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Liposome Nanocarriers Based on γ Oryzanol: Preparation, Characterization, and *In Vivo* Assessment of Toxicity and Antioxidant Activity

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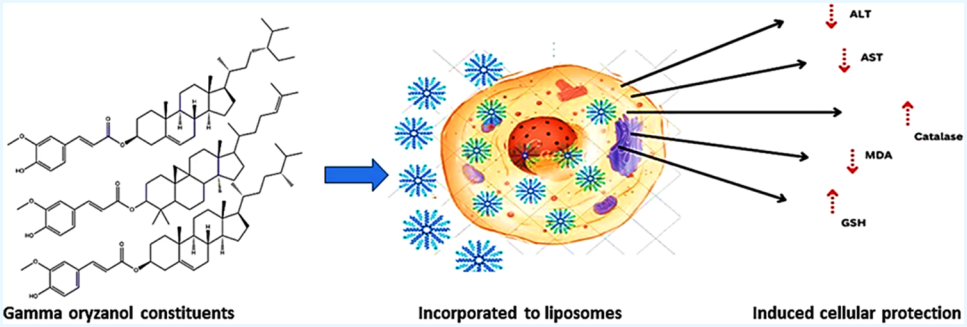

ABSTRACT: The present study aimed to develop and characterize liposome nanocarriers based on γ oryzanol and evaluate their potential *in vitro* and *in vivo* toxicity and antioxidant effects. The liposomes were physicochemically characterized using various techniques, including dynamic light scattering (DLS) for size and polydispersity index (PDI) measurements and ζ-potential analysis. The *in vitro* toxicity assessments were performed using hemolysis and MTT assays on the HS5 cell line. *In vivo*, acute oral toxicity was evaluated by using LD_{50} assays in mice. Additionally, antioxidant activity was assessed through biochemical analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and liver tissue catalase, malondialdehyde (MDA), and glutathione (GSH) levels. The results revealed that the liposomes exhibited a uniform and spherical morphology with suitable physicochemical properties for drug delivery applications. The *in vitro* cytotoxicity and hemolysis assays and the *in vivo* LD_{50} experiment indicated the potential safety of γ oryzanol liposomes, especially at lower concentrations. In addition, the assessment of liver enzymes, i.e., ALT and AST, and the antioxidant markers further revealed the safety of the formulation, particularly for the liver as a highly sensitive soft organ. Overall, the liposome nanocarriers based on γ oryzanol were successfully formulated and expressed potential safety, supporting their application for the purposes of drug delivery and therapeutic interventions, particularly for hepatocellular and antioxidant therapies; however, further investigations for preclinical and clinical studies could be the future prospects for liposome nanocarriers based on γ oryzanol to explore the safety and efficacy of these nanocarriers in various disease models and clinical settings.

KEYWORDS: *Gamma oryzanol, Liposomes, Nanotechnology, Drug delivery, In vivo and in vitro Safety profile, Antioxidant, Cytotoxicity*

1. INTRODUCTION

Liposomes are sphere-shaped vesicles that are made up of one or more phospholipid bilayers constructed of synthetic or natural materials, and their spherical design boasts a lipid double layer that safeguards hydrophobic fatty acids from water while presenting polar head groups to the surrounding media.[1] This character of liposomes enables them to encapsulate polar compounds in their aqueous core and to dissolve hydrophobic molecules within their bilayers. The primary lipid phase of liposomes consists of phospholipids and surfactants that create a bilayer system similar to the bilayers of the biological membranes.[2] Dimyristoyl-phosphatidyl-glycerol (DMPG) and dimyristoyl-phosphatidylcholine (DMPC) are common examples of naturally occurring surfactants.[3] There is a growing interest between researchers and pharmaceutical production in enhancing the delivery and improving the bioavailability of drugs to improve their therapeutic efficacy. Particularly, the self-

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assembly approach holds great promise for designing effective delivery systems.[4,5] Liposomes are one of the drug delivery systems that are frequently used as a carrier for the lipophilic and hydrophilic antioxidant agents to evaluate their antioxidant activities.[6] Moreover, liposomes are also used as an efficient delivery system for water-soluble (e.g., glutathione, N-acetylcysteine, and quercetin) and lipid-soluble (e.g., tocopherol and CoQ10) antioxidant medicines to various organs and tissues for the treatment of oxidative stress-associated damage.[7−8910] Recently, there has been a growing interest in exploring innovative approaches to enhance drug delivery systems for improved bioavailability and therapeutic efficacy.[11,12] Among these approaches, the incorporation of bioactive compounds into the structure of liposomes has gained significant attention.[13] Liposomes, as versatile and biocompatible nanocarriers, have shown great potential for encapsulating a wide range of therapeutic agents. The current research expressed the potential novel use of γ oryzanol, the rice bran oil bioactive ingredient, as a replacement for the conventional lipid-based liposome structure.[14] Several phytosteroidal esters of ferulic acids, e.g., cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, and campesteryl ferulate, have been detected as a major (about 80% of the bran rice oil) composition in the γ oryzanol.[15] The health benefits of γ oryzanol related to its effect on cholesterol levels have been reported. γ oryzanol has also been reported for its applications as an antioxidant and anti-inflammatory agent.[16] Integrating the γ oryzanol into the liposome, with its unique chemical structural mixture and biological importance, might impart additional therapeutic advantages for the developed drug delivery nanocarrier system. Our current study aimed to replace conventional lipids with γ oryzanol in the synthesis of liposome nanocarriers. The study also aimed to investigate the stability and physicochemical properties of the formulated γ oryzanol liposomal preparation. The *in vivo* and *in vitro* safety profile investigations were conducted in the current study for the formulated γ oryzanol liposome using cell-line-based hemolysis assays as well as the lethal dose determination on the animal model. Further safety and antioxidant assessments were also conducted by measuring the levels of liver enzymes, i.e., ALT and AST, the antioxidant enzyme, i.e., catalase (CAT), and the levels of glutathione and malonaldehyde (MDA) in the experimental animals. The findings from this study hold the potential to advance the field of liposome-based drug delivery by introducing γ oryzanol as a novel component of the liposome structure.

2. MATERIALS AND METHODS

γ Oryzanol (1479202), phosphatidylserine (PS; P7769), dimethyl sulfoxide (DMSO; 67-68-5), tris (252859), Triton-X 100 (9036-19-5), and phenylmethanesulfonyl fluoride (PMSF; 6-98-329) were obtained from Sigma Company. RPMI-1640 medium, FBS, trypsin-EDTA, and penicillin−streptomycin were purchased from Gibco, Rockville, MD. (ALT; BXC0215) and (AST; BXC0205) assay kits were obtained from Biorexfars Co., Iran. Lipid peroxidation (MDA) and catalase activity kits were from KiaZist Co., Iran. All additional chemicals and solvents were commercially available and of analytical quality.

2.1. Preparation of Liposomal NCs. Multilamellar liposomes based on γ oryzanol were prepared using the thin-film hydration method.[17] To prepare the liposomes, 80 mg of γ oryzanol and 20 mg of phosphatidylserine (PS) were dissolved in the $CHCl_3$−CH_3OH mixture in a 9:1 ratio. Then, the mixture was poured into a round-bottom flask of a rotary vacuum evaporator, where the organic solvent was evaporated at 60 °C for 15 min at 90 rpm. The thin-lipid layer, which formed on the inner surface of the flask, was then vortexed for 10 min in PBS with a pH of 7.4 and left to hydrate for an hour at 70 °C and 90 rpm. The resulting liposome suspension was then sonicated for 15 min at 65 °C to obtain small unilamellar vesicles (SUVs).[18] While liposomes can be susceptible to higher temperatures, the sonication parameters used in our investigation were chosen based on previous research and methodologies.[19−22] Visual inspection of the prepared liposomes incorporated gamma-oryzanol for changes to their physical characteristics, including color, transparency, and the presence of any visible particles or aggregates. Alteration in these visual attributes could indicate changes in the physical state of the liposomes, such as aggregation or degradation.

2.2. Physicochemical Characterization of Liposome NCs. The physicochemical properties of the liposome NCs were characterized by various techniques such as FT-IR, DLS, FESEM, and EDS. The chemical compositions of BSA, NISM, NISM@B, Zn NPs, and NISM@BSA-Zn NPs were examined using an FT-IR spectrometer (Bruker, Tensor 27, Biotage, Germany). To generate potassium bromide (KBr) disks, a mixture of 10% of the sample and 90% of KBr was mechanically ground and pressed into a plate form under a pressure of 10 ton. DLS was utilized to determine the polydispersity index (PDI), the average hydrodynamic size of nanoparticles, and ζ-potential using the Nano-Zeta sizer apparatus (Malvern Instruments, Worcestershire, U.K., model Nano ZS). For each sample, 0.5 mL was diluted with ultrapure water and transferred into a Malvern sample vial before being analyzed for PDI, hydrodynamic diameter, and ζ-potential. FESEM was used to determine the morphology and size of the nanoparticles. The samples were coated with gold, and a FESEM (MIRA TESCAN, Czech Republic) was operated at an acceleration voltage of 15 kV and a scale of 35k× magnification. Furthermore, the EDS technique was employed to estimate the element distributions of the prepared liposome nanocarriers.

2.3. *In Vitro* Experiments. *2.3.1. Blood Compatibility Assay.* The hemolysis test assesses the damage to the red blood cells (RBCs) and the subsequent release of hemoglobin into the surrounding media. The test was conducted according to previously reported protocols.[23] Standardization of the National Institute of Health (NIH) and Food and Drug Administration (FDA) and Helsinki's declaration and regulation are statements of ethical principles. Permissions were obtained from the hospitals of the medical city in Baghdad, Iraq, and approved by the institutional ethical committee of the University of Technology, Baghdad, Iraq (ref AS 24/2020). Briefly, healthy human blood was collected into a heparinized tubule and then centrifuged at 4000 rpm for 5 min to remove the upper plasma. Cold PBS was added slowly to the removed plasma in equal amounts, and this process was repeated three times. The RBC pellet was then diluted 1/10 with cold PBS. Liposomes NCs for the hemocompatibility assay were prepared at concentrations of 50, 100, 150, 200, 250, and 300 μg/mL based on the total weight of the NCs. For the negative and positive controls, phosphate buffer (PBS) and sodium dodecyl sulfate (SDS), respectively, were used. Next, the various concentrations of the liposome NCs were added to the RBCs and incubated in a shaking water bath for 4 h. After incubation, the solution was collected in a tube and centrifuged at 4000 rpm for 10 min. Subsequently, 200 μL of the centrifuged media was transferred to a 96-well plate, and the absorbance of hemoglobin was recorded at 540 nm. This

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experiment was replicated ($n = 3$). The percentage of hemolysis was calculated by applying the following formula

$$\text{hemolysis (\%)} = \frac{A_{\text{treated sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100$$

In this equation, $A_{\text{treated sample}}$, $A_{\text{negative control}}$, and $A_{\text{positive control}}$ are representative of the mean absorbance of the sample, negative control, and positive control, respectively.

2.3.2. Cell Culture. The human bone marrow-derived stromal fibroblast cell lines (HS5, ATCC CRL-11882) were purchased from the ATCC. The cells' free-of-contamination status was established. At 37 °C in a humidified atmosphere of 95% air and 5% CO_2, HS5 cells were cultured in RPMI-1640 media supplemented with nonessential amino acids, 10% FBS, 100 U/mL of penicillin, and 100 g/mL of streptomycin (Gibco, Invitrogen). Cells that were in passages 2 and 7 were conducted for testing.[24]

2.3.2.1. MTT Assay. To assess the biocompatibility of the liposome NCs, an MTT assay was conducted on the HS5 cell line. The cells were incubated at a concentration of 1×10^4 cells/well in a complete growth medium for 24 h. Next, the cells were treated with the test samples (γ oryzanol and liposome NCs) at varying concentrations (10, 20, 40, 80, 160, and 320 μg/mL). To evaluate the effect of radiation exposure, a parallel procedure was carried out in which, 5 h after treatment with the test samples, the medium was removed, and the cells were washed with PBS. After 24 h, 20 μL of MTT solution with a concentration of 5 mg/mL was added to each well. Following a 4 h incubation, 100 μL of DMSO was added to each well, and the absorbance at 570 nm wavelength was measured.

2.4. *In Vivo* Experiments. *2.4.1. Median Lethal Dose (LD_{50}) Assay.* The utilization of different compounds in liposome NCs, such as γ oryzanol and phosphatidylserine, may result in various dangerous effects. One way to determine the acute toxicity of the prepared liposome NCs is to calculate the LD_{50}. An acute oral toxicity trial was conducted on adult mice to investigate the safety of the produced NCs. Mice weighing between 25 and 30 g were selected and housed in ideal laboratory settings, in accordance with OECD criteria. For the LD_{50} experiment, γ oryzanol and liposome NCs were orally administered to each animal at doses of 1750 and 5000 mg/kg. Every animal was weighed prior to treatment as well as 1 and 7 days after. Throughout the test, the animals' movements and alterations in behavior were also noted. If all animals remained alive after 24 h, two more animals were given the best care possible. If any of these animals survived, the LD_{50} would be higher than the dose ceiling, and the test would be terminated.[25]

2.4.2. Experimental Design. For this experiment, male mice weighing 30 ± 2 kg were used. The mice were kept in plastic cages with a 12/12 light cycle at room temperature, a regular lab feed, and water for the duration of the experiment. The mice were randomly divided into three groups of three, and 24 h before the experiment, they were subjected to food deprivation. To assess the antioxidant activity of γ oryzanol and liposome NCs, two groups received a daily single dose of 20 mg/kg of γ oryzanol and liposome NCs intraperitoneally for 10 days. The third group served as the control group and did not receive any treatment. The mice were given ketamine/xylazine anesthesia on the 9th day, and 2 mL of blood was drawn. The mice were then executed by guillotine. Livers were isolated and homogenized with 5 volumes of ice-cold homogenization buffer (50 mM tris pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl) using several strokes with a Teflon pestle. The homogenate was then centrifuged at 8000 rpm for 20 min under refrigeration, and the obtained supernatants were stored at −80 °C for the examination of antioxidant activity. The collected blood was centrifuged for 15 min at 3000 rpm to separate the serum, which was then stored at −20 °C for further analysis.[26,27]

2.4.3. Biochemical Evaluation. The Biorexfars diagnostic kit was used as described by the manufacturer to measure the activity of AST and ALT. Additionally, the catalase (CAT) and MDA activities in liver tissues were estimated using the Kiazist diagnostic kit, also following the manufacturer's protocol. Furthermore, changes in the glutathione (GSH) content in liver tissue were measured according to the method of Hu et al. using 5 5′-dithiobis (2-nitrobenzoic acid) (DTNB) as a substrate. The principle of the assay involved the reaction of the sulfhydryl group of glutathione with 5 5′-dithiobis (2-nitrobenzoic acid) (DTNB), resulting in the production of a yellow 5-thio-2-nitrobenzoic acid (TNB). Simultaneously, the mixed disulfide, GSTNB (GSH and TNB), produced during the reaction was reduced by glutathione reductase, recycling the GSH and generating more TNB. The level of GSH in the sample had an immediate connection with the rate at which TNB formed. GSH at the concentration range of 0.2−1 mM was used to prepare a standard curve. To begin the reaction, 230 mL of phosphate buffer (pH 7.6) was mixed with 20 μL of each of the serial dilutions and tissue lysate. This was followed by the addition of 50 mL of a 1 mM DTNB solution (19.8 mg of DTNB in 50 mL of phosphate buffer, pH 7.6). The mixes were thoroughly shaken before being left to incubate for 5 min at room temperature. After that, each sample mixture's absorbance was measured at a fixed 412 nm wavelength, and a standard curve was established. The protein concentration of GSH was given as g/mg.[28,29]

2.5. Statistical Analysis. The GraphPad Prism (version 8) software was used to analyze the data. The one-way analysis of variance test and the independent samples t test were used for normally distributed data. The presentation of data was as mean ± standard deviation (S.D.), and the p value <0.05 was considered significant.[30]

3. RESULTS AND DISCUSSION

The rationale of this study returned to our interest in developing innovative drug delivery systems for the incorporated antioxidant material γ oryzanol to enhance their therapeutic potential. The usefulness of liposomes as biocompatible nanocarriers offers an excellent platform for encapsulating and delivering various therapeutic agents. The natural rice bran oil-derived γ oryzanol is a well-known material for several health characteristics. Reduction of the serum cholesterol levels, anti-inflammatory effects, and antioxidant activity are part of the γ oryzanol health benefits. Gamma oryzanol is a lipid-like component integrated into the liposome structure rather than a traditionally encapsulated drug, and its release behavior may not conform to the conventional patterns observed with drug-loaded liposomes. Therefore, incorporating γ oryzanol as part of the liposomal nanoparticles was our potential approach to produce and optimize the efficient liposomal drug delivery system for therapeutic applications.

3.1. Physicochemical Characterization of Liposome NCs. *3.1.1. FT-IR Spectroscopy.* The FT-IR spectra provide notes for the possible chemical interactions after the incorporation of γ oryzanol into the liposome nanocarriers (Figure 1). Therefore, γ oryzanol and liposome NCs FT-IR

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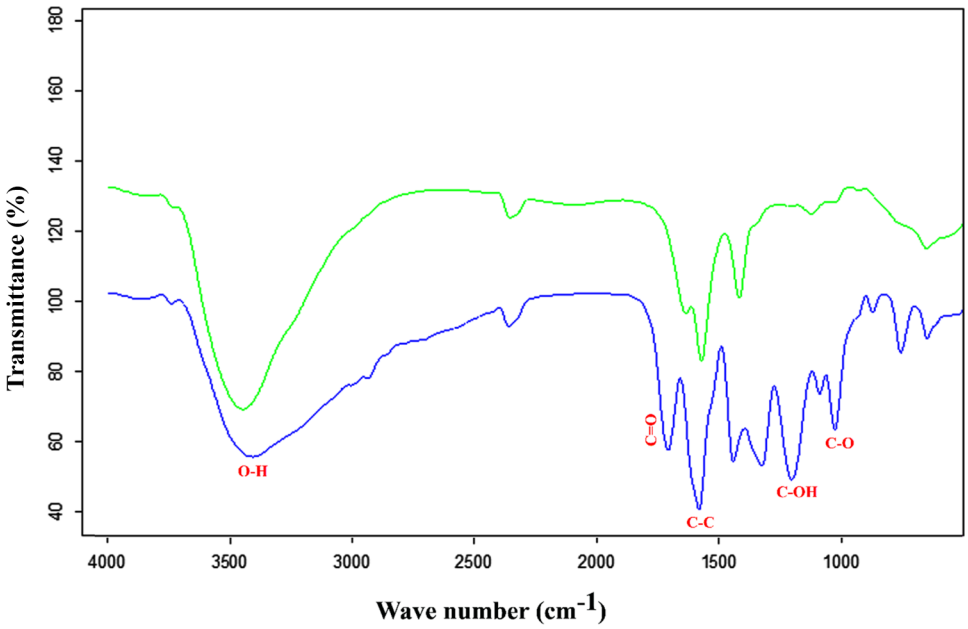

Figure 1. FT-IR absorption spectra. (Green line) FT-IR absorption spectrum of γ oryzanol, highlighting key peaks indicative of its molecular composition. (Blue line) FT-IR absorption spectrum of liposome NCs, illustrating characteristic peaks and shifts, providing insights into the liposomal nanocarriers' chemical structure and molecular interactions.

spectra were plotted together to investigate the possible chemical changes that might result after the γ oryzanol liposome formulation. FT-IR spectrum of liposome NCs (liposome nanocarriers) showed a broad peak at 3500 cm^{-1} related to the O−H stretching vibrations of hydrogen bonding. This stretching is attributed to the O−H functional groups of the liposomal phospholipids. The hydrophilic heads and hydrophobic tails of phospholipids are represented by the presence of phosphate O−H groups and hydrocarbon chains, respectively. These O−H groups are involved in hydrogen bonding with water and other materials in the liposome structure. Furthermore, the FT-IR spectrum of γ oryzanol exhibited characteristic peaks at 1680 cm^{-1} (assigned to the C═O stretching), 1590 cm^{-1} (assigned to the C−C aromatic ring stretching), 1261 cm^{-1} (C−O bond), and 1108 cm^{-1} (C−OH stretching). These characteristic peaks have been reported for the γ oryzanol.[31] On the other hand, the spectrum (FT-IR) of liposome NCs showed distinguishing peaks in the range of 1600−1400 cm^{-1}. By comparing both FT-IR spectra of liposome NCs and γ oryzanol, a reduction in the intensity of certain bonds in the liposome NC spectrum was noticed. The absence of some peaks present in the γ oryzanol spectrum was also observed in the spectrum of liposome NCs. These changes advocate for molecular interactions between γ oryzanol and other liposomal components and support the successful incorporation of γ oryzanol into the liposome nanocarriers.

3.1.2. Hydrodynamic Average Size and ζ-Potential. The hydrodynamic average size, polydispersity index (PDI), and ζ-potential are crucial parameters that influence the stability and colloidal behavior of liposome nanocarriers. According to our findings, liposome NCs have a hydrodynamic average size of 134.83 ± 0.21 nm with a low PDI of 0.19 ± 0.03, suggesting a relatively uniform size distribution (Figure 2A,B). The negative (−ve) ζ-potential of −28.43 ± 0.05 mV indicates that the liposomes have a net −ve charge on their surface. The negative ζ-potential of liposome NCs at −28.43 mV is an indicator of the surface charge of these nanocarriers. It suggests the presence of functional groups or chemical moieties on the liposome surface that can ionize in an aqueous environment, resulting in a net negative charge. The negative charge of the ζ-potential in liposomes is mostly attributed to the presence of phosphatidylserine phosphate groups with negative ionization. The negatively charged functional groups contributed to the electrostatic repulsion between liposomes and helped to stabilize their colloidal dispersion and prevent aggregation. This repulsion is an important characteristic of liposomal nanocarriers as drug delivery systems for various applications. In addition, the phenolic and carboxylic acid moieties in γ oryzanol are likely responsible for the negative ζ-potential of the γ oryzanol liposomes. The hydrodynamic average size of 134.83 nm was in the reported range of nanoscale liposomes (50−200 nm). This small size of the obtained γ oryzanol liposomes is preferred to enhance the cellular uptake and bioavailability of liposomes.[32] In addition, the low PDI value of 0.19 indicated the narrow size distribution range of the liposomes, so the long-term stability of the product during storage is expected.[33,34] Furthermore, stable dispersion and low aggregation tendency are also expected based on the negative ζ-potential value of −28.43 mV.[35] A more negative ζ-potential is generally associated with increased stability in colloidal systems.[36]

3.1.3. FESEM and EDS Analyses. The findings obtained from FESEM and EDS analyses described the morphological characteristics and elemental composition of the liposome nanocarriers, highlighting their suitability for drug delivery applications. Several advantages have been reported for the spherical shape of liposomes as drug delivery systems; these advantages include an increase in the contact area of liposomes with the targeted cells and enhance their cellular uptake, which subsequently improve their therapeutic efficiency.[37] The reproducibility and stability of liposomes were also improved by their uniformity and well-size distributions. In our study, the FESEM scan indicated that prepared liposomes were in a uniform and spherical shape (Figure 3A). The elemental composition of the prepared liposome nanocarriers was assessed

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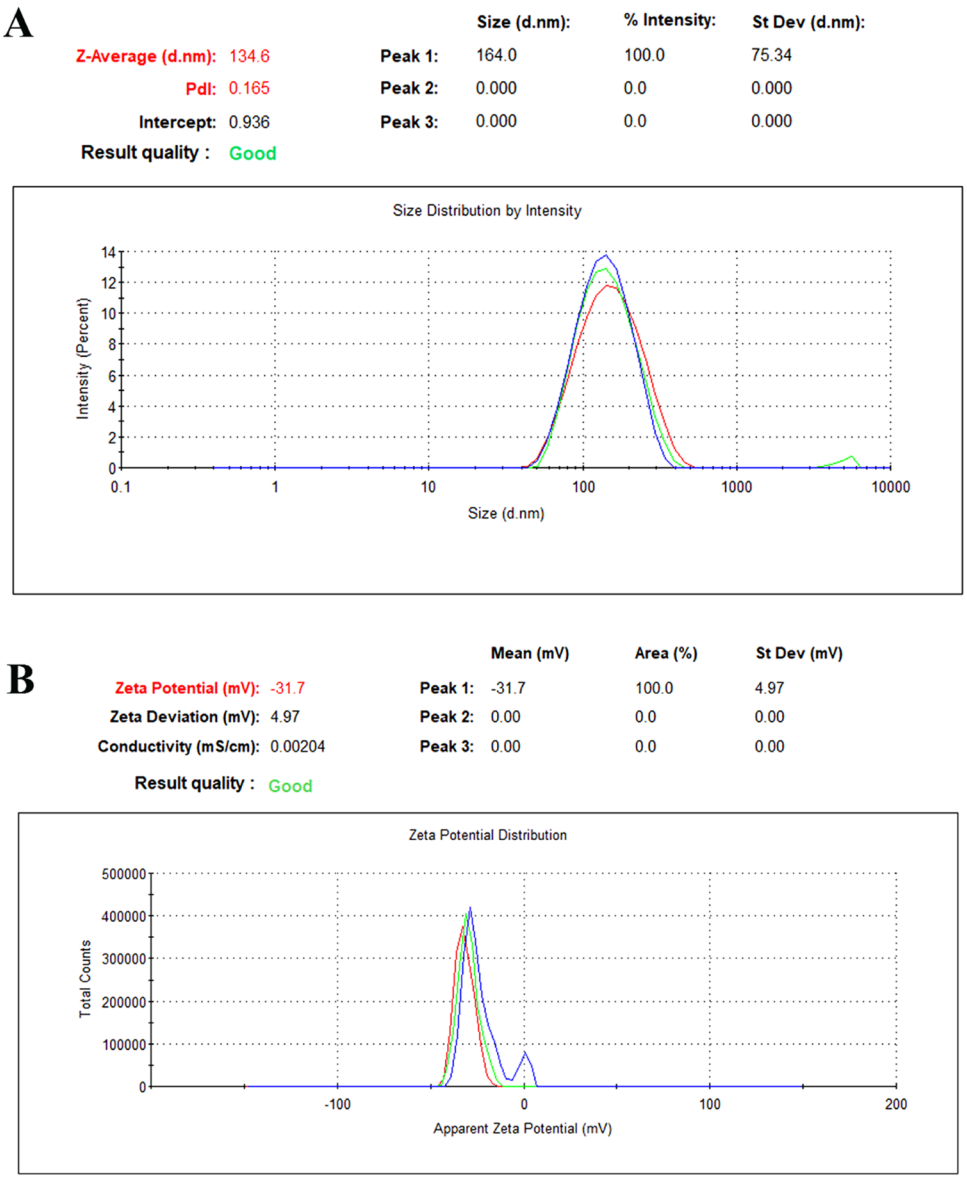

Figure 2. (A) Hydrodynamic average size and (B) ζ-potential of liposome NCs.

using an EDS profile (Figure 3B). Strong signals for carbon (C), oxygen (O), and nitrogen (N) were detected, with weight percentages of 78.01, 19.79, and 2.19%, respectively. The presence of C and O is attributed to the presence of phospholipids, which constitute the liposome structure. In addition, the presence of nitrogen in the EDS profile indicated the incorporation of γ oryzanol, which contains nitrogen in its molecular structure. Therefore, EDS results indicate the incorporation of γ oryzanol into the liposome nanocarriers. The results obtained from FESEM and EDS were consistent with previous reports for the well-prepared liposomes[38,39] and supported the incorporation of the γ oryzanol into the liposomal structure.

3.2. Hemocompatibility and Cytotoxicity Assay. In this study, a hemolysis assay was used to evaluate the potential toxicity of prepared liposomes to red blood cells (RBCs). The results presented in Figure 4A indicate that both γ oryzanol and liposome NCs exhibit minimal hemolytic activity. The hemolysis percentages ranged from 5.29 ± 1.37 to 12.9 ± 3.18% for concentrations ranging from 50 to 300 μg/mL of the samples. These low hemolysis results indicated that the prepared liposomal formulation did not affect integrity and did not induce significant lysis in the RBCs. These results also indicated good biocompatibility between the prepared liposomes and the RBCs. The high hemolytic effect might raise concerns about the safety of the nanocarriers for intravenous administration or other applications involving interactions with blood components. Several reports have documented the *in vitro* and *in vivo* biocompatibility profiles of the phospholipid-based liposomes.[40,41] Besides, liposomal formulations have been investigated as a drug carrier due to their ability to minimize hemolysis and reduce side effects when used as drug delivery systems.[42]

It was essential to test the cytotoxic effect of the formulated γ oryzanol liposome against normal cell lines as part of the safety profile of the formulation. Therefore, we conducted the cytotoxic test using the HS5 cell line as a model for normal human cells. The results indicated the limited cytotoxic effect of both γ oryzanol and prepared liposomes against HS5 cells at concentrations up to 40 μg/mL. The concentrations below 40

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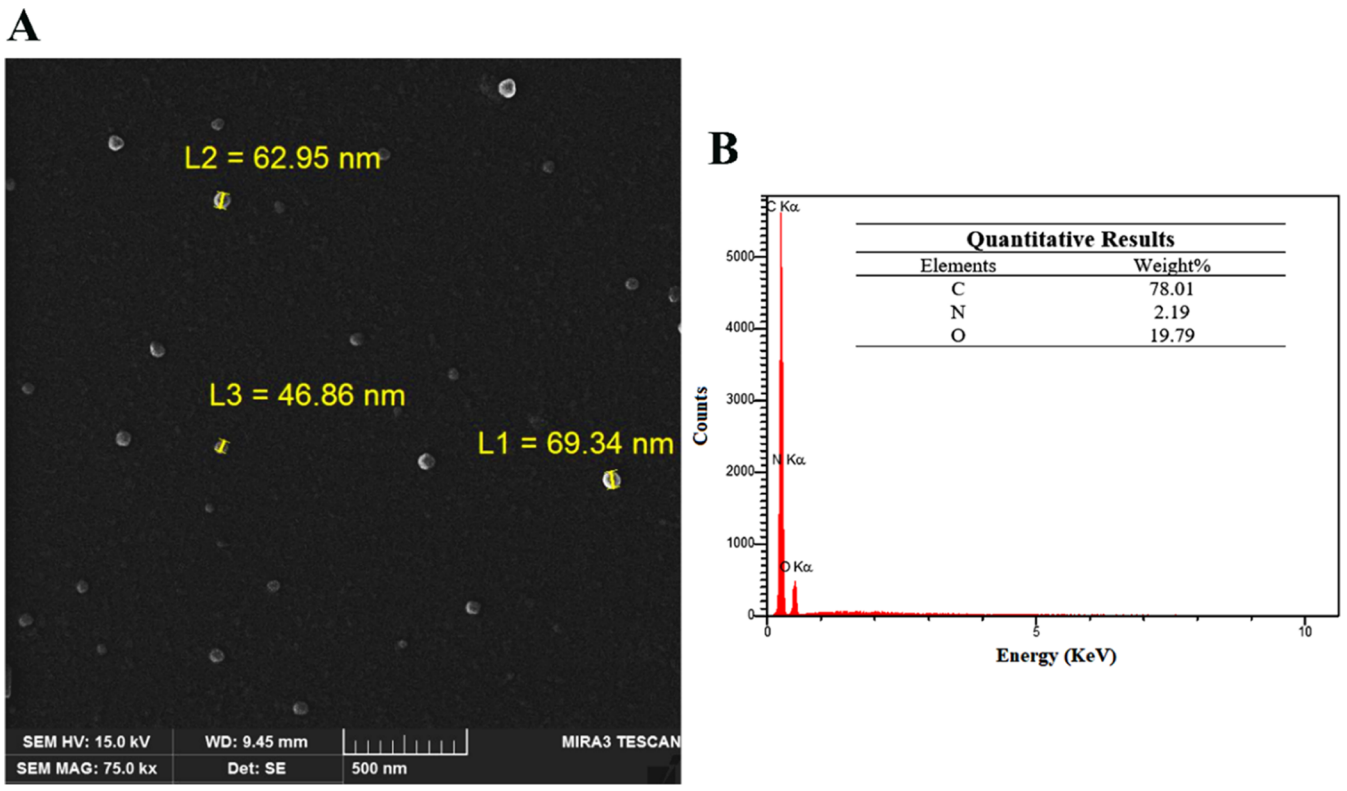

Figure 3. (A) FESEM image and (B) EDS analysis of synthesized liposome NCs.

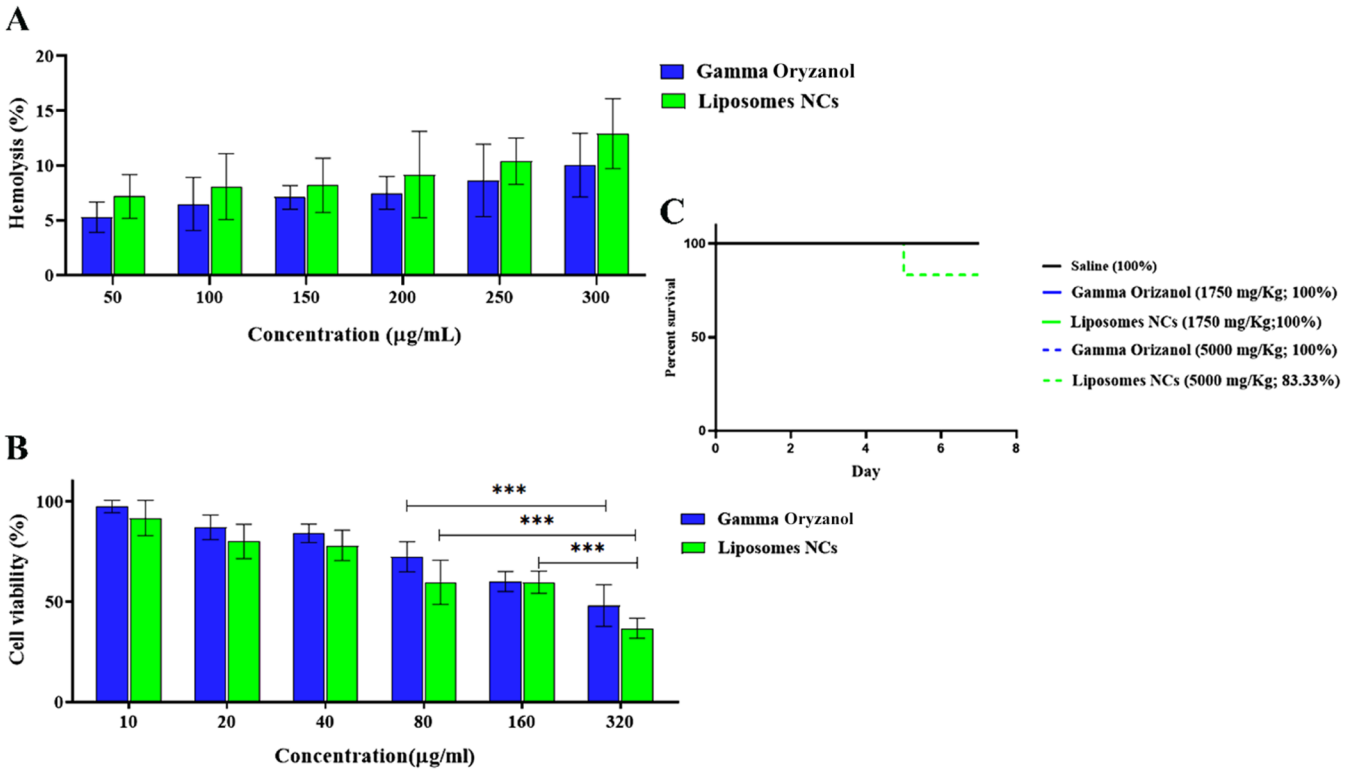

Figure 4. (A) Hemocompatibility assay, (B) cytotoxicity assay against the HS5 cell line, and (C) LD_{50} assay of synthesized liposome NCs. A comparison of the results was performed by a one-way ANOVA statistical test. Symbols marked with *** show significant differences with $p < 0.001$ (between γ oryzanol at 320 and 80) and $p < 0.001$ (between liposome NCs at 320 and 80 and between liposome NCs at 320 and 160).

μg/mL have induced a cell viability of 78−97%, which means that the formulation at that concentration has a limited impact on cell life. The cytotoxic effect of γ oryzanol and liposomes was pronounced at high concentrations; for example, at a concentration of 320 μg/mL, γ oryzanol induced a 48% toxicity within the cell (viability of the cell was 58%). However,

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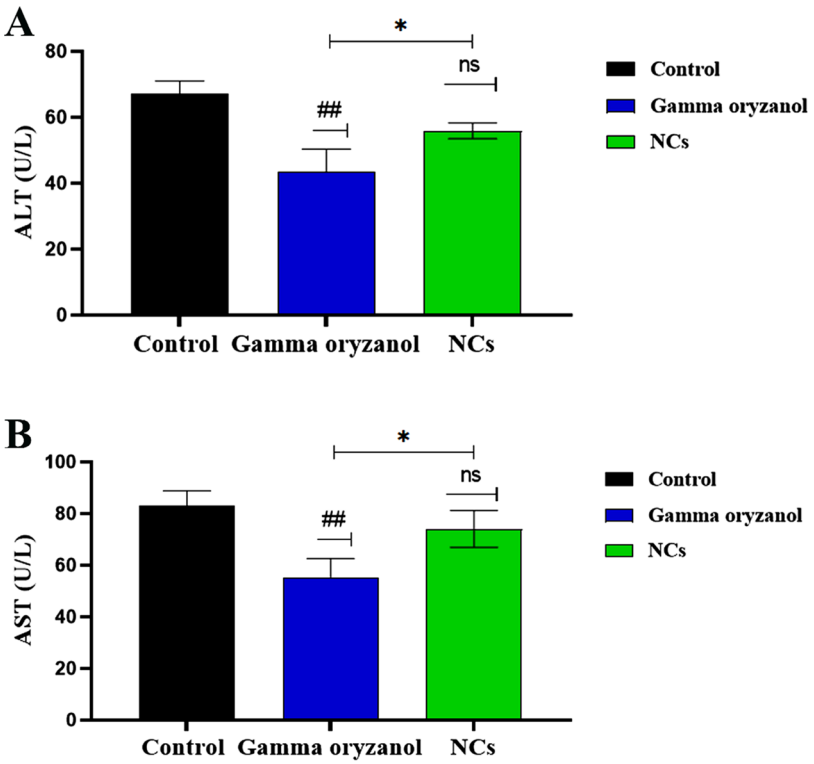

Figure 5. Effect of γ oryzanol and liposome NC treatments on (A) ALT and (B) AST serum levels (U/L) in mice. The symbol marked with * shows significant differences with $p < 0.05$ (ALT: between γ oryzanol and NCs) and $p < 0.05$ (AST: between γ oryzanol and NCs), the symbol marked with ## shows significant differences with $p < 0.01$ (ALT: between γ oryzanol and NCs) and $p < 0.001$ (AST: between γ oryzanol and NCs), and "ns" represented not significant.

liposomes induced 63.33% cell toxicity (viability of 36.67%). The results shown in Figure 4B indicated that both γ oryzanol and liposomes induced limited toxicity at the lower concentrations with dose-dependent cytotoxicity against the fibroblast HS5 cells. The results also pointed out the importance of concentration selection and precise dosage when considering these nanocarriers for potential therapeutic applications. The current findings for the cytotoxicity effect of prepared liposomes were in line with the previous study for the toxicity of liposomes against various cell lines.[43] With the reported cybotactic and our current results, further in-depth studies are still necessary to understand the mechanisms of cytotoxicity and evaluate the overall safety and efficacy of these nanocarriers in a broader biological context. As these nanocarrier systems have potential therapeutic applications, their safety profile, including both *in vitro* and *in vivo* testing using preclinical and clinical examination, needs more extensive studies and documentation.

3.3. *In Vivo* Acute Toxicity Assay. The LD_{50} (lethal dose of 50%) is an important assay used to determine the acute toxicity of substances in living animals. Therefore, we conducted the LD_{50} assay to evaluate the possible toxic effects of the oral administration of γ oryzanol and liposome NCs. The substances, γ oryzanol and liposome NCs, were tested in a mouse animal model at two different doses, i.e., 1750 and 5000 mg/kg.[44] The obtained results indicated that both substances, γ oryzanol and liposome NCs, at both concentrations, 1750 and 5000 mg/kg, did not induce acute toxicity in the mice. However, a higher dose of the liposome NCs (5000 mg/kg) affected the survival rate of the mice, which was decreased to 83.33%. These results revealed that liposome NCs could induce a slightly higher acute oral toxicity compared to γ oryzanol at this dose (Figure 4C).

The LD_{50} tests provide important data for safety concerns when assessing the possible risk of substances used in pharmaceutical and biomedical applications. The LD_{50} values with doses greater than 5000 mg/kg usually indicate the low toxicity of the tested substances. These substances of low toxicity fall into the globally harmonized classification systems (GHS) grade 5.[44] Overall, the current findings of the LD_{50} assay indicated the safety and low acute oral toxicity of γ oryzanol and liposome NCs in mice, with LD_{50} values exceeding the highest dose, i.e., 5000 mg/kg, tested in this study.

3.4. Liver Enzymes, ALT and AST, Serum Levels. The levels of ALT and AST are considered to be important signals for liver health and function. Dramatical alteration of the ALT and AST levels is an indicator of liver stress and injury.[45] Therefore, we evaluated the effect of γ oryzanol and liposome NCs on serum levels of ALT and AST after their intraperitoneal injection (20 mg/kg) for 10 days. The results revealed that γ oryzanol significantly reduced the serum level of ALT by 40.50% compared with the control group. This reduction could indicate the protective effect of γ oryzanol for hepatocytes, as decreased serum levels of ALT are linked to improved liver function.[46,47]

The results also indicated that liposome NCs have induced a reduction in the ALT level by 15.85% with a nonsignificant value compared to the control group of animals. With the nonsignificant reduction in the ALT level obtained by the treatment of animals with liposome NCs, the substance was still hepatoprotective or at least neutral toward hepatocytes (not induced oxidative stress or elevation in the liver enzymes). Furthermore, γ oryzanol induced a significant reduction in the AST levels by 33.40% compared to the control group of animals (Figure 5A,B). This effect indicated the protective ability of γ oryzanol, as decreased serum AST level is a sign of liver health.[48]

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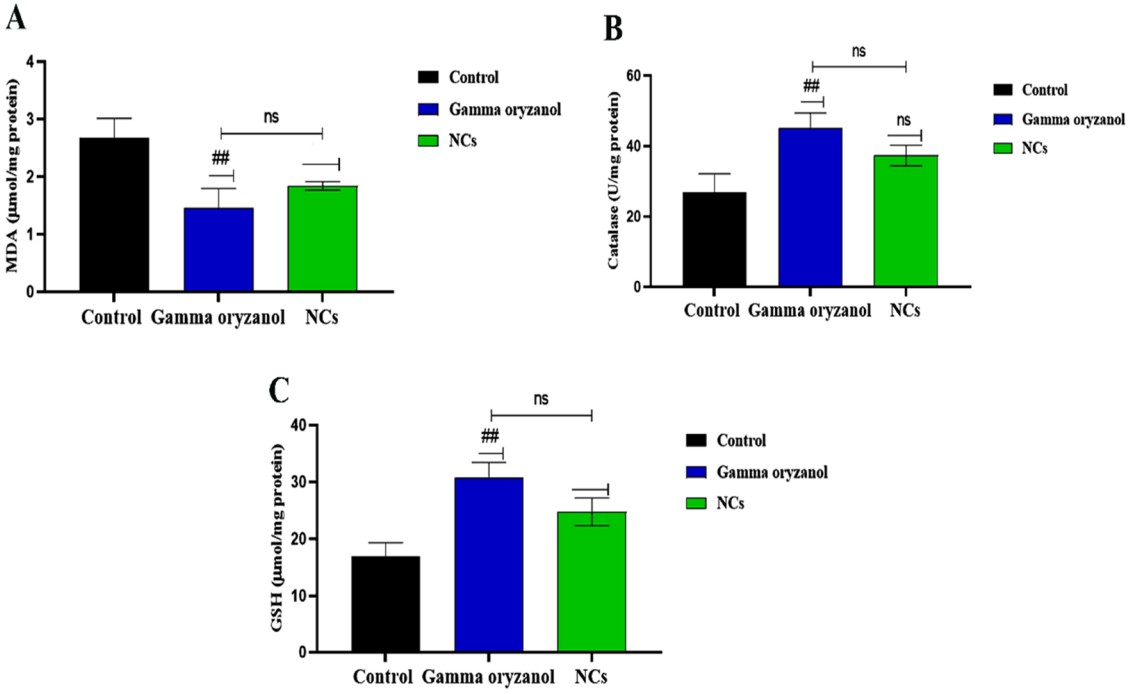

Figure 6. Malonaldehyde (MDA), catalase, and glutathione (GSH) levels in mice liver homogenate of the control and treated animals with γ oryzanol and liposome NCs. (A) MDA level (μmol/mg protein). Columns labeled with ## show significant differences ($p < 0.01$). (B) Catalase level (U/mg protein). Columns labeled with ## show significant differences ($p < 0.01$). (C) GSH level (μmol/mg protein). Columns labeled with ## show significant differences ($p < 0.01$).

Similar to its effect on ALT, liposome NCs induced a nonsignificant reduction in the level of AST by 10.86%, compared to the control group of animals. However, this decrease was not statistically significant but still suggests a potential trend toward reduced AST levels with liposome NC treatment. Previous research has indicated that γ oryzanol possesses hepatoprotective properties and may help reduce liver injury caused by oxidative stress, inflammation, or toxic agents.[49] Similarly, liposomes have been explored as potential carriers for hepatoprotective agents, and certain liposomal formulations have shown promising results in protecting liver cells from damage.[50]

3.5. Lipid Peroxidation Process, Catalase, and GSH Level in Mice Liver Homogenate. The results obtained from the analysis of lipid peroxidation, catalase activity, and GSH levels in mice liver homogenates provide valuable insights into the effects of γ oryzanol and liposome NCs on antioxidant activity and oxidative stress. The lipid peroxidation process results from the attack of cell membrane lipids by the free radicals, the effect that consequently induces cellular damage. The oxidative stress biomarker malondialdehyde (MDA) is a byproduct of lipid peroxidation. The level of MDA in the mice liver homogenate indicated the antioxidant and hepatoprotective effects of γ oryzanol and liposome NCs, which was demonstrated by the significant reduction of MDA in their treated groups as compared to that of untreated animals (Figure 6A). Moreover, γ oryzanol significantly elevated the catalase concentration compared to the untreated animals, which further indicates its ability to enhance the endogenous antioxidant levels in the treated mice and combat oxidative stress (Figure 6B). The current findings are consistent with the reported antioxidant activity of γ oryzanol and its ability to scavenge free radicals. This character of γ oryzanol leads to its ability to reduce oxidative stress in liver cells and promote the efficient functioning of the antioxidant defense system.[15,16] γ oryzanol has also been reported to have an anti-inflammatory effect.[15,16] Therefore, part of the γ oryzanol activity as a suppressor of oxidative damage might be attributed to its anti-inflammatory activity, as oxidative stress increased at higher levels of inflammation. The enhancing levels of catalase induced by γ oryzanol can be considered a mechanism for the substance hepatoprotective effect through enhancing the antioxidant capacity of the liver and reducing lipid peroxidation and free radicals, leading to enhanced hepatocellular viability and reducing cellular damage and death. The findings demonstrated in Figure 6B also indicated that liposome NCs increased the activity of the enzyme but with nonsignificant level compared to the control untreated animals. These results indicated a lower activity of liposome NCs compared to that of γ oryzanol but still showed interest in the formulation for improving antioxidant defense levels in the liver (Figure 6B).

The effect of γ oryzanol and liposome NCs on the glutathione (GSH) level was measured as part of the substances' antioxidant and liver protection evaluation. GSH is one major antioxidant component of the body and is considered an important indicator of the health of the liver.[51] The level of GSH was increased significantly by the administration of γ oryzanol and liposome NCs compared to that of the control group (Figure 6C). These results support again the safety, antioxidant activity, and hepatoprotection effect of γ oryzanol and liposome NCs. In the literature, γ oryzanol has demonstrated potent antioxidant activity, and its protective effects against oxidative stress have been investigated in animal experimental models.[52,53] In addition, antioxidant materials incorporated in the liposomal structure have also been reported to alleviate oxidative stress and protect cells from damage.[54,55] The results of this study are consistent with previous research and provide further support for the antioxidant effects of γ oryzanol and liposome NCs. This

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antioxidant effect is evidenced by the reduction of lipid peroxidation (reducing the MDA activity), the elevation of catalase activity, and the increase in GSH levels. The results also integrated the reported therapeutic potential of liposome-incorporated antioxidant compounds and proposed a potential liver-protective effect for the prepared liposome-incorporated γ oryzanol.

4. CONCLUSIONS

This research was conducted to formulate γ oryzanol liposomal nanocarriers and evaluate their physicochemical properties. The toxicity of the prepared liposomes, including *in vitro* and *in vivo* toxicities, and their antioxidant activity were also evaluated. The γ oryzanol liposomes have been prepared successfully and well-characterized by FT-IR spectroscopy, FESEM images, and EDS analysis. The results of the *in vitro* and *in vivo* biological assays indicated potential applications of γ oryzanol liposomes as a nanocarrier system. Current results also supported that liposome nanocarrier incorporated γ oryzanol can be effectively used as a drug delivery system with enhanced therapeutic properties. The antioxidant and hepatoprotective effects of the prepared γ oryzanol liposomal nanocarriers also indicated their potential clinical applications for antioxidant and liver-targeted therapies. Further research, including preclinical and clinical studies, could be useful to explore the therapeutic potential, safety, and efficacy of γ oryzanol-loaded liposome nanocarriers. The development of liposome nanocarriers incorporating γ oryzanol represents a promising avenue for advancing drug delivery technologies and therapeutic interventions.

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Notes

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