

Resveratrol and Puerarin loaded polymeric nanoparticles to enhance the chemotherapeutic efficacy in spinal cord injury

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Abstract

Introduction:

Spinal cord injury includes inflammation and apoptosis of neurons, which is difficult to cure by systemic drug administration. Administration of natural active compounds (resveratrol and also Puerarin) by advance drug delivery technology improves the patient's conditions.

Material and Methods: Oil-in-water emulsion method was utilized to prepare resveratrol as well as puerarin loaded PLGA nanoparticles. The nanoparticles were subjected to mean zeta potential, mean particle size, encapsulation efficiency as well as *in vitro* drug release studies. The biochemical parameters i.e. malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), advanced oxidation products (AOPP), catalase (CAT) and nitrite/nitrate levels were tested for the loaded nanoparticles. Reperfusion injury induced rats treated with 10 mg/kg resveratrol and puerarin loaded nanoparticles protects spine from ischemia injury and supports biological parameters.

Results: The mean particle size varies from 238 nm to 274 nm and also particle size distribution was mono-dispersed (0.239 to 0.318). Zeta potential value of nanoparticles was observed to be -12.6 ± 2.1 mV. Optimized nanoparticles reveals 72% -79% of drug release over 36 h by diffusion mechanism. Significantly, lowers the levels of plasma nitrite/nitrate level as well as phosphorylation of p38MAPK pathways in reperfusion injury induced rats.

Conclusion: The resveratrol and puerarin loaded nanoparticles decreases free radicals produced by reperfusion injury induced rats, as well as decrease of oxidative stress because of IRI. Resveratrol and puerarin loaded nanoparticles decreases GSH, SOD and CAT antioxidant level, which helps in overall health improvement of patients.

1. Introduction

Spinal cord connected with brain by peripheral tissue and nervous system[1]. Spinal cord primarily comprise of neurons, glial cells, astrocytes and oligodendrocytes cells. Spinal neurons, poses cutaneous sensory input are mainly located in the dorsal spinal cord and interconnected in circuits; while neurons responsible for motor control are located in ventral spinal cord. The loss of neurons and axonal degeneration occurs at the lesion site due to spinal cord injury. In some cases severe functional impairment, paraplegia, or tetraplegia may occurs due to motor deficits, loss of sensory inputs and chronic pain [1, 2]. Secondary spinal cord injury is a degenerative response that includes inflammation, ionic imbalance, excitotoxicity, neurotransmitter accumulation and apoptosis [3, 4]. The severity of the secondary injury response slowly to systemically administered drug within therapeutic window.

Pain relief, spasticity treatment, rehabilitation and prevention of complications are most focus area in long term therapy for spinal cord injury. The glial scars are developed in the trauma region, which limits the regenerative capacity of central nervous system. [5–7]. Special methods are required to reconstruct

grey and white matter of CNS, which helps in promote axonal growth and reduce scar formation. [8]. The nanoparticles facilitate passage through the blood-spinal cord barrier and extend therapeutic delivery and/or imaging in the brain and spinal cord to protect it from toxins.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenol has antioxidant and anti-inflammatory activity [9, 10]. Resveratrol helps in free radical scavenging, increases the antioxidant enzymes, promote neuroprotection and neutralizes oxidative stress. Puerarin (Daidzein-8-C-glucoside) is the major bioactive ingredient in kudzu root [8]. Many researchers show that Puerarin has anti-apoptotic, anti-oxidant [11], anti-inflammatory [2], anti-arrhythmic[1], and anti-fibrosis [12] activity. These therapeutic effects of Resveratrol and Puerarin have much useful for treatment of spinal cord injury [13]. The major constrains for oral administration of Resveratrol and Puerarin orally is its short half-life, in which it is rapidly metabolized by first pass metabolism in liver. The nanocarriers useful for significant prolong residence time of the dosage; improve the efficacy of drugs as potential treatment of spinal cord. The main objective of this work was to evaluate the capability of Resveratrol and Puerarin encapsulated in Poly(DL-lactic-co-glycolic acid) nanoparticles as treatment to reduce the oxidative stress in spinal cord injury [14].

2. Materials And Methods

2.1. Materials

Resveratrol (RES) and Puerarin (PUE) purchased from Sigma Aldrich (St. Louis, MO, 454 USA). Poly(lactic-co-glycolic acid) with 50:50 ratio was purchased from Sigma-Aldrich. Polyvinyl alcohol, 6 kg/mol and 80%mol hydrolyzed, was purchased from Polysciences Inc. (Warrington,USA). Acetonitrile (HPLC grade) was obtained from BDH Laboratory Supplies (Pools, UK). All chemicals were of analytical grade and were used as received. DMSO, ethylene diamine tetraacetic acid (EDTA) were purchased from Merck co. (Darmstadt, Germany). All other chemical reagents were of analytical grade. All buffers were made with ultrapure water collected from Millipore at 18 MΩ resistances (Millipore, Bedford, USA).

2.2. Animals

Male wistar albino rats weighing 210–250 g were purchased and acclimatised to laboratory environment by exposed them to free access of food and water for 24 hrs. Prior to run the animal experimentation, all the procedures were taken to account for consideration and supervision by the Animal ethical clearance committee.

2.3. Preparation of RES-PUE-PLGA nanoparticles

Oil-in-water emulsion techniques were used for the drug loading in PLGA nanoparticles and PVA used as co-solvent to stabilized the emulsion. RES-PUE loaded nanoparticles were prepared with aqueous outer phase (PVA and NaCl) covers the inner organic phase of PLGA (0.25 dL/g), Resveratrol (RES) and Puerarin (PUE) in DCM. Micrio-tip probe sonicator at 39 W (VCX750, Sonics and materials Inc, USA) was use as energy source for emulsion preparation over ice bath for 20 s. The nanoparticles formed in emulsion were stabilized by addition of 40 mL of PVA solution (25 mg/mL) and stirred for 18 hrs at room

temperature. The RES-PUE nanoparticles were collected by ultracentrifugation at 40,000 rpm for 20 min at 4 °C (Sorvall-WX, ThermoFisher Scientific, USA), washed thrice with ultrapure water to remove free drugs and unbound PVA, lyophilized for 2 days (- 80 °C and < 10 µm mercury pressure, Lab-conco Lyophilizer, USA) to get the powdered NPs.

To prepare an optimum dosage form, with surplus entrapment, the impact of drug to polymer ratio was studied. Herein, all the variables were found to be constant except that the drug to PLGA ratio showed variations by 20, 30, 40, 50 and 60% (w/w) on dry weight basis.

2.4. Characterization of Resveratrol and Puerarin loaded nanoparticles (Res-Pue nanoparticles)

RES-PUE loaded nanoparticles synthesis was optimized for Drug-polymer ratio, and thus encapsulation and thus encapsulation efficiency varied. Yield of nanoparticle was calculated from the weight of PLGA nanoparticles recovered, divided by the initial weight of PLGA taken for formulation [15, 16].

2.5. Drug encapsulation efficiency (DE)

Encapsulation efficiency was calculated from the ratio of theoretical drug content to fraction of drug detected per unit mass of particle. RES-PUE loaded nanoparticles sample (100 mg) was dissolved in 1 mL of HPLC grade methanol in a microcentrifuge tubes. The solution was mix for 5 min by vortexing (MX-S, Vortex mixer, Biobase) followed by centrifugation (BKC-TH20RL, Biobase) at 35,000 rpm for 20 min at 4 °C to collect the supernatant. The collected supernatant was filtered through 0.2 µm syringe filter and 20 µl was injected to RP-HPLC (e2695 separation module, Water's Alliance, Milford, MA, USA). The area under the curve (AUC) and retention time (t_R) of the peaks were recorded by extracting the chromatogram at 270 nm and 304 nm wavelength for Resveratrol and Puerarin respectively, using operating software Empower3 pro. Resveratrol and Puerarin encapsulation efficiency and loading capacity was calculated using regression equation obtained from concentration vs AUC of standard Resveratrol and Puerarin respectively.

2.6. Size of Particles and Zeta Potential of RES-PUE loaded nanoparticles

The particle size analyzer (Zetasizer, Malvern Panalytical, UK) based on dynamic light scattering technique used for average particle size determination. Zeta potential of nanoparticles shows surface properties of nanoparticles and it was measured by electrostatic or charge repulsion/attraction between particles (Nano ZS analyzer, Malvern Panalytical, UK). Briefly RES-PUE loaded nanoparticles sample solution was prepared with water and solution was placed in separate polystyrene cuvettes meant for particle size and zeta potential measurement.

2.7. Scanning Electron Microscopy

Surface morphological evaluation of RES-PUE loaded nanoparticles were carried out by scanning electron microscope (EVO, ZEISS Germany) for which lyophilized powder samples were spread on the carbon

adhesive sample holder and coated with gold and used in scanning electron microscope[14].

2.8. Atomic Force Microscopy

Atomic force microscope (AFM) (Axio Observer Z1, Carl Zeiss Microscopy GmbH) was used for visualization of individual as well as groups of particles. It offers visualization in three dimensions, unlike other microscopy techniques. The samples were dispersed in optimum quantity of deionized water by sonication. Small amount of the dilution were put on a cleaned mica plate and incubated to dryness by a stream of nitrogen. This method was followed for all samples.

2.9. Fourier Transmission Infrared spectroscopy (FTIR) analysis

The chemical integrity of the polymer and drug was analysed via Fourier Transmission Infrared Spectroscopic technique (SPECTRUM RX I, Perkin Elmer, USA). Approximately 2 mg of native Resveratrol, Puerarin and RES-PUE loaded nanoparticles samples were mixed separately in 300–400 mg of anhydrous KBr and ground properly in a mortar pestle. The sample mixture was compressed by applying hydraulic pressure of 2000 Kg/ cm² (Jasco MP2 mini press) for 2 min. The FTIR spectrum was obtained by scanning all samples under 2 cm⁻¹ resolution power in 4000 – 400 cm⁻¹ spectral [17].

2.10. Differential Scanning Calorimetry

The physico-chemical properties and drug polymer compatibility of the drugs in the polymer were analysed via Differential Scanning Calorimetric technique (DSC-60, Shimadzu, Japan). The samples RES-PUE loaded nanoparticles, native Resveratrol native Puerarin (2–4 mg) were sealed separately in standard aluminium pans and scanned 10°C/min rate of heat supply at a temperature ranging from 50–350°C with continuous nitrogen gas flow of 65 mL/ min [17].

2.11. In-vitro drug release study

RES-PUE loaded nanoparticles, native Resveratrol and Puerarin (each containing 5 mg of Resveratrol and Puerarin) were kept in two separate hermetically sealed dialysis bags (12000–14000 Dalton, Sigma-Aldrich). The dialysis bags were hanged in a beaker that was filled with saline buffer of phosphate, maintained at pH = 7.4 (100 mL) as dissolution medium maintained at 37 ± 2 °C with continues stirring at 100 rpm. The entire assembly was covered with aluminium foil to prevent loss of dissolution media. 1 mL of samples was collected at specified time intervals. The release media was replenished with 1 mL of fresh dissolution media after every sample withdrawal. The collected samples were subjected to centrifugation at the rate of 13,800 rotations per minute for 30 min and the supernatant was lyophilized (Lab-conco Lyophilizer, USA) for a day. Dissolution was affected by dissolving the lyophilised, dried powder in methyl alcohol and this was analysed via RP-HPLC technique. The entire analysis was repeated thrice [18].

2.12. Instigation of ischemia–reperfusion injury in spinal cord

Induction of ischemia in spinal cord according previously published article. Mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) used as interperitoneal injection to anesthetize rats. Small aneurysm clip was placed between left kidney arteries and cross clamping used to cause ischemia. After surgical treatment animals were preyed on a common diet plan as well as water *ad libitum*. All animals were anesthetized and give up after 48 h of ischemia-reperfusion treatment. The 3, 4, 5 lumbar segments of spinal cord and blood samples were collected.

2.13. Nitrite/Nitrate levels in blood plasma

The UV-Vis spectrophotometer is used for determination of enzymatic conversion of nitrate to nitrite by nitrate reductase at 540 nm. This shows nitrite/nitrate levels based on Griess reaction.

2.14. Western blot analysis

Total protein was extracted from tissues of treated and controlled group by utilizing a lysis buffer consisting of 1M Tris-HCl (pH 7.5), 1ml 0.5M EDTA, NP-40 (1% v/v), Trizma base with protease inhibitor. The protein concentration of the supernatant was identified making use of BCA as well as kept at -80 C. The equivalent quantity (50 Ig/ well) of proteins were divided in 10% SDS-PAGE, moved to a nitrocellulose membrane layer. The membrane layers were nurtured overnight at 4 °C with key antibodies of iNOS, p-p38, overall p38 and β -actin. The equivalent amount of IgG antibodies in alkaline phosphatase were used for incubation for 2 h at RT. The membrane layers were established utilizing ECL reagent and protein signals evaluated by ChemiDoc after being normalizd to cognate β -actin signals.

2.15. Quantification of advanced oxidation products (AOPP) and malondialdehyde (MDA)

AOPP and MDA in lipid peroxidation were determined by following the reported method of Ohkawa. Briefly, Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) was used for preparation of tissue homogenates and absorbance was recorded at 532 nm. Addition of $MgCl_2$ and phosphor tungstate to tissue homogenates helps in determining AOPP in the supernatant at 340 nm absorbance read in acidic conditions. The level of AOPP and MDA was shared as nM of MDA or AOPP reactants/100 g of wet tissues.

2.16. Quantification of reduced glutathione (GSH)

The elevated levels of glutathione were determined by previously reported method of Ellman. The tissue homogenates were combined with 5% TCA and 0.3 M disodium hydrogen phosphate. Add 1.0 ml of Ellman's reagent to the homogenates mixture. Record the absorbance of sample against blank containing TCA at 420 nm. The quantity of glutathione is expressed as mg/g of protein.

2.17. Quantification of superoxide dismutase (SOD) activity

The SOD of samples was determined following by reported method by Misra and Fridovich. Briefly, 1.8 mM epinephrine was used for initiation of the response as well as absorbance read at 480 nm. Non-polar solvents were added in tissue homogenates followed by 0.6 mM EDTA solution as well as 0.1 M carbonate buffer solution. The superoxide dismutase activity revealed as 50% inhibition of epinephrine in one minute/mg protein.

2.18. Quantification of catalase (CAT) activity

The CAT activity was performed by previously reported method by Takahara. Briefly, the tissue homogenate was mixed with 50 mM phosphate buffer and absorbance was recorded by the addition of 30 mM H₂O₂ solution at 240 nm. The enzyme activity was revealed as I moles of H₂O₂ decomposed/min/mg protein.

2.19. Statistical analysis

Analyses were performed in triplicate (n=3) and the outcomes were expressed as mean ± SD. For the statistical analysis the experimental data was tested by on-way analysis of variance (ANOVA). The values of p < 0.05 were characteristic of statistical significance.

3. Results

3.1. Characterization of RES-PUE loaded nanoparticles

3.1.1. Loading capacity and Drug entrapment efficiency

The drug loading and entrapment efficiency of PLGA nanoparticles is depends on the solvent affinity of drug, polymer and surfactant. It's previously reported that PLGA have encapsulation efficiency of ~ 82%w/w. We have prepared five different RES-PUE loaded nanoparticles formulations taking different ratio of drug, PLGA and keeping same PVA content [5, 6]. The RES-PUE loaded nanoparticles fabricated with a ratio of 1:3 drug-polymer shows, higher entrapment efficiency of 74.85% and loading capacity of 15.50% (Table-1).

3.1.2. Particle Size and Zeta potential of RES-PUE loaded nanoparticles

The photon correlation spectroscopy is one of the easy and rapid method used for measurement of particle size. In RES-PUE loaded nanoparticles the mean particle size was ranges from 238 nm to 274 nm and particle size distribution was mono-dispersed (0.239 to 0.318) (**Figure-1a**). The stability of RES-PUE loaded nanoparticles in water can be attributed from single peak at particle size distribution plot [5, 6]. Polymers and additives used in formulation influence the zeta potential of particle surface charge. Zeta potential of RES-PUE loaded nanoparticles was found to be -12.6 ± 2.1 mV (**Figure-1b**).

3.1.3. Scanning Electron Microscopy

Scanning electron microscopic studies were performed for surface morphology and particle size measurement of nanoparticles. The SEM images of particles shown in **Figure-1c**, obtained directly by visualization through scanning electron microscope, so it appears smaller than the hydrodynamic layer obtained by Zetasizer.

3.1.4. Atomic Force Microscopy

Three dimensional surface morphology studies of the RES-PUE loaded nanoparticles were carried out by AFM on cleaned Si substrates (**Figure-1d**). The lateral sizes of the nanoparticles varies from image to image because of the variation in the tip shape [19]. The height of nanoparticle were varies from 9 to 11 nm.

3.1.5. Differential scanning calorimetry (DSC) studies

The physical state of drug incorporated in PLGA nanoparticles was determined by DSC studies. The DSC curve of native Resveratrol, Puerarin, PLGA and RES-PUE loaded nanoparticles were shown in **Figure-2a**. The sharp endothermic peak at 262 °C and 189 °C in native Resveratrol and Puerarin respectively shows the melting point of native drug. The formulation mixture shows the endothermic peak at 183 °C, that of PLGA. In addition it shows less intense peak of Resveratrol and Puerarin, which may be due to surface bound drugs [17, 20].

3.1.6. FTIR spectroscopy

The FTIR spectrum of Resveratrol, Puerarin, PLGA and RES-PUE loaded nanoparticles were presented in **Figure-2b**. The FTIR spectrum of Resveratrol shows a major olefinic band at 965 cm⁻¹ and 3293 cm⁻¹ show due to O-H stretching vibration. The band at 1383, 1586 cm⁻¹ shows presence of C-O stretching in Resveratrol. Two characteristic peaks of puerarin at 1630 and 1260 cm⁻¹ shows presence of C-O stretching in puerarin. FTIR analysis of PLGA polymer showed distinct peaks at wave numbers 692 cm⁻¹, 1008 cm⁻¹, 1370 cm⁻¹, 1627 cm⁻¹, 1960 cm⁻¹, 2257 cm⁻¹. In addition peaks at 2441 cm⁻¹ and 3406 cm⁻¹ in both PLGA and RES-PUE loaded nanoparticles conforms the presence PLGA in the formulation. The presence of hydrogen bonding between hydroxyl groups of Resveratrol and carbonyl group of PLGA shifts O-H stretching band to higher wave number [10, 17, 20].

3.1.7. *In vitro* drug release studies

In the present study cumulative percentage release data of drug from native Resveratrol, Puerarin and RES-PUE loaded nanoparticles divulges sustained release action of formulation (**Figure-2c**). The native Resveratrol and Puerarin shows 96% and 98% of drug release respectively in just 6 hrs of study, whereas the optimized formulation of RES-PUE loaded nanoparticles shows only 72% -79% of drug release over 36 hrs by diffusion mechanism.

3.2. Pharmacokinetic study

3.2.1. Effect of RES-PUE loaded nanoparticles on plasma nitrite/nitrate levels

RES-PUE loaded nanoparticles alter the circulating plasma nitrite/nitrate levels induced by reperfusion injury. The plasma nitrite/nitrate level in control group was less than the reperfusion injury induced group [21]. Administration of RES-PUE loaded nanoparticles decreases the nitrite/nitrate level in plasma of reperfusion injury induced rats (**Figure-3a**). However, the control group rats did not show any change in nitrite/nitrate levels in plasma even after treated with RES-PUE loaded nanoparticles.

3.2.2. Effect of RES-PUE loaded nanoparticles on protein expressions

Effect of RES-PUE loaded nanoparticles on IRI-induced, iNOS protein expression was measured by western blot. The protein (**Figure-3b**) expression was not altered in control, RES-PUE loaded nanoparticles and surgery groups [22]. The increased level of iNOS protein expression was normalized after treatment with RES-PUE loaded nanoparticles in reperfusion injury induced rat groups.

3.2.3. Effect of RES-PUE loaded nanoparticles on p38MAPK pathway

Effect of RES-PUE loaded nanoparticles on reperfusion injury induced group was studied by activation of p38MAPK pathway indicating p38MAPK phosphorylation. As shown in **Figure-4**, the p38MAPK phosphorylation did not alter in control [16]. But, p38MAPK was distinctly phosphorylated in reperfusion injury induced group treated with RES-PUE loaded nanoparticles.

3.2.4. Effect of RES-PUE loaded nanoparticles on Malondialdehyde and AOPP

The level of Malondialdehyde (**Figure-5a**) and AOPP (**Figure-5b**) formed from lipid peroxidation/ oxidation was used to investigate the effect of RES-PUE loaded nanoparticles [23]. The reperfusion injury induced groups treated with RES-PUE loaded nanoparticles shows decreased level of Malondialdehyde and AOPP as compared to control group.

3.2.5. Effect of RES-PUE loaded nanoparticles on GSH, SOD and CAT antioxidant activities

The GSH non-enzymatic antioxidant activity level (**Figure-6a**) alters in RES-PUE loaded nanoparticles treated reperfusion injury induced group [13]. Similarly, the SOD (**Figure-6b**) and CAT (**Figure-6c**) enzymatic level didn't alter in control group, but significantly increases in RES-PUE loaded nanoparticles treated reperfusion injury induced group [11].

4. Discussion

The increase in Drug to polymer ratio increases the encapsulation efficiency. It was observed that increase in the drug to PLGA ratio from 20–40% increases the drug encapsulation efficiency of nanoparticles. The increases in drug loading saturate the surface matrix of the PLGA nanoparticles, which favour the leaching, process of drug. So, the formulation with 1:3 drug to PLGA ratio was selected and used for all further studies.

The higher zeta potential value of the particles shows higher stability of nanoparticle formulations. Negative charge can be attributed to the presence of free carboxyl groups of PLGA on the nanoparticle surface [5, 6]. The projected PEG chains of PVA prevent aggregation of the particle despite of lower zeta potential. The SEM and AFM image of RES-PUE loaded nanoparticles was found to be spherical in shape with smoother surface [6, 14]. The DSC and FTIR studies also reveal that Resveratrol and Puerarin were present as molecular dispersion form in the PLGA matrix.

In vitro drug release studies shows that the formulation shows a burst release of drug at initial stage (0.25 hrs and 0.5 hrs), attributes the presence of surface bound drugs. Low melting point and hydrophobic nature of PLGA provides better sustained release action on solidification [17, 20].

In vivo pharmacokinetics studies shows no change in nitrite/nitrate levels and increased level of iNOS protein expression in plasma after treated with RES-PUE loaded nanoparticles. The reperfusion injury induced group treated with RES-PUE loaded nanoparticles shows decrease in p38 phosphorylation, Malondialdehyde and AOPP level as compared to control group.

Cytokines production and neutrophils activation causes loss of membrane lipid functions [24], which generates free radicals and results in cell death [25]. The free radical causes reoxygenation and changes the pathology of neurologic dysfunction in reperfusion injury induced group. The group treated with 10 mg/kg of RES-PUE loaded nanoparticles protects spinal cord from ischemia.

Oxidative stress induced in injured spinal cord tissues due to over production of nitric oxide. Similarly, several studies have been focused on inhibition of p38MAPK pathway by suppressing oxidative stress in reperfusion injury induced group [3, 26]. Significant decrease in p38MAPK expression and plasma nitrite/nitrate levels were observed in RES-PUE loaded nanoparticles treated reperfusion injury induced group [27]. The Malondialdehyde and AOPP levels were increased in reperfusion injury induced group and it correlated with cytokines levels [12].

The administration of RES-PUE loaded nanoparticles to reperfusion injury induced group significantly increases the antioxidant level i.e. GSH, SOD and CAT, which are acts as defence.

The multifunctional resveratrol and puerarin loaded nanoparticles were developed to enhance the oral bioavailability of resveratrol and puerarin. RES-PUE loaded nanoparticles protect spinal cord injured rat models. The RES-PUE loaded nanoparticle blocks the p38MAPK signalling pathway and expressed antioxidant effects. The further investigation may be acceptable to determine the efficacy of RES-PUE loaded nanoparticles on human spinal cord reperfusion injuries. Reperfusion injury induced free radicals were reduced by RES-PUE loaded nanoparticles treatment by increasing enzymatic and non-enzymatic antioxidant levels.

Declarations

Ethics approval and consent to participate

The animal experiment study protocol was approved by the institute's committee on animal research, Linyi central hospital, Shandong province, China. The legal and ethical approval was obtained prior to initiation of the research work on animals, and the experiments were performed in accordance with the animal ethical committee guidelines and regulations of Republic of China.

Consent for publication

Not applicable.

Availability of data and material

The supporting data for present findings are under ethics laws of university and is hence not presented here.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

WC, ZZ re associated planning the work The experiments were carried out by SZ, LZ whereas QS and LZ were also involved in data handling and processing. All the authors contributed equally in preparing the manuscript. All authors read and approved the final manuscript.

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Table

Table-1: Physico-chemical characterization of RES-PUE loaded nanoparticles (data represents mean \pm SD).

Batch No.	Drug- Polymer ratio	Loading capacity (% w/w) ^a	Entrapment efficiency (% w/w) ^b
RES-PUE-NP1	1:2	21.91 \pm 0.09	69.63 \pm 0.68
RES-PUE-NP2	1:3	15.50 \pm 0.08	74.85 \pm 0.89
RES-PUE-NP3	1:4	10.71 \pm 0.86	72.72 \pm 0.80
RES-PUE-NP4	1:5	8.60 \pm 0.17	61.43 \pm 0.64
RES-PUE-NP5	1:6	7.78 \pm 0.20	54.24 \pm 0.42

$$^a\text{Loading Capacity} = \frac{\text{Weight of Drug in Nanoparticles}}{\text{Weight of Polymer and Drug added}} \times 100$$

$$^b\text{Entrapment efficiency} = \frac{\text{Weight of Drug in Nanoparticles}}{\text{Weight of Drug added}} \times 100$$

Figures

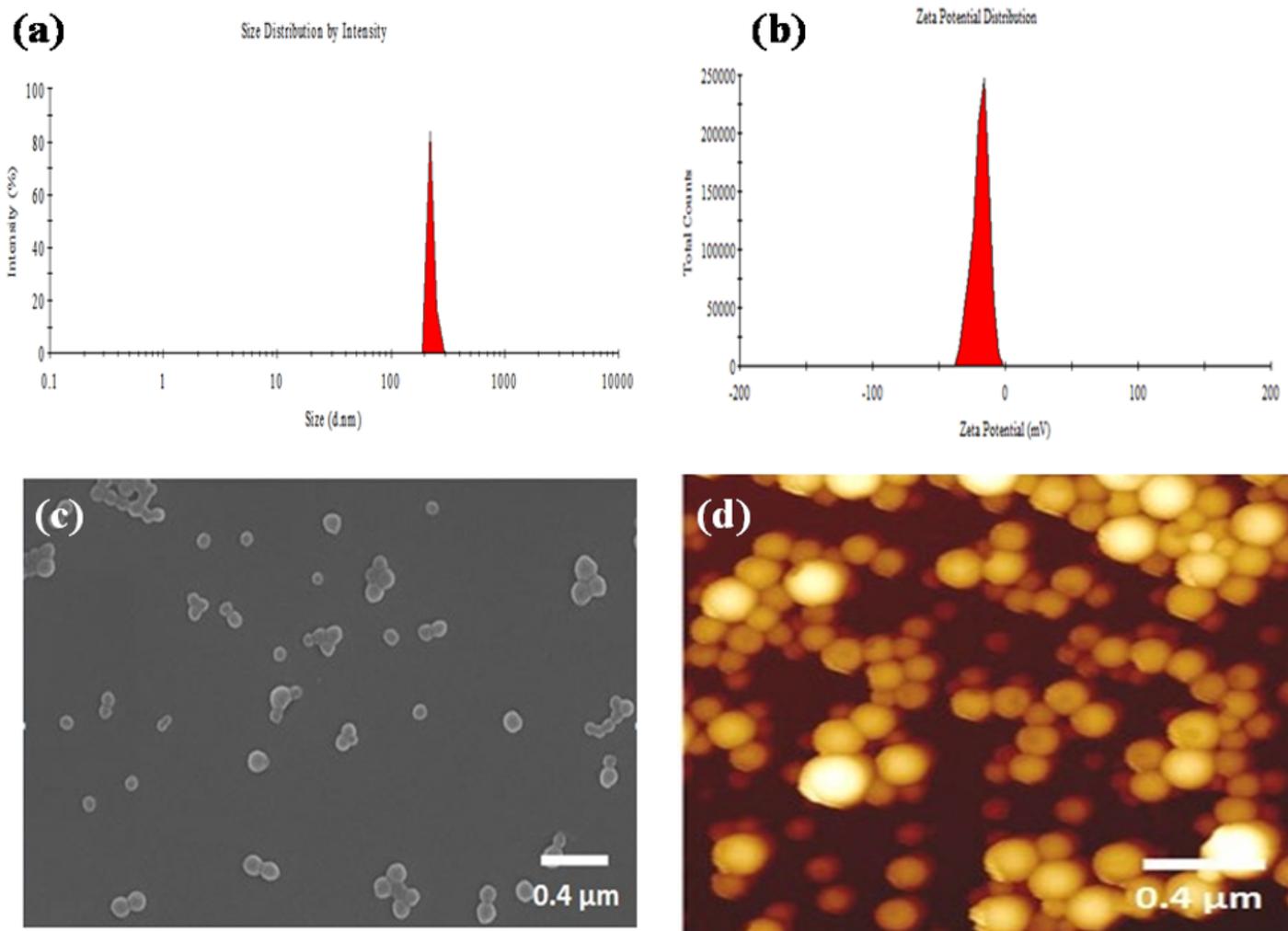


Figure 1

Surface morphology of RES-PUE loaded nanoparticles (a) Mean particle size distributions of RES-PUE loaded nanoparticles, (b) Zeta potential of RES-PUE loaded nanoparticles, showing negative values, (c) Scanning electron microscopy images of RES-PUE loaded nanoparticles (bar = 400 nm). (d) Atomic force microscopy images of RES-PUE loaded nanoparticles (bar = 400 nm).

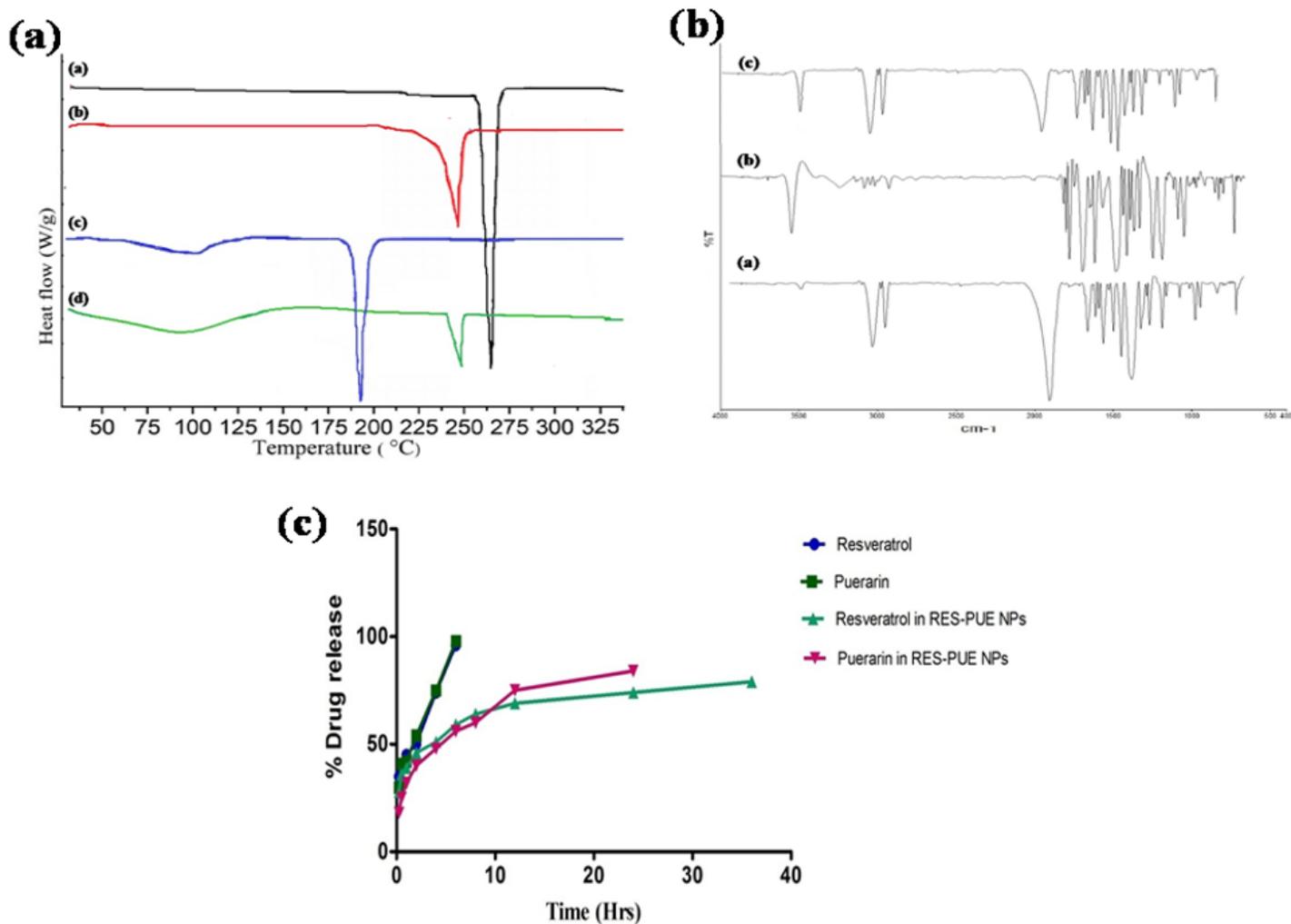


Figure 2

(a): Differential scanning calorimetry (DSC) thermogram of (a) Native resveratrol, (b) PLGA, (c) Native puerarin and (d) RES-PUE loaded nanoparticles. (b): Fourier transforms infrared spectra of (a) Native resveratrol, (b) Native puerarin and (c) RES-PUE loaded nanoparticles. (c): In vitro drug release study of Native resveratrol, Native puerarin and RES-PUE loaded nanoparticles. Data as mean \pm standard error of mean (n = 3).

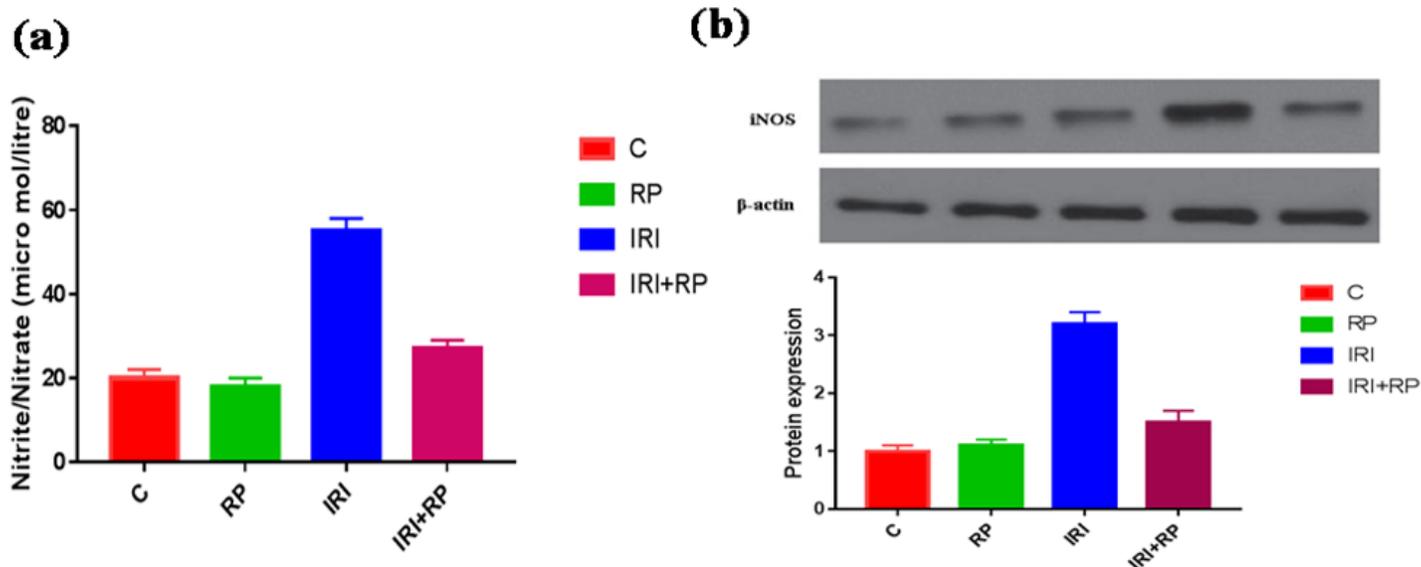


Figure 3

Effect of RES-PUE loaded nanoparticles on plasma nitrite/nitrate and protein expressions. (a) Estimation of levels of nitrite/nitrate in rats plasma. (b) β -actin normalized protein levels in spinal cord tissue homogenates. (C: Control, RP: Resveratrol and Puerarin nanoparticles, IRI: Ischemia-reperfusion injury, IRI + RP: Ischemia-reperfusion injury treated with Resveratrol and Puerarin nanoparticles) P value < 0.001 compared with IRI group

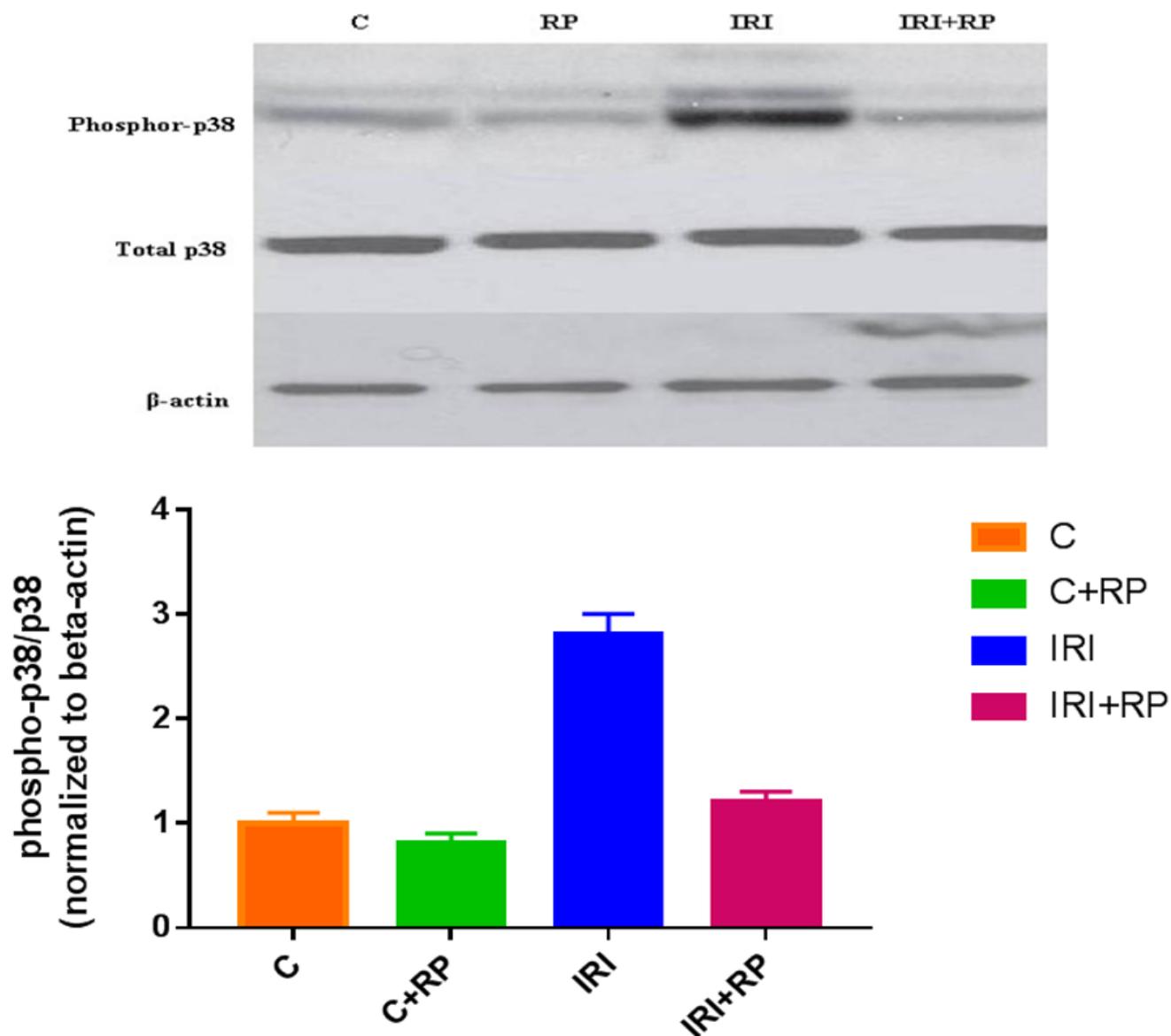


Figure 4

Effect of RES-PUE loaded nanoparticles on p38MPAK pathway. Estimation of protein expressions of phospho-p38 MPAK and total MAPK in spinal cord tissue homogenates. (C: Control, RP: Resveratrol and Puerarin nanoparticles, IRI: Ischemia-reperfusion injury, IRI + RP: Ischemia-reperfusion injury treated with Resveratrol and Puerarin nanoparticles) P value < 0.001 compared with IRI group

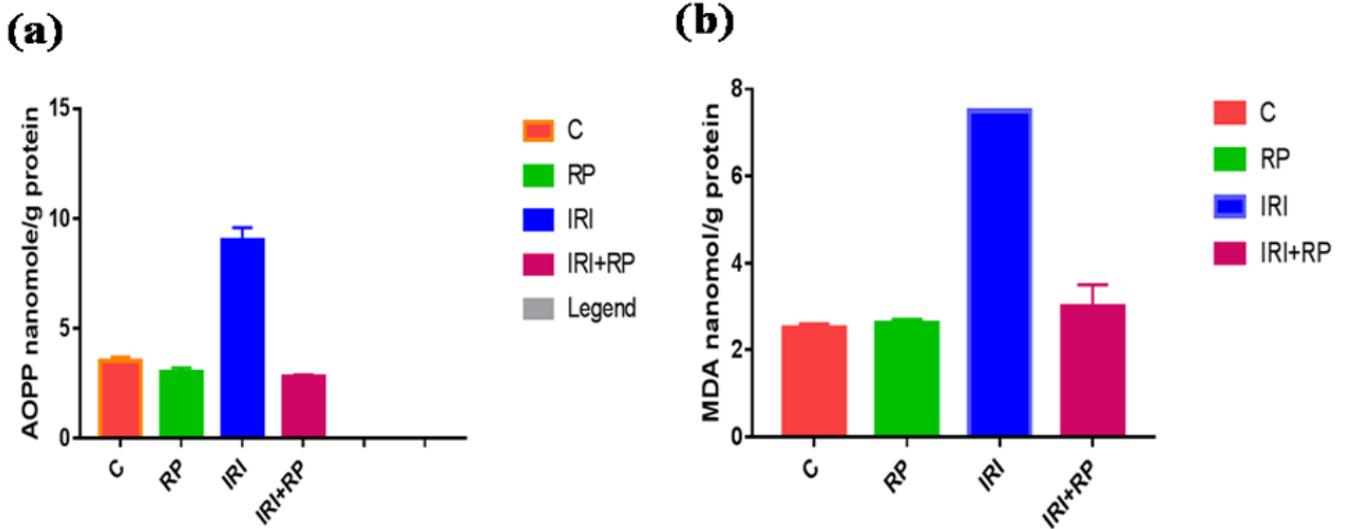


Figure 5

Effect of RES-PUE loaded nanoparticles on lipid peroxidation (MDA) and oxidation (AOPP) production. (a) Malondialdehyde (MDA) and (b) Advanced oxidation products (AOPP) in spinal cord tissue homogenates. (C: Control, RP: Resveratrol and Puerarin nanoparticles, IRI: Ischemia-reperfusion injury, IRI + RP: Ischemia-reperfusion injury treated with Resveratrol and Puerarin nanoparticles) P value < 0.001 compared with IRI group

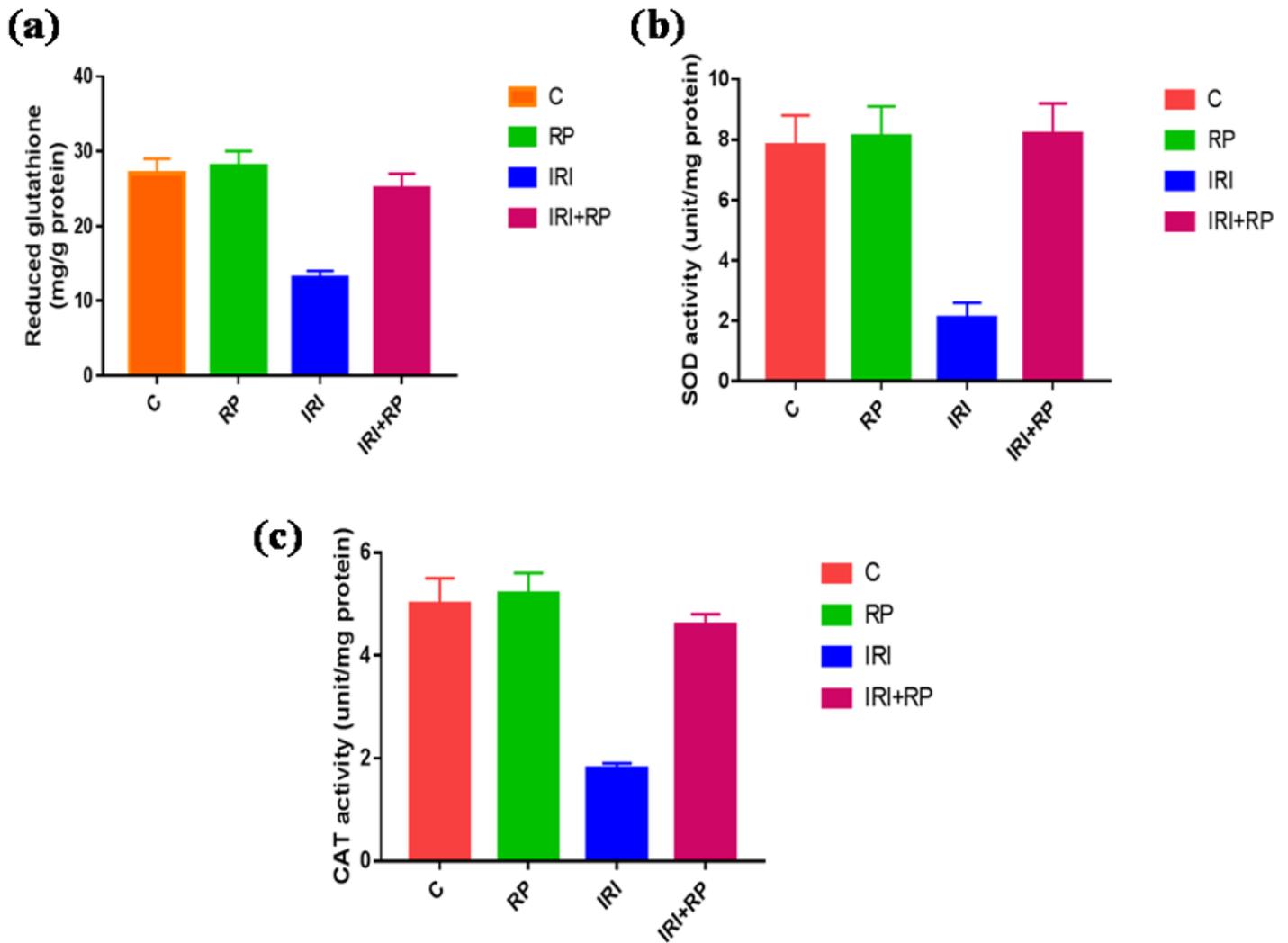


Figure 6

Effect of RES-PUE loaded nanoparticles on the level of reduced GSH, SOD and CAT. (a) Level of reduced glutathione (GSH), (b) Activity of SOD enzyme and (c) Activity of CAT enzyme levels in spinal cord tissue homogenates. (C: Control, RP: Resveratrol and Puerarin nanoparticles, IRI: Ischemia-reperfusion injury, IRI + RP: Ischemia-reperfusion injury treated with Resveratrol and Puerarin nanoparticles) P value < 0.001 compared with IRI group.