Research Article

Formulation, Characterization, And Antibacterial Evaluation Of A Novel Drug Delivery System Incorporating Selected Medicinal Plant Extracts

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ABSTRACT:

The growing challenge of antimicrobial resistance necessitates the development of alternative therapeutic approaches. Although Holoptelea integrifolia and Withania somnifera have been extensively utilized in traditional medicine for their therapeutic properties, the antibacterial potential of their combined extracts has not yet been systematically investigated.

Plant extracts were obtained through successive solvent extraction and subjected to preliminary phytochemical screening. The antibacterial efficacy of individual extracts and their combinations (1:1, 1:2, and 2:1) was assessed against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae using the agar well diffusion method, minimum inhibitory concentration (MIC) determination, and checkerboard assay. The most effective synergistic combination was subsequently incorporated into chitosan-based microparticles using the ionotropic gelation technique and optimized employing a 3^2 factorial design. The optimized microparticles were further characterized and evaluated for their in vitro drug release profile and antibacterial performance.

Among the prepared extracts, the ethanolic extracts exhibited the highest extraction yield along with the greatest diversity of phytochemical constituents. The 1:2 extract combination demonstrated synergistic antibacterial activity, with fractional inhibitory concentration index (FICI) values ranging from 0.5 to 0.75, while the most pronounced synergistic effect was observed against P. aeruginosa (FICI = 0.5). The optimized microparticle formulation, containing 1.5% chitosan and 1.0% tripolyphosphate (TPP), exhibited a mean particle size of 42.36 µm, an encapsulation efficiency of 78.42%, and a sustained drug release profile extending over 24 hours. Furthermore, the developed formulation significantly enhanced antibacterial efficacy, producing a 2–4-fold reduction in MIC values and achieving complete eradication of S. aureus within 24 hours. Stability studies confirmed that the formulation remained stable for six months under accelerated storage conditions.

To the best of our knowledge, this study represents the first report demonstrating the synergistic antibacterial interaction between H. integrifolia and W. somnifera. The developed sustained-release chitosan microparticle formulation exhibited enhanced antibacterial efficacy against clinically significant bacterial pathogens, with particularly promising activity against P. aeruginosa.

Keywords: Antimicrobial resistance, Holoptelea integrifolia, Withania somnifera, synergy, microparticles, chitosan, NDDS.

INTRODUCTION:

Antimicrobial resistance (AMR) has emerged as one of the most critical global public health challenges of the twenty-first century. The World Health Organization (WHO) recognizes AMR as one of the ten most significant threats to global health, underscoring the urgent need for effective interventions and innovative therapeutic approaches [1]. In this context, medicinal plants and natural products have gained considerable attention as promising sources of new antimicrobial agents. The plant kingdom constitutes an extensive reservoir of structurally diverse bioactive compounds that have evolved over millions of years to protect against microbial pathogens. In contrast to conventional synthetic antimicrobial agents, phytochemicals frequently exert their antimicrobial effects through multiple mechanisms of action, thereby potentially minimizing the emergence and spread of antimicrobial resistance [6]. Moreover, the extensive history of traditional medicinal use associated with numerous plant species provides valuable evidence regarding their therapeutic efficacy and safety, facilitating their translation into modern pharmaceutical applications and potentially expediting the drug development and regulatory approval process.

Among these medicinal plants, Holoptelea integrifolia and Withania somnifera have been widely recognized for their therapeutic properties in traditional systems of medicine. However, despite their established individual pharmacological potential, the antibacterial efficacy of their combined extracts has not been systematically investigated. The escalating burden of AMR, coupled with the diminishing pipeline of new antibiotics and the inherent limitations of existing antimicrobial therapies, highlights the pressing need to explore innovative therapeutic alternatives. Consequently, investigating the synergistic antibacterial potential of these medicinal plants may provide a promising strategy for the development of effective plant-based antimicrobial formulations capable of addressing clinically relevant drug-resistant pathogens [2].

MATERIALS AND METHODS

i. Collection and Authentication of Plant Material

The stem bark of Holoptelea integrifolia (Roxb.) Planch. was collected in March 2025 from the Medicinal Plant Garden of Krishi College, Indore, India. The collection was performed during the flowering season, when the tree was at full bloom, as this stage is considered optimal for the accumulation of bioactive phytoconstituents.

The roots of Withania somnifera (L.) Dunal were also collected in March 2025 from the certified Medicinal Plant Garden of Krishi College, Indore, India. Root samples were obtained from plants aged approximately 6–8 months during the flowering stage, as this developmental phase has been reported to contain the highest concentration of withanolides.

ii. Authentication of Plant Materials

The collected plant materials were authenticated by Dr. Prof. S. K. Sharma, Retired Professor, Govt. Mata Jijabai Girls P.G. College, Moti Tabela, Indore, Madhya Pradesh, India. Voucher specimens of both plant species were prepared and preserved in the institutional herbarium to facilitate future identification and reference.

iii. Chemicals and Reagents

All chemicals and reagents employed throughout the study were of analytical reagent (AR) grade or high-performance liquid chromatography (HPLC) grade, depending on the specific experimental requirements.

iv. Processing and Extraction of Plant Materials

For Holoptelea integrifolia bark: The fresh bark was washed thoroughly under running tap water to remove any adhering dust, dirt, and other contaminants. It was then rinsed with distilled water. The cleaned bark was cut into small pieces (approximately 2-3 cm in size) using a stainless-steel knife and spread in a thin layer on clean paper sheets. The bark pieces were shade-dried at room temperature (25±2°C) for 15 days with occasional turning to prevent fungal growth. Complete drying was confirmed by constant weight measurement.

For Withania somnifera roots: The washed roots were cut into small pieces (1-2 cm) and shade- dried at room temperature for 12 days. Drying was continued until constant weight was achieved.

v. Antibacterial Activity of Extracts

Antibacterial Activity of Individual Extracts

The antibacterial activity of all extracts (petroleum ether, chloroform, ethanol, and aqueous) from both plants was evaluated against four bacterial strains: Staphylococcus aureus (MTCC 737), Escherichia coli (MTCC 443), Pseudomonas aeruginosa (MTCC 424), and Klebsiella pneumoniae (MTCC 109). The activity was assessed by measuring the zone of inhibition (ZOI) at different concentrations (5, 10, 25, and 50 mg/mL) using the agar well diffusion method. Ciprofloxacin (10 µg/well) was used as positive control, and 10% DMSO was used as negative control.

vi. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Based on the findings of the agar well diffusion assay, the ethanolic extracts of both medicinal plants, which exhibited the highest antibacterial activity, were selected for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the broth microdilution method.

vii. Synergistic Activity of Extract Combinations

Based on the MIC values obtained for the individual ethanolic extracts, combinations of H. integrifolia and W. somnifera were prepared in ratios of 1:1, 1:2, and 2:1 relative to their respective MIC values. The antibacterial activity of these combinations was subsequently evaluated using the checkerboard assay, and the Fractional Inhibitory Concentration Index (FICI) was calculated to determine the nature of the interaction between the extracts.

The observed synergistic antibacterial activity may be explained through several complementary mechanisms:

1. **Complementary mechanisms of action:** H. integrifolia is rich in tannins and flavonoids that can disrupt bacterial membranes and chelate metal ions, while W. somnifera contains withanolides that inhibit bacterial enzymes and modulate host immune responses [7]. The combination attacks bacteria through multiple mechanisms simultaneously, making it more difficult for bacteria to develop resistance.
2. **Inhibition of resistance mechanisms:** Some flavonoids from H. integrifolia may inhibit bacterial efflux pumps, enhancing the intracellular accumulation of withanolides from W. somnifera [8]. This is particularly relevant for P. aeruginosa, which is known for its active efflux pumps.
3. **Membrane permeabilization:** Tannins from H. integrifolia can disrupt the outer membrane of Gram-negative bacteria, facilitating the entry of withanolides that might otherwise be excluded [9].
4. **Multi-target effects:** The diverse array of compounds from both plants may target different bacterial enzymes and pathways, producing combined effects that exceed the sum of individual activities [10].

viii. Formulation and Optimization of Microparticles

Based on the synergistic antibacterial activity results, the 1:2 combination of Holoptelea integrifolia and Withania somnifera ethanolic extracts was selected for incorporation into microparticles. Chitosan and alginate were selected as the polymers for microparticle preparation using the ionotropic gelation method.

ix. Preliminary Trials

Preliminary trials were conducted to identify the suitable concentration ranges for polymers and cross-linkers that would produce spherical, discrete microparticles with acceptable characteristics.

x. Selection of Optimized Formulation

The optimized formulation was selected based on the desirability function approach, aiming to minimize particle size (for better patient acceptability and potentially improved bioavailability) and maximize encapsulation efficiency (to ensure maximum drug loading and minimize drug loss during preparation).

xi. **Composition of Optimized Formulation (F5)**

Component	Quantity
Chitosan	1.5% w/v (in 1% acetic acid)
Extract combination (HI:WS 1:2)	10 mg/mL of polymer solution
TPP	1.0% w/v (in distilled water)
Stirring speed	1000 rpm
Needle gauge	24G
Dropping distance	10 cm
Curing time	30 minutes

Batch F5 was selected as the optimized formulation and was subjected to comprehensive characterization.

xii. **Surface Morphology by SEM**

Scanning electron microscopy was performed.

xiii. **Fourier Transform Infrared (FTIR) Spectroscopy**

FTIR analysis was performed to assess any possible chemical interactions between the extract and polymers.

xiv. **Differential Scanning Calorimetry (DSC)**

DSC analysis was performed to study the thermal behavior and physical state of the extract within the microparticles.

xv. In-vitro Release Profile

The in-vitro release of the extract combination from the optimized microparticle formulation was studied in phosphate buffer saline (PBS) pH 7.4 at 37 ± 0.5°C using the dialysis bag method. The release profile was compared with that of the free extract combination.

xvi. Antibacterial Efficacy of the Formulation

MIC and MBC of Microparticle Formulation

The antibacterial efficacy of the optimized microparticle formulation was evaluated by determining the MIC and MBC against the test organisms. The results were compared with those of the free extract combination.

xvii. Stability Studies

Stability studies were conducted as per ICH guidelines to assess the physical and chemical stability of the optimized microparticle formulation under accelerated (40°C/75% RH) and long-term (25°C/60% RH) conditions for 6 months.

RESULT AND DISCUSSION

i. Antibacterial Activity of Extracts

The antibacterial activity of the extracts showed concentration-dependent effects, with higher concentrations producing larger zones of inhibition. For both plants, the ethanolic extract exhibited the highest antibacterial activity against all tested bacterial strains, followed by chloroform, aqueous, and petroleum ether extracts.

Table 1: Zone of Inhibition of H. integrifolia Extracts (mm)

Extract	Conc. (mg/mL)	S. aureus	E. coli	P. aeruginosa	K. pneumoniae
Petroleum ether	5	7.2 ± 0.4	6.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	10	8.6 ± 0.5	8.2 ± 0.4	7.4 ± 0.4	7.0 ± 0.3
	25	10.4 ± 0.6	9.8 ± 0.5	8.6 ± 0.5	8.2 ± 0.4
	50	12.8 ± 0.7	11.6 ± 0.6	10.2 ± 0.5	9.8 ± 0.5
Chloroform	5	8.4 ± 0.5	7.8 ± 0.4	7.2 ± 0.4	7.0 ± 0.3
	10	11.2 ± 0.6	10.4 ± 0.5	9.6 ± 0.5	9.2 ± 0.5

	25	14.6 ± 0.7	13.8 ± 0.6	12.4 ± 0.6	11.8 ± 0.6
	50	17.8 ± 0.8	16.4 ± 0.7	15.2 ± 0.7	14.6 ± 0.7
Ethanol	5	10.2 ± 0.6	9.4 ± 0.5	8.8 ± 0.5	8.4 ± 0.4
	10	14.8 ± 0.7	13.6 ± 0.6	12.4 ± 0.6	11.8 ± 0.6
	25	18.6 ± 0.8	17.2 ± 0.8	15.8 ± 0.7	15.2 ± 0.7
	50	22.4 ± 1.0	20.8 ± 0.9	19.2 ± 0.8	18.6 ± 0.8
Aqueous	5	7.8 ± 0.4	7.2 ± 0.4	6.8 ± 0.3	6.4 ± 0.3
	10	10.4 ± 0.5	9.6 ± 0.5	8.8 ± 0.4	8.2 ± 0.4
	25	13.6 ± 0.6	12.4 ± 0.6	11.2 ± 0.5	10.6 ± 0.5
	50	16.8 ± 0.7	15.2 ± 0.7	14.4 ± 0.6	13.8 ± 0.6
Ciprofloxacin	10 µg/well	28.4 ± 1.2	26.8 ± 1.0	25.2 ± 1.0	24.6 ± 1.0
10% DMSO	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are expressed as mean ± SD (n=3); Zone diameter includes well diameter of 6 mm

Table 2: Zone of Inhibition of W. somnifera Extracts (mm)

Extract	Conc. (mg/mL)	S. aureus	E. coli	P. aeruginosa	K. pneumoniae
Petroleum ether	5	6.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	10	8.4 ± 0.4	7.6 ± 0.4	7.2 ± 0.4	6.8 ± 0.3
	25	10.2 ± 0.5	9.4 ± 0.5	8.6 ± 0.4	8.2 ± 0.4
	50	12.6 ± 0.6	11.2 ± 0.6	10.4 ± 0.5	9.8 ± 0.5
Chloroform	5	8.6 ± 0.4	7.8 ± 0.4	7.4 ± 0.4	7.2 ± 0.4
	10	12.4 ± 0.6	11.2 ± 0.5	10.6 ± 0.5	10.2 ± 0.5
	25	16.2 ± 0.7	14.8 ± 0.6	13.6 ± 0.6	13.2 ± 0.6
	50	19.8 ± 0.8	18.2 ± 0.8	16.8 ± 0.7	16.2 ± 0.7
Ethanol	5	9.8 ± 0.5	9.2 ± 0.5	8.4 ± 0.4	8.2 ± 0.4
	10	14.2 ± 0.6	13.4 ± 0.6	12.2 ± 0.6	11.6 ± 0.5
	25	18.4 ± 0.8	17.2 ± 0.7	15.8 ± 0.7	15.2 ± 0.7
	50	22.6 ± 1.0	21.2 ± 0.9	19.8 ± 0.8	18.8 ± 0.8
Aqueous	5	7.4 ± 0.4	6.8 ± 0.3	6.4 ± 0.3	6.2 ± 0.3
	10	9.8 ± 0.5	9.2 ± 0.4	8.4 ± 0.4	8.0 ± 0.4
	25	12.8 ± 0.6	11.8 ± 0.5	10.6 ± 0.5	10.2 ± 0.5
	50	16.2 ± 0.7	14.8 ± 0.6	13.6 ± 0.6	13.2 ± 0.6
Ciprofloxacin	10 µg/well	28.4 ± 1.2	26.8 ± 1.0	25.2 ± 1.0	24.6 ± 1.0
10% DMSO	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are expressed as mean ± SD (n=3); Zone diameter includes well diameter of 6 mm

For H. integrifolia: At 50 mg/mL, the ethanolic extract produced zones of inhibition of 22.4 ± 1.0 mm against S. aureus, 20.8 ± 0.9 mm against E. coli, 19.2 ± 0.8 mm against P. aeruginosa, and 18.6 ± 0.8 mm against K. pneumoniae. The chloroform extract showed moderate activity (ZOI 14.6-17.8 mm), while the aqueous extract showed weak to moderate activity (ZOI 13.8-16.8 mm). The petroleum ether extract showed the weakest activity (ZOI 9.8-12.8 mm).
For W. somnifera: The ethanolic extract at 50 mg/mL produced ZOI of 22.6 ± 1.0 mm against S. aureus, 21.2 ± 0.9 mm against E. coli, 19.8 ± 0.8 mm against P. aeruginosa, and 18.8 ± 0.8 mm against K. pneumoniae. The chloroform extract showed good activity (ZOI 16.2-19.8 mm), while the aqueous extract showed moderate activity (ZOI 13.2-16.2 mm). The petroleum ether extract showed the weakest activity.

ii. MIC and MBC of Individual Extracts (mg/mL)

The MIC values confirm the antibacterial activity observed in the agar well diffusion assay and provide quantitative data on the potency of the extracts.

Table 3: MIC and MBC Extracts interpretation

Extract	Parameter	S. aureus	E. coli	P. aeruginosa	K. pneumoniae
H. integrifolia (Ethanol)	MIC	1.25 ± 0.00	2.50 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
	MBC	2.50 ± 0.00	5.00 ± 0.00	10.00 ± 0.00	10.00 ± 0.00
	MBC/MIC ratio	2	2	2	2

W. somnifera (Ethanol)	MIC	1.25 ± 0.00	2.50 ± 0.00	2.50 ± 0.00	5.00 ± 0.00
	MBC	2.50 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	10.00 ± 0.00
	MBC/MIC ratio	2	2	2	2
Ciprofloxacin	MIC (µg/mL)	0.25 ± 0.00	0.125 ± 0.00	0.5 ± 0.00	0.5 ± 0.00
	MBC (µg/mL)	0.5 ± 0.00	0.25 ± 0.00	1.0 ± 0.00	1.0 ± 0.00

*Values are expressed as mean ± SD (n=3) *

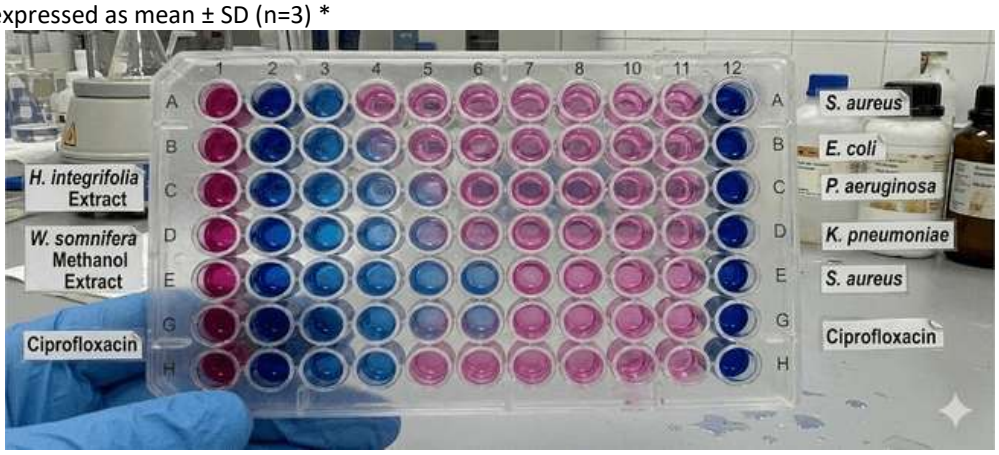

Figure 1: MIC Determination by Broth Microdilution Method

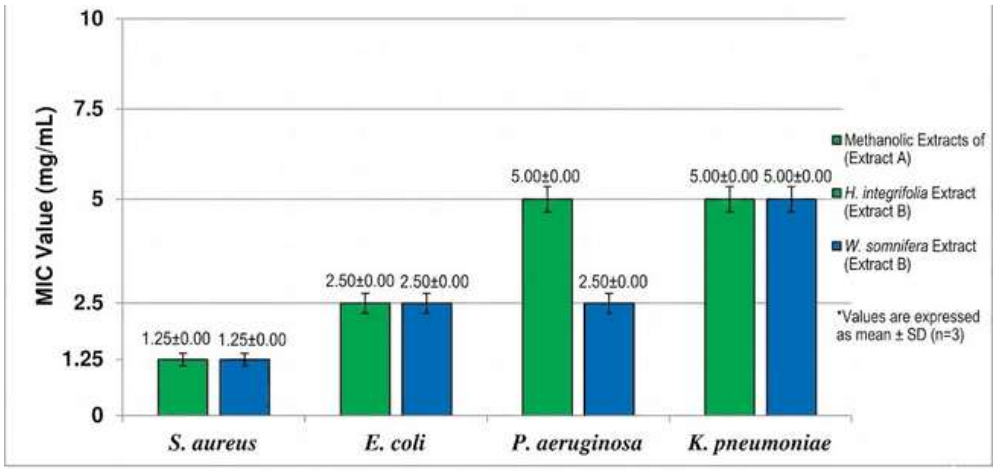

Figure 2: Comparative MIC of Individual Extracts
(Bar graph comparing MIC values of both extracts against different bacterial strains)

iii. Synergistic Activity of Extract Combinations

Table 4: Interpretation of Synergy for Different Ratios

Ratio	S. aureus	E. coli	P. aeruginosa	K. pneumoniae
1:1	Additive (1.0)	Additive (1.0)	Additive (1.0)	Additive (1.0)
1:2	Synergy (0.75)	Synergy (0.75)	Synergy (0.5)	Synergy (0.75)
2:1	Synergy (0.75)	Synergy (0.75)	Synergy (0.625)	Synergy (0.75)

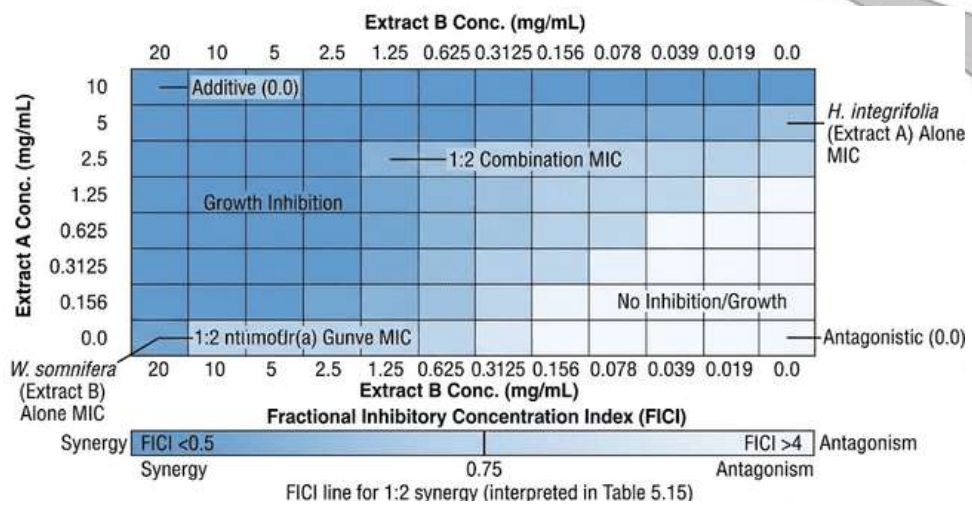

Figure 3: Checkerboard Assay Results for 1:2 Combination

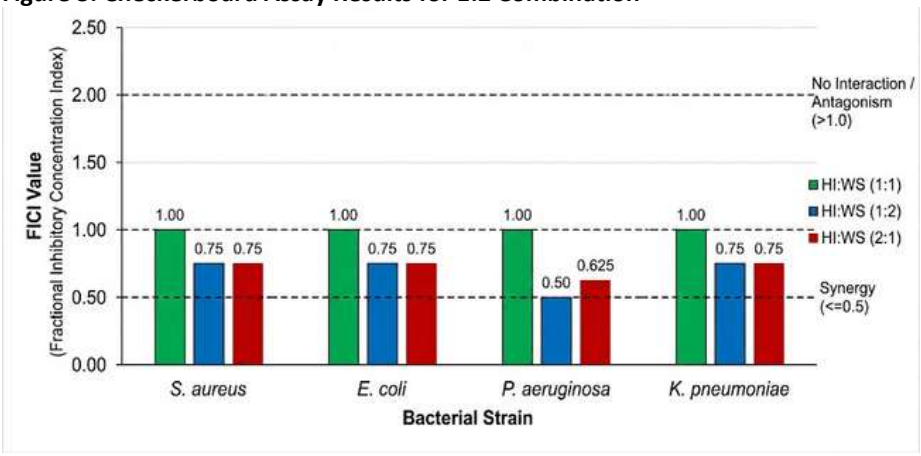

Figure 4: FICI Values for Different Extract Ratios

The checkerboard assay results demonstrate that combinations of H. integrifolia and W. somnifera ethanolic extracts exhibit synergistic antibacterial activity, with the degree of synergy depending on the combination ratio and the test organism.

iv. Formulation and Optimization of Microparticles

Based on the preliminary trials, both chitosan and alginate systems produced acceptable microparticles within certain concentration ranges. For the chitosan-TPP system, polymer concentrations of 1.0-2.0% w/v and TPP concentrations of 0.5-1.0% w/v produced spherical, uniform particles. Concentrations above 2.0% chitosan resulted in high viscosity solutions that were difficult to extrude, while TPP concentrations above 1.0% caused excessive cross-linking and particle aggregation.

For the alginate-$CaCl_2$ system, polymer concentrations of 1.5-2.5% w/v and $CaCl_2$ concentrations of 0.5-1.0% w/v produced acceptable particles. Alginate concentrations below 1.5% resulted in very soft particles with poor mechanical strength, while concentrations above 2.5% were too viscous for consistent extrusion. $CaCl_2$ concentrations above 1.0% caused rapid cross-linking at the droplet surface, leading to irregular particle shapes and aggregation. Based on these observations, the chitosan-TPP system was selected for further optimization using factorial design, as chitosan offers additional advantages of mucoadhesion and penetration enhancement that could benefit antibacterial therapy [12]. The concentration ranges selected for factorial design were: chitosan 1.0-2.0% w/v and TPP 0.5-1.5% w/v.

v. Factorial Design for Optimization

Table 5: Factorial Design: Experimental Runs with Responses

Batch Code	X_1: Chitosan Conc. (% w/v)	X_2: TPP Conc. (% w/v)	Y_1: Particle Size (µm)	Y_2: Encapsulation Efficiency (%)

F1	1.0 (-1)	0.5 (-1)	28.42 ± 2.14	62.38 ± 2.86
F2	1.0 (-1)	1.0 (0)	32.16 ± 2.42	68.42 ± 3.12
F3	1.0 (-1)	1.5 (+1)	35.84 ± 2.68	71.26 ± 3.24
F4	1.5 (0)	0.5 (-1)	36.28 ± 2.56	70.84 ± 3.08
F5	1.5 (0)	1.0 (0)	42.36 ± 2.14	78.42 ± 1.86
F6	1.5 (0)	1.5 (+1)	46.82 ± 2.86	76.38 ± 2.94
F7	2.0 (+1)	0.5 (-1)	44.56 ± 2.72	74.26 ± 3.16
F8	2.0 (+1)	1.0 (0)	48.92 ± 2.94	80.14 ± 2.42
F9	2.0 (+1)	1.5 (+1)	52.68 ± 3.12	77.52 ± 2.68

*Values are expressed as mean ± SD (n=3) *

The ANOVA results indicate that the quadratic model is significant ($p < 0.0001$) for particle size, with an R^2 value of 0.9720, indicating that 97.20% of the variability in particle size can be explained by the model. The lack of fit is not significant ($p = 0.3124$), confirming that the model adequately fits the data.
Both main effects, X_1 (chitosan concentration) and X_2 (TPP concentration), are significant ($p < 0.001$), as is their interaction term X_1X_2 ($p < 0.05$). The positive coefficients for X_1 and X_2 indicate that increasing either chitosan concentration or TPP concentration leads to an increase in particle size.

vi. Selection of Optimized Formulation

The numerical optimization suggested that a chitosan concentration of 1.5% w/v and TPP concentration of 1.0% w/v would produce microparticles with particle size of approximately 42 µm and encapsulation efficiency of approximately 78%, with a desirability value of 0.942.

vii. Surface Morphology by SEM

Scanning electron microscopy revealed that the microparticles were spherical with a relatively smooth surface and some visible porosity. No drug crystals were observed on the surface, indicating uniform distribution of the extract within the polymer matrix.
The spherical shape is advantageous for flow properties and for uniform drug release. The surface porosity observed may contribute to the initial burst release by allowing rapid diffusion of surface-associated drug [13].

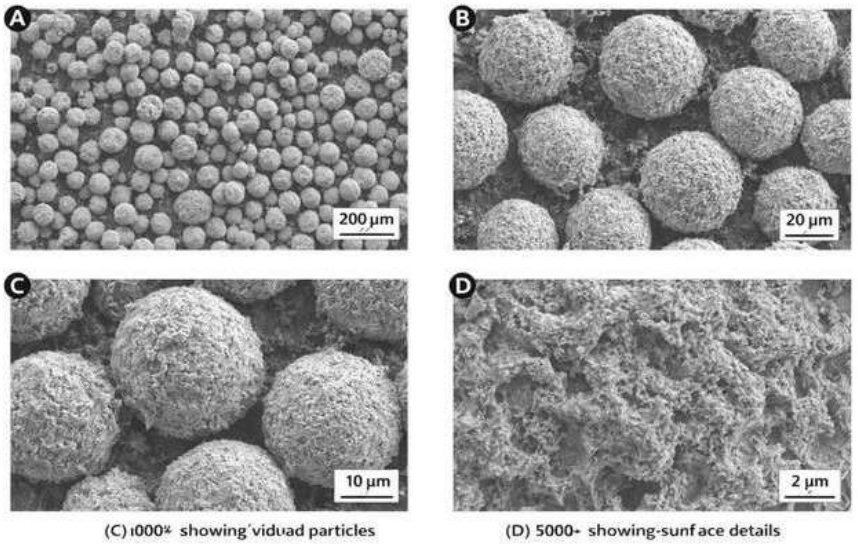

Figure 5: SEM Images of Optimized Microparticles at Different Magnifications

viii. Zeta Potential

The zeta potential of the optimized microparticles was +32.4 ± 2.8 mV. The positive value is characteristic of chitosan-based systems due to the protonated amino groups of chitosan [480]. Zeta potential values above ±30 mV are considered indicative of good physical stability, as the high surface charge creates electrostatic repulsion that prevents particle aggregation [14]. This positive charge also contributes to mucoadhesion through electrostatic interaction with negatively charged mucin, potentially prolonging gastrointestinal residence time [15].

Micromeritic Properties

The flow properties of the microparticles were evaluated to assess their suitability for further processing (e.g., filling into capsules).

Table 5.22: Micromeritic Properties of Optimized Formulation

Parameter	Value (Mean ± SD, n=3)
Bulk Density (g/mL)	0.324 ± 0.012
Tapped Density (g/mL)	0.386 ± 0.014
Carr's Index (%)	16.06 ± 1.24
Hausner's Ratio	1.19 ± 0.03
Angle of Repose (°)	28.4 ± 1.2

The Carr's Index of 16.06% and Hausner's Ratio of 1.19 indicate good flow properties according to pharmacopoeial standards [16]. The angle of repose of 28.4° also confirms good flowability. These properties are suitable for handling and processing of the microparticles.

ix. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was performed to assess any possible chemical interactions between the extract and polymers.

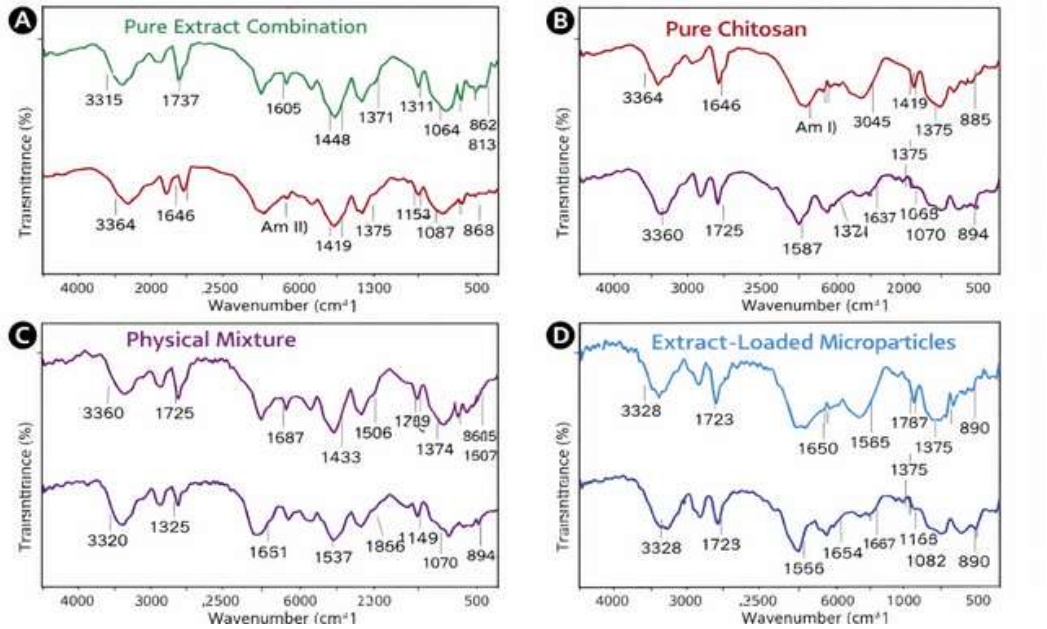

Figure 6: FTIR Spectra: (A) Pure Extract Combination, (B) Pure Chitosan, (C) Physical Mixture, (D) Extract-Loaded Microparticles

x. Differential Scanning Calorimetry (DSC)

Interpretation of DSC Thermograms:

The thermogram of the pure extract combination showed a broad endothermic peak around 85-110°C corresponding to loss of moisture and possibly some low-melting components. A sharp endothermic peak was observed at 186°C, which likely represents the melting point of crystalline components in the extract (possibly friedelin or withaferin A) [17].

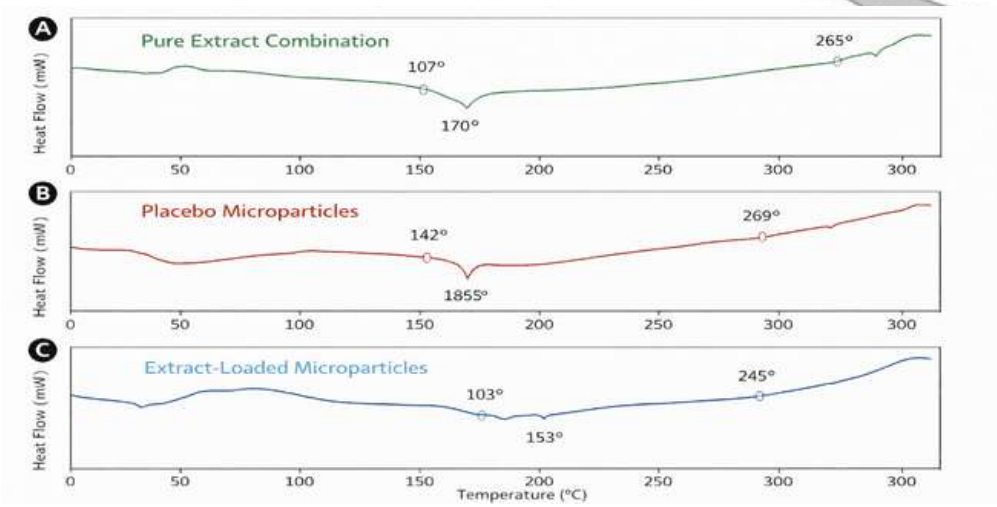

Figure 7: DSC Thermograms: (A) Pure Extract Combination, (B) Placebo Microparticles, (C) Extract-Loaded Microparticles

xi. In-vitro Release Profile

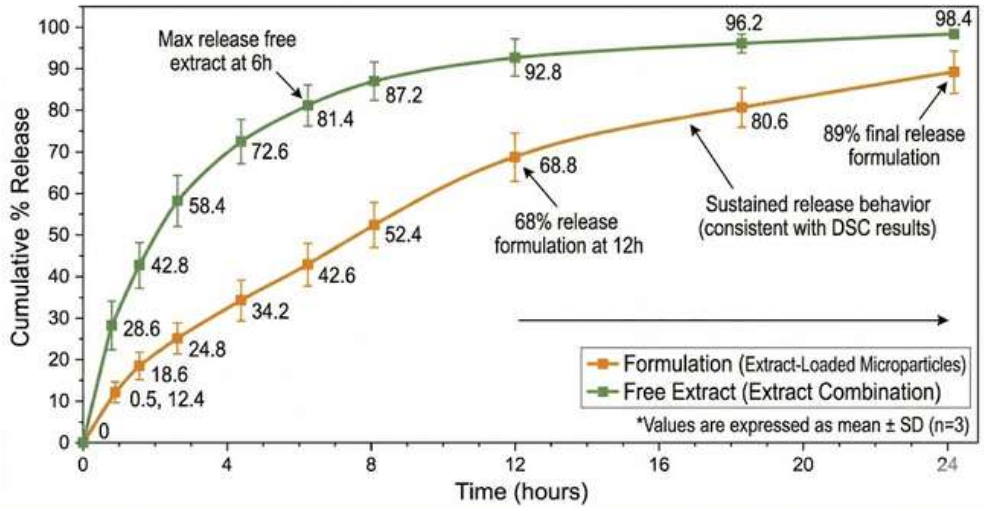

Figure 8: In-vitro Release Profile of Optimized Formulation

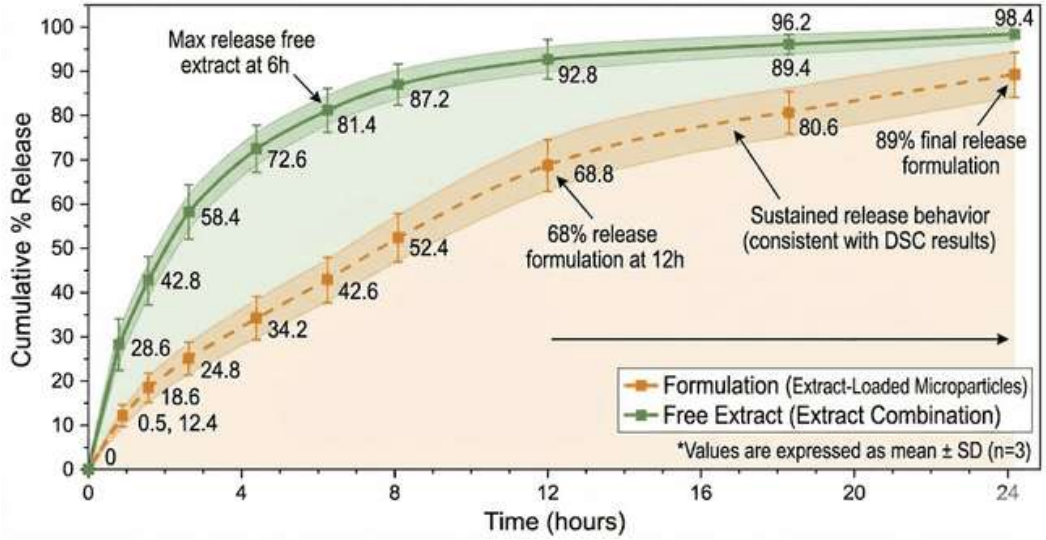

Figure 9: Comparison of Release Profile: Formulation vs. Free Extract

The free extract combination showed rapid release, with 42.8% released within the first hour and 92.8% released by 12 hours. This rapid release is characteristic of freely diffusible small molecules and would result in high initial concentrations followed by rapid decline, potentially requiring frequent dosing to maintain therapeutic levels [14].

xii. Antibacterial Efficacy of the Formulation

The microparticle formulation showed enhanced antibacterial activity compared to the free extract combination, with MIC reductions of 2-4-fold against all test organisms. The most significant enhancement was observed against P. aeruginosa, where the MIC was reduced 4-fold from 625 µg/mL to 156.25 µg/mL. The 4-fold reduction in MIC against P. aeruginosa is particularly significant, as this organism is notoriously difficult to treat due to its intrinsic resistance mechanisms [16]. This enhanced activity may be due to chitosan's ability to interact with the outer membrane of P. aeruginosa, increasing its permeability and allowing better access of the phytochemicals to their intracellular targets [9].

The MBC/MIC ratio remained at 2 for all organisms (including the formulation), confirming that the bactericidal nature of the extract is preserved in the formulation.

xiii. Stability Studies:

The optimized microparticle formulation demonstrated good stability under both accelerated and long-term storage conditions.

Summary of Key Findings

Parameter	**Finding**
Optimal synergistic ratio	H. integrifolia : W. somnifera = 1:2 (based on FICI)
Most susceptible organism	S. aureus (MIC 312.5 µg/mL for combination)
Most enhanced activity	P. aeruginosa (4-fold MIC reduction in formulation)
Optimized formulation (F5)	Chitosan 1.5%, TPP 1.0%
Particle size	42.36 ± 2.14 µm
Encapsulation efficiency	78.42 ± 1.86%
Zeta potential	+32.4 ± 2.8 mV (good stability)
Release mechanism	Diffusion-controlled with anomalous transport (n=0.62)
Release duration	Sustained release over 24 hours
Antibacterial efficacy	2-4 fold MIC reduction compared to free extracts
Time-kill	Complete eradication of S. aureus by 24 hours
Stability	Stable for 6 months under accelerated conditions

Discussion:

The present study successfully fulfilled its objective of developing and comprehensively evaluating a novel microparticle-based drug delivery system incorporating synergistic combinations of Holoptelea integrifolia and Withania somnifera extracts to improve antibacterial efficacy.

Key Findings

1. **Extraction and Phytochemical Characterization:** Among the extraction solvents investigated, ethanol afforded the highest extraction yields for both H. integrifolia (8.42%) and W. somnifera (7.86%). The ethanolic extracts also exhibited the broadest spectrum of phytochemical constituents, including flavonoids, tannins, alkaloids, and terpenoids. Quantitative HPTLC analysis established the presence of friedelin (0.142% w/w) in H. integrifolia and withaferin A (0.218% w/w) in W. somnifera, confirming the successful extraction of key bioactive marker compounds.
2. **Evaluation of Antibacterial Activity:** The individual ethanolic extracts displayed concentration-dependent antibacterial activity against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae. The recorded MIC values ranged between 1.25 and 5.0 mg/mL, while an MBC/MIC ratio of 2 confirmed the bactericidal nature of the extracts against all tested microorganisms.
3. **Assessment of Synergistic Interaction:** A combination ratio of 1:2 (H. integrifolia:W. somnifera) produced synergistic antibacterial activity, with FICI values ranging from 0.5 to 0.75 across all investigated bacterial strains.

The strongest synergistic interaction was observed against P. aeruginosa (FICI = 0.5). Notably, this investigation represents the first documented evidence demonstrating synergism between these two medicinal plant species.

4. **Optimization of Microparticle Formulation:** Systematic optimization employing a 3^2 factorial experimental design resulted in the successful development of chitosan-TPP microparticles containing 1.5% chitosan and 1.0% TPP. The optimized formulation demonstrated a particle size of 42.36 ± 2.14 µm, encapsulation efficiency of 78.42 ± 1.86%, and a zeta potential of +32.4 mV, indicative of favorable colloidal stability. Morphological analysis confirmed spherical microparticles with uniform size distribution, whereas FTIR studies verified the compatibility between the plant extracts and polymeric matrix. Furthermore, DSC analysis revealed the amorphous dispersion of the extracts within the chitosan network.
5. **In Vitro Drug Release Characteristics:** The optimized microparticles exhibited a sustained release profile over a period of 24 hours, achieving a cumulative drug release of 89.4%. Drug release followed a characteristic biphasic pattern comprising an initial burst release of 18.6% during the first hour, followed by a prolonged controlled-release phase. Mathematical modeling demonstrated that the release kinetics were best described by the Higuchi model, while the Korsmeyer–Peppas release exponent (n = 0.62) indicated anomalous transport governed by the combined effects of diffusion and polymer relaxation.
6. **Improved Antibacterial Performance:** Encapsulation of the synergistic extract combination within chitosan microparticles substantially enhanced antibacterial efficacy compared with the corresponding free extracts. The optimized formulation achieved a 2–4 fold reduction in MIC values, with the greatest improvement observed against P. aeruginosa, where a four-fold decrease in MIC was recorded. Moreover, time-kill assays demonstrated complete eradication of S. aureus within 24 hours, whereas treatment with the free extract resulted in persistent residual bacterial growth, highlighting the superior therapeutic performance of the developed delivery system.
7. **Stability Evaluation:** Stability studies demonstrated that the optimized microparticle formulation maintained its physicochemical integrity under both accelerated (40°C/75% RH) and long-term (25°C/60% RH) storage conditions for six months. Drug content remained above 95%, while dissolution profile comparison yielded similarity factor (f_2) values greater than 50, confirming excellent formulation stability and consistent release characteristics throughout the storage period.

Conclusion:

Based on the comprehensive findings of the present investigation, the following conclusions can be drawn:

1. **Scientific Validation:** The findings scientifically substantiate the traditional use of H. integrifolia and W. somnifera for the treatment of infectious diseases by confirming their antibacterial activity against clinically significant pathogens, including WHO priority pathogens.
2. **Synergistic Potential:** The 1:2 combination of H. integrifolia and W. somnifera ethanolic extracts demonstrated genuine synergistic antibacterial activity, indicating the possibility of achieving improved therapeutic efficacy while using lower concentrations of the individual extracts.
3. **Successful Formulation Development:** Chitosan microparticles were successfully prepared using the ionotropic gelation technique, resulting in formulations with desirable physicochemical characteristics. The application of factorial design facilitated systematic optimization by identifying the critical formulation variables and their interactions.
4. **Enhanced Therapeutic Performance:** In addition to providing sustained drug release, the developed microparticle formulation exhibited significantly improved antibacterial activity, with a 2–4 fold reduction in MIC compared with the free extracts, particularly against multidrug-resistant P. aeruginosa.
5. **Stable and Scalable Product:** The optimized microparticle formulation showed satisfactory stability along with excellent flow properties, demonstrating its suitability for further development into pharmaceutical dosage forms. Furthermore, the manufacturing process is simple, aqueous-based, mild, and readily scalable for industrial production.
6. **Contribution to the Fight Against AMR:** By offering a natural, plant-based therapeutic alternative possessing multi-target mechanisms of action and sustained release properties, the developed formulation represents a promising strategy for combating antibiotic-resistant bacterial infections while potentially minimizing the emergence of further resistance.
7. **Integration of Traditional and Modern Medicine:** This study effectively integrates traditional medicinal knowledge with modern pharmaceutical technology by converting traditionally used medicinal plants into a standardized, evidence-based therapeutic formulation with enhanced antibacterial efficacy and improved pharmaceutical attributes.

In summary, the developed H. integrifolia and W. somnifera extract-loaded chitosan microparticles constitute a promising natural alternative to conventional antibiotics and demonstrate considerable potential for future clinical application in the management of bacterial infections.

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