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Cytotoxic and Antioxidant Effects of Curcumin Nanoparticles on Hepatic Cell Lines Exposed to Acetaminophen-Induced Toxicity: An In Vitro Experimental Study

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ABSTRACT

Acetaminophen (APAP), which is safe at therapeutic levels, is a major cause of drug-induced hepatotoxicity globally. In this study, curcumin nanoparticles (Cur-NPs) were tested for their cytotoxic and antioxidant effects on HepG2 hepatic cells after APAP injury. Nanoprecipitation produced Cur-NPs, which were characterised by FTIR, XRD, and FESEM. FTIR shows curcumin's functional groups, while XRD shows a semi-crystalline structure with much lower peak intensities than bulk curcumin. Spherical nanoparticles with 60–90 nm diameters were seen in FESEM micrographs. After pretreatment with Cur-NPs (5, 10, 20, 40, and 80 µg/mL), HepG2-cells were subjected to 20 mM APAP over 24 hours. MTT assay-measured cell viability revealed dose-dependent protection, peaking at 40 µg/mL Cur-NPs. Pretreated groups had dramatically restored antioxidant biomarkers SOD, GSH, and MDA to near-control levels. Based on bioavailability and cellular uptake, Cur-NPs appear to reduce APAP-induced oxidative damage and promote hepatic cell survival more than free curcumin. Before clinical translation, Cur-NPs must be validated in vivo, but these in vitro results suggest hepatoprotection.

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INTRODUCTION

Paracetamol (acetaminophen, APAP) is among the most widely consumed over-the-counter analgesic and antipyretic drugs across the globe [1]. At therapeutic doses, it is considered reasonably safe, yet accidental or deliberate overdose remains a well-documented cause of acute liver injury, and in several Western countries it accounts for nearly half of all cases of acute liver failure [2]. The hepatotoxic action of APAP is largely attributed to its reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is formed through cytochrome P450 (mainly CYP2E1) biotransformation. Once generated, NAPQI quickly depletes hepatic glutathione (GSH) and covalently binds to cellular macromolecules, triggering oxidative stress, mitochondrial dysfunction and, eventually, hepatocyte necrosis [3,4].

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Although N-acetylcysteine (NAC) is still regarded as the standard antidote, its delayed administration and the cost-related issues in low-income regions have motivated the search for alternative hepatoprotective agents from natural origins [5]. Curcumin, the yellow polyphenol obtained from Curcuma longa, is a well-studied phytochemical with recognized anti-inflammatory, anticancer and potent antioxidant properties [6,7]. However, its clinical utility is frequently restricted by poor aqueous solubility, rapid first-pass metabolism and limited systemic bioavailability [8].

These constraints can be circumvented by nanotechnology. Nanoscale curcumin improves solubility, stability, and cellular absorption, improving biological performance compared to bulk curcumin [9,10]. Recent mouse studies have shown that curcumin nanoparticles (Cur-NPs) protect against chemical- and drug-induced liver damage [11]. Nevertheless, mechanistic in vitro assessments in human hepatic cell lines are still relatively limited, and direct side-by-side comparisons between free curcumin and its nanoformulation under the same experimental conditions remain scarce. The present study was therefore undertaken to synthesize and characterize Cur-NPs and to evaluate their cytotoxic and antioxidant behaviour on HepG2-cells exposed to APAP-induced toxicity, with a special focus on their ability to restore cellular antioxidant defences and to improve overall cell survival.

MATERIALS AND METHODS

Chemicals and Reagents

Curcumin (≥95% purity), acetaminophen (≥99%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethanol (analytical grade), Tween-80 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution and trypsin–EDTA were obtained from Gibco (USA). All other chemicals used were of analytical grade, and double-distilled water was used throughout the experiments.

Preparation of Curcumin Nanoparticles

Cur-NPs were prepared by the nanoprecipitation (solvent–antisolvent) technique with slight modifications of the procedure described by Bhawana et al. [12]. In brief, 100 mg of curcumin was dissolved in 10 mL of absolute ethanol to

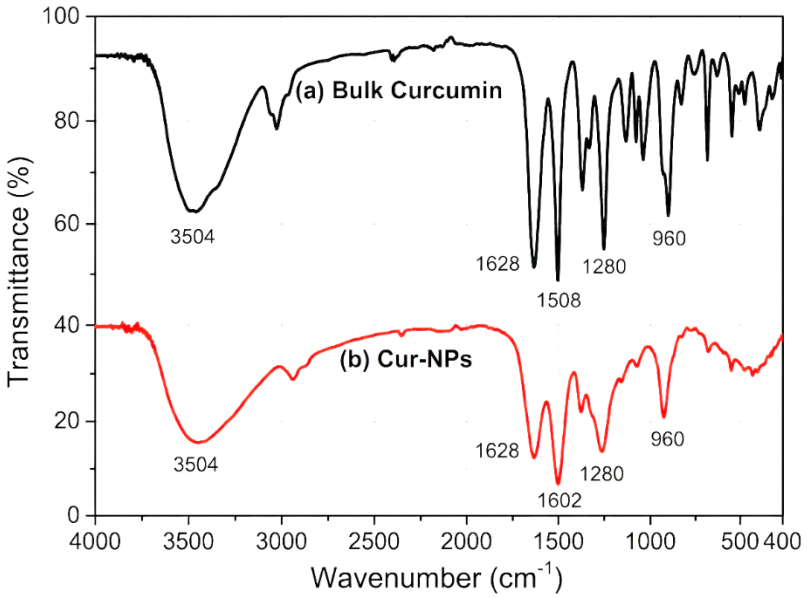

Fig. 1. FTIR spectra of (a) bulk curcumin and (b) curcumin nanoparticles (Cur-NPs), showing the preservation of characteristic functional-group vibrations with slight broadening and intensity reduction in the nanoparticle sample.

obtain a clear yellow organic solution. This organic phase was then injected drop-wise at a controlled rate of ~1 mL/min into 100 mL of double-distilled water containing 0.2% (w/v) Tween-80, under vigorous magnetic stirring (1200 rpm). Stirring was maintained for 4 h at room temperature to allow complete evaporation of ethanol and to produce a stable colloidal suspension. The suspension was then centrifuged at 12,000 rpm for 20 min at 4 °C, and the resulting pellet was washed twice with distilled water to remove excess surfactant. The final product was lyophilized and stored at 4 °C in amber glass vials until further use.

Characterization of Cur-NPs

FTIR analysis

Functional-group verification was carried out using a Shimadzu IRAffinity-1S spectrometer. About 2 mg of each sample was mixed with dry KBr and compressed into a transparent pellet. Spectra were recorded over the 4000–400 cm^{-1} range at a resolution of 4 cm^{-1}.

XRD analysis

The crystallinity of bulk curcumin and Cur-NPs was examined using a Shimadzu XRD-6000 diffractometer with Cu-Kα radiation (λ = 1.5406 Å) operated at 40 kV and 30 mA. Samples were scanned over a 2θ range of 5–60° with a step size of 0.02°.

FESEM analysis

Surface morphology and particle size of the lyophilized nanoparticles were determined using a FEI Inspect F50 FESEM instrument. A thin layer of the dry powder was mounted on an aluminium stub with double-sided carbon tape and sputter-coated with gold before imaging at different magnifications.

Cell Culture and Treatment Protocol

Human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C under a humidified 5% CO_2 atmosphere. Cells were subcultured at 70–80% confluence using 0.25% trypsin–EDTA. For the experiment, HepG2-cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. Cells were then divided into the following groups: (i) untreated control, (ii) APAP-only (20 mM, 24 h), (iii) Cur-NPs alone at 5, 10, 20, 40 and 80 µg/mL, and (iv) pretreatment

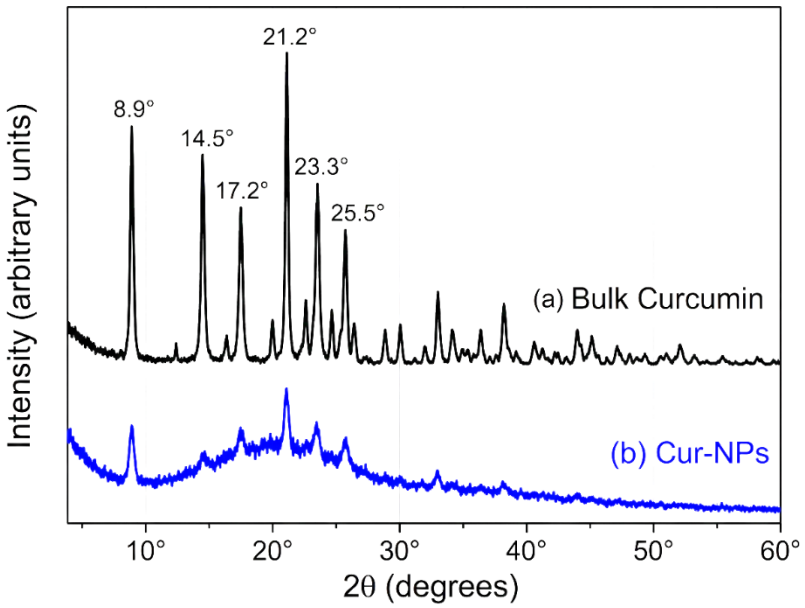

Fig. 2. X-ray diffraction patterns of (a) bulk curcumin and (b) curcumin nanoparticles, showing a clear reduction of peak intensities and the appearance of a broad amorphous halo in the nanoparticle sample.

groups, where cells were exposed to Cur-NPs for 2 h before the APAP challenge.

Cytotoxicity Assay (MTT)

Cell viability was assessed by the standard MTT colorimetric method as originally described by Mosmann [13]. After the corresponding treatment period, the medium was aspirated and 100 µL of MTT solution (0.5 mg/mL in fresh medium) was added to each well. Plates were incubated at 37 °C for 4 h in the dark. The formazan crystals formed were then dissolved in 100 µL of DMSO, and the absorbance was recorded at 570 nm using an ELISA microplate reader (BioTek, USA). Percentage of viable cells was calculated as:

$$\% \text{ Viability} = (\text{Atreated} / \text{Acontrol}) \times 100$$

IC_{50} values were calculated from the dose–response curves using GraphPad Prism 9.0.

Antioxidant Biomarker Assays

After the 24-h treatment, HepG2-cells were harvested, lysed in ice-cold PBS containing 0.1%

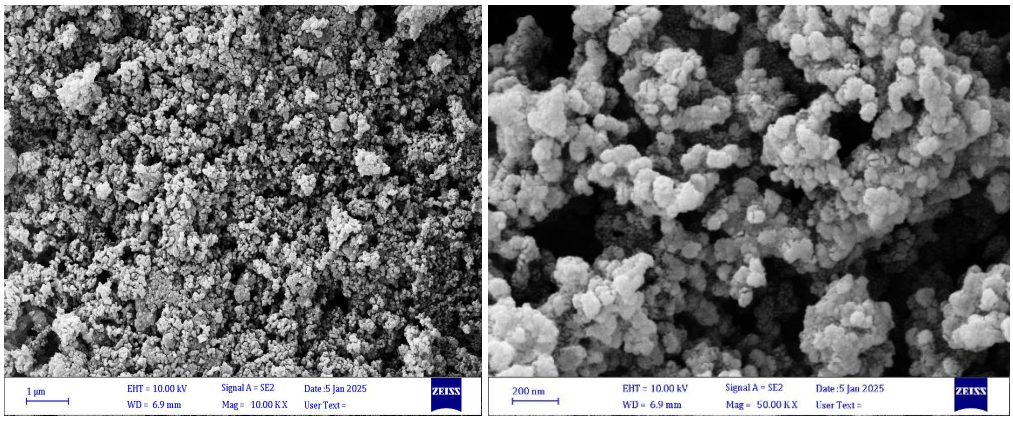

Fig. 3. FESEM micrographs of curcumin nanoparticles at ×10,000 (left) and ×50,000 (right) magnification, showing spherical, smooth-surfaced particles with an average diameter of ~74 nm.

Table 1. Physicochemical characterization parameters of the prepared curcumin nanoparticles (Cur-NPs).

Parameter	Value
Method of preparation	Nanoprecipitation (solvent–antisolvent)
Stabilizer	Tween-80 (0.2% w/v)
Appearance	Bright yellow, free-flowing powder
Morphology (FESEM)	Spherical, smooth surface
Average particle size (FESEM)	74 ± 12 nm
Size range	~ 50 – 100 nm
Crystalline nature (XRD)	Semi-crystalline (partial amorphization)
Main XRD peaks (2θ)	8.9°, 14.5°, 17.2°, 21.2°, 23.3°, 25.5°
Characteristic FTIR bands (cm^{-1})	3504, 1628, 1602, 1508, 1280, 960
Yield (%)	78.6 ± 3.2

Values are expressed as mean ± SD (n = 3).

Triton X-100 and centrifuged at 10,000 × g for 10 min at 4 °C. The resulting supernatant was used for the following biochemical measurements:

Reduced glutathione (GSH): Ellman's DTNB method at 412 nm.

Superoxide dismutase (SOD): pyrogallol autoxidation assay at 420 nm.

Malondialdehyde (MDA): as an index of lipid peroxidation, by the thiobarbituric acid-reactive substances (TBARS) method at 532 nm, following the procedure of Ohkawa et al. [14].

Protein content of cell lysates was measured by the Bradford assay in order to normalize enzymatic activities.

Statistical Analysis

All data are presented as mean ±SD from three independent, duplicate trials. Group differences were analysed using one-way ANOVA and Tukey's post-hoc test in GraphPad Prism 9.0, with significance set at $p<0.05$.

RESULTS AND DISCUSSION

FTIR Characterization

The FTIR spectra of bulk curcumin and the prepared Cur-NPs are presented in Fig. 1. Bulk curcumin showed the characteristic vibrations at 3504cm^{-1} (phenolic –OH stretching), 1628 cm^{-1} (C=O stretching overlapping with C=C alkene), 1602cm^{-1} (aromatic C=C), 1508 cm^{-1} (C=O conjugation), 1280 cm^{-1} (C-O phenolic) and 960cm^{-1} (benzoate trans-CH vibration). The nanoparticle sample had similar-peaks with slight shifts and lower intensities, especially in O-H and -C=O. Hydrogen bonding between curcumin's phenolic groups and Tween-80 may cause the expansion and slight displacement without damaging the molecular skeleton-Pandit et al.[15] obtained comparable results with nanoprecipitated curcumin, showing that nanonization protects its chemical integrity.

XRD Analysis

Fig. 2 shows XRD diffractogram peaks at 8.9°,

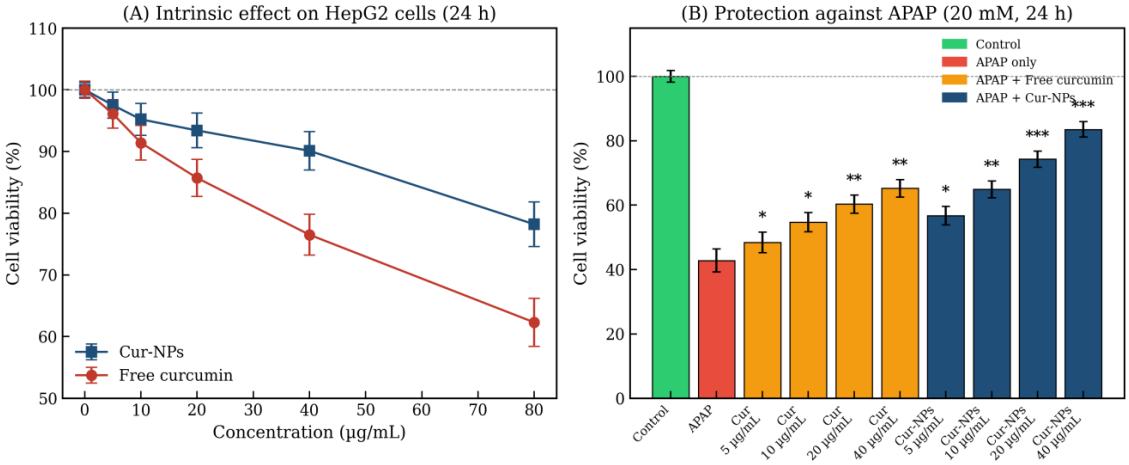

Fig. 4. (A) Effect of increasing concentrations of free curcumin and Cur-NPs (5-80µg/mL) on HepG2 cell viability after 24h. (B) Protective effect of Cur-NPs versus free curcumin pretreatment against APAP (20mM)-induced cytotoxicity. Data are mean ± SD (n = 3).*p<0.05,**p<0.01, ***p<0.001 vs APAP-only group.

Table 2. IC_{50} values of free curcumin and Cur-NPs against APAP-induced toxicity in HepG2-cells.

Treatment	IC_{50} (µg/mL)	95% CI	R^2
Free curcumin	28.4 ± 2.1	26.1 – 30.8	0.962
Cur-NPs	11.7 ± 1.3	10.4 – 13.1	0.981
Fold improvement	~ 2.4 ×	—	—

IC_{50} values were calculated from the dose–response curves using non-linear regression in GraphPad Prism 9.0. Values are expressed as mean ± SD (n = 3); $p < 0.001$ vs free-curcumin group.

14.5°, 17.2°, 21.2°, 23.3°, and 25.5°, indicating its crystalline nature [16]. But the Cur-NPs sample had a broad diffuse halo with much lower peak intensities, indicating a partial change from crystalline to quasi-amorphous during nanoprecipitation. Due to their increased solubility and faster dissolving rates, amorphous forms are preferable over crystalline ones [17]. We found a semi-crystalline pattern in curcumin nanoparticles generated by antisolvent precipitation, as did Kakran et al. and Ahmad et al.[17,18].

FESEM Imaging and Morphology

FESEM micrographs (Fig. 3) indicated Cur-NPs were predominantly spherical with smooth surfaces and a narrow size distribution. Using 100particles, ImageJ software found an average particle diameter of 74±12nm. Some locations showed modest aggregation, possibly from drying before imaging rather than suspension agglomeration. The size range (50-200nm) is suitable for endocytosis [19]. A summary of the main characterization parameters is provided in Table 1.

Intrinsic Cytotoxicity of Cur-NPs on HepG2-cells

Before assessing the protective effect, the inherent cytotoxicity of the prepared Cur-NPs on HepG2-cells was determined after 24-h exposure to concentrations ranging between 5 and 80 µg/mL. As shown in Fig. 4A, Cur-NPs alone did not produce any significant cytotoxic effect at concentrations up to 40 µg/mL, with cell viabilities remaining above 90%. A mild reduction to around 78% was observed only at the highest concentration tested (80 µg/mL). This slight decline at very high doses could be attributed to a pro-oxidant behaviour of curcumin, which is a well-known biphasic effect of polyphenolic compounds [20]. Free curcumin at the same concentrations produced slightly greater reductions in viability, probably due to its limited solubility and tendency to precipitate out of the culture medium. Therefore, the protective experiments were performed within the safe 5–40 µg/mL range.

Protective Effect Against APAP-Induced Cytotoxicity

Exposure of HepG2-cells to 20 mM APAP for 24 h caused a marked drop in viability down to 42.8 ± 3.6% of the untreated control, which confirms successful induction of hepatotoxicity. Pretreatment with Cur-NPs significantly improved viability in a dose-dependent manner. The maximum protective effect was observed at 40 µg/mL, where cell viability increased to 83.5 ± 2.4%, nearly double the value of the APAP-only group ($p < 0.001$). Free curcumin at the same concentration raised viability to only ~65%, clearly showing the superior performance of the nanoparticulate formulation (Fig. 4B). Table2 shows that Cur-NPs have significantly lower IC_{50} values than free curcumin. Farghali et al. and Nabavi et al. [21,22] found that curcumin nanoparticles are more hepatoprotective than bulk curcumin in numerous toxicity studies.

The enhanced protection is likely complex. Cur-NPs can be rapidly internalised by hepatocytes via endocytic pathways due to their sub-100 nm size, increasing active curcumin concentrations. Due to their semi-amorphous nature, they dissolve faster and provide more curcumin molecules to scavenge NAPQI-derived free radicals before they damage vital cellular targets. The surfactant coating of Tween-80 around the particles stabilises them and may decrease premature drug metabolism in the extracellular environment, increasing apparent potency. The decline in viability after APAP exposure was consistent across three separate replicates (SD < 4%), supporting the observed protective trend.

Effect on Oxidative Stress Biomarkers

APAP treatment caused oxidative stress in HepG2-cells, with GSH content decreasing by 63%, SOD activity decreasing by~54%, and MDA levels rising to roughly 2.8-fold of control values (Fig. 5, Table 3). The NAPQI-mediated glutathione depletion, mitochondrial ROS overproduction, and membrane lipid peroxidation pathway is well supported by these changes [23].

Cur-NP pretreatment dose-dependently reversed these changes. GSH was recovered to ~89% of control at 40µg/mL, SOD activity increased to ~82%, and MDA dropped to 1.3-fold, close to baseline. The nanosized formulation's pharmacological benefit was further demonstrated by partial restoration with equivalent free-curcumin.

Our findings are in agreement with the observations of Rahimi et al. [24], who reported that curcumin nanoparticles efficiently preserve the glutathione pool and enhance antioxidant-enzyme activity in chemically injured hepatic models. The ability of curcumin to donate

hydrogen atoms from its phenolic groups is central to its radical-scavenging activity, while the keto–enol moiety also contributes to Fenton-type iron chelation [25]. Encapsulation or nanonization reinforces these effects by stabilizing the molecule and by protecting it from premature metabolic inactivation. Furthermore, curcumin is known to modulate several antioxidant-related transcription factors, most notably the Nrf2/Keap1 axis, which upregulates the expression of phase-II detoxifying enzymes such as HO-1, NQO1 and glutathione-S-transferase. Although these pathways were not directly analyzed in the present experiments, the coordinated restoration of SOD and GSH observed here is fully compatible with Nrf2 activation reported in previous studies, and may partly explain the strong protective performance of the Cur-NPs.

Comparison with Previous Studies

Several earlier reports have investigated curcumin-based nanosystems for hepatoprotection. Ghosh and colleagues [26] were among the first to demonstrate that curcumin nanoparticles (~20 nm) offered remarkably better protection against CCl_4-induced hepatic injury in rats compared with free curcumin. Later, Abo-Zeid et al. [27] showed that PLGA-encapsulated curcumin nanoparticles attenuated APAP-induced oxidative stress in mice through the activation of the Nrf2/HO-1 pathway. Our present in vitro data are in good agreement with these in vivo observations, and additionally indicate that even a simple surfactant-stabilized nanoprecipitation

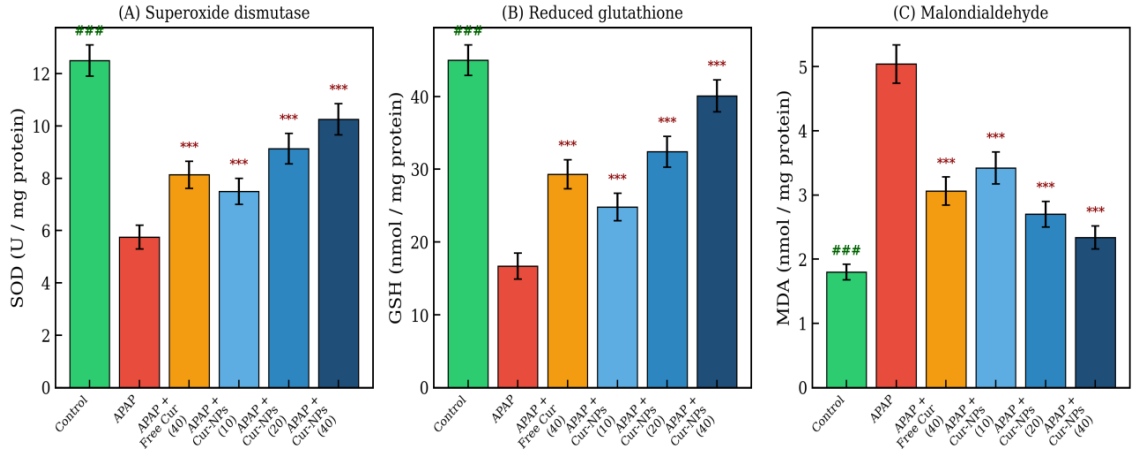

Fig. 5. Effect of free curcumin and Cur-NPs pretreatment on oxidative-stress biomarkers in HepG2-cells challenged with 20 mM APAP for 24 h: (A) superoxide dismutase (SOD), (B) reduced glutathione (GSH), (C) malondialdehyde (MDA). Data are expressed as mean ± SD (n = 3). ### p < 0.001 vs APAP; *** p<0.001 vs APAP-only group.

Table 3. Antioxidant biomarker levels in HepG2-cells after APAP challenge with or without Cur-NPs pretreatment.

Group	SOD (U/mg protein)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Control (untreated)	12.50 ± 0.60	45.00 ± 2.10	1.80 ± 0.12
APAP (20 mM)	5.75 ± 0.45 ###	16.70 ± 1.80 ###	5.04 ± 0.30 ###
APAP + Free Curcumin (40 µg/mL)	8.13 ± 0.52 **	29.30 ± 2.00 **	3.06 ± 0.22 **
APAP + Cur-NPs (10 µg/mL)	7.50 ± 0.50 *	24.80 ± 1.90 *	3.42 ± 0.25 *
APAP + Cur-NPs (20 µg/mL)	9.13 ± 0.58 **	32.40 ± 2.10 **	2.70 ± 0.20 **
APAP + Cur-NPs (40 µg/mL)	10.25 ± 0.60 ***	40.10 ± 2.20 ***	2.34 ± 0.18 ***

Values are mean ± SD (n = 3). ### p < 0.001 vs Control; * p < 0.05, ** p < 0.01, *** p < 0.001 vs APAP-only group (one-way ANOVA followed by Tukey's test).

route can yield formulations with functionally superior performance. A more recent study by Alizadeh et al. [28] on HepG2-cells likewise reported a protective IC_{50} value in the low-µg/mL range after nanoformulation, which is in the same range as the values obtained in the current work. Minor differences between studies can be explained by variations in particle size, surfactant choice, treatment duration and cell confluency.

Despite this promising profile, the HepG2-cell line, a common hepatic in vitro model, does not fully replicate primary human hepatocyte metabolic competence, notably CYP450 expression [29]. Further research using primary cells or metabolically competent HepaRG cells and in vivo validation would strengthen clinical translation. Long-term colloidal stability, tailored distribution to hepatocytes, and Cur-NPs' effects on phase-II drug metabolism should also be studied [30].

Nanonization improves curcumin's drug-induced liver damage protection. Cur-NPs showed negligible intrinsic cytotoxicity, strong antioxidant activities, and considerable cell viability restoration post-APAP treatment, suggesting they may be a promising natural supplementary hepatoprotective drug. This nanoprecipitation process is simple and cheap, thus it can be scaled up in excellent manufacturing practice without polymeric carriers or special equipment. HepG2 is a transformed cell line with changed metabolic pathways, therefore optimal protection in vitro may not match plasma levels in vivo. This work shows Cur-NPs' bioactivity, however more physiologically relevant models are needed to confirm and refine.

CONCLUSION

This study demonstrated that curcumin nanoparticles synthesised via straightforward nanoprecipitation exhibit more cytotoxicity and antioxidant properties compared to free curcumin in the context of acetaminophen-induced toxicity in HepG2-cells. FTIR, XRD, and FESEM confirmed the formation of semi-crystalline, spherical nanoparticles with an average diameter of less than 100nm, while maintaining the chemical integrity of curcumin. Cur-NPs exhibited minimal intrinsic cytotoxicity up to 40µg/mL and effectively safeguarded hepatic cells from APAP-induced injury, reinstating glutathione levels, enhancing SOD-activity, and reducing markers of lipid peroxidation. Nanoscale curcumin formulations can enhance bioavailability and antioxidant activity, indicating a potential adjunctive approach to mitigate drug-induced hepatotoxicity. This work is limited by its single cell line and acute exposure strategy. In vivo study, mechanistic studies, gene-expression analysis, and complete pharmacokinetic evaluations are needed to prove Cur-NPs' clinical significance and therapeutic use.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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