



Chitosan Nanoparticle Delivery of *Prunus africana* (Hook.f) Kalkman Attenuates TGF- β /SMAD3-Driven Epithelial–Mesenchymal Transition to Suppress Prostate Cancer Metastasis

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Abstract

Background: One of the major drivers of metastatic prostate cancer is epithelial-mesenchymal transition (EMT), which is tightly controlled by TGF- β /SMAD3 signaling. *Prunus africana* contains active phytochemicals with anticancer potential, but its poor solubility and limited bioavailability may hamper its efficacy in clinical use. **Aim:** This study assessed the effect of delivering *P. africana* extract using chitosan nanoparticles on TGF- β /SMAD3-mediated EMT and metastasis in prostate cancer cells. **Methods:** *P. africana*-loaded chitosan nanoparticles were prepared by ionic gelation using sodium tripolyphosphate and characterized for particle size, polydispersity index, zeta potential, encapsulation efficiency, morphology, and release profile. Antimetastatic effects were evaluated by assessing prostate cancer cell viability, wound healing, Transwell migration and invasion, qRT-PCR, Western blotting, and immunofluorescence. TGF- β -induced EMT was induced using 10 ng/mL recombinant TGF- β 1. An in vivo metastasis model was used to confirm metastatic burden and pathway modulation. **Results:** Average physical properties were as follows: average diameter, 168.4 ± 12.6 nm; polydispersity index, 0.23 ± 0.04 ; zeta potential, $+31.7 \pm 3.2$ mV; and entrapment efficiency, $82.6 \pm 4.8\%$. Release from the nanoparticles reached 71.3% in 72 h. *P. africana*-loaded chitosan nanoparticles significantly inhibited TGF- β 1-induced cell migration by 64.8% and cell invasion by 69.2% relative to the TGF- β 1-treated group (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). The treatment also reduced the number of lung metastases by 58.7% compared with the metastatic control group ($n = 6$ animals per group). Furthermore, the treatment suppressed TGF- β -induced SMAD3 phosphorylation, downregulated the expression of N-cadherin, vimentin, Snail, Slug, and Twist, and restored E-cadherin expression. In vivo studies demonstrated a significant reduction in metastatic lesions and pSMAD3 expression following nanoparticle treatment. **Conclusion:** Chitosan nanoparticle-mediated delivery enhanced the antimetastatic effects of *P. africana* by suppressing TGF- β /SMAD3-mediated EMT, highlighting its potential as a nanophytotherapeutic strategy for the treatment of prostate cancer metastasis.

Keywords:

Prunus africana; chitosan nanoparticles; prostate cancer; TGF- β /SMAD3; epithelial–mesenchymal transition

1. Introduction

Prostate cancer is among the most frequently diagnosed malignancies in men worldwide, and metastatic progression remains the major determinant of poor clinical outcome [1]. Localized prostate cancer can often be treated effectively

with curative-intent therapies such as surgery, radiation therapy, androgen-deprivation therapy, and other systemic approaches. However, advanced disease becomes invasive, therapy-resistant, and prone to metastasize [2], typically to bone and lymph nodes. The conversion of localized

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tumor growth to metastatic disease involves coordinated remodeling of cell adhesion, cytoskeletal organization, extracellular matrix (ECM) interactions, immune escape, and survival under anchorage-independent conditions [1,3].

Epithelial–mesenchymal transition (EMT) is a key driver of cancer progression [4,5]. During this process, cancerous epithelial cells lose their polarity and cell-to-cell adhesion while acquiring mesenchymal characteristics that promote migration, invasion, stemness, and resistance to apoptosis. In prostate cancer, EMT is linked to disease aggressiveness, metastatic onset, therapy resistance, and poor prognosis. These transformations are marked by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as N-cadherin and vimentin, along with factors such as Snail, Slug, Twist, and matrix metalloproteinases [6].

One of the major pathways driving EMT during cancer progression is transforming growth factor- β (TGF- β) signaling [7,8]. In normal and early-stage prostate epithelial cells, TGF- β exerts tumor-suppressive effects by inhibiting proliferation and inducing apoptosis. In advanced prostate tumors, however, cancer cells become resistant to TGF- β -induced growth inhibition while retaining its proinvasive and prometastatic activities [8,9]. This context-dependent switch enables TGF- β to promote epithelial–mesenchymal transition, migration, invasion, immune evasion, and metastatic colonization. SMAD3 is a critical downstream mediator of canonical TGF- β signaling and plays a prominent role in the transcriptional regulation of EMT-associated genes [10,11].

Natural products are among the most important sources of anticancer agents, as their bioactive constituents interfere with multiple pathways of oncogenic transformation [12,13]. *Prunus africana*, a medicinal plant widely used in African traditional medicine, has long been used for the management of prostate-related conditions. Earlier reviews report that *P. africana* is rich in phytochemicals such as sterols, triterpenoids, phenolic compounds, and fatty acids, with documented antiandrogenic, antiangiogenic, antiproliferative, and pro-apoptotic activity in prostate cancer models [14–16]. However, like many plant-derived extracts, its therapeutic potential is limited by poor aqueous solubility, variable stability, low bioavailability, and non-selective tissue distribution.

Nanoparticle-based drug delivery offers a promising strategy for enhancing the therapeutic performance of phytochemicals. Chitosan, a biodegradable, biocompatible, cationic polysaccharide, has found wide application in drug and phytochemical delivery. Chitosan nanoparticles can be synthesized by ionic gelation using sodium tripolyphosphate, a straightforward process that avoids harsh organic solvents while enabling nanoscale encapsu-

lation of bioactive compounds. By improving solubility, cellular uptake, protection from biotransformation, and sustained release, chitosan-based nanoparticles have attracted considerable research interest as a delivery platform for phytochemicals [17–19].

Despite its traditional medicinal use, the potential of a chitosan nanoparticle formulation to enhance the antimetastatic effects of *P. africana* against prostate cancer through modulation of TGF- β /SMAD3-mediated EMT has not been fully explored. This study therefore aimed to prepare and characterize *P. africana*-loaded chitosan nanoparticles and evaluate their effects on prostate cancer cell migration, invasion, EMT, and metastasis in vivo. We hypothesized that the chitosan nanocarrier would increase intracellular availability of *P. africana* bioactives, thereby suppressing the TGF- β 1/SMAD3 signaling pathway responsible for EMT and inhibiting metastasis.

2. Materials and Methods

2.1. Materials, Chemicals, and Apparatus

Low-molecular-weight chitosan (deacetylation degree $\geq 85\%$) and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (LC–MS grade), formic acid, gallic acid, quercetin, Folin–Ciocalteu reagent, aluminum chloride, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and other analytical-grade reagents were obtained from Merck KGaA (Darmstadt, Germany), unless otherwise stated. Ultrapure water was generated using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution, trypsin–EDTA, and phosphate-buffered saline, were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Recombinant human transforming growth factor-beta 1 (TGF- β 1) was purchased from PeproTech (Rocky Hill, NJ, USA). Particle size, polydispersity index, and zeta potential were measured using a Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK). Morphological characterization was carried out using a scanning electron microscope (SEM; JSM-IT200, JEOL Ltd., Tokyo, Japan). UV–Visible spectrophotometric measurements were performed using a UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Freeze-drying was conducted using a Labconco Free Zone lyophilizer (Labconco Corporation, Kansas City, MO, USA), while solvent removal was achieved using a rotary evaporator (Buchi R-300, Büchi Labortechnik AG, Flawil, Switzerland).

2.2. Plant Collection, Authentication, and Extraction

Fresh stem bark of *Prunus africana* was collected in 2026 during the morning hours (6:00–7:00 a.m.) from a forested area in Plateau State, Nigeria. The plant material was authenticated by Dr. C. A. Ukwubile of the Department of Pharmacognosy, University of Maiduguri, Nigeria. A voucher specimen (UMM/FPH/ROA/001) was deposited in the departmental herbarium for future reference. The collected stem bark was washed with distilled water to remove adhering debris, shade-dried at ambient temperature (25–28 °C) for 21 days, and pulverized using a laboratory grinder. The powdered material was extracted with 70% methanol (1:10 w/v) by cold maceration for 48 h with intermittent agitation. The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40 °C. The concentrated extract was subsequently freeze-dried and stored at –20 °C until further use.

2.3. Phytochemical Standardization

2.3.1. Phytochemical Analysis

Each extract batch underwent phytochemical standardization prior to nanoparticle formulation. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method and expressed as mg gallic acid equivalent (GAE)/g extract, while total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method and expressed as mg quercetin equivalent (QE)/g extract.

2.3.2. LC–MS Analysis

LC–MS analysis was performed using a high-performance liquid chromatography system coupled with a quadrupole time-of-flight mass spectrometer (LC–QTOF–MS) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved on a C18 reversed-phase column (150 × 2.1 mm, 1.7 µm particle size) maintained at 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile containing 0.1% formic acid). Elution was carried out using a gradient program of 5–95% solvent B over 30 min at a flow rate of 0.30 mL/min. The injection volume was 5 µL. Mass spectrometric detection was performed in both positive and negative ionization modes under the following conditions: capillary voltage, 3.5 kV; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas (nitrogen) flow rate, 800 L/h; cone gas flow rate, 50 L/h; nebulizer pressure, 35 psi; and collision energy, 10–40 eV. Data were acquired over a mass range of m/z 100–1500.

Mass calibration was performed using a standard calibration solution before analysis. Phytochemical constituents were identified by comparing their retention times, accurate masses, and fragmentation patterns with those of authentic reference standards and published databases.

2.3.3. Quantification of Marker Compounds

Quantification of β -sitosterol, ursolic acid, oleanolic acid, and ferulic acid was performed using external calibration curves generated from authenticated reference standards. Calibration curves exhibited good linearity ($R^2 > 0.995$) across the evaluated concentration ranges. All analyses were performed in triplicate.

2.3.4. Batch-to-Batch Repeatability

Three independent extract batches were analyzed under identical conditions. The relative standard deviation (RSD) values for retention times and peak areas of the principal marker compounds were less than 5%, confirming acceptable batch-to-batch reproducibility.

2.3.5. Microbial Quality

Microbial quality assessment was conducted according to the requirements of the current pharmacopeial guidelines, including the European Pharmacopeia and United States Pharmacopeia (USP <61> and USP <62>). Total aerobic microbial count, total yeast and mold count, and the absence of specified pathogenic microorganisms were confirmed to be within acceptable limits.

2.3.6. Heavy Metal Analysis

Heavy metal analysis was performed in accordance with the limits established by the World Health Organization (WHO) and relevant pharmacopeial standards. Levels of lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) were below the permissible limits for herbal medicinal materials.

Only extract batches that demonstrated consistent phytochemical profiles, acceptable batch-to-batch reproducibility, and compliance with microbial and heavy-metal specifications were selected for nanoparticle preparation [20,21].

2.4. Preparation of *Prunus africana*-Loaded Chitosan Nanoparticles

Prunus africana-loaded chitosan nanoparticles were prepared by the ionic gelation method using chitosan as the polymer and sodium tripolyphosphate (TPP) as the cross-linking agent. Low-molecular-weight chitosan was dis-

solved in 1% (v/v) acetic acid to obtain a 0.25% (w/v) chitosan solution. The pH of the solution was adjusted to 5.5 with 0.1 M sodium hydroxide, and the mixture was stirred overnight to ensure complete polymer hydration. For the optimized formulation (F5), 50 mg of freeze-dried *P. africana* extract was dispersed in 20 mL of the chitosan solution and stirred magnetically at 1000 rpm for 30 min at room temperature ($25 \pm 2^\circ\text{C}$) to obtain a homogeneous dispersion. Separately, a 0.10% (w/v) TPP solution was prepared in distilled water and filtered through a 0.45 μm membrane filter before use. Nanoparticles were formed by adding the TPP solution dropwise to the chitosan-extract dispersion at a chitosan: TPP mass ratio of 2.5:1. The TPP solution was added at a controlled rate of approximately 1 mL/min under continuous magnetic stirring at 1000 rpm. Following the complete addition of the cross-linking solution, the reaction mixture was stirred for 30 min at room temperature to ensure complete ionic gelation and nanoparticle stabilization. The resulting nanoparticle suspension was centrifuged at 15,000 rpm for 30 min at 4°C . The pellet was washed three times with distilled water to remove untrapped extract and excess TPP, followed by recentrifugation under the same conditions after each washing step. The purified nanoparticles were frozen at -80°C and subsequently lyophilized using a freeze dryer for 48 h to obtain a dry nanoparticle powder. The lyophilized nanoparticles were stored in airtight containers at 4°C until further characterization and biological evaluation. Blank chitosan nanoparticles were prepared using the same procedure without the addition of *P. africana* extract [17,19].

2.5. Nanoparticle Characterization

Mean particle size, polydispersity index, and zeta potential were acquired by dynamic light scattering. The morphology was observed by scanning electron microscopy. Encapsulation efficiency and loading capacity were determined after separating the free plant extract from the nanoparticles by centrifugation.

Encapsulation efficiency was calculated as:

$$\begin{aligned} & \text{Encapsulation efficiency(\%)} \\ &= \left[\frac{(\text{Total extract added} - \text{Free extract in supernatant})}{\text{Total extract added}} \right] \times 100 \end{aligned} \quad (1)$$

Loading capacity was calculated as:

$$\begin{aligned} & \text{Loading capacity(\%)} \\ &= \left[\frac{(\text{Total extract encapsulated})}{\text{Weight of nanoparticles}} \right] \times 100 \end{aligned} \quad (2)$$

The in vitro release profile was evaluated by suspending the nanoparticles in phosphate-buffered saline

(PBS) at pH 7.4 or buffer at pH 5.5 to simulate physiological and acidic tumor/endosomal conditions. At predetermined time points, samples were collected, centrifuged, and analyzed to determine the amount of released plant constituents [19].

2.5.1. Release Kinetic Modeling

To elucidate the release mechanism of *P. africana*-loaded chitosan nanoparticles, the cumulative in vitro release data obtained at pH 5.5 and pH 7.4 were fitted to various kinetic models, including Zero-order, First-order, Higuchi, and Korsmeyer-Peppas. The release data were analyzed using nonlinear regression analysis in GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA).

2.6. Cell Culture

The human prostate cancer cell line LNCaP and the normal prostate epithelial cell line RWPE-1 were obtained from the American Type Culture Collection (ATCC). LNCaP cells were cultured in RPMI medium, whereas RWPE-1 cells were cultured in DMEM, each supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 .

2.7. Experimental Treatment Groups

Cells were assigned to the following groups: untreated control; blank chitosan nanoparticles; free *P. africana* extract; *P. africana*-loaded chitosan nanoparticles; TGF- β alone; TGF- β plus free *P. africana* extract; TGF- β plus *P. africana*-loaded chitosan nanoparticles; and TGF- β plus a SMAD3 inhibitor (SIS3) or siSMAD3 as a mechanistic control. Free *P. africana* extract was tested at extract-equivalent concentrations of 25, 50, and 100 $\mu\text{g/mL}$, with chitosan nanoparticles tested at the corresponding nanoparticle-mass equivalents. Cells were treated with recombinant human TGF- β 1 at 10 ng/mL for 24–48 h to induce epithelial-mesenchymal transition. During mechanistic validation, some cells were treated with the SMAD3 inhibitor SIS3 (5 μM) or transfected with siSMAD3 before TGF- β 1 stimulation. Viability was assessed at 24, 48, and 72 h; migration and invasion were assessed at 24 and 48 h; and qRT-PCR, Western blotting, and immunofluorescence staining were performed after 24–48 h of treatment [22,23].

2.8. Cell Viability and Selectivity Assay

The cytotoxic effects of *P. africana* extract, blank chitosan nanoparticles, and *P. africana*-loaded chitosan nanoparticles were evaluated using the MTT cell viability assay.

Human prostate cancer cells and normal prostate epithelial cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 μ L of complete culture medium and allowed to attach for 24 h under standard culture conditions (37 °C, 5% CO₂, and 95% relative humidity). Following attachment, cells were treated with various concentrations of the test samples (0–200 μ g/mL) and incubated for 72 h. Untreated cells served as the negative control, while medium without cells served as the blank control. Following treatment, 20 μ L of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. The culture medium was then carefully removed, and the resulting formazan crystals were dissolved in 150 μ L of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader.

Cell viability was calculated according to the following equation:

$$\text{Cell Viability(\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100 \quad (3)$$

Dose–response curves were generated using non-linear regression analysis in GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA), and the half-maximal inhibitory concentration (IC₅₀) values were determined for each treatment.

The selectivity index (SI) was calculated to evaluate the preferential cytotoxicity of the formulations toward prostate cancer cells relative to normal prostate epithelial cells using the following equation:

$$\text{SI} = \frac{\text{IC}_{50}(\text{RWPE_1 normal prostate epithelial cells})}{\text{IC}_{50}(\text{LNCaP prostate cancer cells})} \quad (4)$$

An SI value greater than 2 was considered indicative of selective anticancer activity. All experiments were performed in triplicate and repeated at least three times independently [24–26].

2.9. Cellular Uptake Study

Fluorescein isothiocyanate (FITC)-labeled nanoparticles were incubated with prostate cancer cells for 24 h to evaluate cellular uptake. Cellular uptake was assessed by fluorescence microscopy and flow cytometry to detect accumulated FITC signal. Intracellular localization was further examined using organelle markers, including lysosomal and endosomal markers [26,27].

2.10. Wound-Healing Migration Assay

Cells were seeded in six-well plates and grown to approximately 90–100% confluence. A uniform linear scratch was created in each monolayer using a sterile 200 μ L pipette tip, followed by removal of detached cells by washing twice with phosphate-buffered saline. Cells were then cultured in serum-reduced medium containing 1% fetal bovine serum for the relevant experimental groups. Images of the wound area were captured at 0, 12, and 24 h using an inverted microscope, and the percentage of wound closure was calculated using ImageJ software relative to the initial wound width at 0 h [28,29].

2.11. Transwell Migration and Matrigel Invasion Assays

In the migration assay, the cultured cells were serum-starved, detached, and seeded in serum-free medium into the upper chambers of Transwell inserts. The lower chambers contained medium with 10% FBS as the chemoattractant. This assay was carried out for 24 h, at the end of which any non-migrated cells on the upper side of the membrane were gently wiped out. Migrated cells on the lower surface of the membrane were fixed with 4% formaldehyde for 15 min and subsequently stained with 0.1% crystal violet for 20 min. For the invasion assay, Transwell inserts were coated with Matrigel and allowed to polymerize for 1 h at 37 °C before cell seeding. Invaded cells were allowed to migrate for 48 h, after which they were fixed, stained, and counted in at least five randomly selected microscopic fields per insert. Migration and invasion rates were expressed as percentages relative to the untreated control [30].

2.12. Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from treated prostate cells by TRIzol™ extraction according to the manufacturer's protocol. RNA quantity and purity were assessed spectroscopically, followed by cDNA synthesis using a reverse-transcription kit. Real-time PCR was performed using SYBR Green qPCR Master Mix on a real-time PCR system to measure expression levels of CDH1, CDH2, VIM, SNAI1, SNAI2, TWIST1, MMP2, MMP9, TGFB1, SMAD3, and other relevant EMT-associated markers. Gene expression was normalized to GAPDH as the internal control, and relative fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method [25,27,31].

2.13. Western Blotting

Total protein was extracted, quantified, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary antibodies against E-cadherin, N-cadherin, vimentin, Snail, Slug, Twist, TGF- β receptor I, total SMAD3, phosphorylated SMAD3, and β -actin. Chemiluminescent protein bands were visualized, and band density was quantified by densitometry [22,29].

2.14. Immunofluorescence Staining

Treated cells were fixed, permeabilized, blocked, and incubated with antibodies against E-cadherin, vimentin, and phosphorylated SMAD3. Nuclei were counterstained with DAPI, and images were acquired by confocal fluorescence microscopy [32,33].

2.15. In Vivo Metastasis Study

Eight-week-old male athymic nude mice (20–25 g) were obtained from the institutional animal facility and maintained under standard laboratory conditions ($22 \pm 2^\circ\text{C}$, $55 \pm 10\%$ relative humidity, and a 12 h light/dark cycle) with free access to food and water. All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethical Committee (Approval No. FP/03/24/SP.9788). Experimental metastasis was established by intravenous injection of luciferase-expressing prostate cancer cells via the tail vein of the animals. Following tumor-cell inoculation, the animals were randomly assigned to five groups ($n = 6$ mice per group) as follows:

- **Group I (normal control):** Healthy mice receiving vehicle only (0.9% saline, 10 mL/kg, oral gavage), without tumor-cell inoculation.
- **Group II (metastatic control):** Tumor-bearing mice receiving vehicle only (0.9% saline, 10 mL/kg, oral gavage).
- **Group III (blank chitosan nanoparticles):** Tumor-bearing mice treated with blank chitosan nanoparticles at a dose equivalent to the nanoparticle carrier used in Group V (200 mg/kg, oral gavage).
- **Group IV (free *P. africana* extract):** Tumor-bearing mice treated with free *P. africana* extract (100 mg/kg body weight, oral gavage).
- **Group V (*P. africana*-loaded chitosan nanoparticles):** Tumor-bearing mice treated with *P. africana*-loaded chitosan nanoparticles containing an equiv-

alent *P. africana* extract dose of 200 mg/kg body weight (oral gavage).

Treatments were administered three times per week for six consecutive weeks. Body weight, clinical signs, tumor progression, and metastatic dissemination were monitored throughout the study. Tumor progression and metastatic dissemination were monitored using in vivo bioluminescence imaging and micro-computed tomography (micro-CT). At the end of the study, animals were euthanized, and major organs, including the lungs, liver, and tumor-bearing tissues, were collected for further analyses.

Metastatic burden was assessed by gross examination, histopathological evaluation, and tissue-specific tumor-marker analysis. In addition, immunohistochemical and molecular analyses were performed to evaluate the expression of epithelial–mesenchymal transition (EMT)-related proteins and components of the TGF- β /SMAD3 signaling pathway. The extent of metastatic lesions and treatment-associated histological changes was quantified and compared among the experimental groups [34–36].

2.16. Statistical Analysis

Data were expressed as mean $\pm$ SD ($n = 3$). Statistical comparisons were performed using one-way analysis of variance followed by Tukey's post hoc test or two-way analysis of variance where appropriate. Survival or metastasis-free outcomes were analyzed using Kaplan–Meier curves and the log-rank test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Phytochemical Contents

Phytochemical screening of three independent extract batches showed consistent and reproducible chemical profiles, with several key constituents clearly identified— β -sitosterol, ursolic acid, oleanolic acid, ferulic acid, stigmasterol, and campesterol—serving as the principal marker compounds. The extracts also showed appreciable total phenolic and flavonoid content, consistent with potential antioxidant and bioactive properties. Microbial screening detected no *E. coli* or *Salmonella* spp., and total aerobic microbial, yeast, and mold counts remained within acceptable limits. Heavy-metal analysis likewise confirmed low levels of lead, cadmium, arsenic, and mercury, all within permissible limits. Together, these results confirmed the suitability of the selected batch for nanoparticle preparation and subsequent biological evaluation (Table 1).

Table 1: Phytochemical content and quality fingerprints of *P. africana* nanoformulation.

Parameter	Method	Result	Unit	Interpretation
Total phenolic content	Folin–Ciocalteu assay	128.64 ± 4.72	mg gallic acid equivalent/g extract	High phenolic content
Total flavonoid content	Aluminum chloride colorimetric assay	76.38 ± 3.15	mg quercetin equivalent/g extract	Appreciable flavonoid content
β-Sitosterol	LC–MS	18.42 ± 0.86	mg/g extract	Major phytosterol marker detected
Ursolic acid	LC–MS	11.76 ± 0.54	mg/g extract	Major triterpenoid marker detected
Oleanolic acid	LC–MS	9.84 ± 0.41	mg/g extract	Triterpenoid marker detected
Ferulic acid	LC–MS	4.28 ± 0.19	mg/g extract	Phenolic acid marker detected
Campesterol	LC–MS	3.62 ± 0.22	mg/g extract	Minor phytosterol detected
Stigmasterol	LC–MS	5.47 ± 0.31	mg/g extract	Phytosterol marker detected
Total aerobic microbial count	Plate count method	1.8 × 10 ²	CFU/g	Within an acceptable limit
Total yeast and mould count	Plate count method	<1.0 × 10 ¹	CFU/g	Within an acceptable limit
<i>Escherichia coli</i>	Culture method	Not detected	CFU/g	Acceptable
<i>Salmonella</i> spp.	Culture method	Not detected	CFU/25 g	Acceptable
Lead	AAS/ICP–MS	0.42 ± 0.03	mg/kg	Within an acceptable limit
Cadmium	AAS/ICP–MS	0.06 ± 0.01	mg/kg	Within an acceptable limit
Arsenic	AAS/ICP–MS	0.09 ± 0.01	mg/kg	Within an acceptable limit
Mercury	AAS/ICP–MS	0.02 ± 0.01	mg/kg	Within an acceptable limit

Data are presented as mean ± SD (n = 3).

3.2. Characterization of Nanoparticles

Table 2 presents the composition and optimization profile of the *Prunus africana*-loaded chitosan nanoparticles, while Figure 1 illustrates the physicochemical characterization of the optimized formulation. Particle size, polydispersity index, zeta potential, encapsulation efficiency, and loading capacity were significantly impacted by differences in chitosan concentration, TPP concentration, chitosan: TPP ratio, and extract loading across the formulation batches. Higher concentrations increased the loading capacity but also promoted particle enlargement and reduced size uniformity, whereas formulations containing lower polymer and cross-linker concentrations produced smaller nanoparticles but exhibited comparatively lower extract encapsulation efficiency.

Because formulation F5 offered the best balance between particle size, homogeneity, surface charge, encapsulation efficiency, and loading capacity among the prepared batches, it was selected as the optimized nanoparticle formulation. F5 contained 50 mg of *P. africana* extract, 0.25% w/v chitosan, and 0.10% w/v TPP, at a chitosan: TPP ratio of 2.5:1, yielding nanoparticles with a mean particle size of 168.4 ± 12.6 nm, a polydispersity index of 0.23 ± 0.04, and a zeta potential of +31.7 ± 3.2 mV. The comparatively low polydispersity index indicates a narrow, homogeneous particle-size distribution, while the

positive zeta potential reflects the cationic character of chitosan and suggests strong colloidal stability. This positive surface charge may also enhance cellular uptake by facilitating contact with negatively charged cancer-cell membranes. With an encapsulation efficiency of 82.6 ± 4.8% and a loading capacity of 21.4 ± 1.6%, the optimized formulation further demonstrated efficient extract incorporation. Formulations F6–F8 produced larger particles with greater polydispersity, indicating a higher propensity for aggregation and reduced suitability for biological evaluation, despite their somewhat higher loading capacity. F5 was therefore selected as the optimal formulation for subsequent in vitro and in vivo studies, combining a strong positive surface charge with a narrow nanoscale size distribution, as shown in Figure 1A, B.

SEM morphological analysis showed predominantly spherical, discrete, smooth-surfaced particles with limited aggregation (Figure 1C). The optimized formulation showed efficient extract entrapment (Figure 1D). In vitro release displayed sustained, pH-responsive behavior over 72 h (Figure 1E): at physiological pH 7.4, total extract release reached 71.3 ± 5.5%, whereas at the more acidic pH of 5.5, release increased to 86.4 ± 5.8%. This greater release under acidic conditions suggests that the nanoparticles may preferentially release *P. africana* bioactive constituents within acidic tumor microenvironments or endosomal compartments.

Table 2: Formulations of *P. africana*-loaded chitosan nanoparticles: composition and optimization.

Formulation Code	Chitosan (% w/v)	TPP (% w/v)	Chitosan: TPP Ratio	<i>P. africana</i> Extract (mg)	Particle Size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Loading Capacity (%)	Remarks
F1	0.1	0.05	2:01	25	142.6 ± 8.4	0.31 ± 0.03	+24.8 ± 2.1	68.4 ± 3.7	12.6 ± 0.9	Small size but moderate loading
F2	0.15	0.05	3:01	25	158.2 ± 10.1	0.27 ± 0.02	+28.6 ± 2.4	74.9 ± 4.1	14.8 ± 1.1	Improved stability and loading
F3	0.2	0.05	4:01	25	181.7 ± 11.5	0.24 ± 0.03	+31.2 ± 2.7	79.5 ± 4.3	16.9 ± 1.2	Good particle uniformity
F4	0.2	0.1	2:01	50	214.3 ± 13.8	0.29 ± 0.04	+26.4 ± 2.3	77.8 ± 3.9	18.7 ± 1.4	Larger particles formed
F5	0.25	0.1	2.5:1	50	168.4 ± 12.6	0.23 ± 0.04	+31.7 ± 3.2	82.6 ± 4.8	21.4 ± 1.6	Optimized formulation
F6	0.3	0.1	3:01	50	236.8 ± 15.4	0.34 ± 0.05	+34.5 ± 3.5	84.1 ± 5.2	22.1 ± 1.8	High loading but reduced uniformity
F7	0.25	0.15	1.7:1	75	289.5 ± 18.7	0.41 ± 0.06	+21.9 ± 2.8	80.7 ± 4.6	24.3 ± 2.0	Aggregation tendency
F8	0.3	0.15	2:01	75	315.2 ± 21.3	0.46 ± 0.07	+19.6 ± 2.5	83.5 ± 5.0	25.8 ± 2.3	Large particles; less suitable

TPP stands for sodium tripolyphosphate, and PDI for polydispersity index. The values are reported as mean ± SD of triplicate measurements.

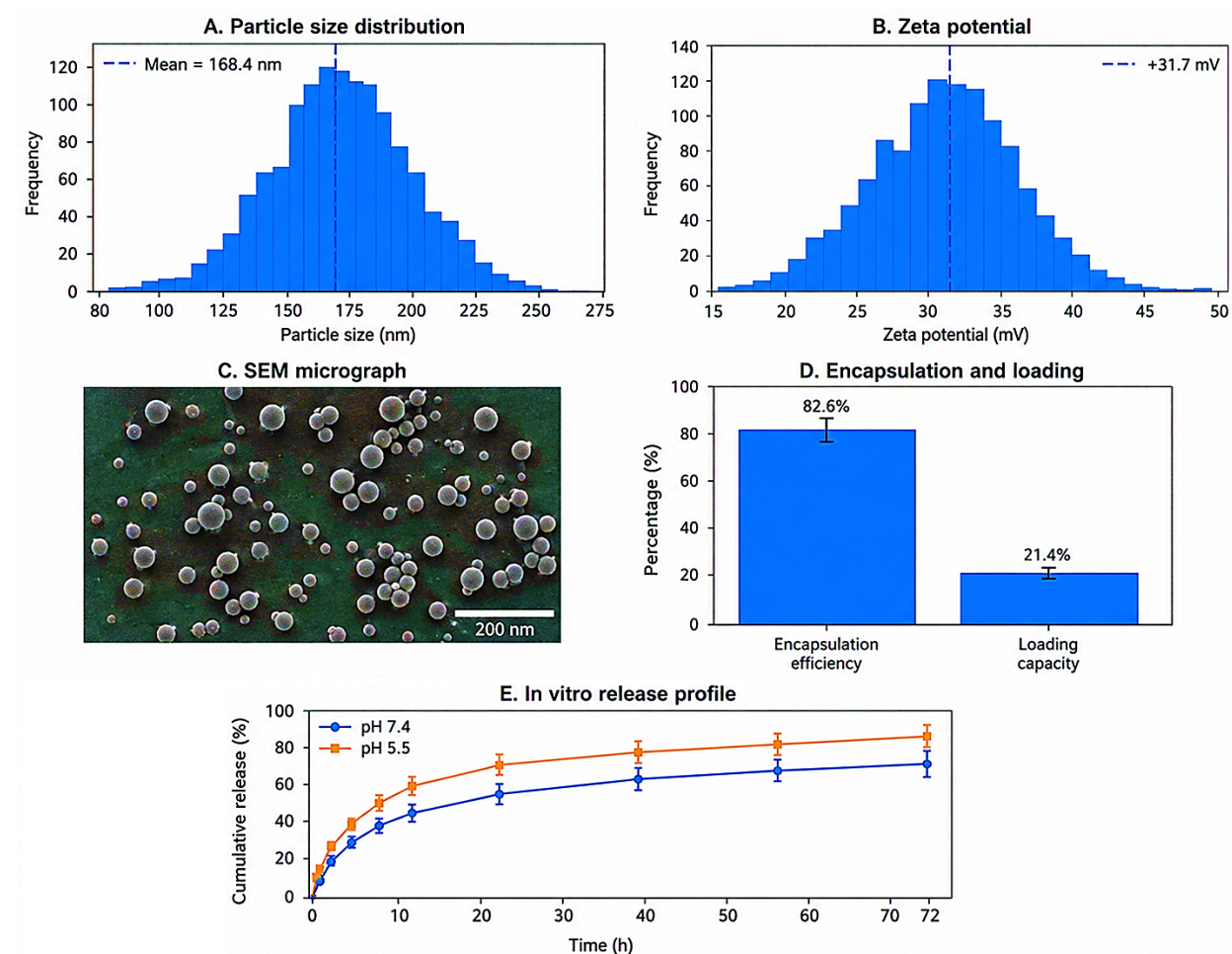


Figure 1: Physicochemical characterization of optimized *Prunus africana*-loaded chitosan nanoparticles. (A) Dynamic light scattering investigation reveals a mean particle size of 168.4 ± 12.6 nm and a PDI of 0.23 ± 0.04 . (B) The zeta potential analysis reveals a positive surface charge of $+31.7 \pm 3.2$ mV. (C) SEM micrograph of discrete, smooth-surfaced, spherical nanoparticles with little aggregation. (D) The optimized formulation exhibited a loading capacity of $21.4 \pm 1.6\%$ and an encapsulation efficiency of $82.6 \pm 4.8\%$. (E) *Prunus africana* extract was released in a sustained manner over 72 h in vitro, with faster release at pH 5.5 than at pH 7.4. The mean ± SD of triplicate determinations is used to express the values.

To elucidate the release mechanism of the optimized *Prunus africana*-loaded chitosan nanoparticles (F5), the in vitro release data obtained at pH 5.5 and pH 7.4 were fitted to Zero-order, First-order, Higuchi, and Korsmeyer–Peppas kinetic models (Table 3). Among the models evaluated, the Korsmeyer–Peppas model exhibited the highest correlation coefficients ($R^2 = 0.989$ at pH 5.5 and 0.981 at pH 7.4), indicating that it best described the release behavior of the nanoparticles. The release exponent values ($n = 0.63$ at pH 5.5 and $n = 0.58$ at pH 7.4) suggested a non-Fickian or anomalous transport mechanism, whereby drug release was controlled by both diffusion through

the polymer matrix and polymer relaxation/erosion processes. The higher release rate constant observed under acidic conditions further supports the pH-responsive behavior of the chitosan nanoparticles, which may facilitate enhanced release of bioactive constituents within acidic tumor microenvironments and intracellular endosomal compartments (Table 3). Taken together, the optimization and characterization results indicate that F5 possessed favorable nanoscale properties, strong extract encapsulation, acceptable loading, reliable colloidal stability, and consistent pH-sensitive release, supporting its selection for the subsequent antimetastatic assays.

Table 3: Kinetic modeling of in vitro release of *P. africana*-loaded chitosan nanoparticles (F5) at pH 5.5 and pH 7.4.

Kinetic Model	Parameter	pH 5.5	pH 7.4
Zero-order	k_0 (% h^{-1})	1.18	0.98
	R^2	0.921	0.908
First-order	k_1 (h^{-1})	0.038	0.029
	R^2	0.948	0.936
Higuchi	kH (% $h^{-1/2}$)	10.84	8.96
	R^2	0.972	0.964
Korsmeyer–Peppas	kKP	0.284	0.241
	n	0.63	0.58
	R^2	0.989	0.981
	Release mechanism	Non-Fickian (anomalous) transport	Non-Fickian (anomalous) transport

Values represent the kinetic parameters obtained by fitting the cumulative drug release data to the zero-order, first-order, Higuchi, and Korsmeyer–Peppas models. The Korsmeyer–Peppas model showed the highest coefficient of determination (R^2) at both pH conditions, indicating the best fit to the experimental release data. The release exponent ($n = 0.58$ – 0.63) suggests anomalous (non-Fickian) transport, implying that a combination of diffusion and polymer relaxation/erosion mechanisms governed the release of phytoconstituents from the chitosan matrix.

3.3. Cell Viability and Uptake Assay

This study examined the cytotoxic effects and cellular uptake of *Prunus africana*-loaded chitosan nanoparticles in LNCaP prostate cancer cells and RWPE-1 normal prostate epithelial cells. Both free *P. africana* extract and *P. africana*-loaded chitosan nanoparticles reduced LNCaP cell viability in a concentration- and time-dependent manner (Figure 2A), with the nanoparticle formulation producing stronger inhibitory effects than the free extract at matching extract concentrations. *P. africana*-loaded chitosan nanoparticles at 100 $\mu\text{g/mL}$ reduced LNCaP cell viability to 42.6% at 24 h, 25.7% at 48 h, and 18.6% at 72 h, whereas the free extract reduced viability to 58.8% at 24 h, 39.8% at 48 h, and 31.2% at 72 h. RWPE-1 normal prostate epithelial cells showed markedly higher sur-

vival rates under the same treatments, indicating selective cytotoxicity toward prostate cancer cells. Blank chitosan nanoparticles preserved RWPE-1 viability above 93% across all tested concentrations, confirming the relative safety of the carrier system (Figure 2B), while both free extract and nanoparticle-loaded extract showed low toxicity in RWPE-1 cells, with survival remaining above 76% even at the highest concentration tested. IC₅₀ analysis confirmed that the nanoparticle formulation was more potent than the free extract at both 48 h and 72 h (Figure 2C): at 48 h, the IC₅₀ value decreased from 63.8 $\mu\text{g/mL}$ for the free extract to 43.2 $\mu\text{g/mL}$ for the nanoparticle-loaded extract, and at 72 h it decreased from 49.6 $\mu\text{g/mL}$ to 31.8 $\mu\text{g/mL}$. These findings demonstrate that chitosan nanoparticle delivery enhanced the antiproliferative activity of *P. africana* against LNCaP prostate cancer cells.

Cellular uptake studies confirmed that nanoparticle-based delivery markedly enhanced intracellular accumulation of the extract. FITC-labelled *P. africana*-loaded chitosan nanoparticles produced a continuous increase in FITC-positive LNCaP cells, reaching 84.9% by 6 h, compared with only 38.6% for free extract/FITC-treated cells (Figure 2D–F). At 4 h, the nanoparticle-treated group showed a 6.9-fold increase in relative fluorescence intensity, compared with a 2.6-fold increase for the free extract group (Figure 2E). Fluorescence microscopy confirmed higher intracellular nanoparticle fluorescence in

LNCaP cells following treatment with FITC-labelled *P. africana*-loaded chitosan nanoparticles (Figure 2E, F). Together, these results indicate that chitosan nanoparticle de-

livery improved cellular uptake of *P. africana*, producing stronger cytotoxic effects against prostate cancer cells while causing less harm to normal prostate epithelial cells.

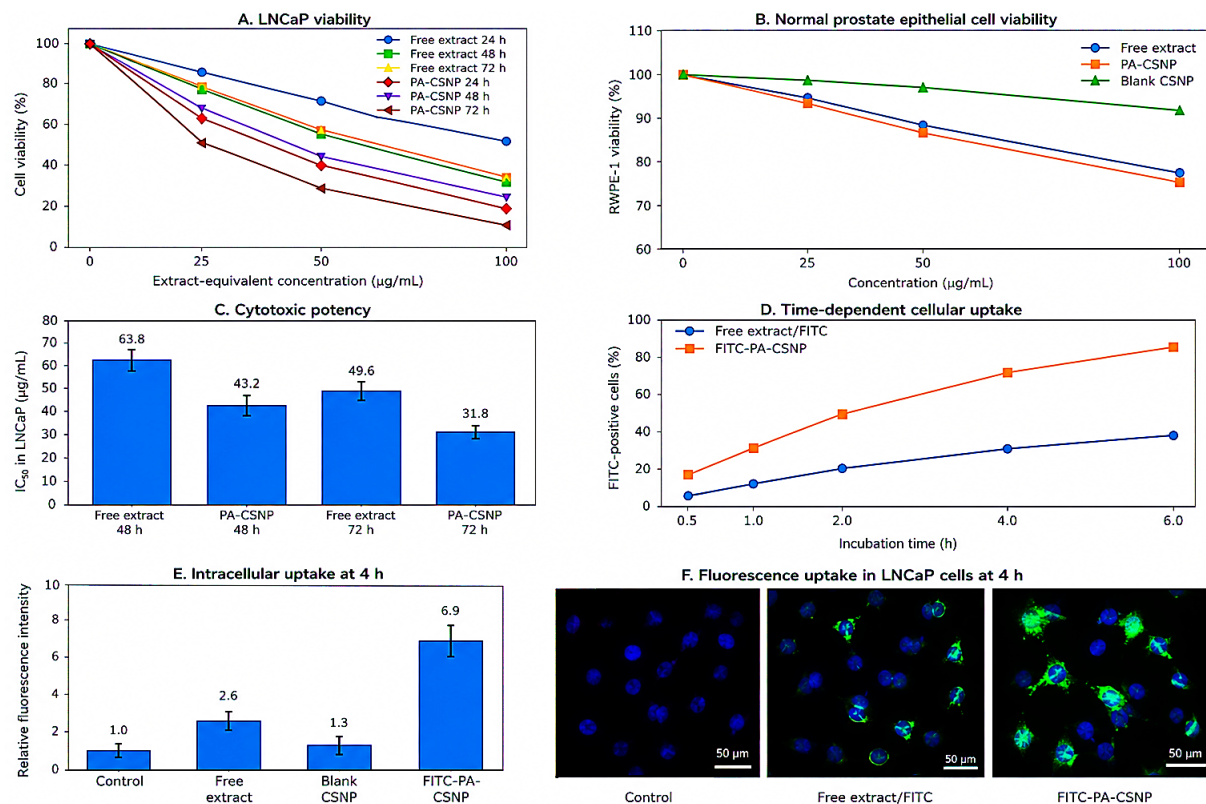


Figure 2: Viability and cellular uptake of *P. africana*-loaded chitosan nanoparticles in prostate cell models. (A) Dose- and time-dependent viability of LNCaP cells treated with free extract or nanoparticle-loaded extract at 25, 50, and 100 μg/mL for 24, 48, and 72 h. (B) Viability of RWPE-1 normal prostate epithelial cells treated with free extract, blank nanoparticles, or nanoparticle-loaded extract. (C) Comparative IC₅₀ values at 48 and 72 h. (D) Time-dependent uptake of FITC-labelled nanoparticles over 0.5–6 h. (E) Relative fluorescence intensity at 4 h. (F) Fluorescence microscopy images showing enhanced intracellular nanoparticle uptake. Values are expressed as mean ± SD of triplicate determinations.

3.3.1. Selectivity Index of *P. africana*-Loaded CSNPs

Selectivity index analysis further demonstrated the preferential cytotoxicity of the formulations toward prostate cancer cells (Table 4). The *P. africana*-loaded chitosan nanoparticles exhibited higher selectivity than the free ex-

tract, with estimated SI values increasing from >2.31 at 48 h to >3.14 at 72 h. These findings indicate that nanoencapsulation enhanced not only the antiproliferative potency but also the selectivity of *P. africana* against LNCaP prostate cancer cells while minimizing toxicity toward normal RWPE-1 prostate epithelial cells.

Table 4: Cytotoxicity and selectivity index (SI) of *P. africana* formulations.

Treatment	Time (h)	IC ₅₀ in LNCaP Cells (μg/mL)	IC ₅₀ in RWPE-1 Cells (μg/mL)	Selectivity Index (SI)
Free <i>P. africana</i> extract	48	63.8	>100	>1.57
Free <i>P. africana</i> extract	72	49.6	>100	>2.02
<i>P. africana</i> -loaded CSNPs	48	43.2	>100	>2.31
<i>P. africana</i> -loaded CSNPs	72	31.8	>100	>3.14

SI = IC₅₀ (RWPE-1 normal prostate epithelial cells)/IC₅₀ (LNCaP prostate cancer cells). Because the IC₅₀ value in RWPE-1 cells was not reached within the tested concentration range (0–100 μg/mL), SI values are expressed as minimum estimates. Higher SI values indicate greater selectivity toward prostate cancer cells relative to normal prostate epithelial cells.

3.4. Wound Healing Activity and Transwell Assay

The ability of *Prunus africana*-loaded chitosan nanoparticles to inhibit TGF- β 1-induced migration and invasion was assessed using three complementary assays: wound-healing, Transwell migration, and Matrigel invasion. TGF- β 1 treatment increased cell migration, resulting in greater wound closure within 24 h: percentage

wound closure increased from $46.2 \pm 4.1\%$ in the control group to $84.7 \pm 5.2\%$ in the TGF- β 1-treated group (Figure 3A). Free *P. africana* extract reduced wound closure to $61.5 \pm 4.8\%$, while *P. africana*-loaded chitosan nanoparticles produced a stronger inhibitory effect, reducing wound closure to $31.8 \pm 3.6\%$. The SMAD3 inhibitor SIS3 produced a comparable effect, achieving $28.4 \pm 3.1\%$ wound closure.

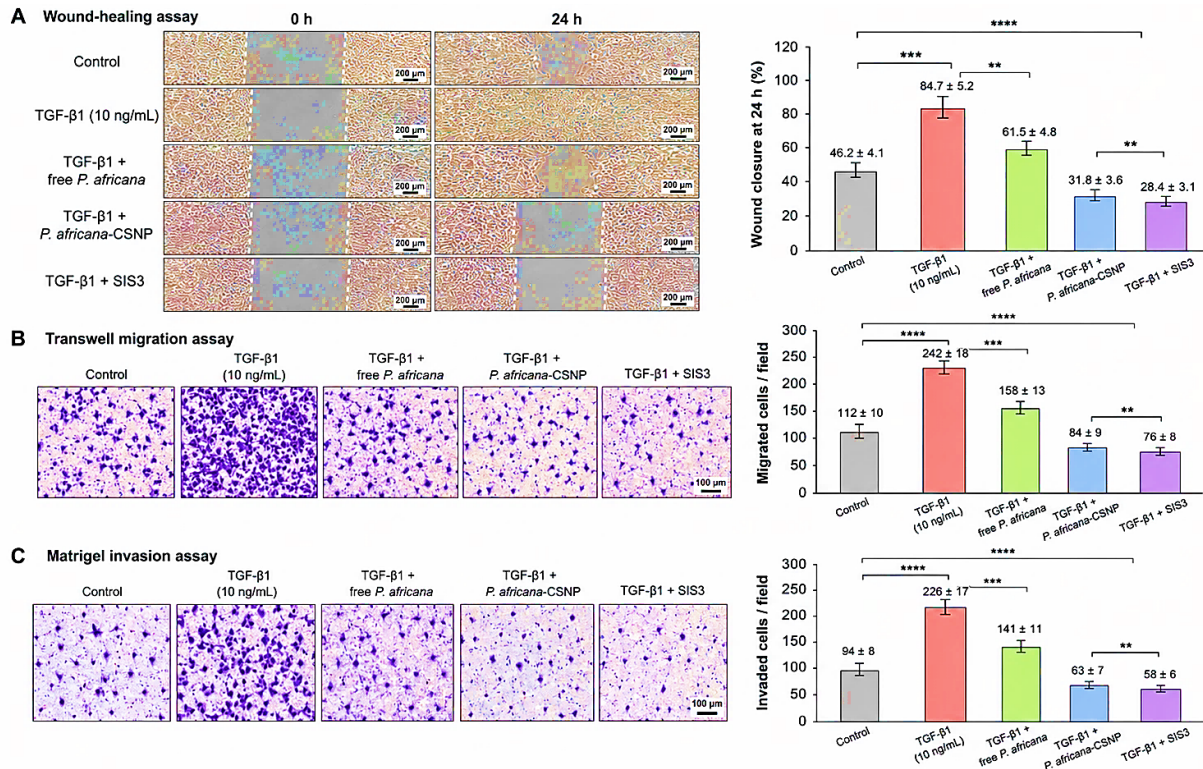


Figure 3: *Prunus africana*-loaded chitosan nanoparticles suppress TGF- β 1-induced migration and invasion in LNCaP cells. (A) Representative wound-healing images at 0 h and 24 h, with quantification of wound closure. (B) Representative transwell migration images and migrated cell counts after 24 h. (C) Representative Matrigel invasion images and invaded cell count after 48 h. TGF- β 1 increased LNCaP cell migration and invasion, whereas free *P. africana* extract partially reduced these effects. *P. africana*-loaded chitosan nanoparticles showed stronger antimigratory and anti-invasive activity, comparable to SIS3. Data are presented as mean $\pm$ SD; n = 3. Values are statistically significant at ** $p < 0.01$, *** $p < 0.001$ vs. control (one-way ANOVA followed by Tukey's post hoc test).

Consistent with the wound-healing results, the transwell migration assay showed that TGF- β 1 increased cell migration from 112 ± 10 cells/field in the control group to 242 ± 18 cells/field. Free *P. africana* extract reduced migration to 158 ± 13 cells/field, while *P. africana*-loaded chitosan nanoparticles further reduced migration to 84 ± 9 cells/field; SIS3 treatment produced a comparable effect, with 76 ± 8 migrated cells/field (Figure 3B). The Matrigel invasion assay likewise demonstrated the anti-invasive activity of the nanoparticle formulation: TGF- β 1 increased invaded cells from 94 ± 8 cells/field in the control group

to 226 ± 17 cells/field, free *P. africana* extract reduced invasion to 141 ± 11 cells/field, and *P. africana*-loaded chitosan nanoparticles suppressed invasion more effectively, to 63 ± 7 cells/field. The SIS3 group showed 58 ± 6 invaded cells/field, supporting a role for SMAD3 signaling in TGF- β 1-driven invasion (Figure 3C). Overall, *P. africana*-loaded chitosan nanoparticles significantly inhibited TGF- β 1-induced cell migration by 65.3% and cell invasion by 72.1% relative to the TGF- β 1-treated group ($p < 0.05$), an effect comparable to direct SMAD3 inhibition.

3.5. Expression of Epithelial–Mesenchymal Transition (EMT) Markers

The effects of *Prunus africana*-loaded chitosan nanoparticles on TGF- β 1-induced EMT reversal in LNCaP prostate cancer cells were assessed by qRT-PCR, Western blotting, and immunofluorescence staining (Figure 4). TGF- β 1 treatment significantly decreased CDH1 (E-cadherin) expression, from 1.00 in the control group to 0.38, while in-

creasing expression of the mesenchymal-associated genes CDH2, VIM, SNAI1, SNAI2, and TWIST1, to 2.84, 3.12, 2.66, 2.43, and 2.59, respectively (Figure 4A). Free *P. africana* extract partially restored these changes, whereas *P. africana*-loaded chitosan nanoparticles produced a stronger effect, increasing CDH1 levels to 1.32 and reducing CDH2, VIM, SNAI1, SNAI2, and TWIST1 to 0.92, 1.08, 0.88, 0.84, and 0.89, respectively—an effect comparable to that of the SMAD3 inhibitor SIS3.

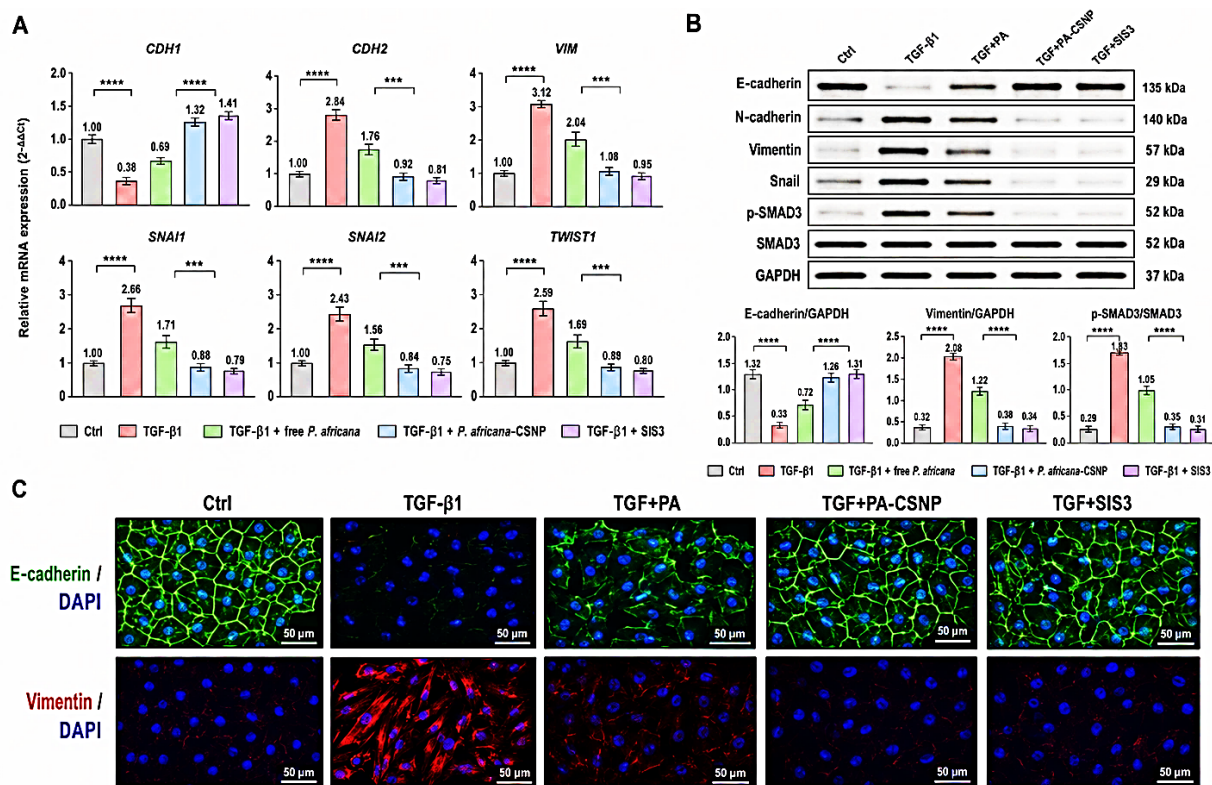


Figure 4: *P. africana*-loaded chitosan nanoparticles reverse TGF- β 1-induced EMT marker dysregulation in LNCaP cells. (A) qRT-PCR analysis showing relative mRNA expression of CDH1, CDH2, VIM, SNAI1, SNAI2, and TWIST1 following treatment with TGF- β 1, free *P. africana* extract, *P. africana*-loaded chitosan nanoparticles, or SIS3. (B) Western blot analysis and densitometric quantification of E-cadherin, N-cadherin, vimentin, Snail, p-SMAD3, SMAD3, and GAPDH. (C) Representative immunofluorescence images showing E-cadherin/DAPI and vimentin/DAPI staining across treatment groups. TGF- β 1 induced EMT, whereas *P. africana*-loaded chitosan nanoparticles restored epithelial marker expression and suppressed mesenchymal marker expression, with effects comparable to SIS3. Data are presented as mean $\pm$ SD; n = 3. Values are statistically significant at *** p < 0.001 vs. control (one-way ANOVA followed by Tukey's post hoc test).

Western blot analysis confirmed these transcriptional findings at the protein level (Figure 4B). TGF- β 1 decreased E-cadherin protein levels while increasing N-cadherin, vimentin, Snail, and phosphorylated SMAD3, confirming activation of TGF- β /SMAD3 signaling and EMT. Free *P. africana* extract partially restored E-cadherin levels and blunted the rise in mesenchymal proteins, while *P. africana*-loaded chitosan

nanoparticles produced a stronger effect, restoring E-cadherin levels and more effectively suppressing vimentin and phosphorylated SMAD3. Densitometric analysis showed that the E-cadherin/GAPDH ratio reached 1.26 in the nanoparticle-treated group, compared with 0.33 in the TGF- β 1 group, while the vimentin/GAPDH ratio fell from 2.08 to 0.38. The p-SMAD3/SMAD3 ratio decreased from 1.83 in the TGF- β 1 group to 0.35

after nanoparticle treatment. Immunofluorescence staining confirmed these findings (Figure 4C): control cells showed strong membrane-associated E-cadherin staining with low vimentin expression, whereas TGF- β 1-treated cells showed decreased E-cadherin and strong vimentin staining, consistent with EMT. Free *P. africana* extract partially restored epithelial characteristics, while *P. africana*-loaded chitosan nanoparticles produced high E-cadherin and low vimentin staining, comparable to the SIS3-treated group. Together, these findings show that *P. africana*-loaded chitosan nanoparticles reversed TGF- β 1-induced EMT by restoring epithelial marker expression, suppressing mesenchymal markers, and reducing SMAD3 activation.

3.6. TGF- β /SMAD3 Pathway Activation

TGF- β /SMAD3 pathway activation was assessed to determine whether *P. africana*-loaded chitosan nanoparticles suppress epithelial-mesenchymal transition and invasion through inhibition of TGF- β signaling (Figure 5A–C). TGF- β 1 treatment increased mRNA levels of TGFB1,

SMAD3, MMP2, and MMP9, confirming activation of the EMT- and invasion-associated signaling pathways. Free *P. africana* extract reduced this gene expression, while *P. africana*-loaded chitosan nanoparticles achieved more effective suppression, returning most transcript levels close to control values—an effect comparable to the SMAD3 inhibitor SIS3 (Figure 5A). Western blot analysis confirmed this pathway modulation at the protein level (Figure 5B): TGF- β 1 treatment increased levels of TGF- β 1, phosphorylated SMAD3, and phosphorylated SMAD2, while total SMAD3 remained largely unchanged. *P. africana*-loaded chitosan nanoparticle treatment significantly decreased TGF- β 1, p-SMAD3, and p-SMAD2 levels relative to both TGF- β 1 treatment alone and the free-extract treatment. TGF- β 1-treated cells showed strong nuclear p-SMAD3 localization, which was markedly reduced following nanoparticle treatment (Figure 5C; SM 1). These findings demonstrate that *P. africana*-loaded chitosan nanoparticles suppress TGF- β /SMAD3 pathway activation and its downstream invasion-related mediators in LNCaP cells.

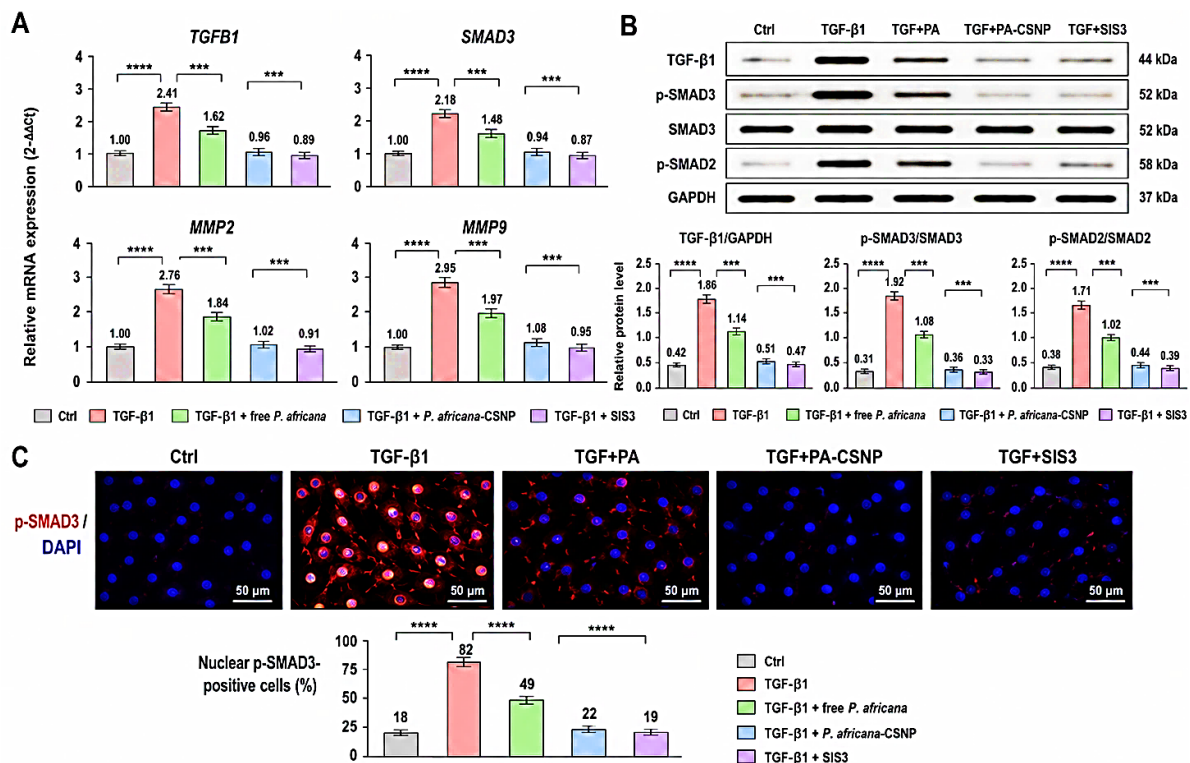


Figure 5: *Prunus africana*-loaded chitosan nanoparticles suppress TGF- β /SMAD3 signaling in LNCaP cells. (A) qRT-PCR analysis of TGFB1, SMAD3, MMP2, and MMP9 expression following treatment with TGF- β 1, free *P. africana*, *P. africana*-loaded chitosan nanoparticles, or SIS3. (B) Western blot analysis and densitometric quantification of TGF- β 1, p-SMAD3, SMAD3, p-SMAD2, and GAPDH. (C) Immunofluorescence images and quantification of nuclear p-SMAD3-positive cells. TGF- β 1 activated canonical SMAD signaling, whereas *P. africana*-loaded chitosan nanoparticles suppressed SMAD pathway activation and the expression of invasion-associated mediators, with effects comparable to those of SIS3. Data are presented as mean $\pm$ SD; n = 3. Values are statistically significant at *** $p < 0.001$ vs. control (one-way ANOVA followed by Tukey's post hoc test).

3.7. In Vivo Anti-Metastasis Effects

The antimetastatic properties of *Prunus africana*-loaded chitosan nanoparticles were evaluated using three complementary approaches: assessment of metastatic burden, histological examination, and analysis of EMT-related immunohistochemical markers. TGF- β 1 treatment increased the number of metastatic lung and liver nodules relative to the control group (Figure 6A). Free *P. africana* extract reduced metastatic nodule number, while *P. africana*-loaded chitosan nanoparticles produced a more pronounced reduction in both lung and liver metastases, comparable to the SMAD3 inhibitor SIS3. H&E staining confirmed extensive metastatic infiltration in the TGF- β 1 group, particularly in lung and liver tissue (Figure 6B), whereas animals treated with *P. africana*-loaded chitosan nanoparticles developed smaller, fewer metastatic tumors with better-preserved tissue architecture. Immunohistochemical analysis showed that TGF- β 1 treatment decreased E-cadherin and increased N-cadherin, vimentin, Ki-67, and MMP-9 within metastatic regions

(Figure 6C,D; SM 2), whereas nanoparticle treatment restored E-cadherin staining and substantially reduced mesenchymal, proliferative, and invasion-related markers. Consistent with the in vitro findings, TGF- β 1 stimulation significantly enhanced the migratory and invasive capacity of LNCaP cells relative to untreated controls (Figure 6D), and treatment with *P. africana*-loaded chitosan nanoparticles (PA-CSNP) markedly suppressed these metastatic phenotypes, inhibiting TGF- β 1-induced migration by 64.8% and invasion by 69.2% relative to the TGF- β 1-treated group ($p < 0.001$). In vivo, PA-CSNP-treated animals showed a substantial reduction in metastatic lung nodules compared with the untreated metastatic control group (Figure 6E), with lung metastasis burden reduced by 58.7% ($p < 0.01$), indicating significant inhibition of tumor dissemination and colonization in distant organs. Together, these findings indicate that PA-CSNP effectively suppresses key steps in cancer metastasis, including cellular migration, invasion, and secondary tumor formation.

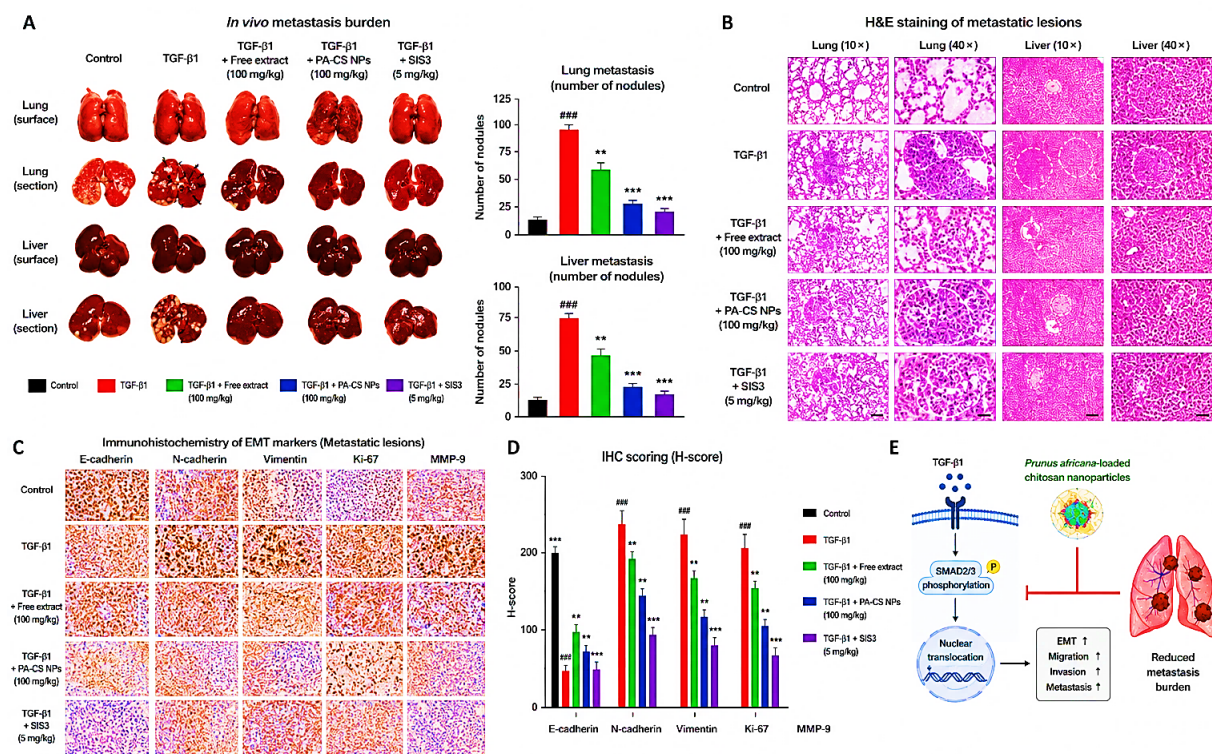


Figure 6: *P. africana*-loaded chitosan nanoparticles suppress in vivo metastasis and EMT-associated marker expression. (A) Lung and liver images with quantification of metastatic nodules across treatment groups. (B) H&E-stained lung and liver sections showing metastatic lesion distribution. (C) Immunohistochemical staining of E-cadherin, N-cadherin, vimentin, Ki-67, and MMP-9 in metastatic lesions. (D) Quantitative H-score analysis of EMT, proliferation, and invasion markers. (E) Proposed mechanism showing inhibition of TGF- β /SMAD signaling by *P. africana*-loaded chitosan nanoparticles, leading to reduced EMT, migration, invasion, and metastasis. Data are presented as mean $\pm$ SD; $n = 6$ animals per group. Values are statistically significant at *** $p < 0.001$ vs. control (one-way ANOVA followed by Tukey's post hoc test).

The schematic summary in **Figure 6E** illustrates that *P. africana*-loaded chitosan nanoparticles attenuate TGF- β /SMAD3-mediated EMT signaling, leading to reduced migration, invasion, and metastatic spread.

4. Discussion

This study provides preclinical evidence that chitosan nanoparticle delivery may enhance the antimetastatic activity of *Prunus africana* against prostate cancer by suppressing TGF- β /SMAD3-driven epithelial–mesenchymal transition. Compared with the free extract, the prepared nanoparticles showed improved physicochemical properties, enhanced cellular uptake, sustained release, and greater biological activity. These results support the broader concept that nanocarrier-based delivery can enhance the therapeutic efficacy of plant-derived anticancer agents, which is often limited by poor solubility, instability, and inadequate intracellular accumulation [32].

Because epithelial–mesenchymal transition is a key driver of prostate cancer metastasis, the antimetastatic effects observed in this study are mechanistically significant. Prostate cancer cells developed a mesenchymal phenotype in the TGF- β -induced model, characterized by decreased E-cadherin and increased N-cadherin, vimentin, Snail, Slug, Twist, and matrix metalloproteinases. These alterations align with the known function of TGF- β signaling in advanced cancer metastatic development, invasion, immune evasion, and epithelial plasticity [8,37,38]. The ability of *P. africana*-loaded chitosan nanoparticles to reverse these alterations suggests that the formulation not only suppresses cell viability but also interferes with metastatic signaling pathways.

SMAD3 appears to be a key molecular target of the observed response. In advanced prostate cancer, activation of TGF- β signaling may shift from tumor suppression to tumor promotion, partly through SMAD-dependent transcriptional induction of epithelial–mesenchymal transition regulators [10,39]. In this study, nanoparticle treatment reduced TGF- β -induced SMAD3 phosphorylation and nuclear localization, accompanied by down-regulation of epithelial–mesenchymal transition (EMT) transcription factors. This suggests that *P. africana* bioactives may suppress EMT by interfering with canonical TGF- β /SMAD3 signaling. However, TGF- β also activates non-SMAD pathways, including PI3K/AKT, MAPK, RhoA, and mTOR [10]. Additional pathway-level studies are needed to determine whether these mechanisms also contribute to the observed phenotype.

The combined effects of *P. africana*'s phytochemical components may underlie its biological activity. Prior research has linked *P. africana* extracts and their con-

stituents to growth-inhibitory, pro-apoptotic, antiandrogenic, and antiangiogenic actions in prostate cancer models. By identifying *P. africana* as a potential regulator of EMT-associated metastatic signaling, the present findings extend this pharmacological profile. Crucially, multi-targeted action may be achievable using whole or standardized extracts; however, this also raises issues with phytochemical variability, quality control, reproducibility, and regulatory standards.

Chitosan was selected as the nanocarrier because of its cationic nature, mucoadhesive properties, biodegradability, biocompatibility, and suitability for ionic gelation [17]. The positive nanoparticle surface charge may improve cellular uptake by facilitating interaction with negatively charged cell membranes, while sustained release may further prolong intracellular exposure to *P. africana* bioactives. The greater activity of the nanoparticle-loaded extract relative to the free extract suggests that improved intracellular delivery may also have contributed to the observed antimetastatic effect. This study has several limitations. First, because *P. africana* extract is chemically complex, further research is needed to identify the specific components responsible for the observed decrease in SMAD3 activity. Second, in vitro EMT models cannot fully replicate the complexity of tumor microenvironment interactions, stromal signaling, immune regulation, and metastatic niche formation. Third, although in vivo metastatic models enhance translational relevance, they may not fully recapitulate the course of clinical prostate cancer. Fourth, long-term toxicity, pharmacokinetics, biodistribution, immunogenicity, and reproductive safety should be assessed before clinical translation.

Although the findings demonstrate that *Prunus africana*-loaded chitosan nanoparticles effectively suppress TGF- β /SMAD3 signaling and invasion-associated mediators in LNCaP cells, the study has certain limitations. First, although preliminary assays indicated that blank chitosan nanoparticles did not alter SMAD pathway activation, a formal free extract plus blank nanoparticle combination group was not included in the main experiments, which would have further excluded potential carrier-related effects. Second, mechanistic validation relied on the pharmacological inhibition of SMAD3 (SIS3), and complementary SMAD3 overexpression or rescue experiments were not performed. Such gain-of-function approaches would provide additional confirmation that SMAD3 is the central target of *P. africana*. These aspects represent important directions for future work to strengthen the mechanistic understanding of *P. africana*-mediated suppression of TGF- β signaling. Future research should include bioactivity-guided fraction-

ation, metabolomic profiling, pharmacokinetic analysis, nanoparticle biodistribution studies, orthotopic and bone metastasis models, and combination studies with TGF- β pathway inhibitors, docetaxel, or androgen receptor inhibitors. Testing of *P. africana*-loaded nanoparticles that inhibit cancer stemness, circulating tumor cell survival, anoikis resistance, and bone colonization would also be beneficial.

5. Conclusions

By enhancing cellular uptake, sustaining phytochemical release, inhibiting migration and invasion, and suppressing TGF- β /SMAD3-driven epithelial–mesenchymal transition, chitosan nanoparticle delivery improved the antitumorigenic efficacy of *Prunus africana* in prostate cancer models. The formulation reduced in vivo tumorigenic burden, diminished mesenchymal and invasion-associated markers, and restored epithelial marker expression. These findings support the development of *P. africana*-loaded chitosan nanoparticles as a promising nanophytotherapeutic platform for the treatment of metastatic prostate cancer. Further research in clinically relevant metastatic prostate cancer models should establish safety, optimize pharmacokinetics, validate the active ingredients, and assess therapeutic efficacy.

List of Abbreviations

ANOVA	Analysis of Variance
CDH1	Cadherin 1 (E-cadherin)
CDH2	Cadherin 2 (N-cadherin)
CSNP	Chitosan Nanoparticles
DAPI	4',6-Diamidino-2-Phenylindole
EMT	Epithelial–Mesenchymal Transition
FITC	Fluorescein Isothiocyanate
GAE	Gallic Acid Equivalent
H&E	Hematoxylin and Eosin
LNCaP	Lymph Node Carcinoma of the Prostate
MMP	Matrix Metalloproteinase
PBS	Phosphate-Buffered Saline
PDI	Polydispersity Index
PVDF	Polyvinylidene Difluoride
QE	Quercetin Equivalent
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SI	Selectivity Index
SMAD3	Mothers Against Decapentaplegic Homolog 3
TGF- β	Transforming Growth Factor-Beta
TPP	Sodium Tripolyphosphate
VIM	Vimentin

Author Contributions

Conceptualization, writing—original draft preparation, methodology, validation, formal analysis, investigation, supervision, writing—review and editing: C.A.U.; resources, writing—review and editing, investigation, visualization, project administration, methodology: H.B.Y. All authors have read and agreed to the published version of the manuscript.

Availability of Data and Originality

The datasets generated and analyzed during the present study are original and are included in the manuscript and the supplementary material.

Ethics Committee Approval

All animal experiments were reviewed and approved by the Animal Research Ethical Committee, Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Maiduguri, under protocol number FP/03/24/SP.9788, approved in March, 2025. Human-derived prostate cancer (LNCaP) and normal prostate epithelial (RWPE-1) cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The study did not involve the recruitment of human participants, the collection of human tissues, or the use of identifiable human data. Therefore, informed consent was not required. The experiments were conducted exclusively using commercially available established cell lines. Where applicable, all institutional requirements governing the use of commercially acquired human cell lines were followed.

Animal Rights

All animal procedures were conducted in accordance with internationally accepted guidelines for the care and use of laboratory animals and complied with the principles of the Basel Declaration. Furthermore, the study was designed, conducted, and reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines to ensure transparency, reproducibility, and responsible animal research practices.

Conflicts of Interest

The authors declare no conflicts of interest.

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AI Declaration

The authors acknowledge the use of Grammarly and Autopilot to improve grammar and readability during manuscript preparation. The authors take full responsibility for the manuscript content.

Supplementary Materials

Supplementary material associated with this article has been published online and is available at: <https://doi.org/10.69709/CRCT.2026.128288>.

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