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Antibacterial and Antioxidant Activities of Various Fraction of *Leea rubra* (Leeaceae)

Sarinya Kadchumsang[1] Panee Sirisa-ard[1] Siriwoot Sookkhee[2] Suntara Eakanunkul[1]
Sunee Chansakaow[1*]
1. Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200 Thailand
2. Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
*E-mail of the corresponding author: chsunee@gmail.com

Abstract
The present study was designed to evaluate the antibacterial and antioxidant activities of hexane, ethylacetate and ethanol extracts of *Leea rubra* (Leeaceae) roots and stems, which has been used as a Lanna Traditional Medicines for Màhòog. Each extract was tested for antibacterial activity by agar diffusion method and microbroth dilution method and antioxidant activity by 2,2′-azino-bis(3-ethyl- benzthiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. The ethylacetate extract of *L. rubra* root showed the highest antibacterial activity against gram-positive (IZD=15.5.0±0.5 to 17.5±0.5 mm, MIC=0.098-1.562 mg/ml). While the ethanolic extract of root showed the strongest antioxidant activity in ABTS, DPPH and FRAP method (TEAC=0.888±0.001, 0.849±0.020 and 0.733±0.037, respectively). The data obtained from this study confirms the traditional use of *L. rubra* for treatment Màhòog.
Keywords: *Leea rubra*, antioxidant activity, antibacterial activity

1. Introduction
Leea rubra Blume ex Spreng is and erect or suberect soft-wooded shrub of family Leeaceae. Root and stem of this plant has been used as a Lanna Traditional Medicines for Màhòog. Màhòog is a group of intestinal diseases (Brun *et al*., 1987). The exact cause of Màhòog disease is not well established, however there are many factors that provoke this disease including half-cooked and spicy food consumptions, drinking less water and hard work. Although the disease is not severe and occurs much less frequently, this disease is a chronic disease if left untreated, and it may result in death. The characteristic symptoms of Màhòog are pain, inflammation and wound infection. Especially, when wound is occurred, it is accompanied with pain, reddening and edema within a short time, which are the classical symptoms of inflammation. These symptoms are caused by releasing of eicosanoids, prostaglandins, leukotrienes, and reactive oxygen species (ROS). Not only is ROS produced in large amount at the site of wound as a defense mechanism against invading bacteria, but also at the same time, the presence of free radicals may be hampered the process of wound healing, resulting in wound damage or microbial infection (Houghton *et al*., 2005; Srinivas *et al*., 2008). Therefore, the objectives of the present study were to investigate the antibacterial and antioxidant activities of various fraction of *Leea rubra*. The data obtained from the study will be used as a scientific evidence to support the pharmacological properties of Lanna medicinal plants.

2. Experimental
2.1 *Plant materials*
The plants used in the work were collected from Chiang Mai provinces, Thailand. The identity of the Lanna medicinal plants were verified by a taxonomist at Faculty of Pharmacy and the voucher specimens were deposited in the Herbarium of Faculty of Pharmacy, Chiang Mai University.
2.2 *Plant extract*s
The stem and root samples of *Leea rubra* were cut into small pieces, dried at 50°C for 24 hours and then ground into powder. Each powder was successively extracted with hexane, ethylacetate and ethanol by soxhlet apparatus, respectively. The extract solutions were filtered through Whatman filter paper No. 1 and then the solvents were removed by using rotary evaporator.
2.3 Antibacterial activity
2.3.1 Microorganisms
Four bacteria; 2 Gram-positive and 2 Gram-negative were used for the antibacterial assays. The following strains of microorganisms were used: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027.
2.3.2 Agar diffusion method
Diameter of zone of inhibition was determined using agar well diffusion technique modified from Kirby-Bauer method (Bauer *et al*., 1966). A swab of the bacteria suspension containing 1×10^8 cfu/ml was spread on to sterile

Petri dish (*Greiner* bio-one, Austria) containing Tryptic Soy Agar (TSA; Difco, MI) media. Each extract was dissolved in propylene glycol to concentration 50 mg/ml and autoclaved at 121°C, 15 psi for 15 min. Wells were cut with sterile borer (6 mm) and 50 µl of the extracts were added into the wells. The plates were incubated at 37°C for 24 hours. The propylene glycol used as negative control while the standard chloramphenicol 10 mg/ml and gentamicin 1 mg/ml were used as positive controls. Antibacterial activity was indicated by the presence of clear inhibition zone around the wells. Tests were performed in triplicate.

2.3.3 Minimum inhibitory concentration (MIC)

The MIC was determined using microbroth dilution method. The extracts were dissolved in 50% DMSO (*Sigma-Aldrich*, Germany). The extracts were diluted by two-fold to obtain a concentration range 100–0.05 mg/ml with Tryptic Soy Broth (TSB; Difco, MI) in the 96-well microplates (*Greiner* bio-one, Austria). The microorganism suspension ($1x10^5$ cfu/ml) of 50 µl was added to the broth dilutions. These were incubated for 24 hours at 37ºC. MIC of each extract was taken as the lowest concentration that did not permit any turbidity of the tested microorganism.

2.4 Antioxidant Activity

2.4.1 ABTS free radical scavenging assay

The ABTS assay was performed using a modified method (Re *et al*., 1999). ABTS•+ stock solution was generated by oxidation of 7.0 mM ABTS (*Sigma-Aldrich*, Germany) with 2.45 mM potassium persulfate (UNILAB, Austria). The solution was protected from light and stored at room temperature for 12-16 hrs. The 2.0 ml of ABTS•+ working solution was mixed with 100 µl dilute extract, comparing it to Trolox (*Sigma-Aldrich*, Germany). After 3 min incubation at room temperature, the color reaction was measured at 734 nm using a UV/VIS spectrophotometer (*Varian Cary 1E* UV/Visible Spectrophotometer, USA). The results of ABTS assay were expressed as Trolox equivalent antioxidant capacity (TEAC). This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

2.4.2 DPPH free radical scavenging assay

The DPPH free radical scavenging assay was done according to the method of Brand-Williams *et al*., (1995) with some modifications. The 2.1 milliliters of reaction mixture containing 2.0 ml ethanolic DPPH (*Sigma-Aldrich*, Germany) and 100 µl diluted extract. The mixture was incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm using a UV/VIS spectrophotometer. The results were calculated in terms of TEAC. This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

2.4.3 FRAP assay

The FRAP assay was done according to Benzie and Strain, (1996) with some modifications. The 3.1 milliliters of reaction mixture containing 3.0 ml FRAP reagent and 100 µl diluted extract. The mixture was incubated in the dark for 4 min at 37°C. The absorbance was measured at 593 nm. The results were calculated in terms of TEAC. This index is defined as gram of standard is equivalent to 1.0 gram of the extract.

3. Results and discussions

3.1 Antibacterial Activity

In this study, the antibacterial activities of hexane, ethylacetate and ethanol extracts of *L. rubra* root and stem were evaluated against 2 gram-positive and 2 gram-negative bacterial. Chloramphenicol and gentamicin were used as standards for bacteria at concentration 0.5 and 0.05 mg/well, respectively. The results showed that the ethylacetate extracts of root and stem, ethanol and hexane extracts of root were active against the gram-positive bacteria: *B. subtilis* and *S. aureus* (IZD=9.0±0.5 to 17.5±0.5 mm). None of extracts showed activity against the gram-negative bacteria: *E. coli* and *P. aeruginosa*. The zone of inhibition values are summarized in Table 1.

Table 1: Antibacterial activity of *L. rubra* extracts in agar well diffusion method

Fraction	Inhibition zone diameter (mm)							
	B. subtilis		*S. aureus*		*E. coli*		*P. aeruginosa*	
	Root	Stem	Root	Stem	Root	Stem	Root	Stem
Hexane	10.5±0.5[b]	-	10.5±0.5[a]	-	-	-	-	-
Ethylacetate	17.5±0.5[c]	9.0±0.0	15.5±0.5[c]	9.0±0.0	-	-	-	-
Ethanol	12.0±0.0[a]	-	12.0±0.0[b]	-	-	-	-	-
Chloramphenicol	27.5±0.5		25.0±0.5		19.0±0.5		-	
Gentamicin	38.0±0.5		33.5±0.5		31.5±0.5		28.5±0.5	

Diameter of well 6 mm , (-) no inhibition, Chloramphenicol 10 mg/ml and Gentamicin 1 mg/ml are the standards for bacteria (values are mean ± S.D. of three replicates, in difference in letter on column represented different statistic at 99%).

The MIC values of different microbes, tested in a concentration range 100–0.05 mg/ml, are given in Table 2. All of root extracts showed activity against gram-positive bacteria, *B. subtilis* and *S. aureus*, with MIC ranging from 0.098-12.5 mg/ml. The ethylacetate extract of stem showed MIC against *B. subtilis* and *S. aureus* were 50 mg/ml while the MIC of hexane and ethanol extracts of stem could not be observed even at a concentration of 100 mg/ml.

Table 2: The minimum inhibitory concentration (MIC)

Fraction	MIC (mg/ml)							
	B. subtilis		*S. aureus*		*E. coli*		*P. aeruginosa*	
	Root	Stem	Root	Stem	Root	Stem	Root	Stem
Hexane	12.5	>100	6.25	>100	ND	ND	ND	ND
Ethylacetate	1.562	50	0.098	50	ND	ND	ND	ND
Ethanol	3.125	>100	1.562	>100	ND	ND	ND	ND
Chloramphenicol (µg/ml)	25.0		12.5		3.125		3.125	
Gentamicin (µg/ml)	0.50		0.25		1.00		1.00	

ND= Not detected

3.2 Antioxidant Activity

The antioxidant activity of hexane, ethylacetate and ethanol extracts of *L. rubra* root and stem were evaluated by using ABTS, DPPH and FRAP method. The results of antioxidant activity were expressed as Trolox equivalent antioxidant capacity (TEAC) (Table 3). It was found that the antioxidant activity of ethanol extract (TEAC=0.790±0.001 to 0.888±0.001) and ethylacetate extract (TEAC=0.733±0.037 to 0.884±0.001) of *L. rubra* root were higher than ehanol and ethylacetate extracts of *L. rubra* stem 7-8 times. While the hexane extracts showed low antioxidant activity in all method.

Table 3: The antioxidant activity of hexane, ethylacetate and ethanol extracts of *L. rubra* root and stem

Fraction	Antioxidant activity (TEAC)					
	ABTS		DPPH		FRAP	
	Root	Stem	Root	Stem	Root	Stem
Hexane	0.028±0.005	0.060±0.002	0.011±0.003	0.026±0.001	0.020±0.000	0.036±0.003
Ethylacetate	0.884±0.001	0.136±0.015	0.819±0.020	0.149±0.021	0.733±0.037	0.124±0.003
Ethanol	0.888±0.001	0.110±0.003	0.849±0.020	0.123±0.001	0.790±0.001	0.086±0.003

Each value is mean ± S.D. of three replicates

4. Conclusions

In conclusion, the ethylacetate extract of *L. rubra* root showed the highest antibacterial activity against gram-positive (IZD=15.5.0±0.5 to 17.5±0.5 mm, MIC=0.098-1.562 mg/ml). While the ethanolic extract of root showed the strongest antioxidant activity in ABTS, DPPH and FRAP method (TEAC=0.888±0.001, 0.849±0.020 and 0.733±0.037, respectively). The results of this study indicated *L. rubra* to have potential antibacterial and antioxidant activities that could be used as a scientific evidence to support the pharmacological properties of Lanna medicinal plants. In this study, the hexane extracts of this plant indicated less active than the ethylacetate and ethanol extracts. This trial for these activities guided isolation led us knows that, the active principle(s) belongs to moderately polar fraction.

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