Structural Identification and Evaluation of Some Biological Activities of Cyclo (D-Pro-L-Val) from the *Pseudomonas aeruginosa* Schroeter.

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Abstract

This research deals with the studies of identification and evaluation of some biological activities of bioactive secondary metabolite compound of *Pseudomonas aeruginosa*isolated from the clinical soil sample. The soil sample was collected from Insein General Hospital, Yangon Region. The isolated *P. aeruginosa* strain was characterized by microscopic examination, biochemical tests and confirmed by 16S rRNA sequencing technique. The chloroform extract was prepared from the *P. aeruginosa*cultured in a large scale of nutrient broth medium.Diketopiperazines, such as, cyclo-(D-Pro-L-Val) was isolatedfrom the silica gel chromatographic separation on the chloroform extract. The isolated secondary metabolite was structurally identified by using modern NMR spectroscopic techniques such as ^{1}H NMR**(***Proton Nuclear Magnetic Resonance)*, ^{13}C NMR (Carbon-13 Nuclear Magnetic Resonance spectroscopy), COSY (^{1}H-^{1}H Correlation Spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), ROESY (Rotating frame Overhause Effect Spectroscopy), HMBC spectroscopies (Heteronuclear Multiple Bond Correlation), and MS (mass spectrometry). The isolated compound, cyclo(D-Pro-L-Val) (IC_{50} = 0.93 µg /mL) was found to exhibit good *in vitro* cytotoxic activity against human lung cancercell A-549. In addition, cyclo(D-Pro-L-Val) (IC_{50} = 64.12 µM) has mild inhibited in anti-inflammatoryactivity.

Keywords: cyclo(D-Pro-L-Val), modern NMR spectroscopic techniques,*in vitro* cytotoxicity activity and anti-inflammatoryactivity

Introduction

Many bacteria isolated from soils are the most important assets of specialized metabolites. Secondary (or “specialized”) metabolite compounds are produced through microorganisms and some of these compounds, consisting of antibiotics, also are beneficial in medication and biotechnology. *Pseudomonas aeruginosa*species encompass gram-negative, rod-shaped, aerobic (facultative anaerobic) and polarly flagellated microorganism and colonize a huge range of niches. Some *Pseudomonas* species may be pathogenic to humans, animals and plant life. *Pseudomonasaeruginosa* is a large group of free-living bacteria that live primarily in soil and water. Secondary metabolitecompounds isolated from *Pseudomonas* have been detected and investigated by their antibiotic activity. The soil isolated bacteria *Pseudomonasaeruginosa*presence

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of bioactive compounds like phenazine (pyocyanin, 1-hydroxy-phenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid) and diketopiperazine compounds (Sharrar*et al.*, 2018). In this research, isolation and identification of bioactive secondary compound was isolated from chloroform extract of *P.aeruginosa* and it was applied to investigate some biological activities such as *in vitro* cytotoxicity activity and anti-inflammatory activity.

Materials and Methods

Collection of Soil Sample

In this research, soil sample was collected from Insein General Hospital, Yangon Region.

Isolation and Identification of the Bacterium

The bacterial strain was isolated from the clinical soil sample. After three days, soil bacteria were isolated by the serial dilution method on nutrient agar medium. Among several selected colonies, a unique bacterial colony with a faint creamy color, and a round thin surface was isolated. The isolated bacteria, a rod shaped and gram negative bacteria, were characterized by microscopic examination and by conventional biochemical tests and the bacterium was identified as *Pseudomonas aeruginosa*strain S 04 by phylogenetic analysis of the 16S rRNA sequence.

Preparation of Chloroform Extract of *P. aeruginosa*

The isolated bacteria *P.aeruginosa*was cultured on nutrient broth medium in the large scale subsequently, the culture was centrifuged (20 min, 4 ºC, 3500 rpm).The cell-free supernatant was extracted with chloroform for 10 times to get the chloroform soluble compounds by liquid-liquid partition between chloroform and the culture solution 1:1 (v:v).This process was done according to the ultrasound-assisted extraction to give 0.03 % (w/v) of chloroform extract. The crude extract was applied to investigate the chemical constituents and some biological activities. (Su Swe Su *et al.,* 2020).This experiment was carried out at Department of Chemistry, University of Yangon.In this research, the bioactive compound was isolated from chloroform extract of *P.aeruginosa* and it was evaluated some biological activities.

Isolation and Identification of Bioactive Compounds from the Chloroform Extract

The chloroform extract (5 g) prepared from the selected bacterial strain was separated by using silica gel column chromatographic method and different polarities of solvent systems of pet-ether, PE:EA in 9:1, 4:1, 7:3, 3:2 and 1:1 v/v. On chromatographic separation, six main fractions (F-I to F-VI) were collected after examining on precoated TLC plates. The isolated compound was obtained as a colourless solidin 3 mg (0.06 % yield) from the sub-fraction FVI-b of F-VI. The yield percentages were calculated based on the chloroform extract.

Identification of the Isolated Compound

The isolated secondary metabolite was structurally identified by using modern NMR spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, ROESY and Mass spectroscopies, and also compared with the reported data. The NMR and Mass spectra of the isolated compound were measured at Department of Chemistry and Center of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Mahidol University, Bangkok, Thailand.

Investigation of Some Biological Activities of the Isolated Compound

Determination of *In Vitro* Cytotoxic Assay of the Isolated Compound

The cytotoxic activities of the isolated compound was determined by using the standard *in vitro*sulforhodamine B assay with 96-well microliter plates. The cytotoxic activities were measured at Department of Chemistry and Center of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Mahidol University, Bangkok, Thailand. In cytotoxic activities, four cell lines were employed, including FaDu; human squamous cell carcinoma (skin cancer), HT-29; human colorectal adenocarcinoma (colon cancer), A-549; human lung carcinoma (lung cancer), KKU-M213; human intrahepatic cholangiocarcinoma (liver cancer) (Jaipetch*et al*., 2019). Ellipticine was used as a positive control. *Invitro* cytotoxic activity of the isolated compound was determined against cancer cell line by using CCK-8 Assay (Cell Counting Kit-8).

Determination of Anti-inflammatory Activities of the Isolated Compound AgainstRAW264.7 CellsLinesby MTT Assay

Anti-inflammatory activity of chloroform extract from the selected bacteria and isolated compound were investigated in *in vitro* by using RAW 264.7 cells lines at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. Anti-inflammatory activity of the samples was evaluated by NO inhibition assay according to the method of Jin *et al*. (Jin *et al*., 2012) with some modifications.

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 biochemical tests and DNA sequencing technique (Su Swe Su *et al*., 2018**).** In this research,the isolated compound was separated by column chromatographic separation from chloroform extract of the isolated *P. aeruginosa*and it was characterized by their physical properties such as melting points, R_f values, solubilities in some solvents. The isolated compound was structurally identified by using 1D and 2D NMR spectroscopic techniques and mass spectrometry.

Identification of the Isolated Compound

The isolated compoundwas observed as a colourless solid (mpt. 210-216 °C)in 0.06 % (3 mg) of yield by silica gel column chromatographic separation of chloroform extract of *P.aeruginosa*. Its $R_f$value was observed to be 0.6 (PE:EA:MeOH, 1.8:8:0.2 v/v). It is soluble in chloroform, pet-ether, ethyl acetate, methanol and ethanol but insoluble in water. The integration of ^{1}H NMR (500 MHz, $CDCl_3$) spectrum (Figure 1) indicated the presence of sixteen protons in the isolated compound. The two doublet signals appeared at δ_H 1.07 ppm (J = 7.5 Hz) and δ_H 0.92 ppm (J = 6.9 Hz) were assigned to be two methyl groups adjacent to-CH-. The signals appeared at δ_H 2.08 ppm (1H, m), 2.39 ppm (1H, m), 1.91 ppm (1H, m), 2.02 ppm (1H, m), 3.55 ppm (1H, m), 3.65 ppm (1H, m), δ 2.64 ppm (1H, m), δ_H 4.08 ppm (1H, m) and δ_H 3.94 ppm (1H, m) were attributed to nine protons. A broad signal appeared at δ_H 6.04 ppm was appeared due to a NH- proton. The ^{13}C NMR (125 MHz, $CDCl_3$) spectrum (Figure 2) of the isolated compound revealed the presence of ten carbon signals including two methyl carbons at the chemical shifts of δ_C 16.02 and 19.21 ppm, four sp^3 methylene carbons at the chemical shifts of δ_C 22.33 ppm, 28.33 ppm, 28.50 ppm and 45.12 ppm, two sp^3 methine carbons at δ_C 58.79 ppm and 60.36 ppm and two carbonyl carbons at δ_C 164.89 ppm and 170.03 ppm.From the study on one bond H-C correlation by HSQC spectrum (Figure 3) the results showed that there were three sets of diastereotopic protons (-CH_2-) such as the protons of δ_H 1.91 ppm and 2.02 ppm attached to the carbon δ_C 22.33 ppm, that of δ_H 2.08 ppm and 2.3 ppm attached to the carbon δ_C 28.50 ppm, and the protons of δ_H 3.55 ppm and 3.65 ppm attached to the carbon δ_C 45.12 ppm. Furthermore, ^{1}H-^{1}H correlations and the long range proton-carbon correlations were also examined by using ^{1}H-^{1}H COSY (Figure 4) and HMBC (Figure 5) spectra. According to the ROESY NMR spectrum of isolated compound (Figure 7), it was not observed through space interactions between the methine proton at δ_H 3.94 ppm (δ_C 60.36 ppm) as well as the methine proton at δ_H 4.08 ppm (δ_C 58.79 ppm) with any other protons. Therefore these two methine protons could be assumed not to be in the same space with other protons, and these two protons could be assigned as the alpha hydrogens. The 1D and 2D NMR spectral data of isolated compoundwere observed to be consistent with the reported data of cyclo(D-Pro-L-Val) (Kwon *et. al.*, 2001) and the isolated compound was structurally elucidated as shown in Figure 8. The molecular weight of the isolated compoundwas found to be *m/z* 196 determined by ESI-MS spectrometry (Figure 6). According to NMR spectroscopic techniques, 10 carbons and 16 protons together with two carbonyl carbons, resulting the partial molecular formula of $C_{10}H_{16}O_2$, *m/z* 168. The remaining mass *m/z* 28 may be due to two nitrogen atoms. Therefore, the complete structural formula of this compound must be assigned as $C_{10}H_{16}N_2O_2$ with the molecular weight 196. The DBE (Double Bond Equivalence) equals to 4 which confirmed the presence of two rings and two double bonds in the isolated compound. Therefore, isolated compound wasfinally assigned as cyclo(D-Pro-L-Val) ($C_{10}H_{16}N_2O_2$) (Figure 9).

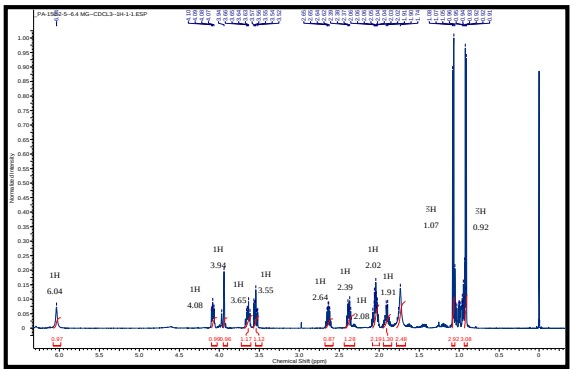

Figure 1. Expanded ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the isolated compound (δ_H 0.0 ~ 6.5 ppm)

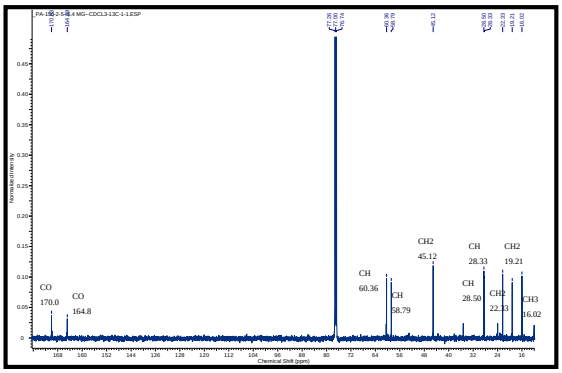

Figure 2. Expanded ^{13}C NMR spectrum (125 MHz,$CDCl_3$) of the isolated compound (δ_C 8.0 ~ 184 ppm)

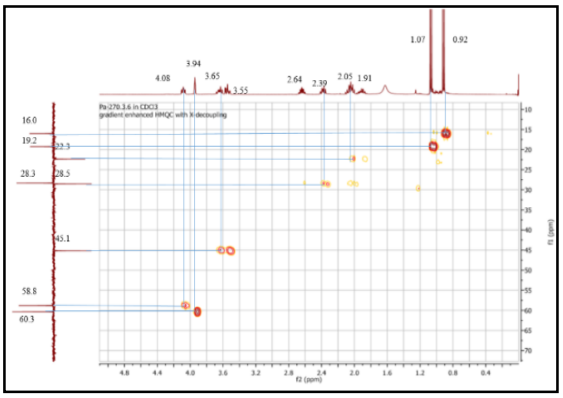

Figure 3. Expanded HSQC spectrum (500 MHz, $CDCl_3$) of the isolated compound (δ_H 0.5 ~ 4.5 ppm)

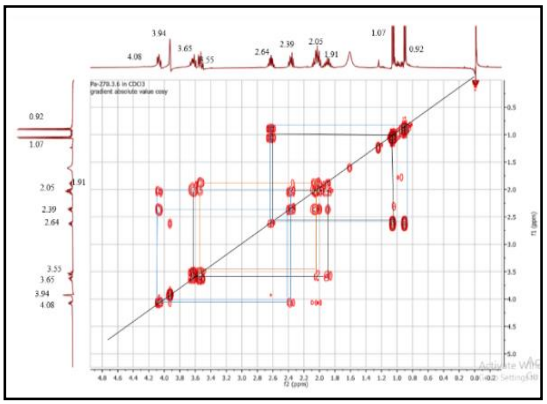

Figure 4. Expanded ^{1}H-^{1}H COSY spectrum of the isolated compound (^{1}H-^{1}H correlation at δ_H 0.5 ~ 4.5 ppm)

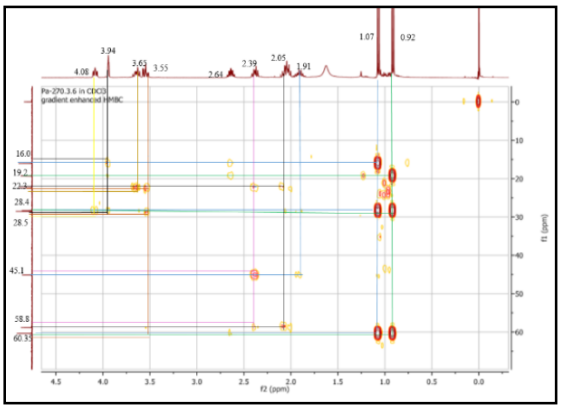

Figure 5. Expanded HMBC spectrum of the isolated compound (^{1}H-^{13}C correlation at δ_H 0.0 ~ 4.5 ppm)

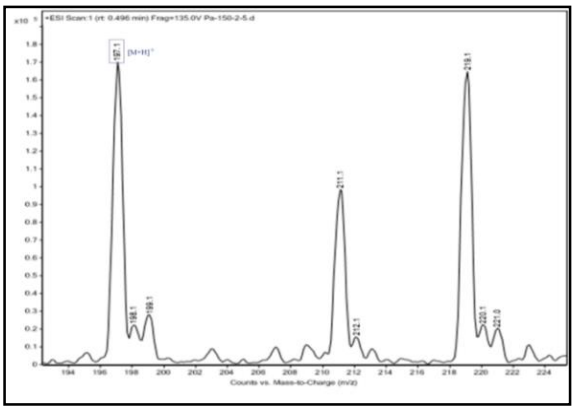

Figure 6. ESI-Mass spectrum of the isolated compound

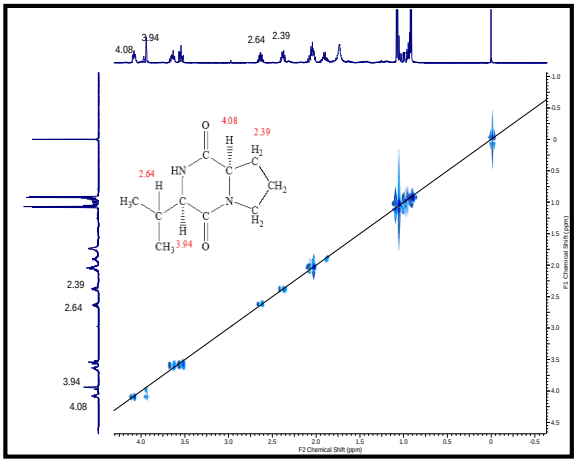

Figure 7. Expanded ROESYspectrum of the isolated compound (δ_H 0.0 ~ 4.5 ppm)

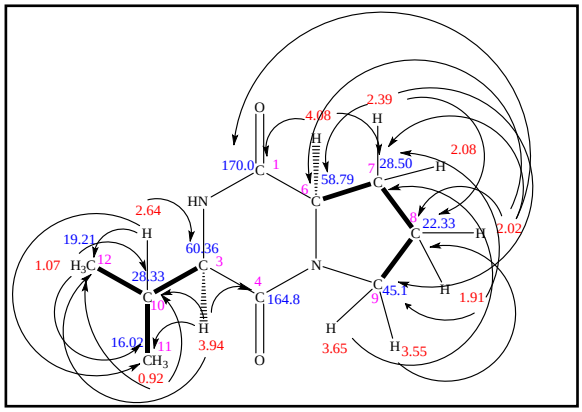

Figure 8. Structure of cyclo(D-Pro-L-Val) COSY (▬) andHMBC (H→C) correlation

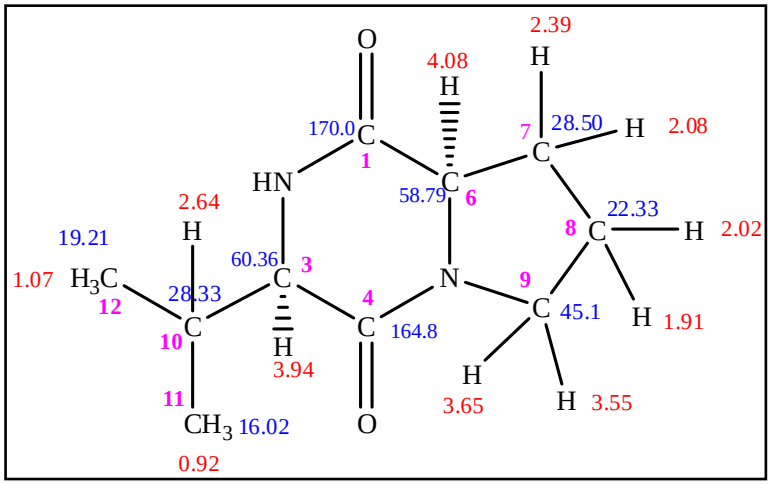

Figure 9. Chemical structure of Cyclo (D-Pro-L-Val) ($C_{10}H_{16}N_2O_2$)

Biological Activities of the Isolated Compound of *P. aeruginosa*

***In vitro* cytotoxicity activity of isolated compound against human cancer cell lines**

Cytotoxicity activity of the sample was studied in *in vitro* against human cancer cell lines. Screening of cytotoxicity activities of isolated compound was done against four human cancer cell lines such as FaDu; human squamous cell carcinoma (skin cancer), HT-29; human colorectal adenocarcinoma (colon cancer), A-549; human lung carcinoma (lung cancer), KKU-M213; human intrahepatic cholangiocarcinoma (liver cancer). Cytotoxicity activity was expressed as the IC_{50} (50 % inhibitory concentration) value. Ellipticine was used as a positive control. The cytotoxic activity of isolated compound is summarized in Table 2. It was observed that the isolated compound has the cytotoxicity activity against human lung cell A549 (IC_{50} = 0.93 µg/mL) comparable to the standard Ellipticine (IC_{50} = 0.36 µg/mL). Then, the isolated compound has the cytotoxicity activity against human skin cancer cell FaDU (IC_{50} = 1.21 µg/mL) and human colon cancer cell HT-29(IC_{50} =

1.87 µg/mL) and human liver cancer cell KKU-M213 (IC_{50} = 13.60 µg/mL) (Table 2). However, since the lower the IC_{50} values, the higher the antiproliferative activity, the isolated compound was weaker than standard Ellipticine in cytotoxicity activity against FaDU (IC_{50} = 0.46 µg/mL) and HT-29(IC_{50} = 0.46 µg/mL) and KKU-M213 (IC_{50} = 0.43 µg/mL) (Table 3).

Table 2. Cytotoxicity Activity of the Isolated Compound of *P.aeruginosa*against Human Cancer Cell Lines

Human Cancer cell lines	% Cell survival in different concentrations of isolated compound				IC_{50} (µg/mL)
	0.1 µg/mL	1 µg/mL	10 µg/mL	20 µg/mL	
A-549	55.90± 0.2	50.91 ±0.1	45.01 ± 0.2	33.10± 0.1	0.93
FaDU	63.12± 0.1	55.13 ± 0.2	47.35 ± 0.1	40.13±0.1	1.21
HT-29	67.12 ± 0.1	57.32 ± 0.1	41.35 ± 0.2	37.10 ± 0.2	1.87
KKU-M213	75.16 ± 0.2	63.22 ± 0.1	55.16 ± 0.1	45.03 ± 0.2	13.60

A-549 = Human lung cancer cell, FaDu = Human skin cancer cell,
HT-29 = Human colon cancer cell, KKU-M213 = Human liver cancer cell

Table 3. Cytotoxicity Activity of the Standard Ellipticine against Human Cancer Cell Lines

Human cancercell lines	% Cell survival in different concentrations of Ellipticine				IC_{50} (µg/mL)
	0.1 µg/mL	1 µg/mL	10 µg/mL	20 µg/mL	
A 549	54.35 ± 0.2	42.53 ±0.1	37.01 ± 0.1	35.20± 0.2	0.36
FaDU	57.23 ± 0.5	47.03 ± 0.2	35.08 ± 0.1	31.05± 0.1	0.46
HT 29	55.11 ± 0.1	46.06 ± 0.1	38.05 ± 0.2	35.15 ± 0.1	0.46
KKU-M213	53.23 ± 0.1	45.33 ± 0.1	35.02 ± 0.2	33.13 ± 0.1	0.43

Ellipticine = positive control

Anti-inflammatory Activities of the Isolated Compound

The anti-inflammatoryactivityof the isolated compound was investigated with Raw 264.7 cells. Antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. L-*N*MMA monoacetate was used as positive control. The anti-inflammatoryactivityof thecyclo(D-Pro-L-Val) was exerted the inhibition of cellular NO production (IC_{50} = 64.12 µM). The isolated compound has mild inhibited NO production than the positive control, L-NMMAmonoacetate (IC_{50} = 10.16 µM) in the cellular NO production assay. The results are summarized in Table 4.

Table 4. Anti-inflammatory Activities of the Isolated Compoundof *P.aeruginosa*against Raw 264.7 Cells

Human cancer cell lines	% Cell survival in different concentrations				IC_{50} (μM)
	5 μM	10 μM	50 μM	100 μM	
Cyclo (D-Pro-L-Val)	14.88± 0.06	30.56 ±0.01	44.58 ± 0.10	63.77± 0.05	64.12
*L-NMMA	33.02 ± 0.02	49.53± 0.01	65.01 ± 0.1	74.33 ± 0.01	10.16

*L-NMMA monoacetate = positive control

Conclusion

The present study reveals that *P. aeruginosa* bacterial strain isolated from the clinical soil sample of Insein General Hospital, Yangon Region was identified by biochemical test and the DNA sequencing technique using PCR amplification of 16S rRNA. In addition, diketopiperazines derivatives such as cyclo(D-Pro-L-Val) (0.06 % based on chloroform extract, m.pt 152-157 °C) could be isolated from the chloroform extract of the isolated *P. aeruginosa*strain. The isolated compound was structurally elucidated by 1D and 2D NMR spectroscopic techniques and ESI-MS spectrometry, and also by comparing with their respective reported data. According to the results of cytotoxicity activity, it was observed that the isolated compound has high activity against A549 human lung cancer cell line (IC_{50} = 0.93 μg/mL), comparable to the standard Ellipticine (IC_{50} = 0.36 μg/mL) followed byFaDU human skin cancer cell line (IC_{50} = 1.21 μg/mL), HT-29 human colon cancer cell line (IC_{50} = 1.87 μg/mL) and KKU-M213 human liver cancer cell line (IC_{50} = 13.60 μg/mL) have mild activity. However, the isolated compound was weaker than standard Ellipticine in cytotoxicity activity against FaDU (IC_{50} = 0.46 μg/mL), HT-29(IC_{50} = 0.46 μg/mL) and KKU-M213 (IC_{50} = 0.43 μg/mL). Furthermore, cyclo(D-Pro-L-Val) was also found to possess in the anti-inflammatory activities exerted mild inhibition of cellular NO production (IC50 = 64.12 μg/mL) and it has low activity than the positive control, L-NMMAmonoacetate (IC50 = 10.16 μM) in the cellular NO production assay.

From the present work, it can be inferred that the isolated compoundcyclo(D-Pro-L-Val) may be useful in the formulation of anticancer agent for the treatment of human lung cancer cell. Hence, the finding of this research work will contribute to some extent in the development of anticancer agent and anti-inflammatory agent from the source of soil bacteria *P. aeruginosa.*

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