Evaluation of antibacterial potential of bilirubin nanoparticles against *Staphylococcus aureus* and *Escherichia coli*

Dhaval J. Kamothi[1], Vinay Kant[1], Vinod Kumar[1], Vinay G. Joshi[2], Rajesh Chhabra[3]

[1]Department of Veterinary Pharmacology & Toxicology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

[2]Department of Animal Biotechnology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

[3]College Central Laboratory, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Corresponding author: Vinay Kant, drvinaykantluvas@gmail.com

ABSTRACT Antimicrobial resistance is a major emerging clinical and public health issue across the globe. Nanotechnology is an emerging field and developing area of scientific interest in the world. Earlier reports showed *in vivo* antibacterial activity of bilirubin. Therefore, in present study, bilirubin nanoparticles (BNP) were synthesized of $\sim 100 - 150$ nm, spherical shape and negative charge. Antibacterial activity of BNPs at different concentrations (0.03, 0.1 and 0.3 %) was evaluated by agar well diffusion method against the gram-positive i.e. *S. aureus* and gram-negative i.e. *E. coli* bacteria. Tetracycline and enrofloxacin used as positive control. Tetracycline and Enrofloxacin at different concentrations exhibited zones of inhibition against both *S. aureus* and *E. coli*. However, BNPs as well as bulk bilirubin showed no *in vitro* antibacterial effect on *S. aureus* and *E. coli*, however, testing by broth method and at higher concentration as well as against other microorganisms can be explored in future.

KEYWORDS Bilirubin nanoparticles, *S. aureus*, *E. coli*, antibacterial activity

1. Introduction

The discovery of antibiotics is considered to be one of the most important medical achievements during the twentieth century [1]. In the years between 1930 and 1962, more than 20 new classes of antibiotics were produced, however, as a result of the evolution of new resistant bacterial strains, a major challenge has been set up against the pharmaceutical industries for the discovery of new molecules with antibacterial activity [2]. In the present scenario, an emerging major clinical and public health issue across the globe is the development of bacterial resistance to antibiotics. Indiscriminate use of antibiotics, lack of new strategies against antibacterial resistance development has mainly lead to the development of bacterial resistance to antibiotics. Microorganisms acquire the ability to produce resistance against antibacterial agents through different modifications either by inactivating them or by causing a decrease in their therapeutic efficacy. Antibiotic resistance is developed by decreased influx and increased efflux of antibiotic by microorganisms, alteration in binding target sites, production of some enzymes etc. These resistances can appear spontaneously in microorganisms through genetic modifications over a period of time. Such modifications are favored by the inappropriate use and abuse of antibiotics [2]. In the present situation, the development of new antibacterial agents is required to overcome antimicrobial resistance. Bilirubin belongs to the superfamily of tetrapyrrolic compounds, which is one of the most highly conserved groups of molecules. Bilirubin, due to its strong antioxidant, immunomodulatory and anti-inflammatory responses has gained wide attention in biomedical research [3]. Bilirubin exhibited protective effects in rat model of endotoxemia [4]. Bilirubin also showed antiviral activity when added to infected cell cultures [5]. Bilirubin levels are also elevated during conditions of sepsis, intra-abdominal abscesses from urological, gynecological or gastroenterological origins, and antiviral therapy [6]. Bilirubin has been suggested to be an effective antibacterial, but little data is available. In the previous studies, the antibacterial effects of bilirubin on gram-negative bacterial agents have been evaluated [7]. However, bilirubin did not exhibit *in vitro* antibacterial effect. The present study was proposed to synthesize bilirubin nanoparticles, as

the nanoformulations have the advantage of requiring low dosage or drug usage, nano particle size which enables better penetration of drug, increased drug solubility, sustained release property and decreased degradation of drug. On the contrary, a bulk or dissolved form of drug have large particle size, requires higher concentration to produce desired effect and increases the risk of toxicity. Therefore, to exploit the full potential of bilirubin, the present study was proposed to evaluate the antibacterial potential of bilirubin nanoparticles and its comparison with its bulk form, as such work has not been reported previously.

2. Materials and methods

2.1. Preparation of bilirubin loaded pluronic F-127 (PF-127) nanoparticles

Bilirubin (mixed isomers) and pluronic F-127 (PF-127) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Bilirubin and eudragit were dissolved in dimethyl sulfoxide (DMSO) and were stirred for 20 min on a magnetic stirrer. Thereafter, 1% Pluronic F-127 (1% PF-127) was prepared by dissolving pluronic F-127 in distilled water. After that, using a 25-gauge needle, bilirubin solution was then added dropwise into PF-127 (1 %) aqueous solution and stirred for 2 h on magnetic stirrer at 1000 rpm for antisolvent precipitation and encapsulation. The suspension was further sonicated for 5 min at 4 °C. Three different concentrations of bilirubin nanoparticles i.e. 0.03 % bilirubin nanoparticles [BNP (0.03 %)], 0.1 % bilirubin nanoparticles [BNP (0.1 %)] and 0.3 % bilirubin nanoparticles [BNP (0.3 %)] were prepared using this method. The ratio of bilirubin to eudragit was kept at 1.0 : 0.5 for the synthesis of bilirubin nanoparticles.

2.2. Characterization of nanoparticles

2.2.1. Hydrodynamic diameter and polydispersity index (PDI) of bilirubin nanoparticles. Hydrodynamic diameter and polydispersity index (PDI) of the nanoparticles were determined by Zetasizer (Malvern, UK), as per the instructions of the instrument.

2.2.2. Zeta potential measurement of bilirubin nanoparticles. Zeta potential of the nanoparticles was determined by Zetasizer (Malvern, UK), as per the instructions of the instrument. Zeta potential measurement was carried out to determine the surface charge of the nanoparticles. For this the bilirubin nanosuspension was injected into the zeta cell with the help of 1mL disposable syringe and then the syringe plunger was gently pressed to fill the zeta cell with the nanosuspension, thereafter the zeta cell was placed in the instrument to determine the zeta potential of the bilirubin nanoparticles.

2.2.3. Transmission electron microscopy (TEM). Size and morphology of bilirubin nanoparticles were determined by transmission electron microscopy studies. TEM grid was used for the study on which the dispersed nanoparticle suspension was placed and then it was transferred to the microscope for imaging.

2.3. In vitro evaluation of antibacterial activity of bilirubin nanoparticles

Antibacterial activity of bilirubin nanoparticles at different concentrations (0.03, 0.1 and 0.3 %) were evaluated against the gram-positive i.e. *Staphylococcus aureus* (*S. aureus*) (ATCC 12600) and gram-negative i.e. *Escherichia coli* (*E. coli*) (ATCC 10536) bacterial strains. These bacterial strains were gifted by the Department of Veterinary Microbiology, COVS, LUVAS, Hisar, Haryana, India. The PF-127, blank NP, 0.3 % bulk bilirubin were also evaluated along with bilirubin nanoparticles for antibacterial activity. Standard antibacterial i.e. tetracycline (Cat. no. MB178, HiMedia) (at 62.5, 125, 250 μg/ml) and enrofloxacin (Cat. no. CMS8318, HiMedia) (at 31.25, 62.5, 125 μg/ml) were used as positive control to compare the efficacy of the nanoparticles. The antibacterial activity was determined on Muller–Hinton agar by the agar well diffusion method [8]. The autoclaved Muller–Hinton agar was poured (12 ml) in petri plates and smeared with overnight cultures of different bacterial strains (10^8 cfu/ml). The agar plates were allowed to solidify and, wells of 6 mm diameter were made in each plate with the help of sterile gel puncher. Thirty microlitres of bilirubin nanoparticles of different concentrations, other preparations (i.e. pluronic F-127, blank nanoparticles and 0.3 % bulk bilirubin) and different dilutions of standard antibiotic (tetracycline and enrofloxacin) were poured into separate wells using a micropipette. After overnight incubation at 37 ∘C, the different diameters of zone of inhibition were measured to quantify the antibacterial activity. The same experiment for all was conducted in triplicate.

2.4. Statistical analysis

The values are expressed as mean $\pm$ SEM with n equal to the number of replicates and calculated using GraphPad Prism v8.0.2 software program (San Diego, CA, USA).

3. Results

3.1. Characterization of synthesized bilirubin nanoparticles

The hydrodynamic diameter of the blank PF-127 nanoparticles was 87.41 ± 4.98 nm and that of bilirubin loaded nanoparticles in three different concentrations i.e. 0.03, 0.1 and 0.3 % was 119.83 ± 7.28, 121.03 ± 6.21 and 139.03 ± 6.21 nm, respectively (Fig. 1A).
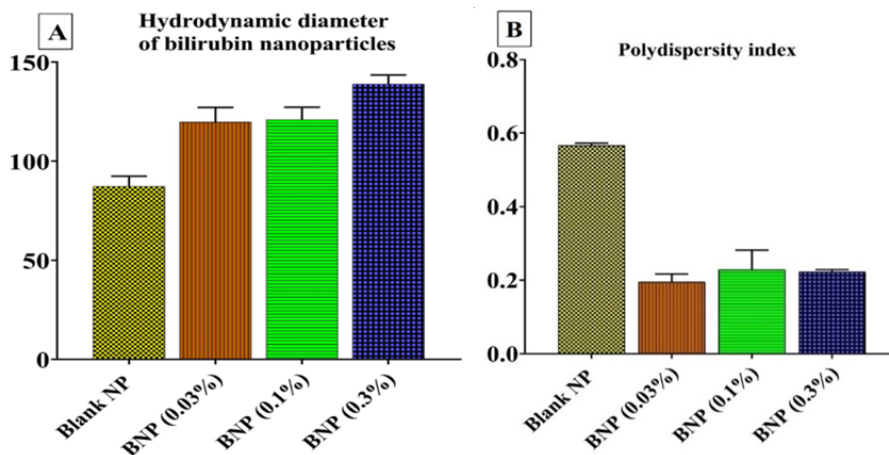
FIG. 1. (A) Hydrodynamic diameter and (B) Polydispersity index (PDI) of blank nanoparticles, and bilirubin nanoparticles (BNP) of different concentrations (0.03, 0.1, 0.3 %) ($n = 3$)

The PDI of blank PF-127 nanoparticles was 0.57 ± 0.01, that of the bilirubin nanoparticles of three different concentrations i.e. 0.03, 0.1 and 0.3 % was 0.19 ± 0.02, 0.23 ± 0.05 and 0.22 ± 0.01, respectively (Fig. 1B). The zeta potential of the three different concentrations of bilirubin nanoparticles i.e. 0.03, 0.1 and 0.3 % was -11.47 ± 0.17, -13.57 ± 0.20 and -15.43 ± 0.15 mV, respectively ($n = 3$) (Fig. 2).
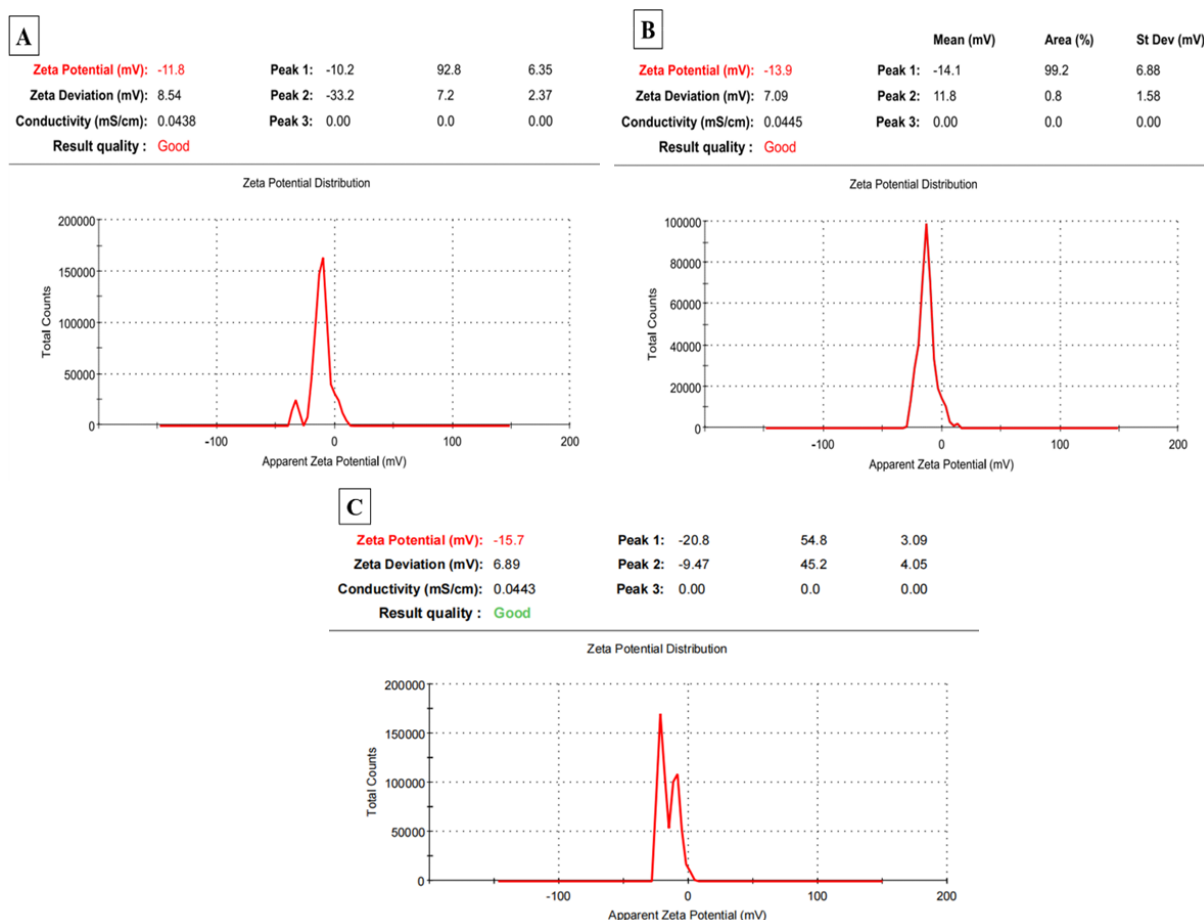

FIG. 2. Graphs showing the zeta potential of nanoparticles. (A) 0.03 % bilirubin nanoparticles, (B) 0.1 % bilirubin nanoparticles and (C) 0.3 % bilirubin nanoparticles

3.2. TEM of bilirubin nanoparticles

The representative TEM image of bilirubin nanoparticles is given in Fig. 3. The image revealed that the size of particles was in nano range. The TEM study revealed that the bilirubin encapsulated pluronic F127 nanoparticles have spherical morphology. There was no presence of agglomeration/aggregation of bilirubin nanoparticles and the size of the nanoparticles was found to be in agreement with that of the hydrodynamic diameter measured using zetasizer.
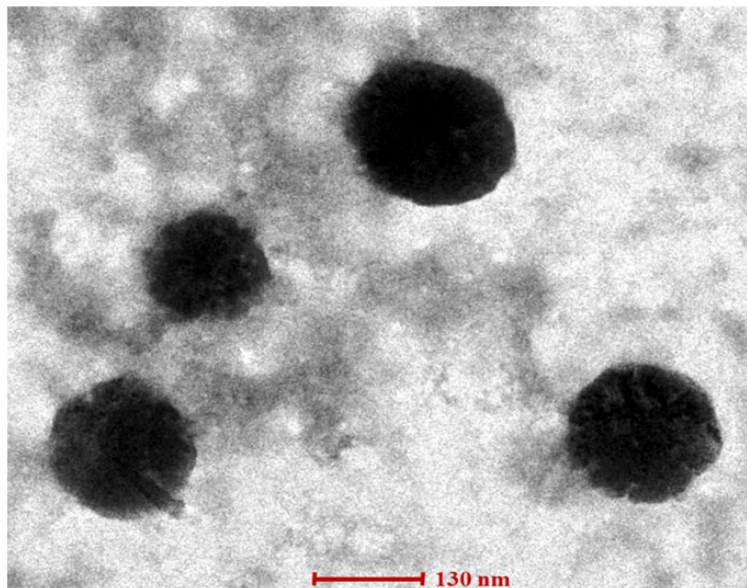

FIG. 3. TEM image showing the spherical shape of the synthesized bilirubin nanoparticles

3.3. Antibacterial effect of bilirubin nanoparticles

The representative images of zone of inhibition produced by different concentrations of antibiotics i.e. tetracycline and enrofloxacin, and different formulations i.e. pluronic F-127, blank nanoparticles, 0.3 % bulk bilirubin, bilirubin nanoparticles (0.03, 0.1 and 0.3 %) against *S. aureus* and *E. coli* is shown in Fig. 4(A). The value of zone of inhibition are presented in Fig. 4(B,C). The different concentrations of tetracycline i.e. 250, 125, 62.5 μg/ml exhibited zones of inhibition of 22.67 ± 0.33, 19.33 ± 0.67 and 17.33 ± 0.67 mm against *S. aureus*, and 19.00 ± 0.58, 16.33 ± 0.33 and 13.67 ± 0.33 mm against *E. coli*, respectively. In the case of enrofloxacin, the different concentrations i.e. 125, 62.5, 31.25 μg/ml exhibited zones of inhibition of 21.67 ± 0.88, 19.00 ± 0.58 and 17.00 ± 0.58 mm against *S. aureus*, and 19.00 ± 0.58, 18.00 ± 0.58 and 15.00 ± 0.58 mm against *E. coli*, respectively. Bilirubin nanoparticles and other formulations exhibited no zone of inhibition against these bacterial strains.

4. Discussion

Nanomaterials have been identified as an emerging area of research that are highly in demand for multiple practical applications. Nanoparticles provide an efficient carrier for the delivery of drugs and have a particle size ranging from 1 to 1000 nm. Polymers such as pluronic F-127 have been commonly used for encapsulation of active compounds by entrapping within the polymeric core. In our previously published work, we synthesized bilirubin nanoparticles by our novel method and the bilirubin nanoparticles were in nano range and had spherical morphology which was observed in TEM study and had negative zeta potential. The Fourier transform infrared microscopy (FTIR) of bilirubin nanoparticles showed that there was no intermolecular interaction between the bilirubin and the pluronic F127 and the entrapment of bilirubin by pluronic F127 was estimated by determining encapsulation efficiency [9]. In view of our previous work, the present study was proposed and the bilirubin nanoparticles were synthesized and characterized revealing spherical morphology of the bilirubin nanoparticles as observed in TEM study, also the hydrodynamic diameter of the bilirubin nanoparticles (120 – 140 nm) estimated by the zetasizer was in accordance with that of the TEM study and the zeta potential of the bilirubin nanoparticles was in the range of -11 to -16 mV which is in agreement with the literature value [10], considering the three different concentration of bilirubin nanoparticles.

In the *in vitro* antibacterial study, bilirubin nanoparticles (0.03, 0.1 and 0.3 %), bulk bilirubin and other prepared formulations of present study did not show antibacterial effect against *S. aureus* and *E. coli* at the tested concentrations. There is no report of evaluation of antibacterial effect of bilirubin against *S. aureus*, which has been recognized as one of the most important bacteria that cause diseases in humans, which has been tested in the present study. The earlier study has also reported that bilirubin had no antibacterial effect on *E. coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*
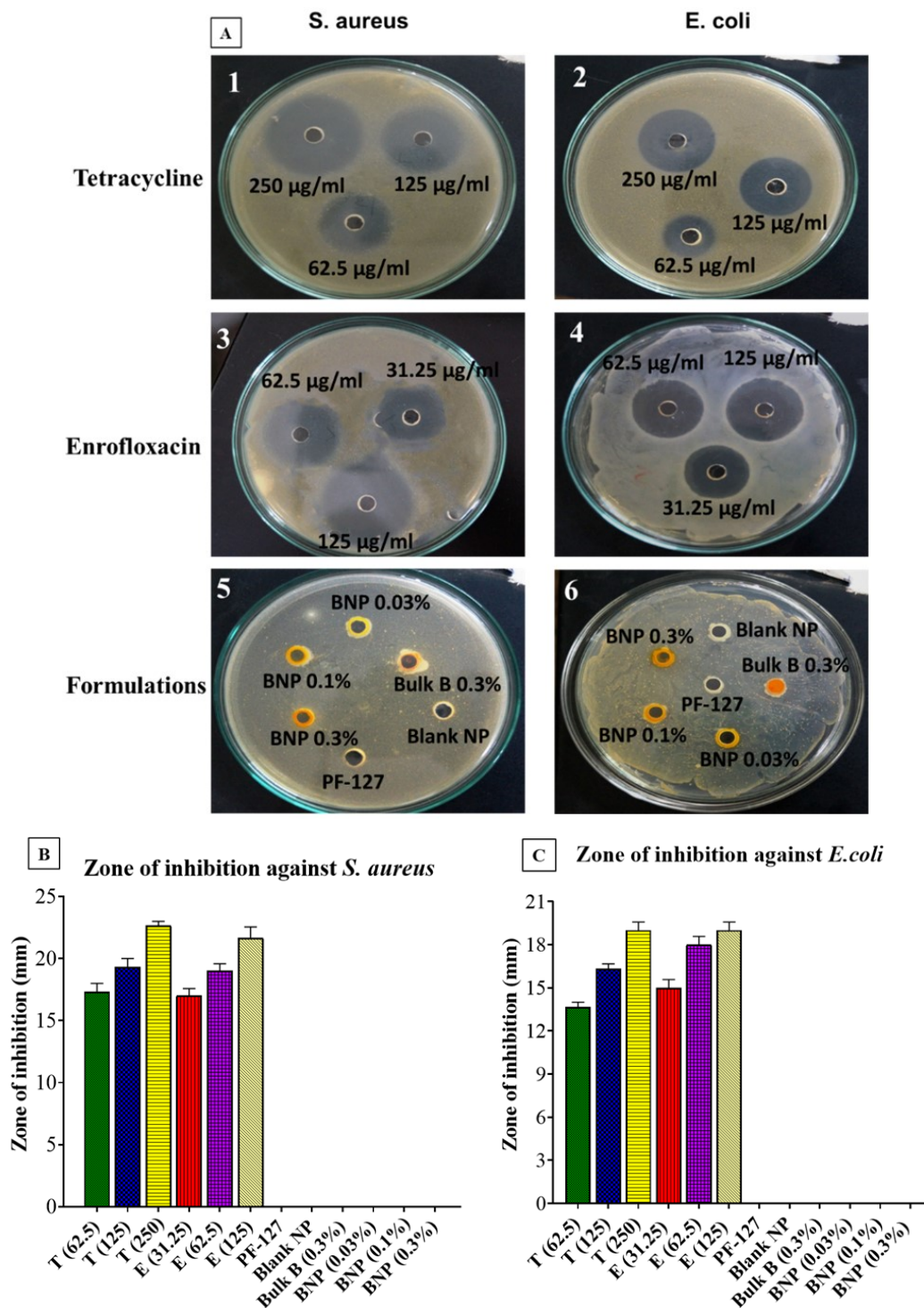
FIG. 4. (A) Images showing zone of inhibitions by tetracycline, enrofloxacin and different formulations against *S. aureus* and *E. coli*. (B–C) Zone of inhibitions of tetracycline (T; 62.5, 125 and 250 μg/ml), enrofloxacin (E; 31.25, 62.5 and 125 μg/ml) and different formulation against (B) *S. aureus* and (C) *E. coli*. Data are expressed as mean $\pm$ SEM ($n = 3$). PF-127: (Pluronic F-127); Blank NP: (Blank nanoparticles); Bulk B 0.3 %: (0.3 % bulk bilirubin); BNP 0.03 %: (0.03 % bilirubin nanoparticles); BNP 0.1 %: (0.1 % bilirubin nanoparticles) and BNP 0.3 %: (0.3 % bilirubin nanoparticles)

and *Pseudomonas aeruginosa* [7]. In contrary to our *in vitro* antibacterial study, *in vivo* study has revealed that mice injected with *E. coli* endotoxin were protected from mortality and liver dysfunction by a single intravenous injection of bilirubin [4]. Further, it has been reported that mouse mortality caused by endotoxins is reduced in the presence of increased bilirubin levels [11]. Protective action of bilirubin against endotoxins is due to its potential of attenuating nitric oxide synthase-2 (NOS2) induction secondary to inhibition of NADPH oxidase activity, which is involved in the reduction of oxygen to form superoxide radicals [11]. These studies suggested that bilirubin might have no direct effect on endotoxins or bacteria, but attenuates the damage caused by radicals formed during bacterial infection as a result of its antioxidant potential. This might be the reason for the failure of bilirubin nanoparticles to show antibacterial activity during their *in vitro* evaluations.

In a recent study, antibacterial effect of bilirubin was evaluated against *Streptococcus agalactiae*, which is involved in early-onset neonatal sepsis [12]. The study involved a transcriptomic and proteomic assessment of *Streptococcus agalactiae* cultured in the presence/absence of bilirubin. The transcriptome analysis revealed that 19 genes were expressed/upregulated in presence of bilirubin and in case of proteomic analysis, 12 different proteins were expressed of which half were over-expressed due to the bilirubin. The expressed genes and proteins in the bacterial cells were involved in transport mechanism of sugar or toxic substance. This indicated that in presence of bilirubin, bacterial cells enhance their transport mechanism in order to eliminate the bilirubin out of the cell or increase the uptake of sugar in presence of bilirubin to compensate increased metabolic rate. In presence of bilirubin the bacterial cell showed downregulation of certain genes which are responsible for the production of N-acetyl neuramic acid synthetase NeuB, transketolase, and ornithine carbamoyltransferase leading to functional alteration of bacterial cell.

These reports indicate that the antibacterial mechanism of bilirubin can be indirect i.e. due to its antioxidant potential and ability to upregulate or downregulate certain bacterial genes and expression of bacterial proteins. Although, bilirubin having such potential, it is able to inhibit bacterial growth or exert bacteriostatic effect to a certain limit.

However, the antibacterial effect of bilirubin was evaluated in a liquid model which allowed interaction of bacteria and the bilirubin in free suspension, partially representing an *in vivo* environment. In the present study, *in vitro* antibacterial study was carried out using agar well diffusion assay. The failure of nanoparticles to diffuse through the agar might be another reason for no antibacterial activity of bilirubin nanoparticles. So, testing these nanoparticles for antibacterial activity in the broth might be a more effective approach providing better interaction between the bilirubin nanoparticles and the microbes in a suspension and their testing can also be attempted at concentrations higher than 0.3 % against more bacterial strains.

5. Conclusion

On the basis of findings of this study, it might be concluded that the synthesized spherical bilirubin nanoparticles showed no antibacterial activity against *S. aureus* and *E. coli* by the agar well diffusion method. The testing of these nanoparticles for antibacterial activity in the broth might be a more effective approach. Further, their testing for antibacterial activity at higher concentrations and against other microorganisms may be explored in the future. However, in view of searching for a novel formulation having antibacterial activity, we synthesized bilirubin nanoparticles and investigated its antibacterial effect against *S. aureus* and *E. coli*, in order to understand the effect of bilirubin nanoparticles compared to its bulk form, a novel approach which is not yet adopted and reported.

Authors' contributions

D.J.K., V.K.*, V.K. and R.C. designed the experiment. D.J.K. and V.G.J. did the synthesis and characterization studies of nanoparticles. D.J.K. and V.K.* did the *in vitro* experimentation. All authors analysed the data and contributed in writing the manuscript. V.K.* supervised the whole study.

References

[1] World Health Organization. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. No. WHO/EMP/IAU/2017.11, World Health Organization, 2017.

[2] Aslam B., Wang W., et al. Antibiotic resistance: a rundown of a global crisis. *Infect. Drug Resist.*, 2018, **11**, P. 1645–1658.

[3] Lee Y., Kim H., et al. Bilirubin nanoparticles as a nanomedicine for anti?inflammation therapy. *Angew. Chem. Int. Ed.*, 2016, **55** (26), P. 7460–7463.

[4] Wang W. W., Smith D.L., Zucker, S. D. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. *Hepatology*, 2004, **40** (2), P. 424–433.

[5] Santangelo R., Mancuso C., et al. Bilirubin: An Endogenous Molecule with Antiviral Activity in vitro. *Front. Pharmacol.*, 2012, **3**, 36.

[6] Buyukasik Y., Akman U., et al. Evidence for higher red blood cell mass in persons with unconjugated hyperbilirubinemia and Gilbert's syndrome. *Am. J. Med. Sci.*, 2008, **335**, P. 115–119.

[7] Terzi H.A., Kardes H., et al. The antibacterial effects of bilirubin on gram-negative bacterial agents of sepsis. *Biomed. Res.*, 2016, **27**, P. 207–209.

[8] Perez C., Pauli M., Bazerque P. An antibiotic assay by agar-well diffusion method. *Acta Biol. Med. Exp.*, 1990, **15**, P. 113–115.

[9] Kamothi D.J., Kant V., et al. Novel preparation of bilirubin-encapsulated pluronic F-127 nanoparticles as a potential biomaterial for wound healing. *Eur. J. Pharmacol.*, 2022, **919**, 174809.

[10] Shaarani S., Hamid S.S., Mohd Kaus N.H. The Influence of Pluronic F68 and F127 Nanocarrier on Physicochemical Properties, *In vitro* Release, and Antiproliferative Activity of Thymoquinone Drug. *Pharmacogn. Res.*, 2017, **9** (1), P. 12–20.

[11] Lanone S., Bloc S., et al. Bilirubin decreases nos2 expression via inhibition of NAD (P) H oxidase: implications for protection against endotoxic shock in rats. *Faseb J.*, 2005, **19** (13), P. 1890–1892.

[12] Hansen R., Gibson S., et al. Adaptive response of neonatal sepsis-derived Group B Streptococcus to bilirubin. *Sci. Rep.*, 2018, **8** (1), 6470.

Information about the authors:

Dhavak J. Kamothi – Department of Veterinary Pharmacology & Toxicology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India; ORCID 0000-0003-2072-2855; kamothidhaval@gmail.com

Vinay Kant – Department of Veterinary Pharmacology & Toxicology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India; ORCID 0000-0001-9600-4417; drvinaykantluvas@gmail.com

Vinod Kumar – Department of Veterinary Pharmacology & Toxicology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India; drvinodk10@gmail.com

Vinay G. Joshi – Department of Animal Biotechnology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India; ORCID 0000-0001-8872-1838; vinaygjoshi18@gmail.com

Rajesh Chhabra – College Central Laboratory, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India; rchhabra59@rediffmail.com

Conflict of interest: the authors declare there is no any potential conflict of interest related to this article.