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Sonication-based Nanosuspension formation of Microbial Extract to assess their Antibacterial properties

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The emergence of antibiotic-resistant bacteria has become a significant public health concern worldwide, necessitating the development of alternative antibacterial agents. In this context, the use of secondary metabolites derived from natural sources is gaining attention as a potential alternative to conventional antibiotics. However, the poor solubility and bioavailability of these compounds limit their clinical use. Nanoparticle formation of secondary metabolites is a promising approach to overcome these limitations, enabling their efficient delivery and targeted action against bacterial pathogens. In this study, we develope a nanoformulation of secondary metabolites with antibacterial properties against WHO-listed priority pathogens using a sonication probe. The nanosuspension is synthesized by combining the secondary metabolites with a biocompatible polymer and is sonicated using a probe sonicator to achieve a uniform nanoparticulate suspension. The resulting nanosuspension is characterized using scanning electron microscopy (SEM), UV-spectroscopy, and Fourier Transform Infrared Spectroscopy (FTIR). The antibacterial properties of the nanosuspension are examined against selected WHO-listed priority pathogens using the zone-inhibition method and by calculating their minimum inhibitory concentration (MIC). The results demonstrate the potent antibacterial effect of nanosuspension against the tested bacterial strains. Additionally, the nanosuspension shows improved solubility, stability, and bioavailability of the secondary metabolites, which are essential factors for their clinical applications. In conclusion, this study highlights the potential of sonication probe-assisted nanoparticle formation as an effective approach for the delivery of secondary metabolites with antibacterial properties against priority pathogens. The findings of this study provide a promising avenue for the advancement of novel antibacterial agents to combat antibiotic-resistant bacterial infections.

Keywords: Actinobacteria, Antibacterial, Multi-drug resistance, Nanosuspension, Secondary metabolites

1 Introduction

In order to target the global escalation of multi-drug resistance in pathogenic microorganisms, there is a requirement to enable development of novel therapeutic interventions and targeted drug delivery systems. The utilization of microbial extracts as a natural source of bioactive compounds has garnered significant attention in recent years. In this context, Actinobacteria are being explored extensively as they are known to produce therapeutic secondary metabolites possessing various pharmacological properties like anti-bacterial, anti-fungal, anti-oxidant, anti-cancerous, anti-malarial, etc.[1] Despite Actinobacteria being a producer of plethora of therapeutic secondary metabolites, only a few make it to the market level because of their physicochemical limitations such as low solubility in an aqueous medium which affects their bioavailability and undesirable toxicities that must be addressed before progressing through the drug development pipeline. So, to tackle these limitations and develop effective and safer drugs, principles of nanotechnology are being applied.[2] In nano-form, the physicochemical properties of the material change due to increase in surface area to volume ratio.[3] Therefore, nanotechnology for synthesizing nanosuspensions of bioactive compounds is a fast-growing domain. Amongst various approaches, utilization of microbial secondary metabolites as a natural source of nanosuspension has garnered significant attention in recent years. However, the conversion of these secondary metabolites into nanosuspension poses inherent challenges attributed to their complex formulation process and limited physicochemical properties such as instability, aggregation, low solubility, that might lead to decrease in their biological activity. Also, careful decisions need to be made in selecting the processing parameters and appropriate stabilizers to develop stable and potent nanosuspension. Sonication offers a feasible solution by leveraging ultrasonic waves to facilitate the synthesis of nanosuspension from microbial extracts

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through cavitation.[4] During the sonication process, the microbial secondary metabolites are subjected to high-frequency ultrasonic waves, leading to the disruption of the molecules which in turn serve as a nucleation site for the nanoparticle formation tailored with physicochemical attributes.[5] This technique may offer precise control over nanoparticle size, morphology, and surface characteristics, enabling fine-tuning of their properties to meet specific biomedical requirements. The present study aims to elucidate the potential of sonication-assisted formation of nanosuspension of microbial secondary metabolites as a versatile and innovative approach for the development of novel therapeutics against WHO-listed priority pathogens such as *Staphylococcus aureus* and *Enterococcus faecalis* to drive forward the frontier of nanomedicine.[6]

2 Materials and Methods

Mueller Hinton Broth, Methanol (HPLC Grade), and Poly-ethylene-glycol with molecular weight 400(PEG-400) were directly purchased from Sigma-Aldrich. Standard drug Vancomycin hydrochloride (CMS217), and 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride(INT) were purchased directly from Himedia. Dimethyl sulphoxide(DMSO) of Emparta grade was purchased from Merck Life Science private limited. Autoclaved distilled water was used in all experiments.

2.1 Bacterial strains and growth conditions

WHO-listed priority gram-positive pathogens *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were used as reference strains. They were grown and maintained in Mueller Hinton Agar at 37˚C overnight.

2.2 Production of secondary metabolites from Actinobacteria

The production of secondary metabolites was done by following the protocol reported by Singh et al, 2020 where Actinobacteria was grown in SCN (Starch Casein Nitrogen) media for 7 days. Extraction of secondary metabolites from SCN broth was done using a liquid-liquid solvent extraction technique.[7]

2.3 Synthesis of nanosuspension

The secondary metabolites were dissolved in autoclaved distilled water (1mg/ml) using DMSO (0.02%) to prepare a stock solution. A reaction mixture was prepared by mixing 1 ml of the secondary metabolites from the stock solution (1mg/ml) and 1 ml of polymer solution (1mg/ml of PEG-400 dissolved in aqueous medium). The total volume of the reaction mixture (rxn mix) was made to 10 ml by adding autoclaved distilled water as the solvent medium. This rxn mix was subjected to high-frequency sound waves using a probe sonicator at an amplitude of 50 Hz with 5 seconds pulse for 20 minutes. The process was done by maintaining the sample in ice to prevent the degradation of biological molecules due to heat generated by the sonication method. After 20 minutes, the rxn mix was used for further characterization of the nanosuspension.

2.4 Characterization of the synthesized nanosuspension

The nanosuspension of the secondary metabolites was characterized using various bioanalytical instruments such as scanning electron microscope (SEM) - Zeiss EVO40 to gain valuable insights into the surface morphology and size of the nanosuspension of secondary metabolites. UV-Visible spectra of nanosuspension and gross material were obtained using UV-Visible spectrophotometer (Cary-60, Agilent technologies). Fourier transform infrared (FTIR) spectroscopy of synthesized nanosuspension and gross material was done from range 4000 to 400 cm^{-1} using FTIR spectrometer (Cary-630, Agilent Technologies).

2.5 Antibacterial activity assessment

The nanosuspension was tested against *Staphylococcus aureus* and *Enterococcus faecalis* using a well-diffusion assay as per Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentration was also determined using various concentrations of nanosuspension in MHB by broth micro-dilution method with 0.5 McFarland cell suspension prepared using overnight grown culture.[8]

3 Results and Discussion

3.1 Synthesis and characterization of nanosuspension

The large particles of irregular shapes and sizes of the gross secondary metabolites (Fig. 1a) began to disintegrate in presence of PEG, when subjected to high-frequency sonic waves at an amplitude of 50 Hz for 10 min shown in Fig. 1(b). After 15 minutes of treatment with high-frequency sonic waves, there was formation of uniform spherical size nanosuspension. SEM analysis showed development of spherical nanosupension of secondary metabolites with an average diameter of 200 nm shown in Fig. 1(c).

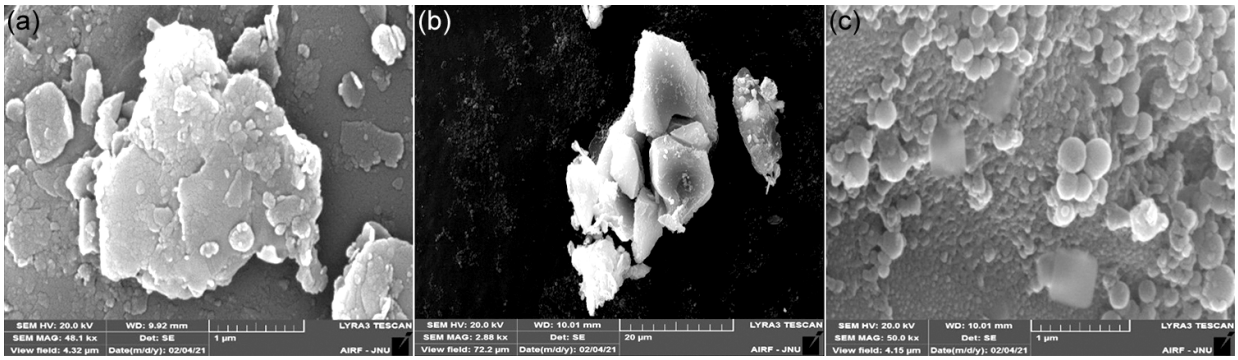

Fig. 1 — Scanning Electron Microscope (SEM) images of (a) gross secondary metabolites, (b) secondary metabolites after sonicatinf at an amplitude of 50 Hz for 10 min, & (c) nanosuspension formation of the secondary metabolites after sonication at an amplitude of 50 Hz for 15 min.

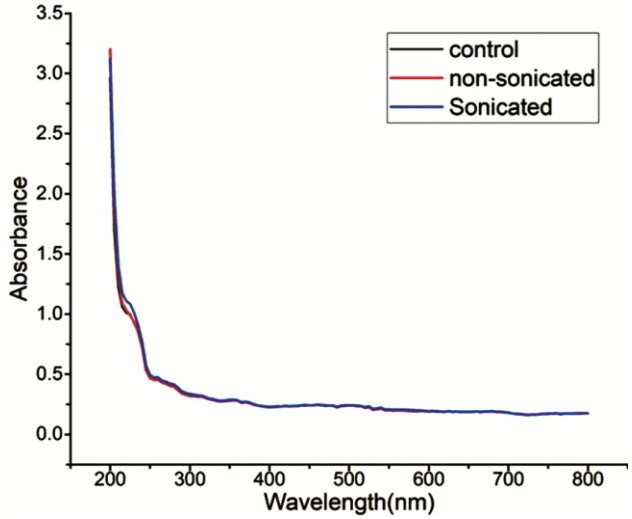

Fig. 2 — UV-Vis spectra depicting the optical behaviour of the control i.e. gross secondary metabolites, non sonicated rxn mix, and sonicated rxn mix.

The UV-Vis spectra as shown in Fig. 2, depicted that there was no significant change in the optical absorption and scattering of the secondary metabolites nanosuspension in comparison to the gross material which may imply that the integrity of the material was maintained even after application of high-frequency sonic waves.

FTIR spectra of gross secondary metabolites, developed nanosuspension without, and after sonication are shown in Fig. 3(a-c). It was observed that there was no change in absorption bands at 570 cm^{-1}, 1636 cm^{-1}, and 3335 cm^{-1} that correspond to C-I stretching, C=C stretching, and O-H stretching, respectively. A slight change at 1084 cm^{-1} was observed in nanosuspension which corresponds to surface modification in the nanosuspenison due to strong C=O stretching.

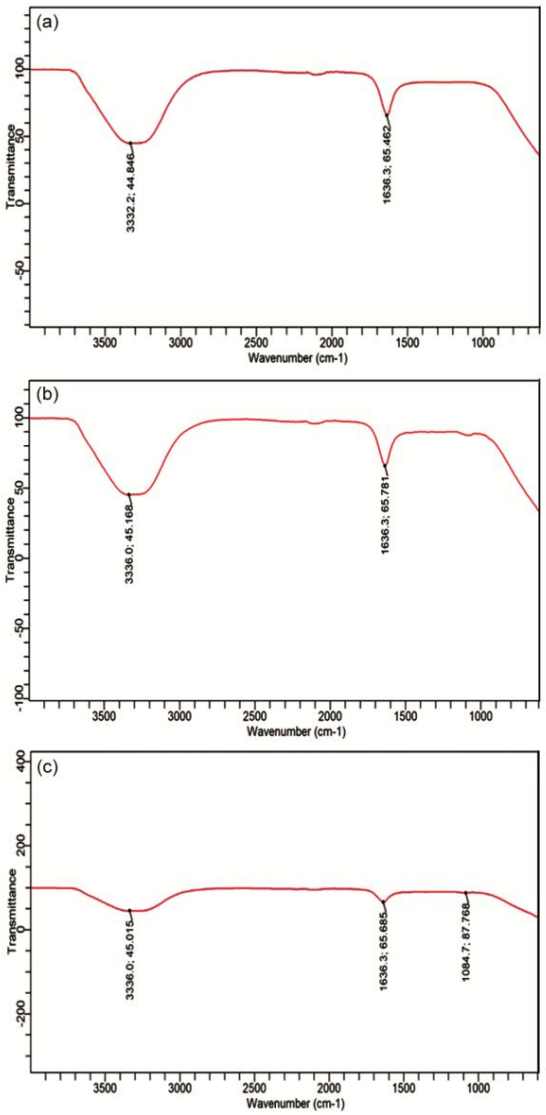

Fig. 3 — Fourier transform infrared (FTIR) of (a) secondary metabolites without polymer, (b) rxn mix without sonication, & (c) rxn mix after sonication.

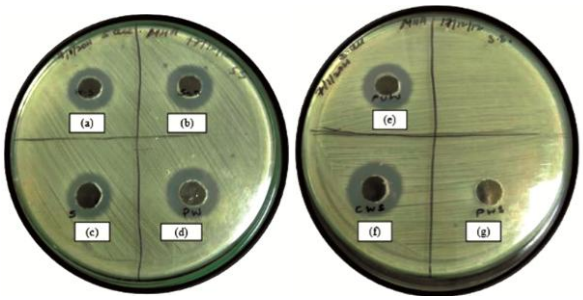

Fig. 4 — Zones of Inhibitions against *S. aureus* ATCC 25923 (a) Gross secondary metabolites, (b) Rxn mix without polymer with sonication, (c) Rxn mix with polymer but non-sonicated, (d) The supernatant of the centrifuged sonicated rxn mix, (e) Pellet of the centrifuged sonicated rxn mix, (f) Sonicated rxn, & (g) Only polymer.

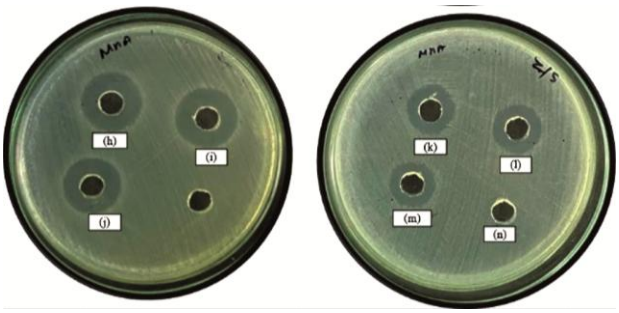

Fig. 5 — Zones of inhibition against *E. faecalis* ATCC 29212 (h) Pellet of the centrifuged sonicated rxn mix, (i) Sonicated rxn mix, (j) Gross secondary metabolite, (k) Rxn mix with polymer but unsonicated, (l) Rxn mix without polymer with sonication, (m) The supernatant of the centrifuged sonicated rxn mix, & (n) Only Polymer.

Table 1 — Zones of inhibition against *S. aureus* and *E.faecalis*

Sample number	Sample Name	Zones of inhibition (mm)
(a)	Gross secondary metabolites	15 ± 1
(b)	Rxn mix without polymer with sonication	14 ± 1
(c)	Rxn mix with polymer but non-sonicated	14 ± 1
(d)	The supernatant of the centrifuged sonicated rxn mix	15 ± 1
(e)	Pellet of the centrifuged sonicated rxn mix	17 ± 1
(f)	Sonicated rxn mix	16 ± 1
(g)	Only polymer	No zone observed
(h)	Pellet of the centrifuged sonicated rxn mix	16 ± 1
(i)	Sonicated rxn mix	17 ± 1
(j)	Gross secondary metabolites	16 ± 1
(k)	Rxn mix with polymer but unsonicated	14 ± 1
(l)	Rxn mix without polymer with sonication.	14 ± 1
(m)	The supernatant of the centrifuged sonicated rxn mix	13± 1
(n)	Only Polymer	No zone observed

Table 2 — MIC values of nanosuspension in comparison to gross material against *S. aureus* and *E. faecalis*.

Sample name	Culture Name	MIC_{90} value (ug/ml)
Gross secondary metabolites	*S.aureus*	2.7 ± 0.2
Nanosuspension	*S.aureus*	0.76 ± 0.2
Gross secondary metabolites	*E.faecalis*	25 ± 0.1
Nanosuspension	*E.faecalis*	12 ± 0.2

3.2 Antibacterial susceptibility assessment

Antibacterial susceptibility testing was performed against WHO-priority bacterial pathogens namely *Staphylococcus aureus* ATCC 25923 shown in Fig. 4 and *Enterococcus faecalis* ATCC 29212 shown in Fig. 5 using well-diffusion assay and calculating the minimum inhibitory concentration (MIC).

Table 1 depicts zones of inhibition. Table 2 presents MIC_{90} of nanosuspension in comparison to gross material against both *S. aureus* and *E. faecalis*. It was observed that the developed nanosuspension showed enhanced antibacterial activity in comparison to gross secondary metabolites, with more efficacy against *Staphylococcus aureus* as compared to *Enterococcus faecalis.*

4 Conclusion

Nanosuspension of Actinobacteria secondary metabolites with an average diameter of 200 nm is developed using sonication method. It is found to exhibit excellent antibacterial properties against both *Staphylococcus aureus* and *Enterococcus faecalis*, in comparison to gross seocondary metabolites, with more efficacy against *Staphylococcus aureus.* Our results indicate promosing potential of nanosuspension of secondary metabolites as novel antibacterial agent against Gram positive priority pathogens.

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