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Original Article

Chemotherapeutic profiling of lupeol encapsulated chitosan nanoparticles by attenuating molecular events, glycoprotein, and mast cell population in an animal model of mammary carcinoma

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Abstract

Breast cancer is a major public health issue that rouses the interest and concern of physicians of all categories. A plant-based triterpenoid has potential therapeutic properties and activity against breast cancer, but establishing the mechanism of action in cancer therapy requires a greater understanding of the genes and cellular pathways dictated by phytocompounds. Lupeol is a phytosterol present in edible fruits and vegetables, and possesses a wide range of biological activities against various diseases, including cancer. Unfortunately, clinical trials on this lupeol are significantly restricted by its low solubility and poor bioavailability. Nanoparticle-based systems for drug delivery have established the way for an explosion in cancer therapy by boosting the efficacy of the drug. The purpose of this study was to inquire into the effects of LUP-encapsulated chitosan nanoparticles (LUP-CSNP) on mast cell population, glycoprotein, and western blotting in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis. After 10 weeks of tumor development, rats were given an oral administration of LUP and LUP-CSNP. The oral consumption of LUP-CSNP suppressed the Bax, Bcl-2, and BRCA-1 protein expressions in DMBA-induced rats, as well as glycoprotein and mast cell population more strongly than LUP. Furthermore, our findings demonstrate that LUP-CSNP has the potential to impede inappropriate levels of apoptotic markers, breast cancer gene markers, glycoprotein, and mast cell population.

Keywords: breast cancer, lupeol, mast cell population, glycoprotein, BRCA-1

1. Introduction

Breast cancer is the most common cause of morbidity and mortality in women. It is the second most prevalent leading cause of cancer deaths worldwide (The & Wilson, 1998). According to reports, one in four new cancers diagnosed worldwide each year is breast cancer. It is estimated that 43,700 deaths from breast cancer occurred in the United States in 2023. The impact has been steadily rising despite extensive research and various treatment modalities. Besides their age, hormonal status, and sedentary lifestyle, continuous direct and indirect exposure to chemicals in the environment makes women more vulnerable to cancer (Yau *et al.*, 2022).

Polycyclic aromatic hydrocarbons (PAH) are used to produce pharmaceuticals, dyes, plastics, and pesticides. 7,12-dimethylbenz(a)anthracene (DMBA) is a PAH and a well-known chemical carcinogen that has been used to induce cancer in rats. DMBA, a procarcinogen, is activated by liver elimination enzymes to ultimately become a carcinogen (Yunker *et al.*,2002). These oxidation and reduction reactions also produce large amounts of free radicals, which tip the oxidant-antioxidant balance in favor of the oxidant, resulting in oxidative stress linked to breast cancer. The desired outcome of chemotherapy is to shrink primary tumors, slow tumor development, and kill cancer cells that have spread (metastasized) from the initial tumor to other regions of the body (Zheng & Obbard, 2002).

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Chemotherapeutic drugs are also toxic to normal cells, which limits their use. Chemotherapy is characterized as the use of pharmacological agents, either in particular drugs or as naturally occurring components, to treat cancer. Recent laboratory studies and evidence from epidemiology have also demonstrated that certain pharmacologically active compounds found in food may reduce the risk of cancer development (Van *et al.,* 2001). Lupeol is a popular triterpene that is typically obtained through extraction from natural sources such as edible fruits and vegetables. LUP is currently recognized as a compound that promotes breast cell proliferation and migration, thereby boosting harmed breast rehabilitation (Geetha & Varalakshmi, 2001). An enormous obstacle in the clinical application of LUP is its low aqueous solubility and limited bioavailability, which can lead to reduced effectiveness as a treatment. However, novel approaches are available for boosting the solubility and bioavailability of effective drugs (Mirunalini & Susmitha, 2021).

In recent years, nanoscale drug delivery systems made of biocompatible and biodegradable polymers have presented an entirely novel approach to drug delivery and tumor targeting. Biodegradable drug carriers are currently purposefully designed and built with nanometer aspects (Champion *et al.,* 2007). The encapsulation of drugs with hydrophobic properties into an aqueous nanoparticle system has been tested, for delivering drugs to enable their full potential (Macartney, 2011). Chitosan (CS) is composed of two naturally appearing biopolymers that have significant applications in the food and pharmaceutical industries. Because of their faster degradation rate, CS formulations with a high level of deacetylation are recommended in drug delivery systems (Grenha, 2012).

Therefore, the current study was carried out to investigate the chemo-modulatory effect of lupeol encapsulated in chitosan nanoparticles (LUP-CSNP) on modifications to the genome comprising the activation of oncogenes and the depletion of tumor suppressor genes or an amalgam of both (Susmitha & Mirunalini, 2022). In these scenarios, cancerous tissue undergoes rapid proliferation, deregulation of tumor suppressor genes, decreased cell death (apoptosis), the depreciation of apoptotic genes, metastasis, and eventually dysfunction of the organ-like mammary tissues. Numerous studies have explored the intrinsic and extrinsic markers of apoptosis that evolved as tumorigenesis-establishing mechanisms (Hengartner, 2000). Furthermore, apoptosis in breast cancer would be created by the signaling of proapoptotic and anti-apoptotic genes. The proapoptotic ones like Bax, and Bcl-2, breast cancer gene markers-1 (BRCA-1) protein expression in control and experimental rats, as well as glycoprotein and mast cell population than LUP or alternatively LUP-CSNP treated rats have been compared to control rats (Kim *et al.,* 2015). The current study spans the expression of drugs and boosters of pathways apoptosis using LUP-CSNP, which can be a valuable approach to inhibiting the growth and progression stages of DMBA-induced mammary cancer. In addition, the glycoprotein, and the mast cell population are subjected to staining that is utilized in all tissue specimens, to evaluate these for specific information about the mammary tissue in healthy and cancerous circumstances, as well as the morphology at the intracellular level.

2. Materials and Methods

2.1 Chemicals

Lupeol, DMBA, CS, and sodium tripolyphosphate were purchased from Sigma-Aldrich. Monoclonal antibodies such as Bax and Bcl-2 were purchased from Santa Cruz Biotechnology, USA. All the other chemicals and reagents used were of analytical grade.

2.2 Synthesis of Lupeol loaded chitosan nanoparticles

Lupeol loaded chitosan nanoparticles were synthesized using chitosan of three different molecular weights, produced through ionic gelation as described by Eduarada *et al.,* 2021. The ranges were very low, quite low, and fairly high. Concentrations of 3, 4, and 5 mg/mL were prepared, respectively, for the production of the CS solution using 0.5% acetic acid. Drop-by-drop, 2.5 mL of STPP aqueous solution of the alterantive concentrations was added to 10 mg of LUP dissolved in 5 mL of CS solution. The nanoparticles dispersed after 45 min of moderate agitation at 600 rpm and room temperature. To establish isothermal equilibrium and adsorb excess drug to nanoparticles, moderate agitation at 600 rpm and room temperature was used. After that, Centurion Scientific, UK's K-2015 ambient centrifuge spun the nanoparticle dispersion at 6,000 rpm for an hour and it was rinsed twice with 0.5% acetic acid. Drug encapsulation efficiency was calculated from the combined rinses, and precipitated nanoparticle pellets were examined.

2.3 Animal model

Female Sprague-Dawley rats weighing 130-150 g were obtained from the Biogen Laboratory Animal Facility in Bangalore, India. The experiment was conducted at the Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, India. Before experiments the rats were given a week to acclimate. The animals were housed in six large polypropylene cages lined with husk under standard laboratory conditions: temperature (27±2°C), humidity (55±5%), and a 12-hour light/dark cycle. In the study, the rats were fed standard animal feed and given free access to water.

2.4 Tumour induction

Female Sprague-Dawley rats were given DMBA (25 mg/kg body weight), a dose calculated to cause a significant increase in tumor prevalence in the control group throughout the experiment. The DMBA was dissolved in a 1 mL emulsion of 0.75 mL sunflower oil and physiological saline (0.25 mL) (Arulmozhi & Sankaran, 2013).

2.5 Experimental protocol

The animals were randomly assigned to experimental and control groups before being divided into six groups of six animals each. The control animals will be in Group I. Groups II-IV received a single subcutaneous injection of 25 mg/kg b. wt DMBA during the first week of the experiment. After 10 weeks, Groups III and IV will be

given LUP and LUP-CSNP orally at 5 mg/kg b. wt and 2.5 mg/kg b. wt, respectively. Groups V and VI will be assigned LUP and free LUP-CSNP at varying concentrations orally three times per week (Isabella & Mirunalini, 2016). The current study's dosage is based on previous studies. The investigation was considered completed after 20 days, and all of the rats were sacrificed. The tissues were immediately dissected, washed carefully with ice-cold saline, and preserved in 10% formalin before being paraffin-embedded, split, and fixed on polylysine-coated glass slides for immunohistochemical analysis.

2.6 Western blotting analysis

Western blotting was used to assess the expression pattern of Bax, Bcl-2, and β-actin (control) using the Laemmli method (Laemmli, 1970). Breast cancer gene marker-1 and β-actin in mammary tissue specimens were homogenized with a buffer [5 mM sodium azide, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM NaHCO3 (pH 7.0)]. The homogenates were centrifuged at 12,000g for 30 minutes at 4°C to remove debris. The protein was bursting and divided on a 10% SDS polyacrylamide gel electrophoresis before being electrophoretically transferred to nitrocellulose membranes. To block nonspecific binding sites, the blots were incubated in 0.1% TBST containing 5% dry milk without fat for 1 hour. The blot was incubated overnight at 4 °C with 1:1000 dilutions of primary antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) for Bax, Bcl-2, and β-actin, as well as Breast cancer gene marker-1 β-actin (control). After that, the membranes were incubated with their corresponding secondary antibodies (anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase) for 1 hour before being thoroughly washed, and the bands in the membranes were detected. Protein bands were observed using an ECL kit and a boosting luminescence method. Image J was used to quantify the bands after they were scanned with a scanner.

2.7 Pathological studies

A microtome was used to cut 5 mm portions of paraffin-embedded tissues, which were then rehydrated with xylene and a graded series of ethanol. The specimens were then stained as described below. The histopathological analysis of mast cells used toluidine blue staining (Migliaccio *et al.*, 2003). Glycoprotein in the mammary tissues was stained with Periodic Acid Schiff (PAS) base, according to (Yamabayashi, 1987).

2.8 Statistical analysis

Statistical analysis was carried out using the SPSS V26.0 software package (IBM SPSS, USA). The data were presented as mean ± standard deviation (SD). To assess differences between the treatments, a one-way analysis of variance (ANOVA) was used, followed by Tukey's post hoc test. A $P<0.05$ was considered statistically significant.

3. Results

3.1 Western blotting analysis of mammary tissues from control and experimental rats

The molecular protein alterations that were observed during the process of carcinogenesis are illustrated in Figure 1. With the help of a number of important authorities involved, we attempted to determine which proteins were responsible for inflammation. The levels of Bax, Bcl-2, and BRCA-1 were found to be significantly higher in tumor-bearing rats that were administered DMBA in group II, in comparison to rats that served as controls. On the other hand, it was found that the stages of these proteins were greatly reduced in the LUP 5 mg/kg b.wt. group (III) and the LUP-CSNP 2.5 mg/kg b.wt. group (IV) rats, in comparison to the other tumor-bearing rats. It was discovered that LUP-CSNP 2.5 mg/kg body weight was more effective than LUP 5 mg/kg body weight dosage. There were no significant changes in the expression of these proteins in rats that were treated with LUP alone at a dose of 5 mg/kg body weight or LUP-CSNP at a dose of 2.5 mg/kg body weight. These were compared to rats that served as controls.

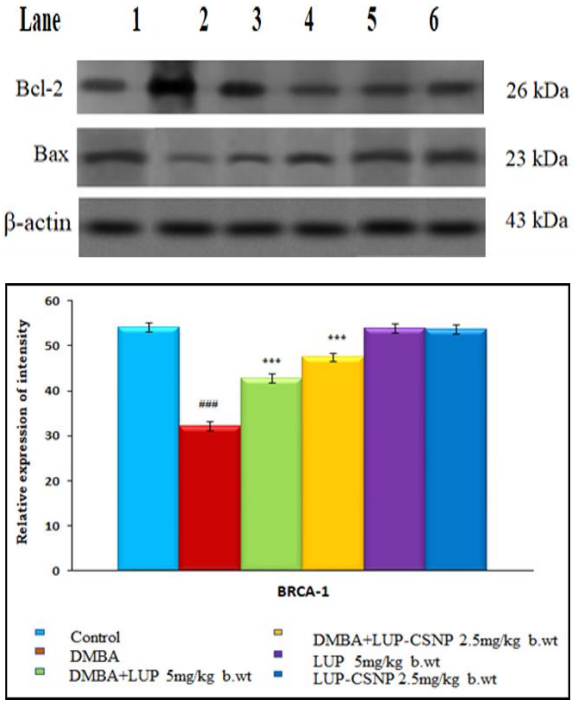

Figure 1. A Representative blot analysis of inflammatory protein expression of Bax and Bcl-2 in mammary tissues 1) Control, 2) DMBA, 3) DMBA+LUP, 4) DMBA+LUP-CSNP, 5) LUP, and 6) LUP-CSNP. B Band intensities were scanned with a densitometer. Histograms of densitometric analysis represent the ratio of Bax and Bcl-2 expressions. Values that do not share a common superscript in the same column differ significantly at $p<0.05$ (DMRT). Statistical significance was compared between these groups: a) Control, b)DMBA, c) DMBA+LUP, d) DMBA+LUP-CSNP, e) LUP, f) LUP-CSNP.

3.2 Effects of LUP and LUP-CSNP on mast cell population in mammary tissues

Figures 2a through 2f illustrate the impact that LUP and LUP-CSNP had on histopathology when the analysis was performed with toluidine blue staining. It was observed that the mast cell populations in the mammary tissues of the rats that were stimulated with DMBA (B) were dramatically increased. When compared to LUP at a dose of 5 mg/kg body weight (C), the treatment with LUP-CSNP at a dose of 2.5 mg/kg body weight (D) gave a considerable reduction in the mast cell population level. The groups of rats that were treated with LUP (E) and LUP-CSNP (F) on their own did not exhibit any significant variations in absorption when compared to the control group (A).

3.3 Effects of LUP and LUP-CSNP on glycoprotein in mammary tissues

A staining technique known as Periodic Acid Schiff (PAS) was utilised to determine the amount of glycoprotein present in the mammary tissues of both the control rats and the experimental rats. There was an excessive buildup of glycoprotein in the mammary tissues of the rats that were treated with DMBA (B). The LUP-CSNP treatment at 2.5 mg/kg body weight (D) resulted in a significant reduction in glycoprotein levels when compared to the LUP treatment at 5 mg/kg body weight (C) in this study. Individual groups of rats were treated with LUP (E) and LUP-CSNP (F), but the control group (A) did not see any significant alterations as a result of the treatment.

3.4 Morphological studies

FESEM was used to investigate the surface morphology of the LUP@CS nanoparticles that were manufactured, and Figure 5 illustrates the morphological characteristics of a LUP-CSNP nanoparticle made from the same material. The LUP-CSNP system is responsible for the creation of nanoparticles that have recognizable forms and frequently exist on the nanometer length scale. The diameter difference between LUP-CSNP and other nanoparticles is just about 12 nanometers, which is a relatively small amount. Synthesized nanoparticles have a tendency to cluster due to the enormous surface area that they possess. This is because of the powerful and long-lasting affinities that they possess. There is a correlation between the environment in which nanoparticles are found and the stability of the nanoparticles as well as their ability to successfully agglomerate with each other. Consequently, the formation of nanoparticles requires the uneven clustering of individual particles. This is a consequence of the fact that nanoparticles are formed.

4. Discussion

The current study demonstrated that LUP-CSNP has an anticancer effect on DMBA-induced mammary carcinogenesis in Sprague-Dawley rats in both pre- and post-initiation stages. It is widely accepted that associated with a cancer diagnosis, the patient suffers excessive consumption of energy, malabsorption, and metabolic changes that contribute to weight loss (Luparello, 2013). However, when compared to

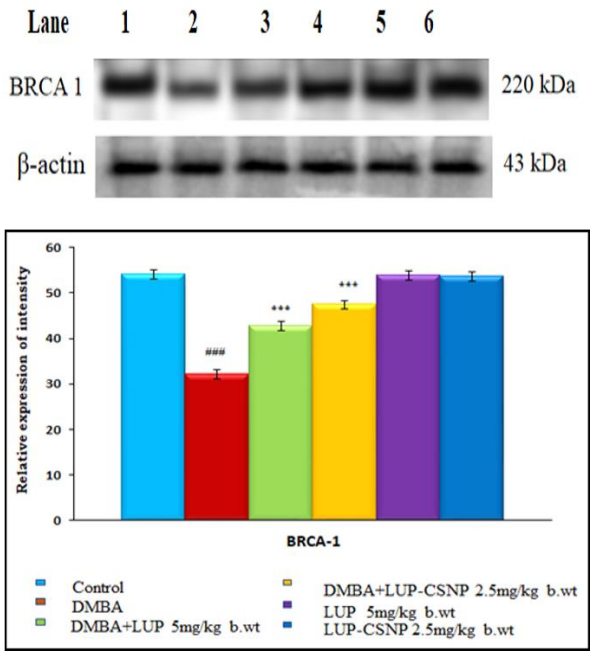

Figure 2. A Representative blot of inflammatory protein expression by BRCA1 in mammary tissues. 1) Control, 2) DMBA, 3) DMBA+LUP, 4) DMBA+LUP-CSNP, 5) LUP, 6) LUP-CSNP. B Band intensities were scanned by using a densitometer. Histograms of densitometric analysis represent the relative BRCA1 expression levels. Values that do not share a common superscript in the same column differ significantly at $p<0.05$ (DMRT). Statistical significance was compared between these groups: a) Control, b) DMBA, c) DMBA+LUP, d) DMBA+LUP-CSNP, e) LUP, f) LUP-CSNP.

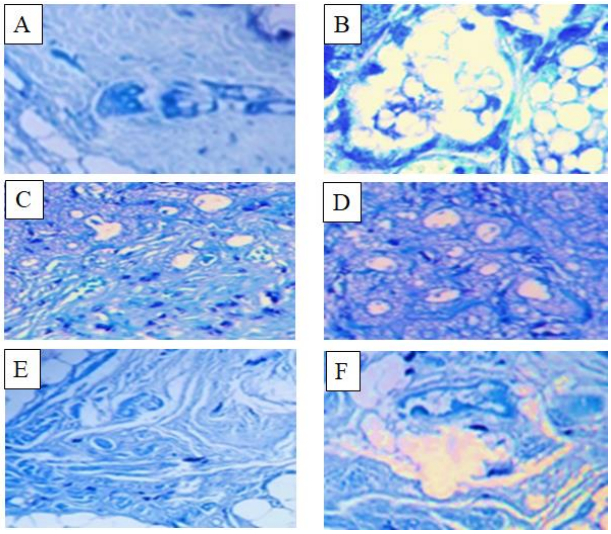

Figure 3. Histopathological analysis by TB staining of mammary tissue from control and experimental rats (A-F). Control (A); DMBA (B); DMBA+LUP 5mg/kg b. wt (C); DMBA+LUP-CSNP 2.5mg/kg b. wt (D); Free LUP 5mg/kg b. wt (E); and LUP-CSNP 2.5mg/kg b. wt (F)

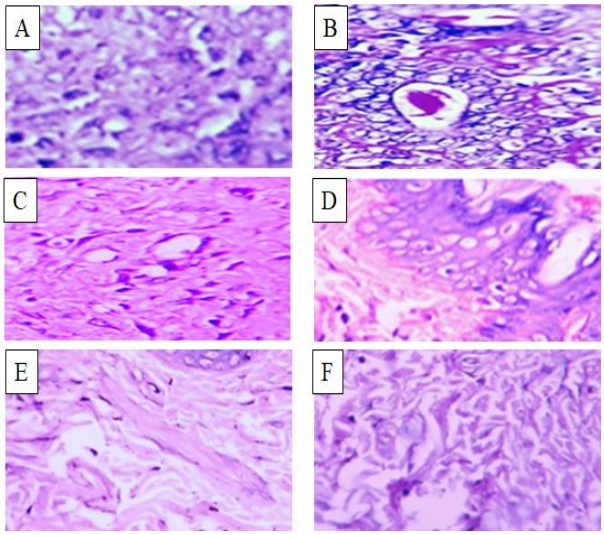

Figure 4. Histopathological analysis by PAS staining of mammary tissue from control and experimental rats (A-F). Control (A); DMBA (B); DMBA+LUP 5mg/kg b. wt (C); DMBA+LUP-CSNP 2.5mg/kg b. wt (D); Free LUP 5mg/kg b. wt (E); and LUP-CSNP 2.5mg/kg b. wt (F)

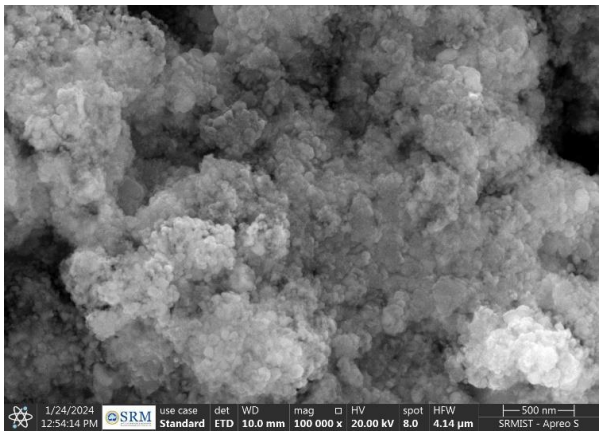

Figure 5. FESEM image of LUP-CSNP nanoparticles.

DMBA-induced tumor-bearing animals, oral administration of LUP and LUP-CSNP proved a progressive increase in body weight. Previous investigations implied that the LUP had a higher nutritional value of these, and edible fruits and vegetables of this genus contain complete proteins with a balanced distribution of amino acids, both essential and non-essential. This could be the key factor in the body weight trend in the LUP and LUP-CSNP-treated rats. Furthermore, LUP-CSNP effectively reduced tumor volume, indicating the drug's inhibitory action on tumor growth in animals. LUP's potential inhibiting activity was also reflected in lower total tumor incidence in DMBA-exposed animals (Susmitha and Mirunalini, 2022). The detected growth of tumors and the detrimental impact of LUP-CSNP may indicate an especially toxic environment for multiplying cells, ultimately slowing the spread of breast cancer. This can advantageous for therapeutic recovery, and LUP-CSNP improved the binding to mammary tissues via exceptional targeted drug delivery.

Apoptosis occurs in an assortment of physiological changes, to eliminate harmful, damaged, or undesirable cells. Apoptosis is attributed to cell-cycle regulators and apoptotic stimuli that disrupt the two progressions; abnormalities and rebellion in apoptosis occurring during mammary cancer pathogenesis. The link between apoptosis and cancer has been emphasized, and growing evidence suggests that each stage of the neoplastic transformation and metastasis requires modifications to the standard apoptotic pathway (Wong, 2011). Furthermore, apoptosis routes are primarily regulated by Bcl-2 family proteins depending on the Bax/Bcl-2 ratio. In normal cells, the accumulation of ROS can alter the mitochondrial membrane, allowing the exacerbated space to leak into the cytosol and activate apoptosis (Shah, Gapor, & Sylvester, 2003). Furthermore, Bcl-2 overexpression has been associated with higher tumorigenicity and metastasis in breast cancer, including invasion, migration, and tumor angiogenesis. Bcl-2 family members that are anti-apoptotic (Bcl-2) can unblock mitochondrial events, whereas proapoptotic Bcl-2 family members (Bax) are capable of triggering those changes (Sarkar *et al.*, 2003). These are intriguing aspects, and the downregulation of the Bcl-2 protein has been suggested as a different treatment approach for breast cancer (Lindsay, Degli Esposti, & Gilmore, 2011). The findings demonstrate that the robust inhibitory effect of LUP on Bcl-2 protein expression can be represented by downregulation of Bcl-2 transcription (Hong, Firestone, & Bjeldanes, 2002). The western blotting analysis of LUP-CSNP-treated rats showed substantially elevated expression of Bax and decreased expression of Bcl-2, which is proven to induce apoptosis and acts as an amplify-cycle barrier compared to LUP.

Breast cancer-associated (BRCA) genes 1 are genes that suppress tumors that play essential functions in DNA integrity. BRCA1 dysfunction causes chromosomal reorganization and gene instability (Futaki & Liu, 2001). Deregulation of BRCA1 alternative gene splicing has also been linked to developing tumors in the breast and ovary. LUP treatment increased BRCA1 expression and antioxidant capacity in endothelial cells. *In vivo* investigations additionally demonstrate that LUP can boost BRCA1 expression, boost antioxidant activity, and lower ROS levels in the colon mucosa (Tassone *et al.*, 2003). In addition, western blotting analysis on DMBA-induced tumor-bearing rats treated with LUP revealed a slight increase in BRCA1. Additionally, LUP-CSNP-treated rats had significantly higher BRCA1 protein expression than LUP-treated rats.

Glycogen is the storage form of glucose in cells and is essential for energy delivery and glucose homeostasis. Cancer metabolism and metabolic remodeling during the adaptation of cells within the tumor microenvironment, as well as persistence against anticancer therapies, are well amplified (Buijs *et al.*, 2004). The results of our study show that the glycogen satisfaction in the mammary tissues of DMBA-induced rats is increased, whereas the LUP-CSNP treatment significantly reduces glycogen content compared to LUP. Mast cells (MCs) are noticed in many tumors. They are associated with the innate immune system and are attracted to and activated in the microenvironment of an establishing tumor (Coussens *et al.*, 1999). MC retention has been linked to increased growth and invasion in multiple human cancers. In contrast, MC permeation has been linked to a favorable

outcome in breast carcinoma (Llaverias *et al.*, 2011). The number of mast cells in the breast tissues of DMBA-induced rats was significantly reduced by LUP-CSNP treatment compared to LUP.

The study on chemotherapeutic profiling of lupeol encapsulated chitosan nanoparticles (LUP-CSNP) presents a significant advancement in cancer therapy research due to its multifaceted approach and innovative nanoparticle delivery system. The encapsulation of lupeol within chitosan nanoparticles enhances its bioavailability and targeted delivery to cancer cells, potentially improving therapeutic efficacy while minimizing off-target effects. Furthermore, the study explores the molecular events underlying the anticancer activity of LUP CSNP, shedding light on the intricate mechanisms involved in its chemotherapeutic action. By elucidating the attenuation of molecular events, glycoprotein expression, and modulation of mast cell populations in an animal model of mammary carcinoma, this study offers valuable insights into the complex interplay between nanoparticle-mediated drug delivery, tumor micro environment, and cancer progression. This comprehensive approach not only contributes to our understanding of the anticancer mechanisms of lupeol but also provides a foundation for the development of novel nanoparticle-based therapies for mammary carcinoma and potentially other types of cancer. The integration of molecular, cellular, and animal model analyses in this study represents a notable contribution to the field, offering a holistic perspective on the therapeutic potential of LUP-CSNP in cancer treatment.

5. Conclusions

Our research established the premise of nanotherapeutic drug delivery for the therapy of mammary cancer. The finding of new molecular targets that drive tumor formation will persist to provide the ideal nano therapy for mammary cancer. Therefore, LUP-CSNP supports greater medicinal properties and effects on tumor-bearing mammary tissues by actively delivering LUP molecules for an extended period of time via continuous release, resulting in a boosted antitumor effect on DMBA-induced mammary carcinogenesis. Our findings suggest that LUP-CSNP-induced apoptosis portions via both intrinsic and extrinsic pathways will provide an effective chemotherapeutic activity for the therapy of mammary carcinoma.

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