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Original Article

Characterisation and Toxicity Evaluation of Synthesized Phloridzin Chitosan Nanoparticles in *in vivo* Model

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ABSTRACT

Background: Phloridzin, a plant compound is of interest in the field of research due to its antioxidant, anti-inflammatory, anticancer and antidiabetic activity. The current study's objectives were to create, characterize and evaluate the toxicity of synthesized phloridzin chitosan nanoparticles in Sprague Dawley rats. **Materials and Methods:** The chitosan nanoparticle was synthesized by ionic gelation method. The synthesized nanoparticles were characterized using different techniques like Dynamic Light Scattering, Fourier Transform Infrared Spectroscopy, X-ray Diffraction, Fourier Emission Scanning Electron Microscopy and %Drug Entrapment Efficiency. The toxicity and cellular uptake of phloridzin chitosan nanoparticles were further evaluated by *in vitro* and *in vivo* methods. MTT and cellular uptake assay were carried out in SH-SY5Y cells. Following in *in vivo* Lorke method and the Organization for Economic Co-operation and Development 407, the sub-chronic toxicity of phloridzin chitosan nanoparticles was investigated. **Results:** Chitosan encapsulated phloridzin nanoparticles showed an average particle size of 246.6 d.nm in Dynamic Light Scattering analysis with a polydispersity index of 0.526 and zeta potential of 30.9mv. Fourier Transform Infrared Spectroscopy and X-ray Diffraction analysis confirmed the presence and stability of phloridzin-encapsulated chitosan nanoparticles. In Fourier Emission Scanning Electron Microscopy analysis, the dehydrated sample size was found to be 186 nm. Drug entrapment efficiency was found to be 50.38%. Cell viability range was above 60% after 48 hr incubation. Cellular uptake of nanoparticles was confirmed. The data obtained from the Lorke method indicated that LD_{50} exceeded 5000 mg/kg. No significant alterations were observed in hematological, biochemical and histopathological studies. **Conclusion:** The findings suggest that the Phloridzin nanoparticles encased in chitosan did not significantly produce toxicity in both *in vitro* and *in vivo*.

Keywords: Phloridzin, Chitosan nanoparticle, Toxicity, Liver, Kidney, Brain.

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INTRODUCTION

Phloridzin is a phenolic phytoconstituent isolated from the bark of the apple tree. Structurally it is a dihydrochalcone, a derivative of flavonoid glycoside. It is known to possess antioxidant, antidiabetic, anticancer and anti-inflammatory properties.[1] Previous studies have shown that phloridzin possesses memory-enhancing and antidepressant activities.[2] One of the significant effects of phloridzin is its ability to inhibit SGLT (Sodium-Glucose cotransporters), managing glucose levels in diabetes.[3] Despite its potential benefits, one of the main drawbacks seems to be its low bioavailability and rapid degradation.[4] Nanotechnology-based advancements have gained attention in recent years and the interaction of nanomaterials with biological molecules has wide applications in medical research and nanomedicine.[5] Recently, polymeric nanoparticles have gained much importance due to their bioavailability, biocompatibility, biodegradability and non-toxicity. Chitin's partial deacetylation yields the biopolymer chitosan. Due to its excellent encapsulation efficiency and sustained release property, this biodegradable polymer is successfully used as a controlled and stabilized delivery system of biological molecules.[6] The chitosan nanoparticles can be synthesized by standard protocols.[7] Due to their biological, antimicrobial and physicochemical properties, they do not cause any adverse effects to human beings.[8] Hence the bioavailability of the phloridzin compound can be enhanced by chitosan encapsulation. The main objective of the present study is to formulate and characterize the chitosan-encapsulated phloridzin nanoparticles and determine its toxicity level in both *in vitro* and *in vivo*.

MATERIALS AND METHODS

All chemicals used were analytical grade. Phloridzin, chitosan low molecular weight (deacetylation>75.0%) and Tripolyphosphate (TPP) (purity: 85%) were purchased from TCI Chemicals.

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Synthesis of Phloridzin Chitosan Nanoparticles

The phloridzin chitosan nanoparticles were synthesized by chitosan and TPP using an ionic gelation technique with slight modification.[9] The chitosan, TPP and compound ratio were selected based on previous studies. 1.75 mg/mL of chitosan was dissolved in 10 mL of 2% acetic acid solution. The solution was stirred overnight at 800 to 900 rpm. To this phloridzin was added in an equal ratio of chitosan, then 4 mL of TPP (2 mg/mL) was added dropwise and centrifuged for 15000 rpm at 10°C for 40 min. The supernatant was discarded and the pellets were collected using a freeze drier.

Characterization of nanoparticles Dynamic Light Scattering (DLS)

The particle size of the chitosan nanoparticles was determined using the DLS (ZetasizerNano ZS90, Malvern, UK). The nanoparticle surface charge and stability were analyzed by zeta potential measurements (ZetasizerNano ZS90, Malvern, UK) using fold capillary cuvettes (Folded Capillary Cell-DTS1060, Malvern, UK).

Fourier Transform Infrared Spectroscopy (FTIR)

The chitosan encapsulated phloridzin functional group and stabilization of synthesized nanoparticles were analyzed using FTIR (Fourier Transform Infrared) spectrometry (FTIR -8400S, Shimadzu, Japan) between 400-4000 cm^{-1} at a resolution of 4 cm^{-1}.

Fourier Scanning Electron Microscope Analysis (FESEM)

The size and structural morphology of the phloridzin-encapsulated chitosan nanoparticle was analyzed using FESEM (SUPRA 55-CARL ZEISS, Germany) analysis.

X-ray Diffractometer (XRD) Pattern analysis

The crystal structure, phase and texture of the synthesized phloridzin chitosan nanoparticle were analyzed using an X'Pert Pro X-ray diffractometer (P analytical BV; Almelo, The Netherlands) operating at a voltage of 40 kV, a running current of 30 mA with Cu Kα radiation.

Percentage Drug Entrapment Efficiency

The encapsulation efficiency of phloridzin chitosan nanoparticles was measured based on slight modification.[10] The formulated nanosuspension was centrifuged at 12000 rpm for 30 mins and the supernatant was quantified spectrometrically at 380 nm. The percentage of Drug Entrapment Efficiency was calculated by:

Total drug

$$\%DEE = \text{Total drug-free drug} X100$$

Evaluation of cytotoxicity

The MTT technique was used to calculate the Inhibitory Concentration (IC_{50}).[11] To attain 80% confluency, the cancer cells were grown in a 96-well plate at a density of 1×10^4 cells per well for 48 hr. The old medium was replaced with fresh media containing sample (Phloridzin and Ph-ChNPs) at various doses and was kept for 24 hr. After the culture media was removed from each well, 100 µL MTT was added to each well and the wells were then incubated at 37°C for 4 hr. After the supernatant was removed from each well, 100 µL of DMSO was added and kept remaining for 10 min to dissolve the formazan crystals. Using an ELISA multi-well plate reader, the optical density was measured at 520 nm.

Based on the findings, the following formula was used to determine the percent viability:

% of viability=OD value of experimental control - OD value of experimental sample/OD value of experimental control×100.

Cellular internalization of Phloridzin-loaded chitosan nanoparticles by confocal microscopy

SH-SY5Y cells were treated with Phloridzin-loaded chitosan nanoparticles for 8 hr, after the treatment, 3% paraformaldehyde (50 µL) was added and left for 10 min at room temperature to fix the cells. After fixation, the cells were washed with PBS twice to remove the excess fixative. The cells were incubated with FITC for 10 min at room temperature.[12] Phloridzin-loaded chitosan nanoparticles complex excited at 496, 546 and 565 nm and emitted at 578 nm. The green emission from FITC was excited at 488 nm and emitted at 505-525 nm.

In vivo toxicity studies Experimental rats

Healthy male Sprague Dawley rats (8 weeks old weighing 200-250 g) were procured for this research. Ethical clearance for the handling of these experimental animals was obtained from the Institutional Animal Ethics Committee (IAEC) of PSG Institute of Medical Sciences and Research, Coimbatore, which acts under the guidance of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (CPCSEA/No: 434/IAEC/2022). The animals were acclimated for three days under conventional laboratory settings with regulated temperature (29°±5°C), humidity (55%±5%) and 12 hr of light/dark cycles and kept under the same circumstances throughout the experimental period.

Acute toxicity study

To determine the LD_{50} value of synthesized phloridzin chitosan nanoparticles, a previously published procedure (Lorke, 1983) was utilized.[13] Nine (9) male Sprague Dawley rats were used in Phase I. Rats were placed into three groups of *n*=3, with each

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	246.6	Peak 1:	373.5	74.3	129.1
PdI:	0.526	Peak 2:	80.14	25.7	24.94
Intercept:	0.952	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

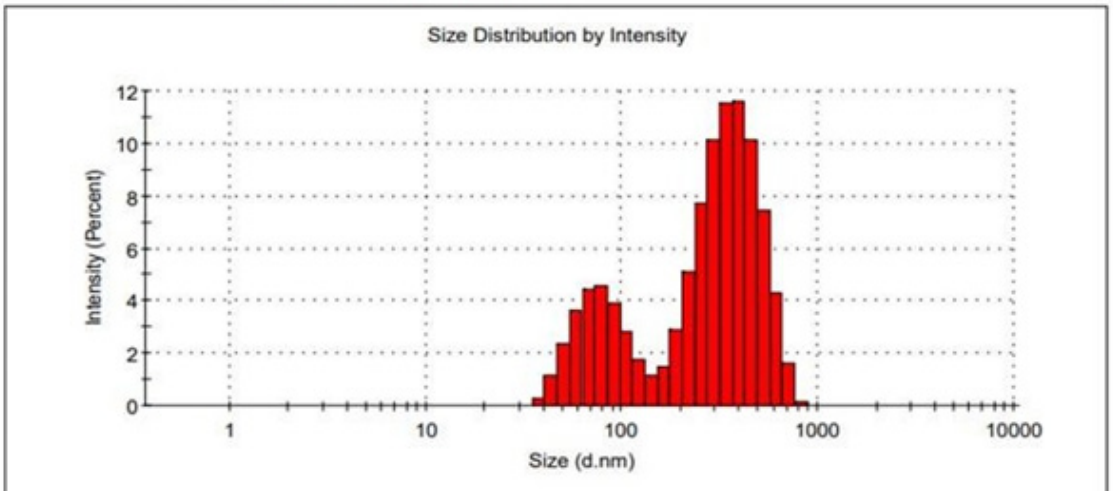

Figure 1a: DLS analysis.

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	30.9	Peak 1:	30.9	100.0	6.31
Zeta Deviation (mV):	6.31	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	1.03	Peak 3:	0.00	0.0	0.00
Result quality	Good				

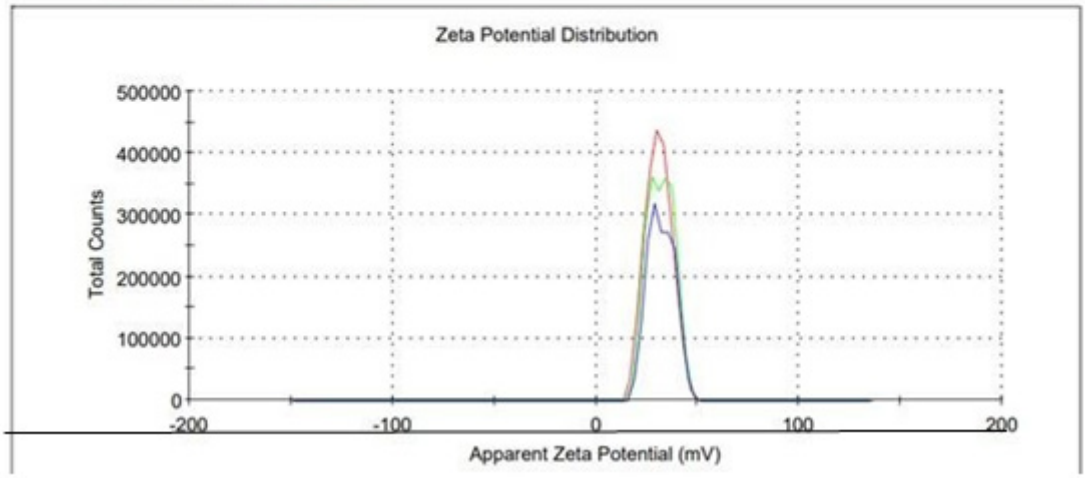

Figure 1b: Zeta potential.

receiving 10, 100 and 1000 mg/kg body weight of phloridzin chitosan nanoparticles, respectively and were monitored for 24 hr for symptoms of behavioral abnormalities and (or) mortality. Three (3) male rats were employed in the post- administration phase (II), separated into three (3) groups of n=1. Each group received dosages of 1600, 2900 and 5000 mg/kg phloridzin chitosan nanoparticles, respectively and was examined for another 24 hr for any signs of toxicity and (or) mortality.

The lethal dose (LD_{50}) was calculated as shown below:

$$LD_{50} = \sqrt{(D0 + D100)/2}$$

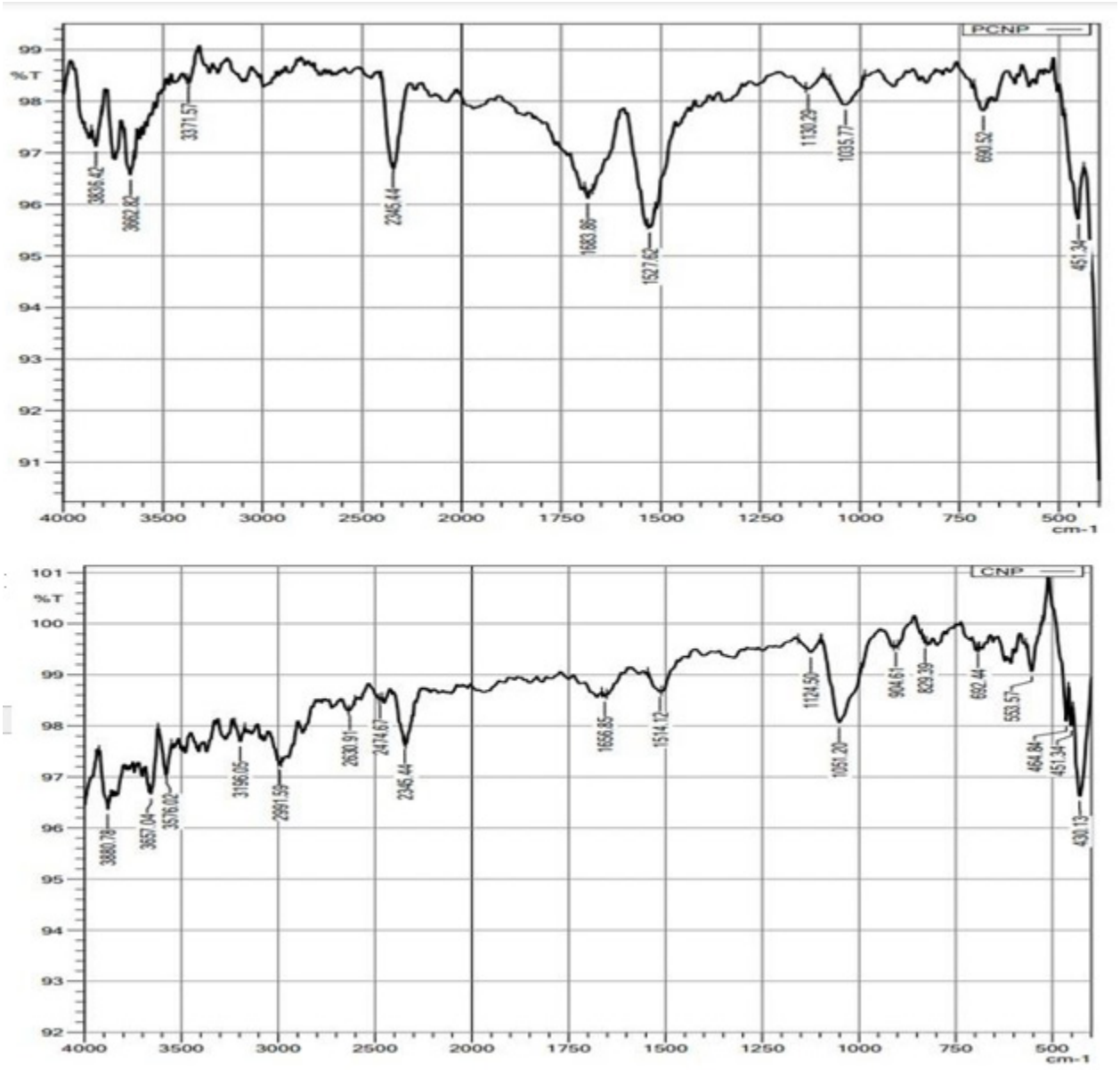

Figure 1c: FTIR analysis of Chitosan Nanoparticle (CNP) and Phloridzin Chitosan Nanoparticle (PCNP).

D0=Highest dose that resulted in no death; D100=Lowest dose that resulted in death.

Sub-chronic toxicity studies

The subacute oral toxicity research of phloridzin chitosan nanoparticles was performed in male Sprague Dawley rats in accordance with OECD guideline 407.[14] The rats were divided into four groups (one control and three experimental groups, *n*=5). The rats in groups 2, 3 and 4 received 250, 500 and 1000 mg/kg of phloridzin chitosan nanoparticles daily for 28 days. During the study, toxic symptoms, body weights and feed intake variations were all tracked. On the 28th day, all of the animals were sacrificed and blood, liver, kidney and brain samples were obtained. Hematology, histology and biochemical markers were assessed.

Hematological and Biochemical Analysis

After 28 days of treatment with chitosan phloridzin nanoparticles, the rats were fasted overnight. The blood sample was collected the next day by cardiac puncture using anticoagulant and non-anticoagulant tubes for analyses of whole blood and serum biochemical parameters. The whole blood was analyzed by an automatic blood cell analyzer (HF-3800; HANFANG 121 Ltd., Jinan, China). The serum biochemical parameters like SGPT, SGOT, ALP, creatinine, %PCV, Hb, TLC, TEC and Blood Urea Nitrogen were also analyzed by ErbaChem 5 Plus V2 Biochemistry Analyzer.[15]

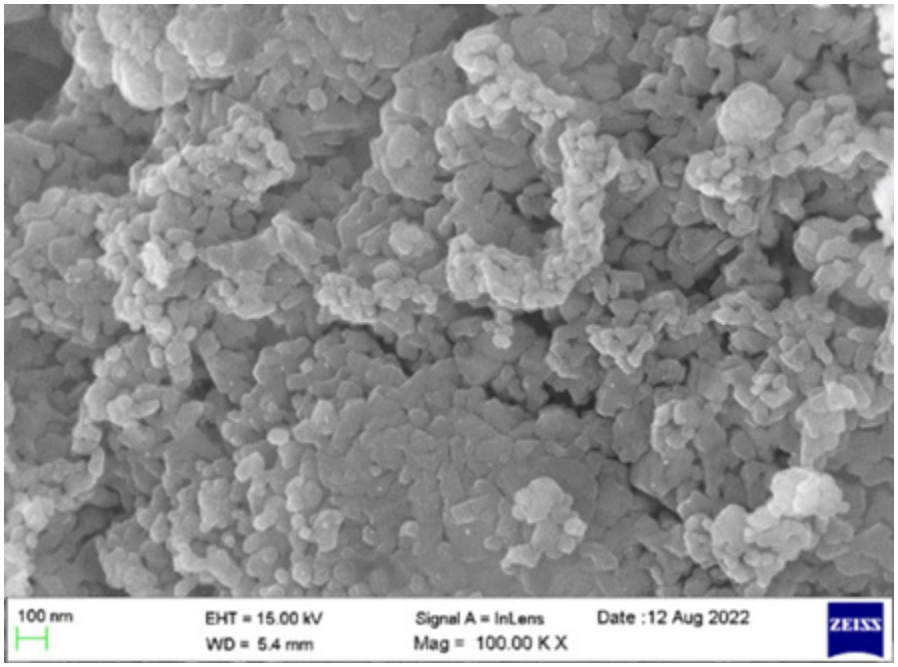

Figure 1d: FESEM analysis of phloridzin chitosan nanoparticle.

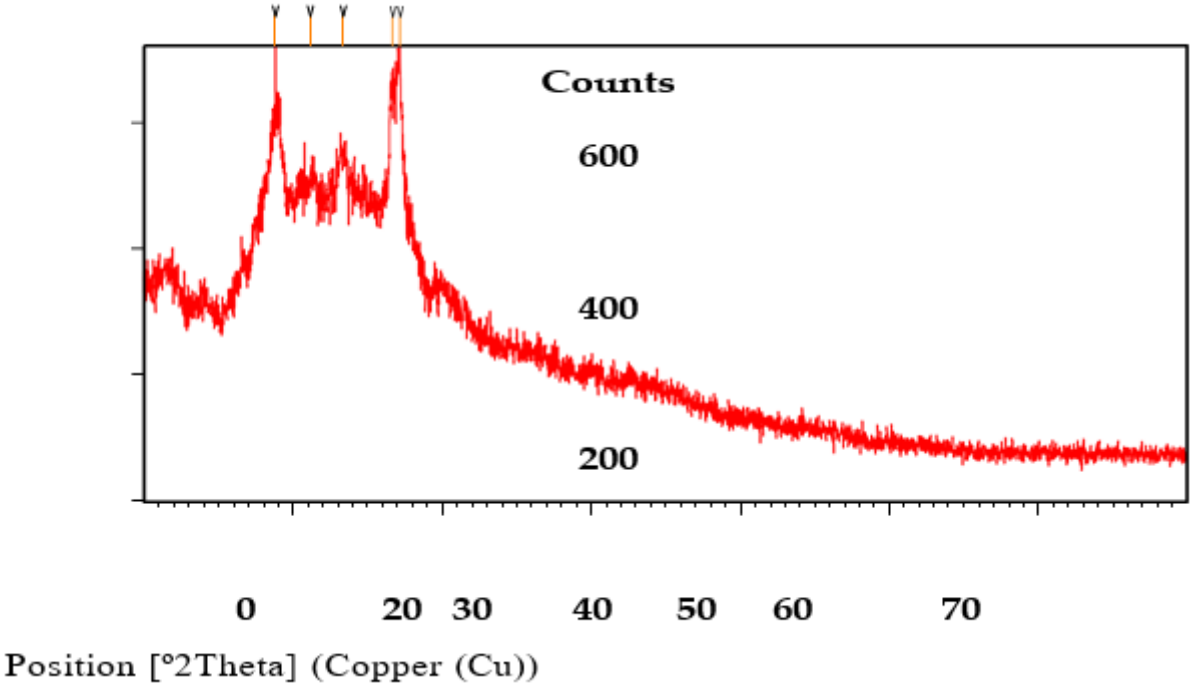

Figure 1e: XRD analysis of phloridzin chitosan nanoparticle.

Histological analysis

On the 29th day after blood collection, the animals were euthanized. The tissues (liver, kidney and brain) were collected, weighed and then fixed in 10% formaldehyde. The samples were processed for paraffin embedding and light microscopic examination. The tissues were sectioned and stained with H and E (Hematoxylin-eosin) and then viewed under the light microscope at 10x magnification.[16]

Statistical analysis

Data are expressed as mean+standard error of the mean. Statistical analyses were performed using one-way ANOVA. Dunnet test analysis was carried out using the least significant difference and $p<0.05$ was considered statistically significant.

RESULTS

Dynamic Light Scattering (DLS)

Chitosan encapsulated phloridzin nanoparticle synthesis was carried out by ionic gelation method. In the DLS analysis average particle size of 246.6 d.nm with a polydispersity index of 0.52 and zeta potential of 30.9 mv was noticed in Figures 1a and 1b.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to determine the functional groups in phloridzin-loaded chitosan nanoparticle and phloridzin-unloaded chitosan nanoparticle interactions. The fundamental vibrations of primary functional groups and their frequency (cm^{-1}) are represented in Figure 1c confirming the phloridzin encapsulation in chitosan nanoparticles.

Fourier Scanning Electron Microscope Analysis (FESEM)

The morphology and particle size of the synthesized nanoparticle were analyzed using FESEM. The nanoparticle showed spherical-like morphological features and was found to be polydispersed and non-agglomerated as shown in Figure 1d. The mean particle size of the nanoparticles was found to be 186 nm.

X-ray Diffractometer (XRD) Pattern analysis

XRD pattern of the synthesized nanoparticle was performed to find out the crystalline nature. The XRD pattern of the chitosan phloridzin nanoparticle exhibited five diffraction signals at (18°, 21°, 23°, 26° and 27°) as shown in Figure 1e.

Percentage Drug Entrapment Efficiency

The percentage drug entrapment efficiency of the prepared phloridzin chitosan nanoparticle was observed to be 50.38%.

Cytotoxicity study

The cytotoxicity of the prepared materials to SH-SY5Y cells was evaluated via an MTT assay and indicates no acute toxicity *in vitro* even at a concentration of 1000 mg/mL as shown in Figure 2a and 2b.

Cellular Uptake

Human neuroblastoma cells (SH-SY5Y) were selected to represent the Ph-ChNPs internalization analysis. Using confocal laser scanning microscopy, the Phloridzin was found efficiently delivered into the SH-SY5Y cells as shown in Figure 3.

Table 1: Acute toxicity studies.

Phase I	Group	Weight of Rats(g)	Dose (mg/kg)	Observations			
				Behavioural Change	Eating Habit	Sleep	Mortality
	I	196.33±3.21	10	No	No	No	No
	II	191.66±2.8	100	No	No	No	No
	III	200±1.7	1000	No	No	No	No
Phase II	I	195	1600	No	No	No	No
	II	190	2900	No	No	No	No
	III	201	5000	No	No	No	No

Table 2: Sub-chronic toxicity studies.

Parameters	Normal	Low (250)	Medium (500)	High (1000)
SGPT (ALT) U/L	80.53±3.73	72±2.94	75.23±1.64	95.73±2.29*
SGOT (AST) U/L	147.86±8.77	148.13±7.86	155.23±1.38	198.13±24.2
Alkaline Phosphatase (ALP) U/L	237.66±31.17	249.33±8.68	274.66±18.81	408±19.67*
Creatinine (mg/dL)	0.58±0.03	0.52±0.05	0.51±0.06	0.49±0.01
Blood Urea Nitrogen (BUN) mg/dL	18.6±0.30	22.4±0.98	21±1.62	20.13±0.97
PCV%	38.5±0.36	38.06±0.27	37.8±0.05	35.5±0.68*
Hb (g/dL)	12.86±0.17	12.53±0.21	12.83±0.20	11.9±0.32
TLC x 10^3 /µL	2.7±0.17	3.6±0.05	3.63±0.44	4.36±0.52*
TEC x 10^6 /µL	6.57±0.12	6.48±0.12	6.52±0.16	5.70±0.18*

The values are expressed in mean±SEM. All the groups were compared to group normal.Statistical significance ($p<0.05$) is indicated by.

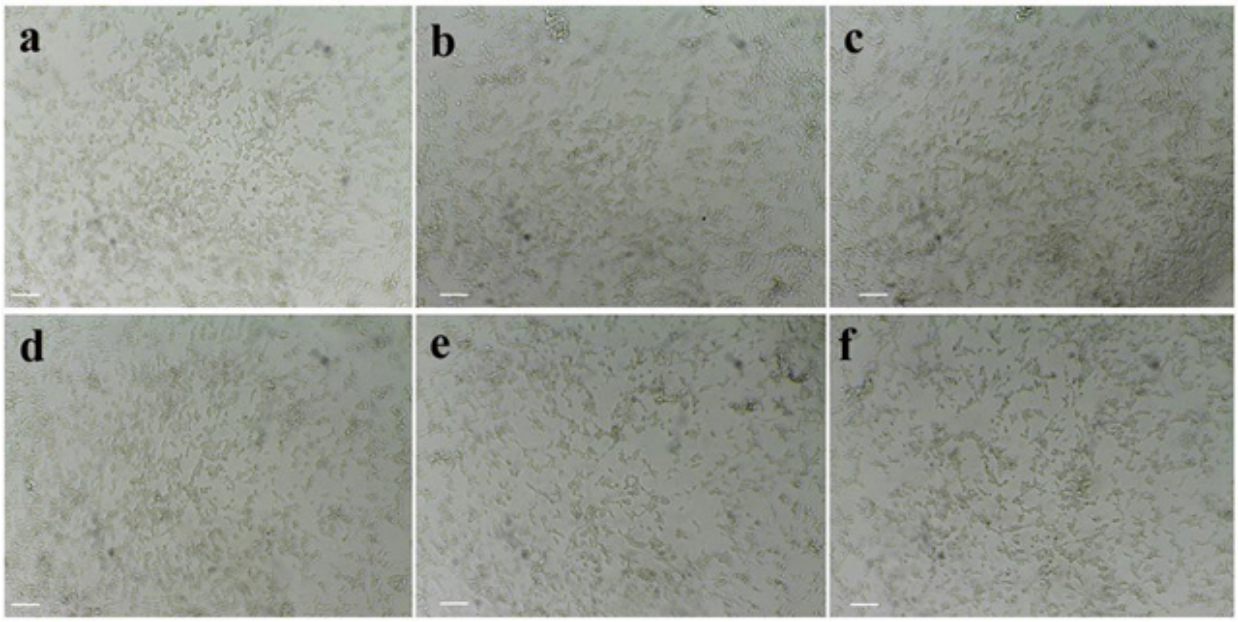

Figure 2a: Cell viability of Phloridzin chitosan nanoparticle.

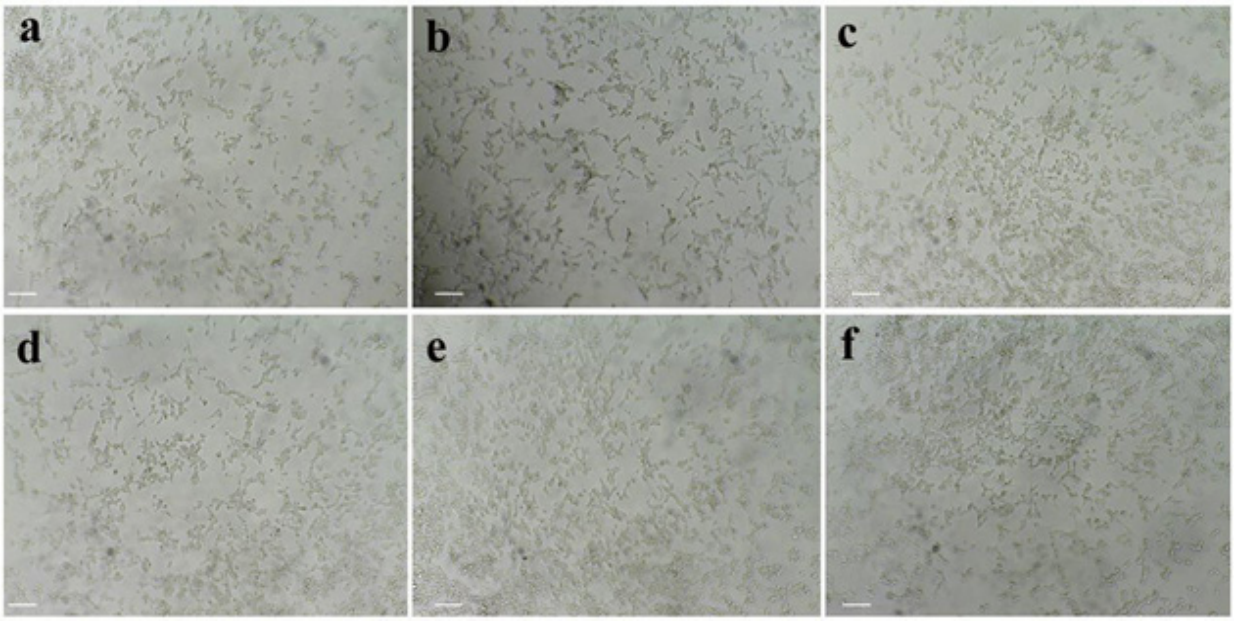

Figure 2b: Cell viability of phloridzin.

Acute toxicity study

In acute toxicity studies administration of the nanoparticle in both phase I and (post-administration) phase II did not show any abnormal symptoms as represented in Table 1. Therefore, from the study, it is evident that the LD_{50} value for chitosan phloridzin nanoparticles exceeded 5000 mg/kg.

Sub-chronic toxicity studies

Hematological and Biochemical Analysis

The systemic effects of phloridzin chitosan nanoparticle exposure were studied using sub-chronic toxicity studies. Exposure to chitosan-encapsulated phloridzin nanoparticles did not reveal any significant hematological and biochemical changes as represented in Table 2.

Histological analysis

The Hematoxylin and Eosin staining of the liver, kidney and brain sections is represented in Figures 4-6. The sub-acute treatment for 28 days did not show any significant alteration in histopathological level.

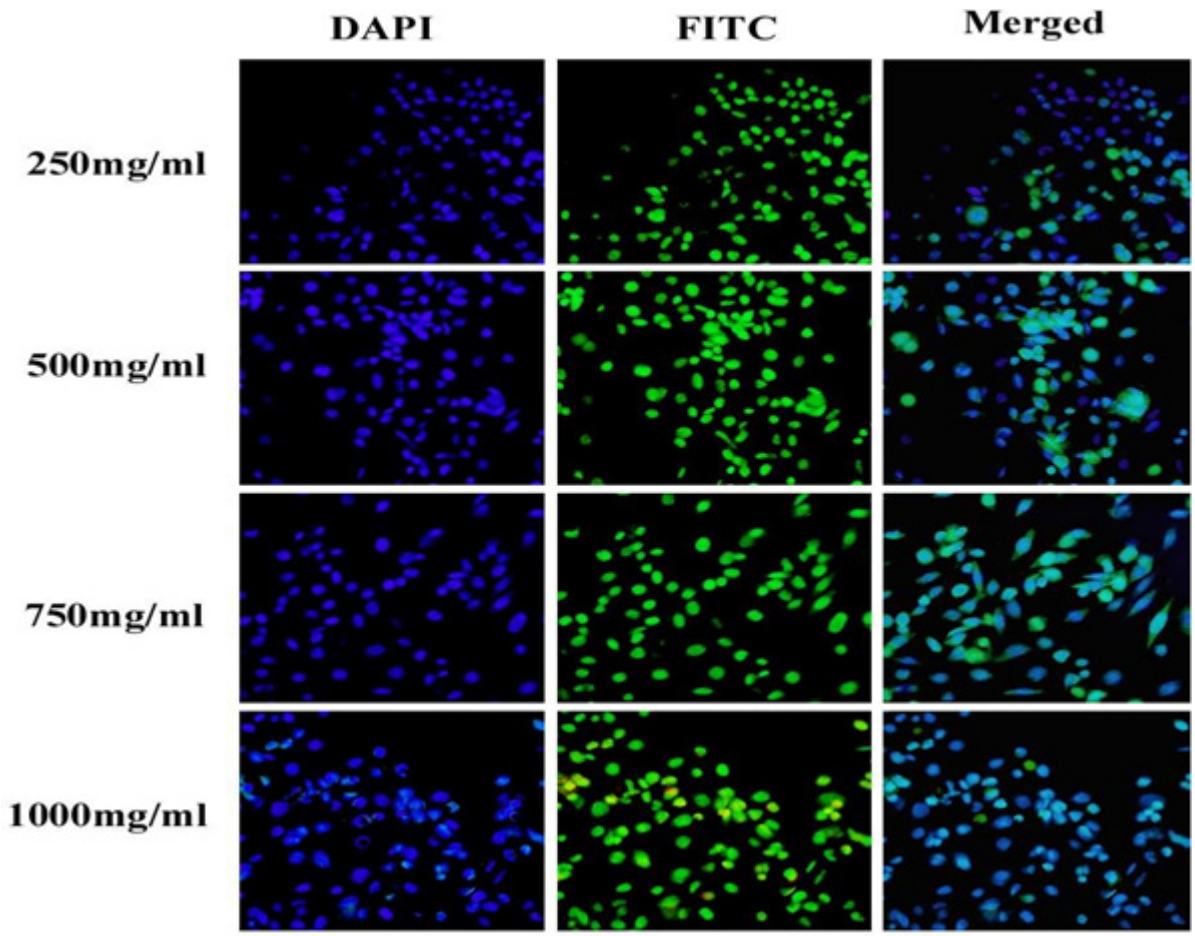

Figure 3: Cellular uptake of Phloridzin nanoparticles.

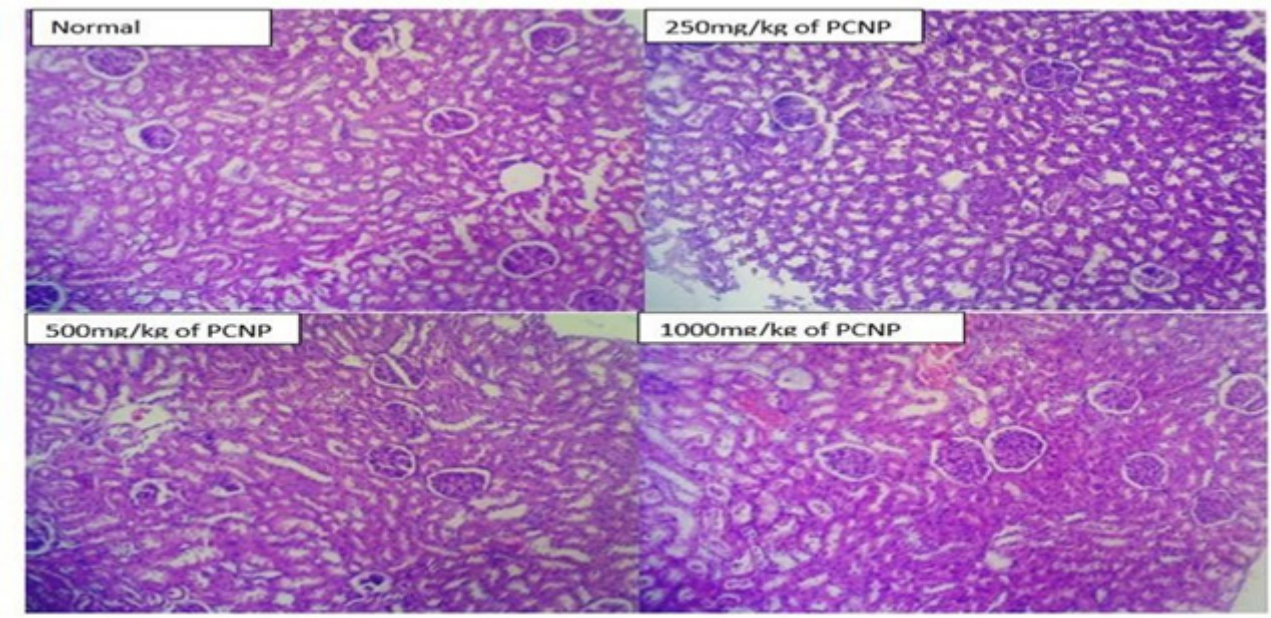

Figure 4: H and E staining of kidney.

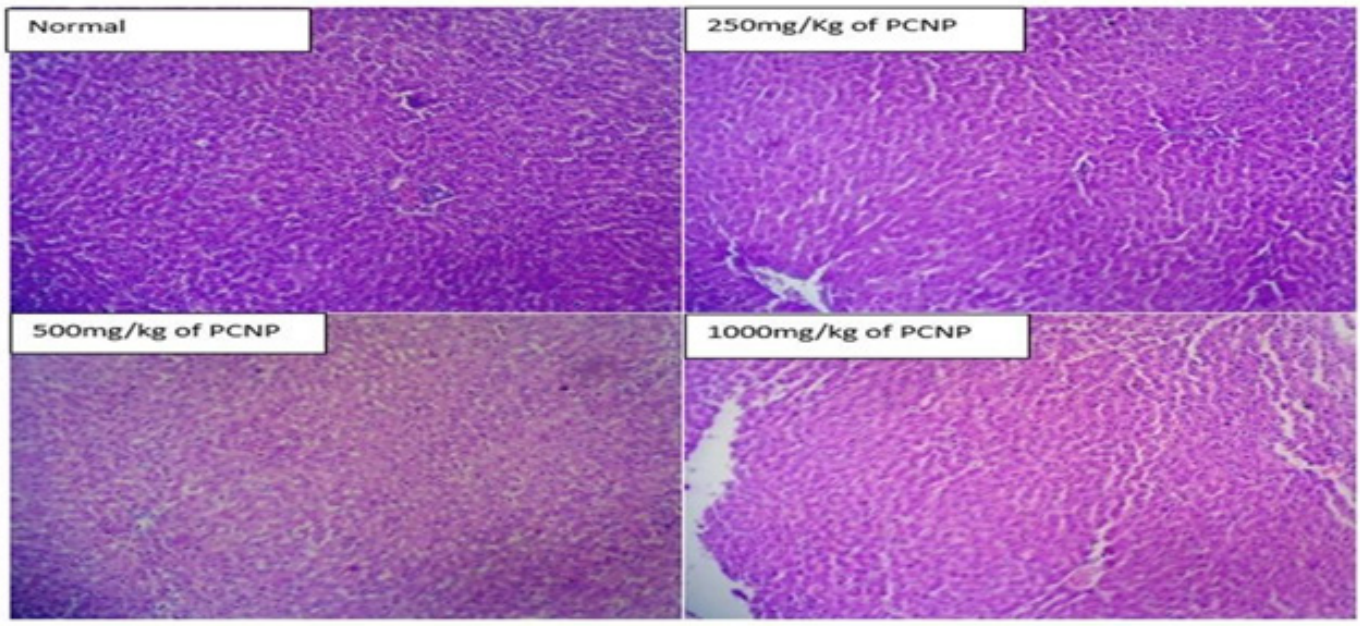

Figure 5: H and E staining of liver.

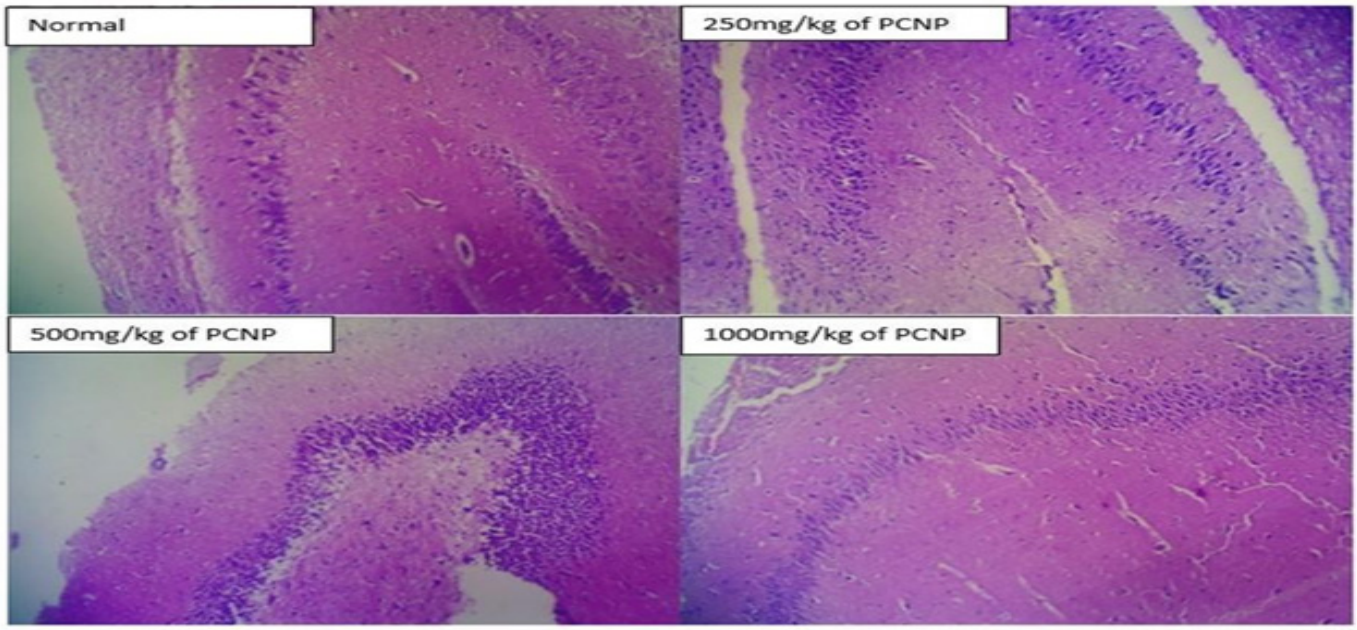

Figure 6: H and E staining of brain.

DISCUSSION

Dynamic Light Scattering (DLS)

The particle size, zeta potential and PDI index were analyzed using DLS. The zeta potential value indicates the stability of the nanoparticles. The values higher than +30 mv and -30 mv demonstrate good stability against coalescence.[17] The particle size ranging between 100 to 500 is considered to be the best suitable for drug delivery.[18] PDI value greater than 0.7 is considered to have a broad size distribution among samples.[19] From the results, it is clear that the synthesized phloridzin chitosan nanoparticle is found to be a suitable particle for drug delivery with good stability and uniform distribution.

Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups present in the synthesized nanoparticle can confirm its formation. Here O-H stretching vibrations of the chitosan nanoparticle were observed at 3576.02 cm^{-1} which might be due to the presence of sugar moieties in the chitosan nanoparticle.[20] C-H stretching vibration at 2991.59 cm^{-1} might be due to the presence of methyl and methylene groups in the chitosan backbone.[21] A band in the region of 1656.85 cm^{-1}

indicates the presence of amide I band C=O stretching. 1514.12 cm^{-1} peak corresponds to N-H bending and C-N stretching vibration in the amide group.[22] 1051.20 cm^{-1} band corresponds to the C-O-C glycosidic linkage present in the chitosan molecule.[23] In phloridzin- loaded chitosan nanoparticles a change in the intensity and shifting of peaks (3371.57, 1683.86, 1527.62 cm^{-1}) was noticed and this might be due to the encapsulation of the phloridzin compound. The peak at 1035.77 cm^{-1} corresponds to the C-O bond stretching, which represents the phenolic structure in phloridzin.[24] This further confirms the encapsulation of phloridzin in chitosan nanoparticles.

Fourier Scanning Electron Microscope analysis (FESEM)

The polydispersed and non-agglomerated nature of the sample might be due to the preparation of the physically dehydrated sample for FESEM analysis. A decrease in the mean size was noticed when compared to that of DLS analysis which might be due to the particles in liquid suspension exhibiting their hydrodynamic property.[25]

X-ray Diffractometer (XRD) pattern analysis

The five diffraction signals at (18°, 21°, 23°, 26° and 27°) indicate the spherical and crystalline nature of synthesized nanoparticles.[26] The cross-links produced by the intercalating polymer chains were responsible for the different peaks in the XRD of chitosan phloridzin nanoparticles (Figure 1e). The sharp peaks denote the presence of the compound in the synthesized nanoparticles.[27]

Cytotoxicity study

The cytotoxicity of the prepared materials to SH-SY5Y cells was evaluated via an MTT assay at different concentrations (range 0-1000 mg/mL). As shown in Figure 2a and 2b, the cell viability of the Phloridzin and Ph-ChNPs of given concentration the cell viability range was above 60% on SH- SY5Y cells after 48 hr incubation, indicating that the prepared compound and formulation do not cause acute toxicity *in vitro* even at a concentration of 1000 mg/mL, which is confirmed that the formulation are biocompatible to the selected cells.

Cellular uptake

Human neuroblastoma cells (SH-SY5Y) were selected to represent the Ph-ChNPs internalization analysis. Using confocal laser scanning microscopy, the colocation of FITC (green fluorescence) and DAPI nucleus staining (blue fluorescence) was evaluated (Figure 3). The cells treated with 250 mg/mL Ph-ChNPs for 8 hr had only a few green fluorescence areas and these were observed in the perinuclear region of SH-SY5Y cytoplasm, as indicated in the merged image. In comparison, a large number of green fluorescent areas were observed for cells incubated with 750 mg/mL Ph-ChNPs for 8 hr and these areas were found to co-localize with the nucleus, indicating that Phloridzin was efficiently delivered into the SH-SY5Y cells.

Acute toxicity study

The toxicity studies were carried out in animals to identify the impact of synthesized nanoparticles on long-term exposure which cannot be immediately noticed in the cell viability assays. The administration of the nanoparticle in both phases I and (post-administration) phase II did not show any abnormal symptoms states that the synthesized nanoparticle has an LD_{50} value greater than 5000 mg/kg.

Sub-chronic toxicity studies

Hematological and Biochemical Analysis

No significant changes were noted in both hematological and biochemical analyses. A slight decrease in the level of PCV, TLC and TEC was noticed which might be due to immunosuppressant activity after continuous administration (1000 mg/kg) of synthesized phloridzin chitosan nanoparticles.[28] An increase in the activity of Alkaline phosphatase was observed after administration of (1000 mg/kg) phloridzin chitosan nanoparticles which might be due to the elevation of any of the several iso-enzymes of ALP that exist in the liver, bones, placenta, kidneys and intestines. The activity of ALP is increased in several clinical conditions (bone and liver disorders).[29] This significant increase might be due to minor inflammation caused by exposure at high dosages.

Histological analysis

Histopathological studies of the kidney section in the normal group showed normal Glomerulus, Bowman's capsule lined with squamous cells and an inner layer filled with podocytes, urinary space, proximal convoluted tubules and distal convoluted tubules. In the phloridzin chitosan nanoparticles treated rats, no significant difference was noticed in the morphology of kidney tissue.[30] Liver tissue histology of the normal group showed normal hepatocytes, central vein and sinusoid appearance. A mild central vein dilation was noticed in the histology of the (1000 mg/kg) chitosan encapsulated phloridzin nanoparticles treated group. The histopathological section of normal brain tissue shows a normal hippocampus region with C- C-shaped cornu amonis and V-shaped dentate gyrus. A slight decrease in the pyramidal layer was observed after the exposure of 1000 mg/kg of phloridzin chitosan nanoparticles. There is no evidence of neuronal degeneration/ sclerosis. However, the sub-acute treatment (28 days) did not show any significant alteration in histopathological level.

CONCLUSION

The chitosan-encapsulated phloridzin nanoparticles a novel drug carrier were successfully synthesized by the ionic gelation method. Characterization of the synthesized nanoparticles done by DLS, FTIR, XRD, %DEE and FESEM which confirmed its formation and indicates good stability, spherical morphology and suitability for drug delivery. The cell viability and uptake assay indicate the formulation is biocompatible and efficiently delivered to the SH-SY5Y cell line. The acute and sub-acute toxicity of the synthesized nanoparticle indicates it is non-toxic in nature and the LD_{50} value exceeded 5000 mg/kg.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SGLT: Sodium Glucose Linked Transporter; **TPP:** Tripolyphosphate; **DLS:** Dynamic Light Scattering; **FTIR:** Fourier Transform Infrared Spectroscopy; **FESEM:** Field Emission Scanning Electron Microscopy; **%DEE:** Percentage Drug Entrapment Efficiency; **LD_{50}:** Median Lethal Dose; **OECD:** Organization for Economic Co-operation and Development; **PDI:** Poly-Dispersity Index; **SGPT:** Serum glutamate pyruvate transaminase; **SGOT:** Serum glutamic oxaloacetic transaminase; **ALP:** Alkaline Phosphatase; **BUN:** Blood Urea Nitrogen; **PCV:** Packed Cell Volume; **Hb:** Hemoglobin; **TLC:** Total Leucocyte Count; **TEC:** Total Erythrocyte Count.

SUMMARY

Phloridzin a phytoconstituent abundantly present in malus species is found to have numerous health benefits. Although some studies have stated its low bioavailability and rapid degradation, it was chitosan encapsulated, characterized and evaluated for toxicity studies. Based on the above analyses the chitosan phloridzin nanoparticles showed stable, nontoxic and suitable material for drug delivery.

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