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Effect of Silymarin and Quercetin in a Miniaturized Scaffold in Wistar Rats against Non-alcoholic Fatty Liver Disease

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Cite This: *ACS Omega* 2021, 6, 20735−20745

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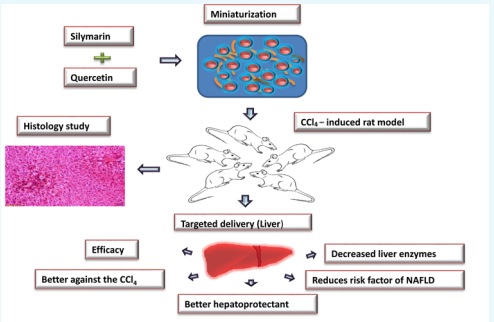
ABSTRACT: Silymarin and quercetin (SQ) are known antioxidants with substantial free radical scavenging activities. The efficacy of SQ activity is restricted due to poor absorption and availability. This study aims to increase the hepatoprotective activity of SQ by a newer delivery technique. We have optimized a technique, miniaturized scaffold (MS), for the delivery of active compounds of SQ. SQ molecules were embedded in MS and characterized by morphology, particle size, miniaturization efficiency, and functional group. Further, the hepatoprotective effects of MSQ were investigated through *in vitro* and *in vivo* methods. Hepatotoxicity was induced in rats by carbon tetrachloride (CCl_4), and subsequently, hepatotoxic rats were treated with the miniaturized scaffold of SQ (MSQ) for 8 weeks. The body weight were significantly high in groups fed with MSQ. A substantial decrease in triglyceride, total cholesterol, low-density lipoprotein, alanine aminotransferase, and aspartate aminotransferase activities were observed in rats treated with MSQ. Similarly, rats treated with MSQ exhibited lower lipid accumulation in the hepatocytes. The experiments clearly demonstrated the efficacy of MSQ as a superior hepatoprotective agent against non-alcoholic fatty liver disease simulated through toxicity induced by CCl_4.

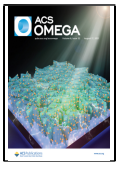


Development of miniaturized scaffold of silymarin and quercetin for targeting NAFLD

1. INTRODUCTION

The liver is a vital organ that facilitates several physiological functions, primarily the metabolism of ingested molecules, especially drugs.[1] Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease having a prevalence rate of 25% worldwide.[2] Many drugs cause damage to hepatocytes and hepatic tissues, leading to NAFLD and has been known to cause prolonged liver disease.[3] NAFLD is characterized by the accumulation of triacylglycerol inside liver cells.[4] Even though it is considered a relatively preliminary form of chronic liver injury, it could lead to liver cirrhosis.[5] Therefore, there is a need to explore ways to reduce the inflammation in the liver caused by NAFLD. Carbon tetrachloride is an effective hepatotoxin that is used to induce liver damage and to involve the increase of inflammatory response.[6] The toxicity of CCl_4 leads to the reactive oxygen species (ROS), and free radicals are produced during the metabolism.[7] Many animal models are used to study liver inflammation to test the potential treatments, including the carbon tetrachloride-induced rat model; Wistar rats are a suitable model for testing the hepatoprotection.[8] Hepatic damage caused by CCl_4 primarily decreases the activities of antioxidant enzymes, which leads to lipid peroxidation and generation of free radicals.[9] Several hepatoprotective agents, including natural substances such as bioactive compounds, have been reported to counter ROS-mediated tissue damage by their antioxidant and free radical scavenging abilities.[10] Hepatic damage can be recovered by bioactive compounds such as silymarin and quercetin (SQ).[11] Silymarin is a mixture of flavonolignans from milk thistle seeds and comprises seven main components: silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin, and taxifolin. Silymarin mainly grows in Africa, South America, Australia, and many parts of Asia.[12] Silymarin has anti-inflammatory activity, antioxidant activity, anti-apoptotic activity, and hepatoprotection activity, and further, it helps in tissue repair and regeneration.[13] A similar compound that benefits hepatoprotection is quercetin (3,3′,4′,5,7-pentahydroxyflavone), which is an essential dietary flavonoid found in red onions, citrus fruits, tea, apples, berries, and grapes. Besides, quercetin exhibits anti-inflammatory properties with the ability of a molecule to scavenge free radicals.[14] Inhibition of lipid peroxidation and chelation are the basic mechanisms behind the antioxidant effects of quercetin, which could prevent mitochondrial oxidative damage of rat hepatocytes.[15] Dietary quercetin can alleviate non-alcoholic steatohepatitis induced by a high-fat diet and has the potential to reduce the inflammatory state that occurs in the body in association with metabolic syndrome.[16] Quercetin protects

Received: January 30, 2021
Accepted: July 26, 2021
Published: August 3, 2021



https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

against NAFLD and shows a positive effect on enzymes such as glutathione reductase (GR), catalase, and superoxide dismutase (SOD) and decreases lipid peroxidation; thus, it acts as a hepatoprotectant and helps in liver regeneration.[17] The therapeutic effect of SQ is restricted due to its low aqueous solubility and poor intestinal absorption.[18] SQ are easily degradable because of their sensitivity to environmental factors such as light, heat, and oxygen.[19] The characteristics of flavonoids may limit the dissolution rate and target delivery, hence resulting in low absorption and availability. Combining two or more bioactive compounds has better efficacy when compared to a single bioactive compound; furthermore, it could slow down the elimination rate and produce a more extended efficiency.[20] Miniaturization technique is a promising and new technique, which can be designed to build diagnostically valuable systems. The study's objective was to improve SQ's efficacy and absorption by a novel technique of miniaturized scaffolding of bioactive molecules and can serve as effective carriers and enhance hepatoprotective activity in CCl_4-induced NAFLD rats. This miniaturized scaffold containing SQ is expected to facilitate better permeability and absorption capacity. Hereby, we report a detailed study of SQ-entrapped miniaturized scaffold for its efficacy toward hepatoprotection against NAFLD.

2. RESULTS AND DISCUSSION

2.1. Morphology and Particle Size of Microspheres. SQs were characterized using light microscopy and a particle size analyzer. A different SQ concentration with various surfactants was optimized to form stable formulations (Figure 1). Among the surfactants, polysorbates 40 and 60 showed aggregation of the microspheres. On the other hand, sorbitan monolaurate and sorbitan monooleate showed coalescence.

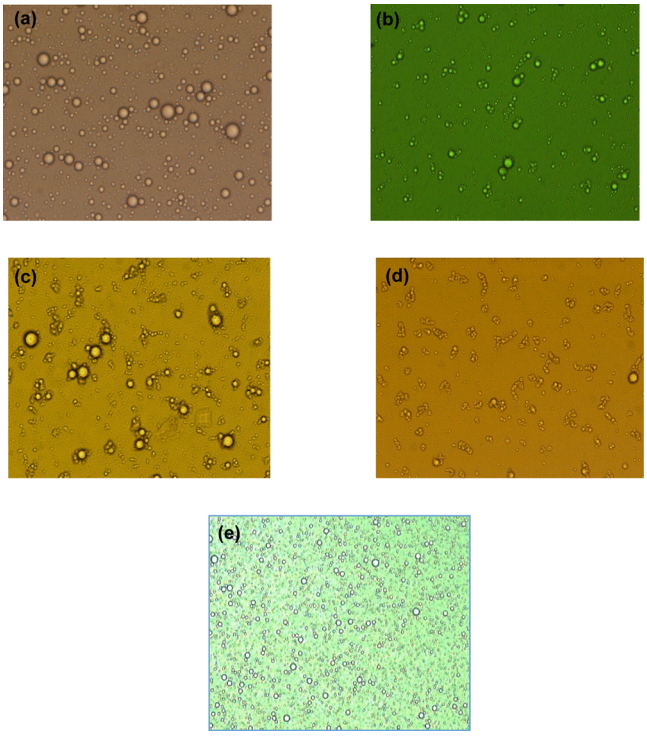


Figure 1. Microscopic images of microspheres with different surfactants. (a) Sorbitan monolaurate, (b) sorbitan monooleate, (c) polysorbate 40, (d) polysorbate 60, and (e) soy lecithin.

The microspheres prepared using soy lecithin exhibited uniform droplet size distribution (Figure 1e). This is due to the surfactant's amphiphilic nature, which offers higher stability to the oil droplets in the microsphere system.

The microspheres of silymarin and quercetin with surfactants were spherical. The mean particle size of soy lecithin was 58.22 ±09.69 μm (Figure 2), which is the lowest among SQ microsphere compared with other surfactants. The small dimensions exhibited by the soy lecithin microsphere could be due to the efficient combination of the bioactive compounds with a surfactant, showing the homogeneous distribution of bioactive compounds in the miniature scaffold. The most stable formulation was seen in soy lecithin, which was stable at room temperature (28 ± 1 °C) for 30 days. The solubility capacity of the surfactant increases in the form of miniature, and the hydrophobic force locates the bioactive compounds in the scaffold.[21]

2.2. Miniaturization Efficiency of SQ. The concentration of quercetin and silymarin after hosting in the miniaturized scaffold are shown in Table S1 was evaluated by a respective standard marker in high-performance liquid chromatography (HPLC). The concentration of quercetin was higher in SQBC (98.84 ± 0.57%). The increase in miniaturization efficiency was found in SQBC in which isosylibin A (9.90 ± 0.53 mg/g) and isosylibin B (4.99 ± 0.23 mg/g) were higher compared with other biopolymers. Similarly, the concentration of silybin B (9.15 ± 0.62 mg/g) is higher in SQBC. The result showed an increase in the efficiency of SQBC biopolymer in retaining compounds in the miniaturized matrix. β-Cyclodextrin encompasses 7-D-glucose units that are connected in conjunction with α-1, 4 linkages. Besides, the structure of β-cyclodextrin seems like a thick-walled bucket with a hydrophobic cavity combined with a hydrophilic exterior. The weak forces such as van der Waals forces, dipole−dipole interaction, and hydrogen bonding have supported them to create an inclusion complex by entrapping the guest molecule within its cavity.[22,23] Therefore, the result confirmed a higher efficiency of SQ in the miniaturized matrix when β-cyclodextrin was used as a biopolymer.

2.3. Fourier Transform Infrared Spectroscopy of MSQ. Fourier transform infrared (FTIR) analysis was carried out to identify the functional group of bioactive compounds in the miniaturized samples. The major characteristic peaks of quercetin at 1100−1600 cm^{-1} and OH phenolic bending at 1200−1400 cm^{-1} are present in the miniaturized scaffold, which is comparable with native quercetin.[24] Figure S1 indicates that there is no chemical interaction occurred in the miniaturized scaffold. A spectrum of silymarin showed bands at 3333 cm^{-1}, (OH), 2926 cm^{-1} (CH), and 1742 cm^{-1} (C═O), which are comparable with native silymarin.[25] Figure 3 illustrates the functional groups of silymarin in SQBC at characteristic peaks of OH stretching at 3293 cm^{-1}, C═O stretching at 1629 cm^{-1}, C═C stretching at 1458 cm^{-1}, and C−Cl stretching at 603 cm^{-1}, respectively. The characteristic bands of free quercetin in all the biopolymer show the aromatic bending and stretching at around 1100 and 1600 cm^{-1} and then −OH phenolic bending at 1200−1400 cm^{-1}.

Spectra of solid dispersions of SQBC, SQC, SQMD, SQP, SQWP, and SQGA have not shown any changes from the standard spectra of SQ (Figure S1). The result suggested that the miniaturization process has not affected the functional groups of the bioactive compounds. Thus, the overall study indicates that there was no chemical interaction between the

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

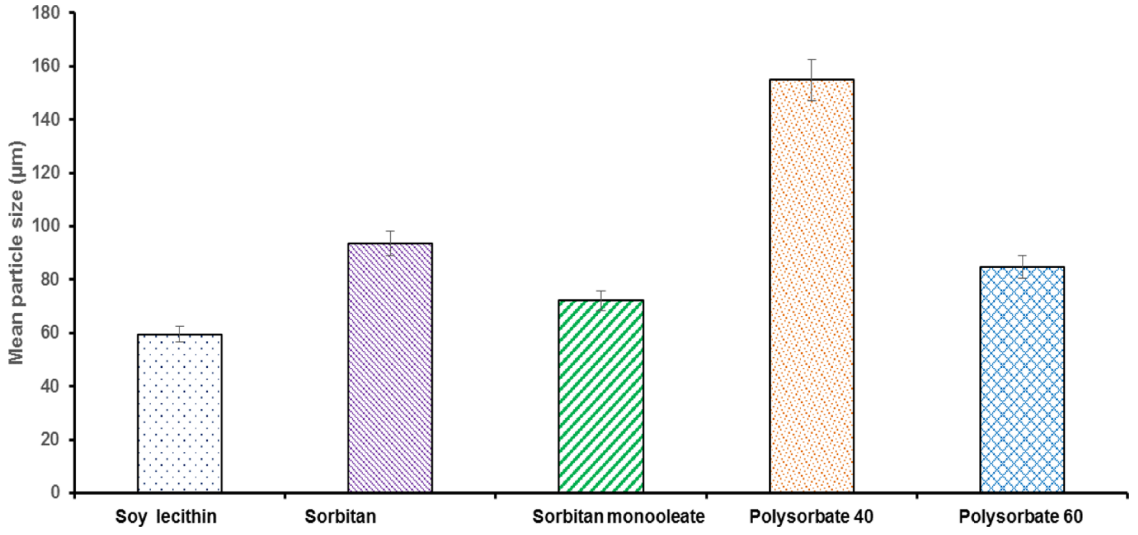


Figure 2. Mean particle size (μm) of microsphere prepared by different surfactants such as soy lecithin, sorbitan monolaurate, sorbitan monooleate, polysorbate 40, and polysorbate 60.

bioactive compound and biopolymer in the miniaturized scaffold form.

2.4. *In Vitro* Evaluation of MSQ by Dissolution and Release Kinetics. *In vitro* dissolution tests, using a pH change method, were carried out to investigate the influence of formulation on flavonoid release from the miniaturized scaffold. The dissolution study of silymarin, quercetin, and miniaturized scaffold of SQ (MSQ) is shown in Figure 4. Pure quercetin solution exhibited a much faster release rate with approximately 75% of quercetin dissolved at the initial 2 h. From the *in vitro* release study, it was found that the miniaturized form had a significant improvement in the release rate when compared with pure silymarin or quercetin. MSQ exhibited slow release of the compounds at a comparable concentration of bioactives within 4.5 h. This may be due to the absorption of the compounds in the miniaturized matrix.[26] The bioactive compound in a miniaturized matrix can be retained and released slowly; the phenomenon has also contributed to increased solubility. The release rate of SQ in the miniaturized scaffold had shown a good resistance at pH 7.4. The combination of SQ in the miniaturized scaffold had shown a slow release in the simulated intestinal conditions than the native form of SQ. The efficiency of miniaturized SQ is very high (97.26%). This is because of the incorporation of bioactive compounds in the miniaturized scaffold, which may have better and sustained release characteristics of bio-actives. The slower and sustained release of SQ may be attributed to the diffusion of SQ entrapped within the miniaturized scaffold. Miniaturization technique has given satisfying efficiency for the target delivery of SQ.

2.5. *In Vivo* Evaluation of a Miniaturized Scaffold of SQs in a CCl_4-Induced Hepatotoxicity Rat Model. Rats were treated with CCl4 to induce hepatotoxicity for evaluating the hepatoprotective effect of MSQ. The average, initial, and final body weights and their relative organ weights of different experimental rat groups, namely, control, CCl_4-treated rat group, and those treated with native form of silymarin, quercetin, miniaturized SQ with low, medium, and high doses are shown in Table 1. In the CCl_4-treated group, the rats had statistically lower body weights (256.23 ± 6.64 g BW) when compared to that of experimental control (278.06 ± 5.71 g BW) and those with HDMSQ (278.25 ± 6.26 g BW) -treated animals. Similar findings were reported such as the bodyweight of the rat group administrated with carbon tetrachloride and it had a lesser bodyweight.[27] The higher dose of miniaturized SQ caused improvement in the bodyweight of rats at the end of 8 weeks of feeding. This result was comparable to the experimental control rat group.

2.6. Hematological Parameters. The hematological parameters of hepatotoxic rats treated with SQ are presented in Table 2. The results indicated that the rats treated with CCl_4 showed decreased hemoglobin (Hb) concentration, red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular Hb (MCH) concentration, platelet count, and lymphocyte and lymphocyte number. The Hb concentration of rat group which received LDMSQ (14.00 ± 0.32 g/dL), MDMSQ (14.98 ± 0.10 g/dL), and HDMSQ (15.23 ± 1.30 g/dL) was significantly higher when compared with those treated with native silymarin (13.02 ± 0.30 g/dL) and quercetin (13.63 ± 0.50 g/dL). An increase in white blood cell (WBC) (16.32 ± 0.13 × $10^3/\mu$L) was observed in rats induced with CCl_4, and WBC was significantly higher ($p < 0.05$) in the CCl_4 group when compared to all the treated groups. The lymphocyte number was higher in HDMSQ-treated rat groups (17.6 ± 1.24 × $10^3/\mu$L) than compared to the control and rat groups treated with the native form of bioactives. The MCV in rat groups treated with MDMSQ (53.53 ± 1.15 fL) and HDMSQ (54.93 ± 1.03 fL) were higher when compared with those treated with the native form of silymarin (52.86 ± 0.69 fL), quercetin (52.16 ± 0.43 fL), and a combination of SQ (50.83 ± 0.41 fL). The results indicated that treatment with miniaturized SQ increased the Hb, MCV, and lymphocyte number, and the values are comparable to the experimental control group. Furthermore, the improved hematological factors in the rats treated with MSQ could be due to effective absorption of bioactive compounds.

2.7. Liver Function Test and Lipid Profile of Rats Treated with MSQ. The enzymes, namely, alanine amino-transferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), are sensitive enzymes, whose assessment reflect the severity of liver damage.[28] The effect of different treatments of

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

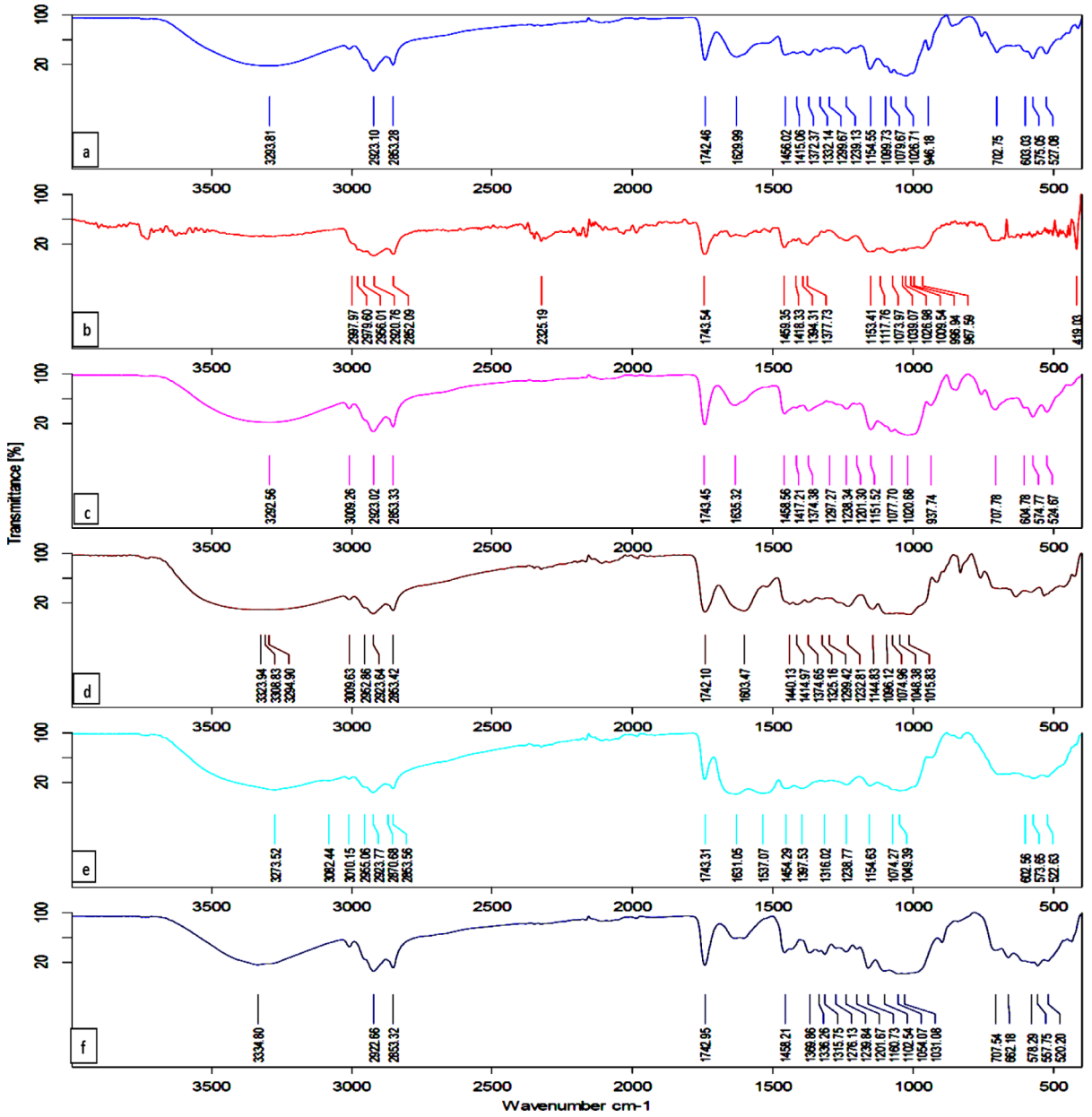


Figure 3. IR spectra of SQ with biopolymers such as (a) SQBC, (b) SQMD, (c) SQC, (d) SQP, (e) SQWP, and (f) SQGA.

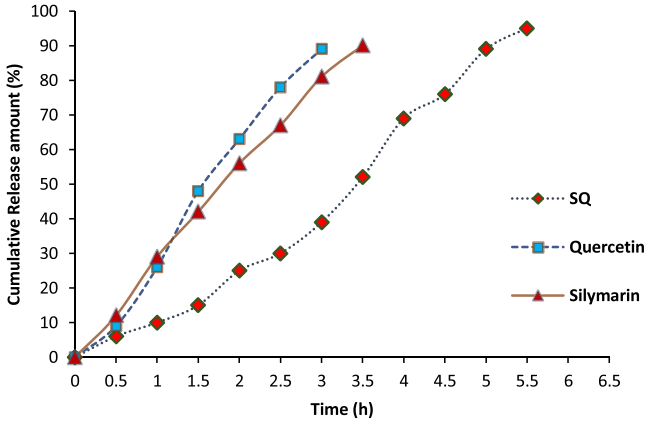


Figure 4. *In vitro* release profile of bioactive compound in the miniaturized scaffold.

bioactive compounds on the liver function test and lipid profile along with few essential biochemical parameters are presented in Table 3. In the serum, ALT and AST levels showed a significant increase ($p < 0.05$) for the rats treated with CCl_4 compared to those treated with bioactives. Similarly, a significant increase in the activity of ALP was seen in the CCl_4-treated group. The native SQ, low, medium, and high doses of miniaturized SQ had considerably reduced GGT compared to that of CCl_4-treated groups. The elevated level of AST and ALT activities in the CCl_4-treated group showed a sign of damage of the liver parenchymal cells, and increased enzyme activity was an apparent response toward the increase in ROS generation.[29] Hence, there was no significant difference in the levels of enzymes such as AST, ALT, ALP, and GGT in MSQ-treated groups compared with the native form of silymarin, quercetin, and a combination of both (SQ).

The HDL levels in the serum of MSQ-treated groups, namely, HDMSQ (45.65 ± 2.47 mg/dL) and MDMSQ (44.31

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

Table 1. Effect of Oral Administration for 8 Weeks of Miniaturized SQ on Relative Organ Weight in Male Wistar Rats[a]

groups	initial BW (g)	final BW (g)	liver (g)	kidney (g)	brain (g)
normal control	237 ± 1.06	278.06 ± 5.71[a]	09.82 ± 0.98[b]	1.77 ± 0.013[b]	1.66 ± 0.06[b]
CCl_4	238 ± 1.19	256.23 ± 6.64[b]	12.89 ± 0.75[a]	1.99 ± 0.14[a]	1.98 ± 0.07[a]
silymarin	239 ± 1.25	270.15 ± 6.92[a]	11.00 ± 0.38[a]	1.70 ± 0.011[b]	1.65 ± 0.07[b]
quercetin	238 ± 1.58	271.46 ± 6.68[a]	10.78 ± 0.52[b]	1.72 ± 0.10[b]	1.44 ± 0.09[b]
SQ	237 ± 1.27	270.33 ± 7.84[a]	10.24 ± 1.16[b]	1.69 ± 0.21[b]	1.58 ± 0.04[b]
LDMSQ	239 ± 1.49	270.66 ± 6.85[a]	09.78 ± 1.65[b]	1.73 ± 0.17[b]	1.54 ± 0.09[b]
MDMSQ	238 ± 1.32	271.06 ± 5.60[a]	09.96 ± 1.23[b]	1.72 ± 0.13[b]	1.51 ± 0.05[b]
HDMSQ	237 ± 1.07	278.25 ± 6.26[a]	10.43 ± 0.54[b]	1.73 ± 0.08[b]	1.58 ± 0.14[b]

[a]Results are expressed as mean ± SEM of the measurements (n = 6). Different subscripts following mean values within a column indicate significantly different groups in Duncan's multiple comparison test with $p < 0.05$.

± 1.39 mg/dL), are significantly higher than those treated with native silymarin (38.94 ± 2.32 mg/dL), quercetin (40.28 ± 3.73 mg/dL), and SQ (39.61 ± 2.54 mg/dL). The LDL level in the HDMSQ (46.42 ± 2.44 mg/dL), MDMSQ (44.93 ± 3.13 mg/dL), LDMSQ (43.86 ± 1.83 mg/dL) was lower compared with the groups treated with native silymarin (59.53 ± 3.90 mg/dL) and quercetin (50.13 ± 4.12 mg/dL), respectively. Results are expressed as mean ± SEM of the measurements (n = 6 animal/group). Different subscripts following mean values within a row indicate significantly different groups in Duncan's multiple comparison test with $p <$ 0.05: ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), GGT (gamma glutamyl transferase), HDL (high-density lipoprotein), and LDL (low-density lipoprotein).

A substantial increase in serum total protein was observed in a rat group treated with miniaturized bioactives LDMSQ (7.871 ± 0.93 g/dL), MDMSQ (7.820 ± 0.64 g/dL), and HDMSQ (7.863 ± 0.36 g/dL), when compared to rats treated with native form of silymarin (6.662 ± 0.50 g/dL), quercetin (6.321 ± 0.18 g/dL), and their combination of SQ (6.591 ± 0.38 g/dL). Lower protein evaluation indicates the diagnostic measurement of liver diseases.[30] Serum albumin improved in rat groups treated with miniaturized bioactives; LDMSQ (3.215 ± 0.18 g/dL), MDMSQ (3.442 ± 0.12 g/dL), and HDMSQ (3.353 ± 0.16 g/dL) than the native form of silymarin, quercetin, and a combination of both SQs. Rat groups administrated with CCl_4 showed lower albumin (2.491 ± 0.10 g/dL) when compared with the control (3.853 ± 0.49 g/dL) group.

The decrease in albumin levels had been related to biliary liver damages and active cirrhosis.[31] A significant decrease in bilirubin was observed in the serum of rat groups treated with miniaturized bioactive LDMSQ (1.122 ± 0.07 mg/dL), MDMSQ (1.136 ± 0.06 mg/dL) and HDMSQ (1.142 ± 0.09 mg/dL), when compared to rats treated with native form of silymarin, quercetin and their combination SQ. The elevated level of bilirubin is an indication of biliary obstruction and hemolysis.[32] The level of glucose showed significant decrease in serum of rats treated with miniaturized bioactives; LDMSQ mg/dL), MDMSQ (85.88 ± 5.10 mg/dL) and HDMSQ (87.64 ± 4.52 mg/dL) than the native form of silymarin, quercetin and a combination of both SQs. However, the rat group administrated with CCl_4 showed a greater increase in the level of glucose when compared with the control group. Uric acid levels showed significant decrease in the rats treated with miniaturized bioactives such as LDMSQ (5.712 ± 0.92 mg/dL), MDMSQ (5.621 ± 0.57 mg/dL) and HDMSQ (5.587 ± 0.65 mg/dL) than compared with native form of silymarin, quercetin and SQ.

Similarly, creatinine levels also decreased in rat groups treated with miniaturized bioactive compound that is LDMSQ (0.880 ± 0.03 mg/dL) MDMSQ (0.892 ± 0.04 mg/dL) and HDMSQ (0.825 ± 0.05 mg/dL) than the native form of silymarin, quercetin and the combination. It was observed that CCl_4 group had a significant elevation in the level of serum uric acid (7.773 ± 0.73 mg/dL) and creatinine (1.196 ± 0.05 mg/dL). The treatment with miniaturized bioactive compound demonstrated hepatoprotection by improvement on liver enzymes and hematological parameters of serum.

2.8. Antioxidant Enzymes and Molecules. ROS are formed during the process of fatty acid oxidation. ROS are minimized by the activity of antioxidant enzymes such as catalase (CAT), SOD and GR.[33] The activity of antioxidant enzymes in rat liver was assayed to reveal the protective effects of the miniaturized scaffold of SQ. The activities of SOD, GR, and CAT in the liver were significantly ($p < 0.05$) decreased in CCl_4 treated rats (95.22 ± 1.68, 08.36 ± 1.22 and 25.8 ± 0.88 U/mg of protein, respectively) than compared to the control rat group (101.71 ± 2.98, 12.93 ± 2.08 and 30.71 ± 0.99 U/mg of protein, respectively) as shown in Table 4.

The activity of SOD in rats treated with different doses of the miniaturized scaffold of SQ (LDMSQ, MDMSQ and HDMSQ) varied in the range of 99.36 ± 2.87 to 102.01 ± 4.82 U/mg of protein, which was higher in the rats with native form of SQ and a combination of SQ, further, it was changed in the range from 95.28 ± 4.59 to 99.91 ± 2.64 U/mg of protein. Similarly, the levels of GR were found higher in rat groups administrated with a high dose of miniaturized bioactives (HDMSQ; 12.99 ± 0.96 U/mg of protein), when compared with LDMSQ, MDMSQ, the native form of silymarin, quercetin and their combination (SQ), which ranged from 10.39 ± 1.06 to 10.97 ± 1.44 U/mg of protein. Similarly, the level of catalase enzyme showed a significant increase in the rat group treated with HDMSQ (30.46 ± 0.84 U/mg of protein) than the other treated groups. The HDMSQ group showed better free radical scavenging property compared to LDMSQ and MDMSQ groups. The obtained results suggested that administration of a high dosage of miniaturized bioactives protects the liver from oxidative damage. Furthermore, it could reduce ROS formation and can act as a better antioxidant. In addition, it was displayed that SQ complex in miniaturized scaffold increased in SOD and CAT activity more significantly ($p < 0.05$) than native SQ. Rat group treated with HDMSQ (0.695 nmol/MDA/mg) decreases significantly for lipid peroxidation, when compared to rat group of LDMSQ (0.798 nmol/MDA/mg), MDMSQ (0.785 nmol/MDA/mg),

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

Table 2. Effect of Miniaturized SQ Feed on Hematological Parameters at the End of 8 Week Study[a]

Particulars	WBC (x10^3/μL)	RBC (x10^6/μL)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (x10^3/μL)	LYM (%)	LYM(#) (x10^3/μL)
control	15.10 ± 0.90^b	7.71 ± 0.85^a	14.43 ± 1.56^a	53.13 ± 1.70^a	18.43 ± 0.68^a	35.35 ± 1.22^a	506.33 ± 17.9^a	84.06 ± 2.06^a	14.7 ± 2.00^a
CCl_4	16.32 ± 0.13^a	6.42 ± 0.25^b	12.52 ± 1.15^b	49.28 ± 1.01^b	15.76 ± 2.10^b	29.53 ± 1.97^b	460.02 ± 19.89^b	72.73 ± 7.69^b	7.30 ± 0.96^b
silymarin	13.81 ± 2.34^b	7.92 ± 0.39^a	13.02 ± 0.30^{ab}	52.86 ± 0.69^a	17.66 ± 0.52^a	33.46 ± 0.15^a	591.66 ± 24.70^a	80.66 ± 5.32^a	14.5 ± 1.56^a
quercetin	13.63 ± 1.62^b	7.36 ± 0.28^a	13.63 ± 0.50^{ab}	52.16 ± 0.43^a	17.16 ± 0.66^a	33.85 ± 0.17^a	579.39 ± 31.43^a	75.28 ± 4.42^a	13.2 ± 0.25^a
SQ	13.96 ± 0.70^b	7.89 ± 0.39^a	13.33 ± 0.90^{ab}	50.83 ± 0.41^b	17.73 ± 0.52^a	31.53 ± 2.44^a	556.36 ± 14.37^a	78.82 ± 3.73^a	14.5 ± 0.95^a
LDMSQ	14.83 ± 0.32^b	7.91 ± 0.35^a	14.00 ± 0.32^a	52.67 ± 1.57^a	17.68 ± 0.52^a	33.46 ± 0.15^a	558.38 ± 32.86^a	80.67 ± 5.36^a	14.4 ± 2.13^a
MDMSQ	15.23 ± 0.50^b	7.77 ± 0.87^a	14.98 ± 0.10^a	53.53 ± 1.15^a	17.76 ± 0.65^a	33.66 ± 0.50^a	559.33 ± 12.50^a	81.33 ± 5.54^a	16.1 ± 1.87^a
HDMSQ	15.18 ± 2.08^b	7.84 ± 0.85^a	15.23 ± 1.30^a	54.93 ± 1.03^a	17.98 ± 1.38^a	33.86 ± 0.15^a	563.01 ± 13.52^a	83.62 ± 5.32^a	17.6 ± 1.24^a

[a]Results are expressed as mean ± SEM of the measurements (n = 6 animal/group). Different subscripts following mean values within a column indicate significantly different groups in of Duncan's multiple comparison test with $p < 0.05$. WBC—WBC count, RBC—RBC count, Hb—Hb concentration, MCV—mean corpuscular volume, MCH—mean corpuscular Hb, MCHC—mean corpuscular Hb concentration, PLT—platelet count, LYM—lymphocyte, and LYM#—lymphocyte number.

native form of silymarin (0.828 nmol/MDA/mg), quercetin (0.838 nmol/MDA/mg) and a combination of both SQs (0.865 nmol/MDA/mg), respectively. The level of thiobarbituric acid reactive substance (TBARS) in the livers of CCl_4 treated rat group was found considerably increased ($p < 0.05$) compared to other rat groups.

2.9. Histological Examination. Histological study was performed using liver to determine the protective effect of the miniaturized scaffold of SQ. The observation of hepatic histology of rat liver cells is displayed in (Figure 5). The liver of control group rats showed a common appearance in portal areas of central veins and hepatic plates. In the CCl_4 treated rats, fat accumulation in the liver and cytoplasm was observed. The hepatocytes were swollen with the presence of vacuoles, which occupied the cytoplasm and the nucleus was in the corner.[34] The CCl_4 has been reported to cause hepatotoxicity with apoptotic hepatocellular injury and necrotic, causing damage to liver function. The rat liver treated with low, medium and high dose MSQ showed improvement in the hepatocytes with less vacuolation, when compared to those administrated with native form of silymarin, quercetin and their combination. In the cases of LDMSQ, MDMSQ and HDMSQ, the hepatocytes appeared oval with homogenous cytoplasm and the nucleus was located in the center; with minimal hepatic damage, scattered and cytological ballooning. However, administration of miniaturized scaffold of SQ to CCl_4-treated rats lowered the destruction in lobule structure compared to the native form of SQ. Furthermore, the histological recovery appeared more superior when applying a combined form of SQ. The administration of complex SQ resulted in a greater decrease in collagen deposition than SQ individually. Thus, the result indicates, the liver cells may regenerate in non-alcoholic induced rats when treated with miniaturized SQ.

3. CONCLUSIONS

The present study investigated the miniaturized scaffold technique to increase the permeability of bioactive compounds, specifically SQ, as hepatoprotective agents. The properties of miniaturized scaffold of various biopolymers with highly active antioxidant molecules focus on the development of better therapeutic properties. High efficiency and slow release of SQ were observed. Oxidative damage induced by carbon tetrachloride in Wistar rats served as an appropriate *in vivo* model to study the hepatoprotective activity of miniaturized scaffold of SQ. The effect of MSQ was pronounced against oxidative stress as demonstrated by an increase in SOD, catalase and GR enzyme activities. Histological observations revealed less cell infiltration and morphological changes when treated with miniaturized scaffold bioactives. Dosage of the bioactive compound SQ (HDMSQ 200:50 mg of SQ/Kg BW of rat) with miniaturization technique showed enhanced hepatoprotective activity. The body weight of HDMSQ treated rats were high and comparable to control. Therefore, the overall MSQ has shown better hepatoprotection against NAFLD than a native form of SQ. The research findings revealed the efficacy of miniaturized bioactives on liver functions. Hence, the formulated SQ complex in a miniaturized scaffold system can be utilized as a novel delivery carrier against NAFLD.

https://doi.org/10.1021/acsomega.1c00555
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Table 3. Effect of Miniaturized SQ in Liver Function and Lipid Profile among Different Rat Groups

particulars	control	CCl_4	silymarin	quercetin	SQ	LDMSQ	MDMSQ	HDMSQ
ALT (U/L)	131.2 ± 2.51^c	170.9 ± 6.85^a	136.6 ± 2.17^b	137.5 ± 2.84^b	138.1 ± 3.21^b	129.9 ± 1.62^c	131.8 ± 2.45^c	130.7 ± 3.37^c
AST (U/L)	130.4 ± 2.26^c	167.5 ± 4.01^a	135.5 ± 2.91^b	136.1 ± 2.34^b	137.0 ± 3.05^c	130.8 ± 3.61^c	132.6 ± 3.47^c	129.1 ± 2.54^c
ALP (U/L)	188.2 ± 5.06^b	240.2 ± 7.12^a	198.2 ± 4.36^b	200.7 ± 3.29^b	198.0 ± 7.27^b	189.7 ± 5.12^b	184.2 ± 7.15^b	187.5 ± 6.84^b
GGT (U/L)	8.407 ± 0.68^b	11.430 ± 0.35^a	8.407 ± 0.34^b	8.522 ± 0.35^b	8.812 ± 0.26^b	8.627 ± 0.42^b	8.652 ± 0.68^b	8.603 ± 0.93^b
HDL (mg/dL)	40.95 ± 7.74^a	28.94 ± 4.47^b	38.94 ± 2.32^a	40.28 ± 3.73^a	39.61 ± 2.54^a	43.64 ± 3.70^a	44.31 ± 1.39^a	45.65 ± 2.47^a
LDL (mg/dL)	40.73 ± 4.40^b	78.33 ± 3.07^a	59.53 ± 3.90^b	50.13 ± 4.12^b	44.86 ± 2.36^b	43.86 ± 1.83^b	44.93 ± 3.13^b	46.42 ± 2.44^b
total cholesterol (mg/dL)	166.6 ± 14.6^b	183.3 ± 12.7^a	162.6 ± 10.3^b	165.0 ± 11.4^b	163.3 ± 7.69^b	157.6 ± 10.3^b	156.3 ± 13.0^b	158.0 ± 10.8^b
triacylglycerols (mg/dL)	124.8 ± 6.56^b	151.4 ± 4.52^a	131.5 ± 6.81^b	128.7 ± 5.42^b	128.7 ± 8.48^b	127.6 ± 6.01^b	124.6 ± 10.1^b	120.0 ± 8.96^b
bilirubin (mg/dL)	0.725 ± 0.27^d	2.634 ± 0.12^a	1.253 ± 0.06^b	1.356 ± 0.10^b	1.206 ± 0.08^b	1.122 ± 0.07^c	1.136 ± 0.06^c	1.142 ± 0.09^c
total protein (g/dL)	7.533 ± 0.21^b	4.992 ± 0.98^c	6.662 ± 0.50^b	6.321 ± 0.18^b	6.591 ± 0.38^b	7.871 ± 0.93^a	7.820 ± 0.64^a	7.863 ± 0.36^a
glucose (mg/dL)	85.82 ± 4.52^b	108.82 ± 4.75^a	89.70 ± 5.62^b	93.52 ± 5.33^b	95.21 ± 4.12^b	87.11 ± 5.10^b	85.88 ± 5.10^b	87.64 ± 4.52^b
albumin (g/dL)	3.853 ± 0.09^a	2.491 ± 0.10^c	3.091 ± 0.09^b	3.045 ± 0.06^b	3.089 ± 0.12^b	3.215 ± 0.18^b	3.442 ± 0.12^b	3.353 ± 0.16^b
uric acid (mg/dL)	4.404 ± 0.95^b	7.773 ± 0.73^a	6.073 ± 0.32^b	6.021 ± 0.69^b	6.146 ± 0.60^b	5.712 ± 0.92^b	5.621 ± 0.57^b	5.587 ± 0.65^b
creatinine (mg/dL)	0.612 ± 0.07^b	1.196 ± 0.05^a	0.912 ± 0.03^b	0.971 ± 0.06^b	0.975 ± 0.06^b	0.880 ± 0.03^b	0.892 ± 0.04^b	0.825 ± 0.05^b

Table 4. Effect of Antioxidant and Enzyme Activities of Miniaturized Bioactive Compounds in Different Rat Groups[a]

particulars	control	CCl_4	silymarin	quercetin	SQ	LDMSQ	MDMSQ	HDMSQ
TBARS (nmol/MDA/mg)	0.868	1.238	0.828	0.838	0.865	0.798	0.785	0.695
SOD (U/mg of protein)	101.71 ± 2.98^a	95.22 ± 1.68^b	99.91 ± 2.64^a	95.28 ± 4.59^a	96.99 ± 5.54^a	99.36 ± 2.87^a	99.66 ± 3.76^a	102.01 ± 4.82^a
GR (U/mg of protein)	12.93 ± 2.08^a	08.36 ± 1.22^b	10.43 ± 1.79^a	10.87 ± 0.99^a	10.39 ± 1.06^a	10.97 ± 1.44^a	10.78 ± 0.40^a	12.99 ± 0.96^a
catalase (U/mg of protein)	30.71 ± 0.99^a	25.89 ± 0.88^b	28.56 ± 0.76^a	29.22 ± 0.98^a	28.87 ± 0.69^a	28.59 ± 0.58^a	28.03 ± 0.36^a	30.46 ± 0.84^a

[a]Results are expressed as mean ± SEM of the measurements (n = 6 animal/group). Different subscripts following mean values within a row indicate significantly different groups in Duncan's multiple comparison test with $p < 0.05$.

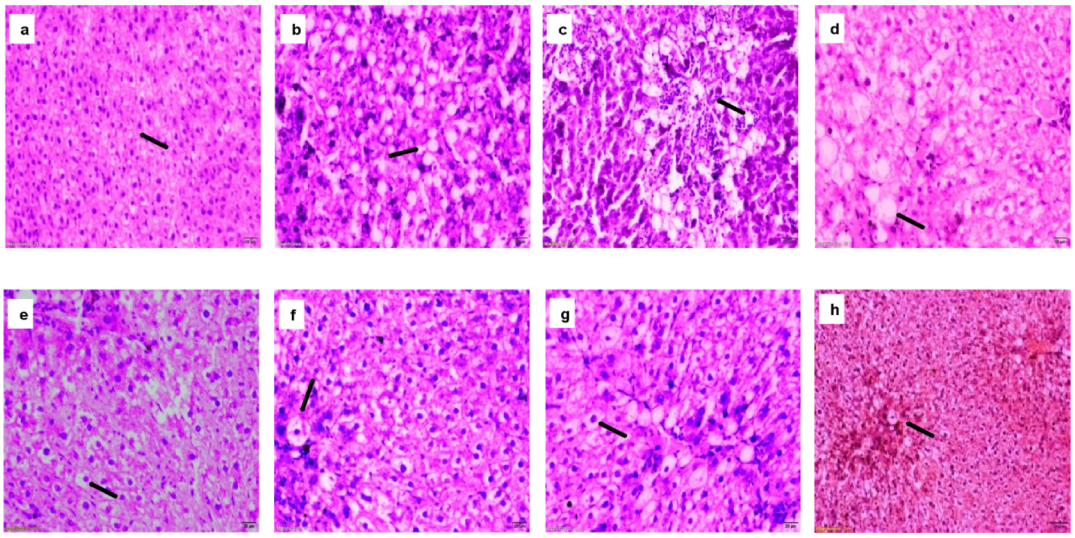

Figure 5. Morphological evaluation of liver histology in different groups (a) Control-shows normal hepatocytes (b) CCl_4-shows hepatocytes ballooning with central nuclei with nuclear vacuolation of hepatocytes (c) silymarin, (d) quercetin & (e) SQ-shows less vacuolation, necrosis and ballooning of hepatocytes, (f) LDMSQ (g) MDMSQ & (h) HDMSQ-shows very few nuclear vacuolation of hepatocytes, hyperactive Kupffer cells and necrotic cells in hepatic parenchyma. (Scale bar = 20 μm with magnification 20×).

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. Silymarin seed powder, Quercetin hydrate (Q) with a purity of 95%, soy lecithin and β-cyclodextrin were procured from Himedia (Bangalore, India). Wheat germ oil was sourced from M/s. Global Merchant (Mumbai, India); groundnut oil from M/s. Shreeya Peanuts (Rajkot, India). CCl_4 were procured from Ragu chemicals Mysore, India. Standard commercial pellet diet was procured from M/s. Sai Durga feeds and foods (Bangalore, India). All reagents used were of analytical grade.

4.2 HPLC Analysis of SQ. SQ standards were prepared in a concentration of 1 mg/mL in methanol (w/v). SQ was separated from miniaturized scaffold by centrifugation of 12,000 rpm for 30 min the concentration of free SQ in the supernatant was calculated by chromatographic analysis, using a stationary phase Shodex C18−250 × 4.6 mm (5 μm) column

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745

(Hitachi, Elite Lachrom 2000 series, Japan). A mixture of 1% acetic acid−methanol−water (1:49.5:49.5 v/v) was served as mobile phase, the injection volume were 10 μL and the elution has been made in gradient mode at a flow rate of 1 mL/min and the detection made at 288 and 366 nm respectively. Program for one analysis the time required was about 60 min.[35]

4.3. Preparation of Microspheres. Bioactive compounds, SQ were hosted in carrier wheat germ oil with surfactants to form microspheres. Microspheres were initially prepared with individual surfactants such as sorbitan monolaurate, sorbitan monooleate, polysorbate (40, 60) with wheat germ oil as a carrier. Considering the hydrophobic nature of SQ, about 200 mg of silymarin and 50 mg of quercetin were dissolved in 0.01% NaOH (w/v) at room temperature (28 ± 1 °C). A optimized mixture composed of soy lecithin (3.5%; w/v), dissolved SQ and 10 mL of wheat germ oil were homogenized to prepare emulsion using a homogenizer (Ultra turrax, T18 basic IKA-T18, Germany) at 16,000 rpm for 30 min and stored at 4 °C for further analysis.[36]

4.4. Size Distribution of Microspheres. The size of microspheres was determined by a trinocular microscope (Olympus BX-5, Japan) with software (Prog Res C-5 software) fixed with a digital camera to capture the images. Microspheres were observed under 100× magnification for analyzing the size distribution.

4.5. Particle Size Analysis. The particle size distribution of the microspheres was measured using a laser light diffraction particle size analyzer (S3500, Microtrac Inc., USA). About 100 μL microsphere sample was taken and was stirred to attain a proper mixture. The analysis was done in triplicates.

4.6. Preparation of Miniaturized Scaffold. Different biopolymers such as maltodextrin, cellulose, pectin, whey protein, beta-cyclodextrin and gum arabica were used for the preparation of miniaturization using a homogenizer at 10,000 rpm for 10 min and the prepared microspheres were added and mixed thoroughly. The mixture was freeze-dried at 0.4 torr at −20 °C using lyophilizer (Scanvac coolsafe—1104 pro, Denmark) to form a miniaturized scaffold. The miniaturized bioactive compound along with biopolymers was further standardized for their physio-chemical properties. After the preparation of miniaturization, the sample was kept in a screw-caped tube and stored at refrigeration (4 ± 0.1 °C) condition for studying the efficacy as hepatoprotectant using the *in vitro* and *in vivo* system.

4.7. FTIR Spectroscopy. Functional properties of bioactive compounds (SQ) were analyzed using an FTIR spectrophotometer (Bruker, Germany/Tensor II). The spectra were recorded in the wavelength region of 4000−400 cm^{-1}. The standards of SQ were analyzed to observe the change in the functional group of miniaturized bioactive compounds.

4.8. Release of Bioactive Compound from a Miniaturized Scaffold. SQ release from the miniaturized scaffold, were studied by incubating the MSQ (30 mg) in phosphate-buffered solution (PBS), at pH 7.4, at 37 °C. 20 mg of MSQ was dispersed in 5 mL of release medium (PBS of pH 7.4 containing 0.1% w/v Tween 80) in a dialysis tube (Sigma dialysis tubes, molecular weight cutoff, 12 kDa), and the closed dialysis bag immersed in 20 mL release medium in a centrifuge tube. Tween 80 was used to increase the solubility of MSQ in the buffer solution to maintain sink condition. The tube was placed in a shaker bath at 37 °C and shaken horizontally at 100 cycles/min. About 15 mL of the sample were withdrawn and replaced with the same volume of fresh medium. The samples were filtered through a 0.22 μM filter and were analyzed for the content of SQ and their release was monitored by measuring the maximum absorption spectrophotometrically at 288 and 366 using a spectrophotometer (Shimadzu spectrophotometer Model UV-1800, Shimadzu, Japan).

4.9. Animals and Experimental Groups. Adult male Wistar rats (n = 48) weighing 250−300 g and aged eight weeks were sourced from institute's animal house facility after approval of institutional animal ethics committee (CFT/IAEC/64/2016) as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India (New Delhi, India).

The rats were randomly divided into eight groups with six animals each. The animals were housed at a room temperature of 21 ± 3 °C, a relative humidity of 50 ± 20%, and light and dark (L/D) cycle of 12 h each. The animals were acclimatized for 6 days before the start of the experiment, provided ad libitum feed and free access to drinking water. Table 5 summarizes the animal grouping and their treatment process with the dosage of bio-actives. Group 1 (control) was fed with normal diet and water. Group 2 (negative control) was given intraperitoneal injection of carbon tetrachloride 1 mL/kg body weight diluted in vegetable oil (1:1) twice a week for inducing NAFLD. Silymarin dose was designed based on previous investigations.[37] The quercetin dose regimen was designed as per the earlier reported animal therapeutic study.[38] Studies are also made on the hepatoprotective effect of quercetin with dose (2 × 250 mg/day) on human clinical trial.[39] The CCl_4-induced rat model described was used for scheduling the dose regimen.[40] The animals were observed daily for mortality and appearance of changes if any. Once in a week, the body weights were recorded, and the dose administered was adjusted weekly according to animal weights to sustain the target dose. Further, routine clinical monitoring was carried out. Besides, food intake was recorded daily. The experiment was carried out for 8 weeks. After 24 h of the last dose, the rats were euthanized and efforts were made to minimize suffering and stress.

Table 5. Animal Grouping and Dose of Administration

s. no.	groups	abbreviation	dosage (per Kg body weight)
1	control group	-	-
2	carbon tetrachloride	CCl_4	1 mL
3	native form of silymarin	S	200 mg
4	native form of quercetin	Q	50 mg
5	a combination of native form of SQ	SQ	200:50 mg
6	low-dose miniaturized SQ	LDMSQ	100:15 mg
7	medium dose miniaturized SQ	MDMSQ	150:30 mg
8	high-dose miniaturized SQ	HDMSQ	200:50 mg

4.10. Blood and Organ Collection. The animals were euthanized with CO_2 at the end of the experimental period (i.e., after 56 days of treatment). About 2 mL of blood was collected into a heparinized container to examine hematological parameters; an additional 5 mL of blood was collected in a non-heparinized container and centrifuged at 3000 rpm for 10 min; the resulting serum was used for bioassays. The animals were quickly dissected, and the liver was excised and weighed to calculate relative organ weight. The samples of the

https://doi.org/10.1021/acsomega.1c00555
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liver were placed in 10% formal saline for histological examination.

4.11. Hematology. The blood sample (approximately 20 μL) was collected with EDTA and used for the estimation of WBCs, RBCs, Hb, mean corpuscular volume (MCV), corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC), platelets (PLT), percentages of lymphocytes (LYM %), and lymphocytes number (LYM#) using a hematology analyzer (Sysmex XP-100).

4.12. Serum Biochemical Markers and Lipid Profile. The effect of miniaturized SQ was evaluated by assaying the serum biochemical parameters associated with liver function. The activities of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma glutamate transferase and concentrations of albumin, total proteins, total cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein, glucose, uric acid, bilirubin, and creatinine were measured in plasma samples obtained from all groups of rats. The analysis was done following the instructions in the diagnostic kits from Agappe Diagnostics (Ernakulam, Kerala, India).

4.13. Antioxidant Enzymes and Molecules in the Liver. *4.13.1. Superoxide Dismutase.* The SOD activity in the liver homogenate was analyzed spectrophotometrically.[41] This method determines the ability of SOD to inhibit the oxidation of nitroblue tetrazolium (NBT). One unit of SOD signifies the amount of enzymes required to hinder the rate of NBT oxidation by 50% at 25 °C. The rate of activity is expressed as units/mg protein.

4.13.2. Catalase. The catalase (CAT) activity was measured as per the method reported by Aebi (1984). The decomposition of hydrogen peroxide (H_2O_2) was monitored kinetically by CAT enzymes at 240 nm. One unit of CAT activity is equal to the micromole of H_2O_2 degraded per minute per milligram of protein.[42]

4.13.3. Glutathione Reductase. About 150 μL of liver serum was prepared in 5% (w/v) trichloroacetic acid and centrifuged at 2000*g* for 10 min, and the glutathione (GSH) content in the deproteinized supernatant was estimated by Ellman's reagent (5,5′-dithio-*bis*[2-nitrobenzoic acid]). GR catalyzes the reduction of GSSG (glutathione disulfide) to GSH (glutathione), and GSH is the sulfhydryl form of a molecule that helps against oxidative stress. The GR activity was expressed as mmol of GSH oxidized/min/mg of protein at 25 °C.[43]

4.13.4. Thiobarbituric Acid Analysis. Lipid peroxidation was measured through TBARSs to detect the level of MDA (malondialdehyde) using fluorescence absorption.[44] Around 0.2 mL of plasma was mixed with 10% sodium dodecyl sulfate, 0.53% TBA, and 20% acetic acid and boiled for 1 h. Butanol: pyridine (15:1) was added, mixed, and centrifuged at 3000 rpm for 10 min. The aliquot was read at 535 nm and recorded.

4.13.5. Histological Analysis. Tissues were taken from the liver of each animal after dissection and fixed using 10% formalin saline. The fixed tissues were processed routinely for paraffin embedding and cut into 4−5 μM thick sections, and the sections of organs were stained by hematoxylin and eosin. Tissue slides were prepared and observed using an optical microscope (Olympus BX-5, Prog Res C-5 software fixed with a digital camera to capture the images).

4.14. Statistical Analysis. Statistical analysis was investigated by analysis of variance using SPSS statistical software version 16. Duncan's multiple comparison test performed the comparison of means. The level of significance used was $p < 0.05$ for all the statistical tests.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00555.

Miniaturized scaffold efficiency of quercetin and silymarin with different biopolymers and FTIR spectra of (a) silymarin (b) quercetin (PDF)

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Author Contributions

J.M.S.R. and A.K.S. conceptualized and proposed the project. J.M.S.R. designed the study, established the protocols, executed the experiments, interpreted the results, and prepared the manuscript. M.S.P. designed and supervised the animal experiments. A.K.S. supervised the *in vitro* studies and revised and finalized the manuscript.

Funding

The work was funded and supported by the Council of Scientific and Industrial Research (CSIR).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The first author acknowledges the Indian Council of Medical Research (ICMR), New Delhi, India, for supporting this work through the award of research fellowship (3/1/2/31/2014-Nut). The authors thank the Director, CSIR-CFTRI, for continuous support and providing the facilities to carry out the research work.

■ ABBREVIATIONS

SQ, silymarin and quercetin; MS, miniaturized scaffold; MSQ, miniaturized silymarin and quercetin; NAFLD, non-alcoholic fatty liver disease; SQMD, silymarin and quercetin with

maltodextrin; SQP, silymarin and quercetin with pectin; SQWP, silymarin and quercetin with whey protein; SQGA, silymarin and quercetin with gum arabica; SQBC, silymarin and quercetin with β-cyclodextrin; SQC, silymarin and quercetin with cellulose; LDMSQ, low dose miniaturized silymarin and quercetin; MDMSQ, medium-dose miniaturized silymarin and quercetin; HDMSQ, high-dose miniaturized silymarin and quercetin

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on August 3, 2021. Superscript a's and b's were inadvertently deleted from Tables 1 and 2 and were added back in. The corrected version was reposted on August 4, 2021.

https://doi.org/10.1021/acsomega.1c00555
ACS Omega 2021, 6, 20735−20745