[PHAR06] Anti *Helicobacter pylori* from extracts of ten species of *Phyllanthus* sp. with special emphasis on chloroform extracts of *Phyllanthus pulcher*

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Introduction

Some *Phyllanthus* species have been traditionally used as remedies for gastric ailments which may be associated with *Helicobacter pylori's* infection (Van Holthoon, 1999). However, no scientific observations on the treatment of this infection using *Phyllanthus* sp. were reported.

Today, treatment for various human upper gastrointestinal tract disorders such as chronic gastritis, peptic ulcer and gastric cancer infected by *H. pylori* are based on seven days triple therapies combining a proton pump inhibitor (PPI) and 2 antimicrobial agents (clarithromycin [C], amoxicillin [A], metronidazole [M]). However, these modern treatments are not 100% efficient (de Corwin, 2004). Now, researches on anti *H. pylori* substances are looking back to the natural products rather than creating new antibiotics or drugs. In this study, plants from genus *Phyllanthus* were selected for their anti *H. pylori* potential agent as an alternative to the current treatments of this bacteria.

Materials and Methods

Plants Material

Ten species of *Phyllanthus* sp. *(P. acidus, P. columnaris, P. debilis, P. emblica, P. myrtifolius, P. niruri, P. oxyphyllus, P. pulcher, P. reticulatus* and *P. urinaria*) were collected around Penang.

Preparation of Extracts

Dried whole plants of *P. debilis, P. myrtifolius, P. niruri* and *P. urinaria* and leaves from *P. acidus, P. columnaris, P. emblica, P. oxyphyllus, P. pulcher* and *P. reticulates* were extracted using soxhlet extraction with non polar solvent hexane, followed by chloroform and methanol. Then, extracts of each solvent were evaporated and the final dry powdered extracts were used for the bioassays. Each sample was diluted with dimethyl sulfoxide (DMSO) in 20mg/ml concentration.

Bacteria's Preparation

H. pylori (S179) stock culture was subcultured on Eugon Agar Blood (EAB) and incubated in 10% CO_2 at 37ºC for 84 hours. Nine other common bacterial pathogens such as *Enterobacter* sp. (E114), *Escherichia coli, Escherichia coli* (ATCC 25920), *Pseudomonas slutzeri, Salmonella* sp., *Shigella bodyii, Sh. dysenteriaceae, Staphylococcus aureus* and *Vibrio cholerae* were subcultured on Mueller Hinton Agar (MHA) and incubated at 37ºC for 24 hours.

Antimicrobial Activity Assay

Disc diffusion method used for screening of the antimicrobial activity assay. All the bacterial cultures were diluted and compared to a standard 5.0 McFarland. The disc was treated with 20µl of the extract. The control experiment was disc diffused with DMSO and distilled water (dH_2O). Clear inhibition zones around the disc on the agar indicated the presence of antimicrobial activity.

Toxicity Test

Brine shrimp (*Artemia salina)* eggs were hatched in artificial sea water (35‰ salinity) using a petri dish and incubated under a 60 W lamp, providing direct light and heat (24-26ºC) for 12 hours. Each sample extracts were diluted in concentrations of 1024µg/ml, 512µg/ml, 256µg/ml, 128µg/ml, 64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml and 1µg/ml in sea water up to 900µl per vials. Then, 100µl of the shrimp solution (containing 10 organisms) were added into each vial. Vials containing DMSO and sea water served as controls. 50% lethality concentrations (LC_{50}) acute and chronic were observed after 6 hours and 24 hours.

Phytochemistry Test

This test was conducted only for the most potential extract. Alkaloids and terpenoids test were conducted by thin layer chromatography (TLC) (Harborne, 1998). Then, compounds in the extract were separated by column

chromatography (CC). Each fraction was screened again onto *H. pylori* (S179) and the bacterial pathogens. Toxicity test was also done on the selected fractions.

Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration test was conducted using the agar dilution method. The best fraction was tested onto 20 strains of *H. pylori* in the petri dish. Serial dilution of the fraction was prepared in concentrations of 256µg/ml, 128µg/ml, 64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml and 1µg/ml. The control experiment for this test was EAB with DMSO and dH_2O. The results were determined by positive or negative growth of the *H. pylori* onto the agar.

Time Kill Assays

Three types of time kill assays experiments design were done on the best fraction. The first experiment was conducted to find out the best concentration fraction to kill *H. pylori* in the Eugon Broth Blood (EBB) compared to concentration in solid agar (EAB) in MIC test. Second experiment was conducted to observe for killing mechanism of the fraction onto *H. pylori* depending to different times. Whereas, third test was planned to reveal the main factor between time factor and media factor in killing the bacteria.

All the experiment designs were prepared in centrifuge tubes containing EBB double stranded, inoculums of *H. pylori* (S179) and the best fraction.

In the first experiment, the fractions were prepared in concentrations 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml and 2µg/ml in each centrifuge tube. At 12, 24, 36, 48, 60, 72 and 84 hours of incubation, samples were removed from each tube and microdilution were made in dH_2O for triplicate to determine the viable counts on EAB. At the same time, one dilution from the microdilution sample was selected to observe for total counts under light microscope. The viable counts were referring to colony of *H. pylori* on the agar. Then, the total counts were to determined amount of spiral form and coccoid form of *H. pylori* in a sample.

For second experiment, only the best concentration from the first experiment was used. The extract at concentration 16µg/ml was added into the centrifuge tubes at different period incubations i.e. 0, 12, 24, 36, 48, 60, 72, 84 and 96 hours. The viable counts and the total counts also were done in the test.

While for third experiment, the samples in the centrifuge tubes were prepared in two sets. In the first set, the extracts were added into tubes at 24 and 48 hour of incubation without to change new EBB. However, in second set, the EBB was changed to fresh media before the extracts were added. Viable counts and total counts were also recorded.

Killing mechanism of *H. pylori* (S179) by the best fraction was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Lastly, photochemical analysis was conducted to detect the major compounds in the fraction.

Results

Anti H. pylori screening test from the Phyllanthus plant extracts

From the screening test by disc diffusion method, 29 out of 30 extracts inhibited *H. pylori* (Table 1). Only methanol extract of *P. acidus* did not inhibit the bacteria. While, hexane extract of *P. debilis, P. myrtifolius, P. niruri* and *P. oxyphyllus,* chloroform extract of *P. acidus, P. myrtifolius, P. niruri, P. oxyphyllus* and *P. pulcher* and methanol extract of *P. myrtifolius* and *P. pulcher* reacted specifically to *H. pylori.* Among all the chloroform extract of *P. pulcher* showed the biggest inhibition zone (24.17 ± 1.2mm) to *H. pylori.*

Most of the samples' extract are not toxic to *A. salina* accept chloroform extract of *P. oxyphyllus* and hexane extract of *P. pulcher* (Table 2).

TABLE 1 Disc diffusion test from30 samples extracts of *Phyllanthus* plants on *H. pylori* (S179) and bacterial pathogens

Species	Solvent	*Entrobacter*	*E. coli*	*E. coli* (ATCC 25922)	*P. stutzeri*	*Salmonella* sp.	*Sh. boydii*	*Sh. dysentry*	*S. aureus*	*V.cholera*	*H. pylori* (S179)
1.*P.acidus*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	6.67±0.47	13.5±0.5
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11.5±0.5
	M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2.*P.columnaris*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	9.17±0.69	11±0
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	7±0	10.5±0
	M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	7±0	10±0
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3.*P.debilis*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	12.83±0.9
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	7.5±0.5	22±2.4
	M	S8.33±0.47	S12.3±0.37	9.17±0.69	10.33±0.47	15.17±0.37	7.67±0.47	16±0.82	7.83±0.37	-ve	22±2.1
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4.*P.emblica*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	7.33±0.47	12±0
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	9±0	12.5±0.5
	M	10±0	S15±0	S15.17±0.37	15.17±0.37	16.17±0.9	10±0	17.83±0.69	10.17±0.37	16.67±0.47	13±0
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5.*P.myrtifolius*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	13±0
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11±0
	M	S7±0	-ve	-ve	7±0	S9±0	7±0	9.67±0.47	9.5±0.5	-ve	8±0
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

TABLE 1 (Continued from previous page)

Species	Solvent	Entrobacter	E. coli	E. coli (ATCC 25922)	P. stutzeri	Salmonella sp.	Sh. boydii	Sh. dysentry	S. aureus	V.cholera	H. pylori (S179)
6.*P.niruri*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	19.67±0.5
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	18.5±0.5
	M	-ve	S12±0	10.5±0.5	9.67±0.47	S11.83±0.37	-ve	14.33±0.75	-ve	10.83±0.69	9±0
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7.*P.oxyphyllus*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11.5±0.5
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	12±0
	M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	8.5±0.5
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8.*P.pulcher*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	S7.5±0.5	20±0
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	24.17±1.2
	M	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	19.5±0.5
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
9.*P.reticulatus*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	8.33±0.47	12.5±0.5
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	7.5±0.5	17±0
	M	-ve	12±0	11.83±0.37	11.83±0.37	14.67±0.47	-ve	16.33±0.94	-ve	15±0.5	12.5±0.5
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10.*P.urinaria*	H	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	9.83±0.37	13±2
	K	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	10.33±0.47	14.83±2.2
	M	7±0	S11±0.75	S10±0	10±0	10.33±0.47	7±0	14.33±0.47	7.83±0.37	S7.17±0.47	13.67±3.7
	D	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

TABLE 2 Toxicity test from 30 sample extracts of *Phyllanthus* sp. plants to *A. salina*

SAMPLE	HEXANE		CHLOROFORM		METANOL	
	ACUTE (mg/ml)	CHRONIC (mg/ml)	ACUTE (mg/ml)	CHRONIC (mg/ml)	ACUTE (mg/ml)	CHRONIC (mg/ml)
1.*P.acidus*	2.831 ± 0	1.697 ± 0.401	2.625 ± 0.730	1.340 ± 0	2.317 ± 0.722	2.176 ± 0.702
2.*P.columnaris*	4.947 ± 1.417	3.397 ± 0.954	5.896 ± 1.615	3.222 ± 0.983	6.810 ± 2.09	4.262 ± 1.464
3.*P.debilis*	3.420 ± 1.046	1.557 ± 0	2.498 ± 0.753	1.541± 0	2.573 ± 0.807	1.535 ± 0
4.*P.emblica*	8.706 ± 1.054	2.737 ± 0	2.378 ± 0.728	1.476 ± 0.406	2.497 ± 0.763	1.417 ± 0
5.*P.myrtifolius*	1.350 ± 0	1.000 ± 0.245	2.628 ± 0.736	1.076 ± 0.404	2.654 ± 0	1.878 ± 0.476
6.*P.niruri*	3.262 ± 0.789	1.805 ± 0.808	2.428 ± 0.799	1.502 ± 0	8.282 ± 2.98	4.362 ± 1.508
7.*P.oxyphyllus*	1.522 ± 0.604	1.926 ± 0.267	3.343 ± 1.076	0.916 ± 0.263	6.524 ± 1.793	5.677 ± 0
8.*P.pulcher*	1.344 ± 0.355	0.450 ± 0.89	2.124 ± 0.752	1.252 ± 0.378	7.674 ± 2.114	2.093 ± 0.907
9.*P.reticulatus*	8.357 ± 3.0	2.504 ± 1.082	2.412 ± 0.726	1.316 ± 0.415	2.371 ± 0.714	1.308 ± 0.428
10.*P.urinaris*	2.198 ± 1.533	1.134 ± 0.367	6.643 ± 1.915	3.131 ± 0	6.105 ± 1.965	1.652 ± 0.429

Anti H. pylori activities from chloroform extract of P. pulcher

Chloroform extract of *P. pulcher* was selected as the best extract for further the bioactivity guided fractionation.

Major compounds in the extract are terpenoids. By column chromatography, the chloroform extract was fractionated into 30 fractions.

Further evaluation by disc diffusion assay revealed that all the fractions specifically inhibited *H. pylori* with one of the fractions (F18) exhibited the widest inhibition zone diameter (30.0 ± 0.0 mm) (Table 3).

This fraction (F18) also exhibited the low toxicity value on *A. salina* (Table 4) with LC_{50} more than 1mg/ml.

TABLE 4 Toxicity test on selected fraction from chloroform extract of *P. pulcher*

Fraction	Acute (mg/ml)	Chronic (mg/ml)
18	1.350 ± 0	1.000 ± 0.245
19	0.774 ± 0.949	5.61 x 10^{-3} ± 0.032
21	0.577 ± 12.83	0.014 ± 0.071

From MIC test, the fraction showed MIC_{50} = 4µg/ml and MIC_{90} = 256µg/ml. While, MIC value for *H. pylori* (S179) was 32µg/ml (Table 5).

In the initial time kill assay test, *H. pylori* (S179) did not grow on EAB within 0 to 12 hours of incubation at 32µg/ml of the fraction (Figure 1). From total counts result (Figure 2), *H. pylori* (S179) was found

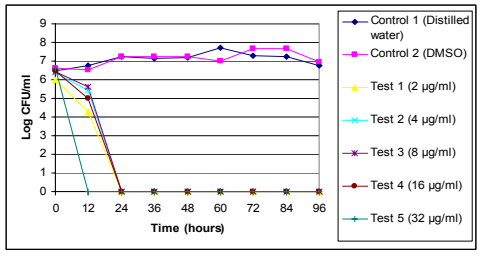
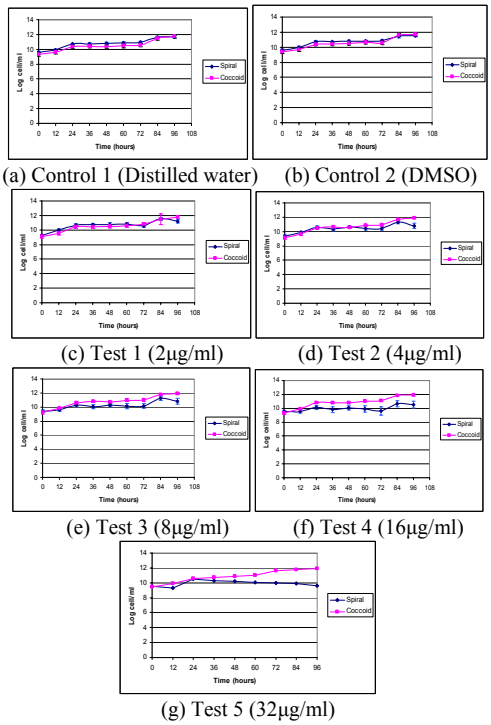


FIGURE 1 Viable counts for first time kill assay test



(a) Control 1 (Distilled water) (b) Control 2 (DMSO)

(c) Test 1 (2µg/ml) (d) Test 2 (4µg/ml)

(e) Test 3 (8µg/ml) (f) Test 4 (16µg/ml)

(g) Test 5 (32µg/ml)

FIGURE 2 Total counts for first time kill assay test

TABLE 3 Disc diffusion test 30 fractions from chloroform extracts of *P. pulcher* on *H. pylori* (S179) and bacterial pathogens

Fraction	*Enterobacter*	*E. coli*	*E. coli* (ATCC 25922)	*P. stutzeri*	*Salmonella*	*Sh. boydii*	*Sh. dysentry*	*S. aureus*	*V. cholera*	*H. pylori* (S179)
1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	9 ± 0
11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11.5 ± 0.5
14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	13 ± 0
17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	19 ± 1.0
18	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	30.0 ± 0
19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	21.5 ± 0.5
20	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	21 ± 0
21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	24 ± 1.0
22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	16.5 ± 0.5
23	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	16.5 ± 0.5
24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	17 ± 1.0
25	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	12 ± 0
26	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11.5 ± 0.5
27	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	12 ± 0
28	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	15 ± 1.0
29	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0
30	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	11 ± 0

TABLE 5 MIC onto 20 strains of *H. pylori*

Sample	69	70	98	109	115	147	164	179	198	205	295	393	396	422	815	879	1036	1040	1042	1046
256 μg/ml	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
128 μg/ml	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	+	-
64 μg/ml	+	-	-	-	-	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-
32 μg/ml	+	-	-	-	-	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-
16 μg/ml	+	-	-	-	-	+	-	+	-	+	-	-	-	+	+	-	-	-	+	-
8 μg/ml	+	-	-	-	+	+	-	+	-	+	-	-	-	+	+	-	-	-	+	-
4 μg/ml	+	-	-	-	+	+	-	+	-	+	-	-	-	+	+	+	-	-	+	+
2 μg/ml	+	-	-	-	+	+	-	+	-	+	-	+	-	+	+	+	-	-	+	+
1 μg/ml	+	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
DMSO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
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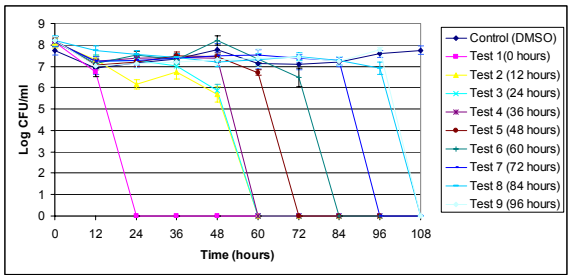


FIGURE 3 Viable counts for second time kill assay test

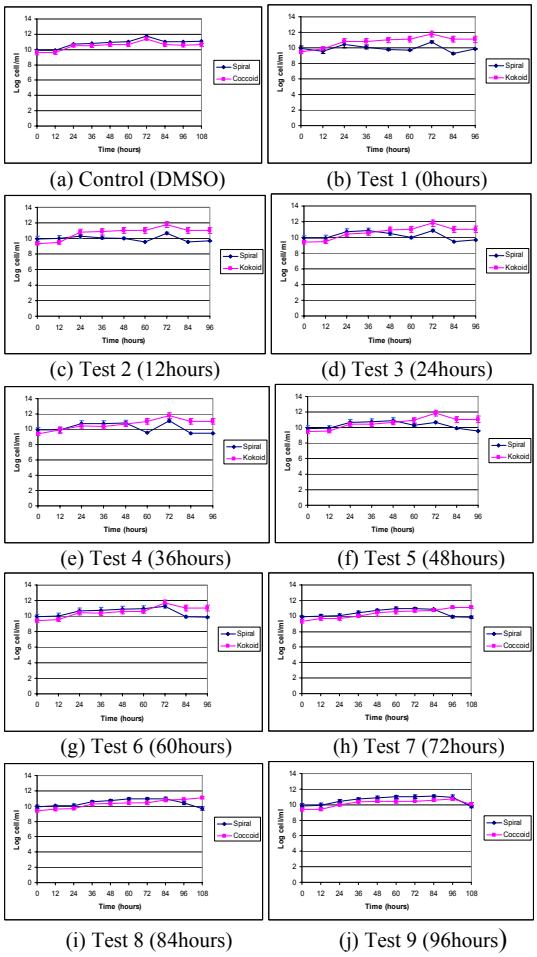


FIGURE 4 Total counts for second time kill assay test

majority in coccoid shape after the fraction was added into the samples. From the second experiments of time kill assay, *H. pylori* (S179) was killed depending to the time of fraction was added into the samples. In viable counts result (Figure 3), *H. pylori* (S179) did not grow immediately after the fraction was added at 0 hours. However, *H. pylori* (S179) remained to grow longer (48-60 hours) when the fraction was added after 12 and 24 hours. *H. pylori* (S179) could maintain in shorter time (12-24 hours) on EAB when the fraction added after 36, 48, 60, 72, 84 and 96 hours of incubation.

In the third experiment, time factor was the main factor in killing *H. pylori* (S179). The viable counts result (Figure 5) showed similar period of time in killing the bacteria for both set of experiments. The total counts result in the second and third experiments (Figure 4 and 6) of time kill assay showed the similar results as the first experiment.

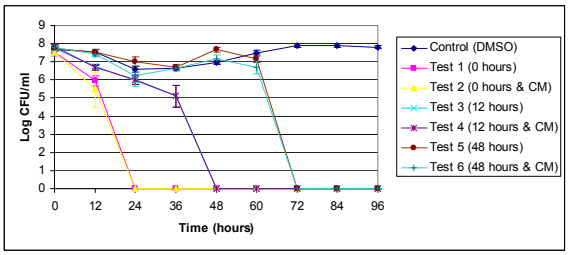


FIGURE 5 Viable counts for third time kill assay test

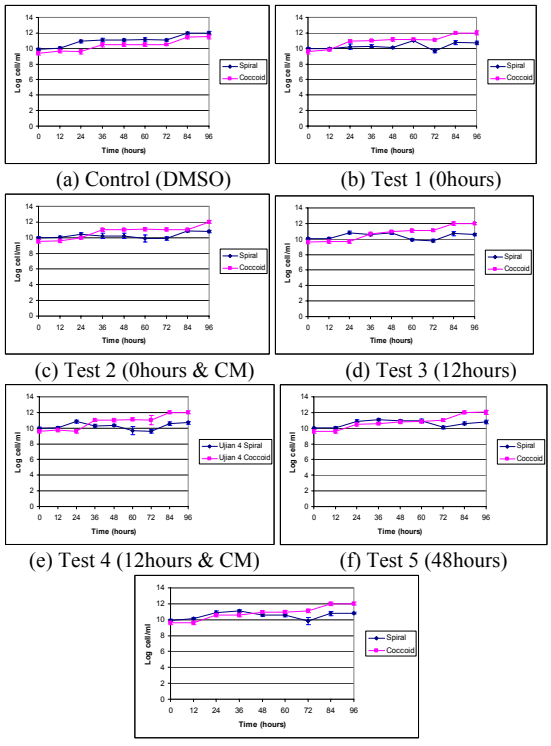
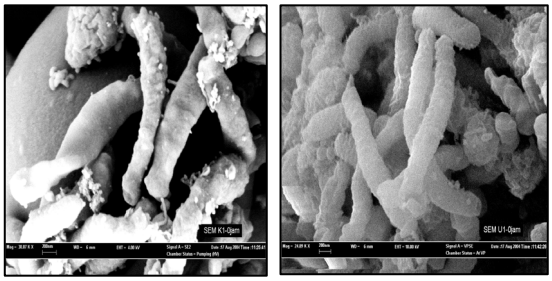
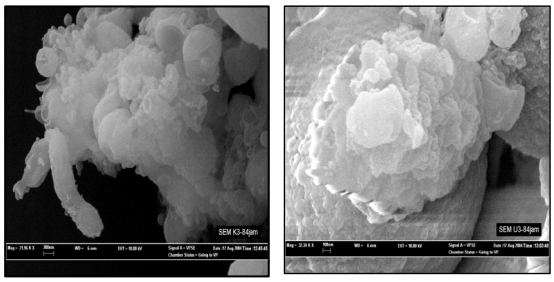


FIGURE 6 Total counts for third time kill assay test

Following expose of *H. pylori* (S179) to the fraction, changed the morphology from spiral form to coccoid form under SEM (Figure 7) and TEM (Figure 8) observations.

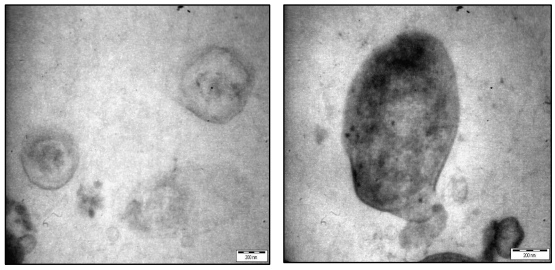


(a) Control (0 hours) (b) Treatment (0 hours)



(c) Control (84 hours) (d) Treatment (84 hours)

FIGURE 7 Micrograph of SEM



(a) Control (0 hours) (b) Treatment (48 hours)

FIGURE 8 Micrograph of TEM

Discussion

The chloroform extract from the leaves of *P. pulcher* exhibited the best anti *H. pylori* activity with the widest inhibition zones. The extract was also highly selective and specific on *H. pylori* (S179). At the same time, the extract is harmless to the other nine common bacterial pathogens and it is not toxic to *A. salina.* These observations suggested the extract had potential as strong anti *H. pylori* agent without producing negative effects to patients and any intestinal bacterial species (Hamasaki *et al.,* 2000). While the other extracts possess relatively lower anti *H. pylori* activity and therefore were not further evaluated.

The extract was separated using column chromatography to find out the main compounds that played the role in inhibiting the bacteria. All 30 fractions found specifically inhibited *H. pylori* (S179) and suggested the extract react as anti *H. pylori* specifically before and after fractionation.

Fraction number 18 (F18) exhibited the biggest inhibition zone diameter on *H. pylori* (S179) and better than crude chloroform extract. From the observation, the fraction was suggested to work better individually than in crude extract. This might be due to the synergistic effect of various terpenoid in the fraction.

From toxicity test, the fraction number 18 is considered as non toxic to *A. salina* and may possibly to use in the clinical evaluation.

From MIC test, value between MIC_{50} and MIC_{90} were quite different. The fraction could not inhibit all the 20 strains because each strain had various pathogenicity values. The MIC_{50} values showed that very low concentration was needed to kill 50% from the strains bacteria (4µg/ml). According to Gadhi *et al.* (2001), plant extracts with MIC below than 100µg/ml, is accept as potential antimicrobial agent.

The best concentration to kill *H. pylori* in broth media (EBB) was 32µg/ml. The results obtained were similar to the MIC test. This result similar to the MIC test i.e. revealed that the type of media do not influenced the efficiency of the fraction.

Killing curve of *H. pylori* (S179) at different incubation periods showed an increment in time of viability of the bacteria on the agar i.e. between 12 to 24 hours after incubation compared to 0 to 12 hours and 48 to 84 hours of incubation.

The result suggested the bacteria had poor motility in lag phase (0-12 hours), became highly motile and active in mid-exponential phase (24-36 hours) and lost motility in the decline phase of growth (48-84 hours) (Worku *et al.,* 1999).

In the third experiment of time kill assay, time factor is more dominant than media factor. It is because no different values obtained either in fresh EBB or in older EBB that were used in the test.

From phytochemistry test, the major compounds detected in the fraction are terpenoids. It was accepted as the compounds that responsibility and played the role as anti *H. pylori* (S179) in this study.

The bacteria were turned from spiral form to coccoid shape in SEM and TEM studied. The morphological changes were due to the disrupting process in membrane cell of the bacteria. At the same time, terpenoids were reacted as the same way (Cowan, 1999). However, more observations must be done to confirm this result.

Chloroform extract of the *P. pulcher* had potential as a new anti *H. pylori* agent in the future. But, more scientific study should be done to prove and candidate the plant for using in eradication therapy of *H. pylori.*

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