CHAPTER 4

Discussion

In Northern Thailand, there are plenty of Lanna Traditional Medicines used for treatments by lots of traditional healers for a long time. Traditional formula of Mahoog is one of a popular Lanna medicine used in treating a group of intestinal diseases and related symptoms. Lanna traditional healers explain that Mahoog was comparable to Ka-Sai in Thai Traditional Medicines. Ka-Sai means physical deterioration, thin and because of consistently chronic diseases or illnesses. Lanna traditional healers defined the symptom of "Mahoog" as follows:

1) Pain in back, lumbar and body (May be called Mahoog by symptom)
2) Change in the consistency and looks of feces, being harder than normal.
3) The intestines are pressed out through the anus. The feces are mixed with mucus and/or blood. In modern medicine calls these symptoms to hemorrhoids.
4) If the patients urinate frequently, this symptom called in Thai "Mahoog Yiao Nak".
5) If the patients feel hot in gastrointestinal tract and burning sensation on defecation and urination, this symptom called in Thai "Mahoog Fai".
6) Frequency in abdominal pain, this symptom called in Thai "Mahoog Khao Sai".
7) Other symptoms such as yellow skinny, swelling with symptom of khaan it called in Thai "Mahoog Kaem Khang".

In my ethnobotanical survey of Mahoog formula in 2006, I gathered information from Lanna medical plants manuscripts and from the in-depth interview with traditional healers who specialize in plants used in the Mahoog formula from Chiang Mai, Chiang Rai and Lampang provinces. They found 85 medicinal plants from 17 formulas. From

ethnobotanical uses, many kinds of plants found to use in Mahoog formula without scientific support for their claims and uses. In this present study, biological activities were evaluated together with chemical constituents in some medicinal plants containing in Mahoog formula. Therefore, plant selection in this study followed the criteria of frequency of uses in formula by traditional healers and lack of scientific support in pharmacological activity.

One Mahoog formula was selected to evaluate the antibacterial and antioxidant activities. The Mahoog formula used in the present study was prepared in Ya-Mud dosage form. Ya-Mud is the combination of crude drug which tied together. The reason for using this formula were this formula had been used in the community from generation to generation, and the traditional healers from San Pa tong district, Chiang Mai consented to disclose the consisting of the plant species and their properties. And this formula contained only 5 major kinds of medicinal plants including: *C. sappan*, *E. cuneatum*, *T. reidioides*, *L. rubra* and *S. oleosa* which the frequently used ingredients of Mahoog formula are *C. sappan* and *L. rubra*. The properties of each plant in Mahoog formula, the data from traditional healers, were shown in Table 4.1.

Table 4.1 The knowledge of Mahoog formula from local wisdom[5]

Plants	Part used	Properties
C. sappan	heartwood	blood tonic, stop bleeding, astringent
E. cuneatum	root, stem	-
T. reidioides	root	-
L. rubra	root, stem	reduce inflammation, relieve pain, tonic
S. oleosa	stem	astringent

4.1 Extraction of 26 medicinal plants and Ya-Mud Mahoog

Medicinal plants containing in Mahoog formula and dosage form were selected following the criteria as described above. Then the medicinal plants were collected and verified by taxonomist at Faculty of Pharmacy and the voucher specimens were

deposited in the Herbarium of Faculty of Pharmacy, Chiang Mai University. The plant samples and Mahoog formula were cut into small pieces, dried and then ground to powder. The dried powder of twenty six medicinal plants and Mahoog formula were extracted with 95% ethanol by using soxhlet's apparatus and water by decoction method followed their local wisdom uses. Each extract solution was filtered and the solvent was removed by using a rotary evaporator for ethanol extract and freeze dryer for water extract. The yields of water and ethanol extract from twenty-six medicinal plants and Ya-Mud Mahoog were shown in Table 3.3.

The yield of water extracts of Lanna medicinal plants varied from 2.77% - 16.65% w/w. The minimum and maximum were *H. integrifolia* (2.77% w/w) and *T. reidioides* (16.65% w/w), respectively. The water extracts yield of top five Lanna medicinal plants as shown in Figure 4.1, were *T. reidioides* (16.65% w/w), *A. marginata* (13.68% w/w), *Piper* sp. (11.76% w/w), *C. deciduum* (11.21% w/w) and *S. obtusa* (10.80% w/w), respectively.

And the yield of ethanol extracts of Lanna medicinal plants varied from 0.78% - 22.59% w/w. The minimum and maximum were *E. cuneatum* (0.78% w/w) and *Piper* sp. (22.59% w/w), respectively. The ethanol extracts yield of top five Lanna medicinal plants as shown in Figure 4.2, were *Piper* sp. (22.95% w/w), *S. siamensis* (22.25% w/w), *L. rubra* (root) (21.95% w/w), *A. dulcis* (16.31% w/w) and *C. sappan* (15.05% w/w), respectively.

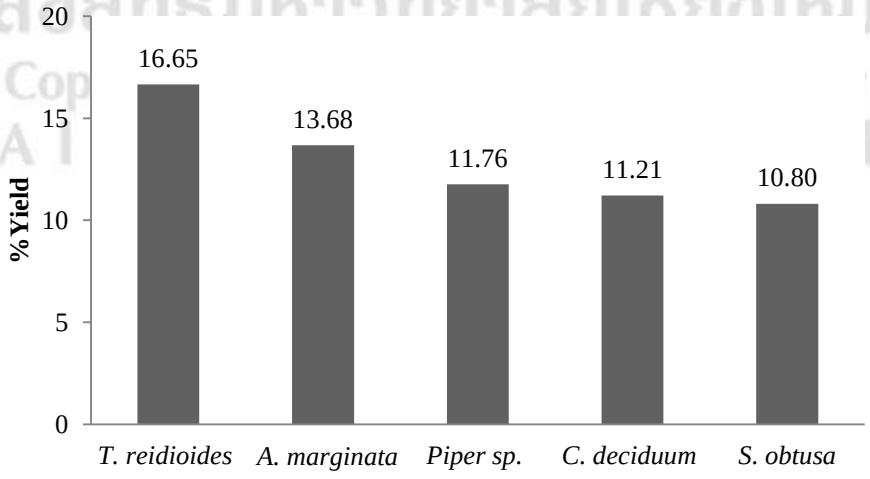

Figure 4.1 % Yield of water extracts of top five medicinal plants

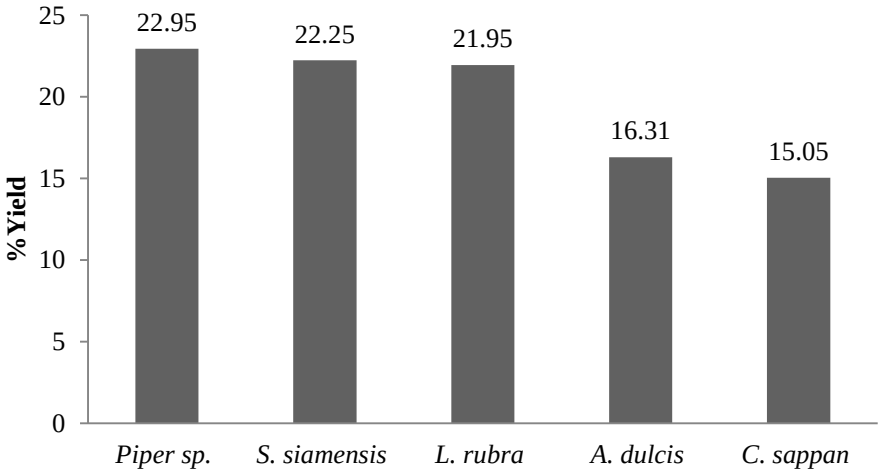

Figure 4.2 % Yield of ethanol extracts of top five medicinal plants

4.2 Antibacterial activity of 26 medicinal plants and Ya-Mud Mahoog

The results of antibacterial activity of 26 medicinal plants in agar well diffusion method were shown in Table 3.4. Chloramphenicol and gentamicin were used as standards for bacteria at concentration 0.5 and 0.05 mg/well, respectively. The inhibition zone diameter of chloramphenicol against *B. subtilis* and *S. aureus* were 27.5±0.5 and 25.0±0.5 mm. The inhibition zone diameter of gentamicin against *E.coli* and *P.aeruginosa* were 31.5±0.5 and 28.5±0.5 mm. The results showed that almost medicinal plant extracts were susceptible against the gram-positive bacteria while no inhibitory effect on gram-negative bacteria except the inhibition of *P. aeruginosa* and *E. coli* from *C. sappan* and the inhibition of *E. coli* from *D. scandens*. It has been reported that gram-negative bacteria had low susceptibility to plant extracts when compared to gram-positive bacteria[124, 154]. The low inhibitory effect of gram-negative bacteria might be due to the lipopolysaccharides in outer membrane of bacteria. Both ethanolic and water extracts of all Lanna medicinal plants were active against *B. subtilis*. While the ethanolic and water extracts of *M. hortensis*, water extract of *C. paniculatus*, *L. rubra* (stem), *L. indica* (stem), *A. marginata*, *M. kityana* and *Z. cambodiana* and ethanolic extract of *E. cuneatum* and *O. horridus* did not show inhibition zone against *S. aureus*.

In the results of antibacterial activity of 26 medicinal plants, they can classify the inhibitory responses as followed: zones of inhibitions greater than 6 mm were considered

susceptible to the extract such that those measuring<10 mm were classified as inactive, 10-13 as mild, 14-19 mm as active, and >19 mm as potent antibacterial activities[155]. The classification of inhibitory responses against *B. subtilis* and *S. aureus* were shown in Table 4.3 and 4.4, respectively.

Table 4.2 The classification of inhibitory responses against *B. subtilis*

<10 mm: inactive	**10-13 mm: mild**	**>13-19 mm: active**	**>19 mm: strong**
WE and EE	WE and EE	WE and EE	WE and EE
M. hortensis	*C. quadrangulare*	*S. siamensis*	*C. sappan*
C. crassifolius	*S. oleosa*	*S. obtusa*	
L. indica (stem)	WE	EE	
T. siamense	*V. denticulata*	*T. bellerica*	
T. reidioides	*Z. mauritiana*	*L. rubra* (root)	
H. integrifolia	EE	*V. denticulata*	
T. laurifolia	*C. paniculatus*	*Z. mauritiana*	
P. macrocarpus	*C. deciduum*		
Piper sp.	*E. cuneatum*		
Z. cambodiana	*L. indica*		
WE	*L. rubra* (stem)		
C. paniculatus	*D. scandens*		
C. deciduum	*Z. oenoplia*		
T. bellerica	*A. dulcis*		
E. cuneatum	*O. horridus*		
L. rubra (stem)			
L. rubra (root)			
D. scandens			
Z. oenoplia			
A. dulcis			
O. horridus			

Table 4.3 The classification of inhibitory responses against *S. aureus*

<10 mm: inactive	**10-13 mm: mild**	**>13-19 mm: active**	**>19 mm: strong**
WE and EE *M. hortensis* *C. paniculatus* *E. cuneatum* *C. crassifolius* *L. rubra* (stem) *L. indica* (stem) *T. siamense* *T. laurifolia* *O. horridus* *H. integrifolia* *Piper sp.* *Z. cambodiana* WE *E. cuneatum* *D. scandens* *V. denticulata* *Z. oenoplia* EE *C. quadrangulare*	WE and EE *A. dulcis* *L. indica* (root) *P. macrocarpus* WE *L. rubra* (root) *Z. mauritiana* *T. reidioides* EE *Z. oenoplia* *D. scandens*	WE and EE *S. siamensis* *C. deciduum* *T. bellerica* *S. obtusa* *S. oleosa* EE *T. reidioides* *L. rubra* (root) *V. denticulata* *Z. mauritiana*	WE and EE *C. sappan*

From the antibacterial activity of Ya-Mud Mahoog formula, it can classify the inhibitory responses of ethanol extract of Ya-Mud Mahoog and their herbal composition against *B. subtilis* and *S. aureus* (Table 4.4). The results showed antibacterial activity of Ya-Mud Mahoog had strong activity similar to *C. sappan*.

Table 4.4 The classification of inhibitory responses of Ya-Mud Mahoog and herbal compositions against *B. subtilis* and *S. aureus*

Extracts	**Inhibitory responses**	
	B. subtilis	***S. aureus***
C. sappan	strong	strong
E. cuneatum	mild	inactive
T. reidioides	inactive	active
L. rubra	active	active
S. oleosa	mild	active
Ya-Mud Mahoog	strong	strong

In this present study, we selected the 5 medicinal plants to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by choosing 3 plants from Ya-Mud Mahoog and 2 plants from 26 medicinal plants. The results were shown in Table 3.3. The ethanolic extract of the *C. sappan* gave very low MIC and MBC values against *S. aureus* and *B. subtilis* (MIC/MBC values = 0.049/0.098 and 0.390/0.390 mg/mL, respectively). *S. siamensis* and *S. oleosa* also revealed low MIC and MBC values against *S. aureus* and *B. subtilis* (MIC/MBC values = 0.049-0.195/6.25-12.500 mg/mL). While MIC/MBC values against *S. aureus* and *B. subtilis* of *T.bellerica* and *L. rubra* are 3.125-12.500/12.500 mg/mL.

The ethanol and water extracts of *C. sappan* heart wood showed the highest inhibitory effect on *S. aureus*, *B. subtilis*, *E.coli* and *P. aeruginosa*. The results were similar to that reported by Kim *et al*[64] and Srinivasan *et al*[156]. The heart wood of *C. sappan* had

been reported it showed an inhibitory effect against *S. aureus*, *E. coli*, *Streptococcus faealis*, *Salmonella typhi*, *Enterobacter aerogenes*, *P. aeruginosa*, *Aspergillus niger* and *Candida albican*[64, 156]. While *S. simensis*, that had the same family of *C. sappan* (Caesalpiniaceae) showed the inhibitory effect on *S. aureus* and *B. subtilis*.

T. bellerica fruit extract had been reported its antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *Streptococcus pneumoniae*, *S. typhi*, *S. typhimurium*, *Yersinia enterocolitica* and *C. albican*[157]. The MIC value of crude and methanol *T. bellerica* fruit extracts against *S. aureus* are 300 and 250 µg/mL, respectively. In our study, the stem extract of *T. bellerica* showed inhibitory effect against only gram-positive bacteria (*S. aureus* and *B. subtilis*). It may be due to the different part of the medicinal plant containing different chemical components[158].

4.3 Antioxidant activity of 26 medicinal plants and Ya-Mud Mahoog

The antioxidant activity of ethanol and water extracts of 26 medicinal plants and Ya-Mud Mahoog were evaluated by using ABTS, DPPH and FRAP method. The results of antioxidant activity were expressed as Trolox equivalent antioxidant capacity (TEAC). (Table 3.4) It was found that the ethanolic and water extracts of all plants showed antioxidant activity. The ethanolic extract of *C. sappan* exhibited the highest antioxidant activity in all method (TEAC = 1.004-1.359), followed by *S. obtusa* (TEAC = 0.666-1.116) and *S. siamensis* (TEAC = 0.652-0.976), respectively. The water extract of *C. sappan* also showed the highest antioxidant activity in all method (TEAC = 0.695-1.148), followed by *S. obtusa* (TEAC = 0.597-0.861) and *S. siamensis* (TEAC = 0.538-0.650), respectively.

In addition to antibacterial and antioxidant activity, the 5 plant species used in this formula were also studied their phytochemical groups. The phytochemical screening was done according to the method of Evans *et al*[153] as shown in Table 3.7. In anthraquinone test, Borntrager's reaction can cause false positive in the compounds that have quinone structure. False positive could observe in the result of *C. sappan* extract.

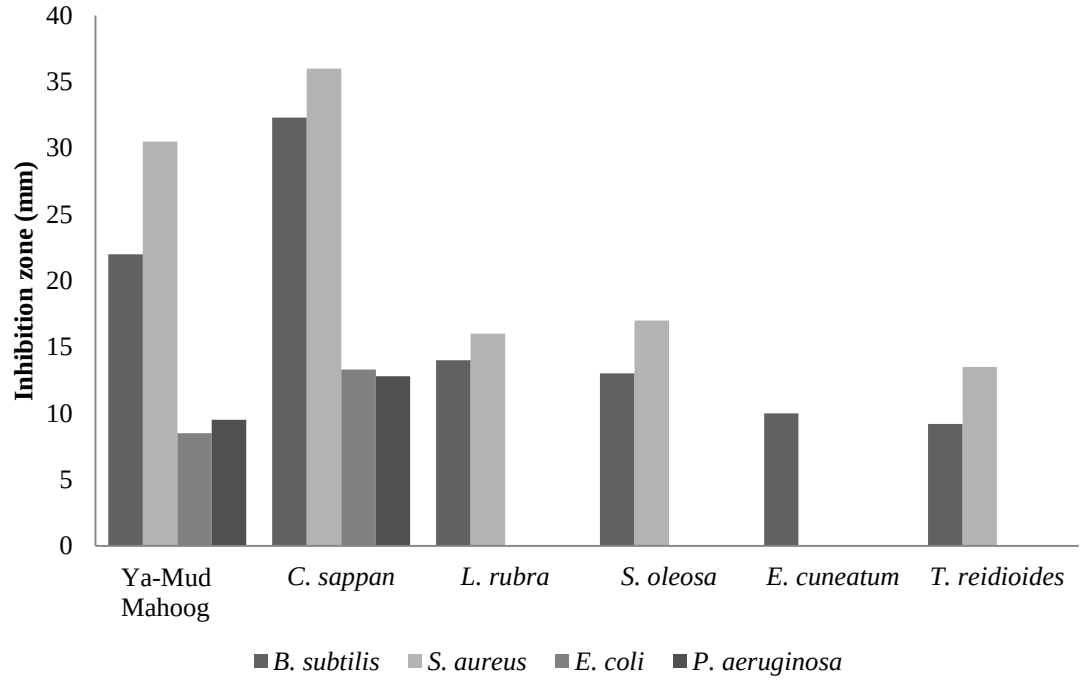

Figure 4.3 Antibacterial activity of Ya-Mud Mahoog and its composition (Ethanol extracts)

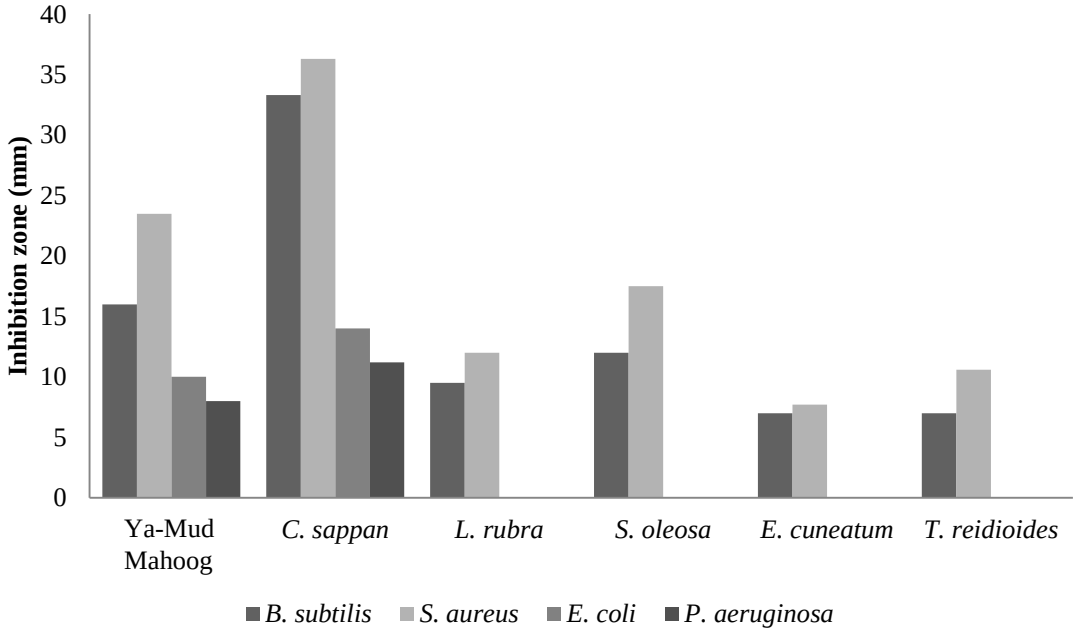

Figure 4.4 Antibacterial activity of Ya-Mud Mahoog and its composition (Water extracts)

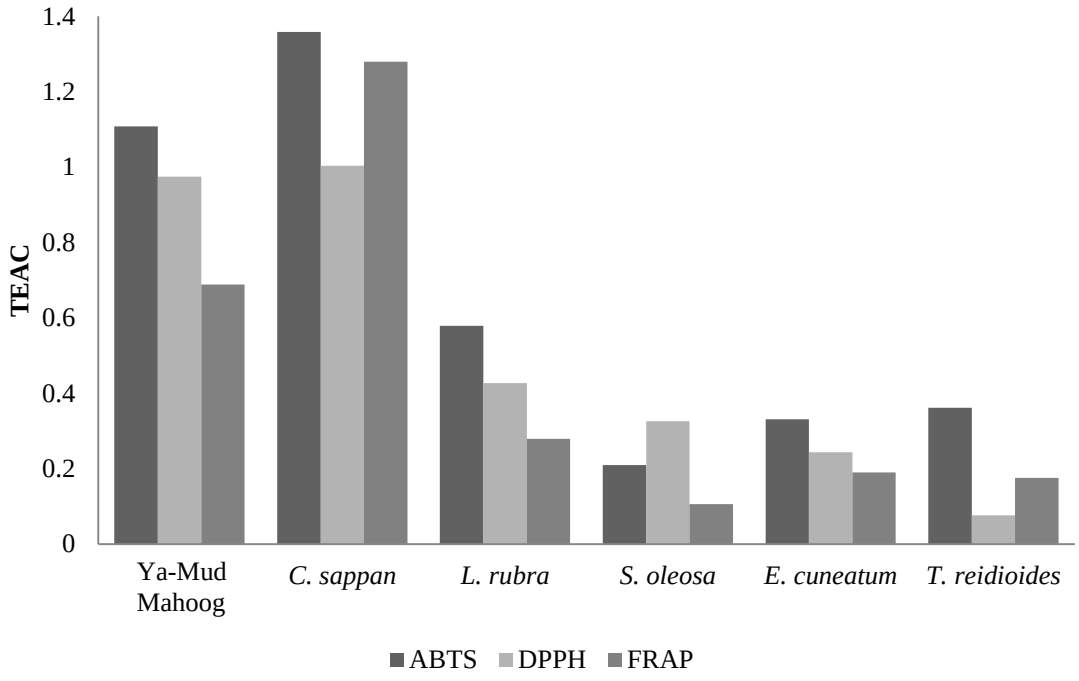

Figure 4.5 Antioxidant activity of Ya-Mud Mahoog and its composition (Ethanol extracts)

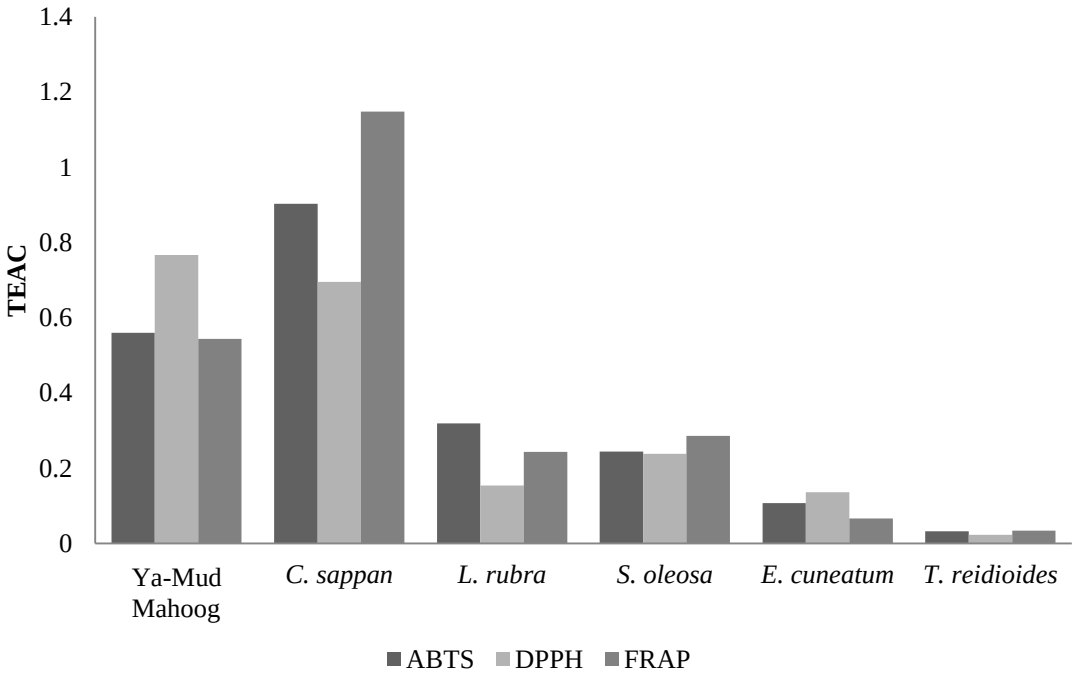

Figure 4.6 Antioxidant activity of Ya-Mud Mahoog and its composition (Water extracts)

The antibacterial activity of water and ethanol extract of Ya-Mud Mahoog were active against gram positive and gram negative and also exhibited high antioxidant activity (TEAC = 0.544-1.018) similar to the *C. sappan* extracts but with less active than *C. sappan* extracts. From the results of both activity studies of Ya-Mud Mahoog extracts, they exhibited high activities may be due to the chemical composition of this formula consisted of tannins and anthraquinones as shown in Table 3.5. In addition to high level antibacterial and antioxidant activity, these both activitites may come from the biological activity of *C. sappan*. *C. sappan* is a well-known medicinal plant belonging to the Caesalpineaceae family. *C. sappan* had been reported in biological activity by many research groups, such as hepatoprotective, anti-inflammatory, antibacterial, antioxidant, immunomodulation and immunosuppression[58, 61-63, 70, 73-75, 156]. Many studies reported the antibacterial and antioxidant activity of *C. sappan* is belong to the phenolic compounds, protosaponin A, protosaponin B, brazilin, brazilein and brazilide A[59, 61, 73, 74]. Brazilin and brazilein were the major components that had high antibacterial and antioxidant activities. From the Lanna wisdom, we found that traditional healers always added *C. sappan* to every Mahoog formula as blood tonic and indicator to identify when completely extraction is. The results from this study and in many review literatures found that *C. sappan* revealed various biological activities, especially anti-inflammation, antioxidant and antibacterial activity which were reasonable for usage *C. sappan* in Ya-Mud Mahoog.

For isolation work, *L. rubra* was selected because it used as a major ingredient in many formulas for Mahoog treatment and from my previous study, we found that this plant showed anti-inflammatory activity and contained flavonoids in the active fraction. And from this present study *L. rubra* also showed a good antibacterial and antioxidant activities. Therefore, we were selected this plant to completely in the study of bioactive compound.

4.4 Isolation and structure elucidation of bioactive compounds of *Leea rubra*

From the results of antibacterial and antioxidant activities of *L. rubra* extracts in Table 3.8 and 3.9, the ethyl acetate extract (LR2) gave the highest activity followed by ethanol extract (LR3) and hexane extract (LR1). Hence, the ethyl acetate extract was selected to bioassay-guided fractionation of silica gel column chromatography by using *n*-hexane, ethyl acetate and methanol as eluting solvent in gradient elution system. The overall of isolation of bioactive compounds was schematically shown in Figure 3.7. Two isolated compounds; LR2-05-A2-1-P and LR2-05-A2-2, were obtained from the ethyl acetate extract.

4.4.1 LR2-05-A2-1-P

LR2-05-A2-1-P was obtained as yellow powder from fraction LR2-05-A2-1-P fraction of ethyl acetate extract by chromatographic techniques using silica gel column and preparative TLC to yield 6.05 mg (0.015% w/w). The important ions in EIMS were found at m/z 420, 405, 377, 365 and 202. The UV spectrum showed absorption band at λmax 291 nm which is a characteristic of 8-prenylated isoflavone. The IR spectrum of compound LR2-05-A2-1-P showed absorption bands at 3331.77 (O-H stretching), 2923.78 (C-H stretching of CH_3, CH_2) and 1644.07 (C=O stretching of carbonyl group) cm^{-1}. In C=O stretching band, it confirmed the carbonyl group of isoflavone which differed from the position absorption band of coumarin.

Four aromatic protons were observed in the ^1H NMR ($CDCl_3$). In ^1H NMR spectrum displayed signals due to the characteristic of isoflavone (δ 7.89, H-2). The prenyl moiety on ring A was indicated by the appearance of doublet signal at δ 3.40 (2H, *d*, *J*=7.3 Hz, H-1´´), triplet signal at δ 5.17 (1H, *t*, *J*=7.3 Hz, H-2´´), singlet signal at δ 1.81 and 1.68. Three signals of three aromatic protons were observed at δ 7.05 (1H, *d*, *J*=1.5 Hz, H-2´), δ 6.88 (1H, *d*, *J*=8.2 Hz, H-5´) and δ 6.85 (1H, *dd*, *J*=1.5, 8.2 Hz, H-6´) on ring B. Moreover, methyl group at δ 1.47 (2 x CH_3, H-5´´´ and H-6´´´) and methine

group at δ 5.63 (1H, *d*, *J*=10 Hz, H-3´´´) and δ 6.74 (1H, *d*, *J*=9.9 Hz, H-4´´´) were also observed.

The HMQC spectrum revealed correlation between the directly coupled ^{1}H and ^{13}C nuclei. Based on the information obtained from the HMQC spectrum, all protons and protonated carbons of compound LR2-05-A2-1-P were assigned, as shown in Table 3.18.

The HMBC spectrum showed correlations of the long-range coupled ^{1}H and ^{13}C nuclei. The spectral data revealed that the prenyl moiety was on C-8, as showed in the Table 3.17 and Figure 4.7.

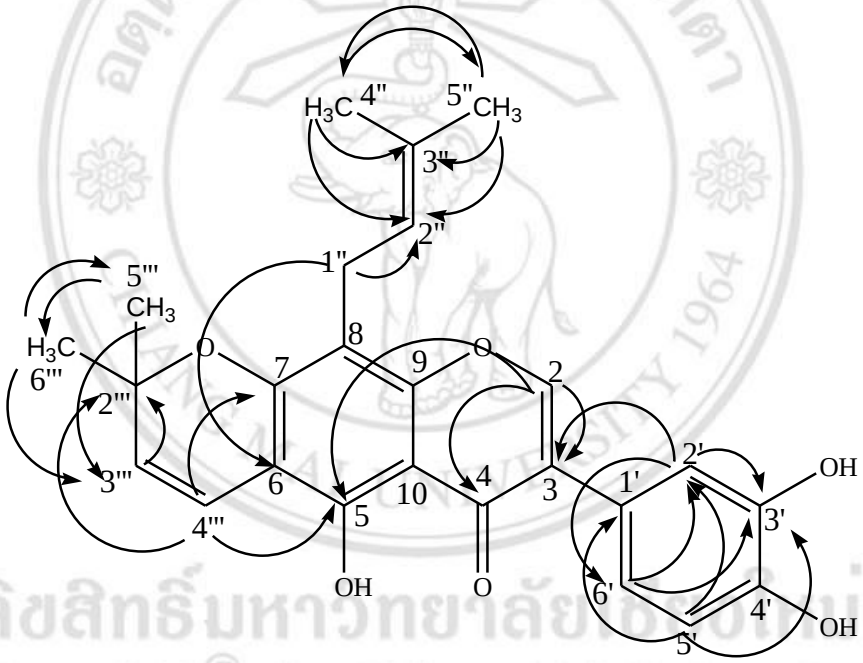

Figure 4.7 Long-range C-H correlations of compound LR2-05-A2-1-P observed in HMBC spectrum

The NMR data of this compound was agreement with the reported in the literature for auriculasin[159, 160]. The comparison of the chemical shifts of this compound reveals that the signals of some protons were significantly diagnostic. This compound has also been found in *Milletia auriculata* (Leguminosae)[161], *Flemingia philippinensis* (Leguminosae)[162], *Erythrina eriotriocha* (Leguminosae)[163] and *Maclura pomifera* (Moraceae)[160].

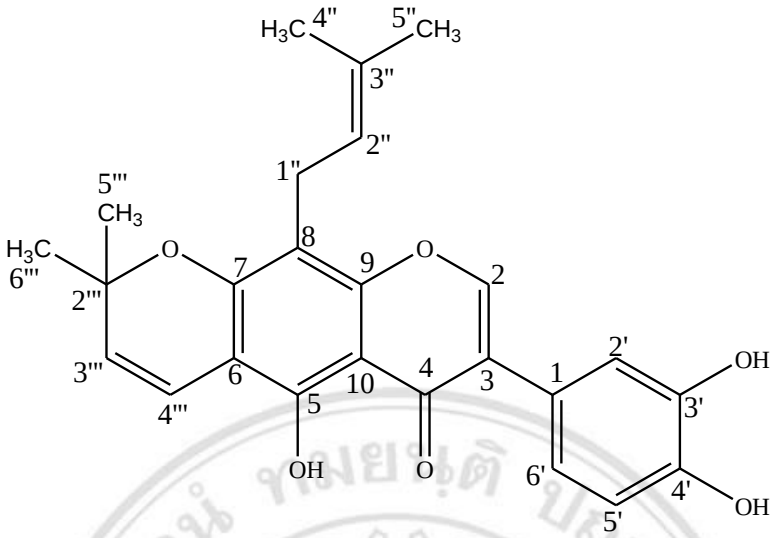

Figure 4.8 Structure of auriculasin (LR2-05-A2-1-P)

4.4.2 LR2-05-A2-2-P

LR2-05-A2-2-P was obtained as yellow powder from fraction LR2-05-A2-2-P fraction of ethyl acetate extract by chromatographic techniques using silica gel column and preparative TLC to yield 7.54 mg (0.019%). The important ions in EIMS were found at m/z 420, 405, 377, 365 and 202. The UV absorption band at λmax 274 nm in methanol showed the characteristic of isoflavone chromophore. The IR spectrum of compound LR2-05-A2-2-P showed absorption bands at 3285.85 (O-H stretching), 2923.34 (C-H stretching of CH_3, CH_2) and 1643.80 (C=O stretching of carboxyl group) cm^{-1}. In C=O stretching band, it confirmed the carbonyl group of isoflavone which differed from the position absorption band of coumarin.

Four aromatic protons were observed in the ^1H NMR ($CDCl_3$). In ^1H NMR spectrum displayed signals due to the characteristic of isoflavone (δ 7.86, H-2). The prenyl moiety on ring A was indicated by the appearance of doublet signal at δ 3.35 (2H, *d*, *J*=7.2 Hz, H-1´´), triplet signal at δ 5.23 5.22 (1H, *t*, *J*=7.2 Hz, H-2´´), singlet signal at δ 1.80 and 1.68 (2x3H, *s*, H-4´´ and H-5´´). Three signals of three aromatic protons were observed at δ 7.04 (1H,*d* *J*=1.8 Hz, H-2´), δ 6.88 (1H, *d*, *J*=8.1 Hz, H-5´) and δ 6.83 (1H, *dd*, *J*=1.8, 8.1 Hz, H-6´) on ring B. Moreover, methyl group at δ 1.48 (2 x CH_3, H-5´´´

and H-6´´´) and methine group at δ 5.59 (1H, *d*, *J*=10 Hz, H-3´´´) and δ 6.70 (1H, *d*, *J*=10 Hz, H-4´´´) was also observed.

The HMQC spectrum revealed correlation between the directly coupled ^{1}H and ^{13}C nuclei. Based on the information obtained from the HMQC spectrum, all protons and protonated carbons of compound LR2-05-A2-1-P were assigned, as shown in Table 3.18.

The HMBC spectrum showed correlations of the long-range coupled ^{1}H and ^{13}C nuclei. The spectral data revealed that the prenyl moiety was on C-6, as showed in the Table 3.17 and Figure.

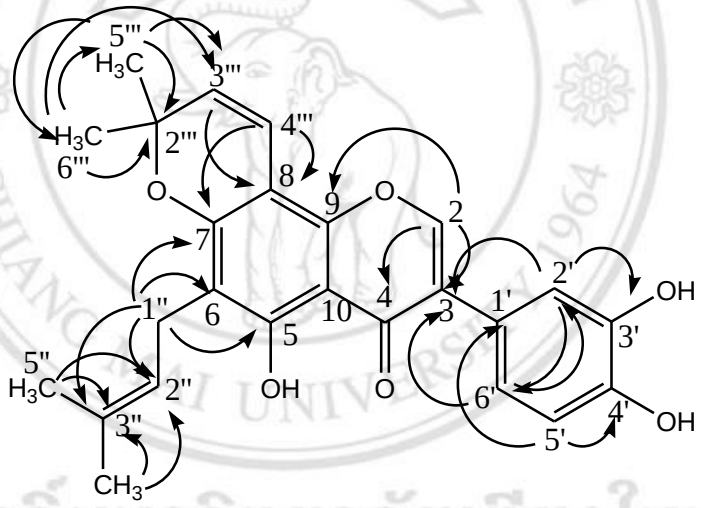

Figure 4.9 Long-range C-H correlations of compound LR2-05-A2-2-P observed in HMBC spectrum

The NMR data of this compound was agreement with the reported in the literature for pomiferin[160]. The comparison of the chemical shifts of this compound reveals that the signals of some protons were significantly diagnostic. The NMR data were shown in Table 3.18. This compound has also been found in *Maclura pomifera* (Moraceae)[160].

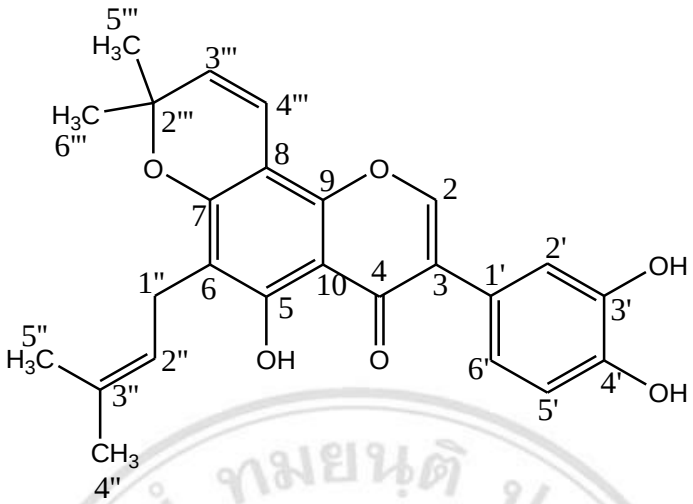

Figure 4.10 Structure of pomiferin (LR2-05-A2-2-P)

3.6 Antibacterial and antioxidant activities of isolated compounds

The antibacterial and antioxidant activities of the isolated compounds were shown in the Table 3.15 and Table 3.16, respectively. Unfortunately, both isolated compounds were obtained 6-7 mg. In the antibacterial activity study, we used 1 mg/well for evaluation their activity. The results showed that both isolated compounds had weak antibacterial activity against *B. subtilis* and *S. aureus* (IZD=8.5-9.5 mm). While in antioxidant activities the LR2-05-A2-2-P or pomiferin showed more potent than LR2-05-A2-1-P or auriculasin. Comparative antioxidant activity between crude extracts and isolated compounds revealed that their isolated compounds showed higher activity than crude extracts. The order of antioxidant activity of crude extracts and their isolated compounds was as follows; LR2-05-A2-2-P > LR2-05-A2-1-P > ethyl acetate extract > ethanol extract > hexane extract.

From the literature reviews, auriculasin had been reported the biological activities such as; bacterial neuraminidase inhibitory activity[163], *in vivo* anti-inflammatory and antinociceptive (inactive)[164], anti-tumor and cytotoxicity[165]. Pomiferin had been reported with the use as ingredient for topical skin and scalp treatments[166], acetylcholinesterase inhibitory[167], antimicrobial[168], antioxidant[169, 170] and anticancer activities[171].