INVESTIGATION OF CHEMICAL COMPOSITION AND SOME BIOLOGICAL PROPERTIES OF CHLOROFORM EXTRACT OF *PSEUDOMONAS AERUGINOSA*

Su Swe Su[1,2], Khine Zar Wynn Lae[3], Nwet Nwet Win[4], Daw Hla Ngwe[5]

Abstract

This research focuses on the chemical and pharmacological studies of bioactive secondary metabolites from *Pseudomonas aeruginosa* isolated by serial dilution plate method from the clinical soil sample collected in the Insein General Hospital, Yangon Region. The isolated bacteria *P.aeruginosa* identified by biochemical tests was cultured on nutrient agar 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 which was applied to investigate the chemical constituents and some biological activities. The preliminary screening of chemical constituents indicated the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, phenolic compounds, polyphenols, steroids, tannins and terpenoids in the chloroform extract of *P.aeruginosa.* The total carbohydrates content (TCC), total tannins content (TTC), total phenols content (TPC), total steroids content (TSC), total flavonoids content (TFC) and total protein content of the chloroform extract determined according to the appropriate reported methods were found to be 283±2.15 mg GE/g ,177.58±5.8 mg GAE/g, 50.64±1.5 mg GAE/g, 50.43±4.01 mg CE/g , 24.70 ± 2.2mg QE/g and 4.43±0.8 mg BSAE/g respectively. The chloroform extract was found to exhibit the high antimicrobial activity against all seven tested microorganisms such as *Bacillus subtilis* (N.C.T.C-8236), *Staphylococcus auerus* (N.C.P.C-6371), *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (N.C.I.B-8982), *Candida albicans* (-), *Agrobacterium tumefacines*(N.I.T.E-09678) and *Escherichia coli* (N.C.I.B-8134) (20 mm ~40 mm) determined by agar well diffusion method. The antioxidant activity of chloroform extract was determined by DPPH radical scavenging activity assay (IC_{50} = 128.6 mg/mL).The chloroform extract was also subjected to investigate the antidiabetic activity via α-amylae and α-glucosidase inhibition activities assays. The extract was observed to possess the α-amylase inhibitory effect (IC_{50}= 3.16 μg/mL) and α-glucosidase inhibitory effect (IC_{50} = 19.5 μg/mL), however, those were weaker than the standard drug acarbose (IC_{50} = 0.02 μg/mL) and (IC_{50} = 0.04 μg/mL). The chloroform extract of *P. aeruginosa* showed significant toxicity against brine shrimp with an LD_{50} value of 1.90 mg/mL after 24 h. In antitumor activity analysis, *Agrobacterium tumerfaceins* (N.I.T.E -09678) cell was used as the tumor cell. In this study, the chloroform extract of *P. aeruginosa* was found to exhibit low value (IC_{50} =147.54 μg/mL) against *A. tumefacines* cell. *In vitro* antiproliferative activity of the chloroform extract was evaluated against A 549 (lung), Hela (cervical) and MCF-7 (breast) human cancer cell lines by using CCK-8 Assay (Cell Counting Kit-8). It was found that, the chloroform extract has the antiproliferative activity against A 549 lung cancer cell lines (IC_{50} = 12.18 μg/mL), Hela cervical cancer cell line (IC_{50} = 49.93 μg/mL) and MCF-7 breast cancer cell line (IC_{50} = 16.59 μg/mL). Furthermore, *in vitro* antiproliferative activity of the chloroform extract was also evaluated against six microorganisms such as *P.aeruginosa* (IC_{50}=98.38μg/mL), *S.auerus*(IC_{50}=155.76μg/mL),*B. pumilus* (IC_{50} = 360.23μg/mL), *B. subtilis* (IC_{50} = 411.03 μg/mL) , *C. albicans* (IC_{50} = 422.16 μg/mL), *A. tumefacines* (IC_{50} = 147.54 μg/mL) and *E. col* (IC_{50}=440.58 μg/mL).

Keywords *:* *Pseudomonas aeruginosa*, chemical constituents, antiproliferative activity, antioxidant activity, antimicrobial activity, cytotoxicity, antitumor activity, antidiabetic activity

[1] Assistant Lecturer, Department of Chemistry, University of Yangon
[2] Second Year PhD Student, Department of Chemistry, University of Yangon
[3] Dr, Lecturer, Department of Chemistry, University of Yangon
[4] Institute of Natural Medicine, University of Toyama , 2630-Sugitani,Toyama 930-0194,Japan
[5] Dr, Professor and Head (Retd.,), Department of Chemistry, University of Yangon

Introduction

Pseudomonas is a genus of gram-negative, aerobic gammaproteo-bacteria that can cause disease in animals, including humans. *Pseudomonas aeruginosa* is one of the main organisms responsible for drug-resistant nosocomial infections, and is one of the leading causes of bacteremia and pneumonia in hospitalized patients. Pseudomonads are of great interest because of their role in plant and human disease and their growing potential in biotechnological applications. The Pseudomonas common last ancestor has encountered a wide range of abiotic and biotic environments which have led to the evolution of a multitude of traits and life-styles with significant overlap among the species (Mena and Gerba, 2009). Pseudomonads are a large group of free-living bacteria that live primarily in soil, seawater, and fresh water. Especially, *P. aeruginosa* is particularly prevalent in environments, such as soil, seawater, sewage, and associated with some plants. Although commonly isolated from the marine environment, the apparent distribution has been restricted to river outfalls and shorelines (Velammal *et al.*, 1994). Contamination of recreational waters and drinking water has been associated with outbreaks of Pseudomonas. With this in mind, it should be emphasized that Pseudomonads are highly versatile and can adapt to a wide range of habitats; they can even grow in distilled water (Hardalo and Edberg, 1997). This adaptability accounts for their ubiquitous presence in the environment and accordingly they have an extensive impact on ecology, agriculture, and commerce (Mena and Gerba, 2009). *P. aeruginosa* is an opportunistic pathogen that is important in the etiology of many infectious diseases seen in humans (Silby *et al*., 2011). Infections with this bacterium are often characterized by blue pus, and the bacterium is commonly isolated from clinical specimens (wounds, burns, and urinary tract infections) (Ullstrom *et al*., 1991). For many degradation studies *Pseudomonas sp*. was selected as it is a well-known biodegrader of agrochemicals. *Pseudomonas* is a versatile genus and suggested that this genus could degrade a number of chemicals like pesticides including carbaryl, malathion, *p*-nitrophenol and parathion, bethoxazin, it is widely present in soil and can be used to clean up different xenobiotics compounds like propiconazole (Sarkar *et al.*, 2009). On the other hand, *P. aeruginosa* has been reported for use in bioremediation and use in processing polyethylene in municipal solid waste (Pathak, 2017). Given the great interest in the genus *Pseudomonas*, there are commercially available media for selective and differential growth of this genus. In this study,*P.aeruginosa* has been isolated from clinical soil samples by serial dilution plate method, followed by cultured in nutrient agar medium. In this research, chloroform extract of *P.aeruginosa* was applied to investigate the chemical constituents and some biological activities.

Materials and Methods

Sample Collection

Soil samples were collected from the Insein General Hospital, Yangon Region. Bacteriological analyses were started within 4 h after collecting the samples.

Isolation and Identification of *P. aeruginosa*

One gram of collected soil samples was weighed and added into a sterile conical flask containing 99 mL of sterile distilled water to make a dilution. The suspension was thoroughly shaken for about 30 min to disperse the individual particles. The conical flask was kept for about

30 min to settle down. Then 1 mL of soil dilution was introduced in the test tube containing 9 mL distilled water to make serial dilution of 10^{-2} to 10^{-6} using a serial pipette each time.

In addition, 100 mL of medium was boiled on a hot plate and sterilized by autoclaving for 15 min at 121 °C. The sterilized media was cooled down. The 20 mL of sterilized medium was poured into the sterilized petri-dishes containing 0.2 mL of serial dilution of each of soil sample.

The inoculate plates were shaken clockwise to make uniform distribution of the inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 37 °C for two days. The separate colonies were appeared and the different types of bacteria colonies were cultured in test tubes. The slants of media were repeatedly sub-cultured to obtain pure cultured (Atlas and Synder, 2006).

The isolated bacteria strains were sub-cultured on nutrient agar slant cultures to check its purity and incubated at 37 °C for 24 h. Then the purified culture was maintained at refrigerator. The isolates were subjected in various physiological and biochemical tests. The isolates were identified by using conventional biochemical tests such as motility test, indole test, methyl red test, gelatin test, citrate test, nitrate reduction test, catalase test, Voges-Proskauer (VP) test, urease test, starch hydrolysis test and sugar fermentation tests. (Atlas and Synder, 2006; Garcia and Isenberg, 2007) (Su Swe Su *et al.,* 2018). Moreover, 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 solution1:1 (v:v).This process was done according to the ultrasound-assisted extraction to give 0.03 % (w/v) of chloroform extract which was applied to investigate the chemical constituents and some biological activities.

Determination of Chemical Composition of the Chloroform Extract of *P. aeruginosa*

(a) Determination of chemical constituents present in the chloroform extract of *P. aeruginosa*

The chemical constituents such as alkaloids, α-amino acids, carbohydrates, flavonoids, phenolic compounds, polyphenols, steroids, tannins and terpenoids were determined according to the appropriate reported methods.

(b) Determination of total phenol content by Folin-Ciocalteu Reagent (FCR) method

The total phenolic content (TPC) of chloroform extract of *P. aeruginosa* was estimated by Folin-Ciocalteu (FC) method according to the procedure described by Song *et al.,* (2010). The extract solution (1000 µg/mL) was mixed with 5 mL of F-C reagent (1:10) in a test tube and incubated for about 5 min. To each test tube, 4 mL of 1 M sodium carbonate was added and the test tubes were kept at room temperature for 15 min and UV absorbance of reaction mixture was read at λ_{max} 765 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total phenol content was estimated as milligram gallic acid equivalent per gram (mg GAE/g) of extract.

(c) Determination of total flavonoid content by aluminium chloride method

The total flavonoid content (TFC) of chloroform extract of *P. aeruginosa* was estimated by Aluminium Chloride method according to the procedure described by Song *et al.* (2010). The extract solution (1000 µg/mL) was mixed with 1.5 mL of methanol, 0.2 mL of 1 % $AlCl_3$ solution

and 2.8 mL of distilled water. The absorbance of reaction mixture was at λ_{max} 415 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total flavonoid content was estimated as milligram quercetin equivalent per gram (mg QE/g) of extract.

(d) Determination of total steroid content by Zak's method

The total steroid content (TSC) of chloroform extract of *P. aeruginosa* was estimated by Zak's method according to the procedure described by Zak *et al.* (1981). The extract solution (1000 µg/mL) was prepared by ferric chloride diluting agent. The test sample solution (5 mL) was added 4.0 mL of concentrated sulphuric acid to each tube. After 30 min incubation, intensity of the colour was read at 450 nm. The blank solution was prepared as the above procedure by using ferric chloride diluting agent instead of sample solution. Total steroid content was estimated as milligram cholesterol equivalent per gram (mg CE/g) of extract.

(e) Determination of total condensed tannin by Broadhurst's method

The tannin contents of chloroform extract of *P. aeruginosa* was determined by method of Broadhurst and Jones (1978) with slight modification, using tannic acid as a reference compound. A volume of 0.4 mL of extract is added to 3 mL of a solution of vanillin and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation the absorbance was read at 500 nm. The blank solution was prepared as the above procedure by using methanol instead of sample solution. The condensed tannin was expressed as milligram of tannic acid equivalent per gram of extract.

(f) Determination of protein content by biuret method

A calibration curve of the standard protein solutions bovine serum albumin (BSA) is used to determine the total protein in the unknown. This (BSA) compound reacts with biuret reagent to give a indigo coloured product with absorption maximum at 560 nm. The sample solution (1 mL) was added with 4 mL of biuret reagent mixed and allowed to stand for 30 min at room temperature. The intensity of colour the violet to indigo developed was read at 560 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. The protein content was expressed as milligram of BSA equivalent per gram of sample solution.

(g) Determination of carbohydrate content by anthrone method

The total carbohydrate content was estimated by the method of anthrone (Hedge *et al.*,1962). Carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxmethyl furfural. This compound forms with anthrone a green coloured product. The extract solution (5 mL) was added with 2.5 mL of anthrone reagent and allowed to stand for 10 min. The intensity of colour the green to dark green developed was read at 630 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. The carbohydrate content was expressed as milligram of glucose equivalent per gram of sample.

Investigation of Some Biological Activity of Chloroform Extract of *P. aeruginosa*

(a) Investigation of antioxidant activity of chloroform extract of *P. aeruginosa*

In this experiment, DPPH (2 mg) was thoroughly dissolved in EtOH (100 mL). This solution was freshly prepared in the brown coloured reagent bottle and stored in the fridge for no longer than 24 h. The chloroform extract of *P. aeruginosa* (1.6 mg) and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. By adding with EtOH, the sample solutions in different concentrations of 160, 80, 40, 20, 10 and 5 μg/mL were prepared from the stock solution. The effect on DPPH radical was determined by using the method of Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 50 μM DPPH solution and 1.5 mL of EtOH using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 50 μM DPPH solutions and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of each solution was measured at 517 nm by using UV-1650 spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation. The capability to scavenge the DPPH radical was calculated by using the following equation: $\%\,\mathrm{RSA} = \dfrac{A_c - (A - A_b)}{A_c \times 100}$

Where, %RSA = Radical Scavenging Activity

A_c = absorbance of the control (DPPH only) solution

A_b = absorbance of the blank (EtOH + Test sample solution) solution

A = absorbance of the test sample solution

(b) Determination of antimicrobial activity of chloroform extract of *P. aeruginosa* by agar disc diffusion method

The screening of antimicrobial activity of chloroform extract of *P. aeruginosa* was carried out by agar disc diffusion method (Perez *et al*., 1990) at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar. Six microorganisms namely *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* were used for this test.

(c) Determination of cytotoxicity

The cytotoxicity of chloroform extract of *P. aeruginosa* was investigated by using brine shrimp lethality bioassay according to the procedure described by Dockery and Tomkins, 2000. The brine shrimp (*Artemia salina*) was used in this study for cytotoxicity bioassay (Ali *et al*., 2016). Brine shrimp cysts (0.5 g) were added to the 1.5 L of artificial sea water bottle. This experiment was carried out at the Department of Chemistry, Yangon University, Myanmar. The suspension was aerated by bubbling air into the funnel and kept for 24 h at room temperature. After aeration had been removed, the suspension was kept for 1 h undisturbed, whereby the remaining unhatched eggs dropped. In order to get a higher density of larvae, one side of the separating funnel was covered with aluminium foil and the other illuminated with a lamp, whereby the phototropic larvae were gathering at the illuminated side and could be collected by pipette. The shrimp larvae were transferred to the chamber of ice cup filled with 9 mL of salt water and the dead larvae counted (number N). One mL of each solution of crude extracts (1, 10, 100, 1000 ppm) was added and the plate was then kept at room temperature in the dark. After

24 h, the dead larvae were counted in each well under the magnifier. The still living larvae (A) were killed by addition of ca. 0.5 mL methanol so that subsequently the total number of the animals (G) could be determined. The control solution was prepared as the above procedure by using distilled water instead of sample solution. The mortality (M) was calculated in %. Each test row was accompanied by a brine solution (number B). The mortality rate M was calculated by using the following formula:

$$M = \left[\frac{(A - B - N)}{(G - N)} \right] \times 100$$

M = percent of the dead larvae after 24 h
A = number of the dead larvae after 24 h
B = average number of the dead larvae in the brine solution after 24 h
N = number of the dead larvae before starting of the test.
G = total number of brine shrimps

(d) Determination of diabetic activity of chloroform extract of *P.aeruginosa*

(i) Determination of α-amylase inhibitory activity

The enzyme inhibitory activity was expressed as a decrease in units of maltose liberated *(Puls et al.1977).* A modified dinitrosalicylic acid (DNS) method was adopted to estimate the maltose equivalent 1mL of the tested samples were pre-incubated with 1 mL of phosphate buffer and 2 mL of α-amylase at 37 °C for 20 min and thereafter 0.4 mL 1% starch solution was added. The mixture was further incubated at 37 °C for 30 min. Then the reaction was stopped by adding 2 mL of DNS reagent and the contents were heated in a boiling water bath for 10 min. A blank was prepared without extract and another without the amylase enzyme, replaced by equal quantities of buffer. The absorbance was measured at 540 nm. The reducing sugar released from starch was estimated as maltose equivalent from a standard graph. Acarbose was used as standard. The anti-diabetic activity was determined through the inhibition of α-amylase which was expressed as a percentage of inhibition and calculated by the following equations:

$$\% \text{ Inhibition} = [A_{control} - (A_{Sample} - A_{Blank}) / A_{control}] \times 100$$

where, % Inhibition = % α-amylase inhibition
$A_{control}$ = absorbance without sample solution
A_{sample} = absorbance of sample
A_{Blank} = absorbance of sample + distilled water solution

The α-amylase inhibition (IC_{50}) is expressed as the test substance concentration (μg/mL) that results in a 50 % α-amylase inhibition of the sample. The IC_{50} values were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation.

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x} - x_1)^2 + (\bar{x} - x_2)^2 + ...(\bar{x} - x_n)^2}{(n-1)}}$$

where, $\bar{x}$ = average % inhibition
$x_1 + x_2 + ... + x_n$ = % α-amylase inhibition of test sample solution
n = number of time

(ii) Determination of α-glucosidase inhibitory activity

The α -glucosidase inhibitory activity was measured the procedure described by (*Puls et al*. 1977). The α -glucosidase was assayed using 0.4 mL of sample extract and 1mL of 0.1 M phosphate buffer (pH 6.9) containing 2 mL of α -glucosidase solution, which was then incubated at 37 °C for 10 min. After the pre-incubation period, 0.5 mL of 0.005M *p*-nitrophenyl- α -D-glucopyranoside solution was added to each well at timed intervals. The reaction mixtures were incubated at 37 °C for 5 min. After incubation, absorbance readings of the sample was recorded at 405 nm and compared with a control that had 0.4 mL of buffer solution in place of the extract. Acarbose was used as standard. The anti-diabetic activity was determined through the inhibition of α -glucosidase which was expressed as a percentage of inhibition and calculated by the following equations:

$$\% \text{ Inhibition} = [A_{control}-(A_{Sample} - A_{Blank})/ A_{control}] \times 100$$

where,% Inhibition = % □-glucosidase inhibition

$A_{control}$ = absorbance without sample solution

A_{sample} = absorbance of sample

A_{Blank} = absorbance of sample + distilled water solution

The α - glucosidase inhibition (IC_{50}) is expressed as the test substance concentration (μg/mL) that results in a 50% α -glucosidase inhibition of the sample. The IC_{50} values were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation.

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x}-x_1)^2+(\bar{x}-x_2)^2+.....(\bar{x}-x_n)^2}{(n-1)}}$$

where, $\bar{x}$ = average % inhibition

$x_1 + x_2 + \ldots + x_n$ = % α-glucosidase inhibition of test sample solution

n = number of times

(e) Determination of antiproliferative activity of chloroform extract of *P.aeruginosa* by turbidimetric assay

According to turbidimetric assay *in vitro* antiproliferative activity of chloroform extract of *P.aeruginosa* was determined against the tumor cell *A. tumerfaceins* (Elian and Herida (2015) at Department of Chemistry, University of Yangon. The diluted cell solution (9 mL) was mixed the sample solution (1 mL) was incubation in an incubator for 12 h. After incubation, absorbance readings of the samples were recorded at 600 nm and compared with a control that had the diluted cell culture and 1 mL of fresh nutrient medium was used in place of the extract as the blank. Fluorouracil (5-FU) was used as standard. The anti-proliferative activities were determined clindamycin, ciprofloxacin, ampicillin, flucloxacillin, fluconazole, aziythromycin used as a standard antibiotic through the inhibition of *Agrobacterium tumerfaceins* cell, *B. subtilis, B. pumilus, P. aeruginosa, S. aureus, C. albicans* and *E. coli*, respectively which was expressed as a percentage of inhibition and calculated by the following equations:

$$\% \text{ Inhibition} = [A_{control}-(A_{Sample} - A_{Blank})/ A_{control}] \times 100$$

where,

% Inhibition = % cell inhibition

$A_{control}$ = absorbance without sample solution

A_{sample} = absorbance of sample

A_{Blank} = absorbance of sample + fresh nutrient broth medium

The cell inhibition (IC_{50}) is expressed as the test substance concentration (µg/mL) that results in a 50% cell inhibition of the sample. The IC_{50} values were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x}-x_1)^2+(\bar{x}-x_2)^2+.....(\bar{x}-x_n)^2}{(n-1)}}$$

where, $\bar{x}$ = average % inhibition

$x_1 + x_2 + \ldots + x_n$ = % cell inhibition of test sample solution

n = number of times

(f) Investigation of antiproliferative activity of chloroform extract against Human Cancer Cell Lines

Antiproliferative activity of chloroform extract of *P.aeruginosa* was investigated in *in vitro* by using cancer cell lines at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. The cell lines used were Hela (human cervix cancer), A549 (lung cancer) and MCF 7 (human breast cancer). 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% antiobiotic antimycotic solution (Sigma). For MCF 7 cell, 1 % 0.1 M non-essential amino acid (NEAA, Gibco) and 1% 1mM sodium pyruvate (Gibco) were also supplemented. The *in vitro* antiproliferative activity of the crude extracts 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 % CO_2 and 95 % air for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the tested samples were added. After 72 h incubation, the cells were washed with PBS and 100 µL of medium containing 10 % WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. The concentrations of the crude extracts were 200, 100, 10 µg/ mL and 10, 1, 0.1 mM for positive control 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.

$$\text{(\%) Cell viability} = 100 \times \frac{\left\{Abs_{(\text{test samples})} - Abs_{(\text{blank})}\right\}}{\left\{Abs_{(\text{control})} - Abs_{(\text{blank})}\right\}}$$

In vitro antiproliferative activity of the chloroform extract was evaluated against A 549 (lung), Hela (cervical) and MCF-7 (breast) human cancer cell lines by using CCK-8 Assay (Cell Counting Kit-8).

Results and Discussion

In this study, *P. aeruginosa* bacterial strain, identified by the biochemical characteristics was isolated from the clinical soil sample of Insein General Hospital, Yangon Region (Su Swe Su *et al.*, 2018). The isolated bacteria *P.aeruginosa* was cultured on nutrient agar medium in the large scale subsequently, the culture was centrifuged(20min,4ºC,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 which was applied to investigate the chemical constituents and some biological activities. In order to find out the types of chemical constituents present in the chloroform extract of *P.aeruginosa* was observed that various secondary metabolites such as alkaloids, α-amino acids, carbohydrates, flavonoids, phenolic compounds, polyphenols, steroids, tannins and terpenoids were observed in the chloroform extract of *P. aeruginosa* but cardiac glycosides, glycosides, organic acids, reducing sugars and starch were not found. These secondary metabolites contribute significantly towards the biological activities of chloroform extract of *P.aeruginosa* such as antidiabetic, antioxidant, antimicrobial, cytotoxicity, antiproliferative activities etc. The sample for screening was found to possess the flavonoids, phenols and tannis. Flavonoids are also present as a potent water-soluble antioxidant and free radical scavengers, which prevent from the oxidative cell damage and also have strong anticancer activity. It also helps in managing diabetes induced oxidative stress (Yadav *et al*., 2014).

Total Phenol, Total Flavonoid, Total Steroid, Total Condensed Tannin, the Protein and Total Carbohydrate Contents of the Chloroform Crude Extracts of *P.aeruginosa*

The total phenol content of the chloroform extract of *P.aeruginosa* was determined with spectrophotometric method by using Folin-Ciocalteu reagent. The total phenol content of the chloroform extract of *P.aeruginosa* was 50.64 ± 1.5 mg GAE/g. The total flavonoid content of sample was determined with spectrophotometric method by aluminium chloride reagent and was found to be 24.70 ± 2.2 mg QE/g. The total steroid content of the chloroform extract of *P.aeruginosa* was determined by Zak's method and was observed to be 50.43 ± 4.0 mg CE/g. The total tannin content in the chloroform extract of *P.aeruginosa* was estimated by Broadhurst's method and was found to be 177.58 ± 5.8 mg TAE/g. The protein content in the chloroform extract of *P.aeruginosa* was estimated by Biuret method was found to be the same content of 4.43 ± 0.8 mg BSAE/g and total carbohydrate content of the chloroform extract was determined by anthrone method was observed to be 283.0±2.15 mg GE/g. The results are shown in Table1.

Table 1 Total Phenol, Total Flavonoid, Total Steroid, Total Tannin, Total Protein and Total Carbohydrate Contents of Chloroform Extract of *P.aeruginosa*

Chemical Constituents	Contents
Total Phenol Content (mg GAE ± SD)/g of extract	50.64 ± 1.5
Total Flavonoid Content (mg QE ± SD)/g of extract	24.70 ± 2.2
Total Steroid Content (mg CE ± SD)/g of extract	50.43 ± 4.0
Total Tannin Content (mg TAE ± SD)/g of extract	177.58 ± 5.8
Total Protein Content (mg BSAE ± SD)/g of extract	4.43 ± 0.8
Total Carbohydrate Content (mg GE ± SD)/g of extract	283.0±2.15

In the experimental results, phenol, flavonoid, steroid and tannin compounds were observed in chloroform extract of *P.aeruginosa*. Tannins and carbohydrate have amazing stringent properties. They are known to hasten the healing of wounds and inflamed mucous membranes. Flavonoids are also present as a potent water-soluble antioxidant and free radical scavengers, which prevent from the oxidative cell damage and also have strong anticancer activity. It also helps in managing diabetes induced oxidative stress. The steroids are among the most widely used class of drugs and their role in the therapy of pulmonary, inflammatory, dermatological and oncological diseases has been well described (Meikle and Tyler, 1977). Protein molecule is composed of amino acids which are characterized by containing nitrogen and sometimes sulphur. There are twenty two amino acids that can be found in the human body, and about ten of these are essential, and therefore must be included in the diet. A few amino acids from protein can be converted into glucose used for fuel through a process called gluconeogenesis; this is done in quantity only during starvation (WFP, 1998).

Some Biological Activities of Chloroform Extract of *P.aeruginosa*

Antioxidant activity of the chloroform extract of *P.aeruginosa*

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability of the chloroform extract of *P.aeruginosa* by using the stable radical DPPH. The results are shown in Table 2. The chloroform extract of *P.aeruginosa* was found to be the concentrations (IC_{50}) of 128.6 μg/mL, however, it was weaker than the standard gallic acid (IC_{50} = 0.75 μg/mL)

Table 2 Antioxidant Activity of Crude Extracts of *P.aeruginosa*

Sample	% RSA (mean ±SD) in different concentrations (μg/mL)				IC_{50} (μg/mL)
	20	40	80	160	
Chloroform Extract	16.00±2.88	17.60±2.12	40.53±3.23	56.27±2.01	128.6

Antimicrobial activity of the chloroform extract of *P.aeruginosa*

The chloroform extract of *P.aeruginosa* was subjected to screening of antimicrobial activity against six different pathogenic microbes such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans and Escherichia coli* using agar well diffusion method. This method is based on zone diameter including the well diameter, in millimeter (mm). The chloroform extract of *P.aeruginosa* was found to be high in antimicrobial activity against all tested microorganisms with the inhibition zone diameter ranged between 20 ~ 40 mm. The results are shown in Table 3.

Table 3 Inhibition Zone Diameters of the Chloroform Extract of *P.aeruginosa* against Seven Microorganisms by Agar Well Diffusion Method

Organisms	Inhibition zone diameter (mm) of extract	
	$CHCl_3$	Control
Bacillus subtilis	20 (+++)	-
Staphylococcus aureus	40 (+++)	-
Pseudomonas aeruginosa	38 (+++)	-
Bacillus pumilus	40 (+++)	-
Candida albicans	40 (+++)	-
Escherichia coli	36 (+++)	-

Agar well – 10 mm, 10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm and above (+++)

This high antimicrobial activity (20 mm ~ 40mm) against *Bacillus subtilis*(N.C.T.C-8236),*Staphylococcus aueru* (N.C.P.C6371) , *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (N.C.I.B-8982), *Candida albicans*(-) , *Escherichia coli* (N.C.I.B-8134) and *Agrobacterium tumefacines* (N.I.T.E – 09678) may be the present of the flavonoids, phenols and tannis.

Cytotoxicity of the chloroform extract of *P.aeruginosa*

The cytotoxicity of chloroform extract of *P.aeruginosa* was evaluated by brine shrimp cytotoxicity bioassay. This bioassay is general toxicity screening for bioactive phytoconstituents and their derivatives. A model animal that has been used for this purpose is the brine shrimp, *Artemia salina* (Tawaha, 2006). The cytotoxicity of the extract was expressed in term of mean ± SEM (standard error mean) and LD_{50} (50% Lethality Dose) and the results are shown in Table 4. In this experiment, standard potassium dichromate ($K_2Cr_2O_7$) and caffeine were chosen because $K_2Cr_2O_7$ is well-known toxic for this assay (Salinas and Fernendez, 2006) and caffeine is a natural product. According to the results of brine shrimp cytotoxicity bioassay, the tested sample has cytotoxic effect with the LD_{50} value of 1.90 µg/mL and it was observed to be toxic camparable to $K_2Cr_2O_7$ (LD_{50} = 1.50 µg/mL).

Table 4 Cytotoxicity of Chloroform Extract of *P.aeruginosa*

Crude extract	Dead % in different concentrations (µg/mL) of samples				LD_{50} (µg/mL)
	1	10	100	1000	
$CHCl_3$ Extract	46.66 ± 0.57	80 ±0.10	90±0.10	100±0.10	1.90
*Caffeine	0±0	0±0	9.58±0.91	12.73±0.41	>1000
*$K_2Cr_2O_7$	48.63±0.19	73.13±0.47	74.67±0.11	100±0.10	1.50

*****standard

Anti-diabetic activity of chloroform extract of *P.aeruginosa*

Based on the *in vitro* assay model, the chloroform extract of *P.aeruginosa* occurred to inhibited α-amylase and α -glucosidase enzymes. The chloroform extract of *P.aeruginosa* was demonstrated a relatively high α -amylase and α -glucosidase inhibitory activity with the values of IC_{50} values of 3.16 µg/mL (Table 5) and IC_{50} values of 19.5 µg/mL (Table 6) respectively, however, weaker than that of standard acarbose IC_{50} = 0.02 µg/mL(Table 7) and IC_{50} = 0.04 µg/mL(Table 8) . α -Amylase and α-glucosidase are key enzymes involved in starch breakdown and subsequent glucose release leading to rapid intestinal absorption. When inhibited by a high

carbohydrate diet, these enzymes are beneficial in maintaining the postprandial blood sugar level and managing hyperglycemia (Puls *et al*., 1977). A major drawback of currently used α -amylase and α -glucosidase inhibitors, such as pharmacologically beneficial acarbose, are reported to have side effects, such as abdominal distention, flatulence, tympanitis, and diarrhea. These side effects are possibly due to complete inhibition of α -amylase, leaving the carbohydrate to ferment in the colon (Bischoff, 1994). Thus, integrating natural inhibitors from dietary edible plants with strong inhibitory effects against α-amylase and α-glucosidase can be potentially targeted as a more complimentary and effective therapy for postprandial hyperglycemia control with minimal side effects.

Table 5 α-Amylase Inhibition and IC_{50} of Chloroform Extract of *P.aeruginosa*

Tested sample	% Inhibition in different concentrations (μg/mL)					IC_{50} (g/L)
	0.625	1.25	2.5	5	10	
$CHCl_3$ Extract	24.95±1.14	27.43±3.74	37.041±5.79	86.18±17.69	107.56±2.92	3.16

Table 6 α-Glucosidase Inhibition and IC_{50} of Chloroform Extract of *P.aeruginosa*

Tested sample	% Inhibition in different concentrations (mg/mL)					IC_{50} (μg/mL)
	6.25	12.5	25	50	100	
$CHCl_3$ Extract	5.62 ±1.6	24.94±0.7	69.74±0.2	84.26±0.3	98.26±1.5	19.5

Table 7 α-Amylase Inhibition and IC_{50} of Standard Acarbose

Tested sample	% Inhibition in different concentrations (μg/mL)						IC_{50} (μg/mL)
	0.009765	0.019531	0.03906	0.0781	0.156	0.31	
Acarbose	36.8±6.8	58.39±4.9	141.61±3.2	154.58±2.5	162.2±1.3	173.69±5.4	0.02

Table 8 α-Glucosidase Inhibition and IC_{50} of Standard Acarbose

Tested sample	% Inhibition in different concentrations (μg/mL)						IC_{50} (μg/mL)
	0.0390625	0.078125	0.15625	0.3125	0.625	0.31	
Acarbose	48.68±1.3	54.98±0.8	66.13±0.8	96.09±1.0	115.79±1.2	125.39±1.1	0.04

Antiproliferative Activity of Chloroform Extract of *P.aeruginosa* on *Agrobacterium tumerfaceins* cell

Antiproliferative activity of chloroform extract of *P.aeruginosa* was determined by the turbidimetric method. The turbidimetric assay technique is based on the fact that within a certain range of antibiotic concentration, the partial inhibition of the growth of the test organism occurs and there is a gradient in bacterial growth with increasing concentration of antibiotics. Peptone was used to culture for bacterial cell. Peptone is a semi-digested protein which is soluble in water and easily metabolized by the bacterial cell, provides the rich source of protein to the bacterial cell of the rapid growth. *In vitro* antiproliferative activity of the chloroform extract was evaluated against sixmicroorganisms such as *B.subtilis* (N.C.T.C8236), *S.auerus* (N.C.T.C6371),

P.aeruginosa, *B. pumilus* (N.C.I.B-8982), *C.albicans* and *E. coli* (N.C.I.B-8134). Based on the results, the chloroform extract of *P. aeruginosa* showed the highest antiproliferative activity (IC_{50} = 98.38 µg/mL), that followed by *A.tumefacines* (IC_{50} = 147.54 µg/mL),

S. auerus (IC_{50} = 155.76 µg/mL), *B. pumilus* (IC_{50} = 360.23 µg/mL2), *B.subtilis* (IC_{50} = 411.03 µg/mL) , *C.albicans* (IC_{50} = 422.16 µg/mL) , and *E. col* (IC_{50} = 440.58 µg/mL) (Table 9). However, the chloroform extract was weaker than standard antibiotics.

Table 9 Antiproliferative Activity of *P.aeriginosa* Chloroform Extract by Turbidometric Method

Tested sample	50 % Inhibition concentrations IC_{50} (µg/mL)						
	P.aeruginosa	***A.tumefacines***	***S.aureus***	***B.pumilus***	***B.subtilis***	***C.albicans***	***E.coli***
$CHCl_3$ extract	98.38	147.54	155.76	360.23	411.03	422.16	440.58
Antibiotics	70.69	0.17	143.71	212.15	98.38	118.33	196.64

Antibiotics = Clindamycin for *B.subtilis* , Flucloxacillin for *S. auerus*, Ampicillin for *P. aeruginosa*, Fluconazole for *C.albicans*, Ciprofloxacin for *B. pumilus* , Azithromycin for *E. col*, 5 FU for *A.tumefacines*

Antiproliferative Activity of the *P. aeriginosa* Chloroform Extract against Human Cancer Cell Lines

Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. Antiproliferative activity of the sample was studied in *in vitro* against human cancer cell lines. Screening of antiproliferative activities of chloroform extract of *P.aeruginosa* was done against three human cancer cell lines such as A 549 (human lung cancer), MCF7

(human breast cancer) and Hela (human cervix cancer). Antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-Fluorouracil was used as positive control. The antiproliferative activity of crude extracts is summarized in Table 8. Since the lower the IC_{50} values, the higher the antiproliferative activity, in antiproliferative activity. It was observed that the chloroform extract has the antiproliferative activity against human lung cell A 549 (IC_{50} = 12.18 µg/mL) comparable to the standard 5FU (IC_{50} = 10.2 µg/mL) (Table 10) . Then, the chloroform extract has the antiproliferative activity against human breast cancer MCF7 (IC_{50} = 49.93 µg/mL) and human cervix cancer Hela (IC_{50} = 16.59 µg/mL). However, the chloroform extract was weaker than standard 5FU.

Table 10 Antiproliferative Activity of Chloroform Extract of *P. aureginosa* against Human Lung (A549), Breast (MCF7), and Cervix (HeLa) Cancer Cell Lines

Samples	50 % Inhibition (IC_{50} μg/mL) of the sample against human cancer cell lines		
	Lung A549	**Breast MCF7**	**Cervix HeLa**
ChloroformExtract	12.18	49.93	16.59
*5-Fluorouracil	10.2	11.5	6.93

A 549 = Lung cancer cell line; MCF 7 = Breast cancer cell line; Hela= Cervix cancer cell line,
*5 FU = 5 Fluorouracil

Conclusion

From the overall assessment concerning with the investigation of chemicals and biological activities on the chloroform extract 0.03 % (w/v) of *P.aeruginosa* isolated from the clinical soil sample collected in the Insein General Hospital, Yangon Region the following inferences could be deduced. Various types of secondary metabolites such as alkaloids, α-amino acids, carbohydrates, flavonoids, phenolic compounds, polyphenols, steroids, tannins and terpenoids were found to be present in chloroform extract of *P. aerugenosa.* No harmful constituent cyanogenic glycosides was observed. The chloroform extract contains significant high TCC content (283±2.15 mg GE/g),TTC content (177.58±5.8 mg GAE/g) compared to TPC content (50.64±1.5 mg GAE/g), TSC content (50.43±4.01 mg CE/g), TFC content (24.70 ± 2.2 mg QE/g) and total protein content (4.43±0.8 mg BSAE/g). It showed high antimicrobial activity (20 mm ~ 40mm) against *Bacillus subtilis* (N.C.T.C-8236), *Staphylococcus aueru*(N.C.P.C6371), *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (N.C.I.B-8982), *Candida albicans* (-) and *Escherichia coli* (N.C.I.B-8134) due to contain the flavonoids, phenols and tannis. The chloroform extract also showed DPPH free radical scavenging activity assay (IC_{50} = 128.6 mg/mL) has antioxidant activity. The chloroform extract possessed the antidiabetic activity due to its α-amylase inhibitory effect (IC_{50}= 3.16 µg/mL) and α -glucosidase inhibitory effect (IC_{50} = 19.5 µg/mL).It exhibited the cytotoxicity effect against brine shrimp (LD_{50} value of 1.91 mg/mL) indicating that it may possess the anticancer property. The chloroform extract was found to possess the antiproliferative activity against tested microorganisms in the order of *P.aeruginosa* (IC_{50} = 98.38µg/mL), *A.tumefacines* (IC_{50} =147.54 µg/mL), *B. pumilus* (IC_{50} = 155.75 µg/mL), *S.aureus* (IC_{50} = 360.23 µg/mL), *B. subtilis* (IC_{50} = 411.02 µg/mL), *E. coli* (IC_{50} = 440.58 µg/mL) and *C. albicans* (IC_{50} = 442.163 µg/mL) as well as human cancer cell lines such as A 549 lung cancer cell line (IC_{50} = 12.18 µg/mL) Hela cervical cancer cell line (IC_{50} = 49.93 µg/mL) and MCF-7 breast cancer cell line (IC_{50} = 16.59 µg/mL). It was observed that the chloroform extract has the antiproliferative activity against human lung cell A549 (IC_{50} = 12.18 µg/mL) comparable to the standard 5FU (IC_{50} = 10.2 µg/mL) among the three human cancer cell line. These data indicate that *P. aeruginosa* microorganism is the good source of bioactive compounds and this information will be beneficial for further utilization and development of anticancer compounds from soil microorganisms and as lead compounds for pharmaceutical industry in the future.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education (Lower Myanmar), Ministry of Education, Yangon, Myanmar, for provision of opportunity to do this research and Myanmar Academy of Arts and Science for allowing to present this paper. Grateful thanks are also to Professor Dr Hiroyuki Morita, Institute of Natural Medicine, University of Toyama for his help and suggestions throughout course of antiproliferative activity .

References

Ali, N., Zada, A., Ali, M. and Hussain, Z. (2016). "Isolation and Identification of *Agrobacterium tumefaciens* from the Galls of Peach Tree". *Journal of Rural Development and Agriculture*, vol. 1 (1), pp. 39-48.

Aldona, B. and Raymond, S. (1994). "*Pseudomonas aeruginosa*: Infections and Treatment", *Journal of Information Health Care*, vol.4, pp. 83-84.

Atlas, R. M. and Snyder, J. W. (2006). "*Handbook of Media for Clinical Microbiology*". New York: 2nd Ed., CRC Publisher, pp. 278-339.

Bischoff, H. (1994). "Pharmacology of Glucosidase Inhibitor". *Eur. J. Clin. Invest.* vol.24, pp. 3–10.

Broadhurst, R. B. and Jones, W. T. (1978). "Analysis of Condensed Tannins using Acidified Vanillin". *J. Sci. Food Agr.*, vol. 29, pp. 788-794.

Devnath. P., Md. Kamal Uddin, F. Ahamed, Md. Towhid Hossain and M. Abul Manchur. (2011). "Extraction, Purification and Characterization of Pyocyanin Produced by *Pseudomonas aeruginosa* and Evaluation for its Antimicrobial Activity" .*International Research Journal of Biological Sciences*, **6**(5), 1-6

Dockery, M. and Tomkins, S. (2000). *Brine Shrimp Ecology*. London : 1st Ed., The British Ecology Society, pp. 92-93.

Elian, G. and Herida , R. .(2015). " Rapid Turbidimetric Assay to Determination the Potency of Daptomycin in Lyophilized Powdwer". *Journal of Pharmaceutics*, vol **7** , pp. 106-111

Garcia, L. S. and Isenberg, H. D. (2007). "*Aerobic bacteriology: Clinical Microbiology Procedures Handbook*". Washington, D.C.: 2nd Ed., vol. 3(1) , pp. 3-8.

Hardalo, C. and Edberg, S. C. (1997). "*Pseudomonas aeruginosa*: Assessment of Risk from Drinking Water". *Critical Reviews in Microbiology*, vol.23 (1), pp. 47-75.

Hedge, J. E., Hofreiter ,B.T. Whistler, R.L. and BeMiller, J.N.(1962). "*Methods in Carbohydrate Chemistry*".New York: Academic Press, vol.17, pp.420.

Marinova, G. V. and Batchvarov, V. (2011). "Evaluation of the Mehthods for Determination of the Free Radical Scavenging Activity of DPPH". *Bulgarian Journal of Agricultural Science*, vol. 17, pp. 11-24.

Meikle, A. W. and Tyler. F. H. (1977). "Potency and Duration of Action of Glucocorticoids. Effects of Hydrocortisone, Prednisolone and Dexamethasone on Human Pituitary Adrenal Function". *Am J Med*., vol. 63, pp. 200- 207.

Mena, K. D. and Gerba, C. P. (2009). "Risk Assessment of *Pseudomonas aeruginosa* in Water". *Reviews of Environmental Contamination and Toxicology*, vol.201, pp.71-115.

Pathak, V. M. (2017). "Review on the Current Status of Polymer Degradation: a Microbial Approach". *Bioresources and Bioprocessing. vol.4(15), pp.* 2197-4365

Perez, C., Paul, M. and Bazerque, P. (1990). "Antibiotic Assay by Agar Well Diffusion Method.". *Alta Bio Med Group Experiences*,vol. 15, pp. 113-115.

Puls, W., Keup, U., Krause, H. Thomas, P.G and Hoffmeister, F. (1977). "Glucosidase Inhibition: A New Approach to the Treatment of Diabetes, Obesity and Hyperlipoproteinemia". Naturwissenschaften vol, 64, pp. 536– 537.

Sarkar, S., Sreenivasan, S. and Premkumar, R. (2009) "Biodegradation of Propiconazole by *Pseudomonas putida* Isolated from Tea Rhizosphere". *Plant Soil Environ*. vol, 55,pp. 196-201.

Salinas, M. M. G. and Fernandez, S. S. (2006). "A Modified Microplate Cytotoxicity Assay with Brine Shrimp Larvae (*Artemia salina*)".*Pharmacology*, vol. 3, pp. 633–638.

Silby, M. W., Winstanley, C, Godfrey, S. A. C, Levy, S.B. and Jackson. R.W.(2011). "*Pseudomonas genomes*: Diverse and Adaptable". *FEMS Microbiol Rev*., vol , 35, pp. 652–680.

Song, F. L., Gan, R. Y., Zhang, Y., Xiao, Q., Kuang, L. and Li, H. B. (2010). "Total Phenolic Contents and Antioxidant Capacities of Selected Chinese Medicinal Plants". *Int. J. Mol. Sci.*, vol. 11, pp- 2367-2372.

Su Swe Su, Khine Zar Wynn Lae, Nwet Nwet Win 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.

Susan R., M. and Eduaro, C. (1960). "*Bioanalytical Chemistry"* New Jersey, 2nd Ed., vol. 3, pp. 4-7

Tawaha, K. A. (2006). "Cytotoxicity Evaluation of Jordanian Wild Plants Using Brine Shrimp Lethality Test".*J. J. Appl. Sci*., vol. 8, pp. 12–17.

Ullstrom, C. A., Siehnel, R, Woodruff, W, Steinbach, S. and Hancock, R. E. (1991). "Conservation of the Gene for Outer Membrane Protein OprF in the Family Pseudomonadaceae: Sequence of the *Pseudomonas syringae* oprF gene". *J. Bacteriol.*, vol. 173(2), pp.768-775.

Velammal, A., Aiyamperumal, B. Venugopalan,V. K. and Ajmalkhan, S. (1994). "Distribution of *Pseudomonas aeruginosa* in Pondicherry Coastal Environs". *Indian J Mar Sci*, vol. 23, pp. 239–241.

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

WFP. (1998). "*Food and Nutrition Hand Book, In; Macronutrients*", Italy, pp.1–3.

Yadav, M., Chatterji, S., Gupta, S. K. and Watal, G. (2014). "Preliminary Phytochemical Screening of Six Medicinal Plants used in Traditional Medicine". *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 6 (5), pp. 539-542.

Zak, B., Dickenman, R. C., White, E. G., Burnett, H. and Cherney, P.J. (1981). "Rapid Estimation of Free and Total Cholesterol". *Life Sciences*, vol. 24 (18), pp. 16.