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Protective efficacy of crocetin and its nanoformulation against cyclosporine A-mediated toxicity in human embryonic kidney cells

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ABSTRACT

Aim: This study is aimed to formulate crocetin-loaded lipid Nanoparticles (NPs) and to evaluate its antioxidant properties in a cyclosporine A-mediated toxicity in Human Embryonic Kidney (HEK-293) cells in vitro.
Main methods: Crocetin-loaded NPs were prepared followed by physicochemical characterization. In vitro protective efficacy of crocetin and crocetin loaded NPs was investigated in cyclosporine A-mediated toxicity in HEK-293 cells by assessing free radical scavenging, DNA Nicking, cytotoxicity, intracellular Reactive oxygen species (ROS) inhibition, Mitochondrial membrane potential (MMPs) loss and evaluating the activity and expression of antioxidant enzymes and non-enzyme level. Further, we have studied the mechanism of protective activity of crocetin either native or in NPs by studying the expression of phase II detoxifying proteins (HO-1) via Nrf2 mediated regulation.
Key findings: Our results showed that pretreatment with crocetin and crocetin-loaded NPs attenuated the cyclosporine A-mediated toxicity, ROS production and exhibited enhance free radical scavenging ability and cytoprotective activity. Further, the treatment prevented MMPs loss by directly scavenging the ROS and restored the antioxidant enzyme network with normalization of heme oxygenase-1 (HO-1) expression by inhibiting nuclear translocation of Nrf2.
Significance: Pretreatment of crocetin and crocetin-loaded NPs provided pronounce protective effect against cyclosporine A-mediated toxicity in HEK-293 cells by nullifying the ROS formation and restored antioxidant network through inhibition of Nrf2 translocation and followed by expression of HO-1. Such an approach may be anticipated to be beneficial for antioxidant therapy.

1. Introduction

Cyclosporine A (CsA) is an effective immunosupressor commonly used in organ transplantation and in the treatment of autoimmune disorders during the past 30 years [1]. Cyclosporine exerts its immunosuppressive action by inhibiting the enzyme calcineurin phosphatase and thereby inhibits the expression of cytokines such as IL2 and IFN ϒ and subsequently T cell proliferation [2–4]. However, extensive use of CsA leads to severe side effects such as nephrotoxicity, hepatotoxicity and cardiotoxicity [5,6]. Several lines of evidences indicate towards the role of ROS and decreased antioxidant enzyme activity as one of the major culprits of nephrotoxicity induced by CsA [7,8]. To this end, the antioxidant therapy is an effective strategy, which possesses the ability to protect different vital organs, from damage caused by the ROS. In this context current research is now focusing on natural food derived antioxidants owing to their therapeutic values, no side effects and of their economic availability [9].

Crocetin (8,8′-diapocarotene-8,8′-dioic acid) is a bioactive low molecular weight natural carotenoid compound which originates from saffron stigmas (*Crocus sativus* L.) and gardenia fruit (*Gardenia jasminoides* Ellis) mostly used for the treatment of various diseases in traditional and modern medicine [10]. Crocetin is reported to have a wide range of pharmacological properties, including anticancer [11], antihyperlipidemia [12], anti-atherosclerosis [13] activities, however, its mechanism of action is not clear. In addition to this, study have shown on numerous in vitro and in vivo animal models yet also confirmed that crocetin can also ameliorate the effect of oxidative stress due to its potential antioxidant activity [14]. It can inhibit ROS production and inflammatory cascades towards amelioration of cardiac injury caused by haemorrhage/resuscitation [15]. Ahmad et al. [16] reported that the

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protective effect of crocetin against hemi-parkinsonian in a rat model might be due to reduction in auto-oxidation of dopamine by augmentation of antioxidant enzymes like Glutathione Peroxidase (GPx), Superoxide dismutase (SOD), Glutathione S-transferase [16]. However, despite its several therapeutic applications its pharmacological activities in clinical settings were hindered due to the oxidative degradation of the drug by different external factors which, promotes isomerization of trans-form to cis-form rendering the drug inactive [17,18].

In this regard, novel drug delivery system (NDDS) can significantly improve the ability of drugs in terms of efficacy, solubility, half-life in blood and bioavailability [19]. One of the important NDDS is nanoparticle-based drug delivery system, which is emerging as a highly promising technology in escalating drug delivery by improving the drug pharmacokinetics and enabling to overcome the drawbacks of native drugs. By using nanoparticulate drug delivery system different groups have shown that solubility, bioavailability and therapeutic efficacy of various water insoluble herbal compounds such as ellagic acids [20], quercetin [21], naringenin [22], kaempferol [23] and Coenzyme Q10 [24] can be improved. In this regards, lipid based nanoparticulate systems have drawn significant attention due to their small and narrow size range (10–200 nm), low intrinsic cytotoxicity and biodegradability. Recently much attention was given to glycerol monooleate (GMO), a synthetic non-toxic biodegradable and biocompatible material classified as GRAS (generally recognized as safe) and it is included in the FDA inactive ingredients guide for formulating lipid based drug delivery systems [25].

Here, we have formulated crocetin-loaded lipid NPs to evaluate the antioxidant properties in a CsA-mediated toxicity in Human Embryonic Kidney (HEK-293) cells in vitro. Wide spectrum of in vitro assay was conducted to study the antioxidant potential and protective role of crocetin in crocetin-loaded Nanoparticles (NPs) as compared to native counterpart. We have further studied the mechanism of protection by crocetin either in native or loaded in NPs by studying the expression of phase II detoxifying proteins (HO-1 expression) via Nrf2-mediated regulation in in vitro.

2. Materials and methods

2.1. Materials

Crocetin was purchased from MP, Biomedicals, Inc. (Germany). Polyvinyl alcohol (PVA, average MW = 31,000-50,000), 3-(4, 5-dimethylthiazol 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Pluronic F-127, Diphenyl picrylhydrazyle (DPPH), Sodium nitroprusside, L-Methionine, Triton X-100, hydroxylamine hydrochloride, sulfanilamide, naphthyl ethylenediaminedi hydrochloridein (NED) and all other chemicals used were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification. GMO was procured from Eastman (Memphis, TN). pBR 322 plasmid DNA was obtained from Invitrogen (CA, USA). GPx and Glutathione reductase (GR) antibodies primary were obtained from Abcam (Abcam, Cambridge, MA, USA). Primary Nrf2 and HO-1 and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.2. Preparation of crocetin-loaded NPs

Crocetin-loaded NPs was prepared by a minor modification of our previous protocol [25]. Briefly, 20 mg of crocetin was vortexed with 350 µl of GMO at room temperature. The GMO mixture was emulsified with 5 ml of PVA (0.5% w/v) and 5 ml pluronic F 127 solution (5% w/v) by sonication by using a sonicator (VC505, Vibracell Sonics, Newton, USA) set at 55 W of energy output for 2 min over an ice bath. The emulsified NPs were centrifuged at 1000 rpm for 5 min (Sigma 3K30, Munich, Germany) to remove un-entrapped crocetin. The above supernatant was further subjected to lyophilization by freeze drying methods (−47 °C and < 10 µm mercury pressure, Freezone 12, Labconco, Kansas City, MO) to get lyophilized powder for further use.

2.3. Physical characterization of crocetin-loaded NPs

2.3.1. Particle size analysis and zeta potential measurement

Briefly, ~1 mg/ml of crocetin-loaded NPs solution was prepared in double distilled water (DW). A total of 100 µl of the above solution diluted in 1 ml DW, was sonicated for 30 s in an ice bath and taken for particle size and zeta potential measurement by in a zetasizer (Nano ZS, ZEN3600, Malvern Instrument, UK). All measurements were performed in triplicates.

2.3.2. Transmission electron microscope (TEM)

The size of the crocetin-loaded NPs was evaluated by TEM (Phillips/FEI Inc., Briarcliff Manor, NY). For the analysis, a drop of diluted solution of crocetin-loaded NPs (~1 mg/ml in water) was placed in a carbon-coated copper TEM grid (150 mesh, Ted Pella Inc., CA, USA) and allowed to air dry and the images were visualized at 120 kV under transmission electron microscope.

2.3.3. Assessing the entrapment efficiency of crocetin in crocetin-loaded NPs by high performance liquid chromatography (HPLC) method

The entrapment efficiency of crocetin in crocetin-loaded NPs was assessed by reverse phase isocratic mode high performance liquid chromatography (RP-HPLC) (Waters 600, Waters Co., Milford, MA) followed by a previously described method [26]. The entrapment efficiency was calculated from the equation: Entrapment efficiency (%) = (amount of crocetin in NPs/amount of crocetin used in formulation) × 100.

2.4. In vitro free radical scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined by following the protocol of Wu et al. [21]. The nitric oxide radical (NO·) scavenging activity was measured by following the protocol of Harput et al. [27]. The superoxide anion radical ($O_2\cdot^-$) scavenging activity was determined by following the protocol of Das et al. [28].

The percentages of scavenging of free radicals by the different sample were calculated by the following formula:

$$\%\text{of scavenging activity} = [(A_0 - A_1)/A_0)] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of samples. The scavenging concentration of each sample at 50% (SC_{50}) was used to compare the free radical scavenging activity, and all determinations were performed in triplicate.

2.4.1. DNA nicking assay

The protective activity of crocetin either in native or in NPs at DNA level was performed with plasmid DNA (pBR 322) model by using modified protocol of Park et al. [29]. Briefly, reaction mixture was prepared containing 3 µl (stock 0.25 µg/µl) of plasmid DNA, 7 µl of Fenton's reagent (2 mM H_2O_2 and 1 mM $FeCl_3$) followed by the addition of 5 µl of different concentration of native crocetin or crocetin-loaded NPs (200, 400 and 600 µM). The mixture was incubated at 37 °C for 90 min and DNA was analyzed on 1% agarose gel stained with ethidium bromide, photographed and semi-quantified by ImageJ.

2.5. Cell culture

HEK-293 cell line was purchased from National Center for Cell Science (NCCS), Pune, India and grown using DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin, streptomycin at 37 °C in a humidified, 5% CO_2 incubator (Hera Cell, Thermo Scientific, Waltham, MA). All chemicals for cell culture were purchased from

Himedia Laboratories Pvt. Ltd., Mumbai, India.

2.6. Cell viability assay

Briefly, HEK-293 cells were plated in 96-well plates (Corning, NY) at a density of 5000 cells per well and incubated for 24 h in growth medium at 37 °C for attachment. To study the cytotoxic effect of CsA on HEK-293 cells, HEK-293 cells were treated with different concentration of CsA, incubated for 24 h and measured the cell viability by MTT assay. The concentration of CsA that cause a 50% of cell death (IC_{50}) was calculated by non-linear regression analysis using the equation from sigmoidal plot. Further, to study the cytoprotective activity of crocetin either in native or NPs, cells were pretreated with different concentration of crocetin either in native or NPs and incubated for 24 h. After incubation, CsA at a dose of 10 μM was added and incubated for another 24 h. Medium treated cells served as control in the experiment. Finally, the MTT assay was performed by our previously published protocol [25].

2.7. Intracellular ROS and superoxide measurement

The generation of intracellular ROS and superoxide was measured by using two oxidant sensitive probes DCFH-DA [24] and dihydroethidium (DHE) [30]. Briefly, HEK-293 cells (1×10^5cells/well) were plated in 6 well plates and kept overnight for attachment. Next day, cells were treated with different concentration of native crocetin or crocetin-loaded NPs at a dose of 0.1, 0.5 and 1 μM for 24 h followed by CsA (10 μM) treatment for another 24 h. Cells treated with medium served as control. Following incubation, cells were washed with PBS (pH 7.4) two times, and incubated with 10 μM of DCFH-DA or 5 μM of DHE (Invitrogen, CA) for 30 min followed by washing with PBS (pH 7.4) twice to remove excess dye. The cells were then lysed and mean fluorescence intensity (MFI) of the cell lysate was measured at 488 nm excitation and 530 nm emissions for DCFH –DA and 488 nm excitation and 580 nm emission for DHE by using fluorescence spectrophotometer (PerkinElmer, LS 55, Massachusetts, USA).

2.8. Mitochondrial membrane stability assay

Mitochondrial membrane depolarization study was done using confocal microscopy with Rhodamin 123 dye [31]. Briefly, HEK-293 cells (1×10^5 cells/ml) were seeded in Bioptech tissue culture plates (Bioptechs Inc., Butler, PA). The plates were incubated overnight at 37 °C for cell attachment. Then the cells were treated with crocetin either in native or NPs with different concentration for 24 h and followed by the treatment of 10 μM CsA for another 24 h. Next, cells were incubated with 10 μM rhodamine 123 for 30 min, fixed with 4% paraformaldehyde for 20 min and washed twice with PBS (pH 7.4). The cells were then visualized under a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems GmbH, Germany) equipped with an argon laser (Ex 488 nm, Em 535 nm).

2.9. Determination of stress markers and antioxidant enzymes

2.9.1. Preparation of cell lysate for biochemical assays

In brief, HEK-293 cells (5×10^5 cells) were grown in 25 mm flask (Corning, NY) and kept it overnight for attachment. After this, different concentration of native crocetin or crocetin-loaded NPs were added, and incubated for 24 h followed by treatment with 10 μM CsA for another 24 h. After incubation, the cells were collected by trypsinization and centrifuged for 5 min at 3000 rpm at 4 °C (Sigma 3K30, Munich, Germany). The cells were lyzed with a cocktail containing 200 μl of RIPA buffer (50 mM tris-Cl buffer pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% NP-40) by two cycle of freeze-thawing process [32]. Then the above lysate was centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was collected and stored at −80 °C for further measurement of different enzymatic and non-enzymatic antioxidant level.

2.9.2. Estimation of different antioxidant enzyme activity

The above supernatant obtained after centrifugation was used for the activities of different antioxidant enzymes (SOD and CAT). The activity of SOD was determined according to the method of Das et al. [28] and expressed as unit mg^{-1} protein, where one unit of enzyme activity is defined as the amount of enzyme capable of inhibiting 50% of nitrite formation under assay condition. CAT activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm, according to the protocol of Paital et al. [33] and the activity of CAT was calculated by taking 43.6 M^{-1} cm^{-1} as a molar co-efficient of H_2O_2 and finally expressed as μkat/mg protein.

2.9.3. Estimation of total reduced glutathione levels

The above supernatant was precipitated in ice cold TCA (5% v/v) at 4 °C for 30 min followed by centrifugation at 12,000 rpm for 10 min at room temperature. The supernatant so obtained was used to measure GSH content according to the protocol of Bhardwaj et al. [34]. Result was calculated from the standard curve of reduced glutathione (GSH) and total GSH content was expressed as nmoles/mg protein.

2.9.4. Assessment of lipid peroxidation

LPx in the above cell lysate was assayed by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Subudhi et al. [35]. Concentration of TBARS present in the sample was calculated from its extinction coefficient $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmole TBARS formed per mg protein.

2.10. Cell lysate preparation for Western blotting

The molecular mechanism of protective activity of crocetin (native and its NPs) by modulating primary antioxidant enzymes (GPx and GR) profile, phase 2 antioxidant enzymes (HO-1) and transcription factor (NRf2) were studied by Western blot analysis. HEK-293 cells were treated as described above. The whole cell lysate was prepared according to the protocol of Misra et al. [36] and cytoplasmic cell lysate was prepared according to the protocol of Rosner et al. [37]. Western blot analysis of different proteins were performed using specific primary antibody for recognizing GPx, GR (Abcam, Cambridge, MA, USA) Nrf2, HO-1 and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA.) using our previously published protocol [38]. β actin was used as loading control.

2.11. Statistical analysis

Data are presented as mean ± standard deviation, and analyzed by one-way ANOVA with the Tukey's test applied post hoc for paired comparisons of means (Prism 5.0, GraphPad software Inc., CA,USA).Values of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ were indicative of significant differences and $^{***}p < 0.001$ and $^{\#\#\#}p < 0.001$ was indicative of a very significant difference.

3. Results

3.1. Characterization of crocetin-loaded NPs

In our study, we have successfully developed crocetin-loaded NPs with a high entrapment efficiency of ~80%. The average mean particle size of crocetin-loaded NPs was found to be ~119 ± 4 nm and polydispersity index (measurement of the distribution of molecular mass in a sample) was 0.426 ± 0.026 as determined by dynamic laser light scattering (Fig. 1a) with a negative zeta potential of −18.3 ± 4.21 mV. Topology of the above NPs as observed by TEM analysis confirmed that the NPs are spherical in shape (Fig. 1b).

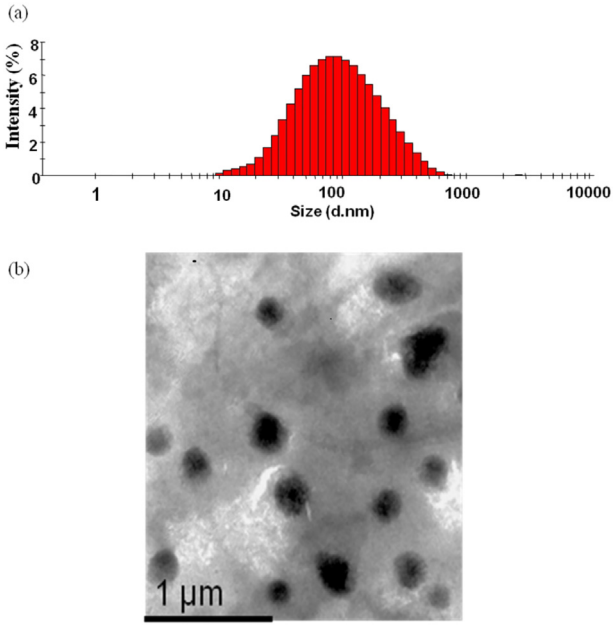

Fig. 1. Size distribution of crocetin–loaded NPs measured by (a) dynamic light scattering (DLS). (b) Representative image of crocetin-loaded NPs through TEM (bar 1 μM).

3.2. Antioxidant activities of crocetin-loaded NPs in response to free radical scavenging capacity

We have evaluated the antioxidant activities of native crocetin (crocetin in DMSO/DW) and crocetin-loaded NPs by different radical scavenging assays (DPPH, NO·, $O_2^{\cdot -}$). Result revealed that crocetin in DMSO or crocetin-loaded in NPs were showing excellent DPPH radical scavenging activity (Fig. 2a). Crocetin-loaded NPs showed 10 fold high DPPH radical scavenging activity than that of crocetin in DW. In Fig. 2b, the result showed that crocetin-loaded NPs and native crocetin in DMSO had similar nitric oxide radical scavenging activity whereas crocetin in DW has shown low scavenging activity. Finally, we have studied the superoxide anion scavenging activity and the result showed crocetin-loaded NPs showed 16 fold stronger superoxide anion scavenging activity than native crocetin in DW (Fig. 2c). In quest to study the ability of crocetin to protect the pBR322 plasmid DNA against damage caused by hydroxyl (·OH) radicals, we have assessed the DNA protective activity of crocetin by tracing the DNA (pBR 322) scission caused by hydroxyl radical generated by Fenton reaction. As shown in Fig. 2d, the supercoiled form of plasmid was converted to nick circular form with the addition of $H_2O_2/FeCl_3$. Upon treatment with either native crocetin or crocetin-loaded NPs the plasmid regained its native form of DNA (i.e. supercoiled form) by protecting DNA from the hydroxyl (·OH) radical induced oxidative damage. It is noteworthy to mention that crocetin-loaded NPs demonstrated significant higher DNA protection activity over native crocetin in a dose dependent manner (Fig. 2e).

3.3. Crocetin-loaded NPs protects HEK-293 cells form CsA-mediated cytotoxicity by inhibiting intracellular ROS generation and preventing mitochondrial membrane potential loss

The protective effect of crocetin and crocetin-loaded NPs on CsA-mediated cytotoxicity towards HEK-293 cells was studied by MTT assay [25]. Initially we have evaluated the cytotoxicity of CsA to HEK-293 cells and found the IC_{50} was ~9.56 μM (data not shown). So, to develop CsA-mediated cytotoxicity, HEK-293 cells were further incubated with CsA at a dose of 10 μM for 24 h. Cell viability assay clearly showed that pretreatment of native as well as crocetin-loaded NPs had a significant ($p < 0.01$) protective effect against CsA-mediated cytotoxicity in a dose dependent manner (Fig. 3a). As the cytotoxicity of CsA is due to the generation of ROS, we further assessed the protective effect of native and crocetin-loaded NPs on CsA-mediated cytotoxicity to HEK-293 cells by measuring the intracellular superoxide generation as well as total ROS production. Elevated ROS level of treated cells was measured by using DCFH-DA dye (Fig. 3b) and DHE (Fig. 3c) dye which are nonfluorescent cell permeable dyes, which were converted to their fluorescent counterpart on encountering ROS species. Treatment of CsA at a dose of 10 μM in HEK-293 cells significantly ($p < 0.01$) increased intracellular ROS level (indicated by high MFI value) in comparison to normal untreated cells. However, pretreatment with native crocetin or

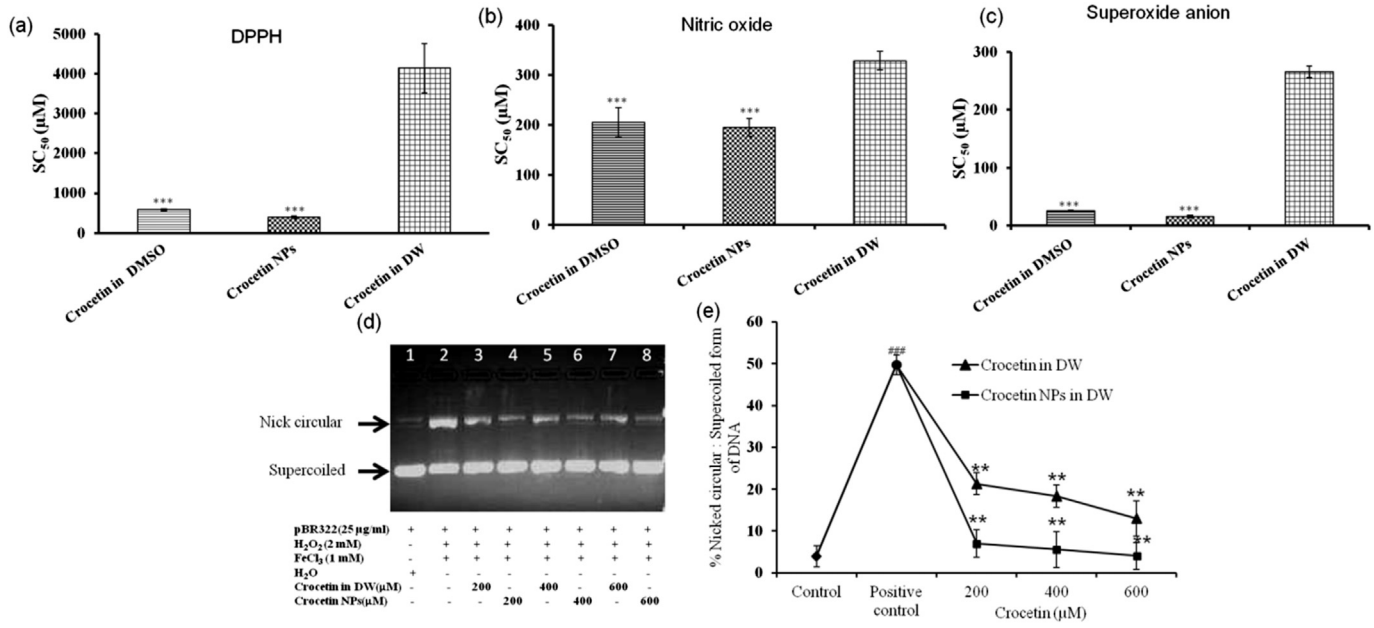

Fig. 2. In vitro antioxidant activities (a) DPPH radical scavenging; (b) nitric-oxide scavenging; (c) superoxide anion scavenging; (d) DNA nicking assay and (e) % of Nicked circular: supercoiled form of DNA. All experiments were performed in triplicate and values were expressed as mean ± S.D. (n = 3), $^{(\#\#\#)}$ $p < 0.001$, Positive control (●) versus control (♦), $^{(**)}$ $p < 0.01$, crocetin in DW versus crocetin in DMSO or crocetin NPs.

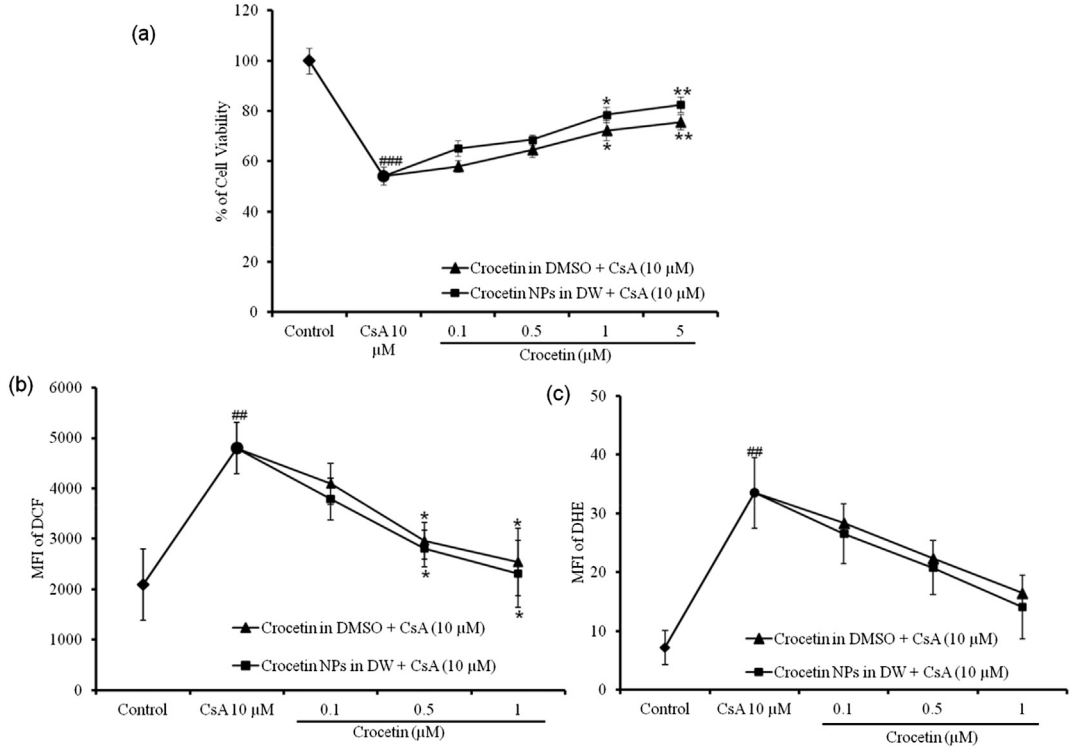

Fig. 3. Effect of crocetin (either in native or NPs) on CsA-mediated cytotoxicity and ROS generation in HEK-293 cells. (a) Cytoprotective effects of native crocetin in DMSO and crocetin-loaded NPs in DW (pretreatment) on CsA (10 μM) treated HEK-293 cells was determined by the MTT assay. The intracellular ROS production was determined by measuring (b) DCF and (c) DHE fluorescence as described in Materials and methods section. Results were expressed as the mean fluorescence intensity (MFI). Data was expressed as mean ± S.D. (n = 3), $^{(\#\#)}$ $p < 0.01$, $^{(\#\#\#)}$ $p < 0.001$. CsA treated group (●) versus control (♦), $^{(*)}$ $p < 0.05$ and $^{(**)}$ $p < 0.01$. CsA treated group versus other treated groups.

crocetin-loaded NPs at different concentrations provided noticeable reduction in fluorescence intensity in comparison to cells treated with CsA only indicating the protective effect of the drug against ROS damage.

Mitochondria are the richest source of intracellular ROS in cells converting 1–2% of reduced oxygen into superoxide, resulting in subsequent mitochondrial membrane potential (MMPs) loss [39]. Due to MMPs loss, release of mitochondrial pro-apoptotic factors such as cytochrome *c* which, promote cell death by apoptosis [40]. To analyze the modulation in MMPs, by regulating superoxide generation (with CsA treatment), accumulation of Rhodamin-123 dye was taken into consideration. The confocal microscopic study demonstrated that in control cells the Rhodamin-123 fluorescence is located in areas around the nucleus that correspond to the distribution of mitochondria (Fig. 4a (i)). After a 24 h exposure to CsA, Rhodamin-123 fluorescence intensity decreased and appeared to bleach out from the cells, with poorly defined and irregular cell morphology correspond to MMPs loss (Fig. 4a (ii)). Loss of MMPs as indicated by leaching of Rhodamin-123 is prevented upon pretreatment of crocetin either in native or in NPs (Fig. 4b). It is noteworthy to be mention that at high concentration (0.5 or 1 μM) of croctin NPs have shown better effect as compared to the native counterpart (Fig. 4a and b).

3.4. Effect of crocetin-loaded NPs on CsA-mediated changes in the antioxidant profile and lipid peroxidation

Oxidative stress was quantified by measuring various antioxidant enzyme activities and non-enzymatic antioxidant level such as SOD, CAT and GSH in CsA-mediated HEK-293 cells. The biochemical studies showed that HEK-293 cells treated with CsA at 10 μM significantly decreased the activities of SOD, CAT and GSH levels as compared to control cells (Fig. 5a, b and c). This reduction was significantly enhanced by the pretreatment of either native crocetin or crocetin-loaded NPs in a dose dependent manner. Further, we have studied the lipid profile of CsA-mediated HEK-293 cells and our result indicated that the level of TBARS in CsA treated HEK-293 cells was considerably increased as compared with that of the control. Moreover, TBARS level was decreased in a dose dependent manner after treatment with native crocetin or crocetin-loaded NPs with the latter being more capable in inhibiting TBARS level in comparison to the former (Fig. 6).

3.5. Effect of crocetin-loaded NPs on CsA-mediated changes in different endogenous and phase II antioxidant enzymes profile

GPx is an important GSH using enzyme and plays an important role in maintaining GSH homeostasis and tissue detoxification. GR is an enzyme that reduces GSSG (oxidized glutathione) to the sulfhydryl form of GSH, which is an important cellular antioxidant. Western blot analysis showed that, the expression of endogenous antioxidant enzymes like GPx and GR were significantly lowered upon CsA treatment in comparison to untreated control cells (Fig. 7a and b). It is noteworthy that cells treated with either native or crocetin-loaded NPs restored the expression of these endogenous enzymes to its basal level in a dose dependent manner (Fig. 7a and b). Transcription factor Nrf2 is a major sensor of oxidative stress in cells [41] and upon sensing any stress stimuli, it translocate from cytoplasm to the nucleus, where it activates transcription of antioxidant and detoxifying genes (like HO-1) by binding to the antioxidant response elements (AREs) in their regulatory regions [42]. To investigate the involvement of Nrf2 mediated cytoprotective effect in CsA-mediated redox instability in HEK-293 cells pretreated with crocetin either in native or in NPs, expression study of Nrf2 and HO-1 proteins were performed. In the present study, we observed a lower expression of Nrf2 protein in cytoplasmic fraction (which indirectly point towards nuclear translocation of the protein) (Fig. 7c)

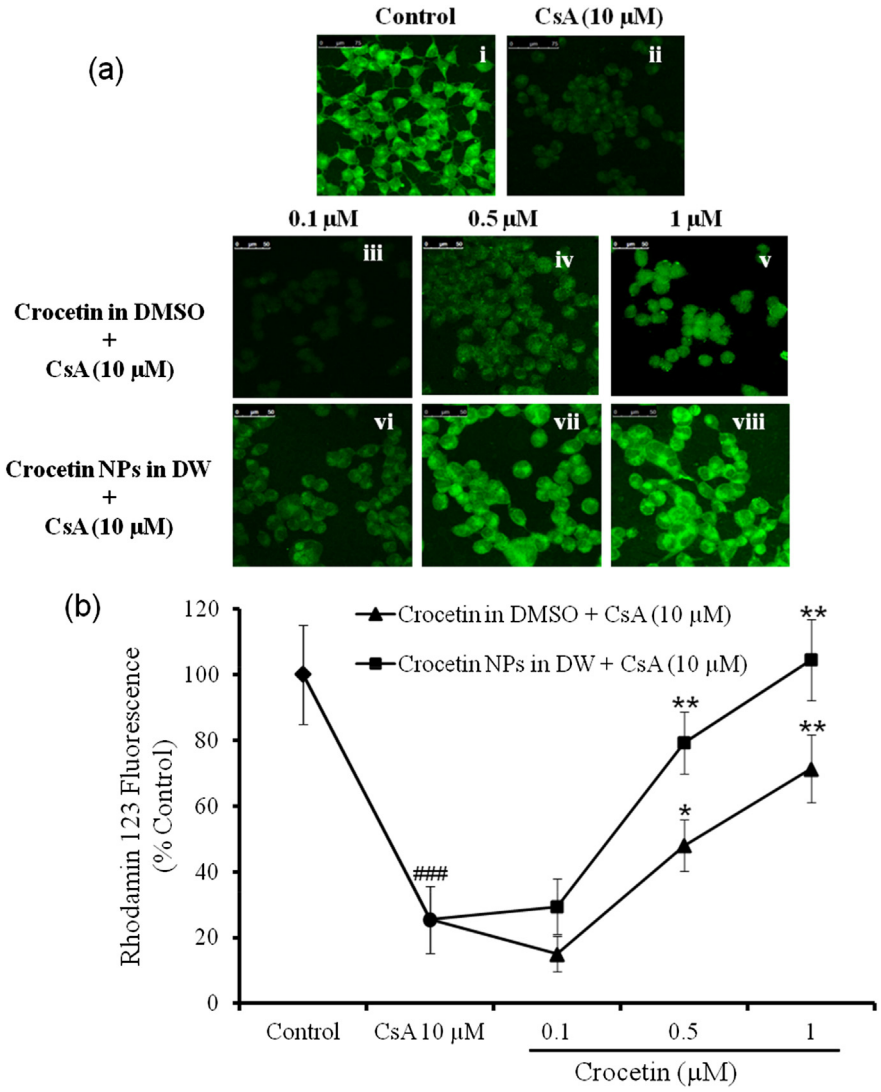

Fig. 4. Effect of crocetin (either in native or NPs) on mitochondrial membrane potential loss in CsA treated HEK-293 cells, studied by using Rhodamin 123 under confocal microscopy. (a) Representative confocal fluorescent image of Rhodamine 123: (i) control: without any treatment, (ii) CsA (10 μM) treatment alone for 24 h, (iii–viii) pretreatment of the indicated concentration of either native crocetin in DMSO or crocetin-loaded NPs in DW for 24 h and followed by CsA (10 μM) treatment for another 24 h. (b) Rhodamin 123 fluorescence intensity, expressed as a percentage of the control group. Data are expressed as the mean ± S.D. $^{(###)}$ $p < 0.001$ CsA treated group (●) versus control (◆); $^{(*)}$ $p < 0.05$ and $^{(**)}$ $p < 0.01$. CsA treated group versus other treated groups.

and increased expression of HO-1 upon treatment with CsA to protect cells from CsA-mediated redox imbalance. However, pretreatment of cells with either native crocetin or its nanoformulation depicted increased level of cytoplasmic Nrf2 and lower expression of HO-1. The above observation indicates towards the fact that, in crocetin treatment endogenous antioxidant enzymes (e.g. GPx, GR, SOD and CAT) are able to efficiently detoxify the redox imbalance mediated by CsA, thereby decreasing the dependency of Nrf2-mediated protection.

4. Discussion

A large number of herbal drugs showed high efficacy in in vitro assays however the results are not reproducible in vivo due to their low absorption and degradation during processing [43]. In this regards, the current approach focuses to formulate a lipid based crocetin nanoparticle system with a view to overcome the limitation associated with crocetin delivery. Several literature have established the fact that lipid based drug delivery system can potentially enhance the solubility, bioavailability, pharmacological activity of the drugs by preventing their physical and chemical degradation [43].

After successful formulation, the NPs were physico-chemically characterized, and found with low polydispersity index (PDI) which infer that particles are uniformly distributed having negative zeta potential that helps the formulations repels to each other which ensure long term stability and avoid particle aggregation. After characterization of crocetin-loaded NPs, its antioxidant and protective efficacy in CsA-mediated toxicity to HEK-293 cells were taken into consideration. In fact, antioxidant or free radical scavenging activity of crocetin may be attributed to its ability to donate hydrogen or may be due to direct quenching of free radicals in the reaction mixture [14,44,45]. In this regards, the free radical scavenging results revealed that crocetin-loaded NPs showed higher free radical scavenging ability than that of its native counterpart, thereby indicating the superior antioxidant activity of crocetin in crocetin-loaded NPs. There are various mechanisms involved in CsA-mediated toxicity, one of the key mechanism is over production of intracellular ROS (as evident from DCFH-DA and DHE experiment result). In fact, Several reports suggested that superoxide (ROS species) not only plays an important role in mitochondrial dysfunction by opening the mitochondrial permeability transition pore but also increases the level of intracellular ROS in cells [46,47]. Thus, we further intend to study whether crocetin treatment could preserve the MMP loss in CsA-mediated cytotoxicity in HEK-293 cells. Our results demonstrated that pretreatment of crocetin either in native or crocetin-loaded NPs prior to exposure of CsA significantly inhibited the MMP loss in HEK-293 cells as compared to cells treated with CsA alone. Our results suggested that crocetin either in native or in NPs might inhibit mitochondrial permeability transition pore opening by directly scavenging the superoxide radicals or by stabilizing the mitochondrial membrane potential, which prevent ROS release from mitochondria to cytoplasm. These results are similar to studies

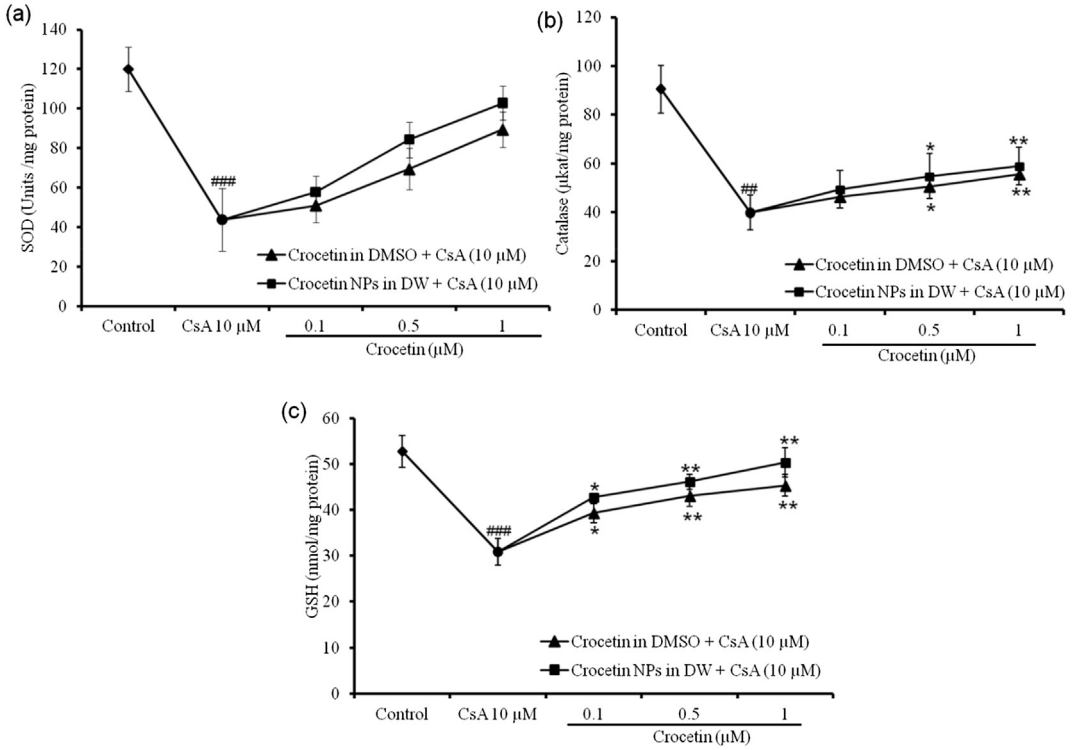

Fig. 5. Effect of crocetin (either in native or NPs) on (a) SOD enzyme activity (b) catalase enzyme activity (c) GSH level in CsA treated HEK-293 cells. In different groups different concentration of native crocetin in DMSO or crocetin-loaded NPs in DW were treated for 24 h. Next, CsA (10 μM) was treated and incubate for another 24 h. Control: without any treatment, CsA: CsA (10 μM) treatment alone for 24 h. Finally the activity and level of enzymatic and nonenzymatic antioxidant was measured and expressed as mean ± S.D. (n = 3), $^{(###)}$ $p < 0.001$ CsA treated group (●) versus control (♦); $^{(*)}$ $p < 0.05$ and $^{(**)}$ $p < 0.01$. CsA treated group versus other treated groups.

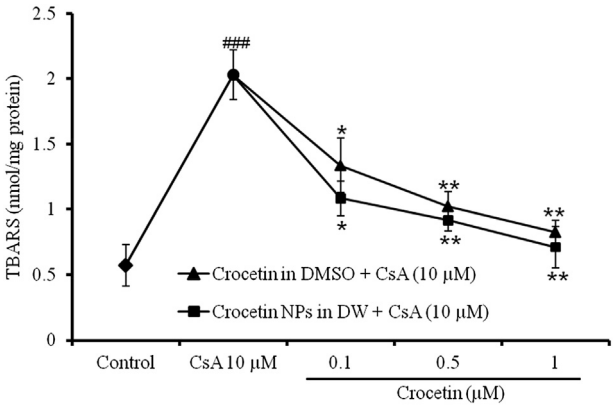

Fig. 6. Effect of crocetin (either in native or NPs) on TBARS levels in CsA treated HEK-293 cells. In different groups different concentration of native crocetin in DMSO or crocetin-loaded NPs in DW were treated for 24 h. Next, CsA (10 μM) was treated and incubate for another 24 h. Control: without any treatment, CsA: CsA (10 μM) treatment alone for 24 h. Finally, the TBARS level was measured by spectrophotometrically and expressed as mean ± S.D. (n = 3), $^{(###)}$ $p < 0.001$ CsA treated group (●) versus control (♦); $^{(*)}$ $p < 0.05$ and $^{(**)}$ $p < 0.01$. CsA treated group versus other treated groups.

conducted by Yang et al. [48] where they have studied the cytoprotective effect of crocetin against cellular apoptosis following Hemorrhagic shock by stabilizing the mitochondrial membrane potential [48].

Studies conducted by several researchers have demonstrated that with CsA treatment endogenous antioxidant enzymes (i.e. SOD, CAT, GPx, GR) and GSH level is depleted which lead to elevation of LPx (an indicator of oxidative stress) thereby inducing nephrotoxicity both in vivo as well as in vitro model [49–51]. In this context reduction of intracellular ROS by enhancement of endogenous antioxidant enzymes and decrease of LPx with antioxidant treatment is a pre requisite for nephroprotection [49–53]. Studies conducted by Venkatraman et al. [54] reported that, crocetin restored the depleted GSH as well as antioxidant enzyme (SOD) level in benzo [*a*] pyrene-induced toxicity which helped to inhibit the production of lipid peroxides in mice [54]. In this regards, our study also suggested that crocetin and its nanoformulation play a detoxifying role in metabolizing the free radicals in CsA–mediated cytotoxic HEK-293 cells through enhancement of endogenous antioxidant enzymes like SOD, CAT and by maintaining nonenzymatic GSH homeostasis. Moreover, these observations also helped us to hypothesize that enhanced levels of these antioxidant enzyme/non-enzyme may be sufficient enough to minimize LPx level (as evident from TBARS level study) in HEK-293 cells.

Transcription factor Nrf2 plays an essential role in antioxidant-response element-mediated expression of phase II detoxifying and antioxidant enzymes in response to oxidative stress [42]. Nrf2 is a transcription factor present in the cytoplasm (as an inactive form) bound to a cytoskeletal associated protein Keap1 [41]. On response to CsA-mediated oxidative stress, Nrf2 pathway gets activated and the transcription factor translocate to the nucleus where it induces the expression of several phase-II detoxifying and antioxidant enzymes, particularly HO-1 [55]. Similar observation was also evident in our Western blot study where CsA activated the Nrf2 signaling pathway by decreasing cytosolic Nrf2 level with an increased HO-1 expression in CsA-treated HEK-293 cells. However, pretreatment with crocetin (either in native or NPs), not only increased the cytosolic Nrf2 level but also decreased the expression of HO-1 protein denoting inhibition of Nrf2 signaling pathway. Such observation helped us to hypothesize that the observed down regulation of Nrf2 might be due to neutralization of free radicals by enhanced expression of other endogenous antioxidant enzymes like SOD, CAT, GPx and GR. Similar results were also observed by Hong et al. [56], where the degree of oxidative stress and

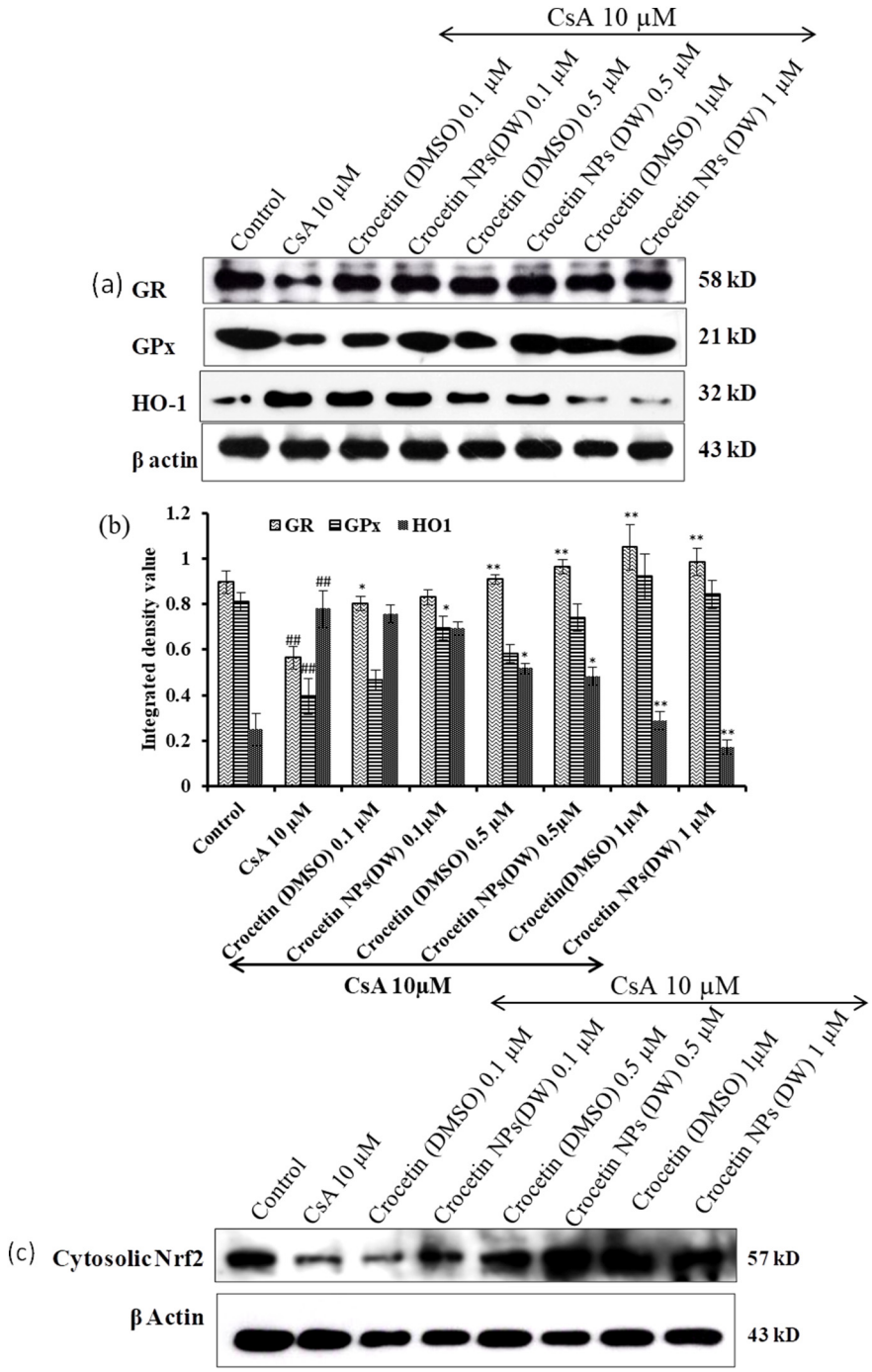

Fig. 7. Effect of crocetin (either in native or NPs) on protein expression in CsA treated HEK-293 cells. (a) Endogenous antioxidant enzymes (GPx and GR) and phase II antioxidant enzyme (HO-1) expression (b) specific band intensity was quantified and normalized to β actin. (c) Phase II detoxifying transcription factor (Nrf2). Data are expressed as the mean ± S.E.M. $^{(\#\#)}$ $p < 0.01$ CsA treated group versus control $^{(*)}$ $p < 0.05$, $^{(**)}$ $p < 0.01$, CsA treated group versus other treated groups.

endogenous antioxidant enzyme system determines the detailed induction of specific antioxidant gene regulated by Nrf2 in response to various oxidative stress [56,57]. In our study, it is noteworthy to mention that inhibition of Nrf2 translocation is more prominent in crocetin-loaded NPs treated cells in comparison to native treatment. This can be attributed to greater accumulation of crocetin delivered by nanoformulation resulting in more pronounced restoration of cytosolic Nrf2 in comparison to native crocetin treated cells.

Thus our study emphasizes on development of a successful lipid based delivery system for crocetin. Further, the enhanced protective effect of crocetin-loaded NPs in comparison to native drug against CsA-mediated toxicity towards HEK-293 cells was analyzed by performing different antioxidant assays. Crocetin-loaded NPs exhibited enhanced free radical scavenging, cytoprotective, LPx inhibition potential compared to native crocetin. Moreover, we anticipate that the superior antioxidant activity of crocetin-loaded NPs can be specified in combination with other therapeutic regimes, where cellular toxicity is a concern.

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Conflict of interest statement

The authors have no conflict of interest.

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