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D Vasavi
Department of Veterinary
Pharmacology and Toxicology,
Nagpur Veterinary College,
Nagpur, Maharashtra, India

Alka Sawarkar
Associate Professor,
Department of Veterinary
Pharmacology and Toxicology,
Nagpur Veterinary College,
Nagpur, Maharashtra, India

Rajesh Limsay
Professor, Veterinary
Department of Pharmacology
and Toxicology, Nagpur
Veterinary College, Nagpur,
Maharashtra, India

Aravind Ingle
Scientific Officer 'H', Tata
Memorial Centre, Mumbai,
Maharashtra, India

Manoj Patil
Professor, Veterinary,
Department of Animal
Reproduction Gynaecology &
Obstetrics, Nagpur Veterinary
College, Nagpur, Maharashtra,
India

Megha Kaore
Assistant Professor,
Department of Veterinary
Pathology, Nagpur Veterinary
College, Nagpur, Maharashtra,
India

Sujit Kolangath
Subject Matter Specialist,
Wildlife Research Training
Centre (WRTC), Gorewada,
Nagpur, Maharashtra, India

Corresponding Author:
Alka Sawarkar
Associate Professor,
Department of Veterinary
Pharmacology and Toxicology,
Nagpur Veterinary College,
Nagpur, Maharashtra, India

Antitumor potential of hydroethanolic extract of *Neolamarckia cadamba* on p53 expression and CA 15-3 levels in DMBA-induced mammary carcinoma in rats

D Vasavi, Alka Sawarkar, Rajesh Limsay, Aravind Ingle, Manoj Patil, Megha Kaore and Sujit Kolangath

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Abstract

Neolamarckia cadamba has shown promising antitumor efficacy due to its rich phytoconstituents such as flavonoids, alkaloids, and triterpenoids, which are associated with antioxidant and anticancer activities. Studies suggest it may help suppress tumor growth by reducing oxidative stress, and influencing cellular proliferation pathways. This study aimed to evaluate the antitumor potential of the hydroethanolic extract of stem bark of *Neolamarckia cadamba* (HENC) by assessing p53 expression and Cancer Antigen 15-3 (CA 15-3) levels in 7,12-dimethylbenz anthracene (DMBA) induced mammary carcinoma in female Sprague Dawley rats. Mammary tumors were induced by a single subcutaneous dose of 7,12-dimethylbenz anthracene (DMBA) @ 20 mg/kg body weight. Animals were divided into five groups: normal control, positive control, standard treatment (tamoxifen @ 3.3 mg/kg body weight), and two treatment groups receiving hydroethanolic extract of *Neolamarckia cadamba* at 500 mg/kg and 600 mg/kg body weight respectively orally for 30 days. Serum CA 15-3 levels were measured using ELISA, while p53 expression in mammary tissue was evaluated by immunohistochemistry.

Keywords: Mammary tumors, (DMBA), *Neolamarckia cadamba*, Sprague Dawley rats

Introduction

Breast cancer is a major global health concern with diverse molecular subtypes, complex risk factors, and varied clinical outcomes, requiring improved understanding for effective prevention and treatment. Continued research is essential to refine targeted treatments and improve long-term patient management [1]. The incidence of breast cancer can rise by up to 40%, or 3 million cases annually, worldwide, and the number of fatalities from the disease might rise from 685,000 in 2020 to 1 million in 2040, accordingly to the journal 'The Breast' [2]. In outbred Sprague Dawley rats, the polycyclic aromatic hydrocarbon-7,12-dimethylbenzanthracene (DMBA) is frequently utilized to cause breast tumors [3]. DMBA is a carcinogenic compound whose tumor-inducing effects are influenced by ovarian hormones. The DMBA-induced rat model is widely used because it produces neoplastic changes such as intraductal proliferation and mammary intraepithelial neoplasia, which closely resemble human ductal carcinoma *in situ* [4]. The p53 tumor suppressor protein, sometimes referred to as the "Guardian of the Genome" is encoded by the TP53 gene. Its main function is to protect the integrity of the cell's DNA. Many malignancies have somatic TP53 mutations, which can result in loss of function, dominant negative, or gain-of-function [5]. By controlling the cell cycle, especially at the G1 to S checkpoint, TP53 helps preserve genomic stability by preventing the replication of damaged DNA. p53 is activated in response to cellular stress or DNA damage, and it can momentarily stop cell division to facilitate repair. It encourages programmed cell death to stop aberrant cell survival if the harm is irreparable. Under normal conditions, p53 levels remain low due to continuous degradation, but they rise rapidly in response to cellular stress [6]. CA 15-3 (Cancer Antigen), also known as MUC1, is the most widely used blood marker for breast cancer. In cancer, the large transmembrane glycoprotein MUC1 is frequently overexpressed and glycosylated improperly. At present, Carbohydrate antigen 15-3 (CA 15-3) is primarily utilized for early detection of recurrent breast cancer and for monitoring therapy in patients with advanced stage of disease.

In a prospective analysis with 368 participants, the study showed that individuals with high preoperative levels of CA 15-3 (>30.4 U/mL) performed worse than those with low levels of the marker [7]. Many people with long-term illnesses, including breast cancer, often turn to herbal remedies as supportive therapy. In fact, over half of cancer patients currently use some form of complementary or alternative medicine along with conventional treatment. A member of the *Rubiaceae* family, *Neolamarckia cadamba* is generally known as "Kadamba" in Ayurveda. In the ancient Indian medical system known as Ayurveda, this evergreen tropical tree has substantial therapeutic value. The stem bark, leaves, root bark, flowers, seeds, heartwood, and other sections of *Neolamarckia cadamba* are a veritable gold mine of phytoconstituents. These bioactive substances include fatty acids, alkaloids, terpenoids, flavonoids, and coumarins. Their documented pharmacological actions are diverse and include anti-inflammatory, anticancer, antidiabetic, antioxidant, and wound-healing properties. There are a lot of different botanical roles that *Neolamarckia cadamba* can play [8].

The search for plant derived anticancer therapies has intensified in recent years, highlighting the potential value of traditional medicinal plants' unexplored therapeutic properties. *Neolamarckia cadamba* is one such plant that has gained attention due to its reported pharmacological activities. Based on this, the present study evaluates the hydroethanolic extract of stem bark of *Neolamarckia cadamba* (HENC) for its influence on key biomarkers associated with breast cancer in DMBA induced mammary tumor model in Sprague Dawley (SD) rats. Particular emphasis is placed on CA 15-3, a commonly elevated serum marker in breast cancer, and p53 gene involved in cell cycle control, DNA repair, and apoptosis.

Materials and Methods

Chemicals and reagents

The chemicals and reagents used in the study included 7,12-dimethylbenz(a)anthracene (DMBA) procured from Sigma Pvt. Ltd.; Tamoxifen citrate (Caditam-20) obtained from Cadila Healthcare; Petroleum ether (60-80 °C, AR grade) supplied by Loba Chemie Pvt. Ltd.; Absolute ethanol (99.9% AR grade) from S D Fine Chem Limited; and a Rat CA15-3 ELISA kit (Catalogue No. ER0789, 98T) obtained from Fine Biotech Co., Ltd.. All other chemicals and consumables used were of analytical grade and procured from standard commercial suppliers.

Collection of plant material and extract preparation

Stem bark of *Neolamarckia cadamba* was collected from the university library of Maharashtra Animal and Fishery Sciences University, Seminary Hills, Nagpur. The plant material was authenticated by a professional botanist from Rashtrasant Tukdoji Maharaj Nagpur University. The collected bark was shade-dried, powdered, and defatted using petroleum ether. The dried material was then extracted with 70% ethanol using a Soxhlet apparatus. The obtained extract was concentrated, dried, and stored in airtight conditions until further experimental use.

Experimental animals

Healthy female Sprague Dawley rats of 8 weeks of age weighing between 150 to 200 g were obtained from the Laboratory Animal Breeding Centre, Pune accredited by CCSEA. The study was conducted at the Department of Veterinary Pharmacology and Toxicology, Nagpur Veterinary College. Ethical approval was obtained from the Institutional Animal Ethics Committee in accordance with Committee for Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Protocol No. NVC/IAEC/20/2025). Animals were housed in polypropylene cages under standard laboratory conditions (12-h light/dark cycle, 22-30 °C temperature, and 45-55% relative humidity). Rats were acclimatized for one week prior to the experiment and provided balanced pelleted feed and filtered water ad libitum.

Induction of mammary tumor

Mammary tumors were induced by a single subcutaneous administration of 7,12-dimethylbenz[a]anthracene (DMBA) at 20 mg/kg body weight in the left flank region. Tumor development was confirmed after eight weeks using ultrasonography, and tumor-bearing rats were included for further study.

Experimental design and treatment

Animals were randomly divided into five groups ($n = 10$ per group): T₁ (normal control), T₂ (positive control), T₃ (standard treatment with tamoxifen at 3.3 mg/kg body weight, orally), and two treatment groups, T₄ and T₅, which received the hydroethanolic extract of *Neolamarckia cadamba* at doses of 500 and 600 mg/kg body weight, respectively, administered orally for 30 days (Table 1).

Table 1: Experimental design of study

Sr. No.	Groups	No. of rats	Treatment	Duration of treatment (Days)
1.	T ₁	10	Normal Control	
2.	T ₂	10	DMBA @ 20 mg/kg BW single dose (Induction of Mammary tumor)	Single dose
3.	T ₃	10	DMBA (20 mg/kg BW S/C single dose) + Tamoxifen orally (3.3 mg/kg B.W)	30
4.	T ₄	10	DMBA (20 mg/kg BW S/C single dose) + Hydro-Ethanolic extract of stem bark <i>Neolamarckia cadamba</i> orally @ 500 mg/kg B. W	30
5.	T ₅	10	DMBA (20 mg/kg BW S/C single dose) + Hydro-Ethanolic extract of stem bark <i>Neolamarckia cadamba</i> orally @ 600 mg/kg B. W	30

Blood collection and CA15-3 estimation

Blood samples were collected at the end of the treatment period (after 30 days), and serum was separated by centrifugation at 2000xg rpm for 10 minutes. Serum carbohydrate antigen 15-3 (CA15-3) levels were quantified using a commercially available sandwich ELISA kit from Fine Test (Catalogue No. ER0789) following the

manufacturer's protocol. All reagents, standards, and serum samples were first brought to room temperature (18-25 °C). The lyophilized CA15-3 standard was reconstituted with sample dilution buffer and serially diluted to obtain concentrations of 250, 125, 62.5, 31.25, 15.625, 7.813, and 0 pg/ml. Antibody-precoated wells were arranged, and 100 µL of standards, serum samples, and blank dilution buffer

were added to respective wells, followed by incubation at 37 °C for 90 min to allow antigen binding. After incubation, the contents were discarded and wells were washed twice with wash buffer. Subsequently, 100 µL of diluted biotin-labeled anti-CA15-3 detection antibody was added and incubated at 37 °C for 60 min, followed by three washes with brief soaking. Then, 100 µL of diluted HRP-streptavidin conjugate was added to each well and incubated at 37 °C for 30 min, followed by five washing cycles to remove unbound conjugate. Colour development was achieved by adding 90 µL TMB substrate solution and incubating the plate in the dark at 37 °C for 10-20 min until adequate colour formation occurred. The reaction was stopped by adding 50 µL stop solution, producing a blue-to-yellow colour change, and absorbance was measured immediately at 450 nm using a microplate reader. Mean absorbance values were calculated after subtracting blank readings, a standard calibration curve was plotted, and CA15-3 concentrations in samples were determined from the curve and expressed as pg/ml.

Immunohistochemical assessment of p53

Immunohistochemistry (IHC) of p53 was performed on paraffin-embedded mammary tissue sections which were initially placed on a hot plate at 55-60 °C for 10-15min to remove excess paraffin, followed by three washes in xylene for 5 min each. The sections were then rehydrated through three changes of absolute alcohol (100%) for 5-8 min each, followed by 90% and 80% alcohol for 6-8 min each, and washed three times with phosphate buffered saline (PBS).

Endogenous peroxidase activity was blocked using 3% hydrogen peroxide, after which sections were washed 3-4 times with distilled water and then with PBS. Antigen retrieval was carried out using Tris-EDTA buffer (10 mM Tris base, 1mM EDTA, 0.05% Tween-20, pH 9.0) by microwave heating in four cycles of 5 min each with a 1min interval between cycles; any loss of buffer volume during heating was compensated with distilled water. The slides were allowed to cool, rinsed with distilled water and washed with PBS. Non-specific binding was blocked by incubating the sections with 0.5% bovine serum albumin (BSA) for 30 min at room temperature. After removal of excess BSA, sections were incubated with primary antibody against p53 (Anti-p53 antibody, Abcam; clone PAb240, catalog no. ab26) at a dilution of 1:400 for 1 h at room temperature or overnight at 4 °C. The sections were then washed with PBS twice for 4 min each and incubated with the appropriate secondary antibody (EnVision FLEX +, Mouse, High pH; Dako catalog no. K800221-2) for 60 min. Following two PBS washes (4 min each), immunoreactivity was visualized using a ready-to-use DAB (3,3'-Diaminobenzidine) substrate-chromogen solution for 5 min. The slides were rinsed under running tap water, counterstained with hematoxylin for 3 min, washed and differentiated using 1% acid alcohol. Finally, the sections were dehydrated through graded alcohols (80%, 90%, and 100%), cleared in three changes of xylene for 5min each, and mounted with DPX (Distrene-Plasticizer-Xylene). Scoring was performed according to the criteria presented in Table 2.

Table 2: Semi quantitative scoring and criteria for p53 Protein expression

Scoring	Staining intensity	Percentage of positive cells	Interpretation
0	Normal	Nil	Normal/no nuclear expression of p53 in the neoplastic cells of epithelial cells type (Tubular/ductular) or mesenchymal cells type (fibroma/sarcoma) in the mammary gland
1	Mild	< 25%	Mild nuclear expression of p53 in the neoplastic cells of epithelial cells type (Tubular/ductular) or mesenchymal cells type (fibroma/sarcoma) in the mammary gland
2	Moderate	< 50%	Moderate nuclear expression of p53 in the neoplastic cells of epithelial cells type (Tubular/ductular) or mesenchymal cells type (fibroma/sarcoma) in the mammary gland
3	Severe	50-75%	Severe nuclear expression of p53 in the neoplastic cells of epithelial cells type (Tubular/ductular) or mesenchymal cells type (fibroma/sarcoma) in the mammary gland
4	Intense	> 75%	Marked nuclear expression of p53 protein in the neoplastic cells of epithelial cells type (Tubular/ductular) or mesenchymal cells type (fibroma/sarcoma) in the mammary gland

Statistical analysis

Experimental data were analyzed using a randomized block design, and statistical significance was interpreted accordingly.

Results

Immunohistochemistry of p53 gene in mammary tissue

The expression of p53 gene in mammary gland tissue was assessed using immunohistochemical analysis (IHC). Nuclear, nucleocytoplasmic staining patterns, staining intensity, and the proportion of positively stained cells were used to evaluate the p53 immunoreactivity (Table 3)

The positive immunoreactivity appeared as a dark brown coloration, indicating positive DAB (3,3'-Diaminobenzidine) staining, whereas negative cells showed blue nuclear staining due to hematoxylin counterstain. T₁ group (Normal control) showed no expression of p53 and scored as 0, in T₂ group (Positive control) intense nuclear expression was evident in neoplastic epithelial cells of solid tumor regions. Furthermore, the stromal cells scattered

throughout the tumor masses showing nucleocytoplasmic expression. The staining intensity and extent corresponded to score 3, indicating severe p53 expression, which is characteristic of breast tumor and reflects the accumulation of mutant p53 protein due to uncontrolled cell proliferation and genomic instability. Similarly, T₃ (Standard control) showed mild nuclear expression of p53 in the neoplastic cells compared to T₂ group confirming the effectiveness of tamoxifen in suppressing tumor progression which was graded as score 1, indicating downregulation. In T₄ group (HENC @ 500 mg/kg BW), mild nuclear and cytoplasmic expression of p53 protein was observed in neoplastic cells of mesenchymal origin indicating downregulation of p53 (graded as score 1) comparable to tamoxifen group (T₃) suggesting a decline in mutant p53 accumulation, likely due to decreased tumor burden and restoration of normal cellular regulation. In the T₅ group (HENC @ 600 mg/kg), moderate expression of p53 was observed in neoplastic cells which is graded as score 2. Figure 1 depicts immunohistochemical expression of p53 in all experimental groups.

Table 3: Immunohistochemical scoring of p53 expression in mammary tissue of experimental rats across different treatment groups

S. No	Group	Score
1.	Normal control (T ₁)	0
2.	Positive control (T ₂)	3
3.	Standard control Tamoxifen 3.3 mg/kg (T ₃)	1
4.	<i>Neolamarckia cadamba</i> @ 500 mg/kg (T ₄)	1
5.	<i>Neolamarckia cadamba</i> @ 600 mg/kg (T ₅)	2

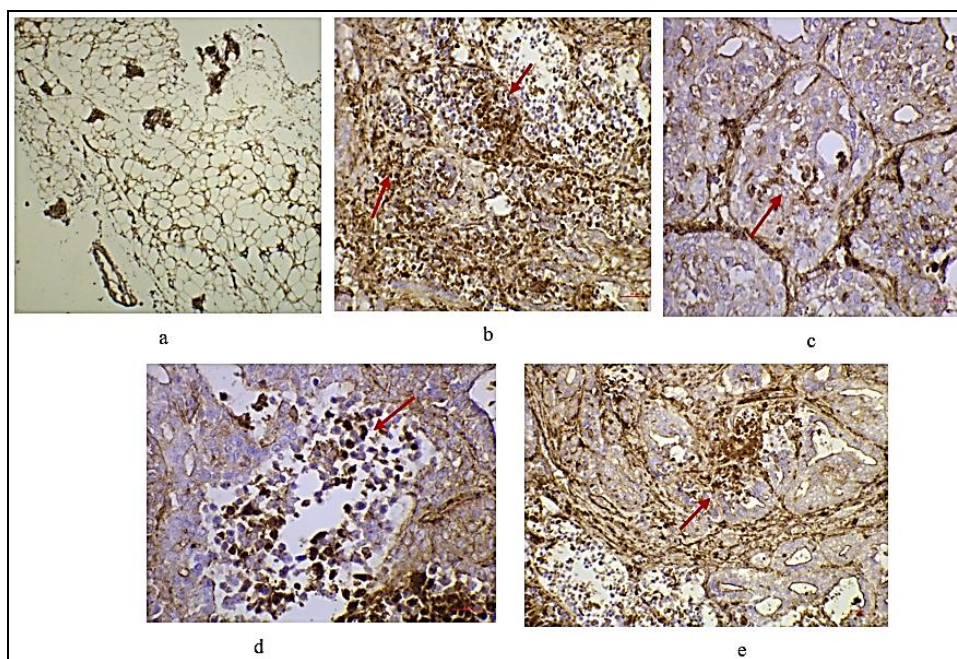


Fig 1: Immunohistochemistry (IHC) of mammary tissue from a) T₁ group showing absence of p53 expression with no detectable nuclear immunoreactivity (200x); b) T₂ group showing intense and severe nuclear expression of p53 in neoplastic cells of solid tumor, appearing as dark brown DAB-positive staining (Upregulation) (400x); c) T₃ group showing mild nuclear expression of p53 (Downregulation) (400x); d) T₄ group showing mild p53 immunoexpression in tumor cells (Downregulation) (400x); e) T₅ group showing moderate nuclear expression of p53 in mammary tumor cells (Downregulation) (400x)

Quantification of CA15-3 protein in rats

Serum samples were taken from each experimental group (T₁-T₅) at the end of treatment period (30 days) and ELISA was carried out in accordance with manufacturer instructions. Optical density (OD) values were measured at 450 nm and serum CA 15-3 concentrations were determined using a standard calibration curve. The serum concentrations of CA 15-3 observed in experimental groups are summarized in table 3.

The T₁ group (normal control) showed CA15-3 level of 36 ± 2.49 pg/ml, indicating a normal physiological concentration. On the other hand, CA15-3 levels were significantly elevated in the T₂ group (positive control) 67.03 ± 1.58

pg/ml, indicating an increased tumor burden and disease progression. The T₃ group (standard control) showed a significant decrease in CA15-3 levels of 43.3 ± 2.3 pg/ml, following treatment with tamoxifen @ 3.3 mg/kg demonstrating its therapeutic efficacy. Among the treatment groups, T₄ group (HENC @ 500 mg/kg) showed near normal concentration of CA 15-3 of 37.12 ± 2.87 pg/ml indicating most pronounced antitumor effect even comparable to or better than standard group. CA15-3 levels in T₅ group (HENC @ 600 mg/kg) were 45.71 ± 2.32 pg/ml similar to standard control group representing moderate efficacy.

Table 3: CA 15-3 concentration across different experimental groups

Groups	CA 15-3 Concentration (pg/ml)
Normal control (T ₁)	36 ± 2.49^a
Positive control (T ₂)	67.03 ± 1.58^c
Standard control Tamoxifen @ 3.3 mg/kg (T ₃)	43.3 ± 2.3^b
<i>Neolamarckia cadamba</i> @ 500 mg/kg (T ₄)	37.12 ± 2.87^a
<i>Neolamarckia cadamba</i> @ 600 mg/kg (T ₅)	45.71 ± 2.32^b

Values are expressed as Mean $\pm$ SE ($n = 7$) significantly different at $p < 0.01$

Figures with common superscript within column does not differ significantly

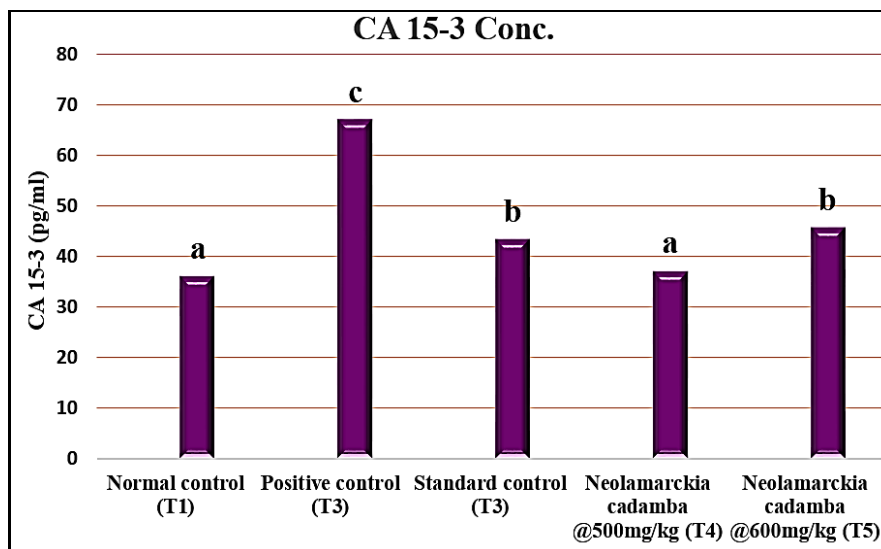


Fig 2: Graph depicting CA 15-3 (pg/ml) levels across all different experimental groups

Results and Discussion

The present study investigated the effect of *Neolamarckia cadamba* extract on p53 gene expression and CA 15-3 protein levels in DMBA induced mammary tumor in SD rats. The results demonstrated a marked alteration in p53 expression in mammary tissues following treatment with Hydroethanolic extract of *Neolamarckia cadamba* (HENC), indicating its potential antitumor activity. Among treatment groups, T₄ group (HENC @ 500 mg/kg BW) showed better results analogous to tamoxifen group (T₃), with both groups exhibiting lower p53 expression compared to T₅ group (HENC @ 600 mg/kg BW). This suggests that by reducing DNA damage and promoting cell cycle regulation, *Neolamarckia cadamba* may prevent the growth of tumors. IHC is useful and reliable technique for determining p53 expression in routine pathology. Abnormal p53 IHC patterns show a strong correlation with Tp53 mutations and are considered crucial prognostic and surrogate markers for tumour characterization [9]. These results are consistent with Amin *et al.*, (2009) who found a significant downregulation of p53 expression in treated groups and strong positive p53 immunostaining in the DMBA control group when compared to the normal control group. This altered expression pattern of p53 in DMBA induced mammary carcinogenesis reflects the accumulation of mutant p53 protein, which is commonly detected by IHC [10]. p53 is mutated in nearly half of the human cancers, and in approximately 80% of triple negative breast cancer (TNBC) cases, contributing to tumor aggressiveness and metastatic potential. In TNBC, positive p53 IHC expression has been associated with adverse clinicopathological features such as higher histological grade, presence of vessel tumor emboli, reduced disease free and overall survival and p53 expression may function as an independent prognostic factor indicating a worse clinical outcome [11]. Approximately 60% positive nuclear expression of p53 was observed in the mammary tissues of DMBA control rats, whereas negative p53 staining in normal mammary tissues. These results provide more evidence that p53 overexpression is a characteristic of mammary tumours [12]. The agreement between the current findings and earlier research emphasizes the sensitivity and dependability of immunohistochemistry as a method for detecting alteration in p53 expression during tumor growth and interventions. The presence of diverse bioactive

phytoconstituents such as alkaloids, flavonoids, glycosides, steroids and tannins in *Neolamarckia cadamba* was confirmed in a study and insilico studies also revealed that some phytoconstituents exhibited strong affinity towards HER2(erb2) receptor, suggesting that *Neolamarckia cadamba* possesses anticancer potential against breast cancer [13]. Quercetin, a flavonoid may reduce the accumulation of mutant p53 protein in breast cancer cells by interfering with its translation rather than its gene transcription. This decline in p53 protein levels is associated with decreased cellular proliferation and alterations in cell cycle progression [14]. However, presence of quercetin in the ethanol extract of *Neolamarckia cadamba* was confirmed based on HPTLC analysis [15]. Preoperative serum CA15-3 levels have a substantial predictive value and elevated concentrations are correlated with higher tumor grade, larger tumor size, and advanced pathological stage [16]. Cancer Antigen 15-3 (CA 15-3) is an FDA-approved breast cancer biomarker used for monitoring treatment response with elevated serum levels correlating with advanced disease stage, larger tumor size [17]. The results of the present study indicate that CA15-3 level significantly increased in mammary carcinoma group and decreased following treatment, with T₄ group showing the best therapeutic response among treated groups ($p < 0.001$). It has been documented by Dharmendra *et al.*, 2014 that CA 15-3 levels were decreased in tumor bearing rats after treatment with herbal medications, an effect attributed to the presence of flavonoid components known for their antineoplastic qualities. Anticancer potential of *Neolamarckia cadamba* as evidenced by its *invitro* cytotoxic activity against MCF-7, A549 and HepG2 cell lines [19]. Overall, this study suggests that the hydroethanolic extract of *Neolamarckia cadamba* possesses promising antitumor potential by modulating p53 expression and reducing CA 15-3 levels in DMBA-induced mammary carcinoma. However, further detailed molecular and clinical investigations are required to confirm its therapeutic efficacy and elucidate the underlying mechanisms.

Conclusion

The present study demonstrated that HENC exhibited notable antitumor activity. It resulted in reduced dp53 immunoexpression and decreased serum CA 15-3 levels,

indicating suppression of tumor progression. Among the treatment groups T₄ group (HENC @ 500 mg/kg body weight) showed effects comparable to the standard drug tamoxifen. These findings suggest that presence of bioactive phytoconstituents could be responsible for its anticancer potential. Overall, the study supports the therapeutic promise of this plant as complementary approach in breast cancer management. Further detailed molecular, toxicological, and clinical studies are required to validate its efficacy and safety.

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