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Original Article

Antioxidant, Anti-quorum Sensing and Cytotoxic Properties of the Endophytic *Pseudomonas Aeruginosa* CP043328.1 's Extract

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ABSTRACT

Background: Novel bioactive compounds are in high demand to combat challenges of microbial resistance. In recent years, secondary metabolites from endophytic bacteria have drawn attention from researchers due to their novel structures and significant biological activities. **Objective:** This study aimed at extracting secondary metabolites from endophytic *Pseudomonas aeruginosa* CP043328.1 from *Anredera cordifolia* leaves for their biological activities. **Methods:** The anti-SMASH was used to predict the biosynthetic gene clusters of *P. aeruginosa* CP043328.1. The bacteria was resuscitated on Nutrient agar. Ethyl acetate was used to extract the secondary metabolites. Chemical composition of the secondary metabolites was evaluated using gas chromatography-mass spectrometry (GC-MS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods were used to analyze the scavenging activity. Anti-quorum sensing activity was investigated using *Chromobacterium violacuem* ATCC 12472 as the biological indicator. Cytotoxicity assay was performed using a tetrozolium-based columetric (MTT) assay. **Results:** The bacterium has 12 gene clusters that encode for secondary metabolites. The extract revealed 15 volatile constituents with diisooctyl phthalate (50.51%) and [1, 2, 4] oxadiazole, 5-benzyl-3 (10.44%) being the major compounds. The extract revealed scavenging capabilities with IC_{50} of 0.625 mg/ml for DPPH and 0.15 mg/ml for ABTS. It displayed anti-quorum sensing activity with 88% violacein inhibition at 3.31 mg/ml. According to MTT assay, the extract was found to be safe for use up to 2000 µg/mL. **Conclusion:** *P. aeruginosa* CP043328.1 shows a potential use as a source of pharmacologically important metabolites.

Key words: *Pseudomonas aeruginosa* CP043328.1, Chemical composition, Antioxidant activity, Anti-quorum sensing activity, Cytotoxicity assay.

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INTRODUCTION

Infectious and metabolic diseases are among the leading causes of death, causing 15.6% and 16.7% death in both women and men respectively.[1] Infectious diseases cause 14 million deaths worldwide annually which has resulted in a high mortality rate of 4.8%. The problem is worsened by the emergence of antibiotic resistance, as well as the occurrence of multiple resistance of pathogens with the potential of global spread.[2] Antibiotic resistance is mainly conferred by randomly mutated genes in pathogens as a means of defense mechanism.[3]

The quorum sensing (QS) activity of bioactive compounds is as important as the antibiotic property to combat bacterial pathogenicity and antibiotic resistance. Both Gram-negative and Gram-positive bacteria use QS to synchronize gene expression in a population density-dependent manner. In Gram-negative bacteria, N-acyl-L-homoserine lactone (AHL) signal molecules called autoinducers (AIs) mainly mediate QS. Since QS regulates expression of several virulence factors, quorum sensing inhibitors (QSIs) can be used to attenuate bacterial virulence. Moreover, QSIs qualify biofilms to be more susceptible to conventional antibiotics and the host immune system, and thus, lower doses and fewer antibiotic treatments would be needed.[4]

Reactive oxygen species (ROS) are a group of free radicals derived from oxygen. They are produced as a result of cellular metabolism, and excessive accumulation of ROS leads to oxidative stress which plays an important role in the pathogenesis of various diseases like; cardiovascular diseases, inflammatory diseases, atherosclerosis, cancer, and in many pathological progression in the central nervous system.[4,5] This leads to the search for novel antioxidant compounds from natural sources such as plant and microbial sources which serve as safe therapeutics.[6]

Microorganisms are recognized as producers of bioactive metabolic compounds of industrial and pharmacological significance.[4] Pseudomonas is a genus of Gram-negative Gammaproteobacteria, belonging to the family Pseudomonadaceae and containing 216 species and 18 subspecies, with the number of species constantly being discovered.[7] All members of the genus denote a great deal of metabolic diversity and consequently can dominate a wide range of niches. The well-known studied species include *Pseudomonas aeruginosa* which is ubiquitous in soil and water. Although there are limited reports of this bacterium as an inhabitant of plants, it has been found as an endophyte in some plant species.[8,9] Endophytic microorganisms are microorganisms

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that inhabit within plant tissues and often occur as symbionts.[10] The ability of *P. aeruginosa* strains to grow in diverse environments, including plant tissues is facilitated by the capability to assimilate a large number of compounds that are recalcitrant to other bacterial species, thus producing secondary metabolites and biopolymers, making these strains useful in medicine, industries, and environment.

In medicine, *P. aeruginosa* strains produce a variety of compounds with bacteriostatic or bactericide activity, which are vital in the control of multiple drug-resistant (MDR) bacteria.[11] They produce compounds with antimicrobial properties, which include a group of peptides called pyocins and other heterocyclic compounds.[12] Apart from the production of antimicrobial compounds, some strains have been reported to synthesize bioactive compounds with anti-inflammatory, neuroprotection, antioxidant, antitumor, antidiabetic, and chemo-modulation properties.[13,14] Although some strains have been isolated from the plants as endophytes, there remain limited studies of endophytic *P. aeruginosa* conducted, leaving this unique niche unexplored. Thus, endophytic *P. aeruginosa* is considered to be a promising source of novel bioactive metabolites of pharmacological importance.

In this context, we focused on the aspects of extraction of secondary metabolites from endophytic *P. aeruginosa* CP043328.1, which was previously isolated from *Anredera cordifolia* CIX1; a medicinal plant widely recognized for possession of diverse pharmacological activities. The ethyl acetate crude extract from *P. aeruginosa* CP043328.1 was investigated for its chemical composition, anti-quorum sensing, antioxidant and cytotoxic properties.

MATERIALS AND METHODS

Chemicals and media

All chemicals and media used were procured from Sigma-Aldrich and Merck (Pty) Ltd. The water used was glass distilled.

Biosynthetic gene clusters

The analysis of the secondary metabolite gene clusters in *P. aeruginosa* CP043328.1 was carried out using the anti-SMASH (antibiotics and Secondary Metabolite Analysis Shell) online tool. The species accession number was submitted to anti-SMASH for the identification of gene clusters. The produced data regarding the type of cluster, most similar known cluster, and percentage similarity was noted.[15]

Metabolites extraction

P. aeruginosa CP043328.1 was previously isolated from *Anredera cordifolia* leaves which were collected on 2nd July 2019 from the KwaDlangezwa area in the city of Umhlathuze, KwaZulu-Natal, South Africa (28 ˚45 'S31 ˚54 'E). The voucher specimen for *A. cordifolia* species, voucher number CIX1, was prepared and deposited in the University of Zululand Herbarium [ZULU], which is available mainly to researchers. The bacterium was resuscitated on nutrient broth and incubated at 28 ˚C, overnight. The endophyte was adjusted to McFarland standard (1.5 x 10^6 colony-forming unit/ml) and 200 µl of bacterial suspension was inoculated into 500 ml of nutrient broth. The bacterial culture was incubated at 28 °C for five days on a rotating shaker at 130 rpm. After incubation, the broth culture was centrifuged at 5000 rpm for 30 minutes to separate the bacterial cells from their metabolites. The supernatant was extracted with an equal volume of ethyl acetate (500 ml) and left overnight. The solvent phase containing the extracted secondary metabolites was separated using a separating funnel and left to air dry to yield the crude metabolites.[16]

Chemical analysis of volatile compounds of the extract

GC-MS analysis of ethyl acetate extract was carried out on a Trace GC Ultra gas chromatograph-DSQ mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) fitted with HP-5 MS capillary column (30 m × 0.25 mm × 0.25 µm). The GC oven temperature was programmed to 40 °C, for 3 minutes, followed by an increase of 5 °C per minute to 105 °C and then increased by 6 °C per minute until 220 °C was reached and held at 220 °C for 10 min. The injector temperature was 250 °C, and the flow rate of carrier gas, helium was set at 1.0 ml per minute, with a 10:1 split ratio. The MS operating parameters were as follows: ionization voltage, 70 eV, and the ion source temperature was 250 °C.[17]

Anti-quorum sensing activity of the crude extract

Quantitative evaluation of quorum sensing inhibitory activity of the extract was carried out based on its ability to inhibit the production of purple pigment violacein produced by *Chromobacterium violaceum* ATCC 12472. The strain was cultured aerobically in Luria broth at 30 °C, 130 rpm with the addition of increasing concentrations of the extract. *C. violaceum* ATCC 12472 without the extract served as a negative control. Thereafter, two millilitres of an overnight culture broth was centrifuged at 13,000 rpm for 15 minutes to precipitate the insoluble violacein. The culture supernatant was discarded and the pellet was evenly suspended in 2 ml of dimethyl sulfoxide (DMSO). The solution was centrifuged at 13,000 rpm for 10 minutes to remove the cells and the violacein was quantified by measuring the optical density (OD) at 585 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The percentage of violacein inhibition was calculated using the following formula: Percentage of violacein inhibition = (control $OD_{585\ nm}$ – test $OD_{585\ nm}$ / control $OD_{585\ nm}$) x 100.[18]

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging activity of the extract was determined in a sterile 96-well plate.[19] The DPPH (0.02 mg/ml) was mixed (1:1 v/v) with different concentrations of the extract. Each mixture was made to stand for 30 minutes in darkness at room temperature (25 ºC) and the absorbance was read at 517 nm using a microplate reader. The extract without DPPH served as blank while ascorbic acid (AA) and butylated hydroxyl anisole (BHA) was used as the positive controls. The percent inhibition of ABTS radical was calculated using the following formula: %DPPH scavenging activity = [Ao – A1 / Ao] × 100 where, A1 and Ao represent the absorbance recorded at 517 nm for the control and the test, respectively. The median inhibitory concentration (IC_{50}) of the extract against DPPH was calculated graphically.

2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

The ABTS free radical scavenging activity of the extract was investigated using the serial dilution method.[20] ABTS solution (0.003 g/ml) was mixed (1:1 v/v) with different concentrations of the extract. The mixtures were made to stand for 15 minutes at 25 ºC and the absorbance was read at 734 nm using a microplate reader. The extract without ABTS solution served as blank. Ascorbic acid (AA) and butylated hydroxyl anisole (BHA) were used as positive controls. The percent inhibition of ABTS radical was calculated by the following formula: %ABTS scavenging activity = [Ao – A1 / Ao] × 100 where, A1 and Ao equal the absorbance recorded at 734 nm of the control and the test, respectively. The median inhibitory concentration (IC_{50}) of the extract against ABTS was calculated graphically.

Cytotoxicity assay of the extract

The cytotoxicity of the extract against human hepatocellular carcinoma (HepG2) cells was investigated using the methylthiazol tetrazolium (MTT) assay. HepG2 were grown to confluency in 25 cm^3 flasks using complete culture medium (CCM: EMEM, 10% foetal calf serum, 1% L-glutamine, 1% Penstrep-fungizone). Confluent cells were trypsinized and seeded into a 96-well plate in triplicate for treatment. Cells were incubated at 37 °C for 24 hours to adhere and adapt. Thereafter, the CCM was removed and treated with different concentrations of the extract. After 24 hours, the treated medium was removed. A hundred microliters of fresh CCM and 20 µl of MTT reagent (5 mg/ml in PBS) were added into the wells and incubated at 37 °C for 4 hours. Tetrozolium-based columetric (MTT) solution was then aspirated from all wells and the formazan crystals were solubilized in 100 µL of DMSO. The MTT reduction was obtained by measuring the optical density (OD) of the samples at 570 / 690 nm using the BioTek µQuant microplate reader (USA). The cell viability percentage was measured by using the formula: Cell viability (%) = (OD treated cells / OD untreated cells ×100. The IC_{50} was calculated from the GraphPad Prism (V5) using linear regression analysis.[21]

Software and statistical analysis

All the experimentations were done in triplicates and the data were subjected to a one-way analysis of variance using Graph Pad Prism ™ 6.1. Arrow bars represented the standard deviation and values with different alphabets represent the significant difference ($p < 0.05$).

RESULTS AND DISCUSSION

Biosynthetic gene clusters

Secondary metabolites from bacteria constitute an important source of effective pharmacological agents. These metabolites are biomolecules that are encoded by different gene clusters. The well-known family clusters are N-$_\gamma$-acetylglutaminylglutamine1-amide (NAGGN), non-ribosomal peptide synthase (NRPS), and polyketide synthase (PKS).[22] The potential production of secondary metabolites by *P. aeruginosa* CP043328.1 was predicted by antiSMASH version 5. There are 12 biosynthetic gene clusters observed (Table 1). The bacterium was predicted to certainly produce L-2-amino-4-methoxy-trans-3-butenoic acid and pyocyanine as the nonribosomal peptide synthetase (NRPS) and phenazine clusters. showed 100% similarity to the gene clusters involved in the production of this metabolites, respectively.[23] Apart from these two clusters, the percentage similarity to most known clusters are very low (≤ 50%). This implies that the metabolites produced by different gene clusters cannot be accurately predicted. In general, the different biosynthetic gene cluster advocated for the ability of the bacterium to produce diverse types of secondary metabolites of pharmacological importance.

Metabolites extraction

Ethyl acetate solvent was used to extract the secondary metabolites and it gave a yield of 0.6g/500 ml. Ethyl acetate is an effective solvent for the extraction of active metabolites from microorganisms as it can dissolve both polar and non- polar active compounds.[24]

Volatile chemical constituents

GC-MC chromatogram profile of the extract showed different volatile constituents. A total of 15 volatile compounds were identified (Table 2 and Figure 1). Two compounds namely diisooctyl phthalate and 9-octadecenamide were found to be major in this fraction with 50.51% and 10.44% peak area respectively. Many other constituents were also identified such as: [1,2,4] oxadiazole, 5-benzyl-3- (thiophen-2-yl) (5.70%), 2-dodecenoic acid (5.51%), trans-2-decenoic acid (4.57%), xanthoxylin (3.74%), L-proline, N-valeryl-, undecyl ester (2.86%), L-proline, N-valeryl-, tetradecyl ester (2.61%), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (2.37%), pentadecanoic acid (2.13%), cis-9-octadecenoic acid (2.13%), cyclohexanone, 4-methyl, O-methyloxime (2.04%), 3-nonynoic acid (1.88%), benzonitrile, 2-(2-pyridinyl) (1.88%) and ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl) (1.68%). Most of the identified compounds have been reported to possess interesting biological activities; xanthoxylin has antimicrobial activity,[25] trans-2-Decenoic acid has anti-tumor and antibiotic activities[26] and dodecenoic acid possesses antioxidant, anti-inflammatory and antimicrobial properties.[27] 3-Nonynoic, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), pentadecanoic acid and diisooctyl phthalate have antimicrobial activities[28-31]. cis-9-Octadecenoic acid has been reported to possess antioxidant properties. [32] Ergotaman-3',6',18 trione,9,10 dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)- is a new chemical and its biological activities are unknown.[33] 9-Octadecenamide has antioxidant and anticancer properties[32] while [1,2,4] oxadiazole, 5-benzyl-(thiophen-2-yl) has anti-inflammatory,[33, 34] antibiotic[35, 36] and anticancer activities.[37, 38] Thus, the extract has potential to serve as a source of pharmacologically active compounds.

Anti-quorum sensing activity

The quorum quenching mechanism seems to be a promising alternative to classical antimicrobial effects and a solution to the constant increase in multidrug resistance among pathogens. Violacein inhibition was evaluated by using *C. violaceum* ATCC 12472 as a bio-indicator against

Table 1: The biosynthetic gene clusters are associated with secondary metabolites production by *P. aeruginosa* CP043328.1. The standard abbreviations signifying the type of clusters as indicated in anti-SMASH are used.

Number of clusters	Types of Clusters	Most similar known Clusters	Similarity (%)
1	NRPS, phenazine	Marinophenazine A / phenaziterpene A	26
2	NRPS-like		
3	Hserlactone		
4	NAGGN		
5	NRPS		
6	Bacteriocin		
7	NRPS	Pyoverdin	24
8	NRPS	L-2-amino-4-methoxy-trans-3-butenoic acid	100
9	Thipeptide	oxalomycin B	6
10	Phenazine	Pyocyanine	100
11	Hserlactone		
12	NRPS-like, betalactone	Pyoverdin	2

Table 2: Volatile constituents of *P. aeruginosa* CP043328.1 extract.

Number of compounds	Compounds	Area (%)
1	Xanthoxylin	3.74
2	Cyclohexanone, 4-methyl-, O-methyloxime	2.04
3	trans-2-Decenoic acid	4.57
4	3-Nonynoic acid	1.88
5	2-Dodecenoic acid	5.51
6	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	2.37
7	L-Proline, N-valeryl-, tetradecyl ester	2.61
8	L-Proline, N-valeryl-, undecyl ester	2.82
9	Pentadecanoic acid	2.13
10	cis-9-Octadecenoic acid	2.13
11	Ergotaman-3',6',18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-	1.68
12	9-Octadecenamide	10.44
13	[1,2,4]Oxadiazole, 5-benzyl-3-(thiophen-2-yl)	5.70
14	Benzonitrile, 2-(2-pyridinyl)-	1.88
15	Diisooctyl phthalate	50.51

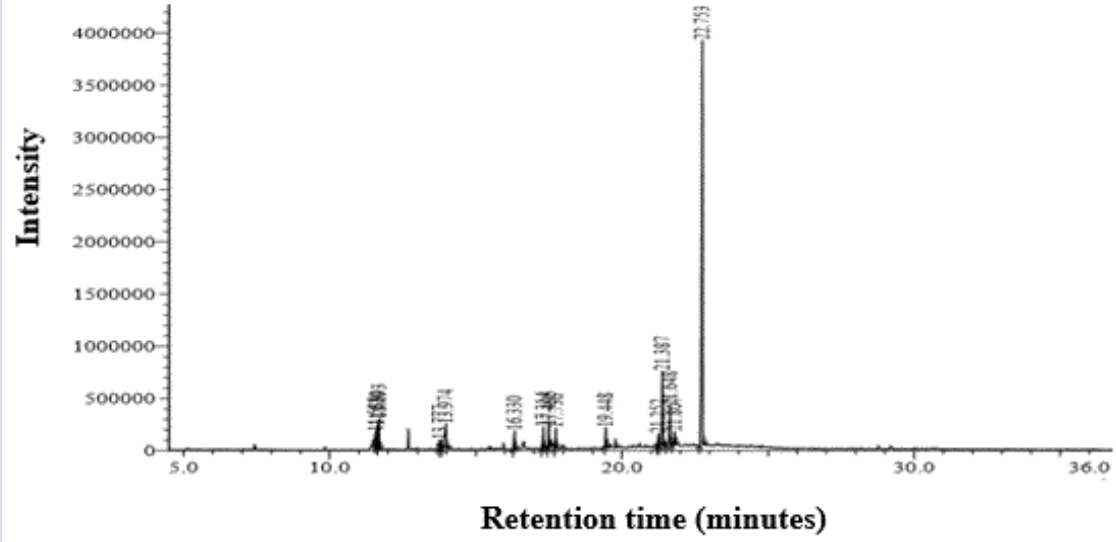

Figure 1: A GC-MS chromatogram peak profile of *P. aeruginosa* CP043328.1 extract.

different concentrations of the extract. The inhibition of the violacein production by the extract is shown in Figure 2. Violacein inhibition increased with an increase in the concentration of the extract up to 88% at 3.21 mg/ml. w. An extract is regarded as highly active when it is ≥ 90%, moderate between 40 - 89%, and inactive when it is < 40%.[39] This implied that the extract has moderate activity and could be used as an anti-quorum agent. Different mechanisms have been reported to elucidate the interference of quorum sensing by natural products. Some of the mechanisms are inhibition of signaling molecules, biosynthesis of acyl-homoserine lactones (AHL) signaling reception, and biodegradation of quorum molecules.[40] It was therefore assumed that the extract exerted one or all of these mechanisms of action. Moreover, the results showed that the extract inhibits quorum sensing without interfering with the growth of the bacterium. It was concluded that the negative effect on violacein production was not caused by the inhibition of *C. violaceum* growth but rather by disruption of the signaling system.[41] This is important because when growth is not affected, there is no selective pressure for the development of resistant bacteria.[38] Thus, this study introduces not only a novel antibiotic but also a potential new therapeutic direction for the treatment of bacterial infections.

Antioxidant activity

Antioxidants have a critical role in negating disease progression caused by excess free radicals. [42] The antioxidant activity of the extract was evaluated by free radical DPPH and ABTS methods and the results are displayed in Figure 3. The extract exhibited maximum DPPH scavenging activity of 63% at 1.0 mg/ml and displayed the IC_{50} value of 0.650 mg/ml, which was higher than that of ascorbic acid (0.200 mg/ml) and BHA (0.188 mg/ml). It also demonstrated a maximum ABTS scavenging activity of 91% at 0.5 mg/ml with an IC_{50} value of 0.150 mg/mL (Figure 3). Its IC_{50} value was lower than of ascorbic acid (0.258 mg/ml) and BHA (0.300 mg/ml). The results imply that the extract has stronger activity against ABTS than the controls but poor action against DPPH in comparison to the controls. Antioxidant compounds are said to be very strong if they have IC_{50} values of less than 0.05 mg/ml, strong for IC_{50} between 0.05 - 0.10 mg/ml, moderate for IC_{50} between 0.10 - 0.15 mg/ml and weak if IC_{50} is greater than 0.150 mg/ml. [38] Thus, the extract revealed moderate activity against ABTS radical and poor activity against DPPH radical. The observed antioxidant activity was attributed to the different identified antioxidant metabolites acting

synergistically. Moreover, the extract has the potential to serve as a source of antioxidant compounds, especially against ABTS radicals. The evaluation of antioxidant activity was of utmost importance in this study as few studies are reporting on the antioxidant activity of extracts or compounds from endophytic bacteria.

Cytotoxicity

Toxicity is the measure of the adverse effects of compounds on the cells or organs in biological systems; it is a crucial parameter to evaluate during drug discovery and development processes. Toxicity issues accounted for approximately 54% of failures in the preclinical stages in drug discovery and development.[43] The cytotoxicity effect of the *P. aeruginosa* CP043328.1`s extract on healthy HepG2 cells was evaluated by MTT assay and the results are displayed in Figure 4. After treatment with the extract, there was an initial increase in cell viability, indicative of metabolism stimulation. The highest viability of 109% was observed at 125 µg/ml and fluctuated at ±100% between 500 - 1500 µg/ml. The lowest viability was observed at 1000 µg/ml (±1%), indicative of the

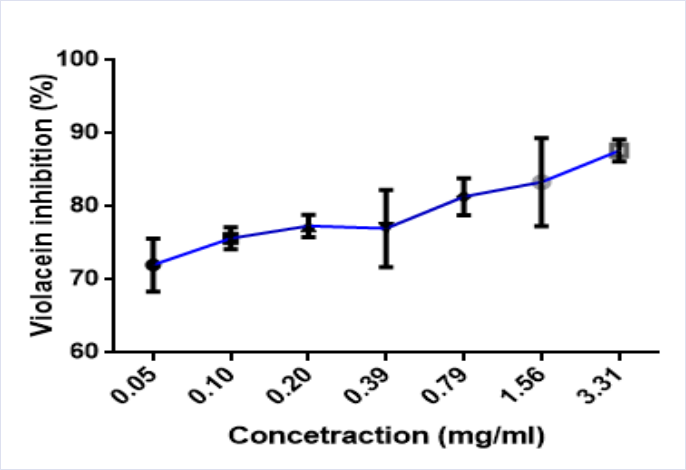

Figure 2: Violacein inhibition of *P. aeruginosa* CP043328.1 extract**.**

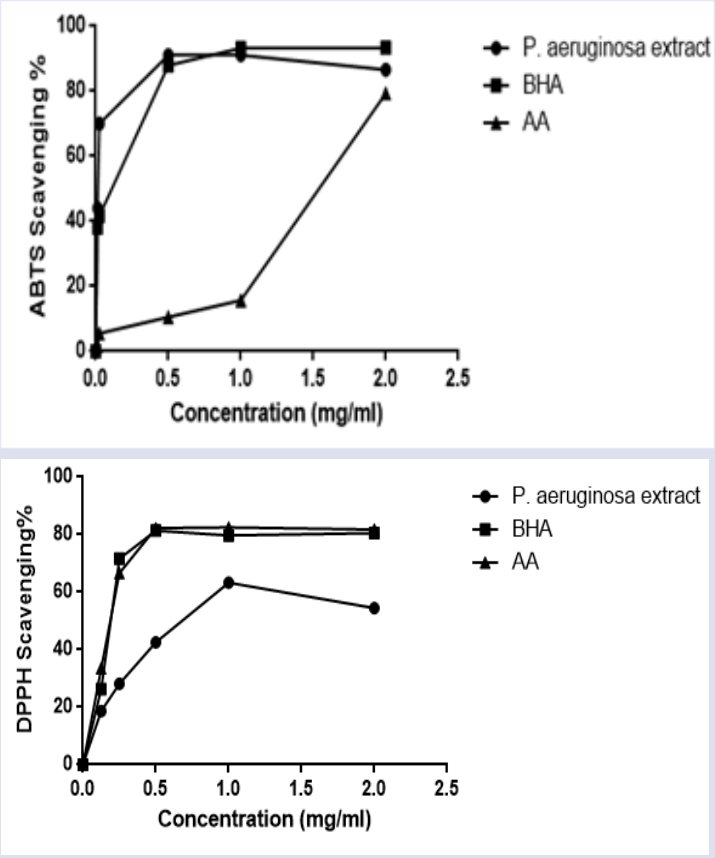

Figure 3: ABTS scavenging activity of the extract, BHA, and AA (left) and DPPH scavenging activity of the extract, BHA, and AA (right)**.** *P. aeruginosa* denotes extract, BHA denotes butylated hydroxyl anisole and AA denotes ascorbic acid.

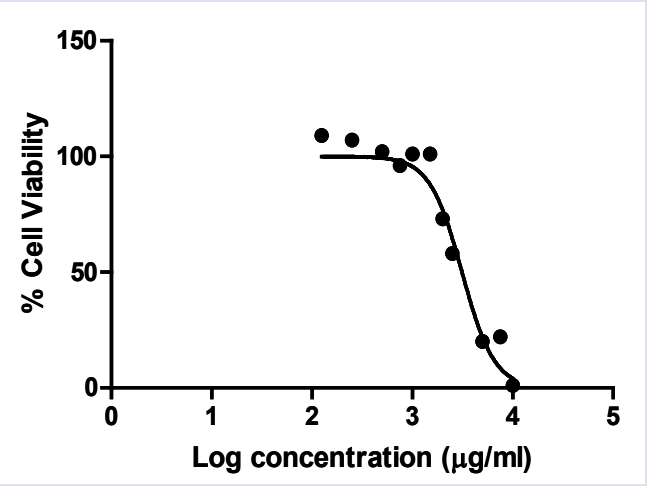

Figure 4: Cytotoxic activity of the extract against HepG2 liver carcinoma cells at different concentrations.

cytotoxic effect of the extract against HepG2 cells. The concentration that was found to inhibit 50% of the metabolic activity of the cells was 3123 µg/ml. According to our observation, the extract is safe for use up to 2000 µg/ml. However, above this concentration (2000 µg/ml), the extract is capable of inhibiting mitochondrial activity and ATP production, suggesting that the concentrations (greater than 2000 µg/ml) can negatively affect cell function. Moreover, it was concluded that the extract could be safe for use as an antioxidant source since its cytotoxic IC_{50} value is higher than the concentration range regarded as significant for antioxidant scavenging agents.

CONCLUSION

P. aeruginosa CP043328.1 has 12 gene clusters that encode for the production of secondary metabolites. Fifteen chemical compounds were identified using GC-MS analysis. The ethyl acetate extract demonstrated the significant antioxidant and anti-quorum sensing activities. Thus, *Pseudomonas aeruginosa* CP043328.1 shows a potential niche in industries for the production of pharmacologically effective and valuable secondary metabolites. For further studies, *in vivo* assays are recommended.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

ATCC: American Type Culture Collection; Anti-Smash: Antibiotic and Secondary metabolite Analysis Shell; DMSO: dimethyl sulfoxide; DPPH: 2.2-diphenyl-1-picrylhydrazyl radical scavenging assay; ABTS: 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay; MTT: Methylthiazol tetrazolium; HepG2: human hepatocellular carcinoma; rpm: revolution per minute; IC_{50}: median inhibitory concentration; µg/mL: microgram/milliliter; g: gram; mL: millimeter; °C: degree Celsius; µL: microliter; %: percent; AA: ascorbic acid; BHA: butylated hydroxyl anisole; EF: extract; GC-MS: gas chromatography-mass spectrometry; GC: gas chromatography; MS: mass spectrometry; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; QS: quorum sensing; v: volume; *A. cordifolia: Anredera cordifolia; C. violeceum: Chromobacterium violeceum; P. aeruginosa*: *Pseudomonas aeruginosa*.

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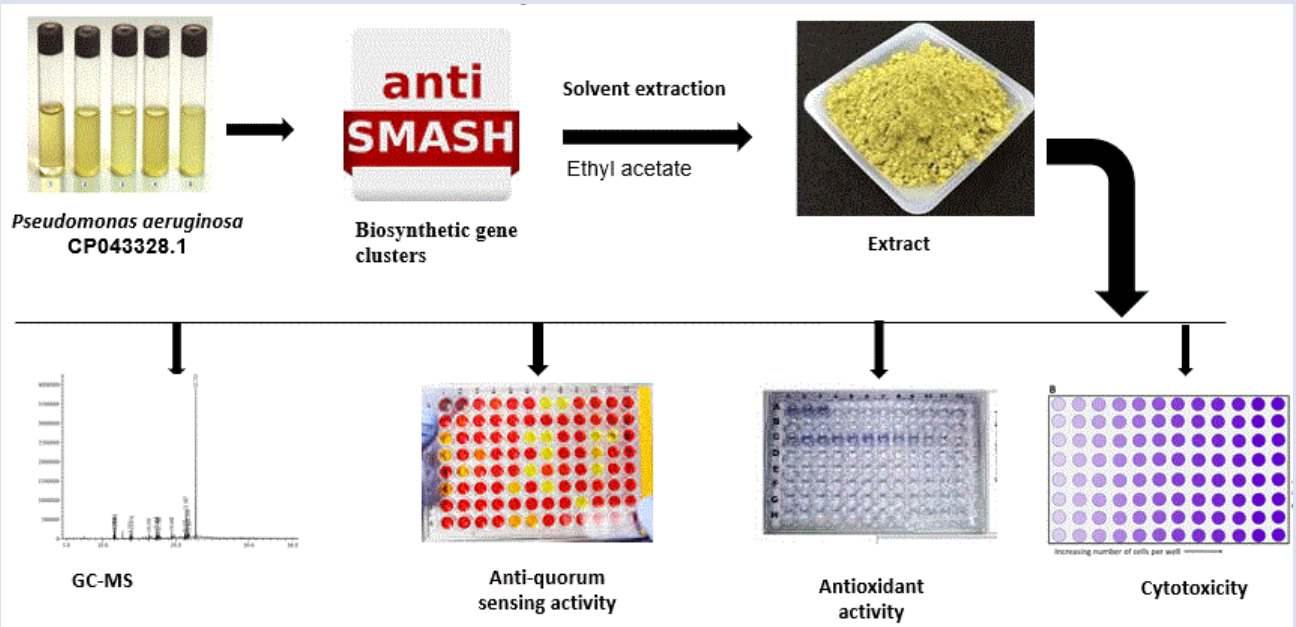
GRAPHICAL ABSTRACT



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