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HESPERETIN ENCAPSULATED PLGA NANOPARTICLES: SYNTHESIS, CHARACTERIZATION AND EVALUATION OF ITS *IN-VITRO* ANTIOXIDANT POTENTIAL AS WELL AS *IN VIVO* TOXICITY IN SWISS ALBINO MICE

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ABSTRACT

Hesperetin (HSP), a bioflavonoid, exhibits wide range of biological and pharmacological activities. It could be used as a prospective therapeutic agent by increasing its bioavailability and hydrophilicity. The aim of the current work is to fabricate hesperetin encapsulated PLGA nanoparticles and to investigate its toxicological effects as well as to assess its *in-vitro* antioxidant capacity. To address these objectives an environmental-friendly HSP loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles (PLGA-HSP NPs) was synthesized. DLS, UV-Vis studies were used to evaluate the properties of nano-hesperetin. The sub-acute toxicity of PLGA-HSP NPs was evaluated at different dose levels in Swiss albino mice after 28-days intraperitoneal administration. Hepatic and renal biomarkers of PLGA-HSP NPs treated mice were not altered significantly compared to free drug and control animals. The effects of nano-hesperetin as an antioxidant agent were also investigated. When PLGA-HSP NPs were compared to conventional ascorbic acid, they exhibited significantly greater free radicals scavenging action than HSP. *In-vitro* nitric oxide, superoxide, lipid peroxidation and hypochlorous acid scavenging tests revealed the potent concentration-dependent scavenging ability of PLGA-HSP NPs. In conclusion, it may be stated that PLGA nanoparticles encapsulated hesperetin is a non-toxic nano-conjugate and possesses a potent *in-vitro* antioxidant efficacy.

KEYWORDS: Hesperetin; PLGA; Toxicity study; *In vitro* antioxidant activity; Swiss albino mice.

INTRODUCTION

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), a flavonoid, is mainly obtained from lemons, grapefruits, and sweet oranges.[1,2] Hesperetin (HSP) possesses a number of biological activities including antioxidant, anti-inflammatory[3], anticancer, and cholesterol-reducing actions.[4,5]

Today it appears possible to create phytochemical based materials at the nanoscale scale for the therapeutic application through the significant advancements in several nanotechnological techniques. Polymeric nanoparticles have numerous brilliant drug delivery designs, as they possess their unparalleled physiochemical properties and have the capacity to precisely target specific sites within the body.[6] Polylactic-co-glycolic acid (PLGA) polymeric nanoparticles protect therapeutic compounds, boost their stability, and enable regulated drug delivery with enhanced pharmacokinetic and pharmacodynamic characteristics.[6,7] For the capacity of loading different kinds of pharmaceuticals (such as hydrophilic or hydrophobic small molecules or macromolecules), PLGA-based nanoparticles offer adjustable drug release, prevent drug degradation, improve interaction with biological components, and may even allow pharmaceuticals to be targeted to certain organs or cells.[7,8] These attractive characteristics of PLGA-based NPs, which can provide better drug delivery systems across a range of biomedical applications, including cancer, inflammation, immunisation, and other illnesses. Additionally, PLGA has been permitted for drug delivery in humans by the Food and Drug Administration (FDA) and European Medicine Agency.[9]

Nanoparticles sometimes show toxicity. It can produce systemic toxicity and oxidative stress. So, before

delivery of any drug for any disease the toxicity of the nanoparticles should be examined.[10]

Oxidative stress is one of the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immune suppression and neurodegenerative diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defence system. A potent scavenger of these reactive species may serve as a possible preventive intervention for free radical-mediated diseases. Several studies showed that a number of phytochemicals including polyphenolic substances (e.g., flavonoids) and various plant or herb extracts exert antioxidant actions.[11] The present study demonstrates the synthesis of hesperetin encapsulated PLGA nanoparticles and to investigate its toxicological effects as well as to assess comparatively the *in-vitro* antioxidant activities of hesperetin (HSP) and hesperetin-encapsulated PLGA nanoparticles.

MATERIALS AND METHODS

Materials

PLGA, poloxamer P123 (Pluronic® P-123), and hesperetin were bought from Sigma-Aldrich. Ascorbic acid, acetone, sodium nitroprusside, sodium tungstate, diacetylmonoxime, sulphanilamide, orthophosphoric acid, reduced nicotinamide adenine dinucleotide (NADH), thiobarbaturic acid (TBA), sodium chloride (NaCl), sulphuric acid (H_2SO_4), hydrogen chloride, ethylene diamine tetra acetic acid (EDTA), hypochlorous acid, hydrogen peroxide (H_2O_2), nitrobluetetrazolium (NBT), sodium citrate, and other chemicals were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India and other reagents utilised in the tests were of analytical grade.

Methods

Synthesis of Hesperetin Loaded PLGA Nanoparticles

With a few modifications, a nanoprecipitation method[12] was used to synthesize hesperetin-loaded PLGA nanoparticles.[13] The organic phase containing PLGA (100 mg) and HSP (10 mg) were dissolved in 10 ml of acetone. The water phase included 20 ml of Millipore water and 1 ml (0.5% v/v) poloxamer. The organic phase was then added drop wise to the water phase, and the mixture was stirred continuously for 24 hours at room temperature (600 rpm). The resultant nanoparticles were cleaned after three washings in deionized water and centrifuged at 15000 rpm for 30 minutes at 40°C. Large aggregate was taken out of the pellet using a hydrophilic syringe filter, and the supernatant was disposed of. The nanoparticles were stored at -20°C until they were lyophilized for 24 hours, which produced freeze-dried nanoparticles.

Characterization of Nanoparticles

Dynamic Light Scattering (DLS)

Measurement of particle size was performed by dynamic light scattering (DLS) and electrophoretic light scattering (Laser Doppler). At first, freshly synthesized nanoparticles were diluted in 40 times Milli-Q water and were added to polystyrene latex cells. Then the mean droplet size was placed in a polystyrene cuvette and calculated at 25°C with a detector angle of 90°.[14]

Ultraviolet spectroscopic (UV-Vis) study

UV-Vis spectroscopy is one of the most important techniques to identify the formation and stability of nanoparticles. UV-Vis spectrophotometer (UV-1800 Shimadzu, Japan) was used to determine the UV-Vis spectra of the solution at the spectral range of 200−750 nm.[15] Analysis of the absorbed light was performed through the resultant spectrum. The recorded spectra were then replotted in Origin 8.5 software.

Sub-acute toxicity study in mice model

Animal maintenance

Healthy male Swiss albino mice (18–25 g) were taken for toxicity test and they were kept under standard temperature (25 ± 2°C) and humidity (60 ± 5%) with 12 h light /dark cycle. The animals were given a standard pellet diet and drinking water *ad libitum.* The experiments were performed in compliance with the guidelines approved by the Institutional Animal Ethical Committee (VU/IAEC/CPCSEA 12/5/2022 dated 11.02.2022) under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Treatment schedule

The sub-acute toxicity study was carried out according to OECD guideline 407. Adult healthy male Swiss albino mice were divided into seven groups, each having six animals and were placed under standard laboratory conditions. Toxicity assays were evaluated in mice at the dose levels of 50 to 4000 µg/kg body weights for HSP, PLGA-HSP NPs (Group II to VII) for 28 consecutive days and Group I was considered as saline control**.** Toxicity studies were performed internationally according to the accepted protocol for Swiss albino mice.[16,17]

Group I: Saline control
Group II: HSP/PLGA-HSP NPs (50 µg/kg body weight)
Group III: HSP/PLGA-HSP NPs (100 µg/kg body weight)
Group IV: HSP/PLGA-HSP NPs (250 µg/kg body weight)
Group V: HSP/PLGA-HSP NPs (500 µg/kg body weight)
Group VI: HSP/PLGA-HSP NPs (2000 µg/kg body weight)
Group VII: HSP/PLGA-HSP NPs (4000 µg/kg body weight).

Biochemical analysis

On 29th day, all animals were anaesthetized under sodium pentobarbital anesthesia. The sample was collected in plastic test tubes and allowed to stand for complete clotting. The clotted blood samples were centrifuged (3000 rpm for 15 min). Then serum samples were stored at -20°C for biochemical estimation.

Estimation of urea

Urea was determined by the modified method[18] of Natelson.[19] To 3.3 ml of water, 0.1 ml of serum, 0.3 ml of 10% sodium tungstate and 0.67 N sulphuric acid were added. The suspensions were centrifuged at 2000 rpm. Then 0.4 ml of diacetylmonoxime, 1.0 ml of water, and 2.6 ml (0.67 N) H_2SO_4-phosphoric acid reagents were added to the supernatant. Using similar way, standard was prepared. All the tubes were heated in a boiling water bath for 30 min and cooled. The developed colour was measured in spectrophotometer at 480 nm (UV-Shimadzu-245, Japan).

Measurement of serum glutamate oxaloacetate transaminase (SGOT) and serum gluamate pyruvate transaminase (SGPT)[18]

For the estimation of SGOT, to 1.0 ml buffer substrate (200mM/L of DL-aspartate and 2 mM/L of α-ketoglutarate, pH=7.4), 0.1 ml serum was added and incubated for 1 h at 37°C. Then 1 ml of dinitrophenyl hydrazine (DNPH) was added and kept at room temperature for 20 min. After 20 min, 10 ml (0.4N) sodium hydroxide was added. After 10 min the colour intensity was measured in a spectrophotometer at 520 nm (UV-Shimadzu-245, Japan). The same procedure was used to prepare the blank and standard. The enzyme activity in serum was recorded as IU/L. For the estimation of SGPT, to 1.0 ml of the buffer substrate (200 mM/L of DL-alanine and 2 mM/L of α-ketoglutarate, pH=7.4), 0.1 ml serum were added and incubated for 1 h at 37°C. The reaction was proceeded by the addition of 1.0 ml of DNPH and kept at room temperature for 20 min. The colour developed by the addition of 10 ml (0.4N) sodium hydroxide was read in spectrophotometer at 520 nm (UV-Shimadzu-245, Japan). The enzyme activity in serum was recorded as IU/L.

Antioxidant Activity

Nitric oxide radical scavenging assay

Nitric oxide scavenging activity was measured by using the Griess reagent.[20] Griess reagent was prepared immediately before use by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid. Briefly, different concentrations of samples (1ml) were mixed with the 5.0 mL of 5 mM of sodium nitroprusside in phosphate-buffered saline (pH 7.4), and was incubated at 25°C for 3 h. Then the solution was mixed with an equal volume of freshly prepared Griess reagent. In this experiment, ascorbic acid was used as standard. The absorbance was measured at 546 nm. The experiment was carried out thrice and % of inhibition was calculated using the following formula.

$$(\%)\ inhibition = \frac{C - T}{C} \times 100 \quad \text{Eq. 1}$$

C = Absorbance of the control sample, T = Absorbance of the test sample.

Superoxide radical scavenging

The superoxide anion scavenging activity was measured by a modified spectrophotometric method depending on the reduction of nitro blue tetrazolium (NBT) to a purple formazan.[21] The reaction mixture contained 1.9 ml of phosphate buffer (0.1 M, pH 7.4), NADH (156 µM), and NBT (25 µM) with various concentrations of sample solutions. After incubation for 5 min at ambient temperature, the absorbance at 560 nm was measured against the appropriate blank. The % of superoxide radical scavenging activity was calculated by the following formula:

$$\%\ of\ inhibition = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{Eq. 2}$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of standard ascorbic acid.[22]

Inhibition of lipid peroxidation

The lipid peroxidation inhibition assay was performed according to thiobarbituric acid-reactive species (TBARS) assay.[23] Briefly, different concentrations of samples and standard (ascorbic acid) solutions were made. Then 0.375% [w/v] 2-thiobarbituric acid, 0.25 N hydrogen chloride, and 15% [w/v] trichloroacetic acid were added to all the samples and standard and heated for 15 min in a boiling water bath. The mixtures were cooled and centrifuged at 3000 rpm for 15 min. Then absorbance of collected supernatants was measured at 532 nm by spectrophotometer. The percentage of inhibition (%) was calculated using equation 1.

Hypochlorous Acid (HOCl) scavenging activity

Diluted sodium hypochlorite was prepared by diluted sulphuric acid (pH 6.2) to form hypochlorous acid (HOCl) for the assay. Prior to use, the concentration of hypochlorite was measured spectrophotometrically at 235 nm.[24] The various ascorbic acid (standard) solution concentrations were employed as the control. A blank and various concentrations of samples were prepared in PBS and then HOCl (50uM) was added. After 15 min, each sample received 20 ml of a 3,3',5,5'-tetramethylbenzidine solution (10 mmol/L). Then the absorbance at 650 nm was measured after 5 min. The experiment was executed three times. From equation 2, the percentage of inhibition was determined.

Statistical analysis

All experiments were performed in triplicate. The results of toxicity studies were expressed as Mean ± SEM (standard error of mean) and the results of antioxidant

parameters were stated as Mean ± SD (standard deviation). The statistical significance and multigroup comparisons of the data was analysed by using one and two-way analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism and Origins 8.5 (Origin Lab, Northampton, USA). All Statistical significance was considered at *p<0.05.

RESULTS

Particles size and morphology analysis

The particles size of synthesized PLGA-HSP NPs was measured at 139.8 nm by Dynamic Light Scattering (DLS) (Fig. 1A) and the nanoparticles was confirmed by taking its absorbance value using UV-Vis spectroscopy. The UV absorption spectra of the PLGA-HSP NPs shows the sharp bands in the visible range within 300-500 nm wavelength interval as shown in Fig. 1B.

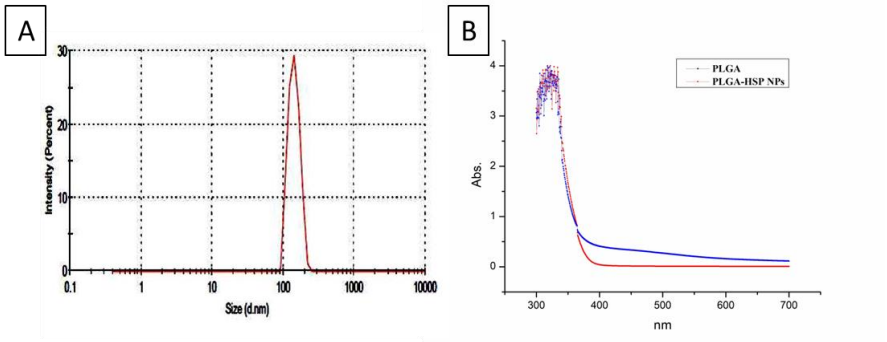

Fig. 1: Characterizations of. The hydrodynamic diameter (139.8 nm) was measured by dynamic light scattering (DLS) study (A), and the sharp bands of PLGA-HSP NPs was observed within 300-500 nm by UV-Vis spectroscopy (B).

Sub-acute toxicity study

Changes in serum urea level

No significant sub-acute toxicity was seen in the level of urea (Fig.2), in mice treated with PLGA-HSP NPs up to the dose level of 500 mg/kg body weight/day for 28 consecutive days when compared with the control animals.

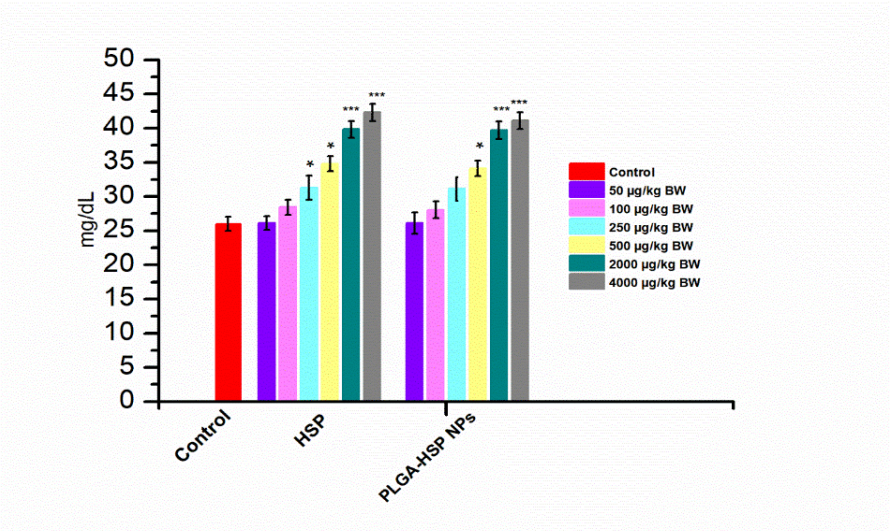

Fig. 2: Shows the effects of HSP and PLGA-HSP NPs on serum urea level in Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one-way ANOVA. '*' and '*' indicate significant difference (*p<0.05, ***p<0.001) compared to control.**

Changes in SGOT and SGPT level

The daily administration of PLGA-HSP NPs for 28 days induced no alteration of SGOT (Fig. 3) and SGPT (Fig. 4) up to 2000 mg/kg body weight.

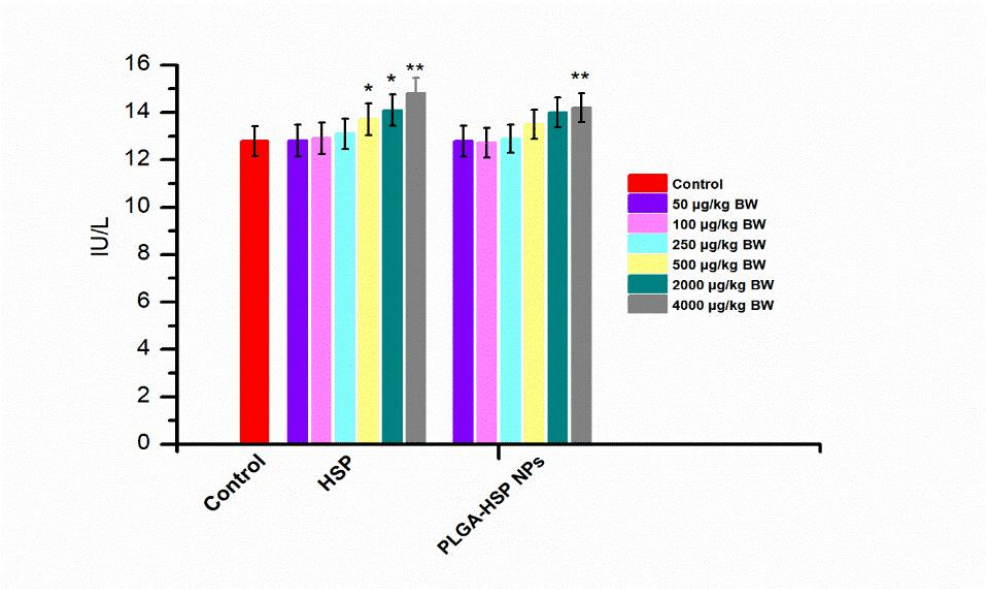

Fig. 3: Shows the effects of HSP and PLGA-HSP NPs on serum glutamic oxaloacetic transaminase (SGOT) of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one-way ANOVA. '*' and '' indicate significant difference (*p<0.05, **p<0.01) compared to control.**

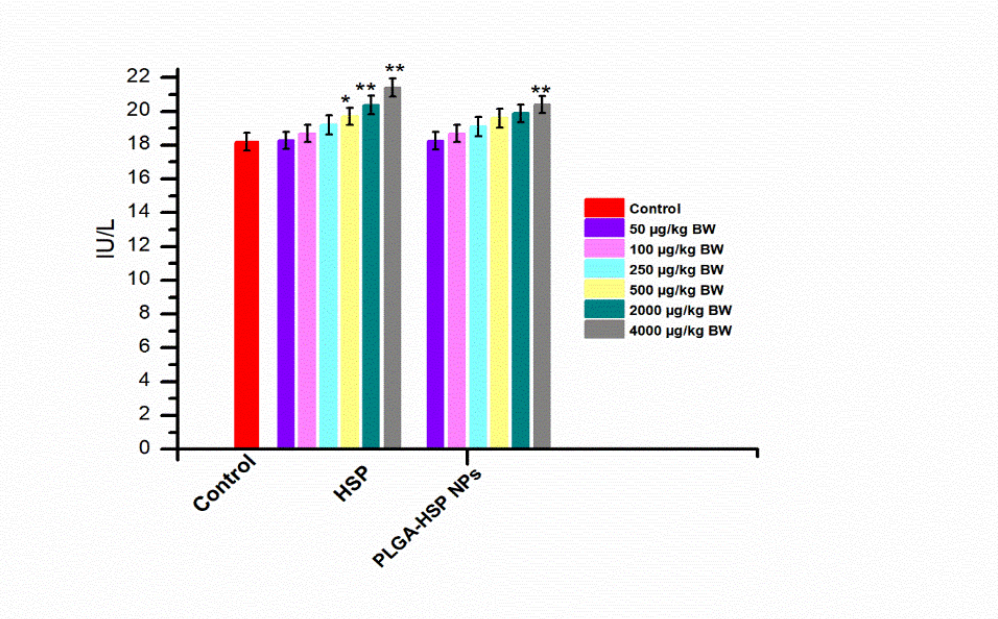

Fig. 4: Shows the effects of HSP and PLGA-HSP NPs on serum glutamic pyruvic transaminase (SGPT) in Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one-way ANOVA. '*' and '' indicate significant difference (*p<0.05, **p<0.01) compared to control.**

***In vitro* Antioxidant study**

Hesperetin (HSP), PLGA-HSP NPs were examined for their *in vitro* antioxidant properties in the current study. For evaluation of *in vitro* antioxidant activity of nanoparticles at various concentrations, nitric oxide scavenging activity, superoxide anion scavenging assay, lipid peroxidation and hypochlorous acid scavenging assay were used. Ascorbic acid was used as the reference standard.

Nitric oxide scavenging activity

PLGA-HSP NPs and ascorbic acid showed nitric oxide scavenging action with an IC_{50} dose of 132.52 µg/mL and 102.29 µg/mL respectfully (Fig. 5).

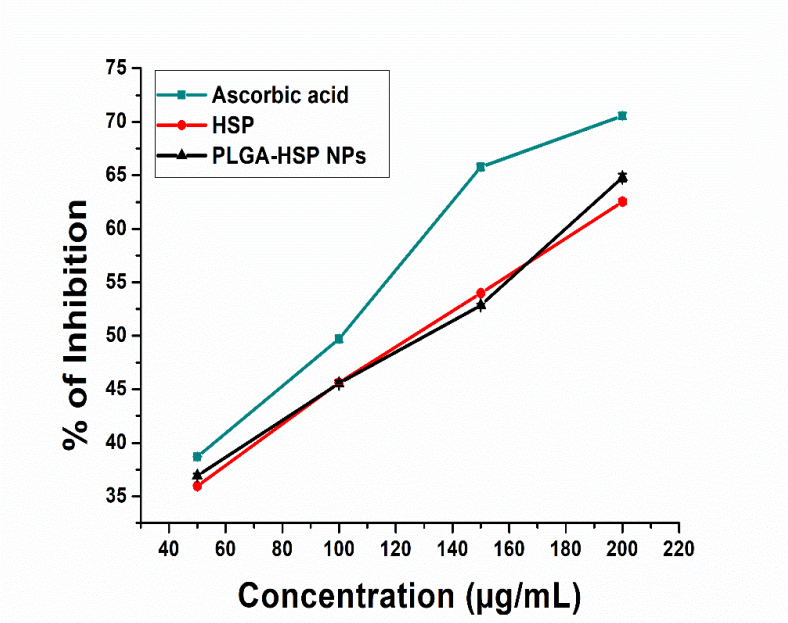

Fig. 5: *In vitro* antioxidant activity of HSP and PLGA-HSP NPs were studied in a concentration dependent manner (50, 100, 150, 200 µg./mL). Nitric oxide scavenging activity assay was performed using Griess reagent. IC_{50} value was of 132.52 µg/mL of PLGA-HSP NPs. Results are expressed as mean ± S.D. (n = 3).

Superoxide radical scavenging

PLGA-HSP NPs exhibited superoxide radicals scavenging activity with IC_{50} value of 161.27 µg/mL. The IC_{50} (161.73 µg/mL) value of PLGA-HSP NPs was indicating its excellent scavenging activity as compared to HSP (IC_{50} = 172.91 µg/mL) (Fig. 6).

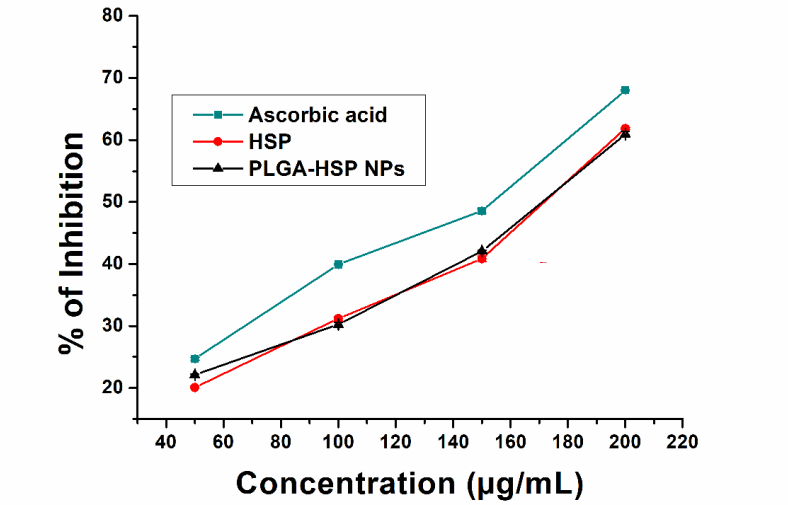

Fig. 6: *In vitro* antioxidant activity of HSP and PLGA-HSP NPs were studied in a concentration dependent manner (50, 100, 150, 200 µg./mL). PLGA-HSP NPs exhibited superoxide radicals scavenging activity with IC_{50} value of 161.73 µg/mL. Results are expressed as mean ± S.D. (n = 3).

Inhibition of lipid peroxidation

Lipid peroxidation was inhibited in all the treated groups but PLGA-HSP NPs exhibited potent inhibitory activity with IC_{50} value of 159.69 µg/mL for lipid peroxidation compared to HSP (IC_{50} = 174.91 µg/mL) (Fig. 7).

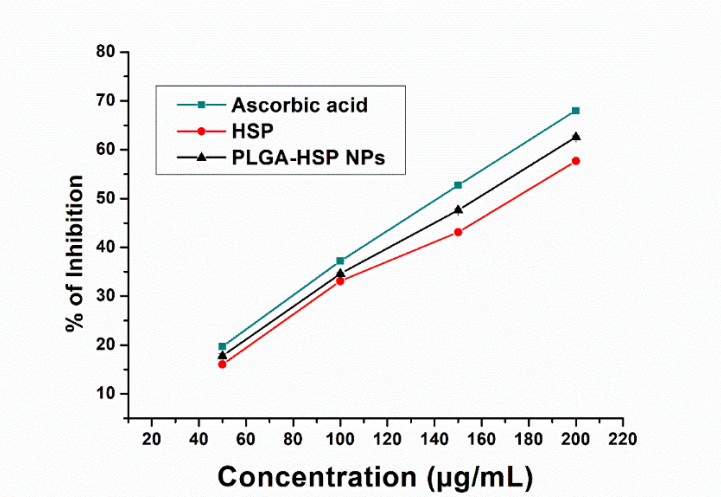

Fig. 7: *In vitro* antioxidant activity of HSP and PLGA-HSP NPs were studied in a concentration dependent manner (50, 100, 150, 200 µg./mL). PLGA-HSP-TF NPs inhibited lipid peroxidation with IC_{50} value of 159.69 µg/mL compared to HSP (IC_{50}= 174.91 µg/mL). Results are expressed as mean ± S.D. (n = 3).

Hypochlorous Acid (HOCl) scavenging activity

The hypochlorus acid scavenging activity was observed (Fig. 8) in a dose dependent manner compared to ascorbic acid. The IC_{50} values for PLGA-HSP NPs and ascorbic acid in this assay were 147.87µg/mL and 118.73 µg/mL respectively.

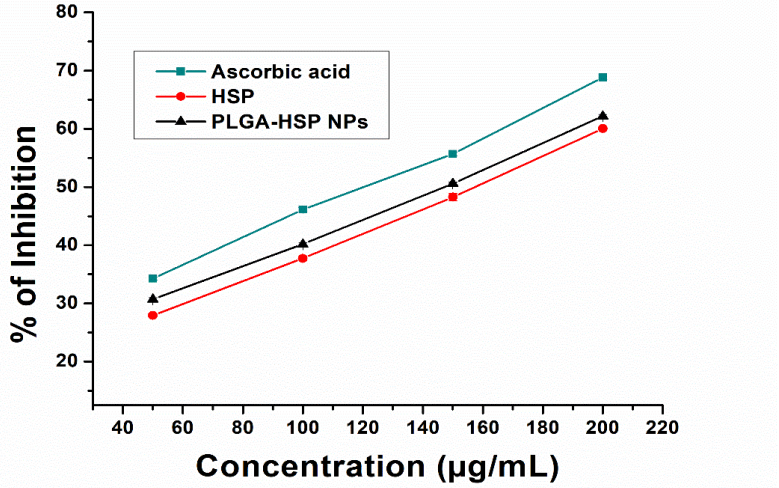

Fig. 8: *In vitro* antioxidant activity of HSP and PLGA-HSP NPs were studied in a concentration dependent manner (50, 100, 150, 200 µg./mL). Hypochlorous acid (HOCL) scavenging activity assay was performed with IC_{50} value of 147.87 µg/mL for PLGA-HSP NPs. Results are expressed as mean ± S.D. (n = 3).

DISCUSSION

In the present study, PLGA-HSP NPs were synthesized using modified nanoprecipitation method. Particle size influences several properties of nanoparticles used in biomedical applications.[25] The DLS results confirmed that the particle size was about 139.8 nm (Fig. 1B) and the UV-Vis spectra (Fig. 1B) of PLGA-HSP NPs exhibited a localized surface plasmon resonance (LSPR) within 300-500 nm wavelength interval. Whereas, HSP was reported to show 288-300 nm wavelength.[26]

Flavonoids are polyphenolic compounds with various pharmacological properties such as antioxidant, anti-inflammatory and antiallergic and act as scavengers of free radicals by OH groups in their molecular structure which have property of preventing a number of chronic diseases and reduced toxicities in animals and humans.[27] Several studies suggested that hesperetin has anti-inflammatory, antioxidant, anticarcinogenic and neuroprotective effects.[28,29]

The sub-acute toxicity study of PLGA-HSP NPs was performed on Swiss albino mice at different dose levels (50, 100, 250, 500, 2000, and 4000 µg/kg body weight) for 28 successive days. In the toxicological assessment, biochemical parameters play a significant role as markers due to their response to clinical signs and symptoms caused by toxicants.[30] Serum urea level was not altered

up to the level of 500 µg/kg body weight (Fig. 2). PLGA-HSP NPs caused a slight increase in serum urea level also at the dose levels of 1000µg and 2000µg/kg body weight. This may be suggestive that PLGA-HSP NPs have possibly no any adverse effect on the kidney up to the highest dose (4000µg/kg body weight). Elevated urea level is a positive risk factor for renal impairment. This study showed that PLGA-HSP NPs produced renal toxicity only at higher doses, not in mild or moderate doses. SGOT and SGPT are indicative biomarkers of liver cells.[31] Serum level of SGOT and SGPT were evaluated as toxic markers and no significant changes are found up to the dose level of 2000 µg/kg body weight (Fig. 3, 4). Elevated levels of SGOT and SGPT may be due to the leakage of these enzymes at 4000µg/kg body weight into the blood stream. All these findings of this study firmly confirmed that PLGA-HSP NPs did not alter the liver or renal function noticeably and indicated the non-toxic nature of PLGA-HSP NPs.

Hesperetin has three hydroxyl groups that maintain a greater antioxidant potency and ability to activate cellular antioxidant potential preventing enzymes due to its ability to penetrate within the lipid biolayer.[27] The production of nitric oxide radical at a sustained levels result in direct tissue toxicity, whereas chronic expression of nitric oxide radical is associated with various carcinomas.[32] PLGA-HSP NPs demonstrated a dose-dependent, substantial nitric oxide scavenging action. As the dose was increased, an increase in the percentage of inhibition was seen (Fig. 5). The present study proved that the nitric oxide scavenging activity of PLGA-HSP NPs is comparatively better like ascorbic acid, the used standard in this study.

Superoxide anion is also a harmful reactive oxygen species. In biological systems, cellular components get damaged in contact with superoxide anions.[32] This study proved that the superoxide scavenging activity of PLGA-HSP NPs is greater than HSP and as good as the standard ascorbic acid (Fig. 6). According to studies, flavonoids have the potential to be powerful radical scavengers and lipid peroxidation inhibitors.[33] Lipid hydro-peroxides break down into peroxyl and alkoxyl radicals, which speeds up the process of lipid peroxidation. Lipid hydro-peroxides are the source of iron-catalyzed formation of hydroxyl radicals or ferrry-perferryl complex.[32]

PLGA-HSP NPs have shown inhibition in lipid peroxidation in a dose-dependent manner. Lipid peroxidation has been inhibited in all the treated groups but PLGA-HSP NPs exhibited potent inhibitory activity for lipid peroxidation compared to the standard ascorbic acid (Fig. 7).

Hypochlorous acid is another harmful ROS. At the sites of inflammation, the oxidation of Cl^- ions by the neutrophil containing enzyme myeloperoxidase results in the production of this ROS. HOCl has the ability to inactivate the antioxidant enzyme catalase through the breakdown of heme prosthetic group.[34] It is anticipated that PLGA-HSP NPs is an efficient scavenger of HOCl like the standard, ascorbic acid (Fig. 8). So, our findings suggest that may be PLGA-HSP NPs has the potential to improve cellular antioxidant capacity.

CONCLUSION

PLGA-HSP NPs were synthesized using modified nanoprecipitation method. The prepared nanoparticles were characterized by DLS and UV-Vis. However, the sub-acute toxicity study indicates that PLGA-HSP NPs did not exhibit any liver and kidney toxicity in mice. Hence, this study suggests that up to the dose level of 2000 µg/kg body weight of PLGA-HSP NPs should be considered safe for use. The *in vitro* antioxidant studies show that PLGA-HSP NPs is greater antioxidant activity than HSP and act like the standard antioxidant, ascorbic acid. This study may be a promising one for the development of PLGA-HSP NPs as an antioxidant agent and could be utilised for therapeutic purpose due to its non-toxic nature.

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