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Research Article

Phytochemical screenings, antibacterial and anti-biofilm activities of *Garcinia cowa* Roxb. leaves extracts and its synergistic effect with antibiotic

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ABSTRACT

The rapidly growing antimicrobial resistance is a global problem. This made it necessary to search for new antimicrobial agents. In present study, agar well diffusion, broth microdilution and time-kill kinetic assays were used to determine the antimicrobial activities of the extracts against seven pathogens. The results showed that 75% acetonic and 95% ethanolic extracts of *Garcinia cowa* Roxb. leaves (GCL) showed the strongest broad-spectrum antibacterial effect towards both Gram-negative and Gram-positive bacteria with 9.67±0.57-22.83±0.45 mm of inhibition zones and MIC/MBC values ranging from 25-100 mg/mL. 95% ethanolic extract revealed the inhibiting and killing actions at rapid first 3 h of incubation, depending on concentration of extracts and had the best capacity to inhibit biofilm formation of all tested bacteria (gastrointestinal and urinary pathogens) with 73.43±0.20-90.14±0.10%. *Pseudomonas aeruginosa* ATCC 27853 displayed the highest sensitivity with all GCL extracts. Interestingly, 95% ethanolic extract and ampicillin combination showed a synergistic effect at FICI value of 0.0313-0.5 (1/16MIC×1/32MIC-1MIC×1/4MIC) against all microorganisms. The bioactive potency of constituents of the 95% ethanolic extract (saponins, tannins, flavonoids, steroids, terpenoids and alkaloids) play an important roles in the observed antibacterial, anti-biofilm and synergistic activities. The findings indicated that *Garcinia cowa* Roxb. leaves extracts had the strong broad-spectrum antibacterial, anti-biofilm and synergistic activities. Crude extracts possess time and concentration-dependent bactericidal actions. Thus, the present results that *G. cowa* Roxb. leaves extracts had potential as a natural alternative antimicrobials for fighting bacterial infections.

Keywords:
Phytochemical screening, Antibacterial and anti-biofilm activities, *Garcinia cowa* Roxb., Time-kill curves, Synergistic effect, *Guttiferae*

1. INTRODUCTION

Recently, *Staphyloccocus aureus*, *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Pseudomonas aeruginosa* have been reported as a group of priority pathogens by the World Health Organization (WHO)[1]. These pathogens have high levels of resistance to most existing antibiotics such as carbapenem, vancomycin, penicillin, ampicillin and the third-generation antibiotic cephalosporin. The incidence of microbial drug-resistance and the limited availability of new antimicrobials gives enough motivation to increase the search for potential drugs or antimicrobial agents from various sources such as plants. Natural resource development is an increasingly important goal in many countries, especially Thailand, where have an abundance of medicinal plants. Medicinal plants are often used in cooking and to treat infections. These plants are often funded by both the public and private sectors in the search for new useful phytochemicals from medicinal plants[2]. *Garcinia cowa* Roxb. is commonly known as Cha-muang in Thailand and is a tropical tree found in Southeast Asia. It belongs to the *Guttiferae* family. The leaves and fruits of this plant are used as vegetables and foods. *Garcinia cowa* Roxb. is rich source of secondary metabolites such as xanthones,

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phloroglucinols, terpenoids, steroids and flavonoids[3]. Many parts of Cha-muang have been used in traditional folk medicine. The leaves are rich in organic compounds such as (-)-hydroxycitric acid and polyphenols[4]. Additionally, the extracts of *G. cowa* roxb. have numerous reported biological activities including anti-diabetes, anticancer, anti-inflammatory, antibacterial and antioxidant[5-9]. Nonetheless, no information on the use of extract of *G. cowa* Roxb. leaves for anti-biofilm and synergistic activities.

Biofilm formation is one of the resistance strategies of many pathogens, which makes it difficult to treat because bacterial strains are resistant to antibiotics. Various bacteria also transfer the resistance genes within members of the biofilm micro-community. Many different bacterial strains are important to humans such as *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Bacillus*, *Salmonella*, etc. Theses strains cause infections that are difficult to treat due to their ability to form biofilms[10]. Currently, antibiotic resistance occurs when bacteria have evolved to avoid the effects of antibiotics through various mechanisms. Antibiotic resistance impairs the ability to treat diseases and infection in humans. Recently, antibacterial effect of plant extracts or natural compounds is found to be synergistic with several antibiotics like ciprofloxacin, tetracycline, gentamycin, etc[11-13]. According to the World Health Organization (WHO), around 80% of the population in developing countries use traditional medicine as primary source of healthcare. Interestingly, the WHO endorses efficacy assessments of plant-based medicine to standardize their application and integration into health care systems[14]. Therefore, this study aimed to screen phytochemical compounds and determine the antibacterial activities of crude extracts of *G. cowa* Roxb. leaves against two Gram-positive bacterial (*Enterococcus faecalis* DMST 4736 and *Staphylococcus aureus* ATCC 25923) and five Gram-Negative bacterial (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212 and *Salmonella typhimurium* ATCC 13311) strains. Furthermore, the anti-biofilm activity of the extracts on the seven pathogens to prevent biofilm formation and synergistic activity of plant extracts with antibiotics was also evaluated to find out the natural/alternative antibacterial substances for controlling pathogenic bacteria.

2. MATERIALS AND METHODS

2.1. Preparation of plant extracts

Fresh leaves of *Garcinia cowa* Roxb. were collected from Mueang District, Chanthaburi Province, Thailand in August, 2019. The plant samples were analyzed and classified by a botanical specialist. The leaves were washed and cut into small pieces, dried with hot air at 40°C and grinded to coarse powder. The coarse powders were macerated with 95% ethanol (ratio=1:6), 75% acetone (ratio=1:6) for 5 days under occasional shaking (room temperature) and boiled in distilled water (ratio= 1:6) at 60°C for 30 min. Afterwards, the extracts were filtered through Whatman No. 1 filter paper and centrifuged at 5,000 rpm for 10 min. Each filtrate was concentrated using a rotary evaporator. All concentrated extracts were kept at -20°C under a dark condition until further analysis. The extraction yield (%; w/v) of all extracts was calculated after evaporation.

2.2. Preliminary phytochemical analysis

Crudes of *Garcinia cowa* Roxb. leave extracts were screened qualitatively the different groups of phytochemical constituents, including saponins, flavonoids, tannins, steroids, alkaloids, terpenoids, anthraquinones and cardiac glycosides[15]. These tests are based on visual observation of color change or precipitate after the addition of specific reagents. The presences of phytochemical compounds in the plant extracts were observed in period of 1-20 minutes after the reactions.

2.3. Microorganisms and culture condition

This study used seven pathogenic strains including Gram-positive bacteria (*Enterococcus faecalis* DMST 4736 and *Staphylococcus aureus* ATCC 25923) and Gram-Negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212 and *Salmonella typhimurium* ATCC 13311). All bacterial strains were obtained from the laboratory of the Department of and were maintained on brain heart infusion (BHI, Difco) agar medium at 37°C.

2.4. Evaluation of antimicrobial activity by agar well diffusion method

Antimicrobial activity of plant extracts was performed using the agar well diffusion method as described Tinrat (2022) with minor modification[16]. Briefly, 100 µL of fresh culture (approximately 10^8 CFU/mL; OD_{600}= 0.5) were inoculated into 5 mL of BHI agar (0.75% agar). The mixture was overlaid on BHI agar plates. Then, well of 6 mm in diameter were dug with a sterile cork borer after solidifying BHI agar. 100 µL of 50, 100, 200 and 400 mg/mL extract solutions was dropped into each wells. After incubating the plates at 37°C for 24-48 h, the inhibition zones around each well were measured in millimetres. The standard positive control (ampicillin; 10 µg) and negative control (DMSO 1%) were included. The results of the diameters of the zones of inhibitions (ZOI) of the extracts were interpreted as a moderate inhibition (6-9 mm); a strong inhibition (10-14 mm) and

a very strong inhibition (>15 mm)[17]. For synergistic activity, the ampicillin, plant extracts and/or combination (ampicillin+plant extract) were filled into each wells.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration values (MBC) of plant extracts

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract were assessed by the broth micro-dilution method by described previously (Tinrat, 2022)[16]. Briefly, the tested concentrations of the extract ranging from 1.56 to 1,600 mg/mL were provided in the 96-well microplate by two-fold serial dilutions in BHI broth. Then, 10 µL of the bacterial suspensions (approximately 10^8 CFU/mL; OD_{600}= 0.5) was added to each microplate well and incubated at 37°C for 24 h. Minimum inhibitory concentration was determined as the lowest concentration that inhibited bacterial growth.

For the MBC values, 5 µL from each well with no visible growth was subcultured on BHI plates, and lowest concentration that showed no visible growth of tested bacteria on the freshly BHI agar plates was considered as MBC.

2.6. Time-kill curves assay

The time taken to give a bactericidal activity after exposure of extracts were assessed using time-kill curves [16]. The bacterial suspension (10^8 CFU/mL) was added into BHI broth containing *Garcinia cowa* Roxb. leave extracts at 1×MIC, 2×MIC and 4×MIC, or 1% dimethyl sulfoxide (DMSO) as a negative control in 96-well plates. The plates were incubated at 37°C. The bacterial growth were conducted at 0, 3, 6, 9, 12, 15, 18, 21, 24, 28 and 32 h by plate count method. Curves were plotted as the viable cells (log_{10}CFU/ml) versus time (h).

2.7. Biofilm inhibition assay

The effects of *G. cowa* Roxb. leave extracts on biofilm formation were evaluated as described by Tinrat (2022) with some modifications[16]. Approximately 200 µL of BHI broth containing pathogenic strains (10^8 CFU/mL; OD_{600}=0.5) were transferred to sterile flat-bottomed 96-well polystyrene microplate. 200 µL of each crude extract (1×MIC value) were added to the microplate and incubated at 37°C for 24 h to culture the biofilms. Broth medium without the inoculum was used as a negative control. The broth culture medium was drained, and each well was washed with 200 µL of phosphate-buffered saline (pH 7.6) to remove all traces of medium and bacterial cells. The biofilm formed was air-dried and stained with 0.1% crystal violet (200 µL/well) for 15-20 min at room temperature. The excess staining solution was washed three times with sterile distilled water. Finally, the adherent cells were re-solubilized with 150 µL of 95% ethanol per well and then the optical density (OD) of each well was measured at 570 nm using a microplate reader. The percentage inhibition of biofilm was calculated as follows:

$$\% \text{ of biofilm inhibition} = \left(\frac{OD_{control} - OD_{sample}}{OD_{control}}\right) \times 100$$

2.8. Synergistic antimicrobial effects

The synergism or antagonism of the combinations was performed by the checkerboard technique in 96-well microplates. Synergistic effects of the combinations were investigated in ampicillin (Amp; 2×MIC) and each crude extract (4×MIC). Each well was inoculated with 10 µL of 10^8 CFU/mL culture (OD_{600}=0.5) of tested microorganisms and then incubated for 24 h at 37°C. 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 in effective MIC of the combination, with the MIC of the antibiotic or extracts alone. FICI values between antimicrobial agents 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)[18].

2.9. Statistical Analysis

All experimental results were expressed as mean± standard deviation (SD) for analysis performed in triplicate. Statistical analysis of the data was performed by analysis of variance (ANOVA). The significant difference between the means was tested using Tukey's multiple comparisons or paired t-test at α=0.05 using Graphpad prism 9.0 software.

3. RESULTS AND DISCUSSION

3.1. % yield of various extracts of *Garcinia cowa* Roxb. Leaves

Biologically active constituents usually occur in low concentration in plants. The extraction technique is that which is able to obtain extracts with high yield and with

minimal changes to the functional properties of the extract required[19]. Maceration technique was used for the extraction in this study. Distilled water and organic solvents (95% ethanol and 75% acetone) were studied for their effects on the extraction yield of *Garcinia cowa* Roxb. leaves. The results showed a significant difference in the extraction yield using different solvents. The crude aqueous extract (12.904% w/v) gave the highest yield, followed by 95% ethanolic (6.509% w/v) and 75% acetonic (6.182% w/v) extracts ($p \leq 0.05$), indicating that extraction efficiency favors the high polar solvents. Based on of the percentage yield, the distilled water was a better solvent for the extraction. The use of water as a solvent is a popular in herbal extraction among folk healers[20].

3.2. Preliminary phytochemical compounds of the *Garcinia cowa* Roxb. leaves extracts

The influence of tested solvents on various phytochemical constituents of *G. cowa* Roxb. leaves (GCL) extracts is depicted in Table 1. According to the observed results, tannins, flavonoids, steroids, terpenoids and alkaloids were found in all crude extracts of GCL, while saponins were present only in 95% ethanolic extract. As with previous research, the major secondary metabolites compounds of *G. cowa* Roxb. had major secondary metabolites in the form of xanthones and phloroglucinols and had minor compounds, including depsidones, terpenoids, steroids and flavonoids[21-22]. These phytochemical compounds are known to support bioactive activities in medicinal plants and thus responsible for the antimicrobial and anti-biofilm activities of this plant extract used in this study. Epidemiological studies reported that flavonoids exhibited antimicrobial, antifungal and antioxidant properties[23], whereas steroids are known to show antibacterial and antifungal activities[24]. Terpenoids are known to have the anti-inflammatory, antibacterial antiviral, antitumor, anti-inflammatory and anti-malarial activities[25]. However, all the extracts showed the absence of anthraquinones and cardiac glycosides, indicating that this plant contain little or no anthraquinones and cardiac glycosides content.

Table 1. Phytochemical screening results of *Garcinia cowa* Roxb. leaves extracts in different solvents.

Plant constituents	*G. cowa* Roxb. leave extracts			Standard of tests
	Distilled water	95% Ethanol	75% Acetone	
Saponins	−	+	−	Froth formation test
Tannins	+	+	+	Ferric chloride test
Flavonoids	+	+	+	Shinoda test
	+	+	+	Lead acetate test
Anthraquinones	−	−	−	Borntrager's test
Steroids	+	+	+	Libermann test
Terpenoids	+	+	+	Salkowski test
Cardiac Glycosides	−	−	−	Keller-Killiani test
Alkaloids	+	+	+	Mayer's test
	+	+	+	Dragendroff's reagent

+: Present, −: Absent

Table 2. Antimicrobial activity of crude extracts of *Garcinia cowa* Roxb. by agar well diffusion method.

Concentration (mg/ml)		Zone of growth inhibition (∅ = 6 mm); mm±SD						
		Gram positive strains		Gram negative strains				
		EF	SA	EC	KP	PA	PM	ST
Dw	**50**	-	-		-	-	-	-
	100	-	-		-	-	-	-
	200	-	-	8.33 ± 0.58^{op}	-	-	-	-
	400	-	10.67 ± 0.28^{kl}	12.83 ± 0.48^{ij}	-	-	-	8.17 ± 0.48^{p}
Eth	**50**	-	-	-	-	8.67 ± 0.75^{op}	-	-
	100	8.67 ± 0.75^{op}	-	6.33 ± 0.49^{q}	-	9.67 ± 032^{mn}	-	-
	200	10.17 ± 0.57^{lm}	-	8.67 ± 0.27^{op}	8.33 ± 0.32^{op}	9.67 ± 0.31^{mn}	8.83 ± 0.88^{no}	-
	400	16.00 ± 0.00^{fg}	9.50 ± 0.21^{mn}	12.83 ± 0.55^{ij}	10.83 ± 0.86^{kl}	11.17 ± 0.12^{jk}	13.83 ± 0.66^{i}	10.00 ± 0.61^{lm}
Ace	**50**	-	-	-	-	-	-	8.33 ± 0.63^{op}
	100	10.83 ± 0.49^{kl}	10.50 ± 1.24^{kl}	9.67 ± 0.57^{mn}	11.33 ± 0.41^{jk}	11.83 ± 0.20^{jk}	10.17 ± 0.54^{l}	13.50 ± 0.98^{i}
	200	13.67 ± 0.49^{i}	12.83 ± 1.72^{ij}	13.67 ± 0.69^{i}	15.00 ± 0.00^{h}	15.33 ± 0.80^{g}	13.00 ± 0.00^{i}	17.83 ± 0.78^{e}
	400	18.50 ± 1.08^{cd}	16.50 ± 0.56^{f}	19.00 ± 0.00^{c}	21.33 ± 0.98^{ab}	22.83 ± 0.45^{a}	18.00 ± 0.00^{d}	20.33 ± 0.63^{b}
Amp (10 µg)		29.00±0.00	21.33±0.043	23.67±0.58	20.33±0.58	22.67±0.58	24.67±0.58	27.00±0.00

^{abcdefghijklmnopqr}: values in the MBC determination with different superscript differed significantly ($p \leq 0.05$) in different extracts; EF: *E. faecalis* DMST 4736, SA: *S. aureus* ATCC 25923, EC: *E. coli* ATCC 25922, KP: *K. pneumoniae*, PA: *P. aeruginosa* ATCC 27853, PM: *P. mirabilis* DMST 8212 and ST: *S. typhimurium* ATCC 13311; Dw : Distilled Water, Eth: 95% Ethanol, Ace: 75% Acetone; –: No zone of inhibition

3.3. Antibacterial activity of agar diffusion method

After the preliminary screening for phytochemical constituents, the crude extracts were assessed antibacterial activity against seven pathogenic strains using agar well diffusion. The varying degrees of the antimicrobial activities of the GCL extracts is shown in Table 2. The results clearly showed that all the extracts showed good dose-dependent antibacterial activity. The 75% acetonic and 95% ethanolic extracts were potentially effective in suppressing microbial growth of tested bacteria. Ampicillin (10 μg/disc) was used as the standard positive control. All tested bacterial strains had more susceptible to ampicillin than crude extracts of GCL within zone of inhibition (ZOI) of 20.33±0.58-29.00±0.00 mm, excepted *P. aeruginosa* ATCC 27853 that showed more sensitive to 75%acetonic extract (22.83±0.45 mm of ZOI) than ampicillin (22.67±0.58 mm of ZOI). Among the tested bacteria, *E. faecalis* DMST 4736, *K. pneumoniae*, *P. aeruginosa* ATCC 27853 and *P. mirabilis* DMST 8212 showed resistance to crude aqueous extract of GCL. *Staphylococcus aureus* ATCC 25923 and *S. typhimurium* was the most resistant strain to 95% ethanolic extracts while *E. faecalis* DMST 4736 (10.17±0.57 mm of ZOI) and *P. aeruginosa* ATCC 27853 (9.67±0.31 mm of ZOI) were the most susceptible strains to the 95% ethanolic extracts at 200 mg/mL, respectively. These results were similar to a previous study of Kaewkod et al. (2022) which reported that the aqueous extract of *G. cowa* had inhibitory effect against *Escherichia coli* and *Staphylococcus aureus* ATCC 25923 with 13.33±2.31 and 20.33± 1.53 mm of ZOI by agar diffusion method. Whereas, ethanol extract of GCL in study of Kaewkod et al. (2022) have more anti-*Escherichia coli* and anti-*Staphylococcus aureus* effect than that of this study with 13.33±2.31 and 22.33±1.53 mm of ZOI[26].

Among the solvents, the most potent antibacterial activity was 75% acetonic (22.83±0.45 mm of ZOI; *P. aeruginosa* ATCC 27853), followed by 95% ethanolic extract (13.83±0.66 mm of ZOI; *P. mirabilis* DMST 8212) and aqueous extract (12.83±0.48 mm of ZOI; *E. coli* ATCC 25922) at 400 mg/ml concentration, respectively. Crude aqueous extract showed the lowest activity with 8.33±0.58-12.83±0.48 mm of ZOI. It did not have any inhibition the all tested bacteria at 50-100 mg/mL indicating the absence of antibacterial activity. 75% acetonic extract of GCL were the most effective extracts and showed a strong antibacterial activity against all bacteria strains at 100-400 mg/mL with inhibition zones of 8.33± 0.63-22.83±0.45 mm. It showed strong antibacterial activity against all tested bacterial strains at 100-200 mg/mL. Moreover, it showed very strong activity in the range of 16.50±0.56-22.83±0.45 mm of inhibition zones against 7 pathogenic strains at the concentration of 400 mg/mL, especially *P. aeruginosa* ATCC 27853. For 95% ethanolic crude extract, it showed the strong inhibitory effects on all bacterial strains at 400 mg/ml (10.00±0.61-13.83±0.66 mm), especially *E. faecalis* DMST 4736 (16.00±0.00 mm; very strong inhibition) and displayed moderate anti-*S. aureus* ATCC 25923 activity (9.50± 0.21 mm). Among tested pathogens, the Gram-negative *E. faecalis* DMST 4736 was more susceptible towards the 95% ethanolic extracts than all tested Gram-positive bacteria. Gram-negative bacteria were more susceptible to the 75% acetonic extract than Gram-positive bacteria which opposed the previous reports[27-28]. In the theory, Gram-positive bacteria are more susceptible than Gram-negative bacteria due to the differences in their cell wall structure. Gram-negative bacteria have outer membrane acting as a barrier to many environmental substances such as antibiotics which Gram-positive bacteria no have. However, in this study revealed that the crude extracts of GCL contain certain constituents like tannins, flavonoids, steroids, terpenoids and alkaloids with significant antibacterial activity which enables the extract to overcome the barrier in Gram-negative cell wall.

3.4. MIC and MBC of plant extracts

The MIC and MBC values of the most effective plant extracts were employed by broth micro-dilution method to evaluate their bacteriostatic and bactericidal properties. The concentration effect of the effective plant extracts were reported in Table 3. The results clearly showed that MIC values of the crude extracts of GCL were ranging from 25-100 mg/mL. The MICs and MBCs of ampicillin (standard control) were showed range from 0.039-1.25 mg/mL and 0.315-2.50 mg/mL for all tested bacterial strains. Crude extracts of *Garcinia cowa* Roxb. represented the higher MIC and MBC values than ampicillin. All crude extracts were exhibited the highest anti-*P. aeruginosa* ATCC 27853 activity with MIC values of 25-50 mg/mL. The 95% ethanolic extract of GCL leaves displayed the strong antibacterial activity against *E. faecalis* DMST 4736, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. typhimurium* ATCC 13311 with 25 mg/mL of MIC values while MIC values against *P. mirabilis* DMST 8212 and *K. pneumoniae* was 50 and 100 mg/mL, respectively. Simultaneously, all pathogenic strains were susceptible to the minimum inhibitory concentration of 75% acetonic extract at 50 mg/mL, except *K. pneumoniae* (MIC value of 25 mg/mL), *P. aeruginosa* ATCC 27853 (MIC value of 25 mg/mL) and *S. aureus* ATCC 25923 (MIC value of 100 mg/mL). Crude aqueous extract showed bacteriostatic activities at 100 mg/ml except for *P. aeruginosa* ATCC 27853 the MIC value was 50 mg/mL. Whereas, the 75%acetonic extract of GCL showed lower inhibitory effect against the growth of all tested microorganisms with 100 mg/mL except for *P. aeruginosa* ATCC 27853 (MIC value of 50 mg/mL).

Table 3. MIC and MBC values of *Garcinia cowa* Roxb. leaves extracts against pathogenic strains by broth micro-dilution assay.

Pathogenic strains	Distilled water		95% Ethanol		75% Acetone	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Gram positive strains						
***E. faecalis* DMST 4736**	100±0.00^{c}	200±0.00^{D}	25±0.00^{a}	50±0.00^{B}	50±0.00^{b}	100±0.00^{C}
***S. aureus* ATCC 25923**	100±0.00^{c}	200±0.00^{D}	25±0.00^{a}	50±0.00^{B}	100±0.00^{c}	100±0.00^{C}
Gram negative strains						
***E. coli* ATCC 25922**	100±0.00^{c}	200±0.00^{D}	25±0.00^{a}	50±0.00^{B}	50±0.00^{b}	200±0.00^{D}
K. pneumoniae	100±0.00^{c}	200±0.00^{D}	100±0.00^{c}	100±0.00^{C}	25±0.00^{a}	50±0.00^{B}
***P. aeruginosa* ATCC 27853**	50±0.00^{b}	200±0.00^{D}	25±0.00^{a}	25±0.00^{A}	25±0.00^{a}	50±0.00^{B}
***P. mirabilis* DMST 8212**	100±0.00^{c}	200±0.00^{D}	50±0.00^{b}	100±0.00^{C}	50±0.00^{b}	100±0.00^{C}
***S. typhimurium* ATCC 13311**	100±0.00^{c}	800±0.00^{E}	25±0.00^{a}	50±0.00^{B}	50±0.00^{b}	100±0.00^{C}

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration

Based on MBC values, crude extracts of GCL represented a bactericidal effect at 25-800 mg/mL which had concentration at >MIC values, except for *P. aeruginosa* ATCC 27853, *K. pneumoniae* (MIC=MBC values in 95% ethanolic extract) and *S. aureus* ATCC 25923 (MIC=MBC values in 75% acetonic extract) (Table 3). All tested bacteria were susceptible to the minimum bactericidal concentration of 95% ethanolic extract (50 mg/mL), excepted *P. aeruginosa* ATCC 27853 (25 mg/mL), *P. mirabili*s DMST 8212 (100 mg/mL) and *K. pneumoniae* (100 mg/mL). Aqueous extract showed a bactericidal activity at MBC value of 200 mg/mL, excepted for *S. typhimurium* ATCC 13311 (800 mg/mL). *Klebsiella pneumoniae* and *P. aeruginosa* ATCC 27853 were the most sensitive bacteria to 75% acetonic extract with MBC values of 50 mg/mL while *E. coli* ATCC 25922 were the least susceptible to this extract (MBC value of 200 mg/mL). It was noted that 95% ethanolic extract had greater antimicrobial activity than aqueous extract, which may be due to the fact that ethanol is the best solvent for bioactive compounds extraction from the plant when compared with distilled water or other solvents[29].

According to the anti-*P. aeruginosa* ATCC 27853 activities, the inconsistency in the antibacterial activity results of 75% acetonic extract by between agar diffusion and broth micro-dilution assay (MICs at 25 mg/ml). It did not show antimicrobial activity at concentrations of 25 and 50 mg/ml of plant extracts by agar diffusion method due to diffusion of plant extracts. The agar diffusion method is unacceptable and cannot be used to determine the MIC values of plant extracts due to its insensitivity. Lack of diffusion of nonpolar molecules to aqueous agar matrix[30].

3.5. Time-kill curves assay

With the screening promoted by the broth micro-dilution test, it was defined that 95% ethanolic and 75% acetonic extracts of *G. cowa* Roxb. leaves were selected for Time-kill kinetics assay. The ability of crude extract of GCL in time-killing the pathogenic strains was evaluated by analyzing the survival cells at 1×, 2× and 4×MIC. From the result of MICs determination, killing curve assay from crude extracts of *G. cowa* Roxb. leaves against 7 pathogenic bacteria, including five gastrointestinal/urinary pathogens (Figure 1A) and two opportunistic pathogens (Figure 1B) were performed. *S. aureus*, *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. mirabilis* were as gastrointestinal pathogens[31] while *E. faecalis* DMST 4736 and *P. aeruginosa* ATCC 27853 were known as an opportunistic pathogens[32]. The result showed that crude 95% ethanolic extracts of *G. cowa* Roxb. leaves had bactericidal effect against all tested microorganisms, except *K. pneumoniae* that was susceptible to 75% acetonic extract of GCL (Figure 1A-B). Moreover, *G. cowa* Roxb. leaves extracts had bacteriostatic (>1.0×10$^{3\text{-}7}$ CFU/ml of survival cell) and bactericidal activity (<2.0×10^{2} CFU/ml of survival cell) against all of the tested microorganisms at all studied concentrations when compared with initial cell (3.0-3.3×10^{8} CFU/ml). All crude extracts exhibited a significant reduction in the viable cell count of the test bacteria after 3 h of incubation at the 1×, 2× and 4×MIC concentrations (p≤0.05). The reduction of viable cells (log cfu/ml) of the tested bacteria were approximately 1-3log$_{10}$, 2-5log$_{10}$, 2-5log$_{10}$, 2-4log$_{10}$, 0.5-2log$_{10}$, 2-4log$_{10}$ and 1-3log$_{10}$ for *S. aureus* ATCC 25923, *E. coli* ATCC 25022, *S. typhimurium* ATCC 13311, *K. pneumoniae*, *P. mirabilis* DMST 8212, *E. faecalis* DMST 4736 and *P. aeruginosa* ATCC 27853, respectively.

At all 95% ethanolic extract of GLC showed significantly reduced at least 1 log$_{10}$ CFU/mL from the initial count at all studied concentrations at the first 3 h of testing and slightly declined to 2-3log$_{10}$ CFU/mL after 6 h of incubation (p≤0.05). At 1×MIC 95% ethanolic extract had bacteriostatic and bactericidal activities

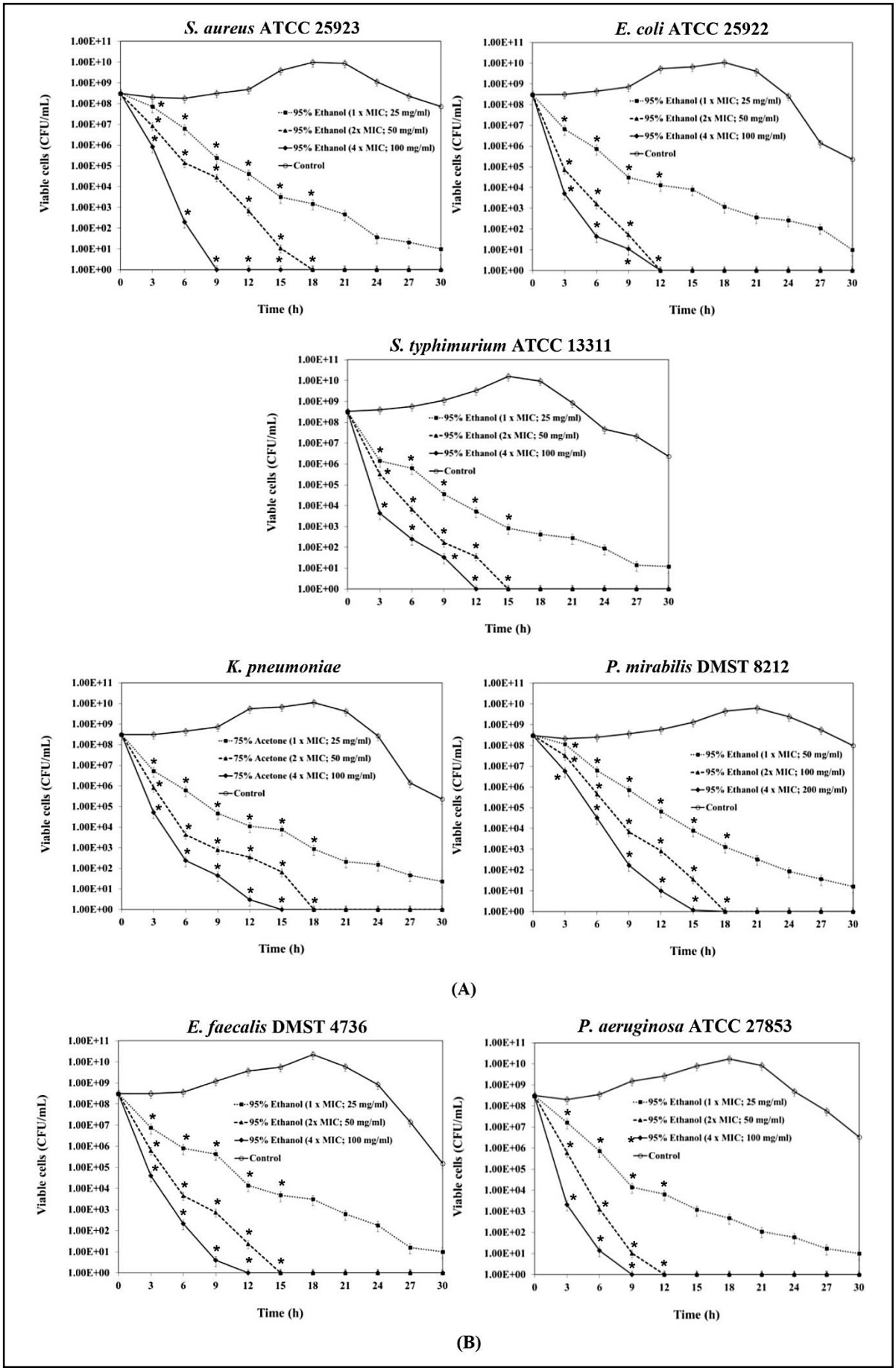

Figure 1. Time-killing curves of gastrointestinal/urinary pathogens (A) and opportunistic pathogens (B) at different concentrations of *Garcinia cowa* Roxb. extracts. CFU=Colony Forming Units; *=Indicate statistically significant difference between groups (p<0.05).

(reducing the $\log_{10}$ CFU/mL by greater than 3 logs) against all of the tested microorganisms after 9 and 12 h of incubation (<2.6×10^2 CFU/ml) when compared with initial cell ($3.0\text{-}3.3\times10^8$ CFU/mL), respectively. Whereas, the viable count ($\log_{10}$ CFU/mL) of *S. aureus* ATCC 25923 (Figure 1A) and *P. aeruginosa* ATCC 27853 had drastically decreased in first 6 h and obvious bactericidal and 100% completed kill effects in the first 9 h of incubation at 2×MIC of 95% ethanolic extract. Moreover, it is observed that *P. aeruginosa* ATCC 27853 was sensitive strains to 95% ethanolic extract of GCL which had 100% complete killed effect for *P. aeruginosa* ATCC 27853 in the first 12 and 9 h of incubation at 2×MIC and 4×MIC concentrations, respectively (Figure 1B). Of 75% acetonic extract revealed the potent bactericidal activity in the first 9 (2×MIC) and 6 (4×MIC) h exposure for *K. pneumoniae* while both concentration had a complete elimination at 18 (2×MIC) and 15 (4×MIC) h of testing (Figure 1A). Time-kill kinetics study has been used to examine many antimicrobial agents and they are also often used as the basis for in-*vitro* investigations for pharmacodynamic drug interaction[33].

3.6. Biofilm inhibition assay

Bacterial biofilms remain a global health threat. Because they can interfere with treatment and exacerbate hospital infections. Therefore, it is an important to find efficacious novel agents to deal with this problem[34]. The crude extracts of *G. cowa* Roxb. leaves were investigated the anti-biofilm activity by the crystal violet retention assay. The results of in *vitro* anti-biofilm activity of GCL extracts against bacteria species are presented in Table 4. The percentage inhibition of biofilm formation values between 0 to 100%. Above the 50% inhibition was good activity, while it is poor when had value of 0-49%. Below the 49% inhibition was slightly activity[35]. The influence on biofilm formation varied among the tested bacterias. *Garcinia cowa* Roxb. leaves extracts had significantly and effectively inhibited the biofilms formation of all tested strains ($p\leq0.05$). Of the three crude extracts of GCL evaluated, all crude extracts had significantly good anti-biofilm activities with values above 75% in all bacterial strains ($p\leq0.05$). The 95% ethanolic extract of GCL had the highest anti-biofilm formation against *P. aeruginosa* ATCC 27853 followed by *E. coli* ATCC 25922 and *E. faecalis* DMST 4736 with values 90.14±0.10%, 84.03±0.40% and 83.99±0.30%, respectively. *Klebsiella pneumoniae* had the lowest percentage of biofilm inhibition by 95% ethanolic extract (73.43±0.20%). *Klebsiella pneumoniae* is an opportunistic pathogen in hospital and causes mainly pulmonary and urinary tract infections[36-37] by developing bacterial biofilms in catheters[38]. *E. coli* ATCC 25922 (72.45±0.20%) and *E. faecalis* DMST 4736 (70.20±0.20%) had the highest and lowest percentage of biofilm inhibition by aqueous extract, respectively. Aqueous extract of GCL had more anti-adhesion activity against Gram-negative bacteria than Gram-positive bacteria while 95% ethanolic and 75% acetonic exhibited remarkable species-dependent inhibition of biofilm formation. Moreover, none of the *G. cowa* Roxb. leaves extracts could completely inhibit biofilm formation at 1×MIC concentration after 24 h of incubation. Of the tested pathogenic strains, *P. aeruginosa* ATCC 27853 were the most susceptible bacterial strain with % anti-biofilm activity of 90.14±0.10 (95% ethanolic extract) and 78.25±0.30 (70% aceonic extract). *Pseudomonas aeruginosa* and *E. faecalis* is a common human opportunistic pathogen. They can produce various virulence factors such as biofilm formation. Both strains were frequently identified bacterial species in biofilm infections. Nevertheless, both bacteria are known to cause serious diseases, interactions between the bacteria in biofilms have rarely been examined. The observed anti-biofilm effects of the active compounds (flavonoids, alkaloids, and saponins) evaluated in this study are supported by research conducted by Apriliany et al. (2013) who reported that flavonoids, alkaloids, and saponins in cinnamon ethanol extract exhibited an anti-quorum sensing and anti-biofilm activities of *Pseudomonas aeruginosa*[39].

3.7. Synergistic antimicrobial effects of plant extracts with antibiotic

Bacterial drug resistance is often associated with

Table 4. Anti-biofilm activity of crude extracts of *Garcinia cowa* Roxb. leaves (1×MIC) against pathogenic strains biofilms.

Pathogenic strains		%Biofilm Inhibition		
		Distilled water	**95% Ethanol**	**75% Acetone**
Gram positive strains	***E. faecalis* DMST 4736**	70.20 ± 0.20^{cC}	83.99 ± 0.30^{aBC}	72.44 ± 0.40^{bD}
	***S. aureus* ATCC 25923**	71.00 ± 0.60^{cB}	83.02 ± 0.30^{aC}	72.37 ± 0.40^{bDE}
Gram negative strains	***E. coli* ATCC 25922**	72.45 ± 0.20^{bA}	84.03 ± 0.40^{aB}	71.32 ± 0.30^{bE}
	K. pneumoniae	71.12 ± 0.40^{cBC}	73.43 ± 0.20^{bE}	77.89 ± 0.20^{aA}
	***P. aeruginosa* ATCC 27853**	71.21 ± 0.20^{cBC}	90.14 ± 0.10^{aA}	78.25 ± 0.30^{bA}
	***P. mirabilis* DMST 8212**	71.19 ± 0.50^{bBC}	78.33 ± 0.30^{aD}	73.94 ± 0.10^{aBC}
	***S. typhimurium* ATCC 13311**	71.57 ± 0.20^{cBC}	78.27 ± 0.40^{aD}	73.37 ± 0.30^{bCD}

^{abc}: Means with different superscript in the same row show significance ($p\leq0.05$).
^{ABCDE}: Means with different superscript in the same column show significance ($p\leq 0.05$).

improper use of antimicrobials. The ability of plant extracts to act synergistically with antibiotics is a new approach that helps in solving the problem of bacterial resistance. The fractional inhibitory concentration (FIC) index (FICI) was obtained by adding the FIC values of plant extract and ampicillin (Amp). Synergistic reaction was also observed in combination of plant extracts and ampicillin against seven pathogenic strains with FICI value of ≤0.5. As shown in Table 5, the combination of ampicillin+plant extracts have synergistic effects on all tested bacterial strains, excepted *E. faecalis* DMST 4736 (50:0.039;2MIC×1MIC) and *S. aureus* ATCC 25923 (50:0.0195;2MIC×1MIC) for ampicillin+95% ethanolic extract which showed additive effect (FICI=1).

Table 5. Synergistic effects of *Garcinia cowa* Roxb. extracts with ampicillin.

Pathogenic Strains	Extracts : Medicine	MIC values (mg/ml : μg/ml)			Interpretation	
		Alone	Combination		FICI value	Effect
		(4MIC:2MIC)	FIC_{ext}	FIC_{ant}		
***E. faecalis* DMST 4736**	GCW : Amp	400 : 0.078	1MIC	1/2MIC	0.5	S
	GCE : Amp	100 : 0.078	2MIC	1MIC	1	AD
	GCA : Amp	100 : 0.078	1/2MIC	1/4MIC	0.25	S
***S. aureus* ATCC 25923**	GCW : Amp	400 : 0.039	1MIC	1/4MIC	0.5	S
	GCE : Amp	100 : 0.039	2MIC	1MIC	1	AD
	GCA : Amp	400 : 0.039	1/2MIC	1/4MIC	0.25	S
***E. coli* ATCC 25922**	GCW : Amp	400 : 0.625	1MIC	1/2MIC	0.5	S
	GCE : Amp	100 : 0.625	1MIC	1/2MIC	0.5	S
	GCA : Amp	200 : 0.625	1/2MIC	1/4MIC	0.25	S
K. pneumoniae	GCW : Amp	400 : 10	1/8MIC	1/16MIC	0.0625	S
	GCE : Amp	400 : 10	1/8MIC	1/16MIC	0.0625	S
	GCA : Amp	100 : 10	1/6MIC	1/16MIC	0.0625	S
***P. aeruginosa* ATCC 27853**	GCW : Amp	200 : 10	1/4MIC	1/8MIC	0.125	S
	GCE : Amp	100 : 10	1/16MIC	1/32MIC	0.0313	S
	GCA : Amp	100 : 10	1/16MIC	1/32MIC	0.0313	S
***P. mirabilis* DMST 8212**	GCW : Amp	400 : 0.078	1MIC	1/2MIC	0.5	S
	GCE : Amp	200 : 0.078	1MIC	1/4MIC	0.5	S
	GCA : Amp	200 : 0.078	1/2MIC	1/4MIC	0.25	S
***S. typhimurium* ATCC 13311**	GCW : Amp	400 : 0.156	1MIC	1/2MIC	0.5	S
	GCE : Amp	100 : 0.156	1MIC	1/2MIC	0.5	S
	GCA : Amp	200 : 0.156	1/4MIC	1/8MIC	0.125	S

GCW: *G. cowa* Roxb. extract in distilled water, GCE: *G. cowa* Roxb. extract in ethanol, GCA: *G. cowa* Roxb. extract in acetone; Syn: Synergy; AD: Addition; Ext: Extracts; Ant: Antibiotic

Among three plant extracts (aqueous, 95% ethanolic and 75% acetonic extracts), mixtures of ampicillin+95% ethanolic extract and ampicillin+75% acetonic extract significantly displayed the best synergistic interactions on *P. aeruginosa* ATCC 27853 with FICI of 0.0313 (1.5625:0.1563;1MIC×1/4MIC) ($p \leq 0.05$) and 31.83±0.66 mm of $ZOI_{synergistic}$ when compared with plant extract (4MIC;9.67±032 mm) and ampicillin alone (2MIC; 22.67±0.58 mm) (Figure 2). The strong synergistic capacity against *K. pneumoniae* was the combination of all plant extract+amplicillin with FICI of 0.0625 (1/8 MIC×1/16MIC). *E. coli* ATCC 25922, *P. mirabilis* DMST 8212 and *S. typhimurium* ATCC 13311 exhibited synergistic reactions to the all plant extracts+amplicillin combinations with FICI of 0.25-0.50 (Extract : Amp; 1-1/2MIC×1/2-1/4MIC) while *E. faecalis* DMST 4736, *S. aureus* ATCC 25923 showed synergistic actions to aqueous/75% acetonic extracts+ampicillin combinations with FICI of 0.25-0.50 (Extracts : Amp; 1-1/2MIC×1/2-1/4MIC). Moreover, the strongest synergistic effect against *S. typhimurium* ATCC 13311 was 75% acetonic extracts+ampicillin combinations with FICI of 0.125 (12.5:0.0098;1/4MIC×1/8MIC). This mixture showed significantly increased antimicrobial activity with 27.50± 0.50 mm of $ZOI_{synergistic}$ when compared with plant extract (4MIC;17.83±0.78 mm) and ampicillin alone (2MIC; 20.50±0.50 mm) (Figure 2).

The interactions between plant extracts and antibiotics were interested in this study. In several previous studies, the synergy of plant compounds with antibiotics have delays drug-resistant bacteria[40-41]. Based on synergistic results, ampicillin is a β-lactam antibiotic that inhibits cell wall peptidoglycan synthesis of bacteria[42]. When the bacterial cell wall is weak, plant compounds of GCL can easily enter the bacterial cell. These compounds can disrupt the bacterial metabolisms of and organelles causing bacterial death. These results are similar to those obtained by Navrátilová et al. (2016) who reported that the medicinal plants effectively increase the antibacterial activity of antibiotics[43].

According to the findings, this study could be useful for the development of new natural products for the treatment of bacterial infection or biofilm-related diseases in the future. Nevertheless, the present study provides an

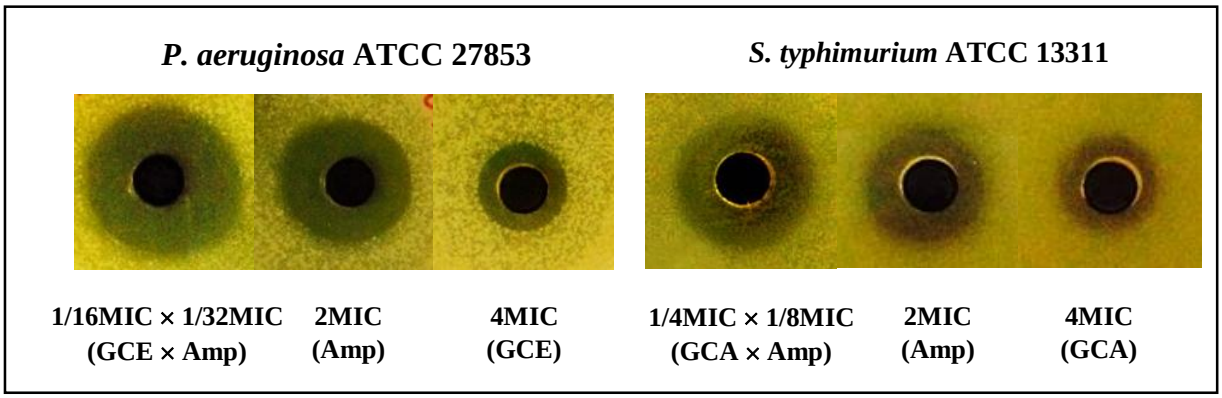

Figure 2. Synergistic antimicrobial activity of plant extracts and ampicillin combinations against pathogenic strains by agar well diffusion.

important basis for further investigations in the characterization of plant constituents from *Garcinia cowa* Roxb. leaves with antimicrobial, anti-biofilm and synergistic activities for drug development. The study used qualitative analysis and screening only. It would be better if a quantitative assay and the infrared spectrum of various phytochemicals could be performed. Assessment of the bioactivities of the active components in pure form is essential to understand the reported effects.

In addition, overuse of antibiotics to treat the bacterial infections tends to grow the problem of antibiotic resistance in the future. The action of the antibiotics affects both beneficial and harmful microorganisms, which the plant extracts can alleviate this concern. From the results of present study, it can be seen that *Garcinia cowa* Roxb. leaves extracts exhibited the bacteriostatic and bactericidal activities towards various bacterial strains, especially *P. aeruginosa* ATCC 27853 which had susceptible to GCL extract (75% acetonic extract) more than ampicillin using agar well diffusion method in this study. Combination of GCL extracts and ampicillin also have synergistic effects on all studied strains. Consequently, *Garcinia cowa* Roxb. leaves extract is an interesting source for future development as a valuable bacterial control agent.

4. CONCLUSION

In present study, preliminary phytochemical screening of three *Garcinia cowa* Roxb. leaves extracts was found to give positive reactions for tannins, flavonoids, steroids, terpenoids and alkaloids, except for saponins was found only in 95% ehanolic extract. All GCL extracts had different antimicrobial, anti-biofilm and synergistic abilities to inhibit the growth of pathogenic strains depending on the solvents of extraction. 95% ethanolic extract exhibited a strong and broad spectrum antimicrobial (MIC/MBC value of 25-100 mg/mL), time killing assay (bacteriostatic and bactericidal effect at first 3 and 6 h of incubation, respectively) and anti-biofilm (73.43±0.20-90.14±0.10% Biofilm Inhibition) and synergistic (0.0313-1.0 of FICI value) activities while 75% acetonic extract showed a strong and broad-spectrum activity against both Gram-positive and Gram-negative bacteria (7 tested bacterial strains) by agar diffusion assay with 9.67±0.57-22.83±0.45 mm of ZOI at 100-400 mg/mL of concentration. 95% ethanolic extract showed strong and rapid bacteriostatic and bactericidal activities against *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. faecalis* DMST 4736 as opportunistic pathogens while 75% acetonic extract showed strong action towards *K. pneumoniae* as gastrointestinal pathogens by time killing assay (Figure 1A). Moreover, it was found that antimicrobial compounds of GCL extracts were added to enhance the synergistic effect with ampicillin, especially aqueous extract. This extract not almost have any antimicrobial properties by agar well diffusion and showed the lowest inhibitory effect against pathogens due to its high concentration of MIC and MBC values by broth micro-dilution method. Simultaneously, ampicillin was also improved antibacterial efficacy against pathogenic strains when supplemented with GCL extracts. Hence, the results of this study have revealed the importance of *G. cowa* Roxb. leaves extracts when used extract alone and combined with antibiotic to bacterial control, which enables the use of a mixture of antibiotics and *G. cowa* Roxb. leaves extracts against bacterial infections, especially, gastrointestinal/urinary and opportunistic pathogens.

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Sirikhwan Tinrat designed an experiment, analyzed the data and wrote the manuscript.

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