Original Article

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Biological activities and antibacterial biomarker of *Sesbania grandiflora* bark extract

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Summary **In the present study, the fractionated extracts of *Sesbania grandiflora* bark were prepared and evaluated for their biological activities. The ethyl acetate fractionate (EAF) showed high antioxidant activity along with free radical scavenging and reducing mechanisms. The free radical scavenging antioxidant activity of EAF was 69.3 ± 3.6% where its Trolox equivalent antioxidant capacity was 13.6 ± 0.7 mM/mg. EAF exhibited the reducing power equivalent to ferrous sulfate at 152 ± 2 mM/mg and equivalent to gallic acid at 1.05 ± 0.01 mM/mg. In addition, EAF presented high potential on inhibition of bacterial growth with the minimum bactericidal concentration less than 1 mg/mL. Further isolation of EAF using normal-phase open column of silica gel 60, showed that the fractions eluted with the mixture of chloroform and methanol at the ratios of 4:1, 3:2, and 2:3 possessed antibacterial activity. The recovery activity of total different active fractions was 5% EAF, 20 times less than that of EAF. The chromatogram of EAF from a high-performance liquid chromatography was compared with caffeic acid, catechin, coumaric acid, ellagic acid, gallic acid, quercetin, syringin, naringic acid, trans-cinnamic acid, and vanilic acid. The result demonstrated that one major compound of EAF was gallic acid. These results suggest that the fractionated extracts of *S. grandiflora* bark contained antioxidant and antibacterial activities.**

Keywords: *Sesbania grandiflora*, antioxidant activity, antibacterial activity, bark extract, HPLC fingerprint

1. Introduction

Sesbania grandiflora (L.) Pers., family Fabaceae, is commonly found in Thailand and many Asian countries. Its leaves and flowers have been reported to possess anticancer (*1*), antioxidant (*2*), anxiolytic (*3*), anticonvulsant (*3*) and antimicrobial activities (*4,5*). Its bark has been used as traditional medicine for treatment of inflammation (*6*), ulcers (*7*) and wound-healing (*8*). It is known that inflammation and oxidation are related mechanisms in the body. Excess endogenous oxidation causes an increase in the formation of the radical oxygen species (ROS) and radical nitrogen species (RNS). The overproduction of ROS and RNS is responsible for damage at inflammatory sites (*9*). In addition, these reactive species play important roles in inflammation by being trigger elements or by being signaling messenger molecules which regulate the expression of key cytokines (*10*). Wounds are injuries that affect in an opening or breaking of the skin, and also disrupting the soft tissue. This symptom is hazard to be occurred inflammation and infection. Contraction and closure of the injury and restoration of functional status of the skin is necessary in treatment of wounds (*11*). Wound healing is interrelated with reactive oxygen species and bacterial infection (*12*). Therefore, antioxidants are necessary for prevention of tissue damage and encourage wound healing process. Furthermore, infection prevention is one of the most important to enhance wound healing (*13*). It was previously reported that the combination of plant extracts possessing antioxidant and antibacterial activities can efficiently enhance wound healing (*14*). Although *S.*

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grandiflora has been applied for treatment in many diseases, the study deeply on which a potential extract of this plant having both antioxidant and antibacterial activities has not been reported. Our previous study reported the comparison of antibacterial activity among different parts such as leaf, branch, stem bark, and stem core of the plant. It was found that the bark of *S. grandiflora* possessed the strongest antibacterial activity against different pathogenic strains (*15*). Therefore, the bark of this plant was selected to use in the current study.

Using medicinal plants for treatment of many diseases is an interesting alternative way (*16*) as there are many phytochemical compounds which enable to relieve ailment (*17*). The pharmacological activities of several plants are from metabolite products existed in the plants (*18*). Most studies reveal that the phytochemical compounds having health promoting properties are phenolic compounds. Thus, these compounds have been widely used for treatment and management of disorders, also explored as model systems of plant research due to ubiquitous in plants of different areas (*19*). The phenolic compounds generally found in plant extracts are phenolic acids, flavonoids, and tannins (*20,21*). However, pre-clinical studies of estimating the phytochemical, toxic, and biological properties of the plant extracts are important before administrating in the clinical studies. It is very essential to know the biomarker of the extracts before establishing further efficacy scientific models in clinical trials. The identical analysis of phytochemical compounds as biomarkers of plant extracts can be primary done by comparing with standard compounds (*22*).

The purpose of the present study is to evaluate antioxidant and antibacterial activities of *S. grandiflora* bark extracts against pathogenic bacteria. Isolation of the most potential extract on antibacterial activity was done in order to search for the bioactive marker existing in the extract. Different standard phenolic compounds were subjected to high-performance liquid chromatography (HPLC) to obtain HPLC chromatograms for comparing with the tested extract.

2. Materials and Methods

2.1. *Chemicals and reagents*

Butylatedhydroxytoluene (BHT), vitamin-E (Vit-E), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazil (DPPH), 2,4,6-tri(2-pyridil)-S-triazine (TPTZ), ferrous sulfate, ferric chloride, sodium chloride, sodium carbonate, dimethyl sulphoxide (DMSO), caffeic acid, catechin, coumaric acid, ellagic acid, gallic acid, quercetin, syringin, naringic acid, trans-cinnamic acid, vanilic acid, and methanol (HPLC grade) were from Sigma-Aldrich (St. Louis, MO). Muller-Hinton broth (MHB) and tryptic soy agar were from Becton Dickinson Labware (Franklin Lakes, NJ). Vancomycin was from Shionogi & Co., Ltd. (Osaka, Japan). Gentamicin was from Sankyo Co., Ltd. (Tokyo, Japan). Organic solvents were from Merck (Darmstadt, Germany) and Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of analytical grade.

2.2. *Plant material*

The bark samples of *S. grandiflora* were collected from Chiang Mai province, a northern area of Thailand. The plant species was identified and the voucher specimen (No. 023207) was deposited at the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

2.3. *Plant extract preparation*

The bark samples of *S. grandiflora* were dried at 50°C for 48 h and ground into powder. The dried powder was sequentially macerated with organic solvents started from hexane followed by chloroform, and ethyl acetate, respectively. The maceration of each solvent was performed for 24 h × 3 cycles at room temperature. The filters from each solvent from the maceration was removed under vacuum by using rotary evaporator at 40°C. Dried fractionate extracts of hexane (HXF), chloroform (CFF), and ethyl acetate (EAF) were kept in the tight containers in the refrigerator for further studies.

2.4. *Antioxidant study*

2.4.1. *Free radical scavenging assay*

This free radical scavenging assay was done using DPPH as free radicals. The performance was according to a method previously developed (*23*) with some modification. Briefly, the extract was mixed with DPPH in ethanol solution. The mixture was left in dark cabinet at room temperature for 30 min. Then the absorbance of the mixture was measured spectrophotometrically at 520 nm using microplate reader. BHT and Vit-E were used as positive controls. The results were calculated and expressed as percentage of radical scavenging antioxidant activity (RSAA) and Trolox equivalent antioxidant capacity (TEAC) for 1 mg extract (*24,25*).

2.4.2. *Reducing power assay*

Reducing power of the extracts was investigated using a method to determine ferric reducing antioxidant power (FRAP) described previously (*26*) with some modification. Briefly, the FRAP reagent was firstly prepared by mixing 10 mM TPTZ solution with 20 mM ferric chloride and 300 mM sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. The extract was added in the

FRAP reagent solution and mixed. The absorbance of the mixture was measured at 595 nm after standing 5 min by microplate reader. BHT and Vit-E were used as positive controls. The result was calculated and expressed as ferrous sulfate equivalent concentration (EC) and gallic acid equivalent concentration (GAE) for 1 mg extract (*27*).

2.5. *Antibacterial activity study*

The *in vitro* antibacterial activity of the extracts was investigated by broth micro-dilution assay in order to determine a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The extracts were dissolved in MHB containing 10% DMSO. The stock suspensions of the tested extracts were centrifuged at 14,000 rpm for 1 min for removing undissolved matters. The supernatant was collected and serially two-fold diluted with MHB. A 100-fold dilution of bacterial suspension of standard strain of *Staphylococcus aureus* ATCC 25923 at a concentration equivalent to McFarland turbidity standard No. 0.5 was added to each dilution of the extracts at the same volume. The mixtures were incubated at 37°C for 24 h. Negative controls were prepared without bacterial suspension, whereas positive controls were received bacterial suspension without the test samples. Acceptable, no bacterial growth in the negative control and complete growth, indicated by turbidity, in the positive control. Comparing with the negative and positive controls, the bacterial growth inhibition of the test samples was visually observed. The lowest concentration of the extracts which could inhibit the bacterial growth at this step was indicated as MIC. Furthermore, the tested broth which presented inhibitory action from MIC assay were taken for MBC determination by streaking on freshly prepared tryptic soy agar plates and further incubated at 37°C for 24 h. After incubating, the bacterial growth on surface of the agar plates was observed. The lowest concentration of the samples which showed no bacterial growth after this subculturing, indicating the bacteria were completely killed, was recorded as MBC. Vancomycin and gentamicin were used as control antimicrobial agents.

2.6. *Isolation of the highest antibacterial extract*

The fractionated extract which demonstrated the highest antibacterial activity was selected for further isolation using normal-phase column chromatography. The extract was dissolved in chloroform and subjected to an open column containing silica gel 60, then eluted with mixture of chloroform and methanol with polarity increasing by stepwise gradient of chloroform to methanol ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5, respectively. Each fraction was collected from the column and removed the solvent using vacuum centrifugal evaporator. The obtained fractions were dissolved in sterile MHB and investigated for antibacterial activity against *S. aureus* as mentioned in section 2.5.

2.7. *HPLC analysis*

HPLC fingerprint of the extract which demonstrated the strongest antibacterial activity and that of 10 standard phenolic compounds including caffeic acid, catechin, coumaric acid, ellagic acid, gallic acid, quercetin, syringin, naringic acid, trans-cinnamic acid, and vanilic acid was performed by HPLC using a Hypersil ODS column (4.6 i.d. × 250 mm) and gradient eluent of solvent A (1% acetic acid in water) and solvent B (methanol). The eluent gradient program started from 100% of solvent A for 1 min then turned to 70% and 40% at 10 and 20 min, respectively. After that, the eluent composition was put back to 100% of solvent A at 25 min and held on 5 min. The extract and the standard compounds were dissolved in methanol (HPLC grade) and filtered through a 0.22 µm filter membrane before injection. The HPLC condition was operated with a mobile flow rate of 1 mL/min, an injection volume of 10 µL, and running time of 30 min. The eluent was monitored with UV/VIS detector at a wavelength of 280 nm.

To confirm the major compound in the extract, the HPLC fingerprint of the extract and the standard which showed the same retention time to the extract was performed again with different eluting ratios of solvent A to solvent B; 90:10, 85:15, and 80:20, and detected at 280 nm. Isocratic conditions were performed with a flow rate of 1 mL/min, an injection volume of 10 µL, and running time of 15 min.

2.8. *Statistical analysis*

All experiments were performed in triplicate and the results are expressed as mean ± SD. The obtained data were analyzed statistically by SPSS statistic 17.0 software. The mean were determined for significance at $p < 0.05$ by ANOVA and Tukey's Multiple test.

3. Results

3.1. *Yield and biological activities of the extracts*

It was found that sequential extraction of dried *S. grandiflora* in powder by hexane, chloroform, and ethyl acetate gave different yield of extracts as shown in Table 1. The yield of CFF was the highest (1.45%) followed by that of EAF and HXF, respectively.

The antioxidant activity of *S. grandiflora* in bark extracts comparison with BHT and Vit-E as the positive controls are presented in Figure 1. Among these three different fractionated extracts; HXF, CFF, and EAF, it was obviously seen that EAF possessed the highest free

Table 1. The percentage yield and antibacterial activities expressed as MIC and MBC values of different fractionated extracts of *S. grandiflora* bark against *S. aureus* by broth dilution method

Extracts	Yield (% w/w)	MIC (mg/mL)	MBC (mg/mL)
HXF	0.23	10.00	> 10.00
CFF	1.45	2.50	2.50
EAF	0.27	0.63	0.63

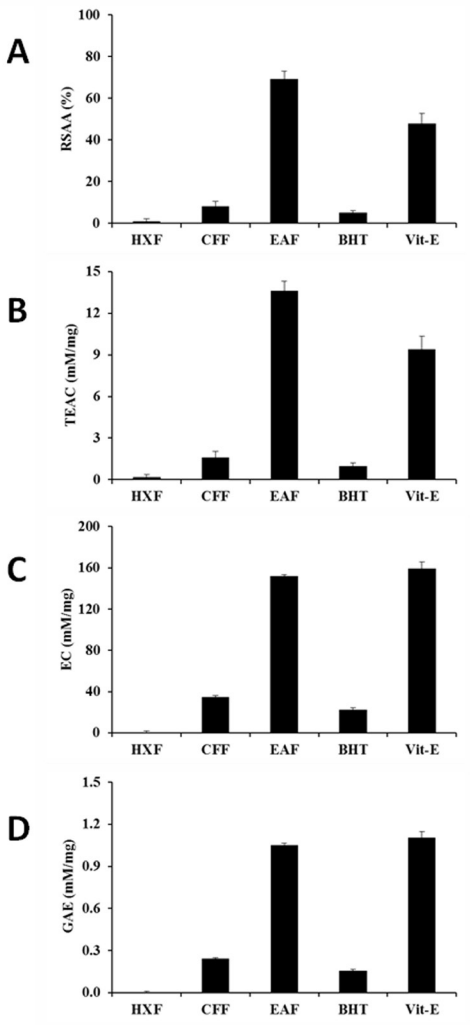

Figure 1. Antioxidant activities expressed as RSAA (A), TEAC (B), EC (C), and GAE (D) of HXF, CFF, and EAF in comparison with BHT and Vit-E.

radical scavenging property with the RSAA value of 69.3 ± 3.6 % and TEAC value of 13.6 ± 0.7 mM/mg. The TEAC values of BHT and Vit-E were found to be 0.9 ± 0.2 mM/mg and 9.4 ± 0.9 mM/mg, respectively. Therefore, the antioxidant activity of EAF was approximately 14 and 1.4 times higher than that of BHT and Vit-E, respectively. EAF also showed the highest reducing power with the EC and GAE values of 152 ± 2 mM/mg and 1.05 ± 0.01 mM/mg, respectively, which was about 6.7 times higher than that of BHT, but was 0.9 times less than that of Vit-E.

The antibacterial activity of the extracts investigated

Table 2. The percentage yield and antibacterial activities expressed as MIC and MBC values of EAF and different fractions of EAF against *S. aureus* by broth dilution method

Sample	Yield (% w/w)	MIC (mg/mL)	MBC (mg/mL)
EAF	100	0.50	0.50
Isolated fraction			
F1	0.05	> 0.02	> 0.02
F2	14.55	8.00	16.00
F3	2.30	1.00	2.00
F4	1.65	2.00	2.00
F5	0.10	> 0.04	> 0.04
F6	0.05	> 0.02	> 0.02

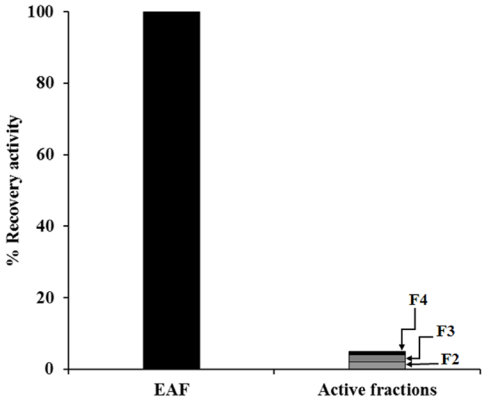

Figure 2. Recovery antibacterial activity of EAF and different active fractions; fraction 2 (F2), fraction 3 (F3), and fraction 4 (F4), from the preparative column chromatography.

by micro-dilution method demonstrated that EAF was the strongest inhibitory activity against *S. aureus* with the MIC and MBC values less than 1 mg/mL. The control antimicrobial agents shows equal values of their respective MIC and MBC. The MIC and MBC values of vancomycin was 0.5 μg/mL whereas that of gentamicin was 1 μg/mL.

3.2. *Isolation of the highest antibacterial extract*

As EAF presented the highest antioxidant and antibacterial activities, this extract was selected for isolation using silica gel 60 column chromatography. The percentage yield, MIC, and MBC values of each fraction from the isolation and EAF were compared in Table 2. Fraction 2 as eluted with chloroform:methanol at a ratio of 4:1 showed the highest yield of 14.6% (w/w) but less antibacterial activity whereas fraction 3 which obtained by elution with chloroform:methanol at a ratio of 3:2 gave the highest antibacterial activity with MIC and MBC values of 1.00 and 2.00 mg/mL, respectively. Considering the antibacterial activity in term of recovery activity as shown in Figure 2, the results indicated that the recovery activity of the sum of total different

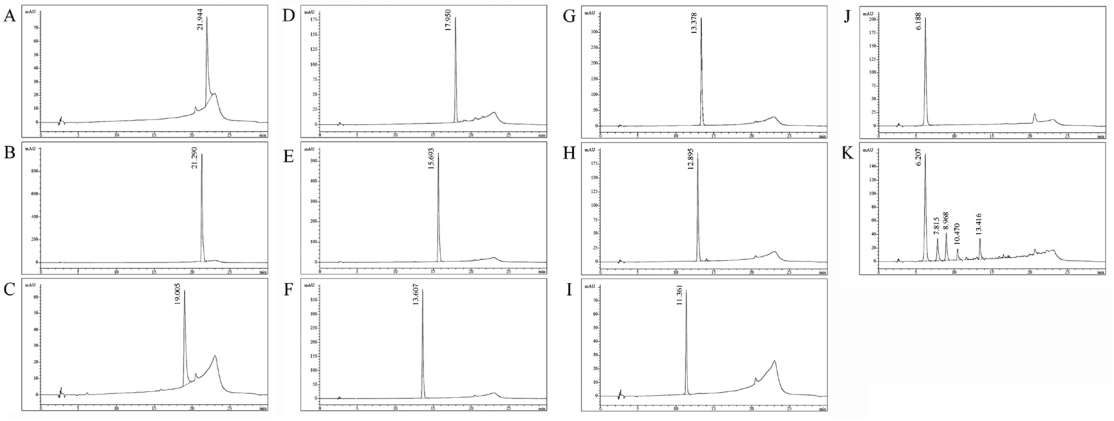

Figure 3. HPLC chromatograms of an individual standard quercetin (A), trans-cinnamic acid (B), ellagic acid (C), naringic acid (D), coumaric acid (E), syringin (F), caffeic acid (G), vanilic acid (H), catechin (I), gallic acid (J) and EAF (K) at 280 nm.

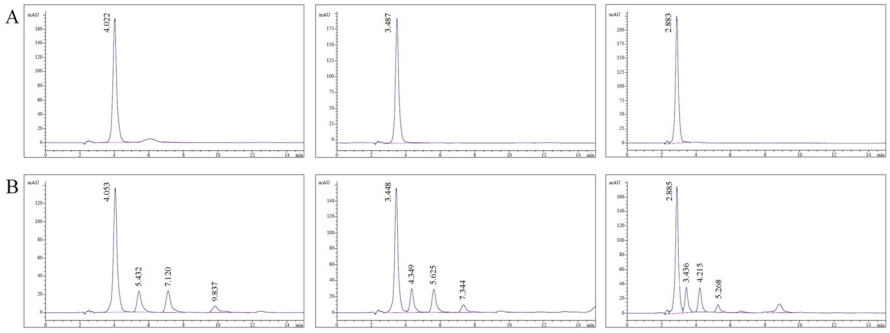

Figure 4. HPLC chromatograms of standard gallic acid (A) and EAF (B) using an eluting mixture containing 1% acetic acid in water and methanol at ratios of 90:10 (left), 85:15 (middle), and 80:20 (right) at 280 nm.

fractions was only 5% of EAF which was 20 times less than that of its original EAF (not isolated).

3.3. *HPLC analysis*

HPLC fingerprint of EAF was performed and compared with that of 10 standard phenolic compounds in order to identify the compounds existed in EAF. For this study, the concentrations of each standard compound and the extract were 100 µg/mL and 1 mg/mL, respectively. It was found that the HPLC chromatograms of 10 standard compounds could be obtained using gradient eluents of 1% acetic acid in water and methanol for 30 min run time and detected at 280 nm when each of these compounds was observed individually. The chromatograms of all standard phenolic compounds in comparison with EAF were shown in Figure 3. The retention time of standard quercetin, trans-cinnamic acid, ellagic acid, naringic acid, coumaric acid, syringin, caffeic acid, vanilic acid, catechin, and gallic acid were 21.944, 21.290, 19.005, 17.950, 15.693, 13.607, 13.378, 12.895, 11.361, and 6.188 min, respectively. The HPLC fingerprint of EAF which obtained by elution with the same gradient condition of those standard compounds gave five distinct peaks at the retention times of 6.207, 7.815, 8.968, 10.470, and 13.416 min. It is noted that a major peak of EAF exhibited at a retention time of 6.207 min. This peak resembled to the peak of standard gallic acid which eluted at a retention time of 6.188 min at the same detection wavelength.

To clarify the major compound existed in EAF whether it is gallic acid, the extract was subjected in various HPLC conditions and compared with HPLC chromatograms of the standard gallic acid. The HPLC chromatograms of EAF from various eluting ratios of 1% acetic acid in water and methanol detected at 280 nm exhibited a major peak which conformed to the peak of gallic acid in the same eluting conditions. As shown in Figure 4, the peak of gallic acid eluted by the ratios of 90:10, 85:15, and 80:20 presented at 4.022, 3.487, and 2.883 min, respectively, whereas the major peaks of EAF at the respective ratios were demonstrated at 4.053, 3.448, and 2.885 min, respectively.

4. Discussion

Bioactive agents from plants is currently interesting for alternative medicine (*16*). The research of effective natural compounds from medicinal plants which are potentiality, safety, and widely utilization has become considerable issue all over the world (*28,29*). The current study explores the antioxidant and antibacterial activities

of the most potential extract of *S. grandiflora*. The antibacterial activity of this plant is interesting because the plant has been used traditionally in the treatment of skin disorders and wound (*30*). In addition, the plant has been reported to have some other activities which related to antioxidant activity (*1,3,31*). Therefore, both antioxidant and antibacterial activities of *S. grandiflora* were investigated. In the antioxidant investigation, the extracts was evaluated by two methods. One is the determination of free radical scavenging activity using DPPH as free radicals and the other is determination of reducing power using FRAP methods. These two different assays are the most potential methods to determine antioxidant capacity of the plant extracts as both of them show high reproducibility and provide better understand the mechanism of action of a tested antioxidant (*26*). Vit-E is natural antioxidant and it is synthesized only by plant (*32*). Its mechanism of action has been reported to be free radical scavenging reaction (*33,34*). In the meanwhile, Vit-E *in vivo* has been reported to have high efficient in transferring a hydrogen atom to a lipid free radical like peroxyl, alkoxyl, and carbon-centered radicals (*35,36*). Therefore, Vit-E possesses both mechanisms of antioxidant action; free radical scavenging and reducing properties whereas the synthetic antioxidant BHT has been reported to predominate in reducing mechanism (*37*). Both Vit-E and BHT were used as positive controls in the current study. Among the three fractions of *S. grandiflora* bark extracts, EAF showed extremely high free radical scavenging action which significantly higher than Vit-E, therefore, the antioxidant activity of this extract was considered to be majorly due to the free radical scavenging mechanism. However, comparing to BHT which is the reducing antioxidant, EAF showed extremely higher EC value than BHT but slightly lower than Vit-E. Therefore, it was considered that reducing action is the minor mechanism of antioxidant action of the extract.

Many researchers report the use of phytochemicals from plant sources as an interesting choice to treat infectious diseases (*38,39*). EAF was therefore investigated for antibacterial activity. The antibacterial assay used in this study was a broth micro-dilution assay. This assay is appropriate for *in vitro* antibacterial activity investigation of plant extracts because it is higher repetition than agar diffusion assay (*40*). The results showed a great interest that among the tested extracts from *S. grandiflora* bark, EAF possessed the highest inhibitory activity against the tested pathogenic bacteria. Therefore, EAF was further isolated by preparative column chromatography. The results notified that the activity of each fraction was not strong inhibitory action. The recovery activity of the sum of total different fractions was only 5% of EAF while that of EAF as original extract (not isolated) was 100%. This low recovery might be due to an inactivation or simple loss of materials during the purification process. Moreover, synergistic effect was also one of the possibilities. Many previous studies have shown that the combination of phytochemical compounds in natural products revealed synergistic effect (*41,42*). The synergistic effect indicate that associating of several compounds in the plant could give the stronger activity than the individual active compound for that activity (*43-45*). The antibacterial potency of EAF that significantly higher than the sum of total different fractions might be explained by the synergistic effect of many compounds existing in the extract.

The results of HPLC fingerprints informed that EAF consisted several elements. One of all constituents in the extract presented the major peak as a main compound which corresponded to a standard phenolic compound of gallic acid. Gallic acid was reported to have an inhibitory action against human pathogenic bacteria, such as *S. aureus* and Corynobacterium accolans, human pathogenic yeast such as *Candida albicans* (*46*), and also other pathogenic microorganisms (*47,48*). From these activities of gallic acid and in accordance with the HPLC consequences, it is considered that the major compound as a bioactive marker of EAF is gallic acid.

In conclusion, EAF of *S. grandiflora* bark possessed the highest biological actions of antioxidant and antibacterial activities. HPLC study confirmed that there are many compounds existed in EAF. Synergistic effect on antibacterial activity of EAF might be occurred from the combination of several compounds existed in EAF. The major bioactive compound in *S. grandiflora* bark possessing an antibacterial activity is gallic acid.

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