Maejo Int. J. Sci. Technol. **2009**, *3*(01), 212-221

Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Antimicrobial property and antioxidant composition of crude extracts of *Pueraria mirifica*, *Butea superba* and *Mucuna macrocarpa*

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Received: 5 June 2008 / Accepted: 18 March 2009 / Published: 10 April 2009

Abstract: In this study, crude extracts of *Pueraria mirifica*, *Butea superba* and *Mucuna macrocarpa* were prepared using sequential extraction with three different solvents (hexane, ethyl acetate and methanol). The extracts obtained were then used to test for their antimicrobial activity by the disc diffusion method, which showed that, against a wide range of Gram-positive and Gram-negative bacteria, only the *P. mirifica* extract obtained with ethyl acetate exhibited antimicrobial activities. The minimum inhibitory concentration (MIC) of the extract was also determined with values between 15 and 50 mg/ml depending on the microbes tested. Thin layer chromatography (TLC) was subsequently used to separate the chemical constituents of the extract. When tested against *B. cereus*, there were only two bands which showed anti-*B. cereus* activity. Additionally, the crude extracts of *P. mirifica*, *B. superba* and *M. macrocarpa* were analysed for some antioxidant compounds using HPLC. Our results showed that all the extracts contained daidzin, genistin, daidzein and genistein, all of which were present in the highest amounts (0.045, 0.037, 0.049 and 0.060 % respectively) in the ethyl acetate extract of *P. mirifica*.

Keywords: antimicrobial activity, antioxidants, Kwao Krua, *Butea superba*, *Mucuna macrocarpa*, *Pueraria mirifica*

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Introduction

Kwao Krua is a generic name of a group of indigenous Thai medicinal plants in the family Leguminosae that have been widely used for a long time by the Thai people. Generally, the term is used for three different plant species, viz. white Kwao Krua (*Pueraria mirifica* Airy-Shaw & Suvatabandhu), red Kwao Krua (*Butea superba* Roxb.), and black Kwao Krua (*Mucuna macrocarpa* Wall.) [1]. There is also a description recorded for a *mor* (grey) Kwao Krua, but its exact identity remains unclear at present. It is suggested [2] that all these plants have rejuvenating properties, although this is found to be highly dependent on the type of Kwao Krua, i.e. black, red or white, and that they are to be taken only by the elderly. In addition to the rejuvenating properties, there are several phytoestrogenic compounds which include deoxymiroestrol, miroestrol, puerarin, daidzein, genistein, kwakhurin and other isoflavonoids, which can be used in medical applications due to their female hormone-like activity. Their estrogenic activity has also been exploited in many commercial products for breast enlargement and body firming [3,4].

Most of the investigations of Kwao Krua plants reported have focused upon their estrogenic and antioxidant activities. Although there have been many claims reporting the antimicrobial properties of these plants, they are still not conclusive due to the lack and/or limitation of scientific documentation. Hakamatsuka et al. [5] reported that *P. lobata* produces pterocarpan phytoalexins, namely tuberosin and glycinol, which prevent microbial attacks. A discovery was made in *B. superba* of a new bioactive flavonol glycoside and revealed its antimicrobial activity against plant pathogenic fungi and Gram-positive and Gram-negative bacteria [6]. There is, however, only one document describing the antimicrobial activity of *M. macrocarpa* [7]. This current study has been performed to investigate the antimicrobial properties of these Kwao Krua plants. The content of some antioxidant compounds in the crude extracts of these plants was also determined.

Materials and Methods

Plant materials

Tubers of *P. mirifica* and *B. superba* (Figure 1) were collected from Chiang Muan, Phayao Province, while *M. macrocarpa* was collected at the same time from Doi Tung, Chiang Rai Province in February 2004. The specimens were then authenticated and kept as voucher specimens Nos. MFLU-307, MFLU-310, and MFLU-311 respectively at Mae Fah Luang University Herbarium.

Microorganisms used

All the microbes used in the experiments were purchased from the Microbiological Resources Centre of the Thailand Institute of Scientific and Technological Research and kept as stock cultures at the Microbiology Laboratory in Mae Fah Luang University (Table 2). For routine culture and maintenance, the bacteria were grown on nutrient agar (NA) or in nutrient broth (NB) at 37°C. Yeasts were grown on the yeast malt agar (YMA) or in broth (YMB) at 30°C. For long term storage, all the microbes were kept either in a slant culture at 4°C or in glycerol stock at -20°C.

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Figure 1. Kwao Krua tubers: left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*

Preparation of Kwao Krua extracts

In this study, sequential extraction was performed as described by Canales et al. [8]. Initially, tuber samples were cut into small pieces, dried at 60°C for three days, and ground into a fine powder. The powdered samples (0.5 kg) were then placed in a closed plastic bag and stored in stainless steel coolers in a desiccator at ambient temperature (~28-30°C) until use.

Three separate extracts from each powdered Kwao Krua were made using 3 L each of hexane, ethyl acetate and methanol in that order. Each extract was left to stand for three days before filtering and then concentrated under reduced pressure at 45ºC using a vacuum rotary evaporator. The residue was dried and frozen with a freeze dryer (Flexi-DryTM, FTS Systems, USA) until a constant weight was obtained (Figure 2).

Antimicrobial assay

The microbial stock samples were cultured in 100 ml of NB or YMB and incubated at 37°C (for bacteria) or 30°C (for yeast) for 20-24 h in a shaking incubator. Each microbial cell sample was then inoculated onto agar plates. Sterile filter papers (diameter 6 mm, No. 3, Whatman, UK) were dipped in solutions of the three different sets of Kwao Krua extracts and placed on the surface of the inoculated agar plates. The plates were then incubated at either 37°C (for bacteria) or 30°C (for yeast) for 24 h. Each antimicrobial assay was carried out in triplicate by observing the clear zone formed on each plate. The antimicrobial activity was observed as the clear zone diameter seen on the plates and recorded in millimeters. Controls were made of pure hexane, ethyl acetate and methanol. The minimum inhibitory concentration (MIC) was determined from the extract samples, which were prepared at various concentrations (5-50 mg/ml). A 15 µl aliquot of each prepared extract was dropped on the sterile filter paper and placed onto the agar plate containing the tested microbes and the plate placed in an incubator at 37°C or 30°C for 24 h. This was also performed in triplicate. The presence of a clear zone at the lowest concentration was expressed as the MIC value.

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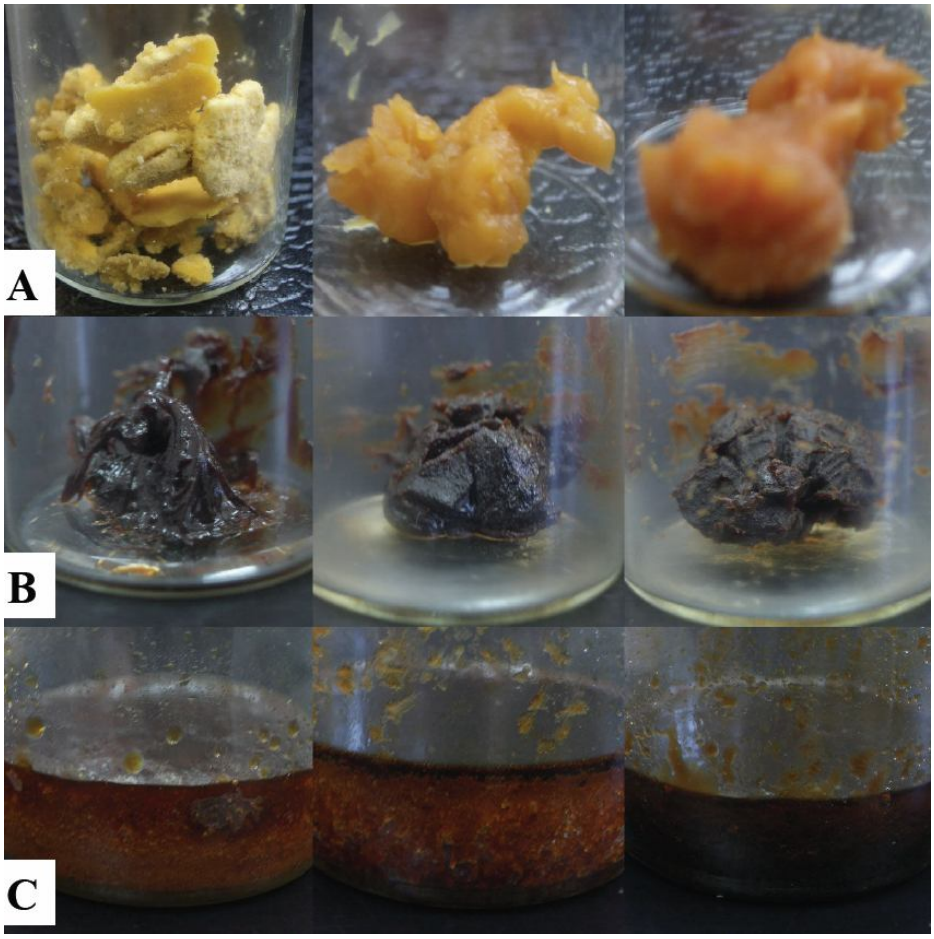

Figure 2. The appearance of Kwao Krua extracts derived from sequential extraction. Rows: A, hexane; B, ethyl acetate; C, methanol. Columns: left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*.

Determination of antimicrobial compounds using thin layer chromatography (TLC)

TLC technique was used to separate the chemical components present in the Kwao Krua extracts. The TLC plates (Silica gel aluminium plate GF254, J. T. Baker, USA) 20 cm x 20 cm in size were used. The extracts and the standard compounds (1 mg/ml of daidzein and genistein) were applied onto the plate, which was then placed in a chamber saturated with hexane and ethyl acetate (6:4) The developed chromatogram was placed under a 254 nm UV light. Each fraction was marked, scraped, dissolved in ethyl acetate (1 ml) and the resulting solution used for the antimicrobial assay above.

Analysis of antioxidant compounds

The analysis of the antioxidant compounds was based on that of Klump et al. [9]. A 100 mg sample of each Kwao Krua extract was dissolved in 40 ml of 80% methanol. The resulting suspension was then placed in an ultrasonic washer (Transsonic 700, Elma) for 5 min and then 2 M NaOH (3 ml) was added and the mixture incubated for 1 min. Subsequently, 1 ml of acetic acid was added to the incubated suspension and the solution was further incubated for 1 min. Finally, the supernatant of the prepared sample was filtered using a syringe filter (Chorm Tech., USA) and thus ready for analysis by HPLC (Water 2695 System). The conditions used were as follows: column, reverse-phase C-18; detector, UV 260 nm; mobile phase, water:methanol:acetic acid (88:10:2) and methanol:acetic acid

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(98:2). Standard antioxidants, namely daidzin, genistin, daidzein and genistein (Sigma-Aldrich Co., USA) were also prepared and used for this analysis.

Results and Discussion

Kwao Krua extracts

After evaporation and freeze-drying, the crude extracts of Kwao Krua were obtained as either a thickened solution or in solid form (Figure 2). The per cent yield of the extract (Table 1) was found to be highly dependent on the type of solvent used. As illustrated in Table 1, the per cent yields of the methanol extracts were the highest.

Table 1. Physical appearance and percent yield of Kwao Krua extracts

Kwao Krua extracts	Appearance	% Yield (w/w)
Hexane extract		
P. mirifica	White-yellow solid	0.21
B. superba	Brown solid	0.13
M. macrocarpa	Brown solid	0.29
Ethyl acetate extract		
P. mirifica	Dark brown solid	0.58
B. superba	Dark brown solid	0.36
M. macrocarpa	Dark brown solid	0.34
Methanol extract		
P. mirifica	Dark brown liquid	11.37
B. superba	Dark brown liquid	15.34
M. macrocarpa	Dark brown liquid	11.46

Antimicrobial activity of Kwao Krua extracts

All Kwao Krua extracts were preliminarily evaluated for their antimicrobial property using the disc diffusion method. In this study, seventeen species of microbes were selected including those that cause food poisoning (i.e. *B. cereus*, *E. coli* and *S. aureus*) and an opportunistic fungal pathogen (i.e. *C. albicans*). The results of this antimicrobial activity test are presented in Table 2.

It was found that only the ethyl acetate extract of *P. mirifica* exhibited antimicrobial activity. The extract was active against various kinds of Gram-positive and Gram-negative bacteria. For yeasts, the extract was able to inhibit *S. cerevisiae* but not *Candida* species. Interestingly, the extract was capable of inhibiting all the Gram-positive bacteria used in this study. However, for Gram-negative bacteria, it could not inhibit *A. faecalis* and *E. aerogenes*.

Further experiment was then carried out using different concentrations of *P. mirifica* ethyl acetate extract. The concentrations were prepared in the range of 25-100 mg/ml and the results are shown in Table 3. It can be seen that the crude extract (100 mg/ml) was most effective when used against *S. lactis*, giving a maximum clear zone of 11.17 ± 0.29 mm. The MIC experiment was then

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performed with various prepared concentrations of the crude extract and the MIC value was expressed as the lowest concentration of the extract that still exhibits antimicrobial activity. The concentrations used in this study were between 15-50 mg/ml and the results are shown in Table 4.

Table 2. Antimicrobial activity of Kwao Krua extracts (100 mg/ml) determined by the disc diffusion method

Microorganisms	C	*P. mirifica*			*B. superba*			*M. macropana*		
		H	E	M	H	E	M	H	E	M
Gram-positive bacteria										
Bacillus cereus TISTR 687	-	-	+	-	-	-	-	-	-	-
B. subtilis TISTR 008	-	-	+	-	-	-	-	-	-	-
Micrococcus luteus TISTR 884	-	-	+	-	-	-	-	-	-	-
Staphylococcus aureus TISTR1466	-	-	+	-	-	-	-	-	-	-
S. epidermidis TISTR 518	-	-	+	-	-	-	-	-	-	-
Streptococcus feacalis TISTR 459	-	-	+	-	-	-	-	-	-	-
Strep. lactis TISTR 457	-	-	+	-	-	-	-	-	-	-
Gram-negative bacteria										
Alcaligenes faecalis TISTR 038	-	-	-	-	-	-	-	-	-	-
Enterobacter aerogenes TISTR 1468	-	-	-	-	-	-	-	-	-	-
Escherichia coli TISTR 780	-	-	+	-	-	-	-	-	-	-
Proteus mirabilis TISTR 100	-	-	+	-	-	-	-	-	-	-
Pseudomonas fluorescens TISTR 358	-	-	+	-	-	-	-	-	-	-
Salmonella typhimurium TISTR 292	-	-	+	-	-	-	-	-	-	-
Serratia marcescens TISTR 1354	-	-	+	-	-	-	-	-	-	-
Yeasts										
Candida albicans TISTR 5239	-	-	-	-	-	-	-	-	-	-
C. utilis TISTR 5001	-	-	-	-	-	-	-	-	-	-
Saccharomyces cerevisiae TISTR 5049	-	-	+	-	-	-	-	-	-	-

Notes: i) C = pure solvent used as control for each extract; H = hexane extract; E = ethyl acetate extract; M = methanol extract

ii) + = presence of inhibition zone; - = no inhibition zone

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Table 3. Antimicrobial activity of *P. mirifica* extract (ethyl acetate fraction) determined by the disc diffusion method. Data shown are mean ± SD (mm) from three separate experiments.

Microorganisms	C	Clear zone diameter (mm)			
		25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml
B. cereus	-	7.00 ± 0.50	7.83 ± 0.29	8.83 ± 0.29	10.50 ± 0.50
B. subtilis	-	-	7.00 ± 0	7.16 ± 0.29	7.83 ± 0.29
E. coli	-	7.16 ± 0.76	7.50 ± 0.50	8.16 ± 0.76	9.67 ± 0.58
M. luteus	-	7.00 ± 0	8.16 ± 0.29	8.83 ± 0.29	10.67 ± 0.76
Pro. mirabilis	-	7.00 ± 0	7.83 ± 0.29	8.33 ± 0.29	10.17 ± 0.29
Ps. fluorescens	-	-	7.33 ± 0.58	8.00 ± 0.50	9.50 ± 0.50
Sac. cerevisiae	-	7.00 ± 0	8.00 ± 0	8.50 ± 0.50	10.17 ± 1.15
Sal. typhimurium	-	-	7.00 ± 0	7.83 ± 0.29	8.67 ± 0.58
Ser. marcescens	-	-	8.00 ± 0	8.00 ± 0	9.33 ± 0.58
S. aureus	-	-	7.83 ± 0.76	8.33 ± 0.76	9.33 ± 0.76
S. epidermidis	-	7.00 ± 0	8.33 ± 0.58	8.67 ± 0.76	10.33 ± 0.58
Strep. feacalis	-	7.00 ± 0	8.00 ± 0	8.17 ± 0.29	9.67 ± 2.02
Strep. lactis	-	7.00 ± 0	8.67 ± 0.76	9.17 ± 0.76	11.17 ± 0.29

Notes: C = control; - = no inhibition zone

Table 4. MIC of *P. miricifa* extract (ethyl acetate fraction) for various tested microbes

Microorganisms	MIC (mg/ml)	Clear zone (mm)
Gram-positive bacteria		
B. cereus	20	7.0
M. luteus	20	7.0
S. aureus	50	7.0
S. lactis	15	7.0
Gram-negative bacteria		
P. fluorescens	50	7.0
P. mirabilis	15	7.0
S. *typhimurium*	45	6.5

It should be noted that the antimicrobial activity of the extract seemed to be more effective on Gram-positive bacteria than the Gram-negative ones. This is probably due to the difference in the cell wall structure of these bacterial groups. Gram-negative bacteria have an outer phospholipid membrane carrying the lipopolysaccharide structure, thus making the cell wall impermeable to lipophilic solutes. Gram-positive bacteria only have an outer peptidoglycan layer, which is not an effective permeability barrier, and thus are more susceptible than Gram-negative bacteria [10].

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There are a few reports of some antimicrobial phytoalexins in the Genus *Pueraria*. These include pterocarpans phytoalexins, namely tuberosin and glycinol, which are found in *P. lobata* [5]. These compounds were synthesised from the basic isoflavones, such as daidzein and genistein, which are also found in *P. mirifica* [11]. Furthermore, Verdrengh et al. [12] reported that genistein can inhibit *B. cereus*, *Helicobacter pylori*, and *S. pasteurianus*, whereas daidzein inhibits the growth of *S. aureus*. In addition, Yadava and Reddy [6] reported the antimicrobial activity of *B. superba* whose stem was used to prepare an extract which was tested for its antimicrobial activity. It was found that this extract was able to inhibit several bacterial and fungal species due to the presence of a novel active compound, chemically called 3,5,7,3',4'-pentahydroxy-8-methoxy-flavonol-3-O-beta-D-xylopyranosyl(1-2)-alpha-L-rhamnopyranoside. However, our study has shown a different result, which could most likely be due to different parts of *B. superba* being used as well as different methods of extraction.

Analysis of bioactive compounds using TLC

The components of the *P. mirifica* extract (ethyl acetate fraction) were separated by TLC and each fraction present under UV was used to test for anti-*B. cereus* activity. Of all the bands appearing on the TLC chromatogram, the ones with R_f values of 0.60 and 0.51 exhibited antimicrobial activity against *B. cereus*. Previous work had suggested the antimicrobial activity of daidzein and genistein against *B. cereus* [12]. According to the TLC analysis, however, our results have indicated that the bioactive compounds of *P. mirifica* extracts are not daidzein and genistein (data not shown). Besides, the standard daidzein and genistein used for antimicrobial purposes did not show any inhibition zone in this study. On the other hand, this might be owing to low amounts of both compounds used. As noted by Mbukwa et al. [13], the isoflavonoid genistein at 100 µg was active against *E. coli* while daidzein at this loading was not.

Antioxidants in Kwao Krua extracts

The presence of antioxidants in Kwao Krua extracts was determined by HPLC, which was performed with daidzin, genistin, daidzein, and genistein being used as chemical standards. The results of this analysis are presented in Table 5. Interestingly, only the *P. mirifica* ethyl acetate extract had the highest amounts of all four compounds among all the Kwao Krua extracts studied.

Generally, plants in the genus *Pueraria* have been known to contain isoflavones, especially in *P. lobata* and *P. thomsonii* [14-16]. In 1999, Zeng [17] reported that the tuber of *P. lobata* collected from Nanchang, Jiangxi, China in February 1995 contained 0.46% daidzin and 0.02% daidzein, while Lian et al. [18] found that the tuber of *P. thomsonii* collected from Pingnan, Guangxi, China in February 1989 contained 0.10% daidzin and 0.02% daidzein. Although *P. lobata* and *P. thomsonii* contain more daidzin than *P. mirifica*, the content of daidzein in *P. mirifica* seems to be higher than in either of those two plant species.

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Table 5. Some antioxidant compounds in Kwao Krua extracts (by HPLC). Data shown are in the unit of % (w/w).

Kwao Krua extracts	Daidzin	Genistin	Daidzein	Genistein
Hexane				
P. mirifica	-	-	0.003	0.054
B. superba	-	-	-	0.055
M. macrocarpa	-	-	-	0.054
Ethyl acetate				
P. mirifica	0.045	0.037	0.049	0.060
B. superba	-	0.010	0.005	0.015
M. macrocarpa	-	-	-	0.016
Methanol				
P. mirifica	0.019	0.011	-	0.054
B. superba	-	0.005	0.001	0.054
M. macrocarpa	-	-	-	0.054

Conclusions

This present study was performed to shed light on whether Kwao Krua plants exhibit any antimicrobial activity on account of the current scarcity of information on the subject. Our results clearly show that of those studied only the *P. mirifica* extract obtained with ethyl acetate exhibits antimicrobial activity against various Gram-positive and Gram-negative bacteria. The same extract is also the one that contains all four antioxidant compounds, viz. daidzin, genistin, daidzein and genistein, and in the highest amounts. Further experimentation on the bioactive compounds in this plant extract is expected to be carried out in the near future for its possible medicinal use.

Acknowledgements

We would like to thank Assoc. Prof. Yuthana Smitasiri for collection of Kwao Krua samples and Mrs. Jantrararuk Towaranonte for the botanical verification of the plants.

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