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Antimicrobial screening and GC-MS analysis of bioactive compounds from strains of *Pseudomonas aeruginosa* isolated from poultry fecal littered soil in Ibadan, Nigeria.

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ABSTRACT

The emergence of pathogens exhibiting multiple antibiotic resistance (MDR) is bringing a thwart to available chemotherapeutic agents thus the urgency in the search for bioactive agents that will be effective against the MDR pathogens. This study screened isolates of *Pseudomonas aeruginosa* from poultry fecal littered soil in two selected locations in northern Ibadan, southwest Nigeria (University of Ibadan Teaching Research Farm and Ayede community) for production of bioactive compounds, evaluated their activities against selected MDR bacteria pathogens and analyzed the crude ethylacetate extract using GC-MS. A total of 178 strains of *P. aeruginosa* were isolated on cetrimide agar and identified presumptively by standard biochemical test. Primary and secondary screening of the whole cell and cell free supernatant ethylacetate extracts were done by perpendicular streaking and agar-well diffusion methods respectively against selected clinical MDR bacteria pathogens. Five out of the 178 strains of the presumptive *P.aeruginosa* with excellent antibacterial activity were further confirmed by 16S rRNA amplification and sequencing. Cell free supernatant ethylacetate extracts of two most active strains among the five (denoted with code APC2-1 and APC2-14) were subjected to GC-MS analysis of the metabolites. The five *P. aeruginosa* strains were identified as *P. aeruginosa* MR24 strains 1, 2, 3, *Pseudomonas* sp SD12 and *P. aeruginosa* Kasamber 1 which were closely related phylogenetically. The bioactive compounds produced by the isolates that were studied had greater activity on Gram positive than Gram negative pathogens. GC-MS analysis revealed a total of 19 different compounds (1-heptadecanamine; 2-ethyl-1-hexanamine; 3-butyn-1-ol; Pent-3-enylamine; Pent-4-enylamine; Propane; Acetaldehyde; Ethylene oxide; hydroxyacetonitrile; Bromo-acetonitrile; Azetidine; 2-methyl-Azetidine; Aminocaproic acid; 18-nonadecen-1-amine; 5-aminovaleric acid; 2,2-dimethyl-1,3-propanediamine; 1-octadecanamine; 1-octanamine and 2-(3-butynyloxy)tetrahydro-2H-pyran) among which are antibacterial, anticancer, antifibrinolytic, and antimalarial compounds. This study therefore revealed the potentials of *Pseudomonas aeruginosa* as producers of secondary metabolites of medical and industrial importance.

Keywords: *Pseudomonas*; Bioactive compound; Poultry littered soil, Antimicrobial activity, GC-MS

Introduction

Soil and microorganisms are related owing to the enormous role of microorganisms inhabiting the soil. The soil comprises bacteria, viruses, fungi, algae, protozoa, worms and insects coexisting together to ensure the continuity of life by decomposing or modifying chemical molecules. The functional role of soil microbes is largely to produce certain enzymes capable of degrading dead

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and waste materials and to recycling such materials (Willey *et al*., 2010; Anderson *et al*., 2011).

Microorganisms are known to play key roles in the formation of soil and nutrient for plant growth, nutrient recycling, contaminants filtration, maintaining a balance within the soil ecosystem, biological control of pathogen etc (Anderson *et al*., 2011; Sonia *et al*., 2011). The integrity and suitability of the soil is dependent on the regional climate, soil structure, texture and constituents. This determines the soil's microbial diversity which are relatively abundant at the surface usually within 10cm deep in to the soil (Bundt *et al*., 2001; Fierer *et al*., 2007; Arifuzzaman *et al*., 2010) and decreases as the depth progresses downward (Eilers *et al*., 2012). Most soil types used for agriculture are usually revitalized by using chemical or organic fertilizers to substitute for the lost nutrients. However, some of these chemical or organic fertilizers are no longer used due to their significant effect on human and animals. They usually precipitate health conditions such as cancer, imbalance in the aquatic ecosystem to surface runoff of the fertilizers, environmental pollution, etc (Nganchamung *et al*., 2017; Sharma and Singhvi, 2017). Therefore, biological methods such as crop rotation and composting are now employed in lieu of organic fertilizers in replenishing or improving the soil fertility. Animal waste such as cattle dungs, poultry waste and plant parts like leaves, flower, stem are disposed on the soil allowing the soil microorganisms to degrade such waste products by producing hydrolytic enzymes that degrade the material to yield metabolites that enhances the fertility of the soil and thus improve the agricultural yield from such soil (Marsh and Wellington, 2007).

The discovery of some soil microorganisms with potentials to produce effective bioactive substances like the antimicrobial compounds as metabolites has paved way for the search of various habitats for microorganisms with potentials to produced novel bioactive compounds with excellent activity against resistant microorganisms, particularly the multidrug resistant pathogens. Multidrug resistance is currently on the increase, globally threatening public health and decreasing the efficacy of the antibiotics available currently (WHO, 2017). This therefore, has precipitated the search for novel compounds from natural sources like the environment, beneficial microorganisms, animals and plants from both aquatic and terrestrial habitats with potentials to produce potent antimicrobial agents against the resistant pathogens (Wolfender *et al*., 2015; Dias *et al*., 2016). Microbial genome mining is an important tool in the discovery of unculturable organisms and screening of isolates with potential secondary metabolites (Bachmann *et al*., 2014; Ziemert *et al*., 2016). With the availability of certain technologies like Ultra-High Performance Liquid Chromatography (UHPLC) and Gas Chromatography and Mass Spectrometry Analysis (GC-MS), an effective separation and identification of different bioactive metabolites from natural products is achievable. Some microorganisms such as bacteria and fungi from ages produce antimicrobials and other bioactive compounds (Butler and Bus, 2006).

Pseudomonas spp.are group of Gram-negative bacilli with polar flagella, Gammaproteobacteria and family, *Pseudomonadaceae*. The species of this genus have a wide array of metabolic diversities capable of colonizing different ecological niches requiring non-specific nutritional requirement for their growth (Meyer *et al*., 2002). *Pseudomonas* spp. are known producers of bioactive metabolites such as Phenazine compounds, volatile organic compounds, thioquinolobactin etc. Some of these metabolites have been reported to possess antimicrobial activity, anticancer property, bio-surfactant, bio-controlling, bio-pesticide/ bio-insecticide and bioremediation effects, hence its application in pharmaceutical, agricultural, cosmetic, industrial and environmental sector is important (Meyer *et al*., 2002; Kumar *et al.*, 2016; Gionco *et al*., 2017). Despite the numerous antibiotics produced from microorganisms most especially the actinomycetes, resistance has taken a great toil in the effectiveness of these antibiotics in combating microbial infection. Pathogens have developed different resistant mechanisms through acquisition of resistant genes by horizontal gene transfer, natural resistance or development of mutation to facilitate their survival and ability to continue their infection cycle (Li and Webster, 2018). It is therefore imperative to search for better chemotherapeutic agent by screening Nigeria's terrestrial environment like the poultry fecal littered soil, for beneficial microorganisms with potentials to produce stronger and potent bioactive metabolites against multidrug resistant pathogens.

MATERIALS AND METHOD

Sample Collection

A total number of 10 poultry fecal littered soil, obtained 5-10cm deep into the soil were collected randomly from different sites in two purposively selected poultry farms located in Ibadan (University of Ibadan Teaching Research Farm: 7°26'30.1"N 3°54'01.0"E and a poultry farm in Ayede community: 6°36'42.0"N 4°32'38.4"E). The samples were collected into sterile universal bottles and labeled appropriately. Samples were immediately taken to the laboratory for microbial analysis and stored at 4°C where necessary.

Collection of Test Isolates

A total of eight already characterized and confirmed multidrug resistant clinical bacteria isolates were collected from the Molecular Laboratory of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan. The identities of the clinical isolates were confirmed using standard biochemical test and were used as test organisms for both primary and secondary screening. They include: 2 strains of methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus saprophiticus, Escherichia coli, Klebsiella pneumoniae, Klebsiella* spp., *Salmonella typhimurium* 14028 and *Acinetobacter baumannii.*

Isolation of *Pseudomonas* spp.

The isolation was done as described by Baserisalehi and Bahador (2013) with some modifications. From each soil sample, 1g of the poultry fecal littered soil was suspended in 9mL of sterile distilled water followed by successive serial dilutions by transferring 1mL of aliquot into another 9mL of sterile distilled water and continues in this manner till 10^{-5} dilution was reached. An aliquot of 0.1mL of 10^{-1} and 10^{-3} each were plated by spread plate method on cetrimide agar and incubated at 37°C for 24-48hrs. Colonies of the presumptive *Pseudomonas* species were isolated, obtained as pure cultures and were stored in 1:1 glycerol-broth at -80°C.

Primary Screening for bioactive metabolites with Antimicrobial activity

The primary screening to determine the production of bioactive metabolites with antimicrobial activity by the presumptive *Pseudomonas* isolates was done using perpendicular streaking method as described by Madigan *et al* with slight modification (Madigan *et al.*, 2008). Briefly, the presumptive *Pseudomonas* isolates were streaked aseptically in a straight line on Muller Hinton Agar (Oxoid Ltd, Germany) and incubated for 48hrs at 37°C after which the eight test bacteria were streaked each at right angle to the *Pseudomonas* isolates. After incubating for 24hrs at 37°C the zones of inhibition of each test clinical bacteria against the *Pseudomonas* metabolites were measured in millimeter from the edge of the *Pseudomonas* growth point to the edge of each test isolate growth point on the Mueller Hinton agar.

Extraction of Bioactive Metabolites with Antimicrobial Activity

All the *Pseudomonas* isolates that showed activity against any of the test clinical isolates in the primary screening stage were subjected to a submerge fermentation process to extract their metabolites as described by Sandeep and Menaka with slight modification (Sandeep and Menaka, 2014). *Pseudomonas* isolates were grown in 500mL of Nutrient broth in sterile conical flasks. The conical flasks were incubated in a rotatory shaker at 150rpm for 72hrs at 37ºC. The culture was later centrifuge at 10,000rpm for 20min. The cell free supernatants were pipetted into sterile tubes mixed vigorously with equal volume of ethyl acetate 1:1 and stored at 4ºC to be used for the secondary screening while the cell debris and biomass were discarded.

Secondary Screening for Bioactive Metabolites with Antimicrobial Activity

The *Pseudomonas* cell free supernatants obtained from the submerge fermentation process was employed for the secondary screening for bioactive metabolites with antimicrobial activity using the agar-well diffusion method. Briefly, two to three colonies of the pure overnight culture of the test clinical bacterial isolates were dispersed in sterile normal saline and the densities of the suspensions adjusted to 0.5 McFarland turbidity standards of approximately 1.0 x 10^7 CFU/ml (Ogah and Osundare, 2015). This was then inoculated on the aseptically dried surface of set 20mL Mueller Hinton Agar plates using sterile cotton swabs after which 6mm cork borer was used to bore wells. To each well 100 µL of the cell free supernatant was introduced and allowed to stand for 20min to facilitate the diffusion of the bioactive compounds into the agar medium before incubating at 37°C for 24 hours. Pure nutrient broth medium was introduced into a well to serves as the negative control. After the incubation the zones of growth inhibition were read and recorded in millimeter (mm).

Whole DNA isolation and 16S rRNA Identification of *Pseudomonas* isolates

The genomic DNA of five of the 178 *Pseudomonas* isolates that gave excellent antibacterial activity was isolated by boiling method. The 16S rRNA gene of the isolates was amplified by PCR technique using the primer sequence 10F-AGTTTGATCATGGCTCAGATTG and 1507R-TACCTTGTTACGACTTCACCCCAG (Mateos *et al*., 2006). The amplicons were purified and sequenced using the forward primer only and the sequence blasted on the National Centre for Biotechnology Information (NCBI) Standard Nucleotide BLAST database for identification. The gene sequences of the isolates were used to construct phylogenetic tree using NCBI BLAST Pairwise alignments.

Identification of Bioactive Compounds by GC-MS Analysis

Bioactive compounds in the cell free supernatants of two of the five *Pseudomonas* isolates that produced metabolites with higher antibacterial activity against the test clinical bacterial isolates were identified by GC-MS analysis. Briefly, 100µL of each cell free supernatant was mixed with ethyl acetate at a ratio 1:1 and this was loaded into GC-MS apparatus for analysis using the protocol of Sengupta *et al*. with slight modifications (Sengupta *et al*., 2015). Analysis was conducted using Agilent 7890A gas chromatograph equipped and coupled to Agilent 5975C Mass Spectrometer with a fused HP-5MS 5% Phenyl Methyl Silox (30 m x 0.25 mm ID x 0.25µm of capillary column). Helium gas was used at a constant flow rate 1.0 mL min−1; and a fix inlet temperature (285 °C); injection volume, 1 µL (LVI). Oven temperature program was set to an initial temperature of 90ºC, then 3 °C min−1 ramp to 180 °C and held for 10 min. The ionization voltage used was 70 eV while a scan of 0.6 s was applied covering a mass range from 50 to 500 amu. The major constituents were identified by matching their minimum similarity with regards to the NIST library.

Results

Antimicrobial Activity of the *Pseudomonas* isolates

Among the 178 *Pseudomonas* isolates screened for antimicrobial activity (primary and secondary screening), five showed broad spectrum activity among which two gave excellent broad-spectrum antimicrobial activity against the test bacteria especially against the Gram positive bacteria (Table 1).

Identification and Phylogenetic Analysis

The five *Pseudomonas* isolates, denoted with the codes APC2-14, APD2-2, U4-2, APA4-3 and APC2-1, were identified as *Pseudomonas aeruginosa* MR24 strains 1, 2, 3 (accession numbers LT969591.1, LT969591.1, LT969591.1) *Pseudomonas* sp SD12 (accession number HQ268805.1) and *Pseudomonas aeruginosa* Kasamber 1 (accession number KY549637.1) respectively when the sequenced 16S rRNA amplicons were compared with the sequences in the NCBI database. Isolate denoted with code APC2-14 has 99% relatedness with *Pseudomonas aeruginosa* MR24 strain 1 and APC2-1 identify as *Pseudomonas aeruginosa* Kasamber 1 having 98% relatedness. Phylogenetic tree constructed showed that all the isolates are closely related to each other with *Pseudomonas aeruginosa* MR24 strain1 more closely related to *Pseudomonas* sp SD (12) than *Pseudomonas aeruginosa* MR24 strain 2 and *Pseudomonas aeruginosa* MR24 strain 3. The tree was rooted with *Bacillus subtilis* UD1022 serving as an out-group (Figure 1).

Gas Chromatography and Mass Spectrometry Analysis

Identification of the bioactive compounds in the cell free supernatants of the two isolates with excellent broad spectrum activity denoted with code APC2-1 (*Pseudomonas aeruginosa* Kasamber 1) and APC2-14 (*Pseudomonas aeruginosa* MR24 strain 1) showed a total of 18 and 12 peaks respectively present at different retention time (Figure 2 and 3). By comparing with NIST library, the peaks correspond to some compounds as presented in table 2. Some compounds like 1-heptadecanamine, 1-hexanamine, 2-ethyl-, 3-butyn-1-ol, Pent-4-enylamine, Propane, Acetaldehyde, Ethylene oxide, Acetonitrile, hydroxy- and Pent-3-enylamine are present in both strains analyzed.

Table 1: Primary and secondary antimicrobial screening of selected Pseudomonads against resistant clinical bacterial isolates

	Selected Pseudomonads strains									
	APC2-14		APC2-1		APD2-2		U4-2		APA4-3	
Test clinical isolates	Pri	Sec	Pri	Sec	Pri	Sec	Pri	Sec	Pri	Sec
	Zones of growth inhibition (mm)									
Gram positive										
Staph. saprophyticus	17	16	13	25	17	17	17	14	14	15
Staph aureus1	20	17	18	17	17	20	12	20	14	22
Staph. aureus2	18	20	20	18	16	15	12	24	20	18
Gram negative										
Acinetobacter baumanii	NZ	10	NZ	8	6	10	12	NZ	3	NZ
Klebsiella sp.	7	12	12	14	10	NZ	NZ	NZ	3	NZ
Kleb. pneumoniae	10	15	8	14	7	NZ	8	NZ	5	NZ
E. coli	10	12	11	9	NZ	NZ	6	11	7	12
S. typhimurium	ND	12	ND	20	ND	NZ	ND	NZ	ND	10

Key: NZ = No Zone; ND = Not Done; Pri = Primary screening; Sec = Secondary screening

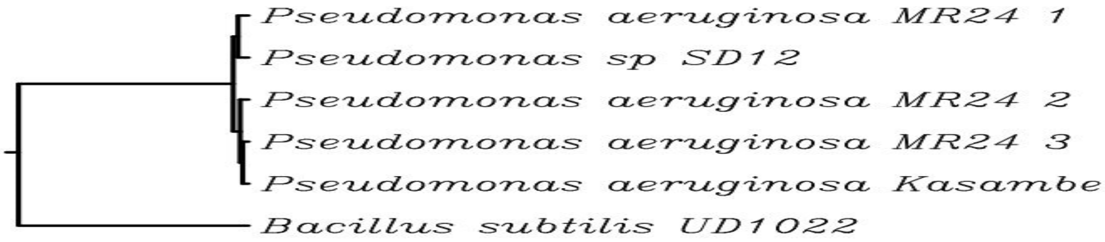

Figure 1: Phylogenetic tree of five selected *Pseudomonas* isolates

Figure 2: Chromatogram of ethyl acetate extract of *Pseudomonas aeruginosa* Kasamber1 metabolites

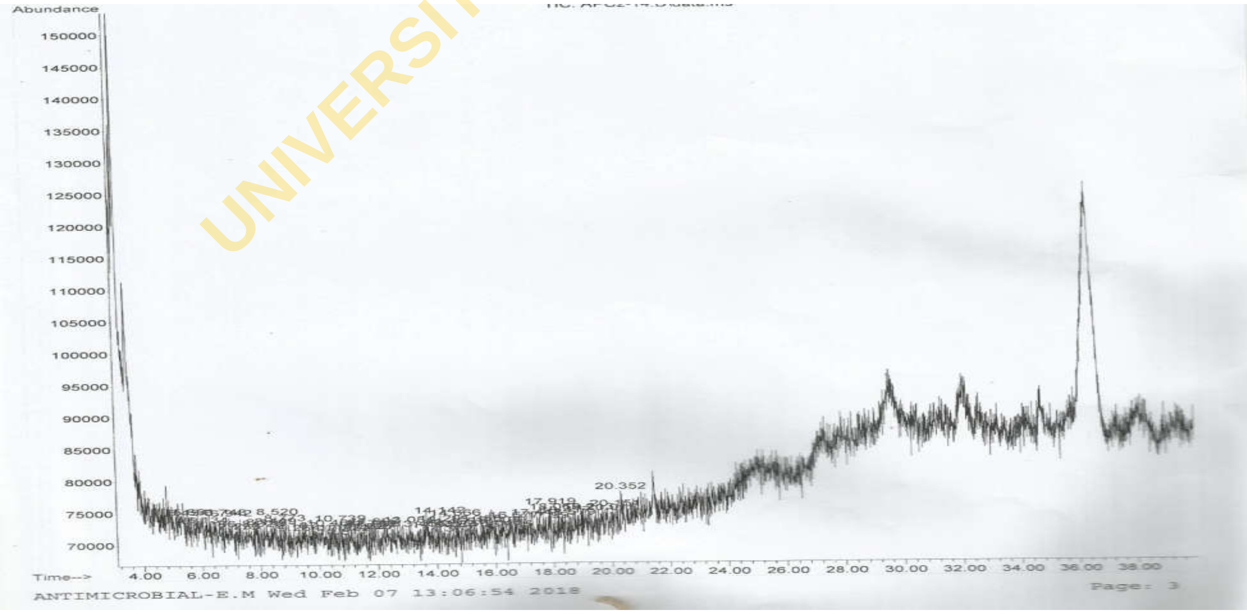

Figure 3: Chromatogram of ethyl acetate extract of *Pseudomonas aeruginosa* MR24 strain 1 metabolites

Table 2: GC-MS profile of ethyl acetate extract of two *Pseudomonas* isolates' metabolites

Components	Retention Time (Min)	Peak Height	Area
Pseudomonasaeruginosa Kasamber1			
1-decanamine	9.491	10575	22227
Azetidine	12.052	6803	11185
Propane	13.087	7117	10824
Bromo-acetonitrile,	13.566	18180	27787
3-butyn-1-ol	14.601	10742	13793
Pent-3-enylamine	16.296	9826	14114
Aminocaproic acid	17.053	11512	10584
18-nonadecen-1-amine	17.900	8227	12792
Pent-4-enylamine	18.042	9192	11657
2-ethyl-1-hexanamine	19.976	7455	9472
5-aminovaleric acid	20.669	8462	19790
2,2-dimethyl-1,3-propanediamine	21.645	8771	12688
1-octadecanamine	23.444	8732	14723
1-heptadecanamine	25.585	7319	12247
1-octanamine	28.282	9557	13625
Ethylene oxide	28.515	9507	9878
Acetaldehyde	31.445	10651	16018
Hydroxyacetonitrile	39.635	7632	10861
Pseudomonas aeruginosa MR24 strain 1			
Pent-3-enylamine	5.454	4485	5867
Hydroxyacetonitrile	6.134	3849	8611
Ethylene oxide	6.386	4481	10020
Acetaldehyde	7.175	2811	5345
Propane	11.742	5185	10966
Pent-4-enylamine	14.465	3628	7424
1-heptanamine	16.199	3756	5448
2-methyl-Azetidine	16.677	4408	5005
3-butyn-1-ol	17.441	4165	7764
2-(3-butynyloxy)tetrahydro-2H-pyran	17.991	4524	7628
1-heptadecanamine	18.579	4355	5256
2-ethyl-1-hexanamine	20.015	3727	5920

Discussion

Most clinically used chemotherapeutic agents were discovered from soil microorganisms ranging from filamentous fungi and bacteria to true bacteria. Over the years, Pseudomonads have been known as good source of various bioactive metabolites (Darabpour *et al*., 2010). Various species have been reported to produce different secondary metabolites active against clinical pathogens (Darabpour *et al*., 2010). This was evident in this study as the strains of *Pseudomonas aeruginosa* isolated produced bioactive compounds with antimicrobial activity against the test MDR clinical bacterial isolates.

The primary screening of the *Pseudomonas* isolates in this study indicated an intracellular production of metabolites with broad spectrum of activity against the tested clinical bacterial isolates. The Gram positive bacteria (namely, methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus saprophiticus*) were more susceptible to the bioactive metabolites compared to the Gram negative bacilli (*Escherichia coli, Klebsiella pneumoniae, Klebsiella* sp., *Salmonella typhimurium* 14028 and *Acinetobacter baumannii*) as seen in the primary and secondary screening, (Table 1) thus, confirming the extracellular production of bioactive metabolites with antimicrobial activity. This was equally reported by Darabpour *et al* (2010) and El-Shouny *et al*. (2011). Darabpour *et al* isolated *Pseudomonas aeruginosa* PG-01 from marine sediment and found that it produced bioactive compounds that were active against Gram positive bacteria including the commonly implicated *Methicillin resistant Staphylococcus aureus*. El-Shouny *et al*. (2011) also studied five different strains of *Pseudomonas aeruginosa* isolated from surgical specimen and minced meat producing pyocyanin and found that the bioactive compounds produced were active on Gram positive bacteria and *Candida* spp. than Gram negative bacteria. Similarly, Mogea *et al.* (2015) studied the effect of cell free supernatant of *Pseudomonas aeruginosa* NS3 containing phenazine derivatives against pathogenic microbes (*Methicillin resistant Staphylococcus aureus*, *Staphylococcus aureus*, Enteropathogenic *Escherichia coli* and *Candida albicans*) and found that the Gram positive bacteria were inhibited more than the Gram negative bacteria. The higher susceptibility of the Gram positive bacteria to the bioactive compounds compared to the Gram negatives may be due to the differences in the cellular composition of the bacteria. Gram negative bacteria are known to possess outer membrane external to the cell wall which prevents easy penetration of substances into the cell (Motamedi *et al*., 2010).

The results of the Gas chromatography and Mass Spectrometry analysis of the ethyl acetate fractions of the APC2-1 (*Pseudomonas aeruginosa* Kasamber 1) and APC2-14 (*Pseudomonas aeruginosa* MR24 strain 1) cell free supernatants revealed a total of 19 bioactive compounds (Table 2), this confirms the abundance of bioactive compounds in *Pseudomonas* spp. Some authors have reported the presence of phenazine and its derivatives, thioquinolobactin, rhamnolipids, certain volatile organic compounds, pyrrolnitrin-type antibiotic, pyo compounds, indole derivatives, peptides, glycolipids, lipids and aliphatic compounds in *Pseudomonas* spp. (Raaijmakers *et al*., 2002; Haas and Keel, 2003; Ligon *et al*., 2003; Raaijmakers *et al*., 2006). While some authors reported the production of 1-heptadecanamine and 2-(3-butynyloxy) tetrahydro-2H-pyran possessing antimicrobial, anticancer and antihypercholesterolemic activity (Dong *et al*., 2011; Sivakumaran *et al*., 2016) in Pseudomonas spp., these were also reported in this study. On the contrary, Sukatar *et al.* (2006) identified 1-heptadecanamine has compound present in

Enteromorphalinza (Linnaeus) extract possessing antimicrobial activity.

Importantly in this study, some compounds of medical importance were produced by the strains of *Pseudomonas*. Among them is Aminocaproic acid, which is medically used as an antifibrinolytic agent marketed under the name Amicar. It is usually synthesized chemically for use clinically in the treatment of excessive bleeding during heart, liver and kidney transplant. This compound was found to have been produced naturally by *Pseudomonas aeruginosa* Kasamber 1. Another chemically produced compound, Azetidine, is a compound having a beta lactam-like structure and has been reported to be rarely present in natural products but possesses antimicrobial, antitumor and antimalarial activity (O'Dowd *et al*., 2008; Yan *et al*., 2016; Maetani *et al*., 2017).

Chemically synthesized bicyclic Azetidine was found effective against *Plasmodium falciparum* while targeting phenylalanyl-tRNA synthetase which affects the three stages of the parasite lifecycle. The lengthy synthetic route in the development of bicyclic Azetidine tends to put a cost constrain in the development of such drug thus utilizing naturally derived Azetidine might be cost effective and more potent than chemically synthesized Azetidine (Maetani *et al*., 2017). In this study Azetidine and 2-methyl Azetidine, was produced by both *Pseudomonas aeruginosa* Kasamber 1 and *Pseudomonas aeruginosa* MR24 strain 1 confirming *Pseudomonas* as a good natural source of the compound.

Conclusion

In conclusion, this study therefore confirms *Pseudomonas* species as one of the soil bacteria with high potentials for bioactive compounds useful in the area of medicine and pharmaceuticals as whole drugs or precursors to the development of stronger drug compounds.

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Disclosure statement

There is no conflict of interest reported by the authors

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