Nanothymoquinone, a novel hepatotargeted delivery system for treating CCl₄ mediated hepatotoxicity in rats

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Thymoquinone (TQ) is the major active principle of Nigella sativa (N. sativa) which is widely being used as a hepatoprotective agent nowadays. However, toxicity at high doses with poor water solubility limit its usage as a therapeutic agent. The idea behind the present study is to design a nanocarrier that exploits the benefit of the antioxidant property of TQ without any toxicity. For this purpose, PAG (p-aminophenyl-1-thio-β-D-galactopyranoside) coated NIPAAM (N-isopropyl acrylamide) nanoparticles are synthesized followed by encapsulation of TQ (NTQ) in their hydrophobic core. PAG is a ligand which directly interacts with asialoglycoprotein receptors (ASGP-R) present on the surface of hepatocytes and delivers the drug directly to the liver. NTQ have a size of ~100 nm and were characterized using IR, NMR, DLS, and TEM. The drug was given in two modes: one as NTQ (3 groups: 0.125 (NTQ₂), 1.25 (NTQ₃) and 12.5 (NTQ₄) µg kg⁻¹ body weight, intraperitoneal (i.p.)) and the other as TQ (12 500 µg kg⁻¹ body weight, i.p.). The best results were obtained with NTQ₂ which is around 1000 times lower than TQ in concentration. Serum and biochemical parameters followed by restoration of histopathology supported this. Expression of inflammatory enzyme COX-2 and NF-kB also gave evidence in support.

Introduction

Chronic injury to liver occurs in response to a variety of harmful effects, including alcohol abuse, drugs, metabolic diseases due to iron or copper overload and autoimmune attack on hepatocytes or bile duct epithelium. CCl₄ administration is a well-recognized model for the production of chemical hepatic injury. It results in the formation of CCl₃ radicals by the hepatic microsomal cytochrome P450 system. The major reactions of CCl₃ with liver microsomes are covalent binding and H-abstraction from polyunsaturated fatty acids (PUFA). The H-abstraction results in CHCl₃ and PUFA formation. PUFA results in cross-linking reactions or lipid peroxidation depending on the O₂ pressure. This CCl₃ also reacts with O₂ rapidly to yield Cl₂COO that causes membrane injury too.

Thus, a promising approach for hepatoprotection is the use of an antioxidant which suppresses the effects of reactive oxygen species (ROS). N. sativa is a very well known plant (in Saudi Arabia) locally known as the black seed, commonly used as a natural food additive. It belongs to the ranunculaceae family, grows in the Middle East, Central Europe and Western Asia. The principal active ingredient isolated from the volatile oil of N. sativa is TQ.6-7 It is known to exhibit a variety of pharmacological actions such as analgesic,8 immunopotentiating,9 anti-inflammatory and anti-oxidant activities10 and is reported to protect organs against oxidative damage induced by a variety of free radical generating agents like doxorubicincardiomyopathy,11 aflatoxin B₁,12 and CCl₄-hepatotoxicity.13 However, toxicity at high doses limits the benefits of its antioxidant activity.14,15 El-Dakhakhany14 and Al-Ali et al.15 reported LD₅₀ values of 10 mg kg⁻¹ and 57.5 mg kg⁻¹ i.p. respectively in rats under different sets of conditions. Since there is difference in LD₅₀ values of TQ under different conditions, it is too difficult to predict the suitable dose to get the benefit of its very good antioxidant properties with low or almost no toxicity.

Here, nano drug delivery systems play an important role as they solve several limitations of conventional drug delivery systems such as nonspecific biodistribution, targeting, lack of water solubility, poor oral bioavailability and low therapeutic indices. Various types of nanocarrier for thymoquinone are already reported in literature that ensure improved properties of thymoquinone when they are administered in nanoform.16-19 The present study involves the synthesis of PAG coated NIPAAM nanoparticles that are encapsulated with TQ for direct hepatotargeting. NIPAAM is a thermosensitive nanopolymer which is widely used as a successful drug delivery system against various diseases20-21 and PAG is a galactosylated moiety that targets the liver22,23 by interacting with asialoglycoprotein receptor (ASGP-R) present on the surface of hepatocytes24 and delivers the drug...
directly to the liver. Previous studies supported that the toxicity of the nanocarrier (NIPAAM) at this concentration is almost negligible. As the size of our particle is smaller than the already reported nanothymoquinone, it gives an added advantage in drug delivery and the drug is directly targeting to the liver as well, so the amount of drug needed for hepatoprotection also is reduced. Thus, it ensures more protection at low concentration with almost no chance of thymoquinone pro-oxidant effect.

**Experimental**

**Materials and method**

Chemicals were purchased from Sisco Research Laboratory, SRL [acryllic acid (AA), ammonium persulphate (APS), glutathione reductase (GR), oxidized glutathione (GSSG), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), sodium hydroxide, trichloroacetic acid (TCA), copper sulphate, ethylene diaminetetraacetate (EDTA), disodium orthophosphate (Na2HPO4), sodium dihydrogen phosphate (NaH2PO4), sodium potassium tartrate, sodium carbonate (Na2CO3), olive oil, phosphate buffer (PBS), hydrogen peroxide (H2O2), Thomas Baker [sodium azide (Na3), ferrous ammonium sulphate (FAS)], Merck [carbon tetrachloride (CCl4)], Spectrochem [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide–hydrochloride (EDC–HCl), thiobarbituric acid (TBA)], SD Fine [epinephrine], Acros [N-isopropyl acrylamide (NIPAAM)], Sigma Aldrich [β-aminophenyl-1-thio-β-D-galactopyranoside (PG), thymoquinone]. All other reagents used were of high purity and were commercially available.

**Preparation of NIPAAM polymeric nanoparticles**

A NIPAAM and AA co-polymer was synthesized through free radical polymerization. Monomers of NIPAAM and AA were dissolved in water and the polymerization was carried out under nitrogen (N2) atmosphere. In order to carry out the experiment, 90 mg of NIPAAM, and 5 μL AA were dissolved in 10 mL distilled water. The dissolved oxygen was removed by passing N2 gas for 45 min. Then, 25 μL of FAS and 35 μL APS solution were added to initiate the polymerization reaction. The polymerization was carried out at 32 °C under N2 atmosphere for 18 h. On completion of polymerization, the polymeric aqueous solution was dialyzed for 48 h using a Spectrope membrane dialysis bag (celluSep®; 12 KD cut off) with continuous change of distilled water after every 4 h. After this, the aqueous solution was lyophilized to obtain dry powder.

**Surface modification of polymeric nanoparticles with PAG**

The conjugation of NIPAAM-co-AA particles with PAG was carried out by the carbamyl-amine reaction. Briefly, 50 mg co-polymeric (NIPAAM-AA) lyophilized powder was dispersed in 10 mL of double distilled water and to this 10 mg PAG was added and allowed to pre-adsorb for 2 h at room temperature. After the pre-adsorb procedure, 0.5 mg of EDC–HCL, a coupling agent for the carbamyl-amine conjugation reaction, was added to the solution described above. The nanoparticle–PAG conjugation reaction was carried out for 4 h with continuous stirring at 25 °C. The resulting PAG conjugated nanoparticles aqueous solution was dialyzed for 12 h using a Spectrope membrane dialysis bag (celluSep®, 12 KD cut off) with continuous change of distilled water after every 4 h. After this, the aqueous solution was lyophilized to obtain dry powder.

**Drug loading**

TQ was purchased from Sigma-Aldrich Chemicals. It was physically entrapped inside the hydrophobic core of the PAG coated co-polymer (NIPAAM-AA) micelles after the complete polymerization reaction. Thus, the process of loading is described as post-polymerization loading. Briefly, 50 mg co-poly(PAG-NIPAAM-AA) micellar lyophilized powder was dispersed in 10 mL of double distilled water. Then, TQ solution in chloroform (5 mg mL−1) was gradually added in the co-polymeric solution and stirred at room temperature till no settling of drug occurred. The drug loaded polymeric nanoparticles were lyophilized to obtain dried powder product for further use.

**Characterization**

**Fourier transform infrared (FTIR) spectroscopy measurement.** FTIR spectra of the nanopolymer showed the bands corresponding to functional groups and the measurements were performed at Jamia Hamdard, New Delhi using a FTS-135, BIO-RAD (USA).

**Nuclear magnetic resonance (NMR).** NMR spectroscopy is one of the principal techniques used to obtain physical, chemical, electronic and structural information about molecules due to the chemical shift on the resonant frequencies of the nuclei present in the sample. Data were recorded at Jamia Hamdard, New Delhi using a Bruker 400 MHz spectrometer.

**Dynamic light scattering (DLS) analysis of encapsulated TQ nanoparticles.** The average size and size distribution of the nanoparticles of co-poly(NIPAAM-AA) micelles were measured by a dynamic light-scattering method using a Nanosize 90ZS (Malvern Instruments, Worcestershire, UK). The lyophilized powder was dispersed in aqueous solution. They were measured at an average value of 12 runs with triplicate measurements within each run.

**Transmission electron microscopy (TEM) of encapsulated TQ nanoparticles.** Size and morphology of the nanoparticles were determined using TEM as follows: one drop of the aqueous dispersion of co-polymeric nanoparticles was put on a carbon-coated copper grid and then air-dried in a vacuum desiccator. The dried grid was examined under a TEM. (JEOL JEM 2000 EX 200 model from the All India Institute of Medical Sciences, New Delhi, India).

**Entrapment efficiency (E%)**

The entrapment efficiency (E%) of thymoquinone loaded in nanoparticles was determined as follows: the nanoparticles were separated from the unentrapped (free) thymoquinone using a NANOSEP (100 kD cut off) membrane filter and the amount of free thymoquinone was measured spectrophotometrically at 296 nm. The E% was calculated as

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**Paper Journal of Materials Chemistry B**

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\[ E% = \frac{\text{thymoquinone}_{\text{total}} - \text{thymoquinone}_{\text{free}}}{\text{thymoquinone}_{\text{total}}} \times 100 \]

where thymoquinone_{total} is the total amount of thymoquinone loaded in the nanoparticle and thymoquinone_{free} is the total amount of unentrapped thymoquinone in the solution.

**In vitro release kinetics of nanoquercetin**

A known amount of thymoquinone encapsulating lyophilized polymeric nanoparticles (100 mg) was dispersed in 10 ml phosphate buffer (pH 7.4) and the solution was divided into 10 microcentrifuge tubes (1000 μl each) and placed on a thermostable water bath at 37 °C. Free thymoquinone is completely insoluble in water. Thus, at specific intervals of time, the solution was centrifuged at 3000 rpm for 10 minutes to separate the released thymoquinone from the nanoparticles which was redissolved in 1 ml of CHCl₃ and the absorbance was measured spectrophotometrically at 296 nm. The concentration of the released drug was then calculated using the standard curve of thymoquinone in CHCl₃.

The percentage of thymoquinone released was determined as:

\[ \text{Release (\%)} = \frac{\text{[thymoquinone]_{release}}}{\text{[thymoquinone]_{total}}} \times 100 \]

where [thymoquinone]_{release} is the concentration of released thymoquinone collected at time \( t \) and [thymoquinone]_{total} is the total amount of thymoquinone entrapped in the nanoparticles.

**Animals.** Male Wistar rats weighing 180–200 g and 6–8 weeks old were obtained from the Central Animal House Facility of Jamia Hamdard University, New Delhi. Rats were housed in an animal care facility at room temperature (25 ± 1 °C) with 12 h light–dark cycles and were given free access to standard pellet diet and tap water. Animals received humane care in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and prior approval was sought from the Institutional Animal Ethics Committee (IAEC no: 173/CPCSEA, 28 January 2000).

**Experimental procedure.** During our present study, we evaluated the preventive efficacy of TQ and NTQ against CCl₄-induced hepatic toxicity. Animals were divided into six groups each having 6 animals and they received the treatment as follows: Group I (N): normal (saline for 7 days i.p.), Group II (D): CCl₄ treated (CCl₄ on 3rd and 4th day, 1.2 ml kg⁻¹, subcutaneous (s.c.)) + diet/water, Group III (NTQ₁): nanothymoquinone low dose (0.125 μg kg⁻¹ body weight, i.p.) + CCl₄ + diet/water, Group IV (NTQ₂): nanothymoquinone medium dose (1.25 μg kg⁻¹ body weight, i.p.) + CCl₄ + diet/water, Group V (NTQ₃): nanothymoquinone high dose (12.5 μg kg⁻¹ body weight, i.p.) + CCl₄ + diet/water, Group VI (TQ): thymoquinone (12500 μg kg⁻¹ body weight, i.p.) + CCl₄ + diet/water. TQ and NTQ (NTQ₁, NTQ₂, and NTQ₃) were administered intraperitoneally for 7 days. Hepatotoxicity was induced in the II, III, IV, V and VI groups by an injection of CCl₄ (1.2 ml kg⁻¹, 1:1 with olive oil s.c.) on the 3rd and 4th days.

At the end of the experiment blood was obtained from all groups of rats by puncturing the retro-orbital plexus. The blood samples were allowed to coagulate for 45 min at room temperature. Serum was separated by centrifugation at 3000 rpm at room temperature for 20 min and used for the biochemical estimation. Rats were sacrificed by cervical dislocation under mild anesthesia and livers were taken at the same time for biochemical, histopathological and immunohistochemistry studies.

**Biochemical estimations**

**Assay of aspartate aminotransferase (AST).** The AST assay in serum was done by using a Span Diagnostic Ltd. Kit, India.

**Assay of alanine transaminase (ALT).** The ALT assay in serum was done by using a Span Diagnostic Ltd. Kit, India.

**Assay of alkaline phosphatase (ALP).** The ALP assay in serum was done by using a Span Diagnostic Ltd. Kit, India.

**Assay of lactate dehydrogenase (LDH).** The LDH assay in serum was done by using a CREST Biosystem kit, India.

**Assay for lipid peroxidation (LPO).** LPO was estimated by measuring the thiobarbituric acid reactive substances (TBARS) in accordance with the method of Utley et al. with some modifications. Briefly, 0.1 ml serum was pipetted into a 2.0 ml flat bottomed Eppendorf tube and incubated at 37 °C in a metabolic water bath shaker stirring up and down. Another 0.2 ml of the same serum was pipetted into an Eppendorf tube and placed at 0 °C for incubation. After 1 h of incubation, 0.5 ml of 10% TCA and 0.5 ml of 0.67% TBA were added to both samples (at 0 °C and 37 °C). The reaction mixture was centrifuged at 3000 g for 15 min. The supernatant was transferred to another test tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535 nm. The rate of LPO was expressed as μmol of TBARS formed h⁻¹ per g tissue using a molar extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹.

**Assay for reduced glutathione (GSH).** GSH was determined as described by Jollow et al. 0.1 ml PMS was precipitated with 0.1 ml of sulfosalicylic acid (4%). The samples were kept at 4 °C for 1 h and then subjected to centrifugation at 1200g for 15 min at 4 °C. The assay mixture contained 0.1 ml of filtered aliquot, 1.7 ml PB (0.1 M, pH 7.4) and 0.2 ml DTNB (4 mg ml⁻¹, 0.1 M PB, pH 7.4) in a total volume of 2.0 ml. The yellow color developed was read immediately at 412 nm. The GSH level was calculated as μmol GSH conjugate formed per min per mg protein, using a molar extinction coefficient of 13.6 × 10⁴ M⁻¹ cm⁻¹.

**Assay of glutathione-S-transferase (GST) activity.** GST activity was measured by the method of Habig et al. The reaction mixture consisted of phosphate buffer (0.1 M, pH 6.5), reduced glutathione (1 mM), CDNB (1 mM), and PMS in a total volume of 1.0 ml. The change in absorbance was recorded at 340 nm and enzyme activity was calculated as nmol of CDNB conjugate formed per min per mg protein, using a molar extinction coefficient of 9.6 × 10⁴ M⁻¹ cm⁻¹.

**Determination of glutathione reductase (GR) activity.** GR activity was assayed by the method of Carlberg and Mannervik as described by Mohandas et al. The assay system consisted of 0.1 M PB pH 7.6, 0.1 mM NADPH, 0.5 mM EDTA, 1.0 mM GSSG.
and 0.1 ml PMS in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmole NADPH oxidized per min per mg protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

**Determination of glutathione peroxidase (GPx) activity.** GPx activity was measured according to the procedure of Mohandas et al. The reaction mixture consisted of 0.05 M PB pH 7.0, 1.0 mM EDTA, 1.0 mM sodium azide, 1.4 U of 0.1 ml GR, 1.0 mM glutathione, 0.2 mM NADPH, 0.25 mM hydrogen peroxide and 0.1 ml PMS in a final volume of 2.0 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmole NADPH oxidized per min per mg protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

**Determination of catalase activity.** Catalase activity was assayed by the method of Claiborne et al. Briefly, the assay mixture consisted of 0.05 M PB (pH 7.0), 0.019 M hydrogen peroxide, and 0.1 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of mmole H$_2$O$_2$ consumed per min per mg protein using a molar extinction coefficient of $40 \text{ M}^{-1} \text{ cm}^{-1}$.

**Determination of superoxide dismutase (SOD) activity.** SOD activity was measured spectrophotometrically according to the method of Stevens et al. by monitoring the auto-oxidation of (−)-epinephrine at pH 10.4 for 3 min at 480 nm. The reaction mixture contained glycine buffer (50 mM, pH 10.4) and 0.2 ml PMS. The reaction was initiated by the addition of (−)-epinephrine. The enzyme activity was calculated in terms of nmol (−)-epinephrine protected from oxidation per min per mg protein using a molar extinction coefficient of $4.02 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

**Determination of protein.** Protein was determined by the method of Lowry et al. using bovine serum albumin (BSA) as standard.

**Histological examination.** Livers were removed and kept in 10% neutral buffered formalin solution. They were processed and embedded in paraffin wax. Sections of 5 μm thickness were cut using a microtome, dewaxed, stained with hematoxylin and eosin and finally images were captured under a microscope (Labovision, India). The liver sections were examined for arrangement of hepatocytes and inflammation around the portal triad (PT) and central vein (CV) in hepatic parenchyma.

**Immunohistochemistry of COX-2 and NF-kB.** Immunohistochemistry was performed to detect the expression of COX-2 and NF-kB proteins. Liver sections (5 μm thick) were dewaxed and processed for immunohistochemical staining. The sections were collected on gelatin coated slides and dipped in 3% H$_2$O$_2$ (in methanol) for 20 min in order to eliminate the endogenous peroxidase activity. They were washed with PBS several times and incubated in 1% bovine serum albumin for 45 min and then incubated with primary antibody: anti-COX-2 polyclonal (dilution 1 : 100) or anti-NF-kB monoclonal (dilution 1 : 100) for 60 min. Then, sections were incubated with universal HRP polymer detection (kit from Neomarker). Slides were finally treated with 3,4-diaminobenzidine, washed, dried and mounted with DPX and studied under a microscope (Labovision, India).

**Statistical analysis.** The level of significance between different groups is based on the analysis of variance test followed by Dunnett’s test. The $P$-values of less than 0.05 were considered significant.

**Results**

**Fourier transform infrared (FTIR) spectroscopy studies of PAG coated NIPAAM nanoparticles**

As seen in Fig. 1, peaks in the range of 800–1000 cm$^{-1}$ corresponding to the stretching mode of vinyl double bonds disappeared in the spectrum of the polymer, indicating that the...
polymerization has taken place (marked by the circle in the lower IR spectrum). Peaks at 1642 and 1540 cm\(^{-1}\) correspond to the amide carbonyl group stretching and the bending frequency of the amide N–H group respectively. The peaks at 2936–2969 cm\(^{-1}\) correspond to –CH stretching vibration of the polymer backbone. Absorption bands in the region 1443–1457 cm\(^{-1}\) are due to the bending vibration of CH\(_2\) group and the bending vibration of CH\(_3\) group can be identified in a slightly higher wavelength region. In the lower IR spectrum (marked as NIPAAM-AA) there is a peak at 1720 cm\(^{-1}\) corresponding to COOH group of acrylic acid (AA) attached in the copolymer. However, on addition of PAG to the polymer, the peak corresponding to COOH disappeared (marked as NIPAAM-AA-PAG) as the NH\(_2\) groups of PAG reacted with COOH groups of AA to form CONH bonds. The absence of this COOH peak indicates the attachment of PAG to NIPPAAM-AA polymer (Fig. 1, upper IR spectrum).

**Nuclear magnetic resonance (NMR) studies of PAG coated NIPAAM nanoparticles**

Fig. 2 shows the typical \(^1\)H-NMR spectrum and the chemical shift assignments of the copolymer formed. Polymerization is
confirmed by the absence of the proton resonance of the vinyl end groups of the monomers in the spectrum of the formed copolymeric micelle. The resonance observed at the upfield region ($\delta = 1.4–1.9$ ppm) is attributed to the saturated protons of the polymeric network and the broad resonance peak at $\delta = 0.8–1.0$ ppm is from the methyl protons of the isopropyl group. The signal peaks for the methyne proton ($-\text{CH}-$) of the NIPAAM group can be observed at 3.91 ppm along with a peak at 11.994 ppm corresponding to COOH of AA (Fig. 2A). On addition of PAG to the polymer, this peak corresponding to COOH disappeared (Fig. 2C) due to the reaction between free COOH of AA and NH$_2$ groups of PAG to form CONH. This confirmed the conjugation of PAG to polymer which is supported by IR as well. Broad peaks in the regions of 2.0 ppm and 3.0–3.5 ppm correspond to the $\sim$OH group and galactoside protons of PAG.

**Dynamic light scattering (DLS) analysis of encapsulated TQ nanoparticles**

Fig. 3 shows the DLS pattern of encapsulated TQ nanoparticles. The average size was found to be 108.5 nm from DLS experiment.

**Transmission electron microscopy (TEM) of encapsulated TQ nanoparticles**

Fig. 4 shows the TEM of encapsulated TQ nanoparticles. The average size was found to be around 90.00 nm with spherical morphology.

![Fig. 3 DLS pattern of encapsulated TQ nanoparticles.](image)

![Fig. 4 TEM analysis of encapsulated TQ nanoparticles.](image)

**Entrapment efficiency and in vitro release kinetics**

The entrapment efficiency of thymoquinone in the nanoparticles was found to be $\sim 90\%$. Thymoquinone loaded nanoparticles (NTQ) show a sustained release of thymoquinone from nanopolymer at a physiological pH of 7.4 in phosphate buffer (Fig. 5).

**Effects of TQ and NTQ on body and liver weight**

Table 1 shows the body weights of the normal and treated animals with different treatment regimes for 7 days. After 7 days of study, rats treated with CCl$_4$ only (Group II, D) showed an increase in body weight ($236.67 \pm 3.33$) when compared with normal rats Group I, N ($213.33 \pm 3.33$). However, the body weights of rats of Group III (low dose of NTQ) i.e. NTQL ($223.33 \pm 3.33$), Group IV (medium dose of NTQ) i.e. NTQM ($216.67 \pm 3.33$) and Group V (high dose of NTQ) i.e. NTQH ($216.67 \pm 3.33$) and Group VI, TQ ($220.00 \pm 0.00$) show decreases in weight, but the decrease in weight was more significant in NTQH along with TQ. Similar results were observed for liver weights (Table 1).

**TQ and NTQ decreased the TBARS level in liver**

TBARS was measured to demonstrate the oxidative damage on lipid peroxidation in CCl$_4$ induced liver injury of wistar rats. A significant increase in TBARS level was found in CCl$_4$ treated group, D ($2.55 \pm 0.018$) when compared with normal, N ($1.36 \pm 0.020$). We have observed that treatment with both TQ and NTQ leads to a decrease in TBARS level, but decreases were more significant in the case of the highest dose of NTQH ($1.45 \pm 0.011$), followed by TQ ($1.51 \pm 0.014$), and finally NTQM ($1.78 \pm 0.013$) and NTQL ($2.22 \pm 0.045$) (Table 1).

**Liver GSH content and antioxidant level restoration**

Animals subjected to CCl$_4$ treatment (D) showed significant decreases in the activities of all glutathione metabolizing enzymes, viz., GST ($29.13 \pm 0.39$), GR ($134.88 \pm 4.82$) and GPx ($109.47 \pm 3.2$) when compared with normal rats (N) GST
Table 1  Effects of treatment with TQ and NTQ on body weight, liver weight, lipid peroxidation and protein content in CCl₄ induced toxicity model

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Body weight [in g]</th>
<th>Liver weight [in g]</th>
<th>Lipid peroxidation [μmole TBARS formed h⁻¹ per g tissue]</th>
<th>Protein (mg per 100 μl tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I saline (normal, N)</td>
<td>213.33 ± 3.33</td>
<td>8.60 ± 0.20</td>
<td>1.36 ± 0.020</td>
<td>0.870 ± 0.022</td>
</tr>
<tr>
<td>Group II, D – CCl₄ (1.20 ml kg⁻¹, 1 : 1 with oil s.c.)</td>
<td>236.67 ± 3.33</td>
<td>11.72 ± 0.39</td>
<td>2.55 ± 0.018</td>
<td>1.430 ± 0.021</td>
</tr>
<tr>
<td>Group III – CCl₄ + NTQ₄ (0.125 μg kg⁻¹, i.p.)</td>
<td>233.33 ± 3.33</td>
<td>10.67 ± 0.12</td>
<td>2.22 ± 0.045</td>
<td>1.325 ± 0.053</td>
</tr>
<tr>
<td>Group IV – CCl₄ + NTQ₅ (1.25 μg kg⁻¹, i.p.)</td>
<td>223.33 ± 3.33</td>
<td>9.940 ± 0.13</td>
<td>1.78 ± 0.013</td>
<td>1.160 ± 0.024</td>
</tr>
<tr>
<td>Group V – CCl₄ + NTQ₆ (12.5 μg kg⁻¹, i.p.)</td>
<td>216.67 ± 3.33</td>
<td>8.833 ± 0.07</td>
<td>1.45 ± 0.011</td>
<td>0.939 ± 0.021</td>
</tr>
<tr>
<td>Group VI – CCl₄ + TQ (12 500 μg kg⁻¹, i.p.)</td>
<td>220.00 ± 0.00</td>
<td>9.140 ± 0.12</td>
<td>1.51 ± 0.014</td>
<td>1.050 ± 0.026</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM of six animals per group. Results obtained are significantly different from control group (**P < 0.01). Results obtained are significantly different from TQ and NTQ treated group (**P < 0.01 and NS P > 0.05). TQ = thymoquinone; NTQ₄ = nanothymoquinone, low dose; NTQ₅ = nanothymoquinone, medium dose; NTQ₆ = nanothymoquinone, high dose.

Table 2  Results of treatment with TQ and NTQ on antioxidant enzymes like GSH, GST, GR, GPx, catalase and SOD on CCl₄ induced liver redox imbalance

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>GSH (μmol GSH min⁻¹ per mg protein)</th>
<th>GST (nmol CDNB conjugate formed min⁻¹ per mg protein)</th>
<th>GR (nmol NADPH oxidized min⁻¹ mg protein)</th>
<th>GPx (nmol NADPH oxidized min⁻¹ per mg protein)</th>
<th>Catalase (μmol H₂O₂ consumed min⁻¹ per mg protein)</th>
<th>SOD (μmol epinephrine protected from oxidation min⁻¹ per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I normal, N (saline)</td>
<td>44.13 ± 1.054</td>
<td>126.69 ± 1.54</td>
<td>577.7 ± 4.0</td>
<td>462.08 ± 3.92</td>
<td>35.32 ± 1.86</td>
<td>168.2 ± 1.57</td>
</tr>
<tr>
<td>Group II – CCl₄, D (1.20 ml kg⁻¹, 1 : 1 with oil s.c.)</td>
<td>12.20 ± 0.18</td>
<td>29.130 ± 0.39</td>
<td>134.88 ± 4.82</td>
<td>109.47 ± 3.2**</td>
<td>8.077 ± 0.43</td>
<td>46.030 ± 0.72</td>
</tr>
<tr>
<td>Group III – CCl₄ + NTQ₄ (0.125 μg kg⁻¹, i.p.)</td>
<td>14.70 ± 0.35</td>
<td>40.390 ± 0.75**</td>
<td>172.90 ± 2.02**</td>
<td>156.83 ± 3.48**</td>
<td>9.850 ± 0.00**</td>
<td>58.720 ± 0.79**</td>
</tr>
<tr>
<td>Group IV – CCl₄ + NTQ₅ (1.25 μg kg⁻¹, i.p.)</td>
<td>18.79 ± 0.42</td>
<td>56.610 ± 0.51**</td>
<td>252.87 ± 3.32**</td>
<td>212.67 ± 2.3**</td>
<td>14.07 ± 0.54**</td>
<td>76.890 ± 0.00**</td>
</tr>
<tr>
<td>Group V – CCl₄ + NTQ₆ (12.5 μg kg⁻¹, i.p.)</td>
<td>38.43 ± 0.62</td>
<td>100.78 ± 0.62**</td>
<td>466.00 ± 9.65**</td>
<td>390.08 ± 7.29**</td>
<td>28.061 ± 1.47**</td>
<td>136.65 ± 0.82**</td>
</tr>
<tr>
<td>Group VI – CCl₄ + TQ (12 500 μg kg⁻¹, i.p.)</td>
<td>33.44 ± 0.16</td>
<td>90.010 ± 1.26**</td>
<td>398.81 ± 8.78**</td>
<td>348.26 ± 3.4**</td>
<td>22.87 ± 0.79**</td>
<td>121.42 ± 1.32**</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM of six animals per group. Results obtained are significantly different from control group (**P < 0.01). Results obtained are significantly different from TQ and NTQ treated group (**P < 0.01 and NS P > 0.05). TQ = thymoquinone; NTQ₄ = nanothymoquinone, low dose; NTQ₅ = nanothymoquinone, medium dose; NTQ₆ = nanothymoquinone, high dose.

(126.69 ± 1.54), GR (577.7 ± 4.0) and GPx (462.08 ± 3.92) (Table 2). GSH content also decreased on CCl₄ treatment, D (12.20 ± 0.18), when compared with normal group, N (44.13 ± 1.05). This depletion of GSH content led to the disruption of other oxidative pathway enzymes like catalase (8.077 ± 0.43) and SOD (46.03 ± 0.72) in CCl₄ treated group, D. TQ and NTQ treatment restored the levels of all glutathione dependent enzymes, but NTQ₆ treatment shows the best results followed by TQ (Table 2).

Effects of TQ and NTQ on serum enzymes AST, ALT, ALP and LDH

Protective effects of TQ and NTQ on serum enzymes like AST, ALT, ALP and LDH levels were observed. On CCl₄ treatment, there is a significant rise in these parameters (D) when compared with normal rat group (N). Treatment with TQ and NTQ was found to be significantly effective in the normalization of these markers when compared to CCl₄ treated group but results were more significant in the case of NTQ₆ along with TQ (Fig. 6).

Histopathological findings

Histological sections of liver (H and E stained) from hepatotoxic group (D) show cell damage in the centrilobular area, vacuolated hepatocytes and inflammatory cells around the central vein, CV (Fig. 7, D-A, D-B). NTQ treated group shows some inflammation around PT and vaculation of hepatocytes in mid and central zones in hepatic parenchyma (Fig. 7, NTQ₄-A, NTQ₅-B, NTQ₆-A, NTQ₇-B, NTQ₈-A and NTQ₉-B). However, NTQ₆ and TQ treated groups show normal arrangement of cells around the central vein (Fig. 7, TQ-A, TQ-B).
Immunohistochemical expression of COX-2 and NF-κB

The immunohistological studies demonstrate that CCl₄ mediated liver toxicity activates a number of inflammatory molecules like COX-2 and NF-κB that result in the progression of cell death. COX-2 enzyme is an important mediator for inflammation. Fig. 8 shows the up-regulation of COX-2 in CCl₄ treated group (D) when compared with normal group (N). The increased expression is observed to be lowered in both thymoquinone (TQ) and nanothymoquinone groups (NTQL, NTQM, NTQH). However, the expression is significantly lowered in NTQH followed by TQ.

NF-κB is a prominent regulator of inflammation and acts as a transcription regulator of inflammatory proteins. Fig. 9 shows the up-regulation of NF-κB in CCl₄ treated group (D) when compared with normal group (N). The increased expression is observed to be lowered in NTQH and TQ groups but with slight positive expression in NTQM and NTQL.

The results confirmed that thymoquinone is a strong anti-inflammatory molecule which regulates the activity of NF-κB and COX-2 significantly in order to down-regulate other pro-inflammatory enzymes.

Discussion

Liver injuries caused by CCl₄ are the best-characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of antihepatotoxic/hepatoprotective activity of drugs. CCl₄ formed from CCl₃ metabolism is the crucial factor involved in pathogenicity of CCl₄ hepatotoxicity. It causes significant increase in liver weight, serum levels of ALT, AST, ALP, centrilobular hepatocellular vacuolar degeneration and necrosis. A previously reported study supports the role of TQ in controlling the oxidative stress through its antioxidant potential and superoxide anion radical scavenging ability in vitro and in vivo. The hepatoprotective activity is also supported by the fact that it prevents the depletion of intracellular glutathione and thus maintains the integrity of cell membranes as evidenced by decreased leakage of ALT and AST in tert-butyl hydroperoxide-induced hepatotoxicity.

But, at higher dose TQ activity gets reversed, i.e. at higher concentration it becomes hepatotoxic instead of hepatoprotectant. It is reported that higher doses of TQ are lethal like other quinones due to its reaction with cellular nucleophiles, including –SH groups of some essential compounds, such as proteins and GSH either spontaneously or catalyzed by glutathione S-transferase. Al-Shabanah et al. showed that the reduced glutathione content of tissues of liver, heart and kidney significantly decreased after oral administration of TQ (2 and 3 g kg⁻¹ body weight). GSH is an important tripeptide and helps in non-enzymatic reduction of free radicals, removal of peroxides via selenium-dependent GSH peroxidase and conjunction of exogenous reactive intermediates either non-enzymatically or catalyzed by glutathione S-transferase. Depletion of cellular GSH reserve in vital organs is lethal and leads to oxidative stress too. Toxicity was also supported by LD₅₀ study of TQ which in rats varies from 10 mg kg⁻¹ to 57.5 mg kg⁻¹ intraperitoneally based on the type of medium used to give TQ.

So, in support of the above findings, a hepatoprotective study mediated by TQ was carried out using nanocarriers. In the present study, TQ was given in two modes, one as naked drug (1% tween80 in water) and another as encapsulated TQ (NTQ)
Depletion in GSH levels (a natural cellular antioxidant) is suggestive of injury by foreign toxic agents as it is being utilized by the hepatocytes in scavenging toxicant metabolites.\(^5\) It has been previously reported that CCl\(_4\) administration leads to changes in membrane functionality and morphology.\(^5\) AST, ALT and ALP are the enzymes that are sensitive to this kind of hepatocellular injury. Release of large quantities of these enzymes in the bloodstream is associated with centrilobular necrosis, degeneration, and reduced performance status of the liver. It has been previously reported that CCl\(_4\) results in increases of these serum markers\(^5,44\) and they are significantly restored by TQ administration at high dose.\(^4\) The present study also demonstrates the same, as these markers are increased in the CCl\(_4\) treated group (Fig. 6, D) and their level is decreased in TQ (Fig. 6, TQ) and NTQH (Fig. 6, NTQH, NTQM, NTQL) treated groups. The decrease is remarkable in NTQH but it is also significant in the TQ treated group.

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oxidative stress that results in decrease in GSH content and the activities of enzymes like GPx, GR and GST\(^{54,55}\) and also of SOD and catalase in liver.\(^{5,47}\) Ismail \textit{et al.}\(^{48}\) and Sheikh and Mohamad\(^{49}\) reported that the GSH content and the activity of these antioxidant enzymes are very much restored by TQ administration. Similar findings are observed in our study as well; the level of GSH and the activity of antioxidant enzymes are clearly restored in NTQ and TQ (Table 2).

TBARS is an indirect indicator of lipid peroxidation of PUFA of membranes. Administration of CCl\(_4\) results in abstraction of H’ from PUFA and forms PUFA.\(^{1} \) This PUFA together with Cl\(_4\)COO results in lipid peroxidation. In support of the previous findings, the present study also shows a decrease in the level of TBARS on TQ administration after CCl\(_4\) mediated hepatotoxicity.\(^{50}\) However, the decrease is more significant in NTQ at highest dose (NTQ\(_{H}\)) followed by TQ, NTQ\(_{M}\) and NTQ\(_{L}\) (Table 1).

Histopathology is a parameter that clearly provides information about the well being of organ morphology and ultimately its functionality. Administration of a toxicant like CCl\(_4\) results in disruption of the whole liver morphology\(^{5,44}\) and administration of an antioxidant like TQ leads to its recovery.\(^{5,51}\) Similar findings are observed in our study as CCl\(_4\) administration clearly results in vacuolation of hepatocytes, centrilobular necrosis, degeneration and inflammation around the CV and PT which is very much restored in NTQ\(_{H}\) along with TQ followed by NTQ\(_{M}\) and NTQ\(_{L}\) (Fig. 7).

Cyclo-oxygenase-2 (COX-2), the key inducible enzyme is responsible for producing prostanoids and nuclear factor-kappa B (NF-kB) activation pathway which regulates the transcription of inflammatory genes. It has already been reported that CCl\(_4\) administration results in an increase in the expression of COX-2\(^{52}\) and is restored to a large extent on TQ treatment.\(^{53}\) The present study also supports these findings as administration of CCl\(_4\) results in an elevation of COX-2 expression, D, and it is clearly restored to normal in the NTQ\(_{H}\) treated group along with TQ (Fig. 8). However, NTQ restores the COX-2 expression to almost normal at a comparatively very low concentration (NTQ\(_{L}\), NTQ\(_{M}\), NTQ\(_{H}\), Fig. 8).

NF-kB is a ubiquitous transcription factor consisting of p50, p65 and I\(\kappa\)B\(\alpha\) that resides in the cytoplasm and is activated in response to various inflammatory stimuli. Administration of a toxicant like CCl\(_4\) cause activation and enhances expression of NF-kB in hepatocytes.\(^{52,54}\) It is reported that TQ suppressed the NF-kB activation pathway through modulation of the p65 subunit of NF-kB and inhibition of IKK, down-regulated the NF-kB regulated gene products involved in cell survival, proliferation and resulted in apoptosis.\(^{55}\) The present study also supports this as expression of NF-kB clearly decreases in the TQ treated group after CCl\(_4\) administration, D. However, the expression is restored near to normal in the NTQ\(_{H}\) treated group indicating that the nanocarrier gives better results than naked TQ and at a very low dose level (Fig. 9).

### Conclusion

The present study clearly demonstrates that the nanoparticles are able to carry bulk amounts of drug to the liver, and their direct targeting to ASGP-R receptors present on hepatocytes results in significant hepatoprotection at such a low dose level (1000 times lower than the naked TQ). Thus, in view of the above findings, this nanocarrier ensures a promising approach to provide significant protection against various liver diseases in the future.

### Acknowledgements

The authors are grateful to the Hon’ble Vice-Chancellor Dr. G. N. Qazi of Jamia Hamdard (Hamdard University) and Department of Science and Technology, government of India, for providing financial support to carry out the study.

### References
