MOLECULAR CONFIRMATION OF THE SOIL BACTERIAL *PSEUDOMONAS AERUGINOSA* AND EVALUATION OF SOME BIOACTIVE SECONDARY METABOLITES*

Su Swe Su[1], Nwet Nwet Win[2], Daw Hla Ngwe[3], Saw Hla Myint[4], Ni Ni Than[5]

Abstract

This research focuses on the molecular confirmation of *Pseudomonas aeruginosa* isolated from the clinical soil sample collected from Insein General Hospital, Yangon Region, and evaluation of some bioactive secondary metabolites from it. The isolated *P. aeruginosa* strain characterized by microscopic examination and biochemical tests was confirmed by 16S rRNA sequencing technique. The chloroform extract was prepared from the *P. aeruginosa* culture in a large scale for the investigation of the chemical constituents. According to silica gel chromatographic separation on the chloroform extract, five nitrogeneous compounds such as 1-hydroxyphenazine, phenazine-1-carboxylic acid, cyclo-(L-Leu-L-Pro), cyclo-(D-Pro-D-Leu) and cyclo-(D-Pro-L-Val) were isolated. These isolated secondary metabolites were structurally identified by using modern NMR spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, COSY, HSQC, ROESY, HMBC spectroscopies, and mass spectrometry. The two isolated phenazines: 1-hydroxyphenazine and phenazine-1-carboxylic acid were found to be potent in antimicrobial activity with the inhibition zone diameters ranged between (21 ~ 32 mm) and (15 ~ 31 mm), whereas the remaining three diketopiperazines showed mild antimicrobial activity (inhibition zone diameters between 12 mm ~ 14 mm). In addition, 1-hydroxyphenazine (IC_{50} = 14.12 µg/mL) and phenazine-1-carboxylic acid (IC_{50} = 15.0 µg/mL) were found to exhibit good antiproliferative activity against human breast cancer cell MCF7 and the remaining three compounds: cyclo(L-Leu-L-Pro) (IC_{50} = 176.4 µg/mL), cyclo(D-Pro-D-Leu) (IC_{50} = 87.7 µg/mL) and cyclo(D-Pro-L-Val) (IC_{50} = 196.5 µg /mL) have mild antiproliferative activity.

Keywords: *Pseudomonas aeruginosa*, 16S rRNA sequencing technique, phenazines, diketopiperazines, antimicrobial activity, antiproliferative activity

Introduction

Pathogenic microscopic organisms can cause extreme and lethal sicknesses in humans, animals and plants. With increase in prevalence of drug resistant pathogenic bacteria, the search for novel antibacterial agents is of most extreme significance. Several microorganisms are known to suppress the growth of pathogenic bacteria and fungi due to production of bioactive compounds. Soil is a broadly investigated natural specialty for sources of microorganisms that produce useful biologically active compounds. Among these microbes, bacteria are prolific source of novel compounds with fascinating antimicrobial activity. *Pseudomonas* is a genus of gram-negative, aerobic bacteria that can cause disease in animals, including humans. *Pseudomonas aeruginosa* bacteria had a potential to produce bioactive compounds belong to the gamma proteobacteria class with these characteristics: gram-negative, rod-shaped, motile, forming smooth spherical colonies with greenish fluorescent colour, wide spread in the environment. These bacteria produce pyocyanin pigment which is soluble in water, chloroform and n-butanol. *Pseudomonas* is a large group of free-living bacteria that live primarily in soil, seawater, and fresh water. In the previous research work, it has been reported that the chloroform extract from *P. aeruginosa* isolated from soil possesses some biological activities such as antimicrobial activity, antioxidant activity, cytotoxic effect and antiproliferative activity (Su Swe Su *et al.,* 2020) and the isolated bacteria was

[1] Dr, Lecturer, Department of Chemistry, University of Yadanabon
[2] Institute of Natural Medicine, University of Toyama, 2630-Sugitani, Toyama 930-0194, Japan
[3] Dr, Professor and Head (Retd.,), Department of Chemistry, University of Yangon
[4] Dr, Part-time Professor , Department of Chemistry, University of Yangon
[5] Dr, Professor and Head Department of Chemistry, University of Yangon
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characterized as a rod shaped and gram-negative bacteria according to the observations from the microscopic examination and biochemical tests (Su Swe Su *et al.*, 2018). Baron and Rowe (1981) reported that some compounds present in *P. aeruginosa*, like phenazines (pyocyanin, 1-hydroxy-phenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid) serve as electron shuttles in reduction and solubilization of Fe(III) function as antibiotics. In the present work, the *P. aeruginosa* bacteria isolated from clinical soil is identified by using DNA sequencing technique. In addition, some bioactive secondary metabolites such as two phenazines and three diketopiperazines are isolated from the chloroform extract of *P. aeruginosa* and structurally identified by modern spectroscopic techniques. Furthermore, some biological activities such as antimicrobial activity and antiproliferative activity of the isolated compounds are also investigated.

Materials and Methods

Sample Collection

Soil samples were collected from the Insein General Hospital, Yangon Region. Bacteriological analyses were started within three days after collecting the samples. *P. aeruginosa* was then isolated from the soil sample and cultured in a large scale of nutrient ager medium and chloroform extract was prepared from the isolated bacteria culture according to the reported method (Su Swe Su *et al.*, 2020).

Identification of the Isolated *P. aeruginosa* by DNA Sequencing Technique

The isolated bacteria *P. aeruginosa* was identified by DNA sequencing technique using bacterial 16S rRNA primer and compared with the reported data (Amuth and Kokila, 2014).

Firstly, about 25 mL of nutrient broth medium at pH 7 was prepared and inoculated with two loops full culture of the isolated bacteria in 250 mL conical flask. It was then incubated at 37 °C in an incubated shaker for overnight providing the bacteria solution. Secondly, the DNA of the *P. aeruginosa* was extracted using Genomic DNA extraction kit (Real Genomics, Canada) according to the bacterial genomic DNA isolation protocol and the purity of the extracted genomic DNA was checked by using agarose gel electrophoresis (Amutha and Kokila, 2014). This experiment was performed in the Pharmaceutical Research Department, Insein, Yangon. Thirdly, the extracted genomic DNA was used as a template in PCR amplification which was carried out in 0.2 mL PCR tubes with 10 µL of reaction mixture volume with the composition as shown in Table 1.

Table 1 Composition of PCR Reaction Mixture

Reaction mixture	**Volume (µL)**
PCR buffer (10X) with $MgCl_2$ (1.5 mM)	2.5
dNTPs mixture (0.5 mM each)	2.0
Taq DNA polymerase (5U/ µL)	0.3
Primer F (10 nM)	0.6
Primer R (10 nM)	0.6
Template DNA (the extracted DNA from *P. aeruginosa*)	4.0
Total	**10.0**

Table 2 PCR Program Used to Amplify 16S rRNA

Stage of PCR		Time (min)	Temperature (°C)	No. of cycle
Pre-PCR	Hot start	3	95	1
PCR	Denaturation	0.5	95	35
	Annealing	0.5	50	
	Extension	2	72	
Post –PCR	Final extension	10	72	1
Hold			4	

The polymerase chain reaction was performed using PCR thermal cycler and a temperature profile standardized for 16S rRNA gene amplification. The PCR program used to amplify the 16S rRNA is shown in Table 2. The PCR products were separated by agarose gel the products obtained through amplification with two universal targeting 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (M = C or A) and 1492R (5'-GGYTACCTTGTTACGACTT-3') (Y = C or T) were subjected to sequencing using same upstream and downstream primers (Amutha and Kokila, 2014) and the present work was carried out in Bio Basic Lab, Singapore. Finally, molecular confirmation of the isolated bacteria was carried out by BLAST (Basic Local Alignment Search Tool) against the (National Center for Biotechnology Information) NCBI data.

Isolation and Identification of Bioactive Compounds from Chloroform Extract of *P. aeruginosa* by Column Chromatography

P. aeruginosa chloroform extract (5 g) was separated with different polarities of solvent systems of PE:EA in the ratios of 9:1, 4:1, 7:3, 3:2 and 1:1 v/v by using silica gel column chromatographic method. On the chromatographic separation, six main fractions (F-I to F-VI) were collected after examining on the precoated TLC plates. Among the fractions, from the sub-fraction F2b2 of fraction F-II, compound **1** was obtained as a yellow crystal in 12 mg (0.24 % yield based on chloroform extract), whereas from the sub-fraction F3b of fraction F-III, 34.6 mg of compound **2** was obtained as a yellow amorphous powder in 0.72 % yield. In addition, two colourless solid compounds: **3** (17 mg, 0.34 % yield) and **4** (4.5 mg, 0.09 % yield) were respectively isolated. Moreover, the sub-fractions F5b and F6a of fractions F-V and F-VI gave compound **5** as a colourless powder (10.4 mg, 0.18 % yield). Some physical properties of these isolated compounds, such as melting points, R_f values and solubility in some solvents such as petroleum ether, ethyl acetate, methanol, ethanol and acetone were determined. The structures of these isolated secondary metabolites were identified by using modern NMR spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, ROESY and Mass spectroscopies, and by comparing with the reported data. The NMR and Mass spectra of the isolated compounds were measured at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

Screening of Antimicrobial Activity of the Isolated Compounds by Agar Disc Diffusion Method

The screening of antimicrobial activity of isolated compounds (**1** to **5**) was carried out by agar disc diffusion method (Perez *et al*., 1990) at Pharmaceutical Research Department (PRD), Yangon, Myanmar and Department of Chemistry, Yangon University, Myanmar. Seven species of microorganisms: *Agrobacterium tumerfaceins* (N.I.T.E -09678), *Bacillus subtilis* (N.C.T.C-8236), *Bacillus pumilus* (N.C.I.B-8982), *Candida albicans* (-), *Escherichia coli* (N.C.I.B-8134) *Pseudomonas aeruginosa* (6749) and *Staphylococcus aureus* (N.C.P.C-6371) were used in this test.

Investigation of Antiproliferative Activity of the Isolated Compounds against Human Breast Cancer Cell Line Using CCK-8 Assay

Antiproliferative activity of the five isolated compounds (**1** to **5**) from chloroform extract of *P. aeruginosa* was investigated in *in vitro* by using CCK-8 assay at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. The cell line used was MCF 7 (human breast cancer cell line). K562 µ-Minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) were used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, Sigma) and 1% of antibiotic antimycotic solution (Sigma). For MCF 7 cell, 1 % of 0.1 M non-essential amino acid (NEAA, Gibco) and 1 % of 1 mM sodium pyruvate (Gibco) were also supplemented. The *in vitro* antiproliferative activity of the isolated compound was determined by the procedure described by (Win *et al.*, 2015). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % of CO_2 and 95 % of air for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial diluted solutions of the tested samples were added. After 72 h incubation, the cells were washed with PBS, and 100 µL of medium containing 10 % of WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added into the wells. After 2 h incubation, the absorbance at 450 nm was measured. The concentrations of the crude extracts were 200, 100, 20, 10 µg/ mL were prepared by serial dilution. Cell viability was calculated from the mean values of the data from three wells using the equation below and antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-Fluorouracil (5FU) was used as positive control.

$$\% \text{ Cell viability} = \left[\frac{A_{\text{test sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right] \times 100$$

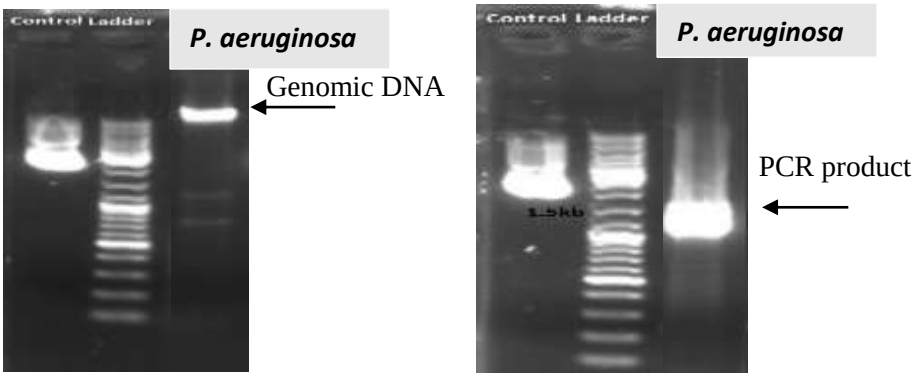
where, $A_{\text{test sample}}$ = absorbance of test sample solution

A_{control} = absorbance of DMSO

A_{blank} = absorbance of CCK–8 reagent

Results and Discussion

In this study, *P. aeruginosa*, a rod shaped and gram negative bacteria isolated from one of the clinical soil sample collected from the Insein General Hospital, Yangon Region was identified by using DNA sequencing technique. The isolated *P. aeruginosa* strain characterized and identified by microscopic examination and biochemical tests (Su Swe Su *et al*., 2018) was used in the present research work. For the molecular confirmation using DNA sequencing technique, genomic DNA was extracted from the isolated *P. aeruginosa* and subjected to check the purity by using agarose gel electrophoresis. It can be clearly seen that the extracted genomic DNA was highly pure and it can also be estimated that the size of DNA may have 10000 bp compared with control as shown in Figure 1 (a). The extracted pure genomic DNA of the isolated *P. aeruginosa* bacteria was used as a template in the molecular confirmation by PCR amplification with two universal 16S rRNA primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (M = C or A) as forward primer F and 1492R (5'-GGYTACCTTGTTACGACTT- 3') (Y = C or T) as reverse primer R according to the reported method (Amutha and Kokila, 2014). The PCR products were photographed under ultraviolet light machine (Transillumnator;Uvite, UK) to detect the specific amplified product by comparing it with 1000 base pairs standard DNA ladder (Figure 1 (b)).



(a) (b)

Figure 1 Agarose gel electrophoresis of genomic DNA and PCR products after amplification of the 16S rRNA gene (a) Gel image showing genomic DNA extracted from the isolated *P. aeruginosa* (250 - 10000 bp) DNA Ladder (b) PCR product

From the PCR result, it can be clearly seen that the size of the PCR product of genomic DNA might be expected to have 1500 bp. The DNA sequence was compared with NCBI gene bank database using BLAST algorithm. Figure 2 shows the consensus nucleotide sequence of the PCR product, amplified segment of 16S rRNA gene of *P. aeruginosa*. The result showed a data gave high similarity (99-100 %) homology of the search sequence with *P. aeruginosa* strain S 04 16S rRNA (accession number MT 626658.1). Therefore the results of phenotypic tests and biochemical tests (Su Swe Su *et al*., 2018) and genotypic confirmation, the isolated bacterial strain was finally identified as a strain of *P. aeruginosa.*

ATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGA
GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAAT
ACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATG
AGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGT
AACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGC
CGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA
GTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCG
TGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGA
ACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGT
AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGG
ACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTG
GCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGT
GCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TG

Figure 2 Nucleotide sequence of the amplified segment of 16S rRNA gene of *P. aeruginosa*

Some Secondary Metabolites of the Isolated *P. aeruginosa*

Five secondary metabolites (**1** to **5**) including two phenazines (**1** and **2**) and three cyclopeptides, diketopiperazines (**3**, **4** and **5**) were isolated from the chloroform extract of *P. aeruginosa* by using silica gel coloumn chromatographic separation technique. Their structures were elucidated using 1D (^{1}H and ^{13}C NMR) and 2D NMR (HSQC, ^{1}H-^{1}H COSY, HMBC, ROESY) spectroscopic techniques. All of these compounds are the chemical constituents found in *P. aeruginosa* (David *et al*., 1986; Kwon *et al*., 2001 and Muhanna *et al*., 2017).

Compound 1: It was obtained as a yellow crystal with melting point of 152-157 °C and has R_f value of 0.6 (PE:EA, 4:1 v/v). It is soluble in all polar and non-polar organic solvents (pet ether, ethyl acetate, methanol, ethanol and acetone) tested. According to the 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, HMBC) NMR spectroscopic data (Table 3), compound 1 was observed to contain eight protons including seven *sp* alkenic methine protons (=CH) and one OH proton together with twelve carbons including seven *sp* alkenic methine carbons (=CH-), one *sp* alkenic methine carbon bearing OH group and four *sp* alkenic quarternary carbons (=C-) resulting the partial molecular formula of $C_{12}H_8O$ with molecular weight of *m/z* 168. The ESI-MS mass spectrum indicated that the molecular weight of compound **1** was *m/z* 196. Consequently, the remaining mass is 28, and it must contain two nitrogen atoms. Thus, the complete structural formula of compound **1** must be assigned as $C_{12}H_8N_2O$ with the molecular weight 196. Since the DBE (Double Bond Equivalent) equal to 10, there will be three rings and seven double bonds present in compound **1**. The COSY and HMBC correlations finally gave the complete structure of compound **1** as described in Figure 3. It was found that the observed NMR data of compound **1** were similar to the reported data of 1- hydroxy phenazine (Sinha *et. al*., 2015) (Table 3). In addition, the melting point (152-157 ºC) of compound **1** was observed to be identical with the reported data of 1-hydroxyphenazine (mpt.156 – 159 ºC) (Tokyo Chemical Industry), one of the constituents of *P. aeruginosa* (Sinha *et. al*., 2015). On the basis of the above information, the compound **1** was identified as 1-hydroxyphenazine.

Table 3 1D and 2D NMR Spectral Data of 1-Hydroxy phenazine and the Reported Data

Carbon Position	1-Hydroxy phenazine ($CDCl_3$)				*Reported data (DMSO)	
	δ_C(ppm) HSQC	δ_H(ppm) (*J* H_z)	COSY	HMBC	δ_C(ppm)	δ_H(ppm) (*J* H_z)
1	151.76, =C-OH	-	-	-	153.9,COH	-
2	108.97, =CH -	7.26,*dd* (2.1,6.5)	H-3	1,3,4a,10a	110.8 CH	7.20,*dd* (1.0,7.5)
3	120.01, =CH -	7.77,*dd* (2.1,8.1)	H-2	1,2,4a,10a	119.4 CH	7.69,*dd* (1.0,8.5)
4	131.92, =CH -	7.87, *m*	H-3	2,4a,10a	132.3 CH	7.97, *m*
4a	143.89, =C(qC)	-	-	-	143.4 qC	-
5a	144.2, =C(qC)	-	-	-	144.1 qC	-
6	130.85, =CH -	7.86, *m*	H-7	5a,9,9a	131.4 CH	7.91, *m*
7	129.25, =CH -	8.26,*dd* (2.3,7.7)	H-6	4,4a,6	129.5 CH	8.22,*dd* (2.5,6.0)
8	130.55, =CH -	7.84,*dd* (6.6,9.5)	H-9	5a,9a,9	130.8 CH	7.79,*dd* (7.5,8.5)
9	129.77, =CH -	8.31,*dd* (2.2,7.8)	H-8	8	129.8 CH	8.27,*dd* (2.5,6.0)
9a	141.25, =C(qC)	-	-	-	141.5 qC	-
10a	134.76, =C(qC)	-	-	-	136.1 qC	-
OH		8.26			-	8.24

*Sinha *et.al*. 2015

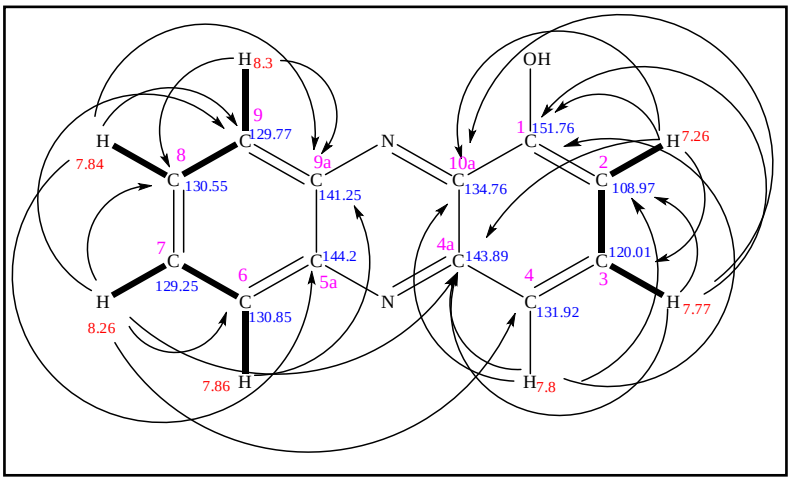


Figure 3 Structure of 1-hydroxyphenazine(1) [COSY (▬) and HMBC (H→C) correlation]

Compound 2: It was obtained as a yellow amorphous powder with melting point of 240- 246 °C and has R_f value of 0.27 (PE:EA, 4:1 v/v). It is also soluble in all polar and non-polar organic solvents tested. It was also found to be a derivative of phenzazine, i.e., phenazine-1-carboxylic acid. Hence, the structure of compound **2** is similar to compound **1** and they differ to each other only in the substituents at C-1. All of the 1D and 2D NMR spectral data were found to be identical with those of the reported data of phenazine-1-carboxylic acid (Sayed *et al*., 2008) (Table 4). The COSY and HMBC correlations gave the complete structure of compound **2** as illustrated in Figure 4. In addition, the melting point of compound **2** ($C_{13}H_8N_2O_2$, m/z = 224 confirmed by mass spectrum) was also observed to be consistent with that of phenazine-1-carboxylic acid (239 ~ 245 °C, $C_{13}H_8N_2O_2$, mol. wt. = 224) (Syn Quest Labs).

Table 4 1D and 2D NMR Spectral Data of Phenazine-1-carboxylic Acid and the Reported Data

Carbon position	Phenazine-1-carboxylic acid ($CDCl_3$)				*Reported data (CD_3OD)	
	δ_C(ppm) HSQC	δ_H(ppm) (*J*,Hz)	COSY	HMBC	δ_H(ppm)	δ_C(ppm)
1	125.04, = C	-	-	2,3	-	124.9 C
2	137.5, = CH	8.99,*dd* (1.44,7.07)	3	1,4,10a,	8.99,*dd* (1.5,7.3)	137.4 CH
3	130.3, = CH	8.05, *m*	COOH		8.05, *m*	130.3 CH
4	135.2, = CH	8.55,*dd* (1.46,8.5)	2,4	1,2,4a	8.54,*dd* (1.5,8.8)	135.1 CH
4a	143.4, = C	-	3	2,10a	-	143.4 C
5a	144.2, = C	-	-	3	-	144.1 C
6	128.0, = CH	8.30,*dd* (1.9,8.3)	-	6,7	8.29,*dd* (1.8,7.7)	128.0 CH
7	133.3, = CH	7.99, *m*	7	5a,8	7.99, *m*	133.2 CH
8	131.8, = CH	8.03, *m*	6	5a	8.03, *m*	131.7 CH
9	130.1, = CH	8.35,*dd* (1.9,8.3)	9	9a	8.35,*dd* (2.6,7.3)	130.1 CH
9a	139.9, = C	-	8	7,9a	-	139.8 C
10a	140.1, = C	-	-	8,9	-	140.1 C
COOH	166.0, C=O	-	-	2,4	-	165.9 C

* Sayed *et al*., 2008

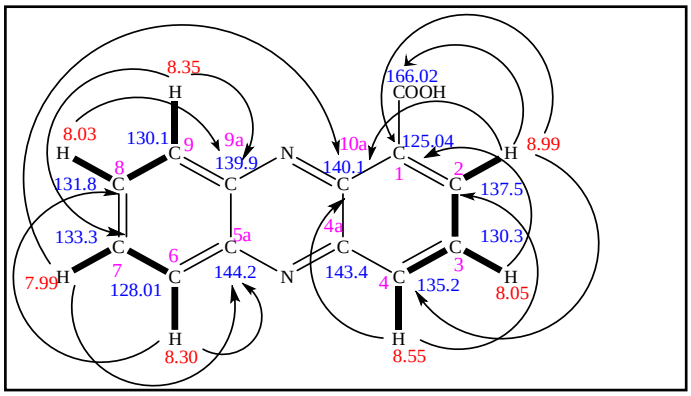


Figure 4 Structure of phenazine-1-carboxylic acid (2) [COSY (▬) and HMBC (H→C) correlation]

Compound 3: It was obtained as a colourless powder (0.34 % yield based on the chloroform extract) with melting point of 161~164 °C and has R_f value of 0.45 (PE:EA, 7:3 v/v). It is soluble in chloroform, ethyl acetate, methanol, ethanol and acetone, and insoluble in non-polar solvent pet ether. 1D and 2D NMR spectral data (Table 5) indicated the presence of 18 protons and eleven carbons related to two sp^3 methyl groups (-CH_3), four sp^3 methylene groups (-CH_2-), three sp^3 methine groups (-CH-), one NH group and two carbonyl groups (C=O). From the NMR and ESI-MS spectroscopic analyses, compound **3** was assigned as a nitrogeneous compound having the molecular formula $C_{11}H_{18}N_2O_2$ with *m/z* 210. These NMR spectral data of compound **3** was observed to be consistent with the reported data of cyclo-(L-Leu-L-Pro) (Youn *et. al.*, 2016) (Table 5) and its melting point was identical with the reported data (mpt. 163 – 165 °C) (ChemSpider). The COSY and HMBC correlationships (Figure **5**) and all of the above observations confirmed the compound **3** as cyclo-(L-Leu-L-Pro), a cyclodipeptide. The ROESY spectral data confirmed the stereochemical configuration of compound **3**, i.e., the beta hydrogens (δ_H 4.11 ppm and 4.01 ppm) on C-6 and C-9 (Table 5).

Table 5 1D and 2D NMR Spectral Data of Cyclo-(L-Leu-L-Pro) and the Reported Data

Carbon position	Cyclo-(L-Leu-L-Pro) (CDCl₃)					*Reported data (CDCl₃)	
	δ_C(ppm) HSQC	δ_H(ppm)	COSY	HMBC	ROESY	δ_C(ppm)	δ_H(ppm)
1	166.21, C=O	-	-	-		168.9(CO)	-
3	45.45, $CH_2(sp^3)$	3.56 (*m*)	4	4,5,6		45.4(CH_2)	3.56(*m*)
4	22.70, $CH_2(sp^3)$	2.02 (*m*)	3,5	4,7		23.6(CH_2)	2.01(*m*)
5	28.04, $CH_2(sp^3)$	2.34 (*m*)	6	4,3,6	6(CHβ)	29.1(CH_2)	2.30, 2.31(*m*)
6	58.93, CH(sp^3)	4.11 (t,8.2)	5	7,5		60.3(CH)	4.27(*dt*,9.3,1.5)
7	170.3, C=O	-	-	-		172.8(CO)	-
8	-	6.36(1H,s, NH)	-	-		-	6.36(1H,*s*,NH)
9	53.35, CH(sp^3)	4.01 (*m*)	10	1,10,11		54.6(CH)	4.13(*m*)
10	38.50, $CH_2(sp^3)$	1.52 (*m*)	11	1,12	9(CHβ)	39.4(CH_2)	1.53(*m*)
11	24.58, CH(sp^3)	1.77 (*m*)	10,1, 12'	10,12		25.8(CH)	1.88(*m*)
12	23.23, $CH_3(sp^3)$	0.99 (*d*,6.59)	11	10,11		23.3(CH_3)	0.99(*d*,6.59)
12'	21.18, $CH_3(sp^3)$	0.94 (*d*,6.52)	11	10,11		22.2(CH_3)	0.95(*d*,6.52)

* Youn *et. al.*, 2016

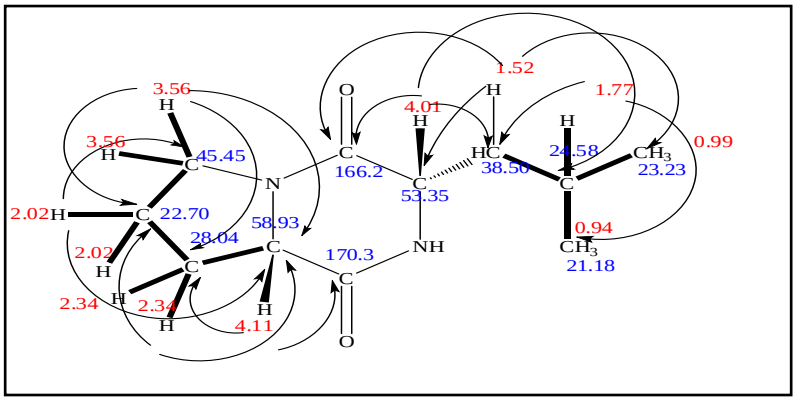


Figure 5 Structure of cyclo-(L-Leu-L-Pro)(3) [COSY (▬) and HMBC (H→C) correlation]

Compound 4: It was obtained as a colourless powder and has R_f value of 0.36 (PE:EA, 7:3 v/v). It is soluble in only in ethyl acetate, methanol, ethanol and acetone, and insoluble in non-polar pet ether solvent. It is also a cyclodipeptide, i.e., a diketopiperazine and structurally elucidated as cyclo-(D-Pro-D-Leu) with the molecular formula $C_{11}H_{18}N_2O_2$ (m/z = 210) by ESI-MS. All of the 1D and 2D NMR spectral data of this compound were found to be similar to the reported data (Youn *et. al.*, 2016) (Table 6). Figure 6 shows the ^{1}H-^{1}H COSY and HMBC correlations of this compound. ROESY spectral analysis on stereochemical configuration confirmed that both hydrogens at δ_H 2.36 and 1.53 ppm respectively attached to C-5 and C-10 were observed to be in alpha positions. Compounds **3** and **4** were found to be the stereoisomers of cyclodipeptide of proline and leucine amino acids.

Table 6 1D and 2D NMR Spectral Data of Cyclo-(D-Pro-D-Leu) and the Reported Data

Carbon position	Cyclo-(D-Pro-D-Leu) ($CDCl_3$)					*Reported data ($CDCl_3$)	
	δ_C(ppm) HSQC	δ_H(ppm)	COSY	HMBC	ROESY	δ_C(ppm)	δ_H(ppm)
1	166.2, C=O	-	-	-		168.9(CO)	-
3	45.49, $CH_2(sp^3)$	3.58 (*m*)	4	4,5,6		45.4(CH_2)	3.56(*m*)
4	22.72, $CH_2(sp^3)$	2.08 (*m*)	3,5	1,4,6,7		23.6(CH_2)	2.01(*m*)
5	28.10,$CH_2(sp^3)$	2.36 (*m*)	6	4,3,6	6(CH,α)	29.1(CH_2)	2.30,2.31(*m*)
6	58.97, $CH(sp^3)$	4.12 (*t*, 8.2)	5	7,5		60.3 (CH)	4.27(*dt*,9.3,1.5)
7	170.3, C=O	-	-	-		172.8(CO)	-
8	-	5.92 (1H, *s*, NH)	-	-		-	6.36 (1H,*s*,NH)
9	53.36, $CH(sp^3)$	4.03(*dd*, 3.29, 3.87)	10	1,10		54.6 (CH)	4.13(*m*)
10	38.61, $CH_2(sp^3)$	1.53 (*m*)	11	1,12	9(CH,α)	39.4(CH_2)	1.53(*m*)
11	24.70, $CH(sp^3)$	1.76 (*m*)	10,12	10,12		25.8 (CH)	1.88(*m*)
12	23.27, $CH_3(sp^3)$	1.01 (*d*,6.59)	11	10,11		23.3(CH_3)	0.99 (*d*,6.59)
12'	21.17, $CH_3(sp^3)$	0.96 (*d*,6.52)	11	10,11		22.2(CH_3)	0.95 (*d*,6.52)

* Youn *et al*., 2016

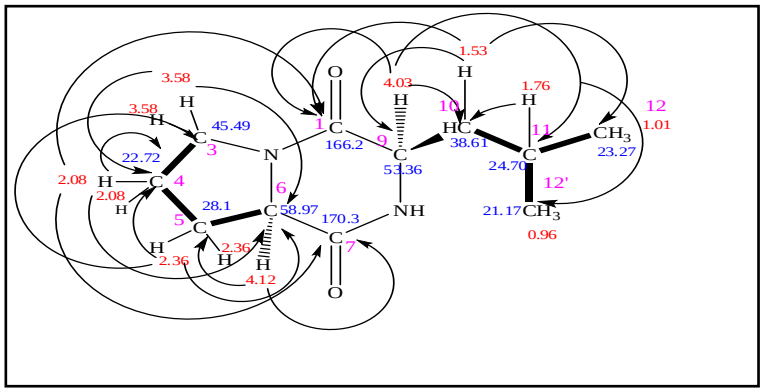


Figure 6 Structure of cyclo-(D-Pro-D-Leu) (4) [COSY (▬) and HMBC (H→C) correlation]

Compound 5: It was obtained as a yellow oil and has R_f value of 0.6 (Hexane:EtOAc:MeOH, 1.8:8:0.2, v/v/v). It is soluble in only in ethyl acetate, methanol, ethanol and acetone, and insoluble in chloroform and pet ether. It is also a cyclodipeptide, i.e., a diketopiperazine and structurally elucidated as cyclo-(D-Pro-L-Val) with the molecular formula $C_{10}H_{16}N_2O_2$ (m/z = 196) by ESI-MS. All of the 1D and 2D NMR spectral data of this compound were found to be similar to the reported data (Kwon *et. al*., 2001) (Table 7). Figure 7 shows the ^{1}H-^{1}H COSY and HMBC correlations of this compound. ROESY spectral analysis on stereochemical configuration confirmed that both hydrogens at δ_H 3.94 and 4.08 ppm respectively attached to C-3 and C-6 were observed to be in alpha positions.

Antimicrobial Activity of the Isolated Compounds (1 to 5)

In the screening of antimicrobial activity on the five isolated compounds (**1** to **5**) against seven different pathogenic microbes such as *A. tumefacines, B. subtilis, B. pumilus, C. albicans, E. coli, P. aeruginosa* and *S. aureus* using agar well diffusion method, compound **1** (1-hydroxyphenazine) was observed to exhibit high activity against all of the tested microorganisms with the inhibition zone diameters ranged above 20 mm. The compound **2** (phenazine-1-carboxylic acid) was observed medium activity against the tested microorganisms. The remaining three isolated diketoperazines: cyclo(L-Leu-L-Pro) (**3)**, cyclo(D-Pro-D-Leu) (**4)** and cyclo(D-Pro-L- Val) (**5**) showed mild antimicrobial activity with the inhibition zone diameters between 12 mm ~ 14 mm but inactive against all of the tested microorganisms. The results are shown in Table 8.

Table 7 1D and 2D NMR Spectral Data of Cyclo-(D-Pro-L-Val) and the Reported Data

Carbon position	Cyclo-(D-Pro-L-Val) ($CDCl_3$)					*Reported data ($CDCl_3$)	
	δ_C(ppm) HSQC	δ_H(ppm)	COSY	HMBC	ROESY	δ_C(ppm)	δ_H(ppm)
1	170.03, C=O	-	-	8		169.9(CO)	-
3	60.36, -CH(sp^3)	3.94 (*m*)	-	4,10,1, 12	3 (CH,α)	60.3(CH)	3.94(1H,m)
4	164.89, C=O	-	-	3		164.8(CO)	-
6	58.79, -CH(sp^3)	4.08(*m*)	7,8	7,1	6 (CH,α)	58.7(CH)	4.09 (1H,*m*)
7	28.50,-CH_2(sp^3)	2.08(*m*),Ha	6,8	6,8,9		28.3(CH_2)	2.05 (1Ha,*m*)
		2.3(*m*),Hb					2.39 (1Hb,*m*)
8	22.33,-CH_2(sp^3)	1.91(*m*),Ha	7,9	7,9		22.2(CH_2)	1.90 (1Ha,*m*)
		2.02(*m*),Hb		1,6,7,8		22.2(CH_2)	2.05 (1Hb,*m*)
9	45.12,-CH_2(sp^3)	3.55(*m*),Ha	8	7,8		44.9(CH_2)	3.55, (1Ha,*m*)
		3.65(*m*),Hb					3.65, (1Hb,*m*)
10	28.33, CH(sp^3)	2.64 (*m*)	11	3,11,12		28.4(CH)	2.64(2H,*m*)
11	16.02,-CH_3(sp^3)	0.92 (*d*,6.9)	10,12	10,12		15.9(CH_3)	0.92 (3H,*d*,6.9)
12	19.21, CH_3(sp^3)	1.07 (*d*,7.5)	11	10,11		19.2(CH_3)	1.07 (3H,*d*,7.5)

* Kwon *et. al*. 2001

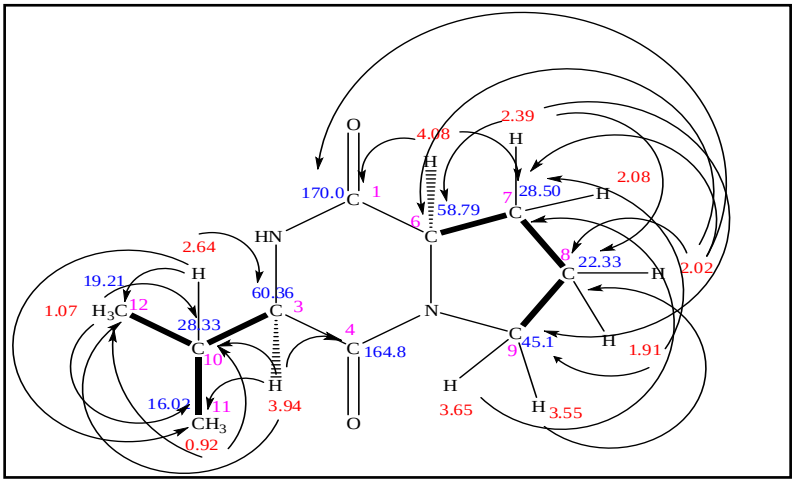


Figure 7 Structure of cyclo-(D-Pro-L-Val) (5) [COSY (▬) HMBC (H→C) correlation]

Antiproliferative Activity of the Isolated Compounds (1 to 5) against Human Breast Cancer MCF7 Cells

The antiproliferative activities of the isolated compounds were investigated with MCF7 (human breast cancer cell line). Antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-Fluorouracil was used as positive control. The results are summarized in Table 9. It was observed that the two isolated phenazines: 1-hydroxyphenazine (**1**, IC_{50}=14.1 µg/mL) and phenazine-1-carboxylic acid (**2**, IC_{50}=15.0 µg/mL) exhibited high antiproliferative activity against MCF 7 cell line whereas the remaining three isolated diketoperirazines: cyclo(L-Leu-L-Pro) (**3**, IC_{50} = 176.4 µg/mL), cyclo(D-Pro-D-Leu) (**4**, IC_{50} = 87.7 µg/mL) and cyclo(D-Pro-L-Val) (**5**, IC_{50} = 196.5 µg /mL) showed mild antiproliferative activity. The compounds **1** and **2** were found to be comparable with the standard 5-fluorouracil (IC_{50} = 11.50 µg/mL) in antiproliferative activity against MCF7 human breast cancer cell line.

Table 8 Inhibition Zone Diameters of the Isolated Compounds of *P. aeruginosa* against Seven Microorganisms by Agar Well Diffusion Method

Microorganisms	Inhibition zone diameters (mm) of the isolated compounds					
	1	**2**	**3**	**4**	**5**	**Control**
A. tumefacines	28(+++)	26(+++)	12(+)	-	-	-
B. pumilus	30(+++)	27(+++)	-	14(+)	14(+)	-
B. subtilis	32(+++)	26(+++)	12(+)	-	14(+)	-
E. coli	32(+++)	20(+++)	12(+)	12(+)	-	-
C. albicans	28(+++)	31(+++)	14(+)	-	-	-
P. aeruginosa	21(+++)	18(++)	14(+)	12(+)	12(+)	-
S. aureus	23(+++)	15(++)	14(+)	12(+)	-	-

Agar well –10 mm, 10 mm ~ 14 mm = (+) low activity, 15 mm ~ 19 mm = (++) medium activity, 20 mm and above = (+++) high activity, 1 = 1-hydroxyphenazine, **2** = phenazine-1-carboxylicacid, **3** = cyclo(L-Leu-L-Pro), **4** = cyclo(D-Pro-D-Leu), **5** = cyclo(D-Pro-L- Val)

Table 9 Antiproliferative Activity of the Isolated Compounds and Standard 5 Fluorouracil against Human Breast Cancer Cell MCF7

Isolated compounds	% Cell survival in different concentrations of each sample				IC_{50} (µg/mL)
	10	**20**	**100**	**200**	
1	51.94 ± 2.40	47.22 ± 1.77	10.29 ± 0.07	9.13 ± 0.14	14.1
2	74.86 ± 0.64	45.55 ± 1.20	13.49 ± 0.07	12.81 ± 0.35	15.0
3	87.97 ± 0.49	77.66 ± 7.92	66.50 ± 0.71	44.93 ± 0.57	176.4
4	78.97 ± 1.48	59.90 ± 3.11	48.21 ± 0.99	44.64 ± 0.64	87.7
5	102.90 ± 3.39	94.28 ± 6.28	67.20 ± 9.97	49.38 ± 1.27	196.5
***5-Fluorouracil	61.94 ± 0.8	37.22 ± 0.7	10.29 ± 1.0	8.42 ± 0.2	11.5

1 = 1-hydroxyphenazine , **2** = phenazine-1-carboxylic acid, **3** = cyclo(L-Leu-L-Pro)

4 = cyclo(D-Pro-D-Leu), **5** = cyclo(D-Pro-L- Val)) * standard

Conclusion

The present study reveals that *P. aeruginosa* bacterial strain isolated from the clinical soil sample of Insein General Hospital, Yangon Region was successfully confirmed by the DNA sequencing technique using PCR amplification of 16S rRNA. In addition, two phenazine derivatives such as 1-hydroxyphenazine (0.24 % based on chloroform extract, m.pt 152-157 °C) and phenazine-1-carboxylic acid (0.7 %, m.pt 240-246 °C) together with three cyclopeptides, diketopiperazines such as cyclo(L-Leu-L-Pro (0.34 %, m.pt 164- 164 °C), cyclo(D-Pro-D-Leu) (0.09 %) and cyclo(D-Pro-L-Val) (0.18 %) could be isolated from the chloroform extract of the isolated *P. aeruginosa* strain. These isolated compounds were structurally elucidated by 1D and 2D NMR spectroscopic techniques and ESI-MS spectrometry, and also by comparing with their respective reported data. Moreover, it can be also evaluated that the two isolated phenazines exhibit high antimicrobial activity against all tested microorganisms with the inhibition zone diameter ranged between 15 ~ 40 mm. The remaining three isolated diketopiperazines showed mild antimicrobial activity with the inhibition zone diameters ranged between 12 mm ~ 14 mm. Furthermore, 1-hydroxyphenazine (IC_{50} = 14.12 µg/mL) and phenazine-1-carboxylic acid (IC_{50} = 15.0 µg/mL) were also found to possess good antiproliferative activity against human breast cancer MCF7 whereas cyclo(L-Leu-L-Pro) (IC_{50} = 176.4 µg/mL), cyclo(D-Pro- D-Leu) (IC_{50} = 87.7 µg/mL) and cyclo(D-Pro-L-Val) (IC_{50} = 196.5 µg /mL) have mild activity. From the present work, it can be inferred that the isolated compounds especially 1-hydroxyphenazine and phenazine-1-carboxylic acid may be useful in the formulation of antibacterial and antifungal agents for the treatment of the diseases infected by the tested microorganisms and may be useful in the formulation of anticancer agents for the treatment of human breast cancer cell. Hence, the finding of this research work will contribute to some extend in the development of antimicrobial agents and anticancer agents from the source of soil bacteria *P. aeruginosa.*

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References

Amutha, K., and V. Kokila. (2014). "PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Pseudomonas aeruginosa*". *Journal of Science and Research*, vol. 3 (8), pp. 2-6

Baron, S. S., and J. J. Rowe. (1981). "Antibiotic Action of Pyocyanin". *Journal of Antimicrobial Agents and Chemotherapy*, vol. 20 (6), pp. 814–820

ChemSpider, ChemSpider.com/Chemical-Structure 5428292. html

David, W., M. John, W. Robert, C. Peter, and W.T. Graham. (1986) "Purification and Structural Analysis of Pyocyanin and 1- Hydroxyphenazine". *Journal of Biochemistry*. vol. 159, pp. 309–313

Kwon, O. S., P. Sang Ho, Y. Bong-Sik, P. Yu-Ryang, and K. Chang-Jin. (2001). "Cyclo (D-Pro-L-Val), a Specific /3-Glucosidase Inhibitor Produced by *Aspergillus* sp. F70609". *Journal of Antibiotics*, vol. 54, pp. 179-181

Muhanna, M. A. N. M., J. Juriyati, S. M. Nik, J. A. Siti, K. Nurkhalida, and E. E. Ru Angelie. (2017). "Isolation, Purification and Characterization of Five Active Diketopiperazine Derivatives from Endophytic Streptomyces SUK 25 with Antimicrobial and Cytotoxic Activities". *Journal of Microbiol Biotechnol*, vol. 27 (7), pp. 1249-1256

Perez, C., M. Paul, and P. Bezique. (1990). *An Antibiotic Assay by Agar Well Diffusion Method*. Alta Biomed., Group Experiences, vol. 15**,** pp. 133-155

Sayed, W. El., M. Abd El-Megeed, A. B. Abd El-Razik, K.H. Soliman, and S. A. Ibrahim. (2008). "Isolation and Identification of Phenazine-1-Carboxylic acid from different *Pesudomonas* Isolates and its Biological Activity Against *Alternaria Solani*". *Journal of Agriculture and Biological Sciences*, vol. 4 (6), pp. 892-901

Sinha, S., X. Shen, F. Gallazzi, Q. Li, J. W. Zmijewski, J. R. Lancaster, and K. S. Gates. (2015). "Generation of Reactive Oxygen Species Mediated by 1-Hydroxyphenazine, a Virulence Factor of *Pseudomonas Aeruginosa*". *Journal of Chemistry Research Toxicol*ogy, vol.28(2), pp. 175-181

Su Swe Su, Khine Zar Wynn Lae, and Daw Hla Ngwe. (2018). "Isolation and Identification of *Pseudomonas Aeruginosa* from the Clinical Soil". *Journal of Research University of Yangon*, vol. 8, pp. 271-275

Su Swe Su, Khine Zar Wynn Lae, Nwet Nwet Win, and Daw Hla Ngwe. (2019). "Investigation of Chemical Composition and Some Biological Properties of Chloroform Extract of *Pseudomonas Aeruginosa*". *Jour. Myan. Acad. Arts & Sc*, vol. 18 (1A), pp. 545-560

Syn Quest Labs. http://synquestlabs.com/product/id/68826.html

Tokyo Chemical Industry. http//: www. tcichemicals.com/US/en/p/H0289#docoments Section PDP

Win, N. N., T. Ito, S. Aimaiti, H. Imagawa, H. Ngwe, I. Abe, and H. Morita. (2015). "Kaempulchraols A–H, Diterpenoids from the Rhizomes of *Kaempferia pulchra* Collected in Myanmar". *J. Nat. Prod.*, vol. 78, pp. 1113–1118

Youn, U. J., M. J. Kim, S. J. Han and J. H. Yim. (2016). "Isolation of Secondary Metabolites from an Arctic Bacterium, *Pseudomonas Aeruginosa* and their Antimicrobial activities". *Journal of Microbiology*, vol. 52(4), pp. 415-420