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Research Article

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The Cytoprotective Effects of Silymarin-loaded Solid Lipid Nanoparticles on Experimentally Induced Tissue Damage: Biochemical Evaluation

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Abstract Silymarin (Sm), known for its anti-oxidative, anti-inflammatory, anti-mitotic and hepatoprotective properties can be successfully incorporated into solid lipid nanoparticles (SLN), which possess multifunctional properties in reducing its pharmaceutics limitations. Here in it is aimed to reduce a new Sm-loaded SLN the limitations of Sm via developing bioavailability formulation for better. For this manner five groups of Balb/c mice were used. The study groups were given saline (0.5 mL), D-galactosamine (D-GaIN)/ tumour necrosis factor alpha (TNF-α), D-GaIN/TNF-α plus Sm, D-GaIN/TNF-α plus Sm-free SLN and D-GaIN/TNF-α plus Sm-loaded SLN respectively. Hepatotoxicity was determined by changes in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), glutathione (GSH) and plasma malondialdehyde (MDA) levels in Balb/c mice. Our biochemical results suggested that the group that received Sm-SLNs for the treatment of D-GaIN/TNF-α-induced experimental hepatotoxicity showed a significantly better improvement than did the group that received commercially available Sm. In conclusion, Sm-loaded SLNs might function as carrier systems on account of their therapeutic effects on D-GaIN/TNF-α-induced hepatotoxicity.

Keywords Silymarin, Solid lipid nanoparticle, liver damage, Balb/c mice

Introduction

Liver disorders are major threats to human health caused by various agents as alcohol, drugs, chemical substances, toxins as acetaminophen, TNF-α, D-GalN and viruses [1]. TNF-α is a pleiotropic cytokine with diverse biological functions, such cell proliferation, inflammation and apoptosis [2]. Although TNF-α is known to activate nuclear factor kappa B (NF-kB) effectively, evidence suggests that TNF-α mainly triggers apoptosis in the cells suffering from NF-kB activation deficiency or defects [3]. Therefore, blocking the expression of such proteins as NF-kB by inhibition of translation or transcription makes cells vulnerable to TNF-α toxicity (4). D-GalN is a specific hepatotoxic agent that blocks protein synthesis and hepatic mRNA transcription due to metabolic depletion of uridine nucleotides [5]. These agents (D-GaIN/TNF-α) are widely used a method in causing experimental liver damage [4].

Sm is an antioxidant flavonoid complex cultivated from the herb milk thistle (Silybum marianum), which has long been used in the treatment of liver diseases [6-7]. Hepatoprotective properties of Sm are attributed to its ability to scavenge free radicals and to chelate metal ions [8]. Sm usage in liver disease treating is employed due to protective effects of Sm on liver cells directly by stabilizing the membrane permeability by means of inhibiting lipid peroxidation [9] and preventing liver glutathione depletion [10]. These effects of Sm cure thanks to the anti-oxidative activity, stimulation of r-RNA polymerase and subsequent protein synthesis, thus leading to enhanced hepatocyte regeneration [11].

On the other hand, Sm has many limitations such as poor solubility in water, uneven distribution within the tissue, and rapid decomposition in the intestine [12], because of the properties of its components with poor

solubility as reported by a number of study in which is reported ng/mL^{-1} concentrations in plasma after oral administration of powdered extracts both in humans and laboratory animals [13]. Many attempts have been made so far in order to minimize these effect-limiting properties of Sm, but pharmacologic success has been reported [14]. In order to minimize the limitations, the present study has considered solid lipid nano-formulation (SLN). SLNs were developed as an alternative carrier system for pharmaceutical applications. SLNs can facilitate the entry of agents into cells or tissues because of their small size, improve the bioavailability of drugs by increasing their diffusion through biological membranes, and protect them against enzyme activation that results in a toxic product [15]. Furthermore, the number of the studies conducted on Sm-SLN application is rather small. Here in, Sm-SLN were prepared via the hot homogenization technique. Afterwards, it is achieved a comparison between dissolution capacity, bioavailability and cytoprotective activity of Sm and Sm-SLN in mice with D-GaIN/TNF-α-induced liver damage.

Materials and Methods

Chemicals

Sm was supplied by Sigma Aldrich (St Louis, MO, USA). Compritol and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Merck Schuchardt (Darmstadt, Germany), Tumour Necrosis Factor-alpha (Human Recombinant *E. Coli*) and D-galactosamine were obtained from Gattefosse (Saint-Priest Cedex, France), Duksan Chemical Company (Ansan, South Korea), CalbioChem and Acros Organics, respectively.

Animals

35 Balb/c male mice with 2 to 2.5 month of age, each one weigh 20 to 25 gr, were used in this study. The mice were fed with standard pellet and tap water in controlled laboratory conditions, kept at daylight and dark for 12 hours each time at a temperature of 22±2 C˚ along with humidity of 45-50% before the experiment was launched. They were randomly categorized into 5 groups per 7 mice. During this adaptation period, all the mice were fed with standard pellet and tap water in polycarbonate transparent cages.

Experimental design

In the control (group 1) animal group 0.5 ml of saline i.p. injected. The group 2 i.p. received only D-GaIN/TNF-α [16]. The group 3 i.p. received Sm (100 mg/kg) 4 hours after D-GaIN/TNF-α injection. The group 4 i.p. received Sm-free SLN 4 hours after D-GaIN/TNF-α administration. The group 5, administration for 4 hours i.p. received Sm-loaded SLN after D-GaIN/TNF-α. The group 1 and group 2 were sacrificed only 4 hours after injection, while the others were sacrificed 24 hours after the final injections [17]. TNF-α (15µg/kg in distilled water) was administered to each mice. The mice were administered with D-GaIN (700 mg/kg in distilled water). Finally, Sm (100 mg/kg in distilled water) was injected to the mice, along with 100 mg/kg of Sm-free SLN and Sm-loaded SLN, which had been prepared in distilled water.

Particle Size and Zeta potential

The zeta potential of the particles was determined using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). For determination of Stern potential measurements were performed in bidistilled water adjusted with 0.9% (w/v) sodium chloride solution to a conductivity of 50 µS/cm.

Preparation of Sm-SLNs

SLNs were prepared through the hot homogenization technique, reported by Muller [18]. Lipid matrix was melted at about 80^{o}C and a certain amount of Sm was added. After distilled, water with surfactants (Tween 80) was heated to the same temperature. The hot lipid phase was poured over the hot water–surfactants solution using an Ultra-Turrax at 20500 rpm (T25, Janke&Kunkel IKA®, Germany), which was followed by cooling at room temperature to obtain the solid lipid nanosuspension.

Characterization of SLNs

The average diameters (particle size) and polydispersity index (PI) of SLNs were determined by photon correlation spectroscopy (PCS) using a Nano Zetasizer (ZS, Malvern, UK) at a fixed angle of 90^{o} and at a temperature of 25 ^{0}C. The PI is known as a measure of the size distribution of a nanoparticle population [19]. To assess colloidal dispersion stability, the zeta potential was measured through a Nano Zetasizer (ZS, Malvern,

UK) at 25ºC. SLNs were then suspended in distilled water (pH 7). Each of the samples was analysed least wise in triplicate. Sm-SLNs have also been characterized by transmission electron microscopy (TEM) (TEM FEI Tecnai BioTWIN). The SLNs were spread over a Cu grid and then stained with uranyl acetate and observed under TEM [20].

***In vitro* dissolution studies**

In vitro dissolution studies the dissolution studies of pure silymarin and silymarin-loaded SLNs were performed according to the USP XXXI pallet method (apparatus II) using a dissolution tester (Aymes, Istanbul, Turkey) (USP 31). The dissolution medium (phosphate buffer pH 6.8) (400 mL) was degassed, the stirring speed was maintained to 75 rpm and the temperature was set at 37±0.5ºC. At each sampling interval, 1.5 mL of the dissolution medium was withdrawn and replaced by an equal volume of fresh medium. The samples were filtered and analyzed by HPLC for silymarin (n=3). In the meantime, an equal volume of the blank medium at the same temperature was added to keep volume constant [21].

Drug loading efficiency (%DL)

Drug loading efficiency (%DL) was calculated from the silymarin in the SLN formulations and the total amount of silymarin and the excipients added in the preparation of SLN according to the following [22].

%DL=[Amount of drug in the SLN precipitations/(Amount of drug added + amount of excipients added)] x100.

Biochemistry Assays

Serums were obtained from the blood samples of all the mice used in the experiment for 10 minutes at 3000 rpm. The samples were then analysed for determination of serum ALT, AST, LDH, ALP, GSH, and plasma MDA via an automated biochemical auto-analyser (HITACHI-917).

Statistical analysis

A package software version of SPSS 12.0 for windows was used in assessing the data obtained in this study. The difference observed for ALT, AST, ALP, LDH, GSH and MDA levels in the groups were evaluated with a one-way ANOVA. The numerical value (p) for the difference was accepted as significant if it was $P<0.05$.

Results

Particle Size and Zeta Potential

In the present study, Sm's particle size ranged from 600 nm to nearly 5 µm, while Sm-SLN's particle size varied between 160 and 200 nm. Moreover, SEM measurement showed that the particle size of SLN formulations was smaller than that of Sm, and that Sm had a cubic shape while SLN formulations had circular shapes. From these results, it could be assumed that SLN formulation has reduced the particle size of Sm. Our study results are in agreement with many other research results [23]. Predictions about the storage stability of colloidal dispersion were achieved by determining zeta potential. Here in, all formulations were negatively charged and varied from -21.2 to -25.9 mV. Sm-SLNs indicated a relatively good physical stability and dispersion quality [24]. The zeta potential value may be affected both by aggregation and storage conditions. However, particle aggregation is less likely to occur for charged particles (high zeta potential) because of electric repulsion [25].

***In vitro* dissolution studies**

Release of Sm and Sm loaded SLN was carried out in phosphate buffer. Sm was released nearly 95% within 4 hour, while Sm loaded SLN formulations were released almost 90% within 50 h. These results show that SLN increased the drug release relative to Sm (Figure 1). So, SLN formulation sare found suitable for sustained release of Sm.

Drug loading efficiency (%DL)

Drug loading efficiency of Sm loaded SLN formulations was found to be satisfactory, that is, 89%.

Biochemical Results

Serum ALT, AST, LDH, and ALP levels were found to have dramatically increased in Group 2 when compared to the control group ($P<0.001$). As to Group 3, serum ALT, AST, LDH, and ALP levels showed a considerable

decrease when compared to Group 2 (P<0.001). As for Group 4, serum ALT, AST, LDH, and ALP levels showed no change of statistical significance when compared to Group 2 (P>0.05). As far as Group 5 is concerned, serum ALT, AST, LDH, and ALP levels decreased in significant levels when compared to Groups 2 and 4 (P<0.001), while it showed a difference with Group 3, but this was of no statistical significance (P>0.05).
A comparison of serum GSH and plasma MDA levels in Group 2 with those of the control group showed a significant decrease in GSH levels while plasma MDA levels increased dramatically (P<0.001). As to Group 3, serum GSH levels increased but plasma MDA levels decreased when compared to those of Group 2 (P<0.001). As for Group 4, there was a difference between serum GSH and plasma MDA levels in Groups 4 and 2 but this difference did not achieve statistical significance (P>0.05). As far as Group 5 is concerned, serum GSH levels had increased significantly as opposed to plasma MDA levels that had decreased significantly when compared to those in Groups 2 and 4 (P<0.001). On the other hand, difference on the MDA and GSH levels between groups 3 and 5 were found to be statistically with no significance (P>0.05).

Discussion

It is a well-documented fact that acute liver damage in animals develops subsequent to an injection of D-GalN/TNF- α. TNF-α is considered to be produced and released in macrophages in reaction to other endotoxins and other stimulants (4). TNF-α is also known to has as an essential effect in different physiological phenomena, of which response to stress, tissue damage, tumour necrosis, stimulation of other cytokines, fever, inflammation, cell division, cell differentiation, regulation of immune functions and apoptosis are many reported (2). D-GalN is a highly selective hepatotoxin that is reported to contribute to intracellular depletion of uridine intermediates *in vivo* [5]. High levels of galactokinase [26] and UDP-glucose: galactose-1-P-uridyltransferase [27] in hepatocyte-derived cells are argued to account for the tissue-specific toxicity of D-GalN. Galactosamine administration in rats upsets the membrane permeability of the plasma membrane, thus causing leakage of the enzymes from the cell, which, in turn, results in a rise in the levels of serum enzymes [28]. Elevated serum enzymes are acknowledged as to be suggestive of cellular leakage and loss of functional integrity of the cellular membrane in the liver [29]. One study by Manabe et al. [30] reported a remarkable increase in the levels of serum ALT, AST, LDH, ALP, and plasma MDA in a study in which they caused liver damage using D-GalN. Nagaki et al. [31] have reported a valuable increase in the serum ALT and AST levels compared to control group after 8 hours of D-GalN/TNF-α administration.
Another study, in which liver damage was caused by a combination of 700 mg/kg of D-GaIN and 15 µg/kg of TNF-α at the 4th hour of the experiment, reported a rise in tissue MDA and LDH levels, as well as in serum ALT and AST levels, but there was a significant drop in GSH levels when compared to the control group [16]. According to our results biochemical results showed that serum ALT, AST, LDH, ALP, and plasma MDA levels had increased significantly when compared to those of control group, but that serum GSH levels had decreased remarkably (P<0.001).
Sm is reported to possess hepatoprotective and anti-carcinogenic effects [32]. Sm has got a hydroxyl group, along with a carbonyl group, which may form a chelate with ferrous iron. This chelation had been reported to raise the activity to the level of most active scavengers, most probably by site specific scavenging [33]. As far as how the mechanism of Sm works, it has been suggested that the hepatoprotective activity of Sm might be due to its effect against cellular leakage and loss of functional integrity of the cellular membrane in hepatocytes. Furthermore, recovery toward normalization suggests that Sm could account for parenchymal cell regeneration in the liver, thus protecting membrane fragility, which, in turn, decreases enzyme leakage [34].
A study into the damaged liver of rats reported that serum ALT, AST, and ALP that had increased due to N-Nitrosodimethylaniline-induced (N-NDMA) liver damage were all significantly reduced on an injection of 50 mg/kg of Sm to the rats [35]. Another study that induced liver damage by using CCl_4 revealed that such markers of liver damage as AST, ALP and lipid peroxidation resulted in the rise of MDA levels, but that these markers significantly decreased upon an injection of 1000 ppm of Sm [36]. Our study results showed that serum ALT, AST, LDH, ALP, and plasma MDA levels significantly decreased in the group given Sm to repair the hepatotoxicity induced by D-GalN/TNF-α (P<0.001), while the group given SLN for the liver damage induced by D-GalN/TNF-α showed no change in serum ALT, AST, LDH, ALP, and plasma MDA levels when

compared with the group given only D-GalN/TNF-α. However, these liver damage markers decreased relatively more when Sm was administered to the rats in combination with Sm-free SLN but still this difference was of no statistical significance (P>0.05). The ability of Sm to scavenge free radicals causes a remarkable rise in the cellular antioxidant defence machinery by ameliorating the harmful effects of free radical reaction and the rise in GSH content, which is assumed to be crucial to maintaining the ferrous state [33,37]. Based on this finding, another study has reported the GSH levels that had decreased in the livers of the rats damaged due to N-NDMA was observed to increase on an administration of 50 mg/kg of Sm [35]. One study by Heba et al. [34] reported the damaged liver of rats reported that serum GSH that had decrease due to 7.5 mg/kg of cisplatin therapy liver damage were increased upon an injection of 100 mg/kg of Sm to the rats. In addition, the results of the present study showed that serum GSH levels significantly increased in the group given Sm to repair the liver damage induced by D-GalN/TNF-α (P<0.001), while the group given Sm–free SLN for the liver damage induced by D-GalN/TNF-α showed no change in serum GSH levels when compared with the group given only D-GalN/TNF-α. However, these liver damage markers increased relatively more when Sm was administered to the rats in combination with Sm–free SLN but still this difference was of no statistical significance (*p>0.05*).

As conclusion, Sm-loaded SLN detected to be more effective in healing to hepatotoxicity in Balb/c mice than Sm that might be attributed to the fact that formulated nanoparticles probably keep releasing Sm slowly and regularly providing Sm with a more effective bioavailability, in return its therapeutic properties proved to be more effective than Sm, which could be due to the fact that nanoparticles probably keep releasing Sm slowly but regularly, in a way that provides Sm with a more sustainable bioavailability, thus raising the chances for its therapeutic properties.

Table 1: Names of the experimental groups, as well as of the chemicals and doses applied.

Group name	Administration
Group 1 (control)	Saline (0.5 ml)
Group 2	D-GaIN (700 mg/kg)/TNF-α (15µg/kg)
Group 3	D-GaIN (700 mg/kg)/TNF-α (15µg/kg)/Sm (100 mg/kg)
Group 4	D-GaIN (700 mg/kg)/TNF-α (15µg/kg)/Sm-free SLN (100 mg/kg)
Group 5	D-GaIN (700 mg/kg)/TNF-α (15µg/kg)/Sm-loaded SLN (100 mg/kg)

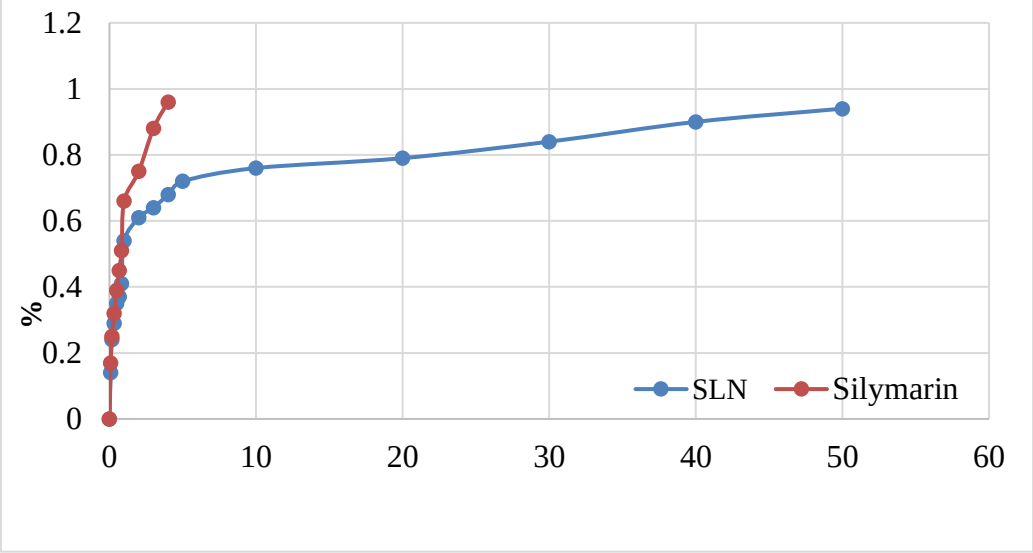

Figure 1: *Drug release profiles of Sm and Sm loaded SLN formulations*

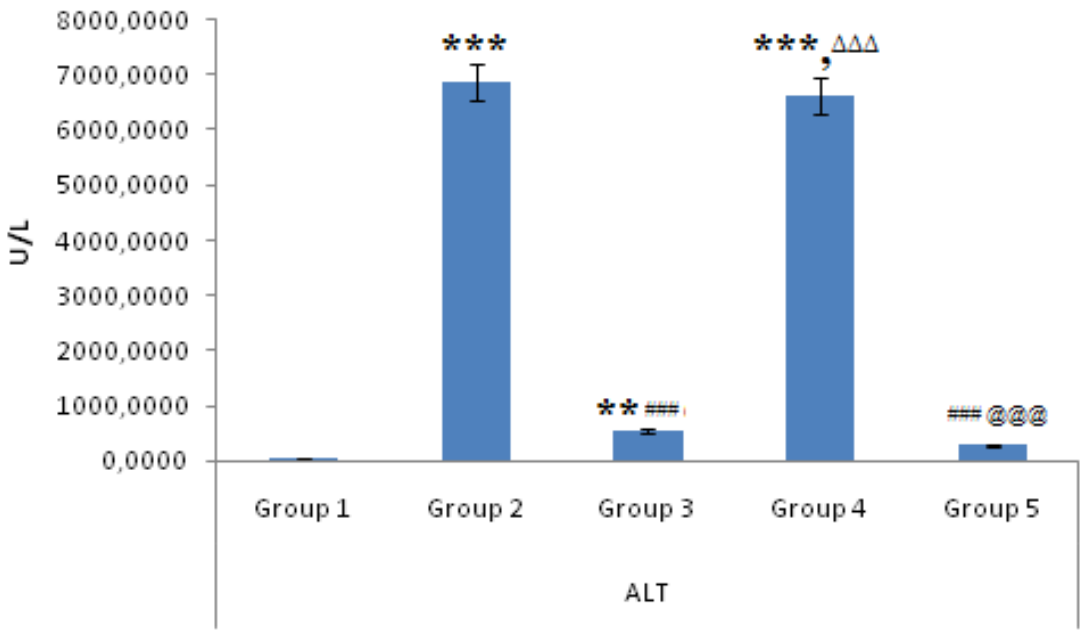

Figure 2: Serum ALT levels of the blood samples taken from Balb/c mice.

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($_p$< *0.05*). Means with the same letter within the same parameter are not significantly different. ***p<0.05* compared to control, ****p<0.001* compared to control, ### *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

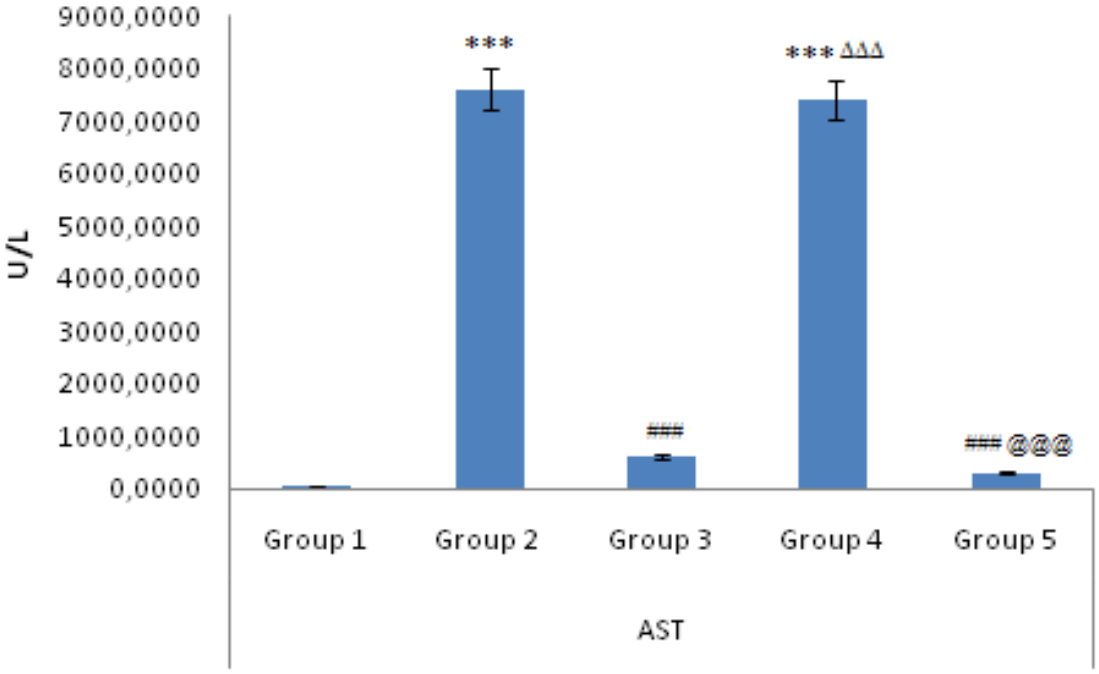

Figure 3: Serum AST levels of the blood samples taken from Balb/c mice.

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test (*p<0.05*). Means with the same letter within the same parameter are not significantly different. ****p<0.001* compared to control, ### *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

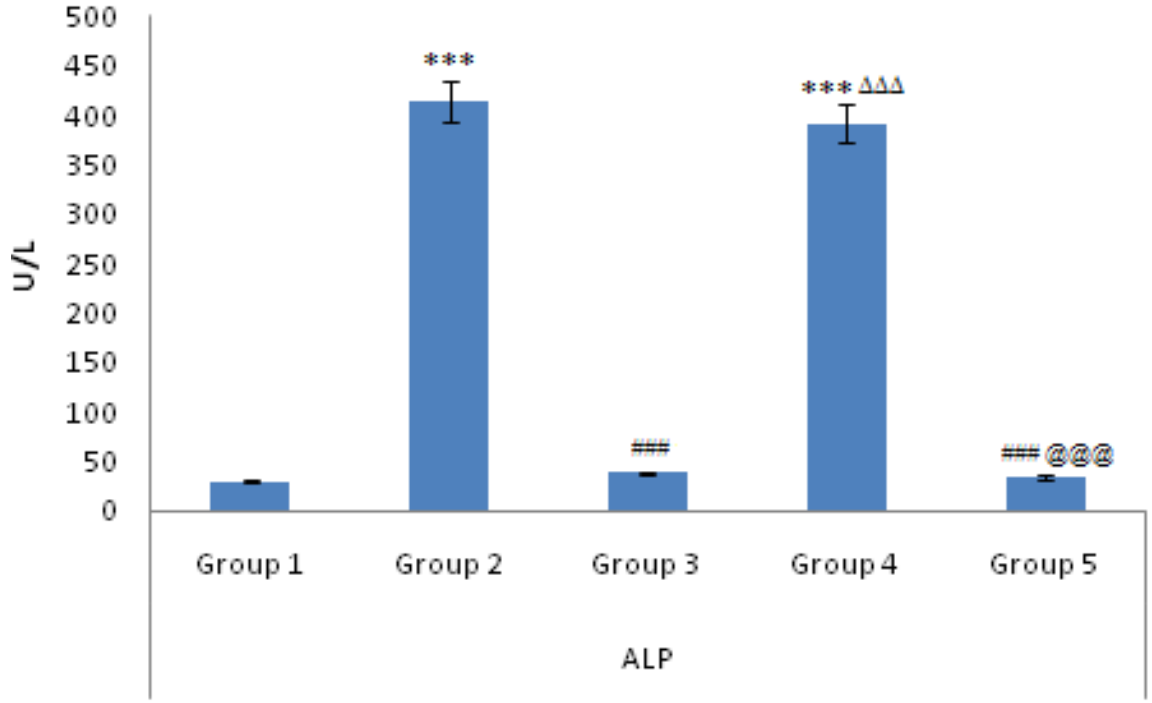

Figure 4: Serum ALP levels of the blood samples taken from Balb/c mice.

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($_p$*<0.05*). Means with the same letter within the same parameter are not significantly different. ****p<0.001* compared to control,### *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

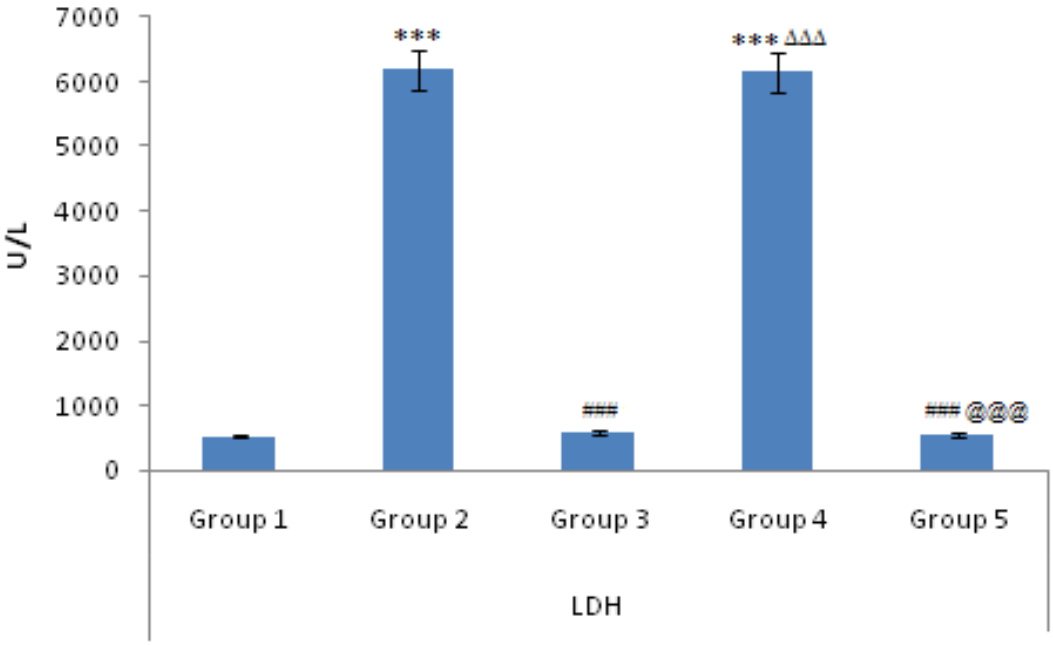

Figure 5: Serum LDH levels of the blood samples taken from Balb/c mice

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($_p$*<0.05*). Means with the same letter within the same parameter are not significantly different. ****p<0.001* compared to control,### *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

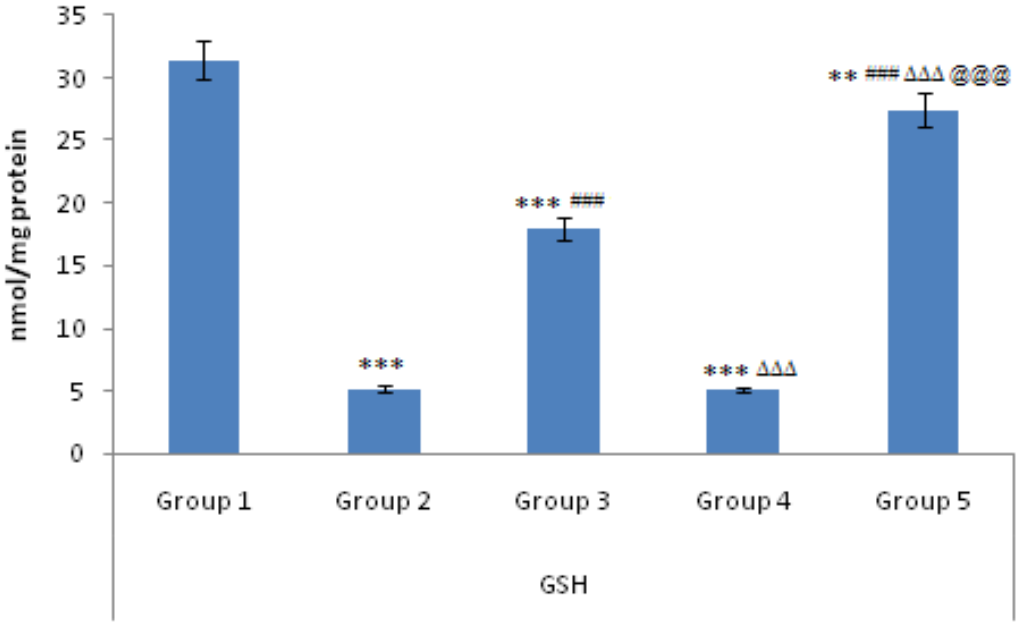

Figure 6: Serum GSH levels of the blood samples taken from Balb/c mice

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($_p$*<0.05*). Means with the same letter within the same parameter are not significantly different. ***p<0.05* compared to control, ****p<0.001* compared to control,$^{###}$ *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

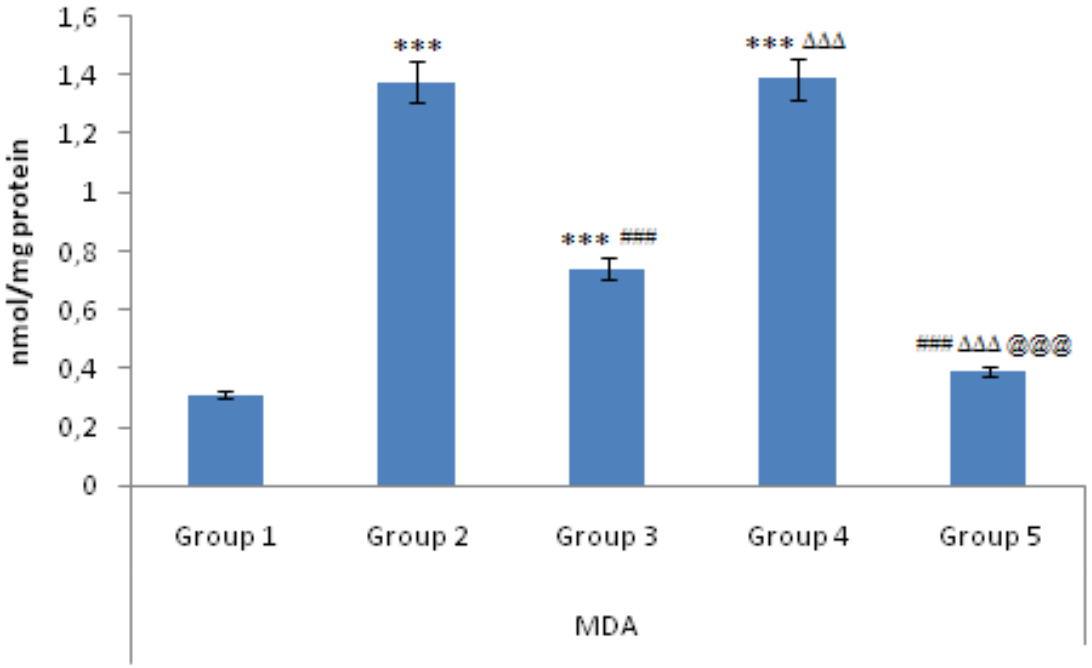

Figure 7: Plasma MDA levels of the blood samples taken from Balb/c mice

All values are the mean ± SD (n =7). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($_p$*<0.05*). Means with the same letter within the same parameter are not significantly different. ****p<0.001* compared to control,$^{###}$ *p<0.001* compared to Group 2, ΔΔΔ *p<0.001* compared to Group 3, @@@ *p<0.001* compared to group 4

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