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Synthesis, characterization, *in silico* prediction and safety evaluation of topical wound healing formulations of hemin and bilirubin nanoparticles

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Abstract
Nanotechnology is an emerging scientific area of research interest. A number of nanomedicine-based therapeutic agents have been synthesized for different ailments. However, safety evaluation of nanoparticles is still a major issue. Present research reports the synthesis of hemin nanoparticles (HNPs) & bilirubin nanoparticles (BNPs) as topical formulations for wound healing based on the information available regarding the pharmacological effects of hemin and bilirubin. *In silico* prediction of hemin and bilirubin revealed that both compounds have high lipophilicity, low water solubility and skin permeation. Hence, HNPs and BNPs were synthesized to overcome these hindrances. Furthermore, safety evaluation of topical formulations of HNPs and BNPs applied for 19 days in diabetic rat wound model was done in terms of haematology and gross necropsy which revealed that HNPs and BNPs treated groups showed no alteration in haematology and no pathological changes in the vital organs were observed.

Keywords: Nanotechnology, viruses, poisons, mechanical, bilirubin nanoparticles

1. Introduction
A variety of noxious stimuli such as viruses, poisons, and mechanical stress against which skin act as an important protective barrier covering the outer body surface [1]. It is especially vulnerable to injuries because of how exposed it is to the environment. A cutaneous wound results in the loss of epithelial continuity, interferes with its typical structure, function, and local environment, and may injure internal organs [2].

Reports suggest that out of 1000 cases there 4.5 cases of wounds which are acute in nature while 10.5 are chronic in nature in Indian population [3]. One to two percent of people in all communities will develop a chronic wound at some point in their lives [4]. Ageing and the rise in diabetic cases contribute to a higher prevalence of non-healing wounds [5]. Therefore, one of the primary needs of the present time is effective novel wound healing medications or formulations.

Innovative bandages made of nanomaterials, like hydrogels, nanofibers, and films, are now widely used. Numerous cutting-edge methods for regenerative medicine are made possible by nanotechnology. There have been many developments in biocompatible self-assembling nanoparticles (NPs) [6]. NPs support delayed wound healing and injury care. Less *in vivo* toxicity and bacteriostatic and bactericidal activity have been seen in the metal NPs [7, 8]. Nanoparticles have size in nanometer range and its use rapidly increasing in modern medicine [9]. The surface area as well as surface area to volume ratio increases upon reducing the size of a material resulting in change in its physiochemical properties. Despite, nanoparticles being such potent agents, safety evaluation of nanoparticles has to be carried out before its potential is exploited. Previous reports have suggested that hemin and bilirubin have wound healing potential. Hemin's structural makeup includes a porphyrin ring. Protoporphyrin hemin has a ferrous ion and a coordinating chloride ligand [10]. Hemin induces heme oxygenase-1, reducing oxidative stress-related tissue damage [11]. Hemin combined with ibuprofen reduced inflammation and pain in arthritis inflicted on Wistar rats [12]. Hemin is hydrophobic due to the presence of a large tetrapyrrolic macrocycle [13]. Hemin's use in a variety of fields has been constrained due to its strong hydrophobic nature and low solubility [14, 15].

Recent studies have demonstrated that the bilirubin produced during heme metabolism has the ability to heal wounds and reduce inflammation [16]. Bilirubin is a unique wound healing agent due to its anti-inflammatory, antioxidant, anti-apoptotic, and cytoprotective properties. In both diabetic and non-diabetic rats, bilirubin has the ability to heal wounds [17]. The diverse *in vivo* pharmacological activities of bilirubin cannot, however, be fully exploited due to its limited water solubility and tissue absorption [18]. The high hydrophobicity and large particle size affect the different pharmacological effects of hemin and bilirubin. Therefore, in the present study aims at studying *in silico* prediction of hemin and bilirubin as well as hemin and bilirubin NPs were synthesized in order to study the safety of the topical formulation of hemin and bilirubin nanoparticles.

2. Materials and Methods

Bilirubin (bilirubin mixed isomers Cat. no. B4126; ≥ 98% purity), Hemin (Cat. no. 51280; ≥ 96% purity), pluronic F-127 (Cat. no. P2443), Chitosan (Cat. no. C3646, ≥ 75% deacetylated) procured from Sigma Aldrich. Sodium tripolyphosphate (STPP) (Cat no. 85124) purchased from SRL, India.

2.1 Synthesis of HNPs and BNPs

HNPs and BNPs were synthesized using ionic gelation technique. Firstly, dissolution of chitosan (0.2%) was carried out in 2% acetic acid and stirred for 12h. Thereafter, filtration of the chitosan was done by 0.45 µm membrane filters and the pH was set to 4.5 with the help of 10N sodium hydroxide (NaOH). Following this, STPP (0.2%) was prepared by dissolving STPP in distilled water. Bilirubin and hemin were dissolved in 0.1M NaOH. For preparation of HNPs and BNPs, bilirubin and hemin solutions were added in dropwise manner in the chitosan solution and kept on stirring for 5 minutes. This was followed by addition of STPP in dropwise manner resulting into formation of translucent suspension. Thereafter, centrifugation was carried out at 12,500g for a time period of 30 min. Obtained pellet was washed thrice to remove the unencapsulated drug particles. Thereafter, the pellet was resuspended in phosphate buffer saline (PBS) and ultrasonicated in order to decrease the particle size. The pellet was dried and weighed accordingly to prepare topical formulation of three different concentrations of hemin (0.02%, 0.1%, 0.5%) and bilirubin (0.03%, 0.1%, 0.3%) nanoparticles in 10% Pluronic F-127 (PF-127) as a vehicle.

2. Characterization of HNPs and BNPs

2.1 Analysis of hydrodynamic diameter and polydispersity index (PDI)

Hydrodynamic diameter and PDI were analysed using zetasizer (Malvern, UK) which measure the Brownian motion of the particles and extrapolate a diameter from this with the help of mathematical equations. The experimentation was carried out in triplicates.

2.2 Zeta potential of HNPs and BNPs

Zetasizer was used in order to establish the charge of the synthesised HNPs and BNPs. The experimentation was carried out in triplicates.

2.3 *In silico* prediction of hemin and bilirubin

In order to study the pharmacokinetics, pharmacodynamics, toxicokinetics and toxicodynamic properties of hemin and bilirubin, Swiss ADME and PASS Online were used. Online platforms with predictive service libraries were employed for *in silico* predictions, and Pub Chem. (https://pubchem.ncbi.nlm.nih.gov/) was used to analyse the chemical makeup of hemin and bilirubin. Swiss ADME (www.swissadme.ch) supports drug discovery by enabling the computation of physicochemical descriptors as well as the prediction of ADME parameters, drug-likeness, and medicinal chemistry compatibility of one or more small compounds. Over 4000 different types of biological activity are predicted by PASS Online (http://www.way2drug.com/passonline/), including pharmacological effects, toxic or adverse effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc.

2.4 *In vivo* experimentation and safety evaluation of HNPs and BNPs

2.4.1 Procurement of animals

Forty-eight male Wistar rats having weight 100-140 g were obtained from LARS, ICAR-IVRI, Bareilly (U.P.), India. The rats were acclimatized for 3 weeks before the initiation of experiment. The experimentation was conducted under the approval of Institutional Animal Ethics Committee (IAEC) of ICAR-IVRI, Bareilly, U.P (IAEC No – 26-1/2022-23/JD(R)/ IAEC) Dated 30.07.2022.

2.4.2 Induction of diabetes

Diabetes was induced using streptozotocin (STZ). Rats were kept off-feed for an overnight period before induction of diabetes, and glucometer was used to measure their fasting blood glucose levels. STZ (45 mg/kg) was given intraperitoneally prepared in 0.1M citrate buffer and pH was set to 4.5. Streptozotocin treated rats were then tested for fasting blood glucose level again seventy-two hours after administration of STZ, and those with a fasting glucose level of higher than 300 mg/dl were chosen for future studies. These rats were observed for 14 days before initiating the experiment.

2.4.3 Experimental protocol

The protocol consists of eight groups consisting of 6 rats in each group. The rats were anesthetized using ketamine (50 mg/kg, I.P.) + xylazine (5 mg/kg, I.P.), following which wound (400mm^2) was created at the thoraco-lumabr portion. Different topical formulation were applied at the wound site twice a day upto 19 days. At the day 19, blood was collected from each rat for haematological study. For gross examination of different organs, rats were sacrificed using thiopentone (I.P.). The details about different groups and formulations for HNPs and BNPs is presented in table 1 & table 2, respectively.

Table 1: HNPs formulation, concentration and animals per group.

Groups	Formulations	Concentration	Animals per group
1	Pluronic F-127 (Vehicle)	10%	6
2	HNPs-1	0.02%	6
3	HNPs-2	0.1%	6
4	HNPs-3	0.5%	6

Table 2: Details of BNPs formulation, concentration and animals per group

Groups	Formulations	Concentration	Animals per group
1	Pluronic F-127 (Vehicle)	10%	6
2	BNPs-1	0.03%	6
3	BNPs-2	0.1%	6
4	BNPs-3	0.3%	6

2.5 Safety evaluation of HNPs and BNPs

2.5.1 Analysis of haematological parameters

At day 19 post-wounding, blood was collected from each rat of different groups and was analysed for different haematological parameters with the help of Hematology Analyzer (URIT 3000 Vetplus) in order to study the systemic effects of HNPs and BNPs, if any.

2.5.2 Necropsy of rats

Rats were sacrificed on day 19 and the different organs such as liver, kidney, heart, lung, spleen, etc. were grossly evaluated to examine whether HNPs and BNPs have any systemic effects.

2.5.3 Statistical Analysis

With n equal to the number of replications, the results are shown as mean ± SEM. Using the GraphPad Prism v8.0.2, the statistical significance between the various groups was examined using one-way analysis of variance (ANOVA) and then a suitable post-test (Tukey's or Dunnet's) was applied. At $p \leq 0.05$, the differences between the various treatment groups were considered statistically significant.

3. Results

3.1 Hydrodynamic diameter & polydispersity index (PdI)

Hydrodynamic diameter of HNPs was found to be 215±5.79nm before ultrasonication [Fig. (1A)], while after ultrasonication, the hydrodynamic diameter of HNPs reduced to 130 ± 7.42 nm [Fig. (1B)]. In case of BNPs, the hydrodynamic diameter of BNPs was found to be 162 ± 4.25 nm before ultrasonication [Fig. (1C)], while after ultrasonication, the hydrodynamic diameter of BNPs reduced to 108 ± 3.36 nm [Fig. (1D)]. The PDI of HNP was found to be 0.22 ± 0.01 and that of BNPs was found to be 0.19 ± 0.02.

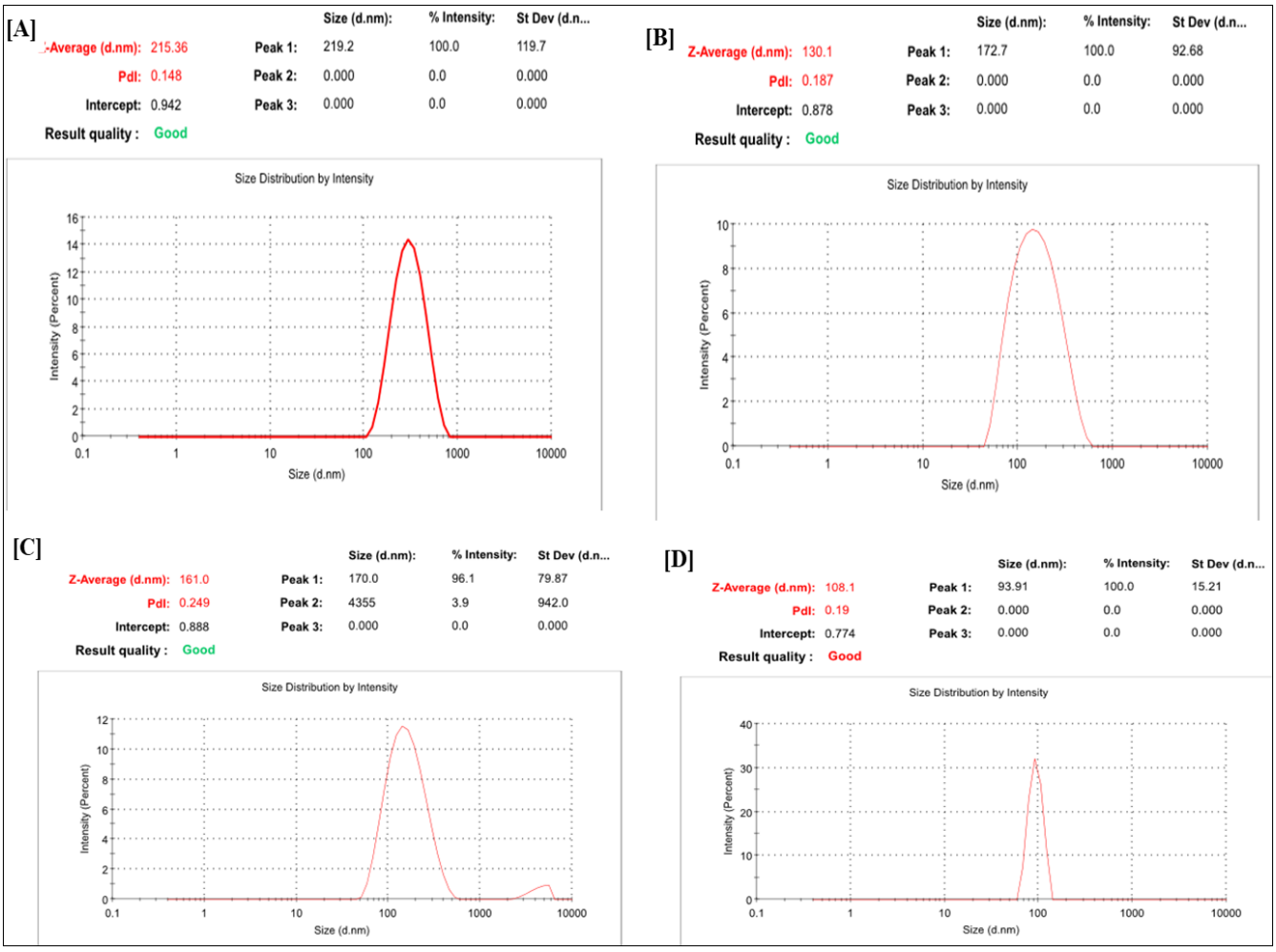

Fig 1: Representative images showing hydrodynamic diameter and PDI of HNPs and BNPs. [A] HNPs before ultrasonication [B] HNPs after ultrasonication [C] BNPs before ultrasonication [D] BNPs after ultrasonication.

3.2 Zeta potential of HNPs and BNPS

The zeta potential of HNPs and BNPs was found to be + 38 ± 0.21mV and +35 ± 0.34mV, respectively.

3.3 *In silico* analysis of hemin and bilirubin

3.3.1 Computed Descriptors of hemin

PubChem-CID- 455658
IUPAC nomenclature: 3-[18-(2-carboxyethyl)-8, 13-bis (ethenyl)-3,7,12,17-tetramethylporphyrin-21,24-diid-2-yl]propanoic acid; Iron (3+);chloride
Molecular Weight- 651.9
Molecular Formula- $C_{34}H_{32}ClFeN_4O_4$
Canonical SMILES-
CC1=C(C2=CC3=C(C(=C([N-]3)C=C4C(=C(C(=N4)C=C5C(=C(C(=N5)C=C1[N-]2)C)C=C)C)C=C)C)CCC(=O)O)CCC(=O)O.[Cl-].[Fe+3]
European Community (EC) Number- 240-140-1
CAS- 16009-13-5

3.3.2 Computed Descriptors of bilirubin

PubChem CID- 5280352
IUPAC nomenclature - 3-[2-[[3-(2-carboxyethyl)-5-[(Z)-(3-ethenyl-4-methyl-5-oxopyrrol-2-ylidene)methyl]-4-methyl-1H-pyrrol-2-yl]methyl]-5-[(Z)-(4-ethenyl-3-methyl-5-

oxopyrrol-2-ylidene)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid
Canonical SMILES-
CC1=C(NC(=C1CCC(=O)O)CC2=C(C(=C(N2)C=C3C(=C(C(=O)N3)C)C=C)C)CCC(=O)O)C=C4C(=C(C(=O)N4)C=C)C
Molecular Weight- 584.7
CAS- 635-65-4
Molecular Formula- $C_{33}H_{36}N_4O_6$
European Community (EC) Number- 211-239-7

3.3.3 SwissADME analysis of hemin and bilirubin

Swiss ADME tool evaluated the physicochemical attributes, water solubility, pharmacokinetics, lipophilicity, medicinal chemistry, drug likeness of hemin and bilirubin. This different parameters are expressed in terms of table (3-8) for hemin and table (9-14) for bilirubin. Hemin showed 8 number of rotatable bonds and 8 H-bond acceptor and topological polar surface area of 125.10 Å².

Table 3: Physicochemical properties

Formula	$C_{34}H_{32}ClFeN_4O_4$
Molecular weight	651.94 gram/mol
Number of heavy atoms	44
Number of aromatic heavy atoms	10
Fraction Csp3	0.24
Number of rotatable bonds	8
Number of H-bond acceptors	8
Number of H-bond donors	2
Topological polar surface area	125.10 Å²
Molar Refractivity	179.05

Table 4: Lipophilicity

iLOGP	-198.36
XLOGP3	5.43
WLOGP	0.15
MLOGP	2.33
SILICOS-IT	8.55
Consensus Log Po/w	-36.38

Table 5: Water Solubility

Log S (ESOL)	-6.94
Solubility	7.43e-05 mg/ml ; 1.14e-07 mol/l
Class	Poorly soluble
Log S (Ali)	-7.81
Solubility	1.00e-05 mg/ml; 1.54e-08 mol/l
Class	Poorly soluble
Log S (SILICOS-IT)	-8.26
Solubility	3.60e-06 mg/ml; 5.52e-09 mol/l
Class	Poorly soluble

Table 6: Pharmacokinetics

Gastrointestinal absorption	High
Blood brain barrier permeant	No
P-glycoprotein substrate	Yes
CYP1A2 inhibitor	No
CYP3A4 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP2C19 inhibitor	No
Log Kp (skin permeation)	-6.42 cm/s

Table 7: Druglikeness

Lipinski	Yes; 1 violation: MW > 500
Ghose	No; 3 violations: MW > 480, MR > 130, #atoms > 70
Veber	Yes
Egan	Yes
Muegge	No; 2 violations: MW > 600, XLOGP3 > 5
Bioavailability Score	0.56

Table 8: Medicinal Chemistry

Pains	0 alert
Brenk	1 alert: heavy_metal
Lead likeness	No; 3 violations: MW > 350, Rotors > 7, XLOGP3 > 3.5
Synthetic accessibility	7.47

Hemin had ESOL value of -6.94 indicating low water soluble. It also showed high gastrointestinal absorption and did not show inhibition of CYP450 enzymes. The log Kp value for hemin was found to be -6.42 cm/s. Bilirubin showed 12 number of rotatable bonds 12 and 6 H-bond acceptor and topological polar surface area of 164.38 Å². Bilirubin showed moderate water solubility with ESOL value of -4.67. It also showed low gastrointestinal absorption and did not show inhibition of CYP450 enzymes. The log Kp value for bilirubin was found to be -7.81 cm/s. Hemin followed lipinski's rule with only one violation while bilirubin did not follow the Lipinski's rule having two violations.

Table 9: Physicochemical properties

Formula	**$C_{33}H_{36}N_4O_6$**
Molecular weight	584.66 gram/mol
Number of heavy atoms	43
Number of aromatic heavy atoms	10
Fraction Csp3	0.27
Number of rotatable bonds	12
Number of H-bond acceptors	6
Number of H-bond donors	6
Topological polar surface area	164.38 Å²
Molar Refractivity	172.81

Table 10: Lipophilicity

iLOGP	3.53
XLOGP3	2.9
WLOGP	3.55
MLOGP	1.37
SILICOS-IT	7.89
Consensus Log Po/w	3.85

Table 11: Water Solubility

Log S (ESOL)	-4.67
Solubility	1.24e-02 mg/ml ; 2.13e-05 mol/l
Class	Moderately soluble
Log S (Ali)	-6.01
Solubility	5.68e-04 mg/ml ; 9.72e-07 mol/l
Class	Poorly soluble
Log S (SILICOS-IT)	-8.37
Solubility	2.52e-06 mg/ml ; 4.31e-09 mol/l
Class	Poorly soluble

Table 12: Pharmacokinetics

Gastrointestinal absorption	Low
Blood brain barrier permeant	No
P-glycoprotein substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP3A4 inhibitor	No
CYP2D6 inhibitor	No
CYP2C9 inhibitor	No
Log Kp (skin permeation)	-7.81 cm/s

3.4 PASS online analysis of hemin and bilirubin

The PASS online anlaysis was carried out to evaluate the activity spectra of hemin and bilirubin for possible pharmacological and adverse effects by indicating Pa value (Probability to be active) and Pi value (Probability to be inactive). Hemin's activity spectra is shown in table 15 while possible adverse or toxic effects is indicated in table 16. Similarly, activity spectra of bilirubin is shown in table 17. The values showing Pa > 0, 7 are taken into account. Bilirubin didn't show any possible adverse effect for Pa > 0, 7.

3.5 Safety evaluation of topical formulations:

3.5.1 Haematological values

The haematological values of different topical formulations are given in Fig. 2. There was no significant difference in haematological values among the different treatment groups as compared to the control (vehicle) group.

Table 13: Drug likeness

Lipinski	No; 2 violations: MW > 500, NHorOH > 5
Ghose	No; 3 violations: MW > 480, MR > 130, #atoms > 70
Veber	No; 2 violations: Rotors > 10, TPSA > 140
Egan	No; 1 violation: TPSA > 131.6
Muegge	No; 2 violations: TPSA > 150, H-don > 5
Bioavailability Score	0.11

Table 14: Medicinal Chemistry

PAINS	0 Alert
Brenk	0 alert
Leadlikeness	No; 2 violations: MW > 350, Rotors > 7
Synthetic accessibility	5.23

3.5.2 Necropsy findings

Representative photographs of necropsy of HNPs and BNPs treated rats for 19 days is presented in Fig. 3. No pathological alteration was observed in the vital organs. In necropsy findings, no evidence of systemic effect of HNPs and BNPs treated groups was observed. The morphological alteration, texture, existence of vascular alterations or EDEMA in important organs like heart, lungs, kidneys, liver etc. was not evident.

4. Discussion

The process of encapsulating substances into fabricated submicronic structures is called nanoencapsulation which has the ability to shield important components from potentially harmful or unfavourable environmental conditions or processes. By using nanoencapsulation techniques, it is possible to increase the bioavailability of bioactive substances that are difficult to absorb while also protecting them during the processing of the matrix in which they are present [19]. The development of site-specific delivery systems centred on polymers has attracted increasing attention in recent years. Polysaccharides are among the most prevalent natural materials; they are also reasonably easy to produce and well-known, making them a natural alternative to manufactured polymers due to their biodegradable, safe, and non-toxic properties. They have countless application possibilities due to their various compositions [20]. Biomaterials known as polysaccharides could be neutral (such as cellulose), cationic (such as chitosan) or anionic (such as alginate) [21]. In the present study, chitosan was used as an encapsulating agent for encapsulation of hemin and bilirubin using ionic gelation technique.

Table 15: PASS: Predicting the activity spectra of hemin (Pa > 0, 7)

Pa	Pi	Activity
0,962	0,000	Ferrochelatase inhibitor
0,869	0,005	Gluconate 2-dehydrogenase (acceptor) inhibitor
0,835	0,002	5-Aminolevulinate synthase inhibitor
0,811	0,003	Radiosensitizer
0,787	0,000	Magnesium-protoporphyrin IX methyltransferase inhibitor
0,783	0,003	Erythropoiesis stimulant
0,742	0,005	HMOX1 expression enhancer
0,702	0,004	Chemosensitizer
0,723	0,043	CYP2J substrate
0,714	0,041	Antieczematic
0,708	0,053	Mucomembranous protector

Table 16: PASS predicting the possible adverse effects of hemin (Pa > 0, 7)

Pa	Pi	Activity
0,910	0,005	Hyperthermic
0,913	0,009	Conjunctivitis
0,826	0,013	Hypertensive
0,821	0,013	Inflammation
0,811	0,015	Consciousness alteration
0,812	0,019	Ocular toxicity
0,816	0,023	Hematotoxic
0,810	0,019	Dizziness
0,782	0,023	Headache
0,758	0,006	Cataract
0,770	0,025	Sensory disturbance
0,765	0,028	Dermatitis
0,763	0,030	Nausea
0,761	0,033	Toxic, gastrointestinal
0,755	0,029	Pain
0,727	0,035	Sleep disturbance

Table 17: PASS: Predicting the Activity Spectra of bilirubin (Pa > 0, 7)

Pa	Pi	Activity
0,867	0,003	Platelet derived growth factor receptor kinase inhibitor
0,769	0,001	Threonine ammonia-lyase inhibitor
0,772	0,024	Gluconate 2-dehydrogenase (acceptor) inhibitor
0,723	0,005	UGT1A1 substrate
0,714	0,014	UDP-glucuronosyltransferase substrate

Therefore in the present study, STPP was used in order to cross link with chitosan and form spherical nanoparticles. Structures between 1-100 nm refers to nanoparticles [22], however, the term is applicable to larger particles below 500 nm. In the present study, HNPs had hydrodynamic diameter of 215 ± 5.79 nm before ultra-sonication while after ultra-sonication, the hydrodynamic diameter of HNPs reduced to 130±7.42 nm, which could be due to the effect of ultrasonic waves on particles. The hydrodynamic diameter of BNPs was found to be 162±4.25 nm before ultrasonication, while after ultrasonication, the hydrodynamic diameter of HNPs reduced to 108 ± 3.36 nm.

In silico analysis of hemin and bilirubin was carried out in order to evaluate the physicochemical attributes water solubility, pharmacokinetics, lipophilicity, action, medicinal chemistry and drug likeness.

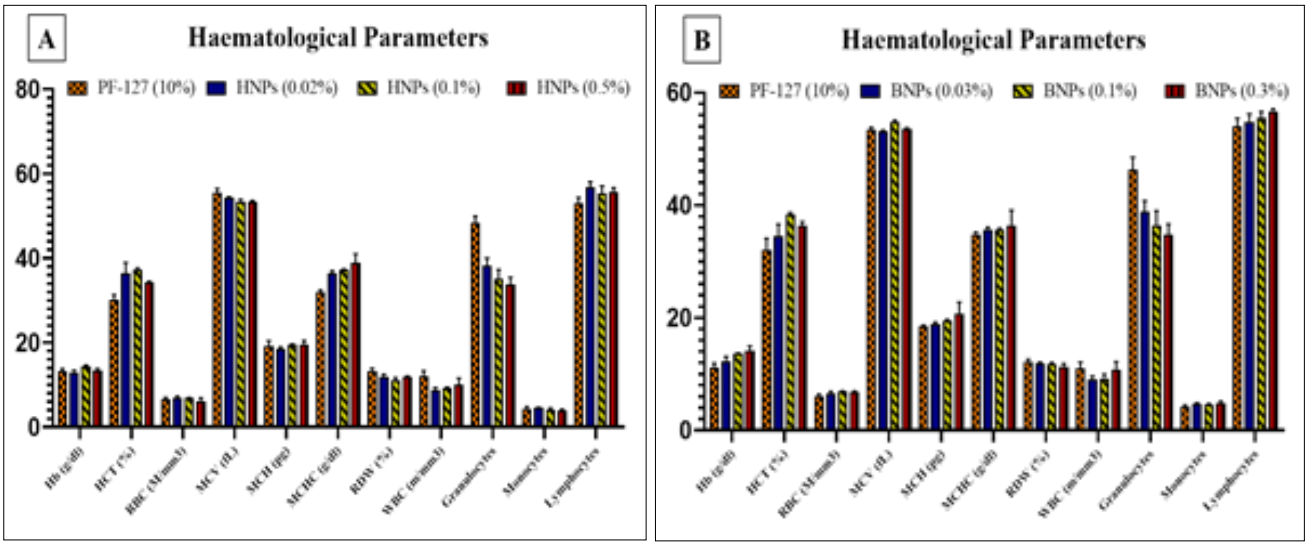

Fig 2: Graphs showing haematological values. [A] HNPs treated groups, [B] BNPs treated groups. Data are expressed as mean ± SEM (n=6)

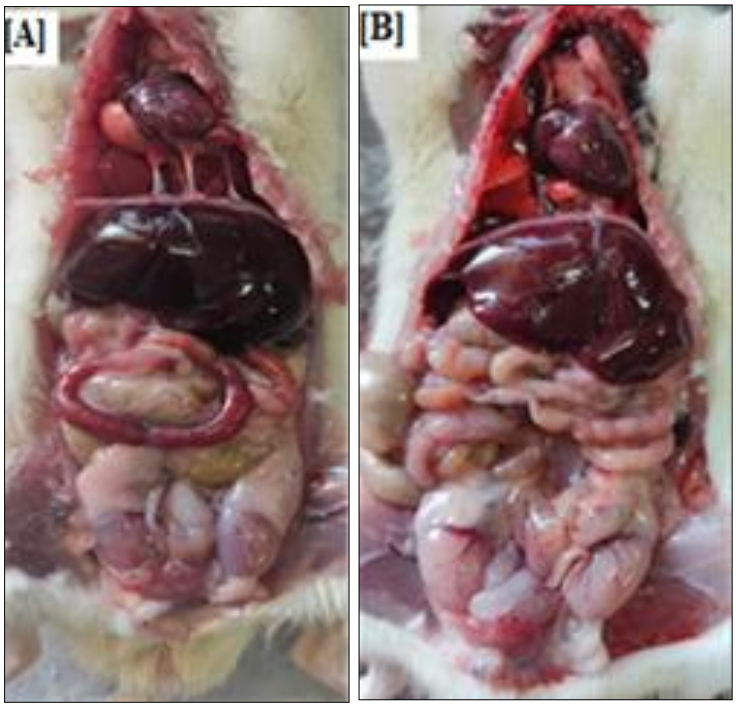

Fig 3: Representative necropsy images, [A] HNPs treated animal [B] BNPs treated animal.

The *in silico* prediction regarding a compound gives idea about whether the compound is an optimum drug candidate or not. Swiss ADME is a tool constructed by Swiss institute of Bioinformatics to validate the properties of drugs [23]. The Lipinski's Rule would be followed by an ideal therapeutic molecule. A drug-like chemical must have a molecular weight (MW) of less than 500 g/mol, a log p value indicating its hydrophobicity of less than 5, no more than five hydrogen bond donors (HBDs), and no more than ten hydrogen bond acceptor (HBA) sites [24]. In the present study, Swiss ADME analysis of hemin revealed that it has molecular weight more than 500g/mol, thus violating one of the rule of Lipinski,

while in case of bilirubin, it violates 2 of the lipinski's rules. Swiss ADME results also revealed that hemin is poorly water soluble with log S value of -6.94 and bilirubin is moderately water soluble with log S value -4.67. The solubility criteria is that the log S should not be more than 6 [25]. The pharmacokinetic parameters showed that both hemin and bilirubin did not have the ability to inhibit major cytochrome P450 enzymes. A model that attempts to predict the skin permeability coefficient (Kp) is multiple linear regression. A linear correlation between Kp and molecule size and lipophilicity exist. The molecule is less skin permeable the more negative the log Kp. Hemin and bilirubin showed log Kp (skin permeation) of -6.42 cm/s and -7.81 cm/s, indicating low skin permeation.

PASS online forecasts biological activity of compounds, even for those that have not yet been synthesised or tested. Based on structure activity relationship, PASS calculates likelihood that a chemical will be active or inactive. The values indicate whether a certain activity type is revealed (Pa) or not revealed (Pi) on a range of 0.000 to 1.000 for each activity type from the biological activity spectrum. The activities having Pa > Pi are only considered [26]. In the present study only those effects whether pharmacological or toxic were considered which showed Pa>0, 7.

In view of this *in silico* analysis, both hemin and bilirubin showing high lipophilicity and low water solubility along with low skin permeation, hemin and bilirubin nanoparticles were synthesized in order to overcome the above mentioned hindrances in drug delivery.

The safety profiles of therapeutic applications using nanoscale materials and formulations for drug delivery systems are major issues of concern. In the current investigation, the safety assessment of the HNPs and BNPs was done, and wounded rats received the HNPs and BNPs topically for 19 days. Blood was taken from rats to analyse the haematological parameters in order to determine whether topically applying hemin and bilirubin nanoparticles had any systemic effects. The haematological readings of HNPs and BNPs treated groups were within the normal range, and there was no discernible difference between them and control group. Furthermore, no significant abnormality was seen in any internal organ, including the lungs, heart, oesophagus, trachea, stomach, small intestine, large intestine, liver, spleen, kidneys, and testes, suggesting that HNPs and BNPs are biocompatible.

5. Conclusion

HNPs and BNPs were synthesized by ionic gelation technique using chitosan and STPP. The synthesized HNPs and BNPs had nano size and were positively charged. *In silico* prediction revealed that both hemin and bilirubin have high lipophilicity and low water solubility as well as skin permeation. Safety evaluation of HNPs and BNPs showed no systemic effects upto to the concentrations of nanoparticles examined in the present study.

6. Acknowledgments

Authors are greatly thankful to administration of ICAR- IVRI, Bareilly, U.P., and India.

7. Authors' contributions

Dhaval J Kamothi, Dinesh Kumar & Anshuk Sharma designed the experiment. Dhaval J Kamothi, Dinesh Kumar & Anshuk Sharma did the synthesis and characterization studies of nanoparticles. Dhaval J Kamothi & Anshuk Sharma did the experimentation. Dhaval J Kamothi & Manju Gari did the haematological analysis and necropsy. All authors analysed the data and contributed in writing the manuscript. Dinesh Kumar supervised the whole study.

8. Conflict of interest

All the authors declare no conflict of interest.

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