CHAPTER 1

INTRODUCTION

A. General background

Kwao Khruea (*Pueraria mirifica* Airy Shaw et Suvatabandhu), family *Laguminosae*, subfamily *Papilionoidcae*, is one of the medicinal plants that predominates in the Northern part of Myanmar, the Northern part of Thailand such as Chiang Mai, Chiang Rai, Mae Hong Son, Tak and Uthaitani provinces, and the Western part such as Rachaburi and Kanchanaburi provinces. It has been used in folk medicine for rejuvenescence by Thai traditional practitioner's medicine for a long time. It was believed that tuberous root of White Kwao is the medicine for long life.

In the Northern part of Thailand, this medicinal plant was prepared by digging up, washing, then pealed, sliced into pieces, and dried in the sun. After that, it was powdered by mortar and pestle, and mixed with the honey, and made up to a small medicinal pill as the size of black pepper. The traditional doctors prescribed the pill of Kwao Khruea to their patients and some people take this medicine for themselves, some take only half pill or one third of the pill daily at bedtime.

White Kwao has also been used long times ago in Myanmar, and it is known as paukse. Women under forty years old were forbidden to take the pill. It is believed that women who had taken Kwao Khruea pill for two to three months will have a good health especially hormonal control and may have a long life. Women who have taken

Kwao Khruea pill should not eat hot food, should take a bath three times a day, and should not expose to cold air. They believed that women who have taken Kwao Khruea pill for about three to six months would cure many diseases, prevent some diseases and have long life (หลวงอนุสารสุนทร, 2474).

The first information of the advantages of using Kwao Khurea was published in a single leaflet, in the 1920s, in the Northern part of Thailand, without date or authors. It was discovered at an old Buddhist temple in Paga, in the ancient capital of Myanmar during the temple was reconstructed. There are many types of Kwao Khruea, but they were used only four types in medicine. There are White Kwao, Red Kwao, Black Kwao and Moh Kwao, from these four types, the Black Kwao is the strongest and the White Kwao is the weakest potency in rejuvination. The Kwao Khruea pills are believed to have anti-aging properties such as improve the function of the brain, expand the breasts, keep black hair, enhance hair growth on bald, nourish the skin, improve memory, prevent the onset of cataracts, improve the sexual efficiency and induce menstruation in women of 60-80 years of age (Radomsuk, 1992).

An English Professor Airy Shaw studied this medicinal plant with Professor Kasin Suvatabandhu, at Chulalongkorn University, Thailand, they announced the name of this medical plant as "White Kwao" officially in 1974. Thus, this plant was named after Airy Shaw and Suvatabandhu for their honor. Lakshnakara et al. (1952) also reported that the tuberous root of White Kwao possesses some active substances, which are similar to estrogens. Miroesterol has been reported as the major estrogenic

compound, isolated from Kwao Khruea(Jones and Pope, 1960). The recent study, using bioassay-guide purification was isolated a new potent phytoestrogen, the deoxymiroesterol. It was found that facile aerial oxidation of deoxymiroesterol to miroesterol suggests the possibility that miroesterol may be artifact. Strong activity was observed for both miroesterol and deoxymiroesterol, but the latter was found to be about 10-folds more potent. (Chansakaow et eal, 2000a).

Nowadays, this plant has been used for the treatment of some diseases such as AIDS, and cancer. It also can be used as a food supplement and cosmetic. White Kwao is known as the "Health Tonic & Longevity Herb" for women. However, White Kwao phytoestrogen, or naturally occurring plant estrogen, is not hormone, but it does share some hormonal property in same benefits of a woman's own estrogen.

At present, the most commonly uses of Kwao Khruea in cosmetic and food supplements are White Kwao and Red Kwao. White Kwao is used for beauty and health in many forms such as tablet, capsule, cream, lotion, gel and also used as hormonal replacement in menopausal women. The general dose of White Kwao powder of both tablet and capsule are approximately 100-600 mg depending on the manufacturer. White Kwao is widely used for relieving menopausal disorders, enhancing breast size, improving memory and prevention of Alzheimer's disease, reducing risks of cataract, decreasing incidence of colon cancer, eliminating arthritic pain, nourishing skin, and keeping hair stronger. It is not recommended for pregnant woman and woman under 25 years old. Red Kwao is used for enhancing male sexual potency by increasing erection strength and timing, enhancing hair growth, curing

gout, preventing blood occlusion, and osteoporosis. It is also not recommended for young male (Cherdcheewasard, 2000; Nawapol, 2000).

B. General characteristic of White Kwao

White Kwao grows in the forest of a high land, or on the mountain, 300 to 800 meters over the sea level. They grow in the soil that has a lot of organic substances, pH approximately 5.5, in humid climate, and the temperature is between 2.8 °C and 39.5 °C (Smitasiri, 1986).

The trunk of White Kwao is approximately 5 meters long and 4 to 16 centimeters in diameter (Figure 1) (Kashemsanta et. al., 1963). White Kwao grows in-groups and is a deciduous woody climber with various size of globular, the tuberous root contains food deposition (Figure 2). Inside, the root is white and its taste can cause dizziness. The sizes of roots are variable depending on the environment and the time of cropping.

When the fresh tuberous root of White Kwao was sliced, a drop of white milk-like gum was seen. The leaf consists of 3 minor leaves, the edge of the leaves is smooth and has sharp end with delicate hair on both sides. The leaf is approximately 6 centimeter long, fully grown up in May to September, and fallen down in December. The flower is small, bluish-purple, and approximately 15.0 to 40.3 centimeters in length and it blossoms in January to March, (Figure 3). When the flowers are fallen down, the shell will be seen. The White Kwao's shell is legume type, long slender, sharp beak, and flat with minimal hairs on it (Figure 4). The length of fully mature shell is approximately1.0 to 7.5 centimeters long and 0.7 centimeters wide. The shell

has 1 to 9 seeds inside. The seed is similar to bean seed, 2 millimeters wide and 4 millimeters long (Wungjai and Smitasiri, 1987).

Figure 1 The tuberous root of White Kwao (Thuppongse, 1984)

Figure 2 The leaves of White Kwao (Chailek, 1988)

Figure 3 The flowers of White Kwao (Chailek, 1988)

Figure 4 The shells of White Kwao (Chailek, 1988)

C. Component of White Kwao

The tuberous root of White Kwao accumulates many substances, they can be classified as the following data

1.Chromenes

Chromenes are importantly active component of White Kwao.These components consist of miroesterol and deoxymiroesterol (Figure 5), they have highest estrogenic activity. There are 2 types of miroestrerol crystal, hydrated and anhydrous forms, colorless, melting point is around 268-270°C, and molecular formula is $C_{20}H_{22}O_6$. Deoxymiroesterol was obtained as colorless primes, melting point 213-216°C, the molecular formula is $C_{20}H_{22}O_5$. Both miroesterol and deoxymiroesterol have a common skeleton with the same stereochemistry in which C-14 hydroxyl group in miroesterol has been replaced by hydrogen atom with retention of configuration (Chansakaow, 2000a).

2. Flavonoids

Besides chromenes, the previous report showed the presence of various isoflavonoids (Ingham et al., 1986; Tahara et al., 1987). There are 2 types of isoflavonoids, the first one is non-glycosidic isoflavonoids such as daidzein, genistein, kwakhurin and tuberosin. The other is glycosidic isoflavonoids; diazin, mirificin, and puerarin. It has been reported that genistein had 10-folds stronger activity than daidzein (Cain, 1960). Kwakhurin was also found to have moderate activity compared with diazin (Martin et al., 1978; Verdeal and Ryan, 1979; Farmakalidis et

al., 1985; Miksicek, 1993; Hsieh et al., 1998). In recent study on the chemical constituents of the tuberous root of White Kwao has reported new isoflavonoids, ptercapene and puemiricarpene. These isoflavonoids may partly cause the rejuvinating action of this plant (Chansakaow, 2000b).

3. Coumarins

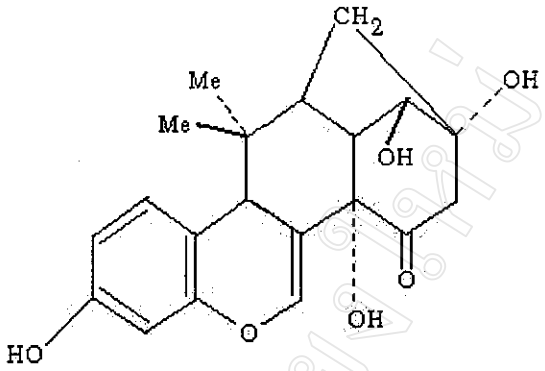
There are some coumarins in White Kwao such as coumestrol (Figure 7), miricoumestan, miricoumestan glycol and miricoumestan hydrate (Ingham, 1988). Coumestrol has been known as a potent estrogenic activity of coumarins (Cain, 1960)

4. Steroids

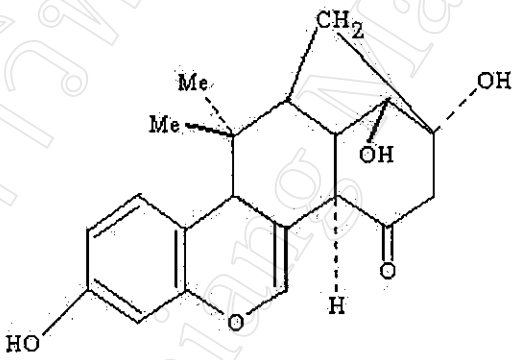
There are some steroids in White Kwao such as β-sitosterol, sticmasterol and pueraria mirifica sterol. Steroids have not been reported to pose estrogenic activity.

5. Others

In addition, there are other components of White Kwao such as lipid,litium, potassium, sodium, phosphorus and calcium oxalate and sucrose (Ingham et al., 1986).

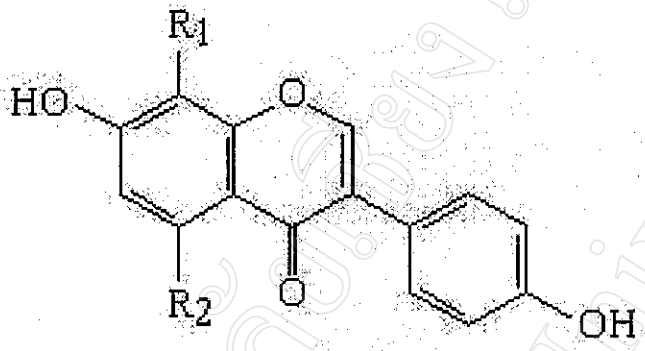


Miroesterol (A)



Deoxymiroesterol (B)

Figure 5 Structure of miroesterol (A) (pharma. chula., 2002) and deoxymiroesterol (B) (Chansakaow et al., 2000a)



Genistein : R_1 = H, R_2 = OH
Daidzein : R_1 = H, R_2 = H
Puerarin : R_1 = -Glucose, R_2 = H
Mirificin : R_1 = -Glucose-Apiose, R_2 = H

Figure 6 Structure of flavonoids (pharma. chula., 2002)

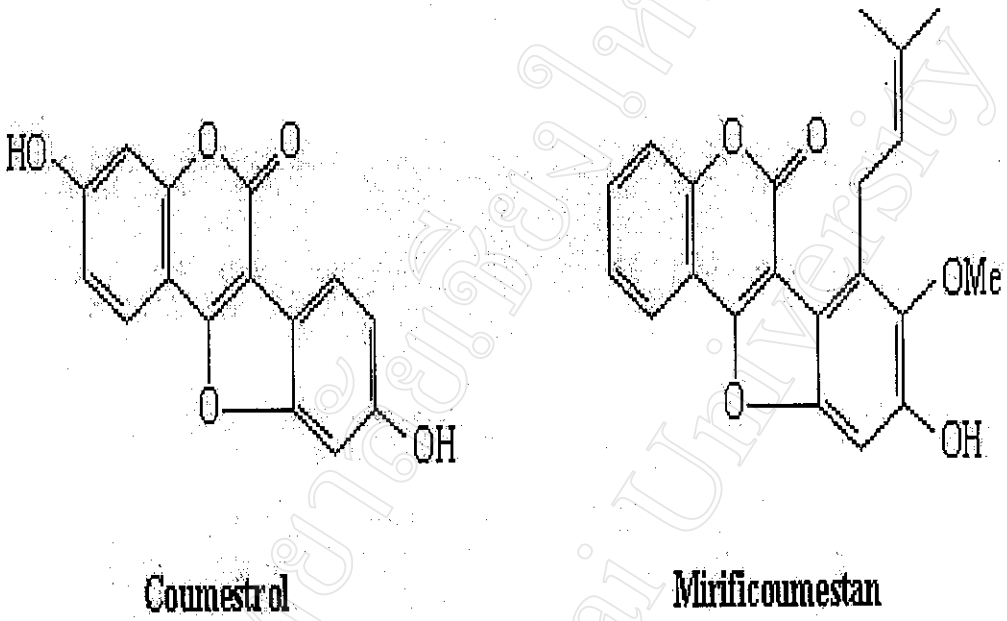


Figure 7 Structure of Coumestrol and Mirificoumestan (pharma. chula., 2002)

D. The pharmacological effect of White Kwao

1.Estrogenic effects

1.1 Effect on mammary gland and lactation in lactating animals

The effect of White Kwao on the development of mammary glands in bilaterally ovariectomized rats and mice showed that White Kwao could stimulate the development of mammary tissue and enlarge the breast size by lengthening and branching the mammary ducts that connect to the nipple (Benson et al., 1961; Sawadipong, 1979). In contrast, White Kwao caused the decrease in the weight of mammary glands in the lactating rat (Chailek, 1988; Smitasiri et al., 1989; Saowakon and Aritajat, 1995). It was noticed that no milk let down from mammary gland was found in both White Kwao, and estrogen-treated rats. These data suggested that White Kwao could inhibit lactation in lactating rats, possibly by inhibiting growth of mammary gland and milk production like estrogen.

1.2 Effect on reproductive organs of animals

It has been reported that mice feeding with White Kwao could cause vaginal cells maturation or cornification similar to that found in the preovulatory phase (Junyatum, 1983; Songkaew, 1987).

Anuntalabhochai et al. (1983) reported that one hundred-fifty five-day old Japanese quials were fed with commercial food contained 5% White Kwao for ten days caused increase in the oviductal weight. This data was in agreement with Chuaychoo (1984); Maungdech (1984); Smitasiri et al. (1992).

Maungdech (1984) and Thuppongse (1984) reported on quails fed with commercial food with White Kwao. They found that the weight of ovaries was decreased and the number of follicles was increased. It was possible that White Kwao inhibited the releasing gonadotropins (FSH/LH) by negative feedback on the anterior pituitary gland. In addition, White Kwao was also found to increase uterine weight (Songkaew, 1987).

Junyatum (1983) reported that White Kwao dissolved in distilled water and given orally to young rats at doses of 25, 50 and 100 mg/day decreased the testicular weight, which was in agreement with Jesrichai (1983); Tanachai and Smitasiri (1987) and Smitasiri and Kaweewat, (1992).

1.3 Antifertility effects

Langkalichan (1984) found that high dose of White Kwao (100 and 200 mg/kg/time) could significantly decrease the number of sperm in the epididymis and the percentage of sperm motility in male rats. High dose of White Kwao treated-male rats were mated with normal females reduced the number and size of implantation in both uterine horns. The gestation was prolonged and the duration of fetal expulsion was reduced. Number and body weight of their offspring were also reduced. No congenital malformation of the offspring was found in this study. White Kwao was given to female rats during different periods of pregnancy, i.e., during the embryo transport period, implantation period and during postimplantation period (Smitasiri et al., 1986). The results clearly showed that White Kwao was effective in preventing pregnancy in rats when given during embryo transport period. This was in an agreement between Tanachai and Smitasiri (1987) who reported that female mice fed

with pellet mixed with White Kwao and caged with male mice since the first day or the tenth day after treatment, could not deliver any offspring in both groups. When laparotomy was made, it was found that only one mouse of each group was pregnant (14.29%). In addition, Radomsuk (1992) has studied the effect of White Kwao extracts on reproduction of American cockroches. Female American cockroaches were fed with ethanolic and aqueous extracts of White Kwao tuber mixed with food for 15 days. The results showed that ovum size of White Kwao treated groups was significantly smaller than the control and abnormal ovaries were usually found in White Kwao-treated American cockroaches. When White Kwao extracts were fed to both sexes of cockroaches for 30 days, it was found that the number of ootheca and non-hatching were quite high in most of the White Kwao -treated groups.

2.Change of calcium, total protein and cholesterol concentration in blood

Japanese quails were fed with commercial food mixed with White Kwao at 5% and 10% (w/w) for 60 days, can increase calcium, protein and cholesterol in blood (Anuntalabhochai et al., 1989). It is possible that miroesterol may increase absorption of calcium, from stomach into blood circulation. It was found that White Kwao had positive effect on increasing total protein, albumin, globulin and cholesterol production.

3.Immunogenic effect

Male albino rats were fed with White Kwao 10, 100 and 200 mg/kg BW, 3 times per day for 14 days. It was found that White Kwao (100 mg/kg BW) could significantly reduce the number of red blood cell, and neutrophillic segmented cell but the number of lymphocytes were increased whereas the number of eosinophil, monocytes and neutrophillic band cell were not different from the control (Pongdom et al., 1987).

It was shown that male quails were fed with commercial food mixed with tuberous roots of White Kwao at 5% and 10%. It was found that both concentration of White Kwao could reduce the percentage of red blood cells, and the hemoglobin. White Kwao had negative effect on decreasing lymphocyte (Thaiyanan et al., 1993). White Kwao could also reduce the immune response to red blood cell in quails treated with White Kwao. But the quails immunized with sheep red blood cell before they were treated with White Kwao were found to have the same immune response as control group.

E. Development of the preimplantation embryo

In mammals, the fertilization takes place in the ampulla of the oviduct. During the following days, the embryo travels down the oviduct to the uterus, and prepares for implantation. In most mammalian species, the study of the preimplantation stages are characterized by a relatively synchronous doubling of cell numbers until the 8-cells stage, followed by a synchronous cell divisions after compaction. At the 8-to 16-

cell stage the embryo enters the uterine environment, developes into a blastocyst, in which the first events of cellular differentiation are observed. At the blastocyst stage the embryo hatches from the surrounding zona pellucida and subsequently implants in the uterine wall (Bowman and McLaren, 1970).

Mouse embryos take about three and a half days to develop from the 1-cell stage to the blastocyst stage containing 32 or more cells. The first, 1-to 2-cell and, 2-to 4-cell cycles of the mouse embryo take between 16 to 20 hours and 18 to 22 hours respectively, depending on the strain of mice (Harlow and Quinn, 1982; Streffer et al., 1980).

Compaction is the first event of morphologic and cellular differentiation. The most significant event occurring at compaction is the emergence of a distinct cell populations: the blastomere and the trophectoderm. The outer cell population of the blastomere develops to form the trophectoderm, while the inner cell population is becoming the inner cell mass (Johnson and Ziomek, 1981). The trophectoderm cells acquire the characteristics of epithelial cells in being flattened and joined together by tight junctional complexes (Ducibella and Anderson, 1975). When the mouse embryo has about 32 cells, trophectodermal cells begin to pump fluid into intracellular spaces and later into extracellular spaces, forming the blastocoelic cavity (Borland et al., 1977). The blastocyst contains two distinct cell types: the inner cell mass, which go on to form the embryo proper, and the trophectodermal cells which are involved in the initial contact with the uterine wall and eventually contribute to the placenta and the extraembryonic membranes.

In conjunction with changes in specific energy metabolites, the development of the embryo is also regulated by amino acids, vitamins and growth factors (Gardner and Sakkas, 1993). Studies relating to the factors affecting metabolism of the preimplantation and development of the embryo are however limited to *in vitro* culture conditions and it is not necessary to identify the actual factors that are critical to the embryo *in vivo*. For example, although glucose is present in the oviductal fluid (Gardner and Leese, 1990), *in vitro* it can be detrimental to the pre-compacted mouse embryo by causing cleavage arrest or retarding the cleavage rate (Brown and Whittingham, 1992; Sakkas et al., 1993).

The rapid increase in cell number post compaction and the changes in the metabolic activity can lead to the question of whether growth factors are involved in the development of the preimplantation embryo development. The transcripts for transforming growth factor α (TGF α), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and insulin-like growth factor II (IGF-II) have been detected in cleavage stage mouse embryos as well as the transcripts insulin receptors, IGF-I receptors, epidermal growth factor (EGF) receptors and PDGF α subunit receptors (Rappolee et al., 1988; Wiley et al., 1992).

F. The effect of estrogen on embryonic development

The naturally occurring estrogens are 17β-estradiol, estrone, and estradiol.

They are C_{18} steroids; i.e., they do not have an angular methyl group attached to the 10 position or a Δ^4-3-keto configuration in the A ring. Primarily the estrogen secretes by the granulosa cells of the ovarian follicles, the corpus luteum, and the placenta.

Estrogen was recognized as essential for embryonic development and maintenance of pregnancy. The function of estrogen was mediated through its specific estrogen receptor. Using reverse transcription polymerase chain reaction, estrogen receptor (ER-RNA) was detected at the one-cell, two-cells, and four-cells stages. The level became undetectable at the five- to eight-cell stages and the morula stage and then reappeared again at the blastocyst stage. The presence of ER mRNA at the blastocyst stage will show estrogen may start to act directly on embryos afterwards and results provide a basis for determining the direct role of estrogen in implantation embryos (Ying et al, 2000).

The mature blastocyst "hatches" out of the zona pellucida before implanting into the uterine endometrium. Deprivation of estrogen causes in a delay in hatching. Although the mechanism of the zona hatching remains to be clarified, these results lead to a hypothetical mechanism by which estrogen stimulates the zona hatching. It may be speculated that estrogen acts on the blastocyst to increase the production of epidermal growth factor receptor on trophoblast cell surface as well as the production of epidermal growth factor in the blastocyst itself and in the uterine epithelium. In the trophoblast, the produced epidermal growth factor binds the epidermal growth factor receptor of its own or neighboring trophoblast, by the paracrine or autocrine mechanism (Paria and Dey, 1990).

G. Principle, modules, rationale, or hypothesis

White Kwao, family *Laguminosae*, is one of the medicinal plants that predominated in the northern part of Thailand. It has been reported to have estrogenic effect on female reproductive tract in many species, *in vivo*, such as Japanese quail (Muangdech, 1984), chick (Wiriya and Smitasiri, 1987), rat (Songkaew, 1987), mice (Tanachai and Smitasiri, 1987), pigeon (Smitarisi and Kaweewat, 1991), American cockroaches (Radomsuk, 1991) etc. However, no scientific information of the direct effect of White Kwao on the development of mammalian embryos was available.

Estrogen is known as the important hormone that influences embryo development and implantation. The previous studies indicated that estrogen could regulate the cell growth and differentiation by stimulation the local expression of peptide growth factors (Murphy et al., 1987; Dickson and Lippman, 1987).

There are many estrogenic substances called phytoestrogen found in White Kwao such as deoxymiroesterol, daizein, etc. The recent study reported that the isoflavones might be playing a role in the embryonic development (Walter and Wheeler, 2000).

Therefore, it is interested to investigate the effect of White Kwao extract on the *in vitro* development and implantation of mouse embryos.

H. Objective

1. To evaluate the effect of White Kwao extract on *in vitro* development of preimplantation mouse embryo

2. To evaluate the implantation rate of mouse embryos which they were cultivated in media containing White Kwao

I. Usefulness

1. To provide an information regarding the effect of White Kwao extract on *in vitro* development and of preimplantation mouse embryo as basic knowledge of furthers White Kwao research

2. To provide an information about the preimplantation rate of the mouse embryos growing in the medium supplemented with White Kwao

3. The knowledge gained from this study can be used for further study in the field of reproductive using White Kwao.