Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 2
Open Access

Comparative Study of Nanosilymarin Loaded on Chitosan versus Free Silymarin in CCl4-Induced Hepatotoxicity in Female Rats

Nisreen Yasir Jasim* and Rashad Fadhil Ghadhban

Department of Physiology, Pharmacology and Biochemistry, College of Veterinary Medicine, University of Basrah, Iraq

*Email: pgs.nisreen.yasir@uobasrah.edu.iq

Article Info

Article type:

Research

Article History:

Received: 2024-03-16

Revised: 2024-05-22

Accepted: 2024-06-28

Keywords:

Silymarin, Nanosilymarin, Nanoparticles, FTIR, XRD, S-CsNPs, Oxidative stress, CCl4 and hepatotoxicity

A B S T R A C T

The current study is aimed to determine the therapeutic efficacy of lower concentration of nanosilymarin(S-CsNPs) loaded on Chitosan compared to higher concentration of free silymarin in ameliorating CCl4-induced liver damage in female rats. 30 female albino rats were divided into groups to evaluate the hepatoprotective properties of silymarin and nanosilymarin (S-CsNPs). The study employed various biochemical assays to measure liver and kidney function markers, oxidative stress markers, and inflammation indicators. The results demonstrated significant improvements in liver enzyme levels, kidney function, and oxidative stress markers in groups treated with both free silymarin and (S-CsNPs), with S-CsNPs showing enhanced efficacy despite being administered in lower concentrations. The findings suggest that S-CsNPs could be a more effective therapeutic agent in mitigating liver damage caused by CCl4. Our study suggest that the oral administration of the (S-CsNPs) enhances the hepatoprotective effects of nanosilymarin against CCl4-induced liver damage in female rats. The S-CsNPs treated group showed significant improvements in liver enzymes (ALT, AST, ALP and GGT), kidney function (serum creatinine and urea levels) and reductions in oxidative stress markers (MDA) and inflammation(TNF-α) and improvement in (SOD and GSH levels) compared to free silymarin. These findings suggest that S-CsNPs could be a more effective treatment for protecting the liver from toxic substances such CCl4.

INTRODUCTION

Liver diseases pose a significant global health challenge, with exposure to toxic substances such as carbon tetrachloride (CCl4) causing substantial liver damage (Hamdy and El-Demerdash, 2012). Silymarin, an extract from the milk thistle plant, is well-known for its hepatoprotective properties, attributed to its antioxidant and anti-inflammatory effects (Macit *et al.,*2023). However, free silymarin faces challenges related to poor bioavailability and distribution within the body (Kesharwani *et al.,*2020). In recent years, nanotechnology has emerged as a promising tool to enhance the efficacy of drugs by improving their physical and chemical properties (Kesharwani *et al.,*2020). In this study the preparation of silymarin in the form of nanosilymarin (S-CSNPs) using chitosan as a carrier, aiming to improve its bioavailability and therapeutic effects.This study aims to compare the effects of free silymarin and nanosilymarin against CCl4-induced hepatotoxicity in female rats. The rats were divided into multiple groups to evaluate the effects of both free silymarin and nanosilymarin on liver and kidney functions, as well as on markers of oxidative stress and inflammation.

MATERIALS AND METHODS

Experimental animal's management

The present study was conducted in the college of Veterinary medicine –at University of Basrah, in the animal house of the department of physiology. A total number of 30 female adult albino rats (RattusRattus), with an average weight between (200±20g) and the ages of animals were ranged (from 8-to 10) weeks, were used in the current study. They were housed for two weeks for an adaptation before the experiment. Every six animals were housed in an individual plastic cage measured as 15x35x50cm. They were fed ad libitum with the meal of standard pellet of diet supplied from IPA (Institute for Public Accuracy), counter for agriculture research. They had free access to water to drink, and they were kept under the exact condition of temperature (22-25) C° and light, the regime of 12hours of light, and 12 hours, of darkness.

DRUGS AND CHEMICALS

The Silymarin (Milk Thistle P.E.) used in this study was purchased from Xi'an Natural Field Bio-Technique Co., Ltd, China. Chitosan was obtained from Sigma-Aldrich, Germany. Sodium Carboxy methylcellulose powder (CMC-Na) from (BDH Chemical Limited Poole, England. Carbon Tetrachloride (CCl4) was manufactured by Shanghai Macklin Biochemical Co., Ltd, China. All the reagents and chemicals used in the study were of analytical grade. Diagnostic kits for the estimation of serum levels of various parameters were sourced from FUJIFILM, Japan. MDA, SOD, GSH, and TNF-α ELISA kits were from Wuhan Fine Biotech Co., Ltd, China.

SILYMARIN SUSPENSION PREPARATION

Since Silymarin is insoluble in water, it is dissolved in a 0.5% CMC-Na solution to facilitate distribution throughout the bloodstream and subsequently into the portal circulation, as described by (Wang *et al.,* 2018) This formulation was prepared to administer it to animal's groups that receive free silymarin.

BIOSYNTHESIS AND CHARACTERIZATION OF NANOSILYMARIN

Chitosan - Silymarin Nanoparticles (S-CSNPs), Cs-Silymarin nanoparticles (S-CsNPs) were prepared using the ionic-gelation method. A solution of 5 mg/ml CS-Silymarin in 1% acetic acid was mixed until clear. Tripolyphosphate (TPP) was then added to the CS-Silymarin solution at a 1:2.5 (w/w) ratio and stirred continuously at room temperature for 6 hours. The nanoparticles were formed, separated, washed, resuspended in water, and dried (Nasti *et al.,* 2009). After preparing the nano-silymarin, several characterization tests were conducted such as UV spectroscopy, FTIR (Fourier-transform infrared spectroscopy), SEM (Scanning Electron Microscopy), AFM (Atomic Force Microscopy), and XRD (X-ray Diffraction).

INDUCTION OF HEPATOTOXICITY BY CCL4 IN FEMALE RATS

Carbon Tetrachloride (CCl4) manufactured by (Shanghai Macklin Biochemical Co., Ltd. China) MW 153.82, HPLC ≥ 99.0%. used in hepatic damage induction. Intraperitoneal injections were given twice a week for four weeks at premeditated doses of 3ml/kg b.w. of a 30% solution of CCl4 in olive oil as vehicle to cause hepatic injury in rats according to (Khan *et al*.,2012). Animals with hepatic toxicity were post treated with Silymarin and Nanosilymarin (S-CsNPs).

EXPERIMENTAL DESIGN

After completing the hepatotoxicity induction period with CCl4 administered intraperitoneally (IP) twice weekly for 4 weeks, 6 rats were euthanized, and full examinations were conducted. The remaining rats were divided into the following groups (6 rats per group): A group treated with silymarin at a dose of 100 mg/kg/day orally via gavage for 3 weeks after the CCl4 intoxication period. A group treated with nanosilymarin at a dose of 0.4 mg/kg/day orally via gavage for the same duration after the CCl4 intoxication period. A group left untreated after

the CCl4 intoxication period. A negative control group was not intoxicated left untreated and did not receive any treatment throughout the study.

COLLECTION OF BLOOD SAMPLE

At the end of the experiments, 24 hours after administrated of the last dose, the rats are anesthetized with Chloroform, then the abdominal cavity and the thoracic cavity are opened and blood was collected by cardiac puncture using 5ml disposable syringe based of the (Hoff and Ralatg, 2000) method of cardiac puncture and then placed in Gel tube. The Blood in Gel tube centrifuged for 20 minutes at (1000 rpm) to extract the serum, which was then transferred to Eppendorf tubes and kept at (-20C) to determine various biochemical parameters

BIOCHEMICAL PARAMETERS

Determination of Liver function markers

Liver function markers was performed by measuring the liver enzymes (Alkaline phosphatase (Alp), Alanine amino-transferase (ALT.), Aspartate aminotransferase (AST.), Gamma-glutamyl Transpeptidase (GGT) by using DRI-CHEM from FUJIFILM based on the manufactured company's guidelines. All determinations were done using the diagnostic test kits prepared by FUJIFILM, Japan.

DETERMINATION OF KIDNEY FUNCTION MARKERS

Which included measurement of Serum urea and Serum Creatinine was performed by using DRI-CHEM from FUJIFILM based on the manufactured company's guidelines. All determinations (urea and Creatinine) were done using the diagnostic test kits prepared by FUJIFILM, Japan.

DETERMINATION OF OXIDATIVE MARKERS

Quantitative detection of Glutathione (GSH) in blood serum was performed using an ELISA kit based on the Competitive-ELISA detection method. Superoxide Dismutase (SOD) was determined using an ELISA kit based on sandwich enzyme-linked immunosorbent assay technology. Lipid peroxidation was determined by measuring Serum Malondialdehyde (MDA) levels using an ELISA kit based on the Competitive-ELISA detection method. Inflammation was estimated by measuring TNF-α levels using an ELISA kit based on sandwich enzyme-linked immunosorbent assay technology. The kits were obtained from Wuhan Fine Biotech Co., Ltd, China.

STATISTICAL ANALYSIS

The data was analyzed using the Statistical Package for Social Scientists (SPSS version 11.0). One-way ANOVA with LSD post hoc analysis was employed to determine significant differences in mean values between groups. dditionally, paired t-test was utilized for mean comparisons when applicable. A significance level of $p<0.05$ was considered statistically significant

RESULTS

Synthesis and Characterization of S-CsNPs:

Nanoparticles were synthesized using the ionic gelation technique, which involves the interaction between positively charged chitosan and negatively charged phosphate groups of TPP. The amine groups of chitosan cross-link with the phosphine groups of TPP, forming nanoparticles. This reaction alters chitosan's molecular structure, affecting its solubility in acidic solutions and resulting in a gel-like or liquid form (Kahdestani *et al.,* 2021). The formation of nanoparticles loaded with standard plant extract (standard silymarin) is indicated by a clear color, as shown in Fig (4-1).

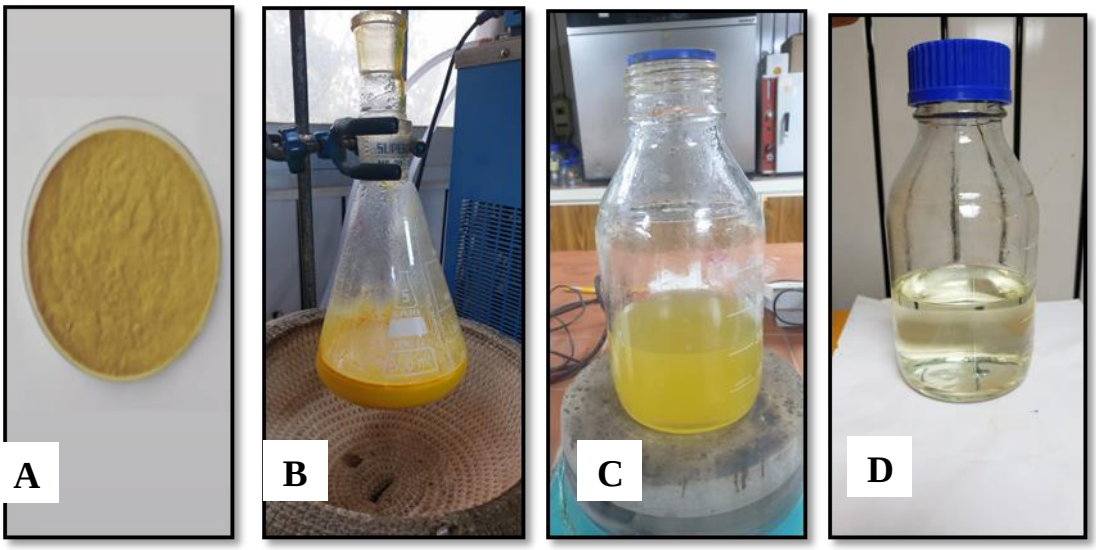

Figure (4-1): Steps of S-CSNPs preparation A: Dried standard, B: Reflux stage of silymarin with chitosan and formation of precipitate on the wall, C: Silymarin /CS adduct, D: S-CSNPs

UV-visible spectroscopy was used to confirm the synthesis of S-CSNPs. Absorbance measurements for both standard Silymarin extract and nanosilymarin (S-CSNPs) were taken by scanning solutions with UV-visible spectrophotometer wavelengths (200-800 nm) at room temperature. For standard Silymarin extract, the highest absorbance was 0.477 at 299 nm, and the lowest was 0.175 at 263 nm while for S-CSNPs, the absorbance was 0.428 at 292 nm the results are displayed in Figures (4-2) and (4-3) respectively.

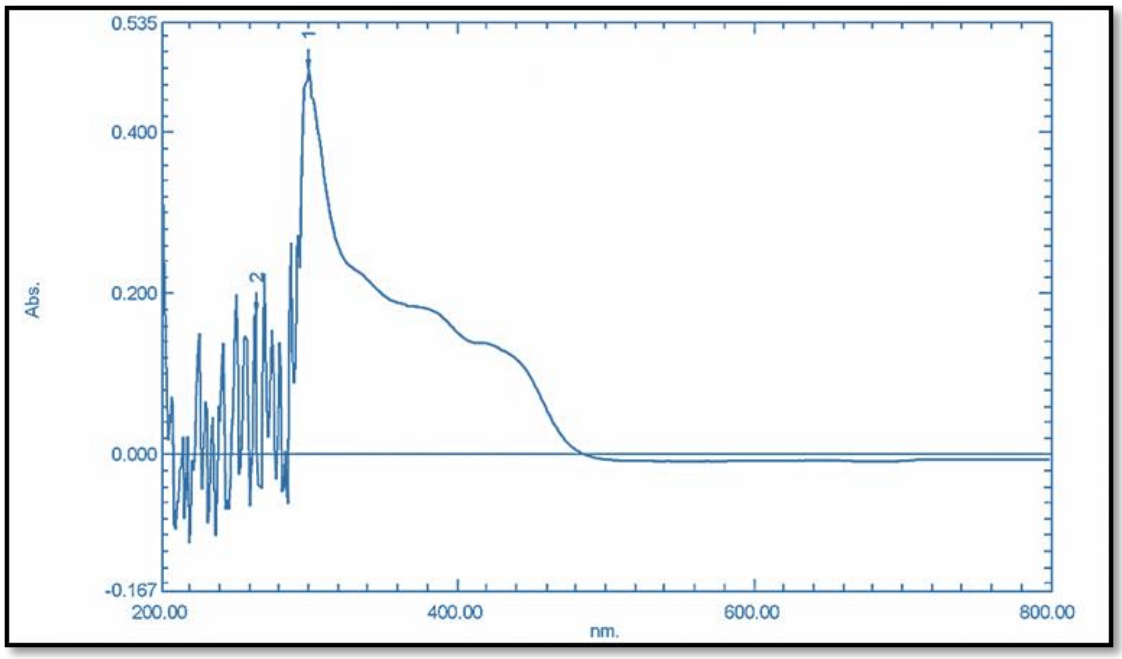

Figure2: UV-Visible spectral analysis of standard Silymarin

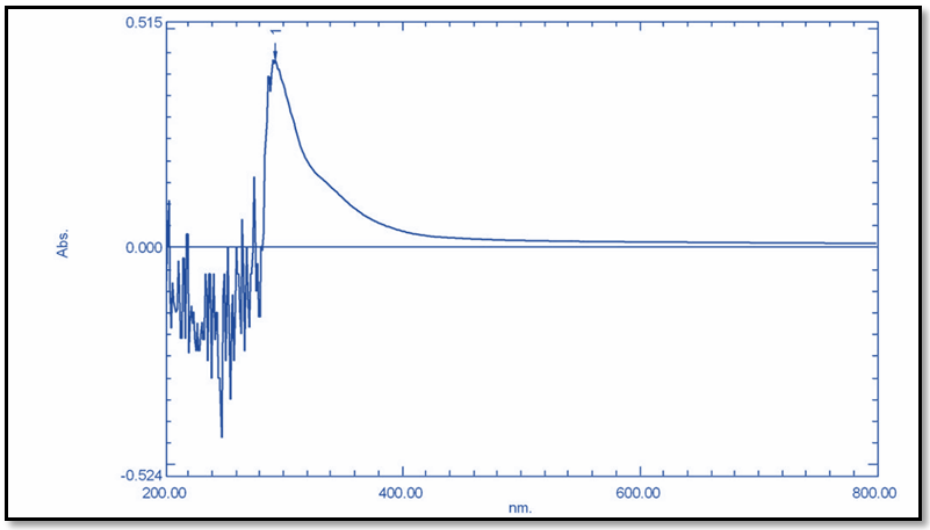

Figure 3: UV-Visible spectral analysis of S-CSNPs

Fourier-transform infrared spectroscopy (FTIR) was used to analyze the chemical composition of S-CSNPs by loading silymarin onto chitosan. The FTIR spectra revealed distinct peaks that indicate the presence of various functional groups and chemical interactions between chitosan and silymarin. FTIR spectra were recorded in the range of 400–4000 cm-1. For chitosan (Figure 4-4): Peak at 3361.93 cm-1: Hydroxyl (-OH) and amino (-NH) groups. Peak at 2875.86 cm-1: Carbon-hydrogen bonds (stretching-CH alkane group). Peak at 1651.07 cm-1: Amide group (stretching C=O carbonyl bond). Peak at 1570.06 cm-1: Secondary vibrations of amides and C=N stretching. For S-CSNPs (Figure 4-5): Peaks at 3757.33 cm-1 and 3433.29 cm-1: Hydroxyl groups (O–H stretching). Peaks at 2924.09 cm-1 and 2856.58 cm-1: Carbon-hydrogen bonds (C–H stretching). Peak at 1730.15 cm-1: Carbonyl groups (C=O stretching, esters). Peak at 1637.56 cm-1: Amide groups (C=O stretching). Peaks at 1454.33 cm-1 and 1390.68 cm-1: C-H bending (alkanes group). Peak at 1136.07 cm-1: Carbon-oxygen bonds (C-O alcohol). Peak at 623.01 cm-1: C-H aromatics group. These results demonstrate the functional groups and chemical interactions in S-CSNPs, confirming the successful loading and stabilization of silymarin on chitosan. The morphology and size of S-CSNPs were investigated using a scanning electron microscope (SEM), the result was presented in Figure (4-6), S-CSNPs have a spherical appearance with a diameter range (25.81-43.03 nm).

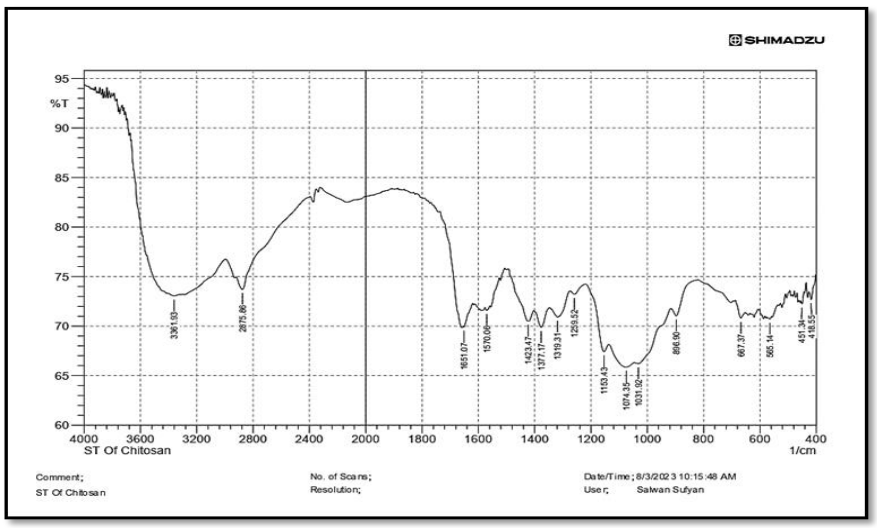

Figure 4: FTIR Spectra Pattern of CS

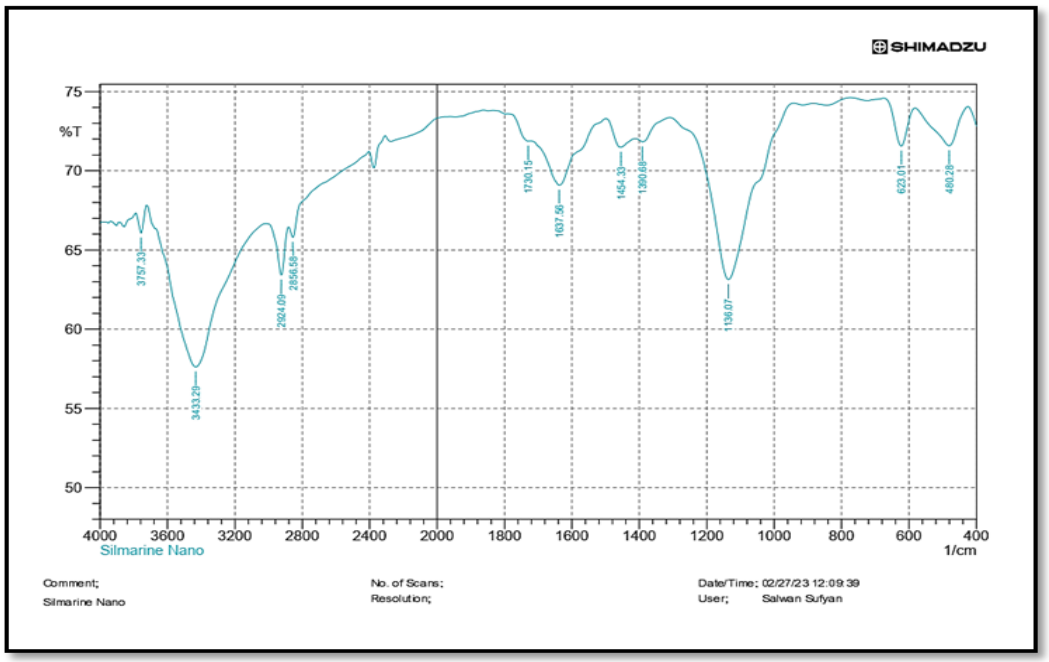

Figure 5: FTIR Spectra Pattern of S-CSNPs

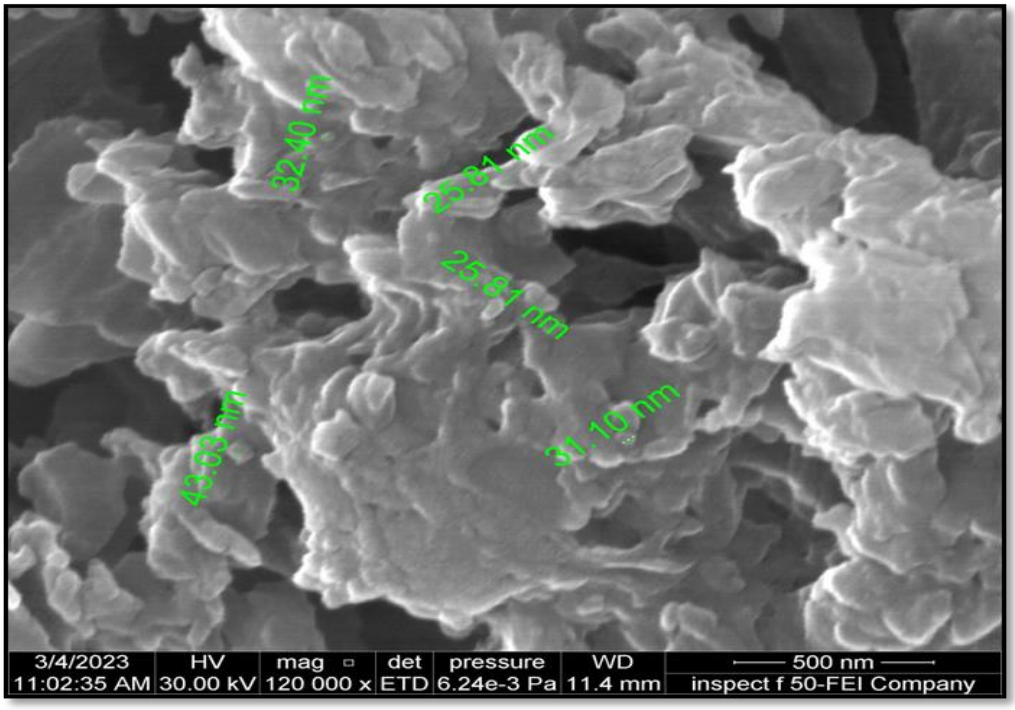

Figure 6: Scanning Electron Microscopy(SEM) image of S-CSNPs

Atomic Force Microscopy (AFM) were used to measure the topography of the surface and particle size of S-CSNPs, the highest size has high frequencies ranging from (40 to 50) nm, extending from 0 to 80 and the mean height (11.40) nm as shown in Figure (4-7) and (4-

A B

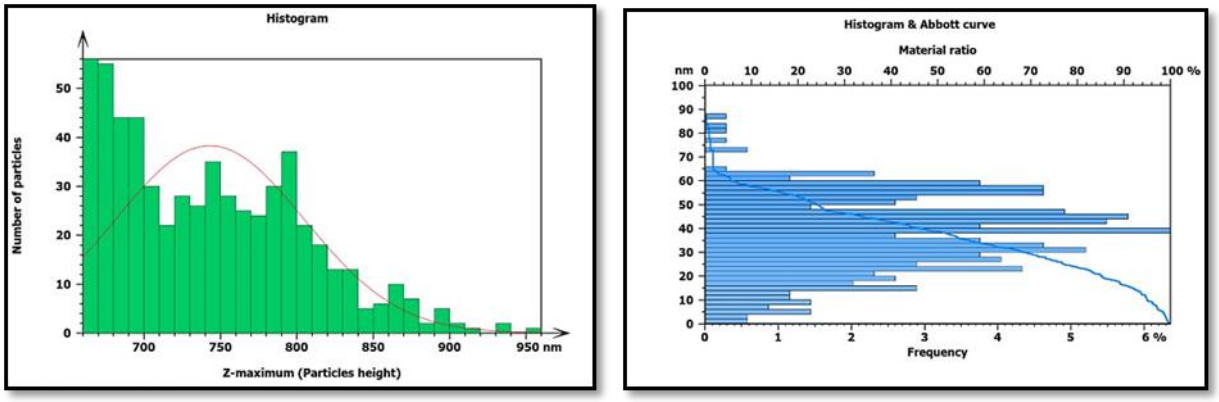

Figure 7: Distribution of S-CSNPs according to particles size (A) Histogram Z -maximum (B) Abbott curve

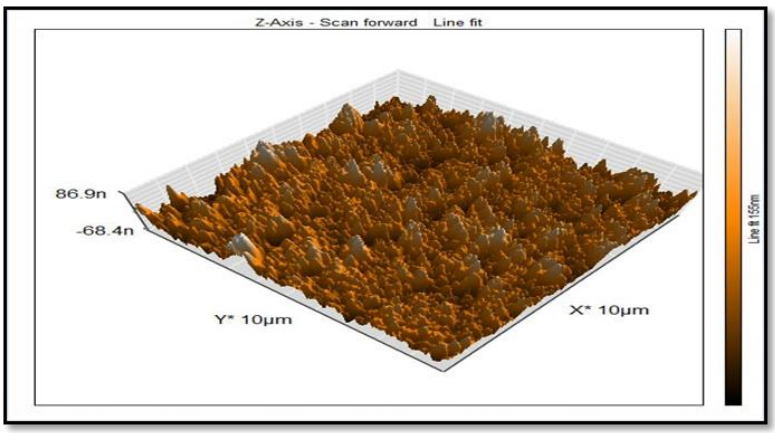

Figure 8: AFM Showed Three-dimensional image of S-CSNPs revealed a population of homogeneous particles with a regular surface shape

The XRD results have been matched with similar & previous studies. The XRD pattern of chitosan exhibited two characteristic broad diffraction peaks at 2θ around 9.63 and 20.53 that are typical fingerprints of semi-crystalline chitosan as indicated in Figure (4-9). On the other hand, Figure (4-10) shows the distinct S-CSNPs peaks, which shows its main peaks of 2**θ** value at (18.71 ◦ , 22.68 ◦ , 23.61 ◦ ,25.62 ◦ ,31.92 ◦ ,33.98 ◦ , 40.19 ◦ , 42.30 ◦ , 45.49 ◦ , 46.24 ◦ , 48.22 ◦ , 52.51 ◦ , 56.72 ◦ , 58.14 ◦ , 64.08 ◦ and 66.74 ◦)

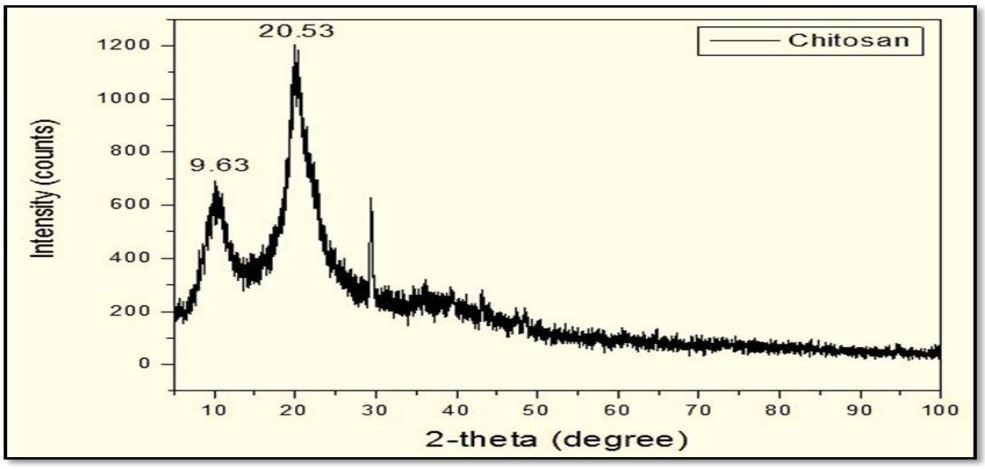

Figure (4-9): Diffractogram of CS (Chandra Dey et al.,2016).

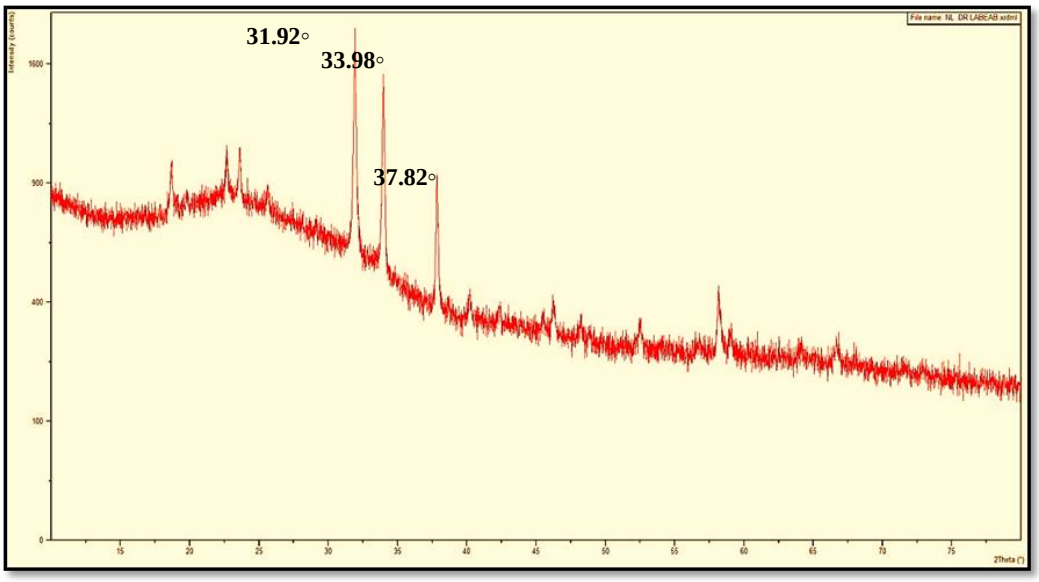

Figure 10: Diffractogram of S-CSNPs

4.2. Effects of S-CsNPs and free silymarin on Liver functions of hepatotoxic rats.

Significant increases (P<0.05) in serum levels of AST, ALT, ALP, and GGT were observed in the groups that received CCl4 and the untreated group compared to the negative control group (Table 1). Both the CCl4 and untreated groups demonstrated significant increases in all liver enzymes, indicating liver toxicity in the CCl4 group and severe liver damage in the untreated group. Meanwhile, the group administered oral silymarin (100 mg) exhibited lower levels of liver enzymes to word control values, suggesting improved liver function. Similarly, the S-CsNPs (0.4 mg) group showed comparable improvements in liver enzymes. Overall, this results indicate that both S-CsNPs and free silymarin effectively reduce elevated liver enzymes associated with liver toxicity, highlighting the therapeutic efficacy of S-CsNPs in improving liver function, despite its lower concentration.

Table 1: Effects of S-CsNPs and free silymarin on Liver functions of hepatotoxic rats

Groups	parameters (Mean ± SD) No=6			
	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)
Negative control	46.50±12.30C	116.16±2.71D	34.16±10.94C	3.75±0.30C
CCl4	88.66±5.39B	181.16±5.77B	71.16±7.13B	14.63±0.42B
Untreated	112.00±5.40A	211.33±3.32A	100.66±4.13A	17.15±0.89A
Silymarin(100mg)	47.33±2.80C	118.66±3.26D	36.50±1.87C	4.40±0.20C
S-CsNPs 0.4 mg	43.00±3.46C	116.54±0.75D	33.33±0.81 C	4.10±0.14C
LSD	7.33	11.66	10.45	2.4

Effects of S-CsNPs and free silymarin on kidney functions

The effect of oral administration of S-CsNPs on renal function (serum creatinine and urea levels) is shown in Table **2** indicate that the exposure to CCl4 induce notable alteration in kidney function indices, noticed there is significant increase (P<0.05) in creatinine and urea levels as compared to the negative control group Elevated

levels of urea and creatinine indicating liver damage. Highest levels of urea and creatinine revealed in untreated group indicating severe liver function deterioration. The oral administration of Silymarin (100 mg) reduced levels of urea and creatinine significantly as compared to CCl4 and untreated groups. While the administration of S-CsNPs (0.4 mg) Similar improvement with urea and creatinine. Both S-CsNPs and free silymarin demonstrate significant improvements in liver function in hepatotoxic rats, with S-CsNPs showing comparable efficacy despite its much lower concentration compared to free silymarin.

Table 2: Effects of S-CsNPs and free silymarin on kidney functions

Groups	parameters (Mean ± SD) No=6	
	Serum Urea (mg/dl)	Creatinine (mg/dl)
Negative control	20.43±4.0C	0.45±0.10C
CCl4	41.75±2.68B	1.30±0.46B
Untreated	72.16±2.78A	2.56±0.16A
Silymarin(100mg)	21.33±1.21C	0.29±0.08D
S-CsNPs 0.4 mg	20.16±1.16C	0.25±0.05D
LSD	3.00	0.4

4.4. Effects of S-CsNPs and free silymarin on Oxidative stress markers

CCl4 group demonstrated significant decreases in SOD and GSH and significant increases ($P<0.05$) in TNF-α (and MDA indicating oxidative stress and liver damage as compared to negative control group. Untreated group similar to the CCl4 group with low SOD and GSH and high TNF-α and MDA indicating severe oxidative stress and liver damage. Silymarin (100 mg) group showed moderate improvement with higher SOD and GSH and lower TNF-α and MDA indicating improved liver function as compared to CCl4 group. S-CsNPs (0.4 mg) group exhibited significant improvement with SOD and GSH levels close to the negative control, and lower TNF-α and MDA indicating strong antioxidant and liver-protective effects despite its lower concentration as shown in Table 3. Overall, this results indicate that both S-CsNPs and free silymarin significantly improve oxidative stress markers and reduce liver damage, with S-CsNPs showing more pronounced effects despite its lower concentration.

Table 3: Effects of S-CsNPs and free silymarin on Oxidative markers

Groups	parameters (Mean ± SD) No=6			
	SOD(ng/ml)	GSH (ug/ml)	TNF-α (pg/ml)	MDA (ng/ml)
Negative control	6.73±0.50A	63.53±3.60A	13.12±1.91C	63.93±11.25E
CCl4	2.29±0.34D	9.98±1.40E	50.41±5.26A	402.26±38.92B
Untreated	2.28±0.69D	9.18±1.39E	53.37±5.10A	471.72±82.98A
Silymarin(100mg)	3.80±0.31C	56.26±1.75B	10.35±1.59C	59.40±11.58E
S-CsNPs 0.4 mg	4.99±0.33B	61.27±2.04A	9.37±1.34C	43.76±12.11E
LSD	0.7	5.00	5.95	37.28

DISCUSSION

The results of this study provide compelling evidence that nanosilymarin (S-CsNPs) exhibits enhanced hepatoprotective effects compared to free silymarin in CCl4-induced hepatotoxicity in female rats. The study

demonstrated significant increases in liver enzymes (ALT, AST, ALP, and GGT) in rats subjected to CCl4-induced hepatotoxicity, indicating liver damage this agreed with (Almatroodi *et al.,*2020).

The administration of CCL4 elevated levels of serum hepatic enzymes indicating liver injury and disturbance of cellular wall integrity (El-Haskoury *et al*.,2018). However, treatment with both free silymarin and S-CsNPs significantly reduced these enzyme levels. Notably, S-CsNPs, even at a lower concentration, showed comparable improvements to free silymarin this indicates the effectiveness of the treatment, as it effectively treated the damage induced by CCl4 and this is consistent with what the (Saller *etal.,* 2001) presents in the review section of their study on the effect of silymarin as a treatment for liver diseases who noticed AST, ALT and GGT levels all fell significantly with silymarin therapy. This suggests that S-CsNPs might be more effective in enhancing liver function recovery due to better bioavailability and sustained release properties of nanoparticles this also aligns with the discoveries made by (Sherikar *et al.,* 2021) showcasing that increased bioavailability of silymarin enhances its anti-inflammatory attributes and liver protection, thereby augmenting its effectiveness. In our study, we utilized nanosilymarin to enhance its solubility and bioavailability, further boosting its efficacy.

Also the kidney function markers (serum urea and creatinine) were also significantly elevated in the CCl4 and untreated groups, reflecting renal stress due to liver injury. The administration of free silymarin and S-CsNPs resulted in substantial reductions in these markers, with S-CsNPs showing slightly better efficacy. This indicates that nanosilymarin not only protects the liver but also ameliorates secondary kidney dysfunction associated with liver damage. Urea and creatinine levels in the blood are used as markers of renal function (Renugadevi and Prabu, 2010). (Olagunju *et al.,*2009 and Al-Yahya *et al.,*2013) Found that, CCl4 significantly increases blood levels of creatinine and urea. while (El-Haskoury *et al*.,2018) noticed that CCl4 shows a high affinity for the kidney and the mechanism of CCl4-induced kidney damage is similar to that in the liver**.** kidney injury is associated with oxidative stress and free radicals. The overproduction of free radicals resulting from oxidative stress can directly damage nephrocellular membrane through lipid peroxidation or other mechanisms. This is followed by a series of cellular events, including the massive release of inflammatory mediators or cytokines, which ultimately leads to liver and kidney damage (Bektur *et al*.,2016).

On the other hand, Oxidative stress markers (MDA, SOD, and GSH) and the inflammatory marker TNF-α were significantly altered in the CCl4 group. The treatment with S-CsNPs led to a marked reduction in MDA levels and TNF-α, and an increase in SOD and GSH levels, indicating a reduction in oxidative stress and inflammation. The superior performance of S-CsNPs can be attributed to its nanoformulation, which enhances antioxidant delivery and activity at the cellular level. Antioxidant enzymes are highly susceptible to cell damage, and a decrease in SOD levels can signify severe liver damage caused by CCl4. The liver damage induced by CCl4 injection results from lipid peroxidation triggered by free radicals derived from CCl4. Consequently, antioxidant activity and the suppression of free radical production play crucial roles in preventing CCl4-induced hepatopathies (Mahmoodzadeh *et al.,* 2017). According to oxidative stress parameters, there was a significant drop in GSH, SOD, and glutathione reductase in the liver of CCl4-injected animals compared to the control group this effect agrees with **(**Abdel-Moneim *et al.,*2015) study, when they investigated CCl4 efficacy on rat models too. The MDA level in liver tissue was assessed as an indicator of lipid peroxidation in oxidative liver damage and is one of lipid peroxidative product and for several decades it has been used as a biomarker of lipid peroxidation. In addition, the increase of MDA has been considered a key feature in liver injury (Mateos *et al.,*2005 and Lien *et al.,*2016). Products of lipid peroxidation can lead to changes in biological membranes, resulting in serious cellular injury. An increase in the formation of MDA was observed in liver cells of rats exposed to CCl4, indicating detrimental effects on the cells (Balahoroğlu *et al.,*2008).

Dong *et al.,* (2016) The increase in TNF-α levels in response to acute liver injury is attributed to its inflammatory effects, as it is regarded as one of the potent inflammatory cytokines crucial for the body's defense mechanism**.**

ACKNOWLEDEGMENTS

The authors would like to express their sincere gratitude to the staff of the Ministry of Science and Technology, Directorate of Environment and Water, especially Dr. Labeeb Ahmed, for his invaluable assistance in the preparation and characterization of nanoparticles.

REFERENCES

1. Abdel-Moneim, A. M., Al-Kahtani, M. A., El-Kersh, M. A., & Al-Omair, M. A. (2015). Free radical-scavenging, anti-inflammatory/anti-fibrotic and hepatoprotective actions of taurine and silymarin against CCl4 induced rat liver damage. *PloS one*, *10*(12), e0144509.
2. Almatroodi, S. A., Anwar, S., Almatroudi, A., Khan, A. A., Alrumaihi, F., Alsahli, M. A., & Rahmani, A. H. (2020). Hepatoprotective effects of garlic extract against carbon tetrachloride (CCl4)-induced liver injury via modulation of antioxidant, anti-inflammatory activities and hepatocyte architecture. *Applied Sciences*, *10*(18), 6200.
3. Al-Yahya, M., Mothana, R., Al-Said, M., Al-Dosari, M., Al-Musayeib, N., Al-Sohaibani, M., ... & Rafatullah, S. (2013). Attenuation of CCl 4-induced oxidative stress and hepatonephrotoxicity by Saudi Sidr honey in rats. *Evidence-Based Complementary and Alternative Medicine*, *2013*.
4. Balahoroğlu, R., Dülger, H., Özbek, H., Bayram, İ., & Şekeroğlu, M. R. (2008). Protective effects of antioxidants on the experimental liver and kidney toxicity in mice. *European Journal of General Medicine*, *5*(3), 157-164.
5. Bektur, N. E., Sahin, E., Baycu, C., & Unver, G. (2016). Protective effects of silymarin against acetaminophen-induced hepatotoxicity and nephrotoxicity in mice. Toxicology and Industrial Health, 32(4), 589-600.
6. Chandra Dey, S; Al - Amin, M; Ur Rashid, T; Zakir Sultan, M;Ashaduzzaman, M; Sarker, M; and Md Shamsuddin, S. (2016).Preparation , Characterization and Performance Evaluation of Chitosan As an Adsorbent for Remazol Red. International Journal of Latest Research in Engineering and Technology, March, 52–62.
7. Dong, Y., Liu, Y., Kou, X., Jing, Y., Sun, K., Sheng, D., ... & Wei, L. (2016). The protective or damaging effect of Tumor necrosis factor-α in acute liver injury is concentration-dependent. *Cell & bioscience*, *6*, 1-10.
8. El-Haskoury, R., Al-Waili, N., Kamoun, Z., Makni, M., Al-Waili, H., & Lyoussi, B. (2018). Antioxidant activity and protective effect of carob honey in CCl4-induced kidney and liver injury. Archives of medical research, 49(5), 306-313.
9. Hamdy, N. M., & El-Demerdash, E. (2012). New therapeutic aspect for carvedilol: antifibrotic effects of carvedilol in chronic carbon tetrachloride-induced liver damage. *Toxicology and Applied Pharmacology*, *261*(3), 292-299.
10. Kahdestani, S. A; Shahriari, M. H; and Abdouss, M. (2021). Synthesis and characterization of chitosan nanoparticles containing teicoplanin using sol–gel. Polymer Bulletin, 78(2), 1133–1148. https://doi.org/10.1007/s00289-020-03134-2
11. Kesharwani, S. S., Jain, V., Dey, S., Sharma, S., Mallya, P., & Kumar, V. A. (2020). An overview of advanced formulation and nanotechnology-based approaches for solubility and bioavailability enhancement of silymarin. *Journal of Drug Delivery Science and Technology*, *60*, 102021.
12. Lien, D. T. P., Hoang, C. T. K., Hanh, N. T., Chu, D. X., Tram, P. T. B., & Toan, H. T. (2016). Hepatoprotective effect of silymarin on chronic hepatotoxicity in mice induced by carbon tetrachloride. *Journal of Pharmacognosy and Phytochemistry*, *5*(5), 262-266.
13. Macit, M., Duman, G., Cumbul, A., Sumer, E., & Macit, C. (2023). Formulation development of Silybum marianum seed extracts and silymarin nanoparticles, and evaluation of hepatoprotective effect. *Journal of Drug Delivery Science and Technology*, *83*, 104378.
14. Mateos, R., Lecumberri, E., Ramos, S., Goya, L., & Bravo, L. (2005). Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress: Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. *Journal of Chromatography B*, *827*(1), 76-82.
15. Nasti, A; Zaki, N. M; De Leonardis, P; Ungphaiboon, S; Sansongsak, P; Rimoli, M. G; and Tirelli, N. (2009). Chitosan/TPP and chitosan/TPPhyaluronic acid nanoparticles: Systematic optimisation of the preparative

process and preliminary biological evaluation. Pharmaceutical Research, 26(8), 1918–1930.https://doi.org/10.1007/s11095-009-99080

16. Olagunju, J. A., Adeneye, A. A., Fagbohunka, B. S., Bisuga, N. A., Ketiku, A. O., Benebo, A. S., ... & Adeleke, A. G. (2009). Nephroprotective activities of the aqueous seed extract of Carica papaya Linn. in carbon tetrachloride induced renal injured Wistar rats: a dose-and time-dependent study. Biol Med, 1(1), 11-9.
17. Saller, R., Meier, R., & Brignoli, R. (2001). The use of silymarin in the treatment of liver diseases. *Drugs*, *61*, 2035-2063.
18. Sherikar, A., Siddique, M. U. M., More, M., Goyal, S. N., Milivojevic, M., Alkahtani, S., ... & Nayak, A. K. (2021). Preparation and evaluation of silymarin-loaded solid eutectic for enhanced anti-inflammatory, hepatoprotective effect: in vitro–in vivo prospect. *Oxidative Medicine and Cellular Longevity*, *2021*, 1-13.
19. Khan, R. A., Khan, M. R., & Sahreen, S. (2012). Protective effect of Sonchus asper extracts against experimentally induced lung injuries in rats: a novel study. Experimental and Toxicologic Pathology, 64(7-8), 725-731
20. Renugadevi, J., & Prabu, S. M. (2010). Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. Experimental and Toxicologic Pathology, 62(5), 471-481.
21. Mahmoodzadeh, Y., Mazani, M., & Rezagholizadeh, L. (2017). Hepatoprotective effect of methanolic Tanacetum parthenium extract on CCl4-induced liver damage in rats. *Toxicology reports*, *4*, 455-462.
22. Wang, L., Huang, Q. H., Li, Y. X., Huang, Y. F., Xie, J. H., Xu, L. Q., ... & Chen, J. N. (2018). Protective effects of silymarin on triptolide-induced acute hepatotoxicity in rats. Molecular medicine reports, 17(1), 789-800.