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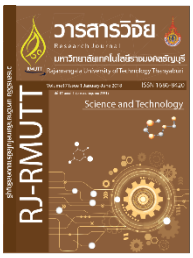

The combination effect of tetracycline with bitter gourd (*Momordica charantia*) fruit extract and gallic acid on antimicrobial against pathogens

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ABSTRACT

Antibiotic drugs resistant of the pathogenic microorganism problem has increased rate in the current. The single antibiotic substances often potential decrease for therapy. Thus, the multiple combinations of antimicrobial agents are a new strategy for improving this problem. Natural products are the sources of antimicrobial substances which are developed for antibiotic combination. In this research, the antimicrobial effect of phytochemical compounds, gallic acid and caffeic acid, bitter gourd fruit extracts and tetracycline which is representative of antibiotic drug on the growth of *Pseudomonas aeruginosa ATCC 27853* and *Staphylococcus aureus ATCC 25923* were determined. Then, the combined effects of bitter gourd fruit extracts and gallic acid with tetracycline were determined using checkerboard assay and analyzed by FIC Index. The result showed that all treatment samples inhibited growth of both pathogenic bacterial cells *in vitro*. The MIC values of bitter gourd fruit extracts, gallic acid and tetracycline

on *Ps. aeruginosa* were 12000, 2000 and 16 µg/ml, respectively. Additionally, MIC dose on S. *aureus* showed 16000, 6000 and 1 µg/ml, respectively. Antagonistic effect was observed with the combination of bitter gourd fruit extracts and tetracycline as evidenced by high values of the FIC index on *Ps. aeruginosa* and *S. aureus* around 5.33 and 6.50, respectively. However, the combination effect of gallic acid and tetracycline showed an additive effect with the values of the FIC index of *Ps. aeruginosa* and *S. aureus* 3.00 and 0.83, respectively.

INTRODUCTION

Drug resistance of pathogenic microorganism is the critical condition of public health of the world wide [1]. In Thailand, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the nosocomial pathogen which has been reported to have high frequency resistance drugs in most hospitals [2-3]. Several mechanisms for resistance against antibiotics of *Ps. aeruginosa* have been offered - which include antibiotic inactivation by enzymatic digestion, altering the efflux pump mechanisms, target mutation, and decreased uptake of antibiotics [4]. Moreover, *S. aureus* has been reported as the superbug [5]. Therefore, the mono-treatment substances are often unsatisfactory for therapy infectious diseases. To solve this situation, the new strategies are developed by using the multiple combinations of antimicrobial agents [3, 6-7].

Since long, there are many natural products which are indicated to have high potential of antimicrobial activity, for example phenolic acids, flavonoids, essential oil and alkaloids [8-9]. The bioactive products from natural resources are a new strategy for developing combined therapies [10-12].

Momordica charantia (Bitter gourd, bitter melon or balsam pear) is a kind of medicinal plant that belonging to the family Cucurbitaceae [13]. It is the economically plant in many countries. Moreover, it is a natural resource which reported to have many biological properties [14]. The ethanoic extracts of bitter gourd leaves showed antimicrobial, antioxidant activity [15], and antitumor activity. Additionally, the extract of bitter gourd fruit is an anti-diabetic substance in normal and alloxan-diabetic rats. Furthermore, bitter gourd fruit extract acted as the antimicrobial, antioxidant, antiviral, anti-hepatotoxic and anti-ulcer genic activities [14].

Tetracycline is an antibiotic drug which inhibited growth of bacterial cell by interruption of protein synthesis of bacterial cells. The mechanism of action of tetracycline has been report by Chopra and Roberts [16]. Bacterial cells up take tetracycline into cytoplasm via transmenbrane protein or efflux pump, after that it interacted on the acyltransferase site (A-site) of 30s ribosome subunit due to aminoacly-tRNA unable to bind at this site and then the process

of protein synthesis stopped and then bacterial cell cannot growth [16].

Thai medicinal herbs and phytochemical agents are determined the combination effect with antibiotic drugs on resistant pathogenic microorganism. For example, *Rhinacanthus nasutus* leaves extract displayed the antimicrobial activity on *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin- resistant *Staphylococcus aureus* (MRSA) [17]. Moreover, the combination results indicated *Rhinacanthus nasutus* leaves extract exhibited the synergistic effect with acarbose [18]. In addition, alpha-mangostin /gentamicin combination against *S. aureus* (MRSA) and MSSA isolate. The synergistic effect was found in MSSA isolate [19].

However, there is small report which studied the combination effect of bitter gourd fruit extract with antibiotic drug on inhibitory bacterial cell growth. Therefore, in this research, in vitro antimicrobial activity of Bitter gourd fruit extracts, gallic acid, caffeic acid and tetracycline were investigated on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. The MIC value of each treatment sample was determined using the broth dilution technique. Finally, the combined effect was investigated using checkerboard test and analyzed data by FIC index.

RESEARCH METHODOLOGY

Extraction process

The process of sample collection was performed in June, 2017. Matured bitter gourd (*Momordica charantia*) fruits were kept from Tha Phae district of Satun province, Thailand. Bitter gourd fruits were washed for 3 times and placed at room temperature until dried. Next, the whole fruits were cut to reduce the area size. The extraction process was briefly applied previously descriptively [13]. Approximately, 15.0 g of small piece fruits were homogenized with 95% ethanol for 5 minutes. The homogenate was shaken 180 rpm at room temperature, for 24 h, after that incubated at 4°C overnight. To make clearing supernatant, the homogenate was filtrated using membrane filter Whatman no. 05. The ethanol solvent was removed under the evaporator machine. Evaporated supernatant was frozen dry by lyophilize. Then, the powder of the extract was balanced and kept in -20°C.

Phytochemical and standard antibiotic agents

Gallic acid, Caffeic and tetracycline were purchased from sigma Addict Company.

Pathogenic microorganism

Pseudomonas aeruginosa ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were supported from Sompong Klaynongsruang laboratory which under the Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.

Antimicrobial susceptibility assay

The mini modified broth dilution method was used to determine the Minimal Inhibitory

Concentration (MICs) of treatment agents. Pathogenic bacteria were cultured in 3 mL of nutrient broth at 37°C, 180 rpm for 4-6 h to observe the optical density at 600 nm (OD600nm) approximate 0.5 (1 x 108 CFU/mL) and then diluted turbidity to 1 x 106 CFU/mL. Next, 1.0 mL of suspended bacterial cells was placed in test tubes. All treatment compounds were prepared under the Serial twofold dilutions using NB medium. Diluted substances (1mL) were added into the suspended bacterial test tube, mixed gentle and incubated at 37°C, 180 rpm for 18-20 h. The MIC of each agent was detected as previously described [3].

Determination of combination effect

The activity of treatment samples and antibiotics in combination effect was determined by the checkerboard broth micro dilution method. The antibiotic and samples were prepared by twofold serial dilutions. Approximately 50 µl of each component (bitter ground fruit extracts and gallic acid) were mixed with tetracycline and incubate at 37°C for 15 min. The combined samples were placed into the sterile 96-well Microtiter plate which contained 100 µL of suspended bacterial cells (1 x 106 CFU/mL). Plates were then incubated at 37°C and MIC was determined after 18-20 h of incubation. The Fractional inhibitory concentration (FIC index) for all the combinations was investigated using the following formula as described by [20].

FIC index = $FIC_A + FIC_B$

$= [A]/MIC_A + [B]/MIC_B$.....(1)

FIC_A, FIC_B - Fractional inhibitory concentration of drug A & B respectively. MIC_A, MIC_B - Minimum inhibitory concentration of drug A & B respectively. [A], [B] - Concentration of drug A & B respectively. FIC index by checkerboard method is interpreted as follows:

FIC index ≤ 0.5; synergy

0.5 < FIC index ≤ 4; additivity and

FIC index > 4; antagonism

EXPERIMENT AND RESULTS

Dried weight of Bitter gourd fruit extracts

Bitter gourd fruits were extracted for three times by the same condition. The lyophilized powder of the extracts was balanced for recording dried weight. The data was showed in Table 1. The average ±SD of dried weight was 0.637±0.040 g. This extract is a treatment sample which was used to treat with pathogenic bacterial cells (*Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923) to determine antimicrobial activity, MIC and combination effects.

Table 1 Dried weight of bitter gourd fruits Extracts

	Dried weight sample (g)			
	Repeat times			Mean ± SD
Bitter gourd	(1)	(2)	(3)	
fruits extracts	0.60	0.68	0.63	0.637 ±0.04

Antimicrobial activity of treatment samples

Four kinds of treatment samples such as bitter gourd fruit extracts, gallic acid, caffeic acid and tetracycline were determined antimicrobial activity on *Ps. aeruginosa* ATCC 27853 and S. aureus ATCC 25923. All treatment samples were able to inhibit growth of both pathogens (Figure. 1-2A, 2B, 2C, 2D). The bitter gourd fruit extracts displayed the antibacterial activity at the high presenting concentration. Approximately 80% of percentage inhibition on *Ps. aeruginosa* ATCC 27853 was proposed at the extracted dose more than 10,000 µg/mL (10.0 mg/mL). Thus, the MIC value of the bitter ground fruit extract was examined from ranging dose 10,000 – 20,000 µg/mL. In addition, gallic acid and tetracycline showed the potential of antimicrobial activity on both pathogens. Additionally, the antibacterial property of these treatment samples on *S. aureus* ATCC 25923 was similar in the results on *Ps. aeruginosa*. However, caffeic acid showed weak inhibition effects on both pathogens. The percentage of inhibition less than 50% of all concentration tests. The antimicrobial property of treatment samples was summarized and showed in Table 2.

Minimal inhibition concentration analysis

Minimal inhibitory concentration is the minimal dose of treatment samples that able to inhibit growth of bacterial cells by appearing clear turbidity. All treatment samples were measured MIC value on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 by broth dilution technique. The results were presented in Table 3. The MIC value of bitter gourd fruit extracts, gallic acid and tetracycline on *Ps. aeruginosa* ATCC 27853 was 12000, 2000 and 16 µg/mL, respectively. In addition, MIC on *S. aureus* ATCC 25923 showed 16000, 6000, 1 µg/mL, respectively. However, caffeic acid unable to determine MIC value in this research

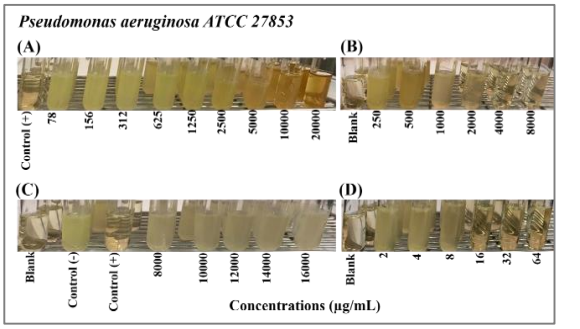

Figure 1 The inhibitory effects of bitter gourd extracts (A), gallic acid (B), caffeic acid (C) and tetracycline (D) on *Ps. aeruginosa* ATCC 27853.

Combination effect

The combination effect between two compounds were studied and analyzed by FIC index. The data showed in Table 4. Tetracycline that combined with bitter gourd fruits extracts on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 presented the FIC value 5.33 and 6.55, respectively. This data indicated that Tetracycline and bitter gourd fruit extracts displayed the antagonistic effect. The result of tetracycline and gallic acid exhibited the FIC 3.00 and 0.83, respectively. Therefore, the combined effect of both agents was the additivity. (Fig. 3).

CONCLUSION AND DISCUSSION

The antimicrobial activity of bitter gourd fruits extracts on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 was indicated that bitter ground fruits were a source of bioactive compounds. Minimal inhibitory concentration value (MIC value) of bitter ground fruits extracts on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 was 12,000 and 16,000 µg/mL, respectively. Previously report, bitter gourd fruits from many areas in Taiwan were extracted by water and methanol. All the extracts (10.0 mg/mL) were determined antibacterial activity on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 3359. The results showed all extracts cannot inhibit growth of both pathogens. On the other hands, these extracts able to induce bacterial cell growth [13] which differenced from our research. Furthermore, there is a report which extracted bitter gourd fruits by 95% ethanol and studied antimicrobial activity on *Ps. aeruginosa* and two species of *S. aureus* (Newman and RomeroΔcrtM). The report indicated that the extract unable to inhibit growth of *Ps. aeruginosa* but inhibited *S. aureus* (Newman) and (RomeroΔcrtM) at the MIC value 12,340 mg/mL and 670 mg/mL, respectively [21]. The differences area sources of bitter gourd fruits also contained the different bioactive compounds due to the extracts displayed the antimicrobial activity difference. Gallic acid was bioactive compound which presented antimicrobial activity of many microorganisms [3]. Moreover, it has been reported to have antioxidant activity [22] and anticancer property [23]. In this report, MIC value of gallic acid *on Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 was 2000 and 6000 µg/mL, respectively. Our report associated with the previous research which showed the antimicrobial activity of gallic acid on *Ps. aeruginosa* DB5218, DR3062, ATCC 15692, PA01 and PT121 at the MIC value 2000 µg/mL [3]. In addition, a recently report showed the MIC dose on *S. aureus* of gallic acid present higher than 5,000 µg/mL which related to this research [24]. Tetracycline is a kind of antibiotic which inhibited growth of microorganism by interruption of protein synthesis. In this report, MIC value of tetracycline on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 was 16 and 1 µg/mL, respectively. However, in another reports found the MIC of tetracycline on *Ps. aeruginosa* and *S. aureus* was 32 and 16 µg/mL, respectively [3, 25]. These data indicated that tetracycline able to inhibit growth of both pathogens and MIC value can different because there are many factors that associated with the experiment; for example, difference of bacterial species and difference of treatment conditions.

The combination effect was analyzed by the FIC index. Tetracycline and bitter gourd fruit extracts on *Ps. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 showed FIC around 5.33 and 6.50, respectively. This result indicated that both substances displayed combination effect as the antagonism. Therefore, bitter gourd fruits extracts should not combination use with tetracycline. The combination effect of tetracycline and gallic

acid on both bacterial pathogens also showed the FIC index in the case of additivity. However, our research different from the report of Jayaraman et al. (2010) which indicated the combination effect of tetracycline and gallic acid as synergism [3]. Gallic acid inhibited growth of bacterial cells by disrupting and produce pore formation on plasma membrane of bacterial cells including intracellular potassium release, physicochemical surface properties, and surface charge [26]. However, the bitter gourd fruit extracts contained many kinds of bioactive compounds leading to show the difference of the combination mechanism.

Although, the synergistic effect is the goal target of combination strategy, but natural compounds in foods or supplement foods also showed the antagonistic effect with many kinds of antibiotic drugs due to the potential of antibiotics are reduced [3]. Therefore, the strategies for using combination should be two possible hypotheses such as (1) the compounds that have synergistic effect with antibiotic drugs should be combined use as well (2) the substances that have an antagonistic effect with antibiotic drugs should not use and avoid.

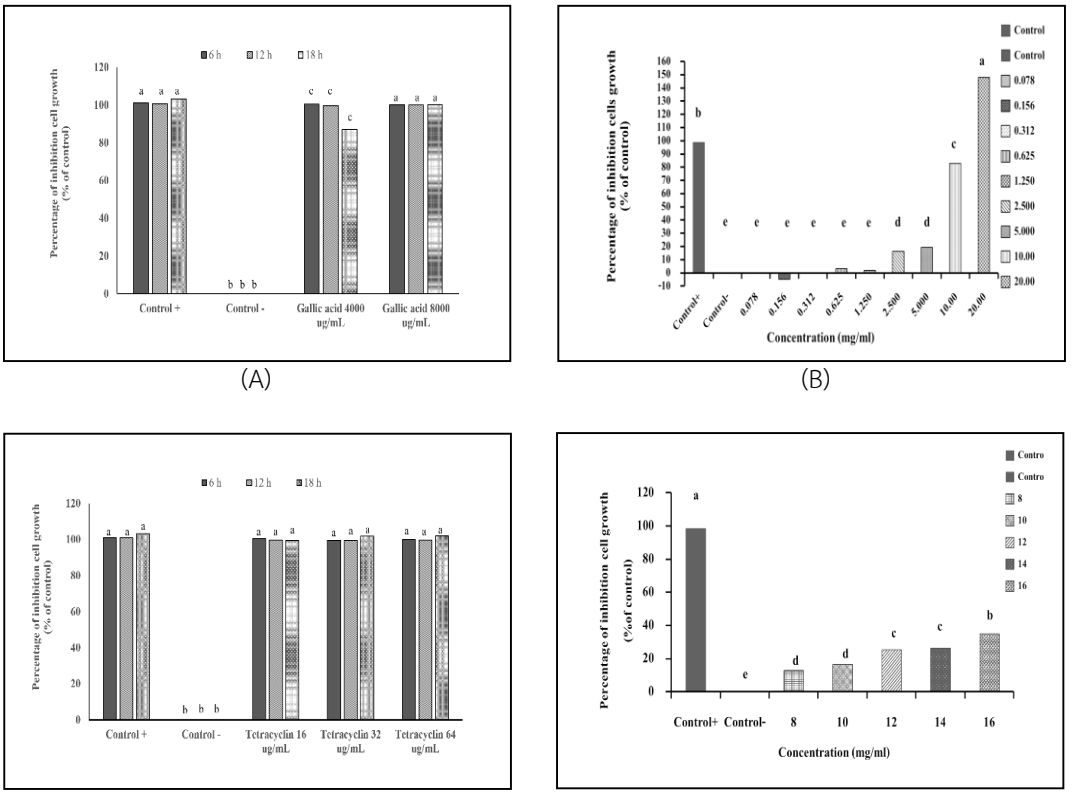

Figure 2. Percentage of inhibition cell growth of *Ps. aeruginosa* ATCC 27853 which treated by bitter gourd extracts (A), gallic acid (B), caffeic acid (C) and tetracycline (D).

Table 2 Antimicrobial activity of treatment samples on *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923)

Treatment samples	Antimicrobial activity on pathogens	
	Pseudomonas aeruginosa (ATCC 27853)	*Staphylococcus aureus (ATCC 25923)*
1. Bitter gourd fruits extracts	+++	+++
2. Gallic acid	+++	+++
3. Tetracycline	+++	+++
4. Caffeic acid	+	+

*High potential of Inhibitory effect "+++" (Percentage of inhibition > 80 %)

Low potential of Inhibitory effect "+" (Percentage of inhibition < 50 %)

Table 3: Minimal inhibition concentration of treatment samples on *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC 25923

Treatment samples	Minimal inhibition concentration (µg/ml)	
	Pseudomonas aeruginosa (ATCC 27853)	*Staphylococcus aureus (ATCC 25923)*
1. Bitter gourd fruits extracts	12,000	16,000
2. Gallic acid	2,000	6,000
3. Tetracycline	16	1
4. Caffeic acid	ND	ND

* ND mean not detectable

Table 4 FIC index and combination effect of antibiotic drugs and treatment samples

Treatment substances	Pathogen	FIC index	Combination effect
Tetracycline + Bitter gourd fruits extract	*Ps. Aeruginosa ATCC27853*	FIC index > 4.0	Antagonistic effect
	S. aureus ATCC25923	FIC index > 4.0	Antagonistic effect
Tetracycline + Gallic acid	*Ps. Aeruginosa ATCC27853*	0.5 < FIC index ≤ 4.0	Additive effect
	S. aureus ATCC25923	0.5 < FIC index ≤ 4.0	Additive effect

* FIC index by checkerboard method is interpreted as follows: synergy ≤ 0.5; 0.5 < additivity ≤ 4.0; and antagonism > 4.0.

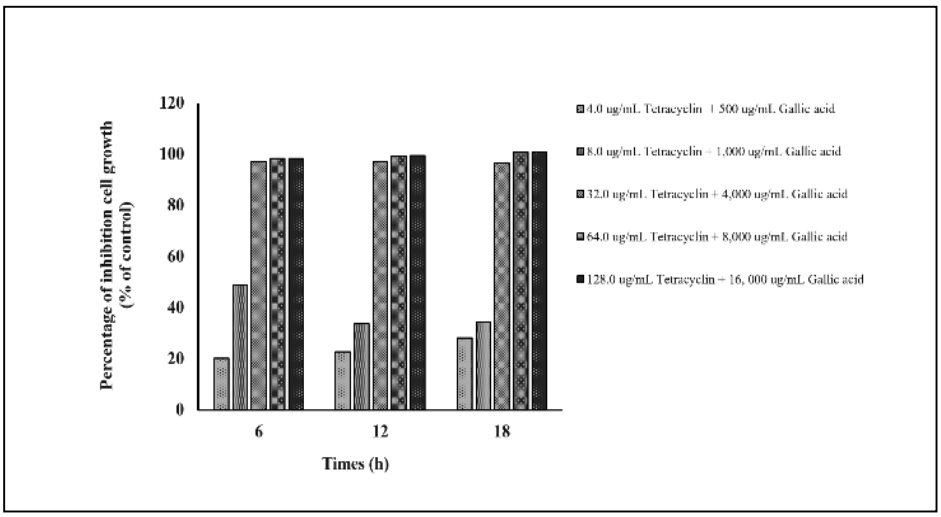

Figure 3 The combination effect of gallic acid and tetracycline on *Ps. aeruginosa* ATCC 27853 which incubated for 6, 12 and 18 h.

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