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ORIGINAL ARTICLE

3, 3′-Diindolylmethane-encapsulated chitosan nanoparticles accelerate molecular events during chemical carcinogen-induced mammary cancer in Sprague Dawley rats

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Abstract

Background 3, 3′-Diindolylmethane (DIM) is a dietary indole compound; its medical application was limited because of poor bioavailability, unsatisfying dispersity, and rapid metabolism. To conquer this problem, nanoformulation of DIM was synthesized and investigated its mechanism-based chemotherapeutic potential.

Methods 7,12-Dimethylbenz(a)anthracene (DMBA) 25 mg/kg b.wt initiated mammary carcinogenesis in rats, the investigational tumor model that closely resembles human mammary cancer. Rats had accessed after 8 weeks of tumor formation, DIM 10 mg/kg b.wt. and DIM@CS-NP 0.5 mg/kg b.wt. were administrated orally for 8 weeks.

Results The treatment with DIM@CS-NP 0.5 mg/kg b.wt. on DMBA-induced tumor-bearing rats was down-regulated Cyclin D1, Bcl-2 expression, and up-regulated proapoptotic proteins such as Bax, p53, Cytochrome-C, Caspase-9, and Caspase-3 as compared to DIM 10 mg/kg b.wt. In addition, the mRNA expressions of Cyclin D1, Bcl-2 decreased and increased Bax, p53 expression, in immunohistochemical analysis decreased expressions of Cyclin D1 and PCNA in the treatment of DIM@CS-NP 0.5 mg/kg b.wt. compared to DIM 10 mg/kg b.wt. Histological analysis of tumor tissues shows abnormal in collagen deposition in with Masson's trichrome (MT) and Picrosirius red (PR) staining, the treatment of DIM@CS-NP 0.5 mg/kg b.wt. reduced the collagen deposition as compared to DIM 10 mg/kg b.wt.

Conclusion Our results clearly provide evidence that DIM@CS-NP exerts chemotherapeutic effect than DIM in DMBA model of mammary cancer by hold back anomalous tumor cell proliferation and inducing apoptosis to intervene through alterations of up-regulated and down-regulated molecules. Taken together, the data provide new evidence for mechanism action of DIM@CS-NP on mammary cancer.

Keywords Mammary cancer · DMBA · DIM@CS-NP · Caspase-9 · Caspase-3

Introduction

Chemotherapy is normally accompanied by toxic symptoms, in this manner for a constrained measure of the medication can be given to a patient, the whole tumor cells may not be exposed to a lethal dose of the medication [1]. Thus, the utilization of nanocarrier such as biopolymer can improves the pharmacological properties of the chemotherapy drug, which can deliver encapsulated cytotoxic agents to tumor cells. The contemporary affirms of nanoparticles drug delivery in cancer, the proposition to shed light on prospect strategies. These encompass the delivery of repositioning agents and genetic material as well as unique strategies for controlling the release of drugs and enhanced uptake in tumor cells [2]. Furthermore, the interesting feature of the biopolymer such as chitosan can be broken down in the body by liposome's to harmless *N*-acetyl glucose amine making it a greatly favorable biodegradable material in forming nanocarrier for a potent drug delivery in malignancy treatment [3].

Among cancers, breast cancer (BC) is the most prevalent malignant tumor in women with its incidence increasing every year, and it is developed from the epithelial component of

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mammary gland [4]. Precise, BC arises from a highly complicated process; in BC condition, various molecular events are involved and altered genetic material functions. These genetic alterations include the activation of oncogenes and inactivation of tumor suppressor genes or an amalgamation of both [5]. In these conditions, the cancerous cell undergoes rapid proliferation, decreased cell death (apoptosis), deregulation of tumor suppressor gene, dilapidation of apoptotic genes, metastasis, and finally dysfunction of affected organs [6]. Moreover, apoptosis is a complex process that proceeds through two main pathways (intrinsic and extrinsic); each of these can be regulated at multiple levels [7]. Numerous studies scrutinized that the intrinsic and extrinsic apoptotic markers have emerged as regulating mechanisms of tumorigenesis and cell proliferation. Moreover, the regulation of proapoptotic and anti-apoptotic genes would be initiated apoptosis in breast cancer [8]. Previously, we scrutinized DIM-loaded chitosan nanoparticles reduced the inflammatory markers such as COX-2, NF-κB, and TNF-α in DMBA-induced rat mammary carcinogenesis [9].

Numerous reports state that increasing vegetable intake is linked to a reduction in the risk of several types of cancer [10]. DIM is a dietary indole compound and normally occurs in glucosinolate conjugates in *Brassica* vegetables, which have been extensively contemplated for its anticancer action [11]. In spite of its well respectable records as an anticancer specialist in many preclinical models, its properties such as poor water solvency and low bioavailability are the most important roadblocks in its advancement as an anticancer agent. Indeed, DIM pharmacokinetics studies reveal that dose-dependent absorption and nonlinear increases in Cmax, indicative of saturation in systemic absorption [12]. On the other hand, DIM by the method for broadened discharge gives better change in bioavailability suggestive of solvency restricted assimilation through the dissolvability improving the microencapsulated formation of DIM [13]. Furthermore, the encapsulation of hydrophobic bioactive in Zein/CMCS nanoparticles is an assured approach to get better their stability adjacent to crucial condition and deliver a controlled release of food/pharmaceutical applications [14]. This present study covers the expression of inhibitors and promoters of apoptotic pathways using DIM@CS-NP can be an excellent approach to inhibit the promotion and progression stages on 7, 12-dimethylbenz (a) anthracene (DMBA) induced mammary cancer. In addition, staining method also used to determine in all tissue specimens who reveals detailed about the breast tissue in normal and cancer condition and morphology in intracellular level [15].

Materials and methods

Chemicals

DIM and DMBA purchased from Sigma-Aldrich. Monoclonal antibodies such as Cyclin D1, p53, PCNA, Bax, Bcl-2, Cytochrome-C, Caspase-9, Caspase-3, β-actin (control), and secondary conjugated anti-mouse were purchased from Santa Cruz Biotechnology, USA. All the other chemicals and reagents used were of analytical grade.

Animal model

Female Sprague Dawley rats weighed 130–150 g were obtained from the National Institute of Nutrition, Hyderabad, India. Rats were housed spaciously individual cages and maintained under standard experimental conditions: temperature (24 ± 2 °C), humidity (50 ± 10%), 12 h light/dark cycle and standard food and water gave in the Central Rat House, Rajah Muthiah Medical College, and Hospital, Annamalai University, Chidambaram, Tamil Nadu. Rats allowed to acclimated a week before beginning the experiment. This experimental method was permitted by the Institutional Animal Ethics Committee by the Committee for the Purpose of Control and Supervision of Experimental Animals (Reg no. 160/1999/CPCSEA and Proposal no. 1123).

Tumor induction using the chemical carcinogen

In the mammary tumor, DMBA was used as a carcinogen-induced by a single subcutaneous injection 25 mg/kg b.wt. dissolved in the 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) [16].

Dose fixation

The experimental period was 16 weeks. DIM (10 mg/kg b.wt. dissolved in the 1 ml of PBS containing 0.1% DMSO/rat) and DIM@CS-NP (0.5 mg/kg b.wt.) were administered orally by every alternative day, after 8 weeks of tumor formation on the rat. The effective dose of DIM and DIM@CS-NP was fixed based on the previous study, and preliminary biochemical and histological studies were shown a remarkable response at the concentration of DIM

10 mg/kg b.wt. and DIM@CS-NP 0.5 mg/kg b.wt., respectively [17]. Hence, these concentrations were selected for this study.

Experimental protocol

Group I	Control
Group II	DMBA
Group III	DMBA + DIM 10 mg/kg b.wt.
Group IV	DMBA + DIM@CS-NP 0.5 mg/kg b.wt.
Group V	DIM 10 mg/kg b.wt.
Group VI	DIM@CS-NP 0.5 mg/kg b.wt.

The rats were divided into six groups with six rats in each group and were given the dose regimen as follows ($n=6$). Group, I rat served as control. Groups II–IV rats were received 25 mg/kg b.wt. of DMBA as a single subcutaneous injection during the first week of the experiment. After 8 weeks of tumor, formation group III and IV were administered with an optimum dosage of DIM, DIM@CS-NP (10 and 0.5 mg/kg b.wt.), respectively. Groups V and VI were received DIM and DIM@CS-NP (10 and 0.5 mg/kg b.wt.) serve as drug control. The tumor weighed weekly once, until end of the experimental period 16 weeks, after rats were euthanized by cervical decapitation the tissues obtained for further studies.

Pathological studies

A piece of the mammary tissues embedded in paraffin, and then, 5 µm tissues were cut using a microtome and rehydrated with xylene and graded series of ethanol. The specimens were stained with Masson's trichrome (MT) and Picrosirius red (PR) staining for analyzing the collagen deposition [18]. Histological scoring quantified by Image J software.

Immunohistochemical (IHC) expression

Paraffin-embedded mammary tissues' section was processed as described earlier and was then immunostained with the primary antibodies for Cyclin D1 and PCNA overnight at 4 °C, followed by incubation with the respective HRP-conjugated secondary antibodies and conjugates diluted 1:200 with 3% BSA in TBS and incubated for 2 h at room temperature. Sections were then washed with TBS and incubated for 5–10 min and the sections were observed (40×) for brown color formation under the bright field in a microscope [19].

Western blotting analysis

Western blotting was carried out to evaluate the expression pattern of Cyclin D1, p53, Bax, Bcl-2, Cytochrome-C, Caspase-9, Caspase-3, and β-actin (control) using the method of Laemmli [20]. The mammary tissue samples were homogenized with a buffer; the homogenates were centrifuged at 12,000*g* for 30 min at 4 °C to remove debits. The protein was loaded and separated using 10% SDS polyacrylamide gel electrophoresis, and then, resolved protein was electrophoretically transferred to nitrocellulose membranes. The blots were incubated in 0.1% TBST containing 5% non-fat dry milk for 1 h to block nonspecific binding sites. The blot was incubated with 1:1000 dilutions of primary antibodies for Cyclin D1, p53, Bax, Bcl-2, Cytochrome-C, Caspase-9, Caspase-3, and β-actin (control) overnight at 4 °C. After this, membranes were incubated with their corresponding secondary antibodies for 1 h membranes were washed extensively; the bands in the membranes were detected. Protein bands were visualized by an enhancing chemiluminescence method using an ECL kit. Bands were scanned using a scanner and quantitated by Image J. Whose control was set to 1.

Reverse transcription–polymerase chain reaction analysis

The mammary tissues total RNA was isolated by Trizol reagent the method of Chomczynski and Sacchi [21] from control and experimental groups of rats. The purified RNA was reverse transcribed using reverse transcriptase enzyme into a single strand cDNA. The cDNA was amplified for the specific primers of Cyclin D1, p53, Bax, and Bcl-2 is below. GAPDH was used as the internal control.

Genes	Primer sequences	bp
Cyclin D1	Forward primer: 5′-CTGGCC ATGAACTACCTGGA-3′ Reverse primer: 5′-CCAGGA AATCATGTGCAATC-3′	331
p53	Forward primer: 5′-GATTCT TTCTCCTCTCCTAC-3′ Reverse primer: 5′-TGTAGA TGGCCATGGCACGG-3′	129
Bax	Forward primer: 5′-CCTGTG CACCAAGGTGCCGGA ACT-3′ Reverse primer: 5′-CCACCC TGGTCTTGGATCCAG CCC-3′	213

Genes	Primer sequences	bp
Bcl 2	Forward primer: 5′-TCTGTG GATGACTGAGTACCT GAAC-3′ Reverse primer: 5′-AGAGAC AGCCAGGAGAAATCA AAC-3′	162
GAPDH	Forward primer: 5′-GAAGGT GAAGGTCGGAGTC-3′ Reverse primer: 5′-GAAGAT GGTGATGGGATTTC-3′	250

DNA fragmentation assay

The protocol provides a strategy for DNA separation of fragmented and intact DNA fractions and for their investigation by the method of Mohanty et al. [22]. The parameter taken to assess cellular DNA damage was tail length (migration of DNA from the nucleus). The images were captured using an epifluorescent microscope at a 40× objective and comet image was analysis by CASP software.

Statistical analysis

Statistical analyses were carried out using SPSS 17 (SPSS, Inc., Chicago) statistical package. The statistical data were shown as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) is followed by Duncan multiple range test (DMRT) and comparison method was used to associate the difference between the variables. The data were expressed significantly if p values are lesser than 0.05.

Results

Tumor volume

Figure 1 shows the rat structure, and the DMBA-induced rats had a large tumor which was adenocarcinomas. Whereas, DMBA-induced treatment with DIM@CS-NP 0.5 mg/kg b.wt. rats showed significantly smaller tumor compared to DIM 10 mg/kg b.wt., in our findings showed that the chemotherapeutic effect of DIM@CS-NP.

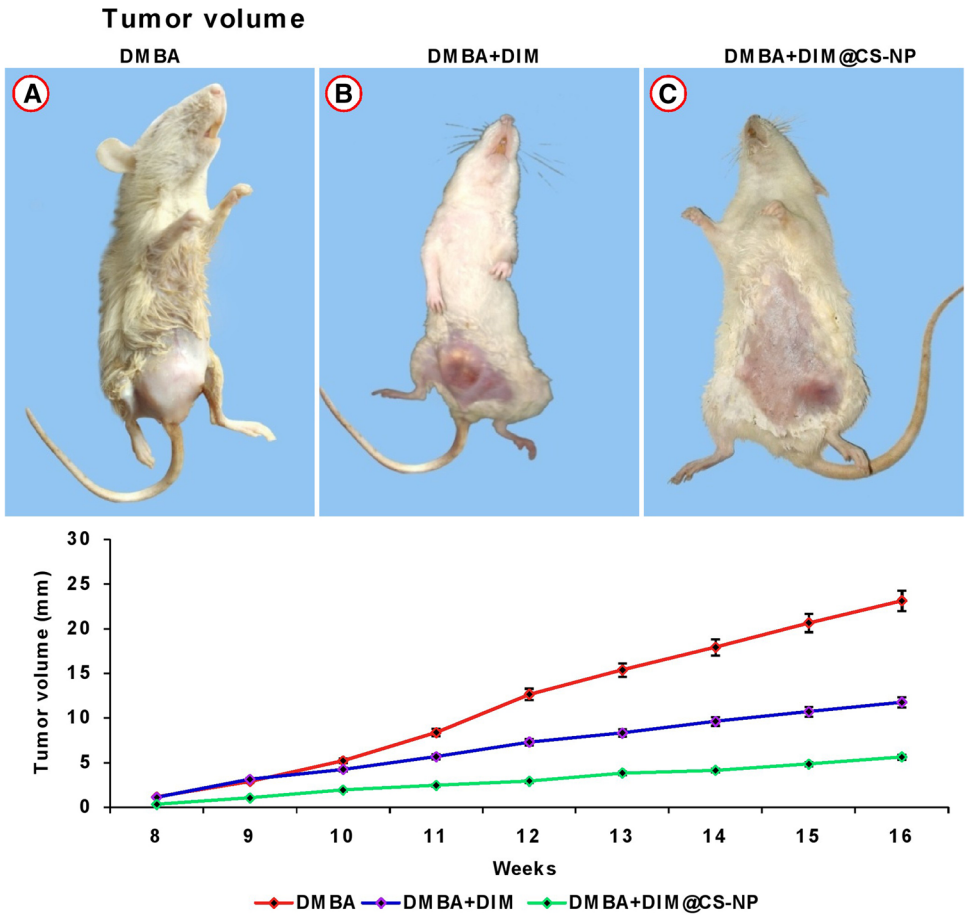

Fig. 1 Representative images **A** show DMBA-induced mammary tumor-bearing rat, **B** show DIM treated with DMBA-induced rat, **C** DIM@CS-NP-treated DMBA-induced rat

Pathological studies in mammary tissues of control and experimental rats

Figure 2 shows the MT (A–F) and PR (G–L) staining in mammary tissues of control and experimental rats, respectively. DMBA-induced tumor-bearing rats (B, H) show increased expression of collagen deposition significantly ($p < 0.05$) compared to control (A, G) rats. The administration of DIM@CS-NP 0.5 mg/kg b.wt. (D, J) to tumor-induced rats showed nearly normal expression of collagen deposition compared to DIM 10 mg/kg b.wt. (C, I). However, the administration of DIM (E, K) and DIM@CS-NP (F, L) did not show any significant ($p < 0.05$) changes in the histopathological markers compared to control rats.

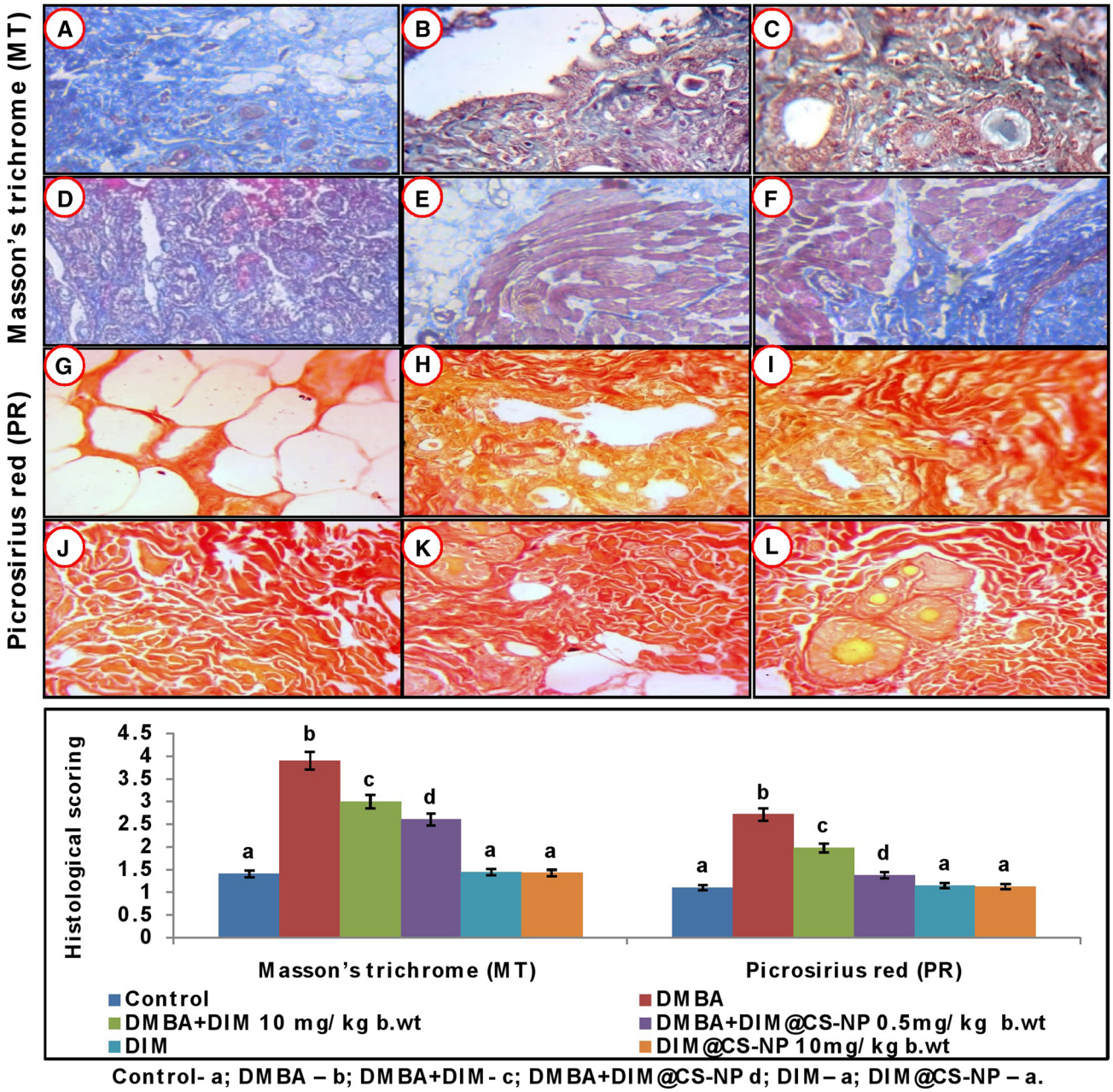

Fig. 2 Masson's trichrome (**A–F**) and Picrosirius red (**G–L**) staining for collagen, respectively, in the mammary tissue of control and experimental rats. Mammary tissue of control (**A, G**), DIM (**E, K**) and DIM@CS-NP (**F, L**) treated rats showed normal levels of regular collagen distribution; mammary tissue of DMBA (**B, H**)-induced rats shows irregular collagen distribution; collagen distribution of DIM@CS-NP (**D, J**) treatment was significantly altered as compared to DIM (**C, I**)-treated rats

Immunohistochemical (IHC) expression of Cyclin D1 and PCNA in mammary tissues of control and experimental rats

The IHC expression of Cyclin D1 (A–F) and PCNA (G–L) in mammary tissues of control and experimental rats is represented in Fig. 3. The expression of Cyclin D1 and PCNA was found to be significantly increased in DMBA-induced tumor-bearing rats (B, H) when compared to control (A, G) rats. Whereas, the levels of Cyclin D1 and PCNA were found to be decreased during treatment with DIM@CS-NP 0.5 mg/kg b.wt. (D, J) compared to DIM 10 mg/kg b.wt. (C, I) significantly. However, there were no significant changes in the expression of DIM (E, K) and DIM@CS-NP (F, L) alone treated rats when compared to control rats.

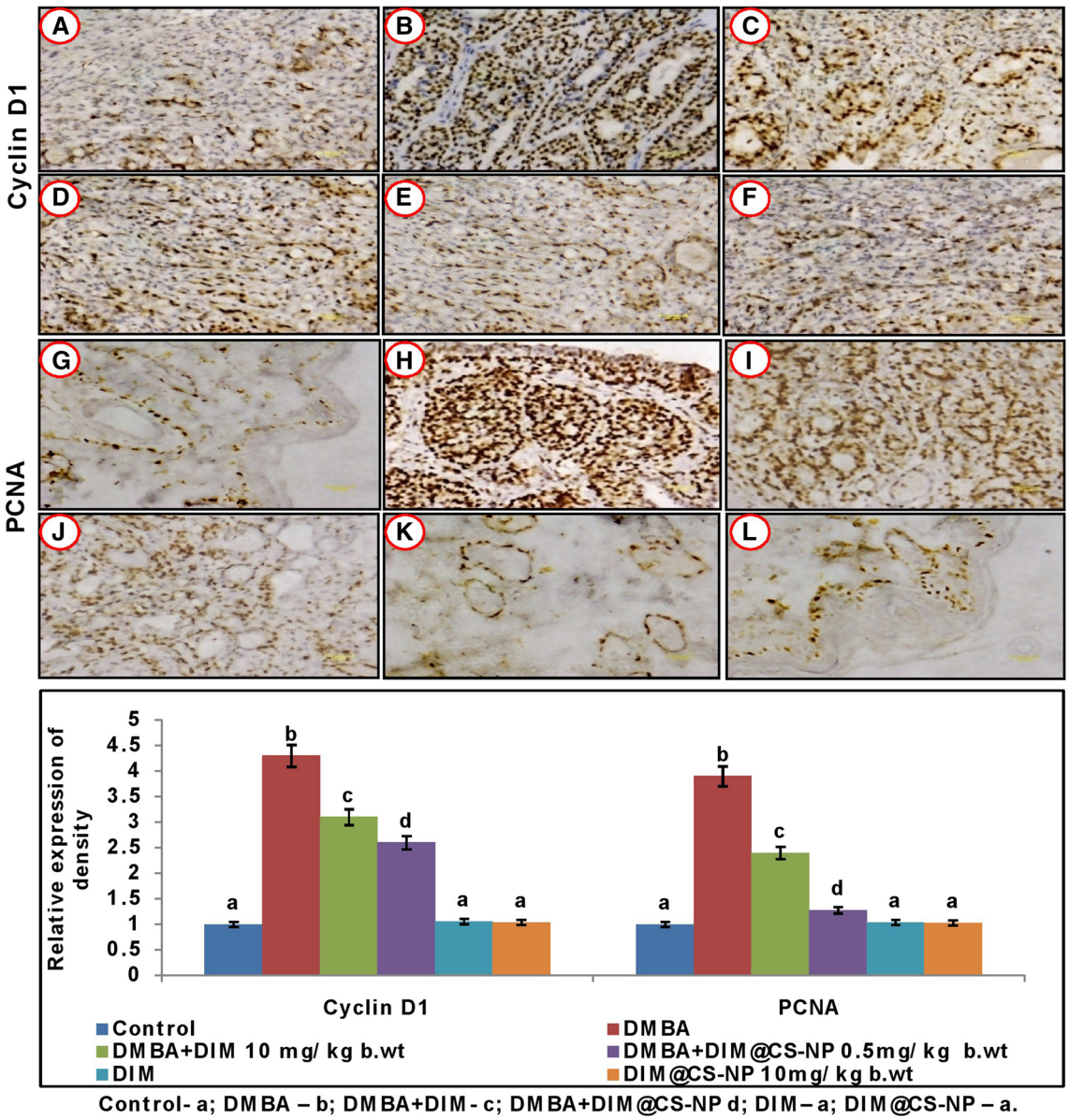

Fig. 3 Effect of DIM and DIM@CS-NP on the immunohistochemical analysis of Cyclin-D1 (**A–F**) and PCNA (**G–L**), respectively, in mammary tissue of control and experimental rats. The control (**A, G**), DIM (**E, K**), and DIM@CS-NP (**F, L**) showed normal mammary tissue staining, the DMBA-induced rats (**B, H**) showed increased expression of Cyclin-D1 and PCNA when compared with control rats. However, oral administration of DIM@CS-NP (**D, J**)-treated rats showed diminished expression levels as compared to DIM (**C, I**) administration

Western blotting analysis in mammary tissues of control and experimental rats

Figure 4 illustrates the molecular protein changes occurred during tumorigenesis. Cyclin D1, Bcl-2 proteins were found to be significantly increased and p53, Bax, Cytochrome-C, Caspase-9, and Caspase-3 proteins' expression was found to be significantly decreased in tumor-bearing rats compared to control rats. Whereas, the levels of Cyclin D1, Bcl-2 proteins were found to be significantly decreased and p53, Bax, Cytochrome-C, Caspase-9, and Caspase-3 proteins, expression significantly increased treatment with DIM 10 mg/kg b.wt. and DIM@CS-NP 0.5 mg/kg b.wt. compared to DMBA-induced rats. Our findings showed that DIM@CS-NP 0.5 mg/kg b.wt. was more effective than DIM10 mg/kg b.wt. However, there were no significant changes in these proteins expression in DIM and DIM@CS-NP alone treated rats compared to control rats.

The p53, Bax, Cyclin D1 and Bcl-2 family mRNA gene expression in mammary tissues of control and experimental rats

The p53, Bax, Cyclin D1, and Bcl-2 mRNA gene expression in mammary tissues of control and experimental rats are presented in Fig. 5. The mRNA expression of Bax, p53 decreased and Bcl-2, Cyclin D1 was increased significantly in DMBA-induced tumor-bearing rats when compared with control rats. In DIM@CS-NP 0.5 mg/kg b.wt.-treated rats, the mRNA gene expression of Bax, p53 increased and Bcl-2,

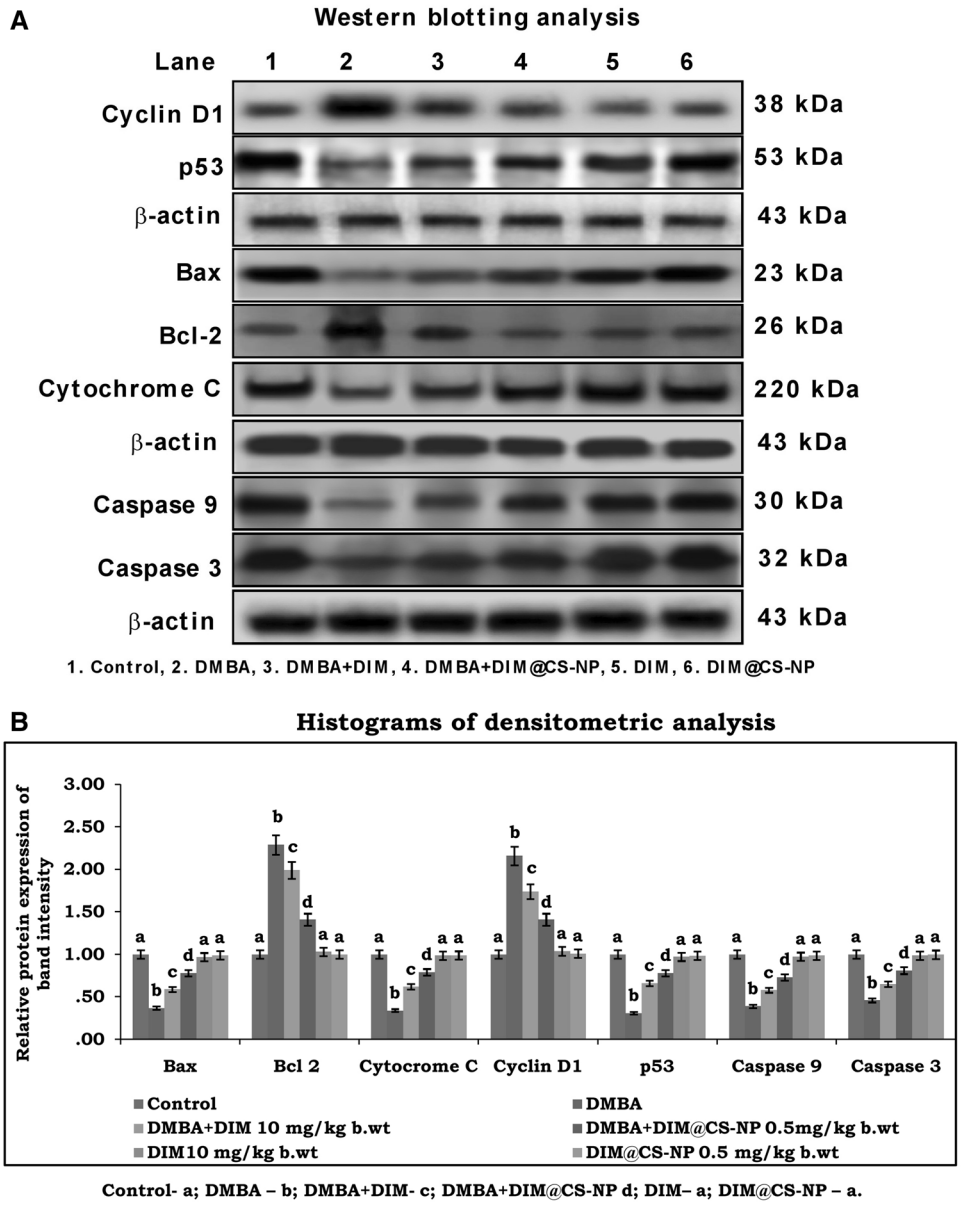

Fig. 4 **A** Effects of DIM@CS-NP on apoptotic protein Cyclin D1, p53, Bax, Bcl-2, Cytochrome-C, Caspase-9, and Caspase-3 in mammary tissue. (1) Control, (2) DMBA, (3) DMBA + DIM, (4) DMBA + DIM@CS-NP, (5) DIM, and (6) DIM@CS-NP. **B** Band intensities were scanned by the densitometer. Histograms of densitometric analysis represent the ratio of Cyclin D1, p53, Bax, Bcl-2, Cytochrome-C, Caspase-9, and Caspase-3 expression. Values that do not share a common superscript in the same column differ significantly at $p < 0.05$ (DMRT). Statistical significance was compared within the groups as follows: Control—a; DMBA–b; DMBA + DIM—c; DMBA + DIM@CS-NP—d; DIM—a; DIM@CS-NP—a

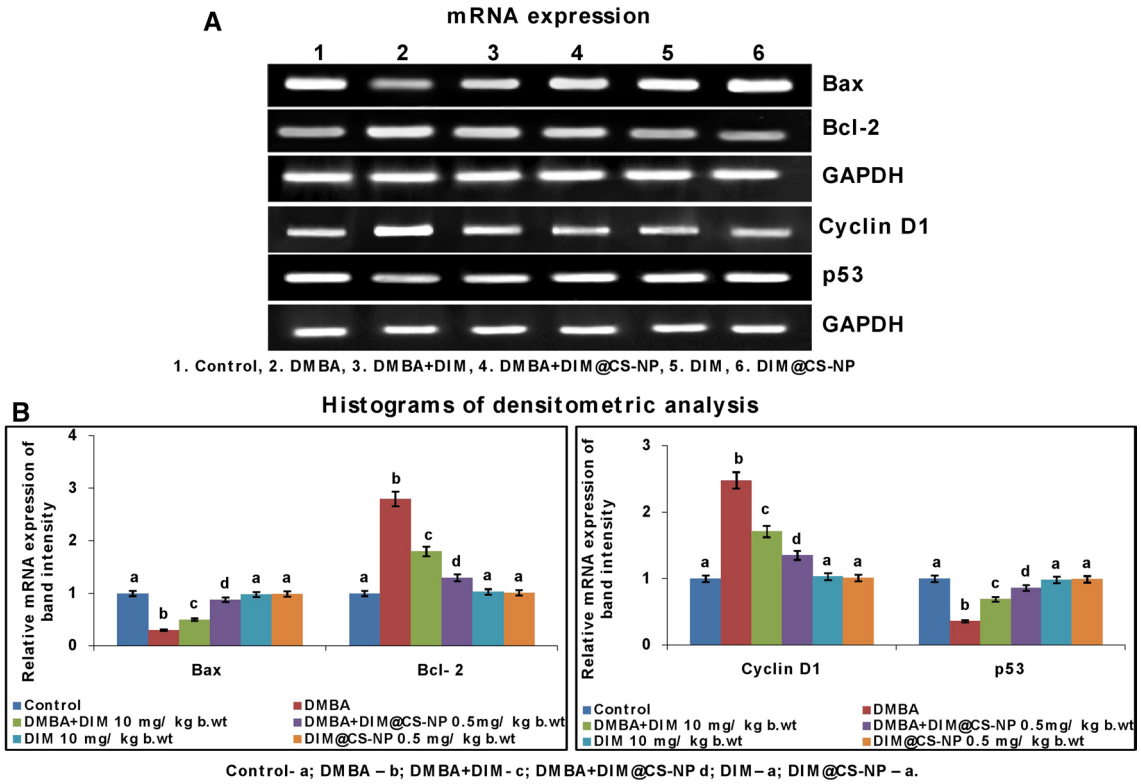

Fig. 5 **A** Effect of DIM@CS-NP on Cyclin D1, p53, Bax and Bcl-2 family mRNA expression in mammary tissues. (1) Control, (2) DMBA, (3) DMBA + DIM, (4) DMBA + DIM@CS-NP, (5) DIM, and (6) DIM@CS-NP. **B** Band intensities were scanned by the densitometer. Histograms of densitometric analysis represent the ratio of Cyclin D1, p53, Bax, and Bcl-2, and values that do not share a common superscript in the same column differ significantly at $p < 0.05$ (DMRT). Statistical significance was compared within the groups as follows: Control—a; DMBA—b; DMBA + DIM—c; DMBA + DIM@CS-NP—d; DIM—a; DIM@CS-NP—a

Cyclin D1 decreased significantly compared to the DIM 10 mg/kg b.wt. However, there was no significant change in the mRNA gene expression of p53, Bax, Cyclin D1, and Bcl-2 in DIM and DIM@CS-NP alone treated rats compared to control rats.

DNA fragmentation by agarose gel electrophoresis

To gain more insight into cell death pathways, DNA fragmentation was detected. As illustrated in Fig. 6, the genomic DNA fragment appears as a series of bands which are described as DNA ladders on agarose gel representing the formation of oligonucleosomes which are the characteristics of apoptosis. The control, DIM and DIM@CS-NP rats showed no DNA fragmentation. On the contrary, DMBA-induced tumor-bearing rats also exhibited that marked DNA fragmentation shows a ladder-like pattern recommend that DMBA induces DNA fragmentation. Whereas, DIM@CS-NP 0.5 mg/kg b.wt. treated rats showed a lesser extent of nuclear DNA damage when compared to the DIM 10 mg/

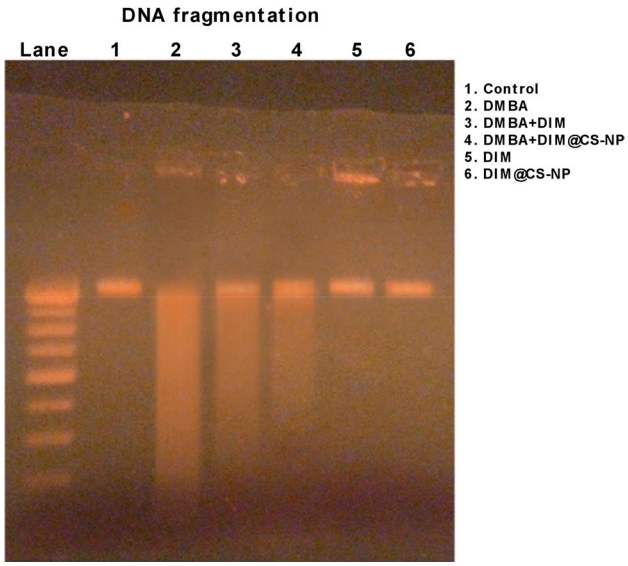

Fig. 6 (1) Control, (2) DMBA, (3) DMBA + DIM, (4) DMBA + DIM@CS-NP, (5) DIM, and (6) DIM@CS-NP

kg b.wt., which is DIM@CS-NP significant improvement in DNA fragmentation towards the control on DMBA-induced mammary tissue.

Discussion

BC is one of the most somber tribulation neoplasms in worldwide; the expanded breast density attributed to collagen deposition is connected with the development of BC [23]. It is normally documented that the extracellular matrix (ECM) of connective tissues plays a vital function in various organic processes such as cell differentiation, promotion of life/death, and carcinogenesis. BC is normally related to drastic massive modifications in architecture and composition of ECM particularly for what concerns its collagen component, in human's collagen is accounting for 33 percentages of total proteins [24]. In the present study, excess accumulation of collagen was observed in DMBA alone treated rats, the administration of DIM@CS-NP decreased collagen deposing in tumor-bearing rats compared to DIM. Similarly, staining method was reported increased glycogen content and mast cell populations in the mammary tissues of DMBA-induced rats; the DIM@CS-NP treatment significantly reduced the glycogen content and mast cell populations than DIM [9].

In BC scenario, cell proliferation is a dangerous process and it changes expression and activity of cell-cycle-associated proteins. Proliferating cell nuclear antigen (PCNA) and Cyclin D1 play a crucial role in mammalian cell-cycle regulation, whereas this gene amplification accounted for 50% of breast cancer patients [25, 26]. Whereas, the up-regulation of PCNA and Cyclin D1 involved in the regulation of cell cycle as well as cell proliferation, it is one of the common phenomena to development of the tumor, up-regulation and amplification of PCNA and Cyclin D1 were shown in breast cancer [27]. Our result shows that IHC analysis of DMBA-induced tumor-bearing rats increased the Cyclin D1 and PCNA expression, which indicates the development of mammary carcinoma. Moreover, DIM@CS-NP-treated rats show decreased expression of Cyclin D1 and PCNA compared to DIM. Which can benefit from the clinical recovery, DIM@CS-NP enhanced the chelating treatment of mammary tissues through outstanding targeted drug delivery.

Apoptosis happens in several physiological changes, where its responsibility is to expel destructively, harmed, damaged, or undesirable cells. Apoptosis and cell proliferation are connected by cell-cycle controllers and apoptotic stimulus that disturb the two progressions; abnormalities and resistance in apoptosis function have been disclosed as a key event taking place during the pathogenesis of mammary cancer [28]. The connection of apoptosis and cancer has been stressed and expanding proof recommends that the process of neoplastic transformation, progression, and metastasis involves modifications of the typical apoptotic pathway. Moreover, apoptosis pathways were mainly regulated by Bcl-2 family proteins depends on the Bax/Bcl-2 ratio. In normal cells, over generation of ROS can alter the mitochondrial membrane resulting in release of Cytochrome-C from the intermembrane space into the cytosol, thereby activate apoptosis in the cells [29, 30].

Furthermore, Bcl-2 overexpression is reported to enhanced tumorigenicity and metastatic potential, including invasion, migration, and tumor angiogenesis in breast cancer [31]. Anti-apoptotic Bcl-2 family members (Bcl-2) can unfetter blocks mitochondrial events, whereas proapoptotic Bcl-2 family members (Bax) can eventually trigger those changes. These are interesting aspects, where down control of Bcl-2 protein has been proposed as another promising treatment procedure for breast cancer [32]. Hong et al. also examined that DIM decreased Bcl-2 protein levels up to 90 and 60% of MCF-7 and MDA-MB-231 also, increased Bax protein levels up to four and sixfold in MCF-7 and MDA-MB 231 cells, respectively. The outcomes recommend that the intense inhibitory impact of DIM on Bcl-2 protein expression can be represented by the down-regulation of Bcl-2 transcripts [33]. Our result also line with the above findings, the western blotting analysis of DIM@CS-NP-treated rats significantly increased the expression of Cytochrome-C, Caspase-9, Caspase-3, Bax, and decreased Bcl-2 expression which is evidenced to induce apoptosis and proves to act as cell-cycle checkpoints compared to DIM. In addition that, the mRNA expression also revealed increased Bax and decreased Bcl-2 expressions on treated with DIM@CS-NP compared to DIM.

In addition, under the normal physiological condition, the tumor suppressor genes like p53 keep in its neutral form. If the DNA gets damaged by the external exposure such as UV, chemical or viruses, DNA repair mechanism would be activated. Failure of the DNA repairs mechanism activating tumor suppressor gene p53 resulting triggers apoptosis [19]. On the other hand, cell-cycle regulatory markers, such as p53 and Cyclin D1, play an important role in apoptosis and cell proliferation. Furthermore, Cyclin D1 is an oncogene encoding a positive controller of G1 phase progression in the course of cell cycle, which controlled the initiation of DNA synthesis. It binds and activates its kinase partners CDK4 and CDK6 resulting in the phosphorylation of the retinoblastoma protein thereby induces transcription of genes that promote progression to the S-phase of the cell cycle [34]. In addition, Carter et al. have reported in a microarray experiment that DIM down-regulates Cyclin D1 gene regulation in human keratinocytes in MCF-7 cells [35]. Similarly, our results also show decreased p53 and increased in Cyclin D1 both protein and mRNA expressions in mammary tissue of DMBA-induced rats. DIM@CS-NP supplemented rats

extremely dwindle down the expression of Cyclin D1 and increased p53 expression as compared to DMBA and DIM-treated rats.

In adding together, DNA fragmentation is observed in various tissues and cells during apoptosis. Apoptosis levels are lower in DMBA treated rats as can be seen by lower intensity of DNA ladders. Mosaad Abdel Wahhab et al. reported that Chitosan nanoparticles plus quercetin (O) modulates DNA fragmentation on against Ochratoxin A (OTA) induced oxidative stress and renal genotoxicity on male Sprague Dawley rats [36]. Over results also line with the above findings, with DIM@CS-NP treatment the apoptosis was restored as seen by increased intensity of fragmented bands of DNA. The molecular targeting effect of DIM@CS-NP on mammary cancer profoundly considered as a potential anticancer drug with the appropriate carrier. The outcome of our study shows that DIM@CS-NP induces apoptotic cell death on DMBA-induced mammary carcinoma at minimal dose with nontoxic to normal rats.

Conclusion

Our study pioneered the concept of drug delivery of nanotherapeutic for the treatment of mammary cancer. The ideal nano therapy in mammary cancer will continue to benefit from the discovery of novel molecular targets that drive the carcinogenesis. Hence, DIM@CS-NP favored superior therapeutic activity in tumor-bearing mammary tissues by actively supplying DIM molecules for a prolonged period through a sustained release manner; thereby exerting an enhanced antitumor effect on DMBA-induced mammary carcinogenesis. Our results emphasize that DIM@CS-NP-induced apoptosis aspects via both intrinsic and extrinsic pathways which will serve as a precise effective chemotherapeutic agent for the treatment of mammary cancer.

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Compliance with ethical standards

Conflict of interest The first author (S. Isabella) and the corresponding author (Dr. S. Mirunalini) declare that there are no conflicts of interest.

Ethical approval This study was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) (Reg No. 160/1999/CPCSEA and Proposal No. 1123).

Informed consent This article does not contain any studies with human participants.

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