RESEARCH PAPER

Screening of Soil-borne Serratia marcescens, Optimization of Prodigiosin production and Assessment of its Antimicrobial and Anti-proliferative activities

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ABSTRACT

The present research synthesized the pigment prodigiosin (PG) using an environmental isolate of the bacteria Serratia and investigated its pharmaceutical potentials. Processing of soil samples of different plains using standard bacteriological techniques yielded bacterial isolates which comprised of 40.6% of Serratia species. Screening of the isolates for antibacterial activity by cross streak method demonstrated that the isolate Serratia marcescens GR1 encompasses superior antimicrobial potential. Production of pigment from the potential isolate was carried out by submerged fermentation using Luria Bertani broth amended with natural substrates. Studies on optimization of culture parameters deciphered that the supplementary nutrient source of peanut seed powder, carbon source of maltose, pH of 7.0, temperature of 32°C and incubation time of 48h facilitate comparatively higher production of the pigment. Purification and characterization of the synthesized pigment by TLC, UV-Vis Spectrophotometry and Mass Spectrometry revealed its identity as PG. Assay of antibacterial activity of PG indicated its efficiency of inhibiting 80% of indicator pathogenic strains with the MIC of 125 µg/mL. Cell viability assay using Hep-2 cell lines revealed the anti-proliferative tumoricidal efficiency of the PG with the respective minimum and maximum IC50 values of 75 and 250 µg/mL under standard conditions.

Keywords: Serratia, Prodigiosin, Fermentation, Optimization, Antibacterial, Anticancer.

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INTRODUCTION

The bacteria of the genus *Serratia* comprise gram negative, motile, non-spore forming facultative anaerobic members of the family *Enterobacteriaceae*. The most widely studied species of this group is *Serratia marcescens*. This species is differentiated from other enteric bacteria by its distinguished red pigmentation. These are commonly found in water, soil, decaying vegetation, meat, and food. Some species are pathogenic to human and animals. Colonies of these bacteria occasionally occur on ice machines, humidifying units and haemodialysis equipment. In distilled water, these organisms may survive for 48 days at room temperature. Serratia species produces a red pigment prodigiosin (PG) under both aerobic and anaerobic conditions. This pigment is shown to be associated with extracellular vesicles or present in intracellular granules of the bacterial cell [1,2]. However, pigmentation is only present in a small percentage of isolated cultures among different *S. marcescens* strains.

Since the dawn of discovery, the antibiotics have found extensive applications in various fields such as treatment of infectious diseases of human and animal origin, preservation of meat

and poultry products and so on [3]. However, the indiscriminate use of antibiotics and their contamination of environment through pharmaceutical effluents rendered them less effective due to the development of drug resistance by the pathogenic microbes. This critical situation necessitates the discovery of antibacterial agents which display novel mechanisms of action. Such a drug is expected to exert its lethality to a wide spectrum of clinically relevant species, demonstrate radically unique mode of action, be convenient to assay and possess key biochemistry.

Cancer is clinically considered as a serious disease and continuous to be a second most killer disease afflicting millions of people worldwide every year [4]. The malignant tumours that cause cancer, if unchecked, can render a complicated clinical state in the affected person through facilitating the metastasis all over the body. Therapeutic approach to cancer aims to control the propagation of tumour cells using chemical agents. These drugs act by anti-proliferative mechanisms over the cells which divide and grow. However, most of these chemotherapeutic drugs, owing to their non-specific targeting, also affect other continually dividing normal cells such as hair follicles, hematopoietic cells and so on. Eventually, the patient is further tormented with immunosuppression, hair loss, defective tissue repair mechanisms and other complications [5]. Besides, due to the short half-life, some of these drugs need repeated administrations. Therefore, there is always a search for a potential drug endowed with target specificity, low toxicity and *in vivo* sustainability for cancer therapy.

The PG produced by the bacteria Serratia is a multifaceted secondary metabolite alkaloid usually having a molecular weight of approximately 323.44 Da. It possesses a unique tripyrrole chemical structure with the molecular formula $C_{20}H_{25}N_3O$ [6]. It is sensitive to light and insoluble in water. It is moderately soluble in alcohol and ether, and soluble in chloroform, methanol, acetonitrile and DMSO [7]. The PG, by virtue of its dynamic bioactive potentials, has attracted considerable interest among the researchers in recent years to develop a drug with versatile pharmaceutical activities [8]. Previous studies have documented several therapeutic properties of PG such as antibacterial, antifungal, anti-protozoal, algicidal and immunomodulatory effects [9-11]. The PG has also been reported to exhibit immunosuppressive and anticancer activities. It induces apoptosis of various tumour cells which promote metastasis of hematopoietic tissues [12]. Interestingly, the PG has no marked toxicity in non-malignant cell lines [13].

In order to extract this bacterial pigment in large volume, several researchers have attempted *in vitro* production of PG by different methods. Among the available techniques, the submerged fermentation has been proved to be an efficient method for the substantial production of PG [14]. However, the production of PG pigment is influenced by various factors such as incubation time, initial pH, temperature, carbon source, sodium chloride concentration, sugar concentration and so on [7].

Most of the contemporary research studies are prompted to design efficacious drugs to treat the dreadful conditions such as diseases caused by emerging and re-emerging multi-drug resistant pathogens, immunodeficiency, neoplastic and neurodegenerative diseases [6]. The natural sources such as plants, marine organisms, soil-borne and endophytic microorganisms are extensively explored in recent years for discovering novel drugs for this purpose. Notably, the bacteriocins of probiotic bacteria, novel peptides of endophytic fungi and enzymatic proteins of actinomycetes are under focus for developing drugs with ideal characteristics for promising clinical applications. Therefore, the present research was carried

out with an objective of advocating the pigment PG as a cost-effective drug for the pharmaceutical applications through its isolation from soil-borne *Serratia sp*., *in vitro* production and demonstration of antibacterial and anti-proliferative activities.

MATERIALS AND METHODS

Sample collection and processing

Soil samples from different places in the premises of the college located in Unaizah city such as garden, playground and backyard were collected in sterile polythene bags by means of aseptic procedures. A total of 15 samples, each containing approximately 5-10g of the soil, were collected at regular intervals over a period of 15 days.

Isolation of soil bacteria

Isolation of bacteria was performed by standard serial dilution technique. Briefly, 1g of the soil was dissolved in 10 mL of sterile distilled water to obtain a dilution of 10^{-1} of the sample. The procedure was continued using series of test tubes containing the diluent until achieving the 10^{-6} dilution. Then, the samples (0.1 mL) from the tubes of 10^{-4}, 10^{-5} and 10^{-6} dilutions were inoculated onto sterile nutrient agar plates supplemented with 1% peanut powder by spread plate technique. These plates were incubated at 37^{o}C for 24h and observed for the growth of bacterial colonies.

Selection of Serratia was carried out by preliminary macroscopic and microscopic techniques. Colonies that were opaque, circular, convex with entire margin and red or pink pigmentation were selected. Microscopic techniques were done with these isolates to confirm that the bacteria are gram negative and motile. For the convenience of study, each isolate was given identification codes and preserved in sterile agar slopes at 5^{o}C until further use.

Screening of bacteria

Isolates of Serratia were screened for antimicrobial activity to assess the pigment production. Each isolate was tested against five reference strains of pathogenic bacteria obtained from IMTECH, Chandigarh (India) viz., *Escherichia coli* (MTCC-443), *Pseudomonas aeruginosa* (MTCC-1688), *Staphylococcus aureus* (MTCC-3160), *Klebsiella pneumonia* (MTCC-7028), *Proteus mirabilis* (MTCC-425). The assay of antibacterial activities of the soil isolates was performed using cross streak method suggested by Lertcanawanichakul and Sawangnop [16]. Briefly, a loop full of overnight culture of the test bacteria was inoculated as a straight line on sterile Mueller Hinton agar (MHA) plate. The cultures of indicator bacteria were streaked perpendicular to that of the test bacteria. After 24h of incubation at 37^{o}C, the plates were observed. Growth inhibition measuring $\geq$5mm was recorded as positive for the antibacterial activity.

Selection and identification of Serratia species

Among the soil bacteria screened for antimicrobial activity, the *Serratia sp*. GR1, based on its ability of inhibiting maximum number of test organisms, was designated as a potential isolate. This isolate was subjected to standard bacteriological techniques involving macroscopic, microscopic and biochemical tests. With reference to the manual of Mackie and McCartney practical medical microbiology (14th Ed.), identification of the isolate as the bacteria *Serratia marcescens* was confirmed.

Production of pigment and process optimization

For the purpose of producing the prodigiosin (PG) pigment using the potential isolate *S. marcescens* GR1, the method of submerged fermentation adopted by Sundaramoorthy et al. [14] was carried out with minor modifications.

Luria Bertani (LB) broth amended with essential substrates and nutrients was used as the fermentation medium. The inoculum was prepared by overnight culture of the potential isolate, separation of pellet of cells by centrifugation at 10,000 rpm for 20 min and suspension of cells in distilled water. The optical density of the suspension was adjusted to 0.250 using UV-Vis Spectrophotometer so as to obtain an inoculum size of 10^5 cells/mL. One mL of this starter culture was inoculated into 100 mL of fermentation medium in a flask and incubated at 37^{o}C under shaking for 48h.

Optimization of fermentation process parameters was carried out to standardize maximum production of the pigment. Supplementary nutrient sources in powder form (1%) such as peanut seed, cashew, chickpea and sesame were added in different setups of fermentation medium and evaluated for their influence on pigment production. In a similar fashion, the initial pH of the medium viz., 6.0, 7.0, 8.0, and 9.0 were tested for their impact on the process. Other parameters evaluated were carbon sources (5% of glucose, maltose, sucrose, and mannitol), nitrogen source (peptone, yeast extract, NH_4Cl and $NaNO_3$), temperature (28, 32, 37 and 42^{o}C) and incubation time (12, 24, 48 and 72h). Fermentation experiment with each parameter was carried out in triplicates and the mean value of concentration of PG production was recorded.

Estimation and characterization of Prodigiosin

Estimation of the pigment produced by the bacterial culture was performed by the method prescribed by Slater et al. [17]. The spent medium was centrifuged at 10,000 rpm for 10 min and the supernatant fluid was decanted to harvest the cells. The resultant pellet of cells was added with 4% acidic ethanol (4mL of 1M HCl + 96 mL of ethanol) and vortexed to obtain uniform suspension. This mixture was centrifuged again at 10,000 rpm for 10 min and the supernatant containing the crude pigment extract was transferred to a fresh vial. The extract was appropriately diluted and measured at 535 nm using UV-Vis spectrophotometer. The quantity of pigment was calculated by graphical method using PG standard curve and determined in mg/L units.

For the purification of the pigment produced by *S. marcescens* GR1, the present research adopted the method followed by Casullo de Araújo et al. [7]. The crude extract of the pigment was separated by thin layer chromatography (TLC) on silica gel along with the standard PG. The pigment eluted from the gel was further examined by UV-Vis spectrophotometry and Mass spectrometry for the characterization of PG.

Assay of antimicrobial and anti-proliferative activities of prodigiosin

Antimicrobial activity of the pigment of *Serratia* species was determined by the assay method prescribed by McArthur et al. [18]. The method of agar well diffusion was followed for this purpose. Briefly, 100 µL of fresh culture of indicator bacterium (10^8 CFU/mL) was grown by swab inoculation on sterile MHA medium on a petri dish. Five wells each with 7 mm dia., maintaining a distance of 20 mm between them, were cut in the agar. Aliquots of PG encompassing pigment concentrations of 50, 75, 125, 250 and 500 µg/mL were prepared from the stock solution using DMSO and loaded into separate wells. One well, designated as control, was added with 50 µg/mL of DMSO suspended of the pigment. Triplicates of plates for each indicator organism were prepared and incubated overnight at 37^{o}C. Growth inhibition viewed as zone formation surrounding each well was measured and recorded. Zone size of $\geq$10 mm dia. was referred as positive for antibacterial activity.

The anticancer activity of the PG, in terms of inhibition of proliferation of tumour cells, was appraised by a

modified MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) based assay described by Freshney [19]. Suspension of cells of human epithelial tumour (Hep-2) were seeded in a 96-well flat bottom micro titration plate preloaded with RPMI 1640 medium at a concentration of 10^4 cells/well. The plate was incubated at 37ºC for 24h under 5% CO_2 atmosphere so as to obtain a confluent monolayer of cell culture. Then, aliquots of test solution each measuring 10, 50, 75, 125 and 250 µg/mL concentrations of PG were pipetted into each well. A separate control well of cell culture devoid of the test solution was made alongside and the plate was further incubated for 48h. Subsequently, 10µl solution of MTT was applied to each well and the plate was incubated at 37°C for 4 hours. Upon completion of treatment process, the optical density of the wells was measured at 495 nm using a multi-plate ELISA reader. The experiment was conducted in triplicates for each pigment concentration and the IC_{50} value of the PG was determined based on 50% of inhibition of the cells with reference to the control well.

RESULTS

Isolation, screening and identification of Serratia

Studies on isolation of bacteria from soil samples yielded a total of 32 isolates with different macroscopic features. Out of these, 13 isolates (40.6%) were preliminarily identified to be of Serratia species based on the colony morphology, pigment production and microscopic techniques. Further to the screening of these soil isolates for antibacterial activity by cross streak method, the isolate *Serratia sp.* GR1 showed a superior lethal activity than its counterparts (Figure 1). This potential isolate demonstrated 80% of bactericidal activity by inhibiting the growth of 4 out of the 5 indicator pathogenic bacteria tested.

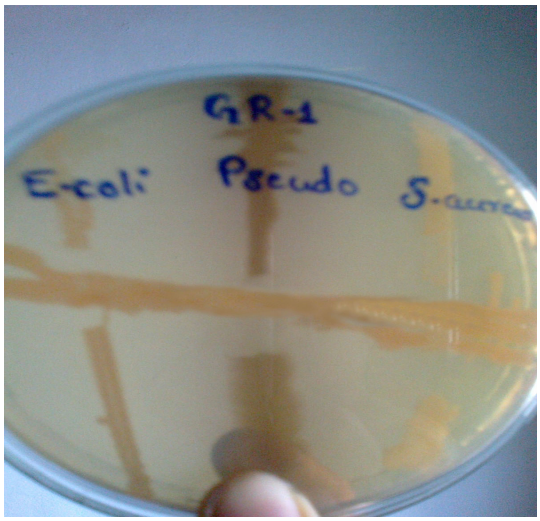

Figure 1: Screening of *Serratia sp.* GR1 for antibacterial activity

The standard bacteriological techniques including macroscopic, microscopic and biochemical tests conducted with the potential isolate indicated its identity as *Serratia marcescens*. The different tests performed for the identification of the bacteria are presented in table 1.

Table 1. Report on the tests for the identification of Serratia sp. GR1

Type of the test	Technique	Observation
Macroscopic test	Colony morphology	Opaque, circular, convex with entire margin
	Pigmentation	Red pigmentation
Microscopic tests	Gram staining	Gram negative rod
	Motility test	+
Biochemical tests	Catalase	+
	Oxidase	-
	Fermentation of Glucose, Fructose, Maltose	+

Fermentation of Lactose, Arabinose, Xylose	-
Indole test	-
Methyl red test	-
Voges-Proskauer test	+
Citrate Utilization test	+
Casein hydrolysis	+

Production, estimation and characterization of prodigiosin

The submerged fermentation of the nutrient medium supplemented with peanut powder substrate, carbon and nitrogen sources caused the *S. marcescens* GR1 biomass accumulation after 48h. Production of the pigment in the culture vessel was determined by photometric method using the prodigiosin (PG) standard graph.

The chromatogram of TLC deciphered the existence of PG in the crude extract as it got separated with the Rf value of 0.9 corresponding to that of the standard material. The absorbance spectrum obtained by UV-Vis spectrophotometry indicated an absorbance value of 534 nm for the purified pigment (Figure 2). Molecular characterization using Mass spectrometry revealed that the pigment possesses a molecular weight of 320 kDa consistent with the ideal PG (Figure 3).

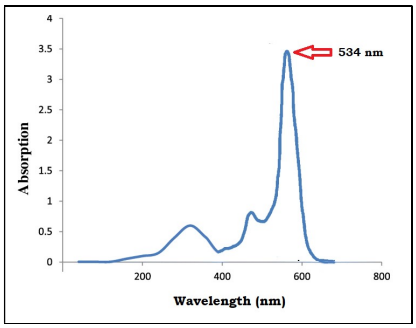

Figure 2: Absorbance spectrum of Prodigiosin synthesized by *S. marcescens* GR1

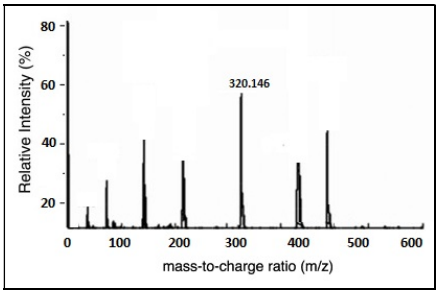

Figure 3: Mass spectrum of Prodigiosin synthesized by *S. marcescens* GR1

Optimization of fermentation parameters

Best culture parameters were determined based on their influence in the fermentation process for enhanced production of the pigment. The concentrations of PG (mg/L) produced with every altered parameter of the fermentation were recorded and compared. The results indicated that among the supplementary nutrient sources tested, the peanut seed prompted higher production of pigment. The carbon source maltose and the nitrogen source NH_4Cl demonstrated better augmenting effects than their counterparts. Other parameters such as pH of 7.0, temperature of 32°C and incubation time of 48h were identified to play boosting roles on the fermentation while maintaining other conditions constant. Table 2 depicts the results of optimization studies carried out with different process parameters. Data from three experiments were analysed using chi-square test for

statistical significance in alignment with the mean and standard error (±10) values.

Table 2. Results of optimization of parameters for Prodigiosin production

Parameter tested	Variables	Production of prodigiosin (mg/L)
Supplement substrate	Peanut seed	545
	Cashew	324
	Chickpea	519
	Sesame	412
Carbon source	Glucose	475
	Maltose	517
	Sucrose	383
	Mannitol	236
Nitrogen source	Peptone	357
	Yeast extract	391
	NH_4Cl	487
	$NaNO_3$	423
Initial pH	6	452
	7	526
	8	518
	9	437
Temperature (°C)	28	498
	33	514
	37	325
	42	146
Incubation time (Hours)	12	187
	24	261
	48	527
	72	378

Evaluation of prodigiosin for antimicrobial and anti-proliferative properties

The PG exhibited varying degrees of antibacterial activities against the indicator bacteria employed in the assay (Figure 4). Results of assay of antibacterial activities of PG are shown in the table 3. The diameter of zone of growth inhibition (mm) caused by each dilution maintained in triplicates were recorded and presented as mean values. Data represent mean ± standard deviation derived from the chi-square analysis for statistical significance. Analysis of results indicated that the PG produced in the present research was effective against 80% of the indicator pathogens with the minimal inhibitory concentration (MIC) of 125 µg/mL. However, the pathogen *K. pneumoniae* stayed resistant to the pigment and showed insignificant susceptibility only at higher concentrations (table 3).

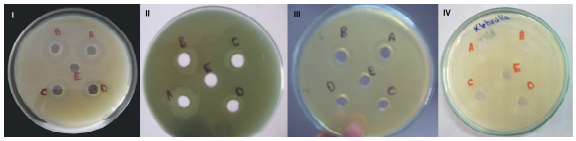

Figure 4: Assay of antimicrobial activity of Prodigiosin

Culture plates of *E. coli* (I), *P. aeruginosa* (II), *S. aureus* (III), IV-*K. pneumoniae* (IV)

Table 3. Results of assay of antibacterial activity of Prodigiosin

Indicator bacteria	Prodigiosin concentration and Zone of inhibition (mm)				
	50 µg/mL	75 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
E. coli	5.21 ±0.30	7.35 ±0.22	12.85±0.43	18.52±0.35	22.13±0.11
P. aeruginosa	7.62 ±0.27	8.53 ±0.31	13.27±0.28	20.33±0.12	25.21±0.24
S. aureus	4.35 ±0.18	6.14 ±0.27	10.82±0.13	15.44±0.22	19.02±0.05
K. pneumonia	Nil	Nil	Nil	1.21 ±0.20	2.35 ±0.15
P. mirabilis	3.43 ±0.12	5.97 ±0.14	10.08±0.18	12.13±0.26	16.09±0.14

The assay of anti-proliferative activity of PG on Hep-2 cell line revealed the conferment of exponential lethality proportionate with the increasing concentrations of the pigment. Figure 5 shows the cytotoxic effects of different concentrations of PG. The IC_{50} values of the pigment obtained from triplicate experiments were derived from the data of survival rate as illustrated in fig. 6, which refer to the mean ± standard deviation considering ±5 standard errors. The chi-square analysis indicated that the concentrations 75 µg/mL and 250 µg/mL correspond respectively to statistically significant minimum and maximum IC_{50} values of the PG.

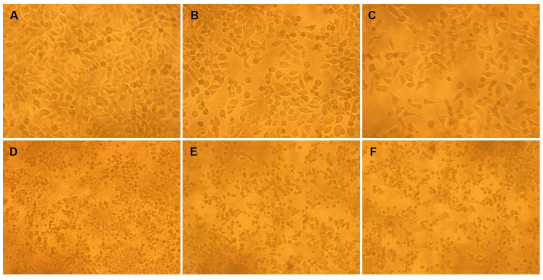

Figure 5: Assay of anti-proliferative activity of Prodigiosin on Hep-2 cell line
Control cell line (A) and cells treated with Prodigiosin concentrations of 10 mg/mL (B), 50 mg/mL (C), 75 mg/mL (D), 125 mg/mL (E) and 250 mg/mL (F)

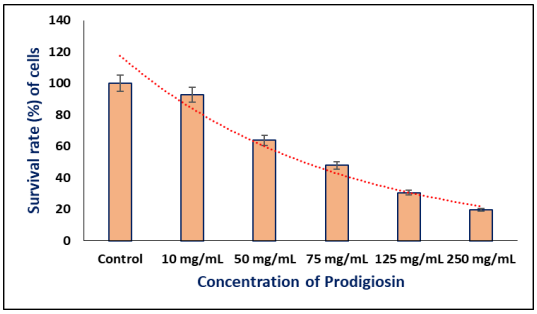

Figure 6: Trend of survival rate of Hep-2 cell line treated with Prodigiosin

DISCUSSION

Twenty first century is earmarked as a significant period in the field of medicine due to the introduction of advanced technologies for the diagnosis, prevention and control of various diseases. However, the continued emergence of antibiotic-resistant pathogens and growing incidences of neoplastic diseases constitute the global threats for ensuring sustainable healthcare. In recent years, the researchers around the world are paying enormous attention on the study of adaptations of microorganisms which enable them to survive in challenging environments. Research studies have demonstrated that the metabolites, which are produced by the microorganisms to combat the unfavourable growth conditions and predatory organisms, could serve as bioactive compounds and therapeutic agents. The prodigiosin (PG) pigment produced by the bacteria Serratia, owing to its unique structural characteristics and biological properties, has gained significance in the field of pharmaceutical sciences.

For the purpose of isolating the bacteria Serratia, soil samples from different sites of the study area were used are source materials. Although the bacteria Serratia has been reported to present in many sources such as fresh water [20], marine water [21], plants, crustaceans [22], human urine [23] etc., it occurs predominantly in soil [14,15]. Since the soil serves as a favourable environment with essential nutrients and optimal physical conditions for growth, the bacteria Serratia tend to occur with high frequency in natural wet soil. Therefore, the present study attempted the isolation of *Serratia* from soil samples. Similar to our study, Casullo de Araújo et al. [7], Parani and Saha [9], Giri et al. [24], Raj et al. [25], Tariq and John Prabakaran [26], Boby et al. [27], Ma et al. [28] have employed soil samples for the isolation of *Serratia*.

In order to identify a high PG yielding bacterial isolate, all the isolates were subjected to screening assay to determine

their antimicrobial activities. Since the isolate *S. marcesans* GR1 demonstrated a superior antibacterial activity (80%) against the indicator bacteria, it was selected and designated as the potential isolate for further studies.

For the purpose of producing PG using the potential isolate, the method of submerged fermentation of Luria Bertani (LB) broth medium, with amended substrates, was followed in the present study. Comparable study by Hardjito et al. [20] has utilized the LB broth medium for the production of PG using the aquatic isolate of *S. marcesans* biovar A2/A6. However, the studies conducted elsewhere have employed different types of media such as nutrient broth [14,23,24,29], Tripticase soya broth [25] and corn steep mannitol medium [7]. Our study chose the LB broth for the fermentation process as this medium comprises of essential nutrients that support the environmental isolates of bacteria. The submerged fermentation is considered as a preferable method of producing secondary metabolites using the aerobic organisms under controlled conditions [30]. Previous studies concomitant to our research have followed the submerged fermentation for the production of PG using *S. marcesans* [7,29]. In contrast, Xu et al. [31] and Han et al. [32] have demonstrated the production of PG by solid state fermentation and fed-batch fermentation methods respectively. The synthesis of PG by *S. marcesans* GR1 in the fermentation medium in the present study was preliminarily confirmed by the spectrophotometric method using a standard prodigiosin. Similar assay method has been adopted by Cang et al. [30], Hardjito et al. [20], Sundaramoorthy et al. [14], Xu et al. [31] and Samrot et al. [23] as it is considered as the most reliable method to estimate the protein (pigment) concentration. Our study attempted the partial purification of PG using TLC method on silica gel as it is recommended as a standard method for the estimation of cellular molecules. Among the various studies conducted for purification of PG, while some have employed the TLC method [7,23,30], the others have utilized the methods of column chromatography [25] and HPLC [29].

The structural characterization of PG produced in the present study using UV-Vis spectrophotometry indicated its maximum absorbance at 534 nm. In conform to our finding, studies conducted elsewhere have reported similar absorbance values such as 499 nm [23], 534 nm [14], 535 nm [24,30,31], 536 nm [7] and 540 nm [20]. The Mass spectrophotometry of PG synthesized by our study indicated its molecular weight of 320 kDa. Our finding resonates with those of Giri et al. [24] and Casullo de Araújo et al. [7] who reported a molecular weight of 324 kDa of PG synthesized independently by *S. marcesans* in their studies.

Our study attempted to standardize the physicochemical factors suitable for the potential isolate so as to achieve the maximum production of PG. For the purpose of optimizing the culture medium, besides the basic medium, a supplementary nutrient source is essential. Contemporary studies on PG production have employed various materials such as cassava waste [33], Trypticase soya [25], ethanol [30], kitchen waste and rice husk [31], L-lactic acid [20], sesame seed and peanut seed [24], peanut oil [14]. However, the previous studies have witnessed substantial production of PG when seeds of sesame and peanut were supplemented with the fermentation medium. The present study utilized the peanut seed powder as the supplementary nutrient source owing to its growth promoting nutrient contents including saturated fatty acid, cost effectiveness and easy availability.

Among the carbon sources added to the fermentation medium, the carbohydrate maltose was observed to enhance the PG production. Our finding agrees with the studies of Sundaramoorthy et al. [14] and Giri et al. [24], who demonstrated maltose

as the best carbon source for PG production. Concomitant studies on synthesis of PG have reported glucose [20,30] mannitol [7], starch [31] and lactose [23] as alternative carbon sources. Earlier researchers have reported that the organic nitrogen sources such as proline [31], beef extract [20], yeast extract [29] have superior potential than their inorganic counterparts for promoting PG production. However, our results indicated the NH_4Cl as the favourable nitrogen source for this purpose. It may be explained that the inorganic nitrogen sources are naturally available and act as simple donors of nitrogen which enable conversion of raw orange pigment to red. In consistent with our finding, Cang et al. [30] have demonstrate better production of PG with the inorganic nitrogen sources such as pharmamedia and polypeptone.

The pH of 7.0 of the fermentation medium was recorded with the higher production of PG in the present study, which is in alignment with the reports of Sundaramoorthy et al. [14], Casullo de Araújo et al. [7] and Samrot et al. [23]. In contrast, some studies have recommended acidic pH such as 5.5 [29] and alkaline pH such as 8.0 [25,31] and 9.0 [20]. Most of the previous studies have recommended the temperatures ranging 25-30^{o}C [7,14,20,23,25,29] for the fermentative production of PG. Interestingly, our study observed that a slightly higher temperature of 32^{o}C is suitable for the production of PG. This may be attributed to the geographical location (Saudi Arabia) wherein the potential isolate *S. marcescens* GR1 has adopted to warmer weather conditions for its existence. An optimal incubation time of 48h was consumed in the present study for the fermentation process, which resonates with the reports of Lazic et al. [29], Cang et al. [30] and Casullo de Araújo et al. [7]. However, some studies have documented shorter durations of fermentation such as 36h [24] and much longer durations such as 60h [31] and 120-164h [25]. This may be due to the physical characteristics of the different process organisms used in those studies.

The antimicrobial property of the purified PG of the present study was determined in terms of its MIC values against the indicator bacteria. Previous studies have suggested that the PG presents a higher lethality against the gram-positive bacteria rather than gram negative counterparts [11]. However, the isolate *S. marcesans* GR1 was observed to equally effective against the indicator bacteria of both categories (table 3). Review of literature inferred that the PG exhibits its antimicrobial action by way of chaotropic-mediated mechanism which causes hydrophobic stress on the plasma membrane of the target bacteria. This eventually disrupts the transport mechanisms and translocation of lipids in the cell membrane. Other microbicidal mechanisms of PG include the induction of apoptosis comprising inhibition of protein synthesis and enzymes such as DNA gyrase and topoisomerase IV [34].

Lapenda et al. [35] have demonstrated that the purified PG could inhibit the oxacillin resistant *S. aureus* with the MIC value of as low as 1 µg/mL**.** Another study by Arivizhivendhan et al. [36] reported the antagonistic activity of PG against the common foodborne pathogens such as *E. coli*, *Bacillus cereus*, *Clostridium botulinum*, *Salmonella enterica* and *Vibrio vulnificus*. Similar study by Boby et al. [27] has demonstrated the inhibition of pathogenic bacteria such as *E. coli*, *S. aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *S. enterica* by PG. However, the pigment produced in this study displayed the bactericidal activity only at higher concentrations (MIC ≥250 µg/mL) than that of the present study (MIC ≥125 µg/mL). More recently, Ma et al. [28] have documented the antibacterial activity of PG against the β-lactum-resistant *P. aeruginosa* with an MBC value of 128 µg/mL. Thus, our results indicate a comparable antibacterial

activity of PG synthesized by the potential isolate *S. marcesans* GR1.

Another significant finding of the present study is the anticancer activity of the purified PG. Previous studies have suggested that the PG stalls the progression of cancer by various mechanisms such as acidification of intracellular pH, disruption of outer membrane mitochondrial permeability, dsDNA cleavage, cell cycle arrest, p53-independent proapoptotic effect, anti-metastasis, etc. [13]. The pigment of our study has been observed to cause marked reduction of tumour cells with its cytotoxic activity (Figure 5). The PG demonstrated its anti-proliferative action against the Hep-2 cells with the IC_{50} values of ≥75-250 µg/mL (Figure 6). Montaner et al. [37] from their study on the cytotoxic effect of PG synthesized by *S. marcescens* 2170 against the cancer cells such as Jurkat, NSO, HL-60 and Ramos have corroborated substantial reduction of cell viability using the techniques such as MTT assay, Hoechst 33342 staining and FACS analysis. Another study has recorded the anticancer activity of PG against human gastric carcinoma cell line HGT-1 with a concentration of 2 µM [38]. Parallel studies on the inhibition of HeLa cancer cells by the PG have reported the IC_{50} values of 50 µg/mL [25] and 700nM [22]. Similar study by Khanafari and Fakhr [2] has portrayed the antineoplastic activity of PG on T-cell acute lymphoblastic leukemia cells, Jurkat at a concentration of 6 µM. Recently, Lazic et al. [29] have documented that the PG produced from *S. marcescens* ATCC 27117 could inhibit the proliferation of a panel of cancer cells of A549, A375, MDA-MB-231, and HCT116 with the IC_{50} values ranging 0.62-1.30 µg/mL. Saleh et al. [39] from their studies with the HCT colorectal cancer cells determined a very low IC_{50} value of 2 µg/mL for PG. Another contemporary study by Muslim et al. [15] has determined an IC_{50} value of ≥62.5 µg/mL of PG for dose dependent inhibition of MDA human breast cancer cells. Overall analysis with the concomitant studies revealed that the PG synthesized in our study displays a moderate *in vitro* anti-proliferative activity against the targeted tumour cells.

Further corroboration with extensive *in vitro* and *in vivo* studies on the synthesized PG would help exploring its diverse bioactive potentials and promising pharmaceutical applications for controlling infectious and neoplastic diseases.

CONCLUSIONS

The soil serves as a favourable habitat for the growth of the bacteria Serratia as it provides essential growth factors and facilitates its bioactive potentials. Screening of the soil isolates based on antimicrobial activities aids in the selection of a potential isolate capable of synthesizing the pigment prodiogicin (PG). Submerged fermentation of Luria Bertani broth medium suits to meet the need of higher production of PG by the potential isolate *S. marcescens* GR1. While the thin layer chromatography is useful for the partial purification of the pigment, the techniques such as UV-Vis spectrophotometry and Mass spectrophotometry can provide information on the structural characterization of the PG. The peanut seed powder serves a suitable supplementary nutrient source for pigment production owing to its richness of saturated fatty acids. Better yield of the pigment can be achieved with the addition of maltose and NH_4Cl as carbon and nitrogen sources in the fermentation medium. A pH of 7.0, temperature of 32^{o}C and the incubation time of 48h serve as the favorable physical conditions that promote the higher production of PG by the potential isolate. The PG of *S. marcescens* GR1 displays comparable antimicrobial properties with the MIC values of ≥125 µg/mL. Testing with the human epithelial cancer cells Hep-2 indicate a moderate anti-proliferative activity of the PG with the IC of ≥75 µg/mL. Extended studies on

the synthesized PG would decipher its overall bioactive and pharmaceutical potentials.

Conflict of Interest

Authors declare that there are no conflicts of interest.

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