

Heptyl prodigiosin from estuarine *Zooshikella* sp. RANM57 induces caspase-dependent apoptosis and suppresses clonogenic survival in androgen-independent prostate cancer cells

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Abstract

Estuarine microbiomes are rich ecological niches for discovering novel secondary metabolites with therapeutic applications. This study focuses on the isolation, purification, structural elucidation, and antineoplastic evaluation of a red pigment from a marine microbial isolate. The bacterium *Zooshikella* sp. RANM57 was isolated from the Netravathi River estuary. Biomass was recovered using an alum-induced flocculation technique and solvent extraction, after which the primary pigment was purified via flash chromatography and structurally characterized using HRLCMS-QTOF and NMR spectroscopy. Anticancer potential was evaluated against human PC-3 prostate cancer cells using MTT and clonogenic assays. The underlying apoptotic mechanism was delineated using Annexin V/PI staining, Caspase-3 quantification, and TUNEL assays. The purified pigment was identified as the tripyrrole alkaloid heptyl prodigiosin based on HRLCMS-QTOF, UV-Vis/FT-IR profiling, and 1D NMR spectral concordance with reported prodiginine features. *In vitro* evaluations revealed significant dose-dependent cytotoxicity against PC-3 cells ($IC_{50} = 9.00 \mu\text{g/mL}$), demonstrating high anticancer efficacy relative to cisplatin. Mechanistic profiling confirmed that cell death occurred exclusively via an intrinsic apoptotic cascade, as evidenced by significant phosphatidylserine externalization (17.60%), executioner Caspase-3 activation (52.45%), and terminal DNA fragmentation (21.25%), without inducing primary necrosis. Furthermore, treatment also reduced clonogenic survival after 24 h exposure and 8-day outgrowth. The successful isolation and characterization of heptyl prodigiosin from *Zooshikella* sp. RANM57 underscores the value of estuarine bacteria in natural product discovery. Our biological evaluations demonstrate that the compound functions as a precise, potent inducer of caspase-associated apoptosis, positioning this tripyrrole scaffold as a highly promising candidate for further anticancer pharmacological development.

Introduction

Marine ecosystems represent a largely untapped repository of structurally complex secondary metabolites that continually provide essential lead compounds for modern oncology, evidenced by FDA-approved therapeutics such as cytarabine, and eribulin [1–3]. The escalating clinical challenge of chemoresistance necessitates the rapid discovery of novel antineoplastic agents. Consequently, marine microorganisms particularly pigmented bacterial taxa are being increasingly targeted due to their vast biosynthetic gene clusters and capacity for scalable metabolite production.

Estuarine microbiomes, such as those within the Netravathi River estuary in Mangalore, are subjected to extreme physicochemical fluctuations [4]. These distinct environmental stressors drive the evolution of highly specialized microbial metabolic pathways, rendering them highly productive biological niches for the discovery of novel therapeutics. Within this context, prodiginines a family of characteristic tripyrrole alkaloids, have emerged as highly promising marine-derived bioactives. Characterized by an intense red color and a unique pyrrolyl pyrromethene skeleton, these metabolites exhibit potent, broad-spectrum antimicrobial, antimalarial, and immunosuppressive properties [5].

The bioactivity of prodiginines is strictly governed by their tripyrrole core and associated alkyl chain dynamics, parameters that dictate lipophilicity and ability to engage biological targets [6]. Of particular interest is heptyl prodigiosin, an analogue bearing an extended aliphatic chain that exhibits markedly enhanced membrane permeability and cytotoxic efficacy relative to the parent molecule [7, 8]. This structural advantage positions heptyl prodigiosin as a promising candidate for inducing apoptosis in androgen-independent prostate cancer cells.

The biological activities of prodiginine alkaloids have been extensively investigated, revealing potent cytotoxic effects that induce apoptosis in cancer cells through multiple mechanisms. These include the disruption of mitochondrial membrane potential, the generation of reactive oxygen species (ROS), and the activation of caspase cascades [9–11]. Furthermore, these compounds exhibit selective toxicity toward multidrug-resistant (MDR) cancer cell lines, suggesting significant potential in overcoming traditional chemotherapy resistance [12]. This is particularly relevant for prostate cancer; while localized disease often responds to androgen deprivation, a significant proportion of patients inevitably progress to metastatic castration-resistant prostate cancer (mCRPC) [13] necessitating the discovery of novel therapeutics. To address this knowledge gap, the present study reports the isolation and structural characterization of heptyl prodigiosin from the Netravathi estuarine isolate *Zooshikella* sp. RANM57 and provides an initial *in vitro* mechanistic evaluation of its antiproliferative and apoptosis-inducing effects in PC-3 androgen-independent prostate cancer cells.

Materials and Methods.

2.1 Microbial Cultivation and Pigment Extraction:

The red-pigmented marine bacterium *Zooshikella* sp. RANM57, previously isolated from the Netravathi River Estuary (Mangaluru, Karnataka, India), was utilized for this study. Pure cultures were maintained as 20% (v/v) glycerol stocks at -20°C. To facilitate pigment production, submerged fermentation was conducted using a custom-formulated Netravathi Estuarine Broth (NEB). A 5% v/v seed inoculum ($OD_{600} = 0.5$) was introduced into 2 L of NEB and incubated at $30 \pm 2^\circ\text{C}$ for 48 h under continuous agitation at 100 rpm.

Following fermentation, biomass recovery was achieved via an alum-induced sedimentation technique (aluminium sulphate, 10 mg/mL of broth) utilizing a proprietary methodology recently published by our group (Indian Patent Application No. 202641026891 A). The recovered biomass pellet was subjected to cellular lysis and initial pigment extraction by the addition of 100 mL of absolute ethanol. Subsequently, liquid-liquid partitioning was performed using an ethanol: dichloromethane (EtOH:DCM) solvent system at a 4:6 (v/v) ratio. The lower organic phase (DCM-rich), containing the enriched crude pigment, was carefully partitioned using a separatory funnel. The solvent was evaporated under reduced pressure using a rotary flash evaporator (IKA RV 3) at 25°C. Finally, the concentrated crude extract was subjected to lyophilization (freeze-drying), yielding a dry crude pigment powder.

2.2 Chromatographic separation and purification

Preliminary chemical profiling of the crude dried pigment was executed using Thin Layer Chromatography (TLC). The lyophilized extract was reconstituted in methanol (MeOH) and spotted onto Merck F254 aluminium-precoated silica gel plates. Chromatographic separation was optimized using a mobile phase consisting of ethyl acetate: hexane (1:1, v/v) to calculate the retention factor (R_f) of the primary red pigment fraction [14]. Guided by the TLC R_f values, scale-up purification was performed using a Pure Chromatography Instrument C-810 (Buchi). A 400 mg aliquot of the crude pigment was loaded onto an Avra silica gel column (230–400 mesh) and eluted utilizing a linear gradient from 100% hexane to 100% ethyl acetate. Target fractions were isolated based on real-time UV absorption monitored by a Diode Array Detector (DAD) across a 200–400 nm range.

2.3. Spectroscopic and Structural Elucidation

2.3.1. High-Performance Liquid Chromatography (HPLC)

The purity and chromatographic behaviour of the pigment were evaluated via reverse-phase HPLC (Shimadzu) utilizing a C18 column (5 μ m particle size). Prior to analysis, HPLC-grade solvents (Merck) were ultrasonically degassed for 5 min. Isocratic elution was performed using a mobile phase comprising Milli-Q water with 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) at a 70:30 (v/v) ratio. The flow rate was maintained at 1.0 mL/min, with UV detection optimized at 535 nm.

2.3.2. UV-Visible and FT-IR Spectroscopy

Preliminary spectral characterization of the flash-purified pigment was conducted utilizing a Shimadzu UV-1780 spectrophotometer, with the sample dissolved in MeOH and scanned across a 200–700 nm range. To identify the functional groups associated with the prodigiosin scaffold, Fourier-transform infrared (FT-IR) spectroscopy was performed using a Shimadzu IRSpirit instrument. Spectra were recorded using the KBr pellet method within the 4000–500 cm^{-1} wavenumber range [15].

2.3.3. High-Resolution Liquid Chromatography-Mass Spectrometry (HRLCMS-QTOF)

Precise molecular mass determination was conducted using a High-Resolution Q-TOF LC-MS system (Agilent Technologies, USA). Separation was achieved on a GOLD C18 column (100 $\times$ 2.1 mm, 3 μ m). The mobile phase consisted of 0.1% formic acid in Milli-Q water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.3 mL/min under a system pressure of 1200 bar. A gradient elution program was applied as follows: 5% B for 1 min, ramped to 100% B from 1 to 25 min, held at 100% B until 30 min, and returned to 5% B from 31 to 35 min. Data acquisition and processing were executed using Agilent MassHunter Qualitative Analysis software (vB.06) [16].

2.3.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

Structural characterization of the purified pigment was achieved via 1D NMR spectroscopy. We acquired the spectra using a Bruker NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C . The purified compound was dissolved in deuterated chloroform (CDCl_3). Chemical shifts were reported in

parts per million (ppm), utilizing tetramethylsilane (TMS) as the internal standard and calibrating against the residual solvent peak [17] .

2.4. *In vitro* Anticancer Bioactivity Evaluation

2.4.1. Cell Line, Culture Conditions, and Sample Preparation

Human prostate cancer (PC-3) cell line was procured from the National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals) and maintained at 37°C in a humidified 5% CO₂ atmosphere (Biobase BSC-1300IIA2-X, China). Stock solutions of the purified pigment were prepared in dimethyl sulfoxide (DMSO) and diluted in culture media to ensure final solvent concentrations remained below 0.1% (v/v), precluding vehicle-induced cytotoxicity. Clinical-grade cisplatin was utilized as a positive chemotherapeutic reference standard across all biological assays.

2.4.2. Cytotoxicity and Viability Assays

Cytotoxicity was determined using the MTT assay. PC-3 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated overnight. Cells were treated separately with heptyl prodigiosin or cisplatin at 5, 25, 50, 100, 200, and 400 µg/mL for 24 h. Untreated cells served as controls, while wells containing only culture medium were used as blanks. After treatment, MTT solution (0.5 mg/mL) was added, and the plates were incubated for 3 h. The formazan crystals formed were dissolved in DMSO, and absorbance was recorded at 570 nm using 630 nm as the reference wavelength. Dose-response curves were generated, and the IC₅₀ value for each compound was calculated independently.

Cell membrane integrity was examined by Acridine Orange/Propidium Iodide (AO/PI) staining. PC-3 cells (2×10^5 cells/well) were exposed to the respective IC₅₀ concentration of heptyl prodigiosin or cisplatin for 24 h, stained with AO/PI (1:1 mixture prepared from 2 mg/mL stock solutions) for 5 min, and observed under a fluorescence microscope (XDFL, Sunny Instruments). Live and dead cells were quantified using ImageJ software (v1.53c, National Institutes of Health, USA).

2.4.3. Flow Cytometric Apoptosis Profiling

To comprehensively evaluate the apoptotic cascade, PC-3 cells (3×10^5 cells/well) were exposed to the established IC₅₀ concentration of heptyl prodigiosin and cisplatin for 24 h. Following exposure, cells were harvested and aliquoted for parallel flow cytometric analyses using a Cytomics FC500 flow cytometer (Beckman Coulter, USA) and analysed using FlowJo X software (v10.0.7; BD Biosciences). To assess early apoptosis via phosphatidylserine externalization, harvested cells were co-stained with Annexin V and Propidium Iodide (PI) for 15 min at room temperature using the Annexin V-AbFlour™ 488 Kit (Abbkine). Activated caspase activity was subsequently quantified by fixing and permeabilizing the cells (0.1% Triton X-100) before a 30-min dark incubation with a FITC-conjugated anti-active caspase-3 antibody (BD Biosciences). Finally, late-stage DNA fragmentation was evaluated utilizing the APO-

DIRECT™ TUNEL Kit (BD Biosciences), wherein ethanol-fixed cells were incubated with terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP for 60 min at 37°C, followed by PI counterstaining to assess cell cycle distribution and DNA strand breaks.

2.4.4. Clonogenic Colony Formation Assay

Long-term reproductive survival was evaluated using PC-3 cells. Cells (1,000 cells/well) were exposed to the established IC₅₀ concentrations of the heptyl prodigiosin and cisplatin for 24 h. The media was subsequently replaced, and cells were incubated undisturbed for 8 days. Macroscopic colonies (≥ 50 cells) were methanol-fixed, stained with 0.4 g/L crystal violet, and quantified via ImageJ to calculate the surviving fraction relative to controls.

2.5. Statistical Analysis

All quantitative experiments were conducted in independent biological triplicates ($n = 3$). Data are expressed as mean $\pm$ standard deviation. Statistical significance ($p < 0.05$) was determined via one-way ANOVA followed by Tukey's HSD post-hoc test.

Result and Discussion

3.1. Isolation and Preliminary Characterization of the pigment.

The estuarine bacterium *Zooshikella* sp. RANM57 produced a red-purple pigment when cultured in Netravathi Estuarine Broth. Approximately 400 mg of the enriched crude extract, recovered via EtOH: DCM liquid-liquid partitioning, was subjected to flash chromatography employing a hexane: ethyl acetate gradient. This fractionation successfully isolated 14 mg of the red-purple primary bioactive constituent, designated as RANM57-1, thereby affording a chromatographic recovery yield of 3.5% (w/w). Preliminary TLC profiling of this purified isolate RANM57-1 revealed a single, well-resolved spot with a retention factor (R_f) of 0.55, which transitioned to brown upon treatment with concentrated H₂SO₄, signifying a prodigiosin-type architecture (Fig. 1a). To quantitatively validate this purity and assess retention behaviour, reverse-phase HPLC was executed. The chromatogram at $\lambda_{\text{max}} = 535$ nm exhibited a single, well-resolved peak corresponding to the isolated RANM57-1 (Fig. 1b).

With chromatographic purity established, the structural framework of RANM57-1 was investigated via multiparametric spectroscopy. UV-Vis spectroscopy analysis demonstrated a maximum absorption at $\lambda_{\text{max}} = 530$ nm, characteristic of the highly conjugated π -electron system inherent to prodigiosin derivatives. This absorption maximum is consistent with previously reported values for prodigiosin and its analogues, which typically range from 525 to 540 nm depending on the specific structure and solvent used [18, 19]. Subsequent FT-IR evaluation provided comprehensive functional group confirmation (Fig. 1c). The spectrum revealed a broad pyrrolic N-H stretching band at 3105 cm⁻¹ and aromatic C-H stretches at 2954 cm⁻¹, accompanied by discrete aliphatic C-H vibrations (2925 and 2854 cm⁻¹).

indicative of the alkyl extension. The presence of the conjugated tripyrrole core was further substantiated by a distinct C = N imine stretch at 1630 cm⁻¹, alongside aromatic/olefinic C = C stretching (1622–1548 cm⁻¹) and C-N stretching (1267 cm⁻¹). These spectral signatures are in strict concordance with established literature for prodigiosin-type architectures [18, 20].

3.2 HRLCMS QTOF

High-resolution electrospray ionization mass spectrometry (HRLCMS-QTOF) analysis of RANM57-1 provided mass-accurate support for the proposed elemental composition. The full-scan ESI-MS spectrum (Fig. 2a) exhibited a prominent protonated molecular ion [M + H]⁺ at m/z 352.2326 as the base peak. This corresponds to the molecular formula C₂₂H₃₀N₃O⁺ with a calculated exact mass of 352.2383 Da (mass error < 5 ppm). The absolute abundance of this ion exceeded 4×10⁶ counts, demonstrating excellent ionization efficiency under positive-ion ESI conditions. This robust ionization is highly consistent with the weakly basic nature of prodiginine alkaloids, wherein the three nitrogen atoms within the tripyrrole scaffold readily undergo protonation in acidified mobile phases.

Beyond the primary molecular ion, the mass spectrum revealed critical structural behaviours at higher m/z values. Most notably, an ion at m/z 739.4363 was tentatively assigned to a non-covalent aggregate [2M + H]⁺. The formation of this aggregate is a well-documented phenomenon for prodiginine compounds, driven by their high propensity for π-π stacking interactions between the extended, highly conjugated planar tripyrrole systems, particularly at elevated analyte concentrations [21]. Additional diagnostic peaks in the lower mass range further corroborated the core architecture. Specifically, the fragment at m/z 322.1854 represents an in-source fragmentation product arising from the neutral loss of formaldehyde (CH₂O, 30 Da) from the methoxy-substituted pyrrole ring [22, 23].

A high-resolution evaluation of the zoomed molecular ion region (m/z 320–380) (Fig. 2b) clearly displayed the expected isotopic distribution pattern. The M + 1 (m/z 353.235) and M + 2 (m/z 354.238) isotope peaks exhibited relative intensities in strict accordance with the natural abundance of ¹³C and ¹⁵N isotopes for a C₂₂H₃₀N₃O⁺ species, validating the molecular formula assignment [24]. Minor background signals in this region are attributable to standard matrix-related ions inherent to the ESI-MS analysis of complex biological extracts.

Collectively, this mass spectrometric data (Fig. 2c) supports a characteristic tripyrrole architecture consisting of a 4-methoxy-2,2'-bipyrrole moiety (rings A and B) connected via a methine bridge to a 2-alkylpyrrole unit (ring C) bearing a linear heptyl substituent. This structural motif is strictly conserved across the prodiginine family, with structural divergence restricted primarily to the alkyl chain variation [25].

3.3 Structural Elucidation by NMR Spectroscopy

The exact topological connectivity of RANM57-1 was conclusively validated via comprehensive 1D NMR profiling. The ^1H NMR spectrum (500 MHz, CDCl_3) confirmed the presence of the hydrogen-bonded tripyrrole architecture, characterized by two highly deshielded broad singlets at δ 12.54 and 12.68 representing the pyrrolic N-H protons (Fig. 3a). The B and C pyrrole rings were indicated by distinct one-proton singlets at δ 6.08 (H-3') and 6.68 (H-3'') while the A ring protons formed a well-defined coupled spin system. Crucially, the presence of the extended linear alkyl side chain was undeniably established. The spectrum displayed a diagnostic downfield triplet at δ 2.39 ($J = 7.5$ Hz) corresponding to the methylene group attached to the aromatic ring (H-7''), culminating in a characteristic terminal methyl triplet at δ 0.89 (3H, $J = 7.0$ Hz, H-13'').

This linear aliphatic progression was corroborated by the ^{13}C NMR spectrum (CDCl_3 , 125 MHz), which revealed 22 distinct carbon resonances (Fig. 3b). Alongside the expected quaternary pyrrolic signals and the methoxy carbon (δ 58.72), the spectrum exhibited a suite of seven consecutive aliphatic carbon resonances (δ 14.14 to 31.44) diagnostic of an unbranched heptyl extension. Based on this robust spectral concordance with established literature [26–28], the isolate RANM57-1 was authenticated as (2Z)-2-[(4-heptyl-5-methyl-1H-pyrrol-2-yl) methylidene]-3-methoxy-5-(1H-pyrrol-2-yl) pyrrole, commonly designated as heptyl prodigiosin. Detailed ^1H and ^{13}C chemical shift assignments are catalogued in the Supplementary Information (Table S1, Table S2).

Assessment of Metabolic Inhibition via MTT Assay

The effect of purified heptyl prodigiosin on the metabolic activity of the androgen-independent human prostate cancer cell line PC-3 was evaluated by the MTT assay. Heptyl prodigiosin caused a concentration-dependent reduction in MTT-derived metabolic activity, with an apparent IC_{50} of 9.00 $\mu\text{g/mL}$ after 24 h of treatment (Fig. 4a). Under the same assay conditions, cisplatin showed an apparent IC_{50} of 30.45 $\mu\text{g/mL}$ (Figure S1). Thus, the apparent IC_{50} of heptyl prodigiosin was 3.4-fold lower than that of cisplatin. The reduction in MTT-derived metabolic activity in PC-3 cells was significant across the tested concentrations ($P < 0.0001$). A marked decrease was observed between 5 and 25 $\mu\text{g/mL}$, with the relative metabolic activity decreasing from $78.03 \pm 0.53\%$ to $24.60 \pm 1.28\%$. At 100 $\mu\text{g/mL}$, the response approached a plateau, and no significant differences were observed at the higher concentrations tested up to 400 $\mu\text{g/mL}$.

The effect of heptyl prodigiosin was also evaluated in the normal human lung fibroblast cell line WI-38 over the same concentration range. After 24 h of treatment, a concentration-dependent reduction in MTT-derived metabolic activity was observed, with an apparent IC_{50} of 16.27 $\mu\text{g/mL}$ (Figure S2). Thus, the apparent IC_{50} in WI-38 cells was approximately 1.8-fold higher than that observed in PC-3 cells under the same experimental conditions.

Colony formation assay:

To evaluate long-term reproductive survival, a clonogenic colony formation assay was performed. Treatment with heptyl prodigiosin at 9.00 µg/mL reduced the clonogenic survival of PC-3 cells, yielding a surviving fraction of 0.67. In comparison, cisplatin at 30.45 µg/mL produced a substantially greater reduction in clonogenic survival, with a surviving fraction of 0.10 (Fig. 4b). These results demonstrate that heptyl prodigiosin reduces the long-term colony-forming ability of PC-3 cells.

AO/PI Dual Staining

To visually determine the mode of cell death, an AO/PI dual-staining assay was utilized (Fig. 5). Untreated control cells exhibited high baseline viability ($99.30 \pm 0.40\%$). Exposure to heptyl prodigiosin (9.00 µg/mL) for 24 hours resulted in $7.30 \pm 0.87\%$ of the population showing apoptotic cell death (EA + LA). The standard chemotherapeutic positive control cisplatin (30.45 µg/mL) showed a $19.40 \pm 2.99\%$ death of cells rate. Heptyl prodigiosin exhibits significant cytotoxicity while largely preserving initial membrane integrity. This combination strongly indicates the induction of a regulated apoptotic pathway rather than rapid primary necrosis [29].

Quantification of Apoptosis via Annexin V-FITC/PI Flow Cytometry

Flow cytometric analysis using Annexin V-FITC and PI dual staining was performed to evaluate the mode of cell death. PC-3 cells were treated with heptyl prodigiosin at the established IC_{50} (9.00 µg/mL) for 24 hours. The treatment triggered a strong pro-apoptotic response. Specifically, the early apoptotic population significantly increased to $17.60 \pm 0.28\%$, compared to the untreated control baseline of $3.92 \pm 0.11\%$. This shift was supported by a substantial rise in Annexin V mean fluorescence intensity (MFI). The MFI elevated from 1017.5 ± 40.3 to 2103.5 ± 37.5 , reflecting high-density phosphatidylserine externalization (supplementary Fig. 1). Notably, the combined late apoptotic and necrotic populations in the treated group remained negligible at $0.75 \pm 0.07\%$. (Table.1). These findings are consistent with regulated apoptotic cell death however, Annexin V signal specificity requires further validation with Ca^{2+} -dependence, autofluorescence, and orthogonal PS-detection control.

Table 1

Quantitative flow cytometric analysis of apoptotic induction in human PC-3 prostate cancer cells following 24 h treatment with Heptyl prodigiosin and Cisplatin.

S. No.	Sample Name	Live Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Debris (%)	MFI (Annexin V)
1.	Control (Untreated)	95.65 ± 0.21	3.92 ± 0.11	0.10 ± 0.01	0.35 ± 0.06	1017.5 ± 40.3
2.	Cisplatin (30.45 µg/mL)	62.00 ± 1.27**	36.05 ± 1.34**	1.77 ± 0.11**	0.20 ± 0.05	3852.0 ± 28.3
3.	Heptyl prodigiosin (9.00 µg/mL)	81.65 ± 0.21**	17.60 ± 0.28**	0.41 ± 0.06**	0.39 ± 0.08	2103.5 ± 37.5

Data represent the mean ± SD of technical replicates (n = 3) analysed via one-way ANOVA and Tukey's HSD post-hoc test (**p < 0.01 vs. untreated control). MFI: geometric mean fluorescence intensity.

Caspase-3 Assay

To evaluate the execution phase of apoptosis, active Caspase-3 expression was quantified using flow cytometry. A 24-hour treatment with heptyl prodigiosin (9.00 µg/mL) significantly increased the active Caspase-3 population to 52.45 ± 0.64%. In contrast, untreated control cells maintained a baseline of just 4.95 ± 0.49%. This proteolytic activation was further supported by a shift in geometric mean fluorescence intensity (MFI). The MFI rose from 1113.0 ± 22.6 in the control to 4174.5 ± 44.5 in the treated group, representing a 3.75-fold increase in active protease abundance (P < 0.01). Notably, heptyl prodigiosin induced caspase activation levels nearly comparable to the cisplatin standard (61.70 ± 0.57%; MFI 4890.0 ± 59.4), despite being applied at a 3.4-fold lower concentration (Table 2). These results demonstrate that heptyl prodigiosin drives cytotoxicity through a structured, caspase-dependent apoptotic pathway. It functions as a potent and precise activator of programmed cell death in recalcitrant, androgen-independent prostate cancer cells.

Table 2

Effect of Heptyl prodigiosin on Caspase-3 activation in PC-3 cells

Sample Name	Caspase-3 + ve Cells (%)	MFI (FITC Caspase-3)
Control (Untreated)	4.95 ± 0.49	1113.0 ± 22.6
Cisplatin (30.447 µg/mL)	61.70 ± 0.57**	4890.0 ± 59.4
Heptyl prodigiosin (9.003 µg/mL)	52.45 ± 0.64**	4174.5 ± 44.5

Data represent the mean ± SD (n = 3) analysed via one-way ANOVA and Tukey's HSD post-hoc test. Statistical significance compared to the untreated control is indicated by ** (p < 0.01). MFI: Mean

Fluorescence Intensity; FITC: Fluorescein isothiocyanate.

TUNEL Assay for DNA Fragmentation Quantification

DNA fragmentation was assessed using the TUNEL assay to determine whether DNA strand breaks were associated with the observed cytotoxic effect. After 24 h of treatment with heptyl prodigiosin (9.00 µg/mL), an increase in TUNEL-positive PC-3 cells was observed compared with the untreated control. The proportion of cells showing late-stage DNA fragmentation increased from $6.75 \pm 0.50\%$ in the control to $21.25 \pm 1.20\%$ following treatment. This increase was further reflected in the dUTP-FITC mean fluorescence intensity (MFI), which increased from 466.5 ± 6.36 to 700.0 ± 11.31 (Table 3). These findings indicate an increase in DNA strand breaks following heptyl prodigiosin treatment and are consistent with increased apoptotic DNA fragmentation in PC-3 cells after 24 h of exposure.

Table 3
Quantitative flow cytometric analysis of DNA fragmentation and mean fluorescence intensity in human PC-3 cells following 24 h treatment.

Sample Name	Healthy Cells (%)	Damaged DNA (%)	MFI (dUTP FITC)
Control (Untreated)	93.25 ± 0.50	6.75 ± 0.50	466.5 ± 6.36
Cisplatin (30.447 µg/mL)	65.10 ± 0.28	34.90 ± 0.28	890.0 ± 38.18
Heptyl prodigiosin (9.003 µg/mL)	78.75 ± 1.20	21.25 ± 1.20	700.0 ± 11.31

Data represent the mean $\pm$ standard deviation of technical replicates (n = 3) analysed via one-way ANOVA and Tukey’s HSD post-hoc test (**p < 0.01 vs. untreated control). MFI; geometric mean fluorescence intensity.

Discussion

Estuarine environments, such as those associated with the Netravathi River, are characterized by variable physicochemical conditions that may influence microbial secondary metabolism. In the present study, heptyl prodigiosin was identified as the major bioactive metabolite produced by *Zooshikella* sp. RANM57. HRLCMS-QTOF and NMR analyses supported the presence of an unbranched heptyl side chain attached to the tripyrrole core. The presence of the heptyl chain is expected to increase the hydrophobic character of the molecule and may influence its interaction with biological membranes. Similar membrane interactions have been reported for prodigiosin [30, 31], although the membrane behaviour of heptyl prodigiosin was not examined in the present study. Heptyl prodigiosin was previously isolated from a marine alpha-proteobacterium during antiparasitic screening [7]. The present study therefore identifies *Zooshikella* sp. RANM57 as an additional microbial source of heptyl prodigiosin and provides an initial *in vitro* assessment of its antiproliferative and apoptosis-associated effects in PC-3 prostate cancer cells.

After 24 h of treatment, heptyl prodigiosin showed a lower apparent IC_{50} than cisplatin under the conditions tested. This finding indicates a greater effect on PC-3 cell metabolic activity under these assay conditions but does not by itself establish greater overall cytotoxic or anticancer activity than cisplatin. Heptyl prodigiosin also reduced the metabolic activity of WI-38 cells, with an apparent IC_{50} of 16.27 $\mu\text{g/mL}$, compared with 9.00 $\mu\text{g/mL}$ in PC-3 cells. Members of the prodiginine family, particularly prodigiosin, have been widely investigated for their anticancer activity, with apoptosis reported in several cancer models [32]. Previous studies have reported inhibition of PC-3 tumour growth by prodigiosin, accompanied by activation of caspase-9 and caspase-3, an increased Bax/Bcl-2 ratio, and reduced expression of inhibitor of apoptosis proteins, including XIAP, cIAP-1 and cIAP-2 [33]. Heptyl prodigiosin has also been reported to induce caspase-dependent apoptosis in leukaemia models and to overcome the anti-apoptotic effects associated with Bcl-2 protein overexpression [34]. However, evidence for the activity of heptyl prodigiosin in prostate cancer models remains limited.

The clonogenic assay further showed a sustained reduction in the colony-forming ability of PC-3 cells following heptyl prodigiosin treatment. However, this reduction was less pronounced than the effect suggested by the lower apparent IC_{50} observed in the 24 h MTT assay. The difference may reflect the distinct biological endpoints assessed by the two assays. While the MTT assay measures cellular metabolic activity at a defined time point, the clonogenic assay assesses the ability of cells to retain long-term proliferative capacity. The reduced colony-forming ability observed in the present study is consistent with the reported effects of prodigiosin and related prodiginines on cancer cell proliferation and survival through multiple mechanisms [35].

Importantly, flow cytometric and morphological data suggest that this cell death occurs predominantly via a controlled apoptotic pathway, rather than non-specific necrosis. Treatment with heptyl prodigiosin induced significant externalization of phosphatidylserine (PS), observed in 17.60% of the cell population. This loss of normal membrane asymmetry is a classical early indicator of apoptosis. While mitochondrial variables were not directly measured in this study, the induction of mitochondrial dysfunction is a well-established characteristic of prodiginine cytotoxicity [36]. Previous literature demonstrates that lipophilic prodiginines rapidly accumulate in mitochondria and induce the release of cytochrome c, thereby triggering the intrinsic apoptotic cascade [37, 38].

Consistent with established upstream mechanisms, a clear progression into the terminal execution phase of cell death was observed. Treatment resulted in a marked activation of Caspase-3 (52.45%) and extensive late-stage DNA fragmentation (21.25%). The cleavage of the inhibitor of Caspase-Activated DNase (ICAD) by active Caspase-3 allows the free CAD enzyme to translocate into the nucleus [39]. This canonical signalling axis provides a mechanistic basis for the internucleosomal DNA breaks detected in the TUNEL assay, aligning with existing reports that extended-chain prodiginines utilize Caspase-3-dependent genomic degradation to induce apoptosis in resistant carcinoma models [40, 41]. These findings establish estuarine-derived heptyl prodigiosin as a potent lead compound capable of activating this apoptotic execution pathway, offering a potential strategy to overcome the survival mechanisms of androgen-independent prostate cancer cells.

Several limitations should be considered when interpreting these findings. The structural assignment of heptyl prodigiosin was supported by HRLCMS-QTOF and 1D NMR data, although 2D NMR analysis and comparison with an authentic standard would provide additional structural confirmation. Because heptyl prodigiosin is pigmented, interference with absorbance- or fluorescence-based measurements cannot be completely excluded. The observed phosphatidylserine externalization, caspase-3 activation, and DNA fragmentation are consistent with an apoptosis-associated phenotype, but the specific apoptotic pathway remains unresolved because mitochondrial membrane potential, cytochrome c release, and initiator caspases such as caspase-8 and caspase-9 were not examined. In addition, the reduction in WI-38 cell viability means that the present data cannot establish cancer-cell selectivity. Further studies using additional prostate cancer models, non-malignant prostate cells, and orthogonal approaches to assess cell death and compound activity will be needed to determine the extent and basis of the observed biological effects.

Conclusion

This study identifies *Zooshikella* sp. RANM57 as a microbial source of heptyl prodigiosin and provides experimental evidence for its biological activity against the androgen-independent PC-3 prostate cancer cell model. The findings link the observed reduction in cellular and clonogenic viability with cellular changes consistent with an apoptosis-associated response, while the broader cellular specificity and underlying molecular mechanism remain to be established. The study therefore provides a foundation for further investigation of heptyl prodigiosin using additional prostate cancer and non-malignant cell models, together with complementary approaches for structural and mechanistic validation.

Declarations

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Generative AI and AI-Assisted Technologies

During the preparation of this manuscript, artificial intelligence models (Gemini, Claude, and ChatGPT) were utilized strictly to improve language fluency, grammar, and spelling. After using these tools, the authors thoroughly reviewed and edited the content and assume full responsibility for the final integrity and accuracy of the publication.

Ethical Approval

No studies involving human participants or animals were conducted as part of this research. Therefore, ethical approval was not required.

Consent for Publication

All authors have approved the final manuscript and consented to its publication.

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by R.M., A.A., and S.P.C. Pigment purification and separation were conducted by S.I., K.R.D., S.R., and P.P. Structural compound elucidation was performed by K.B. The first draft of the manuscript was written by R.M. and S.P.C. All authors critically reviewed the manuscript and approved the final version

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Figures

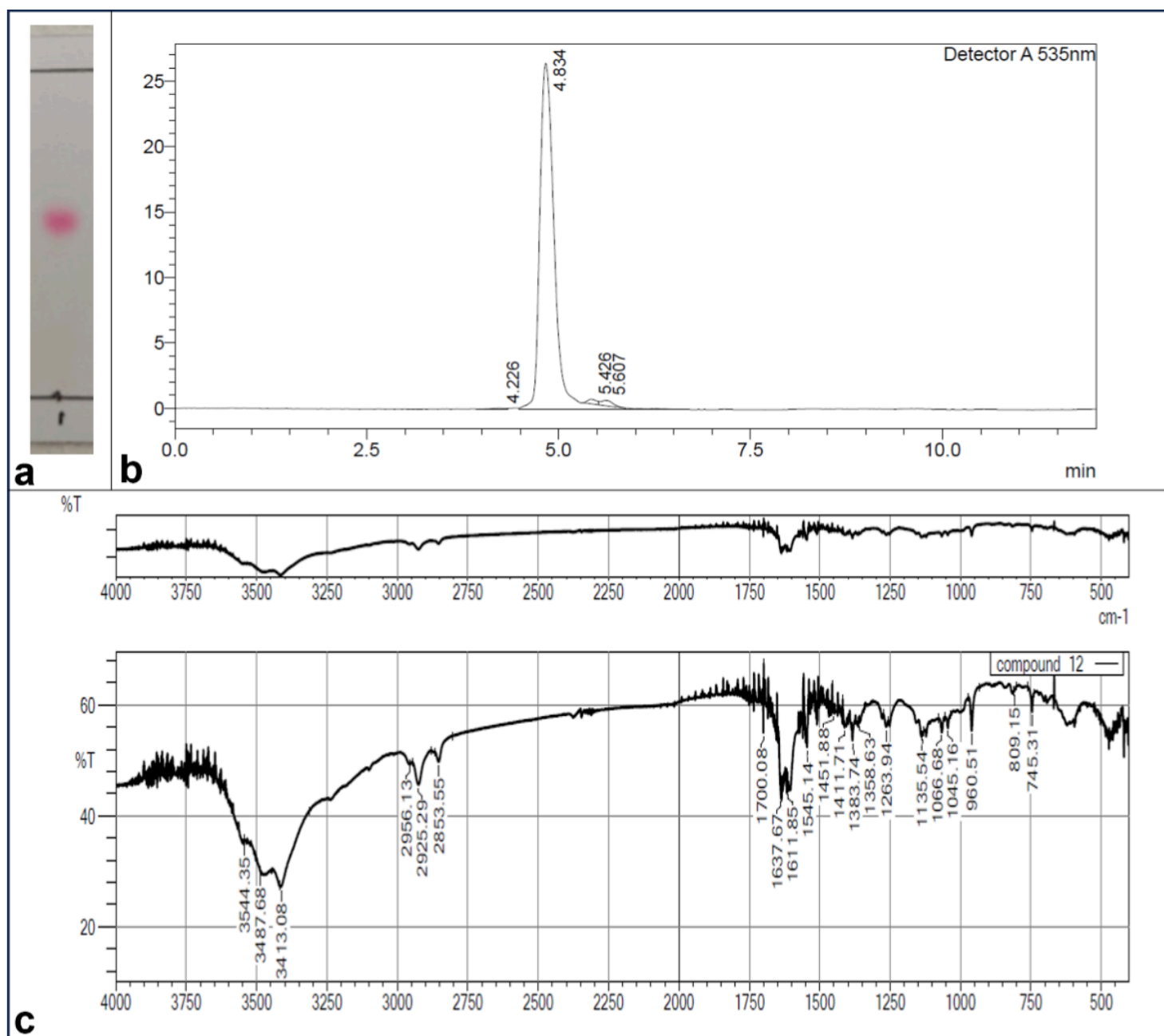


Figure 1

Chromatographic and spectral characterization of RANM57-1. (a) Thin-layer chromatography (TLC) of the purified pigment ($R_f = 0.55$). (b) Reverse-phase HPLC chromatogram demonstrating analytical purity at $\lambda_{\text{max}}535$. (c) FT-IR absorption spectrum illustrating characteristic pyrrolic and aliphatic functional group vibrations.

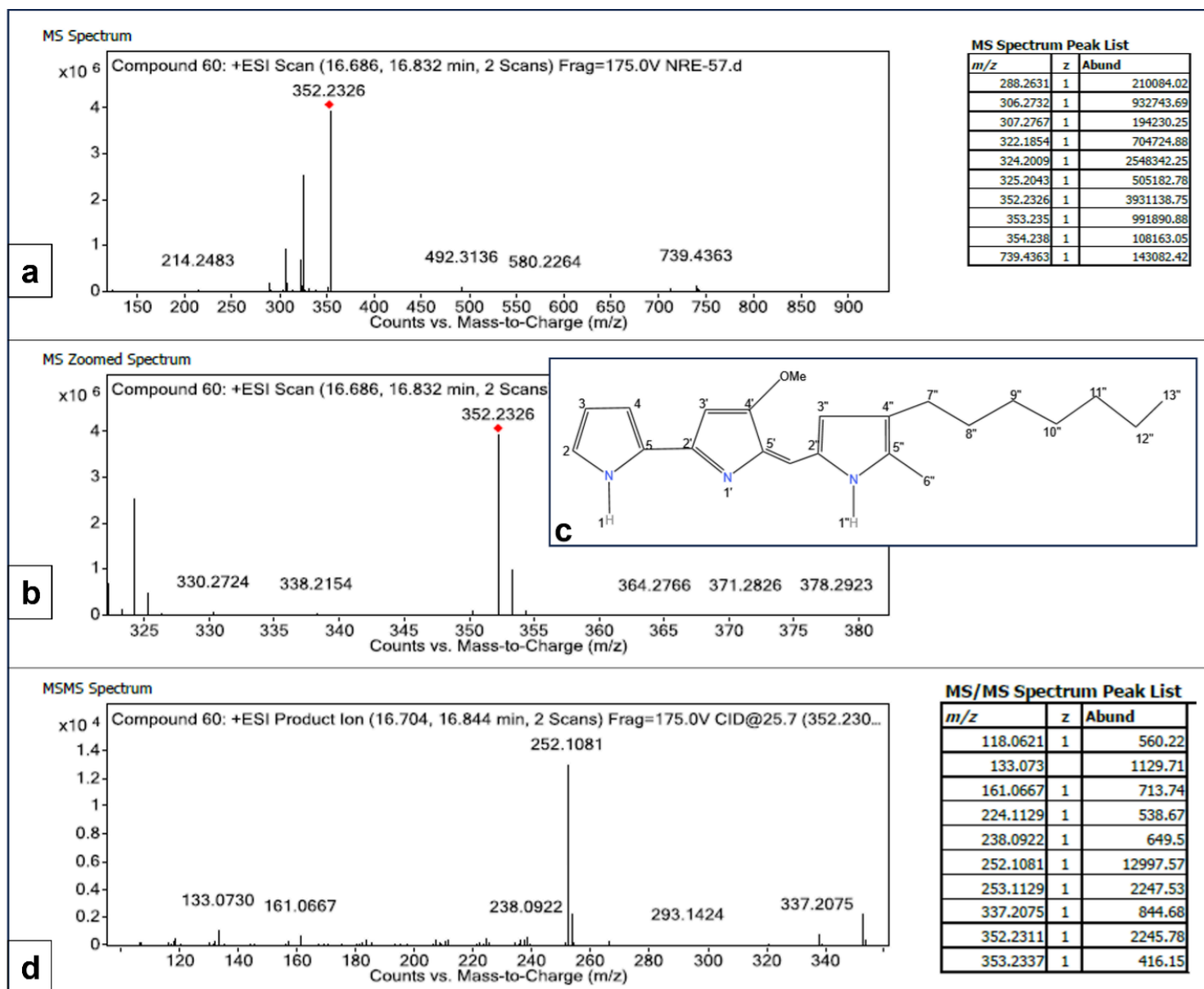


Figure 2

High-resolution mass spectrometric (HRLCMS-QTOF) analysis of RANM57-1. (a) Full-scan ESI-MS spectrum identifying the protonated molecular ion $[M+H]^+$ at m/z 352.2326. (b) Zoomed mass spectrum confirming the expected isotopic distribution. (c) Structural assignment of heptyl prodigiosin detailing the tripyrrole architecture. (d) MS/MS product ion spectrum showing characteristic fragmentation patterns, notably the base peak at m/z 252.1081 indicative of heptyl chain loss.

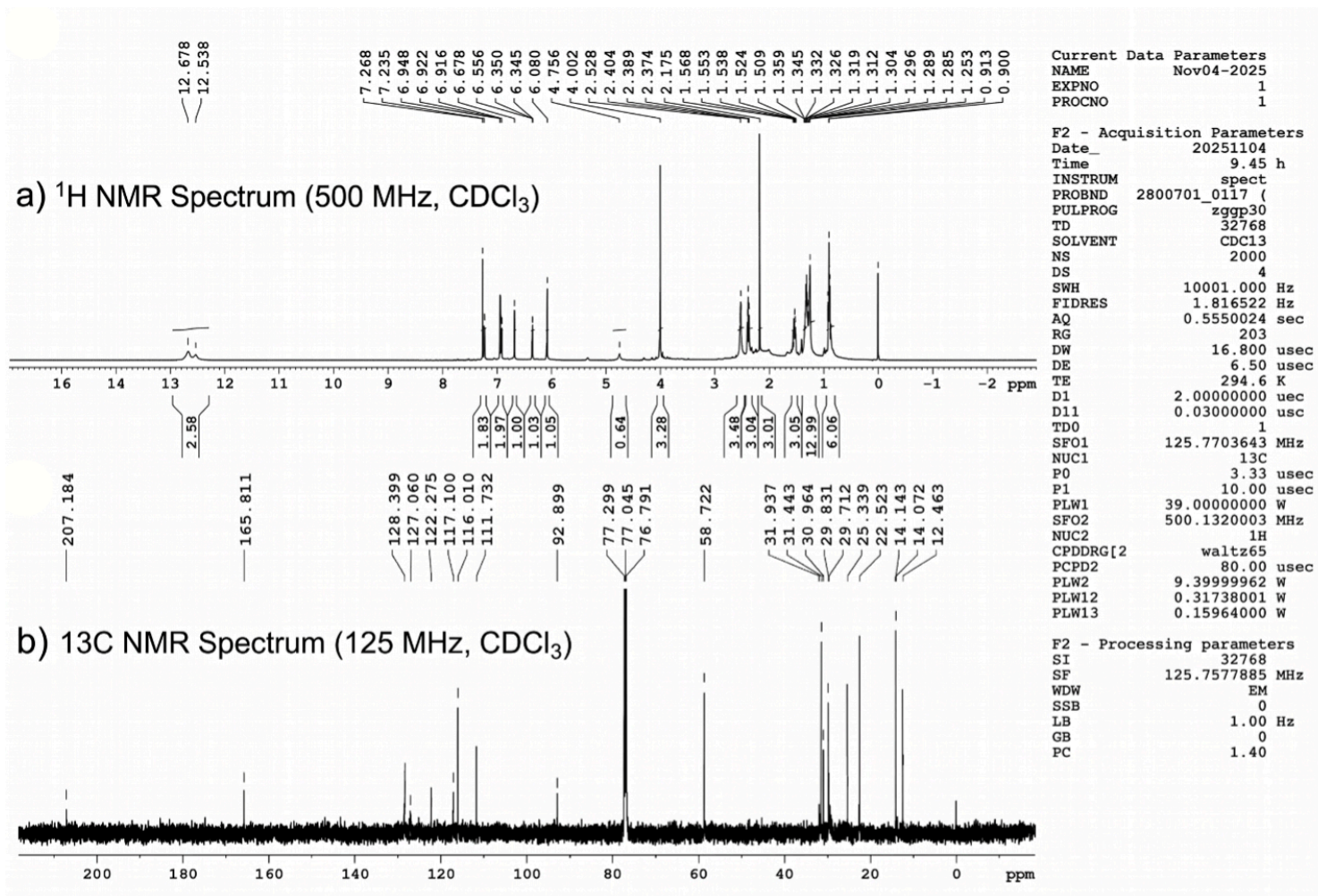


Figure 3

1D NMR spectral profiling of purified heptyl prodigiosin. (a) ^1H NMR spectrum (500 MHz, CDCl_3) and (b) ^{13}C NMR spectrum (125 MHz, CDCl_3) of the primary bioactive isolate derived from *Zooshikella* sp. RANM57. The spectra collectively confirm the conjugated tripyrrole core architecture and the defining linear heptyl extension of the prodiginine scaffold.

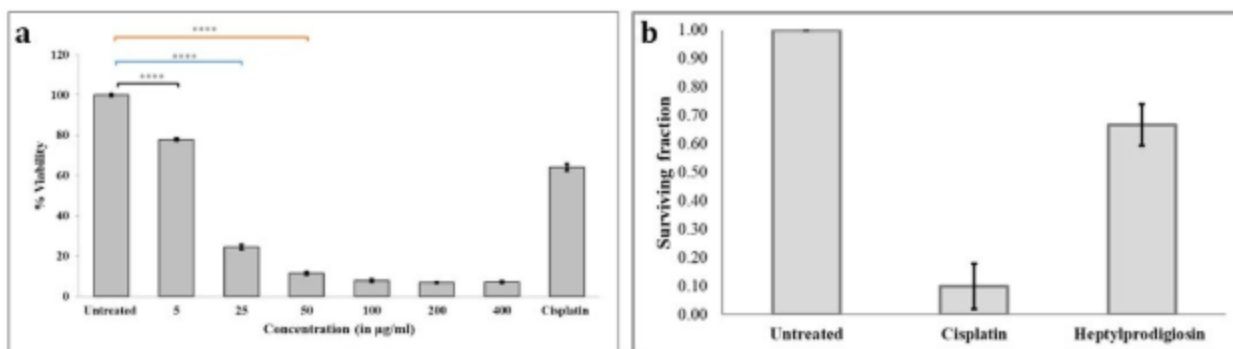


Figure 4

Effect of heptyl prodigiosin on the viability and clonogenic survival of PC-3 prostate cancer cells. (A) Cell viability determined by MTT assay. Cells were treated with the indicated concentrations of heptyl prodigiosin (5-400 $\mu\text{g/mL}$) or the cisplatin reference standard for 24 h. (B) Clonogenic survival assay. Cells were exposed to their respective IC_{50} concentrations for 24 h, followed by an 8-day incubation in drug-free medium to assess colony formation. Data are presented as the mean $\pm$ standard deviation of three independent experiments ($n = 3$). Statistical significance was evaluated using one-way ANOVA followed by Tukey's post-hoc test (**** $P < 0.0001$ versus the untreated control).

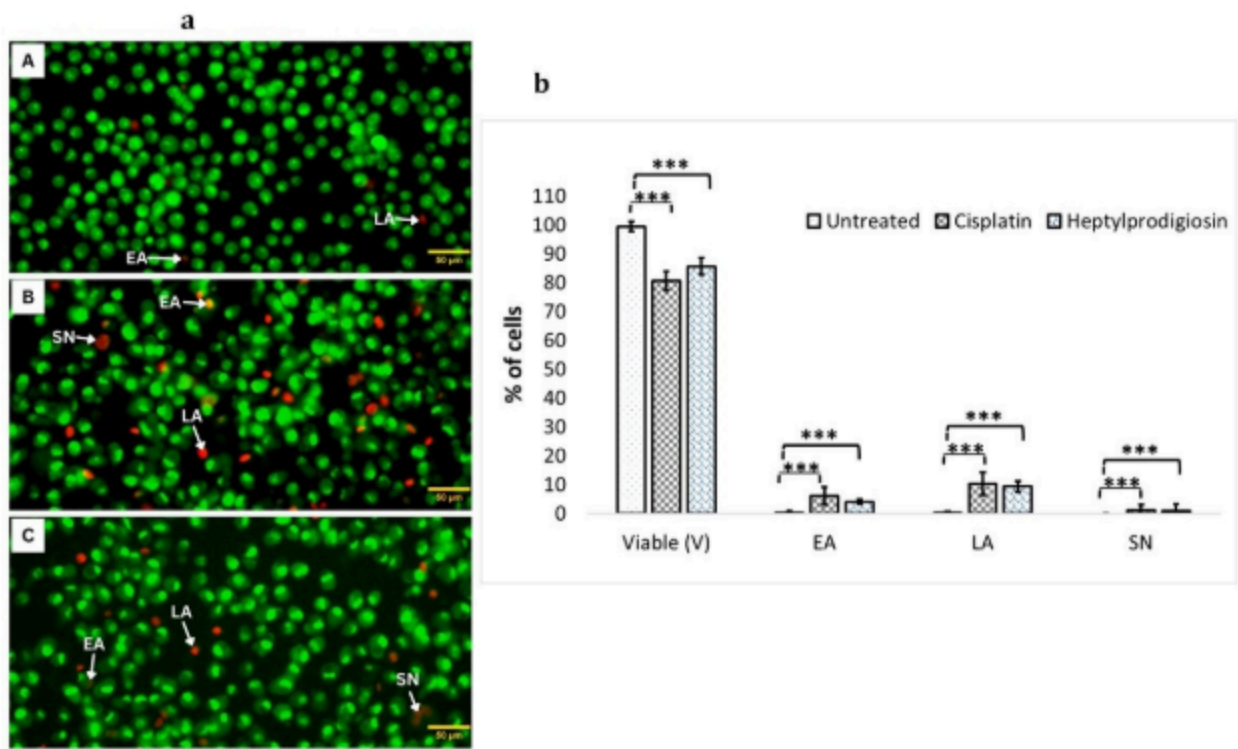


Figure 5

Evaluation of PC-3 cell death induction via AO/PI dual staining. a. Representative fluorescence micrographs of PC-3 cells following 24 h exposure to (A) untreated control, (B) Cisplatin, and (C) Heptyl prodigiosin. b. Quantitative distribution of cell populations across different death stages. Data represent the Mean $\pm$ SD ($n = 3$). Statistical significance was analysed via one-way ANOVA followed by Tukey's HSD post-hoc test, where triple asterisks (***) denote significant differences ($P < 0.001$) compared to the untreated control group

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