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PREPARATION, PHYSICO-CHEMICAL CHARACTERIZATION AND BIOCOMPATIBILITY EVALUATION OF QUERCETIN LOADED CHITOSAN NANOPARTICLES AND ITS NOVEL POTENTIAL TO AMELIORATE MONOCROTOPHOS INDUCED TOXICITY

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Quercetin is a bioflavonoid, used to treat several diseases. The aim of the present work was to estimate the effect of quercetin loaded chitosan nanoparticles (QUER-CHIT) against monocrotophos induced toxicity in rat erythrocytes *in vitro*. Scanning electron microscopy (SEM), atomic force microscopy (AFM), x-ray diffraction (XRD) and fourier transform infrared (FT-IR) characterization and biocompatibility studies like cell viability assay and lactate dehydrogenase (LDH) assay were done for the synthesized nanoparticles. The effect of QUER-CHIT nanoparticles against monocrotophos induced toxicity in rat erythrocytes was investigated. Encapsulation efficiency of QUER-CHIT nanoparticles (1:1 ratio) was 92% with particle size of 232.4 ± 23 nm. *In vitro* drug release at pH 7.4 was found to be 70% at 12 h time period. Cell viability studies in RAW 264.7 cells resulted in inhibitory concentration (IC_{50}) value of 21 µg/ml for QUER-CHIT nanoparticles and 131.2 µg/ml for pure quercetin (QUER). LDH level decreased in QUER-CHIT nanoparticles treated groups compared to QUER. Superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione (GSH) and malondialdehyde (MDA) levels were increased after incubation of monocrotophos treated erythrocytes with QUER-CHIT nanoparticles. This study demonstrates that QUER-CHIT nanoparticles enhance their bioavailability to scavenge the free radicals generated by monocrotophos via an oxidative stress mechanism.

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Keywords: Flavonoid; Monocrotophos; Drug release; Oxidative stress; Antioxidant.

1. Introduction

Nanoparticles are used as drug carriers in pharmacological industries with improved therapeutic effects and reduced toxic side effects [1, 2]. Chitosan nanoparticles are widely used as carriers for drug delivery [3, 4] in pharmaceutical industries. The biological and chemical properties of chitosan are biodegradability, biocompatibility, bioactivity, and poly cationicity [5]. Chitosan based nanoparticles exhibit site-specific delivery of drugs; they solubilize various hydrophobic drugs, increase bioavailability and circulate in the blood for a long time [6]. Chitosan beads were prepared by using tripolyphosphate (TPP), which increases drug-loading efficiency, as well as increases the drug release period [7]. TPP is nontoxic, and it is a multivalent anion [8, 9]. Quercetin is a member of the flavonoid family, commonly found in the human diet. Flavonoids are a large group of phenolic plant constituents, which plays a major role in free radical scavenging activities [10]. Human diets contain flavonoids, which are metabolized by the same pathway as toxic man-made chemicals, such as pesticides and other environmental pollutants [11]. The antioxidant activity of flavonoid quercetin is very high compared to well-known molecules like ascorbyl, trolox, and rutin [12]. Quercetin has several beneficial effects on human health including

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cardiovascular protection, anti-cancer, anti-viral, anti-allergic and anti-inflammatory activity [13]. It has been estimated that intake of quercetin was 20-35 mg/day in western populations [14]. The clinical use of quercetin is very less because of its hydrophobic nature, poor bioavailability, poor permeability, poor solubility, and the metabolism of quercetin occurs in a short time period without entering into the circulation [15]. In the present study, to overcome the disadvantages of quercetin, it is encapsulated in the chitosan nanoparticles and used to treat the monocrotophos induced oxidative stress.

For more than fifty years, organophosphorus group of pesticides are used extensively in the world [16]. Monocrotophos is an organophosphate pesticide used widely for agricultural and household purposes to control insects and spider mites [17]. Monocrotophos usage has been banned in several developed countries but still used in many developing countries. In previous study, monocrotophos toxicity has been extensively investigated on animal models but there are only few reports of cytotoxicity and genotoxicity in humans and *in vitro* models [18]. Monocrotophos causes oxidative damage to erythrocytes with generation of free radicals and thereby shortening the RBC life span [19]. In the present study quercetin was encapsulated in chitosan nanoparticles and its biocompatibility was analyzed in murine alveolar macrophages RAW 264.7 cell line. The mechanism of cytotoxicity was explored for encapsulated quercetin and pure quercetin. The present study was designed to investigate the pharmacological role of encapsulated quercetin nanoparticles against monocrotophos induced toxicity in rat erythrocytes *in vitro*.

2. Experimental

2.1 Materials

Quercetin, chitosan (High molecular weight), monocrotophos and sodium tri polyphosphate has been purchased from Sigma Aldrich. Tween- 80 was purchased from Merck Specialities Pvt. Ltd. Glacial Acetic acid was purchased from Thermo Fischer Scientific India Pvt. Ltd. Murine RAW 264.7 macrophage cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in dulbecco's minimal eagle medium (DMEM) medium, supplemented with 10% fetal bovine serum (FBS), at 37 °C in a humidified atmosphere with 5% CO_2. Ethics approval (551/02/a/CPCSEA/CBT) was obtained from the Institutional Animal Ethics committee of Anna University, India. Antioxidant enzyme kits were obtained from Sigma Aldrich. All the other materials used were of analytical grade.

2.2 Preparation of CHIT and QUER-CHIT nanoparticles

The CHIT-NPS were synthesized by ionic gelation method. Chitosan was dissolved in 2% acetic acid and kept in a magnetic stirrer for 30 min at 450 rpm. Few drops of 0.5% Tween-80 were added to the solution for uniform dispersion of CHIT- NPS and to prevent aggregation at ambient temperature during stirring. To this solution 0.1% of Sodium Tri Poly phosphate was added in drops to the solution as a cross linking agent and the solution stirred vigorously until the opalescence was observed. The final suspension was centrifuged at 12000 rpm for 30 min. The pellet was kept in -80 °C for 12 h, and it was lyophilized at -54 °C and vacuum was maintained at 5 m Torr [20]. To prepare QUER-CHIT nanoparticles, QUER was dissolved in dimethyl sulphoxide (DMSO):water and added to CHIT. The drug was added before the addition of the cross linking agent. The supernatant obtained upon centrifugation was collected and measured.

2.3 Drug encapsulation efficiency

The amount of QUER encapsulated in CHIT-NPS was determined as follows. The λ max of the drug is determined to be 368 nm. Using a spectrophotometer, samples of various known concentrations of QUER dissolved in 1:4 ratio of DMSO:water was measured and a standard

graph between light absorbance at 368 nm and QUER concentration was obtained. Concentration of QUER in supernatant = Absorbance of supernatant / 0.129

2.4 Characterization Studies

2 mg of CHIT-NPS was added to 10 ml of water and sonicated for 15 min. Similarly, 2 mg of QUER-CHIT nanoparticles (Q1, Q2, and Q3) was added to 10 ml of water and sonicated separately. The solutions were poured into disposable sizing cuvettes and their particle size was determined using Malvern Zetasizer Nano Series, USA. The surface morphology of the particles was determined using VEGA3 SB Tescan scanning electron microscope (SEM). A conventional tungsten heated cathode is used to provide accelerating voltage of 15 kv. SEM analysis was done in high vacuum mode that gives a resolution of up to 5.5 nm. The shape and surface morphology of QUER-CHIT nanoparticles were investigated using AFM (Agilent Pico LE Scanning Probe Microscope) under normal atmospheric condition. AFM cantilevers obtained from Nova scan technologies having a scan rate of 5 μm/s. XRD of the standard QUER, CHIT-NPS and QUER-CHIT was done in Miniflex II desktop X- ray Diffractometer (Rigaku, Tokyo, Japan). Cu Kα is used as x-ray source and it emits x-rays of wavelength 1.51 Å. Maximum power of 600 W is applied using tube voltage of 40 kv and tube current of 100 Ma. FT-IR Spectroscopy (ABB-MB3000) was used to identify the functional groups. Spectrum was observed in the scanning range of 4000-600 cm^{-1} with a resolution better than 0.7 cm^{-1} resolution.

2.5 *In vitro* drug release study

The QUER release profile was investigated *in vitro* by incubating QUER-CHIT nanoparticles in a 50 ml glass beaker containing 20 ml medium at 37 °C and at pH 7.4. The medium consisted of phosphate-buffered saline containing 10% ethanol (v/v) (21) because the limit of solubility of QUER in water makes it impossible to study in buffer. At selected times; the incubation medium was completely removed for analysis and replaced with fresh medium. An aliquot of the sampled medium was measured by UV-Vis spectrophotometer. The recorded absorbance was then related to the amount of released QUER using a calibration plot. The absorption of the solutions of QUER was measured at λ max 368 nm. Results were expressed as cumulative percent of released as a function of time compared with standard QUER.

2.6 Cell viability and Lactate dehydrogenase (LDH) assay

Percentage of cell viability for the synthesized nanoparticles and pure QUER was determined by spectrophotometric determination of accumulated formazan derivative in treated RAW 264. 7 cells at 570 nm in comparison with the untreated ones [22]. For the measurement of LDH, different concentrations (12, 25, 50, 100 and 500 μg) of QUER-CHIT and QUER were chosen to identify its effect on macrophage cells. RAW 264.7 cells were seeded into 6-well plates at a density of 2.0×10^5 per ml in 2.5 ml culture medium. Cells allowed to proliferate attach and cover around 80% of the plate surface culture area before the treatment with QUER-CHIT nanoparticles and CHIT for 24 h at 37°C. Upon completion of the incubation, 50 μl of the upper medium were collected from each well. The untreated cells were then lysed with a cell lysis solution for 40 min at room temperature and the lysate collected. LDH level was measured using LDH release quantification (Fischer scientific, India) cytotoxicity assay kit, in accordance with manufacturer's instructions. The percent of LDH released from the cells was determined using the units/mg of protein.

2.7 Blood sampling, preparation of haemolysis and treatment of erythrocytes

Blood sample was collected from wistar rats by puncturing the retero-orbital venous plexus. Ether anaesthesia was given before collecting the sample. Blood samples were collected in heparinised tubes. The erythrocytes were separated from blood plasma and buffy coat by centrifugation at (1600 rpm at 4 °C for 5 min) and the washed three times with a cold isotonic

saline solution. The supernatant and buffy coat was removed after each wash. The packed erythrocytes were suspended in phosphate buffer at pH 7.4 [23]. The supernatant was collected and the test sample (QUER-CHIT nanoparticles) was added at a concentration of 30 µg. After the addition of test sample, monocrotophos was added at various concentrations such as 10, 20 and 30 µg. This mixture was incubated in a shaking water bath for 37 °C for 1 h. The mixture was then centrifuged at 1500 × g for 10 min. Supernatant was collected and various assays were carried out. Erythrocytes that were incubated without the addition of any test samples and monocrotophos serve as non-treated control cells. Erythrocytes were divided into non-treated control and experimental groups. Control groups were incubated for 1 h at 37 °C in 0.9% NaCl. Experimental groups were represented in Table 1.

Table 1 Treatment of erythrocytes.

Groups	**Treatment with QUER-CHIT nanoparticles (µg)**	**Treatment with monocrotophos (µg)**	**Treatment with QUER-CHIT nanoparticles + monocrotophos**	
			QUER-CHIT (µg)	monocrotophos (µg)
P1	30	10	30	10
P2	30	20	30	20
P3	30	30	30	30

2.7.1 Estimation of antioxidant enzymes

SOD activity was measured using Calbiochem® Superoxide Dismutase Assay Kit II. Data is expressed as U of SOD/mg protein. GST kit Sigma, Cat No. CS0410 was used for analysing GST activity. Data is expressed as µmol/ml/min. Sigma-Aldrich, Cat No. CS0260 was used to measures the GSH activity. Data is expressed as µg/mg protein. CAT activity was estimated by the method of Sinha [24]. Data is expressed as U of CAT/mg protein. MDA content was estimated as thiobarbituric acid reactive substances (TBARS) [25]. MDA content is presented as nmol/mg haemoglobin.

2.8 Statistical analysis

Data was analysed using the software program SPSS 11.0 for Windows. The significance was calculated using one-way analysis of variance (ANOVA) followed by bonferroni procedure for multiple comparisons $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Preparation of CHIT and QUER-CHIT nanoparticles

CHIT-NPS are prepared by the addition of TPP solution to the chitosan solution by magnetic stirring; formation of nanoparticles depends upon the ionic interaction between chitosan and TPP. The nanoparticle size depends upon the chitosan and TPP ratio and the biological performance will vary based upon the size characteristics [26]. TPP forms a gel by ionic interaction between the amino groups of chitosan, which are positively charged and negatively charged counterion of TPP. Depending upon the charge density of chitosan and TPP the interaction could be controlled. pH plays an important role in determining the size of the nanoparticles. pH around 4.4 (isoelectric point) leads to faster precipitation and larger size of

nanoparticles. Hence pH of 6.5 was maintained to form smaller nanoparticles. Low molecular weight of chitosan resulted in uniform small particles whereas; High molecular weight chitosan resulted in increased encapsulation efficiency and increased viscosity of internal phase [27]. In this study, high molecular weight chitosan was used. The encapsulation efficiency of the formulation Q1 (1:1) was 92% and it found to be high compared to other formulations. These results were higher than those of reported studies, QUER loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles have encapsulation efficiency of about 79% [28]. Fewer reports identified that QUER loaded polylactic acid (PLA) nanoparticles have encapsulation efficiency of around 95% [29]. In this study, Q1 formulation was selected for further studies due to higher encapsulation efficiency shown in Table 2.

Table 2 Particle Size and encapsulation efficiency of CHIT-NPS and QUER-CHIT nanoparticles

Polymer:Drug ratio	Particle size of CHIT-NPS (nm)	Particle size of QUER-CHIT nanoparticles (nm) (Mean ± SD)	Encapsulation efficiency %
1:1	188 ± 27	232.4 ± 23	92
1:2	188 ± 27	258.3 ± 24	73.3
1:3	188 ± 27	241.2 ± 41	62.5

3.2 Physicochemical characterization of nanoparticles

Particle sizes of the polymeric nanoparticles determine the drug loading, drug release and stability of the drug. QUER is a hydrophobic drug successfully loaded into chitosan nanoparticles. Previous studies reported that encapsulated QUER has a particle size greater than 120 nm [29-31]. QUER loaded PLGA nanoparticles have particle size of around 400 nm, in some cases it can even be higher depending upon the polymer used [28]. In our study, 230 to 260 nm size particles were obtained in all the formulations. Q1 (1:1) ratio had a particle size of about 232.4 ± 23 nm; it was less compared to other formulations. SEM images showed the morphological properties of CHIT-NPS and QUER-CHIT nanoparticles (Figure (1A, 1B)). Well-defined spherical shaped particles were obtained. Under AFM, QUER-CHIT nanoparticles were visualized as spherical nanoparticles, which are an agreement with the result, obtained using SEM (Figure 1 (C)). Previous report of the SEM characterization of PLA and QUER loaded PLA nanoparticles show that nanoparticles are spherical in shape, which is synthesized by solvent evaporation method [29]. Our report differs by synthesis method whereas the obtained nanoparticles are spherical in shape with larger surface to volume ratio and high drug loading capacity.

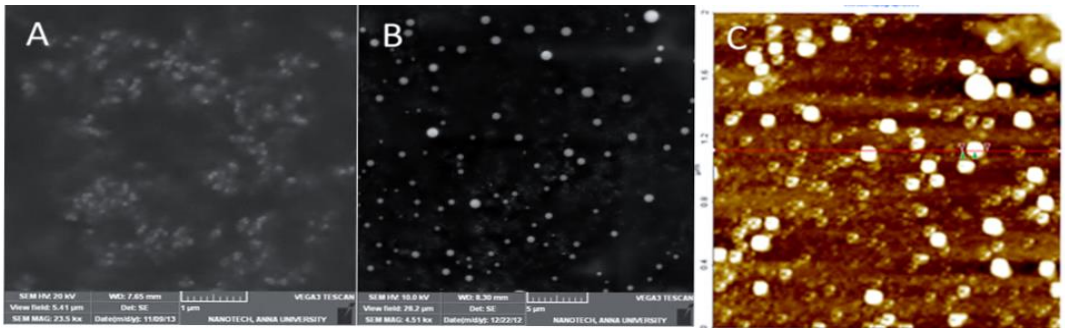

Fig. 1 (A) SEM image showing spherical shaped CHIT-NPS (B) SEM image showing spherical shaped QUER-CHIT nanoparticles (C) AFM image showing well- defined spherical shaped QUER-CHIT nanoparticles

To define the physical nature of the nanoparticles, XRD analysis was performed. Patel *et al.,* [32] reported that crystalline compounds become amorphous in nature once they are coated

with colloidal particles. Standard QUER powder showed a sharp peak at diffraction angles (2θ) of 10.7, 13.5, 17.9, 21.7 23.6, 26, 27.7 and 29.7 suggesting that they are highly crystalline in nature. This result was similar to the result obtained in previous studies; they proved that QUER is crystalline in nature showing sharp peaks at 10.7, 12.3, 16.0, 23.6 and 27.1 in the XRD spectra [30]. In contrast to this, QUER became slightly amorphous once encapsulated into CHIT-NPS, which showed absence of major peaks except some minor peaks at diffraction angles (2θ) of 10.7, 12.5 and 27.3. CHIT-NPS resulted in the absence of sharp peaks, strongly suggesting that it is amorphous in nature (Figure 2).

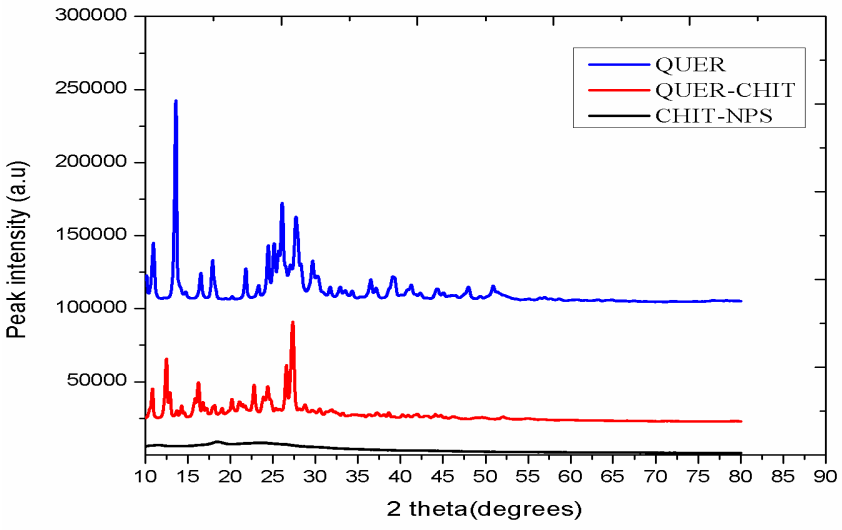

Fig 2. XRD Spectra of pure QUER, QUER-CHIT and CHIT nanoparticles

FTIR is an important study for the quick identification of encapsulated QUER molecules [29]. QUER shows major characteristic peaks, aromatic bonding and stretching at 1100-1600 cm^{-1}, OH stretch at 3300-3500 cm^{-1} and -C-O stretching at 1615 cm^{-1}. -C-C=C asymmetric stretch at 1512 cm^{-1} is present in both QUER and QUER-CHIT nanoparticles. The -C-O stretching at 1615 cm^{-1} also appeared in the QUER-CHIT nanoparticles. The presence of QUER major peaks on the QUER-CHIT nanoparticles shows an indirect confirmation of QUER encapsulation in CHIT-NPS. CHIT-NPS exhibits OH stretch at 3405 cm^{-1} and -N-H stretch at 1043 cm^{-1} (Fig. 3).

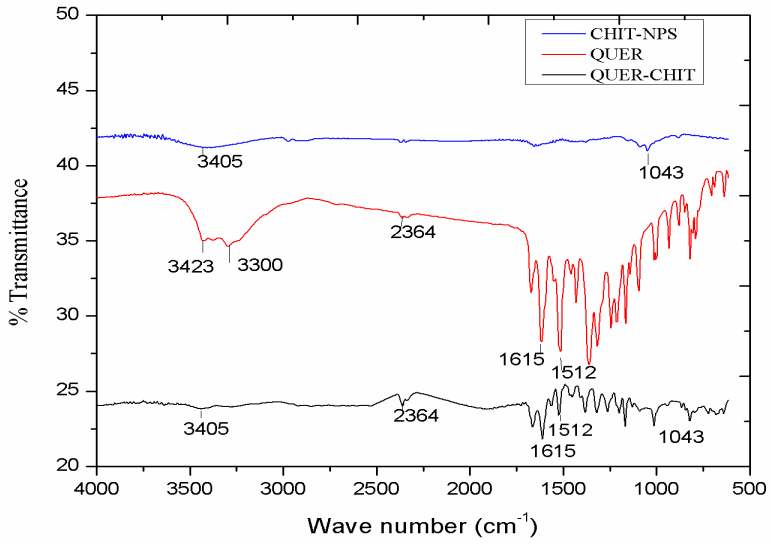

Fig. 3 FT-IR Spectra of QUER, QUER-CHIT and CHIT nanoparticles

3.3 *In vitro* release studies

The release profile at pH 7.4 was evaluated in phosphate buffer for 12 h to determine the release of QUER entrapped within the core of the nanoparticles. Small burst effect was observed in drug loaded by incorporation method with better-sustained release characteristics [33]. In the present study small burst release was seen at pH 7.4 in about 12 h around 20% of QUER was released, the finding here is consistent with their observation. In the previous report, it was identified that initial burst release of about 40-45% within 0-0.5 h was observed in QUER loaded PLA nanoparticles, this is due to the loosely adsorbed QUER on the surface of polymer matrix [29]. At pH 7.4, 70% of the drug was released at 12 h (Table 3). At pH 7.4 sustained releases were observed for 12 h and also it was effectively used for *in vitro* studies to treat the toxicity in rat erythrocytes due to initial burst release.

Table 3 Release curve was obtained by plotting % cumulative quercetin released vs. Time

Time in hour	% of release
0	0
0.4	4.083333
0.6	5.366667
0.8	11.08333
1	20.3
2	28.11667
3	37.8
4	45.61667
5	51.91667
8	58.05
10	69.71667
12	70

3.4 Cell viability and LDH assay

Cell viability assay is widely used to measure the activity of mitochondrial enzymes present in healthy cells and to evaluate the cell viability modulating activity of natural and synthetic compounds [34, 35]. Cytotoxicity study was done to identify effect of encapsulated QUER and pure QUER on cell proliferation under *in vitro* conditions. RAW 264.7 macrophage cells were treated with 12, 25, 50, 100 and 500 µg of QUER-CHIT nanoparticles and pure QUER drug for 48 h. IC_{50} value of QUER-CHIT nanoparticles was found to be 21 µg/ml and for QUER 131.2 µg/ml. The inhibition of cell proliferation by QUER-CHIT nanoparticles was clearly observed in a dose and time dependent manner. No significant sign of cytotoxicity was observed with varying concentration QUER-CHIT nanoparticles; the treated cells exhibit normal morphology as the control. Macrophage RAW 264.7 cells are immune effector cells, plays an important role as antigen presenting cells, phagocytic cells and secretory cells [36], hence we investigated the biocompatibility of QUER-CHIT nanoparticles using macrophage cell line.

Lactate dehydrogenase is a cytosolic enzyme released into the medium upon cell death due to damage of plasma membrane [37]. Number of lysed cells is proportional to the LDH activity in the supernatant of cultured cells. Cell death assays based on LDH activity is more reliable than other enzyme-based cell death assays. In the present study RAW 264.7 cells were treated with different concentration of QUER-CHIT nanoparticles and Pure QUER drug. The LDH activity was measured in IU/mg protein, it was quantified that based upon dose dependent manner the LDH activity was increased in both QUER-CHIT nanoparticles and pure QUER treated groups. QUER-CHIT nanoparticles incubation in RAW 264.7 macrophages resulted in decreased release of LDH compared to pure QUER drug are shown in Fig 4.

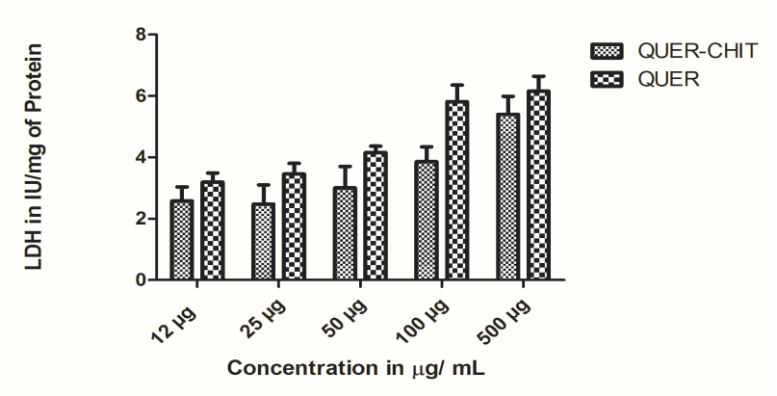

Fig. 4 Measurement of LDH in RAW 264.7 macrophage cell culture supernatant fluids treated with QUER-CHIT nanoparticles and pure QUER drug. Each value is expressed as mean ± SD (n = 3).

3.5 Assessment of oxidative stress parameters

The effect of monocrotophos on oxidative stress in RBC cells was examined by investigating SOD, CAT, GST, GSH, and MDA intracellular enzymes are shown in Fig 5. Various studies declared that pesticide causes oxidative damage in experimental animals depending upon the dosage of pesticide used [38-40]. In the present study dose dependent toxic effect were found in all the parameters. Usage of pesticides in an uncontrolled manner leads to ecological imbalance and it affects public health [41]. Schara *et al*., [42] and Brandao *et al*., [43] identified that toxic chemicals and pesticides target the intact membrane of erythrocyte and disrupt it. In the monocrotophos treated groups SOD, GSH, GST, and CAT intracellular enzymes were decreased. In the previous study, Shvedova *et al*., [44] stated that depletion of SOD, GSH and GST levels were due indication of oxidative stress. Compared to control, monocrotophos treated groups exhibited a significant decrease in the enzyme levels. QUER-CHIT nanoparticles elevated the enzyme level in P1, P2, and P3 groups to scavenge the free radicals generated by monocrotophos. Compared to monocrotophos treated groups, SOD, GSH, GST and CAT levels were significantly increased in QUER-CHIT nanoparticles treated groups. Celik and Suzak [41] reported that depletion of SOD activity is due to the elimination of free radicals. Organophosphate pesticides have been reported to decrease the GPx and GST activity in various rat tissues [45, 46]. Due to toxic effect of monocrotophos the enzyme activity is significantly decreased compared to all the groups. Thus QUER-CHIT nanoparticles play a major role in eliminating the free radicals in a dose dependent manner. Mehta *et al*., [47] reported that LPO involves in the molecular mechanism to ameliorate the toxicity of pesticides. Release of MDA in the medium is due to the cell membrane damage caused by LPO. In the present study the MDA level was significantly increased in the monocrotophos treated groups compared to control, it is due to the cellular damage caused by monocrotophos. In the QUER-CHIT nanoparticles + monocrotophos treated groups the MDA level is decreased due to the protective effect of QUER-CHIT nanoparticles.

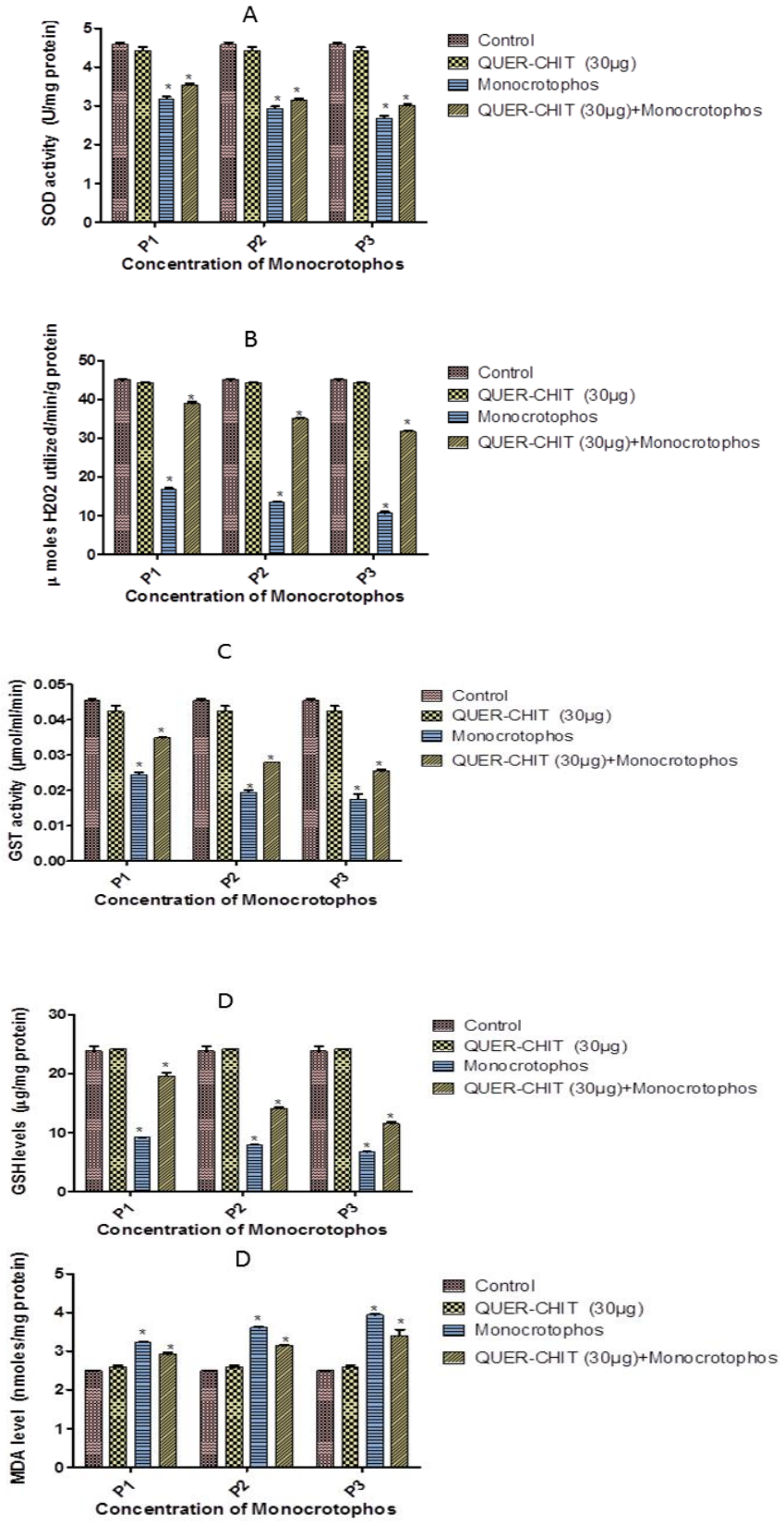

Fig. 5 (A) SOD (B) GST (C) GSH (D) CAT and (E) MDA activity in control and experimental groups of erythrocytes treated with monocrotophos and QUER-CHIT nanoparticles. The asterisk (*) denotes a satistically significance difference from control and monocrotophos treated group ($P < 0.05$).

4. Conclusion

In summary, our *in vitro* data shows that QUER-CHIT nanoparticles can be effectively used for clinical applications to overcome the oxidative damage induced by organophosphate pesticide. Future studies are therefore necessary to understand the mechanism of QUER-CHIT nanoparticles against monocrotophos induced toxicity *in vivo*.

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