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Original Article

Development and Optimization of Cholesterol-Coated Phytosomes of *Asparagus racemosus* Methanolic Extract: Characterization and Antidiabetic Potential

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ABSTRACT

Background: The presence of potential Phytoconstituents in Asparagus racemosus makes it plant of vital therapeutic importance. **Purpose:** The efficacy of the phytoconstituents is less due to poor aqueous solubility and bioavailability. To enhance the bioavailability and efficacy of poorly soluble compounds in methanolic extract, synthesis of cholesterol-coated phytosomes was carried out. **Materials and Methods:** Extraction was carried out using a Soxhlet apparatus using methanol as the solvent. The phytosomes were prepared by the ether injection method. The spectral, morphological and thermal analysis was carried out for the characterization of the nanoparticle. It was further evaluated for *in vitro* release and anti-diabetic activities in comparison to the extract. **Results:** The nanoparticles have a mean particle size of 454.2±8.10 nm, zeta potential -42.80±7.5 mV, and a polydispersity index of 0.33. The spherical morphology with a concentric bilayer structure was confirmed by a microscopic study. FTIR and thermal analyses indicated chemical compatibility and good thermal stability. **Conclusion:** The optimized formulation at 1:1:1 exhibited sustained release behavior. It also enhanced therapeutic efficacy compared to the crude extract, due to improved permeability of the major adenosine-based purine alkaloid.

Keywords: *Asparagus racemosus*, Cholesterol, *In vitro* Anti-Diabetic Activity, Morphological Study, Particle size distribution, Span 60, Surface Charge, Thermo Gravimetric Analysis.

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INTRODUCTION

In the Ayurvedic system of medicine, *Asparagus racemosus* Willd., of the family Asparagaceae, reflects its long-standing reputation for enhancing female reproductive health and vitality. Traditionally, the plant has been employed as a rejuvenating tonic, aphrodisiac, and lactation enhancer. The roots are rich in biologically active constituents, including flavonoids, alkaloids, polysaccharides, and steroidal saponins, most notably shatavarins I-IV, which collectively contribute to its therapeutic properties (Goyal *et al*., 2003).

Pharmacological investigations have demonstrated that Shatavari exhibits potent anti-inflammatory, antioxidant, anti-ulcer, and immunomodulatory activities. Its ability to attenuate stress-induced physiological responses has often been compared with that of Panax ginseng (Sharma *et al*., 2018). Clinically, the plant has shown a favorable therapeutic profile in managing female reproductive conditions such as menopausal symptoms, premenstrual syndrome, and inadequate lactation (Mandal *et al*., 2006). Evidence from randomized controlled trials further supports its galactagogue activity (Alok *et al*., 2013). Additionally, the mucilaginous nature of the roots, along with their antioxidant capacity, has been associated with enhanced gastric ulcer healing (Jeevanandam *et al*., 2018; Sangeetha *et al*., 2021). Immunological studies also suggest stimulation of phagocytic function and lymphocyte proliferation, underscoring its immune-enhancing potential. These multifaceted pharmacological actions have driven growing interest in incorporating Shatavari into modern herbal formulations and functional food products (Meena *et al*., 2022).

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Despite these benefits, many bioactive phytochemicals derived from plant extracts tend to lose efficacy within the harsh gastric environment. The development of phytosomes has attracted considerable attention owing to elevated surface volume ratio, enhanced physicochemical stability, and improved gastrointestinal resilience. They have extensive applications in drug delivery systems, biomedical diagnostics (Sharma *et al.*, 2019). The phytosomes possess distinct functional attributes for their broad-spectrum activity, TDD and photothermal therapy (Xu *et al.*, 2016).

The present study aimed to enhance the anti-diabetic potential of the methanolic extract by phytosomal formulation. Comprehensive characterization was conducted to assess particle size distribution, morphological features, stability and *in vitro* release study. Finally, the therapeutic potential of the optimized phytosomes was investigated through *in vitro* anti-diabetic assays in comparison to the extract.

MATERIALS AND METHODS

Collection of Plant Material and Extraction

Fresh stems of *Asparagus racemosus* were collected from Ibrahimpatnam, Telangana, India (16.5811°N, 77.7489°E; 373 m above mean sea level). The plant material was botanically authenticated by the Department of Botany, Osmania University, Hyderabad.

Preparation of Extract

The Shead dried stem powder was moistened with petroleum ether (60-80°C) and subjected to Soxhlet extraction. Approximately 300 g of the powder was packed in a 100-mesh muslin cloth and extracted with 1 L of methanol until the siphon tube ran clear. The methanolic extract was concentrated using a rotary evaporator (Rotavapor R-300, Buchi, India) at 55°C under reduced pressure with a rotation speed of 50 rpm. The resulting residue was further dried in a desiccator until constant weight, and percentage yield was calculated (Rasheed *et al.*, 2023).

Phytochemical Screening

Qualitative phytochemical analysis of the methanolic extract was conducted following standard literature protocols to identify the presence of bioactive secondary metabolites (Chaudhary *et al.*, 2014).

Synthesis of Phytosomes

The phytosomes were prepared by the ether injection method. In a round-bottom flask, 1g of cholesterol and a varying amount of Span 60 Table 1 were dissolved in 50 mL of diethyl ether. In a 50 mL conical flask, 1 g of extract was dissolved in 20 mL of phosphate buffer (pH 7.4). The ether mixture was transferred at a rate of 1.0 mL / min. Mixed both the solutions using a Remi magnetic stirrer 2 MLH from Mumbai, India. The resulting mixtures were refluxed for 1 hr at 60-65°C with constant stirring. The organic solvent was eliminated using a rotary flash evaporator (Cyberlab Corporation, CR^{2}001, Delhi, India) operating at 65°C and 50 rpm. The aqueous phase was homogenised for 2 hr at 8000 rpm (Remi homogenizer, RQT-1275, Mumbai, India). The mixtures were sonicated at 10°C for 30 min at 30 sec intervals (Citizen Digital ultrasonicator, CD4820). The formulation was kept between 2°C and 8°C in a brown glass container until it was evaluated (Kumar *et al.*, 2023)

Entrapment Efficiency

Using a Remi cooling centrifuge (Remi, India), a freshly made nanoparticle suspension (1 mL) was centrifuged for 10 min at 15,000 rpm. A UV-visible spectrophotometer (Labman LMSPUV-1920) was used to measure absorbance (Kumar *et al.*, 2023). The mean±standard deviation was used to express the results. The formula was used to determine entrapment efficiency (%EE).

$$\% \text{ EE} = \frac{\text{Total drug content} - \text{Drug present in supernatant}}{\text{Total drug content}} \times 100$$

Particle Size, Zeta Potential, and PDI

Particle size distribution, Polydispersity Index (PDI), and zeta potential were evaluated using a Malvern Zetasizer (Malvern Instruments, UK). Measurements were conducted at 25±2°C with a scattering angle of 90°C. Refractive index and absorption values were set at 1.330 and 0.050, respectively (Ramadan *et al.*, 2020). Zeta potential measurements were performed using an electrode cuvette. All analyses were conducted in triplicate.

Environmental Scanning Electron Microscopy

An FEI Quanta 200 (Hillsboro, North America, USA) was used for the ESEM study. The samples were placed on the conducting copper tap in two drops (40 µL) and allowed to air dry. A 10 nm-thick platinum sputter coating was applied to each sample, and it was examined under a microscope at 2500 X magnification (Kumar *et al.*, 2023).

Transmission Electron Microscopy

A JEM-2100F transmission electron microscope (Tokyo, Japan) was used to evaluate the improved nanoparticles' morphology. A drop of nanoparticle suspension was placed on a 200 mesh carbon-coated copper grid prior to imaging, excess solvent was removed with filter paper, and samples were left to air dry (Kumar *et al.*, 2023).

FTIR Analysis

A Bruker Vertex-80 FTIR (Germany) equipped with Hyperion-3000 microscope was used. Approximately 1 mg of extract and 1 mL of nanoparticle sample were analyzed using Micro-ATR mode over a scanning range of 4000-450 $cm^{-1}$with a resolution of 0.2 cm^{-1}.

Thermal Analysis

Thermal stability and weight loss were assessed using a Hitachi NEXTA STA-300 simultaneous thermogravimetric analyzer. A ceramic crucible containing around 28.34 mg of the material was heated from 30°C to 800°C at a rate of 10°C/0.2 s in a nitrogen environment (Maryana *et al.*, 2015).

In vitro Drug Release Study

The dialysis bag diffusion method was used for the *in vitro* drug release study. A dialysis bag diffusion method was used for the *in vitro* drug release investigation. The USP Dissolution Tester (TDT-08L, Electrolab, India) filled with 900 mL of phosphate buffer (pH 6.8) and maintained 37.5±0.5°C, a pretreated dialysis membrane (Dialysis Membrane-60, flat width: 25.27 mm, average diameter: 15.9 mm, capacity approximately 1.99 mL/cm, Mumbai, India) that contained 5 mL of the formulation (equivalent to 250 mg extract) and was agitated at 50 rpm. To maintain sink conditions, 5.0 mL samples were taken out and replaced with new buffer at prearranged intervals. A proven UV spectroscopic approach was used to quantify drug release, and the % CDR was computed. Zero-order, first-order, Higuchi, and Korsmeyer-Peppas models were used to examine release kinetics; the model with the highest correlation coefficient (R^2) was deemed to be the best fit. The results are shown as mean±standard deviation, and each experiment was conducted in triplicate (Kumar *et al.*, 2023).

In vitro Anti-diabetic Activity

The antidiabetic efficacy of the standard (Acrabose), methanolic extract, and nanoparticles was investigated *in vitro* through enzyme inhibition-based screening models. The efficacy was examined at concentrations of 50, 75, and 100 µg/mL (Ononamadu *et al.*, 2019).

The test samples were incubated with the α-amylase (1 U/mL) in phosphate buffer (pH 6.8) for 10 min at 37°C. Starch solution (1%) was added to start the enzymatic reaction, and kept for 15 min. Dinitrosalicylic acid reagent was used to stop the reaction, and heated for 5 min in a water bath. Absorbance at 540 nm was measured after cooling, and inhibitory activity was computed.

The inhibitory assay was conducted using p-nitrophenyl-α-D-glucopyranoside as the chromogenic substrate. The α-glucosidase enzyme solution (1 U/mL) was pre-incubated with varying concentrations of the test samples at 37°C. p-nitrophenyl-α-D-glucopyranoside was introduced to initiate the reaction and kept for a 20 min incubation period. Sodium carbonate solution (0.2 M) was added to terminate the reaction. The amount of liberated p-nitrophenol was quantified spectrophotometrically at 405 nm, and enzyme inhibition was expressed as a percentage relative to the control.

Statistical analysis

Each study was performed in triplicate and data were represented as Mean±SD. The GraphPad Prism software 8.0.2 was used for the statistical analysis. One-way Analysis of Variance (ANOVA). *post hoc* Dunnett's test was used to compare the control and test substances ($p \leq 0.05$ considered significant).

RESULTS

Development of formulations

The ether injection approach was used to create the phytosomes filled with the methanolic extract. Table shows the composition of every phytosome.

Optimization of Phytosomes

The phytosomes exhibited optimal stability at physiological pH (7.2). High-speed homogenization at 10,000 rpm facilitated nanoscale particle formation. The concentrations of lipids, surfactant, and extract at 1:1:1 (w/v) resulted in stable formation.

Entrapment Efficiency

Entrapment Efficiency (EE) is a critical parameter reflecting the extent of bioactive compound encapsulation within the nanoparticle matrix (Shaker *et al.*, 2020). EE values were found to be 56.33±1.75% to 94.00±2.88%. The result confirmed that the increasing concentration of lipid entrapment efficiency increased Figure 1. Increased lipid content influenced particle size, surface morphology, and release kinetics due to stronger ionic interactions.

Particle Size Distribution and Zeta Potential

Particle size and surface charge were strongly influenced by lipid and surfactant concentration. An increase in lipid content led to larger particle sizes and reduced colloidal stability (Refai *et al.*, 2017). The data is represented in Table 2. The study showed an increase in the lipid concentration, the particle size increased, as well as stability and polydispersity. At the high concentration of lipid (1.25 g), the stability and polydispersity decreased. The formulation SMP4 exhibited a mean particle size of 454.2±8.01 nm, zeta potential value- 42.80±7.5 mV, indicating high

Table 1: Composition of Phytosomes.

Ingredients (g)	SMP1	SMP2	SMP3	SMP4	SMP5
Extract	1	1	1	1	1
Cholesterol	1	1	1	1	1
Span 60	0.25	0.50	0.75	1	1.25

Table 2: **Vesicle size, zeta potential and polydispersity index of nanoparticles.**

Formulation	Z average (d.nm)±SD	Zeta potential (mV)±SD	PdI
SMP1	654.4±4.15	- 20.13±3.2	0.29
SMP2	595.5±6.14	- 28.12±3.4	0.30
SMP3	542.1±5.12	- 39.02±4.1	0.31
SMP4	454.2±8.01	- 42.80±7.5	0.33
SMP5	512.3±7.13	-32.08±7.1	0.37

All the studies carried out in triplicate and the data are represented in Mean±SD.

Table 3: **Concentration-dependent *in vitro* antidiabetic activity of standard drug, plant extract and nanoparticles.**

Sample	Concentration (µg/mL)	α-Amylase Inhibition (%)	α-Glucosidase Inhibition (%)	IC_{50} (µg/mL)
Acarbose	50	72.15±1.12***	75.48±1.08***	18.6
	75	80.34±1.05***	83.67±0.97***	
	100	88.45±0.92***	91.28±0.88***	
Methanolic Extract	50	48.62±1.76*	51.30±1.69*	42.3
	75	56.14±1.58**	58.92±1.47**	
	100	64.22±1.33**	67.48±1.25**	
SMP4	50	59.74±1.44**	62.38±1.36**	28.9
	75	68.52±1.31**	71.84±1.24**	
	100	77.90±1.18***	80.65±1.10***	

Each study was carried out in triplicate and data were presented as Mean±SEM. $p<0.001$) was considered as significant.

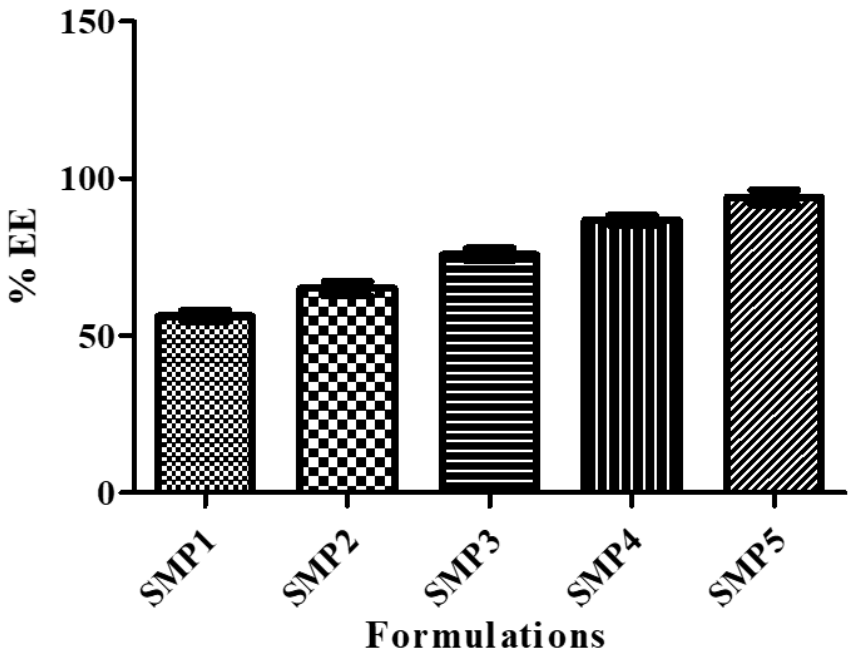

Figure 1: Entrapment efficiency of the phytosomes.

electrostatic stability. The Polydispersity Index (PDI) was 0.33, suggesting polydispersity of the formulation Figures 2 and 3.

SEM and TEM analysis

The morphology of the phytosome is a key determinant of biological performance, particularly cellular uptake and biodistribution. Figures 4a-4b revealed uniform, spherical particles with a concentric bilayer architecture, confirming successful nanoscale formulation and structural integrity (Kumar *et al.*, 2023).

FTIR Spectroscopic Analysis

To examine functional group interactions and verify the production of nanoparticles, FTIR spectroscopy was utilized

Figure 5. The FTIR spectrum of the SPM extract showed characteristic absorption bands at 3366 cm^{-1} (N-H str), 2923 cm^{-1} (C-H stretching), 1636 cm^{-1} (C=O stretching), 1422 cm^{-1} (C-N stretching), and 1027 cm^{-1}(C-O stretching). Cholesterol exhibited prominent peaks at 2921 cm^{-1} and 2852 cm^{-1} (C-H str), 1053 cm^{-1} (C-O str). Span showed a significant peak at cm^{-1} (C-O-C of ester) along with stretching bands between 1100-1240 cm^{-1} (C-O, str). The phytosomal formulation retained all major peaks with slight shifts and reduced O-H intensity, indicating hydrogen bonding and hydrophobic interactions and confirming successful phytosome formation without chemical incompatibility (Haeri *et al.*, 2014).

Thermal Stability Analysis

The TGA profile Figure 6 demonstrated minimal mass loss (2.3%) from an initial weight of 28.34 mg up to 800°C, indicating high thermal stability and negligible volatilization. DTA thermograms Figure 7 revealed an endothermic event at -77.3°C corresponding to the loss of physically adsorbed moisture. Additional endothermic peaks at 145.2°C, -109.9°C, and -150.1°C were attributed to residual solvent removal, dehydration of surface hydroxyl groups, and low-temperature structural rearrangement, respectively. These findings confirm the excellent thermal stability and storage robustness of the optimized phytosomes (Debata *et al.*, 2025).

In vitro release study

The data was represented in Figure 8. The formulations SMP1, SMP2, SMP3 exhibited initial release of 42.39±2.17% to 50.39±1.89%. The initial release was decreased for SMP 4 and SMP 5 to 52.35±1.93 and 57.92±1.87%, respectively. The maximum cumulative release at 12 hr was observed at 83.39±1.93 (SMP1), 86.58±1.29 (SMP2), 90.37±1.64 (SMP3), 95.24±1.91 (SMP4), and 98.38±1.48 (SMP5), respectively. However, because of its low water solubility, the pure extract only showed 51.9±1.93% in 12 hr. At low concentrations of surfactant, the release rate was found to be lower due to a lack of an initial burst. The cumulative release for formulation SMP4 exhibited sustained release property. Because of the higher concentration of surfactant, SMP5 had the fastest and largest release. The increased extract release from phytosomes is due to drug surface deposition and improved solubility due to lipid and surfactant. They reduced the interfacial tension between the aqueous and lipid phases (Gannu *et al.*, 2011).

Drug release kinetics

Plotting the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models in Microsoft Excel. The drug release kinetics of the optimized formulation (SMP4) were carried out Figure 9. Compared to other models, the Higuchi model has a higher maximum R^2 (R^2=0.967). Thus, the kinetic release model that fits the data the best is Higuchi. The diffusion mode of drug release is shown by the release exponent of 0.503 (Kumar *et al.*, 2023).

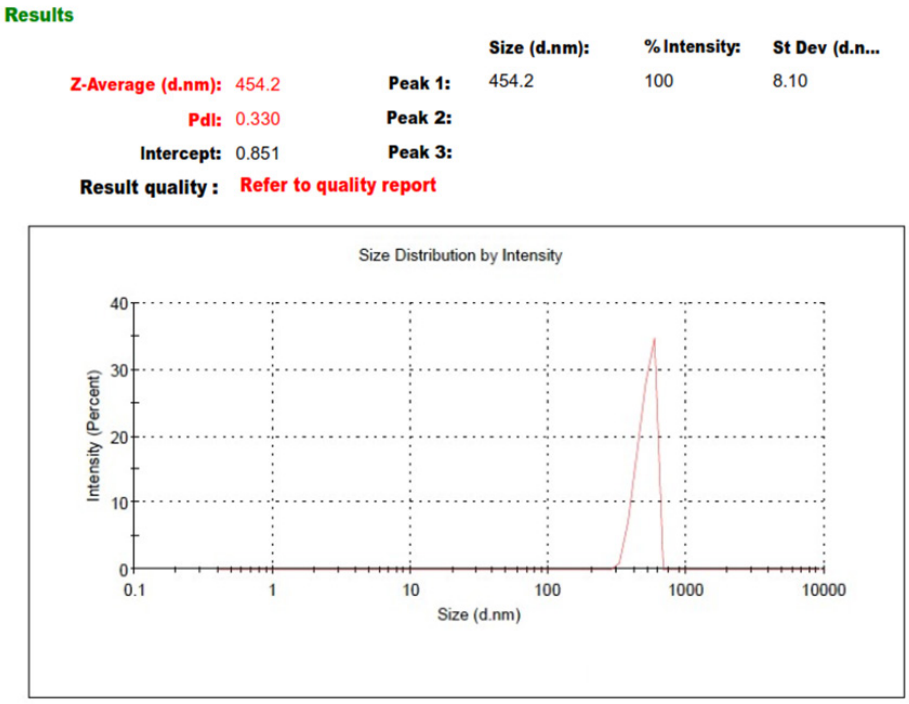

Figure 2: Particle size of the optimized phytosome SMP4.

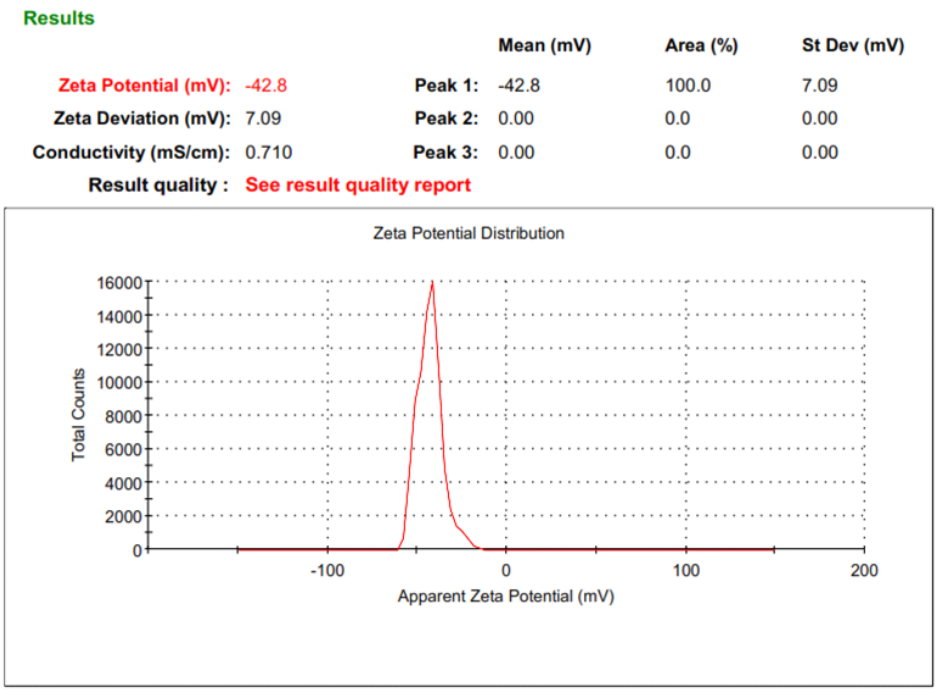

Figure 3: Zeta potential of the optimized phytosome SMP4.

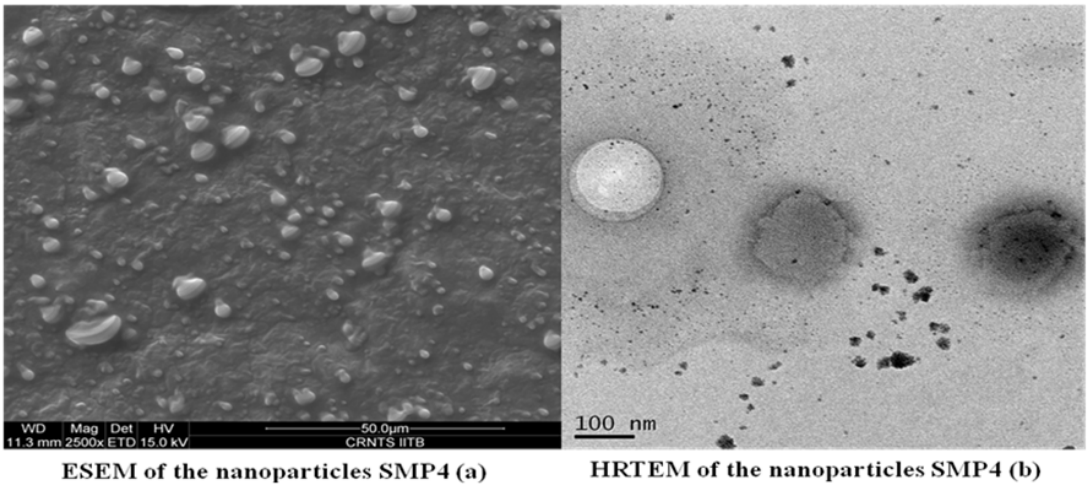

Figure 4: Morphological study of phytosomes.

In vitro Anti-diabetic Activity

Regulation of postprandial glucose excursions through suppression of digestive enzymes involved in carbohydrate breakdown is an established pharmacological approach in diabetes management. In the present work, the inhibitory capacity of a plant-derived extract and its nanoparticle formulation was examined against α-amylase and α-glucosidase and benchmarked using acarbose as a reference inhibitor (Juarez *et al.*, 2015).

The acrabose displayed pronounced inhibitory effects across the tested concentration range, with enzyme suppression exceeding two-thirds at the lowest concentration and approaching complete inhibition at the highest-level Table 3. Its low IC_{50} value (18.6 µg/mL) reflects strong enzyme affinity and confirms the suitability of the assay system. The plant extract exhibited a measurable inhibitory response, with enzyme suppression increasing progressively with concentration. However, the higher IC_{50} value (42.3 µg/mL) indicates reduced inhibitory efficiency, which may arise from physicochemical constraints such as limited solubility, instability of active constituents, or restricted interaction with enzyme catalytic domains.

Encapsulation of the plant extract into phytosome resulted in a marked enhancement of enzyme inhibition. The optimized formulation achieved inhibition levels exceeding 75% for both enzymes at the highest concentration, accompanied by a substantial reduction in IC_{50} (28.9 µg/mL). The improved performance may be attributed to enhanced surface availability

of bioactive molecules, improved dispersion within the reaction medium, and prolonged enzyme inhibitor contact time. These factors collectively favor stronger inhibitory interactions. These findings highlight the potential of nanotechnology-assisted delivery strategies to overcome intrinsic limitations of crude phytochemicals and support further investigation through *in vivo* validation and safety assessment (Patil *et al.*, 2024).

DISCUSSION

Asparagus racemosus was traditionally employed for the management of diverse pathological conditions. Classical and contemporary literature consistently attribute its therapeutic potential to immune-modulatory, anti-diabetic, antioxidant, antiulcer, and anti-diarrheal activities, largely derived from its phytochemical composition. The present investigation, focused on enhancing the therapeutic potential of non-polar bioactive constituents identified from the methanolic extract, with particular attention to improving bioavailability and pharmacological effectiveness (Sen *et al.*, 2024).

GC-MS profiling revealed a high abundance of 4-(((9-(5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-6-yl)amino)methyl) benzoic acid, as purine alkaloid. structurally analogous to nucleoside. *In silico* analyses demonstrated favorable drug-likeness, acceptable pharmacokinetic parameters, and promising bioactivity scores, particularly against G-Protein-Coupled Receptor (GPCR) ligands (Sen *et al.*, 2025).

The substituted purine alkaloid suggests modulation of adenosine-dependent signaling pathways that regulate insulin sensitivity and glucose metabolism. The activation of AMP-activated protein kinase enhanced peripheral glucose uptake and reduced hepatic gluconeogenesis. Structural features such as the modified ribose and benzoic acid moieties are improve metabolic stability and receptor interactions. Moreover, the compound may confer β-cell protection through attenuation of oxidative stress and inflammatory signaling. Together, these mechanistic insights support this molecule as a promising antidiabetic lead and suggest further pharmacokinetic and clinical investigation (Chayah *et al.*, 2024).

The identified molecule exhibited poor aqueous solubility, limited distribution coefficient, and reduced oral bioavailability. To overcome phytosomes were developed using cholesterol as a lipid and Span 60 as a surfactant. Entrapment efficiency increased with increasing Span concentration, reaching a maximum at the 1:1:1 ratio due to optimal vesicle stabilization, while lower ratios showed poor encapsulation and higher levels caused slight leakage. Particle size decreased with increasing surfactant concentration up to 1:1:1, attributed to improved interfacial stabilization, with minor polydispersity observed at 1:1:1.25. Morphological analysis revealed that formulations with moderate Span levels formed well-defined, spherical vesicles, whereas low or excessive surfactant produced irregular structures. Thermal studies indicated enhanced stability at moderate Span concentrations, while very low or high surfactant levels reduced membrane rigidity and thermal resistance (Doost *et al.*, 2020).

The phytosomes SPM 4 (1:1:1) (phytoconstituent:cholesterol:Span) demonstrated optimal structural integrity, indicating balanced lipid-drug complexation and membrane stabilization. The low concentration of surfactant exhibited a reduced release rate due to high sustainability and minimal burst. The high

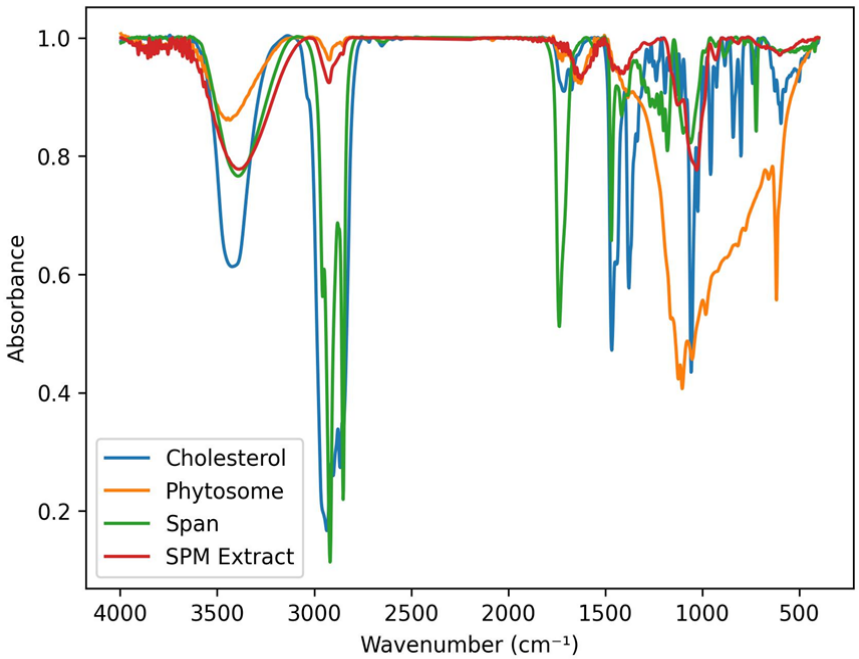

Figure 5: Overlay FTIR spectra of extract, excepient and optimized phytosomes SMP4.

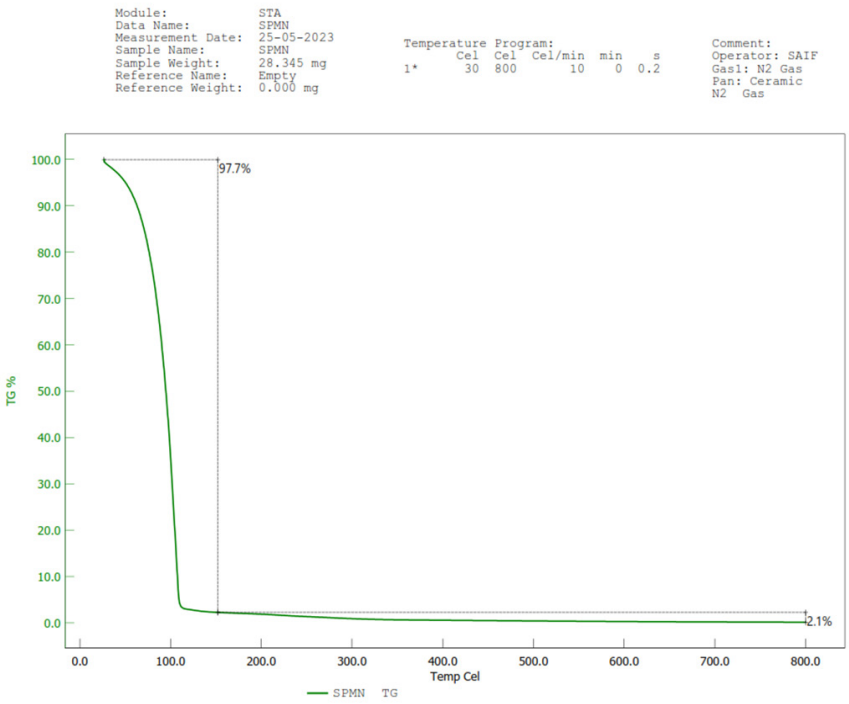

Figure 6: TGA graph of optimized phytosomes SMP4.

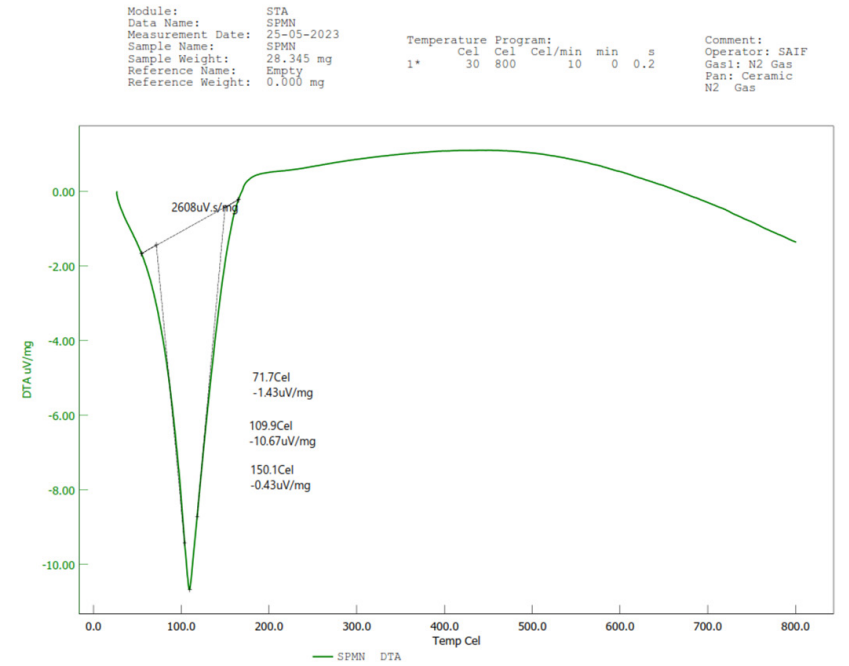

Figure 7: DTA graph of optimized phytosomes SMP4.

surfactant concentration (1:1:1.25) gives faster and higher release rate due to enhanced membrane permeability and increased solubilization of the phytoconstituent. Cholesterol contributed to reduced bilayer permeability, while an equimolar Span concentration ensured uniform dispersion without disrupting the phytosomal matrix. This ratio supported controlled drug release and minimized premature leakage, suggesting suitability for sustained delivery (Singh *et al.*, 2023).

The phytosomal formulation exhibited enhanced anti-diabetic activity compared to extract. The optimized ratio ensured high entrapment efficiency and stable vesicle formation, leading to improved solubility and intestinal permeability of the bioactive constituents. Controlled and sustained release from the phytosome contributed to prolonged hypoglycemic effect, likely through better systemic availability. Overall, the optimized formulation SMP4 demonstrated significant improvement of anti-diabetic efficacy of Satavari extract.

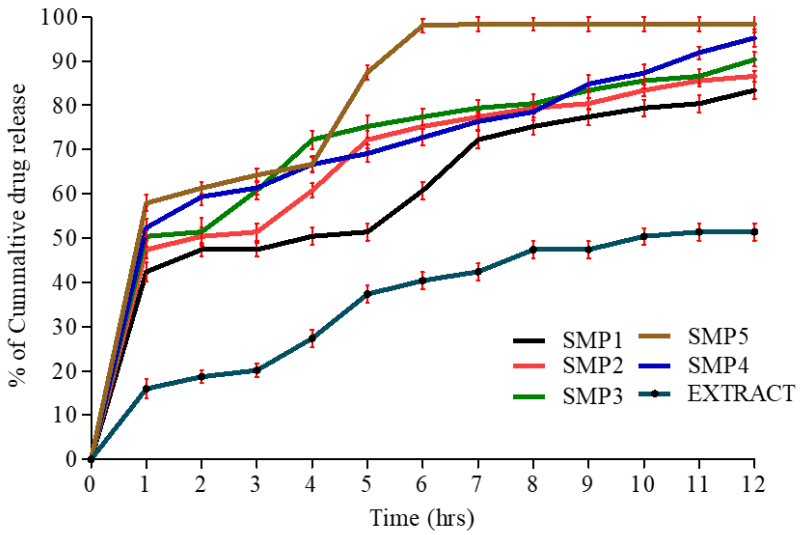

Figure 8: *In vitro* release study of the phytosomes.

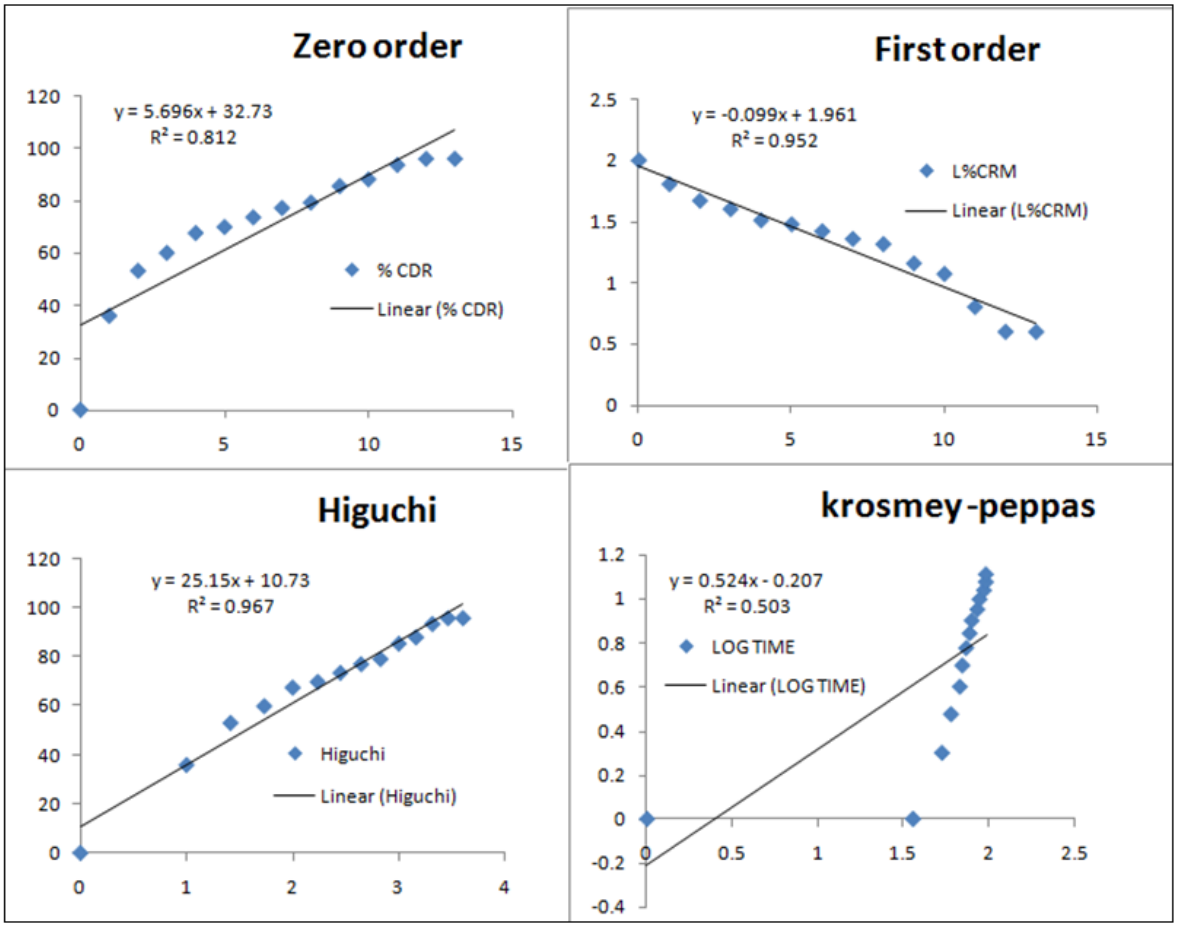

Figure 9: *In vitro* release kinetics of the optimized phytosomes SMP4.

CONCLUSION

This study confirms the enhancement of anti-diabetic potential of *Asparagus racemosus* through phytosomal formulation. Phytosomes prepared using cholesterol and Span 60 significantly improved these limitations. The optimized formulation SMP4 showed optimal entrapment efficiency, uniform spherical vesicles, thermal stability, and controlled drug release. This optimized formulation enhanced solubility, intestinal permeability, and sustained hypoglycemic activity compared to the plain extract. Overall, phytosomal delivery effectively improved the antidiabetic efficacy of *A. racemosus*, supporting its potential for further pharmacokinetic and clinical development.

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ABBREVIATIONS

TDD: Targeted Drug Delivery; **ºC:** Degree Celsius; **L:** Liter; **g:** Gram; **rpm:** Revolutions per minute; **mL/min:** Milliliters per minute; **UV:** Ultraviolet; **ESEM:** Environmental Scanning Electron Microscopy; **HRTEM:** High-Resolution Transmission Electron Microscopy; **FTIR:** Fourier Transform Infrared Spectroscopy; **Mg:** Milligram; **mm:** Millimeter; **USP:** United States Pharmacopeia; **µg/mL:** Microgram per milliliter; **U/mL:** Units per milliliter; **SEM:** Scanning Electron Microscopy; **ANOVA:** Analysis of Variance; **Mv:** Millivolt; **TGA:** Thermogravimetric Analysis; **DTA:** Differential Thermal Analysis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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