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Crocin-loaded niosomes influence serum transaminases and hepatic IL- 6, IL-10, SIRT1 and SIRT2 expression in cadmium-treated rats

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Abstract

Cadmium (Cd) is a persistent environmental toxicant that accumulates in the liver and causes oxidative and inflammatory injury. Crocin, the main water-soluble carotenoid of saffron, protects the liver in several experimental models, but its oral bioavailability is poor. We prepared crocin-loaded niosomes (nanocrocin) and compared them with free crocin against Cd-induced hepatotoxicity in rats. Niosomes were produced by thin-film hydration and probe sonication, and the selected formulation had a mean hydrodynamic diameter of 350.1 nm, a polydispersity index of 0.186 and a zeta potential of + 28.0 mV. Male Wistar rats were assigned to six groups and treated for 10 days: blank niosome control, nanocrocin alone (10 mg/kg), $CdCl_2$ (3 mg/kg, intraperitoneally), and $CdCl_2$ combined with oral free crocin (10 mg/kg) or nanocrocin (2 or 10 mg/kg). Cd increased serum aspartate aminotransferase (AST) from 110.3 to 390.0 U/L and alanine aminotransferase (ALT) from 38.7 to 54.5 U/L, raised hepatic interleukin-6 (IL-6) mRNA and lowered sirtuin 1 (SIRT1) and sirtuin 2 (SIRT2) mRNA. All three crocin treatments reduced AST to a similar extent. Nanocrocin at 2 mg/kg was the only treatment that reduced IL-6 expression ($p = 0.002$ versus Cd) and restored SIRT2 expression ($p = 0.003$), and it lowered ALT. No treatment restored SIRT1, and interleukin-10 (IL-10) expression showed no significant overall difference among groups. Nanocrocin at 10 mg/kg was less effective than at 2 mg/kg. Niosomal delivery allowed a fivefold lower dose of crocin to match or exceed the effect of free crocin on these endpoints in this model.

Introduction

Cadmium (Cd) is a non-essential heavy metal that enters soil, water, and food through industrial emissions, phosphate fertilizers, and tobacco smoke. The International Agency for Research on Cancer (IARC) classifies it as a group 1 human carcinogen [1]. Its elimination from the body is very slow, so it accumulates in soft tissues, and the liver is one of the main sites of Cd deposition, making it susceptible to early damage. Within hepatocytes, Cd disturbs mitochondrial function, depletes glutathione, and interferes with essential metals, resulting in oxidative stress, lipid peroxidation, impaired autophagy, and cell death [2]. Oxidative injury is tightly linked to inflammation, since Cd activates nuclear factor kappa B (NF-κB) in the liver and increases proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, which in turn exacerbates the tissue damage [3, 4].

Sirtuins (SIRT) are NAD^+-dependent deacetylases that link the energy state of the cell to its stress responses. SIRT1 and SIRT2 both deacetylate the p65 subunit of NF-κB and thereby restrain the transcription of inflammatory genes [5, 6]. In the liver, sirtuins participate in the control of bile acid and lipid metabolism and of local inflammation [7–9]. Several metals, Cd among them, disrupt sirtuin signaling [10], and in animals cadmium chloride ($CdCl_2$) suppressed hepatic SIRT1 through a miR-34a-dependent mechanism while promoting fatty liver injury [11]. Far less is known about SIRT2 in Cd hepatotoxicity, although hepatic SIRT2 protects against ethanol-induced liver injury and appears to limit inflammation in liver tissue [9, 12]. Therefore, targeting inflammatory pathways and the SIRT family may provide a protective strategy to combat Cd-induced liver damage.

Crocin, the water-soluble carotenoid that gives saffron (*Crocus sativus* L.) its color, possesses antioxidant, anti-inflammatory and immunomodulatory properties [13]. In Cd-exposed rats, crocin lowered serum transaminases, reduced oxidative stress and limited histological damage in the liver [14, 15]. In other models, it increased the expression of SIRT1 or SIRT3 and dampened inflammatory signaling [16–19]. Oral administration of crocin limits its efficacy, because crocins are largely hydrolyzed to crocetin in the intestine and appear in plasma mainly as crocetin and its glucuronide conjugates [20]. As a result, only a small part of an oral dose reaches the tissues in its original form. In this regard, nanocarriers have been proposed as a way to protect crocin and improve its delivery

[21]. Crocin-coated magnetite nanoparticles were more active than free crocin against hepatoma cells and reduced precancerous liver lesions in mice [22], and crocin-loaded chitosan nanoparticles [23], as well as solid lipid nanoparticles [24] were more effective than free crocin in rat models of stress and epilepsy. Crocin-loaded nanoniosomes also reduced renal ischaemia-reperfusion injury in rats [25].

Niosomes are vesicles formed from non-ionic surfactants and cholesterol. They are chemically stable, inexpensive to prepare, and have the ability to hold hydrophilic molecules such as crocin in their aqueous core [26]. Niosomal formulations of other natural compounds have recently shown protective effects against Cd toxicity [27]. To our knowledge, the effect of crocin-loaded niosomes on Cd-induced liver injury has not been examined, nor has their effect on the hepatic expression of IL-6, IL-10 or of the sirtuins SIRT1 and SIRT2. We therefore prepared and characterized Span 60/cholesterol niosomes containing crocin and compared two oral doses of this formulation with free crocin in rats exposed to $CdCl_2$, using serum transaminases and the hepatic expression of these four genes as endpoints.

Materials and Methods

Chemicals

Crocin, $CdCl_2$, sorbitan monostearate (Span 60) and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform and ethanol were of analytical grade.

Preparation of Crocin-Loaded Niosomes

Crocin-loaded niosomes were prepared by the thin-film hydration method. Span 60 and cholesterol were dissolved in a mixture of chloroform and ethanol, and the organic solvent was removed in a rotary evaporator at 60°C for 20 min to leave a uniform thin film. The film was hydrated with an aqueous solution of crocin at 60°C for 1 h. The suspension was then treated with a probe sonicator to reduce particle size, and the samples were stored refrigerated until analysis. Four formulations (F1 to F4) were prepared with different amounts of Span 60 and cholesterol and a fixed amount of crocin (100 mg) (Table 2). Blank niosomes were prepared in the same way without crocin.

Characterization of Niosomes

The mean hydrodynamic diameter and the polydispersity index (PDI) of each formulation were determined by dynamic light scattering, and the zeta potential was measured with a Zetasizer. The formulation with a particle size in the nanometre range and a PDI below 0.2 was selected for the animal experiment and is referred to below as nanocrocin. The morphology of nanocrocin was examined by scanning electron microscopy. The niosomal suspension was diluted with deionized water, and a small droplet was placed on an aluminium stub and allowed to dry at room temperature. The dried sample was sputter-coated with a thin layer of gold and imaged at an accelerating voltage of 20 kV.

Animals and Ethics

Male Wistar rats weighing approximately 200 g were kept under standard laboratory conditions with free access to food and water. The study protocol was approved by the Animal Ethics Committee of Babol University of Medical Sciences (approval code: IR.MUBABOL.AEC.1404.020). All methods were performed in accordance with the relevant guidelines and regulations, and the study is reported in accordance with the ARRIVE guidelines [28].

Experimental Design

Thirty-six rats were divided into six groups (six rats per group) and treated for 10 consecutive days as follows:

Group 1 received nanocrocin (10 mg/kg) by oral gavage and an intraperitoneal (i.p.) injection of 0.9% normal saline as the vehicle for $CdCl_2$.

Group 2 (control) received blank niosomes by oral gavage and an i.p. injection of 0.9% normal saline as the vehicle for $CdCl_2$.

Group 3 received $CdCl_2$ (3 mg/kg, i.p.) dissolved in 0.9% normal saline, and 0.9% normal saline orally as the vehicle for crocin.

Group 4 received $CdCl_2$ (3 mg/kg, i.p.) and free crocin (10 mg/kg) by oral gavage.

Group 5 received $CdCl_2$ (3 mg/kg, i.p.) and nanocrocin (2 mg/kg) by oral gavage.

Group 6 received $CdCl_2$ (3 mg/kg, i.p.) and nanocrocin (10 mg/kg) by oral gavage.

Sample Collection

Twenty-four hours after the last dose, the rats were anaesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Blood was collected by cardiac puncture, and serum was separated. The animals were then euthanized under deep anaesthesia by an overdose of ketamine and xylazine combined with exsanguination via cardiac puncture, and the liver was removed. The liver tissues were also stored in cryogenic tubes at − 80°C for quantitative real-time PCR (qRT PCR) analysis.

Serum Biochemistry

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercial assay kits (Pars Azmoon) according to the manufacturer's instructions. Enzyme activities were expressed in units per liter (U/L).

Gene Expression Analysis

Total RNA was extracted from liver tissue (Parstous, Mashhad, Iran), and its quality was checked by agarose gel electrophoresis. Complementary DNA was synthesized with a reverse transcriptase kit (Yekta Tajhiz Azma, Iran). DNA amplification was done by RealQ Plus 2× Master Mix Green (Ampliqon, Odense, Denmark). The gene expression levels of *IL-6*, *IL-10*, *SIRT1*, and *SIRT2* were measured by qRT PCR using appropriate primers listed in

Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as the reference gene. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method [29] and is reported as fold change.

Table 1
Primer sequences used for real-time PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Accession no.	Product size (bp)
IL-6	TGATGGATGCTTCCAAACTG	GAGCATTGGAAGTTGGGGTA	NM_012589.2	230
IL-10	CACTGCTATGTTGCCTGCTC	TGTCCAGCTGGTCCTTCTTT	NM_012854.2	174
SIRT1	TTATGCTCGCCTTGCTGTGG	TGCTTCAATGCTGTTTCTTCTTTGC	NM_001372090.1	125
SIRT2	TGGAATCTCCACATCCGCAG	GCAAGGGCAAAGAAGGGTTC	NM_001399631.1	150
GAPDH	GACAGCCGCATCTTCTTGTG	GGTAACCAGGCGTCCGATAC	NM_017008.4	98

Statistical Analysis

Data were analyzed with GraphPad Prism version 10 (GraphPad Software, USA) and are presented as mean ± standard error of the mean (SEM). Groups were compared by ordinary one-way analysis of variance (ANOVA). Pairwise differences were examined with Fisher's least significant difference (LSD) test. A p value below 0.05 was considered statistically significant.

Results

Formulation and Characterization of Niosomes

The composition and physicochemical properties of the four formulations are shown in Table 2. Mean particle size ranged from 237.9 nm (F1) to 504.6 nm (F3). At a fixed Span 60 content of 200 mg, raising cholesterol from 100 mg (F1) to 300 mg (F4) increased particle size from 237.9 to 475.2 nm. At a fixed cholesterol content of 200 mg, raising Span 60 from 100 mg (F2) to 300 mg (F3) increased particle size from 350.1 to 504.6 nm and lowered the PDI from 0.186 to 0.027. F1 and F4 had PDI values of 0.319 and 0.251, respectively. All formulations carried a positive surface charge, with zeta potentials of + 20.5 mV (F1), + 28.0 mV (F2), + 28.5 mV (F3) and + 19.9 mV (F4). F2 had a PDI below 0.2 and a smaller particle size than F3 and F4, and it was selected for the in vivo study.

Table 2
Composition and physicochemical characteristics of the crocin-loaded niosome formulations

Formulation	Span 60 (mg)	Cholesterol (mg)	Crocin (mg)	Particle size (nm)	PDI	Zeta potential (mV)
F1	200	100	100	237.9	0.319	+ 20.5
F2	100	200	100	350.1	0.186	+ 28.0
F3	300	200	100	504.6	0.027	+ 28.5
F4	200	300	100	475.2	0.251	+ 19.9

PDI, polydispersity index. F2 was selected for the animal study.

The scanning electron micrograph of nanocrocin is shown in Fig. 1. The niosomes appeared as rounded particles of submicron size, consistent with the size measured by dynamic light scattering. Some particles were clustered or partly fused, which is likely due to aggregation during drying of the sample.

Serum Transaminases

Serum ALT and AST activities are shown in Fig. 2. $CdCl_2$ exposure (group 3) increased ALT compared with group 2. ALT was lower than in group 3 in groups 4 and 5, but not in group 6, and ALT in group 5 was also lower than in group 6 (Fig. 2A).

AST showed a larger rise after $CdCl_2$ exposure. AST was lower than in group 3 in all three treated groups (groups 4, 5 and 6), which did not differ from one another (Fig. 2B). AST in group 5 remained above the value in group 2, whereas groups 4 and 6 did not differ from group 2. ALT and AST in group 1 did not differ from group 2.

Hepatic IL-6 and IL-10 Expression

$CdCl_2$ increased hepatic IL-6 mRNA compared with group 2 (Fig. 3A). IL-6 expression in group 5 was lower than in groups 3, 4 and 6, whereas groups 4 and 6 did not differ from group 3.

IL-10 mRNA showed no significant overall difference among groups (Fig. 3B). In pairwise comparisons, IL-10 expression in group 6 was lower than in groups 2 and 5, but these differences should be interpreted with caution because the overall ANOVA was not significant.

Hepatic SIRT1 and SIRT2 Expression

$CdCl_2$ reduced hepatic SIRT1 mRNA compared with group 2 (Fig. 4A). None of the treated groups differed significantly from group 3. SIRT1 expression remained below the value in group 2 in groups 4 and 5, whereas group 6 did not differ from group 2.

$CdCl_2$ also reduced hepatic SIRT2 mRNA compared with group 2 (Fig. 4B). SIRT2 expression in group 5 was higher than in groups 3 and 4 and did not differ from group 2, whereas groups 4 and 6 did not differ from group 3. SIRT1 and SIRT2 expression in group 1 did not differ from group 2.

Discussion

In this study, crocin was loaded into Span 60/cholesterol niosomes and tested against Cd-induced liver injury in rats. Ten days of $CdCl_2$ exposure (group 3) raised serum transaminases, increased hepatic IL-6 mRNA and reduced SIRT1 and SIRT2 mRNA. AST was lower to a similar degree in groups 4, 5 and 6. Only group 5, which received one fifth of the free crocin dose, also showed lower IL-6 expression and restored SIRT2 expression. SIRT1 was not restored in any group, IL-10 expression showed no significant overall difference among groups, and group 6 responded less than group 5.

The four formulations showed that both components of the vesicle wall affected particle size. When Span 60 was held constant, raising cholesterol increased the mean diameter by about twofold (F1 compared with F4). Cholesterol inserts between the surfactant chains and makes the bilayer more rigid, which can favour larger vesicles with a larger aqueous core [26]. The direction of this effect is not uniform across studies, and Nowroozi et

al. found that raising cholesterol from 20% to 40% reduced the size of Span 60 niosomes [30]. Their cholesterol fractions were lower than those of F4 in our series, which suggests that the effect depends on the surfactant-to-cholesterol ratio as well as on the surfactant type. Raising Span 60 at a fixed cholesterol content produced larger but much more uniform vesicles (F3). The larger size may reflect the greater mass of wall-forming material, with more bilayers that are harder to break down by sonication, and the low PDI may reflect steric stabilization by the higher surfactant content. In F4, the higher PDI may indicate that excess cholesterol interfered with regular bilayer formation. F2 was chosen because it combined a particle size in the nanometre range with a homogeneous size distribution. Its zeta potential of + 28.0 mV, together with that of F3, was the highest in the series and lies close to the ± 30 mV magnitude usually taken to indicate good electrostatic stability.

The Cd model reproduced the main features reported in earlier rat studies. AST rose about 3.5-fold, while the increase in ALT was modest. Elevated transaminases after $CdCl_2$ have been described together with oxidative stress, inflammatory infiltration and hepatocellular necrosis [14, 31], and Cd has also been reported to cause hepatic oxidative stress and fibrotic changes in rats [15]. The rise in hepatic IL-6 mRNA fits the activation of NF-κB and proinflammatory cytokines by Cd [3, 4] and the increase in hepatic IL-6 reported by Gelen et al. [31]. The fall in SIRT1 agrees with the miR-34a-mediated suppression of hepatic SIRT1 by $CdCl_2$ [11] and with the wider view of sirtuins as early targets of metal toxicity [10]. The 64% reduction in SIRT2 mRNA is less well documented for Cd. Because hepatic SIRT2 limits inflammatory and metabolic liver injury [9, 12], its loss may add to the inflammatory response described here.

AST in groups 4, 5 and 6 was more than 60% lower than in group 3 and reached comparable levels, which agrees with the protection by crocin against Cd hepatotoxicity reported by Hamza et al. and Ibrahim Fouad et al. [14, 15]. In those studies crocin was given at 50 mg/kg, whereas in the present work 10 mg/kg of free crocin (group 4) and 2 mg/kg of niosomal crocin (group 5) produced a similar AST response. Group 5 also showed lower ALT. The ability of a fivefold lower dose to match free crocin is consistent with the idea that encapsulation improves the delivery of crocin, which is otherwise extensively hydrolyzed in the gut [20, 21]. Similar gains over the free compound have been reported for crocin in magnetite, chitosan and solid lipid nanocarriers [22–24]. Crocin concentrations in plasma or liver were not measured here, so this explanation remains to be confirmed.

The clearest difference between the formulations appeared in IL-6 and SIRT2 expression. In group 5, IL-6 mRNA was at the control level and SIRT2 mRNA was restored, whereas group 4 did not differ from group 3 for either gene. SIRT1 and SIRT2 both deacetylate NF-κB p65 at lysine 310 and reduce the expression of NF-κB target genes [5, 6], and IL-6 is one of these targets. A coordinated rise in SIRT2 and fall in IL-6 is therefore plausible, although the present data are correlative and do not show that SIRT2 mediates the effect. Crocin and crocin-I have been reported to increase SIRT1 and SIRT3 in kidney, brain, lung and cultured cells [16–19], and crocin-loaded solid lipid nanoparticles raised SIRT1 expression and lowered inflammatory mediators in the brain [24]. In our study, SIRT1 mRNA in groups 5 and 6 was numerically higher than in group 3, but the difference did not reach significance. SIRT1 may be regulated in Cd-exposed liver mainly at the post-transcriptional level, as suggested by the involvement of miR-34a [11], and changes in SIRT1 protein or activity could have been missed by measuring mRNA alone.

Group 6 responded less than group 5 for ALT, IL-6 and, as a trend, SIRT2, although both groups showed similar AST values. This non-monotonic pattern cannot be explained with the present data. Crocin can affect macrophage cytokine output in different ways depending on the inflammatory state of the cells [32], so the net effect on

hepatic cytokines may depend on dose and timing. A study with more dose levels would be needed to resolve this point.

IL-10 expression showed no significant overall difference among groups. Mean values were lowest in groups 3 and 6 and highest in group 5, a pattern opposite to that of IL-6, but the variability within groups was large and the study was not powered to detect differences of this size. Gelen et al. found higher hepatic IL-10 after Cd exposure [31], whereas the mean value in group 3 was below that in group 2, so the IL-10 response to Cd may depend on dose, duration of exposure and time of sampling. Group 1 did not differ from group 2 in any of the measured endpoints, which indicates that the formulation itself was well tolerated by the liver.

This study has several limitations. Gene expression was measured at the mRNA level only, and liver histology and markers of oxidative stress were not part of this study, so protection at the tissue level cannot be confirmed from the biochemical and gene expression endpoints alone. Only male rats and a single 10-day exposure were studied, with six rats per group. Despite these limitations, the direct comparison of two niosomal doses with free crocin within the same experiment allows the effect of encapsulation to be judged under identical conditions.

In summary, crocin-loaded Span 60/cholesterol niosomes reduced the biochemical and inflammatory markers of liver injury induced by $CdCl_2$ in rats at an oral dose of 2 mg/kg (group 5). At this dose, nanocrocin reduced serum transaminases and hepatic IL-6 expression and restored SIRT2 expression, and it did so at one fifth of the dose of free crocin (group 4), which lowered serum transaminases but did not change the hepatic genes studied. These findings support niosomal encapsulation as a way to lower the effective oral dose of crocin against Cd hepatotoxicity.

Declarations

Funding

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Competing Interests

The authors declare no competing interests.

Ethics Approval

All animal procedures were approved by the Animal Ethics Committee of Babol University of Medical Sciences (approval code: IR.MUBABOL.AEC.1404.020). All applicable international, national and institutional guidelines for the care and use of laboratory animals were followed, and the study is reported in accordance with the ARRIVE guidelines [28].

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Data Availability

The data supporting the findings of this study are available from the corresponding author on reasonable request.

Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Mehdi Hassani Ahangar, Roghayeh Pourbagher, Olia Hamzeh and Seyedeh Melika Ahmadi. Preparation and characterization of the niosomal formulations were carried out by Seyedeh Melika Ahmadi. Biochemical and gene expression analyses were performed by Durdi Qujeq and Roghayeh Pourbagher. Data analysis and interpretation were performed by Mehdi Hassani Ahangar and Hossein Najafzadehvarzi, who also supervised the study. The first draft of the manuscript was written by Mehdi Hassani Ahangar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

The authors declare that all data were generated in-house and that no paper mill was used. AI-assisted tools were used solely to improve English language, grammar, clarity, and readability. No AI tools were used to generate scientific content, data, results, or figures.

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Figures

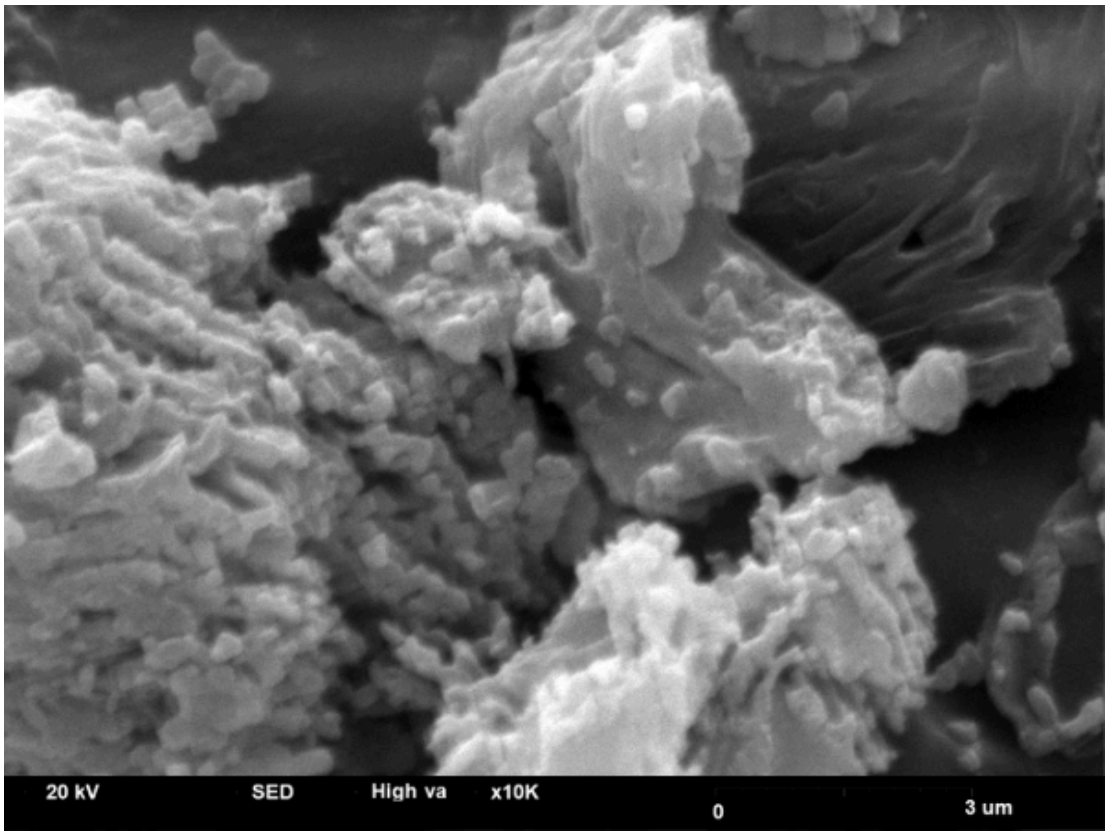

Figure 1

Scanning electron micrograph of the selected crocin-loaded niosome formulation (F2). Magnification ×10,000; accelerating voltage 20 kV; scale bar 3 μm

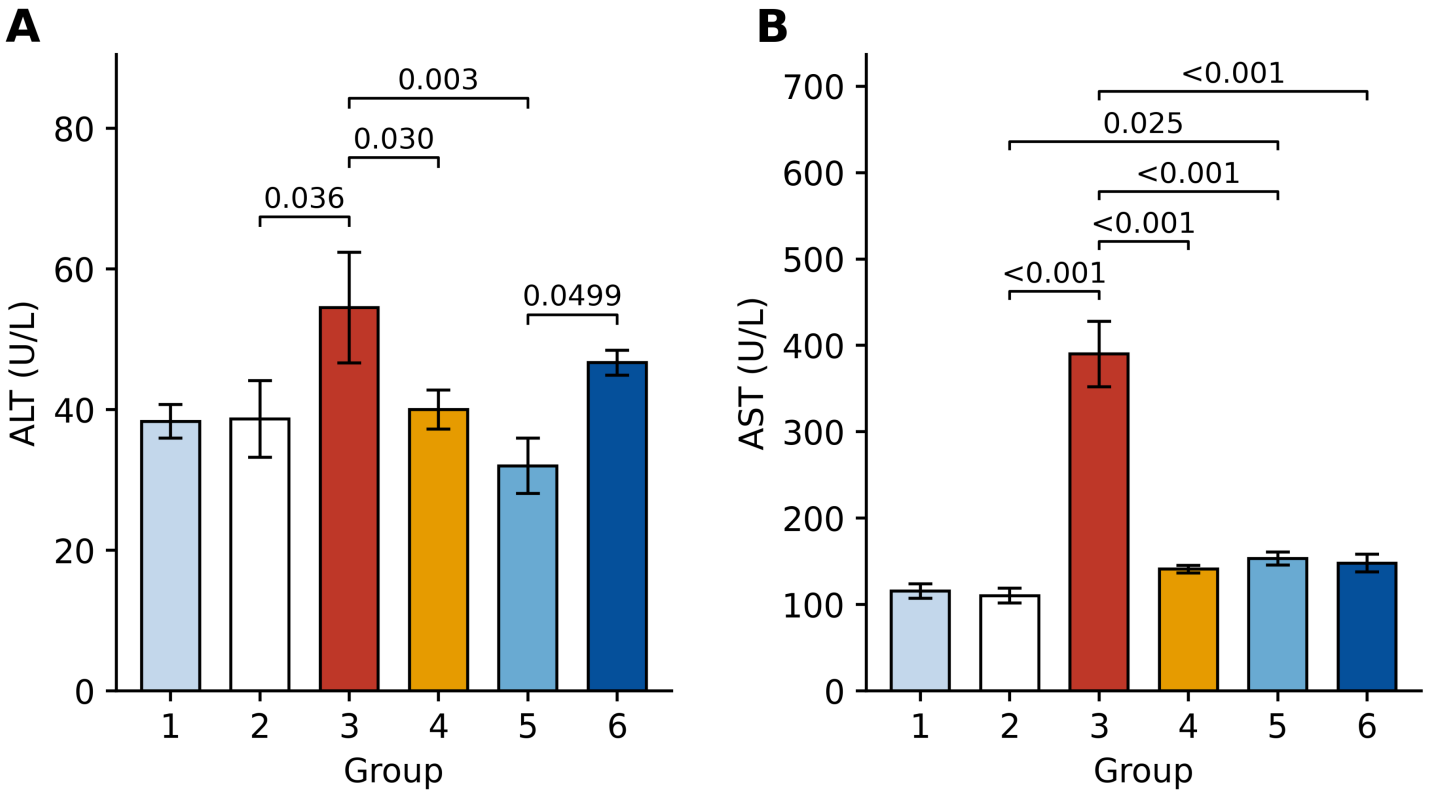

Figure 2

Serum ALT (A) and AST (B) activities. Bars show mean ± SEM. One-way ANOVA: ALT, p = 0.048; AST, p < 0.001. Numbers above the brackets are exact p values (one-way ANOVA followed by Fisher's LSD test) for the significant differences among the following comparisons: each group versus group 2, groups 4, 5 and 6 versus group 3, and groups 4, 5 and 6 versus one another. Group 1, nanocrocin (10 mg/kg); group 2, blank niosome control; group 3, $CdCl_2$ (3 mg/kg); group 4, $CdCl_2$ plus free crocin (10 mg/kg); groups 5 and 6, $CdCl_2$ plus nanocrocin (2 and 10 mg/kg). Blue bars, nanocrocin; orange bar, free crocin; red bar, $CdCl_2$ alone; white bar, blank niosome control

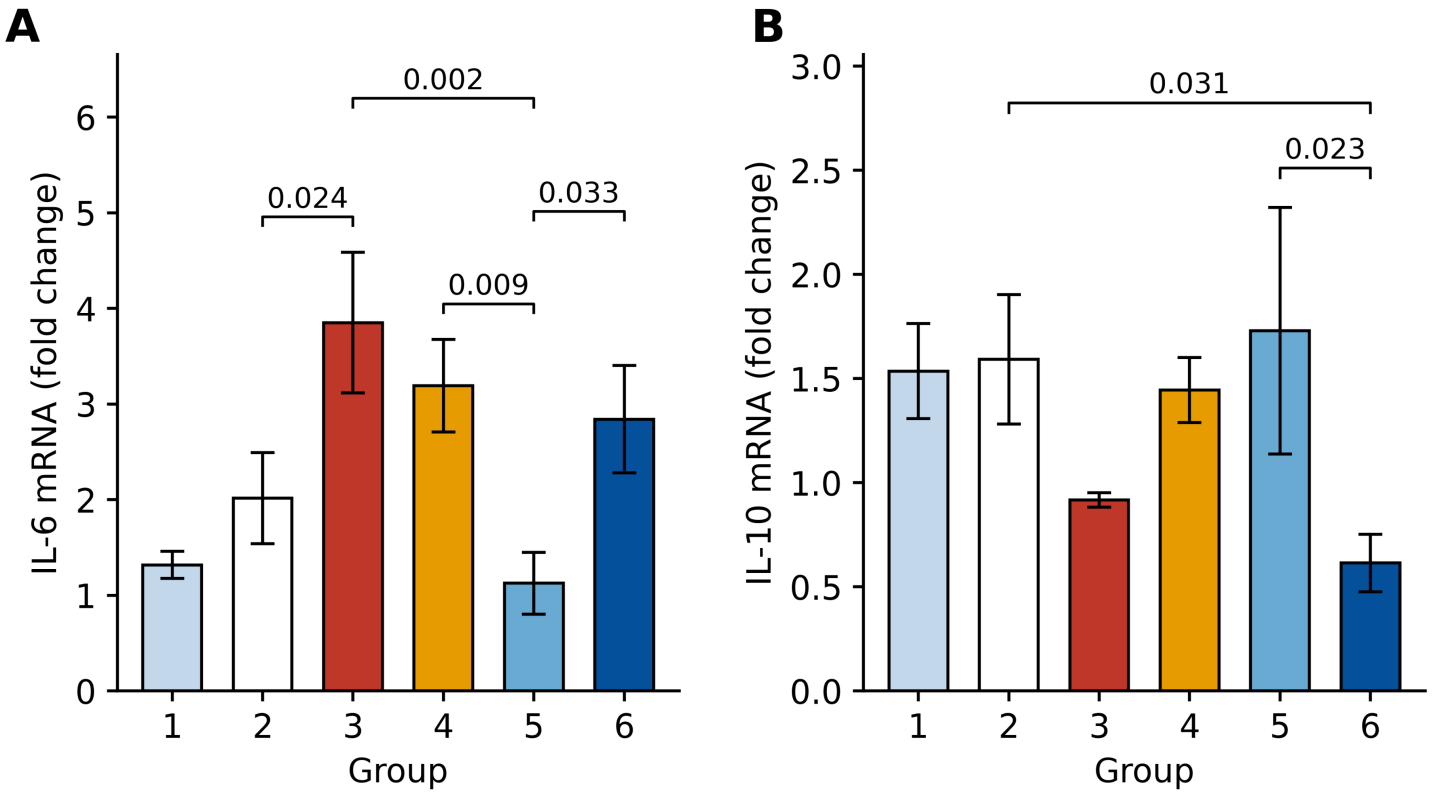

Figure 3

Hepatic IL-6 (A) and IL-10 (B) mRNA expression, shown as fold change ($2^{-\Delta\Delta Ct}$, *GAPDH* as reference). Bars show mean ± SEM. One-way ANOVA: IL-6, p = 0.011; IL-10, p = 0.127. Brackets show exact p values for significant differences; comparisons, groups and colors are as in Fig. 2. In B, the overall ANOVA was not significant, so the pairwise differences shown should be interpreted with caution

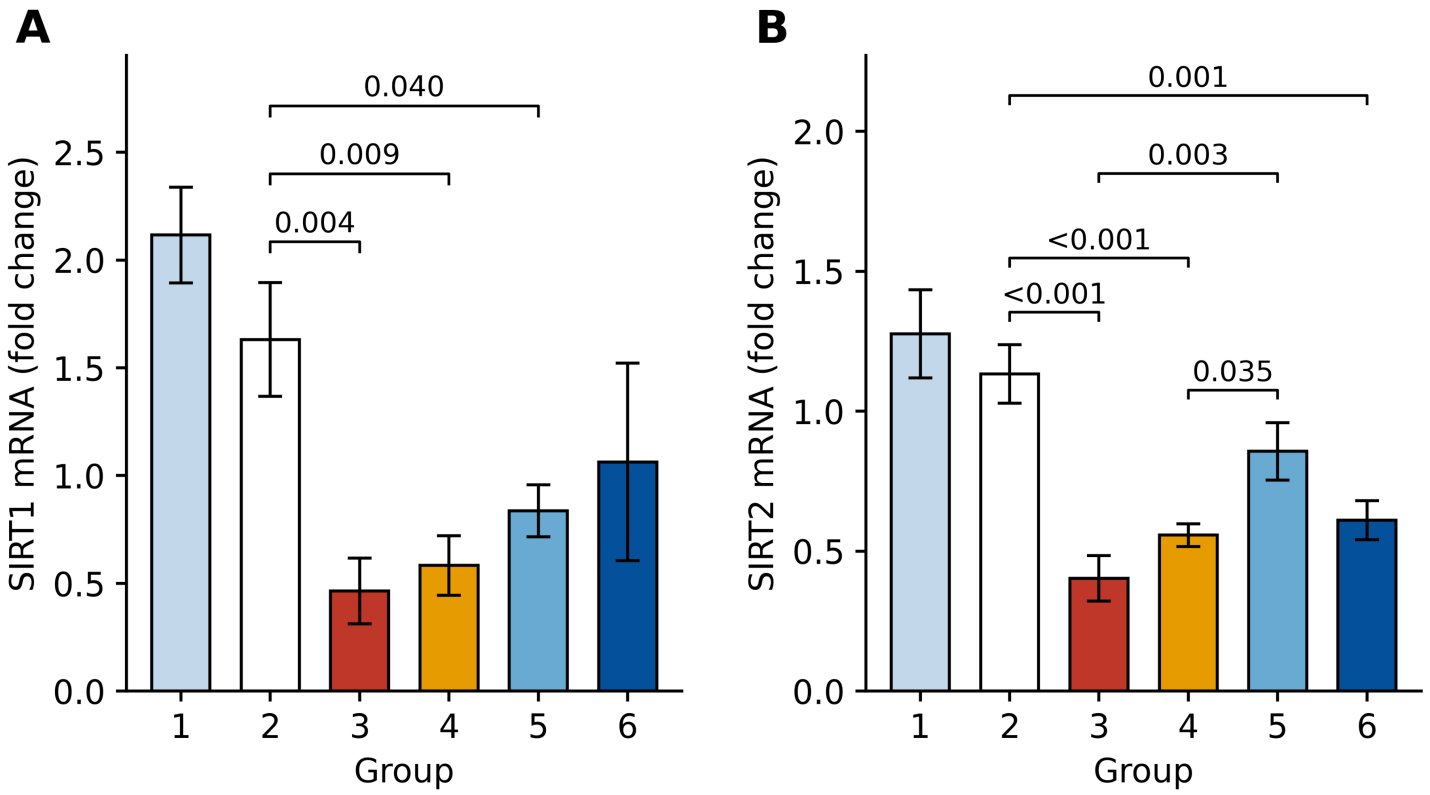

Figure 4

Hepatic SIRT1 (A) and SIRT2 (B) mRNA expression, shown as fold change ($2^{-\Delta\Delta Ct}$, *GAPDH* as reference). Bars show mean ± SEM. One-way ANOVA: SIRT1, p = 0.001; SIRT2, p < 0.001. Brackets show exact p values for significant differences; comparisons, groups and colors are as in Fig. 2