage checkpoint and apoptotic mechanisms, while the reduction in Cyclin D1 expression indicates possible restriction of cell-cycle progression. Berberine has been reported to influence p53-dependent apoptosis is through AMPK-p53 signalling in doxorubicin-resistant breast cancer cells and by inducing p53 activation under nucleolar stress conditions (Sakaguchi *et al*., 2020). Its ability to promote caspase-mediated apoptosis and reduce Cyclin D1 expression further supports its antiproliferative and pro-apoptotic potential (Zhao *et al*., 2017). Similarly, piperine has been shown to inhibit breast cancer cell growth, promote caspase-dependent apoptosis, interfere with cell-cycle progression and reduce MMP-2/MMP-9gene expression, indicating anti-proliferative and anti-invasive activity (Zare *et al*., 2020). Thus, the lower MMP-9 expression observed in treated groups may suggest reduced extracellular matrix remodelling and invasive potential, although confirmation at the protein or enzymatic activity level would provide stronger mechanistic support. Overall, these results indicate that piperine and berberine may attenuate DMBA-induced mammary tumor progression by modulating apoptosis, cell-cycle regulation and invasion-associated molecular pathways.

Histopathological findings further supported the biochemical and molecular evidence of DMBA-induced mammary carcinogenesis. The DMBA control group showed marked disruption of mammary gland architecture, characterized by epithelial proliferation, atypical ductal/acinar changes, hyperchromatic nuclei, stromal reaction and inflammatory cell infiltration, indicating active neoplastic transformation. Similar lesions have been described in DMBA-induced mammary tumor models, where DMBA exposure produced excessive proliferation of acinar and ductular epithelium with hyperchromatic nuclei, ductal proliferation, stromal fibrosis and inflammatory alterations (Adefisan-Adeoye *et al*., 2025). These microscopic changes are consistent with the tumor volume, inflammatory, oxidative stress and gene-expression findings of the present study, suggesting that DMBA promotes mammary tumor progression through sustained epithelial proliferation, oxidative injury, inflammatory activation and tissue remodelling.

Treatment with piperine, berberine and their combinations improved mammary histoarchitecture compared with the DMBA control group, as reflected by reduced epithelial hyperplasia, lower cellular atypia, decreased invasive features and better preservation of glandular organization. The protective effect of berberine is supported by Karnam *et al*. (2017), who reported improvement in mammary gland histology and reduced ductal/invasive carcinoma features in DMBA-induced mammary tumor-bearing Sprague-Dawley rats. Piperine-related improvement may be explained by its reported antitumor, anti-invasive and pro-apoptotic effects; Lai *et al*. (2012) showed that piperine suppressed tumor growth and metastasis in a 4T1 mammary carcinoma model, while *Piper nigrum* extract has been reported to reduce mammary tumor incidence and chemotherapy-associated toxicity in mammary tumor-bearing rats (Mad-Adam *et al*., 2023). Overall, the histological improvement observed in treated groups supports the potential of piperine and berberine to attenuate DMBA-induced mammary tumor progression, probably through combined antiproliferative, antioxidant, anti-inflammatory and apoptosis-modulating mechanisms. In the present study, ultrasonography of DMBA-induced mammary gland tumor-bearing rats showed irregular hyperechoic masses with heterogeneous internal echotexture and disruption of the surrounding mammary tissue pattern. These findings are in agreement with earlier observations that irregular tumor margins and heterogeneous echogenicity are commonly associated with malignant mammary lesions (Feliciano *et al*., 2017). In comparison with the DMBA control group, rats treated with berberine, piperine and doxorubicin, either individually or in combination, showed a relative reduction in lesion severity, improved echotextural uniformity and less irregular tumor borders. The improvement observed in treated groups may be linked to the reported antitumor, antioxidant, anti-inflammatory and anti-invasive properties of berberine and piperine in mammary tumor and breast cancer models (Karnam *et al*., 2017). Therefore, the ultrasonographic observations supported treatment-associated regression of tumor changes and partial recovery of mammary gland architecture.

5. Conclusion

Therapeutic administration of piperine and berberine, alone and in combination with doxorubicin, attenuated DMBA-induced mammary gland tumor progression in rats. Treatment improved general condition, reduced tumor burden, corrected major haematological and biochemical alterations, decreased inflammatory cytokines and oxidative stress, improved mammary gland histoarchitecture and favourably modulated TP53, Caspase-3, Cyclin D1 and MMP-9 gene expression. Although, doxorubicin alone produced the greatest reduction in tumor volume, the combination of piperine, berberine and doxorubicin showed the most consistent overall therapeutic response across multiple endpoints. These findings suggest that this combination may have potential as an adjuvant therapeutic strategy; however, further validation in higher animal models and translational studies is required before clinical application.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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