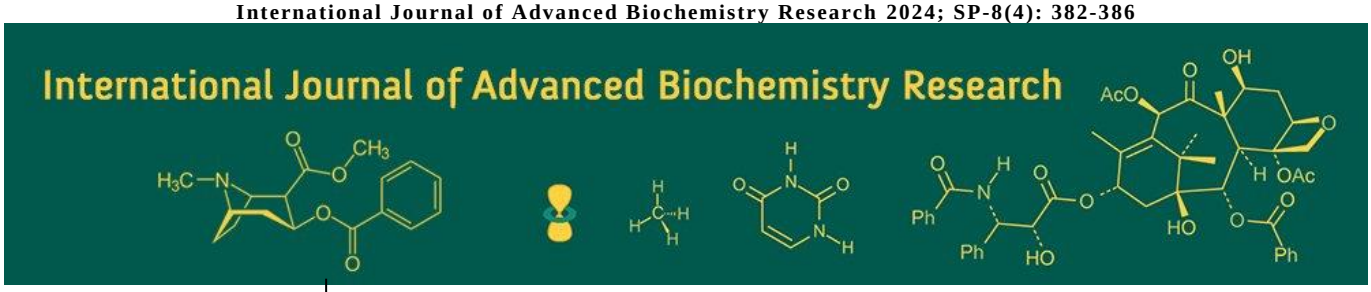

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Mayur Purankar
Department of Veterinary Pharmacology and Toxicology, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

Dr. Alka R. Sawarkar
Assistant Professor, Veterinary Pharmacology and Toxicology, Nagpur Veterinary College, Nagpur Maharashtra Animal and Fishery Sciences University, Nagpur, India

Somkuwar AP
Department of Veterinary Pharmacology and Toxicology, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

Madhuri Hedau
Department of Veterinary Pharmacology and Toxicology, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

Manoj Patil
Department of Veterinary Pharmacology and Toxicology, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

Swati Umap
Department of Veterinary Pharmacology and Toxicology, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

Corresponding Author:
Dr. Alka R. Sawarkar
Assistant Professor, Veterinary Pharmacology and Toxicology, Nagpur Veterinary College, Nagpur Maharashtra Animal and Fishery Sciences University, Nagpur, India

Evaluation of serum biochemical and anti-cancerous activity of *Neolamarckia cadamba* against DMBA induced in Wistar rats

Mayur Purankar, Alka Sawarkar, Somkuwar AP, Madhuri Hedau, Manoj Patil and Swati Umap

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Abstract
Neolamarckia cadamba is a medicinal plant reported for various medicinal properties. Anticancer activity of methanolic extract of stem bark of *Neolamarckia cadamba* on DMBA-induced diabetes and serum biochemical studies were conducted in Wistar rats. Group T_1 is designated as the standard control, Group T_2 was positive control, i.e. orally administered with DMBA at a dose rate of 20 mg/kg body weight three times a week for five weeks, Group T_3 was treated with the standard reference drug Tamoxifen at a dose rate of 3.3 mg/kg body weight and Groups T_4 and T_5 were treated with methanolic bark extract of *Neolamarckia cadamba* at a dose rate of 200 and 400 mg/kg body weight, respectively. The observation obtained from ultrasonography examination of rats after the completion of treatment revealed that all the treatment groups (T_3, T_4, T_5) showed that there was regression of the mammary tumour. On the 30th day, Tamoxifen @ 3.3 mg/kg decreases ($p<0.05$) the BUN and serum protein levels effectively compared to the T_4 and T_5. It is noticed that the T_2 group positive control showed a steady increase in estrogen and progesterone levels, while the T_3 group showed a significant decline as compared to the T_4 and T_5. The Std reference drug tamoxifen group showed effectiveness compared to the *Neolamarckia cadamba* extract dosages 200 mg/kg (T_4) and 400 mg/kg (T_5).

Keywords: Serum, biochemical, *Neolamarckia cadamba*, DMBA, Wistar

Introduction
Cancer is a significant public health problem in many parts of the world. Cancer remains one of the leading causes of death worldwide, with 10 million deaths out of 19.3 million cases in 2020. It is expected to be 28.4 million cases by 2040 if nothing is done, a 47% rise from 2020; with 11.7% of new cases, breast cancer is the most diagnosed cancer in the world, and it affects younger women. In 2018, it is estimated that there will be 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer) and 18.1 million instances of new cancer (17.0 million excluding nonmelanoma skin cancer) (Bray *et al.*, 2018) [2].

Among the animal models, chemically induced rat models are the most widely used model to study human mammary carcinogenesis due to shorter latency periods, reproducibility and flexibility in isolation of tumour tissues during various stages of tumorigenesis. The prototypic polycyclic aromatic hydrocarbons (PAHs), 7,12-dimethylbenzanthracene (DMBA), is the most commonly employed carcinogen for mammary tumour induction in rodents (Russo and Russo., 1996) [11], especially in outbred Sprague-Dawley (SD) rats. SD rats are most sensitive to DMBA, and the mammary gland is a major target organ for DMBA. Beginning in the early 1950s, researchers looked to plants for anti-cancer agents.

Plants are a natural product that has been used to treat a variety of illnesses, including cancer. It is astounding how many different kinds of plants in the world have therapeutic qualities (Patel *et al.*, 2013) [8]. *Neolamarckia Cadamba* is a large tree with a cylindrical bole and a broad, umbrella-shaped crown. The tree's arms or branches are arranged in tiers. It typically reaches a moderate size of 15 to 20 cm and has a rounded crown. However, *cadamba* may grow up to 45 meters in height, with a stem diameter of 100 to 160 cm and a small buttress as high as 2 meters. The branches are layered, horizontally extended, and end simply at the tip. It has deep grey bark with longitudinal fissures and thin, peeling scales.

The oval-shaped leaves have noticeable veins. The flowers are globose, tiny, and orange in colour.

The fruit typically has a fleshy, small, capsulated structure, forms a yellowish-orange infructescence, and contains almost 7,000 seeds. In addition, the fully ripe fruit has a firm, spherical, yellow colour and a sweet and tart flavour. The shape of seeds is irregular or trigonal (Martawijaya *et al*., 1989). The leaves of *Neolamarckia cadamba* (NC) (family: Rubiaceae) are traditionally used to treat breast cancer in Malaysia; however, this traditional claim has yet to be scientifically validated. The ethanol extract of NC leaves has an anticancer impact on MCF-7 human breast cancer cells by inducing apoptosis and cell cycle arrest, validating its traditional use for breast cancer treatment (Razali *et al.,* 2021) [10]. The present study investigated the anti-cancerous and serum biochemical profile of *Neolamarackia cadamba* and DMBA-induced mammary tumours in Wistar rats.

Materials and Methods

The fresh stem barks of the plant *Neolamarckia cadamba* were selected for experiment and procured from the university library MAFSU Nagpur Seminary Hills Nagpur. The plant was identified and authenticated by the expert botanist at the Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur. The *Neolamarckia cadamba* was extracted from the Department of Veterinary Pharmacology & Toxicology. The collected leaves were adequately washed and dried in the shed in hygienic conditions. The powder of stem barks of the plant *Neolamarckia cadamba* was first defatted with petroleum ether in Soxhlet's apparatus. The material was air-dried, weighed, and extracted using methanol in Soxhlet's apparatus. The extract was collected in a clean, Sterilized plate, dried over a water bath at 60 ºC and stored in an airtight desiccator for further use.

The following formula determined the extractability of the extract-

$$\% \text{ Extractability } = \frac{\text{Weight of extract (gm)}}{\text{Weight of powder used (gm)}} \text{ X } 100$$

The physical characteristics of *Neolamarckia cadamba* were recorded. The experimental protocol was duly approved as per the guidelines of CPCSEA. Healthy female Wistar rats of weight 120-150g were divided into five groups: T_1, T_2, T_3, T_4, and T_5, each group comprising ten female rats. A 30-day treatment period is proposed for the study, designed as shown in Table 1. Every animal was kept in (34cm x47cm x18cm) well-ventilated polypropylene cages that offered 148.3–187.0 cm 2 of floor space area/animal, and bedding made of sterilised corn cob was placed inside. Three animals were stocked in each cage. The animals were provided with ad lib standard balanced commercially available pelleted feed containing 17-22% CP, 3-6% fat, 55-65% carbohydrates, and 2.8-3.2 Kcal/gm metabolisable energy, which was procured from Nutrivet Life Sciences, Pune. The anticancer activity of the Methanolic extract of stem bark of *Neolamarckia cadamba* was carried out using induced tumour.

Table 1: Experimental Design

Sr. No.	Groups	No. of rats	Treatment	Route of administration	Duration of treatment (Days)
1.	T_1	10	Normal Control	------	------
2.	T_2	10	DMBA (20mg/kg)	Oral	Three times a week for 5 weeks.
3.	T_3	10	DMBA+Tamoxifen(3.3mg/kg)	Oral	30
4.	T_4	10	DMBA + Methanolic extract of stem bark *Neolamarckia cadamba*@ 200 mg/Kg b wt.	Oral	30
5.	T_5	10	DMBA + Methanolic extract of stem bark *Neolamarckia cadamba*@ 400 mg/Kg b.wt.	Oral	30

(DMBA is a carcinogenic chemical that might cause mortality during the induction of mammary tumours; therefore, each group contains 10 Wistar females).

The female rats were administered DMBA @ 20mg/kg b. wt. Orally three times a week for five weeks of duration. The induced tumour is detected using ultrasonography after eight weeks of induction. Female rats with induced Mammary Tumours were used in the experiment. The blood was collected to separate serum on the 0^{th}, $14^{th,}$ and 28^{th} day to perform biochemical estimation using Autoanalyzer with commercial reagent kits. The following parameters were observed: BUN, serum total protein, estrogen and progesterone.

The results were analysed using a randomised block design. Data were expressed as mean ±SE, and the significance level was $p \leq 0.05$. The data generated will be analysed statistically, and the graphs will be made using the standard statistical procedure WASP 2.0 Software

Results and Discussion

Neolamarckia cadamba fresh stem bark was collected, cleaned, shed dried for seven days, powdered in a mechanical grinder, and defatted with petroleum ether before being used with Soxhlet's apparatus for methanolic extraction. The methanolic extract's colour, consistency, and extractability percentage were displayed in (Table 1) The results for extractability percentage were found to be 22.30%. The colour and consistency were semi-solid dark yellowish brown.

The extractability percentage was similar to the findings of other studies. Moe *et al.* (2020) [7] obtained an 8.07% extract yield of dried powder from the bark of methanolic extract of *Neolamarckia cadamba* using a Soxhlet apparatus. Extracted 23.7% of the bark of *Neolamarckia cadamba* from 180 grams of dried powder to produce 42.8 grams of 95% methanolic extract. Used a Soxhlet apparatus to find a 9.25% extract yield of the dried powder form of the bark of *Neolamarckia cadamba* methanolic extract. Diwthe *et al.* (2023) [5] found that the % extractability of powdered stem bark of *Neolamarckia cadamba* is 13.40%. Dolai *et al.* (2012) [4] powdered stem bark of Neolamarckia cadamba plant material was successively extracted by petroleum ether (60–80 ºC) followed by methanol using a Soxhlet extraction apparatus. The yields of petroleum ether and defatted methanol extract were approximately 8% and 20% w/w,

respectively. The physical attributes and percentage of extractability align with the previously mentioned findings.

Table 2: Extractability percentage and Physical characteristics of methanolic extract of stem bark of *Neolamarckia cadamba*

Sr. No.	Content	Methanolic extract
1	Solvent used	Methanol 100%
2	Quantity	240 gm
3	Consistency	Semisolid
4	Colour	Dark yellowish brown
5	Extractability	22.30%

Serum biochemical parameters

On the 0th day, the mean blood urea nitrogen levels of all groups, i.e., T_1, T_2, T_3, T_4, and T_5, were 26.01±0.49, 47.24±0.44, 54.01±0.36, 56.06±0.28, 58.56±0.42 mg/dl respectively. On the 15th day of the experiment, it was found that the Std. Drug tamoxifen @ 3.3 mg/kg decreases the BUN effectively compared to the T_4 and T_5, while On the 30th day, the BUN level in the positive control group (T_2) was continuously elevated during the study. Throughout the treatment, which lasted 30 days, the BUN levels in the remaining groups (T_3, T_4, and T_5) significantly dropped but did not return to normal but tried to restore normal. The findings were compared with Akhouri *et al.* (2020) [1], who reported that the DMBA-treated group had noticeably higher blood urea nitrogen levels. Similarly, Prakash *et al.* (2023a) [9] studied that the DMBA group had significantly (p<0.05) higher urea indicating renal damage.

Estimation of serum total protein

The mean serum total protein levels on day 0th were 7.15± 0.06, 6.31± 0.13, 6.44 ± 0.14, 5.81± 0.28, and 5.36 ± 0.37 g/dl respectively. Significant differences (P<0.05) occurred on the 15th and 30th days. The positive control group, T_2, shows a declining serum protein level compared to the rest on the 15th day, whereas, on the 30th day, The Std. drug tamoxifen T_3 group is more effective in restoring the serum protein level than T_4 and T_5.

Shaban *et al.* (2023) [12] stated that the rats given DMBA had a non-significantly lower total protein level (about 0.2%) than the control group. The total protein level in the rats treated with KE (kernel extract) and PE (peel extract) after DMBA administration (the DMBA-KE and DMBA-PE groups) decreased non-significantly by approximately 7.3% and 0.42%, respectively, in comparison to the DMBA group. After receiving DMBA, the group treated with both KE and PE (DMBA-KEPE) saw a non-significant 3.8% increase in total protein levels compared to the DMBA group. Compared to the control group, administering KE or PE separately raised the total protein level non-significantly by roughly 2.78% and 1.1%, respectively.

Table 3: Results of mean bun and total protein level in different groups

Groups	BUN (mg/dl)			Total Protein (g/dl)		
Days	0th	15th	30th	0th	15th	30th
Normal Control (T_1)	26.01 ^{a}± 0.49	28.01^{a} ± 0.55	28.22 ^{a}± 0.02	7.15 ^{d}± 0.06	7.28 ^{d} ± 0.04	7.40 ± 0.06
Positive Control (T_2)	47.24 ^{b}± 0.44	53.21^{d} ± 0.40	58.87^{e} ± 0.48	6.31 ^{bc}± 0.13	5.71 ^{a} ± 0.25	5.46 ± 0.13
Std. Drug @ 3.3 mg/Kg b. wt. (T_3)	54.01 ^{c}± 0.36	44.03^{b} ± 0.37	34.42^{b} ± 0.61	6.44^{c} ± 0.14	6.66 ^{c} ± 0.09	7.21 ± 0.31
N. cadamba @ 200 mg/ Kg b.wt (T_4)	56.06 ^{d}± 0.28	52.19 ^{d}± 0.30	44.02^{d} ± 0.57	5.81^{ab} ± 0.28	6.37 ^{bc} ± 0.24	6.70 ± 0.11
N. cadamba @ 400 mg/ Kg b.wt (T_5)	58.56^{e} ± 0.42	48.28 ^{c}± 0.37	38.78 ^{c}± 0.37	5.36 ^{a}± 0.37	6.04 ^{ab}± 0.16	6.76 ± 0.11
CD	1.093	1.278	1.388	0.519	0.547	0.505

ABC within the respective row and abc within the respective column differ significantly (p<0.05)
N. S: Non-significant

Estimation of serum estrogen

As depicted in Table 3, the mean serum estrogen values showed a significant difference in all treatment groups from (T_1 to T_5). On the 15th day, the T_2 group (positive control) had a higher estrogen level than T_1, T_3, T_4, and T_5. The rats in the T_5 group, i.e. Neolamarckia cadamba treated extract @ 400 mg/kg group, showed a significant reduction in estrogen levels compared to the T_3 and T_4 groups. T_3 and T_4 group rats also show a decreased estrogen level compared to the T_2 group. On the 30th of the experiment were 34.74 ± 0.41, 42.47 ± 0.42, 18.67± 2.99, 21.94 ± 0.94, 20.57 ± 0.36 pg./ml, respectively. From the above findings, it is noticed that the T_2 group positive control showed a steady increase in estrogen levels. The T_3 group showed a significant decline compared to the T_4 and T_5.

Shaikh *et al.* (2015) [13] found that progesterone, estradiol, and LH levels in the serum decreased in the (MEAC) methanolic extract of the *Anthocephalus cadamba* extract-treated group in a dose-dependent manner. However, there was no change in the FSH level in all treated groups. Due to altered hormone levels and luteolysis mechanism (MEAC), methanolic extract of *Anthocephalus* cadamba is claimed to be abortifacient. Shaban *et al.* (2023a) [12] reported that compared to the control group, the administration of DMBA dramatically raised the serum E2 level by roughly 29%. As opposed to the DMBA group, the E2 levels in the (DMBA-KE), (DMBA-PE), and (DMBA-KEPE) groups were significantly lower by roughly 53.5%, 27%, and 35.3%, respectively. Compared to the control group, the E2 level was lower in the KE group (significantly 52.2%) and the PE group (non-significantly by roughly 8%).

Estimation of serum progesterone

In groups T_2, T_3, T_4, and T_5, it was observed that serum progesterone was significantly elevated when compared with the standard control T_1. On the 15th day of treatment, the Std. reference drug tamoxifen-treated group showed a significant decrease in the serum level of progesterone compared to other groups. The T_4 and T_5 treated groups showed more or less similar considerable effects. On the 30th day of the experiment, in the T_2 group, the serum progesterone remained elevated compared to other groups. In the case of treatment groups T_3, T_4, and T_5, there is a significant decrease in the serum progesterone level. The Std. reference drug tamoxifen group showed effectiveness compared to the *Neolamarckia cadamba* extract dosages 200 mg/kg (T_4) and 400 mg/kg (T_5).

According to Shaikh *et al.* (2015) [13], the group treated with the MEAC methanolic extract of Anthocephalus cadamba extract showed a dose-dependent decrease in the serum's

progesterone, estradiol, and LH levels. However, the FSH level remained unchanged in all treated groups. The methanolic extract of Anthocephalus cadamba is claimed to have abortifacient properties due to altered hormone levels and the luteolysis mechanism (MEAC).

Table 4: Results of Mean Serum Estrogen and Serum Progesterone in different groups

Groups	Estrogen			Progesterone		
Days	0^{th}	15^{th}	30^{th}	0^{th}	15^{th}	30^{th}
Normal Control (T_1)	$33.70^{a} \pm 0.45$	$34.09^{c} \pm 0.43$	$34.74^{b} \pm 0.41$	$19.283^{a} \pm 0.45$	$20.058^{b} \pm 0.43$	$20.422^{c} \pm 0.41$
Positive Control (T_2)	$41.06^{c} \pm 0.34$	$41.34^{d} \pm 0.35$	$42.47^{c} \pm 0.42$	$21.863^{b} \pm 0.34$	$22.395^{c} \pm 0.35$	$22.970^{c} \pm 0.42$
Std. Drug @ 3.3 mg/Kg b. wt. (T_3)	$41.54^{c} \pm 0.40$	$32.42^{b} \pm 0.49$	$18.67^{a} \pm 2.99$	$22.107^{b} \pm 0.40$	$12.902^{a} \pm 0.49$	$5.853^{a} \pm 2.99$
N. cadamba @ 200 mg/ Kg b.wt (T_4)	$39.62^{b} \pm 0.42$	$31.39^{ab} \pm 0.50$	$21.94^{a} \pm 0.94$	$23.458^{b} \pm 0.42$	$19.290^{b} \pm 0.50$	$14.11^{b} \pm 0.94$
N. cadamba @ 400 mg/ Kg b.wt (T_5)	$39.29^{b} \pm 0.33$	$30.76^{a} \pm 0.48$	$20.57^{a} \pm 0.36$	$22.203^{b} \pm 0.33$	$19.238^{b} \pm 0.48$	$11.90^{b} \pm 0.36$
CD	1.131	1.306	4.084	2.265	2.064	3.047

ABC within the respective row and abc within the respective column differ significantly ($p<0.05$)
N. S: Non-significant

Observations of the tumour by ultrasonography

The suspected rats have undergone ultrasonography to detect mammary tumours. Ultrasonography of normal control rats showed echogenicity, and no angiogenesis was observed. In positive control, T_2, T_3, T_4, and T_5 group rats possess the hypoechoic images and angiogenesis observed in Doppler ultrasonography. At the termination of the experiment after treatment, the angiogenesis was observed to decline in contrast to the start of the treatment. The observation obtained from ultrasonography examination of rats after the completion of treatment revealed that all the treatment groups (T_3, T_4, T_5) showed that there was regression of the mammary tumour. After 30 days of treatment, the positive control group showed increased tumour size. In contrast, the T_3 group Std. reference drug tamoxifen group exhibited effectively regressed tumour growth as compared to the T_4 group *Neolamarckia cadamba* stem bark methanolic extract @ 200 mg/kg, and T_5 group *Neolamarckia cadamba* stem bark methanolic extract @ 400 mg/kg. In comparison between the T_4 and T_5 groups, it was found that the T_5 group showed more regression of mammary tumours than the T_4 group.

Dolai *et al.* (2012) [4] reported that methanolic extract of *Anthocephalus cadamba* MEAC exhibited dose-dependent direct cytotoxicity on the EAC cell line. MEAC significantly ($p<0.01$) reduced the tumour weight, viable cell count, and tumour volume while also lengthening the life span of mice with EAC tumours. Administration of 200 mg/kg and 400 mg/kg methanolic extract of *Anthocephalus cadamba* reduced the tumour size in a dose-dependent manner, i.e. 400 mg/kg dose more effective than 200 mg/kg. In mice treated with methanolic extract of *Anthocephalus cadamba* MEAC, the haematological profile, biochemical estimations, and tissue antioxidant assay returned to normal. Khandelwal and Choudhary (2020) [6] researched that the N1S1 rat hepatoma cancerous cell line was used to test the anticancer activity of hydromethanolic extract of *Neolamarckia cadamba* bark at various concentrations (10, 20, 40, and 80 µg/ml) using the sulforhodamine (SRB) assay. At 40 µg/ml and 80 µg/ml, respectively, the percentage of control cell growth was -37.66 and -34.13. A decrease in the percentage of control cell growth was noted and was dose-dependent.

Conclusion

Based on the given findings, we conclude that the anti-cancerous activity was progressively improved and corrected towards the expected level in mammary tumours in Wistar rats after the treatment of methanolic stem bark extract of Neolamarckia cadamba at doses of 200 and 400mg/kg bd wt. However, rats treated with methanolic stem bark extract of Neolamarckia cadamba at 200 mg/kg were more effective than 400 mg/kg bd wt. The Std. reference drug tamoxifen group showed effectiveness compared to the *Neolamarckia cadamba* extract dosages 200 mg/kg (T_4) and 400 mg/kg (T_5) and showed reduced values in serobiochemical characteristics which had shown promising effects in our studies.

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