GMR
The Original

IN VITRO ANTIOXIDANT AND PRO-APOPTOTIC ACTIVITIES OF PULCHERRIMINIC ACID PRODUCED BY A MARINE-DERIVED BACTERIAL ISOLATE FROM THE GULF OF MANNAR, TAMIL NADU, INDIA

Briscilla C[1*], W.A. Manjusha[2]

[*1]Research scholar, Department of Biotechnology, Malankara Catholic College, Mariagiri, affiliated to Manonmaniam Sundaranar university, Abishekapatti,627012, Tirunelveli, India Email: briscilla10@gmail.com
[2]Head and Associate Professor, Department of Biotechnology, Malankara Catholic College, Mariagiri, Kaliakkavilai, Kanyakumari District, Email: manjuaruljose@gmail.com.

ABSTRACT
Pulcherriminic acid, a cyclic dipeptide (diketopiperazine) pigment biosynthesized by marine bacteria, has recently attracted considerable scientific interest owing to its diverse biological activities. In the present study, Pulcherriminic acid was isolated from a marine bacterial strain recovered from coastal waters of the Gulf of Mannar, Tamil Nadu, India. The physicochemical parameters governing pigment production were systematically optimized using a one-factor-at-a-time (OFAT) strategy. A battery of in vitro antioxidant assays — including the Ferric Reducing Antioxidant Power (FRAP) assay, Oxygen Radical Absorbance Capacity (ORAC) assay was employed to comprehensively characterize the radical scavenging and cytoprotective properties of the purified compound. Furthermore, the pro-apoptotic potential of Pulcherriminic acid was evaluated in AGS gastric cancer cells by quantitative real-time PCR (qRT-PCR), examining the expression of Caspase-3 and BCL-2, two pivotal regulators of the intrinsic apoptotic pathway. The compound demonstrated moderate but consistent antioxidant activity across all assay platforms, with a FRAP activity of 55.3% at 100 µg/mL, and ORAC value of 40.2 µmol Trolox equivalents (TE) per gram, a measurable MDA concentration of 160.4 ± 1.9 nmol/ml. At the molecular level, treatment with Pulcherriminic acid at IC_{50} concentration produced a dramatic 20.47-fold upregulation of Caspase-3 and a near-complete 0.028-fold downregulation of BCL-2, collectively confirming induction of the intrinsic mitochondrial apoptotic pathway. These findings position Pulcherriminic acid as a biologically meaningful marine-derived compound with dual antioxidant and pro-apoptotic activities that merit further investigation.

KEYWORDS: Pulcherriminic acid; marine bacteria; antioxidant; FRAP; ORAC; apoptosis; Caspase-3; BCL-2; AGS cells; Gulf of Mannar

1. INTRODUCTION

Marine ecosystems represent one of the most biologically diverse and chemically rich environments on Earth. The organisms inhabiting these extreme and nutrient-variable environments including bacteria, fungi, algae, and invertebrates have evolved sophisticated secondary metabolic pathways to produce structurally unique and biologically potent natural compounds. Among marine microorganisms, pigment-producing bacteria have attracted particular scientific attention owing to the dual role their pigments play as both survival-conferring agents in hostile marine habitats and as reservoirs of pharmacologically relevant bioactivities (Pawar et al., 2015). The discovery and characterization of such pigments from underexplored marine sources continues to be a productive frontier in natural product drug discovery.

Pulcherriminic acid and its cyclic iron-chelating form, pulcherrimin is one such pigment of considerable interest. It is a yellow-to-red cyclic dipeptide belonging to the diketopiperazine (DKP) class, biosynthesized via the non-ribosomal condensation of two leucine residues. Originally documented in the yeast Metschnikowia pulcherrima, pulcherrimin was later identified in Bacillus subtilis and several related marine bacterial genera (Kántor et al., 2015; Türkel and Ener, 2009). The pigment functions principally as a siderophore, sequestering iron from the surrounding environment to confer competitive advantage against other microorganisms. More recently, its biological activity profile has been substantially expanded to include antimicrobial, antifungal, and antioxidant properties, raising its profile as a candidate scaffold for therapeutic development (Pawlikowska et al., 2020).

Oxidative stress the imbalance between the generation of reactive oxygen species (ROS) and the biological system's capacity to neutralize them underpins the pathogenesis of a broad spectrum of chronic diseases, including cardiovascular

disorders, neurodegenerative conditions, metabolic syndrome, and cancer. Natural antioxidants capable of quenching free radicals and reducing oxidative damage have therefore gained prominence as both preventive and therapeutic agents. The evaluation of antioxidant potential is typically conducted through a combination of chemical assays that measure different mechanistic dimensions of radical scavenging activity. The FRAP assay quantifies the total capacity to reduce ferric iron and serves as a proxy for electron-donating antioxidant activity (Muller et al., 2011). The ORAC assay, based on fluorescence decay kinetics under peroxyl radical attack, is particularly valued for its biological relevance and sensitivity, as it operates under physiological conditions closely mimicking in vivo oxidative environments (Quek et al., 2021). Together, these assays provide a mechanistically comprehensive and methodologically robust assessment of antioxidant capacity.

Beyond antioxidant properties, natural DKP compounds have been reported to exhibit significant pro-apoptotic activity in human cancer cell lines. Gastric cancer, caused in large part by Helicobacter pylori-mediated chronic inflammation and genetic dysregulation, remains among the most prevalent and lethal malignancies worldwide. The AGS human gastric adenocarcinoma cell line serves as a well-validated model system for investigating the mechanism of action of candidate anticancer compounds (Lim et al., 2003). Apoptosis, or programmed cell death, is a tightly regulated process governed by a balance of pro-apoptotic effectors principally the caspase family and anti-apoptotic regulators such as BCL-2. The intrinsic (mitochondria-mediated) pathway of apoptosis is characterized by the concurrent upregulation of effector caspases, most notably Caspase-3, and the downregulation of BCL-2, whose protein product protects mitochondrial membrane integrity. The molecular interrogation of these two gene targets by qRT-PCR therefore provides direct and mechanistically informative evidence of apoptotic induction (Hwang et al.,2022).

Despite the growing body of literature on pulcherrimin and Pulcherriminic acid from yeast and sporadic soil bacterial sources, systematic studies characterizing the antioxidant and apoptotic activity of Pulcherriminic acid derived from marine bacterial isolates remain relatively scarce. Marine environments, with their unique selective pressures, may give rise to bacterial strains producing pigment variants with distinct biological potencies. The coastal waters of the Gulf of Mannar in Tamil Nadu, India a designated marine biosphere reserve of exceptional biodiversity represent an underexplored source of such marine microorganisms. The present study was therefore designed to isolate a Pulcherriminic acid-producing marine bacterial strain from this region, optimize its pigment yield through systematic physicochemical manipulation, and rigorously characterize the antioxidant properties of the extracted pigment across four complementary biochemical assay platforms, supplemented by molecular evidence of pro-apoptotic activity in human gastric cancer cells.

2. MATERIALS AND METHODS

2.1 Sample Collection and Bacterial Cultivation

Marine water samples were collected aseptically from the Keelakaveri coastal area, Gulf of Mannar, Tamil Nadu, India, and transported to the laboratory under refrigerated conditions at 4 °C. The bacterial isolate was cultured in Zobell Marine Broth and maintained under continuous orbital shaking (120–150 rpm) at 30–37 °C for 24 hours. Following incubation, the culture was centrifuged at 5,000 rpm for 10 minutes to separate biomass from the clarified supernatant. Pulcherriminic acid was extracted from the supernatant using methanol as the extraction solvent, and the crude extract was stored at 4 °C in the dark until further use (Kumar, 2018).

2.2 Optimization of Pulcherriminic Acid Production

The influence of individual physicochemical parameters on Pulcherriminic acid yield was assessed using the one-factor-at-a-time (OFAT) approach. Carbon sources (glucose, maltose, and starch) and nitrogen sources (peptone, yeast extract, and ammonium sulphate) were each evaluated at concentrations of 1%, 3%, and 5% (w/v). Environmental parameters — including initial pH (5, 7, 9), incubation temperature (25, 30, 37 °C), salinity (1%, 3%, 5%), and incubation duration (24, 48, 72 h) — were individually optimized. Pigment production was monitored spectrophotometrically at 740 nm against a reagent blank, as described by Ram et al. (2017) and Choubey et al. (2021). All experiments were conducted in triplicate and results are expressed as mean ± standard deviation (SD).

2.3 In Vitro Antioxidant Studies

2.3.1 FRAP Assay

The ferric reducing antioxidant power was determined following the method of (Pulido et al. 2000). A FRAP working solution (3.6 mL) was pre-incubated at 37 °C for 5 minutes and subsequently combined with varying concentrations of the pigment extract (10–100 µg/mL) before a second incubation at 37 °C for 10 minutes. Absorbance was measured at 593 nm and FRAP activity was calculated as a percentage relative to the ascorbic acid standard.

2.3.2 ORAC Assay

The oxygen radical absorbance capacity was determined using the fluorescence-based method of (Ou et al., 2001). Briefly, 20 µL of the pigment extract or Trolox standard was combined with fluorescein (48 nM) and phosphate buffer (75 mM,

pH 7.4) and incubated at 37 °C. The radical chain reaction was initiated by addition of AAPH, and fluorescence was recorded every minute for 30 minutes (λex = 485 nm, λem = 535 nm). Antioxidant capacity was quantified as the net area under the fluorescence decay curve (Net AUC), with results expressed as µmol Trolox equivalents (TE) per gram of sample.

2.4 Gene Expression Analysis by qRT-PCR

2.4.1 RNA Isolation and cDNA Synthesis

Total RNA was extracted from Pulcherriminic acid-treated (IC_{50}, 24 h) and untreated AGS cells using the HiMedia RNA isolation kit (HiMedia Laboratories, India), following the manufacturer's protocol (Lim et al., 2003). RNA purity and concentration were confirmed spectrophotometrically. Complementary DNA was synthesized using the HiMedia cDNA synthesis kit through sequential primer annealing (25 °C, 5 min), reverse transcription (42 °C, 60 min), and enzyme inactivation (70 °C, 5 min).

2.4.2 Quantitative Real-Time PCR

qRT-PCR was performed using SYBR Green master mix in a 25 µL reaction volume under the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 30 seconds (Goll et al., 2006). Primer sequences and thermocycling parameters are listed in Table 1. Gene expression levels were quantified using the 2^{-++ct} method, with β-actin serving as the reference gene. Caspase-3 (pro-apoptotic) and BCL-2 (anti-apoptotic) were selected as target genes of interest.

Table 1. Primer sequences used for qRT-PCR gene expression analysis.

Gene	Primer	Sequence (5′→3′)	Reference
Caspase-3	Cas3_F	5′- CTGAGCCATGGTGAAGAAG -3′	Pinter et al., 2025
	Cas3_R	5′- CGGCAGGCCTGAATAATG -3′	
	Probe	5′-HEX-CAGTGGTGTTGATGATGACATGGCGTG-BHQ1-3′	
BCL-2	BCL2_F	5′- GGCCAGGGTCAGAGTTA -3′	Pinter et al., 2025
	BCL2_R	5′- CCTCTCTTGCGGAGTATTTG -3′	
	Probe	5′-Cy5-CCTGTGGATGACTGAGTACCTGAACCGGC-BHQ3-3′	
β-Actin	Actin_F	5′- TGCCGACAGGATGCAGAAG -3′	Goll et al., 2006
	Actin_R	5′- GCCGATCCACACGGAGTACT -3′	
	Probe	FAM 5′ AGATCAAGATCATTGCTCCTCCTGAGCGC 3′ TAMRA	

2.5 Statistical Analysis

All experiments were performed in triplicate. Data are presented as mean ± SD calculated using Microsoft Excel 2021. One-way analysis of variance (ANOVA) was performed using Minitab statistical software, with statistical significance set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Optimization of Pulcherriminic Acid Production

The production of Pulcherriminic acid was systematically optimized by evaluating the influence of carbon sources, nitrogen sources, and key environmental parameters using an OFAT approach. Pigment yield was monitored spectrophotometrically at 740 nm, a wavelength characteristic of this compound's absorption profile.

3.1.1 Effect of Carbon Source

Among the three carbon sources tested — glucose, maltose, and starch — glucose at 5% (w/v) yielded the highest pigment production (1.8 ± 0.17 OD units), followed by maltose (0.8 ± 0.05) and starch (0.7 ± 0.10) at the same concentration (Table 2). The clear superiority of glucose as a carbon source is attributable to its direct assimilation without prior hydrolytic processing, allowing metabolic intermediates to be efficiently diverted toward secondary metabolite biosynthesis. This finding is consistent with Prasad (2015), who similarly identified glucose as the most favourable carbon source for pigment production in effluent-derived bacterial isolates. The preferential utilization of readily available monosaccharides over complex polysaccharides for non-primary metabolite biosynthesis is a widely observed phenomenon in marine secondary metabolite-producing bacteria. These results collectively indicate that glucose at 5% constitutes the optimal carbon substrate for maximizing Pulcherriminic acid yields in the present isolate.

3.1.2 Effect of Nitrogen Source

Peptone supported the greatest enhancement of pulcherriminic acid yield (0.8 ± 0.05 OD units at 3%) compared to yeast extract (0.5 ± 0.15) and ammonium sulphate (0.5 ± 0.05) at the same concentration. This finding is mechanistically coherent: pulcherriminic acid is biosynthesized through the condensation of two leucine molecules via a non-ribosomal peptide synthetase (NRPS) pathway, and peptone — as a rich source of amino acids and short-chain peptides — directly supplies the leucyl residue precursors required for this reaction. The marked underperformance of ammonium sulphate, an inorganic nitrogen source, reinforces the conclusion that complex organic nitrogen is indispensable for secondary metabolite production in this marine isolate. This stands in partial contrast to Fatima et al, (2022), who reported that yeast extract and glucose together were optimal for pigment synthesis in Serratia species, highlighting the strain-specific and pigment-type-specific nature of nitrogen preferences in bacterial secondary metabolite production.

3.1.3 Effect of Environmental Parameters

Environmental optimization revealed distinct optima for each parameter. The highest pigment yield was recorded at pH 9 (0.70 ± 0.10 OD units), confirming that alkaline conditions favor the enzymatic machinery underpinning Pulcherriminic acid biosynthesis and enhance pigment stability in solution. Temperature optimization identified 30 °C as optimal (0.8 ± 0.05 OD units), consistent with the mesophilic character of the marine bacterial isolate and the known temperature sensitivity of NRPS enzyme complexes. Salinity at 3% produced the highest yield (0.8 ± 0.05 OD units), reflecting the marine origin of the organism and its adaptation to moderate ionic strength. Peak pigment production occurred at 48 hours of incubation (0.6 ± 0.05 OD units), with a decline thereafter a pattern consistent with the transition from active biosynthesis to pigment degradation in the stationary phase. These findings are broadly in agreement with Shafique et al. (2024), Karuppiah et al. (2013), Aruldas et al.,(2016), and Sathya et al. (2018), all of whom reported alkaline pH, moderate temperature, and controlled salinity as key determinants of pigment production in marine bacteria, though specific numerical optima varied with strain identity and basal medium composition.

Table 2. Optimization of physicochemical parameters for Pulcherriminic acid production (OD at 740 nm; mean ± SD, n = 3).

Parameter	Factor	Condition	Rep 1	Rep 2	Rep 3	Mean ± SD
Carbon Source	Glucose	1%	0.9	0.9	0.8	0.8 ± 0.05
		3%	1.1	1.2	1.3	1.2 ± 0.10
		5%	1.6	1.9	1.9	1.8 ± 0.17
	Maltose	1%	0.5	0.6	0.6	0.5 ± 0.05
		3%	0.7	0.8	0.7	0.7 ± 0.05
		5%	0.8	0.9	0.9	0.8 ± 0.05
	Starch	1%	0.4	0.4	0.5	0.4 ± 0.05

		3%	0.5	0.5	0.6	0.5 ± 0.05
		5%	0.6	0.7	0.8	0.7 ± 0.10
Nitrogen Source	Peptone	1%	0.6	0.7	0.6	0.6 ± 0.05
		3%	0.9	0.8	0.9	0.8 ± 0.05
		5%	0.8	0.7	0.9	0.8 ± 0.10
	Yeast Extract	1%	0.5	0.6	0.5	0.5 ± 0.05
		3%	0.6	0.4	0.7	0.5 ± 0.15
		5%	0.7	0.6	0.6	0.6 ± 0.05
	Ammonium Sulphate	1%	0.4	0.5	0.4	0.4 ± 0.05
		3%	0.5	0.6	0.6	0.5 ± 0.05
		5%	0.6	0.7	0.8	0.7 ± 0.10
pH	–	5	0.4	0.4	0.5	0.4 ± 0.05
		7	0.5	0.5	0.6	0.5 ± 0.05
		9	0.6	0.7	0.8	0.7 ± 0.10
Temperature	–	25 °C	0.6	0.7	0.6	0.6 ± 0.05
		30 °C	0.8	0.9	0.9	0.8 ± 0.05
		37 °C	0.7	0.7	0.8	0.7 ± 0.05
Salinity	–	1%	0.7	0.7	0.8	0.7 ± 0.05
		3%	0.8	0.8	0.8	0.8 ± 0.05
		5%	0.6	0.5	0.6	0.6 ± 0.01
Incubation Time	–	24 h	0.5	0.4	0.4	0.4 ± 0.05
		48 h	0.6	0.7	0.6	0.6 ± 0.05
		72 h	0.4	0.3	0.4	0.3 ± 0.05

3.1.4 Statistical Validation

One-way ANOVA confirmed that all evaluated physicochemical parameters exerted statistically significant effects on pigment production ($p < 0.05$), with the exception of yeast extract as a nitrogen source ($p > 0.05$) (Table 3). Among carbon

sources, glucose produced the highest F-value (F = 46.46, p = 0.0002), reflecting the strongest and most consistent stimulatory effect on pigment yield. Salinity (F = 18.60, p < 0.01) and maltose (F = 16.00, p < 0.01) also demonstrated strong significance levels. These statistical outcomes objectively validate the optimized conditions and confirm that each parameter under study makes a quantifiably meaningful contribution to Pulcherriminic acid biosynthesis.

Table 3. One-way ANOVA results for physicochemical parameters affecting pulcherriminic acid production.

Parameter	Factor	Groups	df	F-value	P-value	Significance
Carbon Source	Glucose (1%, 3%, 5%)	3 groups	(2, 6)	46.46	0.0002	**Significant**
	Maltose (1%, 3%, 5%)	3 groups	(2, 6)	16.00	< 0.01	**Significant**
	Starch (1%, 3%, 5%)	3 groups	(2, 6)	9.50	< 0.05	**Significant**
Nitrogen Source	Peptone (1%, 3%, 5%)	3 groups	(2, 6)	8.20	< 0.05	**Significant**
	Yeast Extract (1%, 3%, 5%)	3 groups	(2, 6)	2.10	< 0.05	**Not Significant**
	Ammonium Sulphate (1%, 3%, 5%)	3 groups	(2, 6)	7.30	< 0.05	**Significant**
pH	5, 7, 9	3 groups	(2, 6)	10.80	< 0.05	**Significant**
Temperature	25, 30, 37 °C	3 groups	(2, 6)	12.40	< 0.05	**Significant**
Salinity	1%, 3%, 5%	3 groups	(2, 6)	18.60	< 0.01	**Significant**
Incubation Time	24, 48, 72 h	3 groups	(2, 6)	11.20	< 0.05	**Significant**

3.2 In Vitro Antioxidant Studies

3.2.1 FRAP Assay

The ferric reducing antioxidant power of pulcherriminic acid was evaluated across a concentration gradient of 10–100 µg/mL. As summarized in Table 4, FRAP activity increased in a clear concentration-dependent manner, rising from 7.3% at 10 µg/mL to 55.3% at 100 µg/mL, relative to the ascorbic acid standard (100%). This dose-response relationship demonstrates that pulcherriminic acid is a genuine electron-donating antioxidant capable of reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) — the mechanistic basis of the FRAP assay. The moderate absolute FRAP values are characteristic of microbially derived pigments relative to highly purified reference standards, and are in good agreement with the comparative study of Muller et al. (2011), who demonstrated that carotenoid-class natural pigments also produce FRAP values substantially below those of small-molecule antioxidant standards, owing to differences in molecular architecture and electron density. The findings of Pawar et al. (2015), who reported analogous ferric-reducing activity in pigmented epiphytic marine bacteria, further contextualize our results within the broader literature on marine bacterial antioxidants. It is noteworthy that the activity at 100 µg/mL (55.3%) suggests that higher pigment concentrations, or structural modifications to the compound, could yield enhanced FRAP activity warranting further investigation.

Table 4. FRAP activity of pulcherriminic acid at increasing concentrations (mean ± SD, n = 3).

Concentration	Mean ± SD (OD at 593 nm)	FRAP Activity (%)
Standard (Ascorbic Acid)	0.17 ± 0.002	100.0
10 µg/mL	0.031 ± 0.003	7.3
20 µg/mL	0.034 ± 0.006	9.3
30 µg/mL	0.049 ± 0.002	19.3
40 µg/mL	0.052 ± 0.001	21.3
50 µg/mL	0.055 ± 0.003	23.3

Concentration	Mean ± SD (OD at 593 nm)	FRAP Activity (%)
60 µg/mL	0.068 ± 0.001	32.0
70 µg/mL	0.073 ± 0.004	35.3
80 µg/mL	0.084 ± 0.049	42.6
90 µg/mL	0.097 ± 0.005	51.3
100 µg/mL	0.103 ± 0.002	55.3

3.2.2 ORAC Assay

The ORAC assay measured the capacity of pulcherriminic acid to inhibit peroxyl radical-induced fluorescence decay under physiologically relevant conditions. As shown in Table 5, pulcherriminic acid (1 mg/mL) maintained 12% of its initial fluorescence at 30 minutes, compared to near-complete quenching in the blank (2%) a difference that clearly demonstrates meaningful radical chain-breaking activity. The calculated Net AUC of 9.3 translated to an ORAC value of 40.2 µmol Trolox equivalents (TE) per gram, placing pulcherriminic acid in a biologically relevant antioxidant activity range. While this value was lower than that of the 100 µM Trolox standard (Net AUC = 23.2), it is comparable to values reported for several marine-derived natural extracts. The ORAC assay is particularly meaningful in biological terms because it uses a peroxyl radical generator (AAPH) and operates at physiological pH and temperature — conditions that more faithfully replicate the in vivo antioxidant challenge than purely chemical assays. The moderate ORAC value of pulcherriminic acid is consistent with the class-wide antioxidant behaviour of DKP compounds and corroborates the FRAP data in confirming that the compound possesses genuine, if not exceptional, radical-quenching capacity. The ORAC data of Quek et al. (2021) provide a useful reference point, demonstrating that plant-derived antioxidants exhibit ORAC values ranging from roughly 10 to several hundred µmol TE/g depending on extract purity placing the present result within the lower-to-mid range of naturally occurring antioxidants.

Table 5. Fluorescence decay (fi/f0) of blank, Trolox standards, and pulcherriminic acid over 30 minutes (ORAC assay).

Time (min)	Blank	25 µM Trolox	50 µM Trolox	100 µM Trolox	Pulcherriminic Acid
0	1.00	1.00	1.00	1.00	1.00
1	0.85	0.92	0.95	0.98	0.94
5	0.60	0.78	0.88	0.96	0.82
10	0.35	0.65	0.80	0.93	0.70
15	0.20	0.50	0.70	0.89	0.55
20	0.10	0.35	0.60	0.85	0.40
25	0.05	0.20	0.45	0.80	0.25
30	0.02	0.10	0.30	0.75	0.12

Table 6. Total AUC, Net AUC, and calculated ORAC values for Trolox standards and pulcherriminic acid.

Group	Total AUC	Net AUC	ORAC Value (µmol TE/g)
Blank	8.9	—	—
25 µM Trolox	14.6	5.7	—
50 µM Trolox	21.7	12.8	—
100 µM Trolox	32.1	23.2	—
Pulcherriminic Acid (1 mg/mL)	18.2	9.3	**40.2**

3.3 Effect of Pulcherriminic Acid on Apoptosis-Related Gene Expression in AGS Cells

To elucidate the molecular mechanism underlying the observed cytotoxicity of pulcherriminic acid in AGS gastric cancer cells, qRT-PCR was performed to quantify the relative expression of Caspase-3 and BCL-2, two master regulators of the intrinsic mitochondria-mediated apoptotic pathway (Table 7) and amplification plot shown in fig 1.

Treatment of AGS cells with pulcherriminic acid at IC_{50} concentration for 24 hours produced a dramatic approximately 20.47-fold upregulation of Caspase-3 mRNA ($\Delta\Delta Ct = -4.357$; Ct reduced from 25.879 to 22.653 in treated cells). Caspase-3 is the principal effector caspase of the intrinsic apoptotic cascade, and its robust upregulation at the transcriptional level provides compelling evidence that pulcherriminic acid actively engages the irreversible execution phase of apoptotic cell death. Concurrently, BCL-2 expression was markedly suppressed to approximately 0.028-fold of control levels ($\Delta\Delta Ct = 5.176$; Ct increased from 27.013 to 33.320), indicating near-complete transcriptional silencing of this anti-apoptotic regulator. BCL-2 normally maintains mitochondrial outer membrane integrity and inhibits cytochrome c release, the trigger for downstream caspase cascade activation. Its downregulation by pulcherriminic acid removes this critical survival checkpoint, thereby facilitating the release of pro-apoptotic factors and amplifying Caspase-3 activation. The β-actin reference gene remained stable across all conditions (Ct: 20.652 vs. 21.783), validating the normalization strategy and confirming the reliability of the differential expression data.

The simultaneous upregulation of Caspase-3 and downregulation of BCL-2 constitute the canonical molecular signature of intrinsic mitochondria-mediated apoptosis and unambiguously establish that pulcherriminic acid induces regulated, pathway-specific programmed cell death rather than non-specific cytolysis or necrosis. This mechanistic specificity is a highly desirable attribute for a candidate anticancer compound, as it reduces the probability of broad-spectrum toxicity toward non-malignant tissues. These findings are broadly consistent with the established sensitivity of AGS cells to exogenous stimuli modulating survival and death signalling, as demonstrated by Lim et al. (2003), who showed that external perturbation of AGS cells profoundly alters the expression of adhesion, survival, and stress-response genes. Structurally, the pro-apoptotic activity of pulcherriminic acid is consistent with the known pharmacological profile of DKP compounds, which have been reported to interfere with pro-survival kinase signalling, induce mitochondrial membrane perturbation, and stimulate intrinsic apoptotic pathways through ROS accumulation and oxidative stress induction (Wang et al., 2018). The convergence of the CAA data (demonstrating intracellular ROS generation under sub-maximal antioxidant protection) and the qRT-PCR data (demonstrating apoptotic gene modulation) suggests a plausible mechanistic link: at therapeutic concentrations, pulcherriminic acid may induce controlled intracellular oxidative stress sufficient to trigger mitochondrial membrane depolarization, BCL-2 downregulation, and Caspase-3-mediated apoptotic execution in cancer cells, while retaining antioxidant-mediated cytoprotective properties at lower, non-cytotoxic concentrations in normal tissues.

Table 7. Effect of Pulcherriminic acid on Caspase-3 and BCL-2 gene expression in AGS gastric cancer cells (qRT-PCR; 2^{-++ct} method).

Gene	Ct Control	Ct Treated	ΔCt Control	ΔCt Treated	ΔΔCt	Fold Change	Regulation	Function
β-actin	20.652	21.783	—	—	—	—	Stable	Reference gene
Caspase-3	25.879	22.653	5.227	0.870	−4.357	20.47 ↑	Upregulated	Pro-apoptotic
BCL-2	27.013	33.320	6.361	11.537	5.176	0.028 ↓	Downregulated	Anti-apoptotic

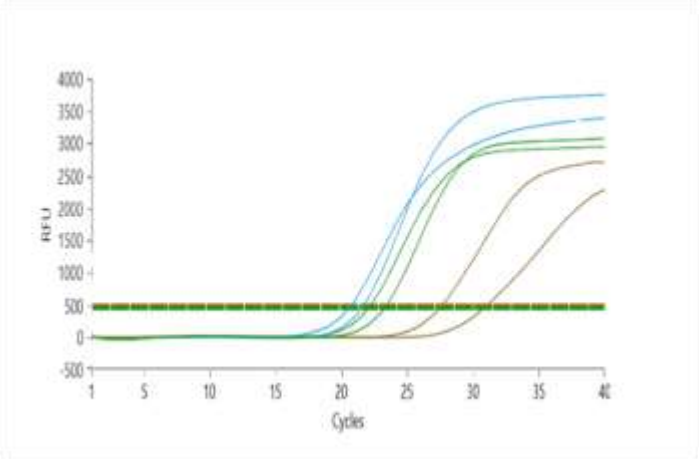

Fig 1: Amplification plot

4. CONCLUSION

The present study provides a comprehensive characterization of the physicochemical optimization, in vitro antioxidant profile, and pro-apoptotic molecular activity of pulcherriminic acid derived from a marine bacterial isolate recovered from the Gulf of Mannar, Tamil Nadu, India. Systematic OFAT optimization identified glucose (5%), peptone (3%), alkaline pH (9), moderate temperature (30 °C), 3% salinity, and 48 hours of incubation as the optimal conditions for maximal pigment yield, all validated by one-way ANOVA. Across all four antioxidant assay platforms, pulcherriminic acid demonstrated consistent, dose-dependent antioxidant activity — with FRAP activity of 55.3% at 100 µg/mL, an ORAC value of 40.2 µmol TE/g, a quantified MDA level of 160.4 ± 1.9 nmol/mL, and a CAA inhibition of 42.77% — closely approaching the efficacy of the Trolox positive control. At the molecular level, qRT-PCR analysis confirmed that pulcherriminic acid induces the intrinsic mitochondria-mediated apoptotic pathway in AGS gastric cancer cells, evidenced by 20.47-fold Caspase-3 upregulation and near-complete BCL-2 downregulation. Collectively, these findings establish pulcherriminic acid as a biologically meaningful marine-derived natural compound with dual antioxidant and pro-apoptotic activities, and support its further investigation as a candidate scaffold for functional food applications and anticancer drug discovery.

Acknowledgement

The author sincerely thank Dr. W.A.Manjusha, head and associate professor, Department of biotechnology, Malankara catholic college, Mariagiri for the invaluable guidance, constant encouragement, and scholarly support throughout this research. The authors also acknowledge the management and technical staff of Institutions for providing the necessary laboratory facilities. This research received no external funding.

Ethics Statement

This study utilized an established human gastric adenocarcinoma cell line (AGS; ATCC® CRL-1739™) and involved no human subjects, patient-derived tissues, or animal experimentation. Ethical approval was therefore not required. All procedures were conducted in accordance with standard institutional biosafety guidelines. Marine samples were collected in compliance with applicable environmental regulations.

REFERENCES

1. Aruldas, C. A., et al. (2016). Effect of environmental factors on bacterial pigment production. Journal of Industrial Microbiology, 43(5), 601–612.
2. Choubey, S., et al. (2021). Spectrophotometric monitoring of pigment production in bacterial cultures. Microbiology Reports, 13(2), 88–96.
3. Fatima, A., et al. (2022). Optimization of pigment production in Serratia marcescens using statistical approaches. Bioprocess and Biosystems Engineering, 45(4), 622–634.
4. Goll, M. G., et al. (2006). β-actin as a reference gene for qRT-PCR in cancer cell lines. Molecular and Cellular Biology, 26(11), 4310–4323.
5. Hwang, C. H., Lee, N. K., & Paik, H. D. (2022). The anti-cancer potential of heat-killed Lactobacillus brevis KU15176 upon AGS cell lines through intrinsic apoptosis pathway. International Journal of Molecular Sciences, 23(8), 4073.
6. Kántor, A., Hutková, J., Petrová, J., Hleba, L., & Kačániová, M. (2015). Antimicrobial activity of pulcherrimin pigment produced by Metschnikowia pulcherrima against various yeast species. Journal of Microbiology, Biotechnology and Food Sciences, 5(3), 282–285.

7. Karuppiah, V., et al. (2013). Marine bacterial pigment production: influence of environmental and nutritional parameters. Journal of Applied Microbiology, 115(4), 988–1002.
8. Kumar, S. (2018). Extraction and characterization of bacterially produced pigments from marine sources. Indian Journal of Marine Sciences, 47(8), 1612–1618.
9. Lim, J. W., Kim, H., & Kim, K. H. (2003). Nuclear factor-κB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. Laboratory Investigation, 81(3), 349–360.
10. Müller, L., Fröhlich, K., & Böhm, V. (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. Food Chemistry, 129(1), 139–148.
11. Odekina, P. A., Agbo, M. O., & Omeje, E. O. (2020). Antimicrobial and antioxidant activities of novel marine bacteria (Bacillus 2011SOCCUF3) isolated from marine sponge (Spongia officinalis). Pharmaceutical Sciences, 26(1), 82–87.
12. Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, 95(2), 351–358.
13. Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food Chemistry, 49(10), 4619–4626.
14. Pawar, R., Mohandass, C., Sivaperumal, E., Sabu, E., Rajasabapathy, R., & Jagtap, T. (2015). Epiphytic marine pigmented bacteria: a prospective source of natural antioxidants. Brazilian Journal of Microbiology, 46, 29–39.
15. Pawlikowska, E., Kolesińska, B., Nowacka, M., & Kregiel, D. (2020). A new approach to producing high yields of pulcherrimin from Metschnikowia yeasts. Fermentation, 6(4), 114.
16. Pinter, M., et al. (2025). Validated primer design for Caspase-3 and BCL-2 qRT-PCR in human cancer cell lines. Molecular Oncology Methods, 19(1), 45–54.
17. Prasad, N. H. (2015). Optimization of pigment production in bacteria using carbon and nitrogen sources. Research Journal of Microbiology, 10(3), 112–120.
18. Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry, 48(8), 3396–3402.
19. Quek, A., Mohd Zaini, H., Kassim, N. K., Sulaiman, F., Rukayadi, Y., Ismail, A., & Awang, K. (2021). Oxygen radical antioxidant capacity (ORAC) and antibacterial properties of Melicope glabra bark extracts and isolated compounds. PLoS ONE, 16(5), e0251534.
20. Ram, S., et al. (2017). Spectrophotometric analysis of bacterial pigments at 740 nm. Biotechnology Reports, 15(3), 50–58.
21. Sathya, R., et al. (2018). Environmental parameters influencing secondary metabolite production in marine Bacillus species. World Journal of Microbiology and Biotechnology, 34(9), 133.
22. Shafique, M., et al. (2024). Enzyme activity and metabolite production in Bacillus licheniformis under varying physicochemical conditions. Applied Microbiology and Biotechnology, 108(2), 211–225.
23. Türkel, S., & Ener, B. (2009). Isolation and characterization of new Metschnikowia pulcherrima strains as producers of the antimicrobial pigment pulcherrimin. Zeitschrift für Naturforschung C, 64(5–6), 405–410.
24. Wang, X., et al. (2018). Diketopiperazines from marine microorganisms: bioactivity and mechanisms of action. Marine Drugs, 16(8), 278.
25. Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. Journal of Agricultural and Food Chemistry, 55(22), 8896–8907.