ผลของสารสกัดกวาวเครือขาว *Pueraria mirifica* ต่อการเจริญของอวัยวะสืบพันธุ์และการเติบโตของกบนา *Hoplobatrachus rugulosus*

นางสาวทาริณี โลนุชิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2553

EFFECT OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACT ON REPRODUCTIVE ORGAN DEVELOPMENT AND GROWTH OF RICE FIELD FROG *Hoplobatrachus rugulosus*

Miss Tarinee Lonuchit

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Thesis Title	EFFECT OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACT ON REPRODUCTIVE ORGAN DEVELOPMENT AND GROWTH OF RICE FIELD FROG *Hoplobatrachus rugulosus*
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ทาริณี โลนุชิต: ผลของสารสกัดกวาวเครือขาว *Pueraria mirifica* ต่อการเจริญของอวัยวะสืบพันธุ์และการเติบโตของกบนา *Hoplobatrachus rugulosus* (EFFECT OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACT ON REPRODUCTIVE ORGAN DEVELOPMENT AND GROWTH OF RICE FIELD FROG *Hoplobatrachus rugulosus*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ผุสตี ปริยานนท์, 108 หน้า

ศึกษาผลของสารสกัดกวาวเครือขาว *Pueraria mirifica* ที่มีสารไฟโตเอสโตรเจนในปริมาณสูงต่อการเจริญของอวัยวะสืบพันธุ์และการเติบโตในกบนา *Hoplobatrachus rugulosus* โดยในการศึกษาครั้งนี้ใช้หัวกวาวเครือขาวจากศูนย์ศึกษาพัฒนาห้วยฮ่องไคร้อันเนื่องมาจากพระราชดำริ จังหวัดเชียงใหม่ (PM-HHK) ก่อนนำกวาวเครือขาวมาศึกษาในกบนาได้ศึกษาลักษณะภายนอกที่ปรากฏและทดสอบฤทธิ์เชิงเอสโทรเจนด้วยวิธี Vaginal cornification ในหนูแรท โดยเปรียบเทียบกับกวาวเครือขาวสายพันธุ์มาตรฐาน คือ กวาวเครือขาวสายพันธุ์วิชัย 3 (PM-Wichai3) และสายพันธุ์มหาวิทยาลัยเกษตรศาสตร์ (PM-KU) และสบู่เลือด *Stephania venosa* พบว่าหัวกวาวเครือขาวทั้งสามสายพันธุ์มีลักษณะทรงกลมหรือรี เปลือกหนาสีน้ำตาลอ่อน เนื้อด้านในมีสีขาวหรือเหลืองอ่อน และมีเส้นใยจำนวนมาก เม็ดแป้งภายในหัวมีขนาด 3.63 - 4.27 ไมโครเมตร PM-HHK มีฤทธิ์ทางเอสโทรเจนใกล้เคียงกับ PM-Wichai3 แต่อ่อนกว่า PM-KU และคงฤทธิ์ได้นานกว่ากวาวเครือขาวทั้งสองสายพันธุ์ ในขณะที่สบู่เลือดไม่มีฤทธิ์เชิงเอสโทรเจน เมื่อนำผงบดละเอียดจากหัวกวาวเครือขาวไปสกัดด้วย 70% เอทานอล ได้สารสกัดปริมาณ 17.7%

จากนั้นทำการศึกษาในกบนาอายุ 4 สัปดาห์ (ระยะหลังการเปลี่ยนแปลงรูปร่าง) แบ่งกบออกเป็น 5 กลุ่ม (กลุ่มละ 30 ตัว) คือ กลุ่มที่ได้รับสารสกัดกวาวเครือขาวในปริมาณ 0, 1.77, 17.7 และ 177 มก. /นน.กก./วัน (กลุ่ม PM-0, PM-10, PM-100 และ PM-1000 ตามลำดับ) และ 17-β estradiol ปริมาณ 100 ไมโครกรัม/นน.กก./วัน (กลุ่ม E_2) ผสมในอาหารเม็ดสำเร็จรูปเป็นเวลา 12 สัปดาห์ ทุก ๆ 2 สัปดาห์ นำกบทุกตัวมาชั่งน้ำหนัก และทุก ๆ 4 สัปดาห์นำกบทุกตัวมาวัดขนาดความยาวตัวและสุ่มกบมา 9 ตัว/กลุ่ม ทำการุณยฆาต เจาะเลือดเพื่อนำไปวัดฮอร์โมนเอสโทรเจน (E_2) และเทสโทสเตอโรน (T) เก็บตับ ไต และอวัยวะสืบพันธุ์มาชั่งน้ำหนัก ศึกษาลักษณะทางสัณฐาน และลักษณะทางจุลกายวิภาค ผลการศึกษาพบว่ากวาวเครือขาวกระตุ้นการเพิ่มขึ้นของน้ำหนักตัวและความยาวตัวของกบนา ($p<0.05$) แต่ไม่สัมพันธ์กับขนาดที่ให้ และไม่มีผลต่อตับและไต ในขณะที่ E_2 เพิ่มเฉพาะความยาวตัว แต่น้ำหนักตัวไม่เปลี่ยน เมื่อศึกษาอัตราส่วนเพศจากลักษณะภายนอกที่ปรากฏของอวัยวะสืบพันธุ์ พบว่ากบนา (กลุ่ม PM-0) ที่เกิดในเดือนกันยายนมีอัตราส่วนเพศผู้สูงกว่าเพศเมีย (88.19/11.11) การให้กวาวเครือขาวทำให้น้ำหนักอวัยวะสืบพันธุ์เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) และแสดงลักษณะที่ปนกันของรังไข่และอัณฑะในร้อยละ 55.56, 59.26 และ 74.07 ในกลุ่ม PM-10 PM-100 และ PM-1000 ตามลำดับ แต่เมื่อนำอวัยวะสืบพันธุ์ที่แสดงลักษณะที่ปนกันของรังไข่และอัณฑะไปตรวจสอบทางจุลกายวิภาค พบว่าเป็นอัณฑะทั้งหมดเนื่องจากพบสเปิร์มในท่อเซมินิเฟอรัสจำนวนมาก แต่ไม่พบเซลล์ไข่ ระดับ E_2 ในซีรัมมีค่าต่ำและไม่แตกต่างกันระหว่างกลุ่มการทดลองทั้ง 5 กลุ่ม ระดับ T ในกลุ่มที่ได้รับกวาวเครือขาวมีแนวโน้มสูงกว่ากลุ่ม E_2 จึงทำให้ระดับ E_2/T ในกลุ่มที่ได้รับกวาวเครือขาวต่ำกว่ากลุ่ม E_2 อย่างมีนัยสำคัญยิ่งทางสถิติ ($p<0.01$) จากผลการทดลองในครั้งนี้สรุปได้ว่ากวาวเครือขาวสามารถเป็นสมุนไพรทางเลือกเพื่อนำไปใช้ในการกระตุ้นการเจริญเติบโตของกบนาได้ และถ้าให้ภายหลังจากที่กบเปลี่ยนแปลงรูปร่างแล้วจะไม่มีผลต่อการแปลงเพศ โดยกลไกการอออกฤทธิ์ของกวาวเครือขาวอาจจะแตกต่างจากของ E_2

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต.....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: GROWTH PROMOTER/ *Pueraria mirifica*/ *Hoplobatrachus rugulosus*
TARINEE LONUCHIT EFFECT OF WHITE KWAO KRUA *Pueraria mirifica* EXTRACT ON REPRODUCTIVE ORGAN DEVELOPMENT AND GROWTH OF RICE FIELD FROG *Hoplobatrachus rugulosus*
ADVISOR: ASSOC. PROF. SUCHINDA MALAIVIJITNOND, Ph. D.,
CO-ADVISOR: ASSOC. PROF. PUTSATEE PARIYANONTH, 108 pp.

This study aimed to determine effects of *Pueraria mirifica* (PM) extract which contains high amount of phytoestrogen on reproductive organ development and growth of Rice Field Frogs *Hoplobatrachus rugulosus*. The tuberous roots of PM used in this study were collected from Huai Hong Khrai Royal Development Study Center, Chiang Mai Province (PM-HHK). Before the PM-HHK was tested in frogs, it was determined the gross morphology and estrogenic activity by vaginal cornification assay in rats compared with those of PM-Wichai3 cultivar, PM Kasetsart University (PM-KU) cultivar, and *Stephania venosa*. The roots of PM were round or oval shape with the yellow brown and thick bark, and the color of the tuberous meat was white with a number of fibers. The starch granules had diameters of 3.63 – 4.27 μm. The potency of estrogenic activity of PM-HHK was similar to that of the PM-Wichai3, but was weaker than that of the PM-KU; however, the estrogenic activity of PM-HHK was kept longer than those of PM-Wichai3 and PM-KU. The *Stephania venosa* did not show any estrogenic activity. The % yield of PM-HHK extracted by 70% ethanol was 17.7%.

One hundred fifty 4 weeks old, at complete metamorphosis stage, Rice Field Frogs were selected, divided into 5 groups (30 frogs/group), and treated with 0, 1.77, 17.7, 177 mg/kg BW/day of PM extract (PM-0, PM-10, PM-100 and PM-1000, respectively) or 100 μg/kg BW/day of 17β-estradiol (E_2) by mixing with frog pellets, for 12 weeks. Frogs were individually measured body weights every 2 weeks. Every 4 weeks, frogs were individually measured body lengths and 9 frogs/group were randomly selected and euthanized. After euthanized, blood sample was collected for estrogen (E_2) and testosterone (T) determinations, and livers, kidneys and gonads were weighed and examined the gross morphology and microscopic histology. PM increased body weight and lengths of frogs ($p<0.05$), but not depended on doses, and showed no toxicological effect on liver and kidney. E_2 could stimulate an increase only in body length, but not on the weight. Determination of the sex ratio of frogs delivered in September, based on the gross morphology, showed the incline to male (88.89/11.11 for male/female). PM treatments significantly increased gonadal weights ($p<0.05$) and induced a mixed type (ovotestis) of gonad by 55.56%, 59.26% and 74.07% for PM-10, PM-100 and PM-1000, respectively. Once the ovotestis was microscopic examined, many sperms were observed in seminiferous tubules, but not oocytes. This indicated a testis type of gonad. There were no significant differences of serum E_2 levels among all five treatment groups, and the levels were low. Serum T levels of all four PM groups tended to be higher than those of the E_2 group. Thus, the serum E_2/T levels in PM treated groups were highly significantly lower than the E_2 group ($p<0.01$). From the results of this study, it can conclude that PM should be an alternative herb of growth promoting of Rice Field Frogs. If the treatment is conducted at the complete metamorphosis stage of frogs, it does not induce a sex reversal. However, the different mechanisms of actions on gonadal development between E_2 and PM are proposed and need to be investigated further.

Department: Biology Student's Signature .....

Field of Study: Zoology Thesis Advisor's Signature .....

Academic Year: 2010 Thesis Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

BW	=	Body weight
◦C	=	Degree celsius
CNS	=	Central nervous system
Co	=	Cornified cell
D	=	Day
DHT	=	Dihydrotestosterone
DW	=	Distilled water
E_2	=	17-β estradiol
ED	=	Endocrine disrupter
EE	=	17α-estradiol
ERs	=	Estrogen receptors
FSH	=	Follicle-stimulating hormone
g	=	Gram
GnRH	=	Gonadotropin-releasing hormone
HPG	=	Hypothalamus-pituitary-gonad axis
kg	=	Kilogram
L	=	Leukocyte cell
LH	=	Luteinizing hormone
µg	=	Microgram
mg	=	Milligram
MS-222	=	Tricaine methane sulphonate
NF	=	Nieuwkoop and Faber stage of development
O	=	Nucleated cell

LIST OF ABBREVIATIONS

Ovx	=	Ovariectomy
PCBs	=	Polychlorinated biphenyls
PM	=	*Pueraria mirifica*
PM-HHK	=	*Pueraria mirifica* Huai Hong Khrai cultivar
PM-KU	=	*Pueraria mirifica* Kasetsart University cultivar
PM-Wichai3	=	*Pueraria mirifica* Wichai3 cultivar
SBP	=	Steroid-binding protein
SV	=	*Stephania venosa*
SVL	=	Snout-vent-length
T	=	Testosterone

CHAPTER I

INTRODUCTION

Rice Field Frogs *Hoplobatrachus rugulosus* are common frog species which distribute throughout Thailand (Nabhitabhata, Chan-ard and Chuaynkern, 2000). Regarding their names, they have been found in the rice field, especially during rainy season. As frogs become an alternative protein source (Teixeira, 1993; Tokur et al., 2007), frog farming becomes one of the livestock in many countries in Europe (Stebbins and Cohen, 1995), America (NOAA, 2005) and Asia (Sardava and Srikar, 1982; Fugler, 1985; Martin, 2000; FAO, 2006). In Thailand, Rice Field Frogs are recently popularly cultured and sold on a commercial basis (Somsiri, 1994).

Frogs and other amphibians are cold blooded animals that grow slowly and not a particularly desirable trait for farming. The rate of growth of the tadpole varies with the climate, length of growing season and size that is available for supply. For the Rice Field Frog, it takes at least 4 months to transform the tadpole into marketable size, young frogs, and requires another 1-2 years to produce mature bleeders (Naksing, 2003; Pariyanonth and Daorerk., 1994). Thus, many frog farmers searched for the ways to promote the growth rate in Rice Field Frogs. Synthetic estrogens are particularly used for that purpose (FDA, 2002; Ferrando, 1982; Preston, 1999; Velle, 1982; Umberger, 1975), however, the estrogenic residues are usually remained in the frog meat and it is banded by the Food and Drug Association (FDA, 2002). To be success in frog farming on a commercial scale, the cost to grow and the socioeconomic factors should be taken into consideration. Thus, the natural products or plants which are inexpensive, promoting growth, technically indifficult to treat to

the frogs and no estrogenic residues remained in the frog meat should be urgently searched.

Pueraria mirifica, a phytoestrogen rich herb, is widely found throughout Thailand. Estrogenic activity of this plant has been scientifically tested in various organ systems e.g. reproductive organ, cancer and bone in rodents and primates (Cherdshewasart et al., 2007, Malaivijitnond et al., 2006, 2007, 2008 and 2010, Urasopon et al., 2007 and 2008, and Trisomboon et al., 2004). It was also reported to increase growth rate in fish (Klaharn et al., 2003), chicken (Tubcharoen et al., 2006) and rabbit (Tubcharoen et al., 2005). To this aim, if *P. mirifica* can stimulate growth rate in Rice Field Frog was tested in this study. Hopefully, the results gained from this study could be beneficial to the frog farmer and this knowledge could also be translated to the marketing scale.

Use of phytoestrogens or *P. mirifica* as a growth promoting agent in amphibian has never been reported either the optimal dosages or side effects. In this study the crude extracts of *P. mirifica* at doses of 1.77, 17.7 and 177 mg/kg BW of frogs were used. These three doses are equal with the *P. mirifica* powder at concentrations of 10, 100 and 1,000 mg/kg BW/day, respectively, which were basically used to stimulate reproduction in rodents as reported in the previous studies (Cherdshewasart, 2003; Cherdshewasart et al., 2007a; 2007b; 2008; Malaivijitnond et al., 2004; 2006; 2010; Urasopon et al., 2008a; 2008b). In addition, sex differentiation of amphibian also depended on genetics and external endocrine disrupters (BÖgi et al., 2002 and 2003; Wallace, Badawy and Wallace, 1999). During a critical period of sex differentiation process, estrogen or phytoestrogens could induce gonad feminization (Li et al., 2006; Mackenzie et al., 2003; Villapando and Merchant-Larios, 1990). Thus, the gonad development of Rice Field Frogs after *P. mirifica*

treatment was also observed in this study. Since the treatment was initiated in complete metamorphosis stage of frogs, after a critical period of sex determination, if *P. mirifica* can induce the sex reversal in post-metamorphosis frogs is one of the focal points. In addition, if the ovotestis characters were found after confirmed by microscopic observation, the functional type of gonad (ovary or testis) was ensuring by sex steroid hormones (estrogen and testosterone) determination.

Objectives

This study aims

1. To determine the estrogenic effect of *Pueraria mirifica* extract on reproductive organ development of Rice Field Frogs.
2. To determine the estrogenic effects of *Pueraria mirifica* extract on growth rate of Rice Field Frogs.
3. To evaluate the effects of *Pueraria mirifica* extract on sex steroid hormones levels in blood serum of Rice Field Frogs.

CHAPTER II

LITERATURE REVIEW

1. Rice Field Frog

1.1. Taxonomy

Rice Field Frog is taxonomically classified as follows;

Kingdom Animalia

Phylum Chordata

Class Amphibia

Order Anura

Family Dicroglossidae

Genus *Hoplobatrachus*

Species *H. rugulosus*

H. rugulosus also has 18 synonyms as follows (Frost, 2010):

1) *Rana chinensis* Osbeck, 1765;
2) *Rana rugulosa* Wiegmann, 1834;
3) *Rana tigrina* var. *pantherina* Steindachner, 1867;
4) *Hydrostentor pantherinus* Steindachner, 1867;
5) *Rana esculenta chinensis* Wolterstorff, 1906;
6) *Rana burkilli* Annandale, 1910;
7) *Rana tigerina* var. *burkilli* Boulenger, 1918;
8) *Rana rugulosa* Annandale, 1918;
9) *Rana (Rana) tigerina* var. *pantherina* Boulenger, 1920;
10) *Rana tigrina rugulosa* Smith, 1930;

11) *Rana tigerina rugulosa* Fang & Chang, 1931;

12) *Rana tigerina pantherina* Taylor & Elbel, 1958;

13) *Rana (Euphlyctis) rugulosa* Dubois, 1981;

14) *Euphlyctis tigerina rugulosa* Poynton & Broadley, 1985;

15) *Limnonectes (Hoplobatrachus) rugulosus* Dubois, 1987;

16) *Tigrina rugulosa* Fei, Ye & Huang, 1990;

17) *Hoplobatrachus rugulosus* Dubois, 1992*;*

18) *Hoplobatrachus chinensis* Ohler, Swan & Daltry, 2002

Rice Field Frog *Hoplobatrachus rugulosus* (Wiegman, 1835) has the morphological appearance of olive brown color with numerous small black spots on skins, the nostril is much nearer tip to snout than to eyes, and lips with dark spots are separated by pale yellow color (Figure 2-1). Adult's size is approximately 6.8 – 8.5 cm in length and weighed about 300 g (Chan-ard, 2003). They are commonly found in every provinces in Thailand, particularly in the lowland and small hills (Nabhitabhata, Chan-ard and Chuaynkern, 2000)

Figure 2-1. *Hoplobatrachus rugulosus*

1.2 Frog culture

Rice Field Frog *H. rugulosus* is a common frog species which is cultivated and found in many frog farms in Thailand (Pariyanonth and Daorerk, 1994). They were recently popularly cultured and selling on a commercial basis (Somsiri, 1994). In 2008, Ministry of Commerce of Thailand reported that the Rice Field Frog was exported by 12,607 tons of 99.93 million baht.

Commonly, frog farmers bought the tadpoles or juvenile frogs (froglets) from the breeding farms. Therefore, the cost of frog farming included the cost of tadpole or froglet, rearing ponds, water treatment and frog pellets. It took at least 4 months of frog culturing before their sizes were reached the market demand (Sangtam, 2005). The farmer had also to feed the frogs daily with frog pellets at amount of 3 – 5% of g BW (Pariyanonth and Daorerk., 1994; Sangtam, 2005; Somsiri, 1994).

Frog farming has been becoming popularly because the market value is higher than the cultured fresh water fishes and especially at the areas where the quantity of water and space were limited. The frog species of *H. rugulosus* also has high potential for cultivation on a commercial scale (Somsueb and Boonyaratpalin, 2001). The frog meats and their products are great of interest in many counties of Europe and America because their taste and color were similar to the chicken meat (Tokur, Gürbüz and Özyurt, 2008). In France, according to Stebbins and Coben (1995), the demand on frog meat was 3.4 tons per year. Moreover, frog has been one of the alternative protein sources with high protein content (Ojewala and Udom, 2005).

Although it showed a positive trend for frog framing and marketing, a major problem for frog farmers was a high cost of production. In fact, frog culture relied on many factors, such as breeding and feeding behaviors of frogs and growth rates. Therefore, many researches had been conducted to search for the way to reduce a cost

of frog farming. Frog nutrition was a main factor that affected on growth and survival rates of adults and froglets. According to previous studies on nutritional requirements of *Rana* species, frogs that fed on optimum protein, lipid and carbohydrate levels, could grow and survive on a better rate than frogs that fed on non-optimal nutrition (Donaghue, 1998; Somsueb and Boonyaratpalin, 2001; Martínez, Real and Álvarez, 2004). Scientists also designed food pellets that were relevant with feeding behavior of frogs. Generally, Ranid frogs, frogs in genus of *Rana*, preferred to consume living and moving preys in natural condition and were not response to a non-movable or inert food in captivity (Modzelenski and Cully, 1974; Holyoak, 2002). The optimization of pellets characteristics, such as modification of texture and color could produce a higher consumption of frogs in captivity.

In 2003, Browne et al. studied the high density effects on the growth, development and survival of *Litoria aurea* tadpoles. The result revealed that use of high density rearing technique with tadpoles could produce consistently high quality metamorphs, shortening rearing time and metamorphosis period, and used water, feed and space more efficiently.

In 2004, Miles et al. investigated biological and mechanical agents to increase a consumption of pelleted food by adult *Rana temporaria*. The inert pellets were moved by mechanical stirrer or by fly larvae. It was found that only fly larvae significantly increased consumption of pelleted food in *Rana temporaria.*

To reduce the culturing period, growth promoting chemicals were also used for frog farming. Generally, synthetic estrogen, a sex steroid hormone which is mainly synthesized from ovary in females, was widely used in aspect of growth promoting agent in many livestock productions (Ferrando, 1982; Preston, 1999; Umberger, 1975; Velle, 1981). However, using of estrogen to promote growth in

livestock was recently banned by the United States Food and Drug Administration (FDA, 2002), because the remaining of estrogenic residues in meats could cause the side effects on the customers. In fact, growth promoter was still important in frog farming on a commercial scale. To avoid the remaining of estrogenic residues in frog meats, the natural products or plants which have had estrogenic activity should be an alternative choice. Furthermore, natural products have many advantages over the synthetic chemicals, that is, they are inexpensive, promoting growth properly and easily to treat to frogs. In Thailand, there have been many endemic herbs or plants containing phytoestrogens (Cherdshewasart, 2003; Cherdshewasart et al., 2007a; 2007b; 2008; Malaivijitnond et al., 2004; 2006; 2010; Urasopon et al., 2008a; 2008b). However, using of estrogen or phytoestrogens as a growth promoter in frogs may have several side effects, particularly on sex organ development or sex differentiation which should be aware (Cong et al., 2006; Kloas and Lutz, 2006; Wallace, Badawy and Wallace, 1999).

2. Sex differentiation in amphibian

Sex determination of amphibian has controlled by both genetics and environmental factors (Wallace, Badawy and Wallace, 1999).

In 1991, Schmid et al. determined chromosomal sex of 50 species of anurans and urodales. They identified both male heterogamete (xx/xy) and female heterogamete (zz/zw) in this group of animals.

In 1999, Wallace, Badawy and Wallace reported heterogamete male (xx/xy) and heterogamete female (zz/zw) appearing in Urodales and Anurans as shown in Table 2-1. They also found that the heat treatment (over 26 ºC) could change the sex ratio in urodales *Pleurodeles poireti* from 1:1 (male: female) to 1: 3.

Genus	Sex chromosome system
Urodales	
Amybystoma	zz/zw
Hynobius	?
Pleurodeles	zz/zw
Triturus	xx/xy
Anurans	
Buergeria	zz/zw
Bombina	xx/xy
Hyla	xx/xy
Pelobates	?
Pseudacris	?
Rana	xx/xy
Xenopus	zz/zw

Table 2-1. Variation of sex chromosome system in 11 amphibian species

In 2001, Chokchaichamnankij studied sex chromosome of *Hoplobatrachus rugulosus* by chromosome banding technique; however, he could not identify the sex chromosome in *H. rugulosus*.

In 2002, Bögi et al. reported that estrogen treatment could increase percentage of female sex in *Xenopus laevis*, a heterogamete male frog (zz/zw). The process of sex differentiation in *Xenopus laevis* was controlled by genetics and sex steroid hormones (Figure 2-2). Sex was first determined by chromosome and was called a genomic sex. Then maternal sex steroid hormones, estrogen or androgens, were elevated in larvae at a stage of development of Nieuwkoop and Faber (NF) 38. The gonad was developed to ovary or testis which was depended on the ratio of androgen and estrogen levels. At NF stage 62 or nearly the end of metamorphosis process estrogen concentration was high in females and androgen concentration was low. Remarkably, the endocrine

disrupter (ED), such as xenoestrogens or phytoestrogens could disturb the normal sex differentiation.

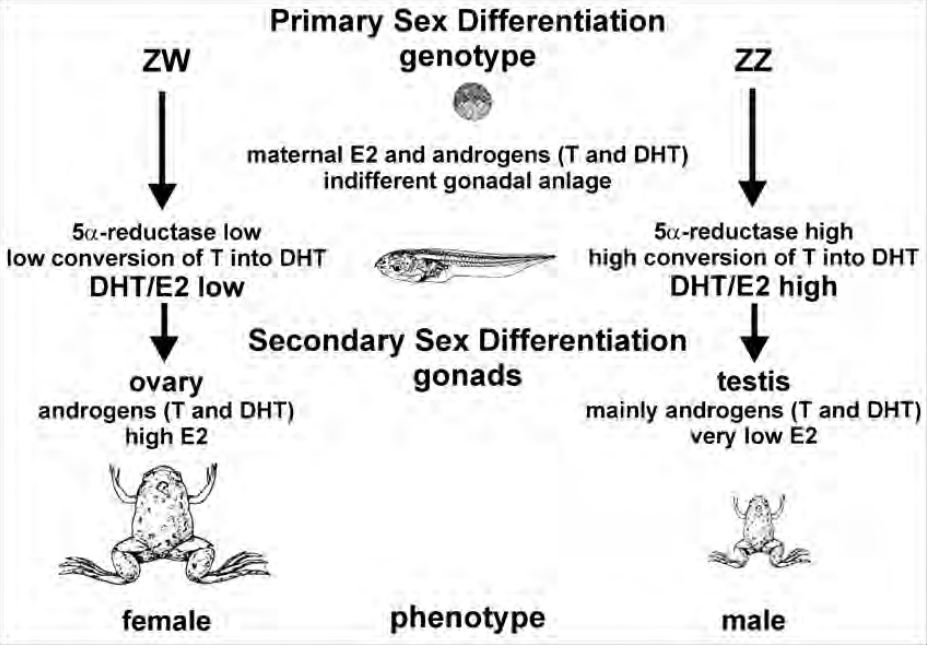


Figure 2-2.The process of sex differentiation in *Xenopus laevis*.

Since sex determination and differentiation in amphibians were depended on environmental factors, temperature and endocrine disrupters could induce sex reversal (Tinsley and Kobel, 1996).

In 2003, Mackenzie et al. reported that the exposure of synthetic estrogens, both 17β-estradiol (E_2) and 17α-estradiol (EE), could induce feminization and increased percentage of female of leopard frog *Rana pipiens* and Woods frog *Rana sylvatica* when the exposure started in the early stage of life at Gosner stage 25 to 42. The exposure of both E_2 and EE could induce both morphological and microscopic appearances in gonads. However, frogs could only be identified as intersexual frogs if changes at the microscopic level was confirmed. If the intersexual gonad was observed only at the morphological levels, it should not be identified as an intersex.

In 2006, Cong et al. reported that quercitin, a phytoestrogen in citrus fruits, could induce sex reversal in *Xenopus laevis* when the treatment was conducted in the early stage of tadpoles. Quercitin at doses of 50, 100 and 200 μg/L treated to 46/47 stage of male tadpoles until 1 month postmetamorphosis stage induced feminization. The oocytes were found in testis tissues of frogs.

Kloas and Lutz (2006) presented the regulation of gonadal function via a hypothalamus-pituitary-gonad (HPG) axis in amphibians (Figure 2-3). The endogenous and exogenous stimuli triggered on the central nervous system (CNS) and then hypothalamus to secrete gonadotropin-releasing hormone (GnRH). GnRH stimulated gonadotroph cells in pituitary gland to secrete luteinizing hormone (LH) or follicle-stimulating hormone (FSH) into the blood circulation. In male, gonadotropins (LH and FSH) stimulated interstitial (Leydig) cells in testis to synthesis and release androgens, mainly testosterone (T) and dihydrotestosterone (DHT). Androgens were carried to the blood and to the target organs (CNS, gonad and liver) by binding with the carrier protein (sexual steroid-binding protein; SBP). The signal pathway included a binding of androgens to cytosolic or nuclear androgen receptor then transfered into the nucleus to induce androgen-specific gene expression (Beato, Herrlich and Schultz, 1995). ED could interrupt the process at all levels. For example, estrogen treatment disturbed the negative feedback mechanism in HPG axis (I) and influenced the rate of sex steroid secretion (V) or acted on the target organs directly (II-IV) which could induce the abnormal sexual differentiation or malformation of testis in early stage of life, and initialized vitellogenin synthesis in liver cells. In female, sex steroids regulated via HPG axis in similar pathways as those in males. The impact of ED, either of estrogens or androgens, on steps I, II, IV and V in females showed the similarity, because androgens or T could be converted to estrogens by aromatase

enzyme. However, the impact on sexual differentiation (III) of generic females showed the differences. The androgenic ED could induce masculinization or malformation of ovary.

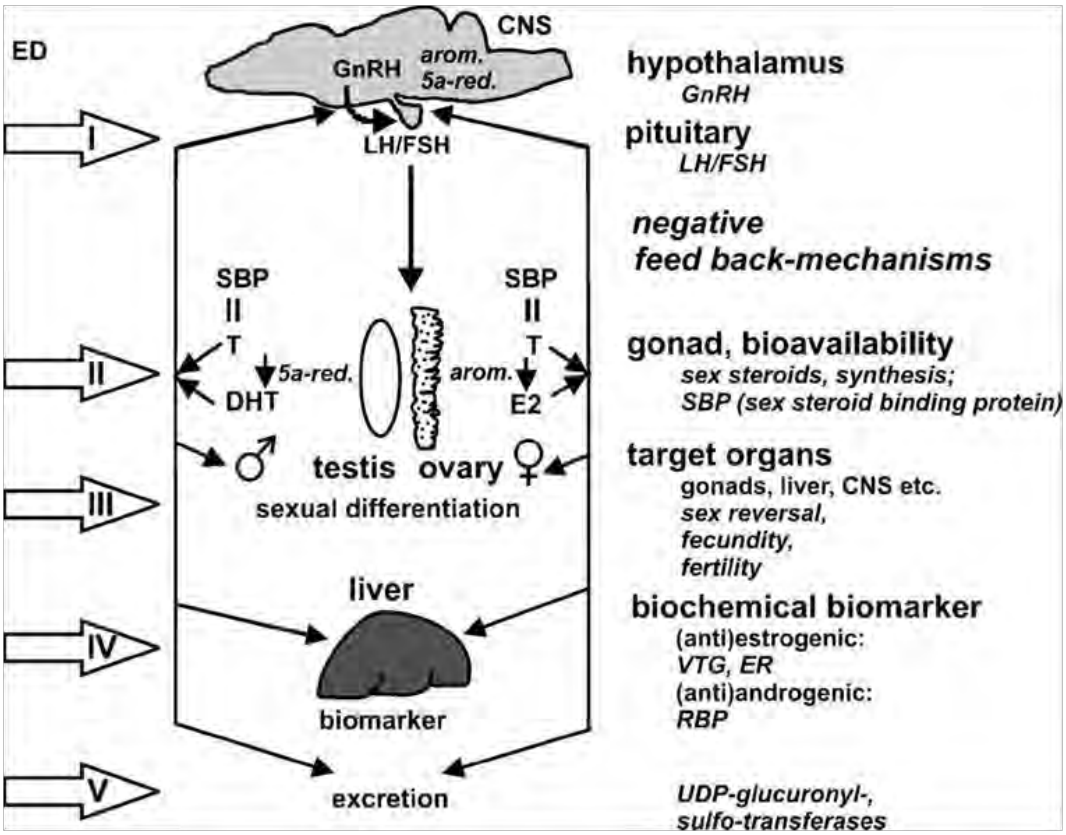


Figure 2-3. Hypothalamus-pituitary-gonad axis and mode of action of endocrine disrupter in amphibian.

Most researches in ED on gonad differentiation were conducted in African clawed frog *Xenopus laevis*. The exposure of herbicide atrazine at low ecologically relevant dose could feminize and induce intersexual frogs (Hayes et al., 2002) and could reduce the number of primary germ cells in both males (Tavera-MendoZa et al., 2002a) and females (Tavera-MendoZa et al., 2002b). Coolants polychlorinated biphenyls (PCBs) could feminize and induce testis malformation (Qin et al., 2007). On the other hand, the treatment of breast cancer Fadrozole induced masculinization on ovary morphology (Olmstead et al., 2009).

From the literatures, the estrogen or phytoestrogen exposures were able to disturb normal sex differentiation and induced sex reversal in early stage frogs. The estrogen or estrogen-like substance also feminized the frog gonad, and induced oogenesis in testis. Those characters were recognized as intersex. Thus, using of estrogens or phytoestrogens as growth promoter should consider these side effects. To decrease the disturbance in sex differentiation, the treatment should be started at the post-metamorphosis stage of frogs.

3. White Kwao Krua

3.1 Taxonomy

White Kwao Krua is taxonomically classified as follows;

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Fabales

Family Leguminosae

Genus *Pueraria*

Species *Pueraria mirifica*

This phytoestrogen rich herb could be found throughout Thailand (Cherdshewasart, Kijsamai and Malaivijitnond, 2007). It was used as an ingredient in Thai traditional medicine for curing the menopausal symptoms (Cherdshewasart, Sriwacharakul and Malaivijitnond, 2008).

Figure 2-4. Leaves (a and b), flowers (www.pueraria-thai.com) (c) and tuberous root of *Pueraria mirifica* (d).

3.2 *Pueraria mirifica* and phytoestrogens

Phytoestrogens are plant derivative compounds which could induce biological responses in vertebrates. Phytoestrogens could imitate the action of endogenous estrogen by binding to estrogen receptors (ERs). Phytoestrogens are commonly found in many plants. *Pueraria mirifica* is one of phytoestrogens rich herbs, it contains at least 22 phytoestrogens (Bounds and Pope, 1960; Chansakaow et al., 2000a; 2000b;

Hayodom, 1971; Inglam et al., 1986; 1988; 1989; Joen et al., 2005; Jones and Pope, 1961; Nilandihi et al., 1957; Scholler et al., 1940) as shown in Table 2-2.

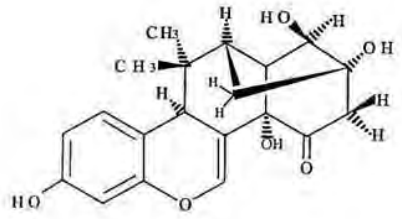
Miroestrol (Figure 2-5a) is the first phytoestrogen extracted from *P. mirifica* and had the strongest estrogenic potency on reproductive organs (Keung, 2002). It could induce cell proliferation in uterus and vagina of immature female rats, the potency was 0.25 times of 17β-estradiol (Jones and Pope, 1960). Deoxymiroestrol (Figure 2-5b) showed strongest estrogenic effects on breast cancer cells (MCF-7) compared with the miroestrol and isomiroestrol (Figure 2-5c). Since deoxymiroestrol could be oxidized in the air during preparing process and restructured to miroestrol, thus, the most recognized effective phytoestrogen in *P. mirifica* is miroestrol. However, the constituents of phytoestrogens of *P. mirifica* were varied and depended on seasonal changes, genetic and metabolic activation of phytoestrogens (Cherdshewasart and Sriwatcharakul, 2008)

In Thailand, *P. mirifica* has been used as a traditional replacement therapy for a long time (Suntara, 1931). Nowadays, it has been used widely in many aspects, such as food supplements for anti-aging, skin rejuvenation and breast enlargement (Dweck, 2002).

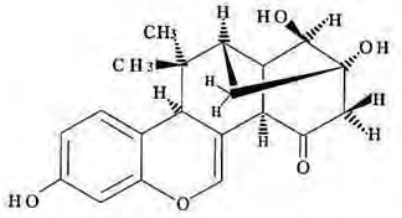
Table 2-2. Phytoestrogens found in *Pueraria mirifica.*

Class	Phytoestrogens
Isoflavonoid	Diadzein
	Genistein
	Kwakhurin
	Kwakhurin hydrate
Isoflavone glycoside	Diadzin (Diadzein-7-o-glucoside)
	Genistin (Genistein-7-o-glucoside)
	Mirificin (Puerarin6'-o-β-apiofuranoside)
	Puerarin (Diadzein-8-glucoside)
	Puerarin 6''-monoacetate
Chromenes	Miroestrol
	Deoxymiroestrol
	Isomiroestrol
Coumestans	Coumestrol
	Mirificoumestan
	Mirificoumestan glycol
	Mirificoumestan
Sterols	β–sitosterol
	Stigmasterol
	Spinasterol
Pterocapans	Puericapene
	Tuberosin
Acid	Tetracosanoic acid

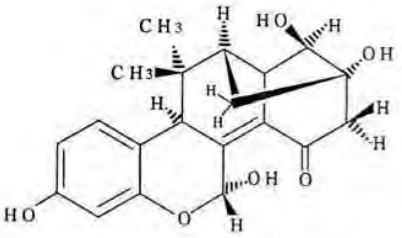
a) Miroestrol



b) Deoxymiroestrol



c) Isomiroestrol



d) Estradiol

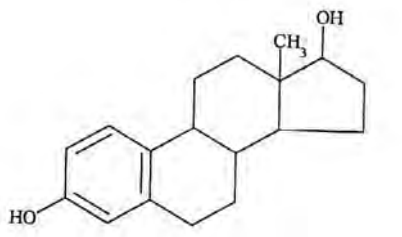


Figure 2-5. Chemicals structure of miroestrol (a), deoxymiroestrol (b), isomiroestrol (c) and 17β-estradiol (d)

The estrogenic activity of phytoestrogens in *P. mirifica* was also tested in various organ systems in animals and humans. Feeding a suspension of 1,000 mg/kg BW/day of *P. mirifica* for 30 days to OVX and orchidectomized rats showed a decrease in both LH and FSH levels in blood serum (Malaivijitnond et al., 2004). *P. mirifica* treatement could suppress gonadotrophin levels in aged menopausal monkeys and depended on doses (Trisomboon et al., 2006). Daily treatment of *P. mirifica* powder could decrease low-density lipoprotein (LDL) cholesterol and increase high-density lipoprotein (HDL) cholesterol in menopause women (Okamura et al., 2008).

P. mirifica could be used as an anti-fertilization and contraceptive agent. The daily treatment of 1 g/day of *P. mirifica* powder could decrease a mating behavior in male dogs and inhibit a pregnancy in mated female dogs during breeding season (Smitasiri, 1988). The diet mixed with *P. mirifica* could control a birth rate of pigeon by inhibiting courtship and mating behaviors. *P. mirifica* also inhibited the reproductive organs development on both male and female pigeons (Smitasiri and Sakdarat, 1995).

After many researchers emphasized that *P. mirifica* contained phytoestrogens and had estrogenic activity, use of *P. mirifica* to induce many livestock productions, for example, pig, rabbit, chicken and fish, was done. Daily treatment of *P. mirifica* at dosages of 25 and 50 g/day could induce a mammary gland development and increase the layer of adipose tissue in immature female pigs. *P. mirifica* also increased the hair and skin development (Smitasiri, 1997). Likewise, mixing a pig diet with 200 ppm of *P. mirifica* powder could increase growth rate, decrease stress and inhibit mating behavior in pig. It also increased a formation of muscle and decreased a fat tissue on dorsal post of pig body (Inthanont, 2005). Treatment of *P. mirifica* powder to immature rabbits could stimulate nipple, mammary gland, uterine, oviduct and vagina

developments (Wongviriya et al., 1998). Mixed a chicken food with 2% of *P. mirifica* powder and fed to a Thai native crossbred chicken could increase a breast weight and an abdominal fat (Tubcharoen, 2007). Treatment of *P. mirifica* powder in hybrid catfish (*Clarias macrocephalus* x *C. gariepinus*) induced a higher growth rate, feeding efficiency, feeding convention, protein efficiency and net protein utilization than control (Kanjanaworakul et al., 2006).

From the literatures cited above, *P. mirifica* had a potential to promote the growth in economic animals, thus the study of the effects of *P. mirifica* on growth of Rice Field Frogs were conducted in the present study.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Animals

1.1. Wistar rat

Sixty female Wistar rats at 8 weeks old and weighing 175-220 g were used in this study. They were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. These rats were used to test the estrogenic activity of *P. mirifica* Huai Hong Khrai cultivar (PM-HHK) compared with those of *P. mirifica* Kasetsart University cultivar (PM-KU) and Wichai3 cultivar (PM-Wichai3) by a vaginal cytology assay.

1.2. Rice Field Frog

One hundred and fifty Rice Field Frogs used in this study were purchased from Somporn Farm, Wang Noi District, Ayutthaya Province, Thailand. They were 4 weeks old and showed a complete metamorphosis, weighing 5-6 g. These frogs were used to test the estrogenic activity of PM-HHK in respects of growth and gonad development.

2. Plant materials

The tuberous roots of *P. mirifica* used in this study were collected from the forest in Huai Hong Khrai Royal Development Study Center, Doi Sa Ket District, Chiang Mai Province, Thailand (hereafter it is named PM-HHK cultivar) (Figure 3-1).

Figure 3-1. The tuberous roots of *Pueraria mirifica* collected from the Huai Hong Khrai Royal Development Study Center and used in this study

It was authenticated as the *P. mirifica* (Airy Shaw & Suvatabhandhu) by comparing with the voucher specimen numbers BCU010250 and BCU011045 which have been deposited at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University (Figure 3- 2).

Figure 3-2. The voucher specimens of leaves of *Pueraria mirifica* (Airy Shaw & Suvatabhandhu) at Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University

Besides the morphological comparison of the tuberous roots of PM-HHK with the voucher specimens from Professor Kasin Suvatabhandhu Herbarium, the other characteristics, such as starch granules and estrogenic activity were also assessed in comparison with other well-established *P. mirifica* (PM-Wichai3 and *P. mirifica* Kasetsart University cultivar: PM-KU) and *Stephania venosa*. The traditional Thai herb *S. venosa* (SV) or Sa Boo Leud in Thai was used as an out group in this study, because the morphological appearances of its tuberous roots were very similar to those of *P. mirifica* and the users were always confused.

Tuberous root of *S. venosa* (Figure 3-3) was purchased from Chong Mek District, Ubon Rachatani Province in northeastern Thailand.

Figure 3-3. The tuberous root of *Stephania venosa* or Sa Boo Leud in Thai.

The 100 mesh powder of PM-Wichai3 collected from Ratchaburi Province was supported by Assoc. Prof. Dr. Wichai Cherdshewasart, Department of Biology, Faculty of Science, Chulalongkorn University. It was used as a standard cultivar or positive control in this study.

The 100 mesh powder of *P. mirifica* Kasetsart University cultivar (or PM-KU), Nakorn Pathom Province was supported by Ajarn Sompoch Tubcharoen, Suwanvajokkasikit Animal Research and Development Institute, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom Province.

3. Preparation of the *P. mirifica* phytoestrogens extract

3.1. Powdering of the *P. mirifica* tuberous root

After tuberous roots of PM-HHK were collected from the Huai Hong Khrai Royal Development Study Center, they were measured in size and weight. Then, thick bark of the roots was peeled off and the sap was washed out by water. The cleaned roots were again weighed, and then sliced into pieces of approximately 5 mm thickness, dried in the hot air oven at 50 ºC for 72 hours. After dryness, pieces of the *P. mirifica* (Figure 3-4a) were ground into powder by grinder and filtered with 100 mesh (or 149 micron) sieve (Figure 3-4b). To avoid the variation of estrogenic activity of *P. mirifica* in different tuberous roots (Urasopon et al., 2008b) powders from all tuberous roots of *P. mirifica* collected were mixed together before the extraction procedure was run.

3.2. *P. mirifica* extraction

A powder of *P. mirifica* was extracted by 70% ethanol following the method of Urasopon et al. (2008b) with a slight modification. The extraction was repeated for 3 times. The process was started with mixing 50 g of the *P. mirifica* powder with 100 ml of 70% ethanol and incubated in the incubator shaker at a speed of 150 rpm and a temperature of 30 °C for 24 hours. The suspension was taken from the shaker and left to stand on the table until it was completely precipitated. Then, the upper liquid part was collected and filtrated with Whatman® filter paper nos. 1 and 41, respectively. The filtrate was stored at -20 °C until used. The lower powder part was repeatedly extracted for another two times by 70% ethanol with the incubation times of 24 and 12 hours, respectively. The extraction products of 3 times were pooled together for the next step of vacuum drying.

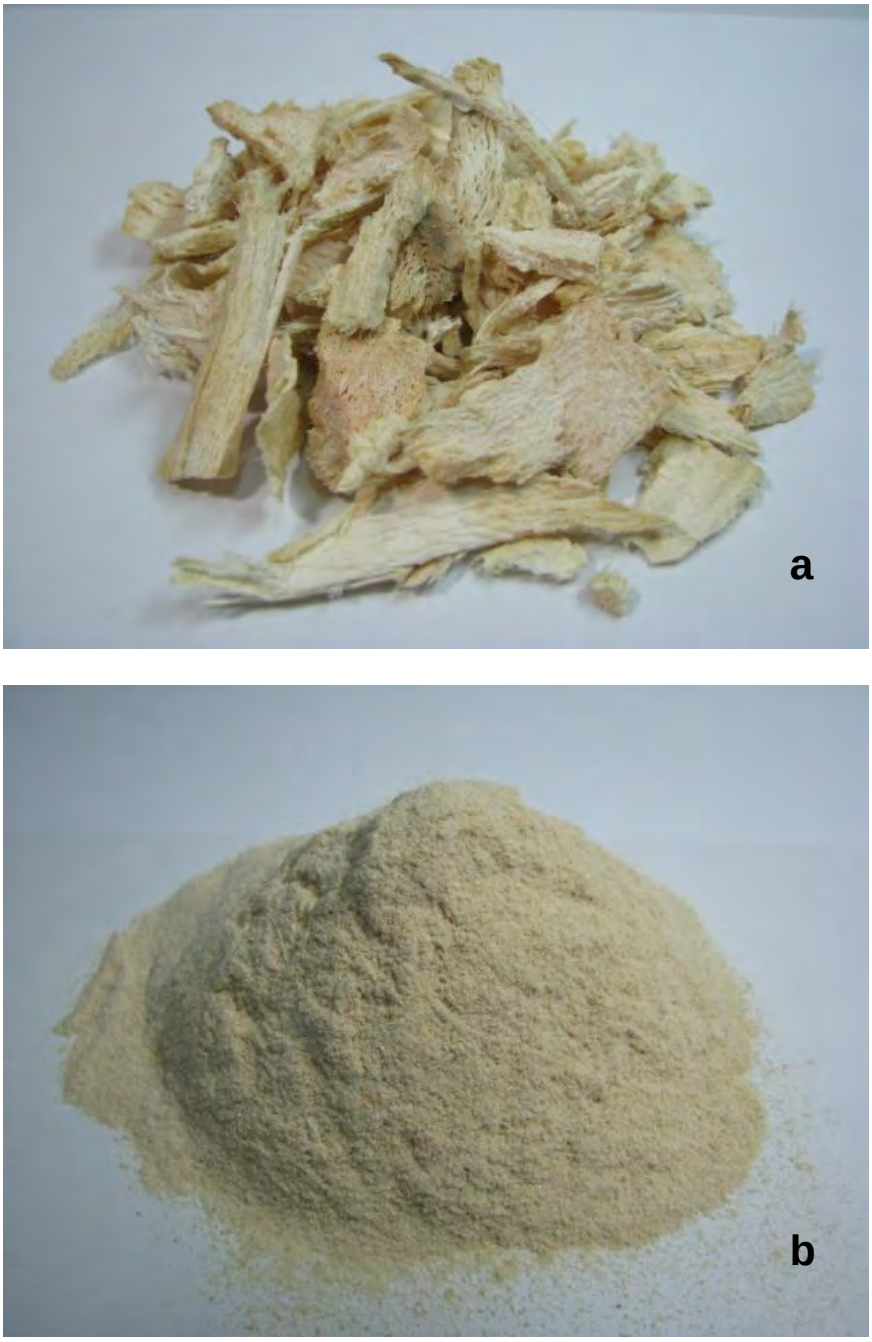


Figure 3-4. Dried pieces of *Pueraria mirifica* (a) and 100 mesh sized powder (b)

3.3. Vacuum Drying

The extraction products from the previous step (3.2. *P. mirifica* extraction) were evaporated the ethanol and water out by rotary evaporator EYELA™ N-1000 model (Tokyo Rikakikai Co., LTD, Japan) and digital hot water bath EYELA™ SB-1000 model (Tokyo Rikakikai Co., LTD, Japan) at 50°C, 40 rpm, until the extract was completely dry (Figure 3-5a and b). The complete dryness of the extract was checked by weighing every 15 min until the weight did not change. To ensure that the water and ethanol were completely evaporated, the extract was once evaporated in 55 °C water bath until the weight did not change. The yield products of the extraction were stored at -20°C.

4. Preparation of the frog pellets

In this study, the *P. mirifica* extract and 17β-estradiol were administered to the Rice Field Frogs by oral route via the frog pellets. The PM extract and 17β-estradiol were first dissolved in 30 ml of 95% ethanol as a stock solution at concentrations of 11.80% and 6.67% (w/v), respectively. Then, the solutions were sprayed on 1 kg of the frog pellets based upon doses of treatment, mixed well, and evaporated the ethanol out by hot air oven at 55 °C for 3 hours. The frog pellets coated with treatment chemicals were freshly prepared in every two days after all frogs were weighed, because the maximum feeding in each frog is approximately 5% of g of body weight/day (Pariyanonth and Daorerk., 1994). The frog pellets were kept in the jars and protected from the sun light (Figure 3-6a and b)

Figure 3-5. Evaporating process of the extraction of *Pueraria mirifica* by rotary evaporator (a) and digital hot water bath (b).

Figure 3-6. Frog pellets (a) coated with various concentrations of *Pueraria mirifica* extracts (PM-0, PM-10, PM-100, and PM-1000) and 17β-estradiol (E-100) and kept in jars (b)

Methods

All experiments performed in laboratory animals (frogs and rats) were approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Science, Chulalongkorn University, with the protocol review number 0823013.

1. Authentication of the PM-HHK by phenotypic characteristics

After the tuberous roots of *P. mirifica* and *S. venosa* were identified by comparison with the *P. mirifica* voucher specimen numbers BCU010250 and BCU011045 kept at Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, they were processed into 100 mesh of powder as described in "3.1. Powdering of the *P. mirifica* tuberous root". The powder of each cultivar of *P. mirifica* (PM-HHK, PM-KU and PM-Wichai3) and *S. venosa* (SV) was mixed with distilled water at a concentration of 10% (w/v). The suspensions were mixed well and observed under the compound light microscope (Olympus Optical Co., Ltd., Japan) at 200x and 400x magnification. The starch granules were identified by staining with Iodine solution. The density of starch granule was observed by counting the number of starch granule in 0.16 mm^2 frame for 10 frames in each sample. Then, 30 rounded shape starch granules for each sample were measured the diameter by ocular micrometer.

2. Testing the estrogenic activity of the PM-HHK cultivar in ovariectomized rats

Before the study of the estrogenic effect of the *P. mirifica* (PM-HHK) on the reproductive organ development and growth rate of the Rice Field Frogs was conducted, its estrogenic activity was first tested in rats. The estrogenic activity of PM-HHK was determined in female Wistar rats by the vaginal cytology assay and compared to those of distilled water, SV, PM-KU and PM-Wichai3 (Malaivijitnond et al., 2008; 2010).

2.1 Animal husbandry

Rats were housed in the environmentally controlled room at a temperature of 25±1 ˚C and lights on 12 hours a day at 0600–1800 h at the Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. They were housed 5 animals per cage in a stainless steel cage with sterile sawdust bedding. The rats were fed with the soybean-free rodent diet (C.P. 082/SBF; Lot No. 050119) and water *ad libitum*. They were allowed to acclimatize to the environment of the animal room for 1 week before used. Rats with regular estrous cycle of 4–5 days for at least 3 consecutive cycles before the experimental period were selected.

2.2 Experimental procedure

Sixty female Wistar rats were bilaterally ovariectomized on the first day (D_1) of the study period. They were ovariectomized under the ether anesthesia in an aseptic room. All surgical equipments were sterilized by 15% Detol®. The ovariectomy was performed by incision of the dorso-lateral side (about 1 cm below the last rib of rat). The skin was cut through the fat and muscle tissue layer, approximately 1 cm long. After the muscle was cut opened, the ovary was searched and removed, the muscle

and skin were sutured, cleaned and disinfected with Betadine® povidine-iodine solution (IDS manufacturing LTD., Thailand). After the operation was done on one side, it was performed again on another side of rat. Rat was housed individually on a sterile bedding paper in the stainless steel cage for the post surgery care. After the rats were completely recovered, they were transferred into their own cages. After ovariectomy, rats were divided into 6 groups (10 rats/group) as follows;

Group 1: negative control or DW group, rats were fed with 0.7 ml of distilled water.

Group 2: positive control or PM-Wichai3 group, rats were fed with a suspension of 100 mg/kg BW/day of PM-Wichai3 powder in 0.7 ml distilled water.

Group 3: PM-HHK powder group, rats were fed with a suspension of 100 mg/kg BW/day of PM-HHK powder in 0.7 ml distilled water.

Group 4: PM-HHK extract group, rats were fed with a solution of 17.7 mg/kg BW/day of PM-HHK crude extract (equal to 100 mg/kg BW/day of PM-HHK powder).

Group 5: PM-KU group, rats were fed with a suspension of 100 mg/kg BW/day of PM-KU powder in 0.7 ml distilled water.

Group 6: SV group, rats were fed with a suspension of 100 mg/kg BW/day of *S. venosa* powder in 0.7 ml distilled water.

The experiment was divided into 3 periods; pretreatment, treatment and post-treatment (14 days for each period) as shown in Figure 3-7. During pretreatment (D_1-D_{14}) and post-treatment (D_{29}-D_{42}) periods rats were fed daily with 0.7 ml of distilled

water. During treatment period (D_{15}-D_{28}), rats were fed daily with distilled water (Gr1), PM-Wichai3 powder (Gr2), PM-HHK powder (Gr3), PM-HHK extract (Gr4), PM-KU powder (Gr5) or SV powder (Gr6), and the feeding was done between 09.00-10.00 h. All rats were monitored the type of vaginal epithelium cells by vaginal smear between 08.00-09.00 h.

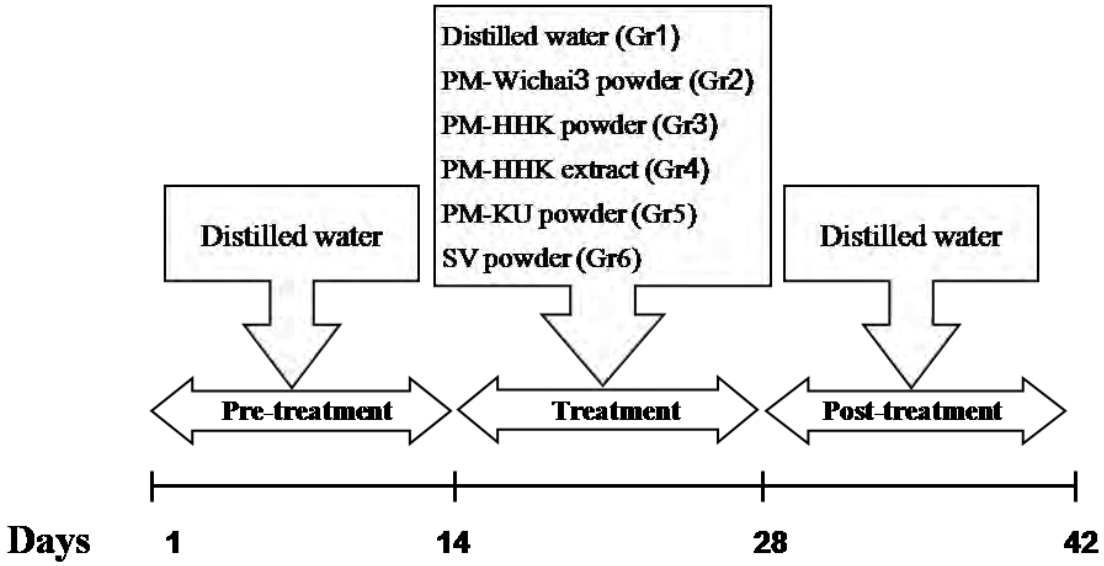


Figure 3-7. The diagram shows an experimental design for each period of the treatment in rats. During pre-treatment and post-treatment periods, all rats were administered with 0.7 ml of distilled water. During 14 days of treatment period, all rats were fed daily with distilled water, PM-Wichai3 powder, PM-HHK powder, PM-HHK extract, PM-KU powder or SV powder, respectively.

2.3 Vaginal cornification assay

Changes of vaginal epithelium cell were checked daily by vaginal smear between 08.00-09.00 h. The glass rod was sterilized by 95% ethanol before used. The glass rod was dipped into 0.9% w/v normal saline solution and inserted into the rat's vagina. The vaginal epithelium cells on the glass rod were smeared on a glass slide

with a drop of normal saline. Subsequently, the vaginal epithelium cells were observed under a compound light microscope (Olympus Optical Co., Ltd., Japan) at 100x magnification.

The vaginal epithelium cells were classified into three types as follows (Figure 3-8);

Leukocytes (L): white blood cells which can be observed in the metestrous and diestrous stages of the estrous cycle.

Nucleated cells (O): the nucleated, oval or round shape cells which can be observed in the proestrous stage.

Cornified cells (Co): the stratified cells without nucleus which can be observed in estrous stage.

Cells were counted and the percentage of Co (an indicator of estrogenic activity of chemicals) was calculated by the following formula.

$$\%Co = \frac{Co \times 100}{(L + O + Co)}$$ (Malaivijitnond et al., 2010)

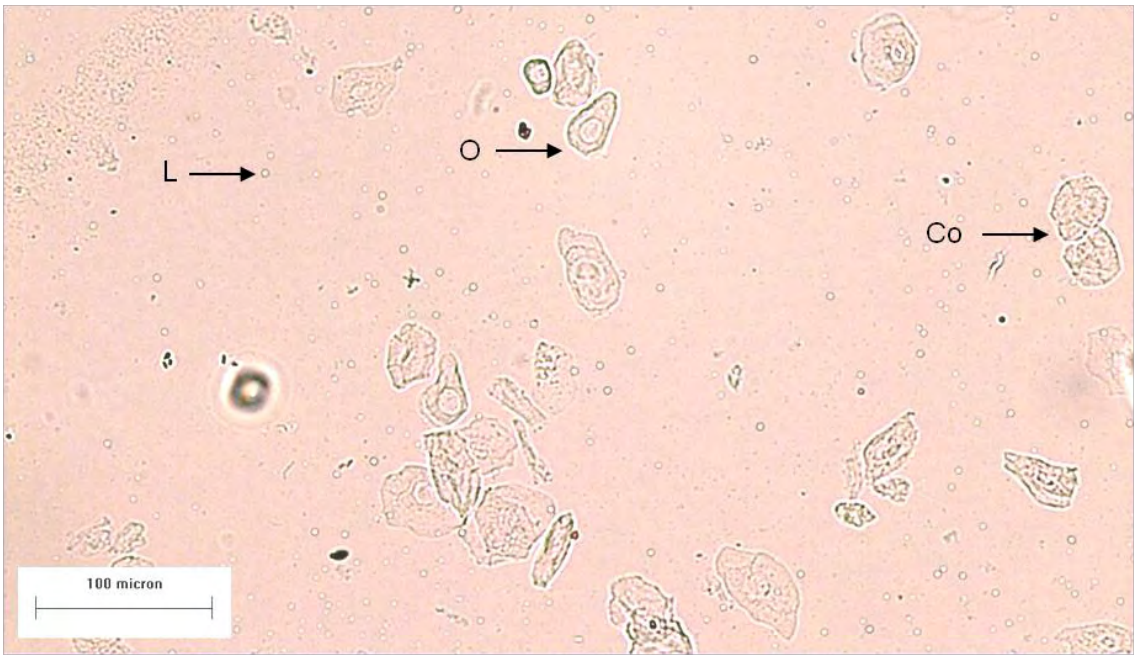


Figure 3-8. Types of vaginal epithelium cells observed under the light microscope at 100x magnification. The arrows indicate leukocyte (L), nucleated cell (O) and cornified cell (Co).

3. Effects of *P. mirifica* extract on gonad development and growth of Rice Field Frogs.

After the estrogenic activity of phytoestrogens in PM-HHK was tested in rats, the effects of PM-HHK extract on reproductive organ development and growth of Rice Field Frogs was subsequently determined.

3.1 Animal husbandry

Frogs were housed in a plastic tank (30x45x45 cm^3), 10 frogs/tank, with 5 L of decholinated tap water. They were housed in the room with the natural condition of approximately 12:12 h light-dark cycle, with 28±2 °C at the Amphibian and Reptile Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. Frogs were fed twice a day with the Hi-grade® (Charoen Pokaphand

Foods PCL, Thailand) standard frog pellet with a composition of 41% of protein and 12% of fat. Frogs were fed with a frog pellet at a dose of 5% of g of body weight per day (Pariyanonth and Daorerk, 1994). Frogs at 4 weeks old and showed a complete metamorphosis were selected for this study.

3.2 Experimental procedure

To determine the effect of estrogenic activity of PM-HHK extract on gonad development and growth of Rice Field Frogs, the doses of PM extracts used in this study were followed those of previous studies in rodents (Cherdshewasart, 2003; Cherdshewasart et al., 2007a; 2007b; 2008; Malaivijitnond et al., 2004; 2006; 2010; Urasopon et al., 2008a; 2008b). The PM-HHK extracts at concentrations of 1.77, 17.70 and 177 mg BW/day which were equal with the PM-HHK powder suspension at concentration of 10, 100 and 1,000 mg/kg BW/day, respectively, were selected.

The complete metamorphosis frogs were divided into 5 groups (30 frogs/group) as follows;

Group 1: negative control or PM-0 group, frogs were fed daily with standard frog pellets.

Group 2: PM-10 group, frogs were fed daily with frog pellets coated with 1.77 mg/kg BW/day of the PM extract.

Group 3: PM-100 group, frogs were fed daily with frog pellets coated with 17.70 mg/kg BW/day of the PM extract.

Group 4: PM-1000 group, frogs were fed daily with frog pellets coated with 177 mg/kg BW/day of the PM extract.

Group 5: positive control or E_2 group, frogs were fed daily with frog pellets coated with 100 μg/kg BW/day of 17β-estradiol.

Treatment was carried on for 3 months. Each experiment was run in 3 replicates (10 frogs/ replicate, 30 frogs/ group). The frogs in each group were fed with the PM extract or 17β-estradiol coated pellets for 2 times a day.

3.3 Determination of growth of Rice Field Frogs

Growth rate of frogs was evaluated by measurement of body weight and length. The body length of frog was determined by means of the snout-vent-length (SVL) which is the length between the tip of the frog's snout to the opening pore of the cloaca (Figure 3-9). SVL was measured by digital vernier caliper (Mitutoyo®, Japan). The body weight was measured by the digital scale (Tanita®, Japan). All frogs were individually measured body weights in every 2 weeks and SVLs in every 4 weeks.

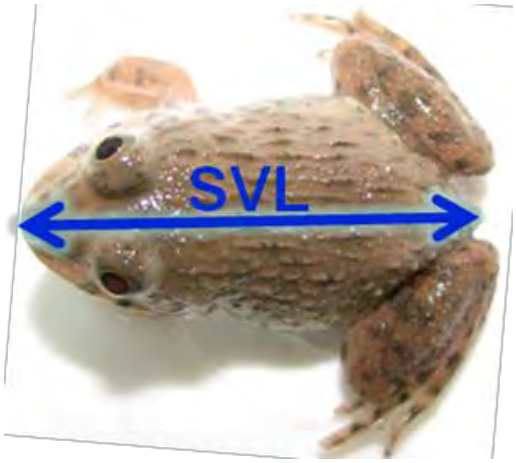


Figure 3-9. The measurement of body length of frog by means of the snout-vent-length (SVL).

3.4 Determination of the gonad development of Rice Field Frogs

To evaluate the effect of PM extract on reproductive organ development of the Rice Field Frogs, 3 frogs in each replicate (or 9 frogs/ group) were randomly selected and euthanized every 4 weeks as shown in Figure 3-10. Frogs were randomly

euthanized by immersion in 0.5% Tricaine methane sulphonate (MS-222) until they were immobilized and sunk into the solution (Close et al., 1997; Torrilles et al., 2009).

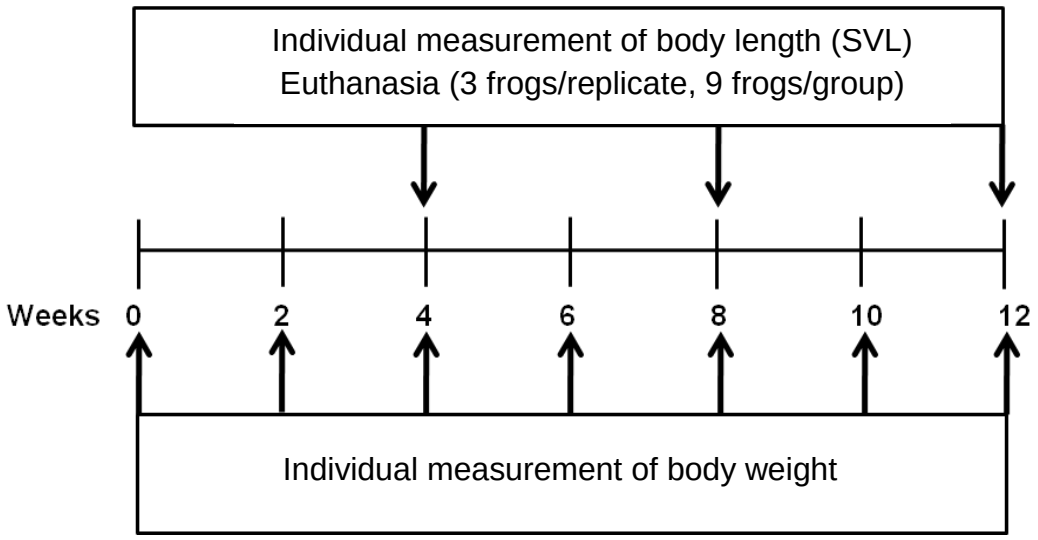


Figure 3-10. A diagram shows an experimental design in frogs. Frogs were individually measured body weights (BWs) and body length (snout-vent-length: SVL) in every 2 and 4 weeks, respectively, and randomly euthanized in every 4 weeks for determination of reproductive organ development.

After euthanized, 1 ml of blood was collected by cardiac puncture at 09.00-12.00 h. One-ml of syringes with heparin coated and 25 G x 1/2" of needles were used. Blood sera were separated by centrifugation at 4000 rpm, 4 °C for 30 minutes, and kept at -20 ºC until sex steroid hormones assayed.

Livers, kidneys, gonads (ovaries or testes) and accessory sex organs (oviduct or vas deferens) were dissected, trimmed all fat tissues, weighed and measured widths and lengths. All organs were fixed in 10% neutral buffered formalin for 3 days and then changed to 70% ethanol until the microscopic observation was done following the standard microscopic technique of Humason (1979). They were dehydrated in

ethanol gradient series, cleared by xylene and then were embedded and blocked in paraffin. The embedded tissues were cut into the serial section of 5 μm thickness by microtome. The sections were stained with hematoxylin and eosin, and subsequently examined under the compound light microscope (Olympus Optical Co., Ltd., Japan) and photographed by a digital camera (Olympus Optical Co., Ltd., Japan). The toxicity of the PM extract in frogs was examined by determining the abnormality of liver and kidney tissues.

3.5 Determination of sex steroid hormones of Rice Field Frogs

At the end of study, sex steroid hormones (estrogen and testosterone) in blood serum were analyzed by RIA techniques. The analysis was done by BRIA Laboratory Ltd., Bangkok, Thailand.

Statistical analysis

The result data were presented as mean ± standard error (SE). Data were analyzed by One-way Analysis of Variance (ANOVA) and LSD Post-Hoc test using SPSS software program (version 17.0). P value less than 0.05 was accepted at a significant level.

CHAPTER IV

RESULTS

1. Authentication of *P. mirifica*

The identification of *P. mirifica* was done by comparing the tuberous roots and leafs with those of voucher specimen numbers BCU010250 and BCU011045 from Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University. Comparing between the roots of *P. mirifica* (PM) and *S. venosa* (SV) indicated that the root of *P. mirifica* was more round shape with the yellow brown and thick bark while the SV root was shapeless with red brown bark (Figure 4-1a and b). There were some differences of the texture of the tuberous meats between PMs and SV. The texture of the tuberous meat of PMs was rough with a number of fibers and the color was white, while the color of SV was obviously yellow and light brown with a circumference pattern on the tuberous meat and the texture was smoother compared with those of the PMs (Figure 4-1c and d).

After the powders of PM-Wichai3, PM-HHK, PM-KU and SV were observed under the light microscope, the differences in powder texture could be recognized. The powders of PMs consisted of small starch granule with many fibers (Figure 4-2). On the other hand, the SV powder consisted of larger starch granules and a few of fibers. Moreover, the PM starch granule was translucent or very pale yellow while the SV granule was yellow to green in color.

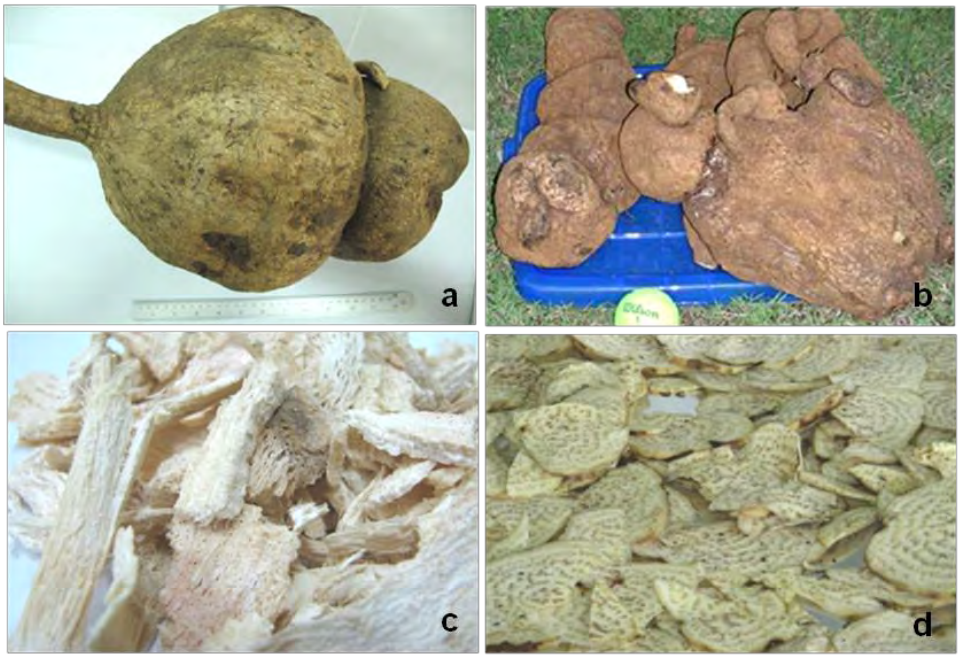


Figure 4-1. The roots of *Pueraria mirifica* (PM) and *Stephania venosa* (SV) (a and b) and the texture of the root meat of PM (c) and SV (d).

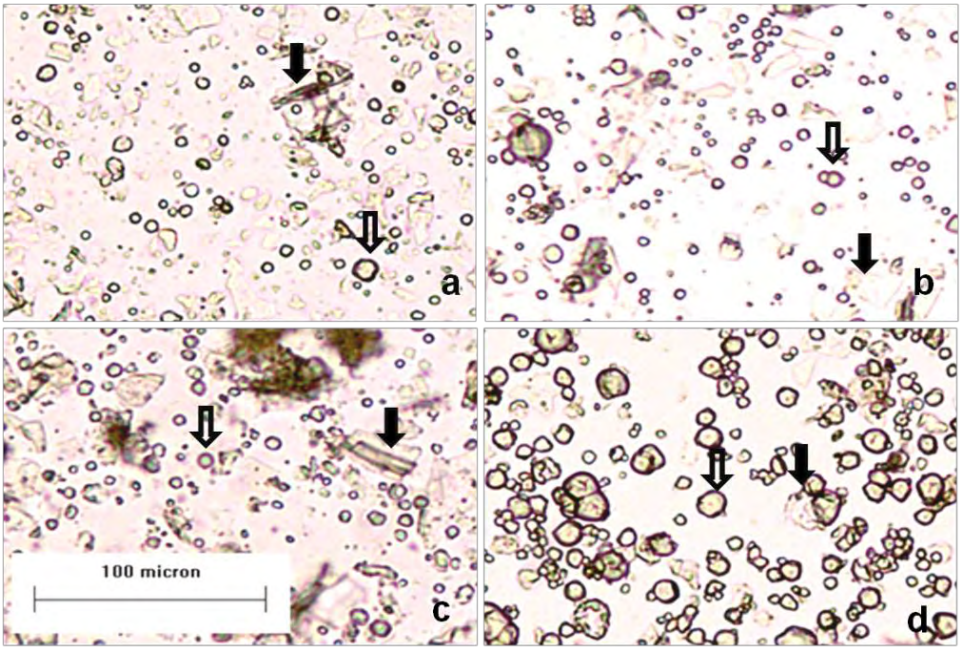


Figure 4-2. Comparison of the differences of numbers and shapes of fiber and starch granule of *P. mirifica* powders (PM-Wichai3 (a), PM-HHK (b), PM-KU (c)) and *Stephania venosa* powder (d). The arrows indicate starch granules (⇑) and fibers (↑).

The starch granule diameter and density of PMs (PM-wichai3, PM-HHK, PM-KU) and SV were analyzed (Table 4-1). The starch granule of SV showed a significantly larger diameter than those of the PMs ($p<0.01$). However, there were no significant differences of the starch granule diameters among the PM groups.

Table 4-1. The starch granules diameter of *Pueraria mirifica* powders (PM-wichai3, PM-HHK, and PM-KU) and *Stephania venosa* powder (SV)

Sample	Starch granule diameter (μm)
PM-Wichai3	3.63 ± 0.28**
PM-HHK	4.27 ± 0.30**
PM-KU	3.73 ± 0.31**
SV	6.13 ± 0.60

** Significantly different ($p<0.01$) with SV

In agreement with the diameter, the density of the starch granules (number of starch granule/0.16 mm^2) of SV was lowest in comparison with the PM-Wichai3 ($p=0.301$), PM-HHK ($p=0.013$) and PM-KU ($p=0.01$) as shown in Table 4-2.

Table 4-2. Density of starch granule of *Pueraria mirifica* powders (PM-wichai3, PM-HHK, and PM-KU) and *Stephania venosa* powder (SV)

Sample	Density of starch granule/ 0.16 mm^2
PM-Wichai3	36.60 ± 0.88
PM-HHK	38.70 ± 1.01*
PM-KU	40.70 ± 0.63**
SV	35.20 ± 1.16

* Significantly different ($p<0.05$) with SV

** Significantly different ($p<0.01$) with SV

2. *P. mirifica* extract

The total weight of fresh tuberous roots of PM-HHK used in this study was 27.5 kg. After the thick bark was peeled and the tuberous roots were sliced into pieces, dried by hot air oven, and ground, the weights were reduced to 1.4 kg (Table 4-3). After extracted with 70% ethanol, the yield product of the yellow brown wax was 0.248 g from 1.4 kg of crude powder or 17.7% yield. Generally, 17.7 mg of *P. mirifica* HHK extract was obtained from 100 mg of the crude powder (Table 4-3).

Table 4-3. The yield product in each step of the extraction of *Pueraria mirifica*

P. mirifica	**Weight (kg)**
Fresh roots	**27.50**
Fresh roots without bark	**19.50**
Dried pieces	**3.00**
100 mesh powder	**1.40**
Extract	**0.248**

3. Estrogenic activity of PM-HHK in ovariectomized rats

3.1 Pre-treatment period

After ovavariectomy and fed with distilled water for 14 days, the percentages of cornified cells (%Co) in all rats were decreased and maintained at the range of 5 - 40 % during the pre-treatment period (Figure 4-3). This confirmed that ovaries were completely removed and no effect of endogenous ovarian estrogen was left. Most of vaginal epithelium cells were leukocyte cells (L).

3.2. Treatment period

Feeding of distilled water to the negative control (DW) group did not affect on proliferation of vaginal epithelium and L cells were mainly observed throughout the treatment period.

Administration of 100 mg/kg BW/day of PM-Wichai-3 powder stimulated vaginal cornification. After 2 days of administration (D_{17}), the Co cells were reappeared and significantly higher than the pre-treatment period ($p<0.05$). The % Co was highest (95.50±3.07) on D_{22} or 7 days after PM-Wichai3 treatment, and kept this high level until the end of treatment period.

Administration of 100 mg/kg BW/day of PM-HHK powder and 17.7 mg/kg BW/day of PM-HHK extract stimulated vaginal proliferation and the %Cos were significantly higher than the pre-treatment ($p<0.05$) period starting from D_{16} or 1 day after treatments. The % Cos were highest on D_{26} (85.75±4.38) and D_{27} (91.89±4.13) for PM-HHK powder and PM-HHK extract, respectively. These high levels of % Cos were kept until the end of treatment period.

Rats treated with PM-KU showed a significant increase in %Co on D_{17} or 2 days after treatment and the highest on D_{20} (100%). This high level was kept until the end of treatment period.

The patterns of increase in %Cos were similar among 4 groups of the PM treatment, except that the high %Co was started earlier in PM-KU than the other 3 PM groups and, on D_{22} the %Co in PM-Wichai3 group was significantly higher than the HHK groups ($p < 0.05$).

SV did not show any significant increase in %Co throughout the treatment period compared with the pretreatment period.

3.3. Post-treatment period

After cessation of the PM treatment and all rats were fed with distilled water, %Cos of all groups of rats were returned to the pre-treatment levels.

Rats in PM-Wichai3 group showed the earliest returning (D_{30}), while PM-KU, PM-HHK powder and PM-HHK extract groups took longer times (D_{31}, D_{32} and D_{33}, respectively). The %Co in the negative control DW group and SV group were kept in the low levels, ranging 5-14% and 8-27% respectively.

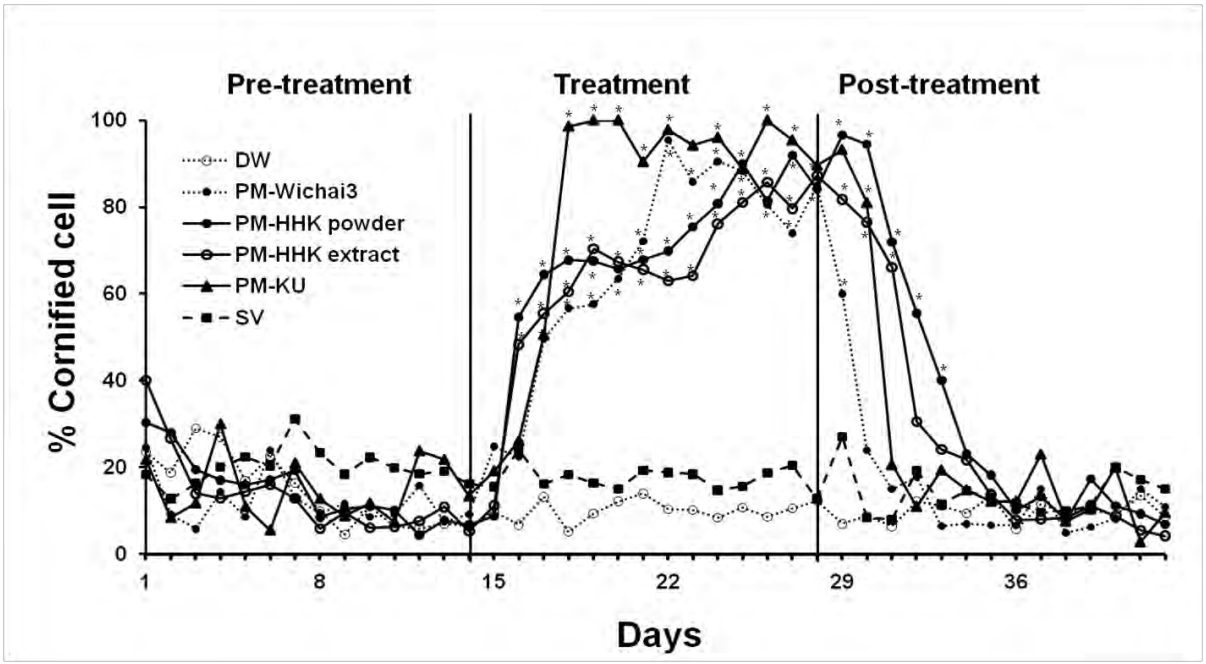


Figure 4-3. Percentage of cornified cells (%Co) in ovariectomized rats fed with distilled water (DW), 100 mg/kg BW/day of PM-Wichai3 powder, PM-HHK powder, PM-KU powder and SV powder, and 17.7 mg/kg BW/day of PM-HHK extract for 14 days during treatment period.

4. Effect of *P. mirifica* extract on growth of Rice Field Frogs

4.1 Body weights

Body weights in all groups of frogs, except the E_2 group, were increased during the study period; however, the growth rate in PM treatment groups was higher than the PM-0 (DW) group (Table 4-4 and Figure 4-4). The body weights of PM treatment groups were significantly higher than the PM-0 group at the 4^{th} - 6^{th} week of the treatment ($p < 0.05$), and significantly higher than the E_2 group starting from the 4^{th} week to the end of treatment period. Although the increases in body weights of frogs were not depended on doses of PM treatment, the body weights of PM-10 (87.79±25.74 g) and PM-1000 (88.48±40.90 g) treated frogs tended to be higher than the PM-100 (75.09±20.67 g) treated frogs ($p = 0.529$ and 0.527, respectively) at the end of the treatment period. Additionally, the increase in body weight of frogs treated with *P. mirifica* was caused by a muscle growth, not fat bodies, regarding the observation during autopsy.

Table 4-4. Body weights of Rice Field Frog treated with PM-0, PM-10, PM-100, PM-1000 and E_2

	Body weight				
Week	PM-0	PM-10	PM-100	PM-1000	E_2
0	7.083 ±1.913	7.593±0.786	7.323±1.489	6.460±0.466	7.173±0.451
2^{nd}	13.277±3.722	16.590±0.257	13.300±2.548	15.243±1.186	12.393±0.559
4^{th}	18.530±1.780	24.560±.0.461**	29.207±4.539$^{++,}$ **	29.053±0.612$^{++,}$ **	12.553±0.879
6^{th}	21.310±1.880	36.930±0.461$^{++,}$**	39.240±3.590$^{++,}$ **	35.927±2.311$^{++,}$ **	12.913±0.358
8^{th}	26.253±1.255	49.740±9.967**	49.333±10.965**	49.867±7.418**	15.010±2.214
10^{th}	40.893±7.853	57.560±16.143*	59.917±14.647*	60.187±13.030*	17.873±3.527
12^{th}	55.767±4.853	87.789±14.859**	75.089±11.934*	88.478±23.614**	15.411±1.797

++ indicates a significant difference $p<0.01$ from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

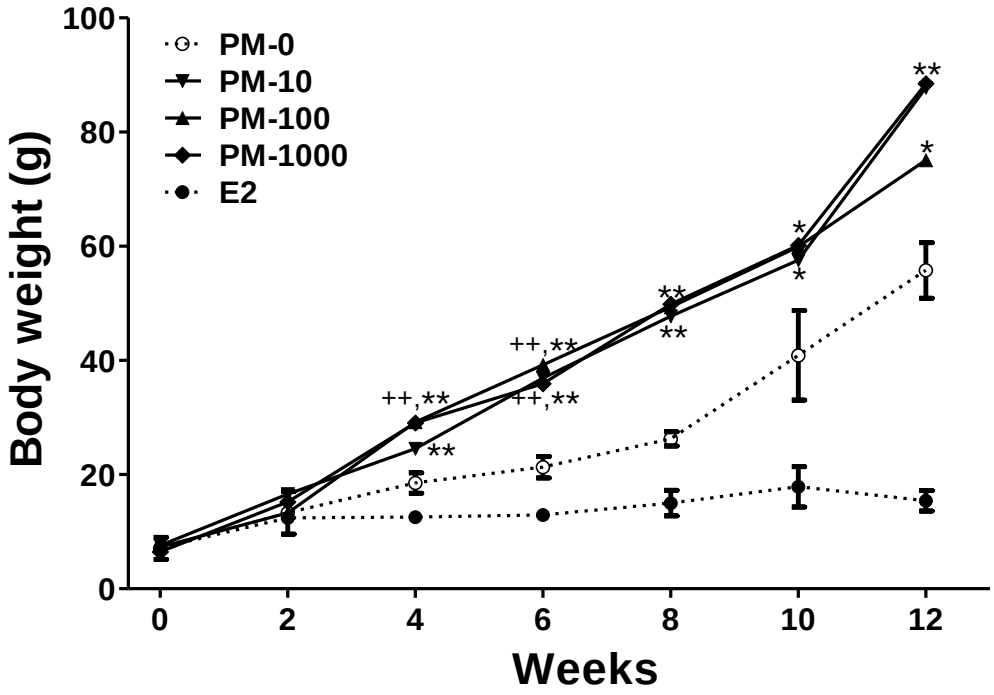


Figure 4-4. Body weights of frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of *Pueraria mirifica* extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 μg/kg BW/day of 17β-estradiol (E_2) for 12 weeks. ++ indicates a significant difference ($p<0.01$) from the PM-0 group. *, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

4.2 Snout-vent-lengths

Patterns of changes of the body lengths (snout-vent-lengths; SVLs) of frogs were differences from those of the body weights (Table 4-5 and Figure 4-5), that is, the increase was also found in the E_2 group. Snout-vent-lengths were markedly increased during the first 4 weeks of treatment period before it seems to reach a plateau at the 4^{th} – 12^{th} week. However, the SVLs of frogs fed with PM-10, PM-100 and PM-1000 were significantly higher than the E_2 group at the 8^{th} – 12^{th} week ($p<0.05$ and $p<0.01$). Only the PM-10 and PM-1000 groups showed a significant increase in SVLs at the 4^{th} week compared to the PM-0 group ($p<0.05$ and $p<0.01$ respectively), however, the SLVs of all three PM treated groups were significantly higher than the PM-0 group on the 12^{th} week ($p<0.05$ for PM-10 and PM-1000 and $p<0.01$ for PM-1000).

Table 4-5. Body length (SVL) of Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2

Body length (mm)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
0^{th}	17.667 ± 0.795	18.722 ± 0.886	20.056 ± 0.517	18.944 ± 0.955	19.944±0.626
4^{th}	49.214 ± 3.162	55.820 ± 1.670^{+}	54.042 ± 1.797	56.820 ± 1.145^{++}	53.638 ± 0.866
8^{th}	58.811 ± 2.754	63.384 ± 3.798*	63.776 ± 2.592*	66.640 ± 2.435**	54.322 ± 2.654
12^{th}	77.040 ± 1.887	89.402 ± 2.877 $^{+}$,**	91.252 ± 5.079^{++},**	88.226 ± 4.735^{+},**	53.2122 ± 1.779

+, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

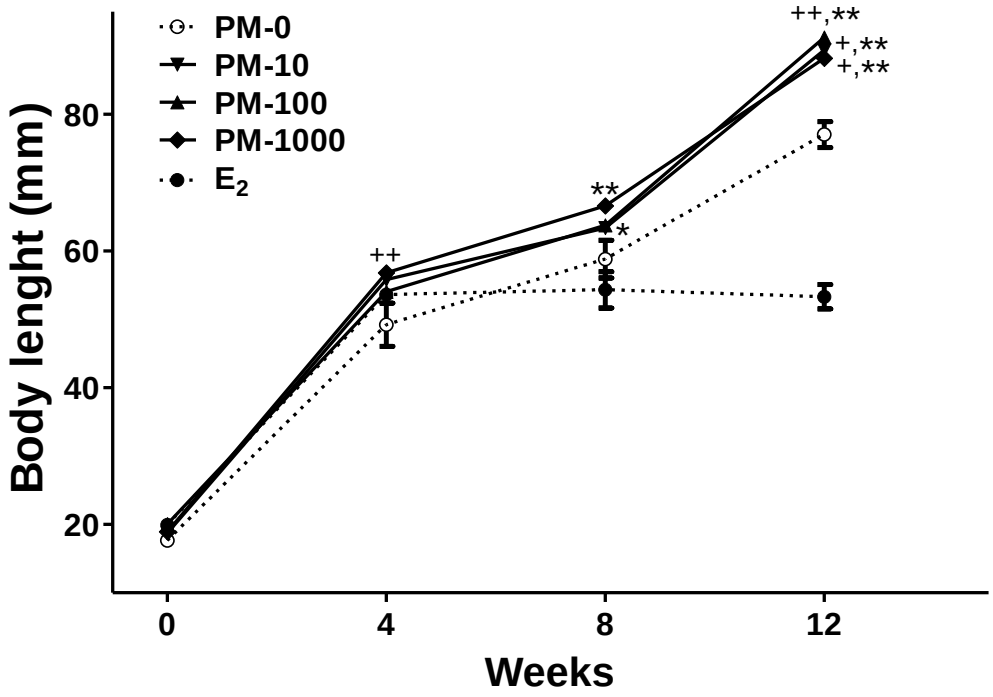


Figure 4-5. Snout-vent-lengths of frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of *P. mirifica* extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 μg/kg BW/day of 17β-estradiol (E_2) for 12 weeks. +, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group. *, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E2 group.

4.3. Liver and kidney weights

1) Liver

Changes of liver weights were similar to those of the body weights; no changes in the E_2 group and increase in the PM-0, PM-10, PM-100 and PM-1000 groups (Table 4-6 and Figure 4-6). The increases were particularly high in the PM treatment groups during the 8^{th} – 12^{th} week of treatment period. All three PM treatment groups showed significant differences from the PM-0 ($p<0.05$ for PM-10 and PM-1000 and $p<0.01$ for PM-100) and E_2 groups ($p<0,01$ for all three PM groups) at 12^{th} week of treatment, and only the PM-10 and PM-1000 were significantly higher than the E_2 group at the 8^{th} week of the treatment ($p<0.05$).

Table 4-6 Liver weights of Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2

Liver weight (g)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4^{th}	0.903 ± 0.272	0.866 ± 0.085	1.040 ± 0.437	1.021 ± 0.157	0.588 ± 0.043
8^{th}	0.977 ± 0.243	1.745 ± 0.586*	1.488 ± 0.371	1.633 ± 0.265*	0.584 ± 0.085
12^{th}	1.972 ± 0.283	4.415 ± 0.896^{+},**	5.437 ± 1.406^{++},**	4.506 ± 1.519^{+},**	0.398 ± 0.040

+, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

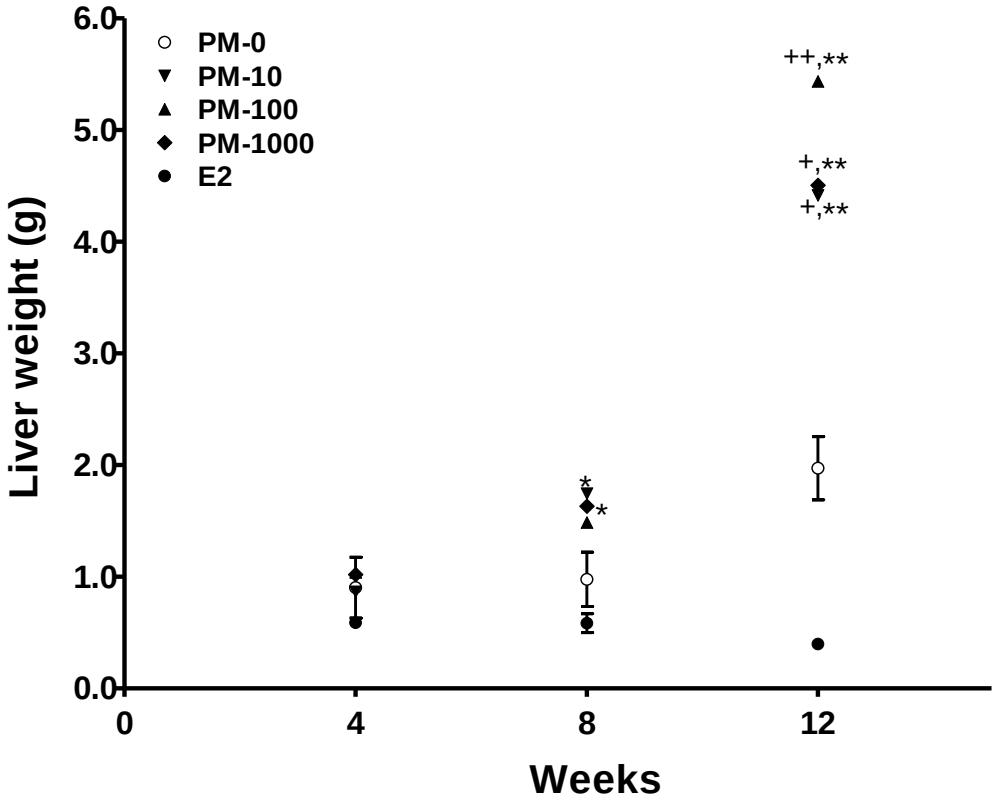


Figure 4-6. Liver weights of frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of *Pueraria mirifica* extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 μg/kg BW/day of 17β-estradiol (E_2) for 12 weeks. +, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E2 group.

2) Kidney

Changes of kidney weights were likely similar to those of the liver weights; no changes in the E_2 and PM-0 groups and increase in the PM treatment groups (Table 4-7 and Figure 4-7). At the 8th week of treatment, only the PM-100 group showed a highly significant increase in kidney weight ($p = 0.006$) compared to the E_2 group. However, at the 12th week of treatment, all three PM treatment groups showed a significant increase ($p<0.05$ for PM-10 and PM-1000 and $p<0.01$ for PM-100) in kidney weights compared to the E_2 group. Only the PM-100 group, the kidney weight was significantly higher than that of the PM-0 (DW) group at the 12th week of treatment ($p<0.01$), whereas the PM-10 and PM-1000 groups tended to be higher, but not significant differences ($p = 0.065$ and 0.060, respectively).

Table 4-7. Kidney weights of Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2

Kidney weight (g)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4th	0.163 ± 0.065	0.180 ± 0.026	0.2125 ± 0.044	0.272 ± 0.038	0.178 ± 0.028
8th	0.296 ± 0.183	0.292 ± 0.066	0.546 ± 0.156**	0.395 ± 0.047	0.183 ± 0.053
12th	0.248 ± 0.021	0.674 ± 0.080*	1.003 ± 0.329^{++},**	0.683 ± 0.101*	0.150 ± 0.037

+, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

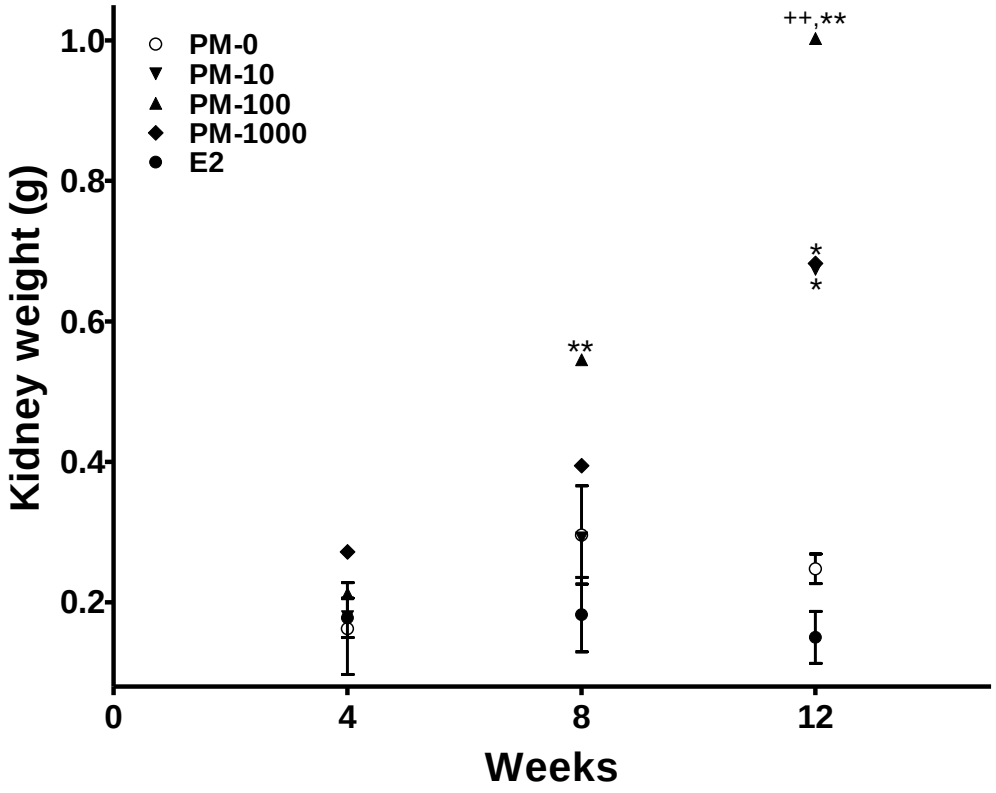


Figure 4-7. Kidney weights of frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of P. mirifica extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 µg/kg BW/day of 17ß-estradiol (E2) for 12 weeks. +, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group. *, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E2 group.

4.4. Microscopic observation of liver and kidney

1) Liver

At the tissue level, after 12 weeks of treatment, no abnormality of liver tissues was detected. However, the Kupffer cells had been appeared on liver tissue in all experimental groups (Figure 4-8)

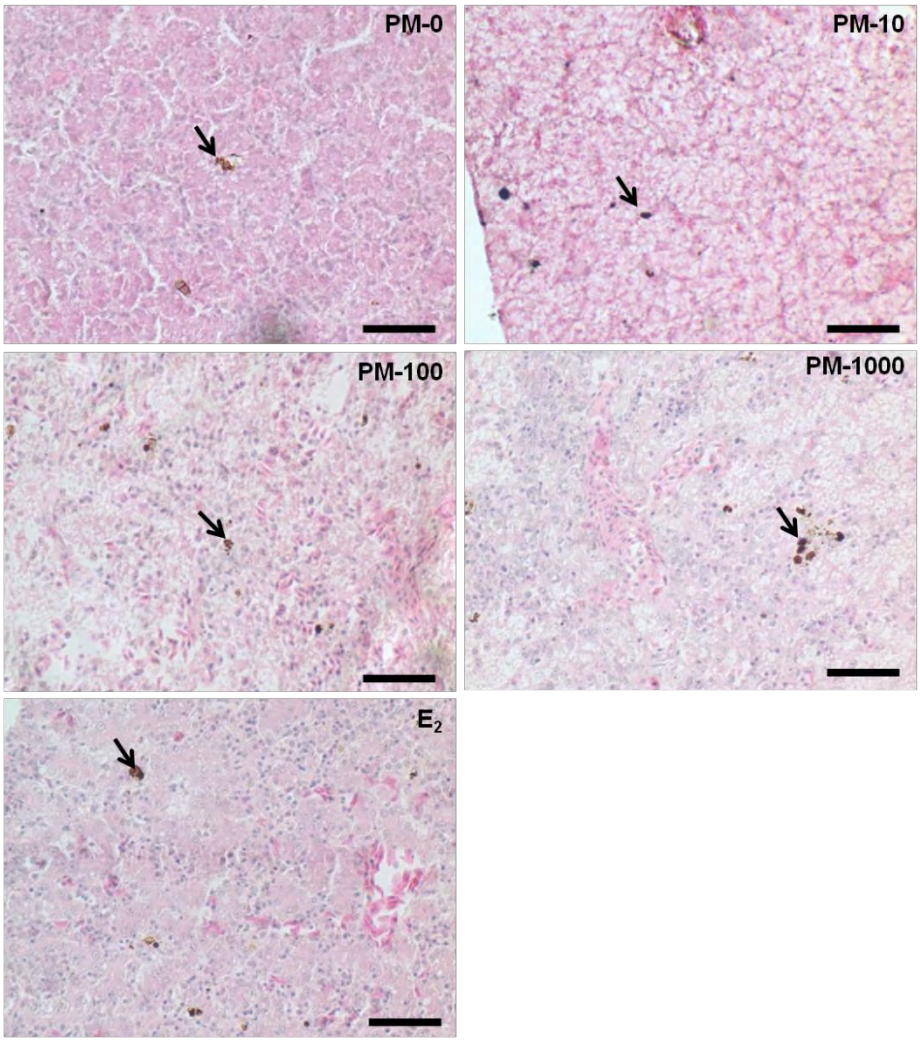


Figure 4-8. Microscopic appearances of liver tissues after frogs were treated with PM-0, PM-10, PM-100, PM-1000 and E_2 for 12 weeks. The arrows indicated the Kupffer cells (bar = 100 μm).

2) Kidney

After microscopic observation, no abnormality of kidney in all PM and E_2 treatment groups was found. (Figure 4-9). A large number of highly convoluted tube-like excretory units called uriniferous tubules (nephrons) was found. These tubules were held together by a highly vascular connective tissue. A dense network of blood capillaries, called glomerulus, residing in the concavity of Bowman's capsule were also observed sparsely.

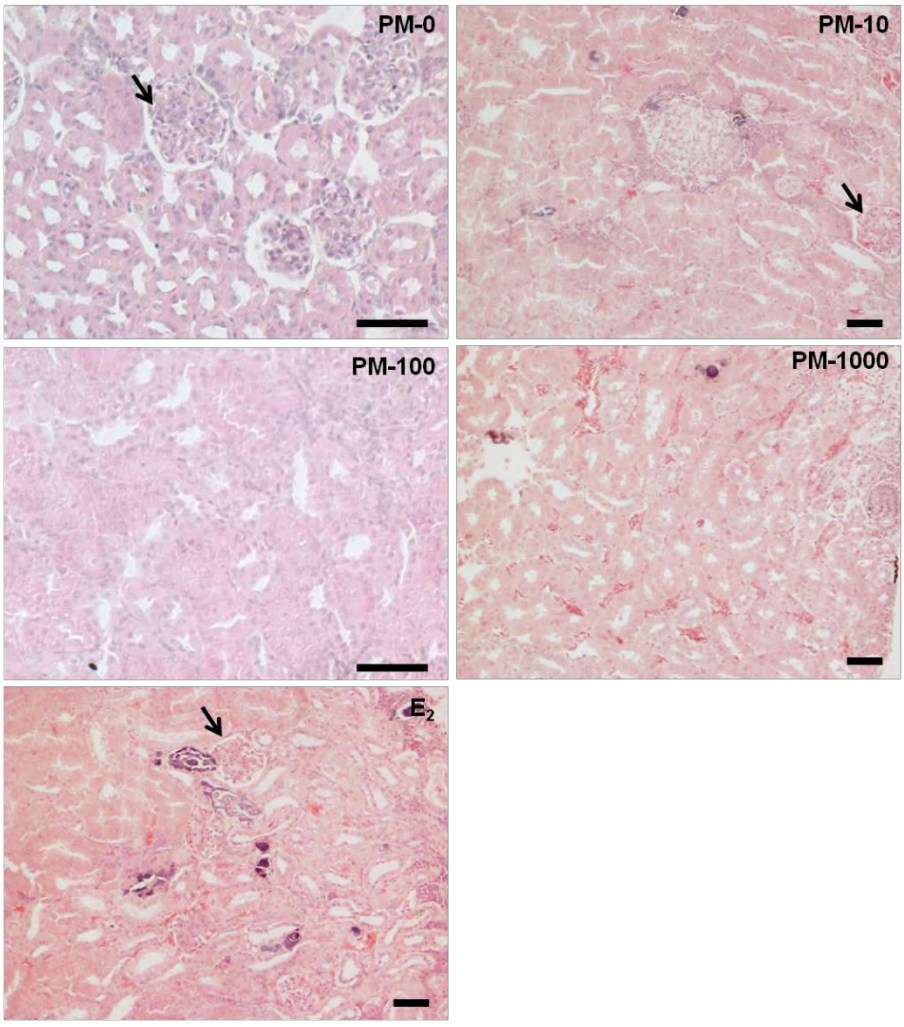


Figure 4-9. Microscopic appearances of kidney tissues after frogs were treated with PM-0, PM-10, PM-100, PM-1000 and E_2 for 12 weeks (bar = 100 μm). The arrows indicate the glomeruluses.

5. Effect of *P. mirifica* extract on reproductive organ development of Rice Field Frogs

5.1. Gonad weights

After the frogs were euthanized, the gonads were collected and determined the weights (Table 4-8 and Figure 4-10). At the 12th week of treatment, only the PM-100 ($p<0.05$) and PM-1000 groups ($p<0.01$) were significantly higher than the E_2 and PM-0, while PM-10 group tended to be higher, but not significant difference ($p = 0.060$ and 0.152 compared to PM-0 and E_2 group, respectively). The gonad weights of the PM-1000 group was also significantly higher than the PM-100 group at the 12th week of treatment ($p = 0.030$).

Table 4-8. Gonad weights of Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2

	Gonad weight (g)				
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4th	0.003 ± 0.001	0.018 ± 0.008	0.015 ± 0.006	0.009 ± 0.003	0.023 ± 0.007
8th	0.013 ± 0.007	0.028 ± 0.012	0.016 ± 0.008	0.012 ± 0.003	0.010 ± 0.001
12th	0.020 ± 0.011	0.048 ± 0.005	0.062 ± 0.009^{+},*	0.106 ± 0.027^{++},**	0.010 ± 0.001

+, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

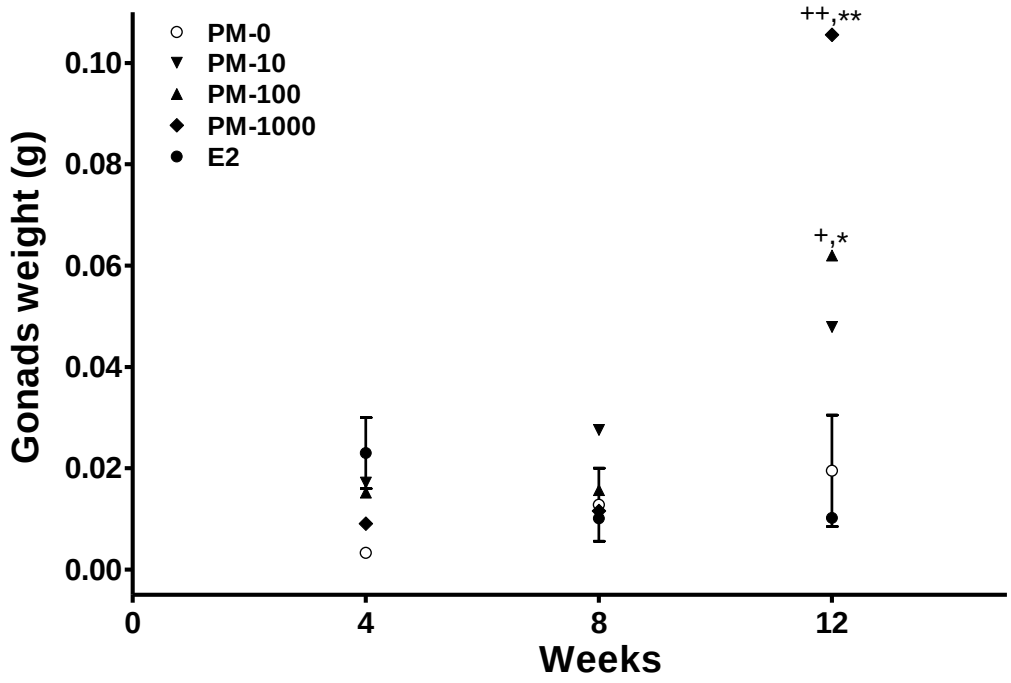


Figure 4-10. Gonad weights of frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of *P. mirifica* extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 μg/kg BW/day of 17β-estradiol (E_2) for 12 weeks. +, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group. *, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E2 group.

5.2. Weights of reproductive tracts

From the urogenital system, the frog reproductive tracts were divided into 2 types; efferent tube (male reproductive tract) and oviduct (female reproductive tract) based on their morphological characters and location (Kelly, 1996). Efferent tubes are connected from posterior of testes to cloaca while oviduct connected from superior of ovaries and along with the kidney to the cloaca.

The appearance of oviduct was occurred on all E_2 treated male frogs. Likewise, the oviduct appearance was also observed on 1 of 8 and 9 of 9 male frogs at 8^{th} and 12^{th} week of PM-1000 treatment, respectively. However, when the reprodutive tracts of frogs were weighed, it was not identified whether it was oviduct or efferent tube.

After 4 weeks of the E_2 treatment, the weights of reproductive tracts were markedly increased and significantly higher than all PM groups ($p<0.01$, Table 4-9 and Figure 4-11). The increases in weights of reproductive tract of E_2 group were kept high up to the 8^{th} week of the treatment and it was decreased at the 12^{th} week of the treatment. The weights of reproductive tract of the E_2 group were higher than those of the PM-0, PM-10 and PM-100 ($p<0.05$) at the 8^{th} week of the treatment. The weight of the E_2 group was lower than that of the PM-1000 group at the 12^{th} week of the treatment. In addition, at the 12^{th} week of treatment the weight of reproductive tract of the PM-1000 was higher than other three PM and E_2 treatment groups ($p<0.01$). The weights of reproductive tract in PM-0, PM-10 and PM-100 were kept low throughout the study period. The increases in weight of the reproductive tracts in E_2 and PM-1000 group was concomitant with the occurrence of oviduct in male frogs. That is, the oviduct weights were heavier than the efferent tube weight.

Table 4-9. Reproductive tract weights in male Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2

Reproductive tract weight (mg)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4th	2.10 ± 1.60**	0.00 ± 0.00**	0.00 ± 0.00**	0.00 ± 0.00**	59.80 ± 17.30
8th	0.00 ± 0.00**	0.00 ± 0.00**	0.00 ± 0.00**	29.50 ± 29.50	58.60 ± 19.70
12th	0.00 ± 0.00**	0.00 ± 0.00**	0.00 ± 0.00**	69.40 ± 29.40++,**	13.00 ± 5.30

+, ++ indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the PM-0 group.

*, ** indicate significant differences, $p<0.05$ and $p<0.01$, respectively, from the E_2 group.

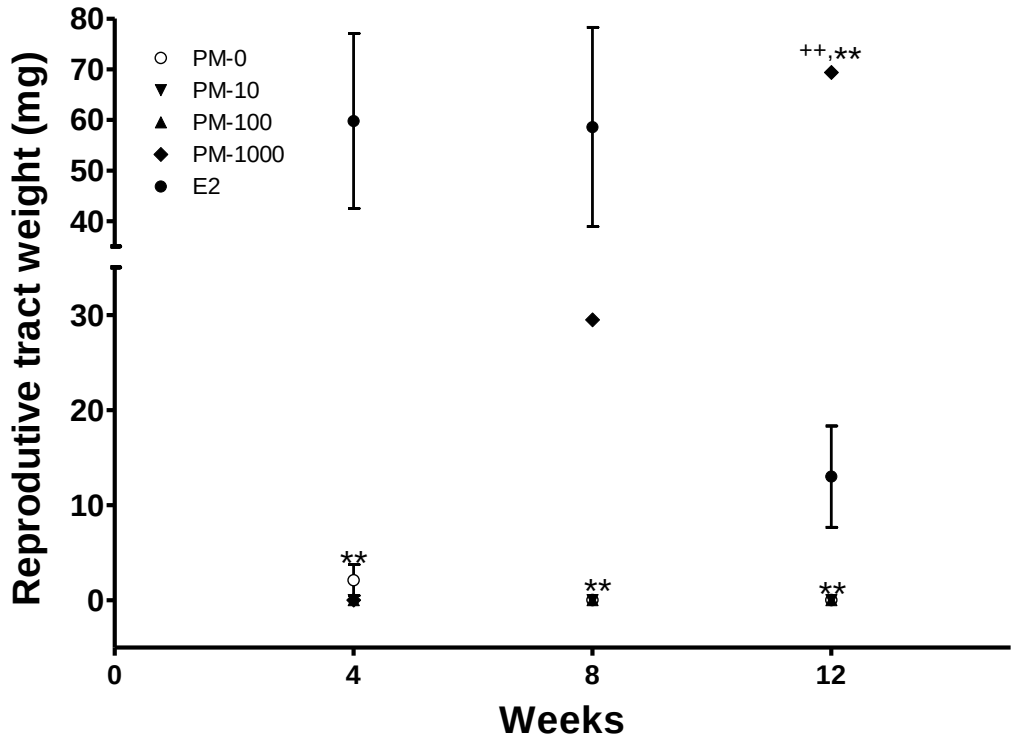


Figure 4-11. Reproductive tract weights of male frogs fed with 0, 1.77, 17.7 and 177 mg/kg BW/day of *P. mirifica* extract (PM-0, PM-10, PM-100 and PM-1000, respectively) and 100 μg/kg BW/day of 17β-estradiol (E_2) for 12 weeks. ++ indicates a significant difference ($p<0.01$) from the PM-0 group. ** indicates a significant difference ($p<0.01$) from the E2 group.

5.3. Microscopic observation of gonads

Gonads could be categorized into 3 types based on their phenotypic characters: testis, ovary and mixed-type. The phenotypic characters of testis were ellipsoidal and cylindrical shape, smooth surface, and white or pale yellow in color (Cong et al., 2006; Hayes et al., 2002 and Qin et al., 2003) (Figure 4-12a). The ovary was distinguished by the greater length with groove structure (Cong et al., 2006; and Qin et al., 2003) and melanin granule on the membrane (Cong et al., 2006 and Hayes et al., 2002) (Figure 4-12b). Thus, the gonad with both characters of testis and ovary was identified as mixed-type or abnormal gonad (Figure 4-13). The mixed-type gonad was further categorized to "ovary-like testis" when more ovarian characters were found than testicular characters or *vice versa* for "testis-like ovary". In this study, all abnormal gonads were identified as ovarian-like testis based on gross morphological examination. The sex identification of frogs was later confirmed by a microscopic observation.

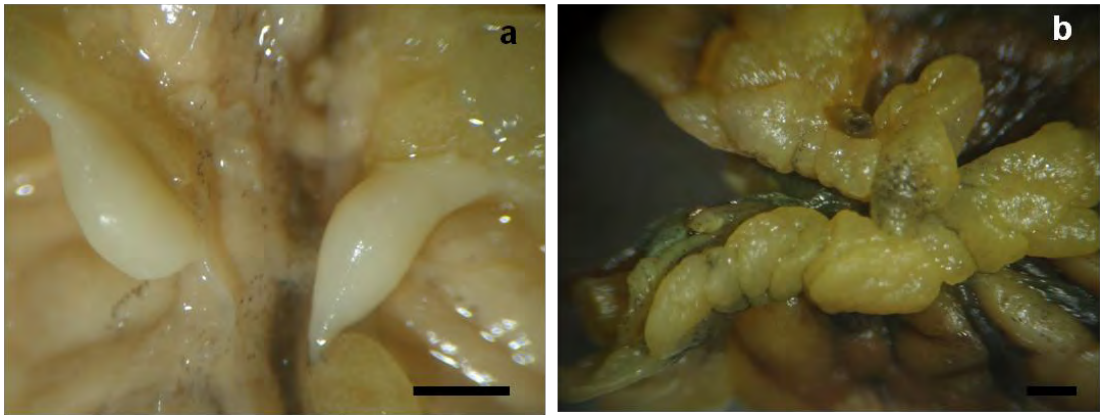


Figure 4-12. Normal testis (a) and ovary (b) of frogs in PM-0 (negative control group) at the 12th week of the experiment (bar = 1 mm).

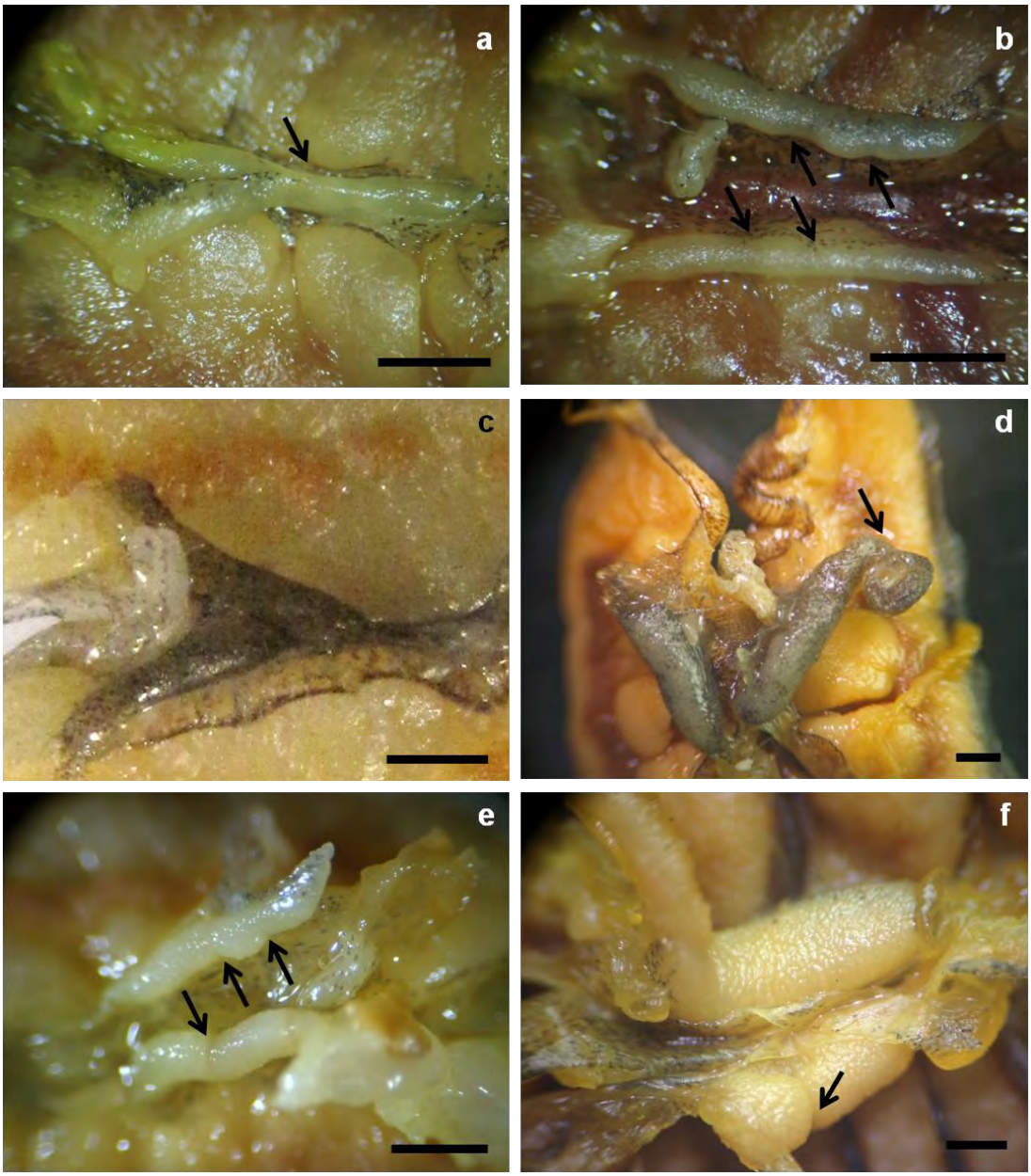


Figure 4-13. Mixed-type or abnormal testes found in E_2 treatment groups at the 4th (a) and 12th (b) week of treatment period, respectively. The melanin pigments found on the testes membrane of the PM-10 group at the 8th (c) and 12th week (d) of treatment period, respectively. The testes with groove structure found in the PM-100 group at the 4th week (e) and in the PM-1000 group at the 12th week (f) of treatment period, respectively. The arrows indicated the fusion of testes (a) and the groove structure on testes (b, d, e and f). Bar = 1 mm.

After the sex of frog was identified by gonadal phenotypic characters, the sex ratio in each treatment group was calculated as shown in Figure 4-14.

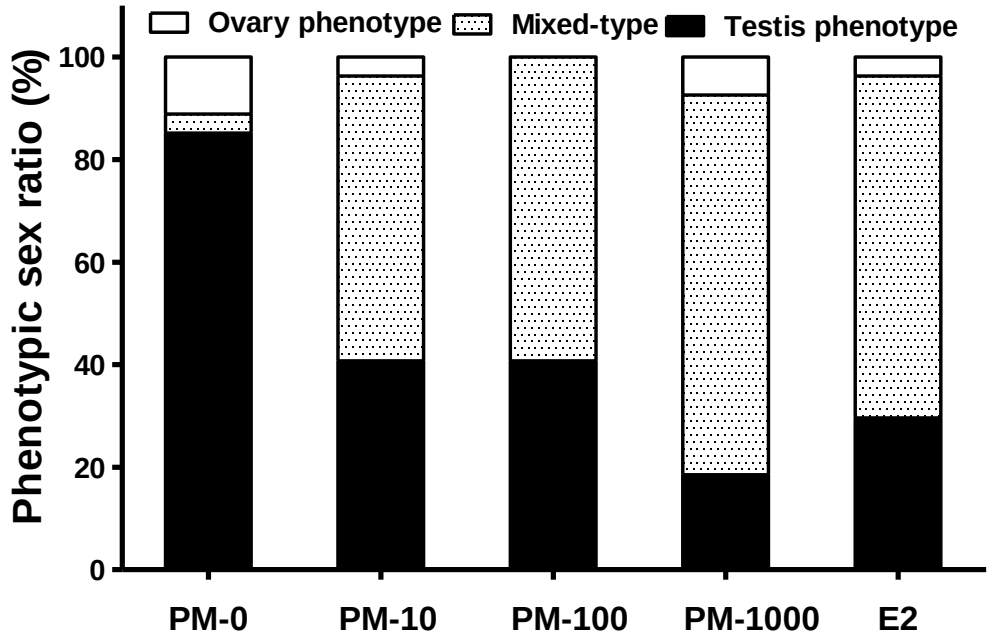


Figure 4-14. Sex ratio of frogs (n=27 in each group) fed with PM-0, PM-10, PM-100, PM-1000, and E_2 for 12 weeks based on phenotypic characters.

In PM-0 (control) group, an abnormal gonad with melanin granule was first found during the 4^{th} week of the treatment. Interestingly, the inclination of the gonadal phenotypic sex of male in the PM-0 group was observed during the study period with testis/ovary ratio of 85.19/11.11. However, after 12 weeks of PM and E_2 treatments, the number of frogs in male was reduced, ranging 18.52 – 40.74% with the increasing of mixed-typed gonad, 55.56%, 59.26% and 74.07% for PM-10, PM-100 and PM-1000, respectively. In E_2 group, 66% of the gonads was identified as abnormal gonad.

Interestingly, the gonad abnormalities found in those PM groups contained with only 2 characters, melanin granule and groove structure on testis, while another abnormality, fusion of testes, was found only in the E_2 group. At the microscopic (histological) level, all abnormal gonads were identified as a testis (Figure 4-16) which had no any characters of ovarian tissues (Figure 4-15) occurred.

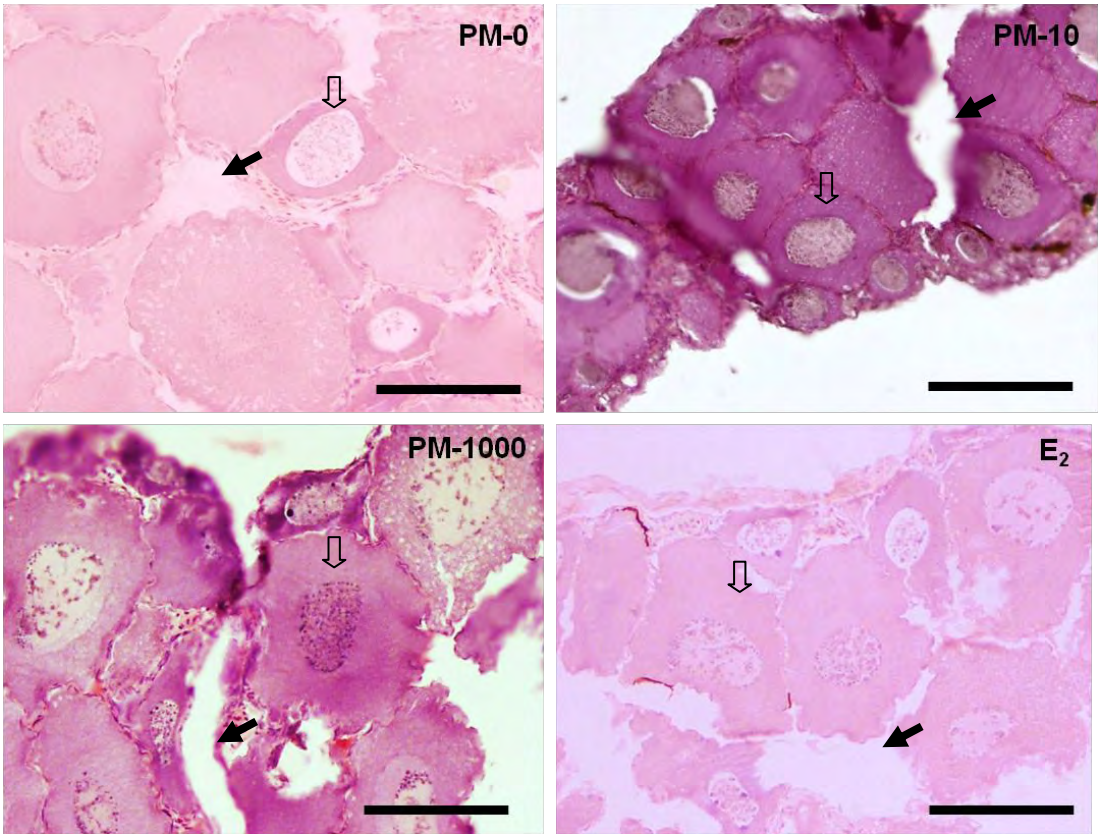


Figure 4-15. Microscopic appearances of ovarian tissue of frogs at the 12th week of the treatment (bar = 50 μm). The arrows indicated the cavity structures (⇧) and oocytes (⇧).

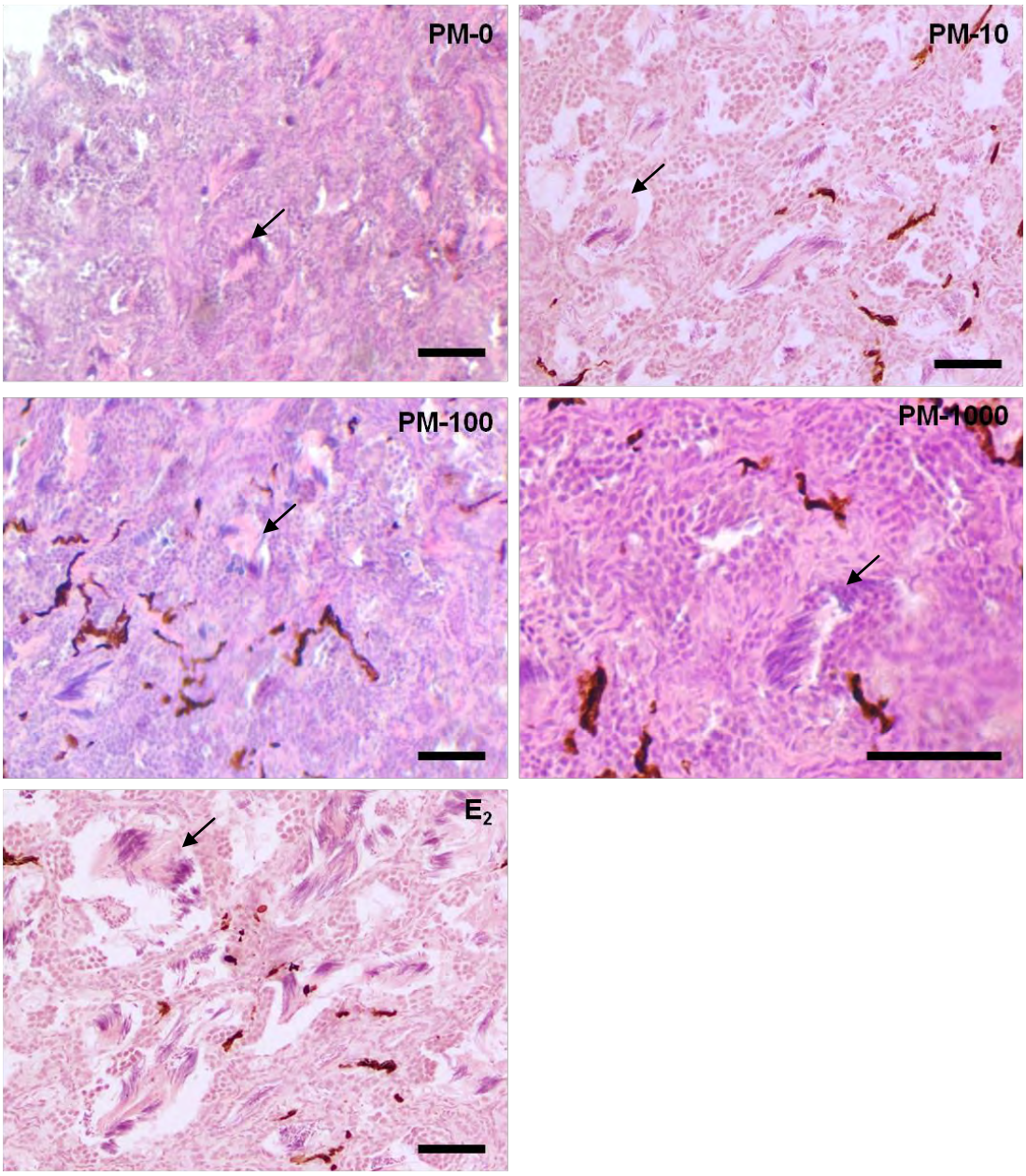


Figure 4-16. Microscopic appearances of normal (PM-0) and abnormal phenotypic testes (PM-10, PM-100, PM-1000 and E_2) at the 12^{th} week of the treatment (bar = 100 μm). However, at the histological level, all abnormal gonads were identified as testes. The arrows indicate the seminiferous tubules filled with spermatozoa.

After all abnormal (mixed-type) gonads were confirmed by microscopic examination, they were identified as testes. The sex ratio based on microscopic examination was done and shown in Figure 4-17.

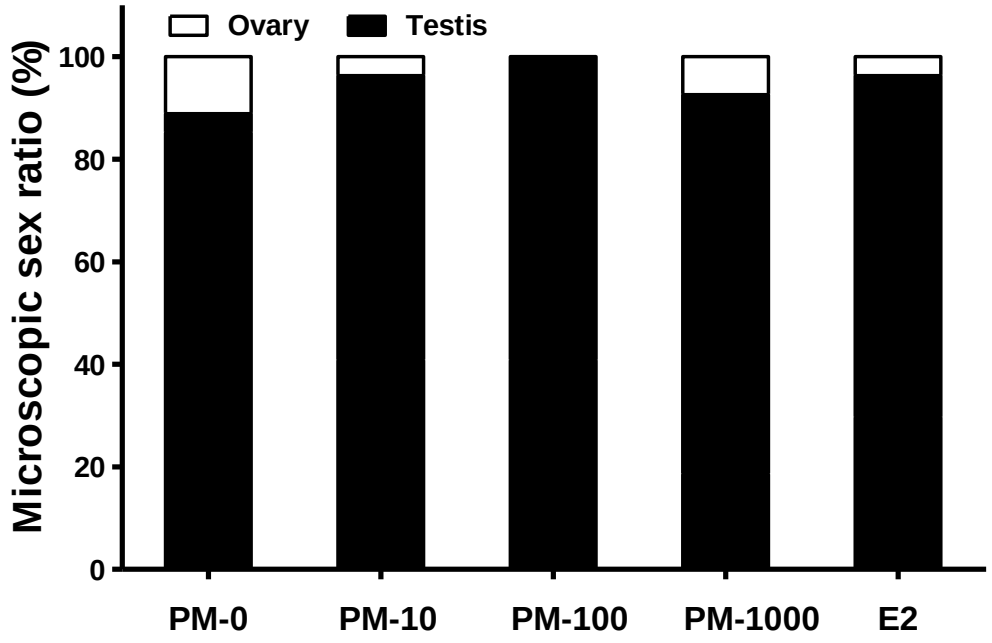


Figure 4-17. Sex ratio of frogs (n=27 in each group) fed with PM-0, PM-10, PM-100, PM-1000, and E_2 for 12 weeks based on microscopic appearance.

5.4. Microscopic observation of reproductive tracts

The reproductive tracts were clearly observed only in the PM-1000 and E_2 treated frogs, and they were identified as oviducts by both morphological (Figure 4-18) and microscopic examination (Figure 4-19).

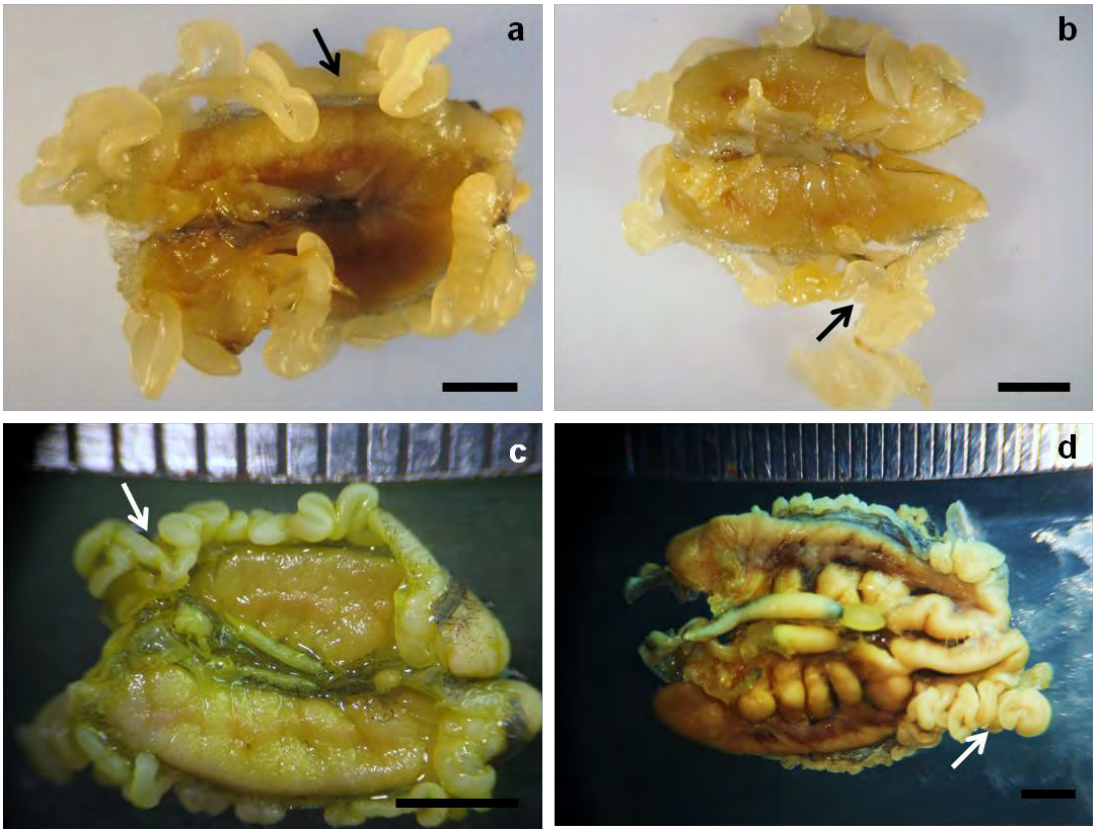


Figure 4-18. The kidney and oviducts of male frogs treated with E_2 for 4, 8 and 12 weeks (a, b and c, respectively) and PM-1000 for 12 weeks (d). The arrows indicated the oviduct. Bar = 3 mm.

At the microscopic level, the oviduct tissue was characterized by longitudinal fold and the lining epithelium columnar cells, ciliated with glandular cells.

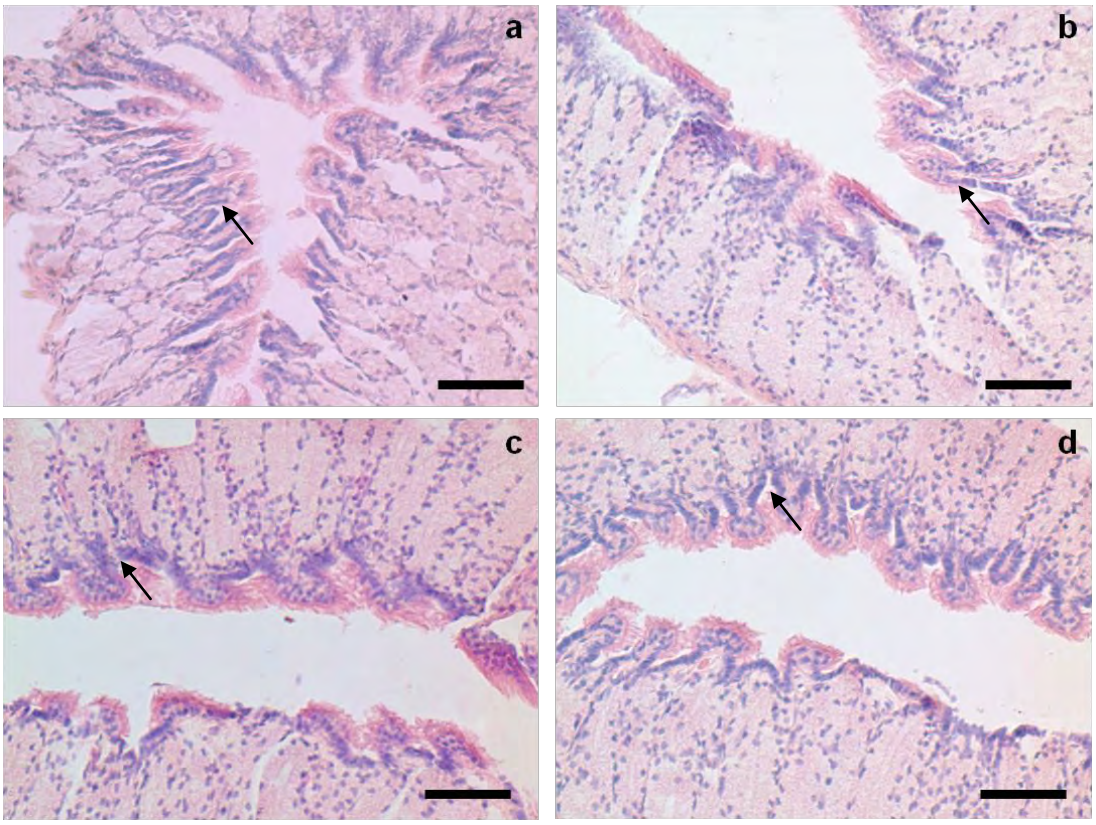


Figure 4-19. Microscopic appearances of oviduct of male frogs treated with E_2 for 4, 8 and 12 weeks (a, b and c, respectively) and PM-1000 (d) for 12 weeks. The arrows indicated oviductal folds. Bar = 100 μm

5.5. Sexual size dimorphism

After sex of frogs was identified by both phenotypic and microscopic appearances to male (testis) and female (ovary), body weights and length (SVL) of PM-0 treated frogs were separated and analyzed due to their sexes. At the 4^{th} and 12^{th} week, the body weights and length of female frogs (36.76 and 73.30 g, 57.92 and 86.52 mm, respectively, n=1) were higher and longer than those of male frogs (12.19±1.16 and 51.36±2.79 g, 45.36±0.80 and 73.02±1.48 mm, respectively, n=8). At the 8^{th} week, female frogs (71.00 g, n=1) showed significantly heavier ($p=0.012$) only in body weight than that of male frogs(29.39±4.14 g, n=8).

5.6. Changes of levels of serum sex steroid hormones

Sex steroid hormones, estrogen and testosterone, in blood serum were analyzed by RIA techniques and shown in Table 4-10 and Figure 4-20 and 4-21. There were no significant differences of serum estrogen levels in PM-10, PM-100 and PM-1000 groups compared to the PM-0 and E_2 groups during the 4^{th}-12^{th} week of treatment. Although there were no differences of serum testosterone levels between PM groups, serum testosterone levels of all four PM groups tended to higher than those of the E_2 group with some significant differences ($p<0.05$). At the end of the treatment period, the PM-1000 treatment tended to show the highest levels of both estrogen and testosterone.

Table 4-10. Serum estrogen, testosterone and estrogen/testosterone levels in male Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2.

Estrogen (pg/ml)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4^{th}	47.67 ± 15.71	34.80 ± 8.09	41.33 ± 4.98	57.80 ± 10.90	63.33±18.66
8^{th}	40.60 ± 11.77	50.20 ± 10.33	31.60 ± 7.47	53.20 ± 9.42	34.40 ± 8.24
12^{th}	53.20 ± 5.19	52.00 ± 8.01	44.40 ± 6.80	88.60 ± 23.14	63.00 ± 42.00

Testosterone (ng/ml)					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4^{th}	1.055 ± 0.775	0.396 ± 0.134	0.347 ± 0.167	0.548 ± 0.136	0.280 ± 0.200
8^{th}	0.366 ± 0.135	0.708 ± 0.158*	0.438 ± 0.168	0.678 ± 0.170	0.242 ± 0.099
12^{th}	1.014 ± 0.787*	0.876 ± 0.189	0.930 ± 0.170	1.362 ± 0.209*	0.310 ± 0.230

Estrogen : Testosterone					
Week	PM-0	PM-10	PM-100	PM-1000	E_2
4^{th}	0.225 ± 0.107	0.228 ± 0.109	0.220 ± 0.118	0.305 ± 0.227	0.604 ± 0.344
8^{th}	0.139 ± 0.286	0.097 ± 0.030	0.139 ± 0.046	0.108 ± 0.033	0.278 ± 0.131
12^{th}	0.053 ± 0.005**	0.064 ± 0.007**	0.051 ± 0.006**	0.064 ± 0.011**	0.229 ± 0.034

*,** indicate significant differences $p<0.05$ and 0.01 from the E_2 group, respectively.

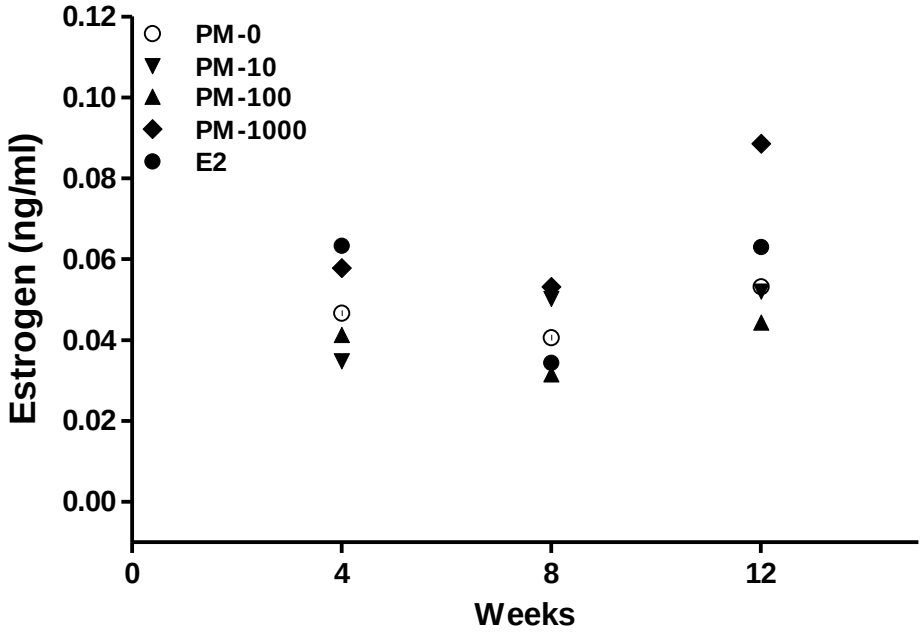


Figure 4-20. Serum estrogen levels in male Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2.

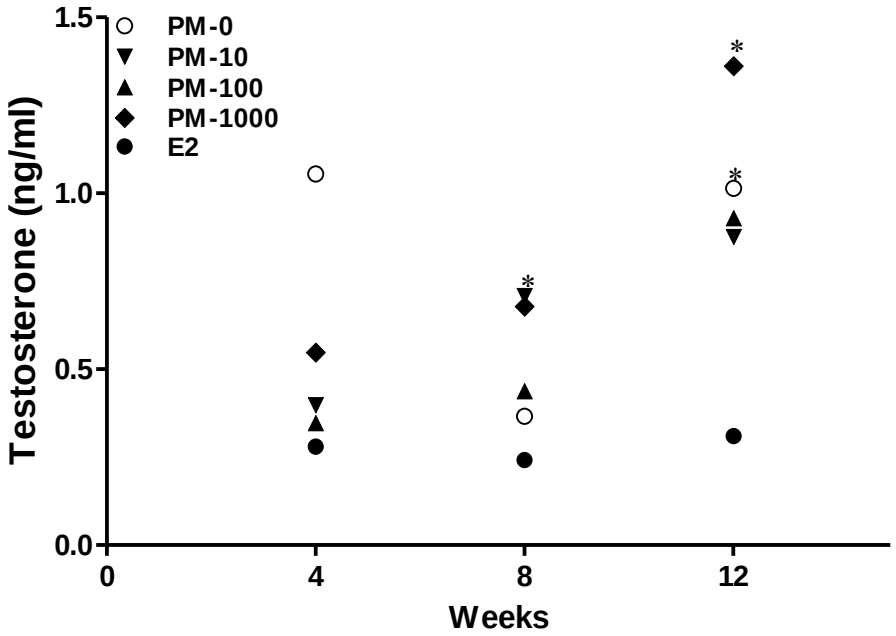


Figure 4-21. Serum testosterone levels in male Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2. * indicates a significant difference ($p<0.05$) from the E_2 group.

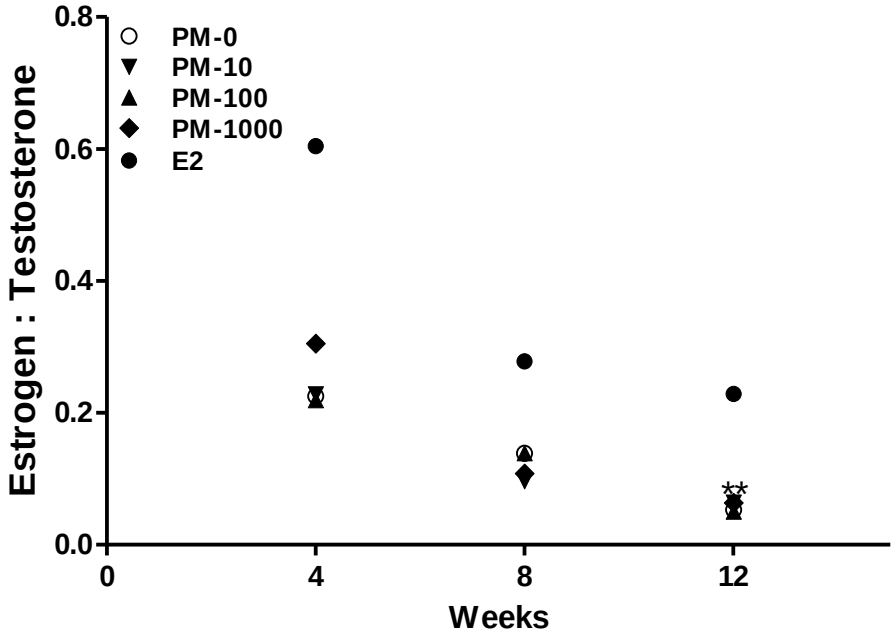


Figure 4-22. Serum estrogen/testosterone levels in male Rice Field Frogs treated with PM-0, PM-10, PM-100, PM-1000 and E_2. ** indicates a significant difference ($p<0.01$) from the E_2 group.

Considering the treatments of *P. mirifica* and E_2, serum estrogen/testosterone (E_2/T) levels tended to be higher in the E_2 group (Figure 4-22). However, the significant differences ($p\leq0.01$) were detected only at the 12th weeks of the treatment.

CHAPTER V

DISCUSSIONS

1. Authentication of *P. mirifica* and its estrogenic activity in rats

P. mirifica is a phytoestrogens-containing herb that could be found in the forests throughout Thailand (Cherdshewasart, Kitsamai and Malaivijitnond, 2007). It has been popularly used for medicinal, food supplementary and cosmetic purposes. Estrogenic activity of phytoestrogen constituents in the tuberous roots of *P. mirifica* was also verified in various species of laboratory animals (Jaroenporn et al., 2006; 2007; Malaivijitnond et al., 2004; 2006; Trisomboon et al., 2005; 2006) and on various organ systems, such as gonads (Trisomboon et al., 2005; 2006; 2007; Malaivijitnond et al., 2006), bone (Urasopon 2007; 2008a), cancer (Cherdshewasart et al., 2009) and brain (Chindewa et al., 2008). *P. mirifica* were also researched on growth promoting and meat exchange rate in economic animals, such as catfishes (Kanjanavarakul et al., 2003), chickens (Tubcharoen, 2007) and pigs (Tubcharoen et al., 2002).

However, various forms of *P. mirifica* were sold in the ordinary or pharmaceutical markets; tuberous roots with flowers and leaves, only tuberous roots, dried pieces of roots or powders. Thus, if the consumers were not a botanist or an expert on this plant, they should be confused on species identification, because there have been many plant species in Thailand that have similar morphological appearances to that of *P. mirifica*, e.g. Sa Boo Leud (*Stephania venosa*). The systemic identification which is simple, inexpensive and quick should be established.

There were 3 steps to identify the *P. mirifica*; 1) gross evaluations of tuberous roots, 2) gross and microscopic evaluation of dried pieces and powder of *P. mirifica*'s

meat, and 3) estrogenic activity of *P. mirifica* powder tested in the OVX rats. It was found that the root of *P. mirifica* was more round shape with the yellow brown and thick bark, the texture of the tuberous meat was rough with white color, and the powders consisted of small, translucent or very pale yellow starch granule (diameter: 3.63 – 4.27 μm) with many fibers. *P. mirifica* could stimulate vaginal cornification in OVX rats (Cherdshewasart et al., 2007; Urasopon et al., 2008b; Malaivijitnond et al., 2010).

The estrogenic activity of PM-HHK on vaginal cornification was first reported here in comparison with those of PM-Wichai3 and PM-KU. It shows that the PM-HHK, both powder and extract forms, could induce proliferation of vaginal epithelium cell, and the potency of activity was similar to that of the PM-Wichai3, but was weaker than that of the PM-KU. However, the estrogenic activity of PM-HHK was kept longer than those of the PM-Wichai3 and PM-KU (Malaivijitnond et al., 2006; Cherdshewasart et al., 2007). As the estrogenic activity of the PM-HHK extract is equivalent to that of the PM-HHK powder, it implies that the phytoestrogens constituents were not lost during the extraction process. Besides, the %yield of the extraction product of the PM-HHK extracted by 70%ethanol (Urasopon et al., 2008b) in this study was also higher than (17.6%) the %yield of the previous report when the *P. mirifica* roots were extracted by methanol (16.8%; Jumpapan et al., 2004).

2. Effect of *P. mirifica* extract on growth of Rice Field Frogs

The effective doses of E_2 and *P. mirifica* on growth promoting in amphibians have not been reported. The dose of E_2 used here (100 μg/L) was followed the previous studies tested the effect of E_2 on reproductive toxicity of *Xenopus laevis* tapole at NF stage 46/47 (Qin et al., 2007; Cong et al., 2006), and on liver

vitellogenesis of mature *Rana catesbian* (Villapando and Merchant-Larios, 1990; Li et al., 2006). They reported that E_2 at concentration of 100 µg/L could also induce sex reversal in tadpoles. The doses of PM-HHK extract tested in the Rice Field Frogs here (1.77, 17.7 and 177 mg/kg BW/day or PM-10, PM-100 and PM-1000, respectively) were followed the studies of the effects of PM-Wichai3 on reproductive organs of rodents (Cherdshewasart, 2003; Cherdshewasart et al., 2007a; 2007b; 2008; Malaivijitnond et al., 2004; 2006; 2010), because the estrogenic activity of PM-HHK extract was equal with that of the PM-Wichai3 as mentioned earlier.

From the previous reports, estrogenic activities of phytoestrogens or estrogens tested in frogs was treated by routes of subcutaneous injection in froglets (Li et al., 2006) or penetrating through the skin and GI tract of tadpoles by dissolving the chemicals in water (phytoestrogen quercitin for Cong et al., 2006; estrogens for Villapando and Merchant-Larios, 1990; Qin et al., 2007; Cong et al., 2006). As the duration of treatment of the PM-HHK extract and E_2 to Rice Field Frogs in the present study was 12 weeks, the subcutaneous injection of chemicals to the frogs should be a tedious work. Additionally, the bioavailability of absorption of E_2 and phytoestrogens through the skin of the complete metamorphosis Rice Field Frogs has not been verified. Frogs did not usually drink water, but they took water only through ventral skin (Iguchi et al., 2001). To ensure that the frogs received the treated chemicals, the PM-HHK extract and E_2 were sprayed on pelleted food. It was well-known that frogs prefer to feed the moving diets or preys (Holyoak, 2002; Mile, et al., 2004; Modezeleiad and Culley, 1974). However, from the present study, it was found that rearing frogs at the density of 1 adult frog/0.01 m^2 could help solving this problem. With this density, when frogs made their movements, the pelleted foods were moved as similar as living preys which could stimulate the frog feeding. It was

found that the PM-HHK extract tended to increase the growth rate, both body weight and length, in Rice Field Frogs which should be a good sign to use *P. mirifica* in frog farming.

Once again, it needs to emphasize that the growth promoter is necessary in frog farming business because the growth of frog is not depended on the composition of the diet. The high percentage of protein or fat could increase formation of fat body but it could not increase growth rate. It has been reported that the suitable % protein of diet of Rice Field Frog was 36.7% (Somsueb and Boonyaratpalin, 2001). From the results of the present study, the recommended dose of the PM-HHK extract that should be used to stimulate growth of the Rice Field Frogs is 1.77 mg/kg BW/day (or PM-10), because it showed the highest growth stimulation and no toxicological effects on livers and kidneys. All frogs were observed healthy throughout the study period. Although, Kupffer cells in liver tissues were observed in PM-10 group, it was also found in PM-0 group. However, it was previously reported that the proliferation of Kupffer cells after estrogen or phytoestrogens treatment was not a hepatotoxic effect (Cattley et al., 2002). Cherdshewasart (2003) also reported that there was no toxicity of PM-Wichai3 on liver, kidney, spleen, stomach, intestine, testis, ovary, and adrenal grand in mice, and the LD_{50} of oral consumption was over 2,000 mg/kg BW which was much higher than the doses used in this study. However, the determination of the remaining of the phytoestrogen residues in frogs' meats should be performed, if the *P. mirifica* extract will be applied on a commercial scale when the safety of the consumers is the major concern (FDA, 2002).

On the other hand, E_2 could stimulate only the increase in length of the complete metamorphosis Rice Field Frogs, not on the body weight. The different results in growth rate of frogs by mean of body weight, between PM groups and E_2

group indicated different mechanisms of phytoestrogens and E_2. Boonchird, Mahapanichkul and Cherdshewasart (2010) reported that the phytoestrogens and E_2 had different binding affinities to ERs. The phytoestrogens binded stronger to ERβ than ERα and vice versa for E_2. Bauer-Dantoin and Meinhardt (2010) reported that treatment of E_2 at concentrations of 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M to the rearing water, at 7-11 day intervals for 43 days, on 48 postfertilization of *Xenopus laevis* tadpoles could stimulate bone ossification. As the increase in body weight is needed for culturing, the use of E_2 as a growth promoter and treats to the complete metamorphosis Rice Field Frogs should not satisfy the frog farmers, because E_2 could not stimulate the increase in body weight of frogs.

3. Effect of *P. mirifica* extract on reproductive organ development of Rice Field Frogs

Interestingly, the sex ratio of the Rice Field Frogs delivered on late September and reared during October-December were moving towards males (88.89/11.11 for male/female). In addition, body size, weight and length of the female Rice Field Frogs in PM-0 group was significantly bigger and heavier than the males. According to the sexual dimorphism basis in various species of animals, amphibians also had a sexual size dimorphism. From the measurement of 589 amphibians species, body size of s was significantly larger than males (Kupfer, 2007). This sexual size dimorphism was also observed in *Hoplobatrachus* spp. (Gramapurohit, Shanbhag and Saidapur, 2004). From the sex ration of Rice Field Frogs hatched in September which was a male bias and had a smaller and lighter of body size and weight, this should impact on the investment of frog culturing. If the chemicals which exhibit characters of growth

promoting as well as sex reversal to female bias can be sought, it should improve the economics of frog culturing. *P. mirifica* might be one of the choices.

Although PM-HHK and E_2 could induce ovary-like testis (ovotestis or abnormal gonad) in the complete metamorphosis Rice Field Frogs, it could not induce sex reversal. One possible explanation is that the critical stage of sex differentiation in Rice Field Frogs is at a pre-metamorphosis stage, estimating from the report of *Xenopus laevis* is at NF stage 51 – 54 (Villapando and Merchant-Larios, 1990). Rice Field Frogs used in this study were at the complete metamorphosis stage of NF stage 66 (Nieuwkoop and Faber, 1994). Similarly, most of the studies on sex-reversal in *Xenopus laevis* were initiated when the frogs were at NF stage 46/47 (Cong et al., 2006; Wolf et al., 2010) or 49/50 (Oka et al., 2006).

PM-HHK extract and E_2 could influence on macroscopic testis development or phenotypic characters, however, the abnormality at the microscopic level was not observed. All of abnormal gonads were identified as testis at microscopic level. In addition, the gonad abnormalities induced by PM-HHK and E_2 were different; only melanin granule and groove structure on testes (Cong et al., 2006; Hayes et al., 2002 and Qin et al., 2003) were found in PM-HHK treated frogs, but the fusion of testes was also found in the E_2 treated frogs. Thus, the different mechanisms of actions between estrogens and phytoestrogens on gonad development need to be investigated further. Similarly, Witschi (1950, 1967) reported that E_2 treatment could induce destruction of testis cortex to form some ovarian characters in *Xenopus laevis* frogs, but there was no abnormality found in microscopic level. Kikuyama et. al. (1986) reported that E_2 treatment in male newts conducted oviduct development which showed some similarity with the E_2 treated frogs in this study. The appearance of reproductive duct (oviduct) in male frogs was a secondary sex differentiation which

was depended on both external (season changes) and internal (sex steroid hormones, mainly estrogen) factors (Bögi et al., 2002). In this study, there was no seasonal change and out of the breeding season, then the female secondary sex characteristic appearance of Rice Field Frogs should be depended only on sex steroid hormones (estrogen or estrogen-like substances). The presence of oviduct is a evidence of estrogen-dependant maturation in amphibian (Witschi, 1971). E_2 could conduct not only oviduct development in female amphibians but it also conducted oviduct development in male amphibians (Kikuyama et al., 1986). However, from the present study, it was found that the oviduct development in male Rice Field Frogs treated with E_2 occurred earlier than the PM-1000 treatment (the 4^{th}-8^{th} week for E_2 and 8^{th}-12^{th} week for PM-1000). Additionally, the oviduct development in male Rice Field Frogs occurred only in PM-1000 group. It can be concluded that the secondary sex differentiation in Rice Field Frogs induced by *P. mirifica* is depended on dose and time of treatment, which should also be different mechanism from E_2.

In agreement with the gonadal gross morphology or phenotypic sex ratio which was mainly in males, the serum E_2 levels were low in all 5 groups of frogs (ranging by 31.60 – 88.60 pg/ml; mean ± SE) as compared to the previous report in *Xenopus laevis* (459.5 ± 51.3 ng/dl; Qin et al., 2007) and no statistical differences among the groups were observed. On the other hand, the serum testosterone (T) levels in the PM –HHK treated frogs were significantly higher than the E_2 group, especially the PM-1000 group. Therefore, the ratio of serum E_2/T levels in all *P. mirifica* treated groups was significantly lower than the E_2 group at the 12^{th} week of the treatment. These results lead to the postulation that the gonadal cells of the E_2 treated frogs could synthesize and release estrogen in a higher level, or on another meaning is that the aromatase enzyme was high in gonadal cells of the E_2 treated frogs. The aromatase

enzyme functions on converting testosterone to estrogen (Beato, Herrlich and Schultz, 1995). Thus, although at the microscopic levels both *P. mirifica* and E_2 treated frogs showed the similar trend of testes-based gonads, changes at cellular levels or mechanisms need to be investigated to confirm this contradictory.

CHAPTER VI

CONCLUSIONS

1. PM-HHK had an estrogenic activity on vaginal proliferation of OVX rats similar to that of PM-Wichai3, however, the effect was lasted longer.
2. PM-HHK extract could stimulate growth rate, body weight and length, in the complete metamorphosis Rice Field Frogs, and the recommended dose of use is 1.77 mg/kg BW/day. Although E_2 could stimulate the growth of the complete metamorphosis Rice Field Frogs, only the increase in length was observed, not on the weight, which should not be satisfy the need of the frog farmers.
3. Rice Field Frogs hatched in late September showed the trend of male-bias in sex.
4. Female Rice Field Frogs hatched in late September and reared during October-December had a heavier body weight and larger body length than those of male.
5. Treatments of PM-HHK extract and E_2 to the complete metamorphosis Rice Field Frogs could not induce sex reversal; however, they showed a particular change in gonadal gross morphology of testes to ovary-like testes.
6. PM-HHK and E_2 could induce oviduct development which is female secondary sex characteristic (oviduct development) in male frogs, but it depended on dose and time of treatment.
7. Based on the gross morphological appearances and high E_2/T levels in E_2 treated frogs compared to the *P. mirifica* treated frogs, the different

mechanisms of actions on gonad development between E_2 and PM should be considered.

8. Thus, *P. mirifica* should be an alternative choice of growth promoters of Rice Field Frogs.

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APPENDIX

APPENDIX

Microscopic study

1. Chemicals

1)	40% Formaldehyde	: E. Merck, Darmstadt, Germany
2)	Absolute ethanol	: E. Merck, Darmstadt, Germany
3)	Ammonium alum	: E. Merck, Darmstadt, Germany
4)	Egg albumin	
5)	Eosin	: E. Merck, Darmstadt, Germany
6)	Ethanol	: E. Merck, Darmstadt, Germany
7)	Glacial acetic acid	: E. Merck, Darmstadt, Germany
8)	Glycerine	: E. Merck, Darmstadt, Germany
9)	Haematoxylin	: E. Merck, Darmstadt, Germany
10)	Na_2HPO_4 (Disodium hydrogen phosphate anhydrous)	: BDH Chemical Ltd., England
11)	$NaH_2PO_4.H_2O$ (Sodium dihydrogen phosphate-monohydrate)	: E. Merck, Darmstadt, Germany
12)	n-Butyl alcohol	: E. Merck, Darmstadt, Germany
13)	Paraffin	
14)	Xylene	: E. Merck, Darmstadt, Germany

2. Equipments

1) Cover glass
2) Hot air oven
3) Hot plate
4) Compound light microscope : Olympus, Japan
5) Microtome : American optical, Scientific Instrument Division, USA
6) Microtome blade : S 35, USA
7) Glass slide
8) Tissue floating bath
9) Digital camera : Olympus, Japan

3. Preparation of histological reagents

solution	compositions	
1. 10% buffered formalin	1) 40% formaldehyde	100 ml
	2) di-distilled water	900 ml
	3) $NaH_2PO_4.H_2O$	4 g
	4) Na_2HPO_4	6.5 g
2. Ehrlich's acid heamatoxylin	1) haematoxylin	8 ml
	2) absolute ethanol	400 ml
	3) Ammonium alum	8 ml
	4) di-distilled water	400 ml
	5) Glycerine	400 ml
	6) Glacial acetic acid	40 ml
3. Eosin	1) Eosin Y	0.5 g
	2) 95% ethanol	100 ml

10% buffered formalin; all chemicals were mixed together in a dark bottle, shaken until completely dissolved and stored in a room temperature.

Haematoxylin was dissolved in absolute ethanol in water bath at 40-50 °C and filtered with filtered paper. Then, ammonium alum was dissolved in warm di-distilled water, and glycerine and glacial acetic acid were added respectively. The heamotoxylin solution needed to be exposed to the sunlight for 6 weeks before used.

Eosin was completely dissolved in ethanol and stored at room temperature.

4. Procedures for microscopic observation

The standard histological techniques of Humason method was used in this study (Humason, 1979). Haematoxylin and eosin were used to stain the tissues of liver, kidney, ovary, testis and reproductive tracts.

4.1. Tissue preparation

After euthanized the frogs, sampling tissues were cut into the cubic shape of approximately 5 x 5 x 5 mm^3 and fixed in 10% neutral buffer formalin for 3 days. Then, all tissues were transferred to preserve in 70% ethanol.

4.2. Tissue dehydration

All sampling tissues were dehydrated by serial concentration of alcohol as follows;

step	solution	duration (hr)
1	90% ethanol	1
2	95% ethanol (1)	6
3	95% ethanol (2)	6
4	N-butyl alcohol	1
5	Xylene	1

4.3. Tissue embedding

Sampling tissues were immersed in the embedded media and incubated in the hot air oven at 55-60 °C as follows;

step	solution	duration (min)
1	Xylene+molten wax (1 :1)	30
2	Wax (1)	30
3	Wax (2)	60

Then, tissues were placed into a mold, poured the wax and allowed to be hardened.

4.4. Sectioning

The blocks of tissue were mounted onto a microtome and were serially sectioned at 5 μm. Subsequently, paraffin sections were mounted onto glass microscopic slides by egg albumin.

4.5. Hydration and staining

The microscopic slides of paraffin sections were deparaffined and stained as follows;

step	solution	duration (min)
1	Xylene (1)	5
2	Xylene (2)	5
3	n-butyl alcohol (1)	3
4	95% ethanol (1)	3
5	70% ethanol (1)	3
6	tap water (1)	3
7	Hematoxylin solution	20
8	Acid alcohol	Dip
9	tap water (2)	10
10	70% ethanol (2)	3
11	90% ethanol	3
12	Eosin staining solution	5
13	95% ethyl alcohol (2)	Dip
14	n-butyl alcohol (2)	5
15	Xylene (3)	5

Then slides were mounted with glass cover slid by Canada balsam, placed on the Table and left it until completely dry.

BIOGRAPHY

Miss Tarinee Lonuchit was born on January 30th, 1982 in Bangkok, Thailand. She finished her Bachelor's Degree of Education from the Department of Curriculum, Instruction, and Educational Technology, Faculty of Education, Chulalongkorn University in 2005. Nowadays, she is a graduate student in Zoology Program at Faculty of Science, Chulalongkorn University.