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Capsaicin Encapsulated Chitosan Nanoparticles Augments Anticarcinogenic and Antiproliferative Competency Against 7,12 Dimethylbenz(a)anthracene Induced Experimental Rat Mammary Carcinogenesis

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Capsaicin is a powerful phytochemical spotted in chilies, starkly tied up with a bunch of health benefits but its clinical applications in cancer therapy are limited due to its poor solubility, and low bioavailability. Nanotechnology offers a strategy to discover new formulations for hydrophobic agent.
Aim: The main intent of the current research was to investigate the effect of Capsaicin encapsulated chitosan nanoparticles (CAP@CS-NP) on 7,12-Dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in rats.
Methodology: Mammary tumor was induced in female rats by injecting DMBA (25mg/kg b.wt) at the first week of the experiment. After 7 weeks, CAP@CS-NP (4mg/kg b.wt) was administered orally to DMBA induced tumor bearing rats for 21 days (thrice per week). The experiment was terminated at the end of the 14th week and their plasma and tissue sections were analyzed.

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Results: We found that significantly elevated levels of lipid peroxidation and diminished levels of antioxidant status in plasma, liver and mammary tissues. Increased levels of detoxification phase I enzymes and dropped levels of phase II enzymes in liver and mammary tissues in DMBA induced tumor bearing rats. As a result, oral administration of CAP@CS-NP suppressed the tumor growth, significantly raised body weight and restored abnormal enzymatic levels to near normal ranges. Additionally, histopathological and immunohistochemical analysis were also confirmed that CAP@CS-NP protects DMBA mediated cellular disruption and also inhibits abnormal cell proliferation.

Conclusion: These findings suggest that nano encapsulation of CAP@CS-NP could be useful in targeted drug delivery and act as a promising chemotherapeutic agent to treat mammary carcinogenesis.

Keywords: Capsaicin; chitosan nanoparticle; 7,12-dimethylbenz(a)anthracene; targeted drug delivery; mammary carcinogenesis; oxidative stress.

1. INTRODUCTION

Breast cancer is the most frequent malignant disease in women and also an enormous public health hazard in both economically developed and developing countries [1]. As per 2019 statistics, nearly 268,600 peoples were diagnosed with breast cancer in the United States and approximately 41,760 women are predicted to die from this disease [2]. One in eight women is at the peril of breast cancer in their lifetime [3]. The detrimental risk factors enrolled in the development of breast cancer includes high fat, calorie diet, obesity, sedentary lifestyle, contraceptive pills, endocrine disruptors, older age, environmental factors, early menarche, late menopause, family history, prolonged hormone replacement therapy, use of tobacco and alcohol consumption. Numerous genes, particularly BRCA1, BRCA2, HER-2/neu, and p53, have also been attributed to breast cancer susceptibility and progression [4].

Heavy occupational exposure to polycyclic aromatic hydrocarbons (PAHs) mixtures brings a major risk of cancer, the key sources being cigarette smoke, automobile exhaust, charbroiled food and oil furnaces [5]. The synthetic PAH, 7,12-Dimethylbenz(a)anthracene (DMBA) is well known as a cytotoxic, carcinogenic, mutagenic and immunosuppressive agent. It triggers the development of free radicals that cause carcinogenesis and is metabolised oxidation converted into DNA attacking electrophiles in the body, as well as releases an excessive amount of reactive oxygen species (ROS) [6,7]. The importance of this DMBA toxin in mammary epithelial cells can be derived from the increased prevalence of DNA adducts [6]. In recent times, DMBA is used to induce various forms of carcinogenesis in ideal models by modifying the metabolic level of enzymes in order to study the therapeutic effect of natural and synthetic agents in the laboratory.

Series of scientific reports have shown that oxidative stress is linked to an escalated the chance of breast cancer. In fact, when the body's ability to detoxify reactive intermediates exceeds the creation of ROS, oxidative stress arises. This disproportion causes oxidative damages to molecules. ROS can also imbalance the antioxidant enzymes like glutathione peroxidase, catalase, superoxide dismutase etc. Changes in these essential antioxidant’s levels may affect the cellular processes such as cell development, differentiation and proliferation. In this regard, the production and accumulation of free radicals, a highly reactive molecule, in the system causes peroxidation of membrane lipids, resulting to cellular damage and derangement in membrane function and integrity [8,9]. Lipids are used to exert the immune regulatory effects. Increase of lipid level in plasma may affect host immune mechanisms, which raise the secretion of estradiol by disrupting gonads so that it can trigger malignancies in the mammary gland and help to improves tumor cells [10,11]. Although numerous drugs are commercially attainable for the treatment of mammary cancer, none has been proven to be ideal due to undesirable side effects. This condition entails the need for new chemotherapeutic drugs with a minimal side effect for the treatment of mammary cancer.

Natural products obtained from plants have always been believed to be suitable sources of prototype medicinal drugs for the treatment of myriad diseases, which have been established to exert anti-cancer activities partially based on ability to quench reactive oxygen species, and protect critical cellular components like DNA,

proteins and lipids from oxidative damage [12]. Phytochemicals present in vegetables, fruits and spices were hot topics in the field of chemotherapy because they exhibit various inhibitory actions against cancer initiation, promotion, progression and metastasis. Capsaicin (trans-8-methyl-n-vanillyl-6-nonenamide) (CAP), a phytochemical available in chili peppers of the *Capsicum* genus, was reported to judiciously inhibit the growth of tumor cells [13,14]. Despite previous conflicting results from research studies that have determined its potential mutation and oncogenic activity, subsequent investigations have shown that CAP induces apoptosis in a variety of tumor cells [15-17]. It also tends to interfere with drug metabolizing enzymes, primarily microsomal Cytochrome P450 and Cytochrome b5 dependent monooxygenases, which are implicated in both the stimulation and detoxification of various chemical carcinogens and mutagens [18]. A major stumbling block in the preclinical and clinical practice of CAP is poor bioavailability due to its low solubility and high lipophilicity that prompts to restricted therapeutic potential and unsatisfactory outcomes [19]. Fig. 1 shows the structure of CAP.

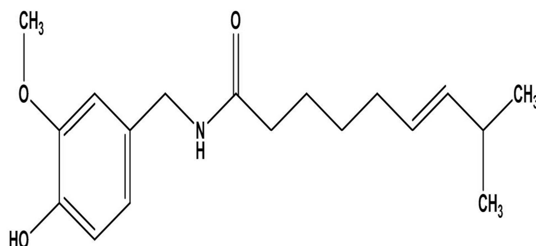

Fig. 1. Structure of Capsaicin

Nanostructures have the proficiency to solve these limitations by acting as carriers for therapeutic agents and nano encapsulation is a crucial technique to optimize the pharmacokinetics of drugs and to target specific regions of the body [20,21]. Chitosan is a biodegradable and bioadhesive polysaccharide. It has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material because of its recognized mucoadhesivity, ability to enhance the penetration and increase bioavailability. So, such approaches made it possible to develop a great interest in scrutinizing the encapsulation of CAP in nanoparticles could endow with better bioavailability, sustained release property with enhanced stability and solubility on awful environmental situation. Hence, our investigation emphasizes the anticarcinogenic and antiproliferative potential of CAP encapsulated chitosan nanoparticles (CAP@CS-NP) against DMBA induced mammary carcinogenesis in female Sprague Dawley rats by analyzing the biochemical, histopathological and immunohistochemical alterations.

2. MATERIALS AND METHODS

2.1 Chemicals

Capsaicin, 7,12-Dimethylbenz(a)anthracene (DMBA), chitosan, sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich Co.Ltd. Thiobarbituricacid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), reduced glutathione (GSH) and dimethyl sulphoxide (DMSO) were purchased from Himedia. All other chemicals used were of analytical grade procured from local commercial sources.

2.2 Preparation and Characterization of CAP@CS-NP

CAP@CS-NP, CS-NP and Free CAP@NP were synthesized *by novel* method *of* ionic gelation with TPP solution *(*Gelling *agent)* [22]. The lyophilized samples CAP@CS-NP, CS-NP and Free CAP@NP were characterized by UV–visible spectroscopy, SEM analysis, FT-IR analysis and *In vitro* drug release.

2.3 Experimental Rats and Diet

The study was conducted on 8 to 10 weeks old healthy female Sprague Dawley rats, weighing approximately 130–150g were obtained from Biogen Laboratory Animal Facility, Bangalore, India and maintained in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Chidambaram, Tamil Nadu, India. The rats were housed in well ventilated large spacious polypropylene cages (six rats /cage) lined with husk under temperature (24 ± 2°C) with relative humidity 50 ± 10% and photoperiod of 12 h light/dark. The rats were given standard pellet diet and water throughout the experimental period.

2.4 Induction of Mammary Carcinogenesis

In current research, DMBA was used as a chemical carcinogenic agent. Mammary tumor was induced in female Sprague Dawley rats by using single subcutaneous injection (near the

mammary gland) of DMBA 25mg/kg b.wt diluted in 1mL emulsion (0.75mL of sunflower oil and 0.25mL of physiological saline) [23].

2.5 Dose Selection

Several literature studies have proven CAP is effective in chronic inflammation, metabolic disorder, oxidative stress and toxicity. Anandakumar et al. suggest that CAP has the possible anticancer potential by the dose of 10mg/kg b.wt in Swiss albino mice model [24]. Based on this literature studies we decided two doses, CAP 8mg/kg b.wt and CAP@CS-NP 4mg/kg b.wt. These doses are administered orally to tumor bearing rats (After 7weeks).

2.6 Experimental Design

Fig. 2 illustrates the schematic representation of experimental design. Experimental rats were assorted into six groups of six rats each. Group I rats served as control (normal untreated rat). Group II to V rats were received 25mg/kg b.wt of DMBA during the first week of the experiment. After 7 weeks, the tumor bearing groups III, IV and V rats were treated with CAP, CAP@CS-NP and CS-NP at the concentration of 8mg/kg b.wt, 4mg/kg b.wt and 5mg/kg b.wt for 21 days (thrice per week). Group VI rats received bare Free CAP@NP administered orally for 21 days (thrice per week). The doses were fixed based on previous research studies [24-26]. The experiment was terminated at end of the 14th week, all the rats were sacrificed. Blood sample were collected in heparinised tubes and then centrifuged at 1000×*g* for 15 min for the separation of plasma. The plasma samples were used for the biochemical analysis. Liver and mammary tissues were dissected out immediately, washed well with ice-cold saline and also homogenized in 0.1M Tris hydrochloric acetic acid buffer (pH=7.4), centrifuged at 3000g for 10 mins at 4°C. The supernatant was collected, which was used to assay various biochemical parameters on the same day of sacrifice. Further, liver and mammary tissues were used for histopathological and immunohistochemical studies.

2.7 Estimation of Lipid Peroxidation

The lipid peroxidation was estimated by measuring the levels of thiobarbituric acid reactive substances (TBARS) in plasma, liver and mammary tissues. The plasma TBARS was assayed by the method of Yagi [27] and tissues TBARS (liver and mammary) were estimated according to the method of Ohkawa et al [28].

2.8 Estimation of Enzymatic and Non-enzymatic Antioxidants

The activities of enzymatic antioxidant such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in plasma and tissues (liver and mammary) were assayed by the methods of Kakkar et al [29], Sinha [30] and Rotruck et al. [31] and non-enzymatic antioxidants such as reduced glutathione (GSH), vitamin C (Vit C) and vitamin E (Vit E) levels in plasma and tissues (liver and mammary) were determined by the methods of Ellman [32], Omaye et al. [33] and Desai [34] respectively.

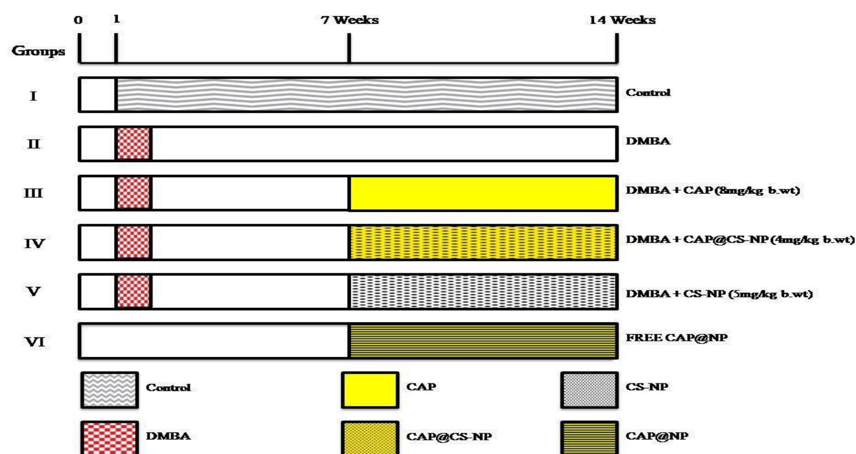

Fig. 2. Schematic representation of experimental design

2.9 Estimation of Phase I and Phase II Detoxification Enzyme

The status of cytochrome P450 (Cyt P450) and cytochrome b5 (Cyt-b5) in liver and mammary tissues were measured according to the method proposed by Omura and Sato [35]. Activities of glutathione-S-transferase (GST) in liver and mammary tissues were determined by Habig et al [36] methods. Glutathione Reductase (GR) levels in liver and mammary tissues were determined by Carlberg and Mannervik [37]. The levels of DT-diaphorase (DT-D) in liver and mammary tissues were assayed by the methods of Ernster [38].

2.10 Histopathological Examination

The histopathology studies of liver and mammary tissues in experimental rats were sliced, immersed in neutral buffered formalin (10% formaldehyde) for fixation and dehydrated with ethanol solutions and then embedded in paraffin wax. Tissues (3-5 μm in thickness) were cut and stained with haematoxylin and eosin (H&E). Then, slides were observed under microscope (40×). All histopathological changes were examined by the pathologist.

2.11 Immunohistochemical Examination

The immunohistochemical of the cell proliferative marker PCNA (proliferating cell nuclear antigen) and Cyclin D1 were analyzed using a labeled Streptavidin-biotin method. Mammary tissues specimens were fixed in 10% buffered neutral formalin, washed, dehydrated, cleared, embedded in paraffin, casted and finally section (5 μm thick) on poly-L-lysine coated glass slides. The tissue slides were deparaffinized by placing the slides in an oven at 60° C for 10 mins and rinsed twice in xylene for 5 mins each. Slides were hydrated in a graded ethanol series for 10 mins each and then finally washed in double distilled water for 5 mins. Then, the sections were incubated in the universal proteinaceous blocking solution for 15 minutes at 37° C for blocking the binding sites then incubated in relevant buffers containing PCNA and Cyclin D1 primary antibodies (Sigma Aldrich, USA) which are utilized for immunohistochemical staining of proteins. Thereafter, it was incubated in the buffer containing secondary antibodies conjugated to horseradish peroxidase (HRP) and 3,3'-diaminobenzidine (DAB) substrate. After reaching the color intensity, slides were cleaned and stained with hematoxylin and covered in a proper medium. Every immunostained slide was investigated under the bright field Olympus microscope (40×).

2.12 Statistical Analysis

The data were expressed as mean ± standard deviation (SD). A statistical analysis was carried out using SPSS V23.0 (IBM SPSS, USA) software package. The comparisons between groups were done using one way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A value of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Effect of CAP and CAP@CS-NP on Body Weight

Table 1 illustrates the mean body weight of control and experimental rats. Initially, there were no significant changes in the body weight of control and experimental rats. Finally, we observed significantly dwindled in the body weight of DMBA induced tumor bearing rats (Group II) when compared with control rats (Group I). On the contrary, in tumor bearing rats treated with CAP 8mg/kg b.wt (Group III) and CAP@CS-NP 4mg/kg b.wt (Group IV) showed significantly increased the body weight when compared to DMBA induced rats (Group II). However, no significant changes were found in CS-NP 5mg/kg b.wt (Group V) treated rats when compared to DMBA induced rats (Group II) and no significant differences were observed in Free CAP@NP (Group VI) alone treated rats when compared to control rats (Group I). Predominantly, CAP@CS-NP 4mg/kg b.wt was shown to be more effective than CAP 8mg/kg b.wt.

3.2 Effect of CAP and CAP@CS-NP on Carcinogenic Parameter

Table 2 illustrates the carcinogenic parameters including tumor incidence, total number of tumors and tumor volume of control and experimental rats. This study demonstrated that 100% of tumor incidence was observed in DMBA induced rats. Whereas, tumor bearing rats treated with CAP 8mg/kg b.wt (Group III) and CAP@CS-NP 4mg/kg b.wt (Group IV) was remarkably reduced the tumor volume and tumor incidence as 50% and 16% respectively, when compared to DMBA induced rats (Group II). However, no changes

were observed in Free CAP@NP (Group VI) alone treated rats when compared with the control rats (Group I). CAP@CS-NP 4mg/kg b.wt was proven to be more powerful than CAP 8mg/kg b.wt in suppressing tumor volume.

3.3 Effect of CAP and CAP@CS-NP on Lipid Peroxidation Marker

Fig. 3 revealed the levels of lipid peroxidation (TBARS) in plasma, liver and mammary tissues of control and experimental rats. The levels of TBARS were significantly elevated in DMBA induced rats (Group II) when compared with control rats (Group I). On the flip side, administration of CAP 8mg/kg b.wt (Group III) and CAP@CS-NP 4mg/kg b.wt (Group IV) significantly curtailed the levels of TBARS when compared with DMBA induced rats (Group II). No changes were noted in CS-NP 5mg/kg b.wt (Group V) treated rats when compared with DMBA induced rats (Group II). Although, no significant differences were detected in Free CAP@NP (Group VI) alone treated rats when compared to control rats (Group I). CAP@CS-NP 4mg/kg b.wt was found to be more efficient than CAP 8mg/kg b.wt in reducing TBARS levels.

3.4 Effect of CAP and CAP@CS-NP on Enzymatic and Non-enzymatic Antioxidants

Figs. 4, 5 and 6 shows the levels of enzymatic antioxidants (SOD, CAT and GPx) and non-enzymatic antioxidants (GSH, Vit C and Vit E) in plasma, liver and mammary tissues of control and experimental rats. The levels of SOD, CAT, GPx, GSH, Vit C and Vit E were significantly diminished in DMBA induced rats (Group II) when compared with control rats (Group I). Contrastingly, administration of CAP 8mg/kg b.wt (Group III) and CAP@CS-NP 4mg/kg b.wt (Group IV) significantly escalated the levels of SOD, CAT, GPx, GSH, Vit C and Vit E when compared with DMBA induced rats (Group II). No alterations were noted in CS-NP 5mg/kg b.wt (Group V) treated rats when compared with DMBA induced rats (Group II). Even though, no significant modifications were noticed in Free CAP@NP (Group VI) alone treated rats when compared to control rats (Group I). CAP@CS-NP 4mg/kg b.wt was proven to be more potent than CAP 8mg/kg b.wt in raising antioxidant levels.

Table 1. Effect of CAP and CAP@CS-NP on body weight changes in control and experimental rats

Groups	Body weight (g)	
	Initial (1st Week)	**Final (14th week)**
I	134.62±13.20	169.02±15.89
II	142.71±13.77	91.72±8.13$^{###}$
III	143.46±14.29	123.64±12.63**
IV	146.25±14.89	155.45±14.07***
V	142.74±13.77	92.28±8.18
VI	135.02±13.63	168.12±15.84

Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{###}P < 0.001$ when compared with control group and $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with DMBA group

Table 2. Effect of CAP and CAP@CS-NP on tumor incidence, total number of tumors and tumor volume of control and experimental rats

Groups	Tumor incidence (%) (14th week)	Number of tumors (N) (14th week)	Tumor volume (mm^3/rat) (14th week)
I	-	-	-
II	100%	(6)/6	21.48±2.95
III	50%	(3)/6	10.62±1.39***
IV	16%	(1)/6	4.52±0.41***
V	100%	(6)/6	21.42±2.94
VI	-	-	-

Tumor volume was measured using the formula $V = 4/3\pi$ (D1/2) (D2/2) (D3/2), where D1, D2 and D3 are the three diameters (in mm) of the tumor; () indicates total number of rats bearing tumors. Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{***}P < 0.001$ when compared with DMBA group

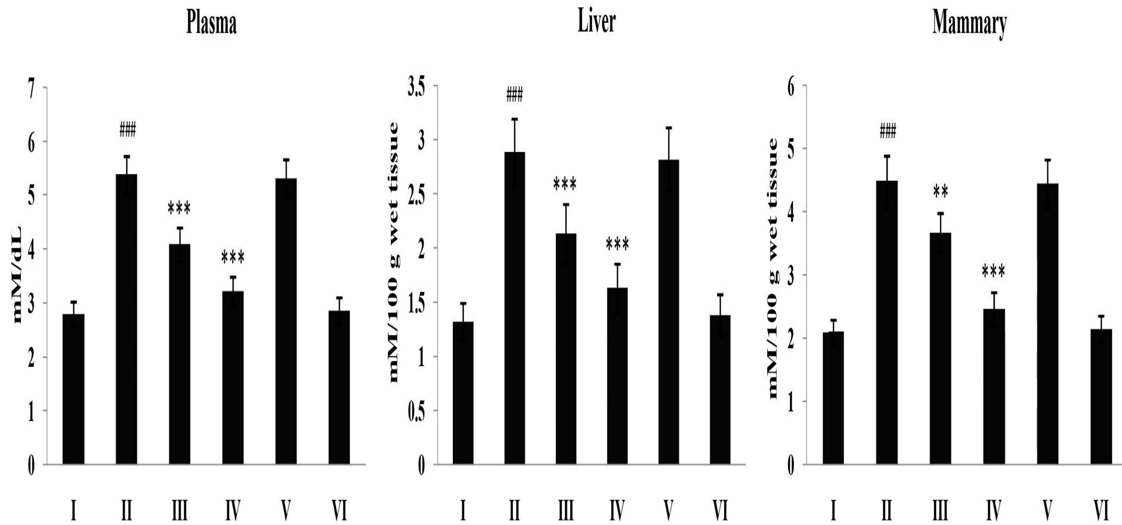

Fig. 3. Effect of CAP and CAP@CS-NP on TBARS levels in plasma liver and mammary tissues of control and experimental rats

Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{\#\#\#}P < 0.001$ when compared with control group and $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with DMBA group

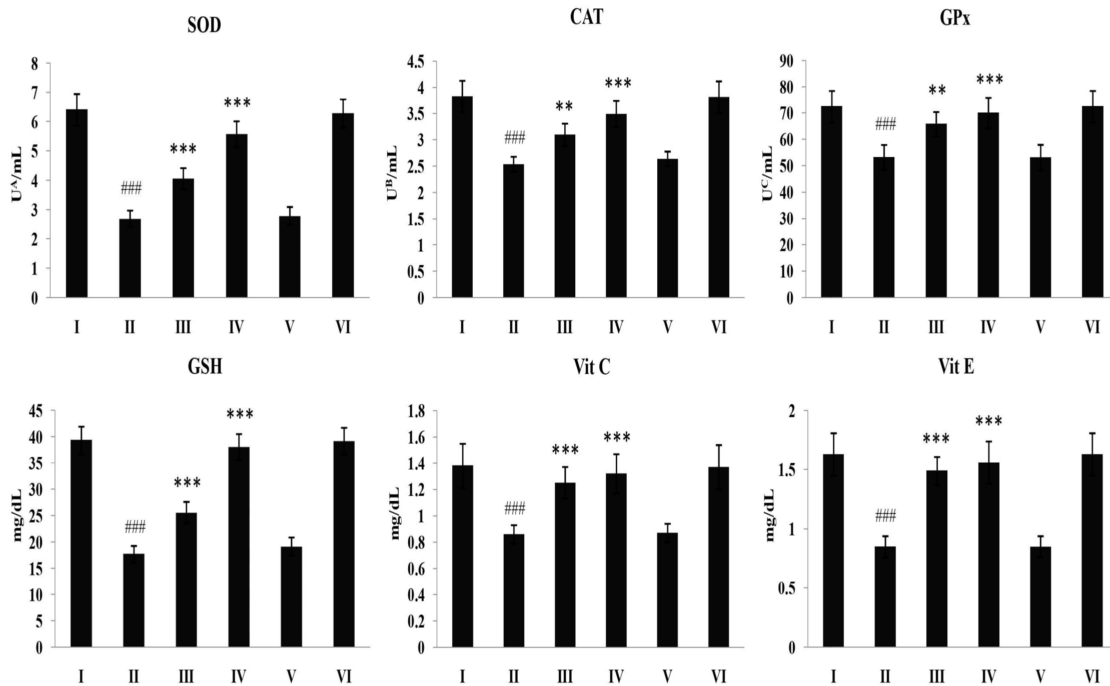

Fig. 4. Effect of CAP and CAP@CS-NP on enzymatic and non-enzymatic antioxidants in plasma of control and experimental rats

U^A: Amount of enzyme to inhibit 50% NBT reduction/min; U^B: µmol of H_2O_2 consumed/min; U^C: µg of GSH consumed/min; Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{\#\#\#}P < 0.001$ when compared with control group and $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with DMBA group

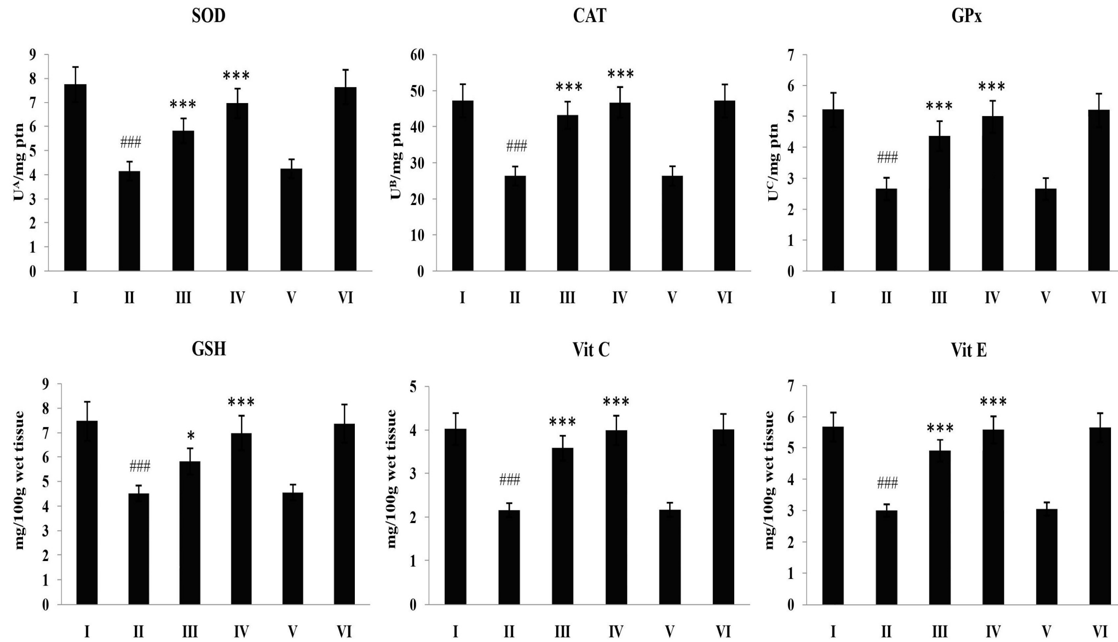

Fig. 5. Effect of CAP and CAP@CS-NP on enzymatic and non-enzymatic antioxidants in liver tissue of control and experimental rats

U^A: Amount of enzyme to inhibit 50% NBT reduction/min; U^B: μmol of H_2O_2 consumed/min; U^C: μg of GSH consumed/min; Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{###}P < 0.001$ when compared with control group and $^{*}P < 0.05$, $^{***}P < 0.001$ when compared with DMBA group

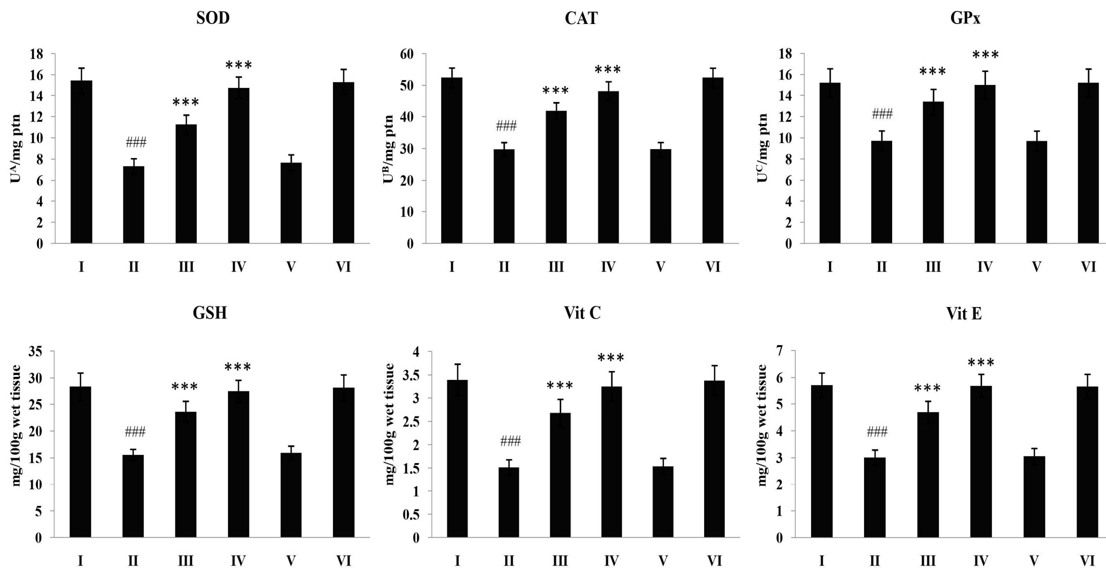

Fig. 6. Effect of CAP and CAP@CS-NP on enzymatic and non-enzymatic antioxidants in mammary tissue of control and experimental rats

U^A: Amount of enzyme to inhibit 50% NBT reduction/min; U^B: μmol of H_2O_2 consumed/min; U^C: μg of GSH consumed/min; Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{###}P < 0.001$ when compared with control group and $^{***}P < 0.001$ when compared with DMBA group

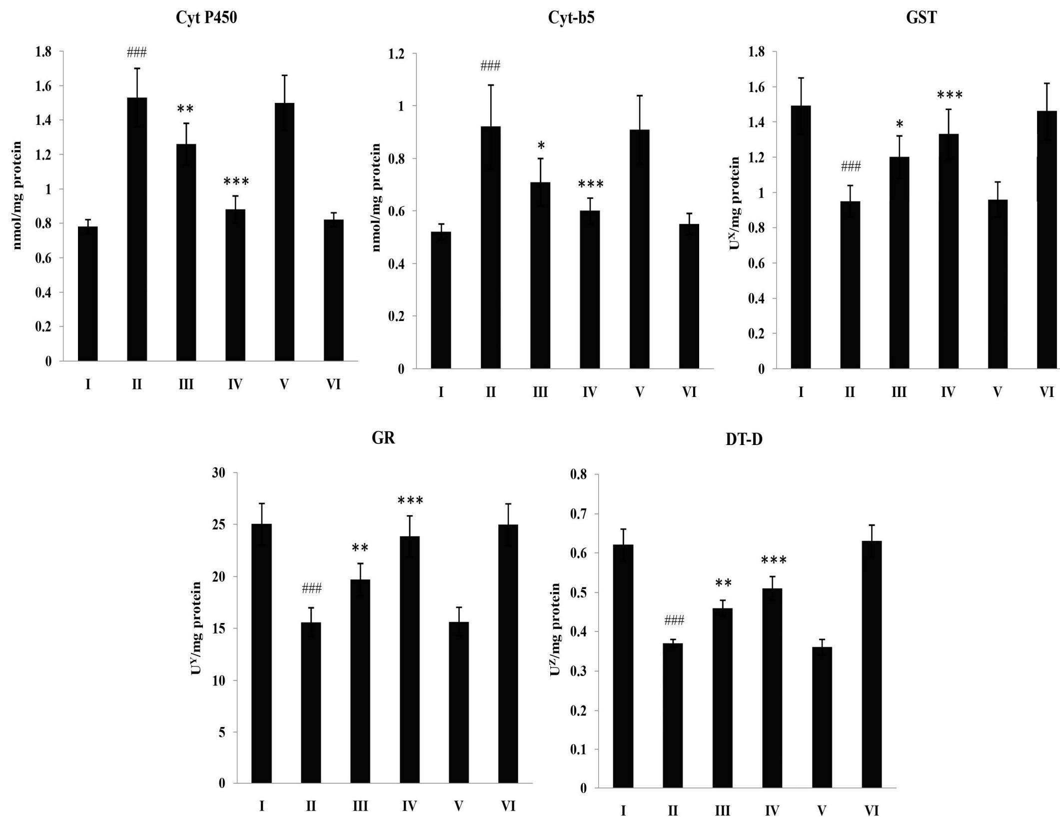

Fig. 7. Effect of CAP and CAP@CS-NP on detoxification enzymes in liver tissue of control and experimental rats

U^X– µmol of CDNB-GSH conjugate formed/mg microsomal protein/min; U^Y– µmol of NADPH oxidized/mg microsomal protein/min; U^Z–µmol of 2,6-dichlorophenol indophenols reduced/min. Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{###}P < 0.001$ when compared with control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with DMBA group

3.5 Effect of CAP and CAP@CS-NP on Detoxification Enzymes

Figs. 7 and 8 depicts the levels of phase I (Cyt P450, Cyt-b5) and phase II (GST, GR and DTD) detoxification enzymes in liver and mammary tissue microsomes of control and experimental rats. The levels of Cyt P450 and Cyt-b5 were significantly raised up, whereas the levels of GST, GR and DTD were significantly lowered in DMBA induced rats (Group II) when compared with the control rats (Group I). On the other hand, administration of CAP 8mg/kg b.wt (Group III) and CAP@CS-NP 4mg/kg b.wt (Group IV) significantly depleted in Cyt P450 and Cyt-b5 enzymes and uplifted in GST, GR and DTD enzymes when compared with DMBA induced rats (Group II). No amendments were specified in CS-NP 5mg/kg b.wt (Group V) treated rats when compared with DMBA induced rats (Group II). However, the administration of Free CAP@NP (Group VI) alone treated rats did not cause any significant emendation in the detoxification enzymes levels compared to control rats (Group I). CAP@CS-NP 4mg/kg b.wt was found to be more dynamic than CAP 8mg/kg b.wt.

3.6 Effect of CAP and CAP@CS-NP on Histopathological Changes in Liver Tissues (Haematoxylin and Eosin staining)

Fig. 9 (A–F) denotes the histological changes in liver tissue sections of control and experimental rats. The control rats (Group I) (A) and Free CAP@NP (Group VI) (F) alone treated rats showed normal architecture of hepatocytes. Contrarily, DMBA induced rats (Group II) (B) and CS-NP 5mg/kg b.wt (Group V) (E) treated rats showed loss of architecture with nuclear

pleomorphism and dilated sinusoids along with feathery degeneration. CAP 8mg/kg b.wt (Group III) (C) treated rats were showed mild periportal inflammation, feathery degeneration along with dilated sinusoids and mild necrosis. CAP@CS-NP 4mg/kg b.wt (Group IV) (D) treated rats were showed almost near normal architecture of hepatocytes with mild dilated sinusoids and mild periportal inflammation. CAP@CS-NP 4mg/kg b.wt was found to be more potent as compare to CAP 8mg/kg b.wt.

3.7 Effect of CAP and CAP@CS-NP on Histopathological Changes in Mammary Tissues (Haematoxylin and Eosin staining)

Fig. 10 (A–F) displays histological changes in mammary tissue sections of control and experimental rats. The control rats (Group I) (A) and Free CAP@NP (Group VI) (F) alone treated rats showed normal architecture of mammary tissue. Antithetically, DMBA induced rats (Group II) (B) and CS-NP 5mg/kg b.wt (Group V) (E) treated rats showed invasive ductal carcinoma with abnormal cellular proliferation. CAP 8mg/kg b.wt (Group III) (C) treated rats were showed moderate ductal hyperplasia and mild tumor infiltration. CAP@CS-NP 4mg/kg b.wt (Group IV) (D) treated rats were showed almost near normal ductal architecture. Notably, CAP@CS-NP 4mg/kg b.wt was shown to be more proficient than CAP 8mg/kg b.wt.

3.8 Effect of CAP and CAP@CS-NP on Immunohistochemical Expression in Mammary Tissues (PCNA)

Fig. 11 (A–F) shows immunohistochemical expression of PCNA in mammary tissue sections of control and experimental rats. The levels of PCNA expression was significantly increased in DMBA induced rats (Group II) (B) when compared with the control rats (Group I) (A). Contradictorily, administration of CAP 8mg/kg b.wt (Group III) (C) and CAP@CS-NP 4mg/kg b.wt (Group IV) (D) significantly decreased the levels of PCNA expression when compared with DMBA induced rats (Group II) (B). No significant alterations in the PCNA expression of CS-NP 5mg/kg b.wt (Group V) (E) treated rats when compared with DMBA induced rats (Group II) (B). However, no significant modifications were noticed in Free CAP@NP (Group VI) (F) alone treated rats when compared to control rats (Group I) (A). Importantly, CAP@CS-NP 4mg/kg b.wt was shown to be more valuable than CAP 8mg/kg b.wt.

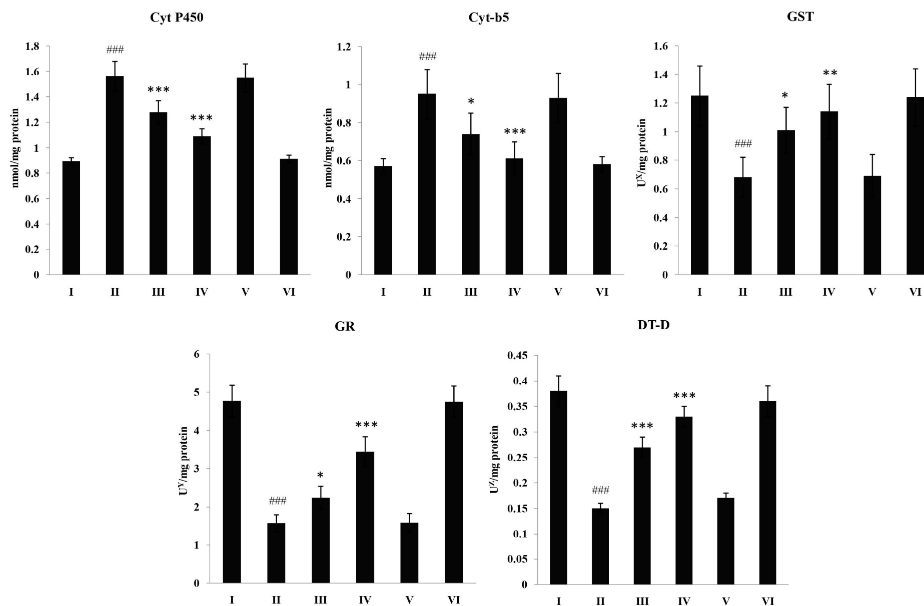

Fig. 8. Effect of CAP and CAP@CS-NP on detoxification enzymes in mammary tissue of control and experimental rats

U^X– µmol of CDNB-GSH conjugate formed/mg microsomal protein/min; U^Y– µmol of NADPH oxidized/mg microsomal protein/min; U^Z–µmol of 2,6-dichlorophenol indophenols reduced/min. Values are expressed as mean ± SD for six rats in each group. Significant levels are $^{###}P < 0.001$ when compared with control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with DMBA group

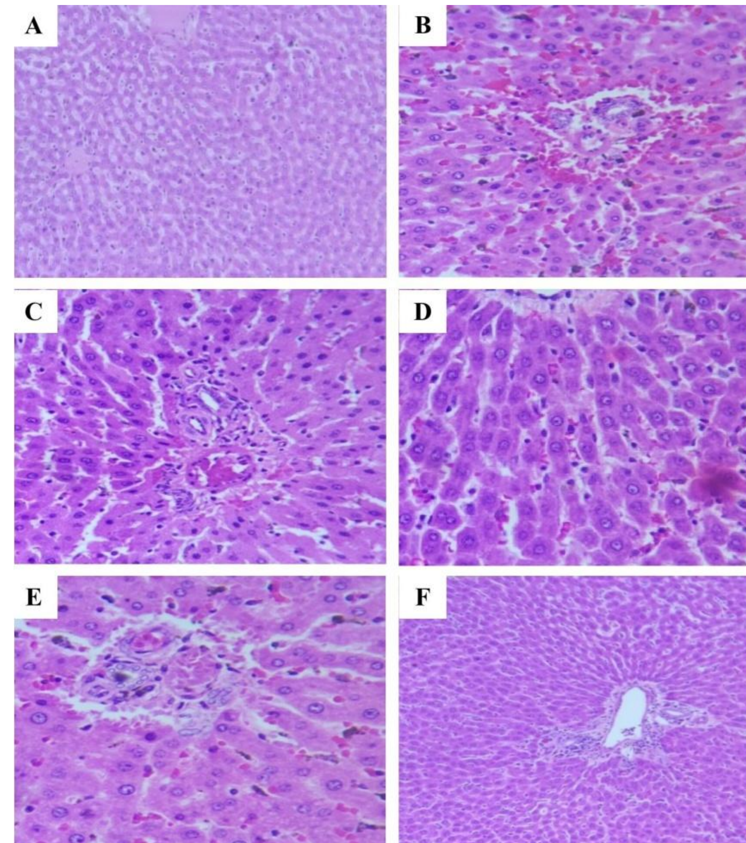

Fig. 9. Photomicrographs of histopathological changes in the liver tissues of control and experimental rats (Hematoxylin and Eosin staining showed at *40x*)

Histology on liver tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal architecture of hepatocytes; Liver tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt (E) treated rats showed loss of architecture with nuclear pleomorphism and dilated sinusoids along with feathery degeneration; Liver tissues of CAP 8mg/kg b.wt (C) treated rats showed mild periportal inflammation, feathery degeneration along with dilated sinusoids and mild necrosis; Liver tissues of CAP@CS-NP 4mg/kg b.wt (D) treated rats showed almost near normal architecture of hepatocytes with mild dilated sinusoids and mild periportal inflammation

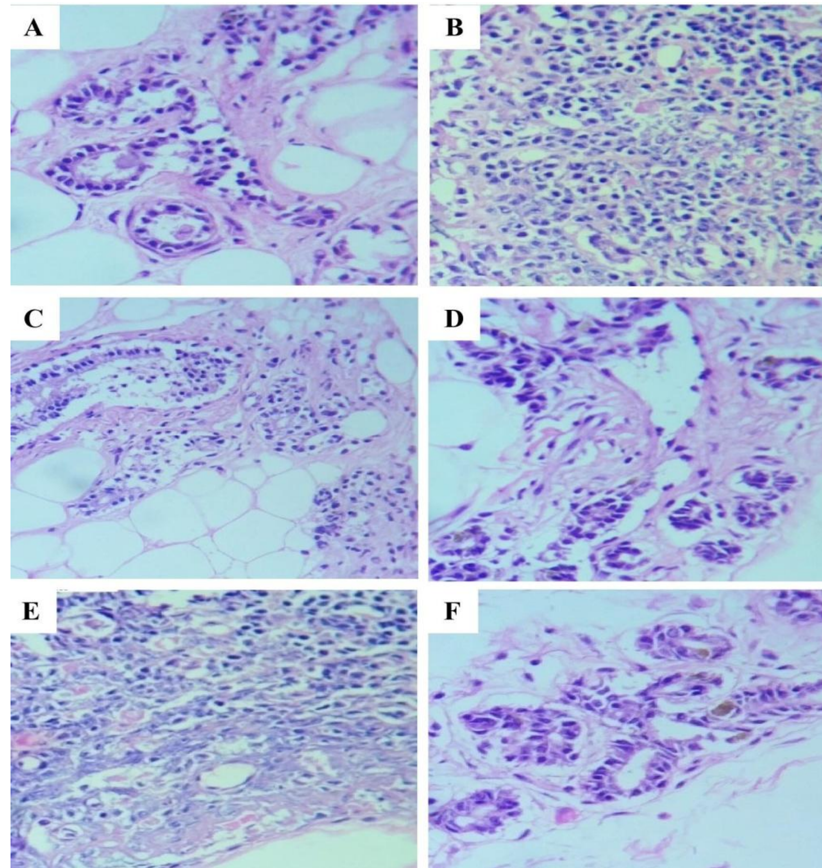

Fig. 10. Photomicrographs of histopathological changes in the mammary tissues of control and experimental rats (Hematoxylin and Eosin staining showed at *40x*)

Histology on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal architecture of mammary tissue; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt (E) treated rats showed invasive ductal carcinoma with abnormal cellular proliferation; Mammary tissues of CAP 8mg/kg b.wt (C) treated rats showed moderate ductal hyperplasia and mild tumor infiltration; Mammary tissues of CAP@CS-NP 4mg/kg b.wt (D) treated rats showed almost near normal ductal architecture

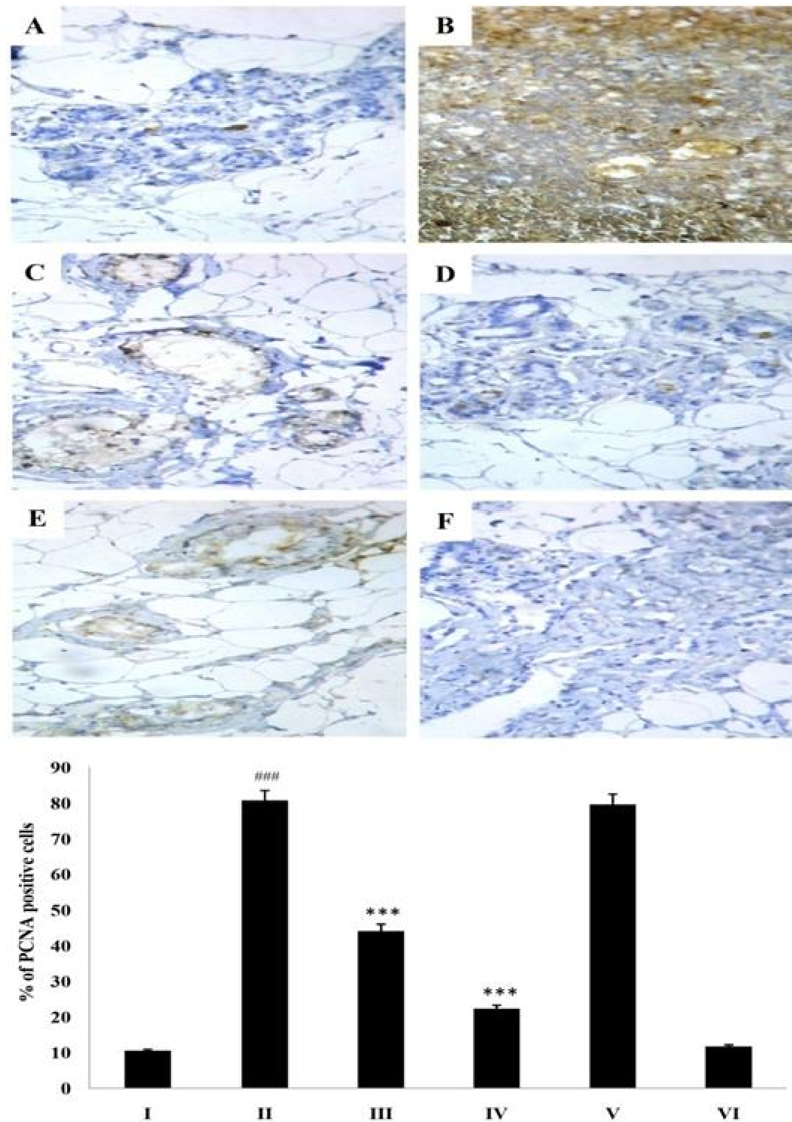

Fig. 11. Photomicrographs of immunohistochemical analysis in the mammary tissues of control and experimental rats (PCNA expression showed at *40x*)

*Immunohistochemical on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal mammary tissue staining; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt (E) treated rats showed increased expression of PCNA; Mammary tissues of CAP 8mg/kg b.wt (C) and CAP@CS-NP 4mg/kg b.wt (D) treated rats showed diminished expression of PCNA as compared to DMBA induced (B). Bar graph represents the percentage of PCNA positive cells in control and experimental rats. Statistical significance levels are ###P < 0.001 when compared with control group and ***P < 0.001 when compared with DMBA group*

3.9 Effect of CAP and CAP@CS-NP on Immunohistochemical Expression in Mammary Tissues (Cyclin D1)

Fig. 12 (A–F) shows immunohistochemical expression of Cyclin D1 in mammary tissue sections of control and experimental rats. The levels of Cyclin D1 expression was significantly increased in DMBA induced rats (Group II) (B) when compared with the control rats (Group I) (A). On the contrary, administration of CAP 8mg/kg b.wt (Group III) (C) and CAP@CS-NP 4mg/kg b.wt (Group IV) (D) significantly decreased the levels of Cyclin D1 expression

when compared with DMBA induced rats (Group II) (B). No significant changes in the Cyclin D1 expression of CS-NP 5mg/kg b.wt (Group V) (E) treated rats when compared with DMBA induced rats (Group II) (B). However, no significant variations were spotted in Free CAP@NP (Group VI) (F) alone treated rats when compared to control rats (Group I) (A). Paramountly, CAP@CS-NP 4mg/kg b.wt was shown to be more considerable than CAP 8mg/kg b.wt.

4. DISCUSSION

Chemotherapy, a most prevalent treatment for breast cancer, delivers anticancer drugs systemically to patients for counteracting the unrestrained proliferation of cancerous cells. Unfortunately, owing to nonspecific targeting by anticancer agents, lots of critical side effects occur and weak drug delivery of such agents cannot bring out the satisfactory outcome in plenty of the cases. Nano-drug delivery systems render the capacity to upgrade the therapeutic index of anticancer agents, either via enhancing the drug concentration in cancer cells or minimizing the exposure in healthy cells [39]. Accordingly, encapsulation of chemotherapeutic drugs in an appropriate nanocarriers platform could be considered a safe way of delivering anticancer drugs to target site. The innocuous biopolymer of chitosan has been used as a smart nanocarrier in drug delivery systems due to its excellent biocompatibility [40]. Among various animal models for investigating the different outlook of breast cancer, mammary tumor induction using chemical carcinogen is most popular approach. In the present report, we evaluate the anticarcinogenic and antiproliferative activity of CAP@CS-NP in DMBA induced rat mammary carcinogenesis.

Cell damage portents the onset of limitless pathophysiological disorders, which triggers in fact due to free radicals generation in the form of ROS [41]. Lipid peroxidation is a complex mechanism that leads in cell destruction and dysfunction when oxygen-free radicals attack polyunsaturated fatty acids in cell membrane lipids. Endogenous antioxidant systems (both enzymatic and non-enzymatic), behaves a pivotal role in the cellular defense mechanism against free radicals [42]. SOD is enzymatic antioxidants plays a crucial role in the frontline of defence against ROS, and it catalyzes the disproportionation reaction of superoxide anion into H_2O_2 and O_2 [43]. Other essential enzymatic antioxidants in the first line of defence are CAT and GPx. These two enzymes promote the conversion of H_2O_2 into H_2O and O_2, a harmless by-product. GPx, a selenium containing antioxidant enzyme that effectively decreases H_2O_2 and lipid peroxides into two molecules (H_2O and lipid alcohol), and convert oxidizes glutathione to glutathione disulfide [44]. Several findings have revealed that there is diminish in the functionality of SOD, CAT, and GPx accompanied by increased levels of lipid peroxidation in numerous cancer cases [45,46]. In this study, DMBA induced rats displayed increased levels of TBARS and decreased levels of SOD, CAT, and GPx activity in plasma, liver and mammary tissues, which upon treatment with CAP@CS-NP significantly altered these levels to near normal range. This portrays the potential role of CAP@CS-NP on the attenuation of oxidative stress in mammary carcinoma of the experimental rats.

The non-enzymatic antioxidants like GSH, Vit C and Vit E betray strong free radical scavenging properties due to their excellent structure, and safeguard the cells from ROS-induced disruption. GSH is the multifunctional intracellular antioxidant, and it performs directly as a free radical scavenger by offering a hydrogen atom to hydroxyl radical [47]. In previous studies, reduction of GSH levels in cancerous rats was significantly enhanced upon treatment with ideal phytochemicals. As a result, it can alleviate the oxidative stress thereby deterring the risk of cancer [48,49]. Vit C, a crucial antioxidant and it works in the second line of antioxidant protection against ROS. Specifically, Vit C acts as a good supplier of electrons for free radicals that are looking out an electron to recover their stability. So, it could contribute electrons to free radicals, thus reducing their vulnerability [50]. Vit E is a lipid soluble antioxidant, which can restrict lipid peroxidation by donating its labile hydrogen atom from phenolic hydroxyl groups. In the present study, the levels of these non-enzymic antioxidants were significantly heightened in the CAP@CS-NP treated rats when compared to tumor bearing cancer rats and this could be continually boosting the antioxidant level and thereby stopping a suitable atmosphere for oncogenesis.

Xenobiotics may provoke the pathological condition through propagation of ROS, which is integrated to cancer etiology. The phase I and phase II enzymes toil jointly to metabolize any xenobiotics, or foreign substances, that penetrate or interact with the body. Phase I enzymes, such

as Cyt P450 and Cyt-b5 acts via inserting polar functional group to original molecule, generate reactive compounds. These reactive substances can bind to DNA, trigger a mutation or become detoxify. The phase II enzymes, including GST, GR and DT-D, concoct the molecule low reactive by conjugation of the functional group. These reactions commonly produce the substrate into aqueous soluble, and conjugated endogenous products are more easily excreted from the body [51]. DMBA induced tumor rats showed raised levels of Phase I enzymes and shortened levels of Phase II enzymes. However, CAP@CS-NP administration to tumor bearing rats significantly altered these enzyme levels to near control rats. Our findings are agree with the prior report of Mariadoss et al. which proved that phloretin encapsulated chitosan nanoparticles (PhCsNPs) minimized the status of phase I enzymes and maximized the status of phase II enzymes [52].This evidence suggested that nanoformulated drug could be used to conjugation or subsequent termination of toxic metabolites developed from DMBA.

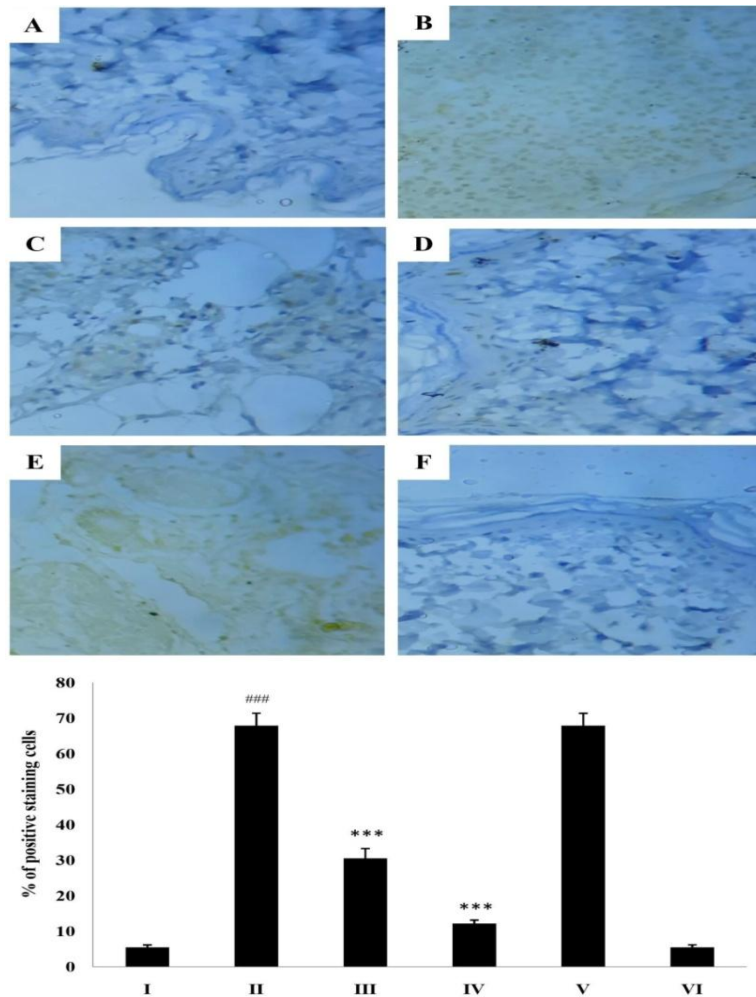

Fig. 12. Photomicrographs of immunohistochemical analysis in the mammary tissues of control and experimental rats (Cyclin D1 expression showed at *40x*)

*Immunohistochemical on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal mammary tissue staining; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt (E) treated rats showed increased expression of Cyclin D1; Mammary tissues of CAP 8mg/kg b.wt (C) and CAP@CS-NP 4mg/kg b.wt (D) treated rats showed diminished expression of Cyclin D1 as compared to DMBA induced rats (B). Bar graph represents the percentage of Cyclin D1 staining cells in control and experimental rats. Statistical significance levels are ###P < 0.001 when compared with control group and ***P < 0.001 when compared with DMBA group*

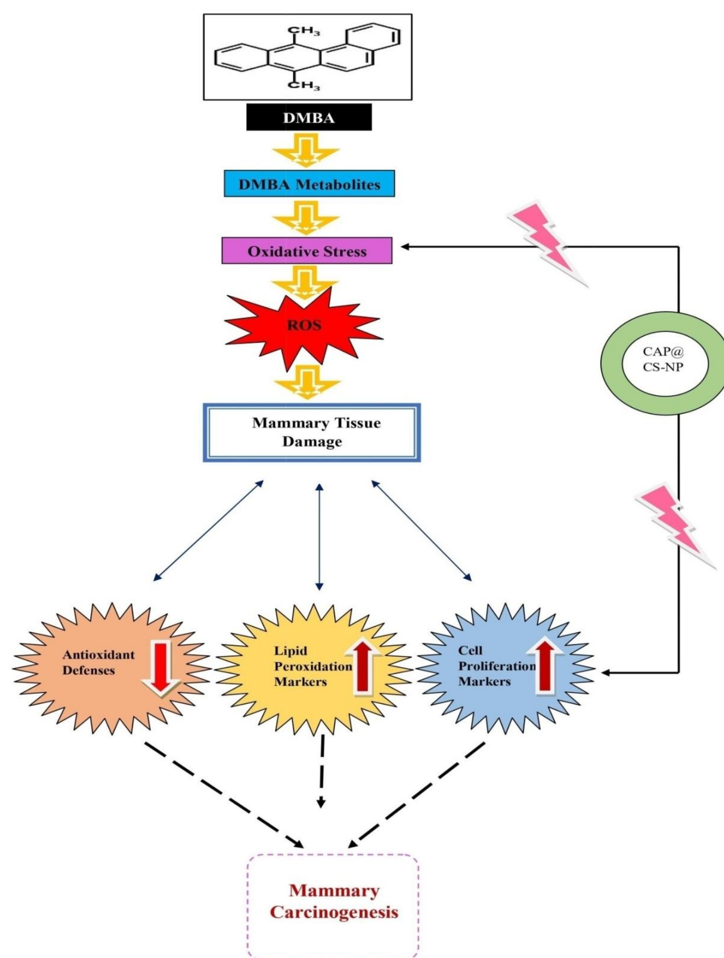

Fig. 13. Schematic diagram represents the possible mode of chemotherapeutic mechanism of CAP@CS-NP against DMBA induced mammary carcinogenesis

Histopathology (histology) is the scientific analysis of ailment at the tissue and cellular level. Hematoxylin and Eosin (H&E) staining reveals a precise morphological characteristic of nucleus and cytoplasm. In the present findings, liver tissues of DMBA induced tumor bearing rats showed defeat of architecture with nuclear pleomorphism and dilated sinusoids with feathery degeneration. These transitions could be owing to the free radical production by the chemical carcinogen. Conversely, CAP@CS-NP treated rats rectified these mutations due to the free radical scavenging antioxidant capacity of this nano formulated compound. The normal architecture of hepatocytes with dilated sinusoids and mild periportal inflammation was noted in CAP@CS-NP treated rats, which had a more pronounced effect. Therefore, our result depicts that the CAP@CS-NP has ability to secure liver from carcinogen, and also pact with the previous report of Arivazhagan et al [53]. In mammary tissues, DMBA induced cancer bearing rats displayed invasive ductal carcinoma with irregular cell proliferation. Contrastingly, CAP@CS-NP treated rats exposed the near normal architecture and no sign of cellular proliferation. Therefore, it proves that CAP@CS-NP exerts chemotherapeutic potency to DMBA induced mammary carcinogenesis via targeted drug delivery system.

Cell proliferation acts a crucial duty in multi stage carcinoma. PCNA, a significant co-factor for DNA polymerase, has been discovered to be a good marker to appraise tumor cell proliferation and progression in breast cancer. Cyclin D1 is also mandatory for normal breast growth and its dysregulated activity promotes abnormal mammary epithelial proliferation. For this reason, we examined the expression levels of PCNA and

Cyclin D1 in mammary tissues by using immunohistochemical (IHC) analysis. The outcome of our study shows that DMBA induced tumor-bearing rats elevated the expression of PCNA and Cyclin D1, which reflects increased cell proliferation. Moreover, CAP@CS-NP treated rats showed a decrease in the PCNA and Cyclin D1 expression that in turn reflects the reduced proliferative activity [54].

5. CONCLUSION

In conclusion, the present research exemplifies that DMBA administration was tied with the progression of mammary carcinoma, increased levels of tumorigenicity and oxidative stress. The new approach nano-formulation of CAP@CS-NP administration reduced tumorigenicity and oxidative stress levels via sustained release and target specific manner. From the results, CAP@CS-NP is capable of shielding the mammary tissue from oxidative harm and also inhibiting cell proliferation and tumor growth (Fig. 13). Overall, findings of our study confirmed that CAP@CS-NP has chemotherapeutic efficacy against DMBA induced mammary cancer in Sprague Dawley rats. However, further analysis is needed at the molecular level to illuminate the role of CAP@CS-NP in DMBA induced mammary carcinogenesis.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study protocol was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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