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Original Article

Antibacterial Effects of Bioactive Boswellic Acids Loaded Chitosan Nanoparticles against Gram-Positive and Gram-Negative Bacteria

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ABSTRACT

Background: Boswellic acids are naturally occurring pentacyclic terpenoids that have revealed valuable anti-inflammatory, antiproliferation and anticancerous activities. Instead of these effects boswellic acids also possess antibacterial potential reported in the literature. However, these phytoconstituents associated with low aqueous solubility and bioavailability restrictions. The present study aimed to explore the antibacterial effects of boswellic acids by means of nano formulations. **Materials and Methods:** Chitosan was utilized as a natural biocompatible material for nanoparticles preparation, employing the ionic gelation technique as an effective approach. Well diffusion method was used to test antibacterial activity against four pathogenic bacteria (Gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis;* Gram-negative *Salmonella typhi*, and. *Escherichia coli*. Micro broth dilution technique was used to determine the minimum inhibitory concentration. **Results:** Boswellic acids-loaded nanoparticles displayed spherical particles with particle size 104.6 nm and 0.081 PDI value, respectively with smooth-surfaced spherical particles. Boswellic acids chitosan nanoparticles have a greater zone of inhibition against *Salmonella typhi* than *Boswellia serrata* extract with MIC values of 3.91 and 7.81 µg/mL, respectively. **Conclusion:** Poorly soluble boswellic acids were successfully encapsulated in chitosan nanoparticles and exhibited improved antibacterial activity compared to *Boswellia serrata* extract.

Keywords: Boswellic acids, Chitosan nanoparticles, Antibacterial activity.

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INTRODUCTION

Boswellic Acids (BAs), which are triterpenoidal pentacyclic moieties and belong to the family Burseraceae, are obtained from the gum resin of the plant. Acids known as 3-Acetyl-11-Keto-β-Boswellic Acid (AKBA), β-boswellic acid, 11-Keto-β-Boswellic Acid (KBA), and α-boswellic acid, are the primary components of *Boswellia* species. The Ayurvedic medicinal structure revealed that BAs possess important pharmacological actions specifically anti-inflammatory effects. BAs are also employed in the management of arthritic conditions,[1-11] ulcerative colitis,[12] Crohn's disease[13] and hyperlipidaemia control.[14] The promising results of *Boswellia serrata* Roxb. can be attributed to antioxidant, antiplatelet aggregation, immunomodulator effect, antibacterial and broad antiviral activity.[15,16] Many human and animal studies were made on BAs and attained data revealed the poor aqueous solubility restrictions. Systems for delivering Nanoparticles [NPs] have a variety of advantages. Primarily increased hydrophilicity of phytoconstituents, particularly drug targeting, which improves absorption, minimizes constraints on drug elimination and metabolism, increases bioavailability, $t_{1/2}$, reduces therapeutic dose, and concerns with drug toxicity. These nanocarrier systems preserve medicine particles from harmful effects of biological fluids eventually causing reduction in drug dosage, justifiable drug release, and more significant cellular uptaking by cells with substantial build-up inside the tissues.[17]

New antibacterial medications are needed due to bacterial resistance. Antibacterial therapies should specifically minimize pathogenic microorganisms without disrupting tissue cells or the environment's microflora. Herbal therapeutics is promising

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because nanotechnology-based delivery technologies may transform unstable, less soluble, and poorly absorbed compounds into active therapeutics. Thus, nanotechnology-based delivery methods may improve herbal efficacy and resolve therapeutic difficulties.[18,19]

Chitosan (Chi) is considered to be one of the most widely accepted biopolymers in the arena of NPs development due to its biocompatibility, nontoxic, biodegradability and cationic polyelectrolyte possessions. Besides, it endorses cross-linking with multivalent anions, like sodium tripolyphosphate (NaTPP) to design an efficient cross-linked network for entrapment of a variety of water-soluble and poorly soluble drug molecules.[20,21]

The literature revealed chitosan and its derivatives antibacterial potential against human infections and food-borne pathogens. Due to its antibacterial characteristics, chitosan is utilized in food preservation, dental, ophthalmic, wound-dressings, and other antibiotic products. Chitosan Nanoparticles (ChiNPs) are prepared through the use of the ionic gelation methodology, which utilizes patented -NH_3- interaction with tripolyphosphate multivalent anions. A number of researchers have used ionic gelation to prepare chitosan NPs for medication purposes.[22]

This study was designed to formulate ChiNPs using Boswellic acids obtained from *Boswellia serrata* and evaluate their physicochemical properties and antibacterial activity against gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Materials

A dry extract from *Boswellia serrata* oleo gum resin, purchased from the local market and verified by the National Institute of Science Communication and Information Resources, was utilized to develop the formulations.

Chitosan (95% deacetylated) with molecular weight of 40-80 KDa was procured from Fluka Chemika, Switzerland. HPLC grade Acetonitrile, orthophosphoric acid, Glacial acetic acid, Sodium tripolyphosphate and other reagents employed were purchased from Central Drug House, New Delhi.

Staphylococcus aureus, Bacillus subtilis, Salmonella typhi and *Escherichia coli* strains were obtained from the Institute of Microbial Technology, Chandigarh, India. Using four pathogenic bacterial strains, BAs were evaluated for antibacterial activity.

Preparation of Boswellic Acids Loaded ChiNPs

Five batches of BAs-loaded Chitosan NPs were prepared to utilize the ionotopic gelation technique using homogenization. Chitosan (0.1-0.5%) was dissolved in 1% (v/v) dilute acetic acid solution with mechanical stirrer. Dropwise, addition of 15 mL surfactant solution (1% Tween 80) to chitosan solution prevents particle agglomeration. NaTPP solution was established using distilled water, whereas BAs were dissolved in acetone. BAs was then poured into the chitosan solution and homogenized at 10000 RPM for 20 min. Subsequently, NaTPP (10 mL) solution was added drop by drop at one drop/sec to that same chitosan solution and homogenized over 20 min. Following 30 min, the sample was centrifuged at 25,000 rpm. After supernatant decantation, NPs sediment then lyophilized and free-flowing powder was kept in a suitable container and placed in desiccator for various investigations.[23]

Characterization of ChiNPs

Particle-Size Estimation

The mean particle hydrodynamic radius (ChiNP-1 to ChiNp-5) and size distribution of freshly created NPs were determined using the zetasizer nano series Nano-ZS90 (Malvern Instruments, UK) with the hydro dispersion unit. Particle size was measured by dissolving NPs in distilled water. In a polystyrene cuvette, the hydro dispensing machine scanned the diluted sample (64 times). The Z-average mean diameter was calculated after scanning.[24]

Loading Capacity and Entrapment Efficiency

BAs entrapment and loading in formulated ChiNPs were calculated directly using equations 1 and 2, respectively. For calculation, dissolve 5 mg of generated NPs in 5 mL of methanol and gently shake. Using binary gradient HPLC, the aliquots were analyzed. Triplicate samples were evaluated.[25]

$$\text{Entrapment Efficiency (\%)} = \left[\frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}}\right] \times 100 \quad \text{.....Eq.(1)}$$

$$\text{Loading Capacity (\%)} = \left[\frac{\text{Total drug} - \text{Free drug}}{\text{Nanoparticles weight}}\right] \times 100 \quad \text{.....Eq.(2)}$$

Zeta Potential Determination

To evaluate NP stability, 2 mg of ChiNPs were added to 5 mL of distilled water using electrophoretic mobility. Zetasizer at 25°C automatically assessed the material in a transparent disposable zeta cell. Tests used 80 V device cell voltages. The reference beam amplitude was 2000-3500 kcps. Zeta potential values were obtained three times for each sample.[17]

FTIR Analysis

FTIR is suggested for excipient-active drug moiety compatibility testing. KBR pellets were used on an Alpha Bruker (Germany) FTIR to scan BAs extract and BAs-excipient and ChiNPs formulation.[26]

DSC Analysis

The drug's compatibility with formulation components was tested using DSC analysis. DSC measured the pure extract, physical mixture, and ChiNPs' physical states from 30 to 300°C.[27]

Surface Morphology by Transmission Electron Microscope (TEM)

NPs surface morphology was examined using a 120 KV TEM (Hitachi -H7500, Japan). Field-emission gun sources improved TEM's sensitivity and resolution. NPs were put on copper-coated carbon grid. Before 120 KV analyses, the carbon grid was preserved on the wax sheet and the excess material was removed and air dried for 25-30 min.[28]

In vitro Dissolution Studies

BAs released from ChiNPs were checked for *in vitro* via dialysis bag process in stomach pH 1.2 and intestinal pH 6.8. 100 mg of formulations were placed in a dialysis membrane bag (specifications: Av. Diameter-21.5 mm, Av. Flat width-32.34 mm, Capacity-3.63 mL/cm^2, molecular weight cut-off 12000 Da) and sealed. USP dissolving apparatus II was employed for investigations at 75 RPM by placing ChiNPs in dissolving media at 37°C ± 0.5°C. After predetermined time breaks (0.5, 1, 2, 4, 6, 8, 12, 18, 24 hr), 2 mL of the sample was taken from the released medium and substituted with the same amount of new media. HPLC was used for analysis.[17]

Antibacterial Activity Determined Using the Well Diffusion Method

Well diffusion technique may determine medicines antibacterial activity *in vitro*. In the investigation, conventional strains of *S. aureus, B. subtilis, E. coli*, and *S. typhi* were used and ciprofloxacin as the reference antibiotic. *Boswellia serrata* Extract (BSE) and ChiNP-2 were used for antibacterial testing. The inoculum was prepared in tryptic soy broth cultures with 1-2x10^5 cells/mL. 1 mg/mL, 10 mg/mL and 100 mg/mL in DMSO were test concentrations and ciprofloxacin 1 mg/mL in water was standard, respectively. DMSO was used as a control. Inoculum was distributed on soybean casein digest agar plates (90 mm). Test cultures were plated on soybean casein digest agar (90 mm). Testing samples (10 µL) and ciprofloxacin (25 µL, 1 mg/mL) were impregnated in 5 mm agar wells. After 24-48 hr of incubation at 35°C, plates were observed for Zone of Inhibition (ZOI).[29-34]

MIC Estimation via Micro Broth Dilution Method

To prepare inoculum, the cell suspension from Tryptic soy broth-grown bacterial cultures at 1-2x10^5 cells/mL was taken. The concentration of ciproflaxacin (0.25-16 µg/mL) was taken in Tryptic soy Broth and test samples (two-fold dilutions) were taken. In Tryptic soy broth, 25 µL samples were diluted twofold. Control was taken as culture-inoculated tryptic soy broth without test substance. In triplicate, mix 100 µL drug/test chemicals with 10 µL inoculum in 96 well plate. In control, mix 100 µL tryptic soy broth with 10 mL inoculum. Bacterial cultures were incubated at 37°C for 24-48 hr. After that, Tecan plate readers detected O.D. at 590 nm on bacterial test plates. MIC is the minimum drug concentration that lowers OD by 50% relative to control.[29-32]

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 104.6	Peak 1:	159.9	91.9	68.09
PdI: 0.127	Peak 2:	38.32	8.1	8.056
Intercept: 0.917	Peak 3:	0.000	0.0	0.000
Result quality : Good				

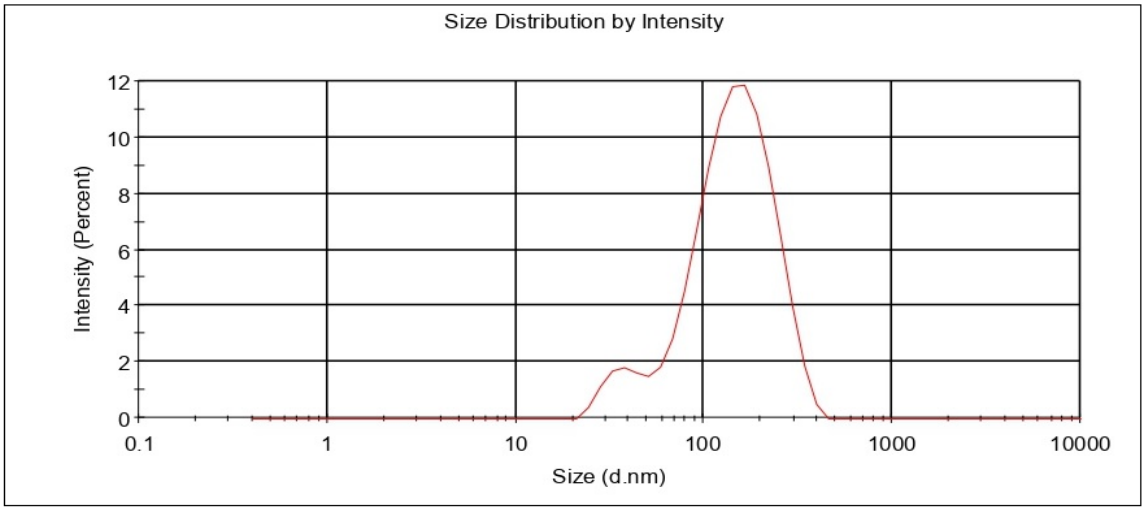

Figure 1: Particle size Analysis of Batch ChiNP-2.

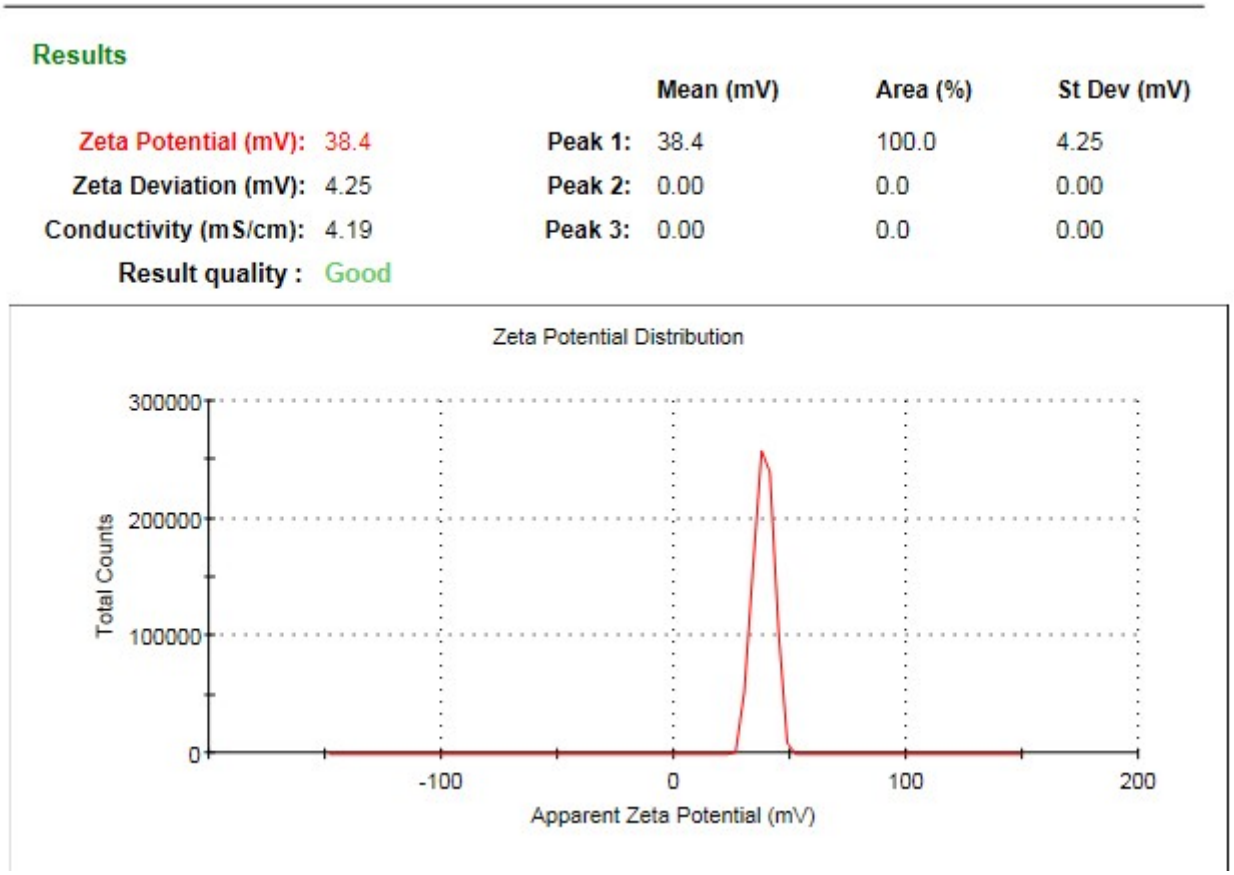

Figure 2: Zeta Potential Analysis of Batch ChiNP-5.

Table 1: Characterization of chitosan nanoparticles.

Batches	Chi conc (%)	NaTPP volume (1%)	Particle size (nm)	PDI	Zeta potential (mV)	Loading capacity (%)	BAs Entrapment (%)
ChiNP-1	0.1	10 mL	202.5	0.512	32.5	58.44±0.17	64.11±0.35
ChiNP-2	0.2	10 mL	104.6	0.127	33.1	76.32±0.47	78.05±0.37
ChiNP-3	0.3	10 mL	124.3	0.222	34.4	64.22±33	69.23±0.21
ChiNP-4	0.4	10 mL	167.2	0.298	35.2	69.45±23	72.11±0.31
ChiNP-5	0.5	10 mL	173.9	0.42	38.4	70.33±45	74.41±0.34

RESULTS AND DISCUSSION

Particle-size estimation

Table 1 illustrates the particle size and Polydispersity Index (PDI) of all ionic gelation-produced chiNPs (ChiNP1-ChiNP5) with particle diameters from 104.6 to 200.5 nm. ChiNP-2 have lowest particle size, 104.6 nm and PDI 0.127 (Figure 1), with 0.2% chitosan concentration. Chitosan-NaTPP ratio determines nanoparticle particle size. Increasing viscosity leads to homogenizer shear capacity and increases particle size with higher chitosan concentrations.

Loading Capacity and Entrapment Efficiency

Direct estimation was used to evaluate ChiNPs entrapment efficiency and loading capacity. Entrapment efficiency and loading capacity were over 50%, as shown in Table 1. Entrapment efficiency and loading capacity ranged from 64.11±0.35 to 78.05±0.37% and 58.44±0.17 to 76.32±0.47%, respectively, for all prepared batches. The batch ChiNP-2 with the optimum chitosan content had the maximum drug entrapment and loading capacity.

Zeta Potential analysis

Zeta potential was used to assess the stability of ChiNPs. NPs with ±30 mV values remain stable for a long time. The batch ChiNP-5 have a positive zeta potential of 38.4 mV (Figure 2). NPs

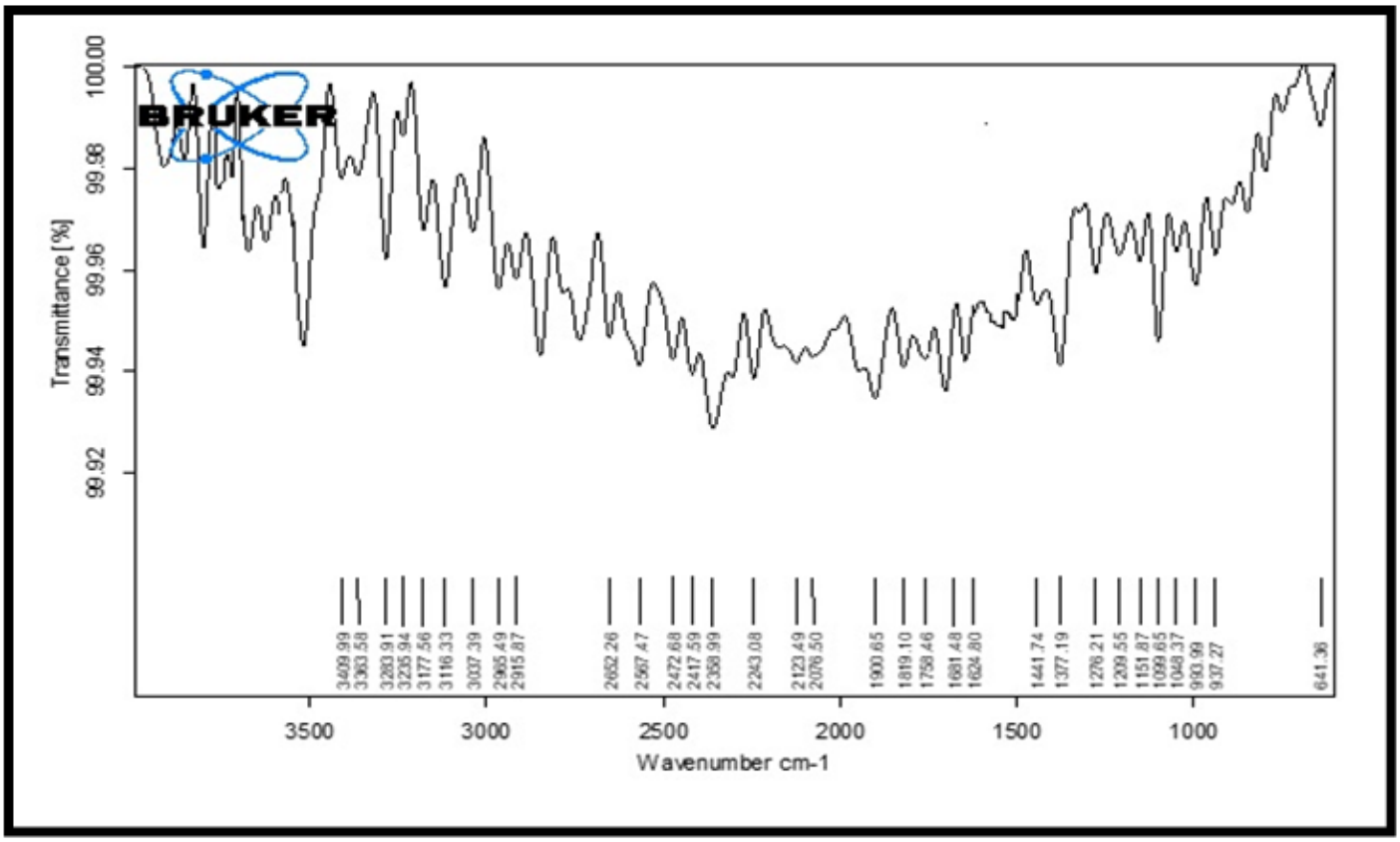

Figure 3: FTIR Spectrum of Batch ChiNP-2.

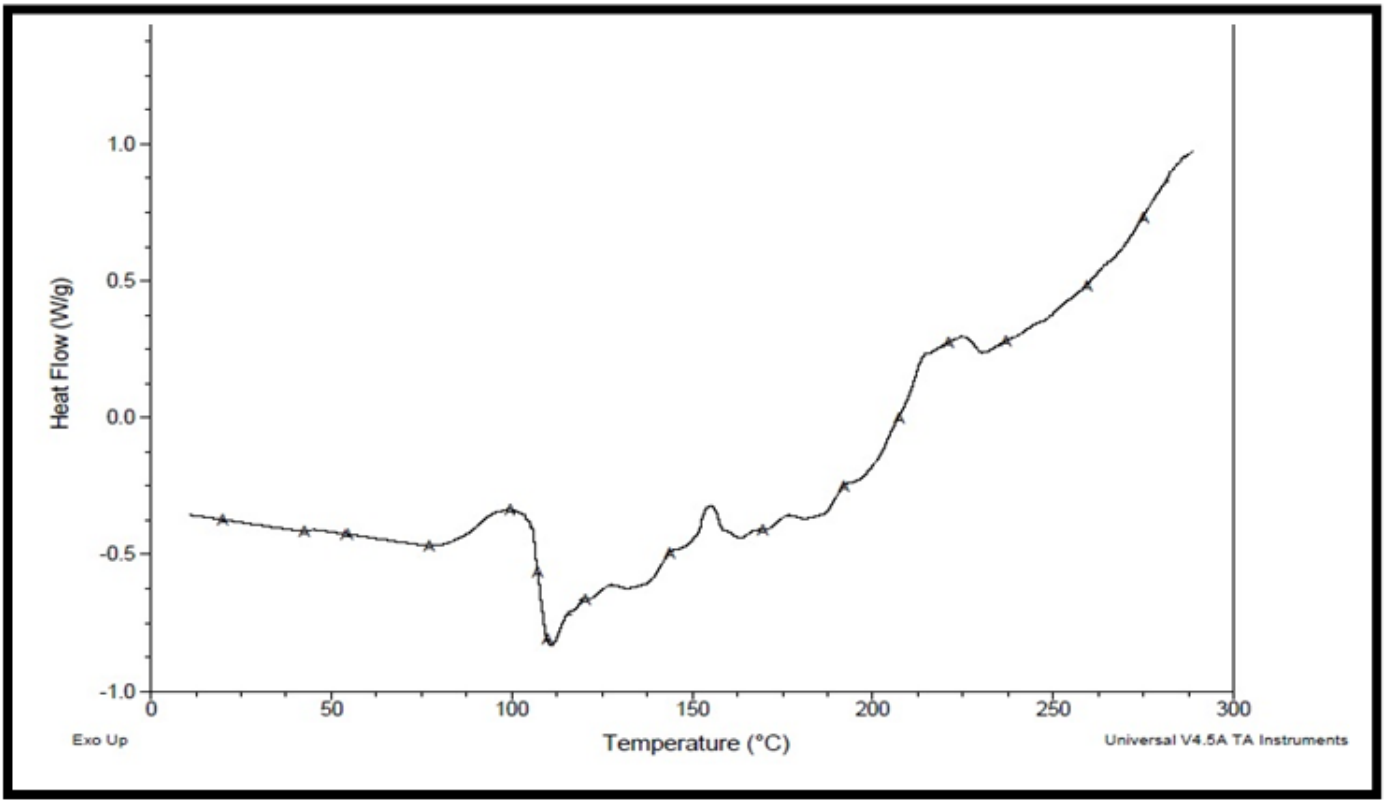

Figure 4: DSC Thermogram of Batch ChiNP2

Table 2: Characteristics Peaks in BAs and BAs Loaded ChiNP-2.

Characteristic Peaks	Bas Peaks (cm^{-1})	Physical Mixture IR Peaks (cm^{-1})	ChiNP5 IR Peaks (cm^{-1})
OH stretch.	3442	3443	3409
CH stretch.	2958	2953	2955
C=O stretch. of aryl acid	1701	1701	1698
Cyclic ketone	1638	1644	1624
CH_2 stretch. of cyclohexane	1454	1453	1441
COO^- symmetric stretching of carboxylates	1375	1376	1377
Ring stretch. of cyclohexane	1054	1027	1049
-	981	980	933

Table 3: Inhibitory activity of BSE against different bacteria.

Sl. No.	Organism	Zone of inhibition				
		Standard 2.5 µg/well	100 µg/well	10 µg/well	1 µg/well	Control (DMSO)
1	*Bacillus subtilis*	30.00±0.2	9.00±0.1	-	-	-
2	*Escherichia coli*	27.00±0.3	11.00±0.2	9.00±0.1	-	-
3	*Salmonella typhi*	38.00±0.3	14.01±0.2	11.00±0.2	-	-
4	*Staphylococcus aureus*	26.00±0.1	14.00±0.3	8.00±0.2	-	-

Table 4: Inhibitory activity of ChiNP-2 against different bacteria.

Sl. No.	Organism	Zone of inhibition				
		Standard 2.5 µg/well	100 µg/well	10 µg/well	1 µg/well	Control (DMSO)
1	*Bacillus subtilis*	31.00±0.2	16.00±0.1	8.00±0.2	-	-
2	*Escherichia coli*	28.00±0.4	16.00±0.3	8.00±0.1	-	-
3	*Salmonella typhi*	38.00±0.15	21.00±0.2	13.00±0.2	-	-
4	*Staphylococcus aureus*	24.00±0.02	17.00±0.2	10.00±0.4	-	-

Table 5: Summary report of minimum inhibitory concentration of test formulations.

Sample Code	MIC (µg/mL)				Test parameters
	Escherichia Coli	*Salmonella typhi*	*Staphylococcus aureus*	*Bacillus subtilis*	
Ciprofloxacin (Std)	1.00	0.50	1.00	1.00	Methodology Microbroth dilution technique using Culture Medium: Tryptone broth Sample test concentrations 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.62 µg/mL, 7.81 µg/mL and 3.91 µg/mL.
BSE	7.81	7.81	7.81	62.50	
ChiNP-2	7.81	3.91	7.81	7.81	

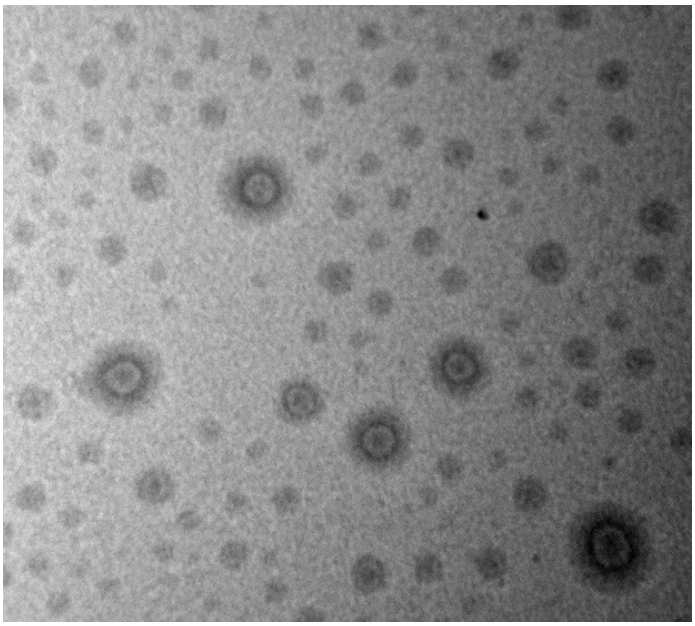

Figure 5: TEM Image of Batch ChiNP-2.

with such ζ values are relatively stable (period of time). Polymeric materials with high cationic charge densities produced NPs with better antibacterial effects than those with low charge densities. Chitosan is a cationic polymer, and its positive surface charge affects its lethal effects owing to the electrostatic ionic interaction between bacterial cells negatively charged groups and Chitosan's amino groups.

FTIR Analysis of NP's

Figure 3 shows ChiNP-2 formulation spectrum. BAs and developed batch showed no significant change in absorption peaks. In contrast to BAs extract, the formulations had no new peaks, no substantial shift of functional peaks, and no overlapping of distinctive peaks. Table 2 shows the distinctive peaks of BAs and BAs-loaded chitosan nanoparticle formulation ChiNP-2. FTIR showed no chemical interactions between BAs functional groups and excipients during ionic gelation entrapment.

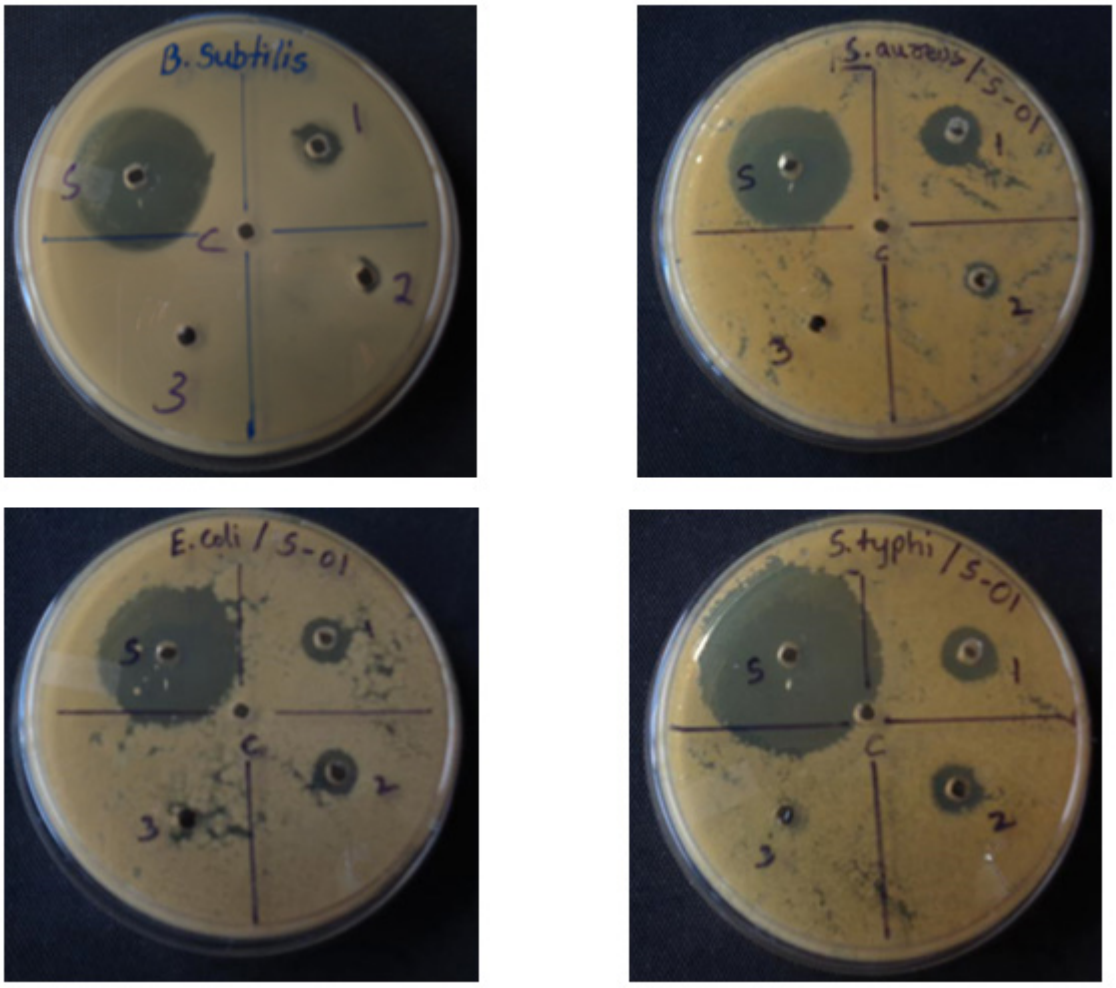

Figure 6: Inhibitory activity of BSE against test bacteria. S: Standard (Ciprofloxacin), C: Control (DMSO), 1-100 µg, 2-10 µg, 3-1 µg.

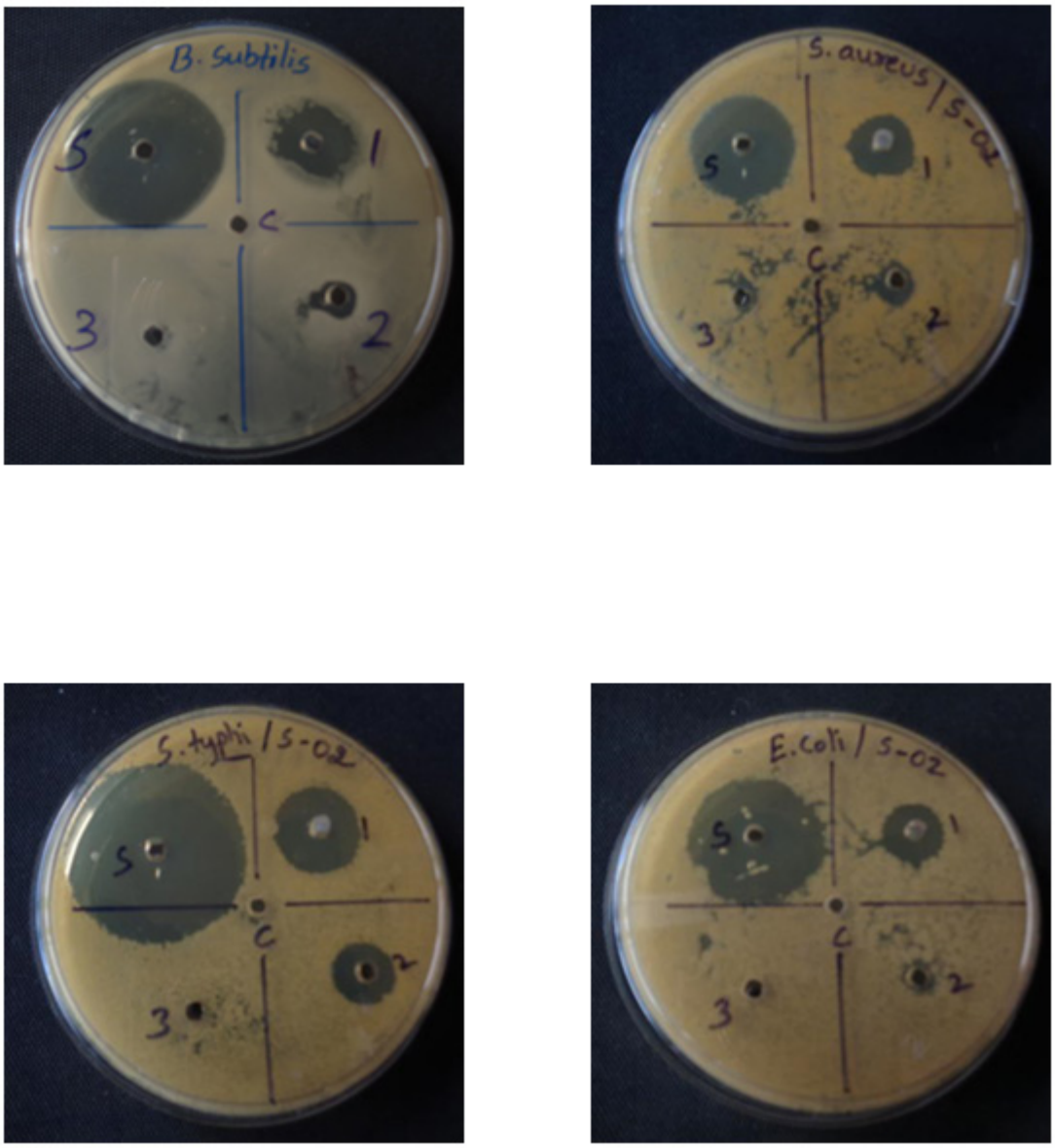

Figure 7: Inhibitory activity of ChiNP-2 against different bacteria.

DSC Analysis of NP's

The DSC thermogram of *Boswellia serrata* exhibited two endothermic peaks for BAs at 193°C and 270°C, and a peak for chitosan at 116.38°C. DSC thermograms of BAs compared to physical combination showed no chemical interaction and compatibility between BAs and study excipients. DSC thermogram of ChiNP-2 presented in Figure 4 and found that ionic gelation converts crystalline BAs into amorphous BAs during NPs formation.

TEM analysis

ChiNPs' cell surface adhesion depends on their morphology. Figure 5 shows the TEM image of optimal BAs-loaded chiNPs formulation (ChiNP-2) generated by ionic gelation. The boswellic acid-loaded chitosan NPs had a smooth surface and were spherical in shape.

In vitro Drug Release

Dialysis bag diffusion was used to study drug release from BAs-loaded nanoparticles *in vitro*. After 24 hr, the percentage cumulative drug release of all BAs-loaded ChiNPs (ChiNP-1 to ChiNP-5) varied from 45.36±0.38% to 55.19±0.27% in pH 1.2 and 69.26±0.35 to 87.17±0.35 in pH 6.8. Phosphate buffer pH 6.8 released more BAs than acidic pH, maybe because BAs dissolves quickly in alkaline medium. Chitosan NPs released boswellic acids at a continuous rate in pH 1.2 and phosphate buffer pH 6.8, with maximal release in the intestinal fluid after 24 hr.

Antibacterial Effects of BAs Loaded ChiNP-2

All the test samples show inhibitory activity against test pathogens. The inhibitory activity of BSE and ChiNP-2 against different test pathogens is summarized in Tables 3 and 4, respectively. The minimum inhibitory concentrations of test samples are summarized in Table 5. The lowest ZOI 8.00±0.2 (Figure 6) was obtained against *Staphylococcus aureus* and highest ZOI 21.00±0.2 (Figure 7) against *Salmonella typhi* (ChiNP-2).

CONCLUSION

Due to newly emerging clinically resistant bacterial strains to one or more antibiotics, there is a rising need for antimicrobial medicines. Promising antibacterial agents are obtained from natural sources. However, their limitations in terms of limited solubility, bioavailability, and pharmacological action, as well as stability issues and readily destructive tendency, restrict their use as medications in a health context. The spherical and uniformly sized NPs (104.6 nm) were visible in TEM photomicrographs, and the NPs had a sustained drug release that was diffusion-controlled and could last up to 24 hr. Boswellic acid containing ChiNPs have more potent antibacterial activity and a greater zone of inhibition (21.00±0.2) against *Salmonella typhi* than extract, which have MIC values of 3.91 µg/mL and 7.81 µg/mL, respectively.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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