

## Some Bioactive Constituents from the Fruits of *Piper longum* Linn. (Peik-Chin) and Their Antibacterial Activities

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### Abstract

The fruits of *Piper longum* Linn. (Peik- Chin) used in the treatment of diarrhoea and dysentery in traditional Myanmar medicinal system were chosen for present study. At first, four crude extracts of this plant were investigated *in vitro* antibacterial activity on 20 microorganisms of bacteria by using agar disc diffusion method. Among the four crude extracts, the most active EtOAc extracts was isolated by using chromatographic method. Two isolated compounds, methyl piperate (0.084%) and piperine (1.80%) were identified by m.pt, TLC and spectrometric method and tested on 11 microorganisms by agar disc diffusion method. In addition Minimum Inhibitory Concentration (MIC) for piperine was determined and found to be 0.16 mg/ml on tested microorganism, *E.coli* 2.

**Key words:** *Piper longum* Linn. , Piperine, Methyl piperate and Antibacterial activities

### Introduction

Diarrhoea and dysentery are important health problems worldwide especially developing countries. So the Government of Myanmar has initiated a national programme for the development of Traditional Medicine System in 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 (Mukerji, 1953).

Dysentery is an inflammatory disorder of the lower intestinal tract, usually caused by bacterial, parasitic, 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 (Khan, 2001).

In this study, Myanmar medicinal plant, *Piper longum* Linn. (Peik-Chin) was selected to find out of active principle for the treatment of dysentery and diarrhoea. It is cultivated in India, Malaysia, Singapore and Bangladesh (Wealth of India, 1950). In Myanmar, Peik-Chin is cultivated abundantly in Mon,Kayin States and hilly regions of Northern Myanmar (Hundley & Chit Ko Ko, 1987). The alkaloid, piperine is a major constituent responsible for the bitter taste of *P.longum* L. (Watt, 1972). It occurs in fruits and roots of *P. longum* Linn. (Joshi *et al.*,1968).

In Myanmar, *P.longum* L. is used in treating diarrhoea, fever, stomachic and asthma (Han Tun, 1993; Pyin Nyar, 1994).

Therefore, antibacterial activity investigation on four crude extracts and some phytoconstituents from *P. longum* L. has been carried out by using agar disc diffusion method. Minimum Inhibitory Concentration (MIC) was determined by microtitre plate dilution method for major constituent from *P. longum* L.

Methyl piperate and piperine have been isolated from *P. longum* Linn. by chromatographic separation method. The structures of these compounds have been identified by melting point determination, UV, FT IR, NMR and Mass spectrometric methods.

### Outstanding Characters of *Piper longum* Linn.

Scientific Name	- <i>Piper longum</i> Linn.
Myanmar Name	- Peik-Chin
English Name	- Long Pepper
Family Name	- Piperaceae
Genus	- Piper
<b>Distribution</b>	- It is cultivated in India, Malaysia, Singapore and Bangladesh. The principal exporters are Afghanistan, Sri Lanka and Pakistan.
<b>Distinguishing characters-</b>	Roots, stems creeping, leaves 5-9 cm long, 3-5 cm wide, ovate, cordate with broad rounded lobes at base, subacute, entire, glabrous; spikes cylindrical pedunculate, male larger and slender, female 1.3-2.5 cm long and 4-5 mm diameter. Fruit is very small, ovoid, completely sunk in solid fleshy spike which is 2.5-3.8 cm, blackish green, shining (Figure 1). The plant flowers in August and September, and the fruit matures in January.
<b>Part used</b>	- Fruits



Figure 1 -The plant of *Piper longum* Linn.

### Materials and Methods

General procedures: Melting point determination;  $^1\text{H}$  (300 MHz) NMR: UNITY-300,  $\text{CDCl}_3$  with TMS as in standard,  $^{13}\text{C}$  (75 MHz) NMR: Shimazu UV-240, (EtOH); FT IR: Perkin Elmer GX system, KBr; CC: Merck Silica gel 60 (70-230) mesh: eluents: Petroleum - ether (PE)-ethyl acetate(EtOAc), (in order of increasing polarity), only ethylacetate; TLC; 0.25 mm pre-coated silica gel 60 (F<sub>254</sub>, Merck), solvent system: (PE:EtOAc) (4:1) (v/v), spots were detected by inspection under UV light (254 nm or 365nm) or by the colour developed with anisaldehyde-sulphuric acid spraying followed by heating.

### Plant materials

The fruits of *P. longum* L. were collected from Mawlamyine, Mon State. The plant was identified at Department of Botany, Yangon University. The fruits of *P. longum* L. were

cleaned and dried at room temperature for one week. Then these were made powder and stored in air-tight container.

### ***In vitro* studies on the antibacterial activity of *P. longum* L.**

#### **Agar disc diffusion method**

This method was used for the detection of antibacterial activity in four crude extracts and some isolated compound from *P. longum* L. The test procedure was as follows. At first, the extracts (1g each for testing 20 species of bacteria) were introduced into sterile petridishes and dissolved in 1 ml of their respective solvents such as, petroleum ether, ethylacetate, ethanol and 50% ethanol.

Discs obtained by filter paper (Toyo No.26, Japan) punched to 6 mm diameter, were used to impregnate the extracts. To obtain approximately 20 $\mu$ g/disc and prior to adherence on the culture plates the discs were allowed to dry at 42°C incubator.

The bacterial suspension from trypticase soy broth was streaked evenly into three planes on the surface of the trypticase soy agar plates with sterile cotton swab (Puritan, USA). After the inoculums had dried for 5 minutes, 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 placed alongside the test discs for control and comparing purposes known antibiotics tetracycline was also used as positive control.

The plates were incubated immediately (or) within 30 minutes after inoculation. After overnight incubation at 37°C, the zones of inhibition diameter including 6 mm discs were measured, by means of a thin transparent ruler or by a divider.

#### **Determination of minimum inhibitory concentration (MIC) by Microtitre Plate dilution method**

Microtitre plate dilution method was done by using trypticase soy broth by dissolving with appropriate soluble compound in 2-fold dilutions. The constant bacterial organisms per ml after culturing in 37°C broth culture was then seeded in 96-well plates.

The active compound was dissolved with ethylacetate and diluted with trypticase soy broth to obtain the following concentration: 5mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.32mg/ml, 0.16 mg/ml, 0.08 mg/ml, 0.04 mg/ml, 0.02 mg/ml, 0.01 mg/ml, 0.005 mg/ml, in 96-well microtitre plates.

Then 0.02 ml of the already prepared inoculum was introduced to its respective wells and the microtitre plates were incubated at 37°C for 18 hours. Prior to taking spectrophotometer readings, contents of all wells were thoroughly mixed with a multi-channel pipetter to resuspend clumped cells at the bottom of the wells in a solution. Growth of the microorganisms was determined by absorbance at 450 nm and automated microplate reader (Bio Rad) as well as confirming by culturing onto trypticase soy agar was subjected to incubation at 37°C for overnight. The last well with no growth of the microorganisms was taken to represent the Minimum Inhibitory Concentration (MIC) of the compound.

The isolated compound, piperine, from *P. longum* L. was tested with, *Escherichia coli* 1, *Escherichia coli* 2, *Escherichia coli* 3, *Staphylococcus aureus* 1, *Staphylococcus aureus* 2 by microtitre plate dilution method. Their Minimum Inhibitory Concentration (MIC) values were reported and discussed in Table 3 and Figure 12. The Minimum Inhibitory Concentration (MIC) values were considered as the lowest concentration of a drug or compound that does not completely inhibit the growth of the microorganism.

## Extraction and isolation of chemical constituents from *Piper longum* Linn.

### Preparation of extracts

The air-dried powder (1000g) was cold extracted with (3000 cm<sup>3</sup>) of various solvent such as petroleum-ether (60-80°C), ethylacetate, ethanol, 50% ethanol, respectively for 7 days and then filtered. The filtrate was evaporated to dryness at normal pressure on a water bath and desiccated. Then the dried extracts weighed which were obtained petroleum-ether extract (1.5%), ethylacetate extract (3.5%), ethanol extract (4.5%) and 50% ethanol extract (5.0%) yielded respectively.

### Isolation of Phytoconstituent from EtOAc Extracts of *Piper longum* Linn.

The column was packed with silicagel (400g) by the wet method using petroleum-ether the column was eluted consecutively with the solvent system (v/v)(PE : EtOAc) (19:1, 9:1, 4:1) and only ethylacetate according to their increasing polarity. The column was completely filled with the solvent system and fractionation was started. Flow rate was adjusted to about 1 drop per second. 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 removed. Finally, four compounds (0.084%, 0.086%, 0.105% & 1.80%) were obtained respectively after combining the similar fractions.

## Results and Discussion

Screening of antibacterial activity of 4 crude extracts has been done by agar disc diffusion method. In the present work it was tested with 20 strains of bacteria as shown in Table 1. Furthermore, isolated compounds, methyl piperate and piperine were investigated antibacterial activity with 11 organisms as shown in Table 2 and Figure 11. According to these results, both compounds showed potent antibacterial activity with respect to various inhibition zone diameters. In addition, piperine, major constituent from *P. longum* L. was employed for Minimum Inhibitory Concentration (MIC) determination with 3 strains of *Escherichia coli* and 2 strains of *Staphylococcus aureus* as shown in Table 3 and Figure 12. The lowest Minimum Inhibitory Concentration (MIC) of 0.16 mg/ml was obtained with *Escherichia coli* 2 that were found to be more effective than the others from this result.

**Methyl piperate** : Yellow crystal (0.12g, 0.084% yield); m.p. 140-141°C;  $\lambda_{\max}$  310,340; FT IR  $\nu$  cm<sup>-1</sup> 2926, 2854, 1707, 1678, 1607, 1496, 1452, 1373, 1265, 1244, 1172, 1442, 1040, 1003, 948, 928, 865, 839, 814, 724; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta_{\text{H}}$  (ppm) ~3.8 (3H, s, OMe) ~5.85 (2H, s, -O-CH<sub>2</sub>-O-) ~6.0 (<sup>1</sup>H, d, H1) ~6.62 (<sup>1</sup>H, d, H4) ~6.67 (<sup>1</sup>H, d, H6) ~6.70 (<sup>1</sup>H, d, H5) ~6.8 (<sup>1</sup>H, d, H3) ~6.85 (<sup>1</sup>H, t, H2) ~7.42 (<sup>1</sup>H, dd, H7); ESI-MS 233.9[M+H<sup>+</sup>] 232.9[M<sup>+</sup>] 232[C<sub>13</sub>H<sub>12</sub>O<sub>4</sub>] (Figure 2-4, 9) (Silverstein *et al.*, 1991).

**Piperine** : Yellow crystal (2.57g, 1.80% yield); m.p. 128-130°C;  $\lambda_{\max}$  309,340; FT IR (cm<sup>-1</sup>) 3009, 2940, 2920, 2862, 1634, 1612, 1584, 1491, 1449, 1253, 1194, 1032, 1018, 1134, 997, 847, 831, 805; <sup>1</sup>H NMR  $\delta$  (ppm); ref; TMS (in CDCl<sub>3</sub>), ~1.55 ~1.68 (6H, m, H2, H3, H4), ~3.55, (4H, broad s, H1, H5), ~5.95 (2H, s, H10), ~6.43 (<sup>1</sup>H, d, J=12Hz, H6), 6.73 (<sup>1</sup>H, d, J=12Hz, H12), ~6.749 (<sup>1</sup>H, s, aromatic H), ~6.77 (<sup>1</sup>H, d, J = 6Hz, aromatic H), ~6.87- 6.90 (<sup>1</sup>H, dd, H7), ~6.955 (<sup>1</sup>H, d, J=6Hz, aromatic H), ~7.37-7.44 (<sup>1</sup>H, dddd, H13); <sup>13</sup>C NMR and DEPT (ppm); ~24.7 (3C, C14, C13, C17), ~26.08 (2C, C15, C16) ~101.19 (1C, C12), ~105.58 (1C, =C=CH aromatic), ~119.91 (1C, CH=C= aromatic), ~122.42 (1C, C2), ~125.26 (1C, C4), ~130.92 (1C, C6), ~138.47 (1C, C5), ~142.97 (1C, C3), ~148.04 (1C, C8 aromatic), ~148.10 (1C, C12), ~165.35 (1C, C1) (amide); FAB MS  $\Rightarrow$  m/z (%) 286.3[M+H<sup>+</sup>], 308.1[M+Na<sup>+</sup>], 594[2M+Na<sup>+</sup>], 877[3M+Na<sup>+</sup>] 285.3 [·M<sup>+</sup>], 285[C<sub>17</sub>H<sub>19</sub>NO<sub>3</sub>] (Figure 5-8, 10).

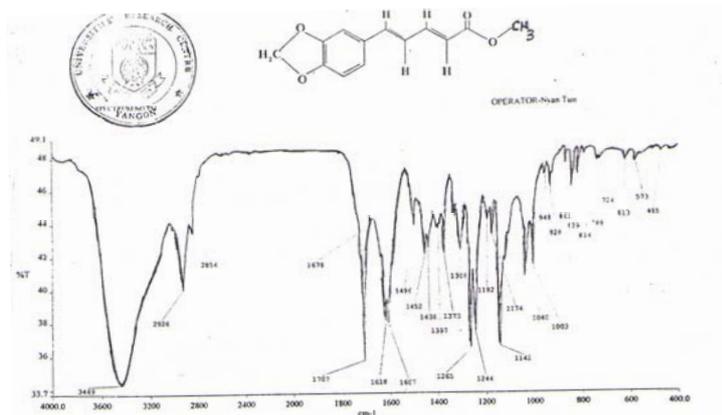


Figure 2 Infrared spectrum of isolated compound A from *P. longum* L.

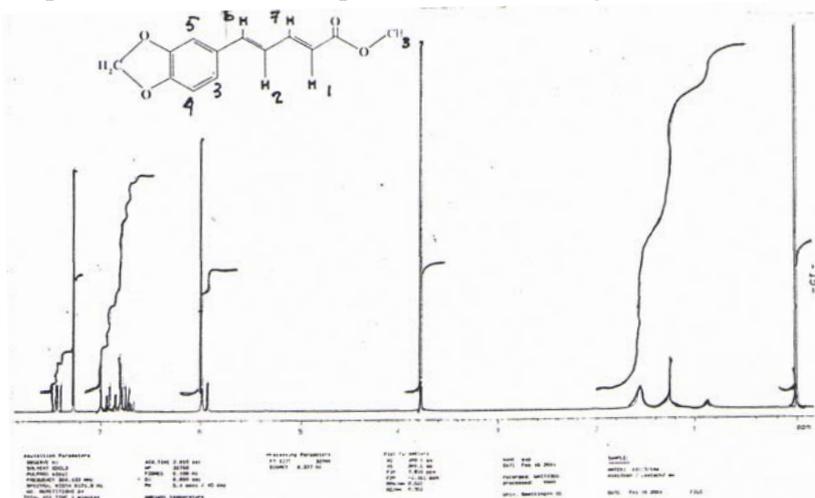


Figure 3 <sup>1</sup>H NMR (300 MHz), spectrum of isolated compound A from *P. longum* L.

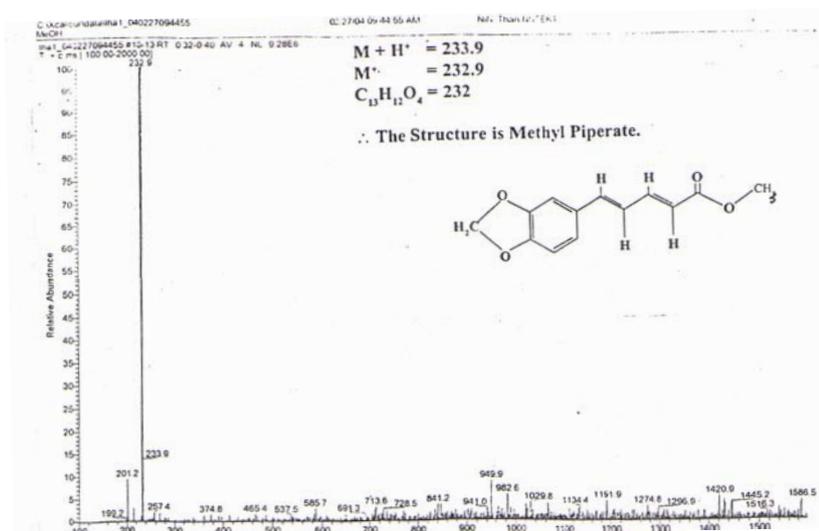


Figure 4 ESI mass spectrum of isolated compound A from *P. longum* L.

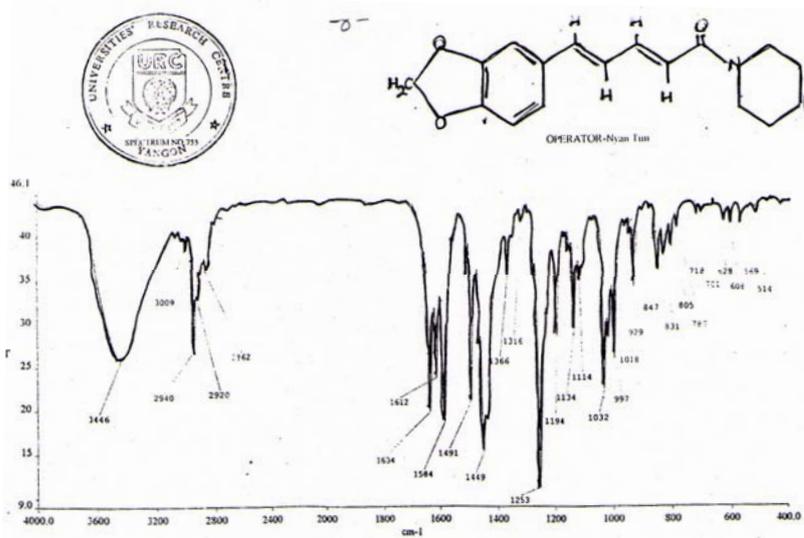


Figure 5 Infrared spectrum of isolated compound D from *P. longum* L.

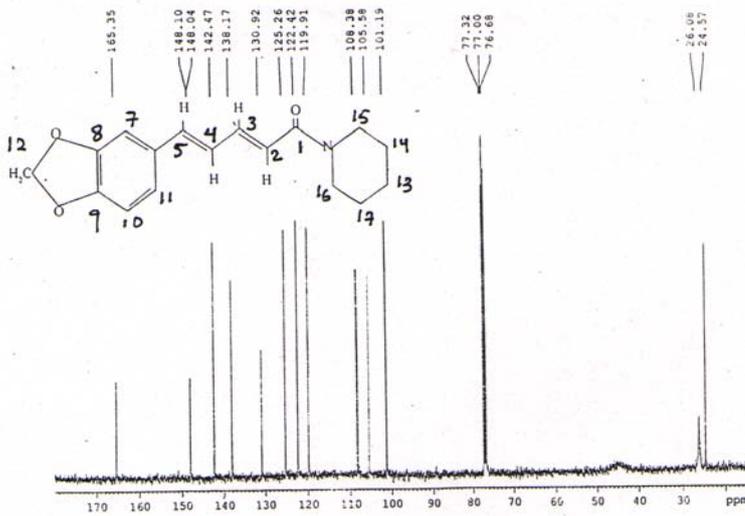


Figure 6 <sup>13</sup>C NMR spectrum of isolated compound D from *P. longum* L.

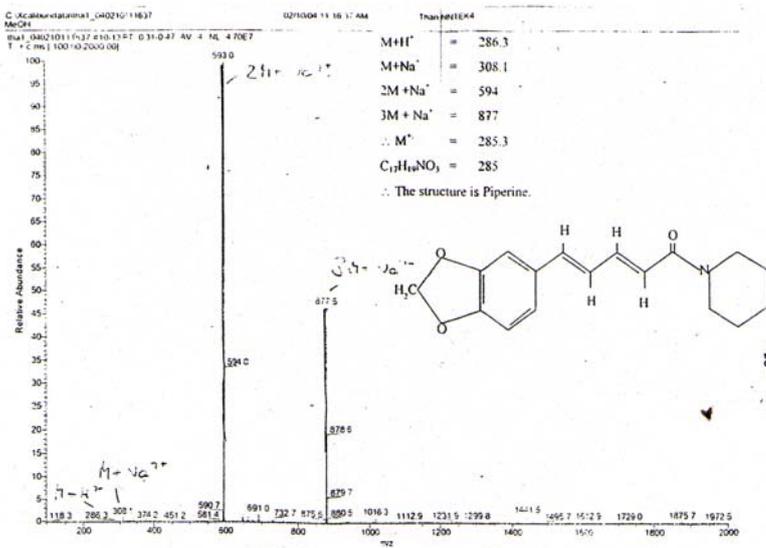


Figure 7 FAB mass spectrum of isolated compound D from *P. longum* L.

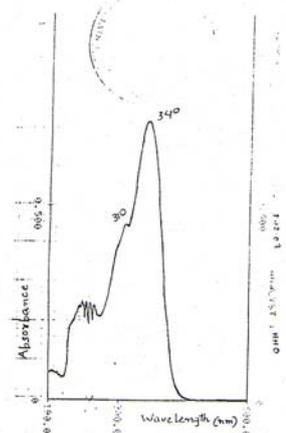
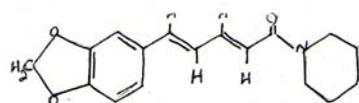


Figure 8 Ultraviolet spectrum of isolated compound D from *P. longum* L.

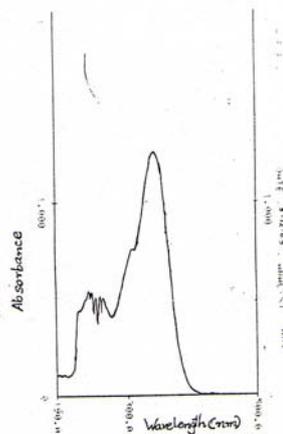
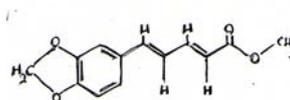


Figure 9 Ultraviolet spectrum of isolated compound A from *P. longum* L.

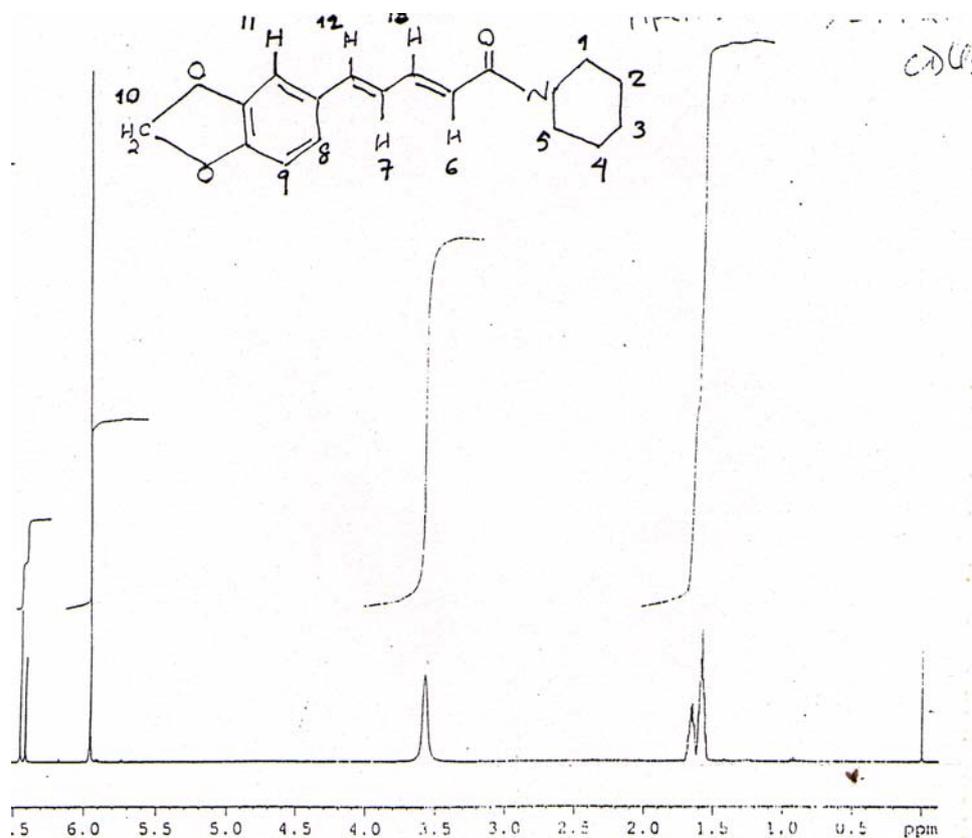


Figure 10 <sup>1</sup>H NMR (300 MHz), spectrum of isolated compound D from *P. longum* L.

Table 1 Results of antibacterial activity of various extracts on different species of bacteria

No.	Type of bacteria	Diameter of inhibition zone(mm)			
		EtOAc ext.	EtOH ext.	50%EtOH ext.	P.E.ext.
1	<i>Salmonella typhi</i>	14	-	14	-
2	<i>Escherichia coli</i> LT	16	16	20	-
3	<i>Escherichia coli</i> 0128	14	7	-	-
4	<i>Escherichia coli</i> EHCC	18	8	12	-
5	<i>Staphylococcus aureus</i>	13	20	17	-
6	<i>Salmonella paratyphi</i>	12	12	-	-
7	<i>Salmonella stanley</i>	12	11	16	-
8	<i>Shigella boydii</i>	17	8	-	-
9	<i>Salmonella pollorum</i>	16	-	12	-
10	<i>Shigella dysenteriae</i>	18	12	-	-
11	<i>Vibrio cholerae</i> Inaba	12	13	13	-
12	<i>Escherichia coli</i> 0125	15	-	-	-
13	<i>Pseudomonas pyocyanea</i>	20	20	12	-
14	<i>Vibrio cholerae</i> 01	20	22	-	12
15	<i>Salmonella typhi</i> 2	14	-	-	-
16	<i>Vibrio cholerae</i> 01	12	-	20	-
17	<i>Shigella flexneri</i>	15	12	-	-
18	<i>Staphylococcus aureus</i>	20	25	-	-
19	<i>Bacillus subtilis</i>	12	10	11	-
20	<i>Staphylococcus aureus</i>	20	20	20	-

(-) = no activity

Disc diameter = 6 mm

Table 2 Antibacterial activity of EtOAc crude extracts and isolated compounds from Peik-Chin

EtOAc Extracts and Isolated Compounds	Mean Zone Diameter (mm) on Tested Organisms										
	1	2	3	4	5	6	7	8	9	10	11
Crude (Peik-Chin)	14	20	19	21	21	18	23	18	13	20	24
Methyl piperate	38	24	31	16	42	42	37	30	19	31	32
Piperine	15	8	19	11	-	24	24	25	-	19	20
Blank disc(control)	-	-	-	-	-	-	-	-	-	-	-
EtOAc solvent (control)	-	-	-	-	-	-	-	-	-	-	-
Tetracycline(control)	13	25	25	25	15	10	25	12	-	29	29

**Tested organisms**

1 =	<i>Klebsiella</i> species	8	=	<i>Shigella flexneri</i>
2 =	<i>Salmonella paratyphi</i> A	9	=	<i>Proteus</i> species
3 =	<i>Citrobacter</i>	10	=	<i>Staphylococcus aureus</i>
4 =	<i>Escherichia coli</i> EPEC	11	=	<i>Vibrio cholerae</i>
5 =	<i>Pseudomonas aeruginosa</i>			
6 =	<i>Salmonella typhi</i>			Disc diameter = 6 mm
7 =	<i>Escherichia coli</i>			- = no activity



Figure 11 Antibacterial activity of EtOAc crude extracts and some isolated compounds from Peik-Chin

- H = *Shigella flexneri*                      J = *Staphylococcus aureus*  
 K = *Vibrio cholerae*                      1 = EtOAc crude extracts. (Peik-Chin)  
 2 = Methyl piperate                      3 = Piperine  
 7 = Blank disc (control)                  8 = EtOAc solvent (control)  
 T = Tetracycline (control)

Table 3 Minimum inhibitory concentration of active isolated compound, Piperine (mg/ml) of *Piper longum* Linn.

No.	Bacteria	MIC(mg/ml)
1	<i>Escherichia coli</i> 1	0.32
2	<i>Escherichia coli</i> 2	0.16
3	<i>Escherichia coli</i> 3	0.32
4	<i>Staphylococcus aureus</i> 1	0.625
5	<i>Staphylococcus aureus</i> 2	0.625

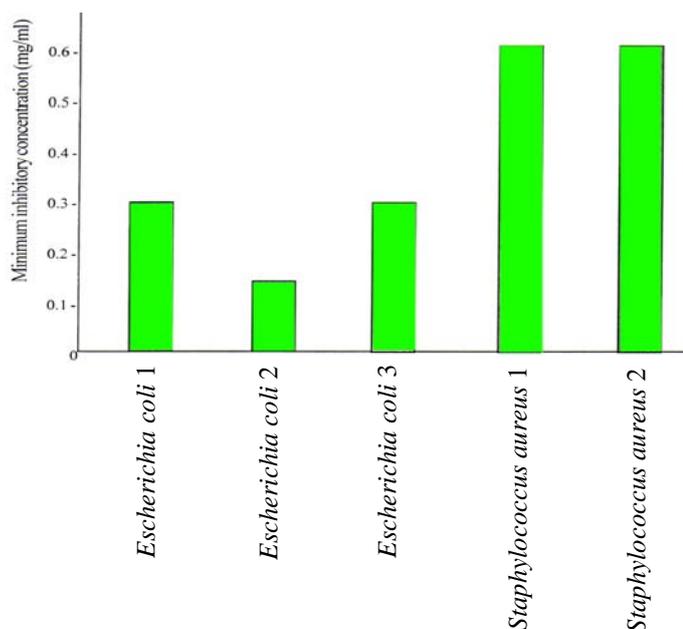


Figure 12 Minimum inhibitory concentration of Piperine from *Piper longum* Linn.(EtOAc) in different bacteria by using microtitre plate dilution method

### Conclusion

Four crude extracts, the isolated compounds: methyl piperate and piperine from *P. longum* L. (Peik-Chin) were investigated *in vitro* antibacterial activity using agar disc diffusion method. Minimum Inhibitory Concentration (MIC) values for isolated compounds (just tested) were determined by using microtitre plate dilution method.

Among the four crude extracts of Peik-Chin, only EtOAc extracts is the most potent to twenty tested bacteria.

Two compounds, methyl piperate (0.084%) and piperine (1.80%) were isolated by chromatographic separation and identified by mpt, UV, FT-IR, NMR, Mass spectroscopy.

The isolated compounds: methyl piperate and piperine from EtOAc extract of *P. longum* L. showed potent antibacterial activity with various zone diameters of the growth inhibition on 11 organisms including *Escherichia coli* and *Staphylococcus aureus*. According to larger zone diameter tested on 11 organisms, methyl piperate was more effective than piperine for the treatment of dysentery and diarrhoea. Then MIC values for piperine were found to be 0.16 mg/ml in *Escherichia coli* 2. From these observations, it may be recommended that the fruit powder or the ethylacetate extract of *P. longum* L. (Peik-Chin) may be used as main materials for the traditional medicine formulation for the treatment against dysentery and diarrhoea.

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