รายงานการวิจัย ฉบับสมบูรณ์

ผลของสารสกัดกวาวขาวต่อการสุกและการการปฏิสนธิภายนอกร่างกายของไข่หนูถีบจักร

Effect of White Gwow (Pueraria Mirifica Airy Shaw et Suvatabandhu) Extract on In vitro Maturation and Feritilization of Mouse oocytes

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สนับสนุนโดยทุนอุดหนุนการวิจัยจากกองทุนพัฒนาคณะแพทยศาสตร์-ส่วนที่ 1
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ABSTRACT

White Gwow (Pueraria mirifica Airy Shaw et Suvatabandhu) is ragarded as a medicinal herb. It has been reported to have some effects on female reproductive tract in many species. However, no scientific information on the effect of White Gwow on the in vitro maturation and fertilizability of mammalian oocyte were available. Therefore, the purpose of this study was to evaluate the effect of White Gwow extract on in vitro maturation and fertilizability of mouse oocytes.

This study was designed into 2 experiments. In the first experiment, approximately 24-day-old female ICR mice were killed and the germinal vesicle (GV) stage oocytes were collected from their ovaries. The immature oocytes were randomly incubated for 24 and 48 hours at 37°C in Waymouth medium containing various concentrations of Lyophilized White Gwow in gas phase of 5% CO_2 and 100% humidified air. The results showed that concentrations of White Gwow ranging from 1.0 to 10.0 mg/ml significantly ($P<0.05$) increased the proportion of GV-stage oocytes developed to metaphase II stage after incubation for 24 hours (7.00±0.85 - 8.35± 1.49 vs 5.70±0.65 for control) and 48 hours (7.70±1.03 - 8.85±1.26 vs 6.10±0.55 for control). However, the concentrations of 15.0 and 20.0 mg/ml significantly ($P<0.05$) inhibited oocyte maturation compared with the control group (at 24 hrs: 4.55±1.14 and 3.30±1.08, respectively vs 5.70±0.65; at 48 hrs: 4.80±1.00 and 3.55±1.10, respectivelyvs6.10±0.55).

The second experiment was conducted, to test the fertilizing ability of the in vitro matured oocytes from each group of experiment I. The mature oocytes were inseminated with sperm collected from the cauda epididymide of mature male mice, and incubated in 5% CO_2 in air at 37°C for 24 hours. The results showed that control group and concentrations of White Gwow ranging from 0.5-10.0 mg/ml had significantly ($P<0.05$) different fertilization rate competence. The concentrations of

White Gwow ranging from 0.5 to 10.0 mg/ml tended to increase the fertilization rate after 24 hours of incubation when compared with the control group (59.05%-65.62% vs 54.78%). However, the concentrations of White Gwow extract at 15.0 and 20.0 mg/ml tended to inhibit fertilization competence (37.63% and 25.58%, respectively vs 54.78%).

This study indicated that White Gwow extract stimulated oocyte maturation in vitro and did increase in fertilizability of the in vitro matured oocytes in a dose response manner. The exact mechanism that White Gwow stimulated oocyte maturation and led to better ferilization rate were unknown. The suspect mechanism may in a manner similar to the mechanism that estrogen affects the oocyte maturation and did increased in fertilizability. Further studies are needed to use the purified White Gwow extract that has estrogenic effect and to investigate the effect of White Gwow on quality of embryo developed in vitro and implantation.

บทคัดย่อ

กวาวขาว (Pueraria mirifica Airy Shaw et Suvatabandhu) จัดว่าเป็นพืชสมุนไพร ซึ่งมีรายงานว่ามีผลบ้างต่อระบบการเจริญพันธุ์ของสัตว์เพศเมียในหลายสปีชีส์ อย่างไรก็ตามยังไม่มีข้อมูลทางวิทยาศาสตร์เกี่ยวกับผลของกวาวขาวต่อการสุกและความสามารถในการปฏิสนธิภายนอกร่างกายของไข่ในสัตว์เลี้ยงลูกด้วยนม ดังนั้นการศึกษานี้มีจุดประสงค์เพื่อประเมินผลของสารสกัดจากกวาวขาวต่อการสุกและความสามารถในการปฏิสนธิภายนอกร่างกายของไข่หนูถีบจักร

การศึกษานี้ออกแบบเป็นสองการทดลอง การทดลองแรกนำหนูเพศเมียอายุประมาณ 24 วัน มาฆ่าเพื่อเก็บไข่ในระยะ GV-stage จากรังไข่ จากนั้นแบ่งไข่ซึ่งยังไม่สุกแบบสุ่มออกเป็นกลุ่มใส่ในน้ำยาเพาะเลี้ยง Waymouth ที่มีสารสกัดจากกวาวขาวที่ความ เข้มข้นต่างๆกัน นำไปเพาะเลี้ยงในตู้อบที่อุณหภูมิ 37°C, 5% CO_2 และความชื้นในอากาศ 100% เป็นเวลานาน 24 ชั่วโมง และ 48 ชั่วโมง จากผลการทดลองพบว่าความเข้มข้นของกวาวขาว ตั้งแต่ 1.0 ถึง 10.0 มก./มล. มีผลเพิ่มสัดส่วนการสุกของไข่ ($P<0.05$) จากระยะ GV-stage ไปเป็นระยะ Metaphase II อย่างมีนัยสำคัญ ภายหลังการเพาะเลี้ยงเป็นเวลานาน 24 ชั่วโมง (7.00±0.85 - 8.35±1.49 เปรียบเทียบกับกลุ่มควบคุมคือ 5.70±0.65) และ 48 ชั่วโมงตามลำดับ (7.70±1.03 - 8.85±1.26 เปรียบเทียบกับกลุ่มควบคุมคือ 6.10±0.55) อย่างไรก็ตามที่ความเข้มข้น 15.0 และ 20.0 มก./มล. มีผลยับยั้งการสุกของไข่อย่างมีมีนัยสำคัญ ($P<0.05$) เมื่อเทียบกับกลุ่มควบคุม (ที่ 24 ชั่วโมง: 4.55±1.14 และ 3.30±1.08 ตามลำดับ เปรียบเทียบกับกลุ่มควบคุมคือ 5.70±0.65; ที่ 48 ชั่วโมง: 4.80±1.00 และ 3.55±1.10 ตามลำดับเปรียบเทียบกับกลุ่มควบคุมคือ 6.10±0.55)

ในการทดลองที่สอง ได้ทำการทดสอบความสามารถในการปฏิสนธิของไข่ที่สุกเต็มที่แล้วจากการทดลองแรก โดยนำไข่จากแต่ละกลุ่มมาปฏิสนธิกับอสุจิซึ่งเก็บมาจากส่วน cauda epididymide ของหนูเพศผู้ที่โตเต็มวัย และเพาะเลี้ยงไว้ในตู้อบที่มี 5% CO_2, อุณหภูมิ 37°C เป็นเวลา 24 ชั่วโมง ผลการทดลองพบว่ากลุ่มควบคุมและความเข้มข้นของกวาวขาว ตั้งแต่ 0.5 ถึง 10.0 มก./มล. มีอัตราการปฏิสนธิที่แตกต่างกันอย่างมีนัยสำคัญ ($P<0.05$) โดยที่ความเข้มข้นของกวาวขาว ตั้งแต่ 0.5 ถึง 10.0 มก./มล. มีแนวโน้มเพิ่มอัตราการปฏิสนธิ ภายหลังเพาะเลี้ยงเป็นเวลา 24 ชั่วโมง 59.05%-65.62% เมื่อเทียบกับกลุ่มควบคุมคือ 54.78%) อย่างไรก็ตามที่ความเข้มข้น 15.0 และ 20.0 มก./มล. มีแนวโน้มยับยั้งความสามารถในการปฏิสนธิ (37.63% และ 25.58% ตามลำดับ เมื่อเทียบกับกลุ่มควบคุมคือ 54.78%)

การศึกษานี้บ่งชี้ว่าสารสกัดจากกวาวขาว มีผลกระตุ้นการสุกของไข่อย่างมีนัยสำคัญและเพิ่มความสามารถในการปฏิสนธิภายนอกร่างกายของไข่ในรูปแบบการตอบสนองต่อความเข้มข้น กลไกที่แท้จริงที่กวาวขาวมีผลกระตุ้นการสุกของไข่และนำไปสู่อัตราการปฏิสนธิที่ดีขึ้นยังไม่สามารถอธิบายได้จากผลการทดลองนี้ แต่กลไกที่อาจเป็นไปได้คือกลไกที่คล้ายคลึงกับเอสโตรเจนที่มีผลต่อการสุกและเพิ่มความสามารถในการปฏิสนธิของไข่ จึงเสนอแนะว่าในการศึกษาต่อไปจำเป็นต้องสกัดบริสุทธิ์สารที่มีผลคล้ายเอสโตรเจนในกวาวขาว จะสามารถพิสูจน์กลไกของกวาวขาวต่อการสุกของไข่ และสืบค้นผลของกวาวขาวต่อคุณภาพของตัวอ่อนที่เจริญในหลอดทดลองและการฝังตัว

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ABBREVIATIONS

ANOVA	analysis of varians
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cm	centrimetre
CO_2	carbondioxide
EKRB	enriched Krebs-Ringer bicarbonate
ER	estrogen receptor
FSH	follicle stimulating hormone
g	gram
hr	hour
GV	germinal vesicle
GVBD	germinal vesicle breakdown
IVM	in vitro maturation
IVF	in vitro fertilization
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium phosphate monobasic
LH	luteinizing hormone
MI	metaphase I
MII	metaphase II
M16	modified Krebs-Ringer bicarbonate
mg	milligram
$MgCl_2.6H_2O$	magnesium chloride hexahydrate

$MgSO_4.7H_2O$	magnesium sulfate hepahydrate
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$Na_2.HPO_4$	disodium phosphate
$NaH_2PO_4.H_2O$	sodium phosphate monobasic
OMI	oocyte maturation inhibitor
OMS	oocyte maturation stimulator
PBI	phosphate buffer saline
PB I	first polar body
PCE	poly chromatic erythocyte by micronucleus test
pg	picogram
PMSG	pregnant mare's serum gonadotropin
PN	pronuclei
rpm	revolution per minute
TAPSO	TAPSO, 3-[N- Tris(hydroxymethy)methyl amino]-2 hydroxypropanesulfonic
µm	micrometre
µl	microlitre
WG	White Gwow
w/v	weight per volume
w/w	weight per weight
ZP	zona pellucida

CHAPTER 1

INTRODUCTION

A. General background

Gwow Khruea is a plant of Leguminosae family, Papilionoidcae subfamily. Gwow Khruea is usually found in the jungles along the northern area of Myanmar and northern and western parts of Thailand. This includes in jungles of Chiang Mai, Chiang Rai, Mae Hong Son, Rachaburi, Kanchanaburi, Uthaitani and Tak (Chilek, 1988; Smithnat, 2000). However there have been reported that the plants of the same family of Pueraria, are also found in other Asian countries but are of different species and may have different biological activities from Gwow Khruea. Gwow Khruea has long been used in Myanmar and Thailand as a rejuvenating folk medicine which is well known to the practitioner of the traditional medicine. Gwow Khruea is also called Paukse in Myanmease language, and has been handed down generation to generation.

In the 1920s, the first document mentioned about Gwow Khruea was discovered as a single leaflet in Yuan character, without date or author, in an old Buddhist temple in Paga, an ancient capital of Myanmar, as the temple was reconstructed. There are four types of Gwow Khruea which are used as herbs; White Gwow (WG), Red Gwow, Black Gwow and Moh Gwow. Of these, the Black Gwow was the strongest and the White Gwow was the weakest potency in rejuvenation. The active principles of this plant are found in the tuberous root. The procedure in traditional use is after uprooting, the root was pealed, and sliced into thin pieces, then they were exposed to sunlight for 1 day and powdered. The dried powdered was

prepared as a pill by mixing with sweetened-condensed-skimmed milk or honey. It is believed that the tuberous root of WG is an elixir for longivity. It has traditionally been used for rejuvenation, brain naurishment, keep up eye sight, increasing breast size, making gray hair back into dark hair, help improve complextion and remove wrinkles, help relieve the post-menopausal syndromes, improve the sexual efficiency in aged women.

From the issue of Luang Anusarnsultorn's pamphlet (หลวงอนุสารสุนทร, 2474), it had different ways to compound Gwow Khruea preparations. In the traditional preparation, it was compound as a small pills as pepper seed. The drug were prescribed and many miraculous virtues of it was proclaimed. Only one pill will be taken daily at bed time. During the use of Gwow khruea, The patients should take a bath three times a day, stop eating pickled food and should not exposure to cold air. A given charm had to be repeated twenty seven times when the drug was compounded, and the five Buddhist precepts must be strictly practised while taking it. These pills were taken for three to six months. People under thirty five years old were forbidden to take the pills. (หลวงอนุ-สารสุนทร, 2474; Koisi, 2000).WG was first discovered by a British Professor Airy Shaw, who jointly studied this woody plant with Professor Kasin Suvatabandhu from Chulalongkorn University, Thailand. WG was definitively identified as Pueraria mirifica in 1952 (Lakshnakara et al., 1952), and an estrogenic phenol miroestrol was first isolated as an active principle eight years later (Jones and Pope, 1960). Therefore, miroestrol has been contemplated to be the actual phytoestrogen of Pueraria mirifica and has the highest activity of all estrogens of plant origin. Todate, using bioassay-guide purification has isolated a new potent phytoestrogen, deoxymiroestrol. It was found that the facile aerial oxidation of deoxymiroestrol to miroestrol suggests the possibility that miroestrol may be an artifact. Strong activity was observed for both

miroestrol and deoxymiroestrol, but the latter was found to be about 10-fold more potent (Chansakaow et al., 2000a).

In the modern time, the potential use of WG in agriculture and public health were discovered in many aspects. It was found that WG induces udder development in economic animals, suppresses bad smell from the boar, induces beautiful fur in some kinds of animals, tends to increase sperm of chicken, reduces sex drive in both sexes of bandicoot, pigeon, dog, house rat and mice. WG could also control the fertility of insects by both induction and suppression. The results of WG depend on the dosage, potency and duration of WG action (Smitasiri et al., 2000).

At present, the most commonly uses of Gwow Khruea in cosmetic and food supplements are White Gwow and Red Gwow. WG 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 WG powder of both tablet and capsule are approximately 100-600 mg depending on the manufacturer. Todate, WG 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 fetal colon cancer, alleviating arthritic pain and warding off the onset of rheumatoid arthritis, naurishing skin, keeping hair stronger and reducing weight. It is not recommended for pregnant woman and woman under 25 years old. Red Gwow is used for enhancing male sexual potency by increasing erection strength and timing, enhancing hair growth, curing gout, preventing blood occlusion, and prevention of osteoporosis. It is not recommended for young male (Cherdcheewasard, 2000; nawapol, 2000).

Oocyte maturation in vitro has been reported in many mammalian species. Pincus and Enzmann (1935) first demonstrated that rabbit oocytes dislodged from their follicular environment undergo spontaneous maturation in vitro even in hormone-free media. They suggested that some follicular components may impart an inhibitory

influence upon the oocyte maturation in vitro which serves to maintain the oocyte in a stage of meiotic arrest (dictyate stage). The observation has been confirmed and extended to other mammalian species examined, i.e., mouse (Moricard and Forbrune, 1937; Gothie and Tsatsatsaris, 1939; Edwards, 1962) , rat and hamster (Edwards, 1962), sheep, cow and pig (Edwards, 1965a), monkey (Edwards, 1962) and man (Pincus and Saunders, 1939; Edwards, 1965a,b; Edwards et al., 1966). Recently, there are many culture media used for in vitro maturation, e.g. for example TCM 199, Earle's salts medium (Tsafriri and Channing, 1974), Eagle's minimal essential medium (Brower and Schultz, 1982; Canipari et al., 1984), L-15 Leibovitz's medium (Feng et al., 1988), Menezo's B_2 and B_3 (Tesarik and mendosa, 1995), Waymouth medium (Eppig et al., 1993), and Ham's F-10 medium (Feng et al., 1988; Das et al., 1992) to which was added the inducer, i.e. growth factor (Das et al., 1992), gonadotropins (Tsafriri et al.,1976) and estrogen (Tsen et al., 1997).

One important problem of in vitro maturation of oocytes is the lower rates of fertilization than in vivo maturation. It has been reported that oocytes cultured in cultured medium cause zona hardening, by changing the structure of ZP_2 to ZP_{2f} (Ducibella et al., 1990). The zona hardening caused the lower number of sperm to penetrate oocyte and could prevent this problem by adding feturin in culture medium (Schroeder et al., 1990).

B. General characteristic of White Gwow

The scientific name of White Gwow is Pueraria mirifica Airy Shaw et Suvatabandhu which is named after the discoverer. It is a plant grown in the group of leguminosae and is a deciduous woody climber with variations of different globular, tuberous roots that have the function of keeping nutrients. Its appearance is similar to

yam root. Inside, the root is white and the taste can cause dizziness. The root has various sizes depending on the soil condition or the environment and the time of cropping. The trunk of WG is approximately 5 meters long, proximal diameters are between 4 to 16 centimeters (Figure 1). The leaf consists of 3 minor leaves put together in one stem. The edges of the leaves are smooth, have sharp ends and has delicate hairs on both sides of the leaf (Figure 2). The full development of leaves occurred during May to September then the leaves became senscence on October and after that the leaves were fallen. Then young leaves were developed very fast during the end of March to April (Jun-ngern and Smitasiri, 1987). The flower is similar to the bean flower, each compose of five petals on both sides that are curved, and the inferior petals wrap an ovary. The color of the flower of WG is bluish-purple. The flower is in a bunch, length approximately between 15.0 to 40.3 centimeters and it blossoms between January and March, for approximately 10 days (Figure 3). When the flower of WG falls down, one can see the shell. The shell of WG is that of a legume type that is slender-long, sharp beak, flat and has minimal hairs covering it (Figure 4). The shell has 1-9 seeds inside. The shape of its seed is similar to been seed, 2 millimeters wide and 4 millimeters long. The length of the fully mature shell is approximately between 1.0 to 7.5 centimeters.

WG grows in the forest with many variety of trees on the height mountain above sea level of 300 to 800 meters, the soil that riches of organic substance, pH approximately 5.5. The development in nature, it was found that during afternoon in the summer season and lack of water, the temperature between 30-37^{0}C, the stem of WG was elongated very quickly but during the falling rain for 2-3 days and the temperature lower than 30^{0}C. The stem was stop for growing by elongation but the change of the size of leaves and petiole development were very fast (Wungjai and Smitasiri, 1987; Smitasiri, 1987).

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

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

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

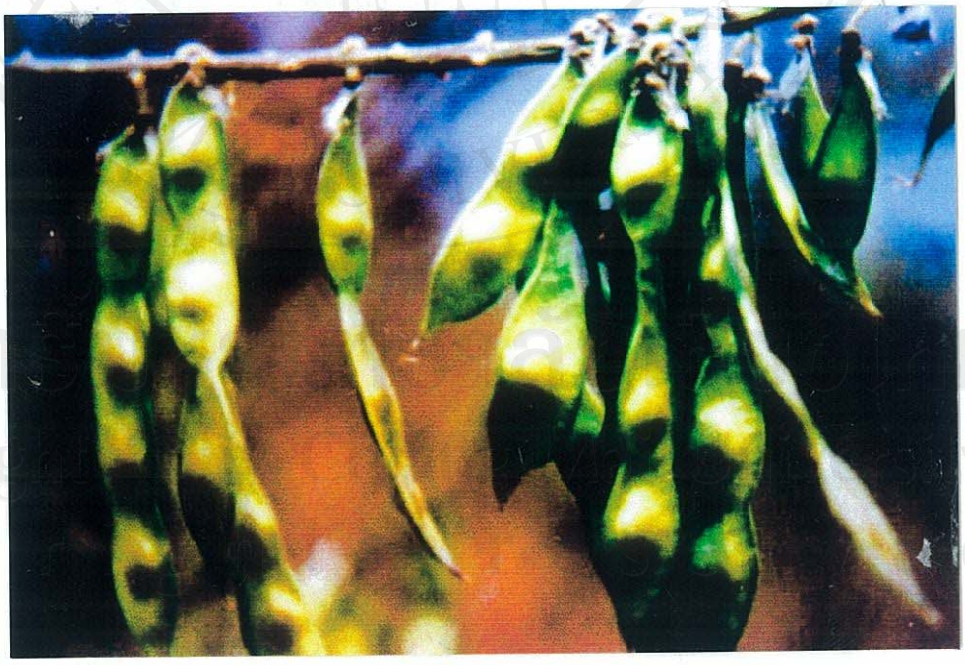


Figure 4 The shells of White Gwow (Chailek, 1988)

C. Components of White Gwow

The tuberous root of WG accumulates many substances as classified follow.

1. Chromenes

Chromenes are importantly active component of WG. These components consist of miroestrol and deoxymiroestrol (Figure 5), which posses highest estrogenic activity. There are two types of miroestrol crystal; have an hydrated form and anhydrous form, colorless, melting point is 268-270^{O}C and molecular formula was inferred to be $C_{20}H_{22}O_6$. Deoxymiroestrol was obtained as colorless prisms, melting point 213-216^{O}C, the molecular formula was inferred to be $C_{20}H_{22}O_5$. Both miroestrol and deoxymiroestrol have a common skeleton with the same stereochemisty in which C-14 hydroxyl group in miroestrol has been replaced by hydrogen atom with retention of configuration (Chansakaow et al., 2000a).

2. Flavonoids

Besides chromenes, the previous reports showed the presence of various isoflavonoids (Ingham et al., 1986; Tahara et al., 1987). There are two types of isoflavonoids, the first one is non-glycosidic isoflavonoids such as daidzein, genistein, kwakhurin and tuberosin. The other is glycosidic isoflavonoid; diadzin, mirificin (Figure 6) and puerarin. It has been reported that genistein had 10-fold stronger activity than daidzein (Cain, 1960). Kwakhurin was also found to have moderate activity compared with daidzein (Martin et al., 1978; Verdeal and Ryan, 1979; Farmakalidis et al., 1985; Mmiksicek, 1993; Hsieh et al., 1998). Recent study on the chemical constituents of the tuberous root of this plant has reported new isoflavonoids, ptercarpene and puemiricarpene. Some isoflavonoids are known as phytoestrogens. The presence of phytoestrogen belonging to the isoflavonoids may partly cause the revejunating action of this plant (Chansakaow et al., 2000b).

3. Coumarins

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

4. Steroids

There are some steroids in WG such as β-sitosterol, stigamasterol and puemarin mirifica sterol. Steroids have not been reported to possess estrogenic activity.

5. Others

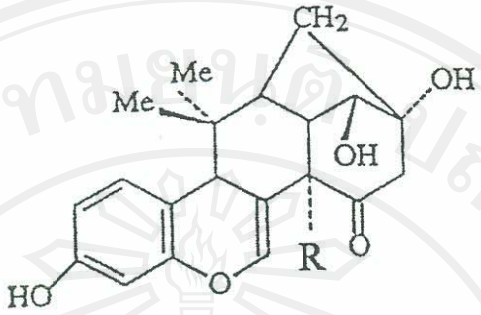
In addition to the above, there are other components of WG such as lipid, lithium, potassium, sodium, phosphorus ,calcium oxalate and sucrose have been reported (Ingham et al., 1986).

D. Action of White Gwow

3.1 Estrogenic effects

3.1.1 Effect on mammary glands and lactation in lactating animals

The effects of WG on the development of mammary glands in bilaterally ovariectomized rats and mice showed that WG could stimulate the development of mammary tissues and enlarge the breast size by lengthening and branching the mammary ducts that connect to the nipple (Benson et al., 1961; Sawatdipong, 1979). In contrast, WG caused the decrease in the weight of mammary glands in 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 WG and estrogen-treated rats. These data suggested that WG could inhibit lactation in the lactating rats possibly by inhibiting growth of mammary gland and milk production like estrogen.



(+)-1: R=OH

(+)-2: R=H

Figure 5 Structures of (+)-miroestrol (1) and (+)-deoxymiroestrol (2) (Chansakaow et al., 2000a)

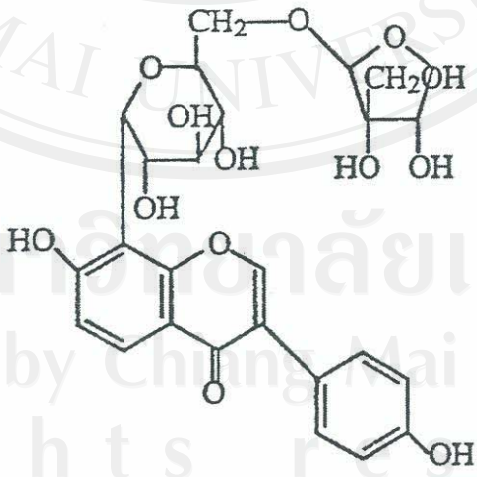


Figure 6 Structure of mirificin (เอมอร โสมนะพันธุ์ และ วีณา จิรัจฉริยากูล, 2542)

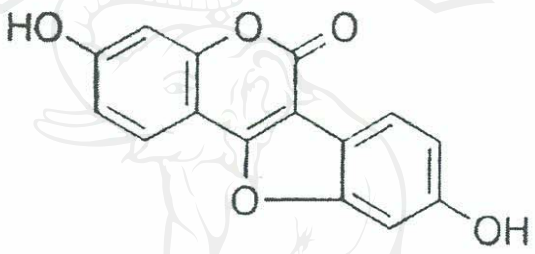


Figure 7 Structure of coumestrol (Chansakaow et al., 2000b)

3.1.2 Effect on reproductive organs of animals

It has been reported that feeding mice with WG 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 5% WG mixed by weight into commercial food to feeds one hundred-fifty five-day old Japanese quails for ten days. causes increase in the oviductal weight after treatment for only two days. This data is in agreement with Chuaychoo (1984); Maungdech (1984); Smitasiri et al. (1992).

Maungdech (1984) and Thuppongse (1984) reported on quials fed with WG mixed with commercial food. WG was found to decrease the weight of the ovary and increase the number of follicles, It was possible that WG inhibited gonadotropins (FSH/LH) released by negative feedback on the anterior pituitary gland. In addition, WG was found to increase uterine weight (Songkaew, 1987).

Junyatum (1983) reported that WG dissolved in distilled water and given orally to young rats at dose of 25, 50 and 100 mg/day decreased testis weight, which is in agreement with Jesrichai (1983); Langkalichan(1984); Tanachai and Smitasiri (1987) and Smitasiri and Kaweewat, (1992).

3.1.3 Antifertility effects

Langkalichan (1984) found that high dose of WG (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 WG in male rats treated and mated with normal females and the number and size of implantation in both uterine horns were significantly reduced. The gestation length 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 young was found in this study. In addition, Smitasiri et al.(1986) reported on the effect of WG given during different periods of pregnancy, i.e., during the embryo transport period, during the implantation period and during postimplantation period. The results clearly showed that WG was effective in preventing pregnancy in rats when given during embryo transport period. This was in agreement with Tanachai and Smitasiri (1987) who reported that female mice fed with pellet mixed with WG and caged with male since the first day or the tenth day of treatment until 60 days of treatment, could not deliver in both groups. When laparatomy was made, it was found that only one mouse of each group was pregnant (14.29%). In addition, the effect of WG extracts on reproduction of American cockroches has been reported by Radomsuk (1992). Female American cockroaches were fed ethanolic and aqueous extracts of WG tuber mixed with food for 15 days. The results showed that ovum size of WG treated groups was significantly smaller than the control and abnormal ovaries were usually found but WG had no effect on the colleterial gland. When WG extracts were fed to both sexes of cockroaches reared together for 30 days, it was found that the number of ootheca and non hatching were quite high in most of the WG-treated groups.

3.2 Change of calcium, total protein and cholesterol concentration in blood

Suthikarn (1985), reported that all doses of WG:1, 3, 5, 7 and 9% by food weight, fed to coturnix quails for 15 days, caused increase in calcium levels, although the duration of this effect did not increase with increasing doses. Jesrichai (1985) also noted that doses of WG of 5% and 7% by weight, administered for 60 days caused increased in calcium levels in quail.

Anantabhochai and Jesrichai (1986) reported on immature corturnix quail fed with 5% and 10% WG by food weight for 60 days. Every 10 days, plasma samples were taken to determine the calcium, total protein and cholesterol concentrations. It was found that the correlation coefficient between the quantity of WG given and calcium and cholesterol concentrations was only significant in the female bird; total protein concentration was not changed. Also, Jesrichai (1985) showed that both of high dose of WG (5 and 7%) could increase serum calcium concentration more than control distinctly. In contrast, Thaiyanan et al. (1992) reported that WG had positive effects on increase of body weight, total protein, albumin, globulin and cholesterol production when quail were treated with starter containing 5% and 10% WG (w/w).

3.3 Immunogenic effect

Anuntalabhochai et al. (1982) and Chuaychoo et al. (1984), reported that Japanese quail were fed with high doses of WG posses inflamatory activity and cause tenderness in some part of body such as head, under wing, legs and claw. The intensity depended on the quantity of WG and duration of feeding. It was suggested that WG may be decrease the immune system of quail that cause bacterial growth.

Pongdum et al. (1987) studied the effects of WG on blood corpuscles in male albino rats. They found that WG 100 mg/kg/time could significantly decrease the number of red blood cells, haematocrit and neutrophillic segmented cells but the number of lymphocytes were significantly increased. The number of eosinophil, monocytes and neutrophilic band cell did not differ from the control.

Tragoonboon et al. (1987) found that WG decreased the immune response to sheep red blood cells in quail treated with WG before and at the same time as immunization with sheep red blood cells was given. But the quail immunized with

sheep and red blood cells before treated with WG were found to have an immune response the same as control group.

E. Mammalian oocyte maturation

The fundamental functions of the mammalian ovary are production of mature oocytes and biosynthesis of sex hormones. These functions are archieved during follicle development and represent a complex process under strict hormonal control.

Meiosis is a process that reduces the number of chromosomes from the diploid number to the haploid number (Figure 8). In human, meiotic maturation of mammalian oocyte is a prolonged process, theme to multiple stop-go controls (Figure 9). Oocytes begin first meiotic devision in the ovary of late fetal life. By the time of birth, They have already became the diplotene stage of meiotic prophase I with the chromosomes still enclosed by a nuclear membrane known as a germinal vesicle (GV) and just prior or shortly there after it is arrested in dictyate stage during prophase (Borum, 1961; Baker, 1972; 1982). At this stage primary oocytes are surround by a few layers of non proliferating flat granulosa cells forming a unit within primordial follicle, here after referred to as primordial oocytes. Once primordial follicles enter growth phase, the morphological modification are an increase in oocyte volume, mitotic division of granulosa cell and zona pellucida formation. This growth of primordial follicle to preantral follicle was independent of gonadotropin influence from anterior pituitary gland. Once a few thousand granulosa follicular fluid-filled cavity follicles have accumulated, they form an antrum. There are two populations of granulosa cells as a consequence of antrum formation; mural granulosa cells line the follicular wall and form an epithelium, while the cumulus granulosa cell surrounds the oocytes (Anderson and Albetini, 1976; Sorensen and Wassarman, 1976; Chaning et al., 1978; Tsafriri, 1978). At this stage, the oocytes are surrounded by several layers of granulosa cells

and the shape is changed to form the cumulus oophorus (Homa, 1995). The growth and differentiation of granulosa cell were regulated by the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the proliferation of granulosa cells and aromatase activity leading to preovulatory follicular growth and estradiol secretion (Daniel and Armstrong, 1980; Hillier and DeZwart, 1981). Estradiol secretion has a peaks in the late follicular phase and triggers midcycle of LH surge which act as a positive feed back on the hypothalamus and anterior pituitary gland. Under the initiation of LH, the germinal vesicles break down (GVBD; the nuclear envelope and the nucleoli disappearance), and meiosis then proceeds into the metaphase stage of second meiotic division (M II; as indicated by extrusion of the first polar body) and arrested at this stage where the oocyte will wait for fertilization (Edward, 1965a; Amsterdam and Rotmensch, 1978). Cytoplasmic events also occur to promote pronuclear formation and preparation for embryogenesis. Cytoplasmic maturation is a process that prepares activation of oocyte, formation of pronuclei and preimplantation development (Eppig et al. 1994).

There are many hormones that affect oocyte maturation such as FSH, LH and estrogen. FSH is the prime inducer of ovarians follicle maturation and responsible for the develpoment of granulosa cell responsiveness to several other hormones. FSH stimulates the granulosa cells to secrete estrogens and progestins as well as various nonsteroidal substance. The action of these multiple granulosa cell products ensures optimal folliculogenesis and oocyte maturation. Since FSH receptors are present exclusively in the granulosa cells, various ovarian effects of FSH are believed to be mediated through granulosa cells (Hsueh et al., 1984). LH stimulates preovulatory follicle growth, induces ovulation, and regulates corpus luteum function. After the FSH induction of LH receptors in cultured rat granulosa cells, these cells are capable of responding to LH in the maintenance of aromatase activity (Wang et al., 1981; Zhuang et al, 1982). There are no LH receptor in oocytes. Therefore, LH probably

induces GVBD by an indirect action mediated by follicular somatic cells, probably granulosa cells (Delkel, 1988). Rapid changs occuring in the multilayered, avascular granulosa cells of the ovarian follicles suggest a necessary for cell-to-cell comunication. In immature hypophysectomized rats, estrogen administration increase intercellular gap junction formation in granulosa cells (Merk et al., 1972). Thus, the coupling of granulosa cells may be estrogen-dependent.

F. Mammalian oocyte fertilization

In vivo fertilization in mammals normally occurs in the ampulla of oviduct and is defined as the sperm-egg interaction, leading to the paternal and the maternal sets of haploid chromosomes. The fusion of spermatozoa with oocyte plasma membrane begin after the spermatozoa pass through the zona pellucida. Only the acrosome reacted spermatozoa penetrate through the cumulus. The granulosa cell is digested by acrosomal contents (acrosin and hyaluronidase), thus spermatozoa can pass towards the zona pellucida. In the mouse, the zona pellucida is composed of three major glycoproteins: ZP_1 , ZP_2 and ZP_3 . It has been reported that binds spermatozoa to ZP_3 and ZP_2 maintains attachment of sperm after acrosome reation (Philpott et al., 1987; Florman and Wassarman, 1980). After zona pellucida penetration occur, the head of the sperm is incorporated and eventually the tail into ooplasm, fusion occurs at the anterior region of the post acrosomal area and the plasma membrane over the equatorial segment of the acrosome (Yanagimachi et al., 1970; 1984; Bedford, 1972). The sperm-egg fusion is followed by the disappearance of nuclear envelope of sperm nucleus and decondensation of the sperm nucleus occurs. The fusion of sperm-egg in mammals results in deporalization of the egg plasma membrane referred to plasma membrane block, followed by fusion of cortical granules in the cortex of the ovum at

the surface of vitelline membrane and releasing contents into the perivitelline space. (Yanagimachi, 1984). The mechanism to prevent polyspermy depends on the species.

Sperm-egg nuclei decondensation will develop to form sperm and egg pronucleus and undergo the first clevage of embryogenesis.

The first successful in vitro fertilization in mammals was demonstrated about 40 years ago, was achieved in the rabbit (Chang, 1959). The fertilization of mammalian oocytes in vitro system has been extended to the hamster (Yanagimachi and chang, 1963), mouse (Iwamatsu and Chang, 1969), chinese hamster (Pickworth and chang, 1969), human (Edwards, 1969), guinea pig (Yanagimachi, 1972), cow (Wright et al., 1976a,b) and monkey (Gould et al., 1973; Kreitmann et al., 1982). The development of the IVF technique has improved our understanding of the inter-specific gamete interaction in mammals.

G. Principle, models, rationale, or hypothesis

WG is regarded as a rejuvenating folk medicine from Thailand. It has been reported to have estrogenic effect on female reproductive tract in many species, in vivo. such as Japanese quail (Muangdech, 1984; Thuppongse, 1984; Kiateadisorn, 1985) chick (Wiriya and Smittasiri, 1987) mice (Tanachai and Samittasiri, 1987) rat (Songkaew, 1987; Chailek, 1988; Samitisiri et al., 1989) pigeon (Smitasiri and Kaweewat, 1991) American cockroaches (Radomsuk, 1991) etc. However, no scientific information on the effect of WG on in vitro maturation and fertilization of mammalian oocyte were available.

Estrogen is known as the important hormone that influences oocyte maturation and fertilization (Tesarik and Mendosa, 1995). Potentially, estrogen produced by cumulus cells and stimulates cumulus cell proliferation and synthesize more estrogen. estrogen increases intercellular gap junction formation in granulosa cells (Merk et al.,

1972). Thus, the coupling of granulosa cells may be estrogen-dependent. Several small gap junctions are present in the region of contact and provide a mechanism for regulating the oocyte maturation (Norton et al., 1978).

The spontaneous meiotic maturation of isolated oocytes lead to basic conclusion that substances in follicular fluid have inhibitory activity (Chang, 1955). The substances are called oocytes maturation inhibitor (OMI) e.g. hypoxanthine, prolactin and inhibin (Tsafriri et al., 1976c; Tsafriri and Bar-Ami, 1981; Eppig and downs, 1987) . However, the spontaneous meiotic maturation of oocytes is limited because the development of germinal vesicle (GV)-stage oocyte to metaphase II oocyte is controlled by other factor; e.g. inducer that may be hormone or oocyte maturation stimulator (OMS) (Downs et al., 1988).

Therefore, it was of interested to investigate the effect of WG extract on oocyte maturation and fertilizability of in vitro matured oocyte.

H. Objectives

1. To evaluate the effect of WG extract on in vitro maturation of mouse oocytes
2. To evaluate the effect of WG extract on fertilizability of in vitro matured oocytes.

I. Usefullness

1. To provide an information regarding the effect of WG extract on in vitro maturation and fertilization of mouse oocytes as basic knowledge of further WG research.

2. The knowledge gained from this study will serve as a basic for further advanced research of WG.

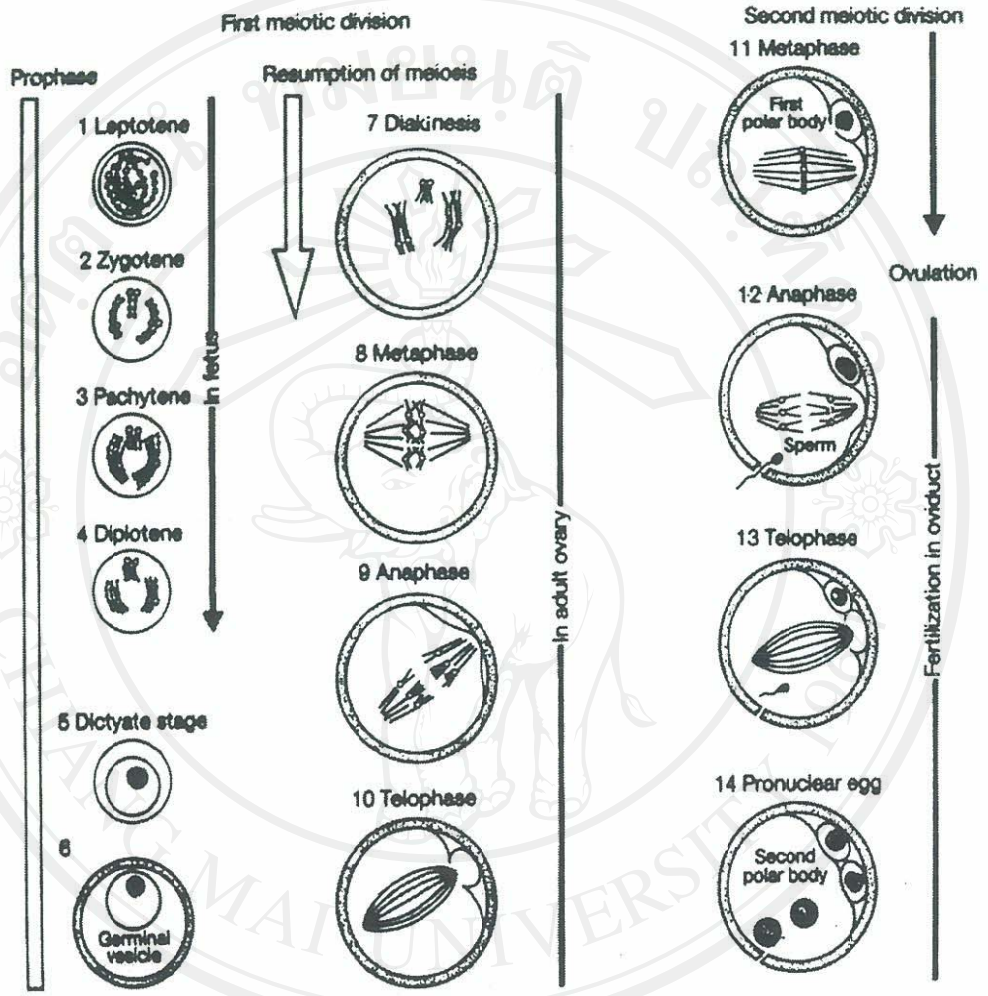


Figure 8 Schematic presentation of the oocyte meiosis (Szollsi et al., 1983)

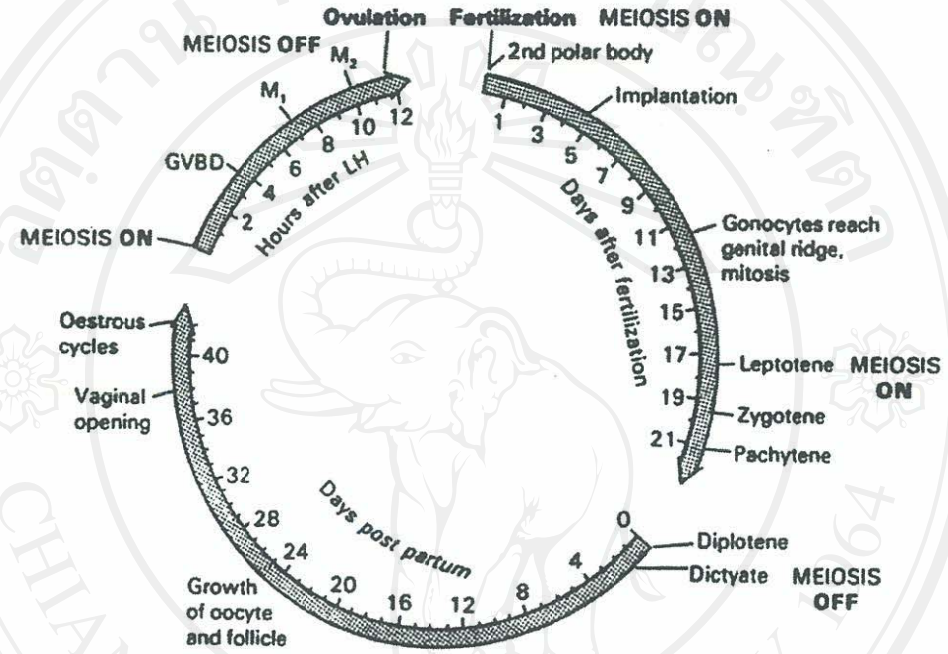


Figure 9 Schematic presentation of multiple stop-go control of oogenesis in rat (Linder et al., 1980)

CHAPTER 2

RESEARCH DESIGNS AND METHODS

A. Research designs

This study was designed into two experiments, In the first experiment, approximately 24 days-old female ICR mice were killed to collect immature germinal vesicle (GV)-stage oocytes from their ovaries. Only immature oocytes surrounded by a well formed cumulus oophorus were selected for subsequent study. The oocytes were washed in 3 changes of PBI medium and transferred to test media. In each group of this experiment, 4 to 5 oocytes were cultureed in 20 µl-drops of medium, at 37°C in 5% CO_2 in air. The culture medium used for this experiment was Waymouth supplemented with 0.23 mM pyruvic acid, 119.7 unit Penicilin G (potassium salt), 38.85 unit Streptomycin sulfate and bovine serum albumin (BSA fraction V) 0.4%. The test media were Waymouth medium containing various concentrations of lyophilized WG (0.5-20 mg/ml) and in Waymouth medium alone which serve as control. Oocytes maturation was recorded at every 24 hours for 2 days.

The second experiment was conducted, to test the fertilizing ability of the in vitro matured oocytes from each group of experiment I. The mature oocytes were inseminated with sperm collected from the cauda epididymide of mature male mice, and incubated in 5% CO_2 in air at 37°C for 4 hours. All fertilized oocytes were washed in 3 drops of M16 medium and transferred into M16 medium for further development.

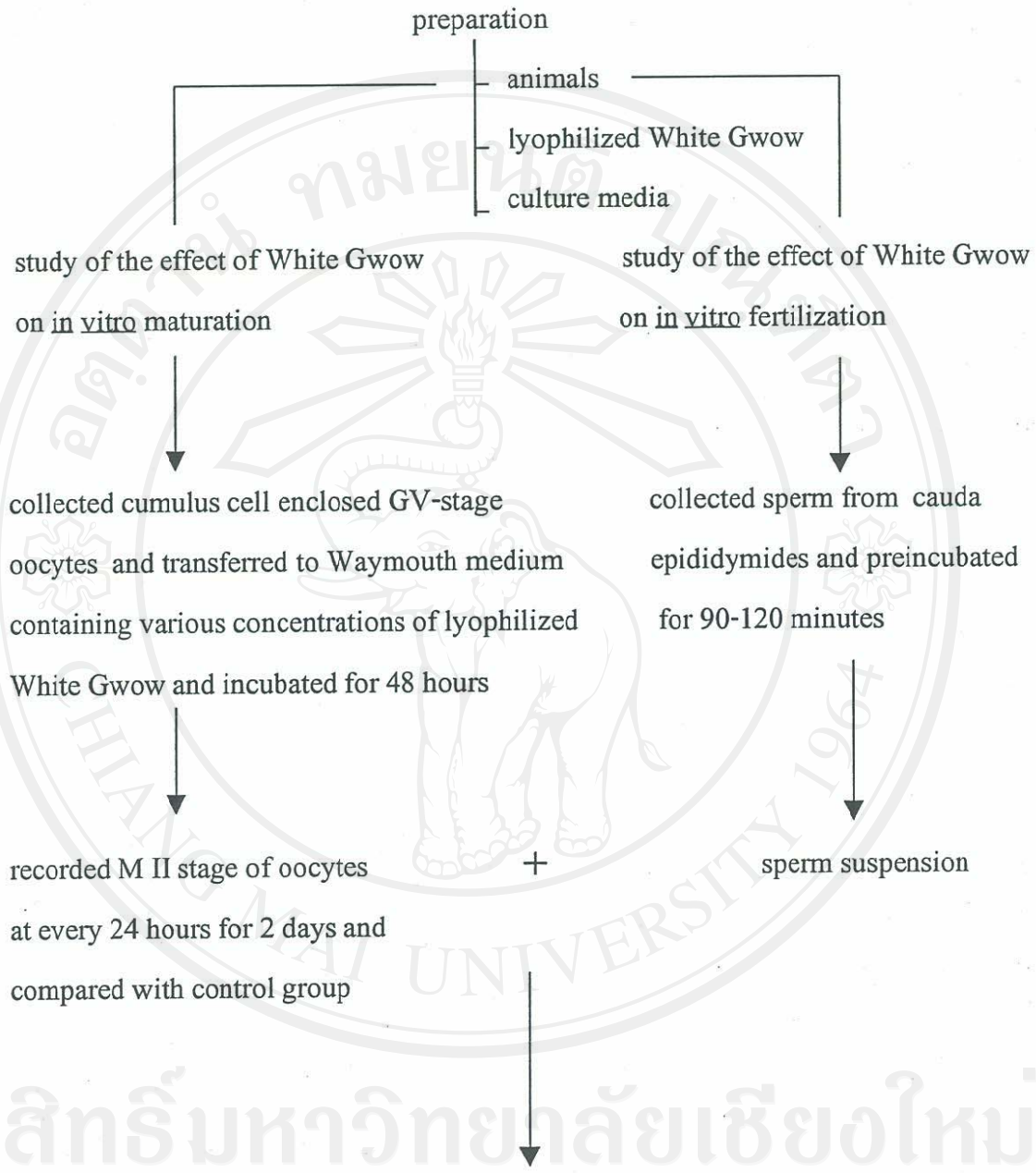


Figure 10 Summary diagram of the procedure in this research

B. Methods

1. Experimental animals

Both male and female random bred ICR-strain mice were purchased from the National Animal Laboratory Center, Mahidol University, Nakornpratom, Thailand and raised at the Animal Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. They were housed in stainless cages and maintained in a controlled room at temperature 24-25^0 C for 14 hours light cycle (5 am-7 pm) and 10 hours dark cycle (7 pm-5 am). The animals were fed with a standard mice chow and tap water ad libitum. The females used in this study were approximately 24 days-old for providence of the GV-stage oocytes and 8 to 16 weeks-old, weighing about 15 to18 g and 30 to 50 g respectively and male mice approximately 12 to 16 weeks-old, weighing about 40 to 60 g, that were mated within 3 to 7 days to prove fertility.

2. Preparation of lyophilized White Gwow

The tuberous root of WG was collected during December 1999 from Mae Tang District, Chiang Mai Province, Thailand. After being dug up, the root was pealed, and sliced into thin pieces.They were then exposed to sun light for 8 hours, and dried again in an oven at 60^{O}C for 48 hours, or until completely dried. The roots were then mashed in to a powder using a grinder. The fine powder of WG was extracted with double distilled water at a ratio of 1:5 (w/v) for 1 day. After filtering through filter paper,the filtrate was heat-evaporated at 60^{O}C and completely dried with a lyophilizer.The dried-residue was weighed and kept at -20^{O}C until use (พรรณิภา ชุมศรี, 2523).

3. Preparation of media

Most of media used in this study were in-house preparation, except the commercially purchased Waymouth medium. The constituents of each medium are shown in Appendix A. All the prepared media were sterile-filtered through the 0.22 μ m membrane filter unit into sterile containers, preincubated at 37^0 C in 5% CO_2 in air for at least 12 hours before used to maintain a pH of 7.35 to 7.40. The media may be kept at 4^0 C and routinely used within 1 month, except the Waymouth medium routinely used within 2 weeks.

3.1 Oocyte collecting and washing medium

Phosphate buffer saline (PBI) supplemented with 0.4% bovine serum albumin (BSA fraction V) was used to collect the oocytes from the ovaries and to wash the oocytes before placing them into the culture medium.

3.2 Oocytes maturation medium

For in vitro maturation of mouse oocytes, Waymouth MB752/1 medium (Appendix A) was supplemented with 0.23 mM pyruvic acid, 38.85 units streptomycin sulfate, 119.7 units penicillin G (potassium salt) and 0.4% bovine serum albumin (BSA fraction V). The media were used for cultured immatured oocytes and can be kept at 4^0 C, routinely used within two weeks (Eppig and Telfer, 1993).

3.3 Embryo culture medium

After in vitro fertilization, all fertilized oocytes were cultured for a further 48 hours in the modified M16 medium supplemented with 0.4% bovine serum albumin (BSA fraction V) (Whittingham, 1971).

3.4 Sperm capacitation medium

The enriched Krebs-Ringer bicarbonate (EKRB) medium (Olds-Clarke and Sego, 1992) was used for recovery and capacitating the epididymal sperm. The pH of the medium was adjusted to 7.35 to 7.40 by incubation with humidified 5% CO_2. Lactate was omitted in EKRB, because it has been shown to have an inhibitory effect on sperm capacitation (Neill and Olds-Clarke, 1987).

3.5 Preparation of lyophilized White Gwow into culture medium

Six concentrations of lyophilized WG (0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 mg/ml) were dissolved in Waymouth medium and then sterilized by passing through the 0.22 μm membrane filter unit. All concentrations were checked for estradiol levels by the electro-chemiluminescent immunoassay. This analysis was repeated three times.

3.6 Microdrop-culture setting

Dispense 20 μl-drops of Waymouth, Waymouth containing lyophilized WG, M16 and EKRB in an array on the bottom of the plastic petri dish (35 mm) and then flood the dish with 2.5 ml of mineral oil (Figure 10). The purpose of putting the oil on

top was to stabilize the drops of medium and also protect the evaporation of medium and CO_2 The petri dish was placed in 5% CO_2 in air, at 37^0 C overnight to maintain a pH of 7.35 to7.40.

4. Preparation of blood samples

Female mice were check for the estrous cycle using the vaginal smear method. Four mice at estrus, metestrus or diestrus stage were killed by decapitation and whole blood was collected in to the heparinized tube for prevention of blood cloting and subsequently centifuged at 1,000 rpm for 10 minutes. The plasma was collected and analysed for estradiol levels using eletro-chemiluminescent immunoassay.

5. Preparation of Pasture pipette

In constructing capillary pipettes, the thin portion of a Pasture pipette was held in a gas flame and rotated for a few seconds. When the glass became soft, it was quickly removed from the flame and immediately pull out a predeterminded distance without breaking the capillary segment. The ends were held steady for a few seconds, then bent it and broken at about 2 cm from the shoulder of pipette. Examination of the pipette under the stereomicroscope was made to be certain broken cleanly to give a perfect flat tip. Aperture diameter was controlled by the initial pull when the glass was soft. For mouse eggs with cumulus, an aperture of approximately 140 μm and for 2-cell embryos an aperture of approximately 120 μm was most desirable. This was achieved with an initial pull of about 12 inches (Figure 11).

6. Study of the effect of White Gwow on in vitro maturation

Female mice (about 3 to 5 mice at a time) were killed by cervical dislocation and the abdominal wall cut open using an aseptic technique. Ovaries were excised and placed into a dish containing approximately 1 ml of warm PBI medium. The ovaries were cut into small 2 to 4 pieces using a 25 G needle attached with a 1-ml syringe and then each piece was pierced upward and downward until immature oocytes were released from antral follicles (approximately 10 to 15 oocytes/ovary). Only cumulus cell-enclosed GV- stage oocytes were collected and transferred to a petri dish containing 50 µl of warm PBI medium by using a fire-drawn Pasture pipette. They were washed three times in PBI medium and then randomly divided and transferred into a petri dish containing the following 20µl-drops culture media (5 oocytes/drop):

1) Waymouth medium (control group)
2) Waymouth medium containing White Gwow 0.5 mg/ml
3) Waymouth medium containing White Gwow 1.0 mg/ml
4) Waymouth medium containing White Gwow 5.0 mg/ml
5) Waymouth medium containing White Gwow 10.0 mg/ml
6) Waymouth medium containing White Gwow 15.0 mg/ml
7) Waymouth medium containing White Gwow 20.0 mg/ml

The petri dish was placed in the incubator with 5% CO_2 in air, at 37^0 C for 48 hours. The maturation of oocytes was evaluated under inverted microscope every 24 hours. The maturation of oocytes was defined as germinal vesicle break down (GVBD) and was proceeded to the metaphase of the second meiotic division (metaphase II), the oocytes has a spindle with chromosomes, an expanding cumulus and first polar body. This experiment was repeated 20 times.

7. Study of the effect of White Gwow on in vitro fertilization

7.1 Sperm suspension

Male mice approximately 12 to 16 weeks of age that have been mated within three to seven days (two mice at a time) were killed by cervicle dislocation. The epididymides were cut and placed in a petri dish containing 0.5 ml of warm EKRB medium. The cauda epididymis was dissected using a pair of forceps to grasp and squeeze out the sperm into the pertri dish containing 100 µl-drops of EKRB flood with 2.5 ml mineral oil. The sperm suspension was then preincubated in 5% CO_2 in air, at 37^0 C for 90-120 minutes. This step was performed about 2.5 to 3 hours before the maturation of oocytes and was evaluated at 24 hours.

7.2 Determination of sperm quality (Makler, 1980)

Before the time of insemination, the percentage of sperm motility and concentration of sperm were determined by the means of a Makler Counting Chamber (Figure 12).

7.2.1 Motility evaluation

Sperm specimens were mixed and formation of bubbles avoided. A small drop of 10 µl of sperm specimens was put in the center of the chamber, cover glass opposite the dark point was grasped and placed it on the four tips. The chamber was lifted by its handles and placed on the platform of the microscope. An ×20 objective lens was used with this chamber. After focusing, the platform of the microscope was moved and the grid locatd in the center of the view area. The numbers of motile sperm

and nonmotile sperm were counted within a strip of 10 squares (Figure 14). The percent of sperm motility was calculated by the following equation.

$$\% \text{ Motility} = \frac{\text{Number of motile sperm}}{\text{(Number of motile + nonmotile)}} \times 100$$

7.2.2 Sperm concentration

The sperm suspension was inserted in water bath at 50^0C- 60^0C for 5 minutes. The small drop of 10 µl was put in the center of the chamber and covered as described above. The chamber was lifted by its handles and placed on the platform of the microscope with ×20 objective lens. Sperm heads within the square was counted within a trip of 10 squares in the same way as blood cells were counted in hemocytometer. The number represented their concentration in millions per ml. This count was repeated in another strip, to determine the average. Five zeros were then added to the number counted and the result was the concentration in millions per ml (10^6).

8. In vitro fertilization of in vitro matured oocyte

Preincubated sperm suspension was removed from the incubator. Three to five µl of sperm suspension (concentration ~ 50,000 cells/ml) were added to the oocyte that matured in vitro from the first experiment preparation, and the culture dish was placed into 5% CO_2 in air, at 37^0 C for 4 hours. Later, all of the fertilized eggs were removed from the inseminated medium and 3 washed in M16 medium and moved into 20 µl-drops of M16 medium and then cultured for 24 hours.The fertilization of oocytes

was evaluated under an inverted microscope at 24 hours and defined as either 2 pronuclei or 2-cell stage embryo. This experiment was repeated 20 times.

C. Statistical Analysis

The effect of WG extract on in vitro maturation was analyzed using repeated-measure two way ANOVA followed by Scheffe test and the fertilizability of in vitro matured oocytes was analysed using χ^2 test. Statistical significance was accepted at $P < 0.05$. All data were expressed as the mean±SD and percentage.

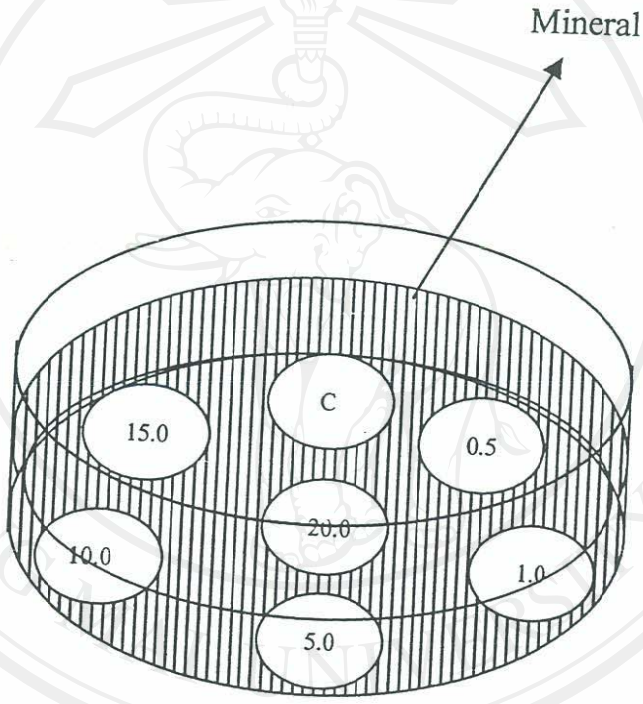


Figure 11 Plastic petri dish containing 20 µl-drops of Waymouth medium containing various concentrations of WG (mg/ml), C means control of Waymouth medium.

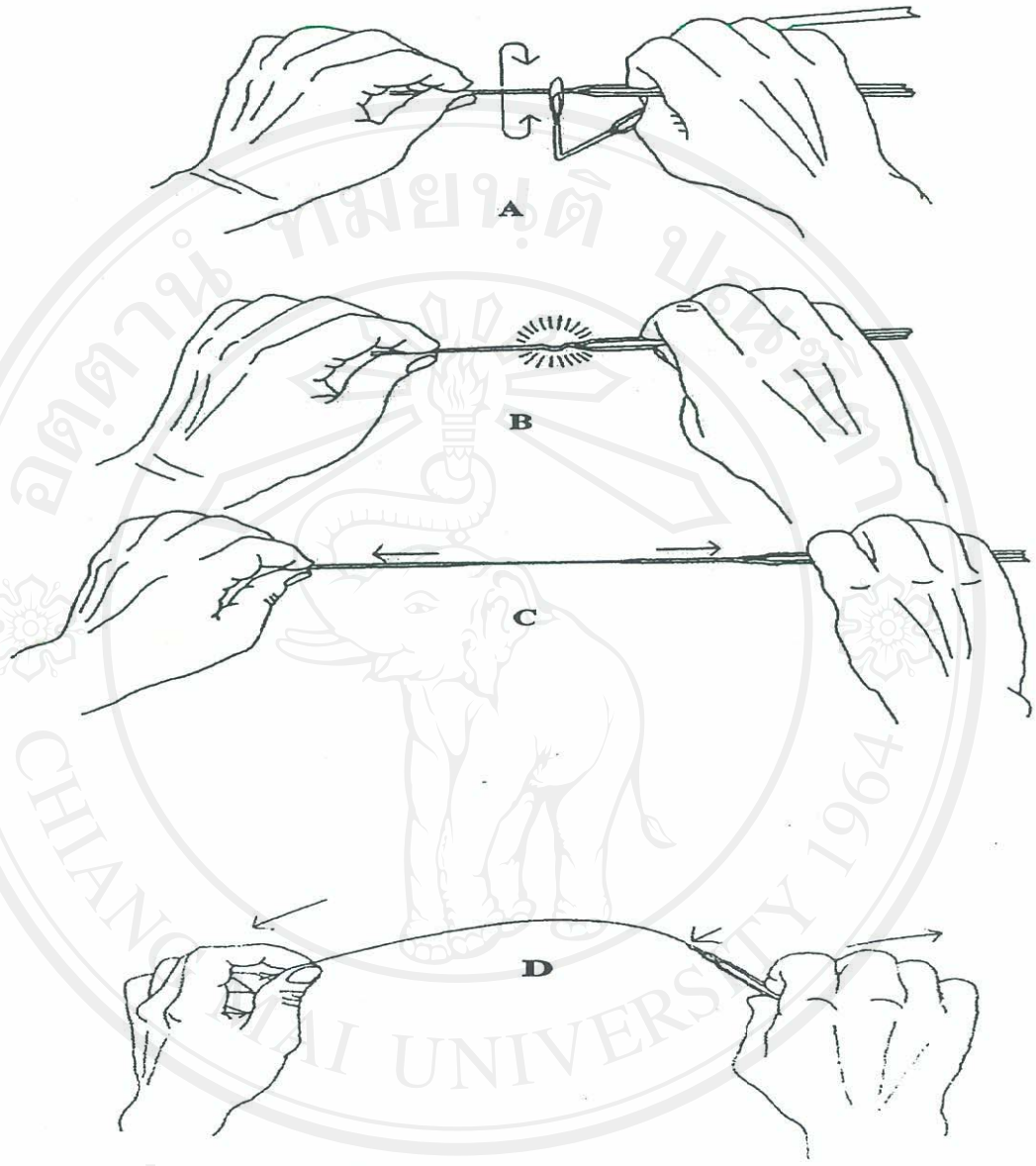


Figure 12 Procedure for drawing Pasture pipette. A Pasture pipette was rotated in a gasflame (A) Untill it was softened locally and quite easy to deform.It was pulled from the flame (B) and immediately drawn out a predetermined distance (C) The ends were held steady for a few seconds, then bent the capillary segment and broken at about 2 cm from the shoulder of pipette (D) (Keen and Rafferty, 1970).

Figure 13 Makler Counting Chamber

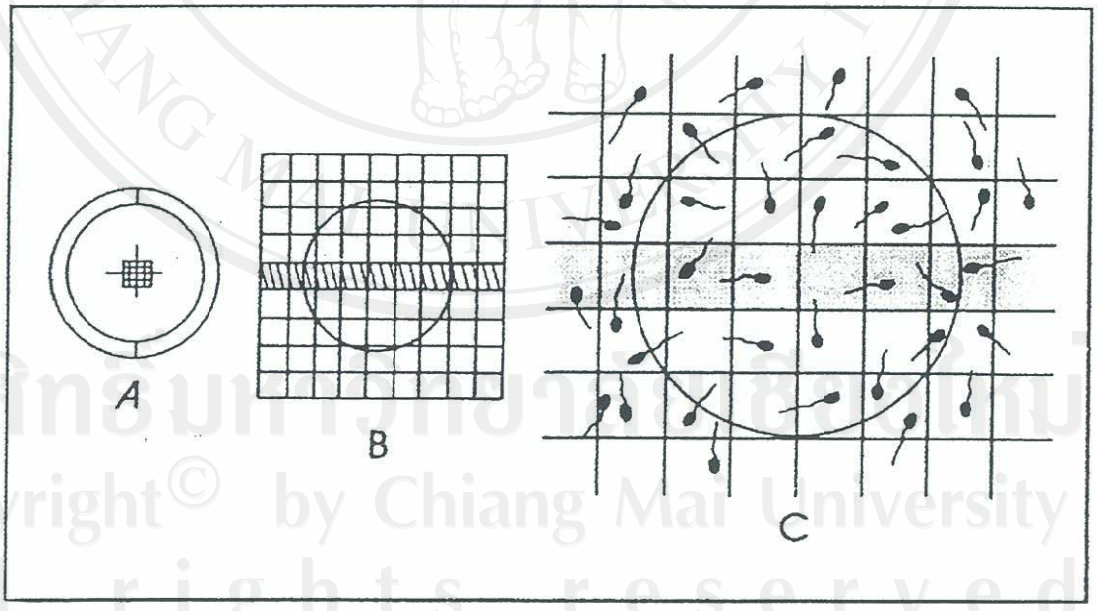


Figure 14 Manner of counting sperm in a trip of 10 squares

CHAPTER 3

RESULTS

A. Electro-chemiluminescent immunoassay for estradiol levels

Waymouth medium containing lyophilized White Gwow extract at various concentrations of 0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 mg/ml and plasma of female mice at estrus, metestrus and diestrus stage of estrous cycle were analysed for estrogen levels using electro-chemiluminescent immunoassay. This analysis was repeated three and four times respectively. The results showed that the average level of estradiol value of lyophilized White Gwow extract at a concentration of 0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 mg/ml were 21.30±3.40, 43.09±3.50, 293.64±7.31, 523.48±35.20, 795.83±35.30 and 949.01±51.97 pg/ml, respectively. The average plasma level of estradiol value in female mice at estrus, metestrus and diestrus stage were 17.48±.93, 14.50±1.83 and 10.69±.76 pg/ml, respectively. A summary of the results are shown in Tables 1 and 2.

Table 1 Estradiol values of lyophilized White Gwow extract at various concentrations

No. of test	estradiol values in waymouth medium containing lyophilized White Gwow extract (pg/ml)					
	0.5	1.0	5.0	10.0	15.0	20.0
	(mg/ml)					
1	17.34	46.99	302.00	518.60	833.34	1009.0
2	22.73	39.93	288.38	561.00	790.92	920.40
3	23.83	42.35	290.56	490.85	763.25	917.63
Mean±SD	21.30±3.40	43.09±3.50	293.64±7.31	523.48±35.32	795.83±35.30	949.01±51.97

Table 2 Plasma levels of estrogen in female mice at estrus, metestrus and diestrus stage of estrous cycle

No. of test	Plasma concentrations of estradiol at		
	estrus stage	metestrus stage	diestrus stage
	(pg/ml)		
1	17.14	12.54	10.00
2	17.13	13.36	10.69
3	18.87	16.15	11.77
4	16.81	15.98	10.33
Mean±SD	17.48± 0.93	14.50± 1.83	10.69±0.76

B. Effect of White Gwow extract on in vitro maturaton of mouse oocytes

To evaluate the effective concentration of White Gwow on the in vitro maturation of mouse oocytes experiments as conducted by culturing the immature GV-stage mouse oocytes in the medium containing concentrations of lyophilized White Gwow between 0.5-20.0 mg/ml for 48 hours in a humidified atmosphere of 5% CO_2 in air at 37^{O}C. The oocyte maturation was recorded at every 24 hours. The 1,319 GV-stage oocytes obtained from 99 females were selected for this study. The maturation of GV-stage mouse oocytes to metaphase II stage after culture for 24 hours was shown in Table 3, Figure 15 and 16. Oocytes cultured in 0.5 mg/ml of White Gwow did not significantly influence oocyte maturation compared with the control group (6.30±.73 versus 5.70±.65 for control). The concentration of White Gwow extract at 1.0, 5.0, and 10.0 resulted in significant ($p<0.05$) increased in the proportion of GV-stage oocytes developing into metaphase II (7.00±0.85, 7.65±0.74, 8.35±1.49, respectively vs 5.70± 0.65 for control) and it is significant different between 1.0 mg/ml and 10.0 mg/ml. However, at a concentrations of 15.0 and 20.0 mg/ml, the maturation of oocytes to metaphase II was significantly ($p<0.05$) decreased by delay development (4.55±1.14 and 3.30±1.08, respectively versus 5.70±0.65) compared with control group.

To investigate the influence of White Gwow extract on the extent of oocyte maturation in vitro. The numbers of GV-stage oocytes that matured to metaphase II after 48 hours of incubation were compared (Table 3, Figure 15 and 16). Oocytes cultured in 0.5 mg/ml of White Gwow did not significantly influence oocyte maturation compared with the control (6.90±0.96 versus 6.10±0.55). Oocytes cultured in at concentration 1.0, 5.0, and 10.0 mg/ml exhibited a higher incidence of

GVBD and MII oocytes (7.70±1.03, 8.10±0.96 and 8.85±1.26, respectively) compared with control (6.10±0.55; P<0.05) and it is significant difference between 1.0 mg/ml and 10.0 mg/ml. However, the concentration of 15.0 and 20.0 mg/ml, White Gwow significantly (p<0.05) decreased the extent of in vitro maturation of oocytes to metaphase II by delay of development (4.80±1.05 and 3.55±1.14) when compared with control groups. It was notable that the concentrations of 15.0 and 20.0 mg/ml increased the degeneration of oocytes compared with control group (at 24 hrs: 0.40± 0.68 and 1.15±1.08, respectively versus .10±.30 for control; at 48 hrs: 1.85±.87% and 2.31±0.17, respectively versus 0.40±0.59 for control).

C. Effect of White Gwow extract on in vitro fertilization of mouse oocytes

This experimemt was carried out to evaluate the fertilizability of the oocytes that matured in vitro in WG extract. The fertilization rate are defined as the number of oocytes that formed 2 pronuclei or proceeded to the 2-cell stage of development 24 hours after insemination with the matured sperm. A total of 805 MII-stage oocytes obtained from experiment I were used in this experiment. The results showed that control group and concentrations of White Gwow ranging from 0.5-10.0 mg/ml significantly (P<0.05) had different fertilization rate competence. The concentrations of White Gwow extract at 0.5, 1.0, 5.0 and 10.0 tended to increase the fertilization rate after 24 hours of incubation when compared with control group (59.05%, 64.46%, 63.43% and 65.62% ,respectively versus 54.78% for control). However, the concentrations of White Gwow extract at 15.0 and 20.0 mg/ml tended to inhibited fertilization (37.63% and 25.58% ,respectively versus 54.78% for control). The summary results is shown in Table 4, Figure 17 and 18.

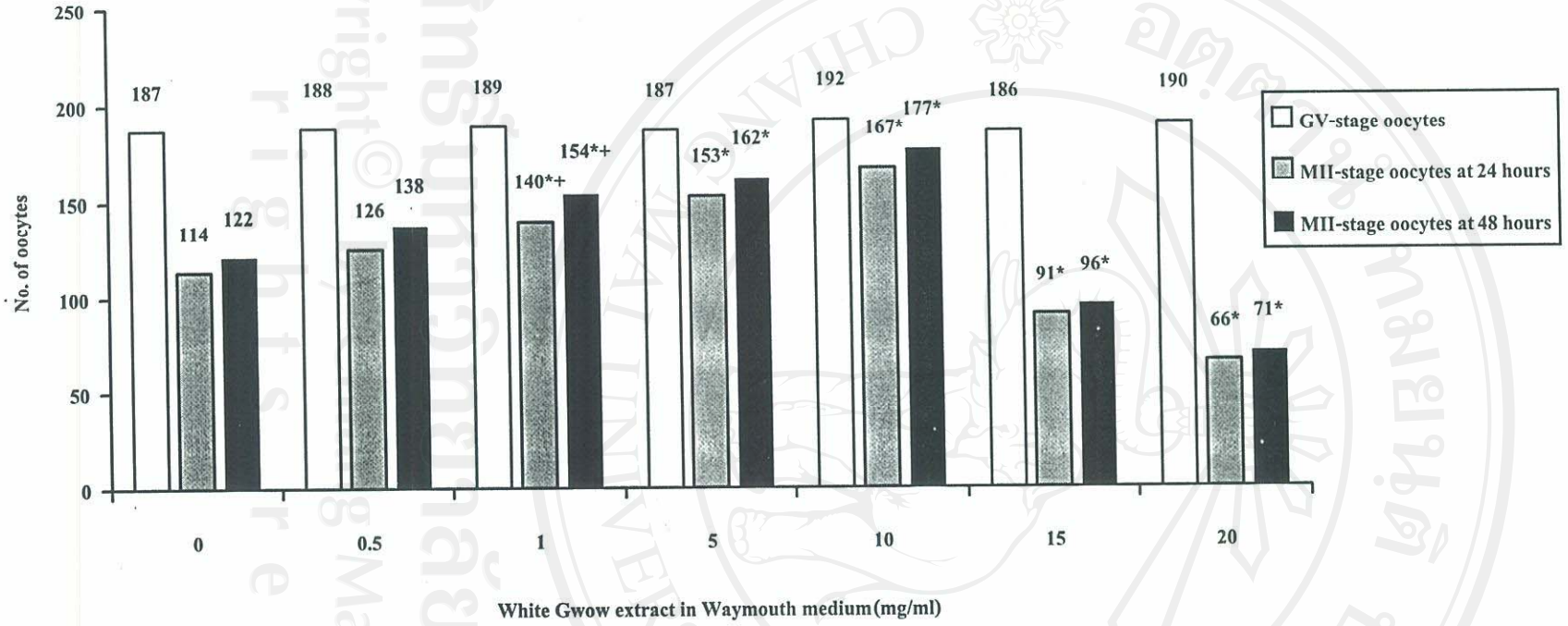
Table 3 Effect of White Gwow extract on in vitro maturation of mouse oocytes.

WG extract in Waymouth medium (mg/ml)	No. of GV-stage oocytes	No. of oocyte maturation in vitro					
		at 24 hours n (mean± SD)			at 48 hours n (mean± SD)		
		MI-stage oocytes	MII-stage oocytes	Degeneration	MI-stage oocytes	MII-stage oocytes	Degeneration
control	187	71 (3.50±0.88)	114 (5.70±0.65)	2 (0.10±0.30)	57 (2.85±0.98)	122 (6.10±0.55)	8 (0.40±0.59)
0.5	188	61 (3.05±1.09)	126 (6.30±0.73)	1 (0.02±0.22)	45 (2.55±0.91)	138 (6.90±.96)	5 (0.25±0.55)
1.0	189	48 (2.40±0.88)	140 (7.00±0.85)*†	1 (0.02±0.22)	33 (1.65±0.74)	154 (7.70±1.03)*✞	2 (0.10±.30)
5.0	187	30 (1.50±0.94)	153 (7.65±0.74)*	4 (0.20±0.41)	13 (0.65±0.67)	162 (8.10±.96)*	12 (0.60±0.68)
10.0	192	22 (1.10±0.71)	167 (8.38±1.49)*	3 (0.15±0.67)	6 (0.25±0.55)	177 (8.85±1.26)*	9 (0.45±0.75)
15.0	186	81 (4.05±1.35)	91 (4.55±1.14)*	8 (0.40±0.68)	53 (2.61±0.23)	96 (4.80±1.05)*	37 (1.85±0.87)
20.0	190	79 (2.80±1.47)	66 (3.30±1.08)*	23 (1.15±1.08)	73 (3.65±1.13)	71 (3.55±1.14)*	46 (2.30±1.17)

* indicates statistically significant difference from control, at $P<0.05$

✞ indicates statistically significant difference between 1.0 mg/ml and 10.0 mg/ml, at $P<0.05$

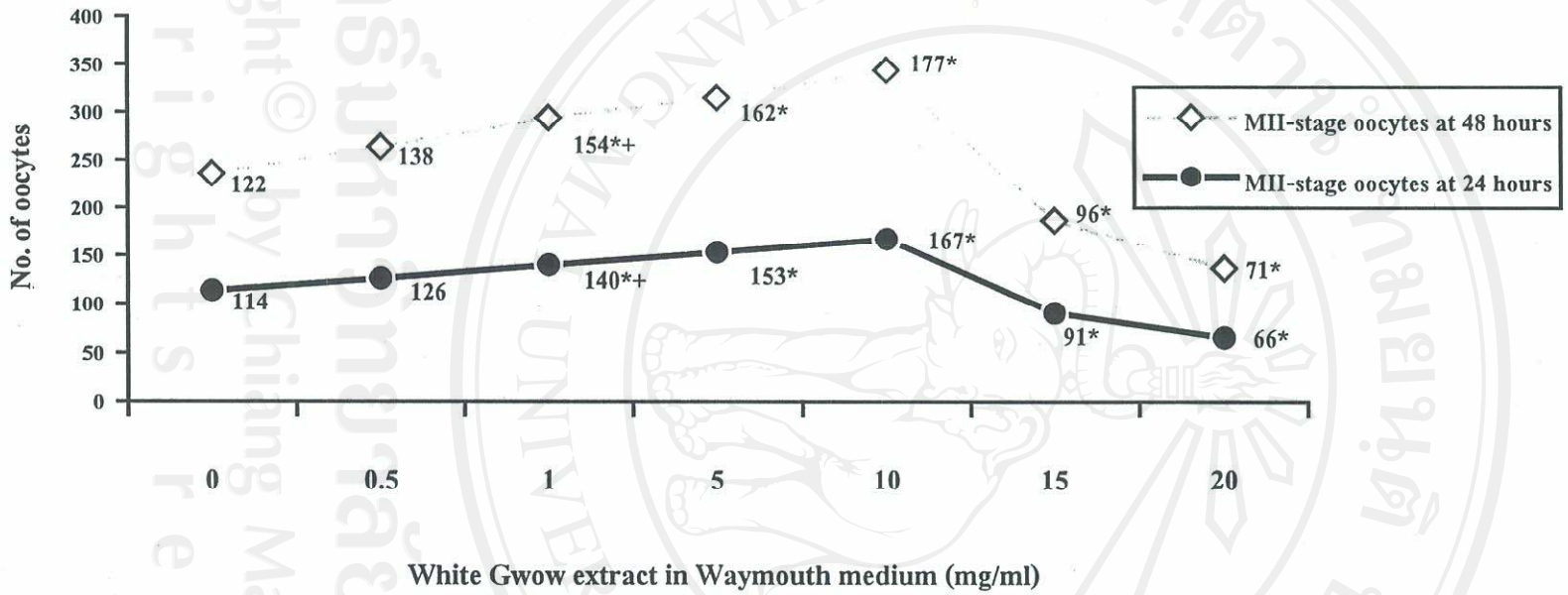
n (mean± SD); n= total oocytes from 20 experiments, mean = average oocytes from each experiment



* indicates statistically significant difference from control group at P<0.05

✞ indicates statistically significant difference between 1.0 mg/ml and 10.0 mg/ml, at P<0.05

Figure 15 Histogram showing the effect of White Gwow extract on in vitro maturation of mouse oocytes



* indicates statistically significant difference from control group, at P<0.05

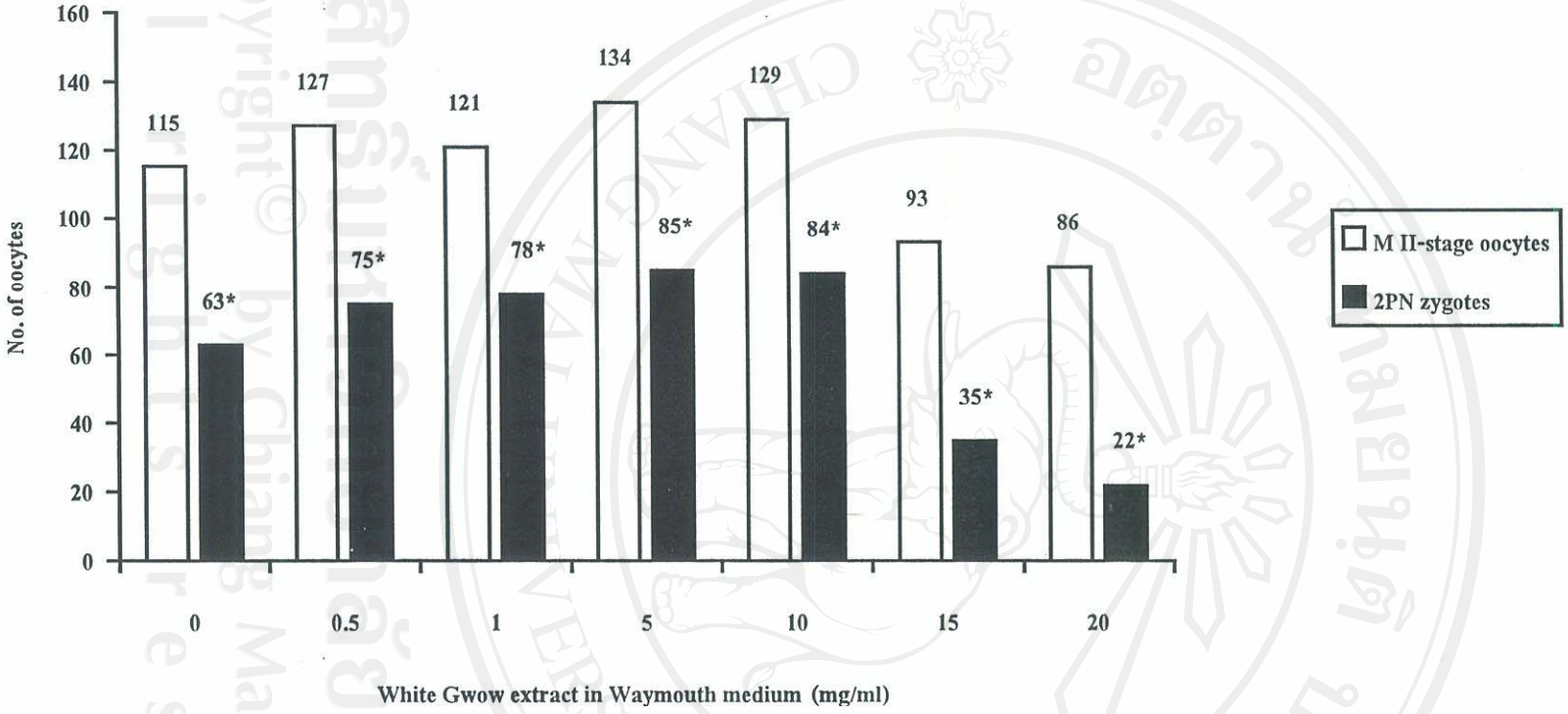
✞ indicates statistically difference between 1.0 mg/ml and 10.0 mg/ml, at P<0.05

Figure 16 Graph showing the effect of White Gwow extract on in vitro maturation of mouse oocytes

Table 4 Effect of White Gwow extract on in vitro fertilization of mouse oocytes after 24 hours of incubation.

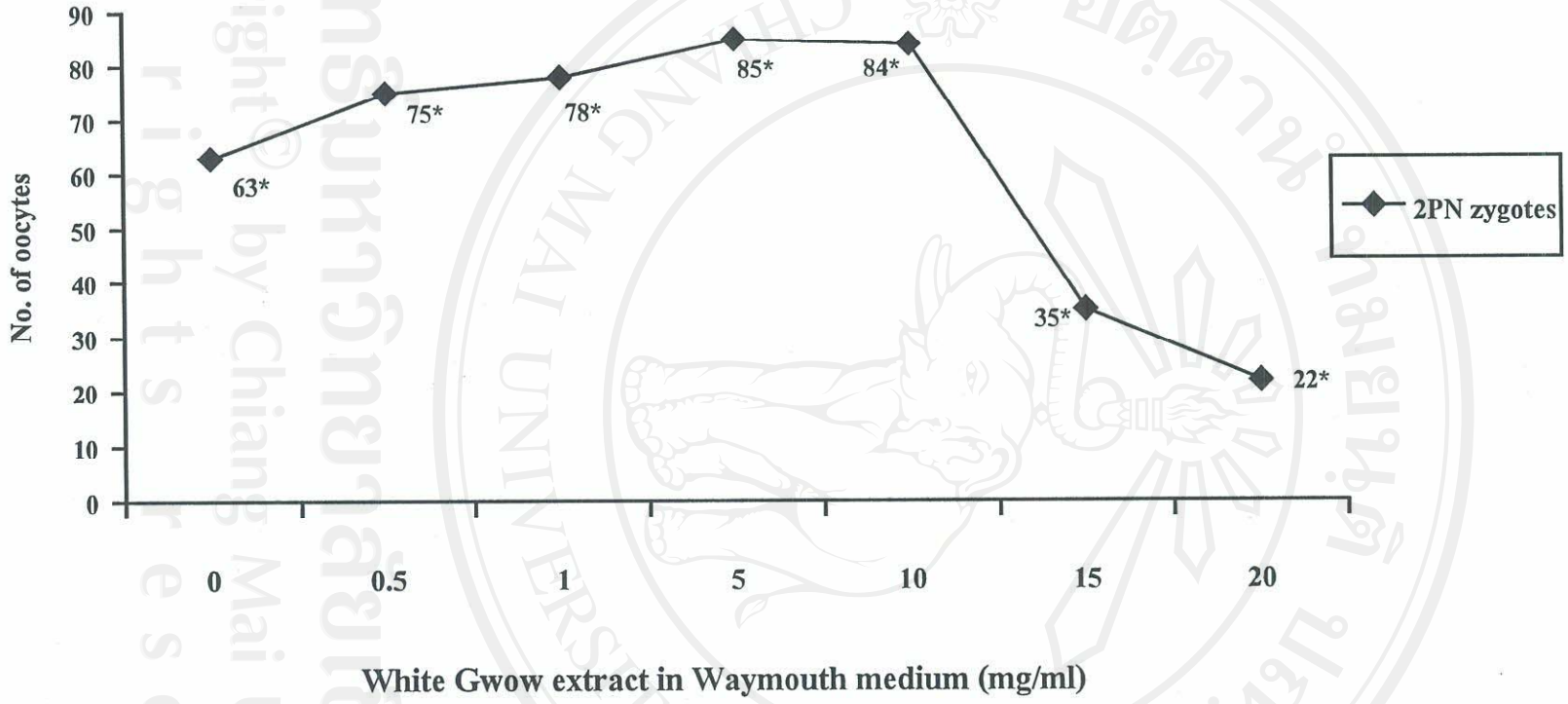
White Gwow extract in Waymouth medium (mg/ml)	No. of MII-stage oocyte	No. of 2 PN zygotes (%)	No. of 2-cell stage	No. of Unfertilization (%)	No. of Degeneration (%)
0	115	63 (54.78)*	0	42 (36.52)	10 (8.69)
0.5	127	75 (59.05)*	1	38 (29.92)	13 (10.23)
1.0	121	78 (64.46)*	3	28 (23.14)	12 (9.91)
5.0	134	85 (63.43)*	6	26 (19.40)	17 (12.68)
10.0	129	84 (65.62)*	5	23 (17.82)	17 (17.82)
15.0	93	35 (37.63)*	1	32 (34.40)	25 (26.88)
20.0	86	22 (25.58)*	0	30 (34.88)	34 (39.53)

* indicates statistically significant difference, at $P<0.05$



* indicates statistically significant difference, at P<0.05

Figure 17 Histrogram showing the effect of White Gwow extract on in vitro fertilization of mouse oocytes.



* indicates statistically significant difference, at P<0.05

Figure 18 Graph showing the effect of White Gwow extract on in vitro fertilization of mouse oocytes.

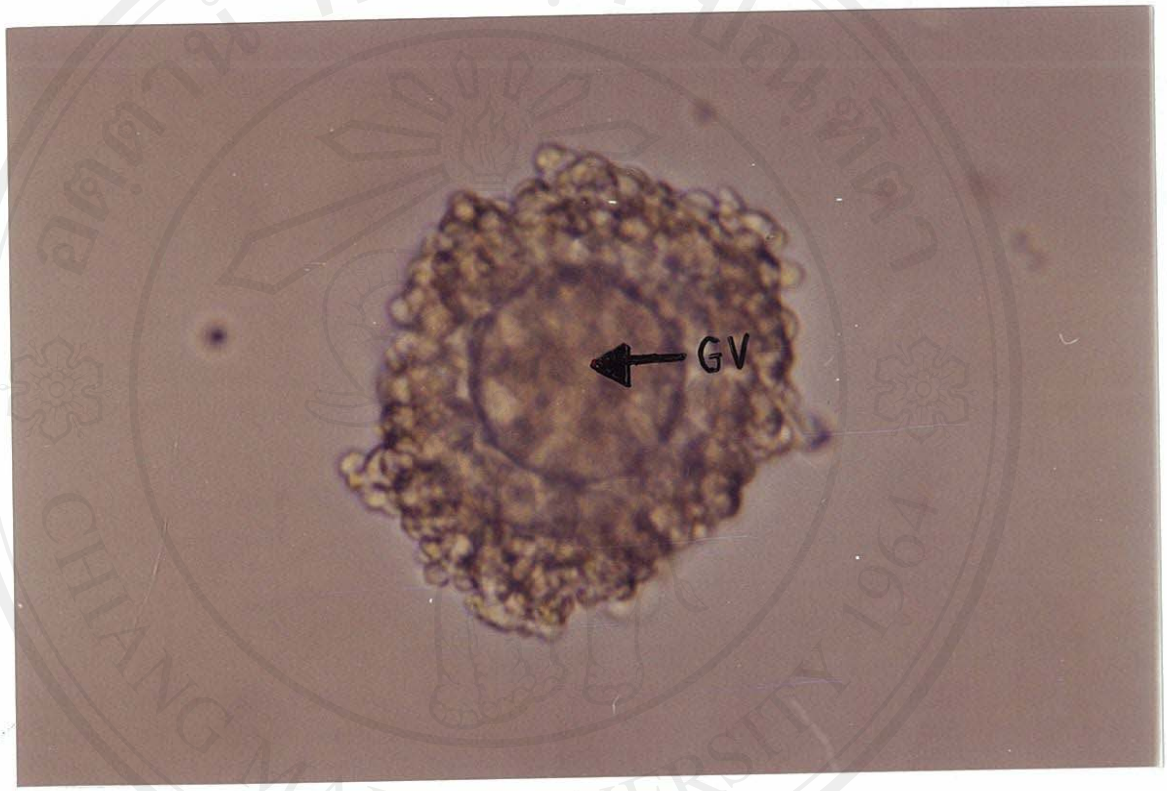


Figure 19 Photomicrograph of representative GV-stage mouse oocytes enclosed with A dense layer of cumulus cells.

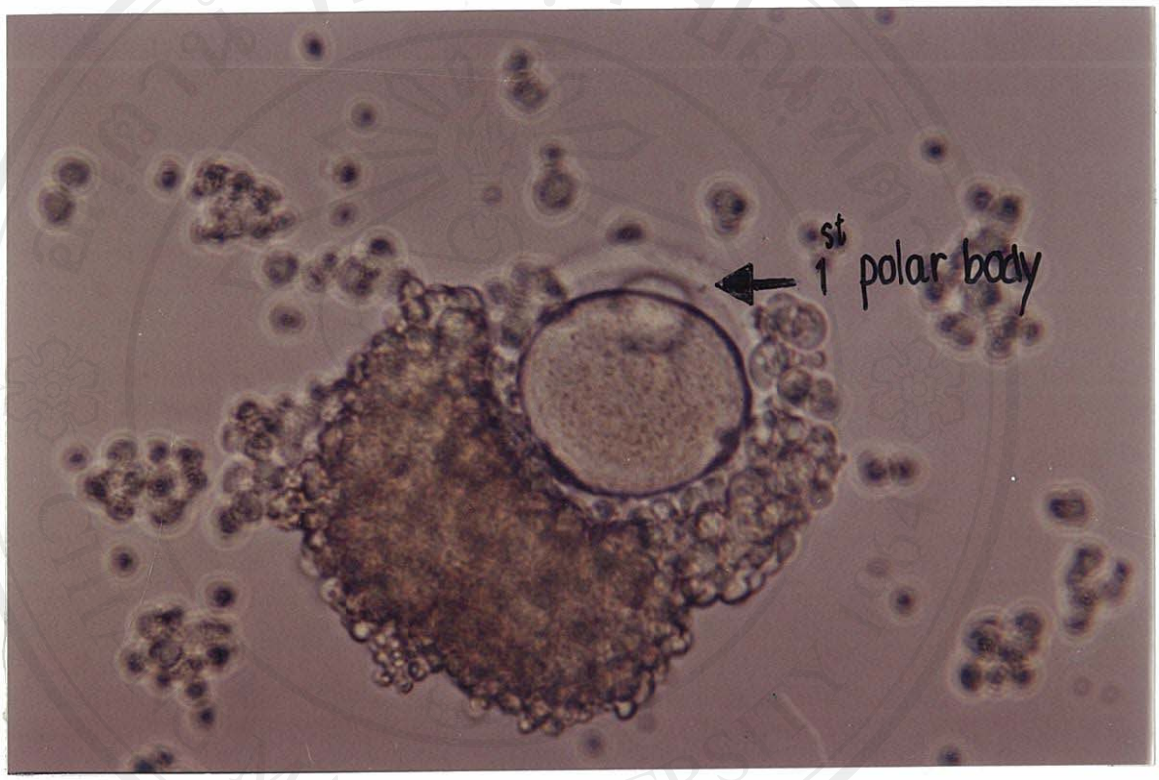
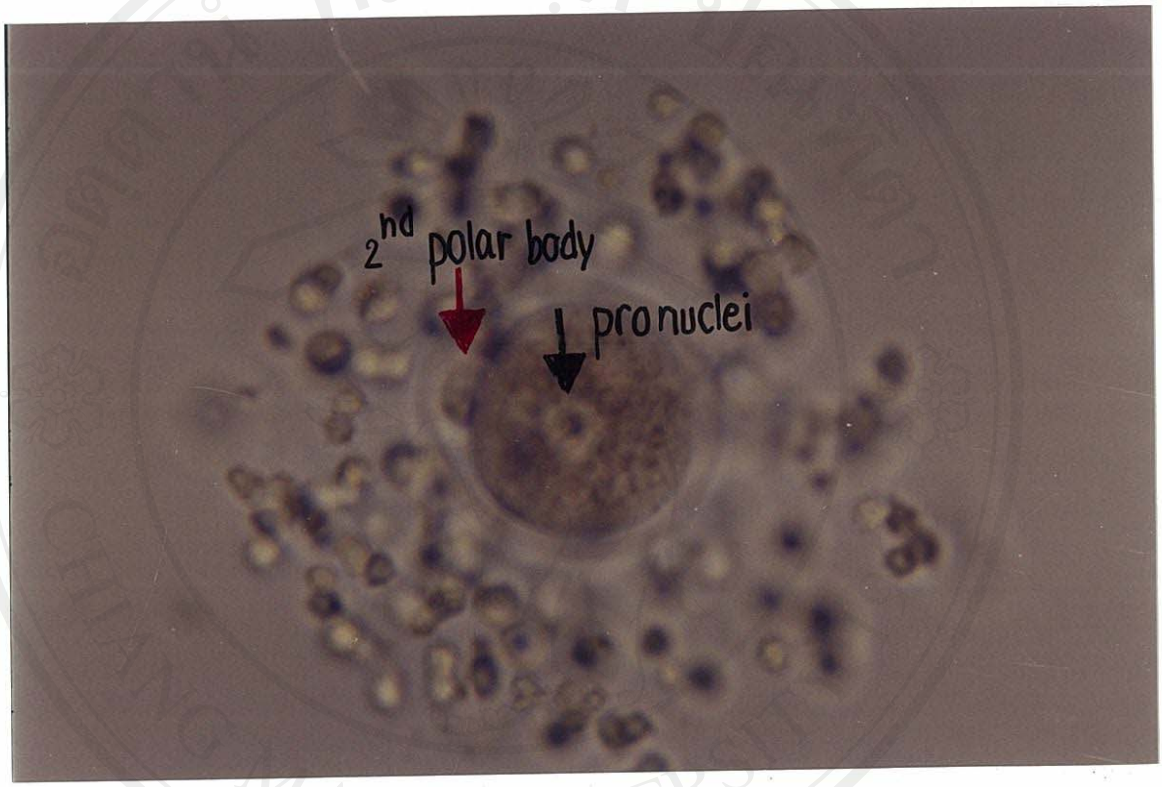
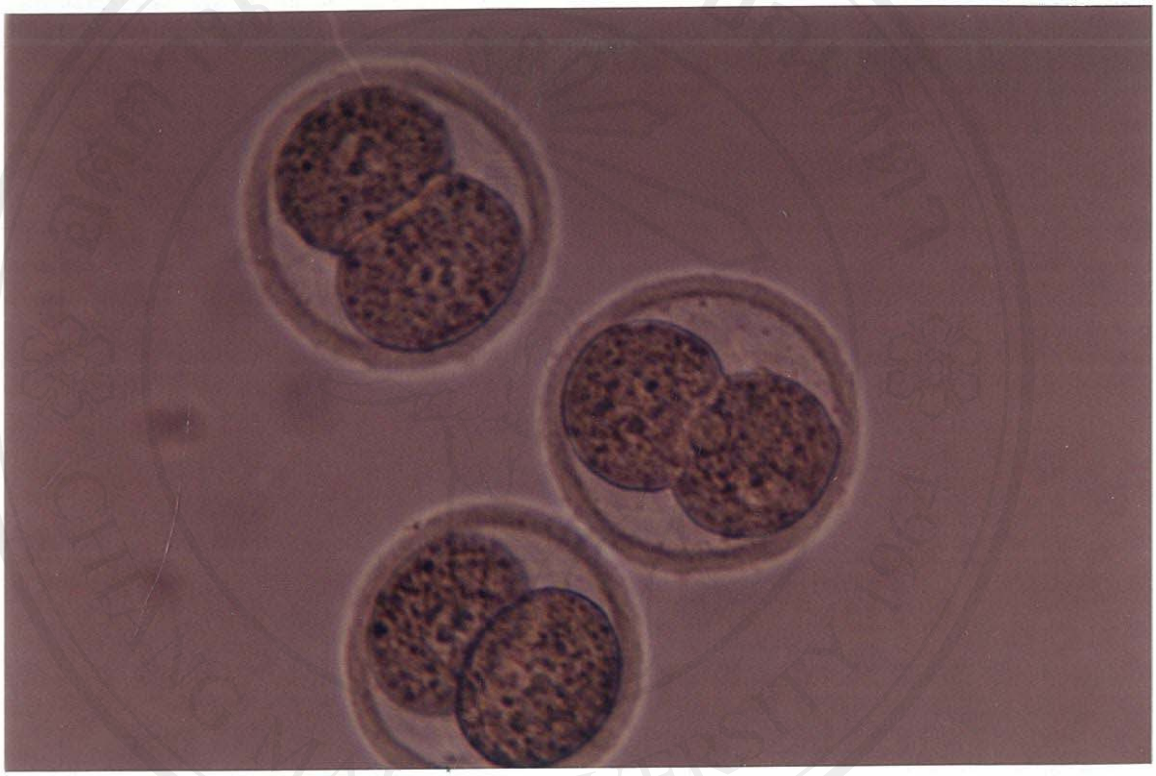


Figure 20 Photomicrograph of representative metaphase II-stage oocyte with one polar body.



Figuire 21 Photomicrograph of representative fertilized oocyte with 2 pronuclei and 2 polar body in the perivitelline space.



Figuire 22 Photomicrograph of representative 2-cell stage

CHAPTER 4

DISCUSSION

A. Effect of White Gwow on in vitro maturation of mouse oocytes

Oocyte maturation is a process of both nuclear and cytoplasmic maturation, undergo fertilization competence and preparation for embryonic development. The meiotic division of mammalian oocyte is a protracted process regulated by several stop-go controls. The mammalian oocyte in the ovary are arrested at the diplotene (dictyate) stage of the first meiotic prophase division just prior to or shortly following birth, at which the nucleolus and nuclear membrane reappear (Borum, 1961; Baker, 1972; 1982). It is characterised by highly diffuse chromosomes surrounded by an intact nuclear membrane, termed the germinal vesicle (GV). The acquisition of meiotic competence is primarily dependent on follicle stimulating hormone (FSH), which probably stimulates the proliferation of granulosa cells and aromatase activity by granulosa cell leading to preovulatory follicular development and estradiol secretion (Ayalon et al., 1972; Dekel et al., 1979; Masui and Clark, 1979; Bar-Ami and Tsafriri, 1986). Preovulatory resumption of meiosis or nuclear maturaton is morphologically identified by germinal vesicle breakdown (GVBD) followed by chromosome condensation and extrusion of first polarbody (PB I) and arrest of meiotic progression at metaphase stage of the second meiotic division (Metaphase II) (Donahue, 1968; Calarco et al., 1972; Wassarman et al., 1976). Moreover, the resumption of meiosis can be mediated by the release of oocytes from their ovarian

follicles into a suitable culture medium in vitro. (Pincus and Enzman, 1935; Donahue, 1972; Eppig and down, 1987).

Cytoplasmic maturation refer to the process occuring in oocyte that prepare it for fertilization, activation, formation of nuclei and preimplantation embryo development (Donahue, 1968; Eppig et al., 1993; 1994). The cytoplasmic maturation may not coincide with nuclear maturation. Nevertheless, some processes of cytoplasmic maturation are coordinated with nuclear maturaton. For example, the sensitivity of the intracellular calcium release system to inositol triphosphate (IP_3) is acquired gradually during the progression of nuclear maturation (Fugiwara et al., 1993). However, the deficiencies of cytoplasmic maturation of oocytes fail to achieve for normal fertilization to take place (Thaibault, 1977; Leibfried and Bavister, 1983; Eppig et al., 1996). Both nuclear and cytoplasmic maturation play important role in achieving successful fertilization. Sorensen and Wassarman (1976) reported that there was a direct relationship between the size of isolated oocytes and their ability to undergo meiotic maturation in vitro. Oocytes from small antral follicles that complete nuclear maturation are rarely competence to fertilize and develope to blastocyst. In contrast, oocytes from large antral follicles that complete nuclear maturation are often competent to develop to the blastocyst stage (Eppig et al., 1994; Crozet et al., 1995). Therefore, oocytes at the GV stage undergo further differentiation during the time of development from small to large antral follicles is directly involves to oocyte conpetence to complete preimplantation development (Pavlok et al., 1992; Eppig et al., 1994; Crozet et al., 1995; Blondin and Sirard, 1995). So, in present study, the oocytes-cumulus complex was collected from large follicles to provide GV-stage oocytes.

In this study, it was noted that the rate of GVBD and first polar body extrusion were greater in cumulus-enclosed oocyte as compared with denuded oocytes that treated in lyophilized WG extract at concentration of 1.0, 5.0, 7.0 and 10.0 mg/ml (data not shown). Evidence indicated that when oocytes are coupled to cumulus, the

maximal growth rate of oocytes occurs in vitro (Brower and schultz, 1981; Eppig, 1982; Eppig and Down, 1987; Dekel, 1987). The previous study showed that oocytes cultured with intact gap junction between the oocytes and granulosa cells grown and become competent to undergo nuclear maturation (Daniel et al., 1989; Hirao et al., 1994). There are many small gap junctions between cumulus cells and oocyte before ovulation. The gap junctions are formed on the surface of oocytes by cumulus process that transverse the zona pellucida and contact the oolemma (Anderson and Albertini, 1976; Everett and Albertini, 1976). The essential metabolic substrates utilize by oocytes, in vitro were required the gap junctions and gap junctons played the important role in supplying nutrients during oocyte growth (Brower and Schurtz, 1982; Highighat and Winkle, 1990). Potentially, estrogen produced by cumulus cell may play an important role in oocytes growth and fertilization competence.

WG (Pueraria mirifica Airy Shaw et Suvatabandhu) has been reported to have estrogenic effects in many species such as Japanese quail (Anuntalabhochai et al., 1983; Muangdech, 1984; Thuppongse, 1984; Kiateadisorn, 1985; Anuntalabhochai and Jesrichai, 1989; Thaiyanan et al., 1992), pigeon (Smitasiri and Kaweewat, 1991), chick (Pongdit, 1985; Wiriya and Smittasiri, 1987), dog (Vaithayachoti et al., 1987), mice (Tanachai and Smittasiri, 1987; Petchang, 1988), rat (Songkaew, 1987; Chailek, 1988; Smitisiri et al., 1989; Kawewat et al., 1994), American cockroaches (Radomsuk, 1991), mosquitoes (Niwasabutr et al., 1987; Tengtriratana et al., 1990), fruit flies (Sommakettarin, 1992) and human (Cherdcheewasard, 2000). However, no scientific information on the direct effect of WG on the mammalian oocyte maturation and fertilizability of in vitro matured oocyte were available. Therefore, the present study has investigated the effect of lyophilized WG extract on in vitro maturation and fertilization of mouse oocytes. The tuberous root of WG used in this study were collected during the winter season. In this study, waymouth medium containing lyophilized WG extract at various concentration were analysed for estrogen level by

the electrochemiluminescent immunoassay. The results showed that the average of estrogen values of lyophilized WG extract at a concentration of 0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 mg/ml were 21.30±3.40, 43.09±3.50, 293.64±7.31, 523.48±35.20, 795.83± 35.30 and 949.01±51.97 pg/ml, respectively. These results indicated that, there were some substances in WG extract that had similar chemical structure to estrogen and may cause estrogenic effect on oocyte maturation. However, in this study an aqueous extract was used which known to have less potency than ethanolic extract (Vanasont, 1989) but ethanolic extract of WG seemed to be more effective mutagen than the aqueous extract (Manoraung, 1996). In present study, not only Waymouth mediun containing lyophilized WG extract were analysed for estradiol level but also the plasma estradiol level in female mice at various stages of estrous cycle were analysed. The results showed that the average plasma level of estradiol values in female mice at the estrus stage is close to the lyophilized WG extract at the concentration of 0.5 mg/ml (21.30±3.40pg/ml and 17.48±.93 pg/ml, respectively).

Many scientists have investigated the effect of hormone on mammalian oocyte maturation. It has been reported that progesterone enhanced the maturation of denuded rabbit and bovine (Roberton and Baker, 1969), oocyte-cumulus complex of rabbit (Bae and Foote, 1975) and oocyte-cumulus complex from gonadotropin treated rat (Dekel and Beers, 1980). Bar-Ami and Tsafriri (1981) and Bar-Ami et al. (1983) showed that FSH and estradiol-17β are required for the acquisition of meiotic maturation by rat oocytes. Similarly, Smith and Tenney (1979) showed that pregnant mare's serum gonadotropin (PMSG) and estradiol-17β is required for completion of first meiotic division (ie, the formation of first polar body) in hypophysectomized mice. Bar-Ami and Tsafriri (1986) also found that the ability of PMSG and estradiol-17β to induce meiotic maturation is subject to age-dependent development of the ovary. The development of germinal vesicle (GV)-stage ocytes to metaphase II is also controlled

by the other factor i.e.. an inducer that may be a hormone or oocytes maturation stimulator (OMS) (Downs et al., 1988).

The previous study demonstrated the expression of the estrogen receptor (ER) gene in mouse oocytes and embryo of various gestational stages (Wu et al., 1992). The results indicated that ER gene is expressed in unfertilization and cumulus-oocyte complexes of mouse oocytes. No ER transcript can be detacted in embryo. This data suggested that estrogen, secreted by granulosa cell may directly influence oocytes growth and maturation in vivo, the absence of ER mRNA in preimplantation embryos suggests that the effects of estrogen on early embryogenesis may be indirect . Wu et al. (1993) examined the estrogen receptor messenger ribonucleic acid (ER-mRNA) in human oocytes and granulosa/cumulus cells and found that there are ER gene expression in human oocytes but not in granulosa/cumulus cell. These data suggest that the regulation of follicular development in human differ from that in non primate species.

The in vivo study found that WG was able to inhibit the growth of follicle and ovulation of rat, in vivo. Substances in WG that cause estrogenic effect may inhibit the growth of follicles and ovulation in rat. This may due to the rise in circulating estrogenic-like substance in WG inhibit the release of gonadotropins at anterior pituitary gland. FSH stimulate of the growth of growing follicle, the lack of FSH inhibit growing of follicle (Daniel and Armstrong, 1980; Hillier and DeZwart, 1981).

In contrast to in vivo effect of WG, in this study, the effects of WG on oocytes maturation in vitro examined. This report is the first evidence of effect of WG on mammlian oocyte maturation and fertilizability of matured oocytes, in vitro. The results showed that the concentration of lyophilized WG extract at 1.0, 5.0 and 10.0 mg/ml was significantly ($P<0.05$) increased in the proportion of GV-stage oocyte developing into metaphase II at 24 hours (7.00± .85, 7.65± .74 and 8.35± 1.49, respectively vs 5.7±.65 for control). Furthermore, the effect of WG on oocytes-

cumulus complexes on the extent of in vitro meiotic maturation at 48 hours was also examined. The results showed that the concentrations of lyophilized WG extract at 1.0, 5.0 and 10.0 mg/ml was significantly increased in the proportion of GV-stage oocytes developing into metaphase II (7.70± 1.03, 8.10±0.96 and 8.85± 1.26, respectively vs 6.10±0.55 for control). It indicated that lyophilized WG extract also had an effects on the extent in vitro meiotic maturation.

These results indicated that lyophilized WG extract significantly stimulated maturation of mouse oocytes and this stimulatory effect may be the direct action that differ from in vivo, in a manner similar to the mechanism that estrogen affects to oocyte maturation.

However, at the concentration of 15.0 and 20 mg/ml, WG sinificantly ($P<0.05$) decreased the rate of oocytes maturation from 51.61% and 37.36%, respectively to 65.24% of control as shown in Table 3. It was notable that the concentration of 15.0 and 20.0 mg/ml also increased the degeneration of oocytes (19.89% vs 24.21%). It may be possible that the higher doses of WG caused toxicity to oocytes. Because the other factors such as osmolality and pH of the culture medium that known to support cellular viability and proliferation (Freshney, 1983) were controlled in the normal ranges in all groups and may be excluded. The studies of toxicological effect of WG demonstrated that Japanese quial fed with high dose of WG posses inflamatory activity and cause tenderness in some part of body such as head, under wing, legs and claw. (Anuntalabhochai, 1982; Chuaychoo, 1984). The preliminary study on the effects of WG on the reproduction of Drosophila sp. indicated that WG might have some toxic effect to the flies (Kumkrue et al., 1991). Manoraung (1996) found that ethanolic extract at 10 mg/day was able to induced micronucleus formation in the poly chromatic erythocyte by micronucleus test (PCE) of male mice lead to abnormal sperm morphology.

B. Effect of White Gwow on in vitro fertilization of mouse oocytes

Experiment II was conducted to examine the effect of WG on the fertilizability of in vitro matured oocytes. All matured oocytes were subsequently to inseminate with cauda epididymal sperm and observed for further development. The results showed that WG extract at concentrations of 0.5, 1.0, 5.0 and 10.0 mg/ml tended to increase the fertilization rate after incubation for 24 hours, when compared with the control group (59.05%, 64.46%, 63.43% and 65.52%, respectively vs 54.78% for control). Tesarik and Mendosa (1995) found that estradiol-17β can directly influence the quality of maturing oocytes and did increased the fertilization. A possible explanation is that WG increased the fertilization rate of mouse oocytes may due to in a manner similar to mechanism that estrogen affects to fertilizability of in vitro matured oocytes. The concentration of WG extract ranging from 1.0 to 10.0 mg/ml may achieve better cytoplasmic maturation, leading to better fertilization.

None of oocytes used in this study showed evidence of polyspermy but parthenogenesis was found at the concentration of WG extract of 15 and 20 mg/ml.

The tuberous root of WG accumulates many substances that is known to have estrogenic effect. Chromenes are importantly active component of WG that made WG differ from other leguminosae plant such as miroestrol and deoxymiroestrol, but the latter was found to be about 10-fold more potent (Chansakaow et al., 2000a). Beside chromene, the previous reported showed the presence of various isoflavonoids such as daidzein, genestin, kwakhurin, diadzin, mirificin and puerarin (Martin et al., 1978; Verdeal et al., 1979; Farmakalidis et al., 1985; Mmiksicek, 1993; Hsieh et al., 1998) and coumarins such as coumestrol mirificoumestan, mirificoumestan glycol and mirificoumestan hydrate (Ingham, 1988 and cain, 1960) which also has estrogenic

effect. Recent studies on the chemical constituents of the tuberous root of this plant have reported new isoflavonoids, new ptercarpene and puemiricarpene. It was found that isoflavonoids may partly cause the revejunating action of this plant (Chansakaow et al., 2000b).

However, to investigate the full maturation of in vitro matured oocytes it is neccessary to determine not only their fertiliaztion competence, but also their potentioal for early embryonic development, thus, further studies are needed to investigate effect of WG extract on the embryonic development.

In conclusion, the present study demonstrated that the concentrations of WG extract ranging from 1.0 to 10.0 mg/ml has significantly stimulated oocyte maturation in vitro in a dose response manner and did increased in fertilizability of the in vitro matured oocytes. The exact mechanism that WG stimulated meiotic maturation and led to better ferilization were unknown. The suspected mechanism may be the direct action of WG that differ from in vivo, in a manner similar to the mechanism that estrogen affects the oocyte maturation and did increase in fertilizability. However, at the concentration of 15.0 and 20 mg/ml, WG sinificantly decreased the rate of oocytes maturation. It was notable that the concentration of 15.0 and 20.0 mg/ml also increased the degeneration of oocytes. It may be possible that the higher doses of WG caused toxicity to oocytes. Further studies are needed to use the purified substances that cause estrogenic effect and clearify the mechanism of WG in vitro maturation, investigate the effect of WG on quality of embryo and implantation.

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APPENDIX A

List of media used in this study

1.The component of Waymouth medium for oocyte growth and maturation

Component	Amount
Waymouth powder MB 752/1	13.8 g/liter
$NaHCO_3$	2.24 g/liter
Pyruvic acid	0.23 mM
Penicillin G (potassium salt)	119.7 unit
Streptomycin sulfate	38.85 unit
Bovine serum albumin (BSA)	0.4 mg/ml

2.The component of PBI, EKRB and M16

Component	PBI (mM)	EKRB (mM)	M16 (mM)
NaCl	136.00	120.00	94.66
KCl	15.42	2.00	4.78
$CaCl_2$	0.89	1.70	1.71
KH_2PO_4	1.47	-	1.19
$MgSO_4.7H_2O$	-	1.20	1.19
$MgCl_2.6H_2O$	0.44	-	-
$NaHCO_3$	-	25.00	25.00
$Na_2.HPO_4$	1.41	-	-
$NaH_2PO_4.H_2O$	-	0.36	-
Sodium lactate	-	-	23.28
Sodium pyruvate	-	1.10	0.33
Glucose	5.56	5.60	5.56
Sucrose	-	18.50	-
TAPSO*	-	10.00	-
Bovine serum albumin	0.4 %	0.4 %	0.4 %
Penicillin G	100 unit	50 unit	100 unit
Streptomycin sulfate	-	50 unit	38.85 unit
Phenol red			
Osmolality (mOsm/kg)	280±5	280±5	280±5
pH	7.35-7.40	7.35-7.40	7.35-7.40

*TAPSO, 3-[N-Tris(hydroxymethy)methylamino]-2 hydroxypropanesulfonic

APPENDIX B

List of the chemicals and materials used in the study

Chemicals / Materials	Source
0.2 μm filtered unit	Satorius, Germany
7x cleaning solution	ICN Biomedical, USA
Bovine serum albumin	Sigma chemical Co., USA
Calcium chloride	Sigma chemical Co., USA
D(+)-Glucose	Sigma chemical Co., USA
Flask	Nunclon, Denmark
Lactic acid	Sigma chemical Co., USA
Magnesium chloride 6-hydrate	Sigma chemical Co., USA
Magnesium sulfate 7-hydrate	Sigma chemical Co., USA
Mineral oil	Sigma chemical Co., USA
Penicilin G potassium salt	Sigma chemical Co., USA
Phenol red	Sigma chemical Co., USA
Plastic petri dish	Nunclon, Denmark
Potassium chloride	Sigma chemical Co., USA
Potassium phosphate monobasic	Sigma chemical Co., USA

Pyruvic acid	Sigma chemical Co., USA
Sodium bicarbonate	Sigma chemical Co., USA
Sodium chloride	Sigma chemical Co., USA
Sodium phosphate monobasic anhydrous	Sigma chemical Co., USA
Streptomycin sulfate	Sigma chemical Co., USA
Sucrose	Sigma chemical Co., USA
Syringe	Nipro, Thailand
TAPSO	Sigma chemical Co., USA
Waymouth's MB 752/1 medium powder	Gibco, USA

APPENDIX C

List of instruments used in the study

Instrument	Source
Beam balance	Satorius, Germany
Centrifuge	Kokusan, Japan
Dissecting microscope	Olympus, Japan
Laminar flow work station	Gelman Sciences, USA
Lyophilizer	Leybold-Heraeus, Germany
Makler couting chamber	Sefi-Medical, Irael
Osmometer	Gonotec, Germanny
pH meter	Orion, England
Water bath	Schupa, Germany
Water-jacketed incubator	Forma Scientific, USA
Water purifier	Elga, England

APPENDIX D

Effect of White Gwow on in vitro maturation of mouse oocytes

Experiment No. 1

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	5	5	0	4	6	0
0.5	10	0	3	6	1	2	6	2
1.0	11	0	3	8	0	3	8	0
5.0	10	0	2	8	0	0	9	1
10.0	8	0	1	7	0	0	8	0
15.0	11	0	4	6	1	2	6	3
20.0	11	2	5	3	1	4	3	4

Experiment No. 2

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	3	6	1	3	6	1
0.5	7	0	1	6	0	1	6	0
1.0	9	0	1	8	0	1	8	0
5.0	8	0	2	6	0	0	6	2
10.0	9	0	2	7	0	1	7	1
15.0	9	0	7	2	0	5	2	2
20.0	8	0	4	1	3	3	1	4

Experiment No. 3

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	3	6	1
0.5	9	0	2	7	0	2	7	0
1.0	10	0	2	8	0	2	8	0
5.0	10	0	1	8	1	1	8	1
10.0	12	0	2	10	0	1	10	1
15.0	8	0	3	4	1	1	4	3
20.0	9	1	3	3	2	1	4	4

Experiment No. 4

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	2	7	1
0.5	10	0	3	7	0	3	7	0
1.0	10	0	3	7	0	2	8	0
5.0	10	0	2	8	0	1	9	0
10.0	12	0	2	10	0	2	10	0
15.0	10	0	6	4	0	3	5	2
20.0	10	1	4	2	3	3	4	3

Experiment No. 5

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	8	0	3	5	0	2	6	0
0.5	8	0	2	6	0	1	7	0
1.0	8	0	2	6	0	1	7	0
5.0	8	0	0	8	0	0	8	0
10.0	8	0	2	6	0	0	8	0
15.0	8	1	3	4	0	3	5	0
20.0	8	1	3	3	1	3	3	2

Experiment No. 6

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	3	7	0
0.5	10	0	3	7	0	2	8	0
1.0	10	0	3	7	0	2	8	0
5.0	10	0	2	8	0	1	9	0
10.0	10	0	1	9	0	0	9	1
15.0	10	0	5	5	0	3	5	2
20.0	10	0	5	4	1	4	4	2

Experiment No. 7

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	6	0	1	5	0	0	5	1
0.5	7	0	2	5	0	1	5	1
1.0	6	0	1	5	0	1	5	0
5.0	8	0	2	6	0	1	6	1
10.0	8	0	1	7	0	0	8	0
15.0	10	0	5	5	0	3	6	1
20.0	7	0	2	5	0	2	5	0

Experiment No. 8

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	3	7	0
0.5	10	0	4	6	0	3	7	0
1.0	10	0	3	7	0	2	8	0
5.0	10	0	2	8	0	1	8	1
10.0	10	0	1	9	0	0	9	1
15.0	10	0	6	4	0	5	4	1
20.0	10	0	4	4	2	4	4	2

Experiment No. 9

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	4	5	0	3	6	0
0.5	8	0	2	6	0	1	7	0
1.0	9	0	3	6	0	1	8	0
5.0	9	0	0	8	1	0	8	1
10.0	8	0	0	8	0	0	8	0
15.0	9	3	2	4	0	3	4	2
20.0	9	4	3	2	0	4	2	3

Experiment No. 10

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	4	5	0	3	6	0
0.5	9	0	3	6	0	2	7	0
1.0	9	0	2	7	0	1	8	0
5.0	9	0	2	7	0	1	8	0
10.0	9	0	0	9	0	0	9	0
15.0	9	0	5	4	0	3	5	1
20.0	9	0	4	4	1	4	4	1

Experiment No. 11

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	4	5	0	3	6	0
0.5	9	0	3	6	0	3	6	0
1.0	10	0	3	7	0	2	8	0
5.0	10	0	2	8	0	1	8	1
10.0	12	0	0	12	0	0	12	0
15.0	10	0	4	5	1	3	5	2
20.0	10	1	4	4	1	4	4	2

Experiment No. 12

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	4	6	0
0.5	10	0	4	6	0	3	7	0
1.0	9	0	2	7	0	2	7	0
5.0	9	0	1	8	0	0	9	0
10.0	10	0	1	9	0	0	9	1
15.0	9	0	4	5	0	3	5	1
20.0	9	0	5	4	0	4	5	0

Experiment No. 13

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	8	0	3	5	0	2	6	0
0.5	8	0	2	6	0	1	7	0
1.0	7	0	1	6	0	1	6	0
5.0	8	0	1	7	0	0	8	0
10.0	8	0	0	8	0	0	8	0
15.0	7	0	2	5	0	1	5	1
20.0	7	1	3	3	0	2	3	2

Experiment No. 14

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	3	7	0	3	7	0
0.5	10	0	4	6	0	3	7	0
1.0	10	0	3	7	0	2	8	0
5.0	9	0	2	7	0	0	9	0
10.0	12	0	2	10	0	1	10	1
15.0	10	0	4	6	0	2	6	2
20.0	10	3	3	4	0	3	4	3

Experiment No. 15

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	4	6	0	4	6	0
0.5	10	0	4	6	0	3	7	0
1.0	10	0	3	7	0	3	7	0
5.0	10	0	2	8	0	2	8	0
10.0	10	0	1	9	0	0	10	0
15.0	10	0	3	6	1	3	6	1
20.0	10	0	4	5	1	4	5	1

Experiment No. 16

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	3	6	0	2	6	1
0.5	9	0	2	7	0	2	7	0
1.0	9	0	1	7	1	0	8	1
5.0	9	0	0	8	1	0	8	1
10.0	9	0	1	8	0	0	9	0
15.0	9	0	3	4	2	2	4	3
20.0	9	2	5	2	0	4	2	3

Experiment No. 17

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	10	0	3	7	0	4	6	0
0.5	12	0	4	8	0	4	8	0
1.0	11	0	3	8	0	2	8	1
5.0	11	0	3	8	0	1	8	2
10.0	9	0	1	8	0	0	9	0
15.0	12	0	4	6	2	3	6	3
20.0	12	1	7	4	0	6	4	2

Experiment No. 18

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	3	6	0	3	6	0
0.5	12	0	5	7	0	3	8	1
1.0	12	0	4	8	0	2	10	0
5.0	12	0	3	9	0	2	10	0
10.0	9	0	2	7	0	0	9	0
15.0	10	0	5	5	0	3	5	2
20.0	12	2	3	4	3	4	5	3

Experiment No. 19

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	11	0	5	6	0	4	6	1
0.5	12	0	5	7	0	3	9	0
1.0	10	0	2	8	0	1	9	0
5.0	9	0	1	8	0	1	8	0
10.0	9	0	1	8	0	0	9	0
15.0	8	0	3	5	0	0	5	3
20.0	10	1	5	2	2	5	3	2

Experiment No. 20

WG extract in Waymouth (mg/ml)	GV	GV at 24 hours	MI at 24 hours	MII at 24 hours	Degeneration at 24 hours	MI at 48 hours	MII at 48 hours	Degeneration at 48 hours
0	9	0	3	5	1	2	5	2
0.5	8	0	3	5	0	2	5	1
1.0	9	0	3	6	0	2	7	0
5.0	8	0	0	7	1	0	7	1
10.0	10	0	1	6	3	1	6	3
15.0	7	2	3	2	0	2	3	2
20.0	10	2	3	3	2	5	2	3

Effect of White Gwow on in vitro fertilization of mouse oocytes

Experiment No. 1

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	3	0	0
0.5	6	3	0	2	1	0
1.0	6	3	1	1	1	0
5.0	7	5	0	0	2	0
10.0	6	3	1	0	2	0
15.0	5	2	1	0	2	0
20.0	4	1	0	1	1	1

Experiment No. 2

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	2	0	0
0.5	6	4	0	2	0	0
1.0	7	4	0	2	1	0
5.0	7	5	0	1	1	0
10.0	5	4	0	1	0	0
15.0	6	2	0	3	1	0
20.0	4	0	0	3	1	0

Experiment No. 3

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	3	0	0
0.5	6	3	0	2	1	0
1.0	7	4	0	2	1	0
5.0	5	4	0	1	0	0
10.0	5	4	0	1	0	0
15.0	3	1	0	2	0	0
20.0	2	1	0	0	1	0

Experiment No. 4

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	4	0	2	0	0
0.5	7	4	0	2	1	0
1.0	7	4	1	1	1	0
5.0	7	5	0	1	1	0
10.0	8	5	0	1	2	0
15.0	4	1	0	2	1	0
20.0	4	0	0	2	2	0

Eeperiment No. 5

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	3	0	0
0.5	7	4	0	3	0	0
1.0	6	4	0	0	2	0
5.0	7	5	0	2	0	0
10.0	8	5	0	2	1	0
15.0	4	1	0	1	2	0
20.0	3	1	0	1	1	0

Experiment No. 6

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	1	1	0
0.5	6	3	0	2	1	0
1.0	5	3	1	1	0	0
5.0	7	4	0	2	1	0
10.0	5	3	0	1	1	0
15.0	4	2	0	2	0	0
20.0	4	1	0	2	1	0

Experiment No. 7

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	4	0	2	0	0
0.5	7	3	0	3	1	0
1.0	6	3	0	1	2	0
5.0	7	5	1	0	1	0
10.0	7	5	0	1	1	0
15.0	5	2	0	1	2	0
20.0	5	1	0	2	2	0

Experiment No. 8

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	2	0	3	0	0
0.5	5	3	0	2	0	0
1.0	4	3	0	1	0	0
5.0	5	4	0	1	0	0
10.0	5	3	0	1	1	0
15.0	5	2	0	3	0	0
20.0	6	2	0	3	1	0

Experiment No. 9

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	3	0	0
0.5	6	4	0	1	1	0
1.0	6	4	0	1	1	0
5.0	7	4	1	1	1	0
10.0	7	3	1	2	1	0
15.0	4	2	0	2	0	0
20.0	5	2	0	2	1	0

Experiment No. 10

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	4	0	1	1	0
0.5	6	3	0	1	1	0
1.0	6	4	0	1	1	0
5.0	7	4	0	1	2	0
10.0	7	5	0	1	1	0
15.0	6	3	0	1	1	1
20.0	6	2	0	1	2	1

Experiment No. 11

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	1	1	0
0.5	6	4	0	1	1	0
1.0	6	5	0	1	0	0
5.0	6	4	0	1	1	0
10.0	6	4	0	0	2	0
15.0	4	2	0	1	1	0
20.0	5	2	0	2	1	0

Experiment No. 12

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	0	2	0
0.5	6	4	0	2	0	0
1.0	6	4	0	2	0	0
5.0	7	4	0	2	1	0
10.0	10	6	0	1	3	0
15.0	5	2	0	0	3	0
20.0	5	2	0	1	2	0

Experiment No. 13

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	3	0	0
0.5	6	4	0	1	1	0
1.0	6	5	0	1	0	0
5.0	7	4	1	1	1	0
10.0	7	5	0	2	0	0
15.0	5	2	0	3	0	0
20.0	5	1	0	3	1	0

Experiment No. 14

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	2	0	0
0.5	6	4	0	2	0	0
1.0	5	4	0	1	0	0
5.0	6	4	0	1	1	0
10.0	6	4	1	1	0	0
15.0	5	2	0	2	1	0
20.0	4	2	0	1	1	0

Experiment No. 15

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	7	4	0	2	1	0
0.5	6	4	0	2	0	0
1.0	6	4	0	2	0	0
5.0	6	4	0	0	2	0
10.0	8	5	1	1	1	0
15.0	6	2	0	1	2	1
20.0	5	2	0	0	2	1

Experiment No. 16

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	5	3	0	2	0	0
0.5	6	4	0	1	1	0
1.0	5	4	0	1	0	0
5.0	7	4	0	1	2	0
10.0	6	4	0	2	0	0
15.0	4	2	0	2	0	0
20.0	3	1	0	1	1	0

Experiment No. 17

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	2	1	0
0.5	7	4	0	2	1	0
1.0	6	4	0	1	1	0
5.0	7	4	1	2	0	0
10.0	6	4	0	1	1	0
15.0	4	1	0	2	1	0
20.0	3	0	0	0	3	0

Experiment No. 18

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	7	2	0	3	2	0
0.5	8	4	1	3	0	0
1.0	7	4	0	2	1	0
5.0	7	4	1	2	0	0
10.0	6	4	0	2	0	0
15.0	4	1	0	2	1	0
20.0	5	0	0	2	2	1

Experiment No. 19

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	3	0	2	1	0
0.5	7	4	0	2	1	0
1.0	7	4	0	3	0	0
5.0	8	4	1	3	0	0
10.0	5	3	1	1	0	0
15.0	5	1	0	1	2	1
20.0	5	1	0	1	3	0

Experiment No. 20

WG extract in Waymouth (mg/ml)	MII	2 PN	2-cell	Unfertilization	Degeneration	Parthenogenesis
0	6	4	0	2	0	0
0.5	7	5	0	2	0	0
1.0	7	4	0	3	0	0
5.0	7	4	0	3	0	0
10.0	6	5	0	1	0	0
15.0	5	2	0	1	2	0
20.0	3	0	0	2	1	0

ประวัติการศึกษาและประสบการณ์โดยย่อ

1. Principal Investigator: Dr. Pornpimon Tangchaisin

1.1 Education

1979-1983 BSc. (nurse and midwifery) Mahidol University

1986-1989 MSc (Physiology) Chulalongkorn University

1993-1997 PhD. (Physiology) Chulalongkorn University

1.2 Field of interest

Assisted Reproductive Technology

Preimplantation genetic diagnosis

Genetic engineering

1.3 Publications

1. **พรภิมล ตั้งชัยสิน** และ ประมวล วีรุตมเสน, การวินิจฉัยโรคทางกรรมพันธุ์ในตัวอ่อนระยะก่อนฝังตัว. ใน การประชุมสัมมนาวิชาการเรื่อง การควบคุมและป้องกันโรคธาลัสซีเมีย ครั้งที่ 4 วันที่ 21-22 พฤศจิกายน 2539 ณ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น, หน้า LS 9/1-11.
2. **Tangchaisin, P.,** Pruksananonda, K., Trirawatanapong, T., and Virutamasen, P. (1996). Reliable Sex determination of Mouse Preimplantation Embryos by PCR Amplification of Male-specific Genes in Single Blastomeres. J. Med. Assoc. Thai. 79 (suppl.1):78-87.
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4. **Tangchaisin, P.,** Pansta, J., Chompurat, D., Ahnonkitpanit, V., Pruksananonda, K., Suwajanakorn, S., and Virutamasen, P. The effects of polyvinylpyrrolidone concentration on the outcome of assissted fertilization. Oral presentation at The Royal Thai college of Obstetricians and Gynaecologists 11th Scientific meeting, at Dusit Resort Pattaya Hotel, October16-18, 1996.
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7. **Tangchaisin, P.,(1996).** Oocyte maturation in vitro and cryopreservation in mouse model. In Syllabus for Hands-on workshop in oocyte maturation in vitro and cryopreservation, pp.1-4. Bangkok: Dept. of OB/GYN, Fact. of Medicine, Chulalongkorn University.

2. Co-investigator: Assoc. Prof. Dr. Amphawan Apisariyakul

2.1 Education

1963-1968 BSc. (Pharmacology) Medical University
1968-1970 MSc. (Pharmacology) Mahidol University
1971-1974 PhD. (Pharmacology) Mahidol University

2.2 Field of Interest

Basic Pharmacology
Applied Pharmacology

2.3 Publications

1. นพพร กันนะเมืองลี้ อัมภารัตน์ ร่มไทรย์ และ **อัมพวัน อภิสริยะกุล.** การศึกษาผลของสาร Stevioside ต่อเม็ดเลือดของกระต่าย บทความทางวิชาการ การสัมมนาเรื่องการวิจัยหญ้าหวาน 9-10 พฤษภาคม 2533 หน้า 77-86, 2534.
2. ไมตรี สุทธจิตต์, **อัมพวัน อภิสริยะกุล** และ รวิวรรณ พัวธนาโชคชัย.การศึกษาความปลอดภัยของหญ้าหวาน. (การรวบรวมองค์กรความรู้) โดยได้รับทุนสนับสนุนจากสำนักงานคณะกรรมการวิจัยแห่งชาติ (สกว.) 3 มีนาคม พ.ศ. 2538.
3. สาลิกา แสงมณี ,นันทนา ชนะรัตน์, **อัมพวัน อภิสริยะกุล,** จีรเดช มโนสร้อย และ อรัญญา มโนสร้อย.การศึกษาผลของน้ำสกัดจากเปลือกต้นนมนาง (Pouteria cambodiana) ต่อค่าทางโลหิตวิทยาและค่าทางเคมีคลินิกในหนูขาว. การประชุมวิชาการวันมหิดลครั้งที่ 23 วันที่ 24 กันยายน 2542 ณ. ชั้น 3 หรือชั้น 4 อาคารเรียนรวม คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่.
4. **อัมพวัน อภิสริยะกุล.** สตีวีโอไซด์. บทความทางวิชาการ การสัมมนาเรื่องการวิจัยหญ้าหวาน 9-10 พฤษภาคม 2533 หน้า 68-71, 2534.

5. อัมพวัน อภิสริยะกุล. การศึกษาฤทธิ์ทางเภสัชวิทยาของสารสติวีโอไซด์ขากหญ้าหวาน. บทความทางวิชาการ การสัมมนาเรื่องการวิจัยหญ้าหวาน 9-10 พฤษภาคม 2533 หน้า 72-76, 2534.
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21. **อัมพวัน อภิสริยะกุล** พาราควอต : พาราควอต (Paraquat) สารกำจัดวัชพืชมีผลกระทบต่อสิ่งแวดล้อมอย่างไร สารสารยาเชียงใหม่ ปีที่ 2 เล่มที่ 1 กันยายน-ธันวาคม 2537, หน้า 36-39.

22. **อัมพวัน อภิสริยะกุล.,** สารอาหารที่มีความสำคัญในร่างกาย., ไภษัชยสาร., ปีที่ 2 ฉบับที่ 1 เดือนพฤษภาคม 2540, หน้า 197-200.

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3. **Co-inveatigator: MS. Preeyalak Rodkaew** (Graduate student)

3.1 Education

1990-1994 BSc. (Occupational Therapy) Chiang Mai University

3.2 Field of Interest:

Reproductive Physiology