ANTIMICROBIAL ACTIVITY AND ANTIOXIDANT COMPOSITIONS OF *KWAO KRUA* EXTRACTS

TANATORN SAISAVOEY

MASTER OF SCIENCE
IN BIOTECHNOLOGY

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A THESIS SUBMITTED TO
MAE FAH LUANG UNIVERSITY IN PARTIAL FULFILLMENT OF
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Title Antimicrobial Activity and Antioxidant Compositions of *kwao krua* Extracts

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ABSTRACT

Crude extracts of *Pueraria mirifica* Airy-Shaw et Suvatabandhu, *Butea superba* Roxb. and *Mucuna macrocarpa* Wall. were prepared by sequential extraction using three different solvents: hexane, ethyl acetate and methanol, and used to test for their antimicrobial activity using the disc diffusion method. It was showed that only the *P. mirifica* extracts prepared from ethyl acetate exhibited antimicrobial activities against *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis* and *S. lactis*. The minimum inhibitory concentration (MIC) of this extract was determined and these values were between 15 and 50 mg/ml depending on the microbes tested. The thin layer chromatography (TLC) technique was subsequently introduced to separate chemical compounds of the *P. mirifica* extracts derived from ethyl acetate. When tested against *B. cereus*, there were only two fractions with the R_f values of 0.51 and 0.60 capable of inhibiting the *B. cereus* cells.

In addition, the *kwao krua* extracts were screened for antioxidants using HPLC. The results showed that all *kwao krua* had daidzin, genistin, daidzein and genistein in which the *P. mirifica* extracts obtained from ethyl acetate showed highest amounts of these antioxidant compounds (0.045% of daidzin, 0.037% of genistin, 0.049% of daidzein and 0.060% of genistein).

Keywords: *kwao krua* / *Pueraria mirifica* / *Butea superba* / *Mucuna macrocarpa* / antimicrobial activity / antioxidants

Contents

Contents

List of Tables

List of Figures

List of Abbreviations

A. faecalis	=	*Alcaligenes faecalis*
A. niger	=	*Aspergillus niger*
A. terreus	=	*Aspergillus terreus*
B. cereus	=	*Bacillus cereus*
B. subtilis	=	*Bacillus subtilis*
B. superba	=	*Butea superba*
C. albicans	=	*Candida albicans*
C. utilis	=	*Candida utilis*
*E. aerogene*s	=	*Enterobacter aerogene*s
E. coli	=	*Escherichia coli*
HPLC	=	High performance liquid chromatography
M. luteus	=	*Micrococcus luteus*
M. macrocarpa	=	*Mucuna macrocarpa*
MIC	=	Minimum inhibitory concentration
NB	=	Nutrient broth
ppm	=	Part per million
P. mirabilis	=	*Proteus mirabilis*
P. vulgaris	=	*Proteus vulgaris*
P. aeruginosa	=	*Pseudomonas aeruginosa*
P. fluorescens	=	*Pseudomonas fluorescens*
P. lobata	=	*Pueraria lobata*
P. mirifica	=	*Pueraria mirifica*
R. oligosporus	=	*Rhizopus oligosporus*
R_f	=	Retention factor
S. cerevisiae	=	*Saccharomyces cerevisiae*
S. typhimurium	=	*Salmonella typhimurium*
S. marcescens	=	*Serratia marcescens*
S. aureus	=	*Staphylococcus aureus*
S. epidermidis	=	*Staphylococcus epidermidis*
S. feacalis	=	*Streptococcus feacalis*
S. lactis	=	*Streptococcus lactis*
S. pyogenes	=	*Streptococcus pyogenes*
SD	=	Standard deviation
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
w/w	=	Weight by weight
YM	=	Yeast malt

Chapter I

Introduction

Luang Anusarnsunthorn (1867-1934), the author of the *kwao krua* drug pamphlet (source, http://www.khonmuang.com/luanganusarn.htm)

kwao krua is a common name of medicinal plants that have been widely used by Thai people for a long time. They use these plants as a rejuvenating drug. Generally, the term "*kwao krua*" is used for three different plant species of the Family Leguminosae. These include white *kwao krua* (*Pueraria mirifica* Airy-Shaw & Suvatabandhu), red *kwao krua* (*Butea superba* Roxb.) and black *kwao krua* (*Mucuna macrocarpa* Wall.). In addition, there is also a description of *mor* (grey) *kwao krua* in which its identity remains unclear at present.From the Anusarnsunthorn's pharmacopeia, he suggested that these plants have the rejuvenating properties; these properties found to be highly dependent on the type of *kwao krua* (black > red > white) and recommends to be only taken by the old people (Anusarnsoonthorn, 1931). In addition to the rejuvenating properties, there are several phytoestrogenic compounds including deoxymiroestrol, miroestrol, puerarin, daidzein, genistein, kwakhurin and other isoflavonoids which can be used in medical applications due to the female-like hormones; their estrogenic activity have been applied to commercial products for breast enlargement and body firming.

Plants synthesise various kinds of chemical compounds that are used for their development, reproduction, defense against attack by many different kinds of organisms and survival in often harsh and ever-changing environments. Humans learn to use these plant natural products for their welfare such as foods, metabolites, chemicals and medicine. Especially in medicine, plants are the important source of many medicines in the pharmacopoeia. In Thailand, there are many medicinal plant products; some Thai medicinal plants that are of great importance are discussed below.

1.1 Overview of Thai medicinal plants and their use

"Every kind of plants can be used in medical treatment" quoted by Shivokkomanpaj (Chumsri, 2001) this sentence remains a fact for all plants, at least due to the availability of chlorophyllin, a water-soluble derivative of chlorophyll, which is currently showed as antimutagenic and anticarcinogenic properties (Negishi *et al*., 1997; Dashwood *et al*.,1998). Since ancient period, human have used plants for disease treatment. They use plants for several purposes such as medicine, cosmetic, spice, food and supplementary food. Especially medicine, the history shows that people of all countries have knowledge about medicinal plants and most medicines are derived from plants. In Thailand, several medicinal plants are well-known for their medical benefit and have used for disease treatments (Table 1.1).

Table 1.1 Benefits of Thai medicinal plants (modified from Tungtrongjit, 1978).

Common name	**Scientific name**	**Part(s) used**	**Use(s)**
bua bok (Asiatic pennywort)	*Centella asiatica*	Leaf	Antidote, antipyretic
kaa ra boon (Camphor tree)	*Cinnamomum camphora*	Stem	Aroma, embrocation
kee lek (Cassod tree)	*Senna siamea*	Leaf, flower	Purgative
krit sa na (Eagle wood)	*Aquilaria crassna*	Stem	Cardiotonic
ma kluea (Ebony tree)	*Diospyros mollis*	Fruit	Parasiticide
yor (Indian mulberry)	*Morinda citrifolia*	Fruit	Emmenagogue
kan ni ka (Night jasmine)	*Nyctanthes arbor-tristis*	Leaf, root	Appetiser, analeptic
ma la gor (Papaya)	*Carica papaya*	Root	Diuretic
kwao krua (Kwao krua)	*Pueraria mirifica*	Root	Panacea
fang (Sappan)	*Caesalpinia sappan*	Stem	Haematinic
pet sang kart (Veld grape)	*Cissus quadrangularis*	Bark	Hemorrhoid treating
ma khaam (Tamarind)	*Tamarindus indica*	Fruit	Cough-remedy

kwao krua is one of the most interesting Thai medicinal plants due to the rejuvenating properties. At the beginning, the research of *kwao krua* was focused on *P. mirifica* in which their chemical compounds were determined. Kerr (1932) and Wanandorn (1933) discover one or more estrogenic compounds from *P. mirifica*, know as phytoestrogens, this experiment was confirmed by Vatna (1939) for some year later, the mice used for *kwao krua* extract injection to observe the estrus activity and the results were showed that the treated mice were found to be in full estrus whereas the controls were unaffected. At present, the scientists find various compounds in the tuber of *P. mirifica* (see Chapter 1.4). From these compounds, genistein, genistin, daidzein, daidzin and coumestrol are a weak phytoestrogen (Ingham *et al*., 1989). Miroestrol demonstrated as a strong phytoestrogen and major activated compound (Bounds and Pope, 1960; Jones and Pope, 1961). In addition, the using of two other *kwao krua*, red *kwao krua* and black *kwao krua* have used for sexual performance maintaining and preventive erectile dysfunction (Cherdshewasart *et al*., 2004). About the researches of *B. superba*, Roengsumran *et al*. (2000) were found that this plant contained rich flavonoids and flavonoid glycosides. Cherdshewasart and Nimsakul (2003) were proved in a clinical test that this plant can use as an erectile dysfunctional treatment. Tocharus *et al*. (2006) were reported that *B. superba* tuber extract can induce penile erection in rats by increasing the inviacavernosal pressure and this mechanism related with c-AMP and c-GMP. However, there are many researches of *P. mirifica* and *B. superba* but the researches about *M. macrocarpa* are limited.

1.2 General characteristics of *kwao krua* plants

kwao krua plants are identified in Family Leguminosae, this family is the second largest Family of seed plants, pea family have around 18,000 species classified into 650 genera of dicotyledonous trees, shrubs, climbers, and herbaceous plants (Polhill and Raven, 1981). People use these plants as crops, forages and green manures. They synthesise a wide range of natural products such as flavors, drugs, poisons and dyes. This family is the major plant food resource next to Graminiae (cereals). They are rich in high quality protein, providing man with a highly nutritional food resource such as mung bean (*Vigna* sp.), soy (*Glycine max*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*) and chick pea (*Cicer arietinum*).

The Leguminosae Family is characterised by the fruit, technically known as a legume, which develops from a single carpel, and when ripe opens along both margins to release the seeds; the seeds are thrown out violently by sudden contraction of the sides of the legume or pod. In some cases the fruit breaks transversely, as in horseshoe vetch, or does not open at all, as in clover. The leaves of the Leguminosae bear stipules (outgrowths at the base of the leafstalk) and are often compound, usually pinnately compound, sometimes with tendrils at the leaf tips. The unique feature of this family is the presence of root nodules containing bacteria of the genus *Rhizobium*, which have a symbiotic relationship with Legumes and fix atmospheric nitrogen, converting it into nitrogenous compounds useful to plants. The Family Leguminosae is divided into three subfamilies such as Papilionoideae, Caesalpinioideae and

Mimosoideae (Duke, 1981). *kwao krua* plants are identified in subfamily Papilionoideae, the detailed information of these plants is described below.

1.2.1 White *kwao krua* (*Pueraria mirifica* Airy-Shaw & Suvatabandhu)

White *kwao krua*, the woody perennial climber, grows in deciduous forests at altitudes between 250 to 800m, in Northern, Western and Northwestern of Thailand. The morphological characteristics are triforiate leaves, inflorescence of small white-purple flowers (Figure 1.1), three brown seeds inside seedpod and round-shape tuber (Figure1.2) contained white liquid inside (Keung, 2002; Sang-Arun *et al*., 2001).

Kingdom Plantae
Division Magnoliophyta
Class Magnoliopsida
Order Fabales
Family Fabaceae (Leguminosae)
Subfamily Faboideae (Papilionoideae)

Genus *Pueraria*	**Genus** *Butea*	**Genus** *Mucuna*
Species *P. mirifica*	**Species** *B. superba*	**Species** *M. macrocarpa*

Figure 1.1 Taxonomy of *kwao krua* plants.

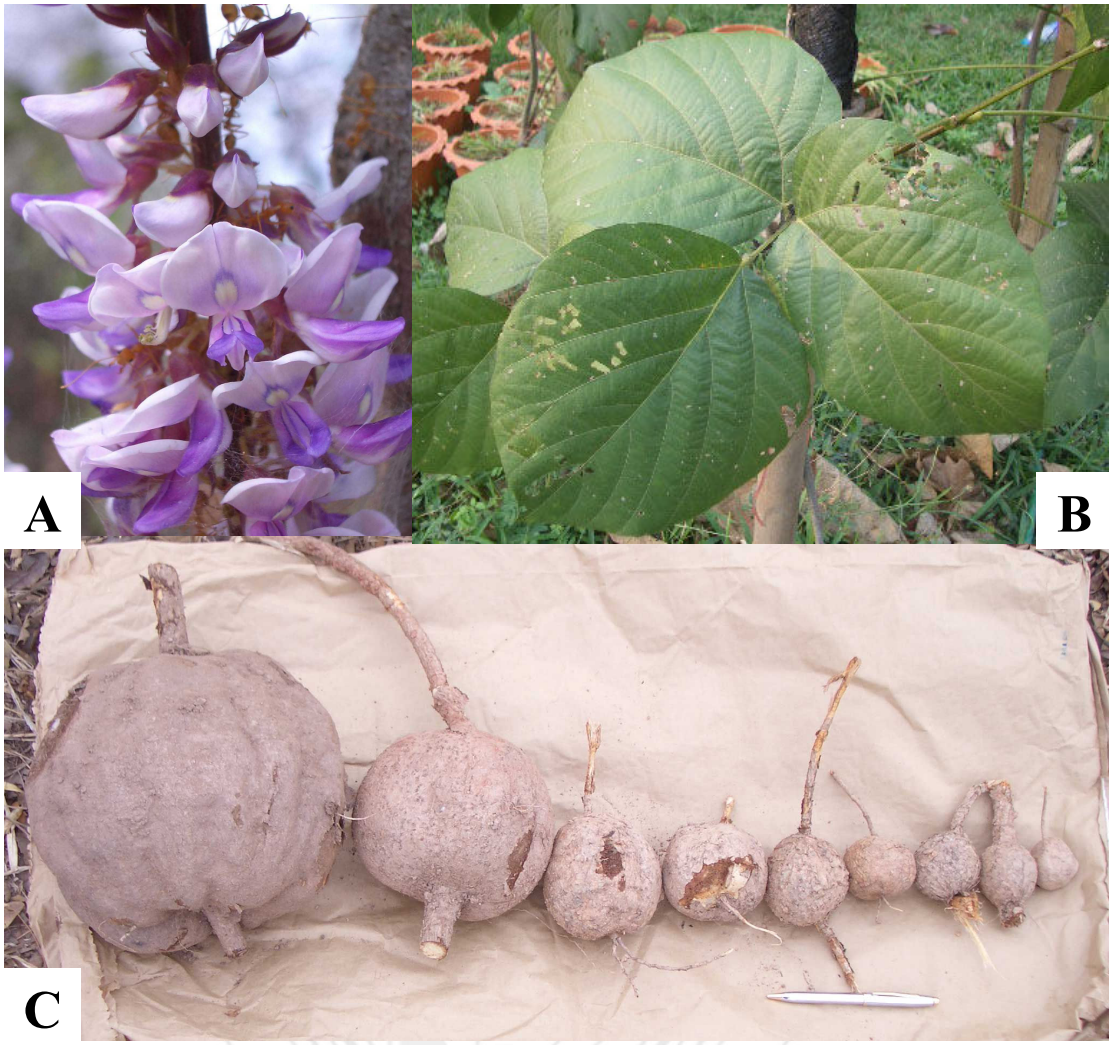

Figure 1.2 Morphological characteristic of *P. mirifica*: A) flowers, B) leaves and C) tubers.

P. candollei (synonym: *P. candollei* var. *candollei*), another woody climber, is similar to *P. mirifica* (synonym: *P. candollei* var. *mirifica*). Both varieties are deciduous and many morphological characteristics are resembled. The only major differences between these two varieties are the size of the corolla and calyx (smaller in var. *mirifica* when compared with var. *candollei*) and the length of the inflorescence (shorter in var. *mirifica*) (Niyomdham, 1992). Therefore, before the plants are collected, we should verify these certain characteristics.

1.2.2 Red *kwao krua* (*Butea superba* Roxb.)

Red *kwao krua* is present in Northern and Northwestern Thailand. The morphological characteristics are huge woody climber (diameter of stem around 15cm), petiole and peduncle have silky pubescent present, large trifoliate leaves, perfect orange flower (Figure 1.3) and fine-shape tuber (Figure 1.4) contained red liquid inside (Sang-Arun *et al*., 2001).

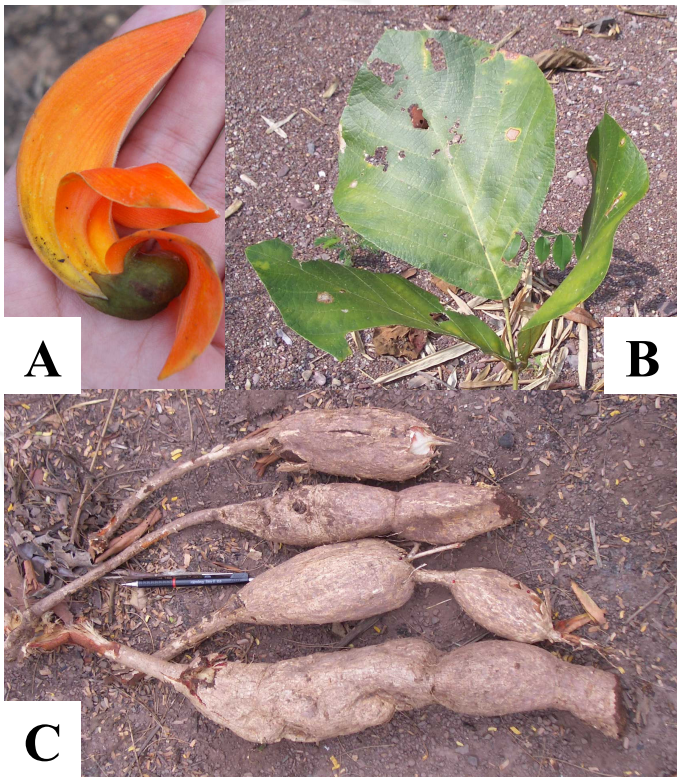

Figure 1.3 Morphological characteristic of *B. superba*: A) flowers, B) leaves and C) tubers.

1.2.3 Black *kwao krua* (*Mucuna macrocarpa* Wall.)

The morphological characteristics of black *kwao krua* are climber, triforiate leaves, purple flowers (Figure 1.5) and stem-like tuber (Figure 1.6) contained black liquid inside (Sang-Arun *et al*., 2001).

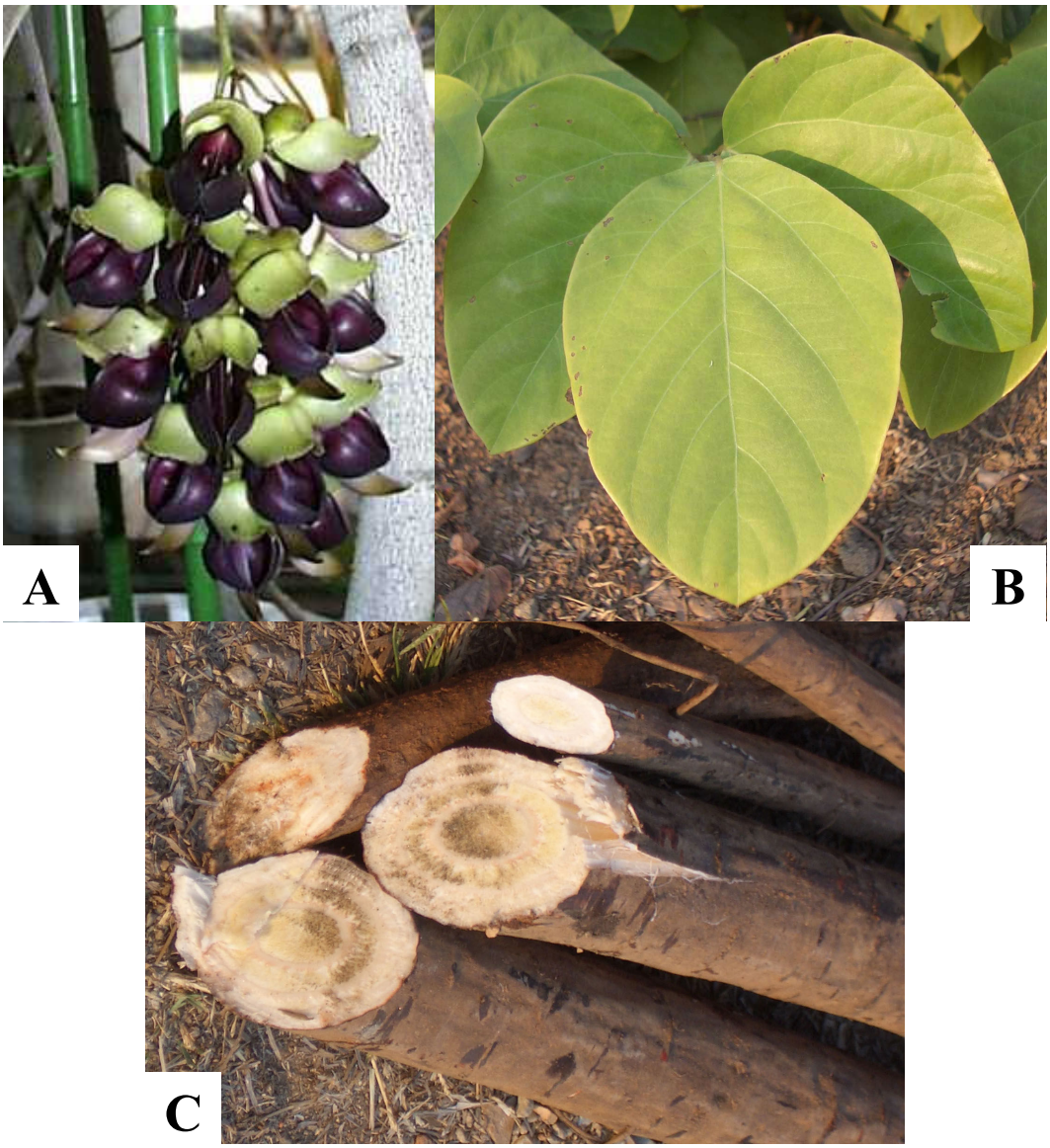

Figure 1.4 Morphological characteristic of *M. macrocarpa*: A) flowers, B) leaves and C) tubers**.**

All *kwao krua* plants have similar morphological parts such as climber, trifoliate leaves, and tuber, but in different size and shape. Therefore, we can identify *kwao krua* from these characteristics and the colours of liquid inside the tuber.

1.3 Biological activity of *kwao krua* phytochemicals

Plant bioproducts are the bioactive compounds from plant known as phytochemicals, which have main structure of carbon, hydrogen and oxygen. It is well-known that most plants have immoveable therefore plant produces these chemicals to protect itself, afterwards recent researches demonstrated that phytochemicals can protect humans against diseases by working in many functions such as antioxidants, hormonal action, stimulation of enzyme, interference with DNA replication and antimicrobial activity. Most of the phytochemicals are secondary metabolites and can be divided into various groups (i.e., alkaroids, phenols, saponins, polysaccharides and vitamins). *kwao krua* also contained phytochemicals and these are presented below.

As shown in Table 1.2 and 1.3, various kinds of phytochemicals can be found in white and red *kwao krua*. However, there is little information relevant to those of black *kwao krua*. Some phytochemicals reported to date include L-dopa, friedelin, hexacosanoic acid 2,3-dihydroxy-propyl ester, lupenone and pentacosanoic acid 2,3-dihydroxy-propyl ester (Hu *et al*., 1994).

Table 1.2 Chemical constituents in the tuber of *P. mirifica* (Ingham *et al*., 1986; Kashemsanta *et al*., 1963; Nilanidhi *et al*., 1963).

Chemicals	Activity
Chromene	
Miroestrol	Estrogen
Deoxymiroestrol	Estrogen
Isoflavones	
Daidzein	Antioxidant, estrogen
Genistein	Antioxidant, estrogen
Kwakhurin	Estrogen
Kwakhurin hydrate	NA
Isoflavone glycoside	
Daidzin	NA
Genistin	NA
Mirificin	NA
Puerarin	NA
Puerarin-6'-monoacetate	NA
Coumestans	
Coumestrol	Estrogen
Mirificoumestan	NA
Mirificoumestan glycol	NA
Mirificoumestan hydrate	NA

Note: NA = not available

Table 1.3 Chemical constituents in the tuber of *B. superba* (Ruksilp, 1995).

Organic acid chain group
Dodecosanoic acid
Tricosanoic acid
Tetracosanoic acid
Pentacosanoic acid
Hexacosanoic acid
Steroid group
Stigmasterol
Campesterol
β-sitosterol
Steroid glycoside group
β-sitosteryl-3-0-β-D-glucopyranoside
Stigmasteryl-3-0-β-D-glucopyranoside
Flavonoid group
3,7,5'-trihydroxy-4'-methoxy flavone
4'-methoxyfisetin
2-(5-hydroxy-4-methoxyphenyl)-3,7-dihydroxy-4H-1-benzopyran-4-one
Flavonoid glycoside group
4'-methoxyfisetin-7-0-β-D-glucopyranoside
3,5'-dihydroxy-4'-methoxyflavone-7-0-β-D- glucopyranoside
2-(5-hydroxy-4-methoxyphenyl)-3-hydroxy-4H-1-benzopyran-4-one-7-0-β-D- glucopyranoside

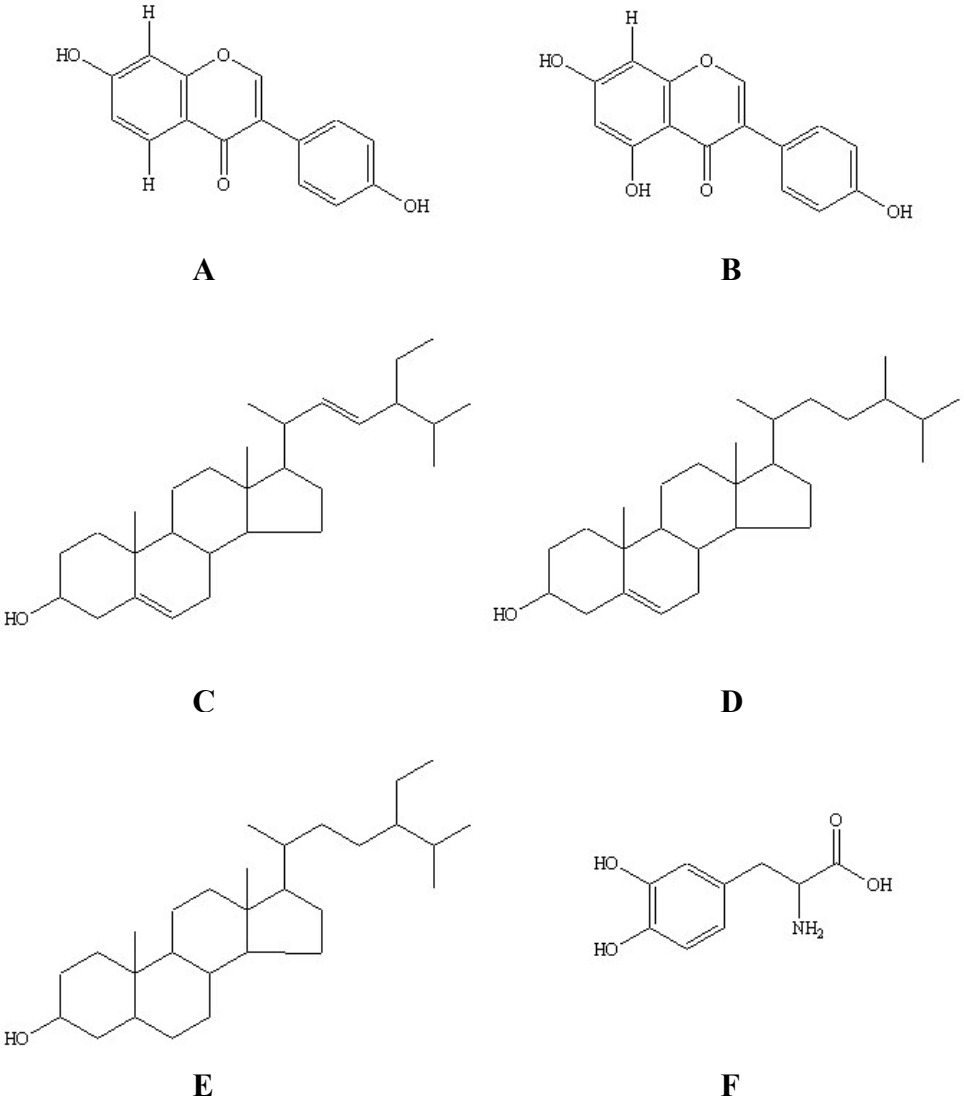

Figure 1.5 Representatives of phytochemicals present in *kwao krua*. A) daidzein and B) genistein in *P. mirifica*; C) Stigmasterol, D) Campesterol and E) β-sitosterol in *B. superba*; F) L-dopa in *M. macrocarpa*.

1.3.1 Antioxidant activity of *kwao krua* phytochemicals

From the chemical reaction in every cell of microorganism, they always produce the free radicals (the unstable molecules that have singlet or unpaired electron), these substances will attack and damage the other molecules by oxidation. However, they also generate the antioxidative agent (antioxidant or free radical scavenger) to keep the equilibrium. The oxidative stress due to high amounts of free radicals can cause aging (Barja, 2004), cancer (Yazdanpanah *et al*., 1997) and diabetes (Oberley, 1988). The compensations with natural antioxidants from the external sources are the way to reduce the balance of this relation. Besides, the natural antioxidants substances are supposed to be safe more than the synthetic antioxidant chemicals. In nature, antioxidants can be found in plants such as vegetable, fruit, cereal, herb or other natural products, these contain several compounds with potential antioxidant activity include vitamin, carotenoids and phenolic compounds (Hunter and Fletcher, 2002). Some antioxidants as well as their sources are shown in Table 1.4.

Table 1.4 Antioxidant sources from plants (Smythies, 1998).

Antioxidants	**Sources**
Beta - carotene	Apricots, carrots, peaches, pumpkin, red pepper
Lutein and zeaxanthin	Kale, broccoli, spinach, celery, leeks, parsley, peas
Lycopene	Tomatoes, mango, oranges, papaya, watermelon
Vitamin C	Citrus fruits, green pepper, kiwi, strawberries
Vitamin E	Avocado, peanuts, sweet potatoes
Lipoic acid	Spinach, broccoli
Flavonoids	Apple, licorice, onion, soybeans, blueberries, tea
Polyphenols	Coffee, grapes, tea, turmeric

kwao krua are also the source of antioxidants with the rich of flavonoids. Daidzein and genistein from soybean have many researches to confirm there antioxidant activity such as inhibit free radical formation, reduce lipid oxidation and stimulate antioxidant enzymes (Davis *et al*., 2001). These compounds found in both white and red *kwao krua* (Ingham *et al*., 1989; Manosroi and Manosroi, 2005) but in black *kwao krua*, there researches are limited. However, Sang-Arun *et al*. (2001) compare the antioxidant activity of *kwao krua* and the results showed that the aqueous extracts of black *kwao krua* are the highest activity among three plants.

1.3.2 Antimicrobial activity of *kwao krua* phytochemicals

In many cases, phytochemicals serve as plant defense mechanisms against aggression by microorganisms, insects, and herbivores, then human use these substances as a medicine, some antimicrobial agents from plants use as antibiotics. In Thailand, there are many plants which have the antimicrobial properties (Table 1.5).

kwao krua are also Thai medicinal plants that have been popularly used for a long time. As described, the researches about *kwao krua* are most focus on phytoestrogenic properties, but the reports of antimicrobial activity are limited. The *kwao krua* reports of antimicrobial activity are concluded below.

Table 1.5 Thai medicinal plants containing antimicrobial activity.

Common name	Scientific name	Compound	Class	Activity	References
van hang ja ra kae (Aloe)	*Aloe vera*	Latex	Complex mixture	*Staphylococcus aureus*	Martinez *et al*. (1996)
bai gra van (Bay)	*Laurus nobilis*	Essential oils	Terpenoids	Bacteria, fungi	Fang *et al*. (2005)
phrik thai (Pepper)	*Piper nigrum*	Piperine	Alkaloid	Bacteria, fungi	Ghoshal *et al*. (1996)
ma muang him ma pan (Cashew)	*Anacardium occidentale*	Salicylic acids	Polyphenols	Bacteria, fungi	Himejima and Kubo (1991)
phrik (Chilli pepper)	*Capsicum annuum*	Capsaicin	Terpenoids	Bacteria	Chichewicz and Thorpe (1996)
goat nam tau (Chinese rhubarb)	*Rheum officinale*	Rhein	Anthraquinone	*S. aureus*	Yanwen *et al*. (2005)
gra thiiam (Galic)	*Allium sativum*	Allicin	Sulfoxide	General	Ankri and Mirelman (1999)
cha em (Licorice)	*Glycyrrhiza glabra*	Glabridin, glabrene	Flavonoids	Bacteria	Fukai *et al*. (2002)
ma la gor (Papaya)	*Carica papaya*	Latex	Mix of terpenoids, organic adcids, alkaloids	General	Osato *et al*. (1993)
ka min (Turmeric)	*Curcuma longa*	Curcumin, turmeric	Terpenoids	Fungi	Apilsarilyakul *et al*. (1995)

Notes: i) General means activity against multiple types of microorganisms (i.e., bacteria, fungi and protozoa).
ii) Bacteria refer to activity against Gram-positive and Gram-negative bacteria.

Most investigations of *P. mirifica* are relevant to estrogenic activity and antioxidant activity but the researches in antimicrobial activity of this plant are limited. However, there is the report about antimicrobial activity of genus *Pueraria*. Hakamatsuka *et al*. (1994) presented that *P. lobata* produces pterocarpan phytoalexins, namely tuberosin and glycinol against microbial attacks. Besides in Family Leguminosae, Verdrengh *et al*. (2004) discovered that the isoflavones, genistein and daidzein containing in soybean (*Glycine max*) exhibited an inhibitory effect against bacteria (i.e., *Bacillus cereus* and *Staphylococcus aureus*). The research of *B. superba* about antimicrobial activity is presented by Yadava and Reddy (1998). They discover a new bioactive flavonol glyside which isolate from the stem of *B. superba* and determine structure by spectral analysis and chemical degradation as 3,5,7,3',4'-pentahydroxy - 8 - methoxy - flavonol – 3 – O – beta – D – xylopyranosyl (1-->2) –

alpha – L - rhamnopyranoside. This compound shows antimicrobial activity against plant pathogenic fungi, Gram-positive and Gram-negative bacteria. The investigations about antimicrobial activity in *M. macrocarpa* are also limited.

1.3.3 Other uses of *kwao krua* phytochemicals

Estrogenic activities are the most interest in *kwao krua* plants. Especially in white *kwao krua*, the phytoestrogens in *P. mirifica* consist of deoxymiroestrol, miroestrol, daidzein, genistein, coumestrol and kwakhurin. The test for estrogenic activity of these compounds was carried out by the proliferation of MCF-7 human breast cancer cells. Strong estrogenic activity observes in deoxymiroestrol and miroestrol. Coumestrol and genistein shows moderate estrogenic activity. Daidzein and kwakhurin presents weak estrogenic activity (Chansakaow, 2000). In addition, *P. mirifica* extracts were tested on many animals such as rats (Smitasiri *et al*., 1986), coturnix quails (Anuntalabhochai and Jesrichai, 1986) and rabbits (Chularojmontri *et al*., 2003) for estrogenic potency. Manosroi and manosroi (2005) were presented that red *kwao krua* contained puerarin, daidzein and genistein which also found in white *kwao krua*, this thing may answer that why these plants have the similar benefits. Besides, *B. superba* found to contain the cAMP phosphodiesterase inhibitor that use for erectile dysfunction treatment as well as sterol compounds, including β-sitosterol, campesterol and stigmasterol (Cherdshewasart and Nimsakul, 2003). Finally, in black *kwao krua*, the specific researches of *M. macrocarpa* are limited but this plant always used to compared with the other two *kwao krua* and the results were often showed the higher than among *kwao krua* plants.

1.4 Aims of this study

This study was undertaken in order to:

i) study the antimicrobial activity of *kwao krua* extracts

ii) characterise the distribution of antioxidants of *kwao krua* extracts

Chapter II

Materials and Methods

The flower of *Pueraria mirifica* Airy-shaw & Suvatabundhu

2.1 Chemicals

Unless otherwise indicated, general chemicals were obtained from the following suppliers; those for extraction and HPLC test from Merck & Co., INC. (Germany) and J.T.Baker SOLUSORB® (USA); those used to prepare media from Difco Laboratories (USA) and Himedia Laboratories (India). Chemical standards for antioxidants were purchased from Sigma-Aldrich Co. (USA).

2.2 Plant materials

Tubers of white *kwao krua* (*P. mirifica*) and red *kwao krua* (*B. superba*) were collected from Chiang Muan, Phayao in February 2004; for black *kwao krua* (*M. macrocarpa*), it was collected from Doi Tung, Chiang Rai in February 2004. The specimens were then authenticated by Mrs. Jantrararuk Towaranonte, a lecturer of the School of Science, Mae Fah Luang University and kept as voucher specimens (*P. mirifica* No. MFLU-307, *B. superba* No. MFLU-310, *M. macrocarpa* No. MFLU-311).

2.3 Preparation of *kwao krua* extracts

In this study, sequential extraction was performed according to the protocol of Canales *et al*. (2005) with details as follows.

In general, tuber samples were chopped into small pieces and dried in the hot air oven (Modell 500, Memmert, Germany) at 60°C for 3 days. Dried tubers were ground into powder using the blender (HR 1721, Philips). The powder was then kept in a closed plastic bag and stored in the desiccator (Model 1344, Boekel) at room temperature (approximately 28 - 30°C) until use.

The powders of *kwao krua* sample (0.5kg) were placed in the stainless steel coolers, initially extracted with 3L of hexane for 3 days and filtrated. The remained *kwao krua* samples were subsequently extracted with 3L of ethyl acetate for 3 days, and filtrated. Finally, the remained *kwao krua* samples were extracted with 3L of methanol for 3 days and filtrated. Each extracted solution was concentrated under reduced pressure at 45ºC using the vacuum rotary evaporator (B-480, Buchi, Switzerland), frozen and dried using the freeze dryer (Flexi-Dry^{TM}, FTS Systems, USA) until constant weights were obtained. The overall procedure of the sequential extraction was shown in Figure 2.1.

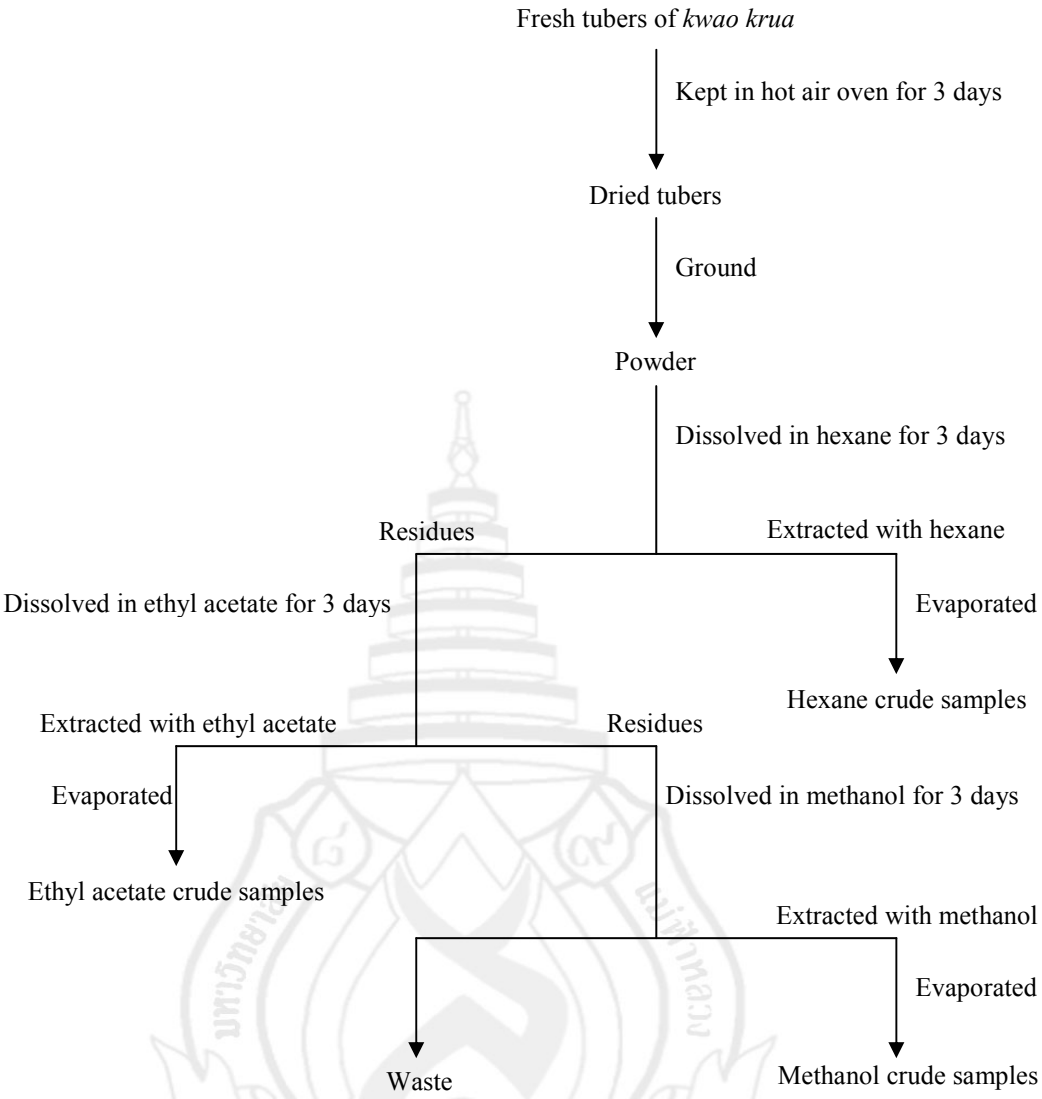

Figure 2.1 Procedure of sequential extraction of *kwao krua*.

2.4 Antimicrobial assay

In this study, *kwao krua* samples were tested for their antimicrobial activity. Typically, the paper disc diffusion was selected to serve this purpose based on the protocol of Arias *et al*. (2004).

2.4.1 Microorganisms used

All microbes used were purchased from Microbiological Resources Center, Thailand Institute of Scientific and Technological Research, and kept as stock culture at Microbiology Laboratory, Mae Fah Luang University (see Table 2.1). For routine culture and maintenance, bacteria were grown on nutrient agar or in broth (NB) at 37°C. Yeasts were grown on yeast malt agar (YM agar) or in broth (YM broth) at 30°C. For long term storage, all microbes were kept either in the slant culture at 4°C or in the glycerol stock at -20°C (20% glycerol for bacteria; 40% glycerol for yeasts).

Table 2.1 Lists of microorganisms used in this study.

Bacteria
Alcaligenes faecalis TISTR 038
Bacillus cereus TISTR 687
Bacillus subtilis TISTR 008
*Enterobacter aerogene*s TISTR 1468
Escherichia coli TISTR 780
Micrococcus luteus TISTR 884
Proteus mirabilis TISTR 100
Pseudomonas fluorescens TISTR 358
Salmonella typhimurium TISTR 292
Serratia marcescens TISTR 1354
Staphylococcus aureus TISTR 1466
Staphylococcus epidermidis TISTR 518
Streptococcus feacalis TISTR 459
Streptococcus lactis TISTR 457
Fungi
Candida albicans TISTR 5239
Candida utilis TISTR 5001
Saccharomyces cerevisiae TISTR 5049

2.4.2 Paper disc diffusion method

The microbial stock samples were initially transferred to 100ml of nutrient broth or YM broth and incubated at 37°C (for bacteria) or 30°C (for yeast) for 20 - 24h in the shaking incubator (180rpm, OM11, Ratek). The cell number was determined using haemocytometer (for detail and calculation, see Appendix I). Typically, the microbial cells used for the assay were between 10^6 - 10^8 cells.

Sterile filter paper (Diameter 6 mm, No. 3, Whatman, UK) was dipped in various concentrations of *kwao krua* extracts (100, 75, 50, 25mg/ml and control), then placed on the agar plates, containing tested microorganisms and kept in the incubator (BED, WTB binder) at 37°C (for bacteria) or 30°C (for yeast) for 24h. This was performed in triplicate for each *kwao krua* extracts. The diameter of clear zone shown on plates was then measured and expressed in millimeters as its antimicrobial activity. Hexane, ethyl acetate and methanol were also used as testing markers for *kwao krua* extracts derived from these solvents performed undertaken tested.

2.5 High Performance Liquid Chromatography (HPLC) analysis of *kwao krua* extracts

2.5.1 Preparation of *kwao krua* extracts

Based on the procedure of Klump (2001), 100mg of each *kwao krua* extract was dissolved in 40ml of 80% methanol; the suspension was then placed in the ultrasonic washer (Transsonic 700, Elma) for 5 min. Then, 3ml of 2M sodium

hydroxide was added and the mixture was further incubated for 1 min. Subsequently, 1ml of acetic acid was added and the solution was further incubated for 1 min. The supernatant of the prepared samples were finally filtered using the syringe filter (0.45µm, Chorm Tech., USA) and thus ready for HPLC analysis.

2.5.2 HPLC analysis

In this study, the HPLC method was used to elucidate the chemical constituents present in the *kwao krua* extracts. The conditions used were shown in Table 2.2.

Table 2.2 Descriptions of the HPLC conditions used to analyse *kwao krua* samples.

HPLC system	: Water 2695 (Waters, USA)
Column	: Reverse-phase C-18, 5µm (Waters, USA)
Mobile phase	: Mobile phase A, H_2O:MeOH:Aetic acid (88:10:2)
	Mobile phase B, MeOH:Acetic acid (98:2)
Injection volume	: 20µl
Flow rate	: 0 - 0.1min (90% Mobile phase A:10% Mobile phase B)
	0.1 - 30min (40% Mobile phase A:60% Mobile phase B)
	31 - 31.5min (100% Mobile phase B)
	37 - 37.5min (90% Mobile phase A:10% Mobile phase B)
Detection	: UV 260nm
Standard antioxidants	: Daidzin (0.08, 0.40 and 2.00ppm)
	Genistin (0.08, 0.40 and 2.00ppm)
	Daidzein (0.16, 0.80 and 4.00ppm)
	Genistein (0.40, 2.00 and 10.00ppm)

2.6 Analysis of *P. mirifica* extracts derived from ethyl acetate

After preliminary screening (section 2.4), only the *P. mirifica* extracts obtained from ethyl acetate were active against the microorganisms tested. Therefore, the ethyl acetate extract of *P. mirifica* was further studied as follows:

2.6.1 Determination of Minimum Inhibitory Concentration (MIC)

The *P. mirifica* extracts derived from ethyl acetate were prepared into various concentrations such as 50, 45, 40, 35, 30, 25, 20, 15, 10 and 5mg/ml. The determination of MIC was tested for the lowest concentration of plant extracts to inhibit the growth of microorganisms. The MIC values were determined using disc diffusion method. 15µl of each diluted extract solution was added to sterile filter paper using micropipette and then placed on the agar plate, containing tested microorganisms and kept in the incubator at 37°C (for bacteria) or 30°C (for yeast) for 24h. This was performed in triplicate for each microorganism. The appearance of clear zone at the lowest concentration was confirmed as MIC value.

2.6.2 Determination of antimicrobial compounds using Thin Layer Chromatography (TLC)

The TLC technique was introduced to separate the chemical compounds present in *P. mirifica* extracts derived from ethyl acetate. For this, the TLC plate (Siliga gel aluminium plate GF_{254}, J.T.Baker, USA) was cut into the size of $4x4cm^2$. The *P. mirifica* extracts and the standards (1mg/ml of daidzein and 1mg/ml of genistein) were then applied into the plate. Daidzein and genistein were selected

due to their antimicrobial activity previously reported (Verdrengh, 2004). The applied TLC plate was then placed in the chamber saturated with hexane and ethyl acetate (6:4) for 1min or until the mobile phase reached the marking line. The developed chromatogram was finally observed under the UV light at 254nm.

Further experiment was also performed to investigate which fraction had antimicrobial activity. For this, the *P. mirifica* sample (100mg/ml) was applied on the TLC plate (20x20cm^2). The applied TLC plate was then put into the chamber saturated with hexane and ethyl acetate (6:4) for 1h or until the mobile phase reached the marking line. The developed chromatogram was observed under UV light at 254nm. Each fraction was marked, scraped and dissolved with 1ml of ethyl acetate. These solutions were then tested with *B. cereus* using the disc diffusion method as described in the section 2.4.2.

Chapter III

Results and Discussion

The illustration of *Butea superba* Roxb.
(source, http://www.illustratedgarden.org)

3.1 *kwao krua* extracts

During February 2004, *P. mirifica* and *B. superba* were collected from Chiang Muan, Phayao and *M. macrocarpa* was collected from Doi Tung, Chiang Rai. Botanical descriptions were verified and kept as reference in the University herbarium. The distinct morphological characteristics of these *kwao krua* plants are as follows: climber, trifoliate leaves and tuber (see further detail in Chapter 1). The photos of tubers collected are also shown in Figure 3.1.

Figure 3.1 The tubers of *kwao krua*. Left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*.

The flesh inside of *kwao krua* tubers was also distinct showing different colours: the so-called "white" *kwao krua* (*P. mirifica*), "red" *kwao krua* (*B. superba*) and "black" *kwao krua* (*M. macrocarpa*) (Figure 3.2).

Figure 3.2 The cross section of *kwao krua* tubers. Left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*.

kwao krua tubers were then prepared in powder form (Figure 3.3) and used to prepare crude extracts using three different solvents: hexane, ethyl acetate and methanol (see Section 2.3). After evaporation and freeze-dry, the crude extracts were obtained in either thickening solution or solid form (Figure 3.4). Percent yields of each extracts were calculated and shown in Table 3.1; these samples were dissolved in appropriate solvent and subsequently tested for their antimicrobial activity.

Figure 3.3 The powders of *kwao krua*. Left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*.

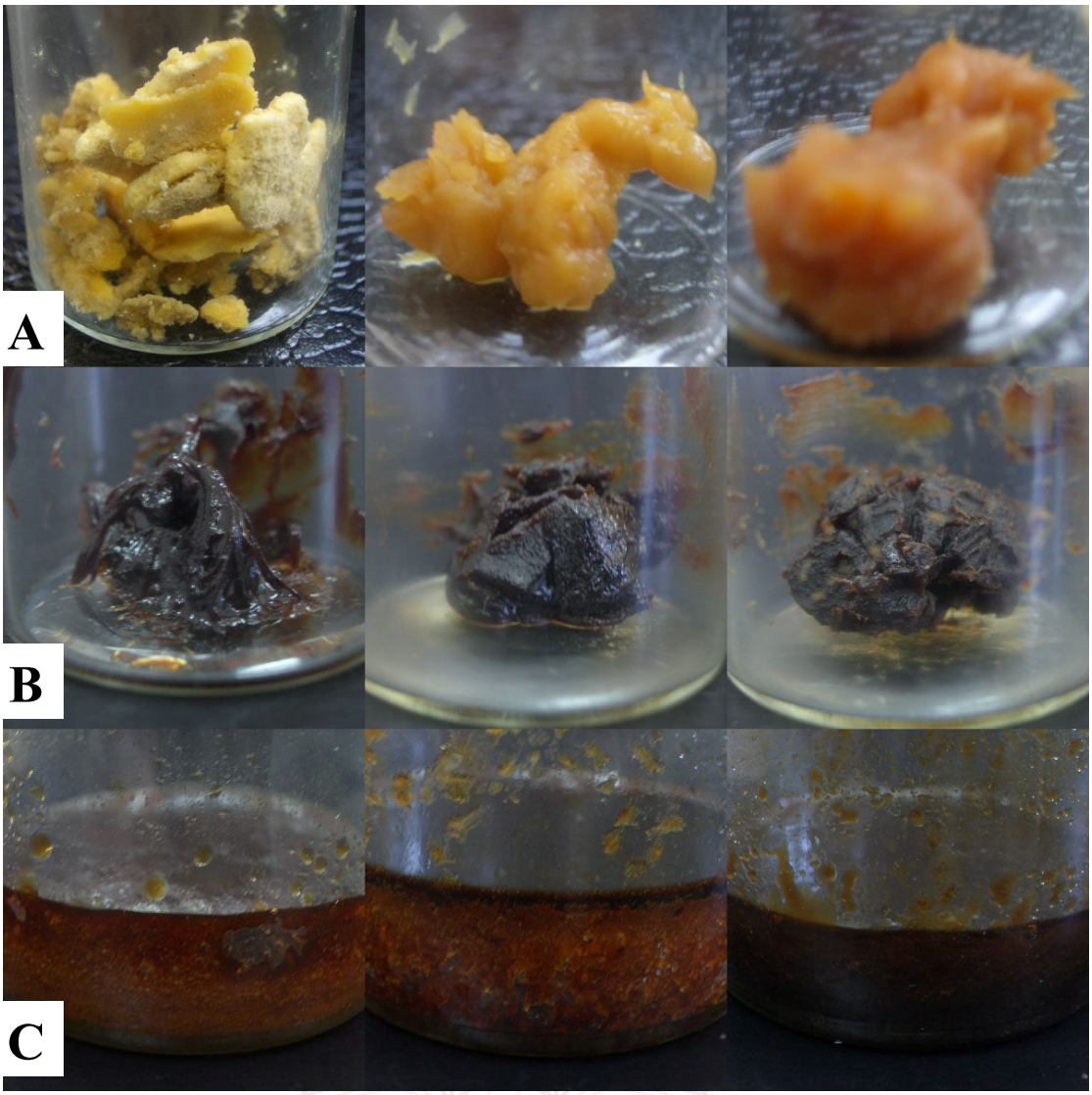

Figure 3.4 *kwao krua* extracts (A, hexane; B, ethyl acetate; C, methanol) derived from sequential extraction. Left, *P. mirifica*; middle, *B. superba*; right, *M. macrocarpa*.

Table 3.1 Physical appearances and percent yields of *kwao krua* extracts.

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

The crude extracts derived from each solvent were similar based on their appearance and colour (Table 3.1). The percent yields of *kwao krua* using sequential extraction were found to be highly dependent on the type of solvents (methanol > ethyl acetate > hexane). As shown in Table 3.1, the percent yield of the methanol *kwao krua* extracts were highest among the three solvents used.

3.2 Antimicrobial activity of *kwao krua* extracts

All *kwao krua* extracts (100mg/ml) were preliminary tested against seventeen species of microorganisms using the disc diffusion method. Some species of microorganisms used are food poisoning (i.e., *B. cereus*, *E. coli* and *S. aureus*) and opportunistic pathogens (i.e., *C. albicans*). The results of this antimicrobial activity test were presented in Table 3.2 and Table 3.3.

As shown in Table 3.2, only the *P. mirifica* extracts obtained from ethyl acetate exhibited antimicrobial activity. The extracts were active against various kinds of Gram-positive and Gram-negative bacteria. For yeasts, the extracts were able to inhibit *S. cerevisiae* but not for the *Candida* species. It should be interesting to note that the *P. mirifica* extracts were capable of inhibiting all the Gram-positive bacteria used in this study. However, for Gram-negative bacteria, the extracts could not inhibit *A. faecalis* and *E. aerogenes*. The control results were pronounced no antimicrobial effect.

Table 3.2 Antimicrobial activity of *kwao krua* extracts determined by disc diffusion method.

Microorganisms	**C**	***P. mirifica***			***B. superba***			***M. macrocarpa***		
		H	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**
Gram-positive bacteria										
B. cereus	-	-	+	-	-	-	-	-	-	-
B. subtilis	-	-	+	-	-	-	-	-	-	-
M. luteus	-	-	+	-	-	-	-	-	-	-
S. aureus	-	-	+	-	-	-	-	-	-	-
S. epidermidis	-	-	+	-	-	-	-	-	-	-
S. faecalis	-	-	+	-	-	-	-	-	-	-
S. lactis	-	-	+	-	-	-	-	-	-	-
Gram-negative bacteria										
A. faecalis	-	-	-	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	+	-	-	-	-	-	-	-
P. mirabilis	-	-	+	-	-	-	-	-	-	-
P. fluorescens	-	-	+	-	-	-	-	-	-	-
S. typhimurium	-	-	+	-	-	-	-	-	-	-
S. marcescens	-	-	+	-	-	-	-	-	-	-
Yeasts										
C. albicans	-	-	-	-	-	-	-	-	-	-
C. utilis	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	+	-	-	-	-	-	-	-

Notes : i) C = pure solvents used for each extract (hexane, ethyl acetate, methanol); H = hexane extracts; E = ethyl acetate extracts; M = methanol extracts.
ii) + = inhibition zone, - = no inhibition zone.

Further experiment was carried out using different concentrations of *P. mirifica* ethyl acetate extracts. The concentrations were prepared ranging from 25 - 100mg/ml and the results were shown in Table 3.3. From this table, the most effective was *S. lactis* in which the clear zone was between 11.17±0.29mm. And the MIC of this extracts were predicted between 25 – 50mg/ml due to the initial clear zones were in these ranges.

Table 3.3 Antimicrobial activity of *P. mirifica* extracts derived from ethyl acetate determined by disc diffusion method. Data shown were mean±SD (mm) of three separate experiments.

Microorganisms	**C**	**Diameter**			
		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 ± 0.00	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 ± 0.00	8.16 ± 0.29	8.83 ± 0.29	10.67 ± 0.76
P. mirabilis	-	7.00 ± 0.00	7.83 ± 0.29	8.33 ± 0.29	10.17 ± 0.29
P. fluorescens	-	-	7.33 ± 0.58	8.00 ± 0.50	9.50 ± 0.50
S. cerevisiae	-	7.00 ± 0.00	8.00 ± 0.00	8.50 ± 0.50	10.17 ± 1.15
S. thiphimurium	-	-	7.00 ± 0.00	7.83 ± 0.29	8.67 ± 0.58
S. macescens	-	-	8.00 ± 0.00	8.00 ± 0.00	9.33 ± 0.58
S. aureus	-	-	7.83 ± 0.76	8.33 ± 0.76	9.33 ± 0.76
S. epidermidis	-	7.00 ± 0.00	8.33 ± 0.58	8.67 ± 0.76	10.33 ± 0.58
S. feacalis	-	7.00 ± 0.00	8.00 ± 0.00	8.17 ± 0.29	9.67 ± 2.02
S. lactis	-	7.00 ± 0.00	8.67 ± 0.76	9.17 ± 0.76	11.17 ± 0.29

Notes : i) C = control
ii) - = no inhibition zone

This extracts showed effective antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria in this study, the reason for different sensitivity between Gram-positive and Gram-negative bacteria is the morphological differences between these bacteria. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide, thus making the cell wall impermeable to

lipophilic solutes. Gram-positive only have an outer peptidoglycan layer which is not an effective permeability barrier, this cause more susceptible than Gram-negative bacteria (Arias *et al*., 2004).

The genus *Pueraria* has been reported to exhibit several antimicrobial phytoalexins. This property is due to the presence of pterocarpans. According to Hakamatsuka *et al*. (1994), it was showed that pterocarpans phytoalexins, namely tuberosin and glycinol found in *P. lobata*, against microbial attacks. These compounds were synthesized from the basic isoflavones, such as daidzein and genistein, which also found in *P. mirifica* (Ingham *et al*., 1986). Furthermore, Verdrengh *et al*. (2004) reported that genistein could inhibit *B. cereus*, *Helicobacter pylori* and *S. pasteurianus* whereas daidzein inhibit the growth of *S. aureus*.

In addition, Yadava and Reddy (1998) reported the antimicrobial activity of *B. superba*. The stem of *B. superba* was used to prepare the extract and tested for their antimicrobial activity. It was found that this extract was able to inhibit Gram-positive bacteria: *B. subtilis*, *S. aureus* and *S. pyogenes*, Gram-negative bacteria: *E. coli*, *Klebsiella pneumoniae*, *P. vulgaris* and *P. aeruginosa* and fungi: *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *Botrytis cinerea*, *Fusarium moniliforme*, *Helminthosporium oryzae*, *Penicillium expansum*, *Rhizopus chinensis* and *R. oligosporus*. Such an inhibitory effect was due to the presence of the novel active compound “3,5,7,3',4' – pentahydroxy – 8 - methoxy-flavonol – 3 – O – beta – D – xylopyranosyl (1-->2) – alpha – L - rhamnopyranoside”. However, our study showed different result because the *B. superba* extracts derived from hexane, ethyl acetate and

methanol did not show any inhibiting effect. This is probably due to i) solvent extract; ii) different parts and varieties used, sources of *B. superba* and iii) low amounts of active compounds in *B. superba* extracts.

3.3 Determination of MIC of *P. mirifica* extracts

From preliminary test (Section 3.2), there was only *P. mifirica* extracts derived from ethyl acetate exhibiting antimicrobial activity. Therefore, this extract was selected to study further regarding its antimicrobial activity by the MIC method based on the disc diffusion method. For this, various concentrations of crude extract were prepared and the MIC values were expressed as the lowest concentration of the extracts that still remain the antimicrobial activity. The concentrations used in this study were between 15 - 50mg/ml and the microorganisms used were *B. cereus*, *M. luteus, P. fluorescens*, *P. mirabilis, S. aureus*, *S. lactis* and *S. thyphimurium*. The results of MIC determination were shown in Table 3.4.

Table 3.4 MIC of *P. miricifa* extracts obtained from ethyl acetate against microorganism.

Microorganisms	Concentrations (mg/ml)	Clear zone (mm)
Gram-positive bacteria		
B. cereus	20	7.00
M. luteus	20	7.00
S. aureus	50	7.00
S. lactis	15	7.00
Gram-negative bacteria		
P. fluorescens	50	7.00
P. mirabilis	15	7.00
S. thiphimurium	45	6.50

In Table 3.4, *S. lactis* and *P. mirabilis* were the most sensitive to *P. miricifa* extract which only required 15mg/ml to inhibit its growth followed by *B. cereus* and *M. luteus* which were inhibited at 20mg/ml. These results were compared with the inhibition zones of antibiotics such as ampicillin, kanamycin, penicillin and tetracycline, shown in Table 3.5 (Johnson and Case, 1995).

Table 3.5 Interpretation of inhibition zones (mm) of antibiotics.

Antibiotics	Disc content	Resistant	Susceptible
Ampicillin tested with			
Gram-negative bacteria	10µg	13 or less	17 or more
Staphylococci	10µg	28 of less	29 or more
Kanamycin	30µg	13 or less	18 or more
Penicillin tested with			
Staphylococci	10units	20 or less	21 or more
Other microorganisms	10units	11 or less	22 or more
Tetracycline	30µg	14 or less	19 or more

To interpret the efficiency based on the data shown in Table 3.5, it was found that the *P. miricifa* ethyl acetate extracts were less efficiency than these antibiotics and thus may not be able to use for controlling these microbial pathogens unless further study has been carried out such as purification of bioactive compounds to increase the concentration of crude extract.

3.4 Analysis of bioactive compounds using TLC

1ml of *P. mirifica* (100mg/ml) derived from ethyl acetate was applied on the TLC plate. Initially, the mixture solvents used and their ratio were experimented including

the use of hexane-ethyl acetate, hexane-methanol and ethyl acetate-methanol. Their ratio was also varied such as hexane-ethyl acetate (3:7), hexane-methanol (4:6) and ethyl acetate-methanol (5:5). Finally, the optimal conditions showing the best separation was the use of the mixture solvent of hexane-ethyl acetate in the ratio 6:4. The chromatogram was developed in a mixture of hexane and ethyl acetate (6:4). The developed chromatogram was then observed under UV light at 254nm. Each fraction present under UV was marked, scraped and dissolved with 1ml of ethyl acetate; these solutions were then tested with *B. cereus* using the disc diffusion method. The results of the TLC analysis were shown in Figure 3.5.

From 20 bands, band 10 and 12 with the R_f values of 0.60 and 0.51 exhibited antimicrobial activity against *B. cereus*. Both fractions were then determined to reveal their identity. Initially, daidzein and genistein were used as chemical standards because previous experiment suggested their antimicrobial activity against *B. cereus* (Verdrengh *et al*., 2004). However, according to the TLC analysis, our results showed that the bioactive compounds of *P. mirifica* extracts were not daidzein and genistein (see Figure 3.6).

Band	Length (cm)	R_f
1	15.6	0.98
2	15.0	0.94
3	14.2	0.89
4	13.2	0.83
5	12.5	0.78
6	11.8	0.74
7	11.2	0.70
8	10.8	0.68
9	10.1	0.63
10	**9.6**	**0.60**
11	8.9	0.53
12	**8.2**	**0.51**
13	7.5	0.47
14	6.7	0.42
15	5.4	0.34
16	3.8	0.24
17	2.5	0.16
18	1.6	0.10
19	0.9	0.07
20	0.3	0.02

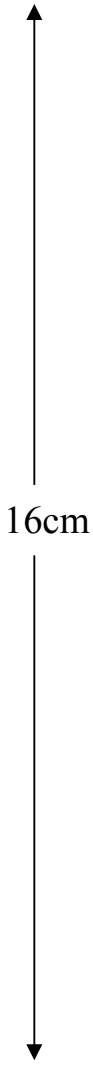

Figure 3.5 The developed chromatogram of *P. mirifica* derived from ethyl acetate.

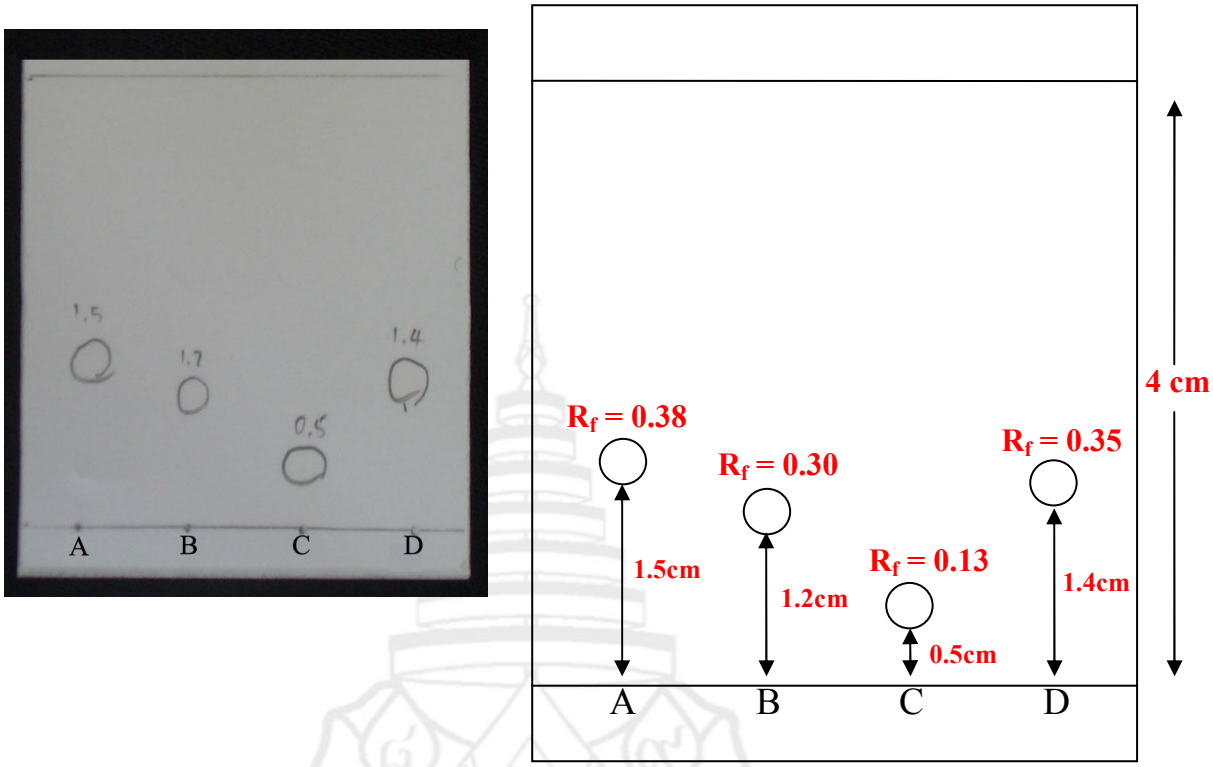

Figure 3.6 Identity of bioactive compounds (band 10, R_f=0.38 and band 12, R_f=0.30) of *P. mirifica* ethyl acetate extracts compared with daidzein and genistein using TLC analysis: A, band 10; B, band 12; C, daidzein; D, genistein.

In this study, daidzein and genistein were also tested against *B. cereus* using the disc diffusion method but these standards did not show inhibition zone. This result is contrary to the report of Verdrengh *et al*. (2004). The possible explanation is due to the low amounts of standard chemicals used.

3.5 Antioxidants in *kwao krua* extracts

Phytoestrogens, the nonsteroidal isoflavones were found in many plants of the Family Leguminosae. Some well known phytoestrogens (i.e., miroestrol, daidzein, genistein

and coumestrol) were analysed and tested for their pharmacological activity. It was showed daidzein functions as an estrogen-like hormone, whereas genistein exhibited anticancer activity (Yu, 2005). Daidzin, genistin, daidzein and genistein were verified in this chemical group and showed the reports about estrogen-like and anticancer activity (Klejdus *et al*., 2004). In this study, the *kwao krua* extracts were prepared and determined the availability of some antioxidants. This was performed using the HPLC test in which daidzin, genistin, daidzein and genistein were used as chemical standards. The results of this analysis were presented in Table 3.6 and Figure 3.7.

Table 3.6 Presence of antioxidant compounds in *kwao krua* extracted analysed by HPLC. Active compounds tested in this study were daidzin, genistin, daidzein and genistein. Data shown are in the unit of ppm; the numbers in parentheses are the unit of %(w/w).

***kwao krua* extracts**	**Daidzin**	**Genistin**	**Daidzein**	**Genistein**
Hexane				
P. mirifica	-	-	0.068 (0.003%)	1.231 (0.054%)
B. superba	-	-	-	1.247 (0.055%)
M. macrocarpa	-	-	-	1.231 (0.054%)
Ethyl acetate				
P. mirifica	1.034 (0.045%)	0.840 (0.037%)	1.115 (0.049%)	1.365 (0.060%)
B. superba	-	0.229 (0.010%)	0.113 (0.005%)	0.345 (0.015%)
M. macrocarpa	-	-	-	0.372 (0.016%)
Methanol				
P. mirifica	0.421 (0.019%)	0.239 (0.011%)	-	1.225 (0.054%)
B. superba		0.127 (0.005%)	0.025 (0.001%)	1.228 (0.054%)
M. macrocarpa	-	-	-	1.219 (0.054%)

From Table 3.6, daidzin appeared only in *P. mirifica*. Genistin and daidzein were found in *P. mirifica* and *B. superba*. In contrast, genistein was present in every *kwao krua* extracts. Besides, it is noteworthy that the *P. mirifica* extracts obtained from ethyl acetate had all four compounds with highest amounts among all *kwao krua* extracts.

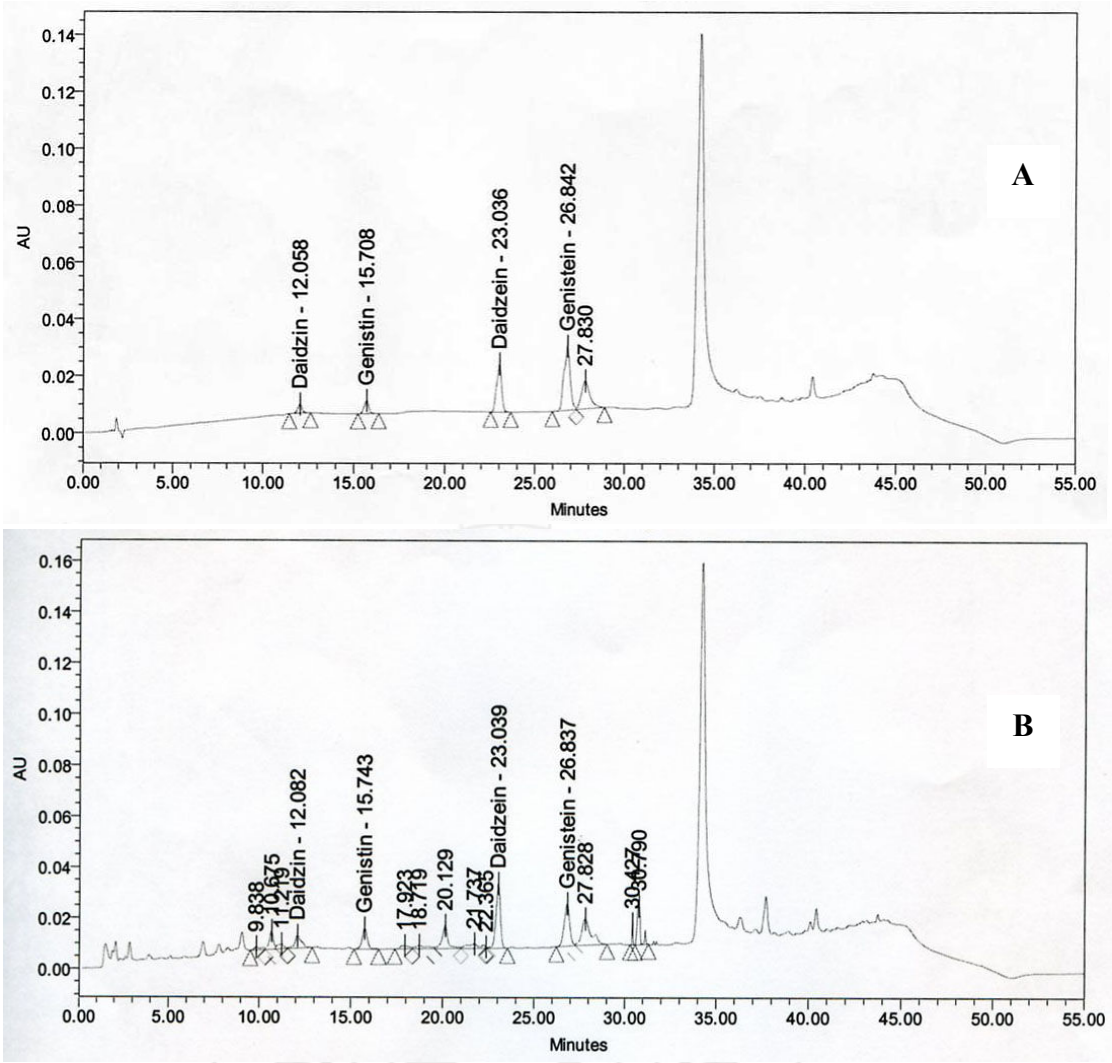

Figure 3.7 HPLC chromatogram A) antioxidant standards (daidzin, genistin, daidzein and genistein) and B) *P. mirifica* extracts obtained from ethyl acetate.

In Figure 3.7, daidzin, genistin, daidzein and genistein were appeared in the HPLC chromatogram of *P. mirifica* extracts obtained from ethyl acetate compared with HPLC chromatogram of antioxidant standards.

Generally, plants in the genus *Pueraria* have been known to contain isoflavones especially in *P. lobata* and *P. thomsonii* (Hayakawa *et al*., 1984; Zhang and Yang,

1984; Zhou *et al*., 1995). In 1999, Zeng noted 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*. (1995) 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* contains content of daidzin higher than *P. mirifica* but the content of daidzein in *P. mirifica* are higher than those of two plants.

Moreover, Manosroi and Manosroi (2005) presented that the tubers of *P. mirifica* harvested at 6 years (source: Chiang Mai, Thailand) gave the highest amounts of daidzein and genistein at 8.9% and 1.6% , tubers that were younger or older than 6 years appeared to contain less amount of these compounds. The contents of these compounds on their report were more than the contents of *P. mirifica* on this study and the ages of *P. mirifica* plants collected were unknown, for that reason, these tubers may be younger or older than 6 years, or the different contents due to the source of *P. mirifica*. Moreover, their report showed that the contents of daidzein and genistein in *B. superba* (source: Chiang Mai, Thailand) were 3.72% and 0.45%, these contents also more than the contents of *B. superba* on this study. They suggested that the contents of isoflavonoids in these plants depended on ages and sources of plants. However, their report affirmed that both *P. mirifica* and *B. superba* contained phytoestrogenic compounds such as daidzein and genistein, therefore, these compounds caused the same properties in *kwao krua* plants.

3.6 Conclusion

The results of this study can be concluded as follows:

a) Only the *P. mirifica* extracts obtained from ethyl acetate were active against the microorganisms tested.

b) The MIC of this extract was determined and these values were between 15 - 50mg/ml depending on the microbes tested.

c) The TLC fractions of this extract were separated into 20 bands and there were only band 10 and 12 with the R_f values of 0.51 and 0.60 exhibiting antimicrobial activity against *B. cereus* cells.

d) This extract showed highest amounts of four antioxidant compounds determined among *kwao krua* extracts.

Chapter IV

General Conclusion

The flowers of *Mucuna macrocarpa* Wall.

(source, http://www.nmns.edu.tw/botany/most/most_detail.php?bid=145&pid=18)

Humans have been using plants for several purposes especially for foods and disease treatment. For medicine, many plants contain a variety of bioactive compounds which have shown to be able to inhibit many microorganisms. It can be assumed that these compounds may be produced as a self-defense mechanism to prevent the microbial attack. As a result, many scientists have been searching the natural products from plants and tested for their activity in an expectation to obtain the novel drugs that can use against serious diseases (including many new resistant microbes).

kwao krua is a generic term for a group of indigenous Thai herbs in the Family Leguminosae. The term *kwao krua* is generally used for three specific plants: white *kwao krua* (*P. mirifica*), red *kwao krua* (*B. superba*) and black *kwao krua* (*M. macrocarpa*). According to traditional Thai medicine, these Thai plants considered as a rejuvenating drug have long been popularly used among Thai people. In addition, tuberous roots of *P. mirifica* are a potent source of phytoestrogen and thus useful for menopausal women. Due to this female-like hormone activity, it can also be used for breast enlargement. Previous work has showed that this plant contains various kinds of chemical compounds including deoxymiroestrol, miroestrol, puerarin, daidzein, genistein, kwakhurin and other isoflavonoids. However, there is limited information of chemical compounds present in *B. superba* and *M. macrocarpa*. Apart from pharmacological activity, it would be of great interest to further investigate whether these *kwao krua* plants exhibit antimicrobial activity. The data related to this issue are currently scarce. Based on the work of Yadava and Reddy (1998), it was showed that flavonol glycoside from the stem of *B. superba* inhibited against microorganisms. As a result, other *kwao krua* plants were also selected for detailed study on this aspect.

The results of this study showed that only the *P. mirifica* extracts obtained from ethyl acetate exhibited antimicrobial activity against thirteen microorganisms (*B. cereus*, *B. subtilis*, *E. coli*, *M. luteus*, *P. mirabilis*, *P. fluorescens*, *S. typhimurium*, *S. marcescens*, *S. aureus*, *S. epidermidis*, *S. feacalis*, *S. lactis* and *S. cerevisiae*). The MIC of this extract was then determined and found between 15 - 50mg/ml depending on microbes tested (*B. cereus*, *M. luteus*, *P. mirabilis*, *P. fluorescens*, *S. aureus*, *S. lactis* and *S. typhimurium*). When using the TLC technique, it was showed that the extracts were compared of at least 20 distinct chemical compounds. After being tested against *B. cereus*, our results showed that band 10 and 12 (R_f = 0.60 and 0.51) were capable of inhibiting the *B. cereus* cells and these active compounds were not daidzein and genistein based on TLC analysis. Furthermore, the *kwao krua* extracts were determined the availability of antioxidant compounds (daidzin, genistin, daidzein and genistein) and it was revealed that the *P. mirifica* extracts derived from ethyl acetate contained all four compounds with the highest amounts of antioxidant compounds among the *kwao krua* extracts tested.

Based on our results obtained in this study, there are some certain points to further clarify and discuss as follows:

a) There is a report of antimicrobial activity of *B. superba* (Yadava and Reddy, 1998). However, this study shows different results. In previous work, the stem of *B. superba* was used, whereas its tuber was studied in this thesis. Besides, other explanations may be due to i) different solvent extracting; ii) different parts and varieties used; iii) sources of *B. superba* and iv) low amounts of active compounds in *B. superba* extracts.

b) The MIC values were significantly lower than the antibiotics. Therefore further study regarding the purification of bioactive compounds to increase the concentration of crude extract should be carried out.

c) In this study, the contents of isoflavonoids in *P. mirifica* and *B. superba* were less than the contents shown in other reports. This is probably due to i) the ages and sources of plants and ii) the solvent extracts.

kwao krua (especially, in *P. mirifica*) are indigenous Thai plants and have been widely used for a long time. This study has been carried out to investigate *kwao krua* in terms of their antimicrobial activity. Several experiments have been performed to characterise the presence of natural products as well as their potential applications. Further work relevant to the use of *kwao krua* is of great importance and, based on this thesis, further investigation is required to complete the project including:

a) Structural elucidation: The bioactive compounds should be purified and tested for their activity. The purified agents are then subject to GC (gas chromatography), HPLC, NMR (nuclear magnetic resonance) spectroscopy and IR (infrared) spectroscopy to reveal their identity.

b) Plants taxonomy: Other plant species in these groups (*Pueraria*, *Butea* and *Mucuna*) as well as these related plants should be verified to screen for the presence of antimicrobial agents.

c) Antimicrobial test: The antimicrobial compounds should be tested with various microorganisms such as pathogenic species (i.e., influenza virus, HIV, and *Bacillus anthracis*), opportunistic species (i.e., *Mycobacterium tuberculosis* and

Pneumocystis jiroveci) and antibiotic-resistant species (i.e., methicillin-resistant *Staphylococcus aureus* (MRSA)).

d) Standardisation: The bioactive compounds in these plants should be standardised for further use in the medicinal aspects. In addition, the toxicity of these bioactive compounds must be evaluated both *in vivo* and *in vitro*. Further study should also be carried out for their potential application in clinical trial.

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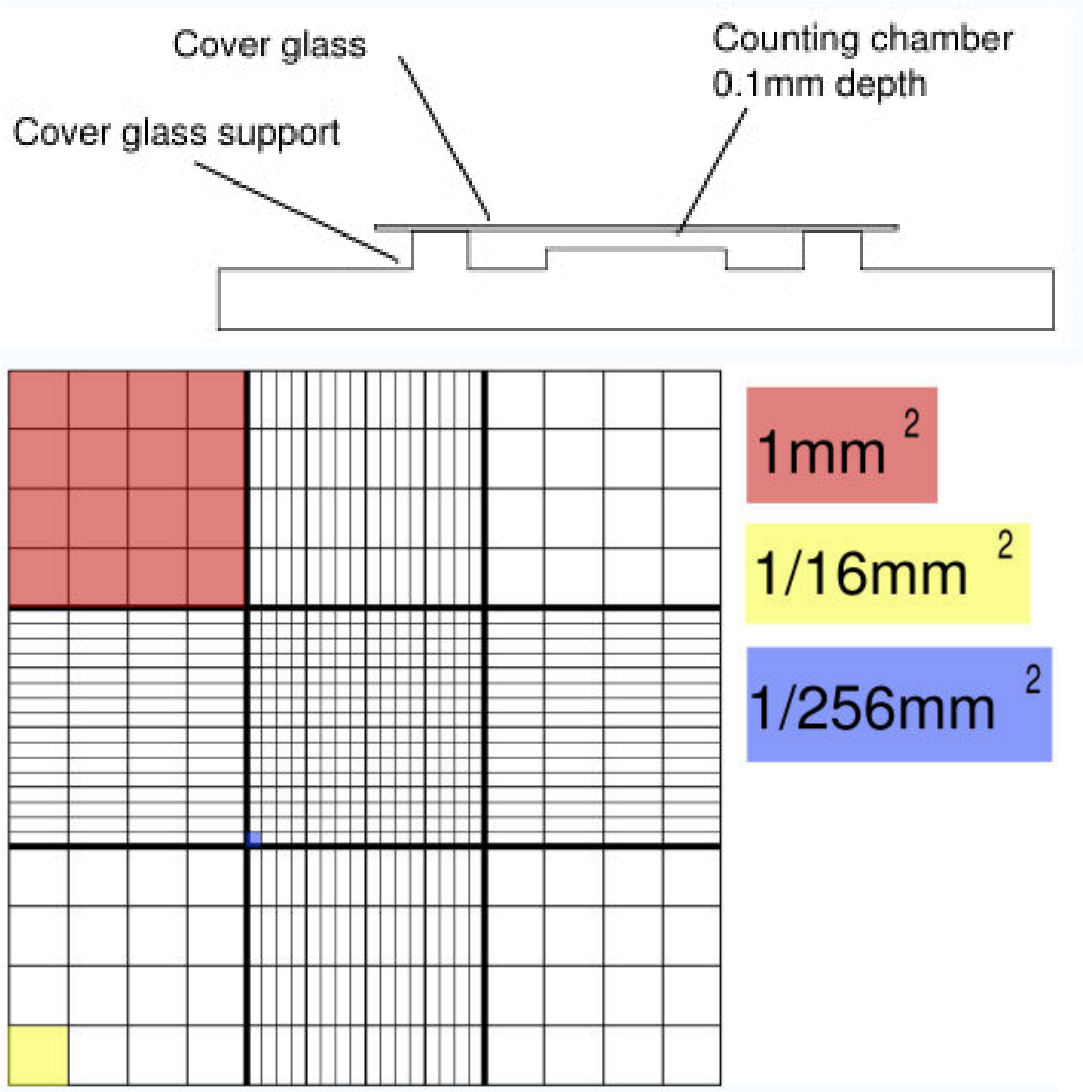
Appendix I

The calculation of haemocytometer



Procedure:

1. Place the coverslip over the haemocytometer counting chamber and using a Pasteur pipette, place a drop of the cell suspension at the edge of the "V" shape of the chamber. Allow the suspension to be drawn into the chamber by capillary action. Care should be taken not to overfill or underfill the chamber. Fill the opposite chamber in the same manner.

2. Place the haemocytometer on the microscope.

3. The haemocytometer consists of nine $1mm^2$ squares divided into smaller squares. One of the $1mm^2$ squares represents a volume of $0.1mm^3$ or 10^{-4}ml. Using the 10X objective, count the number of cells in a $1mm^2$ square area. If there are fewer than 100 cells in a square mm, 2 or more 1-mm^2 square areas should be counted and the results averaged.

4. Use the same procedure to count the cells on the other side of the haemocytometer.

5. To calculate the concentration of the cells, first calculate the average of all $1mm^2$ areas counted and apply this formula:

$$C = n/v$$

where:

C = cell concentration in cells/ml

n = average number of cells/mm^2 area

v = volume counted = 10^{-4}

Thus:

$$C = n \times 10^4$$

Appendix II

Presentation

To be presented at the 1st International Conference on Forensic Science and Medical Science, Naresuan University, Phitsanulok, Thailand, 28 - 29 July 2006.

ANTIMICROBIAL ACTIVITY OF *KWAO KRUA* EXTRACTS

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Crude *kwao krua* extracts (*Pueraria mirifica*, *Butea superba* and *Mucuna macrocarpa*) prepared by sequential extraction using hexane, ethyl acetate and methanol were tested for their antimicrobial activity using disc diffusion method. It was showed that only the *P. mirifica* extracts derived from ethyl acetate exhibited antimicrobial activities against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Streptococcus lactis* and *Saccharomyces cerevisiae*. The minimum inhibitory concentration (MIC) was between 15 and 50mg/ml depending on the microbes tested. Based on the thin layer chromatography (TLC) technique, two chemical compounds with the R_f values of 0.51 and 0.60 were capable of inhibiting the *B. cereus* cells. In addition, *kwao krua* extracts were screened for antioxidants using HPLC. Of these samples, the *P. mirifica* extracts obtained from ethyl acetate showed highest amount of these antioxidant compounds.

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Antimicrobial activity of *kwao krua* extracts

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Abstract

Crude *kwao krua* extracts *(Pueraria mirifica, Butea superba* and *Mucuna macrocarpa)* prepared by sequential extraction using hexane, ethyl acetate and methanol were tested for their antimicrobial activity using disc diffusion method. It was showed that only the *P. mirifica* extracts derived from ethyl acetate exhibited antimicrobial activities against *Bacillus cereus, B. subtilis, Escherichia coli, Micrococcus luteus, Proteus mirabilis, Pseudomonas fluorescens, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus, S. epidermidis, Streptococcus faecalis, S. lactis* and *Saccharomyces cerevisiae*. The minimum inhibitory concentration (MIC) was between 15 and 50mg/ml depending on the microbes tested. Based on the thin layer chromatography (TLC) technique, two chemical compounds with the Rf values of 0.51 and 0.60 were capable of inhibiting the *B. cereus* cells. In addition, *kwao krua* extracts were screened for antioxidants using HPLC. Of these samples, the *P. mirifica* extracts obtained from ethyl acetate showed highest amount of these antioxidant compounds.

Introduction

kwao krua is a generic term for a group of indigenous Thai herbs in the family Leguminoceae. These include *Pueraria mirifica* (white *kwao krua*), *Butea superba* (red *kwao krua*) and Mucuna macrocarpa (black *kwao krua*). According to traditional Thai medicine, these Thai plants (especially for *P. mirifica*) act as a rejuvenating drug and thus have long been popularly used among Thai people. Previous work has showed that there are various kinds of chemical compounds in these herb including miroestrol, puerarin, deoxymiroestrol, kwakhurin and other isoflavonoids. In addition, *kwao krua* tuberous roots are a potent source of phytoestrogen and are hence useful for menopausal women. Due to this female-like hormone activity, *kwao krua* extracts can also be used for breast enlargement (1-2).

Apart from pharmacological activity, it would be of great interest to further investigate whether these *kwao krua* plants exhibit antimicrobial activity. The data related to this topic are currently limited. This study was then performed to determine the antimicrobial activity of *kwao krua*.

Materials and Methods

In February 2004, tubers of *P. mirifica* and *B. superba* were collected from Phayao; those of *M. macrocarpa* from Chiang Rai. To prepare the extracts, *kwao krua* tubers were chopped, dried and initially soaked in hexane for 3 days. The remained samples were then extracted with ethyl acetate for 3 days and finally with methanol for 3 days. Each extracted solutions was concentrated using the rotary evaporator and the freeze dryer.

Various concentrations of *kwao krua* extracts were then prepared and used to test antimicrobial activity using the disc diffusion method. The microorganisms tested were *Alcaligenes faecalis, Bacillus cereus, B. subtilis, Enterobacter aerogenes, Escherichia coli, Micrococcus luteus, Proteus mirabilis, Pseudomonas fluorescens, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus, S. epidermidis, Streptococcus faecalis, S. lactis, Candida albicans, Candida utilis,* and *Saccharomyces cerevisiae*. The MIC was also determined.

The TLC technique was introduced to separate chemical constituents of the extract. Each fraction was subsequently tested for antimicrobial activity. In addition, the HPLC technique was also performed to analyse the available antioxidants.

Conclusion

Only the *P. mirifica* extracts obtained from ethyl acetate exhibited antimicrobial activity against thirteen microbes tested in which two fractions with the Rf values of 0.51 and 0.60 were capable of inhibiting the *B. cereus* cells. Besides, this extract showed the highest of antioxidant compounds among *kwao krua* extracts.

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(1) Ingham JL *et al.* (1986) *Z Naturforsch*, 41a, 403-408.
(2) Cherdshewasart W *et al.* (2004) *J Ethpharm*, 93, 255-260.

Results

Table 1 Antimicrobial activity of *kwao krua* extracts using disc diffusion method.

Microorganisms	*P. mirifica*			*B. superba*			*M. macrocarpa*		
	H	E	M	H	E	M	H	E	M
A. faecalis (G-)	-	-	-	-	-	-	-	-	-
B. cereus (G+)	-	+	-	-	-	-	-	-	-
B. subtilis (G+)	-	+	-	-	-	-	-	-	-
C. albicans (Yeast)	-	-	-	-	-	-	-	-	-
C. utilis (Yeast)	-	-	-	-	-	-	-	-	-
E. aerogenes (G-)	-	-	-	-	-	-	-	-	-
E. coli (G-)	-	+	-	-	-	-	-	-	-
M. luteus (G+)	-	+	-	-	-	-	-	-	-
P. mirabilis (G-)	-	+	-	-	-	-	-	-	-
P. fluorescens (G-)	-	+	-	-	-	-	-	-	-
S. cerevisiae (Yeast)	-	+	-	-	-	-	-	-	-
S. typhimurium (G-)	-	+	-	-	-	-	-	-	-
S. marcescens (G-)	-	+	-	-	-	-	-	-	-
S. aureus (G+)	-	+	-	-	-	-	-	-	-
S. epidermidis (G+)	-	+	-	-	-	-	-	-	-
S. faecalis (G+)	-	+	-	-	-	-	-	-	-
S. lactis (G+)	-	+	-	-	-	-	-	-	-

H = Hexane; E = Ethyl acetate; M = Methanol
+ = Active; - = Not active

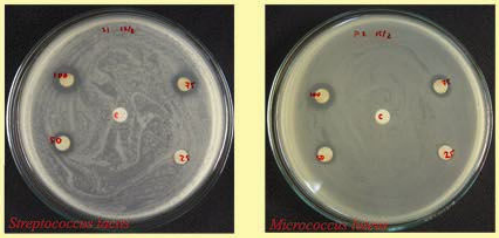


Figure 1 Representatives of clear zones derived from *P. miricifa* extracts

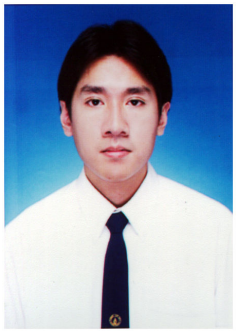
Table 2 MIC of *P. miricifa* extracted from ethyl acetate against microorganisms

Microorganisms	MIC values (mg/ml)
S. lactis (G+)	15
P. mirabilis (G-)	15
M. luteus (G+)	20
B. cereus (G+)	20
S. thiphimurium (G-)	45
S. aureus (G+)	50
P. fluorescens (G-)	50

Table 3 Presence of antioxidants (ppm) in *kwao krua* extracts

Kwao krua extracts	Daidzin	Genistin	Daidzein	Genistein
Hexane				
P. mirifica	-	-	0.068	1.231
B. superba	-	-	-	1.247
M. macrocarpa	-	-	-	1.231
Ethyl acetate				
P. mirifica	1.034	0.840	1.115	1.365
B. superba	-	0.229	0.113	0.345
M. macrocarpa	-	-	-	0.372
Methanol				
P. mirifica	0.421	0.239	-	1.225
B. superba		0.127	0.025	1.228
M. macrocarpa	-	-	-	1.219

Curriculum Vitae



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