Research Article

Antimicrobial and Synergistic Effects with Antibiotics of *Momordica cochinchinensis* Spreng (Gac fruit) Aril against Pathogenic Bacteria

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ABSTRACT

Antibacterial and synergistic activity of *Momordica cochinchinensis* Spreng aril were evaluated. Crude extracts and oils have shown the antibacterial activities against six pathogenic strains by agar well diffusion assay. Crude methanolic extract (ME) and oils by screw press (SP) and supercritical CO_2 fluid (SC) had the broad spectrum of antimicrobial activity. MIC and MBC values of crude extracts and oils vary from 0.78 to > 400 mg/ml. The combination of the ME with crude oils by SP / SC had the highest synergistic activity with the reduction of ≥ 8–64-fold on *E. faecalis* DMST 4736, *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311 (FICI of 0.0313-0.50) and the reduction of MIC values ≥ 4–64-fold in the combination of hexane extract (HE) and crude oils by SP / SC. Combinations of oil by SP (1/64 MIC) with ampicillin and ciprofloxacin had synergistic effect against *E. faecalis* DMST 4736, *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *S. epidermidis*. Hexane extract (1/4MIC) with ampicillin and ciprofloxacin (1/8 MIC) has shown a potent synergistic interaction against *E. faecalis* DMST 4736 and *S. aureus* ATCC 25923. Moreover, oil by SP (1/4 MIC) with ciprofloxacin (1/8 MIC) and HE (1/4MIC) with 2 antibiotics (1/8 MIC) had significantly synergistic bactericidal effect against *E. faecalis* DMST 4736 at 6 and 9 hours, respectively. These findings indicated that the synergistic antibacterial activity of crude *Momordica cochinchinensis* Spreng extracts and oils with antibiotics combination may be beneficial for the development of alternative antibacterial therapy.

Keywords: Gac fruit aril, *Momordica cochinchinensis* Spreng, Olis and crude extracts, Synergistic Effects, Antimicrobial activity, Antibiotics.

INTRODUCTION

During the past to present, Infectious diseases caused by bacteria and fungi affect millions of people worldwide particularly in developing countries, account for one-third of all deaths of infectious diseases in the world.[1] The World Health Organization estimates that nearly 50,000 people die each day throughout the world.[2] The discovery of antibiotics was a significant part in combating bacterial infections that have been ravaging humankind. Various antibiotics exercise their inhibitory activity against various pathogenic strains. The necessary situation for development of new antimicrobial agents occurred in rapid fashion. Many plants are historically used to treat the infectious diseases. In the past, people used to discover remedies from the local herbs because people, at that time, used edible plants as food.

Momordica cochinchinensis Spreng is a tropical plant grown in many countries in tropical regions. It may be called by different name such as Gac (in Viet Nam), Fak kao (in Thailand), Bhat kerala (in India), Moc Niet Tu (in China) and Mak kao (in Laos).[3-4] Many recent studies have demonstrated that gac fruit has a number of biological activities, which are benefits to human health.[5-8] In addition, efforts have focused on the processing of gac fruit to produce gac oil as a natural food additive and for medical uses. Scientific evidence supports the hypothesis that several plants are composed of bioactive compounds entities and several medicines are actually analogues of plant origin substances.[9-11]

The aims of this study were to evaluated the effect of alone and combined antibacterial effects of gac fruit aril extracts and crude oil with 2 antibiotics (ampicillin and ciprofloxacin) against pathogenic strains.

MATERIALS AND METHODS

Sample Preparation and Extraction

The ripe fruit of *Momordica cochinchinensis* Spreng. (gac fruit) were collected from Nakhonpathom Province, in the central region of Thailand, on the 7th date after harvesting (fully ripe; red color).

The gac fruits were thoroughly cleaned with distilled water for 15 min. Gac fruit arils were soaked into methanol, acetone or hexane (ratio 1:2) for 5 days separately.

The mixture was filtered through a filter paper (Whatman No. 1) and centrifuged at 8,000 rpm for 15 min.

Then, the filtrate obtained was subsequently concentrated under vacuum on a rotary evaporator at 50-65^{o}C. The concentrated extracts were kept at -20^{o}C under dark condition until further analysis.

For crude oils, the extraction was done by screw press[12] and supercritical CO_2 fluid technique. The crude extracts were weighted and calculated for the percentage yield.

Microorganisms and Culture Condition

In-vitro antimicrobial activities of all crude extracts and oils at different concentrations were determined by agar well diffusion method. Minimum inhibitory and bactericidal concentration (MIC and MBC) assay was measured for each bacteria strains. This study used nine pathogenic strains including Gram-positive bacteria (*Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis*) and Gram-Negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212 and *Salmonella typhimurium* ATCC 13311). The bacteria strains were obtained from the laboratory of the Department of Biotechnology, King Mongkut's University of Technology North Bangkok, Thailand. All tested strains were maintained on brain heart infusion (BHI, Difco) agar medium at 37°C.

Agar Well Diffusion Method

The antimicrobial activity of the gac fruit aril extracts and oils was carried out by agar well diffusion method against these nine pathogenic strains.[13] Overnight bacterial cultures of tested strains were adjusted the OD_{600} to 0.2 (10^8-10^9 CFU /ml) by spectrophotometer. Briefly, 25 ml of BHI agar was poured into each petri plate. Once the agar solidified, the microorganisms were mixed into 0.75% BHI agar and poured on the surface of the plates. Subsequently, the surface of the agar was punched with a 6 mm-diameter wells by using a sterile cork borer. Each well was filled with 80 µl of each crude gac fruit aril extracts and oils. The concentration of the extracts employed was 80, 100, 200, 300 and 400 mg/ml, respectively. Simultaneously, Ampicillin (Amp, 5 µg/ml) and Ciprofloxacin (CIP, 5 µg/ml) were used as positive control. After 1, 3, 5, and 7 day incubation at 37°C, all plates were observed the inhibition zones, and the diameter of these zones was measured in millimeters.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assay

The microdilution minimum inhibitory concentration (MIC) method was undertaken to quantify inhibitory activity of the sample extracts and oils by using two-fold broth microdilutions in 96 well plates.[14]

All crude extracts and oils were first dissolved in 10% DMSO at the highest concentration of 6,400 mg/ml. Concentration of crude extracts and oils range from 6,400 to 12.5 mg/ml were used.

Then, 5 µl of a standard inoculum of the pathogenic strains was added to each wells. The microtiter plates were incubated under optimal conditions (at 37°C for 24 h; Similar tests were performed simultaneously for growth control (BHI + inoculums) and sterility control (BHI + test sample). The lowest concentration of sample extracts/oils that inhibited the bacterial growth was considered as MIC values. After MIC determination, subcultures were made by spreading visually clear broth dilution MIC well to HBI agar (Difco) at 37°C for 24 h. The lowest concentration which showed the complete absence of bacteria growth, was considered as MBC values.

Synergistic Antimicrobial Effects

Potential antimicrobial synergy was measured by fractional inhibitory concentration (FIC) and indices (FICI). The analysis was performed by the checkerboard dilution method in 96-well microtiter plates.[15] Two antibiotics including amoxicillin (AM) and ciprofloxacin (CIP) were used. Synergistic effects of the combinations were investigated in antibiotics (2xMIC) and each crude extract / oils (4xMIC). The interaction between the two antimicrobial agents was estimated by calculating the fractional inhibitory concentration index (FICI). The FIC of each compound was calculated by dividing the concentration of the plant extracts/oils in effective MIC of the combination, with the MIC of the antibiotic or extracts/oils alone. FICI values were calculated as;

FICI = FIC (A) + FIC (B)

= [A] / MIC (A) + [B] / MIC (B)

[A] **:** MIC value of A in a mixture of A and B substance

[B] : MIC value of B in a mixture of A and B substance

MIC (A) : MIC values of A substance

MIC (B) : MIC values of B substance

FICI values were interpreted as follows: FICI ≤ 0.5 Synergy (S); 0.5 > FICI ≤ 1 Additive (AD); >1.0 < FICI ≤ 4.0 Indifference (no effect: I) and FICI > 4.0 Antagonism (A).[15-16] Each test was repeated three times.

Time-kill Curves

In-vitro bactericidal activities of between crude extracts/oils and antibiotics at synergistic effects of combine concentrations (FICI values) from this study were evaluated by using time-kill curves according to the protocol of NCCLS (1999).[16-18] Crude extracts, oils from gac fruit aril, antibiotic and tested bacteria were mixed and incubated at 37° C. The viable counts were conducted at 0, 3, 6, 9, 12, 15, 18, 21, 24, 28 and 32 hour by plate count method. Cultures with an initial cell density of 1.5-4.0×10^8 CFU/ml were exposed to the MBC of the combination of crude extract and oil with ampicillin or ciprofloxacin. Curves were plotted as the viable cells (log_{10} CFU/ml) versus time. Synergy was defined as ≥2 log 10 decreases in CFU of organisms treated with the antibiotic combination compared to each treatment alone and control (untreated).

Statistics Analysis

The data were reported as mean ± standard deviation (SD) of triplicate measurements. Statistical analyses (ANOVA) were performed with the statistical program MS Excel (Microsoft Office 2010 Professional) to analyze for

significant difference. The P-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Antimicrobial Activities of Plant Extracts by Agar Well Diffusion Method

The crude extracts of gac fruit aril were prepared using 3 different solvents (ethanol, acetone and hexane) and using screw press and supercritical CO_2 fluid technique for crude oils. Table 1 illustrates zone of inhibition of crude extracts and oils against the nine pathogenic strains using agar well diffusion method. The results were presented the broad spectrum antimicrobial activity of methanolic gac fruit extract (ME) and crude oils by screw press (SP) and supercritical CO_2 fluid (SC) against both gram positive and negative organisms. Crude oil by SP showed more prominent inhibition zone against *E. faecalis* DMST 4736 (16.67 ± 0.58 mm), *E. coli* ATCC 25922 14.00 ± 1.00 mm) and *K. pneumoniae* (8.00±0.00 mm) at concentration of 80 mg/ml ($P<0.05$). Crude oil by SC showed outstanding inhibition zone against *Proteus mirabilis* (15.67±0.58 mm; 100 mg/ml) and *S. epidermidis* (12.67 ± 0.00 mm; 200 mg/ml) ($P<0.05$).

Four strains: *E. faecalis* DMST 4736, *E. coli* ATCC 25922, *K. pneumoniae* and *P. aeruginosa* ATCC 27853 were susceptible to crude extracts of gac fruit aril. Significant antimicrobial activity was exhibited in the ME extract when compared with acetone (AE) and hexane extract (HE). However, crude HE of gac fruit aril could not inhibit the growth of *S. aureus* ATCC 25923 in this study, which is consistent with previous our research that had had anti-*S. aureus* (7.83 ± 0.26 (10 mg/ml) and 8.58 ± 0.20 (100 mg/ml)).[8] This may be due to different of during time the harvesting in the testing. Since, the antimicrobial activity of natural compounds could be influenced by various factors including botanical source, time of harvesting, stage of development, and method of extraction in addition to the composition, structure, and functional groups of the natural compounds.[19] From these data, it is clear that the effectiveness of the extracts largely depended on the type of solvent used. Similar observations are reported by many researchers.[20-22]

Antimicrobial Activities of Plant Extracts by MIC and MBC Values

The antibacterial activity of the crude extracts of gac fruit aril by microdilution method was shown in Table 2.

The result indicated that the gac fruit aril extracts and oils were presented antibacterial activities at variable concentrations against 9 tested pathogenic strains, with MIC values varying from 0.78 to more than 400 mg/ml. The MIC analysis of crude gac fruit aril extracts showed the optimal concentration of bacteriostatic using HE of gac fruit aril, showing low-level MIC and MBC value of 1.56-12.50 and 3.125-12.50 mg/ml as compared with the same tested strains. The strains of *B. cereus* DMST 5040, *S. epidermidis* and *P. aeruginosa* ATCC 27853). *E. faecalis* DMST 4736, *K. pneumoniae* and *S. typhimurium* ATCC 13311 were susceptible under HE treatment. Gram negative organisms were much more resistant to these extracts than positive organisms in the this study.

For among crude oils and crude extracts from gac fruit aril, crude oils by SP / SC had shown the higher antimicrobial activities as compared with those by AE and ME due to their bacteriostatic effect at the lower concentration (when considering with the same tested strains). The MIC and MBC values for ampicillin was found at concentration of 0.78 µg/ml, while that of ciprofloxacin was 3.125 to 12.50 µg/ml and 1.56 to 50.00 µg/ml, respectively.

Synergistic Effect

Combination of ME with HE of gac fruit aril displayed an antagonistic effect against *E. faecalis* DMST 4736, *E. coli* ATCC 25922, *K. pneumoniae*, *P. mirabilis* DMST 8212 and *S. typhimurium* ATCC 13311 (Table 3). The combination of ME with crude oils by SP / SC led to a synergistic effect against *E. faecalis* DMST 4736, *E. coli* ATCC 25922 and *S. typhimurium* ATCC 13311. The MIC value was reduced to ≥ 8–64-fold showing an additive effect against *S. aureus* ATCC 25923 and indifferent effect against *B. cereus* ATCC 11778. Moreover, the combinations of the HE and crude oils by SP/SC had a synergic effect against *E. faecalis* DMST 4736, *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *S. typhimurium* ATCC 13311, the MIC for crude extracts were reduced ≥ 4–64-fold. Combination of the HE (1/4 MIC and 1/8 MIC) and crude oils by SC (1/4 MIC and 1/8 MIC) demonstrated the synergistic effect against *P. mirabilis* DMST 8212 and *K. pneumoniae*, respectively. Antibacterial activities of combination between AE and crude oils by SP against *S. epidermidis* were rather shown as an additive effect.

The synergistic effects of the crude gac fruit aril extracts and oils with 2 antibiotics were shown in Tables 4. The best synergism was observed on the combinations of crude oil by SP with ampicillin and crude oil by SP with ciprofloxacin with FICI values of 0.0234. Combinations of crude oli by SP (1/64 MIC) with 2 antibiotics (ampicillin and ciprofloxacin) were shown synergistic effects against *E. faecalis* DMST 4736, *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *S. epidermidis*. Furthermore, synergistic effects in *B. cereus* ATCC 11778 and *P. mirabilis* DMST 8212 were also observed from the combination of crude oil by SP with ciprofloxacin.

Upon combination with the HE of gac fruit aril with ampicillin and ciprofloxacin (1/4MIC), the MIC was reduced -8-fold when tested against *E. faecalis* DMST 4736 and *S. aureus* ATCC 25923. The combinations of ME with ampicillin and ME with ciprofloxacin were presented additive effect in *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (FICI = 0.75; Table 4). However, It was also found the antagonism effect in *P. mirabilis* DMST 8212 from the combination of ME with ciprofloxacin.

Table 1: The inhibition zone of gac fruit aril extracts and oils against pathogenic strains by agar well diffusion assay

Concentration (mg/ml)		Average Inhibition Zone (AIZ); mm±SD								
		Gram Positive Strains				Gram Negative Strains				
		BC	EF	SA	SE	EC	KP	PA^{ns}	PM	ST
Methanol	80	R	R	R	R	R	R	6.58±0.38	R	R
	100	R	6.42±0.26	R	R	R	R	6.67±0.27	R	R
	200	R	6.50±0.00	R	R	R	R	R	R	R
	300	R	R	R	R	R	6.92±0.20^{ab}	R	R	R
	400	R	R	R	R	R	8.75±1.08	R	R	R
Acetone	80	R	R	R	R	R	6.58±0.20^{b}	6.67±0.26	R	R
	100	R	R	R	R	R	7.67±0.26^{a}	6.83±0.26	R	R
	200	R	R	R	R	R	6.58±0.20^{b}	7.17±0.75	R	R
	300	R	R	R	R	R	7.67±0.26^{a}	7.58±0.38	R	R
	400	R	R	R	R	R	8.50±0.45	7.67±0.26	R	R
Hexane	80	R	R	R	R	R	7.75±0.27^{ab}	R	R	R
	100	R	R	R	R	R	7.75±0.27^{a}	6.50±0.32	R	R
	200	R	R	R	R	R	6.67±0.26^{b}	6.50±0.32	R	R
	300	R	R	R	R	R	6.50±0.32^{b}	6.42±0.20	R	R
	400	R	R	R	R	R	6.58±0.38	6.50±0.32	R	R
Screw Press	80	R	16.67±0.58*	R	R	14.00±1.00*^{a}	8.00±0.00^{a}	R	R	R
	100	R	R	R	R	R	6.67±0.29^{b}	R	15.33±0.58^{b}	R
	200	R	R	R	R	R	R	R	R	R
	300	R	19.67±0.58*	R	R	R	R	R	R	R
	400	R	R	R	R	R	R	R	R	R
Supercritical CO_2 fluid	80	R	R	R	R	6.67±0.29^{b}	R	R	R	R
	100	R	R	R	R	R	R	R	15.67±0.58^{a}	R
	200	R	R	R	12.67±0.00	8.00±0.00	8.67±0.29^{a}	R	R	R
	300	R	R	R	6.83±0.29	R	6.67±0.29	R	R	R
	400	R	R	R	R	R	R	R	R	R

BC = *B. cereus* DMST 5040, EF = *E. faecalis* DMST 4736, SA = *S. aureus* ATCC 25923, SE = *S. epidermidis*, EC = *E. coli* ATCC 25922, KP = *K. pneumoniae*, PM = *P. mirabilis* DMST 8212, PA = *P. aeruginosa* ATCC 27853, ST = *S. typhimurium* ATCC 13311 and R = Resistance; *: significantly different (P < 0.05) when compared with all tested strains at the lowest concentrations; ^{abc}: means in the same row (at the same concentration) with different superscript were significantly different (P < 0.05) by one way ANOVA and paired T-test; ^{ABC}: means in the same column (at the same concentration) with different superscript were significantly different (P < 0.05) by one way ANOVA and paired T-test; ^{ns}: = no significant on *P. aeruginosa* (at the same concentration)

Table 2: The MIC and MBC of aril gac fruit extracts/oils against some pathogenic strains by broth microdilution method

Solvents Extraction / Technique			Microorganisms								
			Gram Positive Strains				Gram Negative Strains				
			BC	EF	SA	SE	EC	KP	PA	PM	ST
Methanol		MIC	$400^{A,a}$	$400^{A,a}$	$200^{B,a}$	$0.78^{C,c}$	$400^{A,a}$	$200^{B,a}$	-	$200^{B,a}$	$200^{B,a}$
		MBC	400^{A}	400^{A}	200^{B}	0.78^{C}	400^{A}	200^{B}	-	200^{B}	200^{B}
Acetone		MIC	-	-	-	$0.78^{A,c}$	-	-	-	-	-
		MBC	-	-	-	0.78^{A}	-	-	-	-	-
Hexane		MIC	-	$1.56^{C,c}$	$3.125^{B,c}$	-	$12.50^{A,c}$	$1.56^{C,c}$	-	$3.125^{B,c}$	$1.56^{C,d}$
		MBC	-	6.25^{B}	12.50^{A}	-	12.50^{A}	3.125^{C}	-	12.50^{A}	3.125^{C}
Screw Press		MIC	$100^{A,b}$	$100^{A,b}$	$100^{A,b}$	$100^{A,a}$	$100^{A,b}$	-	-	$100^{A,b}$	$50^{B,c}$
		MBC	100^{A}	100^{A}	100^{A}	100^{A}	100^{A}	-	-	100^{A}	50^{B}
Supercritical CO_2 fluid		MIC	$100^{A,b}$	$100^{A,b}$	$100^{A,b}$	$25^{B,b}$	$100^{A,b}$	$100^{A,b}$	-	-	$100^{A,b}$
		MBC	100^{A}	100^{A}	100^{A}	25^{B}	100^{A}	100^{A}	-	-	100^{A}
Antibiotics	Amoxicillin	MIC	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}	0.78^{A}
		MBC	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}	1.56^{A}
	Ciprofloxacin	MIC	$1.56^{C,c}$	1.56^{C}	25.00^{A}	12.50^{B}	0.78^{D}	1.56^{C}	12.50^{B}	12.50^{B}	1.56^{C}
		MBC	1.56^{D}	6.25^{C}	25.00^{B}	50.00^{A}	1.56^{D}	25.00^{B}	25.00^{B}	25.00^{B}	25.00^{B}

BC = *Bacillus cereus* DMST 5040, EF = *Enterococcus faecalis* DMST 4736, SA = *Staphylococcus aureus* ATCC 25923, SE = *Staphylococcus epidermidis*, EC = *Escherichia coli* ATCC 25922, KP = *Klebsiella pneumoniae*, PA = *Pseudomonas aeruginosa* ATCC 27853, PM = *Proteus mirabilis* DMST 8212and ST = *Salmonella typhimurium* ATCC 13311; (-) = >400 mg/ml; [abcd] : means in the same row with different superscript were significantly different (P < 0.05) by one way ANOVA and paired T-test (Among plant extracts with MIC values); [ABC] : means in the same column with different superscript were significantly different (P < 0.05) by one way ANOVA and paired T-test (crude extracts and oils)

Table 3: Synergistically antimicrobial effects of crude extracts/olis of gac fruit aril

Microorganisms	Extract : Extract	MIC values (mg/ml : mg/ml)				FICI value	Outcome
		Alone		Combination (4MIC : 4 MIC)			
B.cereus **ATCC 11778**	**ME : SP**	400	: 100	400	: 100	2.00	Indifferent
	ME : SC	400	: 100	400	: 100	2.00	Indifferent
E. faecalis **DMST 4736**	**ME : HE**	400	: 1.56	1600	: 6.25	8.00	Antagonistic
	ME : SP	400	: 100	6.25	: 1.56	0.0313	Synergistic
	ME : SC	400	: 100	6.25	: 1.56	0.0313	Synergistic
	HE : SP	1.56	: 100	0.024	: 1.56	0.0313	Synergistic
	HE : SC	1.56	: 100	0.024	: 1.56	0.0313	Synergistic
S. aureus **ATCC 25923**	**ME : HE**	200	: 3.125	200	: 3.125	2.00	Indifferent
	ME : SP	200	: 100	100	: 50	1.00	Additive
	ME : SC	200	: 100	100	: 50	1.00	Additive
	HE : SP	3.125	: 100	0.781	: 25	0.50	Synergistic
	HE : SC	3.125	: 100	0.781	: 25	0.50	Synergistic
S. epidermidis	**ME : AE**	0.78	: 0.78	3.125	: 3.125	8.00	Antagonistic
	ME : SC	0.78	: 25	0.049	: 6.25	0.125	Synergistic
	ME : SP	0.78	: 100	0.78	: 100	2.00	Indifferent
	AE : SC	0.78	: 25	0.049	: 1.56	0.125	Synergistic
	AE : SP	0.78	: 100	0.39	: 50	1.00	Additive
E. coli **ATCC 25922**	**ME : HE**	400	: 12.5	1600	: 50	8.00	Antagonistic
	ME : SP	400	: 100	25	: 6.25	0.125	Synergistic
	ME : SC	400	: 100	25	: 6.25	0.125	Synergistic
	HE : SP	12.5	: 100	0.78	: 6.25	0.125	Synergistic
	HE : SC	12.5	: 100	0.78	: 6.25	0.125	Synergistic
K. pneumoniae	**ME : HE**	200	: 1.56	800	: 6.25	8.00	Antagonistic
	ME : SC	200	: 100	25	: 12.5	0.25	Synergistic
	HE : SC	1.56	: 100	0.195	: 12.5	0.25	Synergistic
P. mirabilis **DMST 8212**	**ME : HE**	200	: 3.125	800	: 12.50	8.00	Antagonistic
	ME : SP	200	: 100	25	: 12.5	0.25	Synergistic
	HE : SP	3.125	: 100	0.781	: 25	0.50	Synergistic
S. typhimurium **ATCC 13311**	**ME : HE**	200	: 1.56	800	: 6.25	8.00	Antagonistic
	ME : SP	200	: 50	25	: 6.25	0.25	Synergistic
	ME : SC	200	: 100	25	: 12.50	0.25	Synergistic
	HE : SP	1.56	: 50	0.195	: 6.25	0.25	Synergistic
	HE : SC	1.56	: 100	0.195	: 12.50	0.25	Synergistic

ME = methanol extract, HE = hexane extract, SP = crude oil by screw press and SC = crude oil by supercritical CO_2 fluid

Table 4: Synergistically antimicrobial effects of crude extracts / oils with antibiotic combinations

Microorganisms	Extract : Medicine	MIC values (mg/ml : µg/ml)				FICI value	Outcome
		Alone		Combination (4MIC : 2 MIC)			
***B. cereus* ATCC 11778**	**ME : AM**	400	: 0.78	400	: 0.39	1.50	Indifferent
	ME : CIP	400	: 1.56	400	: 0.78	1.50	Indifferent
	SP : AM	100	: 0.78	200	: 0.78	1.50	Indifferent
	SP : CIP	100	: 1.56	25	: 0.39	0.50	Synergistic
***E. faecalis* DMST 4736**	**HE : AM**	1.56	: 0.78	0.39	: 0.0975	0.375	Synergistic
	HE : CIP	1.56	: 1.56	0.39	: 0.195	0.375	Synergistic
	SP : AM	100	: 0.78	1.562	: 0.0061	0.0234	Synergistic
	SP : CIP	100	: 1.56	1.562	: 0.0122	0.0234	Synergistic
***S. aureus* ATCC 25923**	**ME : AM**	200	: 0.78	200	: 0.39	0.75	Additive
	ME : CIP	200	: 25	200	: 12.50	0.75	Additive
	HE : AM	3.125	: 0.78	0.78	: 0.0975	0.375	Synergistic
	HE : CIP	3.125	: 25	0.78	: 3.125	0.375	Synergistic
	SP : AM	100	: 0.78	25	: 0.0975	0.375	Synergistic
	SP : CIP	100	: 25	25	: 3.125	0.375	Synergistic
S. epidermidis	**ME : AM**	0.78	: 0.78	1.56	: 0.78	3	Indifferent
	ME : CIP	0.78	: 12.5	0.78	: 6.25	1.50	Indifferent
	AE : AM	100	: 0.78	50	: 0.195	0.75	Additive
	AE : CIP	0.78	: 12.5	0.195	: 1.562	0.375	Synergistic
	SP : AM	100	: 0.78	1.56	: 0.0061	0.0234	Synergistic
	SP : CIP	100	: 12.5	25	: 1.562	0.375	Synergistic
***E. coli* ATCC 25922**	**ME : AM**	400	: 0.78	200	: 0.195	0.75	Additive
	ME : CIP	400	: 0.78	200	: 0.195	0.75	Additive
	HE : AM	12.5	: 0.78	3.125	: 0.0975	0.375	Synergistic
	HE : CIP	12.5	: 0.78	6.25	: 0.195	0.75	Additive
	SP : AM	100	: 0.78	12.50	: 0.049	0.187	Synergistic
	SP : CIP	100	: 0.78	12.50	: 0.049	0.187	Synergistic
K. pneumoniae	**ME : AM**	200	: 0.78	200	: 0.39	1.50	Indifferent
	ME : CIP	200	: 1.56	200	: 0.78	1.50	Indifferent
	HE : AM	1.56	: 0.78	0.39	: 0.0975	0.375	Synergistic
	HE : CIP	1.56	: 1.56	1.56	: 0.78	1.50	Indifferent
***P. mirabilis* DMST 8212**	**ME : AM**	200	: 0.78	50	: 0.0975	0.25	Synergistic
	ME : CIP	200	: 12.5	400	: 25	6	Antagonistic
	HE : AM	3.125	: 0.78	3.125	: 0.39	1.50	Indifferent
	HE : CIP	3.125	: 12.5	3.125	: 6.25	1.50	Indifferent
	SP : AM	100	: 0.78	50	: 0.195	0.75	Additive
	SP : CIP	100	: 12.5	25	: 1.5625	0.375	Synergistic
***S. typhimurium* ATCC 13311**	**ME : AM**	200	: 0.78	200	: 0.39	1.50	Indifferent
	ME : CIP	200	: 1.56	200	: 0.78	1.50	Indifferent
	HE : AM	1.56	: 0.78	0.39	: 0.0975	0.375	Synergistic
	HE : CIP	1.56	: 1.56	1.56	: 0.78	1.50	Indifferent
	SP : AM	50	: 0.78	50	: 0.39	1.50	Indifferent
	SP : CIP	100	: 1.56	50	: 0.39	0.75	Additive

AM = Ampicillin; CIP = Ciprofloxacin

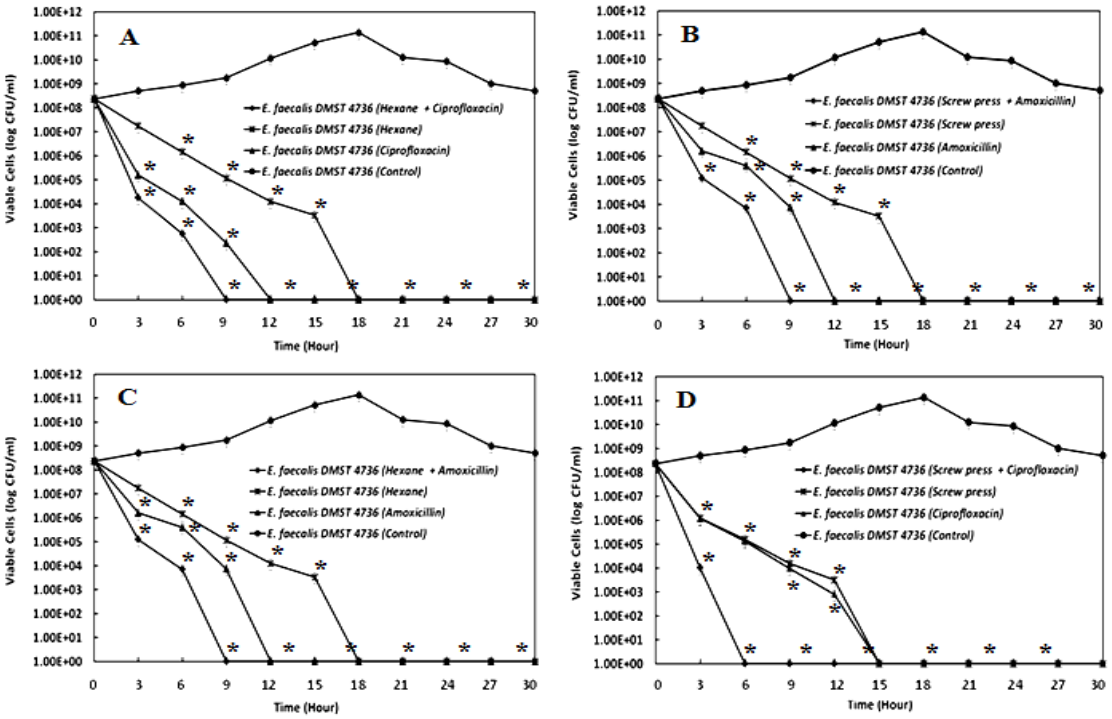

Figure 1: Time-kill curves of hexane extract or screw press extract alone and its combination with ampicillin or ciprofloxacin against *E. faecalis* DMST 4736 (Control = untreated; * = P<0.05 when comparing with control)

Time-Kill Curves

In order to confirm the synergistically antibacterial activity of HE and oil by SP with 2 antibiotics (ampicillin and ciproflaxacin) combinations, Bactericidal activities of these combinations were evaluated using time-kill curves on *E. faecalis* DMST 4736 due to the lowest concentration of MIC values in synergistic interaction. A reduction of >3 $\log_{10}$ and >4 $\log_{10}$ in the cell count obtained in the presence of HE (1/4MIC) with ampicillin (1/8MIC) (Fig. 1A) and crude oil by SP (1/4MIC) with ciprofloxacin (1/8MIC) (Fig. 1D) that was interpreted as synergy in the first 3 hour of incubation.

After 9 hour of incubation, 1/4 MICs of HE was displayed remarkable bacteriocidal synergy in combination with 1/8 MIC of ampicillin and ciprofloxacin on *E. faecalis* DMST 4736 (Figure 1A and 1C).

The combination of 1.562 mg/ml crude oil by SP with 0.0061 µg/ml ciprofloxacin and 0.0122 µg/ml ampicillin had significant bactericidal effect against *E. faecalis* DMST (Figure 1B and 1D) at 6 and 9 hour of incubation, respectively. These findings confirmed the synergistic antibacterial activity of crude extract and antibiotics combination in previous experiment.

The search for bioactive compounds from natural sources has received much attention and efforts of researchers. The suitable antimicrobial agents for replacing synthetic compounds are the goal for identification. Many bacterial strains eg. *S. aureus* and *P. aeruginosa*, the most persistent infectious microorganisms can easily develop their resistance against antibiotics.[23] Thus, the antibacterial drug for these strains is limited. Therefore, attention is then concentrated on alternative or combination agents from edible plants. Increased antibacterial activity of the antibiotic upon combination between crude extracts of gac fruit aril with antibiotics was demonstrated in this study. Gram-negative bacteria was more resistant to gac fruit aril extracts/oils than gram positive bacteria. Many articles have revealed that Gram-positive bacteria was more sensitive to plant antimicrobials than Gram-negative bacteria. This may be due to explanation the different cell wall compositions that limit drug diffusion in concordance with multidrug transporters.[23-24] The different structure of Gram-positive and Gram-negative bacteria and the low affinity between gac fruit aril extracts/oils and lipopolysaccharides may be the main factors for the different susceptibilities to gac fruit aril extracts/oils and to gac fruit aril extracts/oils-drug combination.

Among crude extracts by solvent extraction, crude hexane extracts of *Momordica cochinchinensis* Spreng. (Gac fruit) aril had stronger antimicrobial activity against both gram positive and gram negative bacterial strains than those with methanolic and acetonic extracts. The effectiveness of the extracts depends on the types of solvent used. This observation clearly indicates that the existence of non-polar residues in the crude extracts has better bactericidal and bacteria-static activities. The crude oils of gac fruit aril exhibit higher antibacterial and synergistic effect than crude extracts of that using solvent extractions. Cowan (1999) reported that antibiotic compounds in plants are mostly aromatic or saturated organic molecules which can easily solubilized in organic solvents.[25]

Combination of crude HE and oils of gac fruit aril had synergistic activity on six bacterial strains (*E. faecalis*

DMST 4736, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *K. pneumoniae, P. mirabilis* DMST 8212 and *S. typhimurium* ATCC 13311). Interaction of crude HE and oils by SP has shown synergistic effect against *E. faecalis* DMST 4736, *S. aureus* ATCC 25923. *E. faecalis* DMST 4736, the bacteria of urinary tract infection was sensitive to either combination of crude extracts with oils or separated extracts from *M. cochinchinensis* Spreng aril. The similar finding was found in *S. aureus* ATCC 25923, the bacteria of hospital infections.[26-28] Our study has confirmed that the gac fruit aril extracts and oil and antibiotics could inhibit bacteria. The various mechanisms of inhibition were previously reported.[27] Gac fruit aril contains various bioactive compounds such as carotenoids, fatty acids, α-tocopherol (vitamin E), phenolic compounds and flavonoids,[4,8,29-30] which have been shown to exert profound antibacterial effects against a broad spectrum of pathogenic strains. This paper revealed that *M. cochinchinensis* Spreng aril extracts and oils can be separately used as antibacterial agents or in synergistic combination for overcoming antibiotic resistance.

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