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The antibacterial activity, antibiofilm activity, cytotoxicity, and synergy effect of *Sesbania javanica* Miq. in combination with tetracycline against opportunistic bacteria

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Abstract The result revealed that *Sesbania javanica* flower and leaf extracts exhibited the DPPH radical scavenging activity by IC_{50} of 2.07 and 2.38 and mg/mL, respectively. *S. javanica* flower and leaf extracts showed antibacterial activities against some opportunistic bacteria such as *Acinetobacter baumannii, Escherichia coli*, and *Pseudomonas aeruginosa* ATCC 27853, as MICs ranging between 10 to 80 mg/ml. The synergistic combination of tetracycline and *S. javanica* flower and leaf extracts showed additive effect against drug resistant P. aeruginosa, and indifferent effect against E. coli ATCC 25922, drug resistant *Acinetobacter baumannii* and *P. aeruginosa* ATCC 27853 and FICIs were between 0.5 to 2. Time kill assay was revealed bacteriostatic effect that the flower extract of *S. javanica* was significantly inhibited the growth of *P. aeruginosa* ATCC 27853*,* drug resistant *A. baumannii* and *P. aeruginosa*. The viabilities of bacteria were reduced at least 2log CFU/mL under log phase, when applying with 1/2 MIC tetracycline plus 1/16 MIC to 1/2048 *S. javanica* extract. In addition, *S. javanica* flower extract inhibited biofilm formation of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 as % inhibitions ranging 66.57±1.29 - 69.6±0.92%. Cytotoxicity test of *S. javanica* flower and leaf extracts exhibited no toxicity against RAW-264.7 macrophages. This study demonstrated that *S. javanica* could be developed as a novel supplement of tetracycline to enhance antibacterial and antibiofilm activities.

Keywords: Antibacterial activity, Antibiofilm activity, *Sesbania javanica*, Synergistic effect, Tetracycline

Introduction

Acceleration of incidence of drug resistance to antibiotics was the most troublesome problems in clinical trials because conventional antibiotics such as ampicillin and tetracycline were not effective to treat nosocomial infection (Reygaert *et al.,* 2018). In recent years, nosocomial infectious diseases caused by multidrug resistant bacteria such as *Escherichia coli*, *Pseudomonas*

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aeruginosa and *Staphylococcus aureus* are serious problems and obviously contributed to currently coinfection with SARS-CoV-2 infection especially in prolonged treatment of infection (Manna *et al.,* 2020). Many mechanisms of drug resistance had been revealed such as increasing efflux pumps, modifying peptidoglycan of cell wall, altering membrane permeability and mutation of drug target. Likewise, persistence of forming biofilm on tissue and medical devices contributes to increasing biofilm matrix and permeability barrier of drug resistant bacteria (Rabin *et al.,* 2015). Thus, prevention of biofilm formation may be the first step to reduce infection of multidrug resistant bacteria.

In pharmaceutical approaches, it takes time to recovery new antibiotics of which have best efficacy, less toxicity and cause low side effects. Thus, new strategies like synergism of traditional plants in combination with conventional antibiotics are recommended to remedy infection of drug resistant bacteria (Cheesman, *et al*., 2017). Many reported revealed that clove oil in combination with ampicillin or gentamycin inhibited the growth of oral bacteria (Moon *et al.,* 2011). As the supported of Eumkeb and Chukrathole (2013), combination of ceftazine with apigenin inhibited antibacterial activity of drug resistant *Enterobacter cloacae*. Ciprofloxacin mixed with *Cyclamen coum* extract inhibited biofilm formation of *P. aeruginosa* (Abdi *et al.*, 2015). In addition, medicinal plants have many phytochemical components of which function in many modes of actions, such as maintenance and promotion immunity of human (Dhama *et al*., 2014; Subramani *et al*., 2017).

In developing countries, folk medicine have many sources of phytochemicals for treatment of bacterial and viral infection and contain many pharmaceutical activities such as *Sesbania javanica* Miq., belonged to a family of Fabaceae (Cowan, 2013, Subramani *et al*., 2017). *S. javanica* is commonly harvested and eaten as vegetable in local markets. Likewise, *S. javanica* is a very interested folk medicine found in Asia, China, and India (Kumar and Naheed, 2012). In Thai tradition, properties of *S. javanica* were declared that *S. javanica* has medical properties as diuresis, detoxification, abscess healing, as anti-inflammatory and antipyretic activity and curing menstrual abnormality (Rattanasena *et al*., 2012). In some scientific reports, *S. javanica* contains a great significance in therapeutic compounds such as β-sitosterol, prunetin, genistein, 4-hydroxycinnamic acid, sitosterol-3-*O*--glucopyranoside ,flavonol glycoside and Quercetin 3-2(G) rhamnosylrutinoside (Tangvarasittichai *et al.*, 2005; Laladhas *et al.,* 2010). Quercetin 3-2 (G)-rhamnosylrutinoside found in *S. javanica* flower had antioxidant activity and antimutagenic activity against *Salmonella typhimurium* and anti-apoptotic activity (Laladhas *et al*., 2010; Tangvarasittichai *et al.*, 2005). Likewise, *S. javanica* had an anti- inflammatory activity and antioxidant activity (Ouattara *et al*., 2011). According to the

previous report, *S. grandiflora* had antibiofilm and antibacterial activities against *S. aureus* (Gandhi *et al.*, 2017). *S. grandiflora* also possessed antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus* (Wagh *et al*., 2012). However, pharmaceutical activities of *S. javanica* as the same family of *S. grandiflora* have less information.

To develop a novel medicinal plant, *S. javanica* could be a choice of novel medicinal plant of which may eradicate persistent bacterial cell and decrease incidence of nosocomial infection. *S. javanica* mixed with antibiotics may enhance action of conventional antibiotics as ampicillin and tetracycline and reduce side effects of antibiotics for being used as phytopharmaceuticals.
The objectives were to study antibacterial activity of *S. javanica* extract in combination with antibiotics, antibiofilm activity and cytotoxicity of *S. javanica*.

Materials and methods

Plant materials

Flowers and leave of *S. javanica* were taken from Ongkharak Amphur, Nakhon Nayok Province, Thailand which collected during June to October 2018 and identified by a botanist of Department of Biology, Burapha University, Bangsean, Chonburi Province, Thailand. The voucher specimen as Reference No. ScBuu-Ny601 was deposited at Medicinal planetarium of Department of Biology, Chonburi, Thailand.

Preparation of medicinal plant extract

The dried fine powder (500 g) of *S. javanica* flowers and leave were soaked in 80% (V/V) ethanol (2,000 mL) for 6 days at room temperature (as modified from Chung *et al*. (2011). The extracts were filtrated by Whatman filter paper No. 4 and then evaporated by rotary evaporator at 55°C. The yields (%; W/W) of flowers and leave extracts were 10.28% and 18.14%, respectively.

Bacterial strains

Bacillus subtilis, Escherichia coli ATCC25922*, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* were provided by the Department of Microbiology, Faculty of Science, Burapha university. The drug resistant bacteria**:** *A. baumannii*, and *P. aeruginosa* were provided by Chonburi Hospital, Chonburi Provience, Thailand. All bacterial strains were identified and confirmed by API system. *A. baumannii* was resistant to gentamicin, piperacillin/tezobactam, ceftriaxone, ceftazidime,

imipenem, meropenem, and cefepime. *P. aeruginosa* was resistant to gentamicin, netilmicin, amikacin, sulperazon, piperacillin/tazobactam, ceftriaxone, ceftazidime, imipenem, meropenem, and cefepime.

Flavoniod and phenolic screening

Total phenolic concentration was investigated by method of Misbah *et al.* (2013). A gallic acid was used as reference standard curve, ranged from 0.15 to 1.05 mg/mL (R^2=0.9999). The flavonoid content was estimated by slightly method of Srisawat *et al.* (2010). Quercetin was used as reference standard by equation (R^2=0.9985). Also, thin layer chromatography (TLC) of *S. javanica* extract was analysed under TLC Silica gel 60 F_{254}.

Antioxidant test

The free radical scavenging activity was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay (Mokbel and Hashinaga, 2005). The standard as butyl hydroxyl anisole (BHA) was compared to plant extract and IC_{50} was analysed. Ferric ion reducing antioxidant power assay (FRAP) was conducted by (Mokbel and Hashinaga, 2005).

Antibacterial activity test

Broth microdilution susceptibility assay was performed to determine MICs of plant extract (CLSI, 2017). Briefly as described by following, 80 mg/mL plant extract was diluted as a serial dilution of plant extract (0.625 to 80 mg/mL) by 100 µl Mueller Hinton Broth (MHB) in a microplate. Then, 100 µl of 1 $x10^8$ CFU/mL of bacterial culture was added and incubated at 37°C for 18-24 hours. The viable count of bacteria was measured by optical density at 610 nm by a microplate reader (VersaMax, U.S.A.). The standard antibiotics of 0.625 to 80 mg/mL; ampicillin and tetracycline were tested as positive controls, and deionized distilled water was negative control. Each treatment of sample was conducted in triplicate. Finally, the lowest concentration that reduces 100% O.D. of microorganism was recorded as MIC.

Synergistic effect assay

An interaction of *S. javanica* extract, mixed with antibiotics was tested by synergy effect assay. The checkerboard assay was designed and applied from Chung *et al.* (2011) to measure $MICs_{co}$ synergy effect (MICs of combination of plant extract and antibiotics). The antibacterial activities of plant extracts ($MICs_{medicinal\ plant}$) and MIC of antibiotics ($MICs_{antibiotic}$) were recorded. Fractional

Inhibitory Concentration Index (FICI) calculated by $MICs_{co}/MIC_{antibiotic}$ + $MICs_{co}/MIC_{medicinal,}$ was determined. Synergistic evaluation was interpreted by the following, a synergism as FICI index of ≥ 0.5, a partially synergism, as FIC index of 0.5 < FICI < 1, an additive effect, as FICI index of 1, an indifference, as FICI index of 1 < FICI ≥ 4, an antagonism, as FICI index of > 4 (Chung *et al.,* 2011).

Time kill assay

The 1x 10^8 CFU/mL of bacterial culture in each sample contained 50 µL of 1/16-1/2048 *S. javanica* extract and 50 µL of 1/2MIC antibiotics in 2,800 mL MHB. Each sample was incubated at 37°C for 2 to 48 hours. After incubation for interval times as 2, 4, 8, 16, 24 and 48 hours, the number of bacteria (N_E).in each culture treated with samples was counted. All culture samples were conducted in triplicates. The antibiotics were positive control and bacteria cultured in MHB as negative control (N_0). The effectiveness of antimicrobial activity (EAA) was calculated by the equation 1:

$$\text{(Eq.1)} \quad \text{EAA } \% = \frac{N_0 - N_E}{N_0} X100$$

Where

N_0 was the number of microorganism (CFU/mL) of the control in MHB and

N_E was the number of microorganism (CFU/mL) of the treatments.

Antibiofilm activity

Antibiofilm activity of *S. javanica* extract was designed and adjusted from Awolola *et al.* (2014) and Song *et al.* (2019). A 100 µl aliquot of 1x 10^6 CFU/mL of bacterial culture was filled with 100 µL of Trypticase soy broth (TSB). Then, 100 µL aliquot culture of plant extract, tetracycline (as positive control) or bacteria in TSB (negative control) was added in flat-bottomed 96-well microtiter plates and incubated at 37°C for 4 to 48 hours (without shaking for developing multilayer of biofilm). At the interval time, each well of sample (contained adherent biofilm of bacteria) was fixed and stained by crystal violet. The adherent cell of stained bacteria was solubilized in 150 µL of 33% glacial acetic acid. Then, the soluble biofilm of bacteria was measured using a microplate reader VersaMax (U.S.A.) at an optical density of 600 nm. The mean absorbance and percentage inhibition of biofilm were determined by the equation 2

(Eq.2) (%) Percentage inhibition = $\frac{(\text{OD negative control} - \text{OD experiment})}{\text{OD negative control}}$ X 100**%**

MTT assay

MTT assay *S. javanica* extract was conducted to observe cell viability of RAW 264.7 cell line as described by Shappell, (2003). Cells were plated at a density of 1 x 10^5 cells per well in 96-well tissue culture plates and cultured for 24 hours at 37°C before treatment with 31.25 to 1000 µg/mL in the culture medium and 1% DMSO diluted in the culture medium as a negative control. The absorbance of cell viability was analysed using a microplate reader at an optical density of 690 nm and 570 nm. MTT assay was performed in triplicate times. The percentage of viable cells was calculated as the equation 3.

(Eq.3) % cell viability = $\frac{\text{OD (sample)}}{\text{OD (negative control)}}$ x 100 %

Statistical analysis

The experiment results were indicated as means ± standard error of three replicate. The variance was analysed by ANOVA. Duncan's multiple range tests were chosen to analyse a significant difference among means by using Minitab software version 18 (Minitab Pty Ltd, Sydney NSW, Australia).

Results

Phenolic and flavonoid contents of S. javanica extract

The results of total phenolic and flavonoid of *S. javanica* flower and leaf extracts were not different as shown in Figure 1. The total phenolic content estimated in flower and leaf extracts were 34.81 ± 0.0008 mg GAE/mg extract and 35.93 ± 0.0004 GAE/mg extract, respectively. The total flavonoid contents of flower and leaf extracts were 71.71 ± 0.004 and 72.34 ± 0.002 mg QE/mg extract, respectively. *S. javanica* flower and leaf extracts contained various flavonoids including methyl dihydroflavonoid, flavonol *O*-glycosides and flavonone *O*-glycosides by Thin layer chromatography.

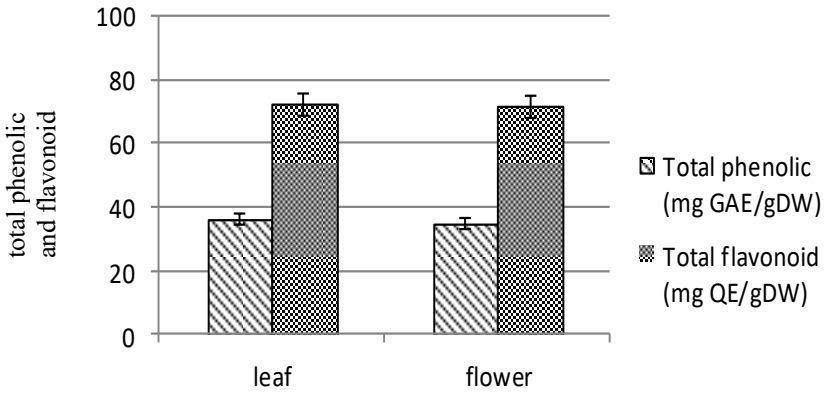

Figure 1. Total phenolic and flavonoid of *S. javanica* flower and leaf extracts

Antioxidant activity of S. javanica extract

S. javanica flower and leaf extracts revealed good DPPH scavenging capacity. Both flower and leaf extracts of *S. javanica* showed dose independent scavenging activity of DPPH free radical. The flowers and leave of *S. javanica* showed 80.83% and 73.37% of DPPH free radical scavenging activity, respectively. DPPH free radical scavenging activity of *S. javanica* extracts were less than 90.78% of BHT activity. For DPPH free radical scavenging activity, *S. javanica* flower and leaf extracts showed EC_{50} of 1.70 and 2.20 mg/mL, respectively. The result demonstrated that *S. javanica* flower and leaf extracts showed indifferently ferric reducing ability as 46.89 ± 0.003 mg and 45.90 ± 0.003 of ferrous sulphate, respectively.

Antibacterial activity of S. javanica extract

The result of antibacterial activities of *S. javanica* flower and leaf extracts were shown in Table 1. Both flower and leaf extracts were able to inhibit the growth of some opportunistic bacteria which were *A. baumannii, B. subtilis, E. coli* ATCC 25922*., P. aeruginosa* ATCC 27853, *S. aureus,* excepted *K. pneumoniae* and drug resistant *P. aeruginosa.* In addition, the MICs of flower extract expressed equal MIC values (10-40 mg/mL) as the MICs of the leaf extract (10 to 40 mg/mL). However, the antibacterial activities *of S. javanica* extracts indicated less antibacterial activities than the antibacterial activities of ampicillin and tetracycline against tested bacteria.

Table 1. Antibacterial activities (MICs) of *S. javanica* flower and leaf extracts, compared to ampicillin and tetracycline

Bacteria	MICs (mg/mL)			
	Flowers	leave	Ampicillin	Tetracycline
B. subtilis	20	20	0.020	0.001
S. aureus ATCC 25922	40	40	0.001	0.001
Drug resistant A. baumannii	40	10	-	20
E. coli ATCC 25922	40	40	0.08	0.01
K.pneumoniae	-	-	0.31	20
P. aeruginosa ATCC27853	10	40	25	1.25
Drug resistant P. aeruginosa	80	80	-	40

Note: - represented had no MIC result

Synergistic effect

The synergistic interaction between *S. javanica* flower and leaf extracts and conventional ampicillin/tetracycline were shown in Table 2**.** The *S. javanica* flower extract in combination with tetracycline revealed the best additive effect against all tested bacteria, as especially against drug resistant *P. aeruginosa* (FICI = 0.50). Also, there were indifferent effect of *S. javanica* extracts in combination with tetracycline against *E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853, drug resistant *A. baumannii* as FICIs were between 1 to 2. In contrast, ampicillin mixed with *S. javanica* flower and leaf extracts showed antagonist effect against *E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853 and *S. aureus* as FICIs ranging 1.06 to 40.25. The results showed the best synergy effect of *S. javanica* flower combined with tetracycline which selected to investigate time kill assay.

Time kill study

From time kill assay, synergistic effect of *S. javanica* flower extract combined with tetracycline showed significantly enhanced antibacterial activity against *P. aeruginosa* ATCC 27853 and drug resistant *P. aeruginosa* and *A. baumannii* as shown in Figure 2-4. The time kill study of *P. aeruginosa* ATCC 27853, drug resistant *A. baumannii* and *P. aeruginosa* were indicated that using 1/16 MIC to 1/2048 MIC *S. javanica* combined with 1/2MIC tetracycline significantly reduced number of bacteria at 6-12 hours after inoculum ($p \leq 0.05$). The efficacious bacterial growth (EAA%) of *P. aeruginosa* ATCC 27853 and

drug resistant *A. baumannii* were significantly reduced by the ranges between 86.55 ± 1.15% and 99.97 ± 2.00% after treatment (data was not shown). Moreover, the most effective synergy effect of *S. javanica* flower extract in combination with tetracycline was shown against drug resistant *P. aeruginosa.* (Figure 3). An appropriated proportion of *S. javanica* flower extract when mixing with 1/2MIC tetracycline was significantly increased an effectiveness of tetracycline and reduced viability of bacteria more than $2\log_{10}$ CFU/mL for all bacteria ($p \leq 0.05$).

Table 2. Synergism of *S. javanica* flower and leaf extracts mixed with some antibiotics

		Synergistic effect (FICIs)		outcome	
Bacteria	Parts	Ampicillin	Tetracycline	Ampicillin	Tetracycline
E. coli	leave	64.43	2	antagonism	indifference
ATCC25922	flowers	32.23	2	antagonism	indifference
S. aureus	leave	10.25	40.25	antagonism	antagonism
	flowers	10	20	antagonism	antagonism
P. aeruginosa ATCC 27853	leave	32	1.13	antagonism	indifference
	flowers	64	1.06	antagonism	indifference
drug resistant	leave	-	2	-	indifference
P. aeruginosa	flowers	-	0.50	-	additive effect
drug resistant *A. baumannii*	leave	-	1.031	-	indifference
	Flowers	-	1.003	-	indifference

Note: - represented could not determine

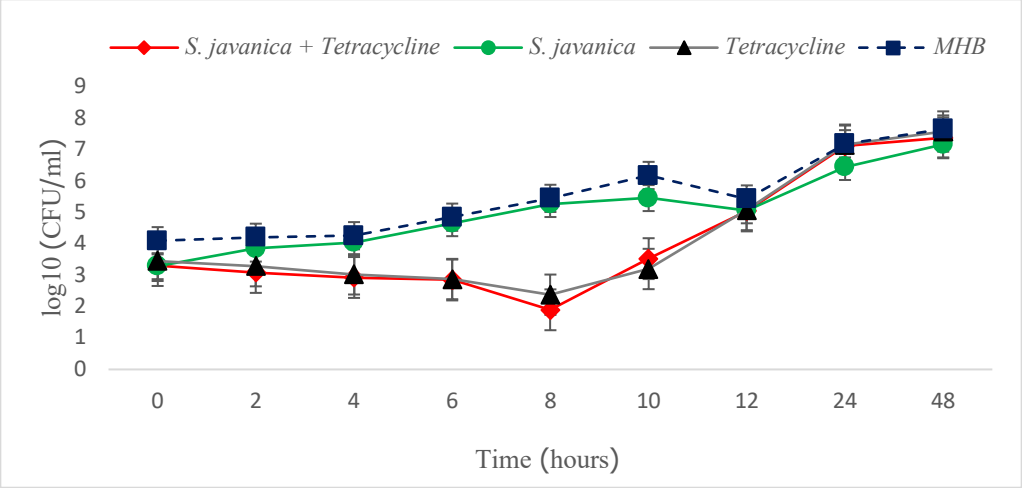

Figure 2. Time-dependent dose of *S. javanica* flower extract mixed with tetracycline inhibited the growth of *P. aeruginosa* ATCC 27853

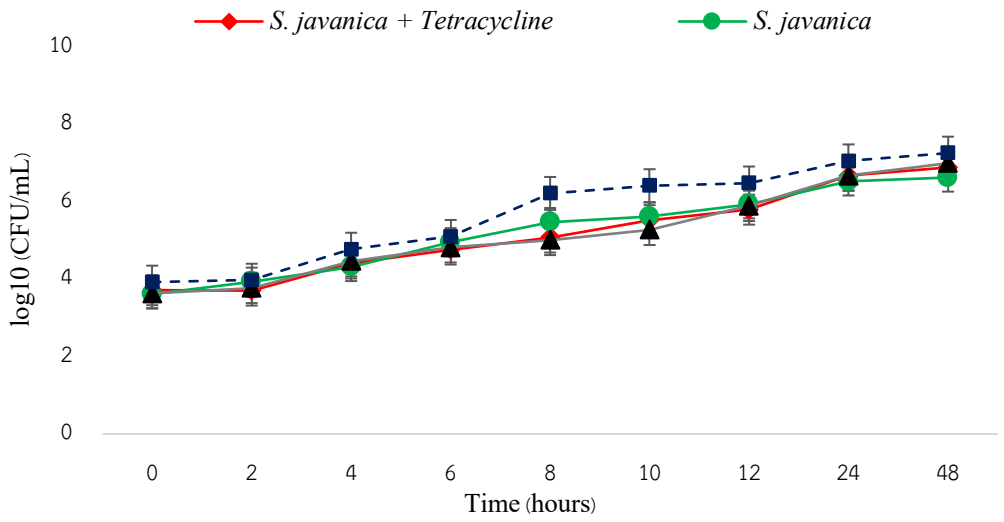

Figure 3. Time-dependent dose of *S. javanica* flower extract mixed with tetracycline inhibited the growth of drug resistant *P. aeruginosa*

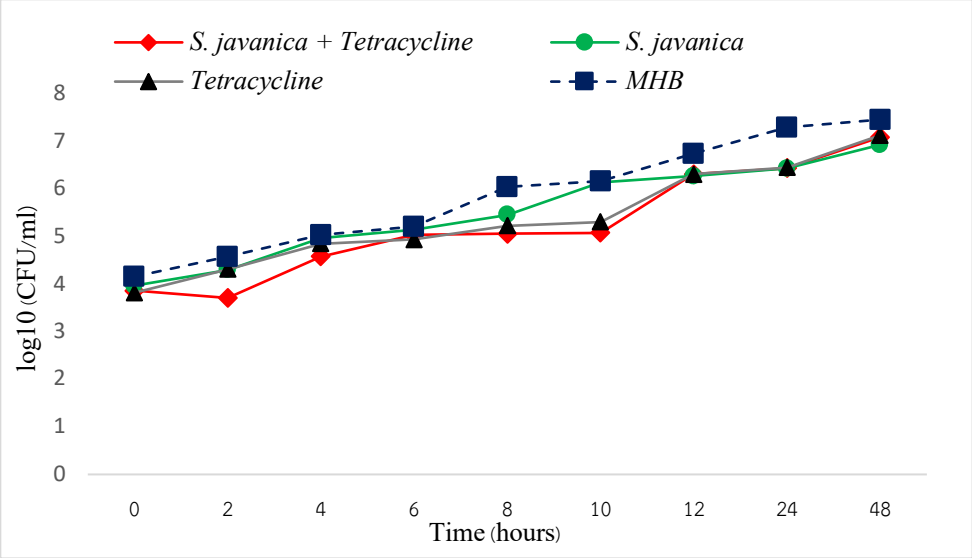

Figure 4. Time-dependent dose of *S. javanica* flower extract mixed with tetracycline inhibited the growth of drug resistant *A. baumannii*

Antibiofilm activity

S. javanica flower extract indicated significantly inhibited the biofilm formation of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 as shown in Figure 5. The 80 mg/mL *S. javanica* extract showed consequently inhibited biofilm development of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923, respectively (Figure 5). Among antibiofilm activity against bacteria, *S. javanica* extract inhibited colonization of biofilm of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 and %inhibition was between 66.57±0.29% to 69.6 ±0.92%. Antibiofilm activity *S. javanica* extract against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 was significantly reduced at 8 to 12 h after treatment. However, antibiofilm activity of *S. javanica* flower extract was less than antibiofilm activity of 80 mg/ml tetracycline activity.

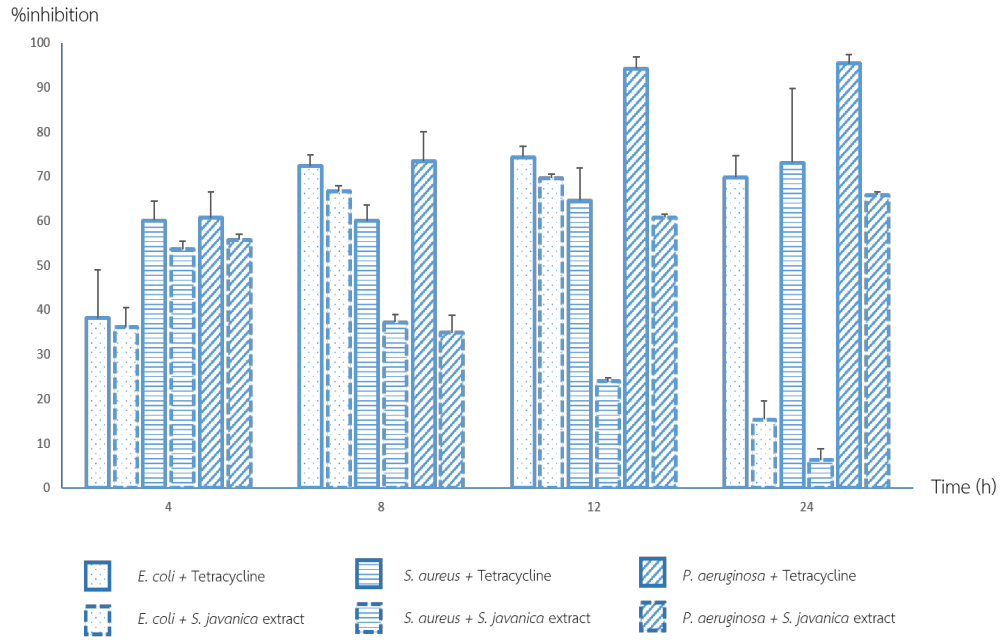

Figure 5. Antibiofilm activity of 80 mg/mL of *S. javanica* flower extract and 80 mg/ml tetracycline against some bacteria

Cytotoxicity of S. javanica extract

Cell viability of macrophage cell line (RAW 264.7) was observed when treatment by different concentration of *S. javanica* flower and leaf extracts in figure 6. The cytotoxicity of *S. javanica* extracts was indicated that *S. javanica* flower and leaf extracts had no toxicity to macrophage cell line. In contrast, *S. javanica* flower and leaf extracts significantly induced proliferation of RAW 264.7 macrophages at $p<0.05$. The concentration for increased viability of RAW-264.7 macrophages ranged from 31.25 to 250 mg/mL. Finally, the *S. javanica* leaf extract showed more slightly increased the rate of proliferation than *S. javanica* flower extract.

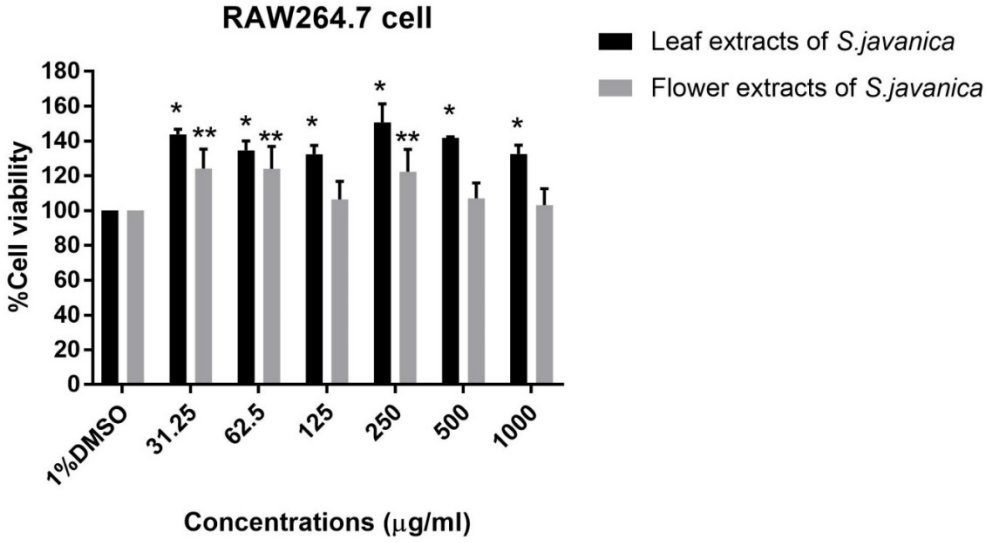

Figure 6. Cytotoxicity by MTT assay of *S. javanica* flower and leaf extracts**.** The different stars in the chart expressed significant difference between individual result ($p<0.05$)

Discussion

Opportunistic bacteria such as *E. coli*, *K. pneumoniae, P. aeruginosa* and *S. aureus* cause infectious disease of which increase mortality among people population in the developmental country (Manna *et al.,* 2020). *S. javanica* could be a choice of an alternative therapy for healing properties such as anti-bacterial, antioxidant, anti-mutagenesis, anti-inflammatory activities (Tangvarasittichai *et al.*, 2005). Thus, antibiotics in combination with *S. javanica* may increase effectiveness to eradicate multidrug-resistance bacteria.

For the reasons above, it encourages us to study antibiofilm activity, antioxidant activity, cytotoxicity and synergistic effect of *S. javanica* in combination with some conventional antibiotics as ampicillin and tetracycline. The result of present study was confirmed that the major contents of phenolic and flavonoids founded in *S. javanica* flower and leaf extracts was expressed as methyl dihydroflavonoid, flavonol *O*-glycosides and flavonone *O*-glycosides. Flavonoid contents found in *S. javanica* flower and leaf extracts showed as the same content as found in the previous report by Tangvarasittichai (2015) who found pure compounds like β-sitosterol, prunetin, genistein, 4-hydroxycinnamic acid, sitosterol-3-*O*-D-glucopyranoside, flavonol glycoside and quercetin rhamnosylrutinoside. Meanwhile, phenolic and flavonoid found in flower and leaf of *S. javanica* was the same as major contents as found in S. *grandiflora* of

which characterized as anthraquinone glycoside, tannin (Kumar and Naheed, 2012). Additionally, our result was consistent with the result of Bunma and Balslev (2019) that β-carotene, β- cryptoxanthin, lutein zeaxanthin were a major chemical contents found in leave of *S. javanica.*

Regarding to an antioxidant activity, it was declared that *S. javanica* flower and leaf extracts expressed significantly higher antioxidant activity than antioxidant activity of leaf extract from *S. grandiflora*, investigated by DPPH scavenging activity. This result was in agree with Ouattara *et al.* (2011) who found antioxidant activity of *S. grandiflora* as 0.75 mg of ascorbic/mg of extract. Also, antioxidant activity tested by FRAP assay revealed that *S. grandiflora* leaf extract showed higher scavenging potential (EC_{50} = 24 µg/mL) than scavenging potential in *S. javanica* leaf extract and flower extract of this study as EC_{50} was 1.70 mg/mL and 2.20 mg/mL, respectively (Ouattara *et al.,* 2011). Our result suggested that the different Ferric reducing activity of *S. javanica* and *S. grandiflora* was depended on the different contents of phenolic flavonoids, coumarins, steroids, triterpenes, and tannin, found in Genus *Sesbania* (Lee *et al.*, 2014).

Antibacterial activity of *S. javanica* flower and leaf extracts displayed indifferently antibacterial activity against *E. coli*, *B. subtilis*, *P. aeruginosa* ATCC 27853, drug resistant *Pseudomonas aeruginosa* and *S. aureus*. The action of antibacterial activity of *S. javanica extract* was depended on contents of flavonoids and phenolic compounds in flowers and leave of *S. javanica*. As a similar report as the study of Mohammed *et al*. (2015), who stated that flavonoid glycoside found in leave of *Leucaena leucocephala* played a role to inhibit the growth of *E. coli* and *S. aureus.* Obviously, the antibacterial activity of *S. javanica* extract was less than antibacterial activity *of S. grandiflora* extract. This result was depended on flavonoid contents in *S. javanica* extract that were different in *S. grandiflora* extract. This result suggested that different method of extraction and various different compounds found in *S. javanica* and *S. grandiflora* may contributed to the difference of antibacterial activity. Phenolic and flavonoid of *S. javanica* may inhibit the permeability of cell membranes and the antibiofilm activity of opportunistic bacteria as possible as the mechanism that was proven by the reported of Silva *et al*. (2019).

An impact on antibacterial activity of S. javanica flower and leaf extracts in combination with antibiotics was confirmed by synergistic assay. The bacteria of which showed the most virulence and the most sensitive to S. javanica extracts were selected to verify as the model of antibacterial action against drug resistant infection. Our result confirmed that S. javanica flower and leaf extracts used in combination with tetracycline was displayed solely additive effect against drug resistant *P. aeruginosa*. In the meanwhile, the work of Eumkeb and Chukratho

(2013) was reported the mechanism of synergism that apigenin in combination with ceftazidime damaged cytoplasmic membrane and subsequently caused leakage of intracellular constituents against ceftazidime-resistant *Enterobacter cloacae* (Eumkeb and Chukrathok, 2013). In agreement of the report of Septama and Panichayupakaranant (2016), this work was urged that *E. coli*, MRSA, and *P. aeruginosa* were sensitive to artocarpin mixed with ampicillin, norfloxacin and tetracycline with FICI between 0.15-0.62. There was a work supported that ApuL (lectin) showed synergistic action with ceftazidime against a multidrug-resistant isolate of *P. aeruginosa* (Ferreira *et al*., 2018). our result had revealed antagonist effect against *E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853 and *S. aureus* when mixing *S. javanica* flower and leaf extracts with ampicillin. The antagonist result of Moussaoui and Alaoui (2016) was also expressed that an essential oil of *C. coronarium* in combination with imipenem indicated antagonistic effect against *E. coli* ATCC 25921. However, the hypothesis of antagonist has not been verified.

Drug resistant *A. baumannii,* and drug resistant *P. aeruginosa* were the good model to observed time-kill assay in this study. It is because the most prevalent opportunistic pathogen found the most infection in hospital care unit in many countries. Time kill results was also confirmed that antibacterial action of S. *javanica* mixed with tetracycline were stabilized in log phase (as 2-12 h after inoculum). Also, our result was implied that flower and leaf extracts of *S. javanica* could enhance antibacterial activity of tetracycline against some bacteria by reducing amount of 1xMIC tetracycline to 1/2MIC tetracycline (0.625 mg/ml-10 mg/ml). Our results corresponded to the work of Eumkeb and Chukrathok (2013) who expressed that 3 µg/ml ceftazidime mixed with 3 µg/ml apigenin was prone to reduce the number of ceftazidime-resistant *E. cloacae* to 10^3 CFU/mL in the log phase. Thus, flower extract *of S. javanica* would be a good choice of antibacterial agent to enhance the action of conventional antibiotic as tetracycline.

Inhibition of biofilm formation can protect bacteria from increasing of growth rate and attachment to host tissue (Rabin *et al.*, 2015). Thus, our hypothesis as *S. javanica* extract can be a good candidate of antibiofilm formation was verified. Our reports indicated that *S. javanica* extract showed consequently inhibited biofilm formation against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. This result was correlated with the report of Gandhi *et al.* (2017) who declared that *S. grandiflora* extract decreased production of biofilm-associated extracellular polymeric substances of *S. aureus*. As a recent study by Song *et al*., (2019), it was also stated that grapefruit seed extract inhibited biofilm formation of *E. coli* ATCC 25922 and *S. aureus.* In addition, some chemical components in medicinal plants like

flavonoid, tannin, terpene and saponin were inhibit biofilm formation and matrix formation of some bacteria (Silva *et al*., 2019). Thus, flavonoid, and phenolic compounds, commonly found in ethanol extract of *S. javanica* may play a role in destruction of bacterial cell membrane and imbalance of electrolytes and preventing biofilm formation (Gupta and Birdi, 2017). So far, pure compounds in *S. javanica* extract will be characterized and mechanism of biofilm eradication will be proved.

Cytotoxicity test revealed that *S. javanica* leaf and flower extracts has no toxicity to RAW-264.7 macrophages. Moreover, leaf and flower extracts from *S. javanica* increased the rate of proliferation of RAW-264.7 macrophages. Our results was correlated to a report that engeletin, astilbin and quercetin from *Smilax corbularia* show low cytotoxicity (IC_{50} >100 μg/mL) to RAW-264.7 macrophages (Ruangnoo *et al.,* 2012). In our experiment, a high concentration as 40-80 mg/mL of *S. javanica* extract of which was active to inhibit formation of drug resistant bacterial biofilm will be confirmed cytotoxicity and anti-inflammatory activity, against RAW-264.7 macrophages in animal model.

In our research, it was concluded that the leaf and flower extracts of *S. javanica* showed antioxidant, antibacterial and antibiofilm activities. *S. javanica* extract in combination with tetracycline was reduced the growth of bacteria for more than 2 log10CFU/mL. However, the *S. javanica* extracts had no toxicity to RAW-264.7 macrophages. Our investigation suggests that *S. javanica* flower and leaf extract may be a good potential choice of therapeutic management for curing infectious disease. *S. javanica* flower and leaf extract in combination with tetracycline may reduce critical side effect and toxicity of liver and kidney of patients who suffered from long term treatment of co-infection of bacteria and virus. According to many therapeutic properties of *S. javanica*, *S. javanica* can be a good candidate as a new antimicrobial agent for reducing microbial population by inhibiting biofilm formation. Also, the continued study of *S. javanica* is to investigate the active compounds in leaf and flower extracts and to study anti-inflammatory effect of active compounds in LPS stimulated RAW-264.7 macrophages and LPS induced in animal model.

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