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Florfenicol-loaded tannic acid-chitosan iron oxide nanoparticles for enhanced efficacy against *Salmonella* Typhimurium

Naglaa A. Ali[1], Sahar A. Abdel Aziz[2*], Ahmed A. Farghali[3], Ahlam G. Khalifa[4]

[1]*Department of Biotechnology, Faculty of Postgraduate Studies for Advanced Science (PSAS), Beni-Suef University, 62511, Beni-Suef, Egypt.*
[2]*Department of Hygiene, Zoonoses and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, 62511 Beni-Suef, Egypt.*
[3]*Department of Materials Science and Nanotechnology, Faculty of Postgraduate Studies for Advanced Science (PSAS), Beni-Suef University, 62511, Beni-Suef, Egypt.*
[4]*Department of Toxicology and Forensic Medicine, Faculty of Veterinary Medicine, Beni-Suef University, 62511 Beni-Suef, Egypt.*

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***Correspondence:**

Corresponding author: Sahar A. Abdel Aziz
E-mail address: abdelaziz.sahar@yahoo.com

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ABSTRACT

Antimicrobial drug resistance caused by *Salmonella* Typhimurium (*S.* Typhimurium) is a noteworthy issue causing morbidity and mortality linked to potential outbreaks throughout the world. The current study aimed to synthesis chitosan, iron oxide nanoparticles (Ch-Fe_3O_4 NPs), tannic acid-Ch-Fe_3O_4 (T-Ch-Fe_3O_4) NPs for the loading of florfenicol, compare the antibacterial efficiency of the free florfenicol and the loaded one against florfenicol-resistant strains of *S.* Typhimurium. Also, the in-vitro bio-toxicity and viability effect of those materials were assessed. It was shown that florfenicol was successfully loaded onto the surface of tannic acid-Ch-Fe_3O_4 NPs. This was determined through measurement of hydrodynamic sizes and zeta potential (+0.120±0.02, -0.372±0.072 and +0.07±0.03 mV for Ch-Fe_3O_4 NPs, T-Ch-Fe_3O_4 NPs, and Florfenicol NPs respectively) and confirmed by FT-IR analysis. Concerning the antibacterial effect, loading florfenicol with T-Ch-Fe_3O_4 NPs resulted in superior antibacterial efficacy against the resistant *S.* Typhimurium strain. This was evident by a decrease in the minimum inhibitory concentration (MIC) from 31.25 µg/ml for florfenicol to 15.62 µg/ml for the loaded florfenicol NPs. This finding suggests that florfenicol NPs possess enhanced antibacterial efficacy compared to the free drug, highlighting the potential of nanotechnology in addressing the challenge of antibiotic resistance.

Introduction

Salmonella infections are a pressing concern for public health and a significant contributor to food-borne illnesses on a global scale. The rise of multi-drug resistant strains of *Salmonella* poses a growing challenge in both human and veterinary medicine. The misuse of antibiotics has fueled the development of antimicrobial resistance in pathogens found in food, necessitating the exploration of alternative treatment methods for *Salmonella* infections in animals raised for food production. Additionally, it is vital to recognize that internal organs of these animals represent a crucial source of *S.* Typhimurium infections in humans, as food animals serve as the primary reservoir for *S. enterica*. Consequently, by preventing colonization in food animals, we can also reduce the occurrence of *Salmonella* infections in humans (Lee and Yoon, 2021).

Antibiotic medications pollute the surroundings where humans, animals, and crops interact. Residues of these medications administered to animals for medical purposes can be detected in various water sources such as surface water, groundwater, and drinking water. Additionally, traces of these drugs can be present in pet waste, livestock manure, and municipal biosolids. The application of antibiotic drugs to safeguard pets and livestock from illnesses plays a role in the existence of drug residues in pet fur and animal skins. Consequently, residues of antibiotic drugs may be present in both processed and unprocessed animal-derived food products (Arsène *et al*., 2022).

A broad-spectrum synthetic amphenicol certified for use in veterinary medicine, florfenicol is one of the antibiotics used to treat animals. It acts well against both Gram-positive and Gram-negative bacteria. Treating intestinal infections is one of its common uses. The issue of antimicrobial medication residues in food products derived from animals has garnered a lot of attention lately. There have been suggestions that antimicrobial drug residues in animal-based food could be harmful to human health (Tikhomirov *et al*., 2021). Furthermore, the European Union has set maximum residue limits for veterinary drugs used in food-producing animals to protect consumer health and confidence in food products. Veterinary drugs must meet quality, safety, and efficacy standards through scientific evaluation in the European Union. Compliance with these regulations has encouraged the development of new drug formulations and administration methods to reduce residue levels in animal food products (Bacanl, 2024).

In recent research, iron oxide nanoparticles (Fe_3O_4 NPs) have emerged as a promising antimicrobial approach to combat *S.* Typhimurium infections. Iron is vital for bacterial growth, and studies suggest that increasing iron levels in animals can heighten their susceptibility to *S.* Typhimurium infection. Fe_3O_4 NPs work by sequestering iron from pathogens and host tissues, leading to iron deprivation and inhibition of bacterial growth. Additionally, these NPs have intrinsic antimicrobial properties by generating reactive oxygen species. Thus, Fe_3O_4 NPs offer a dual mechanism for combating *S.* Typhimurium infections: iron sequestration and ROS generation (Mulens-Arias *et al*., 2020).

Chitosan, being a polycationic compound, possesses a notable attraction towards the negatively charged bacterial cell wall. Studies conducted previously have suggested that the interplay between the cationic groups of chitosan and the anionic components like teichoic acids and phosphate groups present on the bacterial cell wall can result in disruption (Sahariah *et al*., 2023). Tannic acid, a versatile polyphenol, interacts with cell surface proteins through hydrogen bonding. This interaction induces cross-linking and precipitation of proteins, impacting the cellular environment in several ways. Tannic acid can hinder cell wall synthesis, disrupt cell membrane integrity, and promote precipitation of intracellular proteins. Beyond its protein-modifying effects, tannic acid acts as a potent antioxidant, safeguarding delicate drugs from oxidative degradation and preserving their efficacy (Xiong *et al*., 2023).

The objective of the current study was to prepare tannic acid-chitosan coated iron oxide nanoparticles, loading Florfenicol on theses NPs, characterization by zeta potential, FT-IR, and TEM as well as the *in vitro* determination of MICs and MBCs against florfenicol resistant strains of *S.* Typhimurium. Also, the *in vitro* bio-toxicity and viability effect of those materials were assessed. It is expected that the nanoparticles would have

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better antibacterial activity than free drug due to slow and sustained release of drug.

Materials and methods

Chemicals

All chemicals used in the study are lab grade purchased from Sigma, Aldrich company while florfenicol powder was supplied by Pharma Sweed, Egypt.

Coating of Fe_3O_4 nanoparticles with chitosan

Initially, 10 mg of Fe_3O_4 NPs was dispersed in deionized water then sonicated. Also, 16 mg of chitosan was dissolved in a mixture of water/acetic acid at 8:2 ratios at 50°C, separately. Furthermore, the solutions were mixed together and stirred at a speed of 800 rpm at room temperature for 11 h. Finally, the formed Ch-Fe_3O_4 NPs was separated by centrifugation at a speed of 12000 rpm and dried at room temperature (Shagholani *et al*., 2015).

Modification of Ch-Fe_3O_4 NPs with tannic acid

Tannic acid (0.8 g) was dissolved in 10 ml deionized water. Then, 0.12 g of Ch-Fe_3O_4 NPs dispersed homogeneously in 30 ml of deionized water by sonication. The prepared solutions were mixed together, stirred at a speed of 420 rpm at room temperature and dried (Shagholani and Ghoreishi, 2017).

Synthesis and loading of new nano-formulated florfenicol

T-Ch-Fe_3O_4 NPs (7 mg) was added to the florfenicol solution containing 4 mg florfenicol. The mixture was sonicated for 3 min and remained at room temperature for 24 h. During this time, florfenicol can diffuse and can be trapped in branch-like structure of tannic acid in T-Ch-Fe_3O_4 NPs. Then, florfenicol NPs were separated from solution and washed with water for removing the unloaded florfenicol. The supernatant was stored to determine encapsulation efficiency (EE).

Characterization of nanoparticles (Ch-Fe_3O_4 NPs, T-Ch-Fe_3O_4 NPs, and florfenicol NPs)

Dynamic light scattering was used for measurement the hydrodynamic diameter of the Ch-Fe_3O_4, T-Ch-Fe_3O_4 and florfenicol NPs also, zeta potential by a Malvern instrument (Malvern Instruments Ltd). Also, Fourier transform infrared spectra were examined by Fourier Transform infrared spectroscopy (FT-IR, Bruker Vertex 70).

Evaluation of antibacterial efficiency of the tested nanoparticles

S. Typhimurium strains

A total number of the reference strains of *S.* Typhimurium ATCC14028 (n=12) used in this study were obtained from Biotechnology Unit in the Animal Health Research Institute, Egypt, wherever the standard protocols for the isolation and identification of *Salmonella* from their sources were done. The obtained isolates were extremely resistant to various categories of antibiotics used in human and animal field especially florfenicol. Isolates were kept at 4°C while transferred on Columbia slants to Hygiene and Zoonoses Lab., Faculty of Veterinary Medicine, Beni-Suef University, Egypt, where the experiment protocol was initiated.

Inoculum preparation

The inoculum of the tested isolates was picked from the slants and grown on nutrient agar at 37°C for 20-24 h. After that, to reach the final concentration of approximately 10^8 CFU/mL, a loopful of the cultures was transferred into sterile saline and the turbidity was measured at 600 nm according to 0.5 McFarland scale tube.

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

Minimum inhibitory concentrations of tested compounds, Ch-Fe_3O_4, T-Ch-Fe_3O_4, florfenicol and florfenicol NPs were determined using the standard macro-broth two-fold serial dilution technique following the standard procedures of CLSI (2015). In this test, 100 µL of the inoculum obtained from the above step were added to one ml of the Muller Hilton broth containing different concentrations of the tested chemicals which first dispersed in 2 mL propylene glycol (IUPAC name: propane-1,2-diol) and 8 ml of sterilized distilled water to reach concentrations of 1000 µg/mL for each concentration, triplicate tests were performed. The average was taken as the final reading compared to negative control (propylene glycol) and positive controls (oxtetracycline, 30µg, Oxoid). Microbial growth was read as turbidity or as a deposit of cells at the bottom of the tube. The concentration of the tested chemical or drug that prevented the growth of bacteria was recognized as MICs while the lowest concentration that destroyed all bacterial cells was considered as MBCs (Khatami and Pourseyedi, 2015).

Cytotoxicity assessment using viability test

Vero cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well in 100 µL of growth medium. After 24 hours of seeding, fresh medium with various concentrations of the test sample was added. Sequential two- fold dilutions of the tried substance compound were added to blended cell monolayers administered into 96-well, flat-bottomed microliter plates (Falcon, NJ, USA) by a multichannel pipette. The microliter plates were brooded in a humidified incubator at 37ºC with 5.0% CO_2 for a time of 24 h. For every concentration of the tested compound, we used three wells. Control cells were seeded without the tested material and with or without dimethyl sulfoxide (DMSO). The little level of DMSO present in the wells (maximal 0.1%) was viewed as not to influence the analysis. After the cell's incubation, viable cells yield was calculated by MTT test (used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity). The media was discarded and replaced with 100 µL of phenol-free RPMI 1640 fresh medium, then the wells including the untreated ones were received 10 µL of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS). Then incubated for 4h at 37°C and 5.0% CO_2. After removing an 85µL aliquot of the media from each well, 50 µL of DMSO was added to each well, carefully mixed with the pipette, and then incubated for 10 min at 37°C. The number of viable cells was then determined by measuring the optical density at 590 nm using a micro-plate reader (SunRise, TECAN, Inc., USA), and the viability % was calculated as [(ODt/ODc)]x100%. The Graph Pad prism software (San Diego, California, USA) was used to determine the half-maximal inhibitory concentration (IC50) (Mosmann 1983, Gomha, Riyadh *et al*. 2015).

Results

Characterization of Ch-Fe_3O_4, T-Ch-Fe_3O_4 and florfenicol NPs

Droplet size measurements

The average particle size of Ch-Fe_3O_4 (measured by DLS) was 117.1±10.0 nm , while that of T-Ch-Fe_3O_4 was 190.0±8.0 nm and florfeni-

col NPs was 224.7±19.0 nm.

Zeta potential of the NPs

Ch-Fe_3O_4 obtained a negative zeta potential value (+ 0.120±.02 mV), T-Ch-Fe_3O_4 (-0.372±.072 mV), and florfenicol NPs (+0.07±0.03mV). Fourier transform infrared spectra

Florfenicol was showed peaks at 1532 cm^{-1} (attributable to C–N stretching and N–H bending vibrations), 3452 cm^{-1} (assigned to -OH) and 1276 cm^{-1} (-NH streching and -CN bending). In T-Ch-Fe_3O_4 NPs spectra (Fig. 1), a broad absorption band between 3759 and 2927 cm^{-1} was showed the presence of numerous hydrox shown inups. Compared with blank T-Ch-Fe_3O_4 NPs and florfenicol, florfenicol NPs, exhibited two new absorption peaks at 1031 and 1606 cm^{-1} (Fig. 1).

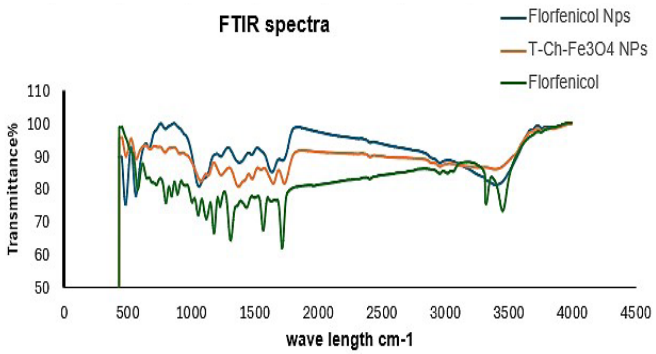

Fig. 1. Fourier transform infrared spectra: (a) T- Ch-Fe_3O_4 NPs, (b) florfenicol, and (c) florfenicol NPs.

Determination of florfenicol loading and encapsulation efficiency

Florfenicol encapsulation efficiency (EE) was found to be 91.091% in T-Ch-Fe_3O_4 NPs. Also, EE is expressed as the percent of the total added florfenicol that is encapsulated in T-Ch-Fe_3O_4 NPs. Moreover, the EE (%) of T-Ch-Fe_3O_4 NPs= 100 x [(total amount of florfenicol added−unbound florfenicol) / total amount of florfenicol], that appeared as EE (%) = 100 x [(348mg–31mg) /348mg] was 91.091%. Florfenicol loading efficiency was found to be 45.54% as the Florfenicol loading is defined as the amount of florfenicol encapsulated in 100 mg of Nps. And the payload (%) = 100x ((total amount of Florfenicol added – unbound Florfenicol) / total amount of nanoparticles (mg)), so, the payload (%) of T- CS-Fe_3O_4 NPs = 100 x [(348mg–31mg) /696mg] was 45.54%

Antibacterial efficiency of loaded form of florfenicol

Relating to the evaluation of antimicrobial efficacy of different loaded forms of Ch-Fe_3O_4, T-Ch-Fe_3O_4, florfenicol and florfenicol NPs using MICs and MBCs. MICs of against *S.* Typhimurium isolates that refers to the lowest concentration of tested chemical and/or NPs that inhibited their growth were, 62.25, 62.25, 31.25 and 15.62 µg/ml, respectively. While MBCs that means the lowest concentration of tested chemicals or nanoparticles NPs that had bactericidal effect against the bacterial isolates were 125.0, 125.0, 62.5 and 31.25 µg/ml, respectively.

Assessment of the Viability

Cytoskeleton changes

Our finding showed that the control cell line was adjacent to a complete healthy layer of Vero cells (Fig. 2a, black arrows). In contrast, the cells exposed to Ch-Fe_3O_4, T- Ch-Fe_3O_4, florfenicol NPs and florfenicol become small, appeared shrunk, and distorted as shown in Fig. 2b, c, d, and e, respectively. Also, there was a reduction in the number with detachment of the cellular sheet.

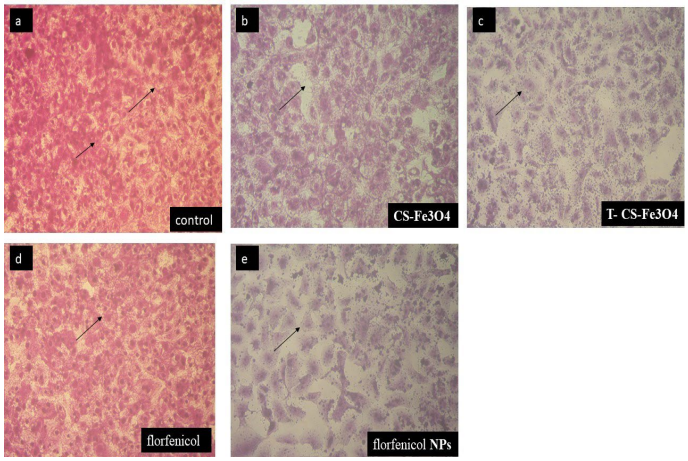

Fig. 2. Optical microscopy images: (a) control group, (b-c-d) and (e) the cytotoxic effect of Ch-Fe_3O_4 ,T-Ch-Fe_3O_4 , florfenicol and florfenicol NPs treated Vero cells at a concentration of 100 mg/mL after 24h of exposure

Cell viability and IC50

Regarding cell viability, it appeared that a concentration of 100 mg/ml Ch-Fe_3O_4 and T-Ch-Fe_3O_4, the viability was 9.81±1.33% and 27.14±1.82%, respectively after 24h of exposure as shown in Table 1.

Table 1. Cytotoxic effects of CS-Fe_3O_4 , T- CS-Fe_3O_4, florfenicol, and florfenicol NPs on Vero cell lines at different concentrations

	Cell viability (%)			
Conc. (µg/mL)	Ch-Fe_3O_4	T-Ch-Fe_3O_4	Florfenicol	Florfenicol NPs
500	9.81±1.33	27.14±1.82	12.95±1.31	6.73±0.65
250	26.05±2.17	65.21±1.75	34.74±0.82	20.68±1.42
125	42.73±1.95	90.68±0.46	78.41±1.93	39.57±2.09
62.5	79.52±2.06	98.07±0.19	92.34±0.62	71.48±1.76
31.25	94.78±0.84	100	99.56±0.40	91.72±0.84
15.6	99.31±0.53	100	100	98.14±0.63
0	100	100	100	100

Discussion

The hydrodynamic sizes of Ch-Fe_3O_4, T-Ch-Fe_3O_4 and florfenicol NPs were gradually increased, which could be interpreted as the successful loading of tannic acid and florfenicol on the surface of Ch-Fe_3O_4. Zeta potential analysis is a measure of the particle's stability in solution. Compared with the Ch-Fe_3O_4 NPs, it was observed that the presence of tannic acid onto the surface of Ch-Fe_3O_4, leading to the decreasing of zeta potential of T-Ch-Fe_3O_4 NPs into negative value -0.372 mV. This indicated that the surface of Ch-Fe_3O_4 NPs was successfully exchanged with the negatively charged tannic acid.

Concerning Fourier transform infrared spectra, it was showed the appearance of peaks at 1532cm^{-1} (attributable to C–N stretching and N–H bending vibrations), 3452 cm^{-1} (assigned to -OH) and 1276 cm^{-1} (-NH streching and -CN bending) could well define the FF (Fang, Li *et al.* 2020). In T-Ch-Fe_3O_4 NPs spectra (Fig. 1), a broad absorption band between 3759 and 2927 cm^{-1} was showed the presence of numerous hydrox shown inups. The peak in 1701 cm^{-1} was related to C=O stretching band of the carbonyl groups. Also, the peak in 1340 cm-1 was due to the vibration of O-H linkage of phenolic and hydroxyl groups (Božič *et al.*, 2012). This showed the adsorbance of tannic acid on the Ch-Fe_3O_4 NPs. Compared with blank T-Ch-Fe_3O_4 NPs and florfenicol, florfenicol NPs, exhibited two new absorption peaks at 1031 and 1606 cm^{-1}. This event demonstrated how successfully modifying of florfenicol on the surface of T- Ch-Fe_3O_4 NPs.

Florfenicol is a broad-spectrum antibiotic used for the treatment of

different diseases. However, the feature of this antibiotic possesses some disadvantages like bacterial resistance, toxicity, poor water solubility and low disposal halftime. From this point of view, the advancement of florfenicol novel preparations will have a promising value.

In our study, iron oxide, chitosan and tannic acid were selected to be incorporated within the drug nanocomposite as they were reported to be safer and possess potential antimicrobials and perfect for drug delivery with lower toxicity (Kim *et al*., 2016; Yiu *et al*., 2012; Youssef *et al*., 2019). The formed polycationic nanocomposite might have high affinity to interact with the negatively charged bacterial surface and the formed large surface area facilitated their tight absorption to the surface and subsequent release of florfenicol to the bacterial cytoplasm leading to disruption of the bacterial membrane and leakage of intracellular compounds and bacterial cell death (Marambio-Jones and Hoek, 2010; Yang *et al*., 2009).

The using of tannic acid which is a phenolic compound found in plants acts as a capping agent and stabilizer in the synthesis of nanoparticles field providing a biocompatible nanocomposite through its antioxidant and antimicrobial activities initiate better antibacterial activity in various applications (Kim *et al*., 2016). Furthermore, phytochemical tannic acid has been evaluated acting as an effective efflux pump inhibitor (EPI) in preventing bacterial resistance induction in prolonged exposure to a sub-lethal dose of the antibacterial agent (Myint *et al*., 2013). Also, Yang *et al*. (2009) denoted that chitosan is unique carrier for poorly absorbed and less soluble materials and the mixing of florfenicol chitosan nanoparticles and bacterial associated with contact of the hydrogen bonding of active sites of protein (COOH and NH2), resulted in destructing bacterial cell integrity subsequent release of florfenicol to the bacterial cytoplasm. From our observations it was denoted that the encapsulation of florfenicol with tannins, iron oxide and chitosan allowing the antimicrobial synergy of florfenicol.

The cells exposed to Ch-Fe_3O_4, T-Ch-Fe_3O_4, florfenicol NPs and florfenicol become small, appeared shrunk, and distorted as shown in Fig. 2b, c, d, and e, respectively. Also, there was a reduction in the number with detachment of the cellular sheet. This was due to the fact that the tested materials probably caused cytotoxicity by generating reactive oxygen species (ROS), which could cause DNA damage and release toxic byproducts that can harm nearby cells and tissues, as well as by disrupting the actin protein, which is an essential component of the cell's cytoskeleton and is crucial for determining cell morphology, division, motility, and tissue organization (Kura *et al*. 2014; Pisanic II *et al*. 2007). The decrease in the cell viability in Ch-Fe_3O_4 may be due to the positive charge (+0.120±.02) while that of T- Ch-Fe_3O_4 (-0.372±.072). The uptake of positively charged NPs is more apparent than negatively charged, resulting more toxic effect for cells (Huang *et al*., 2016). positive Poly (lactic-co-glycolic acid) NPs were more cytotoxic to mouse lymphoma cells than neutral or negative one (Carpentier *et al*., 2016). The calculated IC50 was 112.64 , 349.88 , 206.32 µg/ml and 104.57 µg/ml, respectively, due to their ability at high concentration to produce free radicals and inducing DNA damage and lipid oxidations (Abdel-Tawab *et al*., 2021) through interact with subcellular components including mitochondria, causing oxidative damage and compromising cellular activity in the culture (Martinsone and Baķe, 2010).

Conclusion

Preparation and synthesis of antibiotics loaded on nanoparticles to increase their efficacy and overcome microbial resistance has become nowadays the interest subject of numerous scientists. From our findings, it was denoted that the lowest concentration of T-CS-Fe_3O_4 NPs that inhibit the bacterial growth, was 15.62 mg/mL contains 45.54% of florfenicol. Accordingly, the corporation of both iron oxide, chitosan and tannic acid in the synthesis and loading of florfenicol nanoparticles is one of the promising means for overwhelming antibiotic resistant *S.* Typhimurium with a little toxicity to eukaryotic cells is a novel generation that have found inclusive applications in different fields of biomedicine with worthy biocompatibility *in vivo* and *in vitro* aspects.

Conflict of interest

The authors have no conflict of interest to declare.

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