Original Article

Esculetin Loaded Chitosan Nanoparticles (ESC-CNPs) Ameliorates 7, 12 Dimethylbenz [a] Anthracene (DMBA) Induced Breast Cancer in Rat Model

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ABSTRACT

Aim: This study aimed to explore the chemopreventive potential of Esculetin-loaded Chitosan Nanoparticles (ESC-CNPs) against mammary carcinogenesis induced by 7,12-Dimethylbenz[a]Anthracene (DMBA) in female Sprague-Dawley rats. **Materials and Methods:** DMBA, administered subcutaneously at a dose of 25 mg/rat near the mammary gland, triggered the formation of breast tumors. Female Sprague-Dawley rats were induced with mammary tumors via a single subcutaneous injection of DMBA. The treatment groups received ESC-CNPs orally at dosages of 25, 50 and 100 mg/kg body weight (bw). Biochemical parameters, including lipid peroxidation (TBARS and LOOH), antioxidant status (SOD, CAT, GPX and GSH), phase I (CYP450, Cyt-b5) and phase II (GST, GR) enzymes and lipid profiles (TC, TG, PL, FFA), were evaluated in plasma and breast tissues. Additionally, the expression of VEGF, NF-kB and Cyclin D1 was analyzed through histopathology and western blotting. **Results:** DMBA-induced rats exhibited decreased body weight, increased tumor volume and incidence, elevated lipid peroxidation, reduced antioxidant status, altered liver and mammary tissue enzyme levels and disrupted lipid profiles. ESC-CNPs treatment, particularly at 100 mg/kg bw, significantly reduced tumor occurrence, normalized biochemical markers and downregulated VEGF, NF-kB and Cyclin D1 expression in mammary tissues. **Conclusion:** ESC-CNPs, at a dose of 100 mg/kg bw, demonstrated a potent chemopreventive effect against DMBA-induced mammary cancer, as evidenced by biochemical, histopathological and molecular findings.

Keywords: Antioxidants, Breast Cancer, Chitosan Nanoparticles, DMBA, Esculetin.

INTRODUCTION

Breast cancer is one of the most common malignancies affecting women worldwide, accounting for a significant proportion of cancer-related deaths among the female population. It originates in the cells of the breast, typically in the ducts or lobules and can progress to invade surrounding tissues and metastasize to distant organs, including the bones, liver, lungs and brain. The development of breast cancer is a complex process involving a combination of genetic, environmental and lifestyle factors. Mutations in genes such as BRCA1 and BRCA2 are well-documented contributors to the hereditary risk of breast cancer, while other risk factors include age, family history, hormonal influences, reproductive history and exposure to radiation. Furthermore, certain lifestyle choices, such as alcohol consumption, obesity and lack of physical activity, have been linked to an increased risk of developing the disease.[1] Breast cancer is highly heterogeneous, with various subtypes classified based on the expression of hormone receptors (estrogen and progesterone) and the presence of Human Epidermal growth factor Receptor 2 (HER2). This molecular classification is critical, as it guides treatment decisions and influences prognosis.[2] The management of breast cancer has evolved significantly over the past few decades, with advancements in early detection, surgical techniques, radiation therapy, chemotherapy, hormone therapy, targeted therapy and, more recently, immunotherapy. Early detection through mammography and other screening methods plays a crucial role in improving survival rates, as breast cancer is more treatable and curable in its early stages. Surgical interventions, ranging from lumpectomy to mastectomy, aim to remove the tumor and affected tissues, often in conjunction with reconstructive surgery to restore the breast's appearance.[3]

Adjuvant therapies, including radiation and chemotherapy, are employed to eliminate residual cancer cells and reduce the risk of recurrence. Hormone therapies, such as tamoxifen and aromatase inhibitors, are effective in treating hormone receptor-positive breast cancers, while HER2-targeted therapies, like trastuzumab, have revolutionized the treatment of HER2-positive cases.[4] The advent of precision medicine and personalized treatment strategies has further enhanced the efficacy of breast cancer therapies, offering hope for better outcomes even in advanced stages of the disease. However, despite these advancements, breast cancer remains a major public health challenge, with disparities in incidence and mortality rates observed across different populations, often influenced by socioeconomic factors, access to healthcare and genetic predispositions. Continued research is essential to unravel the underlying mechanisms of breast cancer development, identify novel therapeutic targets and improve preventive measures. Public health initiatives focusing on education, awareness and accessible screening programs are vital to reducing the global burden of breast cancer.[5]

Esculetin, a naturally occurring coumarin derivative, is found in various medicinal plants and has gained attention for its diverse pharmacological properties. Known for its potent antioxidant, anti-inflammatory and anti-cancer activities, esculetin exerts its effects by modulating multiple signaling pathways and cellular processes.[6] It has shown promise in protecting against oxidative stress, reducing inflammation and inhibiting the growth of various cancer cells, including those resistant to conventional therapies. Additionally, esculetin's potential in preventing cardiovascular diseases and managing metabolic disorders underscores its therapeutic relevance, making it a valuable compound for further research and drug development.[7]

Further, nanoparticles synthesized using chitosan as a biopolymer matrix and esculetin as an active therapeutic agent represent a promising approach in drug delivery and biomedical applications. Moreover, Nanoparticles have some limitation and advantages.[8] Chitosan, derived from chitin, is a biodegradable, biocompatible and non-toxic polymer that provides an ideal platform for nanoparticle synthesis due to its ability to form stable nanostructures and enhance drug encapsulation.[9] When combined with esculetin, a natural coumarin with potent antioxidant, anti-inflammatory and anti-cancer properties, the resulting nanoparticles can effectively deliver esculetin to target sites, improving its bioavailability and therapeutic efficacy. The synergy between chitosan and esculetin in nanoparticle formulations enhances the controlled release of esculetin, protects it from degradation and facilitates targeted delivery to specific tissues, such as cancerous cells or inflamed areas.[10] This innovative synthesis approach not only leverages the beneficial properties of both chitosan and esculetin but also opens new avenues for developing advanced nanomedicines with enhanced therapeutic potential. Previously,

we synthesized and characterized Esculetin-loaded Chitosan Nanoparticles (ESC-CNPs) and analyzed their anticancer activity in the MDA-MB-231 breast cancer cell line in an *in vitro* study.[11] This study aimed to evaluate the chemopreventive potential of ESC-CNPs against DMBA-induced mammary carcinogenesis in Sprague-Dawley rats. We investigated changes in biochemical parameters (antioxidant status, lipid peroxidation and lipid profiles) in plasma and liver and breast tissues and histological changes. Additionally, we assessed the impact of ESC-CNPs on the expression patterns of biomarkers such as VEGF, NF-κB and Cyclin D1, during DMBA-induced mammary carcinogenesis in SD rats.

MATERIALS AND METHODS

Chemicals

Esculetin, DMBA and Chitosan were procured from Sigma-Aldrich, USA. All other chemical was purchased as analytical grade from local vendor.

Animal model

Female Sprague-Dawley rats, aged 6-7 weeks and weighing 120-130 g, were purchased from Biogen in Bangalore, India. The rats were housed at the Central Animal House of Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, Tamil Nadu, India. After obtaining approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animals (CPCSEA approval no: 1357), the animals were maintained in controlled conditions, with a 12 hr light/dark cycle, 50±10% humidity and a temperature of 24±2ºC. They had free access to food and water.

Induction mammary carcinoma and ESC-CNPs preparation

To induce mammary cancer, the rats were administered a single dose of DMBA (25 mg/kg body weight) in an emulsion containing 0.75 mL of sunflower oil and 0.25 mL of physiological saline. ESC-CNPs were dissolved in a DMSO solution.

Experimental design

The 36 animals were randomly divided into 6 groups, each consisting of six rats. During the study, Group I received no treatment and had unrestricted access to food and water. Group III rats were given oral administration of ESC-CNPs only. At the end of the first week, DMBA (25 mg/rat) was administered subcutaneously near the mammary gland to animals in Groups II, IV, V and VI. Group II did not receive any additional treatment. Rats in Groups IV-VI began receiving oral ESC-CNPs at varying dosages (25, 50 and 100 mg/kg body weight) once daily by intubation, starting one week before DMBA exposure and continuing until the end of the study period. At the end of the sixteenth week, the rats were fasted overnight and then anesthesias

were administration via intraperitoneal route using overdose of Ketamine hydrochloride to euthanize by cervical dislocation. Blood samples were collected in heparinized tubes for biochemical analysis.

The liver and breast tissues of the rats were promptly excised and placed in ice-cold containers. On the day of sacrifice, the tissues were homogenized with an appropriate buffer, centrifuged at 3000 g and the supernatant was used for biochemical analyses. Additionally, liver and breast tissues were preserved at -80°C and fixed in 10% formalin for subsequent histological examination.

Biochemical analysis

After removal, the liver and breast tissues were rinsed with ice-cold saline. A specified amount of tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4) at 4°C using a Potter-Elvehjem homogenizer equipped with a Teflon pestle, operating for 3 min at 600 g. The homogenate was then centrifuged at 3000 g for 10 min at 4°C. The resulting supernatant was collected and used for various biochemical analyses.

Microsomes from liver and breast tissues were isolated using the method described by Hanioka *et al.* (1997).[12] The microsomal protein content was assessed following the protocol of Lowry *et al.* (1951).[13] TBARS concentration in plasma was measured using the method of Yagi *et al.* (1987),[14] while the TBARS content in mammary tissue was determined using the approach outlined by Ohkawa *et al.* (1987).[15] LOOH concentration in mammary and plasma tissues was estimated using the technique developed by Jiang *et al.* (1992).[16] CAT activity in both plasma and mammary tissues was evaluated according to the method of Sinha *et al.* (1984),[17] and SOD activity was measured using the technique by Kakkar *et al.* (1992).[18] GPX activity was assessed in both mammary and plasma tissues using the method described by Rotruck *et al.* (1972).[19] GSH levels in plasma and mammary tissues were determined using the Beutler *et al.* (1973) method.[20] The levels of cytochrome P450 and cytochrome b5 in liver and mammary tissue microsomes were estimated using the approach of Omura *et al.* (1964).[21] GR activity in liver and mammary tissues was assessed following the protocol of Calberg *et al.* (1985),[22] while GST activity in these tissues was evaluated using the method by Habig *et al.* (1974).[23]

Lipid extraction from breast tissues and plasma was performed using the method outlined by Folch *et al.* (1957).[24] Total cholesterol levels in both breast and plasma tissues were measured using the kit method described by Zlatkis *et al.* (1953).[25] Triglyceride concentrations in plasma and breast tissues were assessed following the method of Foster *et al.* (1973).[26] Free fatty acids in plasma and breast tissues were measured using the protocol established by Falholt *et al.* (1973).[27] Phospholipid (PL) content in both plasma and mammary tissues was determined using the approach of Zilversmit and Davis (1950).[28]

Histopathological analysis

Liver and breast tissues were sliced into thin sections and fixed in a 10% formalin solution for histological analysis. Following fixation, the tissues were dehydrated through a graded series of ethanol solutions, ranging from 50% to 100% and then embedded in paraffin. Sections of 3 to 5 μm thickness were stained with Hematoxylin and Eosin (H&E). The stained slides were then examined under a microscope at 20X magnification.

Western blotting

The Bradford method was employed to quantify the total protein content in the breast tissue homogenate. SDS-PAGE was used to separate 40 μg of protein from each sample, which was loaded onto a 10% polyacrylamide gel with an equal volume of 2×Laemmli buffer. The proteins were then transferred onto 0.2 μm Polyvinylidene Difluoride (PVDF) membranes through electrophoresis. Following transfer, the membranes were blocked for 1 hr at room temperature with 3% BSA in Tris-buffered saline containing 0.2% Tween-20. The membranes were then incubated overnight at 4°C with rabbit monoclonal antibodies against VEGF, NF-κB, Cyclin D1 and GAPDH. After washing with Tris-buffered saline and 0.2% Tween-20, the membranes were incubated for 1 hr with an anti-rabbit HRP-conjugated secondary antibody. The bands were visualized using the 1-Step Ultra TMB blotting solution.

Statistical analysis

Statistical analysis was performed using SPSS statistical package. The data are expressed as mean±Standard Deviation (SD). One-way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) comparison method was used to correlate the difference between the variables. Data are considered statistically significant if $p \leq 0.05$.

Table 1: The initial and final body weights of the animals were recorded and the results are presented as mean±standard deviation. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$ (DMRT).

Groups	Initial body weight (g)	Final body weight
Control	126.06±9.44	174.89±5.3[a]
DMBA (25 mg/rat)	121.48±10.52	113.29±8.78[b]
ESC-CNPs (100 mg)	128.19±10.95	175.25±13.08[a]
DMBA+25 mg ESC-CNPs	125.51±10.61	132.09±10.19[c]
DMBA+50 mg ESC-CNPs	122.41±10.72	161.68±12.80[a]
DMBA+100 mg ESC-CNPs	125.97±11.38	173.42±12.97[a]

RESULTS

Effect of ESC-CNPs on body weight changes in control and experimental animals

The effect of ESC-CNPs on body weight changes in control and experimental animals was evaluated across different treatment groups shown in Table 1. The control group, which did not receive any treatment, showed a significant increase in body weight from 126.06±9.44 g to 174.89±5.3 g by the end of the study. In contrast, animals treated with DMBA alone exhibited a marked decrease in body weight, from an initial weight of 121.48±10.52 g to a final weight of 113.29±8.78 g, indicating the adverse effects of DMBA on overall health. The group treated with 100 mg of ESC-CNPs without DMBA exposure showed an increase in body weight from 128.19±10.95 g to 175.25±13.08 g, suggesting that ESC-CNPs alone support normal body weight gain. In animals co-treated with DMBA and different doses of ESC-CNPs, a dose-dependent protective effect on body weight was observed. Specifically, the group receiving 25 mg of ESC-CNPs alongside DMBA had a slight decrease in body weight from 125.51±10.61 g to 132.09±10.19 g, indicating partial mitigation of DMBA-induced weight loss. The groups treated with 50 mg and 100 mg of ESC-CNPs alongside DMBA demonstrated a more pronounced protective effect, with final body weights increasing to 161.68±12.80 g and 173.42±12.97 g, respectively, from initial weights of 122.41±10.72 g and 125.97±11.38 g. These results suggest that ESC-CNPs, particularly at higher doses, effectively counteract the body weight loss induced by DMBA, highlighting their potential in mitigating DMBA's toxic effects.

Effect of ESC-CNPs on total number of tumors, tumor incidence and tumor volume in control and experimental animals

The data presented in Table 2 demonstrates the effect of ESC-CNPs on tumor formation and progression in control and experimental animals. The control group and the group treated with ESC-CNPs (100 mg) alone showed no tumor formation, with a total number of tumors (n) of 0, tumor incidence of 0% and tumor volume

and burden both at 0. In the DMBA-treated group, all animals developed tumors, with a 100% tumor incidence, a significant tumor volume of 31.51±3.12 mm³/rat and a tumor burden of 189.06±18.72 mm/rat. Co-treatment with 25 mg of ESC-CNPs reduced the total number of tumors to 3 out of 6 rats, decreased the tumor incidence to 50% and significantly lowered the tumor volume and burden to 13.98±0.51 mm³/rat and 41.94±1.53 mm/rat, respectively. A further reduction in tumor formation was observed in the group treated with 50 mg of ESC-CNPs, with only 2 out of 6 rats developing tumors, a tumor incidence of 33.33%, a tumor volume of 7.25±0.4 mm³/rat and a tumor burden of 14.5±0.8 mm/rat. Notably, no tumors were observed in the group receiving 100 mg of ESC-CNPs alongside DMBA, resulting in a total number of tumors of 0, tumor incidence of 0% and both tumor volume and burden at 0, indicating the highest efficacy of ESC-CNPs at this dose in preventing tumor development.

Effect of ESC-CNPs on Lipid Peroxidation levels in Plasma and Mammary Tissue

The data presented in Figure 1 demonstrate the effect of ESC-CNPs on lipid peroxidation in plasma and breast tumor tissue, respectively. The control group and the group treated with ESC-CNPs (100 mg) alone showed normal levels of lipid peroxidation. In the DMBA-treated group, all animals developed tumors, with a significantly increased level of lipid peroxidation. Co-treatment with ESC-CNPs significantly reduced lipid peroxidation in both plasma (Figure 1a and b) and breast tumor tissue (Figure 1c and d) compared to the DMBA-induced group.

Effect of ESC-CNPs on antioxidant status in plasma of control and experimental animals

The data presented in Figure 2 demonstrate the effect of ESC-CNPs on antioxidant (SOD, CAT, GPX and GSH) level in plasma. In the DMBA-treated group, with a significantly reduced level of antioxidant enzymes. Treatment with ESC-CNPs significantly increased antioxidant in plasma (Figure 2a-d) compared to the DMBA-induced group. ESC-CNPs alone there is no changes antioxidant activity compare with control group.

Table 2: Effect of ESC-CNPs on total number of tumors, tumor incidence and tumor volume in control and experimental animals.

Groups	Total number of tumours (n)	Tumor incidence (%)	Tumor volume (mm³/rat)	Tumor burden (mm/rat)
Control	0/6	0	0	0
DMBA (25 mg/rat)	6/6	100	31.51±3.12[a]	189.06±18.72[a]
ESC-CNPs (100mg)	0/6	0	0	0
DMBA+ 25mg ESC-CNPs	3/6	50	13.98±0.51[b]	41.94±1.53[b]
DMBA+ 50mg ESC-CNPs	2/6	33.33	7.25±0.4[c]	14.5±0.8[c]
DMBA+ 100mg ESC-CNPs	0/6	0	0	0

Tumor volume was measured using the formula V=4/3π (D1/2) (D2/2) (D3/2), where D1, D2 and D3 are the three diameters (in mm) of the tumor; n- indicates total number of rats bearing tumors. Tumor burden was calculated by multiplying the tumor volume and total number of tumors. Values are expressed as mean±SD for six rats in each group. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$ (DMRT).
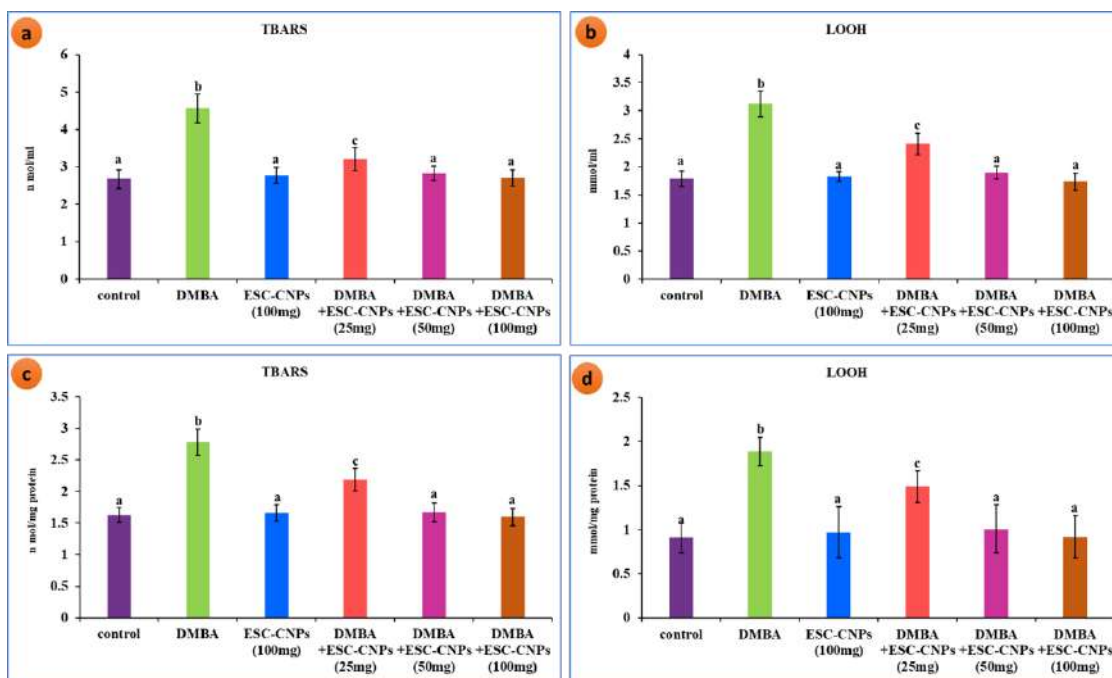
Figure 1: Evaluate the Lipid peroxidation levels in ESC-CNPs treated and untreated groups in plasma (a and b) and tumor tissue (c and d): Data are expressed as the mean±SD for six rats in each group. Values not sharing a common superscript letter in the same row differ significantly at $p \leq 0.05$ (DMRT). TBARS=Thiobarbituric acid reactive substances, LOOH=Lipid hydroperoxides.
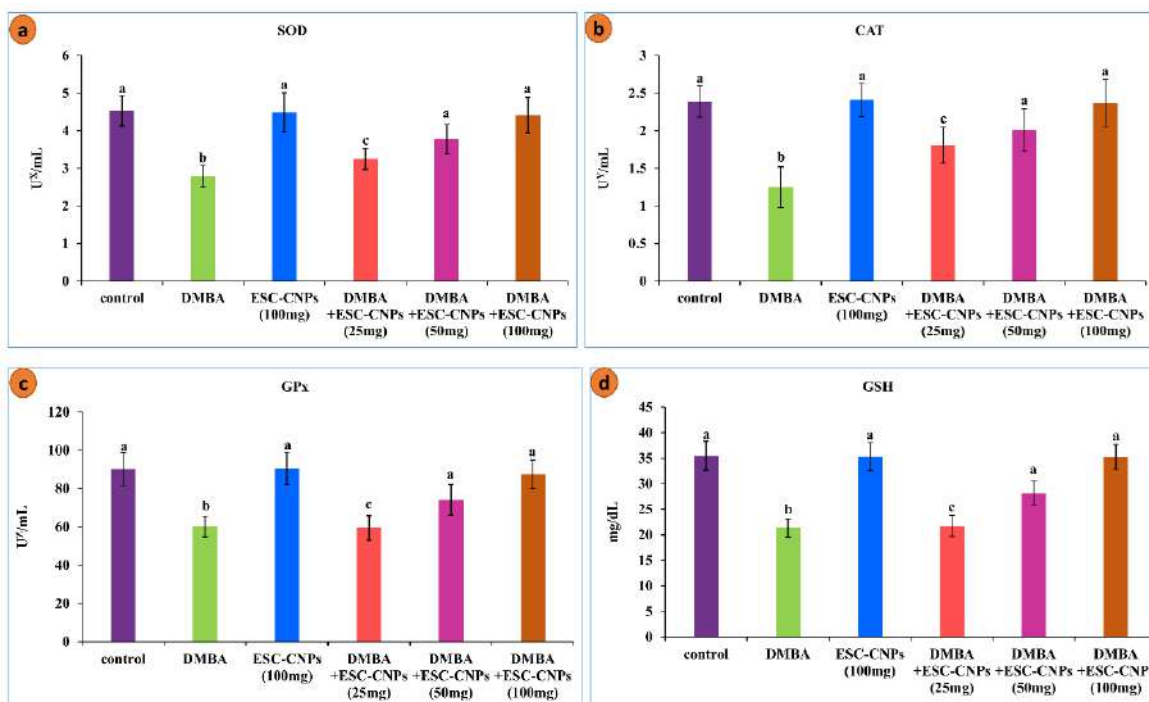

Figure 2: Effect of ESC-CNPs on antioxidant status in plasma of control and experimental animals. Units for SOD[X], CAT[Y], and GPX[Z] expressed as the amount of enzyme required to inhibit 50% of NBT reduction, micromoles of H_2O_2 utilized/second and micromoles of glutathione utilized/minute, respectively. Data are expressed as the mean±SD for six rats in each group. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$ (DMRT).

Effect of ESC-CNPs on antioxidant status in mammary tissue of control and experimental animals

The effect of ESC-CNPs on antioxidant level in breast tumor tissue depicted in Figure 3. In the DMBA-treated group, with a significantly reduced level of antioxidant enzymes. Treatment with ESC-CNPs significantly increased antioxidant in tissue (Figure 3a-d) compared to the DMBA-induced group. ESC-CNPs alone there is no changes antioxidant activity compare with control group.

Effect of ESC-CNPs on biotransformation enzyme levels in liver microsomes of control and experimental animals

The effect of ESC-CNPs on biotransformation enzymes level in liver microsomes depicted in Figure 4. In the DMBA-treated group, with a significantly increased level of CYTP450 and CYTb5 (Figure 4a and b) and while reduced in GST and GR (Figure 4c and d). Treatment with ESC-CNPs significantly reduced in CYT450 and CYTb5 in liver microsomes compared to the DMBA-induced group. ESC-CNPs alone there is no changes enzymes compare with control group.

Effect of ESC-CNPs on biotransformation enzyme levels in mammary tissue of control and experimental animals

The effect of ESC-CNPs on biotransformation enzymes level in tumor tissue depicted in Figure 5. In the DMBA-treated group,

with a significantly increased level of CYTP450 and CYTb5 (Figure 5a and b) and while reduced in GST and GR (Figure 5c and d). Treatment with ESC-CNPs significantly reduced in CYT450 and CYTb5 in tumor tissue compared to the DMBA-induced group. ESC-CNPs alone there is no changes enzymes compare with control group.

Effect of ESC-CNPs on lipid profile in mammary tissue of control and experimental animals

The effect of ESC-CNPs on lipid profiles level in tumor tissue depicted in Figure 6. In the DMBA-treated group, with a significantly increased level of TC and TG (Figure 6a and b) and while reduced in PL and FFA (Figure 6c and d). Treatment with ESC-CNPs significantly reduced in TC and TG in tumor tissue compared to the DMBA-induced group. ESC-CNPs alone there is no changes enzymes compare with control group.

Effect of ESC-CNPs on lipid profile in Plasma of control and experimental animals

The effect of ESC-CNPs on lipid profiles level in plasma depicted in Figure 7. In the DMBA-treated group, with a significantly increased level of TC and TG (Figure 7a and b) and while reduced in PL and FFA (Figure 7c and d). Treatment with ESC-CNPs significantly reduced in TC and TG in tumor tissue compared to the DMBA-induced group. ESC-CNPs alone there is no changes enzymes compare with control group.
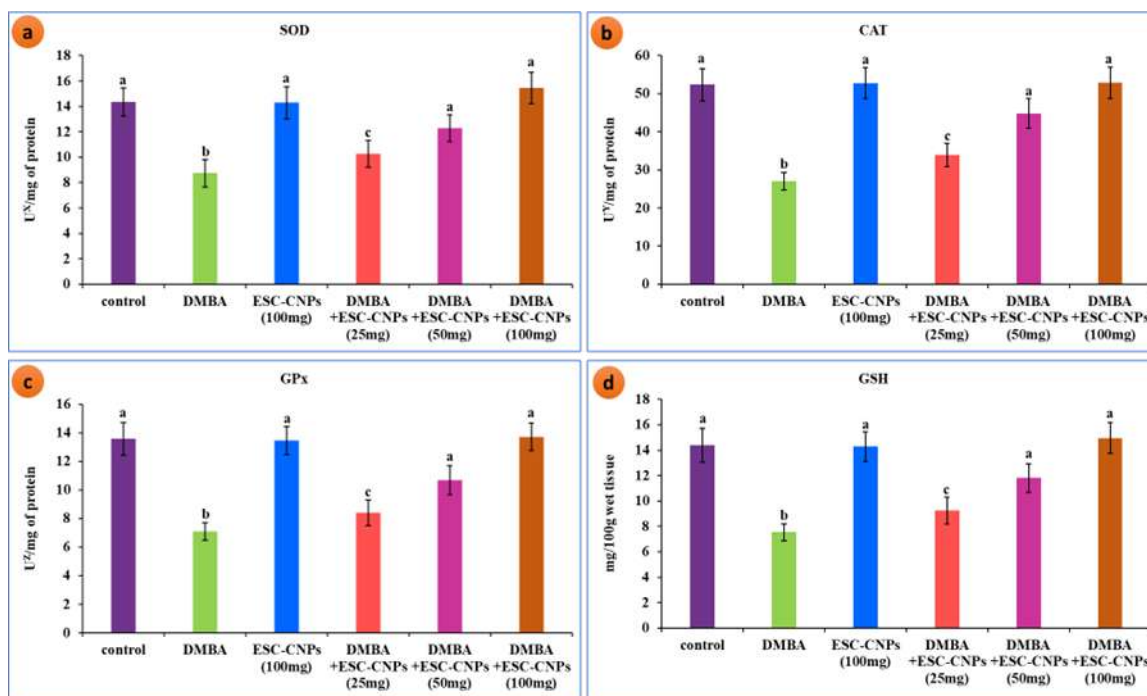

Figure 3: Effect of ESC-CNPS on antioxidant status in mammary tissue of control and experimental animals. Units for SOD[X], CAT[Y] and GPX[Z] are expressed as the amount of enzyme required to inhibit 50% of NBT reduction, micromoles of H_2O_2 utilized/second and micromoles of glutathione utilized/minute/mg protein, respectively. Data are expressed as the mean±SD for six rats in each group. Values not sharing a common superscript (a,b,c) differ significantly at $p \leq 0.05$ (DMRT).
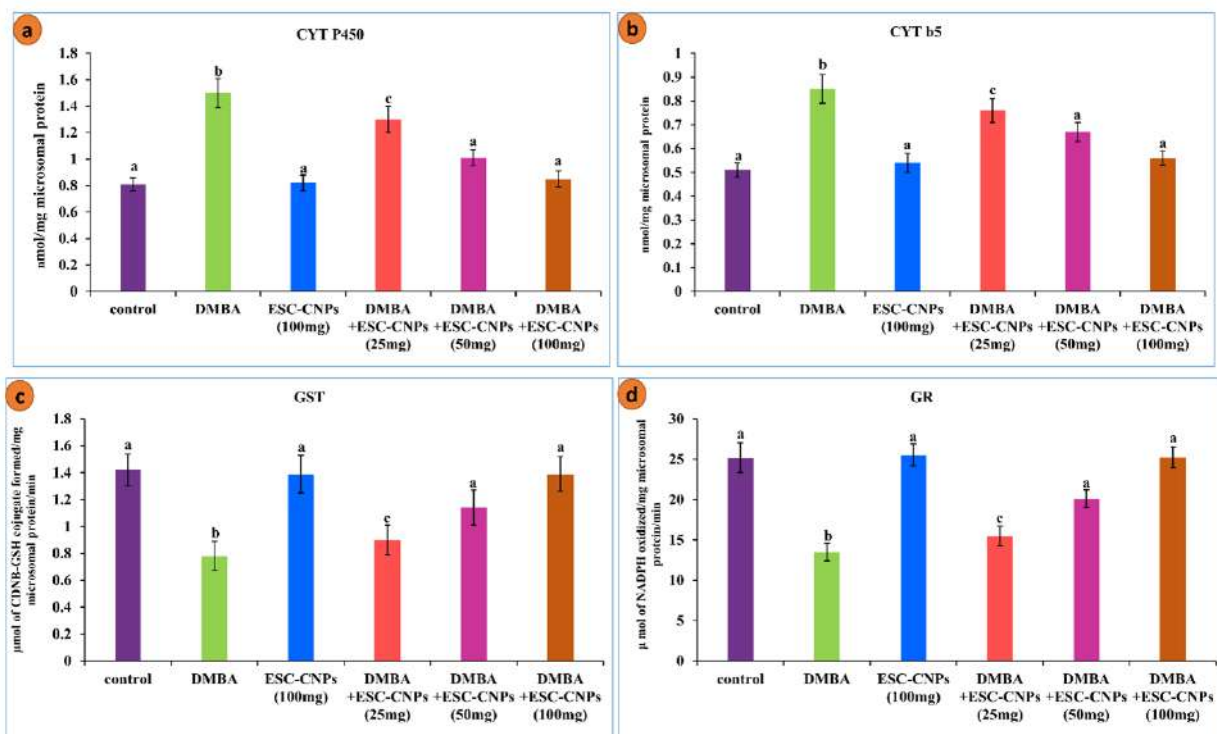
Figure 4: Effect of ESC-CNPs on biotransformation enzyme levels in liver microsomes of control and experimental animals. Values are expressed as mean±SD for six animals in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Figure 5: Effect of ESC-CNPs on biotransformation enzyme levels in mammary tissue of control and experimental animals. Values are expressed as mean±SD for six animals in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
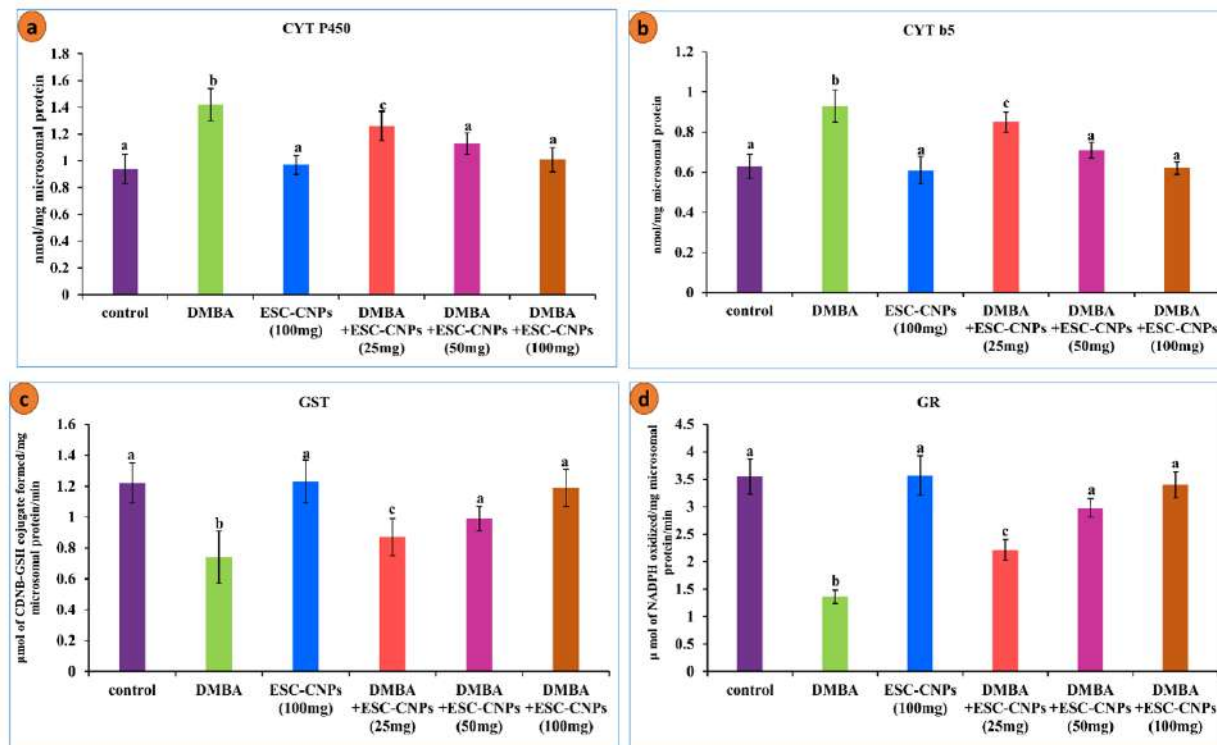
Figure 6: Effect of ESC-CNPs on lipid profile in mammary tissue of control and experimental animals. Values are expressed as mean±SD for six animals in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
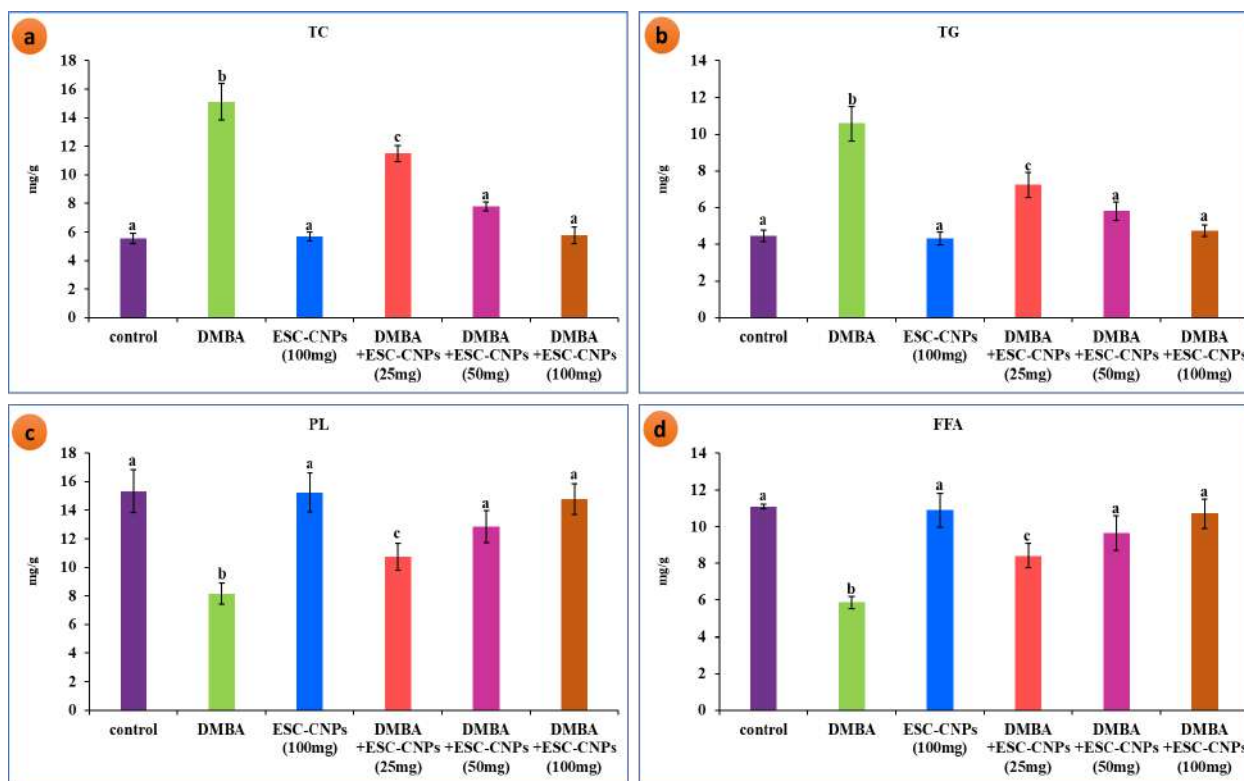
Histopathological changes of mammary tissue

Histopathological analysis was performed to evaluate the effect of ESC-CNPs on breast tumor tissues across different experimental groups. In the control group, breast tissue exhibited normal histological architecture with well-defined lobules, ducts and the absence of any pathological changes in Figure 8a. In contrast, the DMBA-treated group displayed significant histopathological alterations characteristic of malignant transformation, including disrupted tissue architecture, dense cellular infiltration, hyperchromatic nuclei and increased mitotic figures, indicative of aggressive tumor growth in Figure 8b. Treatment with ESC-CNPs showed dose-dependent protective effects against these histopathological changes. The group treated with 25 mg of ESC-CNPs alongside DMBA showed a partial reduction in tumor-associated changes, with some areas of tissue exhibiting reduced cellular density and fewer mitotic figures, though significant abnormalities persisted in Figure 8d. In the 50 mg ESC-CNPs treatment group,the protective effect was more pronounced,with a marked decrease in cellular atypia and mitotic activity,along with the presence of more normal-appearing ductal structures Figure 8e. Notably, the group treated with 100 mg of ESC-CNPs exhibited substantial histopathological improvements, with breast tissue showing near-normal architecture, minimal cellular infiltration and the absence of significant tumor markers, suggesting effective inhibition of tumor progression Figure 8f.

ESC-CNPs alone groups show no significant histopathological changes in breast tissue in Figure 8c.

Histopathological changes of liver tissue

The histological examination of liver tissues, as shown in Figure 9 (A-F), reveals the effects of various treatments on liver architecture in both experimental and control rats. In group I (A), which consisted of control animals not exposed to DMBA, the liver tissue exhibited normal histological architecture with well-preserved hepatocytes. Group II (B), consisting of animals exposed to DMBA, displayed significant alterations, including dilated sinusoids and a marked loss of architectural structure, indicative of liver damage. Conversely, group III (C), which was treated with ESC-CNPs alone, maintained normal hepatocyte architecture, suggesting that ESC-CNPs did not induce any adverse effects on the liver. In group IV (D), where animals were treated with 25 mg/kg body weight of ESC-CNPs, the liver tissue showed signs of vacuolar degeneration in the hepatocytes, indicating some level of cellular stress. Group V (E), treated with 50 mg/kg body weight of ESC-CNPs, demonstrated nearly normal hepatocyte architecture, with only mild alterations such as dilated sinusoids and moderate necrosis. Finally, in group VI (F), treated with 100 mg/kg body weight of ESC-CNPs, the liver tissues displayed noticeably dilated sinusoids, but without significant structural damage. This suggests a protective effect of ESC-CNPs at this dose.
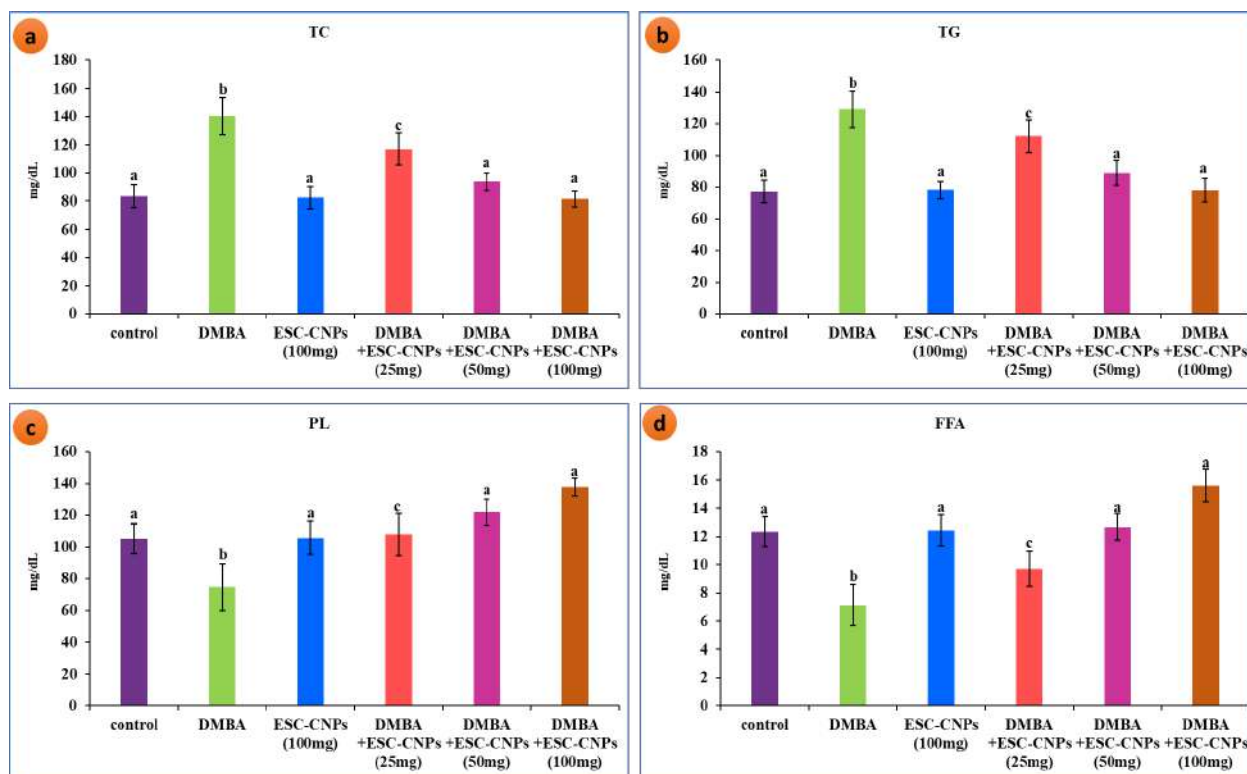
Figure 7: Effect of ESC-CNPs on lipid profile in blood plasma of control and experimental animals. Values are expressed as mean±SD for six animals in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
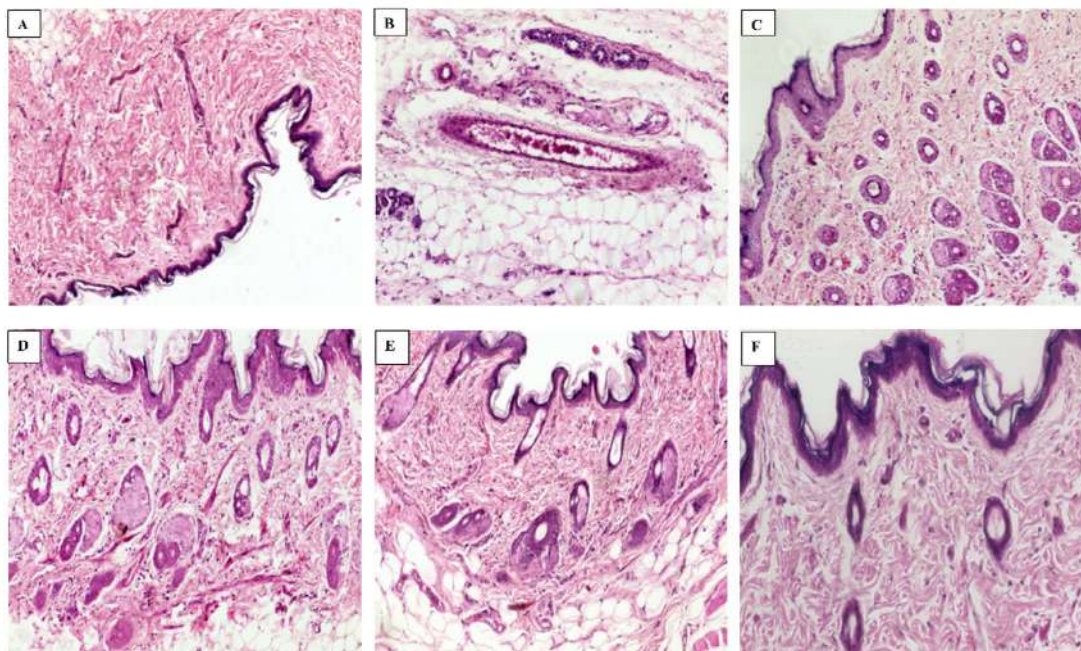

Figure 8: Histopathological changes in breast tumor tissues across different experimental groups stained with Hematoxylin and Eosin (H&E). (A-F) presents the histological examination of mammary tissues from both experimental and control rats. The control group (A) and the DMBA-treated cancer-Bearing group (B) showed significant malignant tumor infiltration. In contrast, rats treated with ESC-CNPs alone in group III (C) displayed normal mammary tissue architecture. Rats treated with 25 mg/kg body weight of ESC-CNPs in group IV (D) showed mild tumor infiltration and some fibrosis. Group V (E) rats, treated with 50 mg/kg body weight of ESC-CNPs, exhibited increased fibrosis but retained nearly normal mammary tissue architecture. Group VI (F) rats, treated with 100 mg/kg body weight of ESC-CNPs, displayed widespread fibrosis.

From the biochemical and histological studies, the effective dose of 100 mg/kg bw of ESC-CNPs were further used for molecular study.

ESC-CNPs alter the gene expression in breast tumor

The results revealed significant differences in protein expression between the groups, reflecting the impact of ESC-CNPs on these key markers involved in tumor progression. In the control group, which received no treatment, low baseline levels of NF-κB, VEGF and Cyclin D1 were observed, consistent with normal cellular activity in Figure 10. In contrast, the DMBA-treated group showed a marked upregulation of all three proteins, with a substantial increase in NF-κB, VEGF and Cyclin D1 expression, indicating enhanced inflammatory signaling, angiogenesis and cell proliferation associated with tumor development. Finally, in the DMBA+ESC-CNPs (100 mg) group, the expression levels of these proteins were significantly reduced, closely resembling those of the control group, further demonstrating the efficacy of ESC-CNPs in suppressing the molecular mechanisms driving tumor progression in lane 3 of Figure 10. Treatment with ESC-CNPs alone (100 mg) resulted in protein expression levels similar to the control group, confirming that ESC-CNPs do not significantly alter these markers in the absence of DMBA in lane 4 in Figure 10.

DISCUSSION

Nanotechnology is employed to enhance drug release and targeted delivery for various conditions, including cancer, by encapsulating natural or synthetic drug molecules.[29,30] Recent advancements in polymeric drug delivery systems have significantly impacted cancer treatment. Chitosan nanoparticles, with their cationic properties, electrostatic interactions and biodegradability, have become a favored choice for targeted drug delivery in cancer therapy. According to Sharifi-Rad *et al.* (2021) and Saravanakumar *et al.* (2020), these characteristics facilitate the effective delivery of carrier drug into host cells via oral and intravenous routes.[31,32] Previously, Ritika Chauhan *et al.*, 2024 has revealed that nano hesperidin has ability to control the Diabetic Nephropathy in a Rat Model.[33] The current study aimed to assess the tumor-preventive potential of ESC-CNPs in an experimental model of mammary cancer, based on these scientific findings. The results indicated that ESC-CNPs effectively reduced DMBA-induced mammary cancer in SD rats.

ROS are crucial for cell signaling and are produced during cellular metabolism. However, an excess of free radicals can lead to oxidative stress, which may damage proteins, induce DNA mutations and cause membrane disruption. Cells have various innate defense mechanisms to manage these free radicals. Natural antioxidants, with their diverse molecular properties,
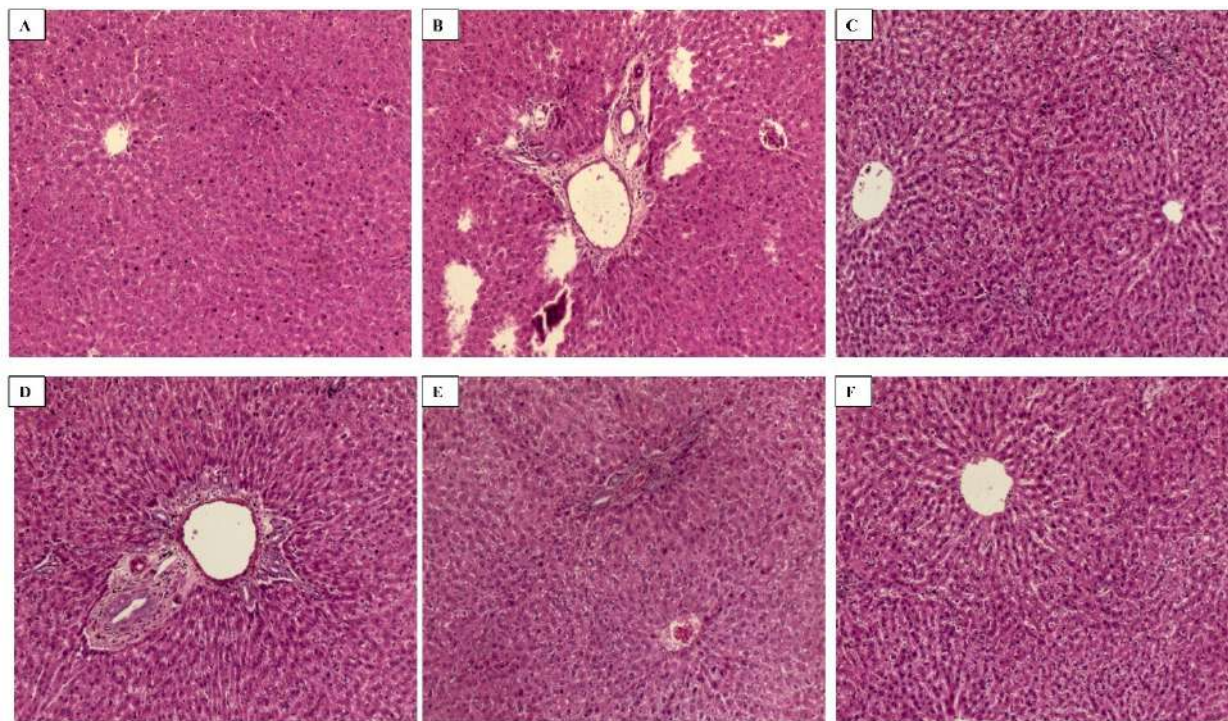

Figure 9: (A-F) illustrates the histological examination of liver tissues from both experimental and control rats. Animals in group I (A), which were not exposed to DMBA and group II (B), which were exposed to DMBA, exhibited dilated sinusoids and a loss of architectural structure. In contrast, animals in group III (C), treated with ESC-CNPs alone, maintained normal hepatocyte architecture. Hepatocytes in group IV (D), treated with ESC-CNPs at 25 mg/kg body weight, showed vacuolar degeneration. Group V (E) rats, treated with ESC-CNPs at 50 mg/kg body weight, had nearly normal hepatocyte architecture with dilated sinusoids and moderate necrosis. Rats in group VI (F), treated with ESC-CNPs at 100 mg/kg body weight, displayed noticeably dilated sinusoids.
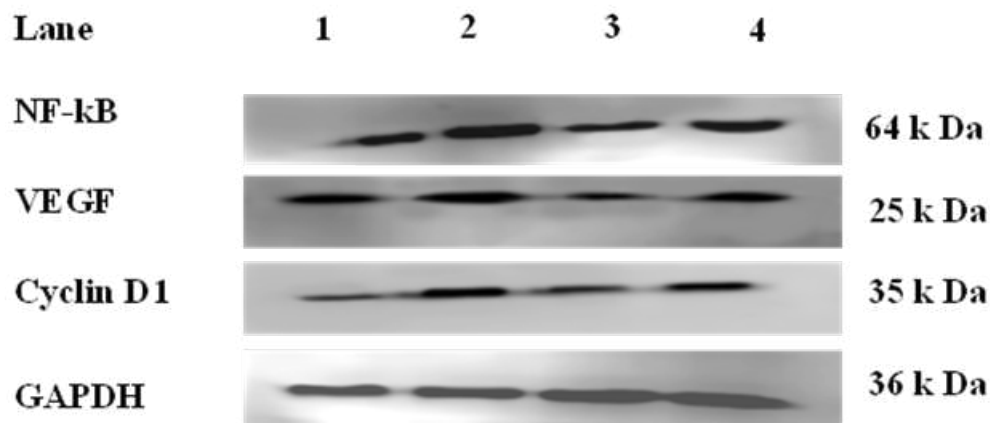
Figure 10: The effect of ESC-CNPs on the expression of VEGF, NF-κB and Cyclin D1. Compared to the control group (lane 1), breast tissue from DMBA-treated animals (lane 2) showed a noticeable increase in the expression levels of VEGF, NF-κB and Cyclin D1. Conversely, ESC-CNPs (100 mg) treatment resulted in reduced levels of VEGF, NF-κB and Cyclin D1 in comparison to DMBA-treated rats (lane 3). No significant difference was observed between the ESC-CNPs control group (lane 4).

play a significant role in altering the intracellular redox state by scavenging free radicals.[34]

Cancer development and progression are linked to increased production of Reactive Oxygen Species (ROS) and lipid peroxidation, which disrupt normal metabolic processes and contribute to weight loss.[35] In this study, DMBA-induced rats experienced a reduction in overall body weight due to alterations in energy metabolism and elevated lipid peroxidation, which were significant factors in tumor growth. In contrast, rats treated with ESC-CNPs gradually gained weight, indicating that the ESC-CNPs ability to neutralize free radicals contributed to lower lipid peroxidation levels. Furthermore, ESC-CNPs significantly reduced tumor size in cancer-bearing rats, likely due to their anti-tumor or inhibitory properties. Esculetin, known for its anti-tumor effects across various cancer cell lines, is a key component of this therapeutic approach. Previous studies have shown that elevated levels of TBARS in tumor cells are linked to excessive free radical production and can serve as an indicator of tissue damage. In this investigation, we observed increased levels of LOOH and TBARS in animals treated with DMBA. Similarly, Lakshmi and Subramanian reported elevated TBARS levels in rats with breast tumors.[36] However, the administration of ESC-CNPs significantly altered lipid peroxidation levels, highlighting its potential anti-lipid peroxidative effects.

Antioxidants are the primary defense against oxidative stress. Previous research indicates that antioxidant enzymes play a crucial role in protecting against tumor-promoting substances. Notably, reduced activity of antioxidant enzymes such as SOD, CAT and GPx is often associated with malignancy or cellular transformation, which increases susceptibility to carcinogens.[37,38] In our study, we observed decreased levels of the non-enzymatic antioxidant GSH, as well as lower activities of enzymatic antioxidants SOD, CAT and GPx in rats treated

with DMBA alone. Treatment with ESC-CNPs in DMBA-treated rats led to a dose-dependent improvement in antioxidant status, though it did not completely prevent tumor incidence or growth. This suggests that while ESC-CNPs antioxidant properties can partially mitigate carcinogenesis, they do not fully eliminate it.

Phase I and Phase II enzymes play crucial roles in detoxifying reactive metabolic intermediates produced by carcinogens. Phase I enzymes, such as CYP450-dependent oxygenases, generate reactive molecules from substances like DMBA. Subsequently, Phase II enzymes, including GST and GR, are responsible for conjugating these reactive compounds with hydrophilic groups. These conjugates can form covalent adducts with DNA bases.[39,40] In our study, we observed increased activities of CYP450 and Cyt-b5 and decreased activities of GST and GR in rats treated solely with DMBA. This finding aligns with Mathivadhani *et al.*, who reported elevated Phase I enzyme activities and reduced Phase II enzyme activities in rats with mammary tumors.[41] However, ESC-CNPs administration significantly altered the activity of these enzymes in our study. Additionally, previous research has shown that ESC-CNPs can stimulate Phase II enzymes in cultured mammary cancer cell lines.

Due to its oxidative nature, cholesterol is prone to contributing to the accumulation of lipid peroxidation products under oxidative stress.[42] Elevated cholesterol levels, which are associated with oxidative stress and hypercholesterolemia, have been shown to activate cells and promote the production of oxygen free radicals.[43] Dhamodharan *et al.* (2021) observed significant increases in plasma levels of Total Cholesterol (TC), Triglycerides (TG), Phospholipids (PL) and Free Fatty Acids (FFA) in rats with DMBA-induced mammary cancer.[44] Bani *et al.*, also reported elevated levels of Total Cholesterol (TC), Triglycerides (TG), Phospholipids (PL) and Free Fatty Acids (FFA) in plasma samples from individuals with breast cancer.[45] In our study, we

observed similar increases in plasma levels. However, mammary tissue samples exhibited lower levels of PL and FFA, likely due to enhanced PL breakdown, which may have led to membrane dysfunction.

Histopathological examination of mammary tissue from cancer-bearing animals revealed the presence of carcinomas with malignant tumors infiltrating the tissue. In contrast, rats treated with ESC-CNPs exhibited no signs of cellular proliferation or necrosis. Notably, the most significant effects were observed in animals treated with 100 mg/kg bw ESC-CNPs, which showed increased areas of fibrosis and preserved mammary tissue architecture. This suggests that ESC-CNPs could potentially be a safe and effective cancer treatment. Histopathological analysis of liver tissue from cancer-bearing animals showed dilated sinusoids and disrupted architecture, likely due to the carcinogen-induced generation of free radicals. In contrast, rats treated with ESC-CNPs exhibited a reversal of these changes, presumably due to the compound's antioxidant and free radical scavenging properties. The most pronounced effects were observed in animals treated with 100 mg/kg bw ESC-CNPs, which showed near-normal hepatocyte architecture with only mild dilation of sinusoids and moderate necrosis. This indicates that ESC-CNPs may offer protective effects for the liver against harmful substances.

Malignant tumors are characterized by chronic inflammation and angiogenesis. NF-κB, a key transcription factor, regulates the expression of various protein classes, including those involved in inflammation. When NF-κB is active, it binds to COX-2 promoters in the nucleus, triggering an inflammatory response and facilitating cancer progression. NF-κB significantly influences the transcriptional activation of apoptotic proteins, cytokines, angiogenic factors and cell adhesion molecules.[46] NF-κB plays a critical role in the development of various cancers, including mammary cancer when it is expressed inappropriately. Research has shown that NF-κB expression progressively increases from healthy tissues through precancerous lesions to cancerous tissues. Numerous studies and accumulating evidence have confirmed that NF-κB is often overexpressed in malignant tissues.

Neovascularization, or the formation of new blood vessels, is a key feature of tumor cells, essential for supplying tumors with nutrients and oxygen. Vascular Endothelial Growth Factor (VEGF) is a crucial growth factor that affects endothelial cells and plays a pivotal role in various biological processes, including vascular permeability, cell migration and angiogenesis. VEGF, a potent mitogen, initiates angiogenesis by binding to tyrosine kinase receptors on endothelial cells, thereby promoting angiogenic sprouting.[47] Several studies have established a connection between angiogenesis and mammary cancer development, with VEGF overexpression being associated with metastasis, advanced disease stages and overall survival in mammary cancer.[48] Additionally, treatments with compounds such as apigenin,[49]

luteolin,[50] and Kalpaamruthaa have been reported to reduce VEGF expression in mammary cancer models.[51]

Cyclin D1 is a key regulatory marker in the cell cycle, essential for managing both cell division and apoptosis. This oncogene encodes a positive regulator that drives the progression of the G1 phase of the cell cycle, initiating DNA synthesis. Cyclin D1 activates its kinase partners CDK4 and CDK6, leading to the phosphorylation of the retinoblastoma protein and the subsequent transcription of genes that promote the transition to the S-phase of the cell cycle.[52] Harakeh *et al.* observed that rats treated with DIM@ CS-NP showed a significant reduction in Cyclin D1 expression compared to rats treated with DMBA and DIM.[53]

CONCLUSION

Our study indicates that ESC-CNPs exhibit chemopreventive effects against DMBA-induced mammary cancer in SD rats. The results reveal that ESC-CNPs significantly inhibited tumor development in both plasma and mammary tissues, reduced lipid peroxidation, improved antioxidant status and modified biotransformation enzyme activities. Additionally, ESC-CNPs positively influenced the lipid profile. Oral administration of ESC-CNPs to DMBA-treated rats led to a marked downregulation of VEGF, NF-κB and cyclin D1 expression in the mammary glands. Overall, the study demonstrates that ESC-CNPs may reduce cellular proliferation, decrease angiogenesis and induce apoptosis during DMBA-induced mammary carcinogenesis in SD rats.

ANIMAL ETHICS

This work carried out after getting ethical approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animals (CPCSEA approval no: 1357).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ESC-CNPs: Esculetin Loaded Chitosan Nanoparticles; **DMBA:** 7,12 dimethylbenz [a]anthracene; **Mg:** Milligram; **b.w:** Body Weight; **LOOH:** Lipid peroxides; **TBARS:** Thiobarbituric acid reactive substances; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GPX:** Glutathione peroxidase; **GSH:** Reduced glutathione; **CYP450:** Cytochrome P450; **Cyt-b5:** Microsomal cytochrome b5; **GST:** Glutathione S-transferases; **GR:** Glutathione reductase; **TC:** Total cholesterol; **TG:** Triglycerides; **PL:** Phospholipids; **FFA:** Free fatty acid; **VEGF:** Vascular endothelial growth factor; **NF-κB:** Nuclear factor kappa-B; **BRCA1:** BReast CAncer gene 1; **BRAC2:** BReast CAncer gene 2; **HER2:** Human epidermal growth factor receptor 2; **SD rat:** Sprague-Dawley rat; **GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase; **PVDF:** Polyvinylidene fluoride.

SUMMARY

This study aimed to investigate the chemopreventive potential of Esculetin-loaded Chitosan Nanoparticles (ESC-CNPs) against 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer in female Sprague-Dawley rats. Rats injected subcutaneously with DMBA developed mammary tumors. Treatment with ESC-CNPs, at 100 mg/kg bw, significantly inhibited tumor growth, restored antioxidant levels and normalized phase I and II enzyme activity and lipid profiles. Moreover, ESC-CNPs downregulated the expression of VEGF, NF-kB and Cyclin D1. These findings highlight ESC-CNPs potential as a chemopreventive agent for breast cancer.

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