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Original Article

Evaluation of Toxicity and Anti-Breast Cancer Potential of Methanolic Leaf Extract of *Anastatica hierochuntica* in DMBA Induced Rat Model

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ABSTRACT

Background: *Anastatica hierochuntica* has been traditionally used in herbal medicine for its therapeutic effects, but its safety and efficacy have not been comprehensively assessed. This study investigated the Methanolic Leaf (ML) extracts of *A. hierochuntica* acute and sub-acute toxicity in Sprague-Dawley rats and its anti-breast cancer potential in a 7,12-Dimethylbenz[a]Anthracene (DMBA)-induced rat model. **Materials and Methods:** Acute and sub-acute oral toxicity studies were conducted following the Organization for Economic Co-operation and Development (OECD) guidelines 423 and 407, respectively. Hematological and biochemical parameters were analyzed in blood and serum respectively. Breast tissues were examined for oxidative stress including Lipid Peroxidation (LPO), Catalase (CAT), and Reduced Glutathione (GSH). Histopathological assessments were performed on liver, kidney, spleen, and mammary tissues. The anti-breast cancer study involved DMBA-induced rats treated with ML (200 and 400 mg/kg) or tamoxifen. **Results:** The acute toxicity study indicated no treatment-related mortality or significant physiological changes at doses up to 2000 mg/kg. Sub-acute toxicity assessment revealed no adverse effects up to 1000 mg/kg. suggest that ML extract has a high safety profile, with a No-Observed-Adverse-Effect Level (NOAEL) above 1000 mg/kg. In the DMBA-induced model, ML treatment significantly ($p<0.05$) reduced tumor incidence and tumor weight, with the 400 mg/kg dose showing the greatest efficacy. ML also improved oxidative stress markers by decreasing LPO levels and increasing CAT and GSH activity. Histopathological analysis revealed a reduced tumour burden and improved mammary architecture in ML-treated groups compared to untreated controls. **Conclusion:** The ML extract of *A. hierochuntica* demonstrated significant anti-breast cancer potential in DMBA-induced rats, alongside a favourable toxicity profile warranting further investigation for potential therapeutic applications.

Keywords: Acute and sub-acute toxicity, DMBA, Anti-breast cancer, *Anastatica hierochuntica*.

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INTRODUCTION

Breast Cancer (BC) is the most prevalent malignancy among women worldwide, representing a significant public health concern due to its high incidence and mortality rates.[1] BC is a highly heterogeneous disease that encompasses various molecular subtypes, each with distinct biological behaviours and therapeutic responses.[2] The progression of BC involves complex interactions between cancer cells and the tumor microenvironment, including immune cells, fibroblasts, endothelial cells, and Extracellular Matrix (ECM) components. These interactions play a critical role in tumor growth, invasion, angiogenesis, and metastasis.[3] Despite significant advancements in treatment modalities such as chemotherapy, radiotherapy, targeted therapy, and immunotherapy, the development of drug resistance and adverse side effects remain major challenges in BC management.[4]

In the pursuit of safer and more effective alternatives, natural products have emerged as promising candidates for cancer therapy due to their diverse bioactive compounds with minimal toxicity.[5] The anticancer potential of these bioactive compounds is attributed to multiple mechanisms, including the inhibition of tumor cell proliferation, induction of apoptosis, suppression of angiogenesis, and modulation of signaling pathways involved in cancer progression.[6] A key aspect of developing plant-based

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therapies lies on assessing their toxicity profiles to determine safe dosage ranges for clinical use as well as to ensure the safety, tolerability, and therapeutic windows of natural products.[7] Toxicity studies are crucial for meeting global regulatory requirements such as Food and Drug Administration (FDA), World Health Organisation (WHO) for drug approval and market access, while also serving as a key step in the drug discovery pipeline to identify lead compounds with optimal safety margins.[8]

Anastatica hierochuntica, commonly known as the "Kaff-Maryam," is widely recognized in traditional medicine to facilitate childbirth, alleviate uterine hemorrhage, and treat respiratory and digestive ailments, as well as fevers and headaches[9] with its therapeutic properties have been comprehensively analyzed.[10] Notably, *in vivo* studies have explored its gastroprotective,[11] hepatoprotective,[12] and nephroprotective[13] potential, highlighting its role in protecting vital organs against damage induced by oxidative stress and toxic agents.

Although preliminary evidence suggests the anticancer potential of *A. hierochuntica*, its role in oncology remains underexplored. Till date, only limited literature on the *in vitro* anticancer activity is available, focusing mainly on its anti-melanogenesis,[14] anti-cervical cancer,[15] and anti-breast cancer[16] effects using methanolic extracts of the plant, where it has been shown to inhibit cancer cell proliferation, induce apoptosis, and suppress angiogenesis and metastasis.

In our recent study, we identified a range of bioactive compounds-including Apigenin-6-C-Glucoside, Luteolin-8-C-Glucoside, Isovitexin 7-O-[Isoferuloyl]-Glucoside, Cis-5-Caffeoylquinic Acid, Naringenin, Taxifolin, Luteolin, Quercetin, Rutin, Hierochin B, Evofolin B, and Balanophonin in the methanolic leaf extract of *A. hierochuntica*,[17] highlighting its therapeutic promise and the need for further investigation. Therefore, the present study aims to evaluate the toxicological and anti-breast cancer potential of this extract in a 7,12-Dimethylbenz[a]Anthracene (DMBA)-induced breast cancer rat model, thereby providing a comprehensive assessment of its safety and therapeutic relevance. To the best of our knowledge, this is the first study to assess the ML extract in a DMBA-induced BC *in vivo* system. The DMBA model is a well-established and biologically relevant preclinical platform that closely mimics key features of human BC and is widely employed for evaluating chemopreventive and therapeutic agents hence its use in this study enhances the translational significance of our safety and efficacy findings.

MATERIALS AND METHODS

Sample Preparation

Kaff-e-Maryam (*A. hierochuntica* L.) plant was procured from local herbal market in Riyadh, Saudi Arabia. The plant was authenticated by Siddha Central Research Institute, Chennai, India under the voucher specimen number code A25072201H and the sample specimen was preserved. The leaves were separated from the whole plant, ground into fine particles, and extracted with methanol in a 1:3 ratio overnight. A reflux condenser was then attached to the flask, gently heated, and the mixture filtered through filter paper. The resulting filtrate was concentrated using a rotary evaporator (IKA, Germany), and the Methanolic Leaf (ML) extracts were obtained in semisolid paste form was weighed and stored at 4°C until further use. The extract was freshly dissolved in distilled water during drug administration.

Experimental Design

All experiments were designed and conducted under the ethical norms approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India under the license number (LVII/02/321/PO/Re/S/03/CPCSEA). Nulliparous and non-pregnant Female Sprague Dawley (SD) rats of 8-9 weeks old weighing between 200±220 g were included in the study. The animals were housed in standard polypropylene cages under controlled environmental conditions (temperature 22±2°C, relative humidity 55±10%, and a 12 hr light/dark cycle). The rats were acclimatized for seven days before the experiment and given a standard diet and water ad libitum. Rats were manually randomized into experimental groups.

Acute Toxicity

The acute oral toxicity test of ML was evaluated according to OECD guideline No. 423.[18] Nine female SD rats were randomly divided into 3 groups (n=3). Group 1 was administered water and acted as a control, and Group 2 was treated with 300 mg/kg body weight of ML as a starting dose. The rats were observed for general behavioural changes, symptoms of toxicity and mortality and other adverse effects after ML treatment for the first 4 hr, then for 48 hr. When no signs of toxicity or mortality showed in group 2 after 48 hr of treatment, Group3 was administered with the next higher dose of 2000 mg/kg body weight of ML. The ML extract was freshly dissolved in distilled water prior to administration, and the dosing volume was maintained at 10 mL/kg body weight, corresponding to an average administered volume of approximately 2 mL per rat. Clinical signs were recorded daily, while body weights, food and water intake were measured every two days during the period of 14 days. In accordance with OECD 423 guidelines, all surviving animals were humanely sacrificed at the end of the 14-day observation period, and a gross necropsy was performed. Internal organs were collected and processed for microscopic histopathological examination to assess potential tissue-level toxic effects.

Sub-Acute Toxicity

The repeated dose 28-day oral toxicity study of ML was executed following OECD guidelines No. 407.[19] Twenty-four female SD rats were randomly assigned into four groups (n = 6); Group 1 was

given water and served as a control, and three treatment groups (Group 2, 3 and 4) were treated with 250, 500 and 1000 mg/kg of ML respectively. The animals were observed twice daily (before and after dosing) for general appearance, behaviour, and mortality for 28 days. The body weight and food/water consumption were measured every 3 days (1, 4, 7, 10, 13, 16, 19, 22, 25 and 28). At the end of the experiment, blood and serum were collected for Haematology and Biochemical analysis. Gross Necropsy was performed, and internal organs were collected for organ: Body weight ratio and carcasses were discarded. Microscopic examination for histopathological analysis was performed on the internal organs.

Anti-breast cancer study

The anti-breast cancer study of ML was performed in DMBA induced rat model. 30 Female SD rats were used in this study, of which normal control Group 1 (n=6) was maintained without treatment. The remaining 24 female SD rats were induced intragastrically with a single dose of DMBA 80 mg/kg body weight diluted in 1 mL of olive oil (Sigma Aldrich, USA) by oral gavage.[20] After DMBA administration, the animals were bred under ideal temperature conditions and had free access to water and food. After 4 weeks of DMBA administration, rats were palpated by touching and examining the tumour formation, and location and size were recorded weekly for confirmation of tumor prevalence and beginning of the treatment. After the induction of first tumour which was after 10 weeks, rats were randomly divided into 4 groups (n=6). Group 2 served as Negative control, receiving 2 mL/kg of water, Groups 3 and 4 received 200 and 400 mg/kg body weight of the ML extract orally for 5 weeks respectively, and Group 5 functioned as positive control receiving 20 mg/kg BW of Tamoxifen orally. All the animals were sacrificed at the end of the treatment. The mammary tumours were counted, weighed, dissected and stored for histopathological, molecular and biochemical analysis. Gross Necropsy was performed and microscopic examination for histopathological analysis was performed on the Breast tissue, liver, heart, spleen, lung, and kidney.

Animal Sacrifice and Blood/organ Collection

All SD rats were anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (7 mg/kg). Blood (5 mL) was collected via cardiac puncture into EDTA-coated tubes for hematological analysis and plain tubes for biochemical assays. After collection, rats were sacrificed by cervical dislocation. Blood samples were centrifuged at 3000×g for 10 min at 4°C, and serum was stored at -20°C until analysis.

Haematological Assay

The blood samples in the heparinised tubes were used for haematological analyses- White Blood Cell count (WBC), Red Blood Cell count (RBC), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), packed cell volume (PCV), hematocrit (HCT), Platelet (PLT), Neutrophils (NE), Lymphocytes (LYM), Basophils (BA), Monocytes (MO) and Eosinophils (EO) numbers in Hitachi 902 Automatic Analyzer® (Hitachi, Japan).

Biochemical Analysis

Serum samples were subjected to measure potassium (K), sodium (Na), cholesterol (CHO), glucose (GLU), Triglycerides (TG), Calcium (Ca), Chloride (Cl), Creatinine (Crea), Alanine Aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TPRO), creatinine kinase (CK), phosphorus (P), urea, lactate dehydrogenase (LDH), and Albumin (ALB) using a COBAS Integra 800 (Roche Diagnostics, Switzerland) assay kit according to the manufacturer's instructions.

Breast tissue homogenate

A portion of the excised breast tissues from the treatment and control group were homogenised in a 50 mM, and 10 mM phosphate-buffered saline (PBS) solution (pH 7.4) (Invitrogen, USA). The homogenates were centrifuged at 4000 rpm for 15 min, and the supernatants were stored at -20°C for downstream application.

Oxidative Stress Biomarkers

Determination of Lipid Peroxidation

The lipid peroxidation in the tissue was performed according to the method described[21] with some modifications. Briefly, 2.5 mL of 10% Tribarbituric Acid (TBA) was mixed with 0.5 mL homogenate and heated in the water bath at 90°C for 15 min. After cooling to room temperature, the mixture was centrifuged at 3000 rpm for 10 min, the supernatant was mixed with 5 mL of n-butanol, and the resultant pink-coloured mixture was measured against a TBA blank (prepared from 5 mL of TBA solution and 5 mL of distilled water) at 532 nm using Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, USA). The amount of MDA formed was expressed as nmol/g weight of the tissue.

Determination of Reduced Glutathione (GSH) level

Reduced glutathione in the tissue was determined according to the method.[22] Briefly, 0.5 ml of the tissue homogenate was premixed with 0.1 mL of 5% Trichloroacetic Acid (TCA) and centrifuged at 3000 g for 10 min at 4°C. About 0.3 mL of the supernatant was separated and mixed with 0.7 mL of 0.2 M sodium phosphate buffer (pH 8) and 2 mL of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8) and incubated at room temperature for 10 min in the dark. The absorbance was measured against a TCA blank (prepared from 5 mL of TCA solution and 5 mL of distilled water) at 412 nm using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, USA). GSH levels were expressed as n moles/mg protein.

Determination of Catalase Activity

The catalase activity was analysed with slight modification as described.[23] Shortly, 0.1mL of the homogenate, 0.3mL of 2mM Hydrogen peroxide and 0.6mL of 10 mM phosphate buffer (pH 7.4) were mixed and incubated at 37°C for 5 min, and then 2mL of Dichromate Acetic Acid reagent (5% Potassium dichromate in water, Glacial Acetic Acid mixed in 1:3 ratio) was added to stop the reaction. The absorbance was read against Dichromate Acetic acid reagent as a blank at 570nm using Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, USA). Catalase activity was expressed as mmol/min/mg protein.

Necropsy and Histopathological Examination

The Necropsy included inspection of the outer surfaces, the orifices, and the thoracic and gastrointestinal cavities. Histopathological assessments were conducted on the kidneys, liver, spleen, and mammary glands were fixed in 10% formalin, trimmed, embedded in paraffin, sectioned (5 µm), stained with hematoxylin and eosin, and examined under a light microscope for histopathological changes. All histopathological evaluations were performed in a blinded manner, with investigators unaware of the treatment groups to ensure unbiased assessment.

Statistical Analysis

All the data are expressed as Mean±SD. Statistical analysis for Student t-test was performed using SPSS software version 24 for Windows (Chicago, IL, USA). Values of $p<0.05^{*}$ and $p< 0.001^{**}$ were statistically considered significant.

RESULTS

Acute toxicity study

The acute oral toxicity study of ML revealed no treatment-related mortality at doses of 300 or 2000 mg/kg body weight. Treated rats showed no abnormal physical or behavioral changes. Additionally, there was no significant ($p>0.05$) difference in mean body weight compared to the control group. Food and water intake also remained unchanged in the treated groups (Figure 1).

Macroscopic examination of the liver, kidneys, and spleen in ML-treated rats showed no visible adverse effects (Figure 2). Histopathology of the liver in the control and 300 mg/kg ML group revealed normal central veins, sinusoids, Kupffer cells, and binucleated hepatocytes. In the 2000 mg/kg ML-treated group, the overall hepatic architecture was largely preserved, with mild to moderate vascular changes were observed, including dilation of central veins and hepatic sinusoids, as well as localized hemorrhage in some areas. Kidney sections from control and 300 mg/kg groups showed normal glomeruli and well-defined tubules, while the 2000 mg/kg group showed mild tubular dilation. Spleen sections in control and 300 mg/kg groups had distinct red and white pulp with a clear central artery, and the 2000 mg/kg group displayed normal structure with slight hemorrhage.

Subacute toxicity

The subacute oral toxicity study of ML showed no treatment-related deaths and no visible changes in the skin, fur, ocular membranes, or behavioral patterns were observed among SD rats treated with either 250, 500 or 1000 mg/kg body weight dosage. There was no statistically significant difference ($p>0.05$) observed in the mean body weight of the ML treated rats compared to control groups (Figure 3).

ML-treated rats showed no significant changes in most hematological parameters (Table 1). However, a significant difference ($p<0.05$) was observed in HCT, MPV and PLT counts across the 250, 500, and 1000 mg/kg ML-treated groups compared to control. Similarly, NE and LYM levels differed significantly ($p<0.05$) in the 500 and 1000 mg/kg groups.

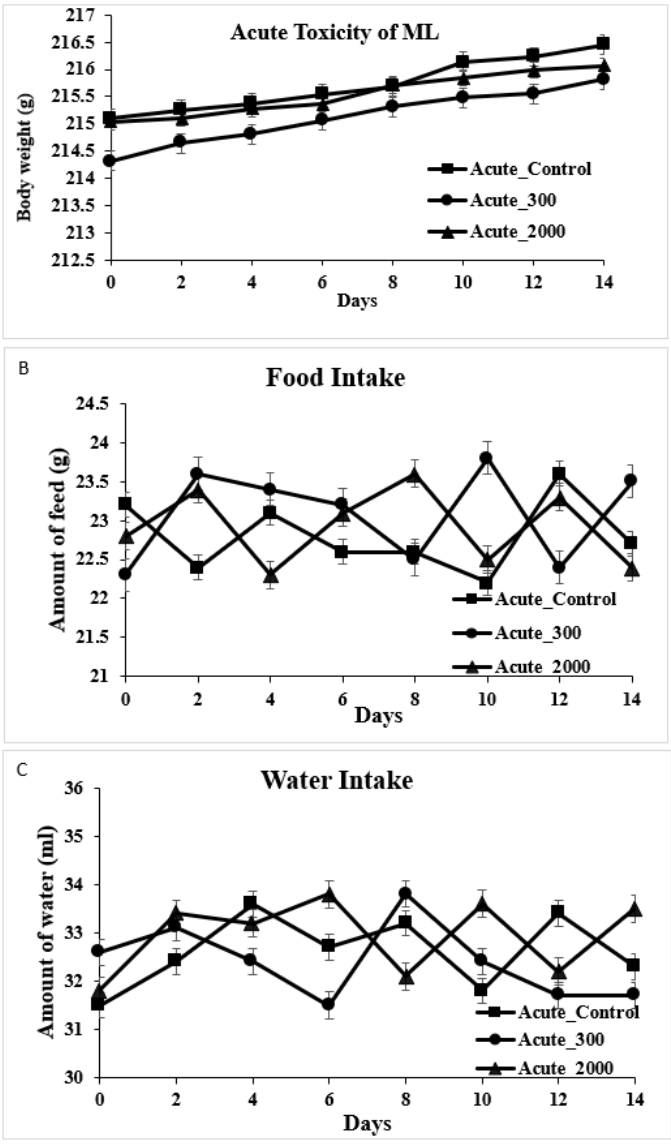

Figure 1: Effect of acute administration of Methanolic (ML) Leaf extract of *A. hierochuntica* on the A) Body weight, Food intake and C) Water Intake of the female SD rats. Error bars represent SD.

Table 1: Effects of sub-acute administration of the Methanolic (ML) Leaf extract of *A. hierochuntica* on the hematological and Serum Biochemical parameters of the SD rats. Each value represents the mean±SD. *Significant difference (p<0.05) compared with the normal.

	Parameters	Control	250 mg/kg	500 mg/kg	1000 mg/kg
Hematological	WBC (10^3/µL)	7.3±1.7	7.2±1.6	7.8±1.5	7.5±1.4
	RBC (10^6/µL)	7.6±1.3	7.335±1.1	7.258±0.6	7.005±0.8
	HGB (g/dL)	14.1±1.8	14.6±1.6	13.3±2.6	13.5±1.8
	HCT (%)	35.55±2.5	44.5±3.6*	48.5±4.2*	52.75±3.2*
	MCV (fl)	57.38±3.2	56.8±4.5	56.85±3.8	56.92±2.9
	MCH (pg)	18.68±1.6	18.63±2.8	18.66±1.9	18.73±2.6
	MCHC (g/dL)	32.53±3.6	35.16±2.9	38.25±1.8*	34.83±2.5
	MPV (fl)	7.48±1.89	6.96±1.07*	7.12±2.5*	7.08±2.1*
	PLT (10^3/µL)	766.83±80.6	868±84.3*	878.2±75.9*	899.6±58.4*
	NE (%)	18.86±1.58	19.96±2.37	21.54±1.72*	23.93±3.65*
	LY (%)	71.91±5.6	72.53±4.9	73.54±3.5	75.78±4.6
	MO (%)	3.8±0.12	4.06±1.03	3.5±0.59	3.71±1.2
	EO (%)	0.55±0.04	0.5±0.1	0.48±0.12	0.35±0.22
	BA (%)	0±0	0.1±0.1	0.1±0.1	0±0
	NE (10^3/µL)	0.70±0.2	0.75±1.2	0.79±0.02*	0.81±0.04*
	LY (10^3/µL)	5.55±2.36	5.9±0.36	6.12±1.8*	6.45±2.8*
	MO (10^3/µL)	0.19±0.2	0.17±0.2	0.21±0.1	0.23±0.1
	EO (10^3/µL)	0.22±0.02	0.21±0.1	0.28±0.98	0.26±0.2
	BA (10^3/µL)	0±0	0±0	0.001±0.01	0.001±0
Biochemical	AST (U/l)	75.333±6.351	80.386±5.78	82.74±7.58	86.166±8.14
	ALT1(U/l)	30.166±5.24	31.666±6.357	35.891±5.64	38.333±5.78
	LDI(U/l)	419.166±89.65	399.833±45.678	386.578±54.23	372.891±46.38
	CKI(U/l)	375.28±59.56	345.872±68.49	319.257±89.2*	295.017±37.6*
	ALP1(U/l)	188.833±85.37	201.8333±26.34	208.36±65.36	216.1666±42.38
	ALB (mg/dL)	3.9471±0.25	3.505±0.148	3.751±0.381	3.914±0.845
	TP (g/dL)	3.276±1.24	3.685±1.845	4.051±1.359	4.3258±1.754
	GLU (mg/dL)	150.82±32.58	157.982±26.258	142.489±36.15*	138.031±25.367*
	CRE2 (mg/dL)	0.412±0.12	0.4254±0.324	0.458±0.21	0.482±0.325
	IP (mg/dL)	5.154±1.58	5.944±2.314	6.214±1.35*	6.429±1.58*
	TBIL (mg/dL)	0.510±0.02	0.517±0.01	0.53±0.05	0.55±0.12
	BUN (mg/dL)	18.486±2.87	19.357±6.57	20.4896±1.98	21.582±3.614
	CHOL (mg/dL)	78.716±6.25	73.308±8.256	71.248±4.15	67.367±4.259
	TRIG (mg/dL)	82.666±14.25	80.151±23.87	74.25±31.56*	68.624±15.478*
	DBIL (mg/dL)	0.133±0.001	0.13248±0.023	0.142±0.058	0.148±0.057
	K (nmol/dL)	4.285±0.587	4.198±0.896	4.523±0.647	4.816±0.951
	Na (nmoldL)	135.479±26.58	139.257±29.74	140.833±35.168	143.789±34.119
	Cl (nmol/dL)	98.591±21.52	101.691±19.39	102.127±22.63	104.847±36.112

In serum biochemistry, the 250 mg/kg group showed no significant changes (p>0.05) versus control (Table 1), while significant differences (p<0.05) in CK, Glu, IP, and TRIG were noted in the 500 and 1000 mg/kg groups. CK, Glu, and IP levels significantly decreased, whereas TRIG levels increased compared to control.

Histological analysis revealed normal liver architecture in control and 250 mg/kg ML-treated rats, with intact central veins, hepatocyte organization, and occasional binucleated cells. At

500 mg/kg, liver sections showed congested central veins and hemorrhage, while the 1000 mg/kg group exhibited inflammatory infiltration, bile duct irregularities, edema, cytoplasmic degeneration, and microvesicular steatosis (Figure 4). Kidney sections from control and 250 mg/kg groups appeared normal, with intact glomeruli and tubules. Mild tubular cell degeneration was observed at 500 mg/kg, and at 1000 mg/kg, glomerular foam cells, tubular degeneration, and cast formation indicated significant damage (Figure 4). Spleen tissue from control and 250 mg/kg groups showed normal white and red pulp separation. The 500 mg/kg group displayed mild vascular changes, whereas the 1000 mg/kg group showed white pulp diffusion into red pulp and hemosiderin deposition (Figure 4).

Anti-breast study of Methanolic (ML) in DMBA-induced rat model

The anti-breast cancer study of ML revealed no treatment-related deaths in DMBA-induced rats treated with either 200 or 400 mg/kg body weight doses. Tumors first appeared in 50% of the rats by the 10th week post-DMBA administration, and by the 14th week, all DMBA-induced rats had developed at least one tumor.

The BC control group developed a total of 20 tumors, averaging 3.3 tumors per animal. In contrast, the 200 mg/kg ML-treated group developed 15 tumors (2.5 tumors per animal), the 400 mg/kg ML-treated group developed 10 tumors (1.8 tumors per animal), and the Tamoxifen-treated group had 8 tumors (1.3 tumors per animal). A significant reduction (p<0.05) in tumor number was

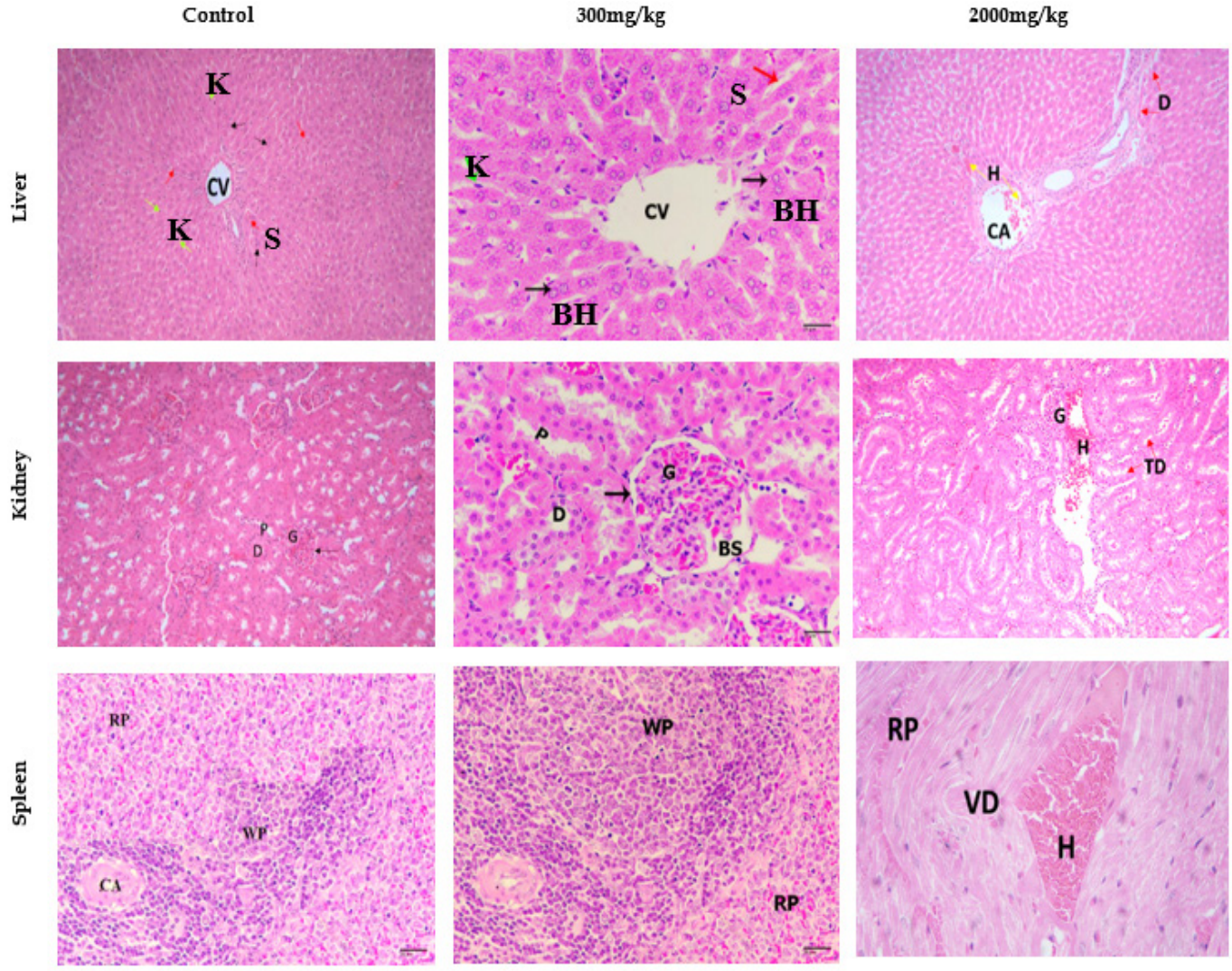

Figure 2: Photomicrograph of liver, Kidney and Spleen section of the SD rats during the acute administration of Methanolic (ML) extract of *A. hierochuntica* (H&E 400×) captured using a Olympus BX53, Olympus Corporation, Tokyo, Japan showing Central vein (CV), Kupffer cells(K), Sinusoids(S), Binucleated hepatocytes (BH), Central Artery (CA), Dilation(D), Haemorrhage(H), Proximal (P), Glomerlus (G), Distal (D), Tubule Dialtion (TD), Cytoplasmic degeneration (CD), Red pulp (RP), White pulp (WP), Central arteriole (CA), mild Vascular dilation (VD)

Table 2: Effects of the Methanolic (ML) Leaf extract of *A. hierochuntica* on the hematological parameters and Serum Biochemical parameters of the female SD rats induced with DMBA. Each value represents the mean±SD. *Significant difference ($p<0.05$) compared with the normal control.

	Parameters	Normal control	BC control	200mg/kg of ML	400mg/kg of ML	Tamoxifen
Hematological	WBC (10^3/µL)	3.7±0.7	6.2±0.06 *	5.9±0.15 *	4.3±1.04	3.6±1.08
	RBC (10^6/µL)	3.2±1.3	2.2±1.1 *	3.4±0.6	3.6±0.8	3.8±1.5
	HGB (g/dL)	10.4±1.8	9.46±1.6	11.6±2.6	12.1±1.8	12.5±2.1
	HCT (%)	40.55±2.5	54.5±3.6 *	50.5±4.2*	45.75±3.2	43.86±3.9
	MCV (fl)	84.2±3.2	70.2±4.5 *	80.1±3.8	82.2±2.9	83.5±3.6
	PLT (10^9/l)	165.6±80.6	144.2±84.3*	206.2±75.9*	184.6±58.4	170.3±24.1
	NE (%)	42.8±1.58	72.5±2.37 *	52.8±1.72 *	48.1±3.65	46.2±1.8
	LY (%)	17.8±5.6	36.6±4.9 *	28.3±3.5 *	25.2±4.6	23.1±1.5
	MO (%)	4.8±0.52	6.9±2.03 *	5.8±1.59	5.1±1.12	4.9±2.4
	EO (%)	0.39±0.04	2.63 ±0.1*	1.82±0.12*	0.82±0.22	0.41±1.7
	BA (%)	0±0	0.1±0.1	0.1±0.1	0±0	0±0
Biochemical	AST(U/l)	122.2±1.2	202.81.08 *	170.1±1.3 *	141.2±2.31	134.6±2.45
	ALT1(U/l)	95.1±2.2	212.41.2 *	165.2±1.1 *	144.4±1.42	123.2±3.2
	LDI (U/l)	399.83±45.67	419.16±89.65 *	402.175±59.5	386.57±54.23	372.89±46.38
	CKI (U/l)	304.26±26.62	375.28±59.56 *	345.82±68.49	319.257±89.2	295.017±37.6
	ALP1(U/l)	58.4±3.2	98.31.4 *	80.1±1.3 *	69.6±1.2	60.2±1.6
	ALB (mg/dL)	4.18±2.6	1.965.4 *	3.4±4.2	3.8±3.2	4.2±2.6
	TP (g/dL)	1.92±2.4	2.22±1.6 *	1.98±1.2	1.442.22	1.88±1.06
	Urea(mg/dL)	36.4±4.2	59.81.7 *	46.1±1.6 *	41.4±1.4	38.2±1.2

observed in the 400 mg/kg ML-treated and Tamoxifen-treated groups compared to the BC control (Figure 5A).

Since tumor incidence is closely associated with tumor weight, the dissected mammary neoplastic tissues showed a significant reduction ($p<0.05$) in tumor weight in the 400 mg/kg ML-treated and Tamoxifen-treated groups compared to the BC control. No significant differences ($p>0.05$) in overall body weight were observed among the DMBA-induced rats treated with ML extracts or Tamoxifen. However, a significant decrease ($p<0.05$) in body weight was recorded in the DMBA-induced BC control group from day 21 until the end of the study compared to the ML and Tamoxifen-treated groups (Figure 5B).

The analysis of the LPO, CAT and GSH antioxidants were assessed in the breast tissue homogenates of the control (normal and BC) and experimental groups, as shown in Figure 5C. A significant reduction ($p<0.05$) in LPO levels was observed in the 400 mg/kg ML-treated and Tamoxifen-treated groups compared to the elevated levels seen in the BC control group. Conversely, a significant increase ($p<0.05$) in GSH and CAT levels was noted in the 400 mg/kg ML and Tamoxifen groups relative to the reduced levels in the BC control. No significant differences ($p>0.05$) were found between the 200 mg/kg ML-treated group and the BC control. Notably, the methanolic leaf extract of *A. hierochuntica* contains bioactive compounds such as luteolin, apigenin, and quercetin, which are known to modulate oxidative stress and may contribute to the observed reduction in LPO and increase of GSH and CAT levels.

A significant difference ($p<0.05$) was observed in all hematological parameters of the BC control group compared to the normal control (Table 2). Likewise, the 200 mg/kg ML-treated group showed a statistically significant difference ($p<0.05$) in WBC, HCT, PLT, NE, LYM, and EO levels compared to the normal control. In contrast, no significant differences ($p>0.05$) were noted between the 400 mg/kg ML- or Tamoxifen-treated groups and the normal control.

An increase in AST, ALT, ALP, and urea levels was observed in the 200 mg/kg ML-treated group, showing a statistically significant difference ($p<0.05$) compared to the normal control. Similarly, all serum biochemical parameters in the BC control group differed significantly ($p<0.05$) from the normal control (Table 2). However, no significant differences ($p>0.05$) were noted between the 400 mg/kg ML- and Tamoxifen-treated groups and the normal control.

Histopathological analysis of the mammary glands (Figure 6) revealed that the control group exhibited a normal structure of Seromucous Glands (SMG) and Mucous Glands (MG). In contrast, the BC group showed cystic papillary adenocarcinoma, muscular invasion, Periductal Stromal Fibrosis (PSF), fatty tissue infiltration, and Atrophy (A) of seromucous glands with

stromal fibrosis (SF). The 200 mg/kg ML-treated group displayed mild fibrotic connective and adipose tissue, Moderate Ductal Dilatation (MDD), and the presence of Dead Cells (DC) within the lumen. In the 400 mg/kg ML-treated group, fewer Cystic Lesions (CL) were observed, with diffuse neutrophil Infiltration (IN), reduced vascular congestion, and minimal fibrocollagenous tissue. Tamoxifen treatment resulted in reduced Collagenous Tissue (LCT), complete absence of mucin, and the presence of dense fibrotic connective tissue.

DISCUSSION

In vivo toxicity is imperative to evaluate the herbs' short- or long-term effects on human health for its assessment of safe consumption. Studies on the acute toxicity of ML at 300 and 2000 mg/kg for 14 days showed that the weight of the animals was within the stipulated range; the body weight changes were acceptable for the female SD rats of this age. Therefore, the current findings suggest that only transient changes were observed, and no abnormal inferences in food, water intake and histopathology were noted. Similar results to the present study were reported showing no obvious behavioural changes when dosed with up to 3 g/kg of ethanolic extract of *A. hierochuntica*, supporting the plant's safety for consumption.[11]

Further, *in vivo* mammalian erythrocyte micronucleus assay results demonstrated no significant reduction in the polychromatic and normochromic ratio in SD rats when dosed with up to 2000 mg/kg of aqueous extract of *A. hierochuntica*.[24] As well as the LD50 cut-off value of ML extracts exceeded 2000mg/kg, it was therefore classified as Category 5 according to the Globally Harmonized System (GHS) of classification and labelling of chemicals and provided direct relevance for protecting animal and human health up to the high dose level that used in this study. It is suggested that ML extracts are safe or non-toxic.

Parallelly, the sub-acute toxicity of Methanolic leaf extract of *A. hierochuntica* after a repeated dose of extract 250,500 or 1000 mg/kg for 28 days reported no death of rats during the studied period. ML administered SD rats showed a normal increase in the body weight of the female SD rats. These increases were not considered related to administering the ML extract, as a gain in body weight was also observed in the control group. The haematology parameters showed significant changes in HCT, MPV and PLT in the ML-treated group compared to the control group. However, these changes remained in the normal range of the reference data.

Nevertheless, the significant difference observed in the lymphocytes and neutrophils among the group treated with 500 and 1000 mg/kg of ML compared to control seemed to increase dose-dependent. The release of natural stress hormones regulates the levels of NE and LY in normal physiology. It helps the body's preparedness to initiate the inflammatory response and respond to and Figure against various diseases.[25] Thus, the increased levels of NE and LY may be due to the release of the stress hormone in the female SD rats and substantially do not harm the SD rats' immune system. Likewise, the serum biochemical parameters showed a significant difference in the CK, Glu, IP and TG levels among the group treated with 500 and 1000 mg/kg of ML compared to the control. A previous study reported that the oral administration

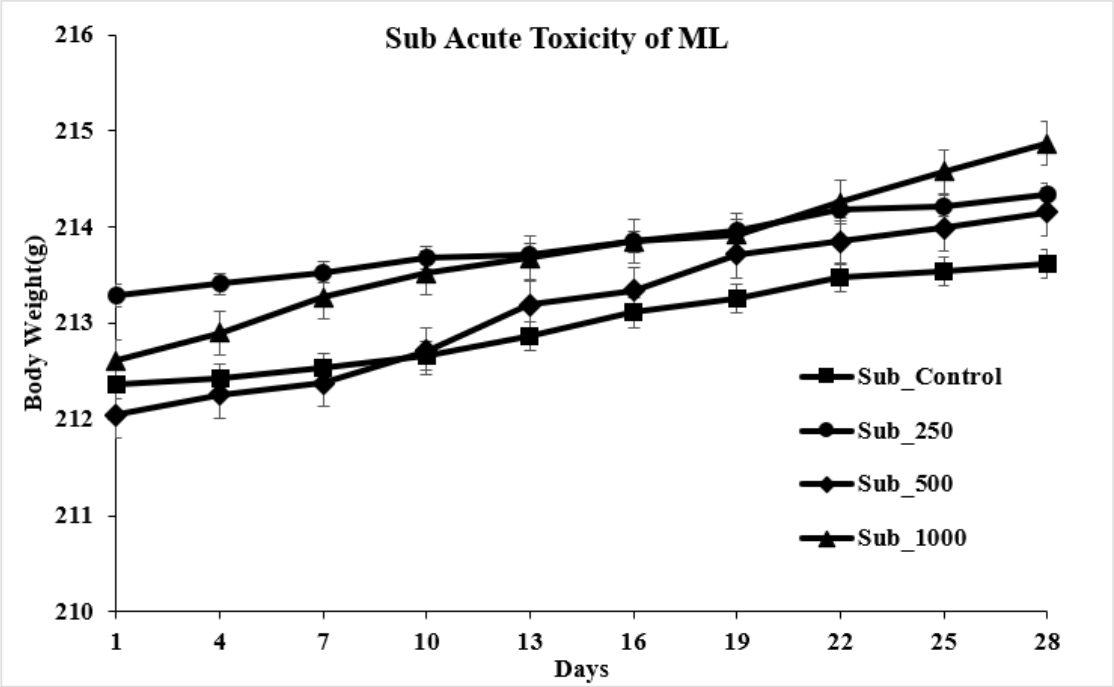

Figure 3: Effect of subacute administration of Methanolic (ML) Leaf extract of *A. hierochuntica* on the Body weight of the female SD rats. Error bars represent SD.

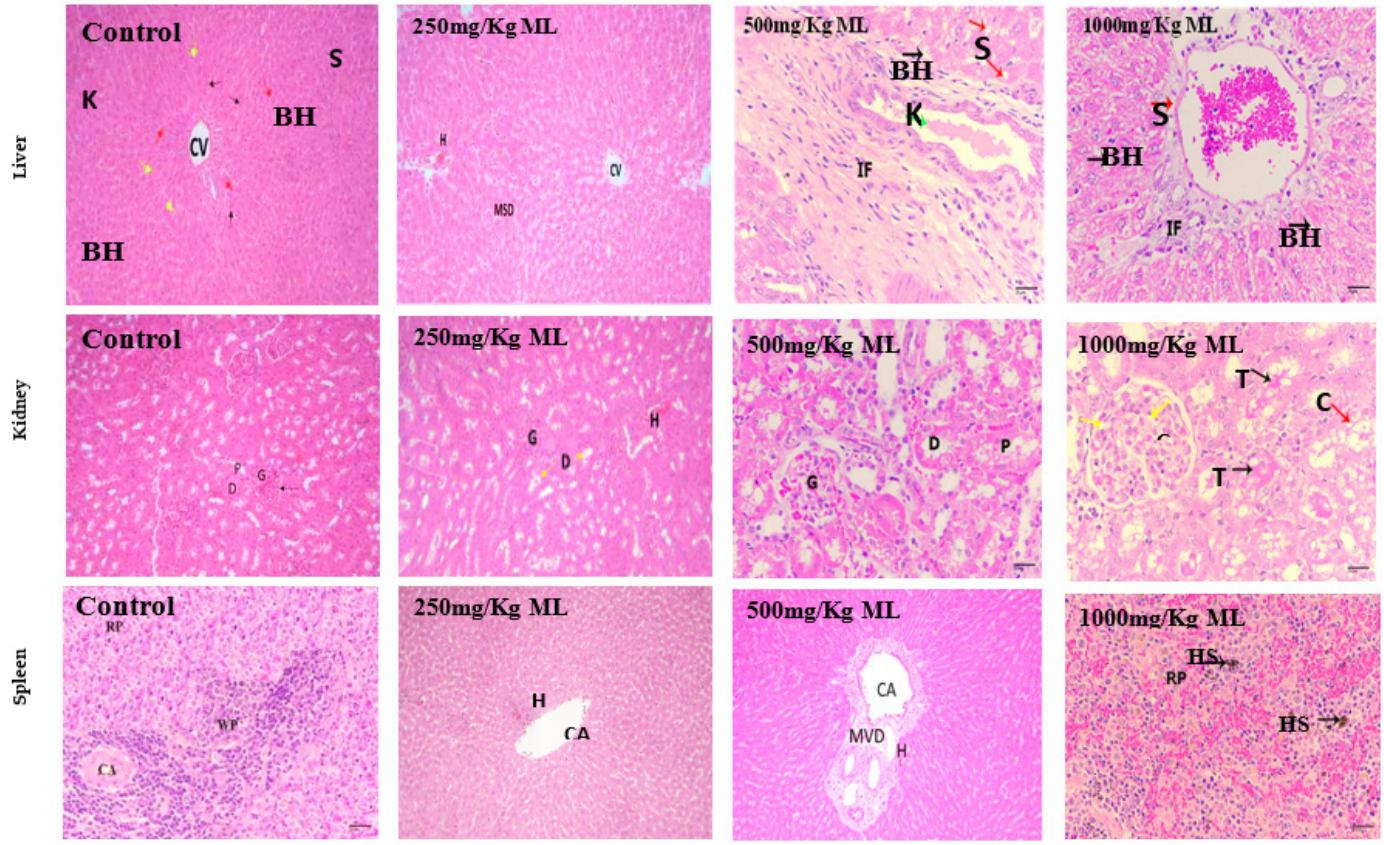

Figure 4: Photomicrograph of liver, Kidney and Spleen section of the SD rats during the Sub Acute administration of Methanolic leaf extract of *A. hierochuntica* (H&E 400×) captured using a Olympus BX53, Olympus Corporation, Tokyo, Japan showing Central vein (CV), Kupffer cells(K), Sinusoids(S), Binucleated hepatocytes (BH), Central Artery (CA), Dilation(D), Haemorrhage(H), Proximal (P), Glomerlus (G), Distal (D), Tubule Dialtion (TD), Cytoplasmic degeneration (CD), Red pulp (RP), White pulp (WP), Central arteriole (CA), mild Vascular dilation (VD), hemosiderin granules (HS).

of 100 mg/kg methanolic extract of *A. hierochuntica* significantly reduced the TG, LDL, VLDL and TC levels in Swiss albino rats.[26]

Correspondingly, the hypoglycemic effect of methanolic extract of *A. hierochuntica* studied reported the restoration of β-cells in the pancreas with a 74% reduction of glucose levels in alloxan-induced diabetic male Swiss albino rats.[27] Consistently, similar effect in Streptozotocin (STZ)-induced male albino Wistar rats administered with the aqueous extract of *A. hierochuntica* reported significant reductions in Glucose and Triglycerides as showed by ML extracts to therapeutically benefit from lowering the lipid and diabetic levels.[28]

Minor histopathological alterations were observed in the 1000 mg/kg ML-administered group. Still, however, these liver, spleen and kidney changes did not alter the normal levels of the liver and hepatic enzymes. Likewise, the aqueous extract of *A. hierochuntica* reported the Nonmonotonous Dose-Response Curve (NMDRC) in prenatal toxicity and presented a No-Observed Adverse Effect Level (NOAEL).[29] Thus, based on the biochemical, haematological parameters, and histopathological examination in the present study, ML was found to be non-toxic and safe to be used at higher concentrations and for an extended period with NOAEL greater than 1000 mg/kg.

In the present study, we evaluated the potential chemo effect of Methanolic Leaf (ML) extract of *A. hierochuntica* against DMBA-induced mammary cancer in SD rats. In our study, the tumour latency period was from 10 to 14 weeks, similar to the outcomes of the previous study reporting the tumour latency period from 8 to 21 weeks for the DMBA-induced rat model showing 100% tumour incidences.[30] In this study, compared to the BC group, we observed a significant ($p<0.05$) reduction in incidences of the tumour with 1.8 tumours per animal and tumour weight of 4.68 g in the treatment group receiving 400 mg/kg of ML, similar to the tamoxifen-treated rats with 1.3 tumours per animal with 3.81 g of tumour weight. Such comparable results were also obtained for natural-based products such as berberine,[31] naringenin,[32] citronellol[33] and Honokiol[34] in DMBA induced mammary cancer due to enriched antioxidant potentials of these compounds.

In addition, the BC control group showed a highly significant decrease ($p<0.05$) in body weight compared to the control and treated group due to the change in energy metabolism because of tumour formation.[35] In other words, this could be explained by the fact that the intake of 200 and 400 mg/kg of ML extract did not induce any toxic effect on the DMBA-induced rats resulting

in body weight gain. Similar results were observed in the 20 mg/kg of resveratrol treated in DMBA rats with no effects on the tissue weights.[36]

The haematological parameters deliver a considerate understanding of the body's physiological conditions. In this study, the 400 mg/kg of ML treated group restored the haematological parameters to normal limits, similar to the normal control and tamoxifen-treated group. BC control group showed a significant difference ($p<0.05$) in almost all the parameters compared to the normal group. A higher incidence of total WBC with lymphocytes, monocytes and neutrophils and decreased platelets indicates the stimulation of the toxic and immunosuppressive potency of the carcinogen.[37] Similarly, the 200 mg/kg of ML also showed variation in WBC, PLT, HCT, NE, and EO levels could be due to the dose-dependent effect of ML to achieve the normal range, which is similar to the results of *Piper aduncum* in the DMBA induced mammary carcinoma.[38]

The analysis of serum biomarkers was considered an ideal to monitor and assess the response of the cancer treatment methods and acts as pathophysiological diagnostic markers. It is evident to report from the present study that the abnormal levels in liver enzymes (AST, ALT, ALP) in the BC group are due to the disruptions caused by the DMBA, leading to the damage of the structural integrity of the cells resulting in the cytoplasmic leakage of enzymes into the blood. The upsurge in the AST and ALP in the blood could be a consequence of tissue injury, while ALP would be due to the release of toxic metabolites resulting in cancer progression. Similarly, the increased LDH due to the high glycolysis rate would promote the energy to cancer cells to attain solid neoplasm.[39]

The liver acts as the main target organ for the metastasis of BC.[40] In the present study, both 200 and 400 mg/kg of ML treated group reinstated the enzymes to normal limits. However, a significant difference ($p<0.05$) was observed for AST, ALP and ALP for 200 mg/kg of ML treated group compared to the normal control and tamoxifen-treated group, but the values are still under the normal limits. These results corroborate previous reports stating the hepatoprotective potential of the *A. hierochuntica* extract.[12,27] Studies have affirmed the presence of novel compounds Anastatin A, B and silybin in the plant extract would have normalised the serum markers and thus ensure the safety and non-toxic nature

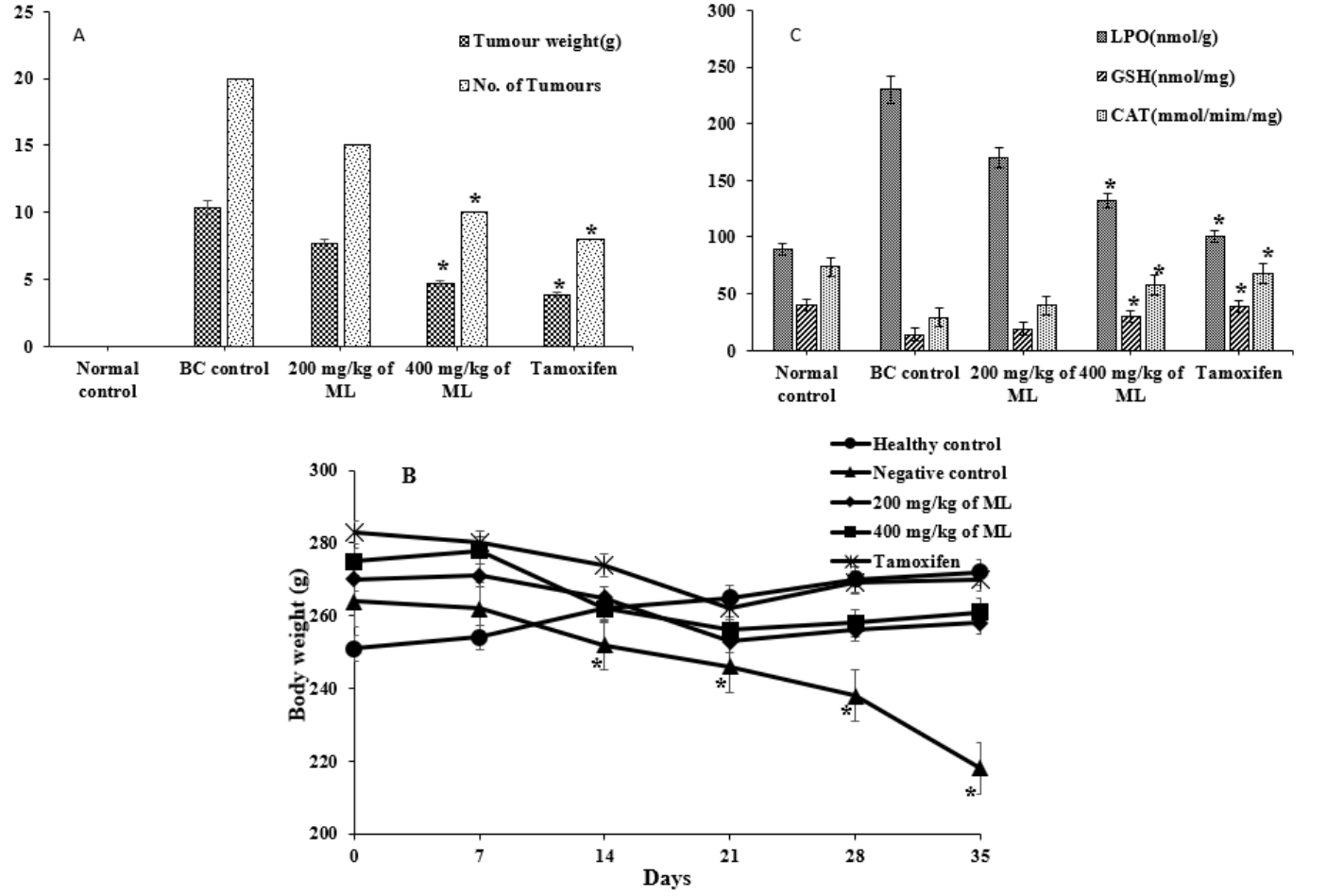

Figure 5: Effects of the Methanolic (ML) Leaf extract of *A. hierochuntica* on A) number and weight of tumours, B) Body weight changes C) antioxidant markers in DMBA-induced SD rats. Error bars represent SD. *Significant difference ($p<0.05$) compared with the BC control.

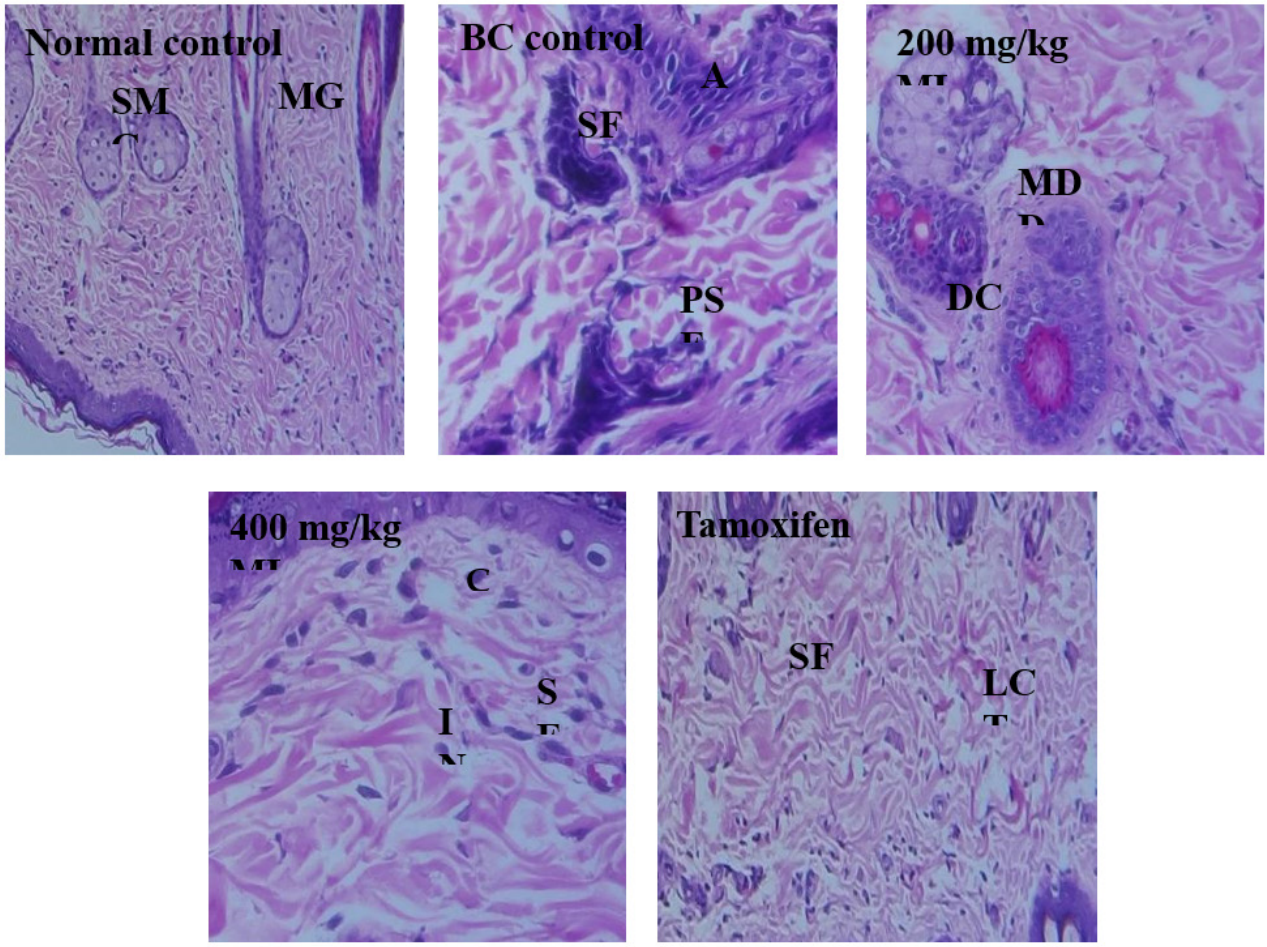

Figure 6: Histopathological changes observed in the mammary gland of the DMBA-induced SD rats treated with Methanolic leaf extract of *A. hierochuntica* (H&E 800×) captured using a Olympus BX53, Olympus Corporation, Tokyo, Japan showing Seromucous gland (SMG), Mucous gland (MG), Atrophy (A), Stromal fibrosis (SF), Periductal Stromal Fibrosis (PSF), Moderate dilatation ducts (MDD), Dead cells (DC), Cystic lesion (CL), Infiltrating Neutrophils (IN) and less collagenous tissue (LC).

of the extract's ability to reduce both oxidative stress and the risk of BC cancer.

The mechanistic carcinogenic action of DMBA involves the activation of Aryl hydrocarbon receptor and induces cytochrome P450 enzyme to convert the DMBA to epoxide mutagenic intermediates, the 7-hydroxymethyl-12-methylbezantracene (7-HMBA), 12-hydroxymethyl-7-metilbezantracene (12-HMBA).[41] Both 7-HMBA and 12-HMBA are toxic compounds commonly found in the environment translocated to the mammary and are capable of forming DMBA-DNA adducts, causing DNA damage and increasing oxidative stress to produce immunotoxicity.[42,43] Therefore, in the present study, the breast tissue homogenate of the DMBA treated with ML extract was analysed to evaluate the antioxidant status of the plant.

The carcinogenesis of DMBA provokes oxidative stress resulting from the excessive production of Reactive Oxygen Species (ROS), which is responsible for the damage of nucleic acids, protein, and lipids in the target cells and tissues, playing a vital role in the initiation and progression of cancer.[44] In the present study, the BC control group observed a significantly high increase in lipid peroxidation level. It was mainly attributed to the oxidation of lipid measured as thiobarbituric acid, resulting in the peroxidation of unsaturated fatty acids augmenting the inhibition of bio-oxidase activities due to free radical propagation.[45] These stress conditions cause DNA damage, resulting in mutated oncogenic genes have reported that lipid peroxidation is altered during carcinogenesis,[46] similar to our result in the current experimental investigation. The increased level of TBARS was significantly reduced upon treatment with 400 mg/kg of ML of *A. hierochuntica* due to the hypolipidemic potential of the plant extract. The flavonoid content in the plant extract was successfully reported to improve rodent lipid levels by regulating adiponectin secretion in lipid metabolism.[26,27]

The enzymatic antioxidant Catalase (CAT) is the main defence system to protect and guard the cells against ROS and oxidative damage during carcinogenesis. CAT (peroxisomal heme) contains the protein catalyses the elimination of Hydrogen Peroxide (H_2O_2) to water and suppresses the free radical formation.[47] The current study observed a significant change in CAT enzyme activity in

400 mg/kg ML-treated DMBA-induced rats to a normal level. The increased CAT enzyme activity was observed in the experimental group in contrast to the BC group representing the deterioration of H_2O_2 by the antioxidant defence system of ML extracts.

The non-enzymatic antioxidant GSH maintains cell viability by regulating the inner membrane permeability of mitochondria. It also acts as a major intracellular reducing agent by sustaining the sulfhydryl groups in a reduced state.[48,49] GSH is a chain-breaking antioxidant that scavenges the ROS and neutralises the peroxidation of polyunsaturated fatty acids generated from the metabolism of the carcinogens through the conjugation process.[50] This study showed reduced GSH levels were significantly upregulated in 400 mg/kg of ML and tamoxifen-treated rats. At the same time, the BC group showed a decreased GSH level due to the oxidative stress exerted by the DMBA, leading to cellular damage and loss of functional integrity of the cellular membrane. These results align with a previous study reporting the increased antioxidant CAT and GSH enzyme activity of the *A. hierochuntica* in Carbon tetrachloride-induced hepatotoxicity rats.[13] These results also suggest that the phytochemical component of *A. hierochuntica* extracts may attenuate oxidative stress by decreasing the lipid peroxidation and increasing the antioxidative wielded by the chemical carcinogen DMBA in the rats.

Luteolin has been shown to activate the Sirt1Nrf2 signaling axis, leading to upregulation of endogenous antioxidant defenses (such as CAT and GSH)[51] and upregulate endogenous antioxidant enzymes and scavenge ROS, thereby reducing LPO.[52] Apigenin, in turn, is known to promote nuclear translocation of Nrf2 and enhance expression of AREdriven antioxidant genes,[53] resulting in elevated CAT and GSH levels, as demonstrated in cell models under oxidative stress.[54] Additionally, quercetin has been reported to modulate the p38 MAPK/Nrf2 pathway, increasing expression of glutathione-related enzymes and restoring intracellular GSH, thereby strengthening cellular antioxidant capacity.[55] The presence of these compounds in the ML extract likely contributes to the chemopreventive potential of the ML extract in our DMBAinduced BC model.

Histopathological analysis of the mammary glands revealed cystic lesions and atrophy of seromucous glands in the DMBA-induced group. These alterations were markedly ameliorated in the 400 mg/kg ML-treated group, with reduced congestion, decreased fibro-collagenous deposition, and restoration of normal tissue architecture. These findings, together with the antioxidant effects, suggest that the phytochemical components of A. hierochuntica mitigate DMBA-induced oxidative damage and tissue pathology, reinforcing its potential as a natural therapeutic agent for BC.

Furthermore, the outcomes from our previous research[16] demonstrated that the antiproliferative nature of the Methanolic (ML) leaf extract of *A. hierochuntica* against BC involved induction of apoptosis and growth arrest, which is reflected in the reduced number of tumors observed in the treatment group. These *in vitro* findings, including activation of caspase-3, provided a mechanistic rationale for evaluating the extract in a DMBA-induced BC model. The results were concordant with other natural product-based interventions validated against breast cancer in DMBA-induced rat models. The study's findings are further supported by the phytochemical identification of key metabolites, such as Apigenin-6-C-Glucoside, Luteolin-8-C-Glucoside, and other flavonoids and phenolic compounds known for their antioxidant and anti-breast cancer properties, suggesting that these bioactive components contribute to the chemopreventive effects of the ML extract. However, we acknowledge that molecular markers of apoptosis and signaling modulation, including Bcl-2, p53, Ki67, and HER2, were not assessed *in vivo*, representing a limitation of the current study and warranting further investigation to fully confirm the extract's pro-apoptotic mechanisms *in vivo*.

CONCLUSION

In this study, the acute oral toxicity of the Methanolic Leaf (ML) extract of *Anastatica hierochuntica* was classified as Globally Harmonized System (GHS) Category 5**,** indicating a low toxicity profile. The sub-acute toxicity assessment demonstrated a No-Observed-Adverse-Effect Level (NOAEL) exceeding 1000 mg/kg**,** confirming the extract's safety at high doses. The ML extract also exhibited significant chemopreventive effects in the DMBA-induced rat model, including dose-dependent reduction of tumor development and enhancement of antioxidant status. Additionally, ML treatment improved serum liver biomarkers and restored mammary gland histopathology damaged by DMBA. Overall, these findings highlight the extract's favorable safety profile and potent chemopreventive potential, supporting its promise as a safe and effective natural therapeutic agent**.** Importantly, the combination of *in vitro* mechanistic evidence, *in vivo* efficacy, and low toxicity underscores the translational relevance of the ML extract, supporting its further development as a safe, natural therapeutic or adjunct for breast cancer prevention and treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, S.R., S.A.M and M.A.W.C; Methodology, S.R., A.B.A., and S.A.M.; Software, K.G. and N.A.R.; Validation, S.A.M.Y., R.E.H., S.A.M and S.R; Formal analysis, S.A.M,

S.A.M.Y, N.A.R. and R.E.H.; Investigation, S.R., and K.G; Resources, S.R. and A.B.A.; Data Curation, S.R. and Z.A.; Original draft preparation, S.R., S.A.M, A.B.A., M.A.W.C; Review and Editing, SS.R., S.A.M, A.B.A, M.A.W.C, K.G., N.A.R, S.A.M.Y., R.E.H; Visualization, S.R., A.B.A, N.A.R, and K.G; Supervision, S.A.M.Y., N.A.R, and S.A.M., Project Administration, K.G. and R.E.H; Funding acquisition, M.A.W.C.

ABBREVIATIONS

BC: Breast Cancer; **CA:** Central Artery; **CAT:** Catalase; **CK:** Creatinine Kinase; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **DMBA:** 7,12-Dimethylbenz[a]anthracene; **EO:** Eosinophils; **GHS:** Globally Harmonized System; **GSH:** Reduced Glutathione; **IN:** Infiltrating Neutrophils; **IP:** Inorganic Phosphorus; **LYM:** Lymphocytes; **ML:** Methanolic Leaf; **NE:** Neutrophils; **PSF:** Periductal Stromal Fibrosis; **SD:** Sprague Dawley; **TRIG:** Triglycerides.

SUMMARY

The study evaluated the acute and sub-acute toxicity, along with the anti-breast cancer potential, of the Methanolic Leaf (ML) extract of Anastatica hierochuntica in Sprague-Dawley rats, using a 7,12-Dimethylbenz[A]Anthracene (DMBA)-induced model. Toxicity assessments indicated that the ML extract has a high safety profile, showing no treatment-related mortality or significant physiological changes up to 2000 mg/kg in the acute study and establishing a No-Observed-Adverse-Effect Level (NOAEL) exceeding 1000 mg/kg in the sub-acute study. In the anti-breast cancer investigation, ML treatment significantly reduced tumor incidence and tumor weight, with the 400 mg/kg dose exhibiting the greatest efficacy. Mechanistically, ML improved oxidative stress markers by decreasing Lipid Peroxidation (LPO) levels and increasing the activity of Catalase (CAT) and reduced Glutathione (GSH). Histopathological findings corroborated the results, showing a reduced tumor burden and improved mammary architecture in the ML-treated groups. Overall, the ML extract demonstrated significant anti-breast cancer potential in DMBA-induced rats, supported by a favorable toxicity profile.

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