ISOLATION OF METHYL PIPERATE FROM THE FRUIT OF *Piper longum* L. (PEIK-CHIN) AND ANTIBACTERIAL SCREENING OF THE CRUDE EXTRACTS AND METHYL PIPERATE

Ei Ei Khaing*

Abstract

The fruit of *Piper longum* L. (Peik-chin) used in the treatment of diarrhoea and dysentery in traditional Myanmar medicinal system was chosen for present study. The aim of the study is to isolate methyl piperate from the fruit of *Piper longum* L. (Peik-chin) and to screen the antibacterial activity of its crude extracts and methyl piperate. At first, four crude extracts of the sample were prepared by using various solvents; petroleum ether, ethyl acetate, 96 % ethanol and 50 % ethanol. *In vitro* antibacterial activity of four crude extracts was investigated against 19 bacterial strains by using agar disc diffusion method. Among the four crude extracts, the most active ethyl acetate extract was selected for isolation of active compound by column chromatographic method by using the solvent systems (v/v) PE : EtOAc (19:1, 9:1, 4:1, 1:1) consecutively. The isolated compound, methyl piperate (0.084 %) was identified by TLC and spectroscopic methods; Ultraviolet, Fourier transform infrared, Proton nuclear magnetic resonance spectroscopy, Electron impact mass spectrometry and then tested on 11 bacteria; *Klebsiella* species, *Salmonella paratyphi* A, *Citrobacter* species, *Escherichia coli* ATCC, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli* YCH 149, *Shigellaflexneri*, *Proteus* species, *Staphylococcus aureu s*and *Vibrio cholerae* O1 by agar disc diffusion method. In addition, minimum inhibitory concentration (MIC) of methyl piperate determined by microtitre plate dilution method was 0.03 mg/mL on six tested bacteria; *E. coli* LT, *S. epidermidis* (MKL-50), *S. epidermidis* (MKL-68), *E. coli* EHEC, *S. aureus* and *B. subtilis*.

Keywords: *Piper longum*, Peik-chin, methyl piperate, antibacterial activity, MIC

Introduction

Diarrhoea and dysentery are important health problems in worldwide especially developing countries. So the Government of Myanmar has initiated a national programme for the development of Traditional Medicine System in

* Dr, Associate Professor, Department of Chemistry, Hinthada University

combating six major types of diseases: namely; malaria, tuberculosis, diarrhoea, dysentery, diabetes and hypertension.

Diarrhoea is the host response to infection of the gastrointestinal tract by a variety of viruses, bacteria and parasites. There are three types of diarrhoea, namely acute diarrhoea, persistent diarrhoea and chronic diarrhoea. Acute diarrhoea is usually defined as the passage of 3 (or) more liquid motions within 7 days. Persistent diarrhoea have a usually long duration, more than 2 weeks, but usually less than 2 weeks duration. Chronic diarrhoea lasts for more than three weeks (Khan, 2001).

Dysentery is an inflammatory disorder of the lower intestinal tract, usually caused by bacteria, parasite, or protozoa infection and resulting in pain, fever, and severe diarrhoea, often accompanied by the passage of blood and mucous. Dysentery is caused by an *Amoeba* or *Bacillus* that infects the colon (Boyd and Marr, 1980).

In this study, Myanmar medicinal plant, *Piper longum* L. (Peik-chin) (Figure 1) was selected to find out of active principle for the treatment of dysentery and diarrhoea. *P. longum*plants are found in the north temperate regions and South East Asia (Fluck, 1976). It is cultivated in India, Indonesia, Malaysia, Sri Lanka, Pakistan, Singapore and Bangladesh. *P. longum* is indigenous and grows wild in Myanmar especially in Mon and Kayin States and hilly regions of Northern Myanmar. The pale yellow crystal, methyl piperate is one of the constituents responsible for medicinal properties of *P. longum* (Jalalpure*et al*.,2003). In Myanmar, *P. longum* is used in treating diarrhoea, dysentery, fever, cough, indigestion, stomachaches and asthma (Mar MarNyein*et al*., 2006).

P.longum contains methyl piperate that shows antioxidant, antibacterial activity as well as antimicrobial activity. The fruit of *P. longum* has been also used in the treatment of gastrointestinal (GI) problems, pneumonia, tumor, flatulence and then used as spice in food industry like a Nga-yoke-kaung (Krool, 2001; Kumar *et al.*, 2011). Therefore, antibacterial activity investigation on four crude extracts (PE, EtOAc, 96 % EtOH, 50 % EtOH) and some isolated phytoconstituents from the fruit of *P. longum*were carried out by using agar disc diffusion method. In this study, Minimum inhibitory concentration (MIC) of active constituent was also determined by microtitre plate dilution method.

Botanical Aspects of *Piper longum* L.

Name : Peik-chin (in Myanmar),
Long pepper (in English)
Botanical Name : *Piper longum*L.
Family : Piperaceae
Genus : *Piper*
Fruit : Fleshy spikes 2.5 - 3.5 blunt, blackish green from small shrub (Kress *et al*., 2003)

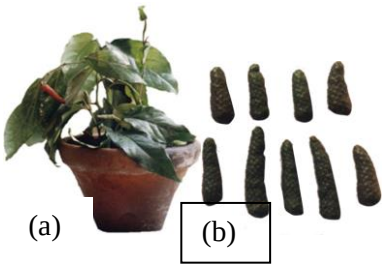


Figure 1: (a) Plant of *P. longum* L.
(b) Fruit of *P. longum* L.

Distribution

P. longum (Peik-chin) is widely distributed in shady places and hilly regions of Myanmar and often planted for its medicinal properties.

Chemical Constituents

The fruit of *P. longum* (Peik-chin) contained a large number of alkaloids and related compounds, the most abundant of which is piperine, followedby methyl piperate, pipernonaline, piperettine, tetrahydropiperlongumine, piperidine, volatile oil, palmitic acid, tetrahydropiperic acid and vitamins A and E (Kumar *et al.*, 2011).

Materials and Methods

Plant Materials

The fruit of *P. longum* L. (Peik-chin) was collected from Mawlamyine, Mon State. The plant was identified at Department of Botany, Yangon University. The fruit of *P. longum* was washed, cleaned and dried at room temperature for three weeks. Then the dried sample was powdered and stored in air- tight container.

Instruments: Shimadzu UV-240 (MeOH), Shimadzu FT IR- 8400 (KBr) (at URC), ^{1}H (300 MHz) NMR, EI MS (at University of Goettingen, Germany).

Chemicals : CC; Merck Silica gel 60 (70-230) mesh, eluents; Petroleum ether (PE), ethyl acetate (EtOAc), TLC; precoated silica gel 60 (F_{254}, Merck)

Extraction and Isolation of Methyl Piperate from Fruit of *Piper longum* L.
Preparation of extracts from fruit of *Piper longum* L.

The air-dried powder (1 kg) was individually cold extracted with (2500 mL) of solvents; petroleum ether (60-80 °C), ethyl acetate, 96 % ethanol and 50 % ethanol, respectively for 7 days and then filtered. The filtrate was evaporated to dryness at normal pressure on a water bath and desiccated. The yield % of petroleum ether extract, ethyl acetate extract, 96 % ethanol extract and 50 % ethanol extract were determined.

Isolation of phytoconstituents from EtOAc extract of fruit of *Piper longum* L.

The EtOAc extract was subjected to isolate the active phytoconstituents from Peik-chin fruit by column chromatography. The column was packed with silica gel (400 g) by the wet method using petroleum ether. The column was eluted consecutively with the solvent systems of PE :EtOAc in the ratio of 19:1, 9:1, 4:1, 1:1 v/v according to their increasing polarity. Five fractions were monitored by thin layer chromatography (TLC). The fractions that gave similar spots on thin layer chromatography (TLC) plates were combined together and the solvent was evaporated. Finally, pure compound obtained from fraction IV, pale yellow crystal (0.12 g, 0.084 %) was characterized as methyl piperate by UV, FT IR, ^{1}HNMR and EI MS spectroscopic methods.

***In vitro* Studies on the Antibacterial Activity of Fruit of *Piper longum* L. by Agar Disc Diffusion Method**
Screening of antibacterial activity of crude extracts against 19 tested bacterial strains

Agar disc diffusion method was used for the detection of antibacterial activity for four crude extracts from *P. longum* fruit. The test procedure was as follows: the extracts (1 g each) were dissolved in 1 mL of their respective solvents; petroleum ether, ethyl acetate, 96 % ethanol and 50 % ethanol, and introduced into sterile petridishes for testing 19 cultural bacterial strains. The discs having 6 mm diameter each with 20 µg extract/disc were allowed to dry at 42 $^{\circ}$C in incubator.

The bacterial suspension from trypticase soy broth was streaked evenly into three places on the surface of the trypticase soy agar plates with sterile cotton swab (Puritan, USA). After the inoculums had dried for 5 min, the dried disc impregnated with extracts were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. A disc impregnated with solvent only was used as control and antibiotics tetracycline was also used as standard for this study.

After overnight incubation at 37 °C, the zones of inhibition diameter including 6 mm discs were measured. Out of four crude extracts, the most active extract (EtOAc) was selected for isolation of active compound and MIC determination.

Screening of antibacterial activity of EtOAc extract and the isolated methyl piperate from active EtOAc extract of fruit (*Piper longum* L.) against 11 tested bacterial strains

The selected most active EtOAc extract and isolated compound methyl piperate (0.12 g, 0.084 %) were subjected to study antibacterial activity against 19 tested bacteria from clinical sources, National Health Laboratory (NHL), Yangon; related to acute diarrhoea (cholera), dysentery, abscess, pneumonia and typhoid.

Determination of Minimum Inhibitory Concentration (MIC) by Microtitre Plate Dilution Method

The MIC value of isolated compound, methyl piperate from *P. longum* was tested with 6 strains; *E. coli* LT, *S. epidermidis* (MKL-50), *S. epidermidis* (MKL-68), *E. coli* EHEC, *S. aureus* and *B. subtilis* by microtitre plate dilution method.

Microtitre plate dilution method was done by using trypticase soy broth by dissolving with appropriate soluble compound, methyl piperate in 2-fold dilutions. First, an inoculum of pure culture of respective bacteria was seeded in 5 mL of trypticase soy broth (TSB) and incubated at 37 °C for 3-4 h to obtain a turbidity of 0.05 by MacFarland nephelometer which corresponded to a bacterial suspension of 10^6 organisms per mL. Prior to the experiment, 50 L of TSB was introduced into all wells of 96-well microtitre plate. The compound (methyl piperate) was dissolved in ethyl acetate and diluted with

trypticase soy broth to obtain the following concentration: 0.12 mg/mL, 0.06 mg/mL, 0.03 mg/mL, 0.015 mg/mL, 0.008 mg/mL, 0.004 mg/mL, 0.002 mg/mL, 0.001 mg/mL, 0.0005 mg/mL, 0.00025 mg/mL, 0.000125 mg/mL and 0.0000625 mg/mL, in 96 - well microtitre plates.

Then 0.02 µL of the prepared inoculum was introduced to its respective wells and the microtitre plates were incubated at 37 °C for 18 h. The contents of all wells were thoroughly mixed with a multi-channel pipetter to resuspend clamped cells at the bottom of the wells in a solution. Growth of the bacteria was determined by automated microplate reader (Bio Rad) at a wavelength of 450 nm as well as confirmed by the growth of culturing onto trypticase soy agar to incubate at 37 °C for overnight. The concentration of the compound in the last well with no growth of bacteria was taken to represent the minimum inhibitory concentration (MIC)of the compound.

Results and Discussion

Isolation and Characterization of Methyl Piperate from Ethyl Acetate Extract of *Piper longum* L. Fruit

The dried fruit powder collected from Mawlamyine, Mon State was extracted with various solvents and the yield % of petroleum ether extract (1.5%), ethyl acetate extract (3.5 %), 96 % ethanol extract (4.5 %) and 50 % ethanol extract (6.2 %) were obtained respectively. Methyl piperate (0.084 %) was isolated from ethyl acetate extract of *P. longum*by column chromatographic separation method using petroleum ether: ethyl acetate solvent system. The isolated compound as methyl piperate was then identified by melting point, TLC determination, UV (Figure 2), FT IR (Figure 3), and ^{1}HNMR (Figure 4) and EI MS (Figure 5) spectrometry. Melting point of the isolated compound was found to be 140-14 °C which was identical with reported value of methyl piperate (Mohring and Necker, 1979). Isolated compound gave R_f value 0.63 with petroleum ether - ethyl acetate (4:1) system and was observed blue colour with anisaldehyde-conc. H_2SO_4 in TLC chromatogram.

Methyl piperate: Pale yellow crystal (0.12 g, 0.084 % yield); λ_{max}^{MeOH} 310, 340 (nm); FT IR ν cm^{-1} 2926, 2854 (ν CHasym & sym of methyl) , 1707 ($\nu_{C=O}$ of ester), 1618, 1607 ($\nu_{C=C}$ asym & sym diene), 1496 ($\nu_{C=C}$ -aro), 1452 (δ_{CH_2}), 1373 (δ_{CH_3}), 1265, 1244 ($\nu_{asym\ C\text{-}O\text{-}C}$), 1142 ($\nu_{sym\ C\text{-}O\text{-}C}$), 948, 928 ($\delta_{CH}$ of trans CH=CH group), 865, 839, 814 (δ_{CH} out of plane bending); ^{1}HNMR (300 MHz,$CDCl_3$) / δ_H (ppm), ~3.78 (3H, s, OC**H$_3$**), ~5.85 (1H, d, -CH=C**H**-CO-), ~6.0 (2H, s, -O-C**H$_2$**-O-), ~6.62 (1H, d, aromatic H), ~6.67 (1H, d, -C**H**=CH-), ~6.70 (1H, d, aromatic H), ~6.80 (1H, d, aromatic H), ~6.85 (1H, t, -CH=C**H**-CH=), ~7.42 (1H, dd, -C**H**=CH-CO-); EI MS, m/z 232.9 [$M^{+\circ}$], 233.9 [$M+H^+$] 201.2 [$M - OCH_3^+$] [$C_{13}H_{12}O_4$] (Figures 2,3,4 and 5) (Silverstein *et al*., 1991)

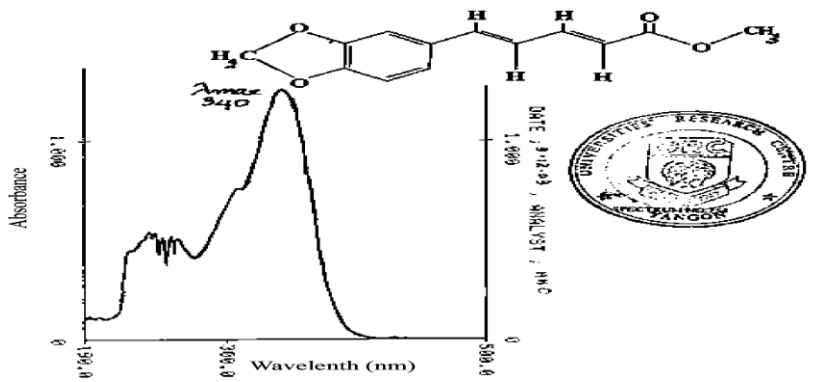


Figure 2 : Ultraviolet spectrum of isolated compound from fruit of *P. longum* (MeOH)

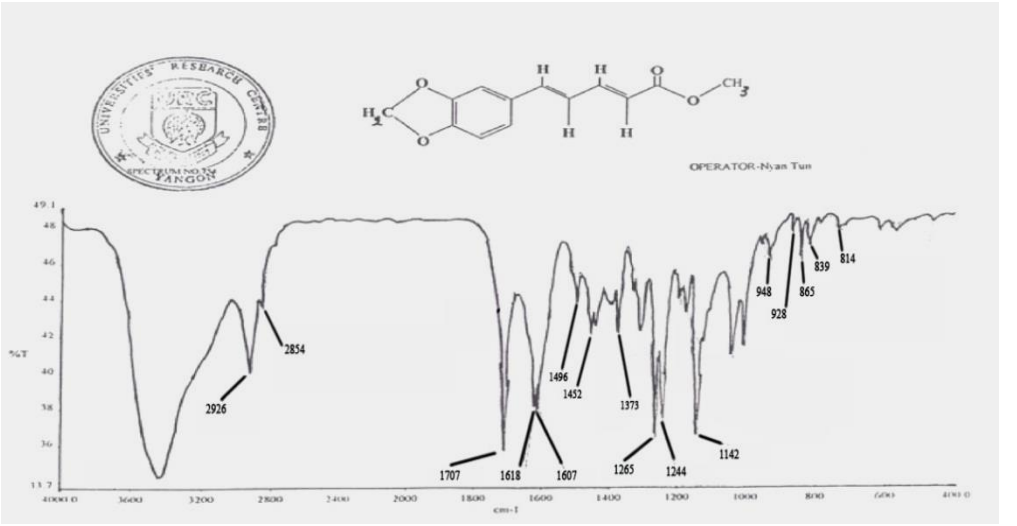


Figure 3: FT IR spectrum of isolated compound from fruit of *P. longum* (KBr)

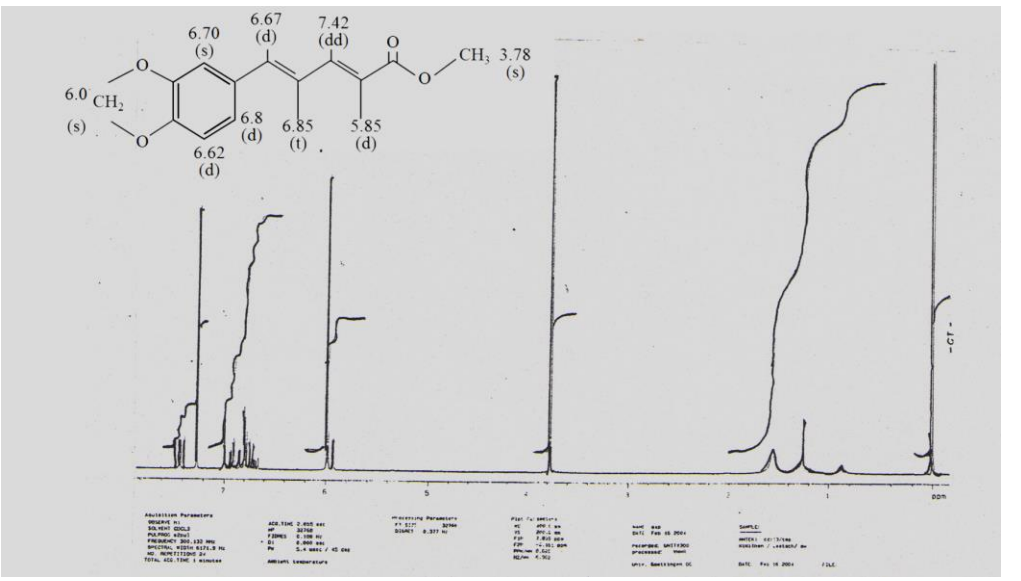


Figure 4: ^{1}HNMR (300 MHz, $CDCl_3$) spectrum of the isolated compound from fruit of *P. longum*

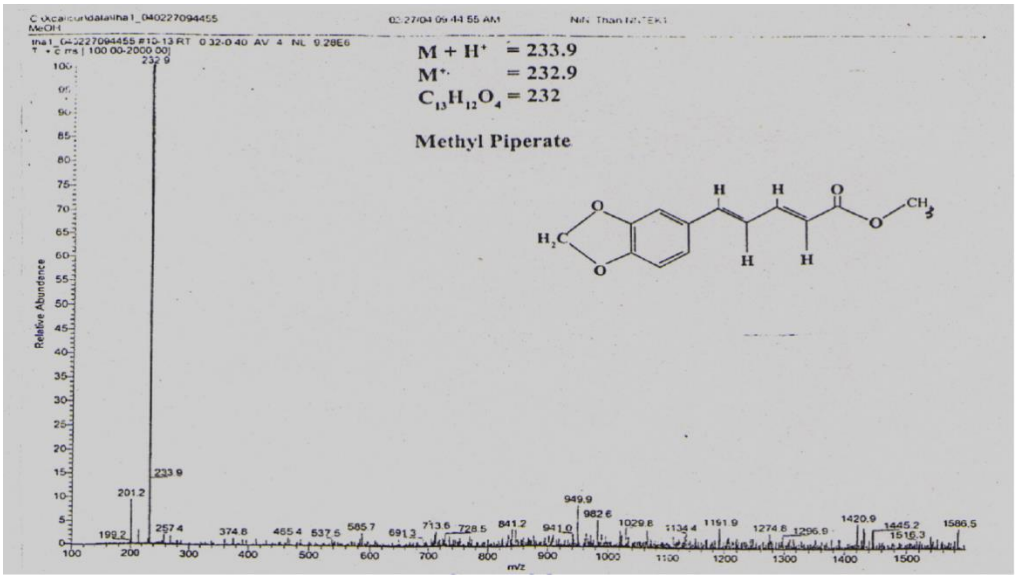


Figure 5: EI MS spectrum of the isolated compound from fruit of *P.longum*

Screening of Antibacterial Activity of Crude Extracts and Isolated Methyl Piperate

Screening of antibacterial activity of 4 crude extracts has been done by agar disc diffusion method. The inhibition zone diameters of extracts tested with 19 strains of bacteria from clinical sources are shown in Table 1. The most active ethyl acetate extract with the range of inhibition zone diameter (12-20) mm against 19 strains; *Salmonella derby*, *Escherichia coli* LT, *Escherichia coli* O128, *Escherichia coli* EHEC, *Staphylococcus aureus*ATCC, *Salmonella paratyphi*, *Salmonella stanley*, *Shigella boydii*, *Salmonella pollorum*, *Shigella dysenteriae*, *Vibrio chlolerae* Inaba, *Escherichia coli* ATCC, *Pseudomonas pyocyanea*, *Vibrio cholerae* O1, *Salmonella typhi*, *Vibrio cholerae* O139, *Shigella flexneri Bacillus subtilis* and *Staphylococcus aureus*was selected for isolation of active compound. But petroleum ether extract only showed activity (12 mm) against *Pseudomonas pyocyanea.* And then antibacterial activity of ethyl acetate extract and isolated methyl piperate were being compared on 11 tested bacteria from clinical sources shown in Table 2 and Figure 6. In Table 2 it was found that the isolated methyl piperate showed more potent activity with inhibition zone diameters (16-42) mm of all

strains but ethyl acetate extract exhibited less potent to 11 strains with inhibition zone diameter (13-24) mm. According to these zone diameters, theantibacterial activity of methyl piperate against *Klebsiella* species, *Citrobacter* species, *Pseudomonas aeruginosa,Salmonella typhi*, *Escherichia coli* YCH 149,*Shigellaflexneri*, *Proteus species, Staphylococcus aureus* and *Vibrio cholerae* O1 are more potent than tetracycline (standard) except *Salmonella paratyphi* A and *Escherichia coli* ATCC. From the screening results, it can be generally deduced as follows. The EtOAc extract and isolated methyl piperate from *P. longum* were found to inhibit the tested bacteria with regard to acute diarrhoea (cholera), dysentery, pneumonia, typhoid, urinary tract infection, sepsis and abscess. In addition, methyl piperate yielded (0.084 %) from *P. longum*was employed by microtitre plate dilution method for minimum inhibitory concentration (MIC) determined with 2 strains of *Escherichia coli*, 2 strains of *S. epidermidis*, each strain of *Staphylococcus aureus* and *B. subtilis* obtained from clinical sources at Bacteriology Research Division, DMR (LM) (Table 3). The microtitre plate dilution method also elaborates the specificity, sensitivity and the least amount required for media, reagents and glassware. It also saves time and working space in conducting the experiments. The minimum inhibitory concentration (MIC), 0.03 mg/mL of methyl piperate with all tested bacteriashowed that the plant possess potent bactericidal activity on them. From the MIC elucidation, methyl piperate isolated from the fruit of Peik-chin would be more effective for the treatment in diarrhoea, urinary tract infection, food poisoning and abscess. It has antibacterial action against *E. coli* responsible for diarrhoea, *S. epidermidis* responsible for urinary tract infection, *B. subtilis* responsible for food poisoning and *Staphylococcus aureus* responsible for abscess occurred in skin, mouth and nose.

Table 1:Results of Antibacterial Activity of Four Extracts of *P. longum* on 19 Species of Bacteria

No.	Type of bacteria	Inhibition zone diameter (mm)			
		EtOAc extract	**96%EtOH extract**	**50%EtOH extract**	**PE extract**
1	*Salmonella derby*	14	-	14	-
2	*Escherichia coli* LT	16	16	20	-
3	*Escherichia coli* O128	14	7	-	-
4	*Escherichia coli* EHEC	18	8	12	-
5	*Staphylococcus aureus*ATCC	13	20	17	-
6	*Salmonella paratyphi*	12	12	-	-
7	*Salmonella stanley*	12	11	16	-
8	*Shigella boydii*	17	8	-	-
9	*Salmonella pollorum*	16	-	12	-
10	*Shigella dysenteriae*	18	12	-	-
11	*Vibrio cholerae* Inaba	12	13	13	-
12	*Escherichia coli* ATCC	15	-	-	-
13	*Pseudomonas pyocyanea*	20	20	12	12
14	*Vibrio cholerae* O1	20	22	-	-
15	*Salmonella typhi*	14	-	-	-
16	*Vibrio cholerae* O139	12	-	20	-
17	*Shigella flexneri*	15	12	-	-
18	*Bacillus subtilis*	12	10	11	-
19	*Staphylococcus aureus*	20	20	20	-

(-) = no activity
Disc diameter = 6 mm

Table 2: Antibacterial Activity of EtOAc Crude Extract and Methyl Piperate, Isolated from Fruit of *P. longum*

Sample	Inhibition zone diameter (mm)										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
EtOAc Crude Extract	14	20	19	21	21	18	23	18	13	2031	24
Methyl piperate	38	24	31	16	42	42	37	30	19	-	32
Blank	-	-	-	-	-	-	-	-	-	-	-
EtOAc solvent (control)	-	-	-	-	-	-	-	-	-	29	-
Tetracycline (standard)	13	25	25	25	15	10	25	12	-		29

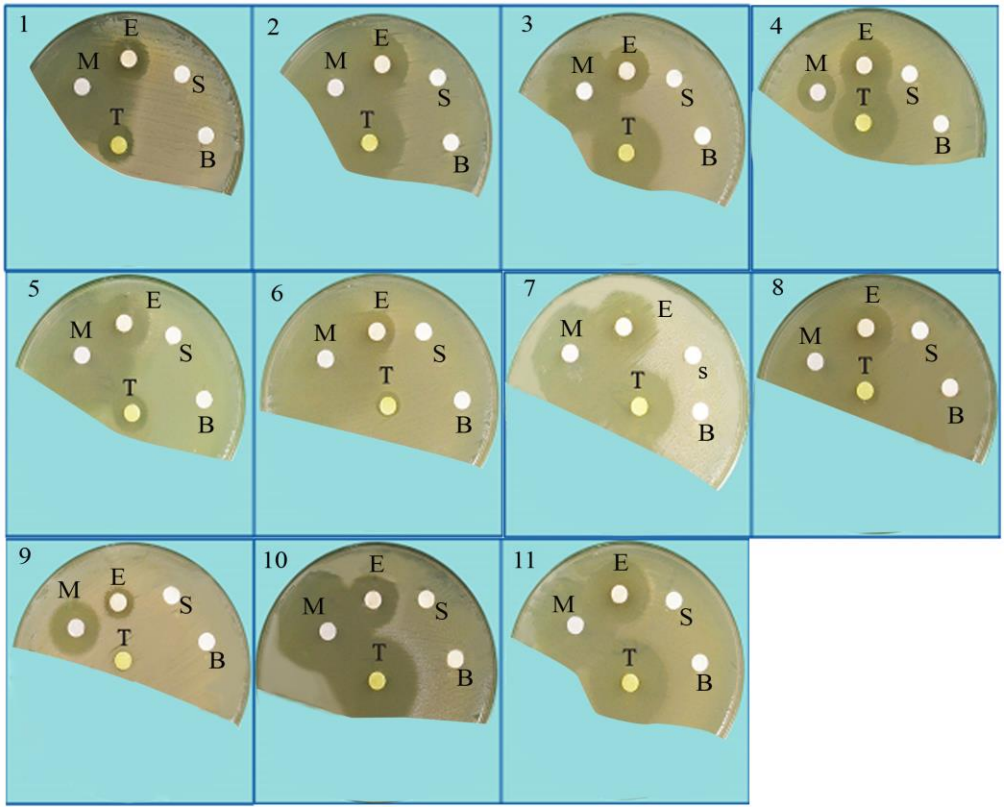
Tested Bacteria (From Clinical Sources*)

1 = *Klebsiella* species
2 = *Salmonella paratyphi* A
3 = *Citrobacter* species
4 = *Escherichia coli* ATCC
5 = *Pseudomonas aeruginosa*
6 = *Salmonella typhi*
7 = *Escherichia coli* YCH 149
8 = *Shigellaflexneri*
9 = *Proteus* species
10 = *Staphylococcus aureus*
11 = *Vibrio cholerae* O1

Disc diameter = 6 mm

- = no activity

***** National Health Laboratory (NHL), Yangon



1 = *Klebsiella* species
2 = *Salmonella paratyphi* A
3 = *Citrobacter* species
4 = *Escherichia coli* ATCC
5 = *Pseudomonas aeruginosa*
6 = *Salmonella typhi*
7 = *Escherichia coli* YCH 149
8 = *Shigellaflexneri*
9 = *Proteus* species
10 = *Staphylococcus aureus*
11 = *Vibrio cholerae* O1

E = EtOAc crude extract (Peik-chin)
M= Methyl piperate
S = EtOAc solvent (control)
B = Blank
T = Tetracycline (standard)

Figure 6: Antibacterial activity of EtOAc crude extract and methyl piperate, isolated from *P. longum* (Peik-chin)

Table 3: Minimum Inhibitory Concentration (MIC) of Active Isolated Compound Methyl Piperate (mg/mL) of *P. longum*

No.	Bacteria	MIC(mg/mL)
1	*Escherichia coli* LT	0.03
2	*Staphylococcus epidermidis* (MKL-50)	0.03
3	*Staphylococcus epidermidis* (MKL-68)	0.03
4	*Escherichia coli* EHEC	0.03
5	*Staphylococcus aureus* (WS)	0.03
6	*Bacillus subtilis* T-34	0.03

Conclusion

From the fruit of *P. longum* L., four crude extracts: PE extract (1.5 %), EtOAc extract (3.5 %), 96 % EtOH extract (4.5 %) and 50 % EtOH extract (6.2 %) were obtained and screened the antibacterial activity against 19 tested bacteria by agar disc diffusion method. Among the four crude extracts of Peik-chin, only EtOAc extract showed the most potent antibacterial activity with the related larger zone diameter (12-20) mm on 19 bacterial strains; *Salmonella derby*, *Escherichia coli* LT, *Escherichia coli* O128, *Escherichia coli* EHEC, *Staphylococcus aureus*ATCC, *Salmonella paratyphi*, *Salmonella stanley*, *Shigella boydii*, *Salmonella pollorum*, *Shigella dysenteriae*, *Vibrio chlolerae* Inaba, *Escherichia coli* ATCC, *Pseudomonas pyocyanea*, *Vibrio cholerae* O1, *Salmonella typhi*, *Vibrio cholerae* O139, *Shigella flexneri*, *Bacillus subtilis* and *Staphylococcus aureus*. Using column chromatographic separation, pale yellow crystal (0.084 %) was isolated from the most active EtOAc extract of *P. longum* (Peik-chin) and identified as methyl piperate by UV, FT IR, ^{1}HNMR and EI MS spectrometry.

In vitro antibacterial activity of ethyl acetate crude extract and methyl piperate was also investigated. It was found that methyl piperate showed the range of inhibition zone diameter between (16-42) mm whereas inhibition zone diameter of EtOAc extract ranged between (13-24) mm. It may be concluded that the antibacterial activity of methyl piperate, pure compound was more potent than that of the crude EtOAc extract against 11 tested

bacteria; *Klebsiella* species, *Salmonella paratyphi* A, *Citrobacter* species, *Escherichia coli* ATCC, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli* YCH 149, *Shigellaflexneri, Proteus* species, *Staphylococcus aureus* and *Vibrio cholerae* O1. They are effective for the treatment of dysentery, urinary tract infection, food poisoning, abscess and diarrhoea.

Minimum inhibitory concentration (MIC) values of methyl piperate against six tested bacteria were determined by using microtitre plate dilution method. Then MIC value of methyl piperate was found to be the same 0.03 mg/mL against all six bacteria; *E.coli* LT, *S. epidermidis* (MKL-50), *S. epidermidis* (MKL-68), *E.coli* EHCC, *S. aureus* (WS) and *B. subtilis* T-34. From these observations, it may be recommended that the ethyl acetate extract of fruit of *P. longum*(Peik-chin) and isolated methyl piperate may be used as main materials for the traditional medicine formulation in the treatment against dysentery, urinary tract infection, food poisoning, abscess and diarrhoea.

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