THE EFFECTS OF ISOLATED FLAVONOIDS FROM THE TUBEROUS ROOTS OF RED KWAO KREU (*Butea superba* Roxb.) ON REPRODUCTIVE SYSTEM OF MALE MICE

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ผลของฟลาโวนอยด์ที่สกัดจากรากกวาวเครือแดง (*Butea superba* Roxb.) ต่อระบบสืบพันธุ์หนูไมซ์เพศผู้

นางสาววณัทกมล นาคณรงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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วณัทกมล นาคณรงค์ : ผลของฟลาโวนอยด์ที่สกัดจากรากกวาวเครือแดงต่อระบบสืบพันธุ์หนูไมซ์เพศผู้ (THE EFFECTS OF ISOLATED FLAVONOIDS FROM THE TUBEROUS ROOTS OF RED KWAO KREU (*Butea superba* Roxb.) ON REPRODUCTIVE SYSTEM OF MALE MICE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เกรียงศักดิ์ เอื้อมเก็บ, 105 หน้า.

กวาวเครือแดงได้รับการอ้างว่าสามารถใช้ในผู้ชายไทยเพื่อการกลับสู่ภาวะหนุ่มในการ เพิ่มสมรรถภาพทางเพศ ป้องกันมะเร็งต่อมลูกหมากและต่อมลูกหมากโต เพราะมีสารแอนโดรเจนจากพืช ในความเป็นจริงการหย่อนสมรรถภาพทางเพศ คือการที่ไม่สามารถรักษา การแข็งตัวของอวัยวะเพศชายในการมีกิจกรรมทางเพศ ที่มีสาเหตุมาจากสภาพร่างกายและจิตใจ หลายประการ ยาในกลุ่มยับยั้งพีดีอี -5 ซิลเดนาฟิล ได้รับการรับรองสำหรับการรักษา อาการหย่อนสมรรถภาพทางเพศ นอกจากนั้นยังพบว่า ยานี้สามารถเพิ่มระดับฮอร์โมนเทสโทสเตอโรน และกระตุ้นเลดิกเซลล์ส่งผลให้เกิดการกระตุ้นกระบวนการสร้างตัวอสุจิ อย่างไรก็ตาม รายงานผลข้างเคียง ที่พบบ่อย ได้แก่ อาการปวดศีรษะ หน้าแดง อาหารไม่ย่อย คัดจมูก และรบกวนการมองเห็น ภาพสี ดังนั้น จุดประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาผลของสารสกัดจากรากของกวาวเครือแดงเปรียบเทียบกับ ซิลเดนาฟิล ต่อระบบสืบพันธุ์ของหนู ไมซ์เพศผู้ จากนั้นผงแห้งของรากพืชกวาวเครือแดง จึงถูกสกัดด้วยเอทานอล และถูก แยกสารสกัด ออกจากกันโดยซิลิกาเจลคอลัมน์ด้วยเทคนิค โครมาโทกราฟี และสารประกอบที่ถูกแยก ถูกพิสูจน์เอกลักษณ์เพื่อให้ทราบชื่อโดยใช้เครื่องมือโครมาโทรกราฟีแบบเฟสเคลื่อนที่เป็นของเหลวสมรรถนะสูง (เฮทพีแอลซี) โดยการเปรียบเทียบกับสารมาตรฐาน ต่อจากนั้น ได้นำสารที่สกัดได้เหล่านี้มา ป้อนหนูเพศผู้โดยให้ทางปาก ในขนาดที่ให้ สารสกัดหยาบ ซิลเดนาฟิล แฟลกชันบี แฟลกชันซี และแฟลกชันอี ได้แก่ 1,250 10 40 50 และ 150 มิลลิกรัมต่อกิโลกรัมต่อน้ำหนักตัวต่อวัน ตามลำดับ ส่วนประกอบของสารสกัดเหล่านี้พบว่า เป็นเจนิสตินและไบโอชานินเอ ทุกกลุ่มได้รับการ ทดลองเป็นเวลา 14 วันติดต่อกัน เลือดและสเปิร์ม ได้ถูกเก็บเพื่อการวิเคราะห์ก่อนและหลังการ ทดลอง ในทุกกลุ่ม นอกจากนี้ เมื่อสิ้นสุด การทดลองได้ ทำการเก็บส่วนต่างๆ ของอวัยวะสืบพันธุ์และอวัยวะที่สำคัญเพื่อการเปรียบเทียบ การเปลี่ยนแปลงน้ำหนักตัวสัมพัทธ์ได้ถูกนำมาวิเคราะห์ด้วย ผลการศึกษาพบว่า น้ำหนักตัวสัมพัทธ์ของหนูในกลุ่มที่ได้รับสารทั้งหมด ไม่แตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ ในขณะที่น้ำหนักม้าม ของหนูในกลุ่มที่ได้รับ แฟลกชันบี ซี และอี จะมีน้ำหนักมากกว่า กลุ่มควบคุม ในทางตรงข้ามกับ น้ำหนักขอ ง (p<0.01) อย่างไรก็ตาม น้ำหนักอัณฑะในกลุ่มที่ได้รับ สารสกัดหยาบและกลุ่มได้รับ ซิลเดนาฟิลนั้นตลอดจนน้ำหนักของเอพิดิไดมิส ของกลุ่มที่ได้รับ แฟลกชันอีและซี มีน้ำหนักมากกว่า กลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (p<0.01) ยิ่งไปกว่านั้นหนูที่ภายหลังการทดสอบสารทุกกลุ่ม มีระดับของเทสโทสเตอโรนสูงขึ้นอย่างมีนัยสำคัญ เมื่อเทียบกับก่อนการให้สาร ยกเว้นกลุ่มควบคุม

นอกจากนั้นระดับของเทสโทสเตอโรนในกลุ่มที่ได้รับแฟลกชันซีและอี ยังสูงกว่ากลุ่มอื่นๆอย่างมีนัยสำคัญและยังพบว่ากลุ่มที่ได้รับแฟลกชันซีมีจำนวนตัวอสุจิสูงที่สุด การค้นพบนี้เป็นหลักฐานที่แสดงว่าเจนิสติน สารที่ยังไม่ทราบชื่อ และไบโอคานินเอ อาจมีบทบาทสำคัญในการเพิ่มระดับเทสโทสเทอโรน จำนวนตัวอสุจิ และเพิ่มการเคลื่อนไหวของตัวอสุจิ หรือการรักษาภาวะการมีบุตรยากในผู้ชาย หลังจากที่ ได้วิจัยระดับของความปลอดภัยแล้ว ผลการวิเคราะห์เลือดพบว่ากลุ่มที่ได้รับแฟลกชันซี อีและซิลเดนาฟิล มีระดับคอเลสเตอรอลสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ยิ่งไปกว่านั้นระดับเฮโมโกลบินในกลุ่มที่ได้รับแฟลกชันอี สูงกว่ากลุ่มที่ได้รับสารสกัดหยาบและกลุ่มควบคุมอย่างมีนัยสำคัญ ($p<0.05$)

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

WANATKAMON NAKNARONG : THE EFFECTS OF ISOLATED FLAVONOIDS FROM THE TUBEROUS ROOTS OF RED KWAO KREU (*Butea superba* Roxb.) ON REPRODUCTIVE SYSTEM OF MALE MICE. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 105 PP.

BUTEA SUPERBA ROXB, TESTOSTERONE, SPERM COUNT, SILDENAFIL, CHOLESTEROL

Butea superba Roxb.(BS) has been claimed to use in Thai men for rejuvenation, improve sexual function, prevent prostate cancer and prostatic hyperplasia, because it contains Phytoandrogen. In fact, Erectile dysfunction (ED) is the inability to maintain penile erection for the successful performance of sexual activity which is caused by many physical and psychological factors. The PDE-5 inhibitors, sildenafil, is approved for the treatment of ED. In addition, sildenafil has increased testosterone level and stimulated Leydig cells resulting in an increase of spermatogenesis. However, the most frequent adverse effects reported for sildenafil is headache, flushing, dyspepsia, nasal congestion, and disturbances in color vision. Hence, the objective of this study was to investigate the effects of the tuberous root of *Butea superba* Roxb extract compared with sildenafil on the reproductive system of male mice. Then, the dried powder of the tuber roots of *Butea superba* Roxb. was extracted with ethanol and separated by silica gel column chromatography The chemical names of the isolated compounds were identified by High performance Liquid Chromatography (HPLC) compared with standard compounds. Then, these compounds were orally administered to male mice. The crude extract, sildanafil, fraction B, C and D were fed at the doses of 1,250, 10, 40, 50 and 150 mg /kg BW/day respectively. The majority of these extract compounds such as Genistein and

Biochanin A were elucidated. All groups were treated for 14 consecutive days. Blood and sperms were collected for analysis before (pre-) and after (post-) treatment in all groups. Also, at the end of the experiments, the selected reproductive and vital organs were collected for comparative measurements. A relative change of body weight was also analyzed. The results showed that the relative body weights in all treated groups were not significant different from the control. Whereas, the spleen weight of fraction B, C and E treated groups were significantly heavier, and stomach weight of fraction E group was lighter than those of the control (p<0.01). However, the testes weight of crude extract and sildenafil treated groups, and the epididymis weight of fraction E and C treated group were significantly heavier than those of the control (p<0.01). In addition, the results exhibited that there were significant increases in testosterone levels of all post-treated groups compared to pre-treatment groups, except for that of the control (p<0.01). In addition, the testosterone levels of fraction C and E treated groups were significantly higher than those of other groups (p<0.01). Also, the sperm number of all treated groups were significantly higher than that of the control group (p<0.01) as well as the fraction C which showed the highest number. These findings provide evidence that Genistein, Unknown compound 1 and Biochanin A may play an important role in increasing of testosterone level, sperm number and motility or infertility treatment in men after safety level is investigated. The blood analysis showed that the cholesterol level of fraction C, E and sildenafil were significantly higher than those of the control (p<0.05). Morover, the hemoglobin level of fraction E treated group was significantly higher than those of the control and the crude extract groups (p<0.05).

School of Biology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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Wanatkamon Naknarong

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF SCHEMES

Scheme **Page**

LIST OF ABBREVIATIONS

AST	=	aspartate aminotransferase
BS	=	*Butea superba* Roxb.
β	=	beta
BUN	=	blood urea nitrogen
CO_2	=	carbon dioxide
$^{\circ}C$	=	Degrees Celcius
cm	=	centimeter
g	=	gram
h	=	hour
HPLC	=	High Performance Liquid Chromatography
Hb	=	hemoglobin
Hct	=	hematocrit
i.p.	=	intraperitoneal
kg	=	kilogram
L	=	liter
LPS	=	lipopolysaccharide
MCV	=	mean corpuscular volume
mL	=	milliliter
mg	=	milligram
mM	=	millimolar
ppm	=	part per million
RBC	=	red blood cell

LIST OF ABBREVIATIONS (Continued)

μg/mL	=	microgram per milliliter
μL	=	microliter
μM	=	micromolar
WBC	=	white blood cell
%	=	percent

CHAPTER I

INTRODUCTION

1.1 Introduction

Medicinal plants have been used in Thailand since at least the Sukhothai period (14th century AD) and the use of traditional drug formulae began during the Ayudhya period. Traditional drugs have been popular in the Kingdom throughout the Ayudhya and Rattanokosin periods. King Rama III (about 200 years ago) ordered the collection of traditional drug recipes, diagnosis of diseases, traditional massage, literature, and poetry, and their subsequent inscription on stone plates installed on the walls of two temples (Wat Phra Chetuphon Vimolmangklaram and Wat Raja Orasaram). Again during the years 1895-1900 King Rama V ordered the collection of all knowledges of traditional medicines from the noble sand doctors, and the printing and distribution for the first time in the two volumes of the official pharmacopoeia called “Tamrapaettayasartsongkroh”. Since the use of herbal medicines is quite often derived from empirical experience, standardization and quality control of these preparations are usually lacking, which hampers an increased utilization of such medicines. Thai pharmacists and scientists have therefore realized that this problem should be solved to upgrade the quality of herbal raw materials and their finished products (Dechatiwongsena Ayudhaya, 1997).

Natural products are organic compounds that are formed by living systems found in nature that usually have a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. It has been estimated that over 40%

of medicines have their origins in these natural products, especially natural products from plants. Natural product research remains one of the main approaches of discovering bioactive compounds. Since little is known about the etiology of many human, animal, and plant diseases, it is difficult to design potentially active molecules for their treatments and therefore leads from natural sources will continue to be sought (Block, 1989).

The traditional herbal medicines have been used for thousands of years in many oriental countries (China, Thailand, Japan, etc). Medicinal plants are widely used as therapeutic drugs or herbal medicines. Some of them are used as pharmaceuticals, fragrances, flavors, colors, stimulants, and cosmetics. The World Health Organization (WHO) estimated that 80% of the world's inhabitants mainly depend on traditional herbal medicines as sources for their health care (Farnsworth, Akerele, Bingel, Soejarto, and Guo, 1985). Over 100 chemical substances, derived from different plants, considered to be important drugs are either currently in use or have been widely used in one or more countries in the world. Approximately, 75% of these substances were discovered as a direct result of chemical studies focusing on the isolation of active substances from plants used in folk medicine (Gad, 2005).

1.2 Research objectives

1.2.1 To extract and isolate chemical constituents and/or flavonoids from tuberous roots of *Butea superba* Roxb.

1.2.2 To investigate the effects of isolated chemical constituents and/or flavonoids from the tuberous root of *Butea superba* Roxb. on the reproductive system of male mice.

1.3 Research hypothesis

Isolated chemical constituents and/or flavonoids from the *Butea superba* Roxb. may show increase in sperm motility, sperm count, testosterone and some reproductive organs.

1.4 Scope and limitation of the study

This research focuses on investigating biological activities of isolated chemical constituents and/or flavonoids from the tuberous root of *Butea superba* Roxb., in male mice. Only active ingredients and/or fraction from extractions has been investigated.

1.5 Expected results

1.5.1 The bioactivity of isolated chemical constituents and/or flavonoids from the tuberous root of *Butea superba* Roxb. in male mice will be elucidated.

1.5.2 The effects of isolated chemical constituents and/or flavonoids from the tuberous root of *Butea superba* Roxb. on sperm count, sperm motility, haematology, blood chemistry, and testosterone in male mice will be investigated.

1.5.3 The results may be useful for development of new drugs to obination against erectile dysfunctions in men.

CHAPTER II

LITERATURE REVIEW

2.1 *Butea superba* Roxb. (BS)

Butea superba Roxb., a plant in the family Leguminosae, is mostly found in the northern region of Thailand deciduous forest and has the domestic name of "Red Kwao Kreu". The plant is one of the four types of Kwao Kreu such as, White Kwao Kreu, Kwao Kreu Mor and Black Kwao Kreu. *Butea superba* Roxb. is a herb with Thai medicinal properties that has been used since ancient times.

B. superba Roxb. has been popularly used among Thai men for rejuvenation because it contains Phytoandrogen that has structure and function similar to testosterone. The effect of *B. superba* Roxb. has been reported that it encouraged more black hair and stimulated more sperms, stimulated vascular growth in the body, decreased hypercholesterolaemia, relieved bone pain and hypertension and improved blood circulation. It has been claimed that *B. superba* Roxb. could improve sexual function, prevented prostate cancer and prostatic hyperplasia (Parnprasong, 1999).

Figure 1 Leaves of *Butea superba* Roxb. [http://www.magnoliathailand.com].

Scientific Name: *Butea superba* Roxb.

Family: Leguminosae

Sub-Family: Papilionaceae

Common Name: Kwao kreu (Phayub), Jan-Kwao (Isaan), Ton-Jom-Thong (Chumporn), Thong-Kwao (Thai), Pho-Ta Ku (Karen-Kanchanaburi), Pho-Mue (Karen-Mae Hong Son) (Samittinan, 2001; Niyumtom, 1995).

2.1.1 Botanical description

This plant has a characteristic of being a crawler about 5-10 meters in length that wraps itself around large trees or scaffolds (Pongbonrod, 1995). The trunk has a diameter of about 15 centimeters, its wood is rather hard, and the bark has dark brown color. One branch has 3 leaves and the flowers are a yellowish orange color, about 4-5 centimeters in length that blossom when the plant sheds its leaves. The shape of *Butea superba* Roxb. looks like *Sesnania grandiflora* (Dok Care Ban). The seed pod is flat and long, about 10-15 centimeters in length, and contains only one large seed of a light brown color. This plant grows out in the open. The long tuber root of this plant is buried under ground like the tuber root of yam. The tuber roots of this plant have a length of 30-50 centimeters (Figures 1-3) (Samittinan, 2001).

Figure 2 Flowers of *Butea superba* Roxb. [http://www.bloggang.com].

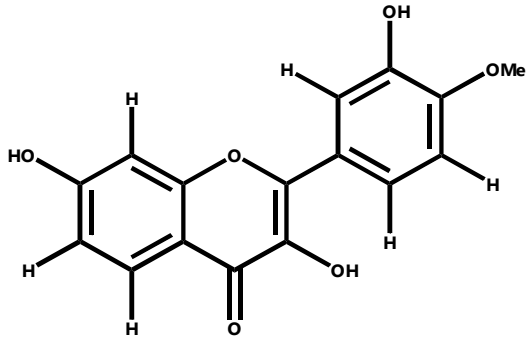
Ecology and distribution: It is mostly found growing in forests in northern regions, eastern regions and northeastern regions of Thailand.

Propagation: They are normally propagated by seeds and roots.

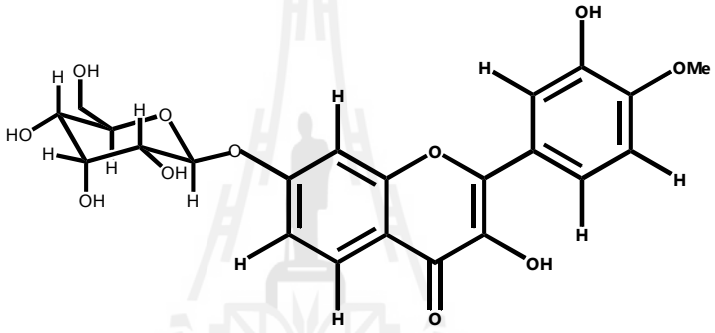
Figure 3 Tuber roots of *Butea superba* Roxb. [http://www.kadnad.com].

2.1.2 Chemical constituents:

Roengsumran et al. (2000) reported that flavonoid and flavonoid glycoside (Figure 4) from *B. superba* Roxb. showed high inhibitory effects on cyclic-adenosine 3',5'-monophosphate phosphodiesterase (cAMP phosphodiesterase). This type of enzyme inhibited the clotting of cock by blooding (or interrupted) the blood flow to the penis and erectile dysfunction for 50% men at 200 µg/ml extract concentration.



3,7,3’-Trihydroxy-4’-methoxyflavone **(1)**

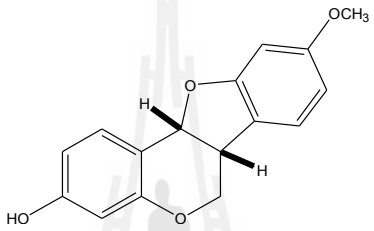


3,5'-dihydroxy-4'-methoxyflavone-7-O-β-D glucopyranoside **(2)**

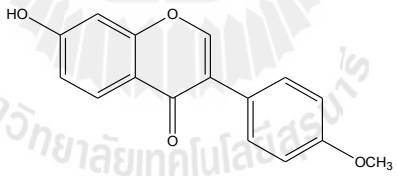
Figure 4 Chemical structure of isolated compounds from *Butea superba* Roxb.

The chemical constituents in the tuberous roots of *Butea superba* Roxb. showed biological activity, toxicology and consumers safety. Flavonoids have been reported that it can prevent the degeneration of cells caused by oxidant that come from interactions oxidation (Winkel, 2001). Ralston et al. (2005) reported that crude extract of *Butea superba* Roxb. had an activity in the preservation of the capillary wall, increased strength and resistance to the capillary by the contraction of blood vessels which results in creasing urination. It was found that anthocyanin may expel carcinogenic and may also have activity to expand blood vessels, reduce the risk of heart disease and paralysis (Hodgson et al., 1999).

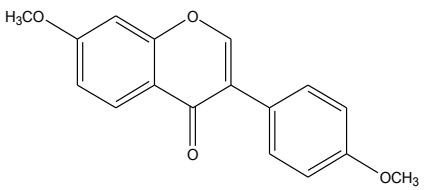
Ngamrojanavanich et al. (2007) reported the isolation of 3-hydroxy-9-methoxypterocarpan (medicarpin) (**3**) and four isoflavones, 7-hydroxy-4´-methoxy-isoflavone (formononetin) (**4**); 7,4′-dimethoxyisoflavone (**5**); 5,4′-dihydroxy-7-methoxy-isoflavone (prunetin) (**6**) and 7-hydroxy-6,4′-dimethoxyisoflavone (**7**) from the chloroform extract of the tuber roots of *B. superba* Roxb. (Figure 5).



3-Hydroxy-9-methoxypterocarpan (medicarpin) **(3)**

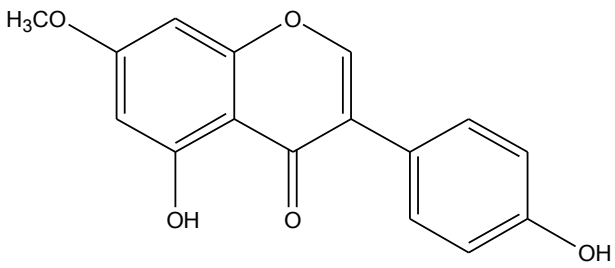


7-Hydroxy-4′-methoxy-isoflavone (formononetin) (**4**)

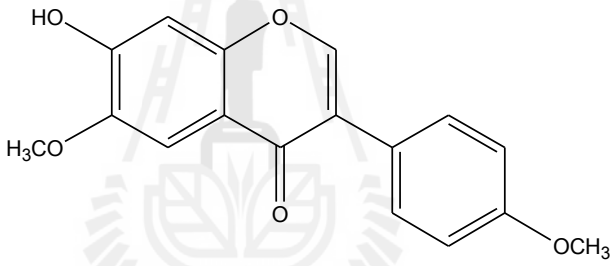


7,4′-Dimethoxyisoflavone (**5**)

Figure 5 Chemical structure of isolated compounds from *Butea superba* Roxb.



5,4′-Dihydroxy-7-methoxyisoflavone (prunetin) (**6**)



7-Hydroxy-6,4′-dimethoxyisoflavone (**7**)

Figure 5 (Continued).

Since *Butea superba* Roxb. helps to enhance human health, it is very important to study the chemical constituents and their biological activity, including flavonoids. Flavonoids are compounds which possess the same C_{15} (C_6-C_3-C_6) flavone nucleus two benzene rings (A and B) linked through an oxygen-containing pyran or pyrone ring (C). This structure is common to 3-deoxyflavonoids (flavones, flavanones, isoflavones and neoflavones) and 3-hydroxyflavonoids (flavonols, anthocyanins, flavan-3,4-diols and flavan-3-ols) as shown in Table 2.1 (Khnau, 1976).

Table 2.1 The Chemical Structures of the Flavonoid Family.

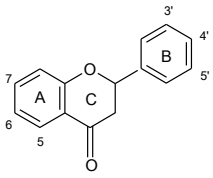
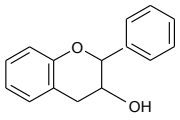
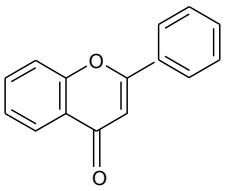
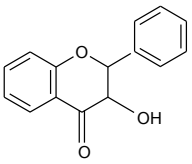
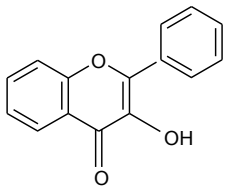
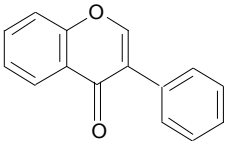
Structural formula	Representative flavonoids	Substitutions 5	6	7	3′	4′	5′
Flavanone							
	Eriodictyol	OH	H	OH	OH	OH	H
	Hesperitin	OH	H	OH	OH	OMe	H
	Naringenin	OH	H	OH	H	OH	H
Flavanol							
	Catechin	OH	H	OH	OH	OH	H
	Gallocatechin	OH	H	OH	OH	OH	OH
Flavone							
	Apigenin	OH	H	OH	H	OH	H
	Chrysin	OH	H	OH	H	H	H
	Luteolin	OH	H	OH	OH	OH	H
Flavanonol							
	Taxifolin	OH	H	OH	OH	OH	H

Table 2.1 (Continued).

Structural formula	Representative flavonoids	Substitutions 5	6	7	3′	4′	5′
Flavonol							
	Kampherol	OH	H	OH	H	OH	H
	Myricetin	OH	H	OH	OH	OH	OH
	Quercetin	OH	H	OH	OH	OH	H
	Galangin	OH	H	OH	H	H	H
Isoflavone							
	Daidzein	H	H	OH	H	OH	H
	Genistein	OH	H	OH	H	OH	H
	Glycitein	OH	OMe	OH	H	OH	H
	Formononetin	H	H	OH	H	OMe	H

Our research group has interested in *Butea superba* Roxb. due to it is widely used in traditional medicine, because of its interesting biological activities, and the fact that report about it in the literature is very rare. Therefore, it is desirable to phytochemical investigate this plant in detail. A literature search for other plants in subfamily *Papilionaceae* give the results shown in Table 2.2.

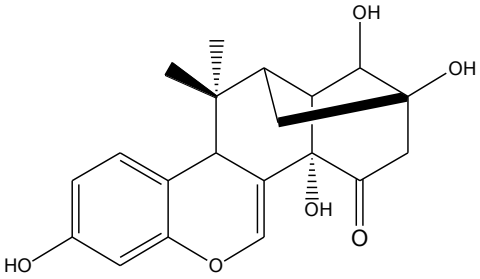
Table 2.2 Chemical constituents of plants in the subfamily *Papilionaceae.*

Scientific name	Plant part	Organic compounds	References
Pueraria mirifica	Root	mioestrol (**8**) β-sitosterol (**9**) stimasterol (**10**)	Hayodom, 1971
Pueraria mirifica	Root	genistin (genistein-7-*O*-glucoside) (**11**) puerain-6"-monoacetate (**12**) mirificoumestan (**13**)	Ingham, Tahara, and Dziedzic, 1989
		mirificoumestan hydrate (**14**) mirificoumestan glycol (**15**)	Ingham, Tahara, and Dziedzic, 1988
		kwakhurin (**16**)	Tahara, Ingham, and Dziedzic, 1987
		daidzein (**17**) daidzin (daidzein-7-*O*-glucoside) (**18**) puerarin (**19**) genistein (**20**) coumestrol (**21**)	Ingham, Tahara, and Dziedzic, 1986

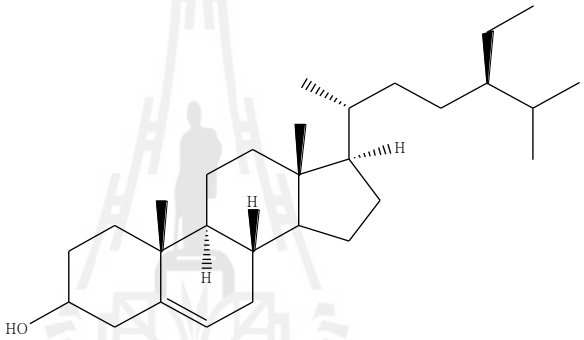
Table 2.2 (Continued).

Scientific name	Plant part	Organic compounds	References
Butea frondosa Roxb.		butrin (**22**) isobutrin (2**3**) coreopsin (**24**) isocoreopsin (**25**) sulfurein (**26**) monospermoside (**27**)	Hildebert, Bettina, Manfred, Yoshinobu, and Hiroshi, 1986. Gupta, Ravindranath, and Seahadri, 1970.
	Seeds	butin (**28**) δ-lactone (**29**) palasonin (**30**)	Dixit, Agarwal, Bhargava, Gupta, and Jam, 1981 Biahnoi and Gupta, 1979. Chandra and Sabir, 1978.

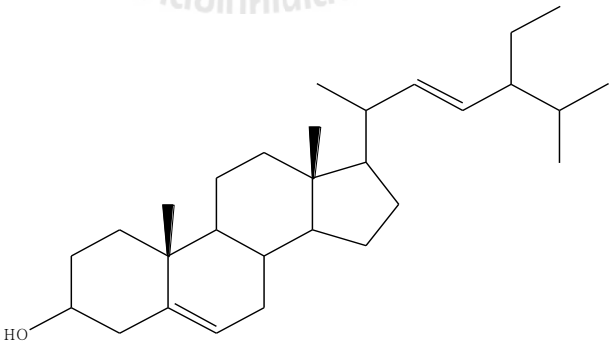
The following structures were reported of chemical constituents of plants in the subfamily *Papilionaceae* (Figure 6).



Mioestrol (**8**)

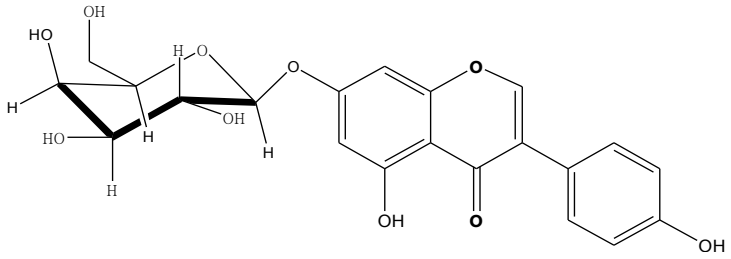


β-Sitosterol (**9**)

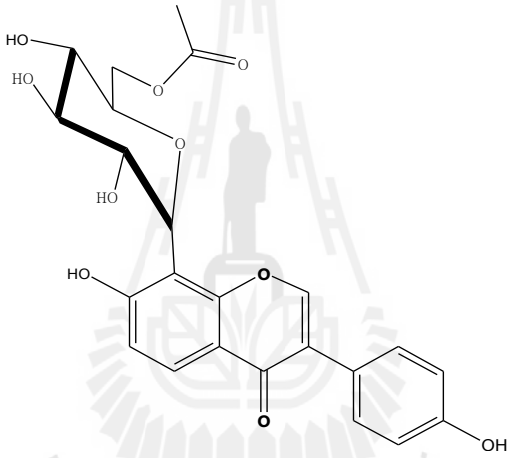


Stimasterol (**10**)

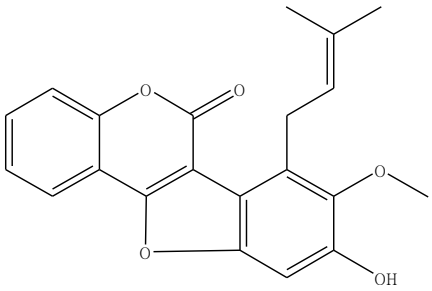
Figure 6 Chemical structure of isolated compounds from subfamily *Papilionaceae*.



Genistin (genistein-7-*O*-glucoside) (**11**)

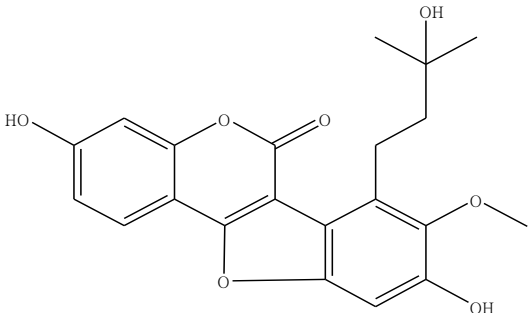


Puerain-6"- monoacetate (1**2**)

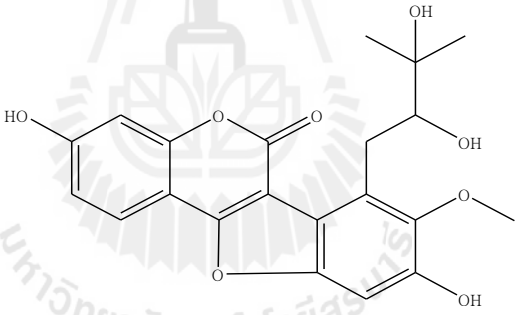


Mirificoumestan (**13**)

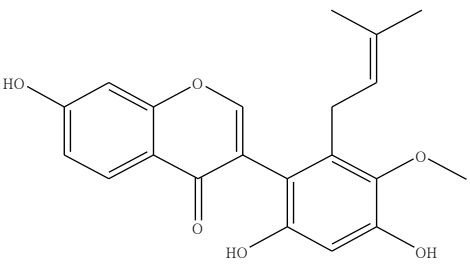
Figure 6 (Continued).



Mirificoumestan hydrate (**14**)

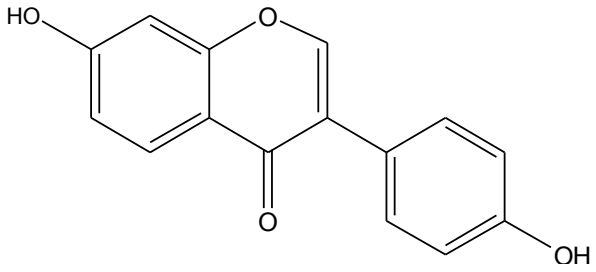


Mirificoumestan glycol (**15**)

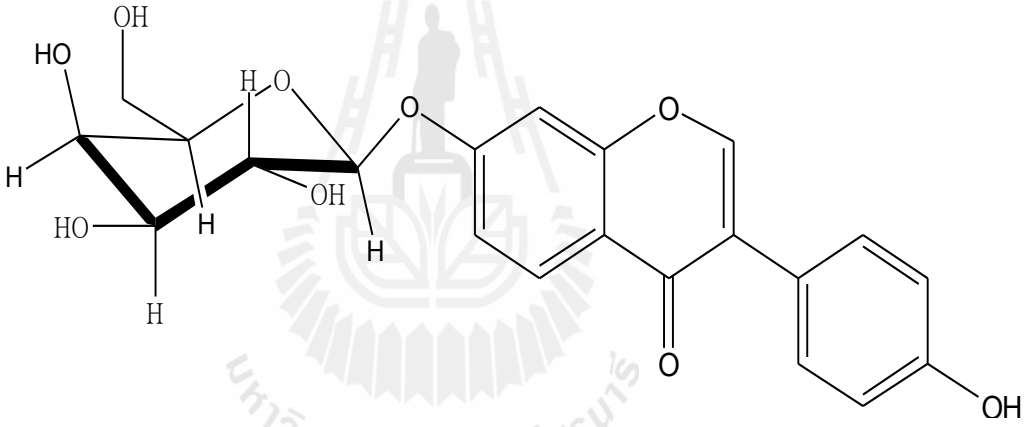


Kwakhurin (**16**)

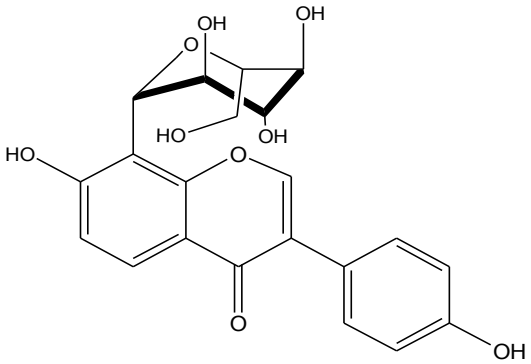
Figure 6 (Continued).



Daidzein (**17**)

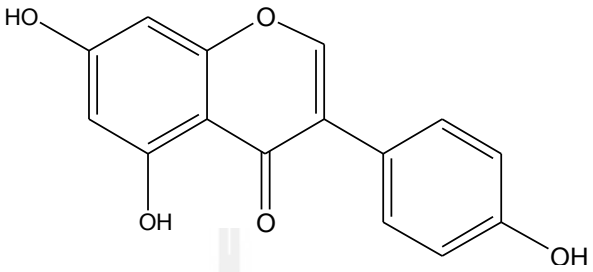


Daidzin (daidzein -7-*O*-glucoside) (**18**)

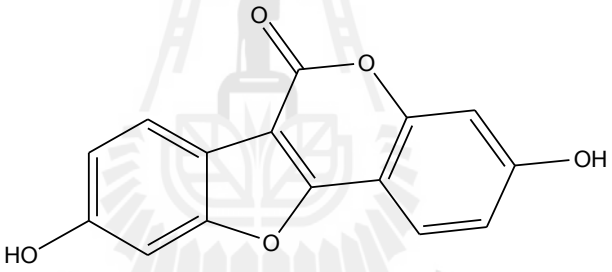


Puerarin (**19**)

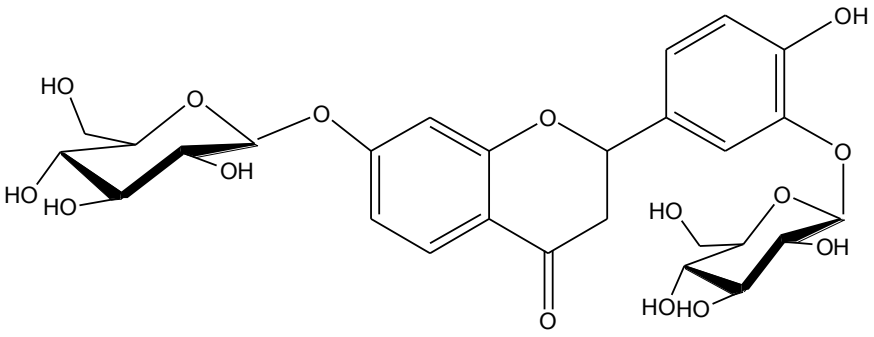
Figure 6 (Continued).



Genistein (**20**)

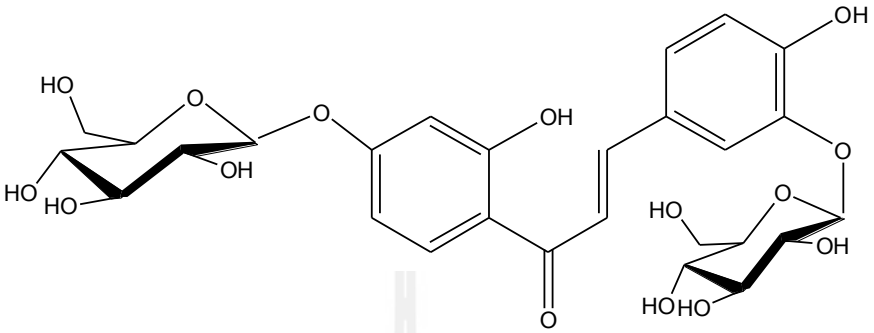


Coumestrol (**21**)

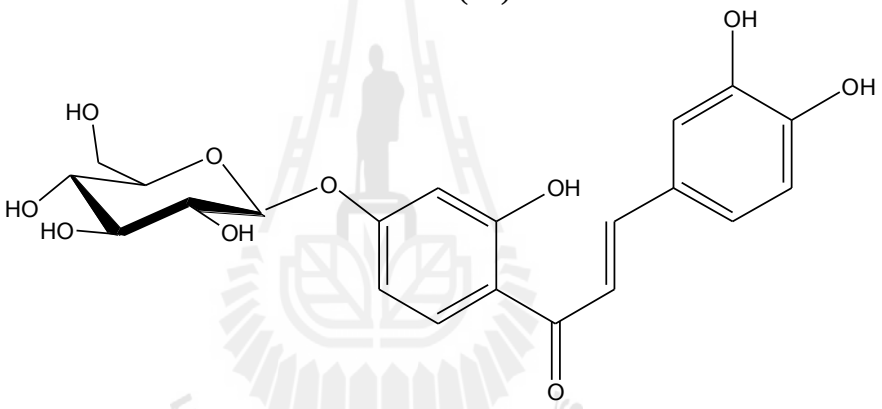


Butrin (**22**)

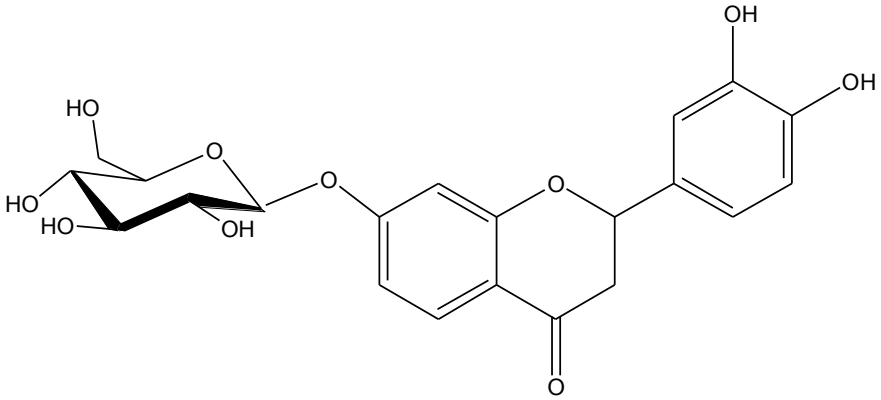
Figure 6 (Continued).



Isobutrin (2**3**)

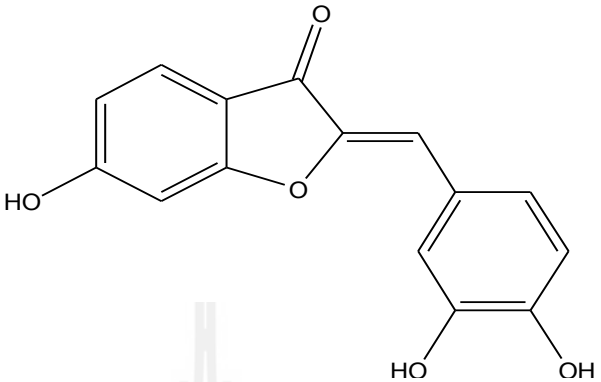


Coreopsin (**24**)

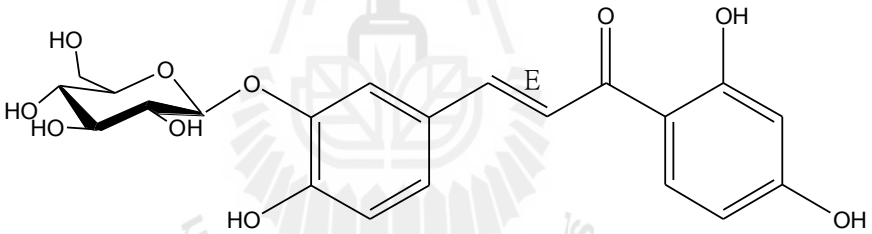


Isocoreopsin (**25)**

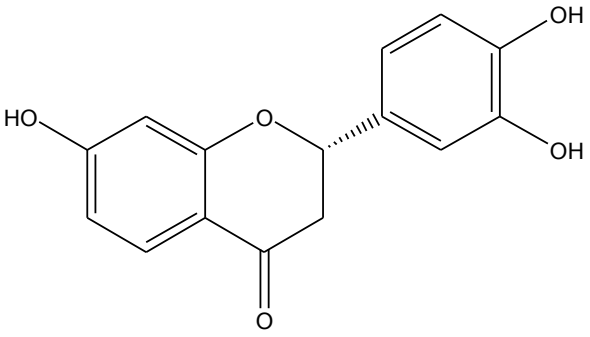
Figure 6 (Continued).



Sulfurein (**26**)

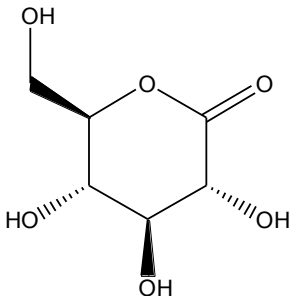


Monospermoside (**27**)

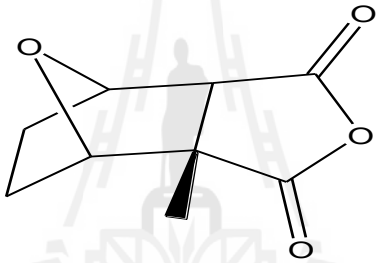


Butin (**28**)

Figure 6 (Continued).



δ-Lactone (**29**)



Palasonin (**30**)

Figure 6 (Continued).

2.1.3 Phamacological of *Butea superba* Roxb.

The effect of *Butea superba* Roxb. on erectile dysfunction (ED) in Thai males has been reported that there was a significant upgrading in 4 of the 5 descriptive evaluations of the IIEF-5 questionnaire. Estimation of the sexual record indicated that 82.4% of the patients exhibited noticeable improvement. Haematology and blood chemistry analysis revealed no apparent change. The plant preparation appeared to improve the erectile function in ED patients without apparent toxicity (Cherdshewasart and Nimsakul, 2003).

Differential anti-proliferation effect of white (*Pueraria mirifica*), red (*Butea superba* Roxb.) and black (*Mucuna collettii*) Kwao Kreu plant extracts on the growth of MCF-7 cells was evaluated after 4 days of incubation. The results demonstrated that only *Pueraria mirifica* showed an estrogenic effect on MCF-7 cell growth and a clear antagonistic effect with E_2 at high concentration. *Butea superba* Roxb. and *Mucuna collettii* exhibited only anti-proliferation effects on the growth of MCF-7 cells in relation with a possible anti-estrogen mechanism or a potent cytotoxic effect (Cherdshewasart et al., 2004). Also, the results of the effect of red Kwao Kreu on male rats showed that the relative body weights of all rat were increased and there was no change on the gross anatomy and histology of heart, kidney, and adrenal gland compared with the control groups (Manasatean, 2001).

More results of red Kwao Kreu on reproductive behavior and erection in male albino rat showed that the weight of the rats from the group given 0.25 mg/ml/time/day of powder and ethanolic extract of red Kwao Kreu were decreased significantly when compared with the control group. While the weight and the sperm count from the group given 0.5 and 5 mg/ml/time/day of powder were increased significantly (Pinmongkongul, 2001). Furthermore, the effects of chronic treatment of *Butea superba* Roxb. on sperm motility and concentration in rats and mice in correlation with testicular damage were investigated. The results of long-term treatment with *Butea superba* Roxb. extract revealed that significantly increased in the sperm concentration and delayed the decrease in motility with time. None of signs of sperm abnormalies and testicular damages were observed. This suggested that chronic use of *Butea superba* Roxb. increased the number of sperm, prolonged sperm motility *in vitro* while produced no changed on sperm morphology. Therefore, chronic use of *Butea superba* Roxb. alcoholic extract may be useful in fertilization

(Tocharus et al., 2005). Apart from this, the isolation and structural determination of the chemical constituents of the tuberous roots of *Butea superba* Roxb. plant and their cytotoxic activity have been investigated. The results showed that these chemicals were a carpin, medicarpin and four isoflavones, formononetin, 7,4-dimethoxyisoflavone, prunetin and 7-hydroxy-6,4-dimethoxyisoflavone. The results of formononetin and prunetin showed moderate cytotoxic activity on KB cell lines with IC_{50} values of 37.3±2.5 and 71.1±0.8 respectively and on BC cell lines with IC_{50} values of 32.7±1.5 and 47.3±0.3, respectively (Ngamrojanavanich et al., 2007). What is more, the potential of red Kwao Kreu and comparison of the effect between two 17-α- mzethyltestosterone (MT) dosage regimens in terms of inducing sex reversal were investigated. The results revealed that MT treatments had a comparable effect in terms of male sex ratio, survival rate (SR), feed conversion ratio (FCR) and gain in weight (GW) and were statistically significant as compare to the control. Red Kwao Kreu treatments did not have significant difference with the control and the MT treatments in some cases (Mengumphan et al., 2006).

In addition, the effects of *Butea superba* Roxb. on reproductive systems of rats were studied. The results showed that the percentage weight ratios of relative weights of seminal vesicles and prostate glands were not different from the control, except that the testis of the group powder crude drug suspended fed with 1250 mg/kg was significantly different from the control and the other treatment groups. All treatment groups gave higher sperm numbers than the control group. In addition, the sperm counts of the group fed with 1250 mg/kg showed that about 16% higher than the control group (Manosroi et al., 2006). In the same way, the effects of the tuberous roots powder of *Butea superba* Roxb. (Leguminosae) on blood testosterone, luteinizing hormone (LH) and toxicity in male rats were investigated. The results

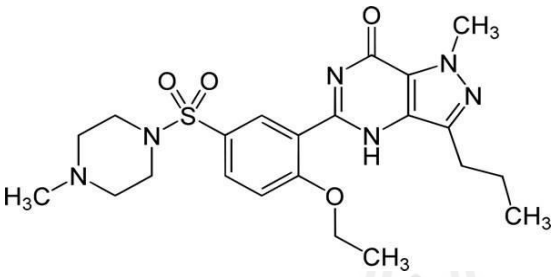
indicated that a plant powder decreased only blood testosterone but did not change luteinizing hormone. This was significantly different from the control in the rats treatment with high doses of plant powder (Cherdshewasart et al., 2008). As well as, the effects of an extract of *Butea superba* Roxb. was compared to sildenafil for treatment erectile dysfunction. The result showed that the patients in the *Butea superba* Roxb. group, 27 (84%) responded positively, compared with 26 (81%) in the sildenafil group.

When assessing the score alone, 12 (38%) had a better score after taking *Butea superba* Roxb. compared to seven (22%) of sildenafil, and eight (25%) had the same score. The results were surprising and could not be repeated in the double-blind part of the study, where no toxicity effect of *Butea superba* Roxb. was recorded (Jeff et al., 2009). Besides, *Butea superba* Roxb. is also well known as Thai female potency herb with anti-estrogenic activities. The result from previous study showed that in intact rats, only *Butea superba* Roxb. with 250 mg/kg BW/day increased the uterine thickness and the number of uterine glands, and could induce a prolonged diestrous phase. In ovariectomized rats, treatment with *Butea superba* Roxb. 50 and 250 mg/kg BW/day exhibited that its increased the uterine thickness and the number of uterine glands in rats (Malaivijitnond et al., 2009).

2.2 Sildenafil (Viagra®)

Erectile dysfunction (ED) is the inability to maintain penile erection for the successful performance of sexual activity that is caused many physical and psychological factors, including vascular disease, diabetes, medications, depression, and sequelae to prostatic surgery. Previous therapies have included penile implants, intrapenile injections of alprostadil. However, because of their efficacies, ease of use,

and safety, oral phosphodiesterase (PDE) inhibitors are now considered to be first-line therapy for men with ED. Three PDE-5 inhibitors, sildenafil, vardenafil, and tadalafil, are approved for the treatment of ED (Harvey, 2012).



(a) **(b)**

Figure 7 Chemical structure of sildenafil (a) and photograph of commercialViagra® (b).

Mechanism of penile erection: Sexual stimulation results in smooth muscle relaxation of the corpus cavernosum eansing an increasing the inflow of blood (Figure 8). The mediator of this response is nitric oxide (NO). NO activates guanylyl cyclase, which forms cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. cGMP produces smooth muscle relaxation through a reduction in the intracellular Ca^{2+} concentration. The duration of action of cyclic nucleotides is controlled by the action of PDE. Sildenafil, inhibit PDE-5, the isozyme responsible for degradation of cGMP in the corpus cavernosum. The action of PDE-5 inhibitors is to increase the flow of blood into the corpus cavernosum at any given level of sexual stimulation (Figure 9). At recommended doses, PDE-5 inhibitors have no effect in the absence of

sexual stimulation. PDE-5 inhibitors are indicated for the treatment of ED due to organic or psychogenic causes (Harvey, 2012).

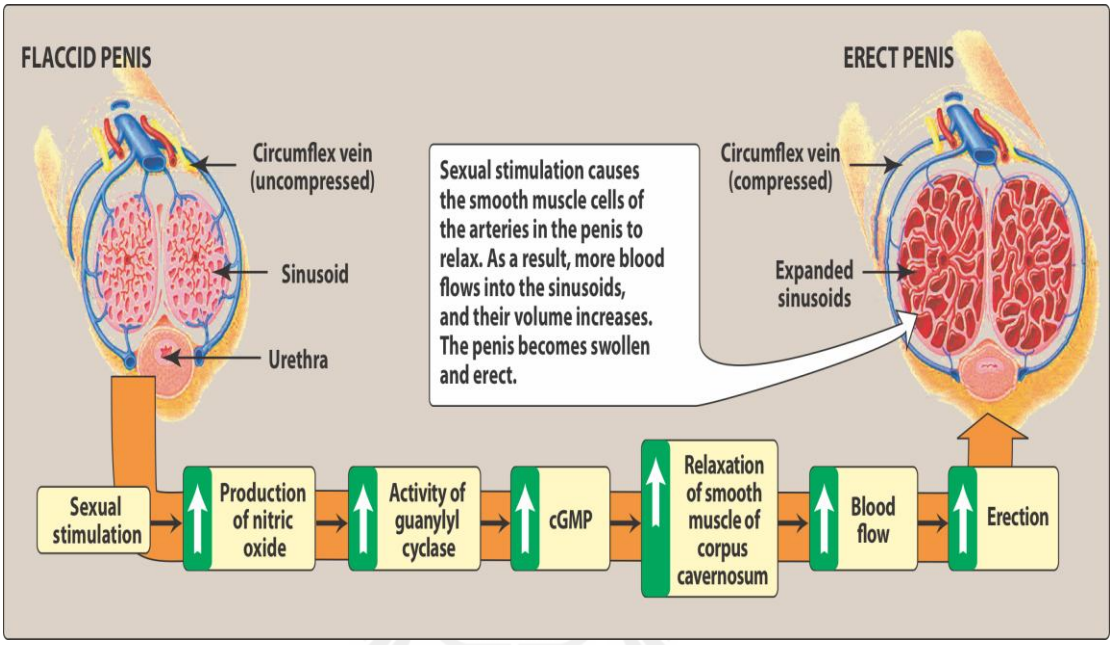


Figure 8 Mechanism of penile erection. cGMP = cyclic guanosine monophosphate (Harvey et al., 2012).

Pharmacokinetics: Sildenafil should be taken approximately 1 hour prior to anticipated sexual activity, with erectile enhancement observed up to 4 hours after administration. Thus, administration of sildenafil must be timed so that sexual activity occurs within 1 to 4 hours. The absorption of this drug is delayed by consumption of food, particularly high-fat meals. Sildenafil is metabolized by the cytochrome P450 3A4 (CYP3A4) enzyme. Dosage adjustments are recommended in patients with hepatic dysfunction (Harvey, 2012).

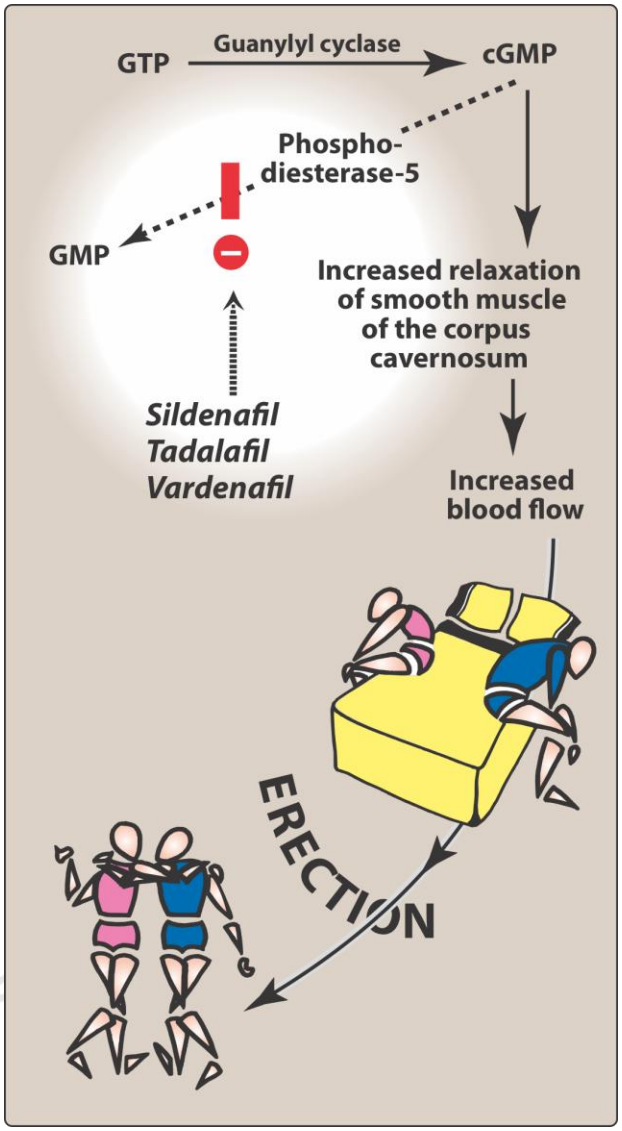


Figure 9 Effect of phosphodiesterase inhibitors on cyclic guanosine monophosphate (cGMP) levels in the smooth muscle of the corpus cavernosum. GTP = guanosine triphosphate (Harvey et al., 2012).

Adverse effects: The most frequent adverse effects reported for sildenafil is headache, flushing, dyspepsia, and nasal congestion. These effects are generally mild, and men with ED rarely discontinue treatment because of side effects. Disturbances in color vision (loss of blue/green discrimination) occur with sildenafil, probably

because of inhibition of PDE-6 (a PDE found in the retina that is important in color vision). The incidence of these reactions appears to be dose dependent. Because there is an inherent cardiac risk associated with sexual activity, PDE-5 inhibitors should be used with caution in patients with a history of cardiovascular disease (CVD) or those with strong risk factors for CVD. PDE-5 inhibitors should not be used more than once per day (Harvey, 2012). More serious adverse effects of sildenafil include myocardial infarction, when used alone or with nitrates, hypertrophic subaortic stenosis obstruction, priapism, and optic ischemia (Flomenbaum, 2006).

Drug interactions: Because of the ability of PDE inhibitors to potentiate the activity of NO, administration of these agents in patients taking any form of organic nitrates is contraindicated. PDE-5 inhibitors may produce additive blood pressure lowering effects when used in patients taking α-adrenergic antagonists (used to alleviate symptoms associated with benign prostatic hyperplasia) (Harvey, 2012).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials chemicals and equipments for extraction and isolation of *Butea superba* Roxb.

3.1.1 *Butea superba* Roxb. were

Butea superba Roxb. were collected from Chiang Rai province in the north of Thailand.

3.1.2 Solvents

The organic solvents used for extraction and as eluent for thin-layer and column chromatography were commercial grade and distilled before use. Other chemicals were analytical and HPLC grade as listed below:

Ethyl acetate	Carlo Erba
Hexane	Carlo Erba
Methanol	Carlo Erba
Dichloromethane	Carlo Erba
Chloroform	Carlo Erba
Acetone	Carlo Erba
Acetonnitrile	Carlo Erba
Acetone-d_6	Aldrich
Chloroform-d_1	Aldrich
Methanol-d_4	Aldrich

3.1.3 Equipments

Soxhlet apparatus, rotary evaporator (Buchi B-850), mixer (Buchi Model 5000), thimble, hot air oven, column chromatography, filter paper, silica gel, cotton, HPLC with column and analysis was used at F1 at Suranaree University of Technology.

3.1.4 Silica Gel

3.1.4.1 Merck silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for normal column chromatography.

3.1.4.2 Merck silica gel 60 G Art. 7731 and 60 GF_{254} Art. 7730 was applied as adsorbent for preparative TLC.

3.1.4.3 Merck TLC aluminum sheet, silica gel 60 F_{254} precoated 20 cm x 20 cm in size with layer thickness of 0.2 mm was used to identify the identical fractions.

3.1.5 Instrumentation

Rotary evaporator	Büchi
Heating bath: Büchi heating bath B-490	
Rotavapor: Büchi rotavapor R-200	
Controller: Büchi vacuum controller V-800	
UV/VIS Spectrophometer series CARY 1E	Varian
UV-Cabinet II	Camag
HPLC (High Performance Liquid Chromatography)	Agilent

3.2 Methods

3.2.1 Plant materials and preparation of extracts

Fresh tuberous root of *Butea superba* Roxb. were collected from Chiang Rai province, Thailand. The plant specimen were authenticated by the Forest Herbarium, National Park, Wildlife, and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand and compared to BCU 11046 at the herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University. The tuberous roots were washed thoroughly and dried in an oven at 50 ^{o}C and the dried samples were ground to powder.

3.2.2 Extraction

Dried powdered tuber roots of *Butea superba* Roxb. (25 kg) was extracted continuously with ethanol by Soxhlet extraction for 12 hours. The extracted solutions were then filtered through filter paper (Whatman No. 1). The filtrates were concentrated to remove solvent by evaporation under reduced pressure on a rotary evaporator leaving 375 g of syrupy dark brown gum. The ethanol extract (375 g) was separated by silica gel column chromatography. The column was eluted sequentially with hexane, chloroform, acetone, and methanol. All fractions were concentrated to give hexane crude extract (60 g), chloroform crude extract (71.5 g), acetone crude extract (97.8 g), and methanol crude extract (135.7 g). The extraction sequence is shown in Figure 10 and Scheme 3.1.

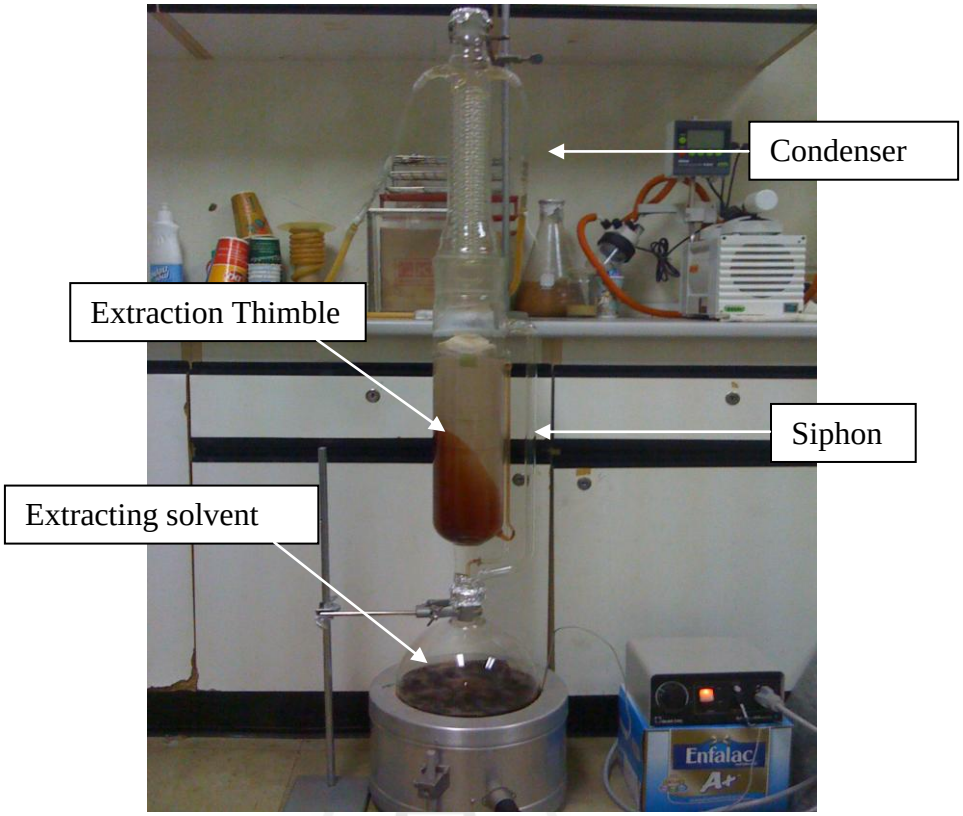


Figure 10 Soxhlet extraction apparatus.

3.3 Chromatography techniques

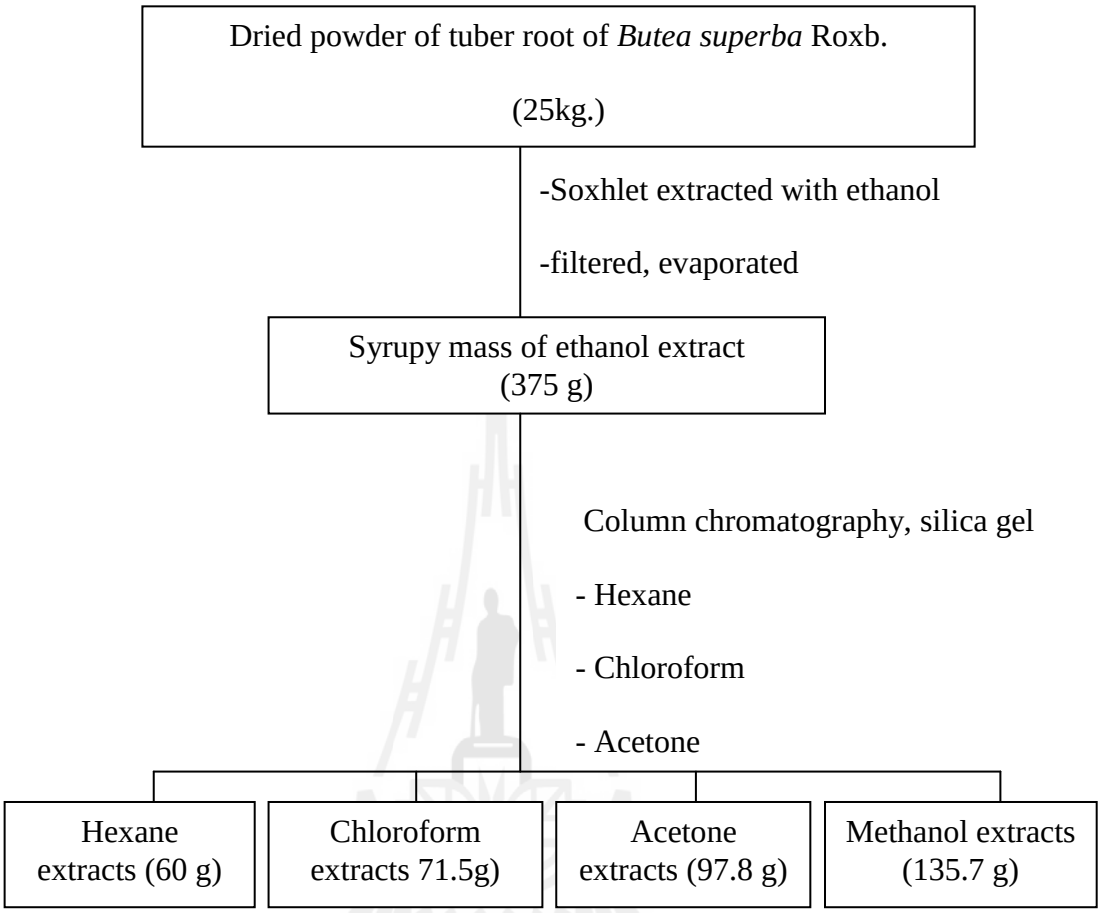
The separation of the components of a mixture by chromatography involves a stationary phase (chromatographic support) through which a mobile phase (chromatographic solvent) is flowing. When a mixture is applied to the stationary phase and allowed to flow with the mobile phase, the various components of the mixture move at different relative rates. These differences in rates of movement are a function of their relative affinities for the stationary phase and the mobile phase. For instance, if substance A has a high affinity for the stationary phase and a low affinity for the mobile phase, it will move slowly, or not at all, as the mobile phase flows over the stationary phase. Conversely, if substance B has high affinity for the mobile phase but a low affinity for the stationary phase, it will move rapidly (Wade, 1987).

3.3.1 Thin-Layer Chromatography (TLC)

TLC is an easy, quick, and inexpensive procedure that gives the scientist a quick answer for how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture. It involves the use of particulate solid adsorbent (usually silica or alumina) coated on glass, metal, or plastic as a stationary phase. A small amount of the mixture to be analyzed was spotted near the bottom of plate. The TLC plate was then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate was in the liquid. This liquid, or the eluent, was the mobile phase, and it slowly rose up the TLC plate. For visualizing the spots, viewed the TLC plate under UV light (254 nm and 366 nm) and the compounds was appeared as dark spots.

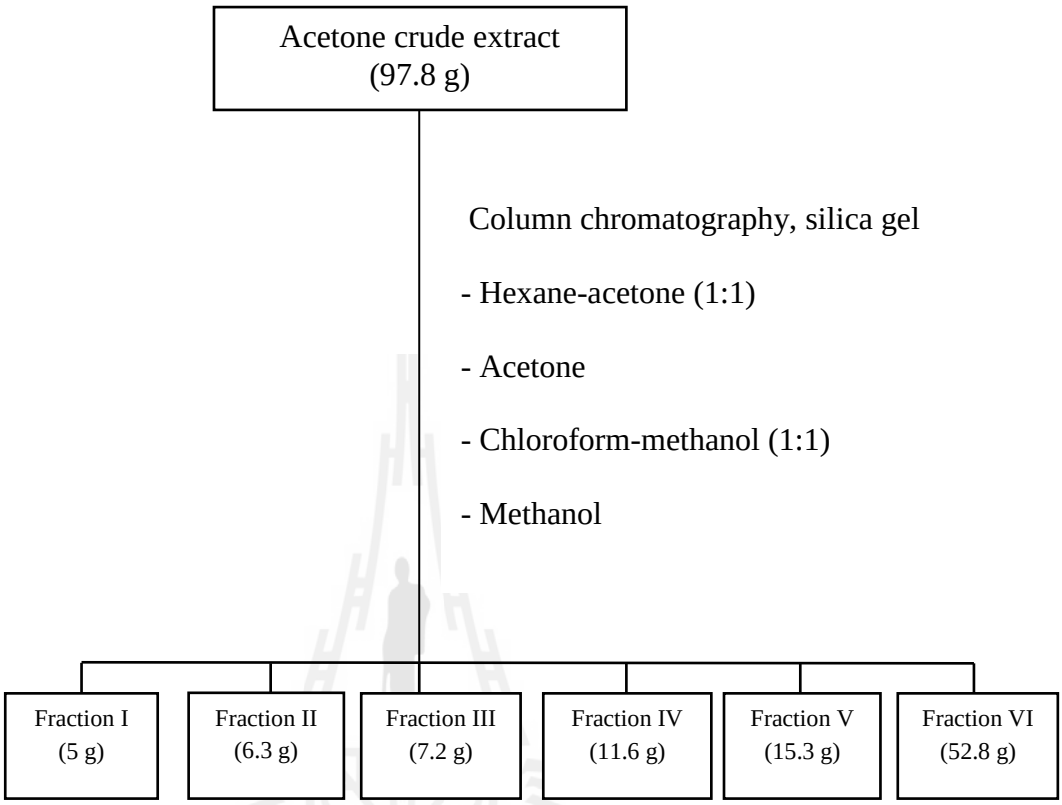
3.3.2 Column chromatography

In column chromatography, the stationary phase, a solid adsorbent, was placed in a vertical glass column and the mobile phase, a liquid, was added to the top and flowed down through the column. Column chromatography is generally used as a purification technique: it isolates desired compounds from a mixture. The mixture to be analyzed by column chromatrography was applied to the top of the column. The eluent was passed through the column by gravity or by the application of air pressure. Equilibrium was established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they was out with the mobile phase to varying degrees and a separation was achieved.



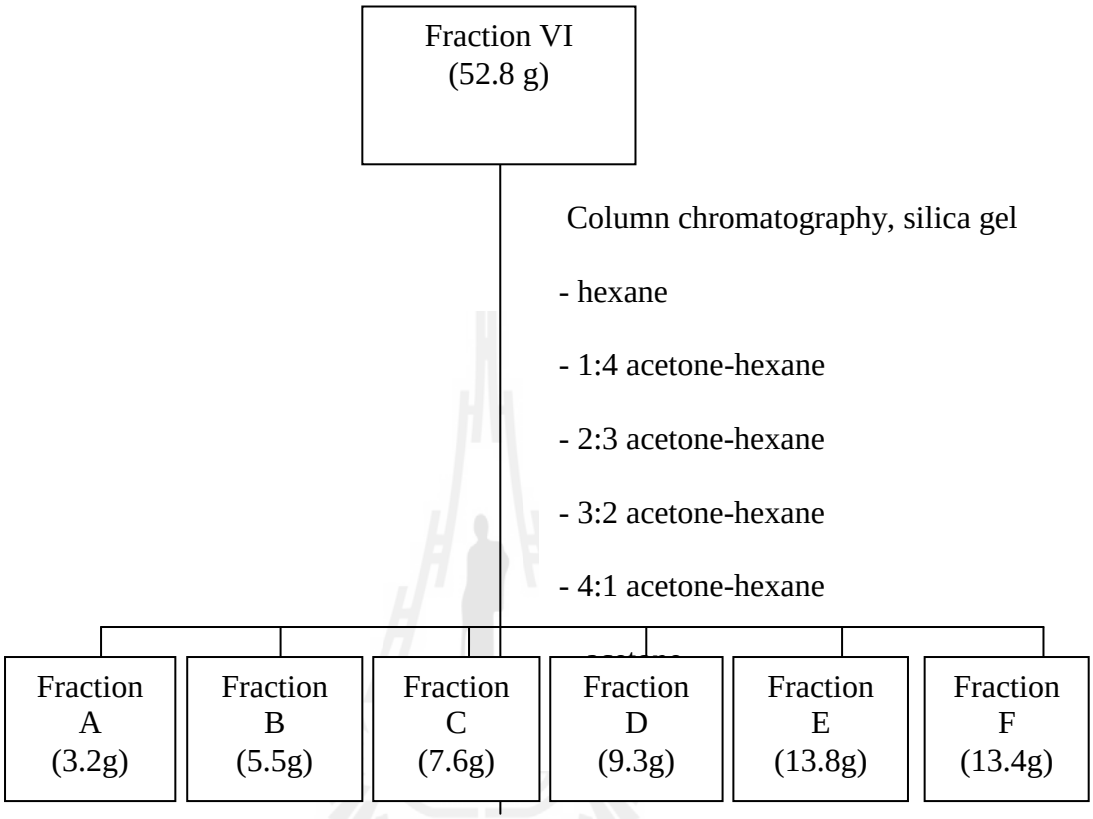
Scheme 3.1 The extraction of the tuber roots of *Butea superba* Roxb.

The acetone crude extract was subjected to silica gel column chromatography. The column was eluted successively with hexane-acetone (1:1), acetone, chloroform-methanol (1:1), and methanol. Every fraction of 1000 mL was collected and concentrated to a small volume and four major fractions (I 5.86 g, II 7.13 g, III 10.46 g and IV 6.97 g) were separated by monitoring with TLC (2 cm x 5 cm in size with chloroform:methanol, 9:1 as developing solvent) in order to combine the fractions which had the same compounds. The separation sequence is shown in Scheme 3.2.



Scheme 3.2 The separation of acetone crude extract.

A portion of fraction VI (52.8 g) was chromatographed on silica gel 60 column. The column was eluted successively with hexane, 1:4 hexane-acetone, 2:3 hexane-acetone, 3:2 hexane-acetone, 4:1 hexane-acetone, and acetone respectively. Every fraction of 500 mL was collected and concentrated to a small volume and six fractions were separated by monitoring with TLC (2cm x 5cm in size with hexane:acetone, 1:1 as developing solvent) in order to combine the fractions which had the same compounds. In all fraction carry to freezdry powder. The separation sequence is shown in **Scheme 3.3**



Scheme 3.3 The separation of fraction I from acetone crude extract.

3.4 Quantitative HPLC

Methods for isoflavonoid analysis were modified from those previously described by setting the linear gradient system for 50 min from 100:0 to 55:45 with 1.5 % acetic acid:acetonitrile, with the flow rate of 1ml/min for 45 min and analyzed at the wavelength of 254 nm. The standard isoflavonoids (Genistein and Biochanin A) were serially diluted from 1:1 to 1:16 with methanol to establish the concentrations of ¼, 1/8, 1/16, 1/32 and 1/64 mg/ml, to generate a five point calibration curve. Calibration curves were obtained for all isoflavonoids by plotting and the standard concentrations of standard were chosen to cover the range of isoflavonoid

concentrations in the samples. The analyses of the samples were run in triplicate and identified by comparing the retention times and quantified for the amount using standard curves of peak area of the isoflavonoid standards (Cherdshewasart et al., 2007).

3.5 Bioactivity assay

3.5.1 Experimental animals

Sixty adult male mice, aged about 130 days, weighing 30-40 g, were obtained from the Animal Care Building, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The experimental protocol was approved in accordance with guideline for the care and use of laboratory animal by animal care and use committee (ACUC), Suranaree University of Technology. The animals were housed at room temperature (25 ± 0.5 ºC) on a reverse day-night cycle (06:00 AM to 06:00 PM).

3.5.2 Experimental procedures

Mice were divided into 6 groups with 10 animals each. Before treatment (Pre-treatment), blood was drawn from tails of all mice and sperms were abdominal surgery for comparison with after treatment (Post-treatment). All mice were cardiac puncture in blood. During treatment period, the first group was fed with 0.5 ml of distilled water and used as negative control. The second group was fed with *Butea superba* Roxb. crude extract at the dose of 1,250 mg/kg BW/day. The third group was intraperitoneally injected with sildenafil at the dose of 10 mg/kg BW/day. The forth group was fed with fraction B at 40 mg/kg BW/day in distilled water. The fifth group was fed with fraction C at 50 mg/kg BW/day in distilled water. The six group was

given fraction E at 150 mg/kg BW/day in distilled water. The experiment was performed throughout 14 consecutive days. At the end of treatment period, all mice in these groups were collected blood and sperms again for comparison with pre-treatment in each mouse.

3.5.3 Sperm motility, sperm count and morphology assay

Sperm motility was done according to the method of Bavister and Andrews (1988).

$$\text{Motility (\%)} = \frac{\text{Number of motility}}{\text{Number of all}} \times 100$$

The cauda epididymis was cut and weighed. A cell suspension was prepared by macerating the cauda in 1.0 ml of 0.85% saline. The cell suspension was kept for 24 hrs at 4 ºC. The suspension was then filtered through a double gauze layer and an aliquot of the sample was used for sperm count in a Makler counting chamber (Figure 11). An aliquot of the epididymal sperm suspension was smeared and stained with hematoxylin and eosin and then examined under a light microscope (CH-2, Olympus, Japan) at magnification of 100X. The head and tail abnormalities (200 sperms per animal) were recorded.

Figure 11. Makler counting chamber.

3.5.4 Haematology and blood chemistry

At the end of the experiment, blood samples were collected by cardiac puncture under ether anesthesia from 9.00 to 10.00 A.M. and partly used for haematology. The remainder blood serum was prepared by centrifugation at 1000×*g* for 30 min and kept at −20 ºC for blood chemistry and cholesterol analysis (Cherdshewasart et al., 2008).

3.5.5 Histology of testis and testosterone level determination

After 14 days, the mice were sacrificed under thiopental sodium anesthesia and subjected to necropsy. The heart, liver, spleen, kidney, and stomach were removed and weighed. Body weight measured on the day of necropsy was used to calculate the relative organ weight. All organs were preserved in 10% (w/v) neutral phosphate buffer formaldehyde. Heart, liver, spleen, kidney, stomach and the reproductive organs (testis, seminal vesicle and prostate glands) fixed-tissue were embedded in paraffin and prepared for microtome sectioning at 5 µm and hematoxylin and eosin were used for staining. The histopathology of the organ tissue slides were examined under light microscope. Concentrations of mice testosterone were measured by radioimmunoassay techniques using reagents obtained from the National Hormone and Pituitary Program (Sharma et al., 2013).

3.5.6 Body weight

The animal's weight was recorded every day throughout the experimental period.

3.5.7 Statistical analysis

All data are presented as the mean ± S.E.M. Significant differences between the relative selected organ weight and body weight of control and treatment groups were analyzed by ANOVA. The difference of haematology, blood chemistry, growth rate and sperm analysis between pre- and post- treatment groups were calculated by paired student's *t-test*. Then, significant difference between each group was compared using ANCOVA. The Tukey HSD post hoc test at $p<0.05$ and $p<0.01$ were also considered statistically significant difference between each group.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Calibration curve of standard isoflavonoids

Calibration curve of standard isoflavonoids were obtained for all standard isoflavonoids with high linearty, R^2 = 0.995, by the established HPLC analysis for isoflavonoids of *Butea superba* Roxb. (Figure 12). In this study, with a limit quantitation of 0.5 mg/100g, could demonstrate the difference of isoflavonoids among of *Butea superba* Roxb. It could be of practical use to screen for *Butea superba* Roxb. with high isoflavonoids content.

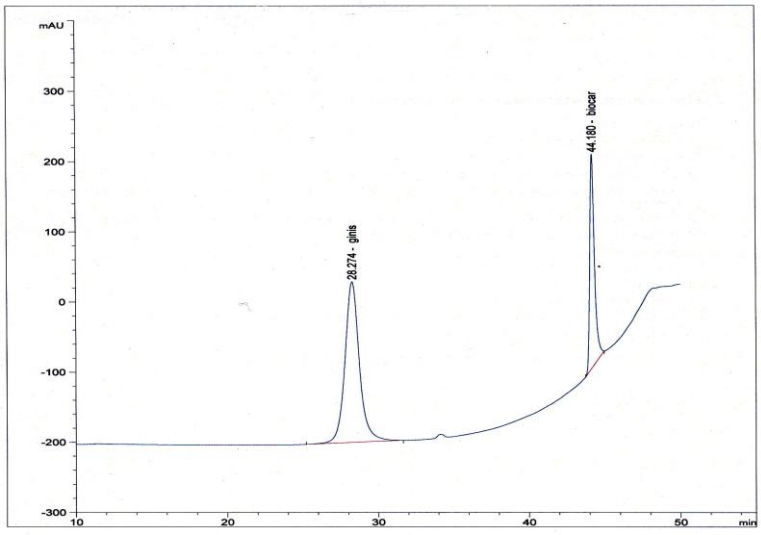


Figure 12 HPLC of isolated flavonoids from the *Butea superba* Roxb. showing the standard of genistein and biochanin A.

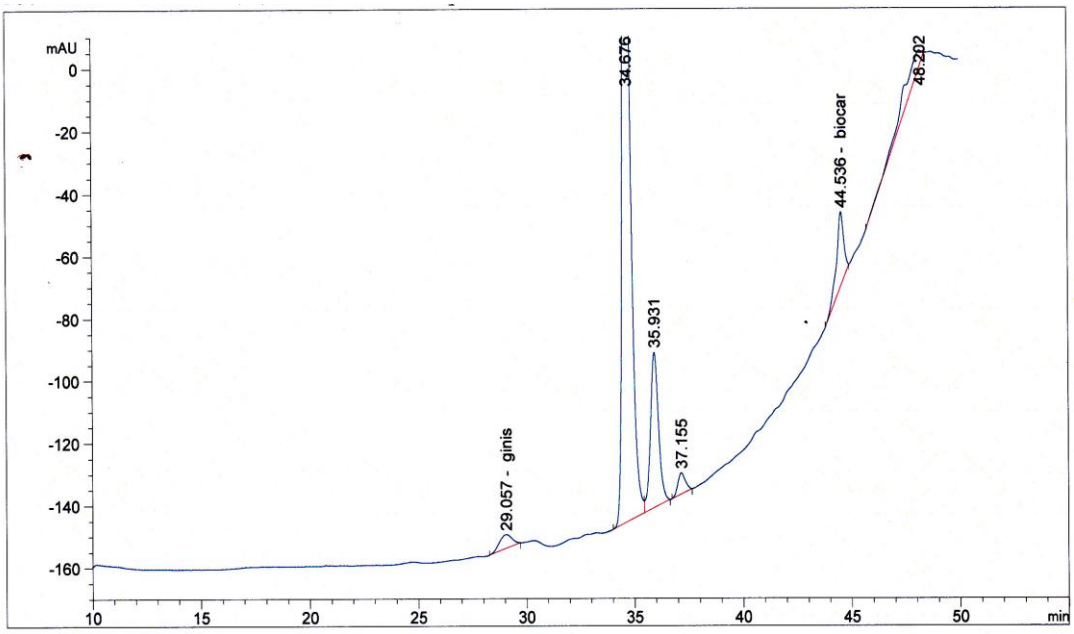


Figure 13 HPLC of isolated flavonoids from the *Butea superba* Roxb. showing the crude extract of *Butea superba* Roxb.

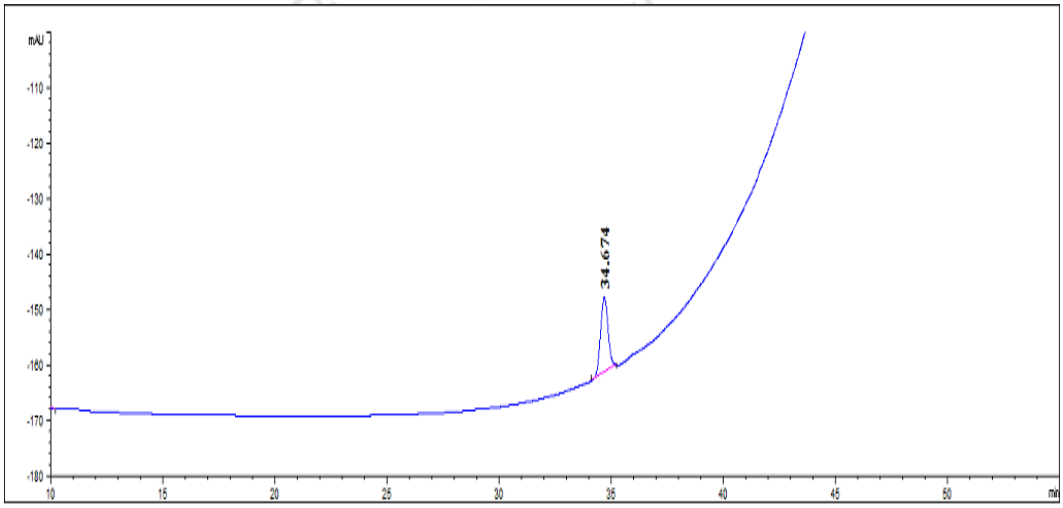


Figure 14 HPLC of isolated flavonoids from the *Butea superba* Roxb. Fraction B.

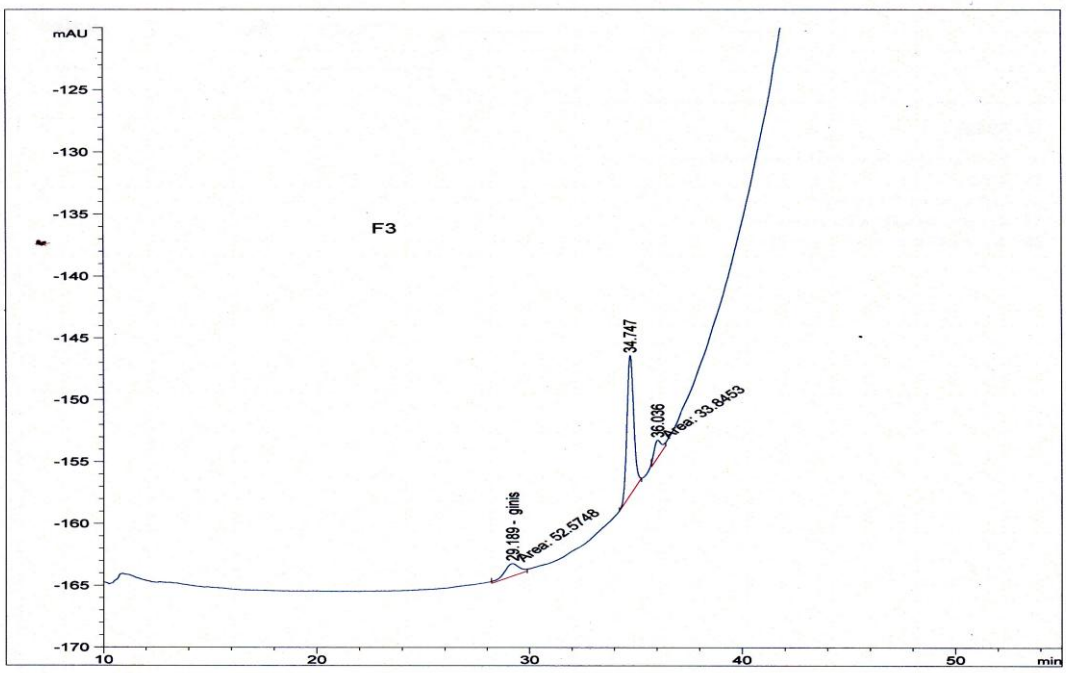


Figure 15 HPLC of isolated flavonoids from the *Butea superba* Roxb Fraction C.

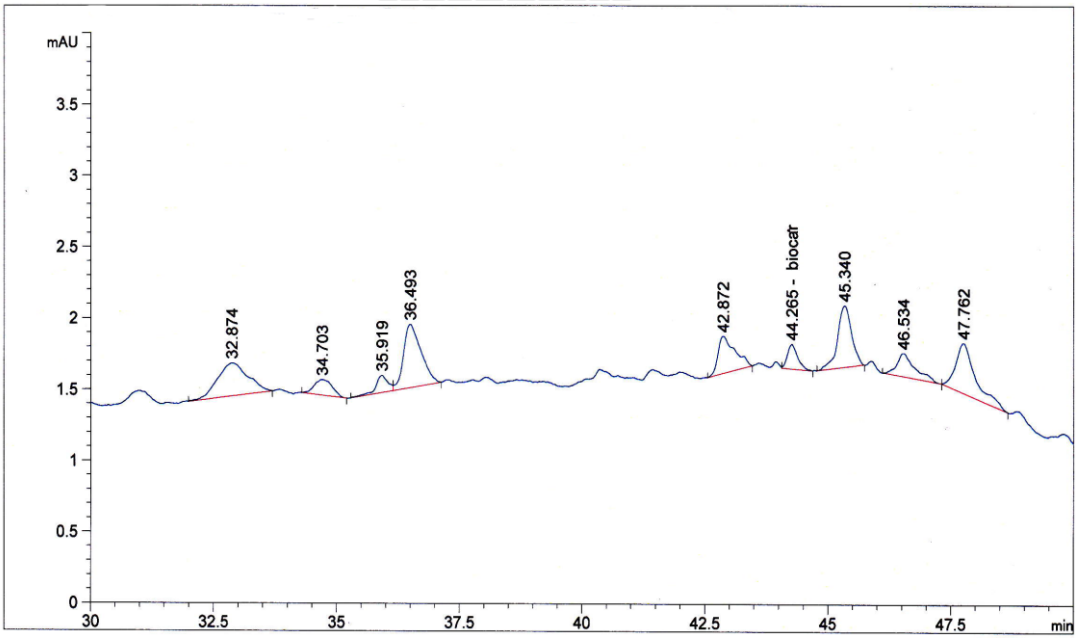
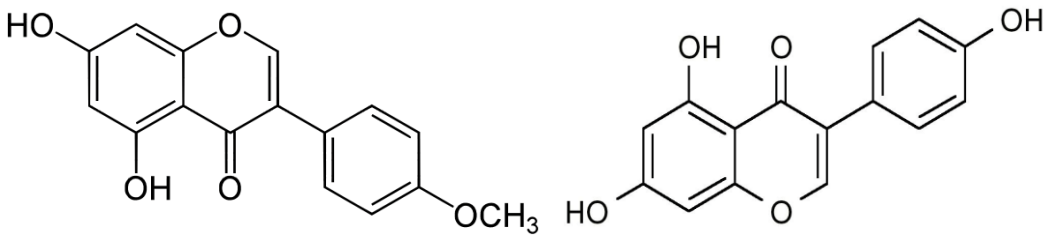


Figure 16 HPLC of isolated flavonoids from the *Butea superba* Roxb. Fraction E.

4.1.1 The quality and quantity of crude extract and each fraction from *Butea superba*

The crude extract and each fraction that orally fed into mice were analyzed. The quality and quantity were revealed as follows; The 375 g. of crude extract was isolated from 25 kg of the dried powder of the tuber roots of *Butea superba* Roxb. The percent yield of 1.5% (w/w) from dried powder was calculated. The crude extract (Cru) in distilled water at the dose of 1,250 mg /kg BW/day consisted of 53.57μg Genistein, 312.95 μg Biochanin A, and (Un1) 396.59 μg unknown compound 1. Fraction B was isolated from 375 g of sequentially elution of crude extract. The percent yield of 0.022% (w/w) from dried powder was obtained. Fractions B (FrB) at the dose of dose 40 mg/Kg BW/day consisted of unknown compound 1 (Un1) 1,260.00 μg. Fraction C was also isolated from 375 g of sequentially elution of crude extract. The percent yield of 0.030% (w/w) from dried powder was got. Fraction C (FrC) at the dose of 50 mg/Kg BW/day consisted of Genistein 6.78 μg plus unknown compound 1 (Un1) 31.59 μg. In addition, Fraction E was isolated from the same amount of sequentially elution of crude extract. The percent yield of 0.054% (w/w) from dried powder was happened. Fraction E (FrE) at the dose 150 mg/Kg BW/day consisted of Biochanin A 66.92 μg that was the main compound plus 9 unkown compounds. The compound structures isolated from *Butea superba* Roxb. are shown in figure 17. The unknown compound 1 that contained in fraction B was appeared at retention time about 34.674 minutes (Figure 16). It will be elucidated by GC-MS later.



Biochanin A **Genistein**

Figure 17 Compound structures isolated from *Butea superba* Roxb.

4.2 Sperm motility and sperm count assay

4.2.1 Sperm motility

The effects of BS extracts and sildenafil on sperm motility (%) of mice are presented in figure 18. The results exhibited that there were significant increase in sperm motility of all of post-treated groups compared to pre-treatment ($p<0.05$) except for control. Apart from this, the highest motility level was found in the fraction C and E compared to others including sildenafil ($p<0.01$). Moreover, the sperm motility of fraction B treated groups was significantly higher than the sildenafil and control groups ($p<0.05$). These findings provide evidence that Genistein, Un1 and Biochanin A may play an important role in increase sperm motility.

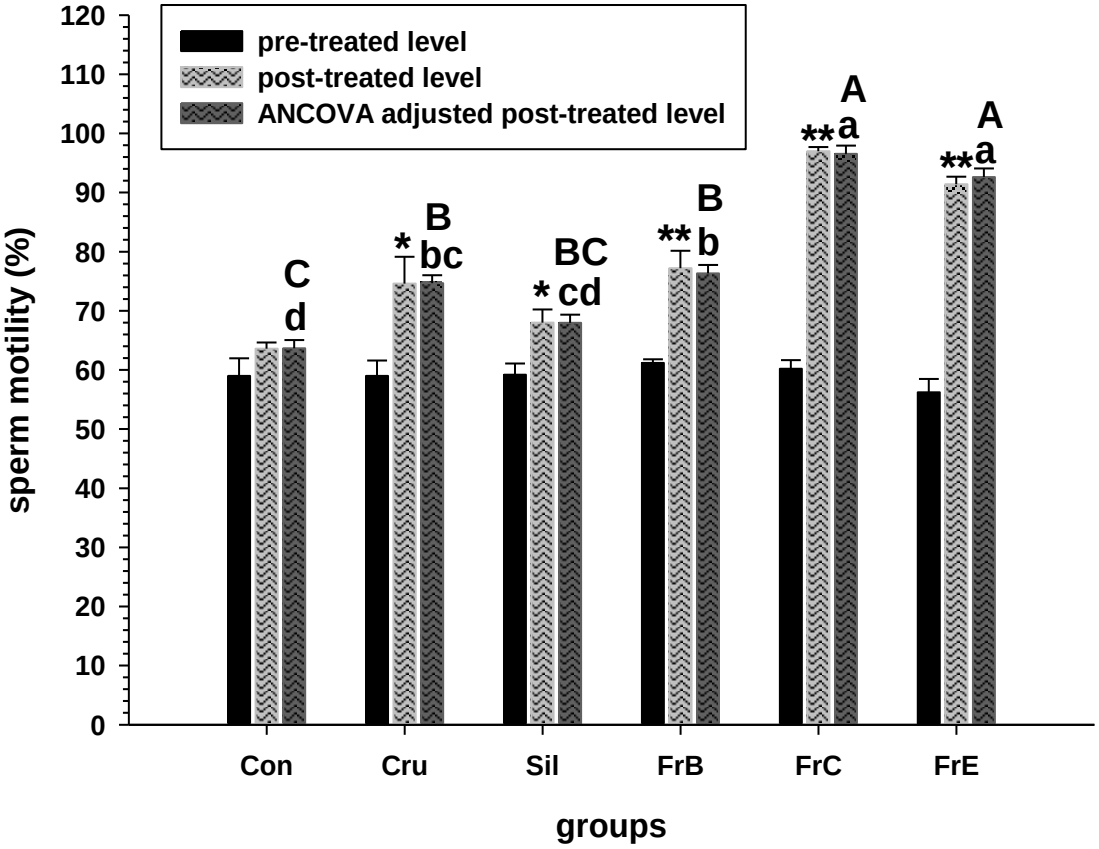


Figure 18 Effects of BS extracts and Sildenafil on sperm motility (%) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

4.2.2 Sperm count and morphology

Figure 19 shows the effects of BS extracts and sildenafil on sperm number (x100,000 n/ml) of mice. The results exhibited that there were significant increase in sperm number of all post-treated groups compared to pre-treatment (p<0.01) except for control. Also, fraction C showed the highest sperm number compared with others. Besides, the sperm number of crude extract, sildenafil, fraction B and E treated groups were significantly higher than control group (p<0.01).The morphology of these sperms reveal normal appearance compared with control (Figure 20). This result is in substantial agreement with Manosroi et al., (2006) that the sperm counts in male rats fed with 1,250 mg/kg BS crude drug group showed about 16% higher than the control group. In fact, the result of sperm count was almost the same as sperm motility result. These results lead us to believe that both fraction C and E can improve both sperm number and motility.

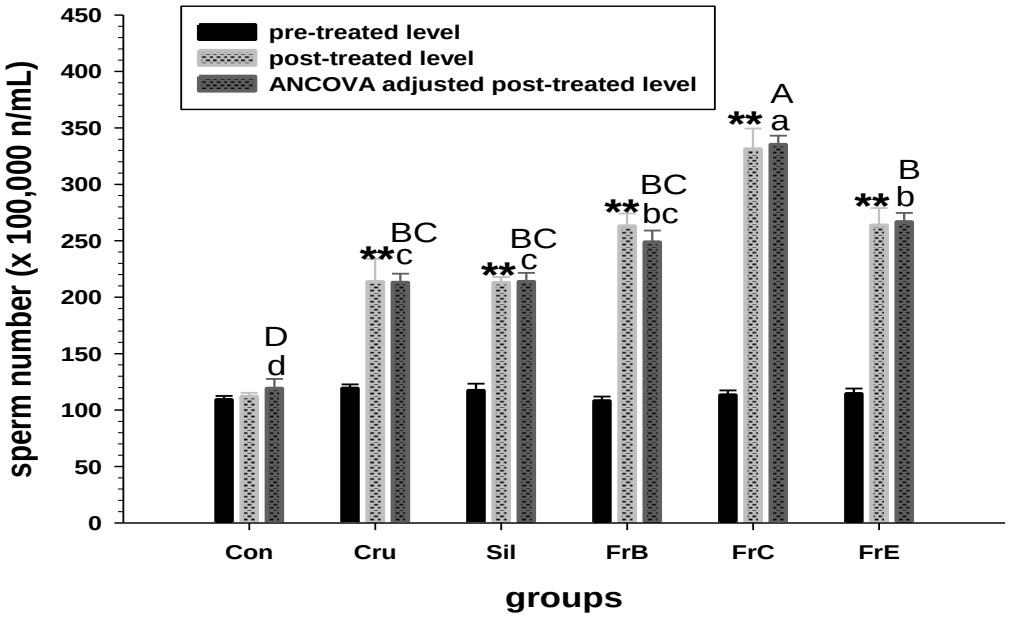


Figure 19 Effects of BS extracts and Sildenafil on sperm number (x100,000 n/ml) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

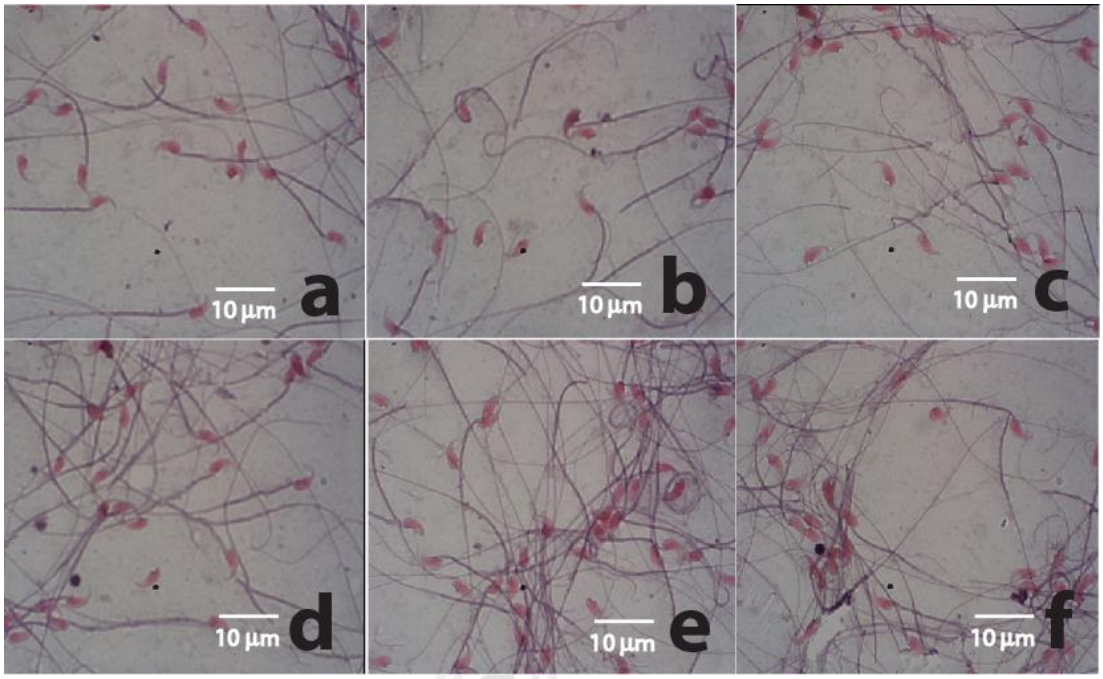


Figure 20 Micrographs of sperm morphology of mice; a = control DW 0.5 ml/kg BW/day, b = crude extract 1,250 mg/kg BW/day, c = Sildenafil 10 mg/kg BW/day, d = fraction B 40 mg/Kg BW/day (Un1 1,260 µg), e = Fraction C 50 mg/Kg BW/day (Genistein 6.78 µg plus Un1 31.59 µg), f = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 µg plus 9 unknown compounds). All groups were treated for 14 days. All micrographs displayed at X100 magnifications. Bar = 10 µm.

4.3 Haematology and blood chemistry

Results of haematology and blood chemistry are showed in table 4.1 and figures 21-24. The cholesterol level of post-treated groups of fraction C, E, B and sildenafil were significantly higher than pre-treated at $p<0.01$, 0.01, 0.05 and 0.05 respectively. The cholesterol level of fraction C, E and sildenafil were significantly higher difference from those of control ($p<0.05$). Whereas, hemoglobin level of sildenafil and crude extract post-treated groups were significantly lower than pre-treated at $p<0.01$ and 0.05 respectively. Although, the hemoglobin level of fraction E

treated group was significantly higher than those of control and crude extract groups (p<0.05). Obviously, Only post-treated sildenafil group exhibited significantly lower hematocrit level than pre-treated (p<0.01). Although, the hematocrit level of all treated groups revealed not significantly different compared in each group including control group (p<0.01). Furthermore, the PMN level of fraction B and C post-treated group were significantly higher than those of pre-treated at p<0.05 and 0.01 respectively. However, these levels were not significant difference from those of control (p<0.01). In the same way, the MCHC level of fraction C post-treated group was significantly higher than pre-treated (p<0.05) whereas not significant from those of control. Apart from this, the MPV level of fraction E, sildenafil and control post-treated group were significantly higher than those of pre-treated at p<0.05 whereas significantly lower in crude extract treated group. In addition, the MPV level of crude extract and sildenafil treated groups showed significantly lower than other treated groups includind control (p<0.05). These results seem inconsistent with Cherdshewasart et al. (2008) that orally administered BS powder suspension at 200 mg/Kg BW/day in male rats did not change cholesterol level compared to control. These findings suggest that these extract compounds from BS and sildenafil at these doses can increase cholesterol level whereas reduce hemoglobin in fraction E. One reason could be that cholesterol, a precursor of testosterone, is prepared to synthesize testosterone (Maqdasy et al., 2013; Midzak et al., 2009).

Table 4.1 Effects of BS extracts and Sildenafil on haematology and blood chemistry of mice. Con = control (5%DW) 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

	Con		Cru		Sil		FrB		FrC		FrE	
Parameter	**Pre-test**	**Pos-test**	**Pre-test**	**Pos-test**	**Pre-test**	**Pos-test**	**Pre-test**	**Pos-test**	**Pre-test**	**Pos-test**	**Pre-test**	**Pos-test**
Cholesterol (mg%)	116.4±6.2	123.8±9.9^{b}	88.4±3.42	134±15.6^{ab}	86.0±6.4	130.8±9.2^{*a}	108±4.04	142.2±13.1^{*ab}	112±17.64	155.2±15.82^{**a}	97.0±8.60	134.8±6.68^{**a}
RBC count (x10^{6} /μL)	8.3±0.2	7.5±0.3	8.1±19	8.1±1.02	9.0±0.3	7.7±0.2	9.23±0.34	8.14±0.10	9.50±0.11	8.71±0.54	9.66±0.10	9.03±0.37
Hemoglobin (g/dL)	14.2±0.5	12.8±0.5^{b,AB}	14.7±0.4	12.8±0.5^{*b,B}	17.4±0.2	14.1±0.2^{**ab,AB}	14.8±0.80	13.8±0.20^{ab,AB}	15.8±0.20	14.4±0.67^{ab,AB}	16.0±0.00	15.4±0.67^{a,A}
Hematocrit (%)	42.0±14	37.8±1.5	44.2±1.0	37.8±2.3	49.8±0.5	42.0±0.8**	45.6±2.15	39.8±0.58	50.2±1.20	44.0±3.86	50.0±0.70	47.2±2.70
WBC count (x10^{3} /μL)	9.3±1.3	7.8±1.3	7.3±0.9	6.9±0.5	8.7±1.3	6.8±1.7	7.34±1.85	6.08±0.78	10.1±0.46	8.80±0.71	12.9±1.56	12.3±2.08
PMN (%)	15.2±4.0	17.6±3.7^{abc,ABC}	11.4±1.7	9.6±1.4^{c,C}	13.8±1.9	15.0±1.4^{ab,AB}	5.40±1.60	13.40±2.42^{*b,ABC}	4.80±0.86	9.40±1.43^{**abc,ABC}	4.00±1.09	9.40±2.11^{abc,ABC}
Lymphocyte (%)	84.6±4.0	84.8±4.3	88.6±1.7	94.2±1.8	85.4±1.9	82.8±4.3	90.2±2.87	84.0±2.70	95.2±0.86	91.2±2.13	95.8±0.96	92.6±3.23
Platelet count (x10^{3} /μL)	821.4±21.7	793±32.5	821.5±21.7	792±32.5	820.4±21.7	792±32.5	636±19	826.6±50.08	912.2±22.71	859.8±17.04	880.6±44.69	806.4±91.1
MCV (fL)	50.0±0.6	50.2±0.3	54.1±0.2	51.2±0.3	52.5±0.6	51.2±0.7	49.1±0.53	48.96±0.65	51.7±0.85	50.5±1.24	50.8±1.02	51.5±0.72
MCH (pg/cell)	17.0±0.05	17.5±0.2	16.6±1.3	17.8±0.3	18.3±0.1	18±0.3	16.2±0.27	16.6±0.18	16.5±0.20	16.88±0.21	16.3±0.30	16.6±0.27
MCHC (g/dL)	34.1±0.09	34.0±0.18^{ab}	31.1±0.6	34.7±0.6^{a}	34.8±0.2	35.1±0.3^{ab}	33.0±0.37	34.0±0.52^{ab}	31.9±0.35	33.6±0.51^{*ab}	32.0±0.90	32.3±0.89^{b}
RDW (%)	23.8±3.0	21.9±2.3	14.6±0.2	18.0±1.3	16.0±0.8	16.8±1.0	21.8±2.48	18.5±0.64	16.6±0.60	17.5±0.79	17.4±0.65	17.1±0.33
MPV (fL)	5.86±0.3	6.96±0.2^{*b,AB}	6.2±0.03	5.8±0.1^{*c,D}	5.2±0.1	5.6±0.1^{*c,CD}	6.82±0.11	7.22±0.24^{b,ABC}	6.60±0.13	6.9±0.40^{b,ABC}	6.48±0.06	7.00±0.10^{*ab,ABC}

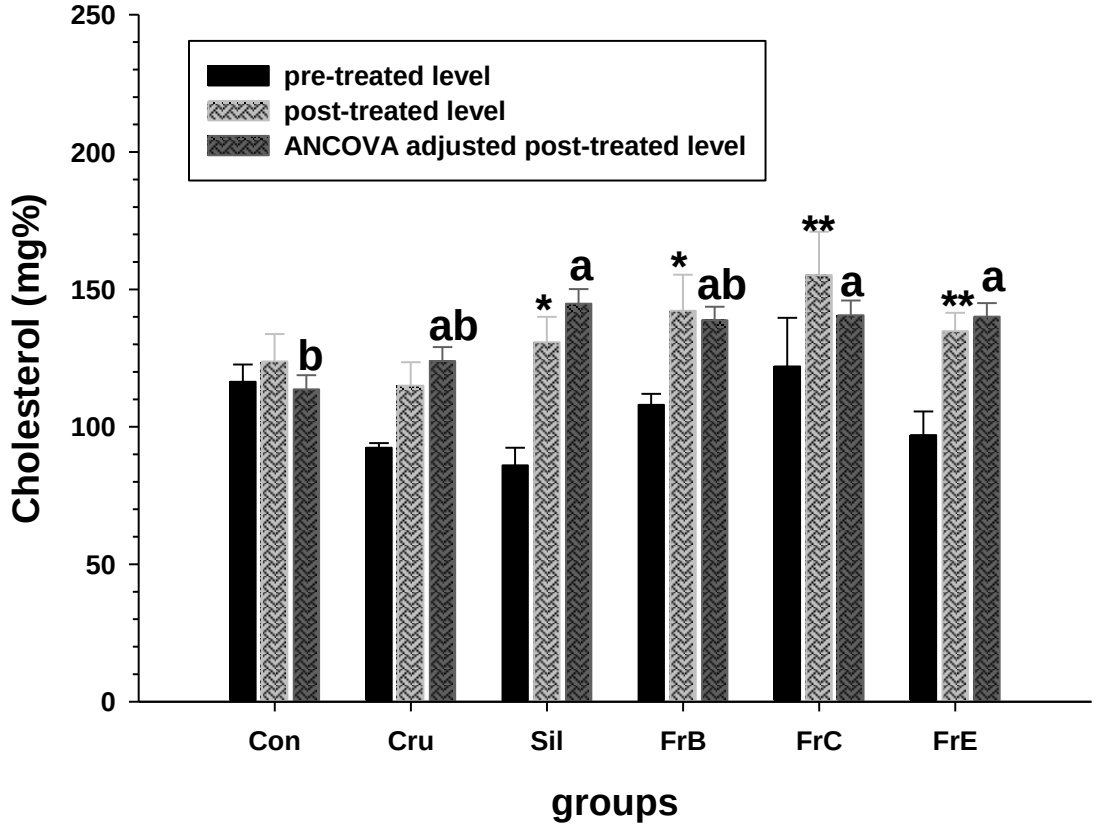


Figure 21 Effects of BS extracts and Sildenafil on cholesterol of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

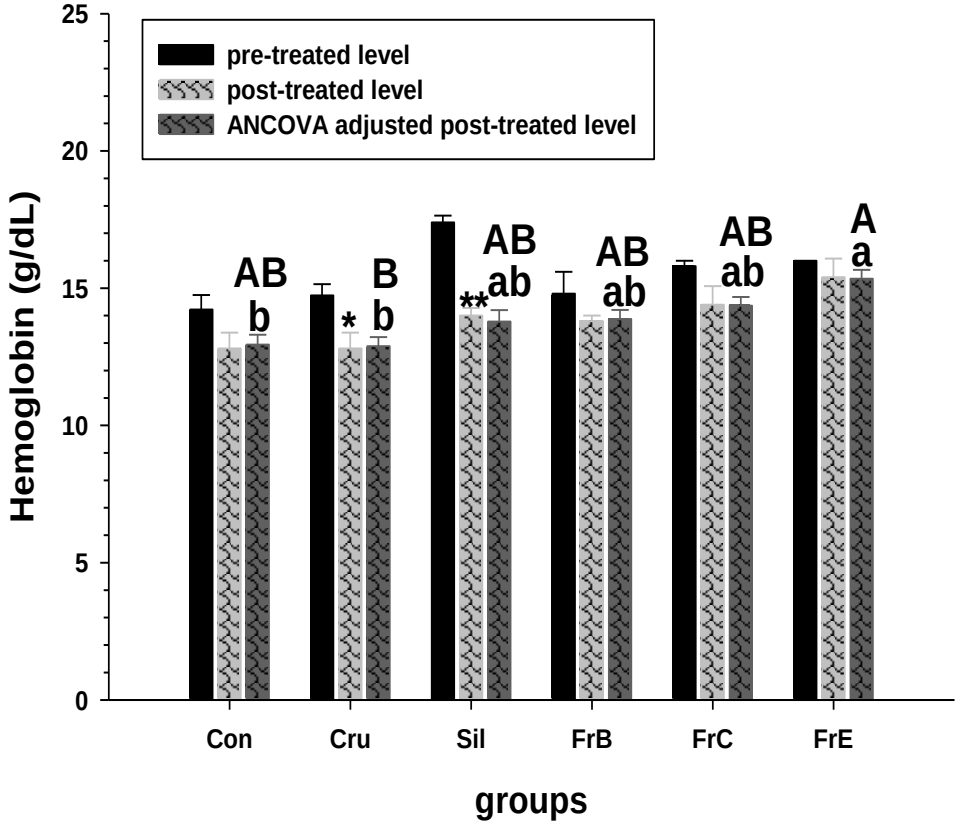


Figure 22 Effects of BS extracts and Sildenafil on hemoglobin (Hb) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

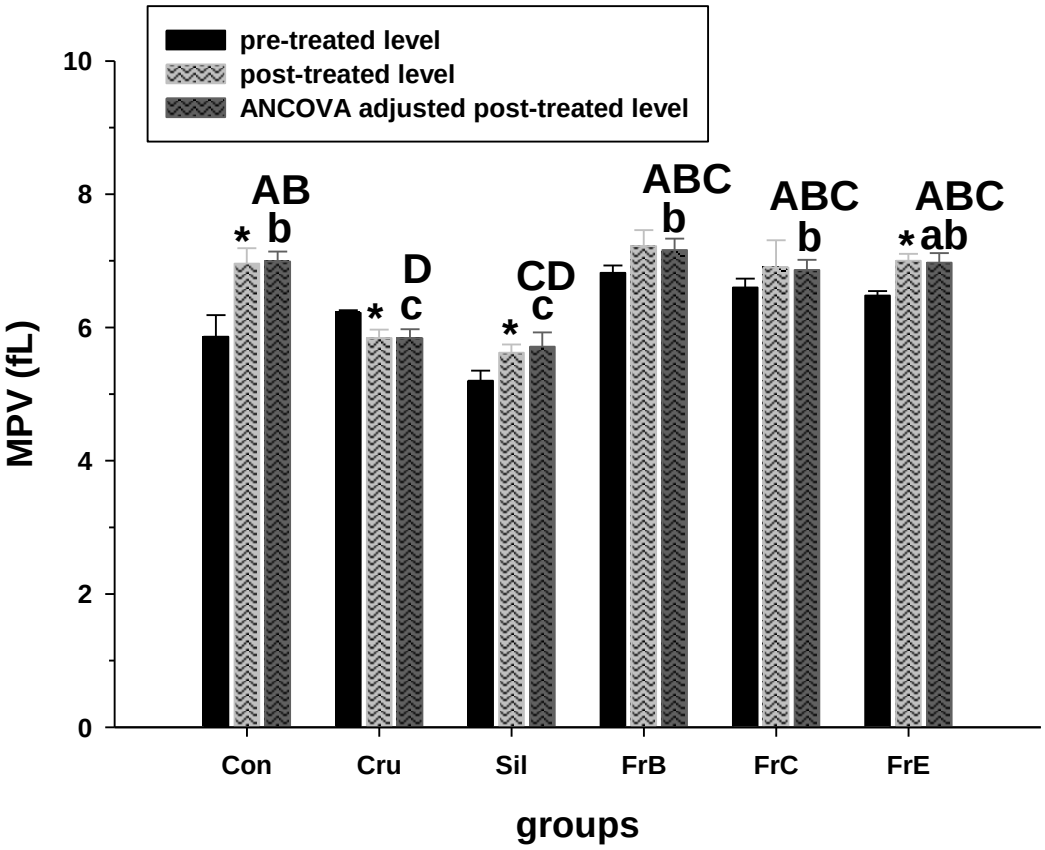


Figure 23 Effects of BS extracts and Sildenafil on mean platelet volume (MPV) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

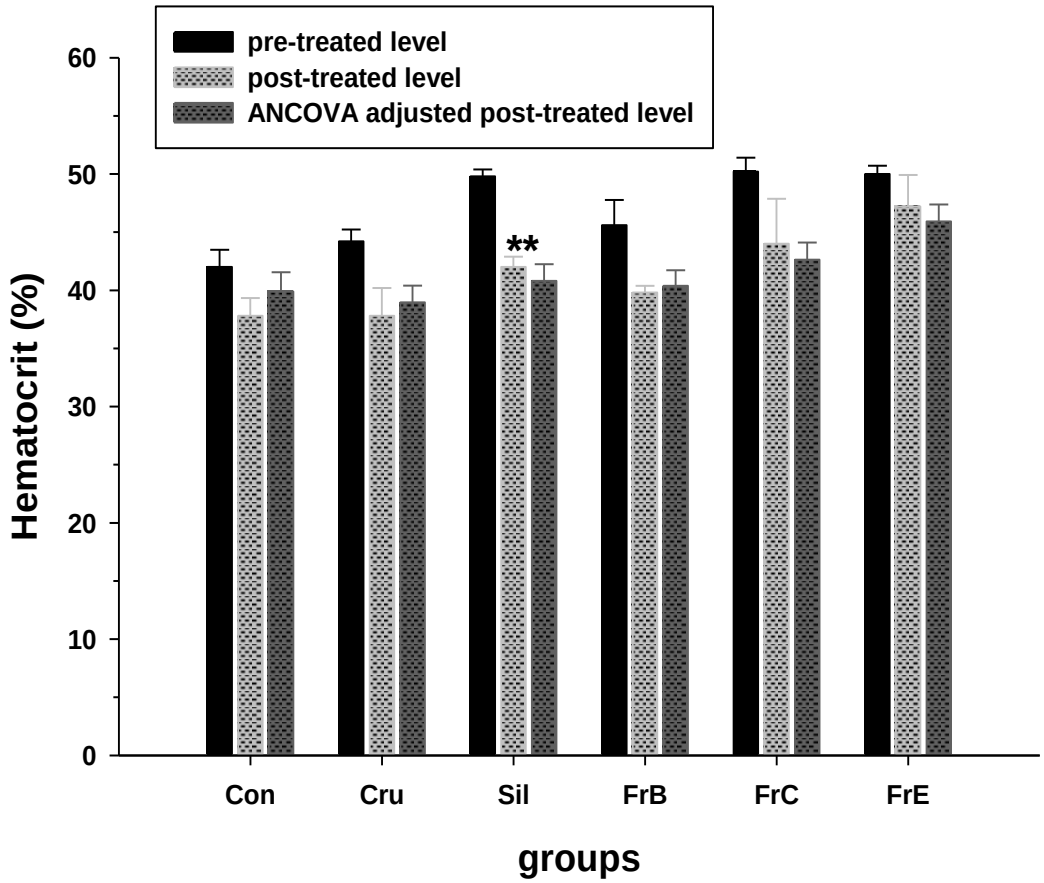


Figure 24 Effects of BS extracts and Sildenafil on hematocrit (Hct) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day. FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

4.4 Histology of testis

It can be seen from Figure 25 that the spermatogenesis of all treated groups had more maturation process of spermatids than those of the control. These spermatogenesis processes were higher in both spermatogenesis levels and spermatid numbers. These findings lend support to the sperm count and motility that extract compounds from BS can increase both sperm number and motility.

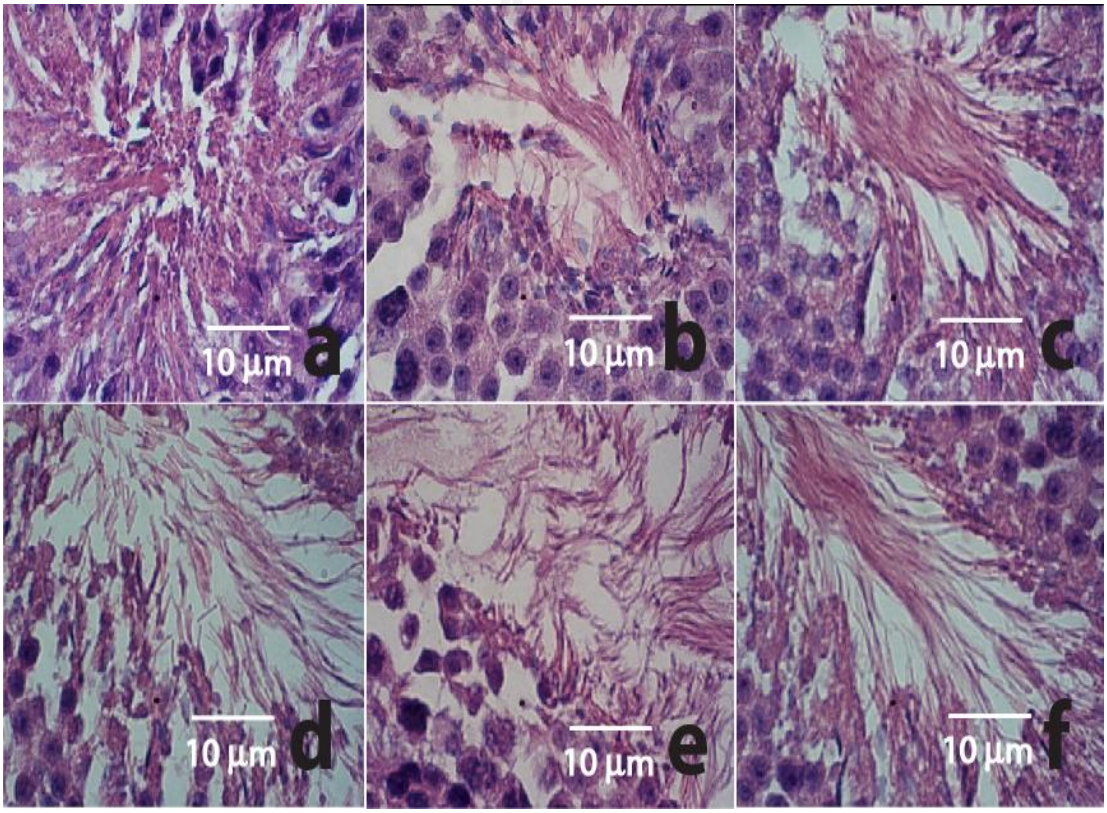


Figure 25 Micrographs of testicular (seminiferous tubules) section of mice; a = control DW 0.5 ml/kg BW/day, b = crude extract 1,250 mg/kg BW/day, c = Sildenafil 10 mg/kg BW/day, d = fraction B 40 mg/Kg BW/day (Un1 1,260 µg), e = Fraction C 50 mg/Kg BW/day (Genistein 6.78 µg plus Un1 31.59 µg), f = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 µg plus 9 unknown compounds). All groups were treated for 14 days. All micrographs displayed at X100 magnification. Bar = 10 µm.

4.5 Testosterone level

The effects of BS extracts and sildenafil on testosterone level of mice is shown in figure 26. The results exhibited that there were significant increase in testosterone level of all post-treated groups compared to pre-treatment ($p<0.01$) except for the control. In addition, the testosterone level of fraction C treated groups were significantly highest increase compared to other groups. Furthermore, serum testosterone level of both fraction C and E treated groups were significantly higher than those of other groups ($p<0.01$).

In the same way, the crude extract, fraction B and sildenafil treated groups also showed significantly higher than those of control ($p<0.01$). These results are in substantial correspondence with Saraiva et al. (2009) that sildenafil-treated mice showed significant increased levels of total testosterone compared to control and Leydig cells presented noteworthy ultrastructural alterations, such as a vesicular smooth endoplasmic reticulum, large vacuoles, enlarged discontinue cristaes of mitochondria and vesicles of whorle membranes at the periphery, which are typical characteristics of an activated steroid-secreting cell. Nevertheless, the rats treated with 150 and 200 mg/Kg BW/day BS powder revealed a dose dependent decrease in testosterone level (Cherdshewasart et al., 2008).

Previous findings of Leydig cell which were stimulated by sildenafil can be explained by theory that Leydig cells produce male sex hormone, such as testosterone. Testosterone functions to stimulate spermatogenesis, promote the physical and functional maturations of spermatozoa, maintain the accessory organs of the male reproductive tract and etc. (Martini, 2006). Testosterone is synthesized from a precursor, cholesterol (Maqdasy et al., 2013; Midzak et al., 2009). In fact, these

findings provide evidence that cholesterol was increased in these treated groups. As a consequence, testosterone was rose up. These results can be explained by assuming that the increase in testosterone often results in higher sperm number and motility as a result of spermatogenesis and mature of spermatozoa are stimulated by this hormone (Martini, 2006). In fact, recent studies have also reported that blood testosterone concentrations were lower in male patients suffering from Alzheimer's disease (AD) (Hogervorst et al., 2001; Moffat et al., 2004). For this reason, these BS extracts may be useful to increase testosterone level in AD patients.

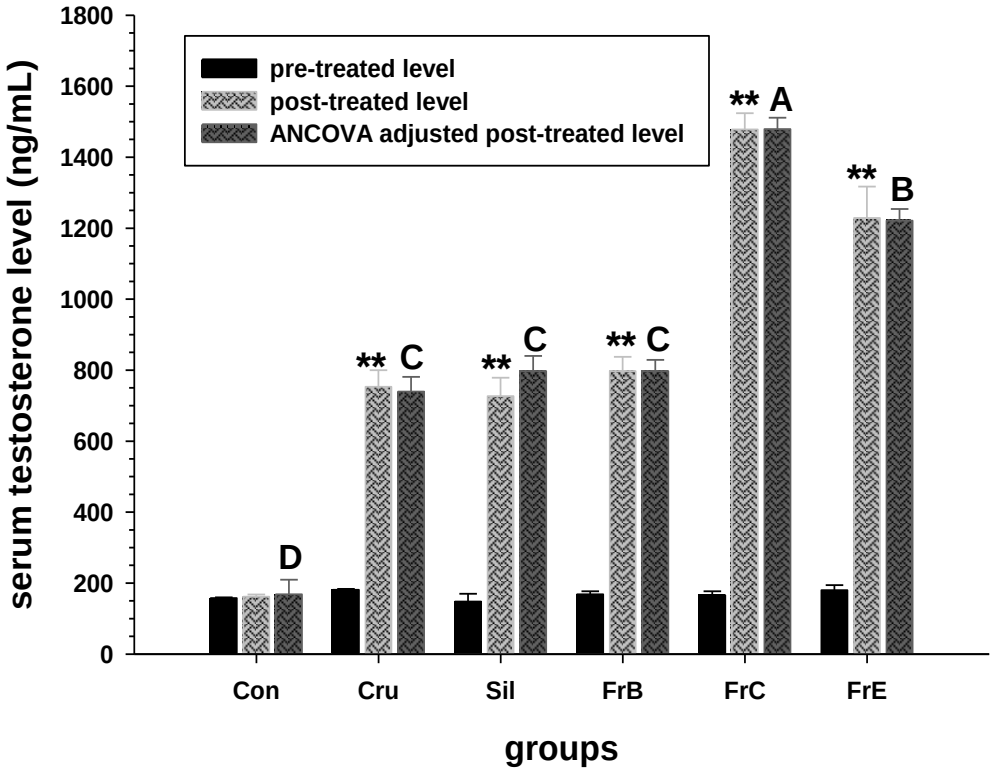


Figure 26 Effects of BS extracts and Sildenafil on testosterone level (ng/ml) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$ Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

4.6 Body weight

There was no significant difference in the relative growth rate measured by living body weight of male mice treated with BS extract compounds and sildenafil groups when compared to the control (p<0.01) (Figure 27). These results are substantial agreement with those of Cherdshewasart et al., (2008) that the rats treated with 150 and 200 mg/Kg BW/day BS powder were not significant different in the growth rate measured by living body weight compared to the control.

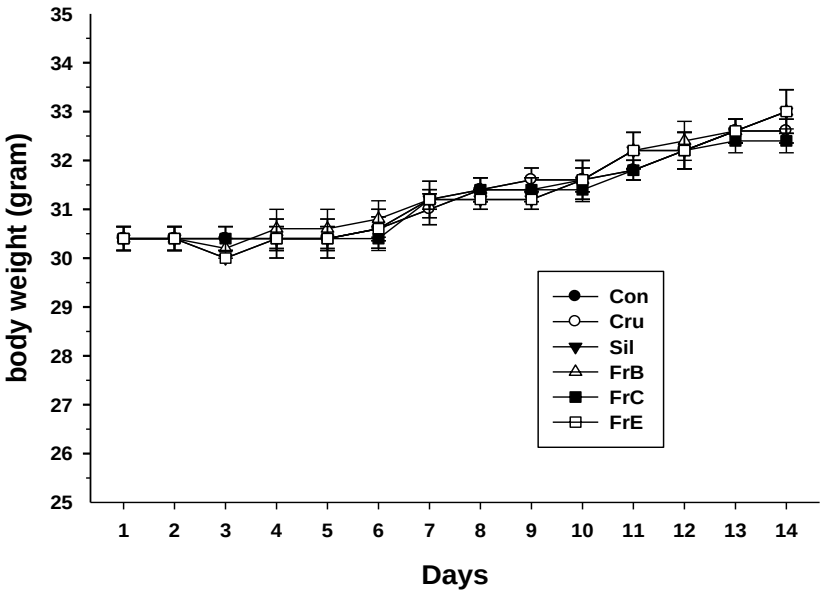


Figure 27 Effects of crude extract and sildenafil on body weight (gram) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} p<0.05, ^{A} p<0.01.

4.7 Selected reproductive and vital organs

The effects of BS extract compounds and sildenafil on selected reproductive organ weight (per 100g body weight) in mice are presented in table 4.2 and figure 28-31. The testes weight of crude extract and sildenafil treated groups were significantly heavier than those of control and fraction B groups (p<0.01) (Figure 28). Also, the epididymis weight of fraction E and C was significantly heavier than others whereas crude extract, fraction B and sildenafil treated groups were significantly lighter than control (p<0.01) (Figure 29). Furthermore, the seminal vesicle weight of fraction B, C and E treated groups were significantly heavier than the remainder of these groups (p<0.01) (Figure 30). As well as, the prostate gland weights of control and crude extract groups were significantly heavier than other groups (p<0.01) (Figure 31).

However, the spleen weight of fraction B, C and E treated groups were significantly heavier than those of control (p<0.01). On the contrary, the stomach weight of fraction E treated group was significantly lighter than sildenafil, crude extract and the control groups (p<0.01) (Table 4.3). These results seem consistent with Manosroi et al. (2006) that *Butea superba* crude drug at the dose of 1,250 mg/kg showed significantly (p<0.05) higher percentage of testis/BW than the control.

The histopathology of the heart, liver, spleen, kidney, and stomach of all treated groups revealed normal appearance compared to the organs of control group (Figure 32-41).

This investigation provides evidences that *Butea superba* extract could increase testosterone level, sperm number and motility of mice compared to the control. This extract had effects on lower stomach weight, whereas it increase, spleen weight, testes, seminal vesicle, epididymis and cholesterol level in fraction E

compared to the control. This finding may be applied that this extract could developed to increase testosterone, sperm number and motility in men.

Table 4.2 Effects of BS extracts and Sildenafil on selected relative reproductive organ weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$

Reproductive Organ (g/100BW)	Con	Cru	Sil	FrB	FrC	FrE
Testes	0.18±0.023^{B}	**0.29±0.014^{A}**	**0.30±0.045^{A}**	0.19±0.010^{B}	0.24±0.013^{AB}	0.25±0.008^{AB}
Epididymis	0.08±0.002^{B}	**0.09±0.01^{C}**	**0.08±0.01^{C}**	**0.09±0.003^{C}**	0.14±0.007^{AB}	**0.16±0.003^{A}**
Vas deferens	0.03±0.004	0.03±0.003	0.02±0.003	0.03±0.003	0.02±0.004	0.03±0.004
Seminal vesicle	0.12±0.004^{B}	0.12±0.005^{B}	0.13±0.004^{B}	**0.22±0.034^{A}**	**0.30±0.022^{A}**	**0.31±0.001^{A}**
Prostate gland	**0.06±0.005^{A}**	**0.06±0.003^{A}**	**0.04±0.005^{B}**	0.03±0.002^{B}	0.02±0.002^{B}	0.02±0.002^{B}

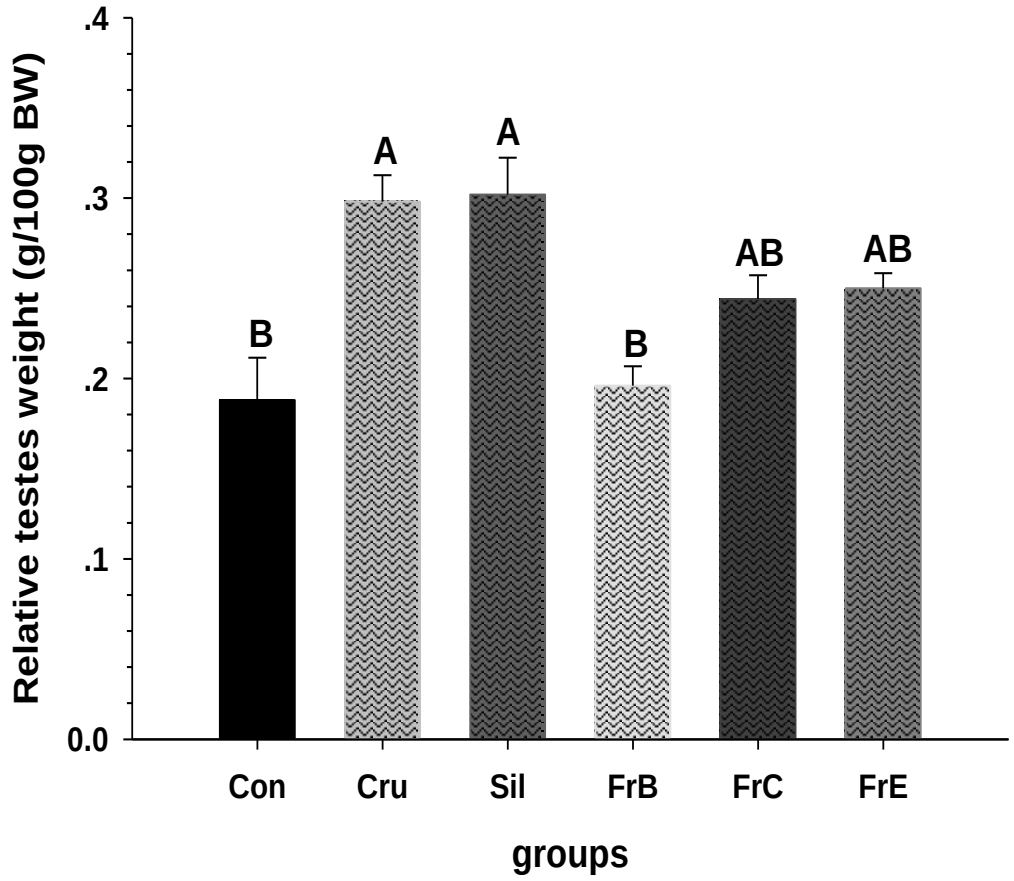


Figure 28 Effects of BS extracts and Sildenafil on relative testes weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at [a] $p<0.05$, [A] $p<0.01$.

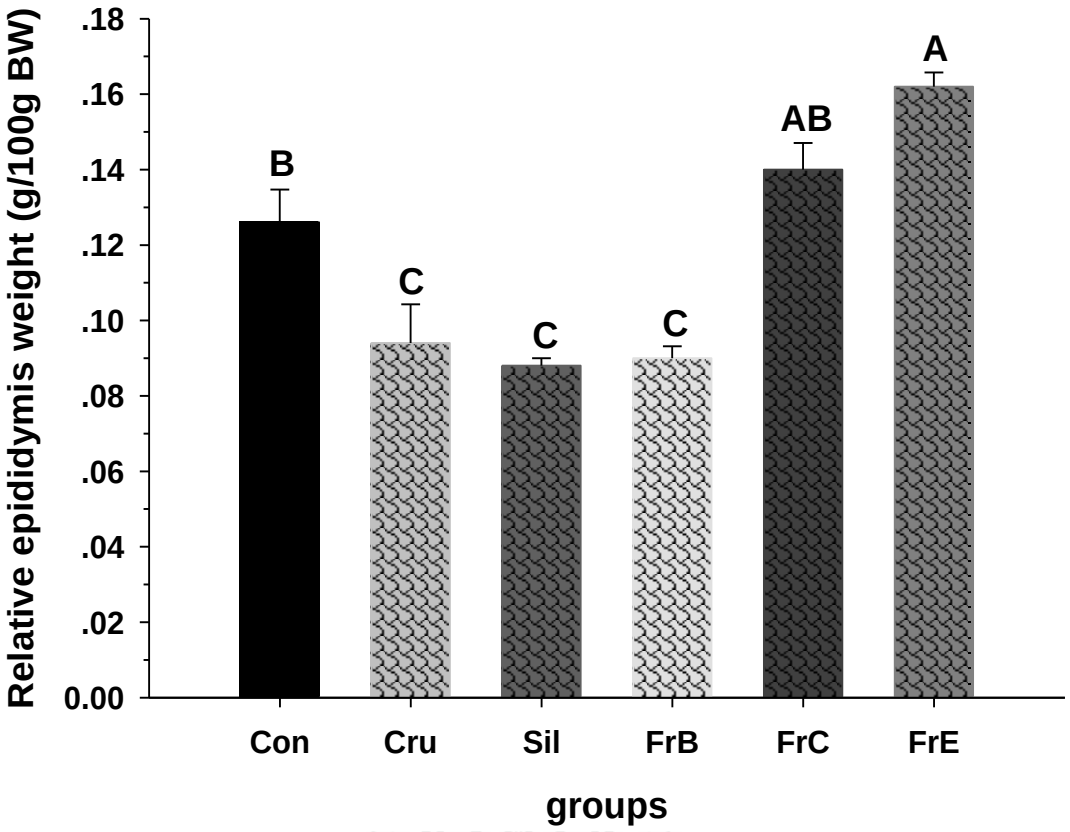


Figure 29 Effects of BS extracts and Sildenafil on relative epididymis weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

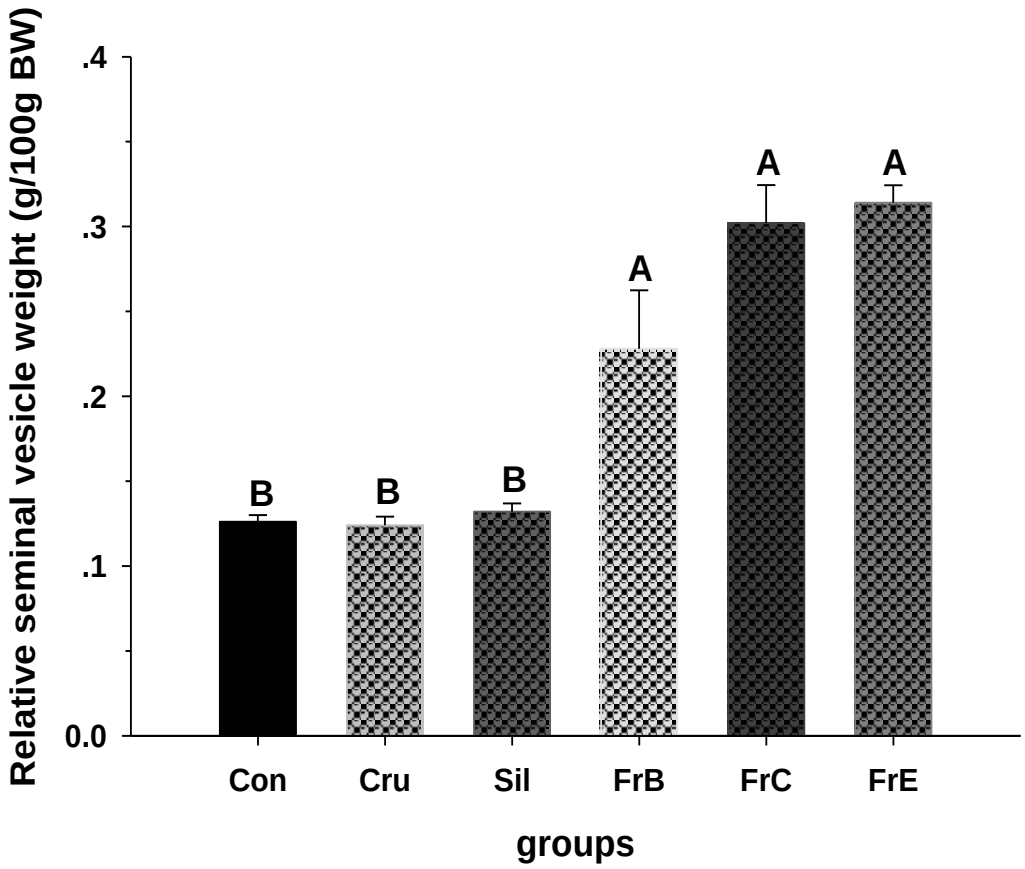


Figure 30 Effects of BS extracts and Sildenafil on relative seminal vesicle weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

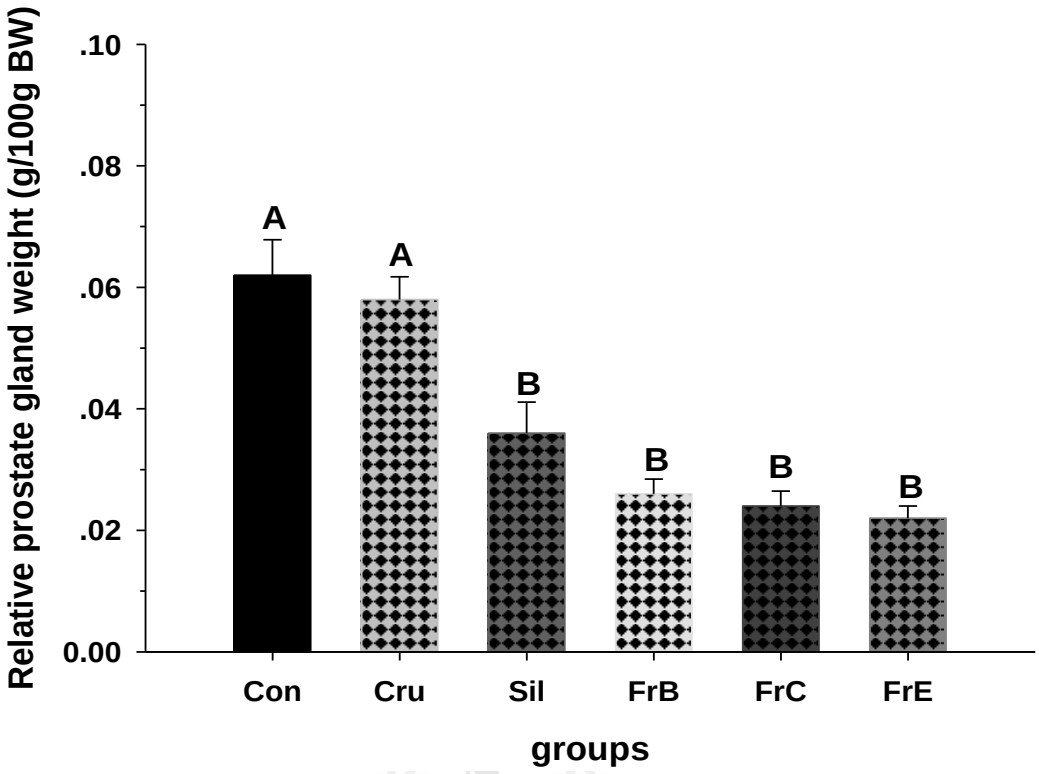


Figure 31 Effects of BS extracts and Sildenafil on relative prostate gland weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

Table 4.3 Effects of BS extracts and Sildenafil on selected relative vital organ weight (per 100g body weight) of mice. Con = control DW 0.5 ml/kg BW/day, Cru = crude extract 1,250 mg/kg BW/day, Sil = Sildenafil 10 mg/kg BW/day, FrB = Fraction B 40 mg/Kg BW/day (Un1 1,260 μg), FrC = Fraction C 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg), FrE = Fraction E 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds). All groups were treated for 14 days. Significant difference between each group was compared using ANOVA and Tukey HSD post hoc test at ^{a} $p<0.05$, ^{A} $p<0.01$.

Vital organ (g/100BW)	Con	Cru	Sil	FrB	FrC	FrE
Heart	0.14±0.002	0.13±0.003	0.14±0.003	0.13±0.010	0.15±0.011	0.13±0.005
Liver	1.74±0.005	1.71±0.022	1.76±0.005	1.70±0.05	1.73±0.017	1.64±0.042
Spleen	**0.05±0.005^{c,C}**	**0.04±0.003^{c,C}**	**0.07±0.007^{bc,BC}**	**0.11±0.025^{ab,AB}**	**0.12±0.007^{a,AB}**	**0.15±0.005^{a,A}**
Lung	**0.19±0.01^{AB}**	**0.17±0.02^{AB}**	**0.21±0.004^{A}**	**0.16±0.012^{AB}**	**0.16±0.011^{AB}**	**0.14±0.004^{B}**
Kidney	0.8±0.06	0.6±0.01	0.73±0.003	0.59±0.048	0.63±0.026	0.61±0.032
Stomach	**1.04±0.06^{AB}**	**1.19±0.008^{A}**	**1.09±0.06^{AB}**	**0.70±0.109^{BC}**	**0.72±0.089^{BC}**	**0.49±0.038^{C}**

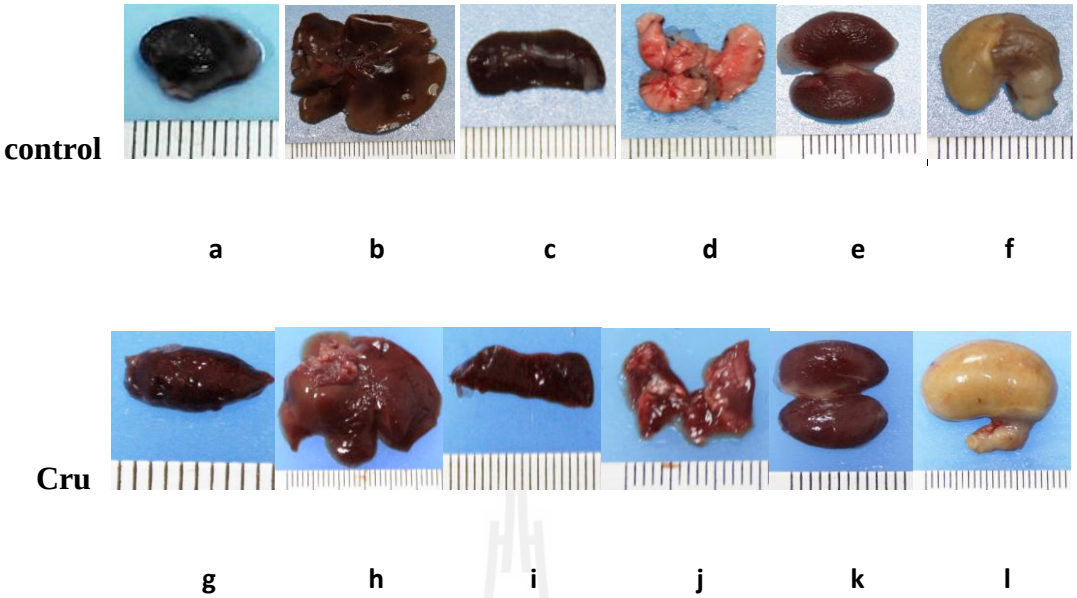


Figure 32 Morphology of main body organs of mice treated with crude extract (Cru) of *Butea superba* at 1,250 mg/kg BW/day for 14 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach. One hole scale = 1 mm.

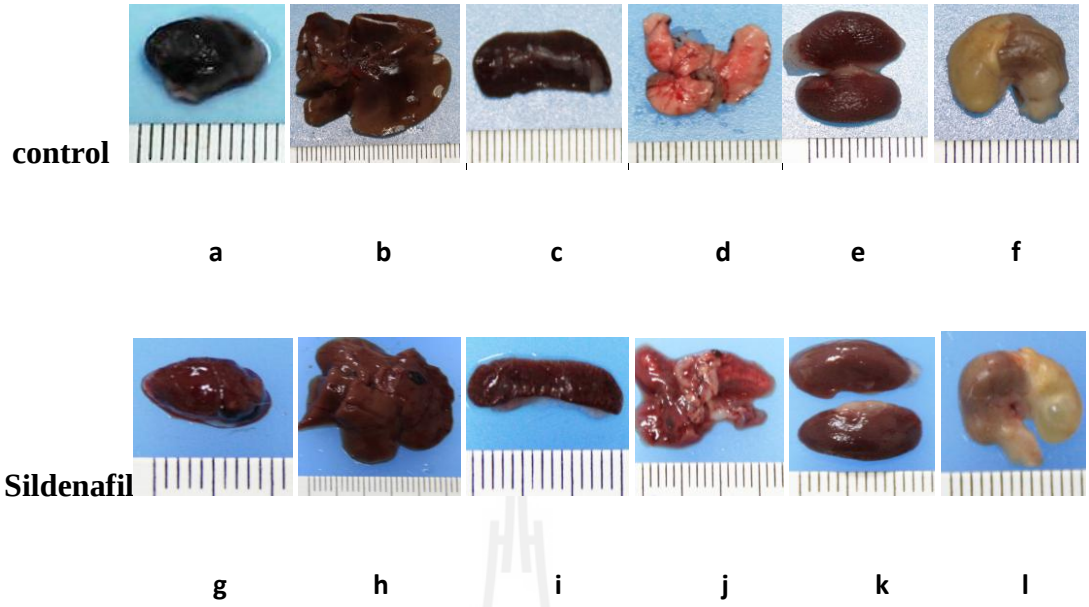


Figure 33 Morphology of main body organs of mice treated with Sildenafil at 10 mg/kg BW/day for 14 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach. One hole scale = 1 mm.

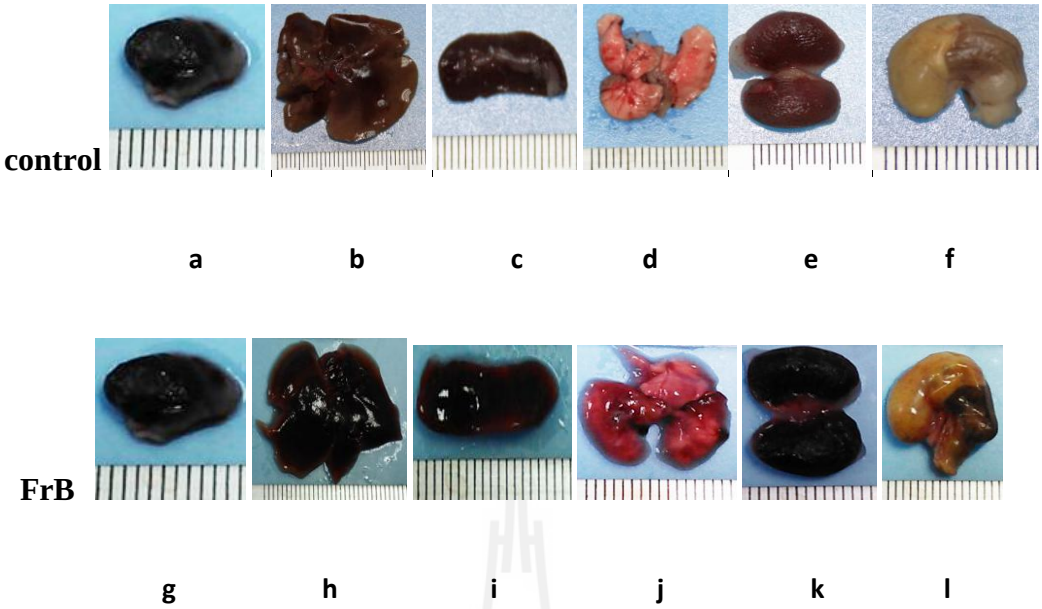


Figure 34 Morphology of main body organs of mice treated with fraction B (FrB) at 40 mg/Kg BW/day (Un1 1,260 µg) for 14 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach. One hole scale = 1 mm.

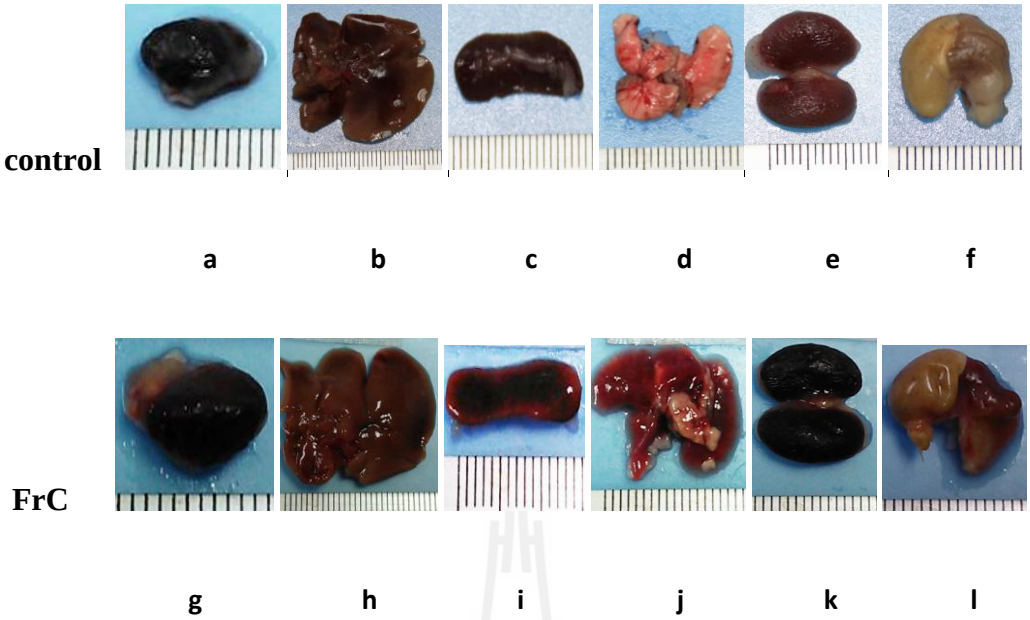


Figure 35 Morphology of main body organs of mice treated with Fraction C (FrC) at 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg) for 14 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach. One hole scale = 1 mm.

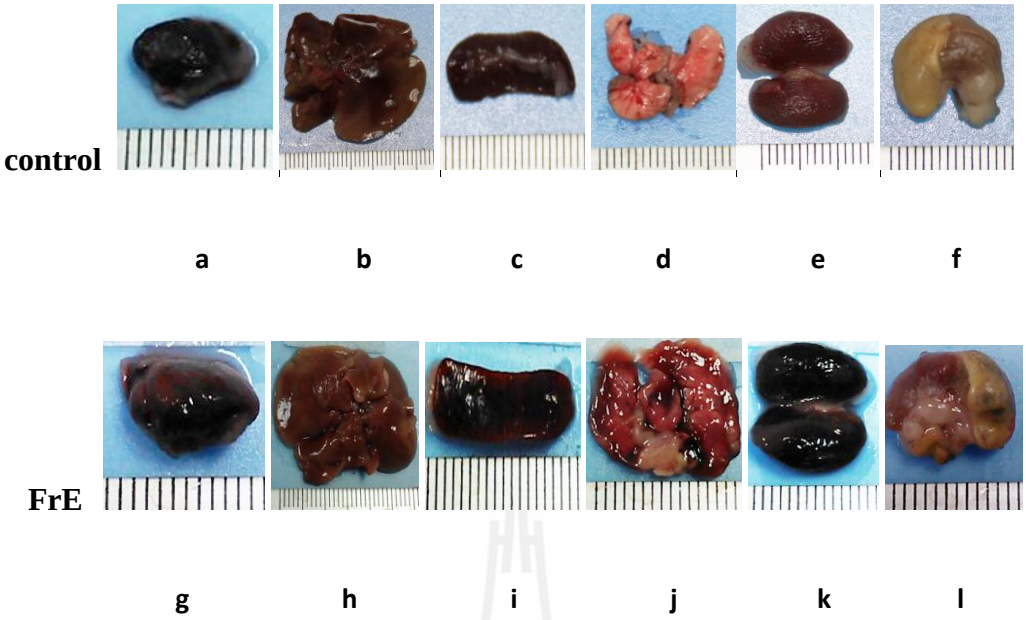


Figure 36 Morphology of main body organs of mice treated with fraction E (FrE) at 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds) for 14 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach. One hole scale = 1 mm.

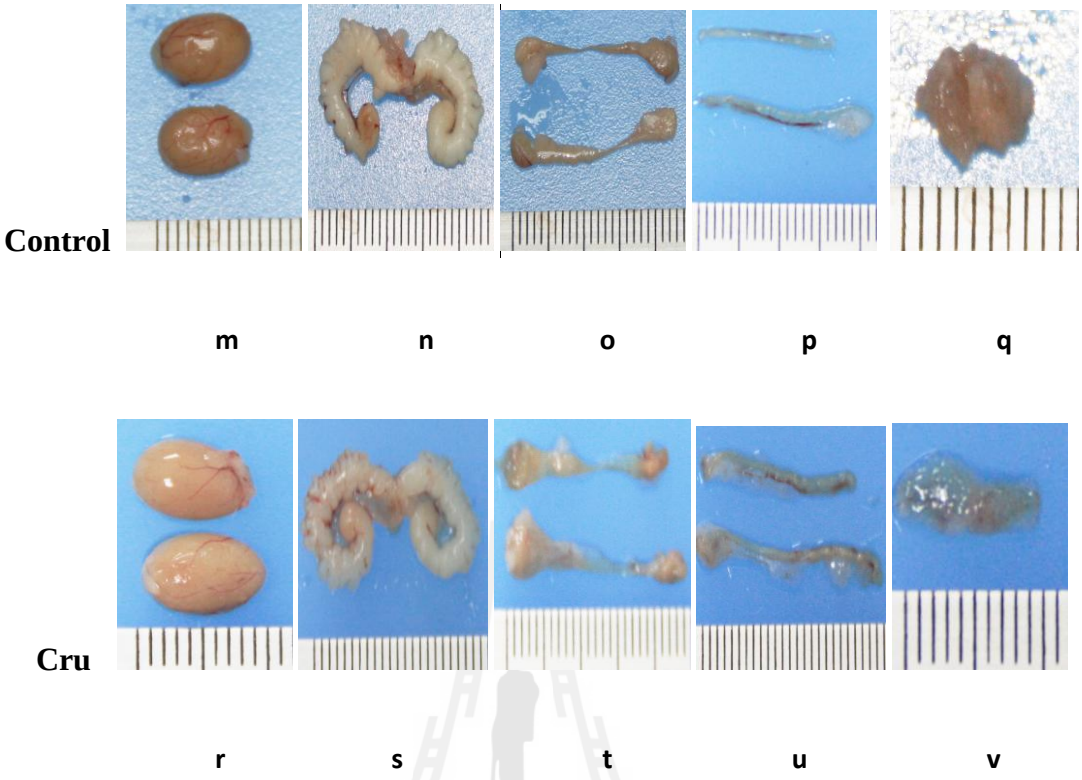


Figure 37 Morphology of main reproductive organs of mice treated with crude extract of *Butea superba* (Cru) at 1,250 mg/kg BW/day for 14 consecutive days in comparison with the negative control. Control group; m = testes; n = seminal vesicle; o = epididymis; p = vas deferens; q = prostate gland. Treated group; r = testes; s = seminal vesicle; t = epididymis; u = vas deferens; v = prostage gland. One hole scale = 1 mm.

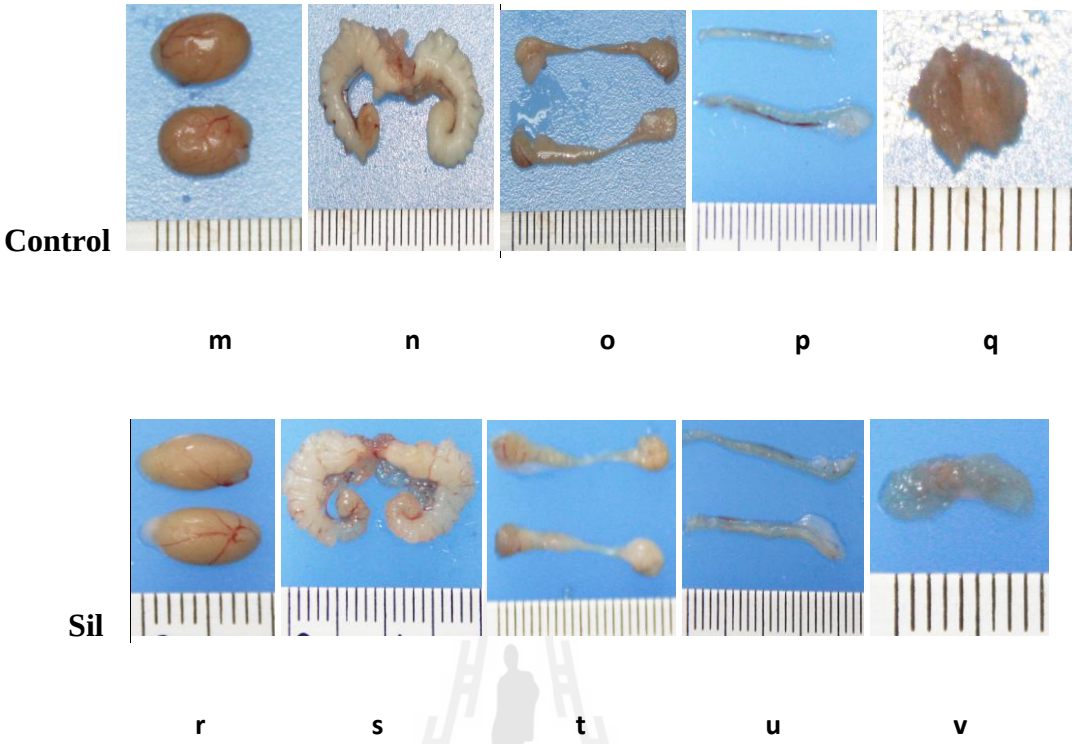


Figure 38 Morphology of main reproductive organs of mice treated with sildenafil at 10 mg/kg BW/day for 14 consecutive days in comparison with the negative control. Control group; m = testes; n = seminal vesicle; o = epididymis; p = vas deferens; q = prostate gland. Treated group; r = testes; s = seminal vesicle; t = epididymis; u = vas deferens; v = prostage gland. One hole scale = 1 mm.

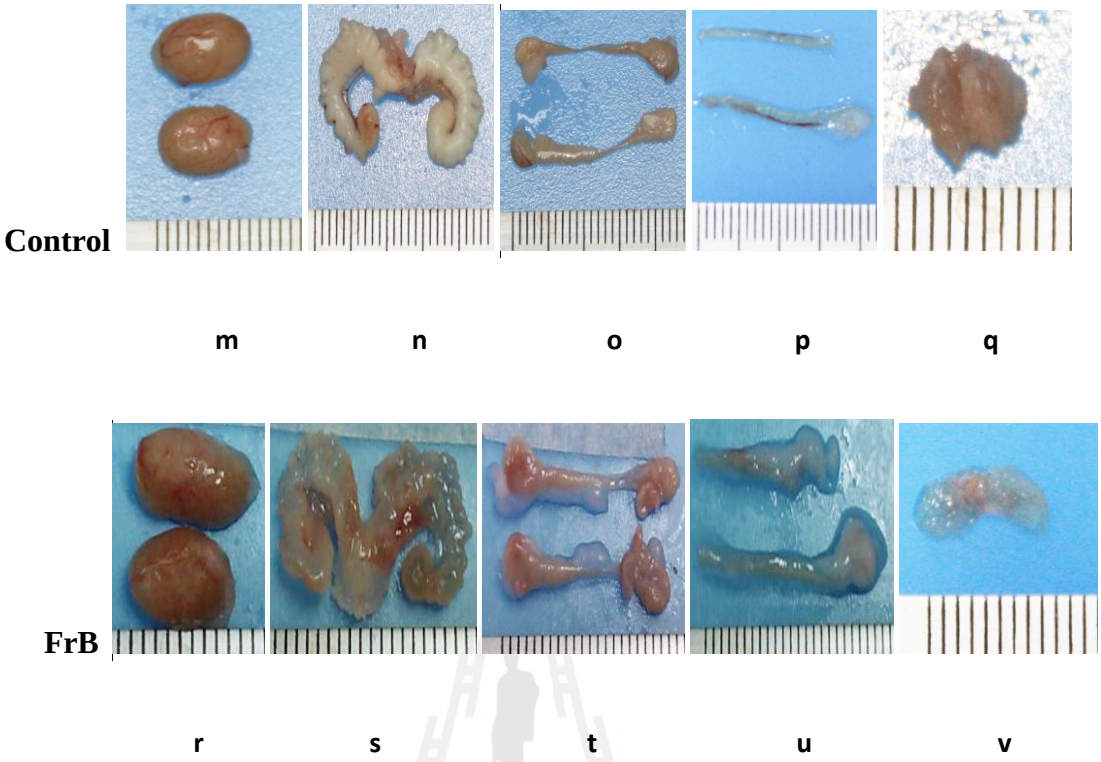


Figure 39 Morphology of main reproductive organs of mice treated with fraction B (FrB) at 40 mg/Kg BW/day (Un1 1,260 μg) for 14 consecutive days in comparison with the negative control. Control group; m = testes; n = seminal vesicle; o = epididymis; p = vas deferens; q = prostate gland. Treated group; r = testes; s = seminal vesicle; t = epididymis; u = vas deferens; v = prostage gland. One hole scale = 1 mm.

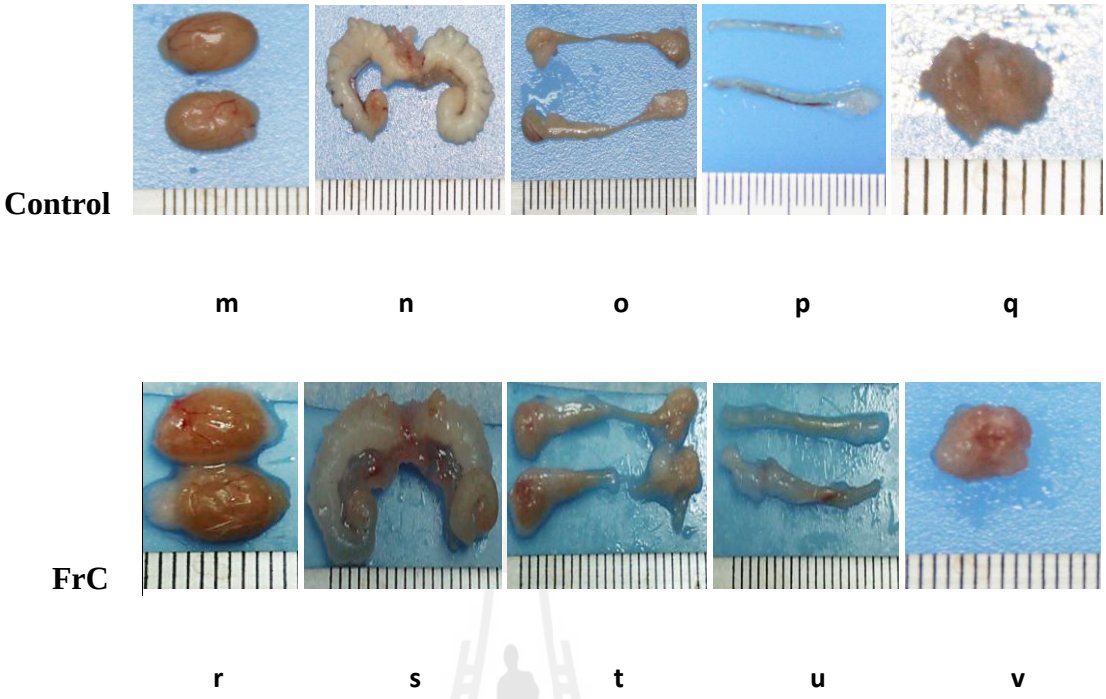


Figure 40 Morphology of main reproductive organs of mice treated with fraction C (FrC) at 50 mg/Kg BW/day (Genistein 6.78 μg plus Un1 31.59 μg) for 14 consecutive days in comparison with the negative control. Control group; m = testes; n = seminal vesicle; o = epididymis; p = vas deferens; q = prostate gland. Treated group; r = testes; s = seminal vesicle; t = epididymis; u = vas deferens; v = prostage gland. One hole scale = 1 mm.

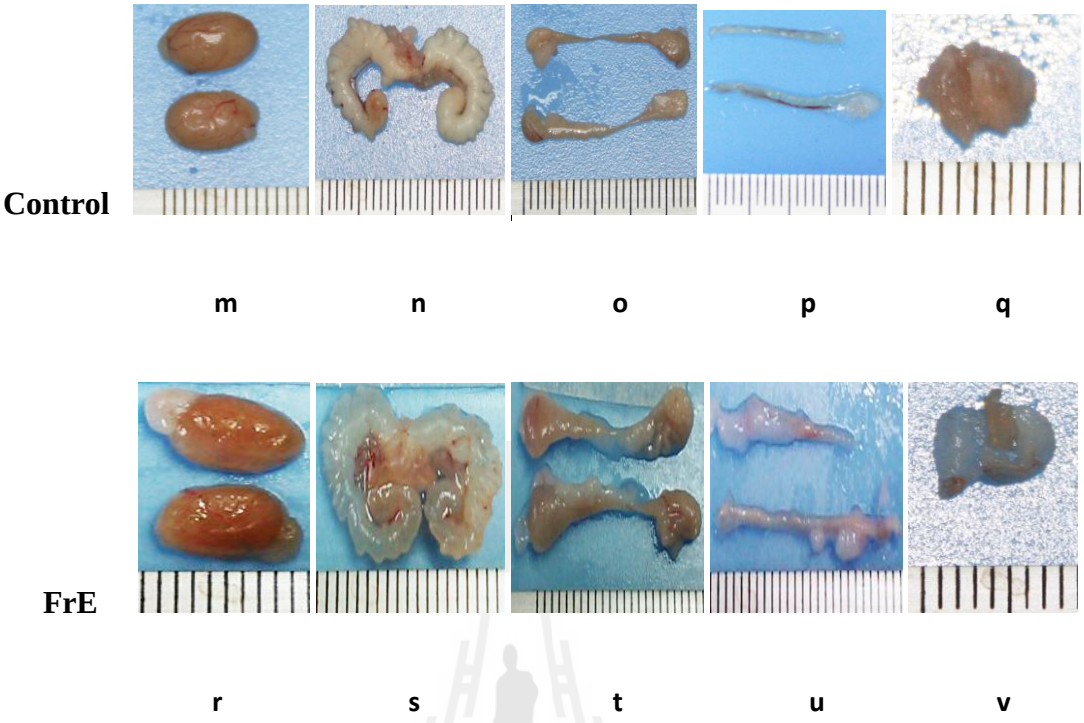


Figure 41 Morphology of main reproductive organs of mice treated with fraction E (FrE) at 150 mg/kg BW/day (Biochanin A 66.92 μg plus 9 unknown compounds) for 14 consecutive days in comparison with the negative control. Control group; m = testes; n = seminal vesicle; o = epididymis; p = vas deferens; q = prostate gland. Treated group; r = testes; s = seminal vesicle; t = epididymis; u = vas deferens; v = prostage gland. One hole scale = 1 mm.

CHAPTER V

CONCLUSION

Butea superba Roxb. has been popularly used among Thai men for rejuvenation because it contains Phytoandrogen that has structure and function like testosterone. It has been claimed that *Butea superba* Roxb could improve sexual function, and prevented prostate cancer and prostatic hyperplasia. In fact, erectile dysfunction (ED) is the inability to maintain penile erection for the successful performance of sexual activity that has many physical and psychological causes, including vascular disease, diabetes, medications, depression, and sequelae to prostatic surgery. The oral phosphodiesterase (PDE) inhibitors are now considered to be first-line therapy for men with ED. The PDE-5 inhibitors, sildenafil, are approved for the treatment of ED. However, the most frequent adverse effects reported for sildenafil is headache, flushing, dyspepsia, and nasal congestion. These effects are generally mild, and men with ED rarely discontinue treatment because of side effects. Also, disturbances in color vision (loss of blue/green discrimination) occur with sildenafil. Hence, the objective of this study was to investigate the effects of the tuberous root of *Butea superba* Roxb extract compared with sildenafil on the reproductive system of male mice. Then, the dried powder of the tuber roots of *Butea superba* Roxb. was extracted with ethanol. The ethanol extract was separated by silica gel column chromatography by sequential elution with hexane, chloroform, acetone, and methanol. The acetone crude extract was separated by column chromatography and to give functional group compounds. The chemical structures of the isolated

compounds were identified by High performance liquid chromatography (HPLC) compared with standard compounds. The major components of each fraction were elucidated and orally administered to male mice. The 1.5% (w/w) percent yield from dried powder of crude extract was fed at the dose of 1,250 mg /kg BW/day that consisted of Genistein 53.57 μg plus Biochanin A 312.95 μg and unknown compound 1 (Un1) 396.59 μg. Fractions B (FrB) (0.022% yield) at the dose of dose 40 mg/Kg BW/day consisted of unknown compound 1 (Un1) 1,260.00 μg. Fraction C (FrC) (0.030% w/w yeild) at the dose of 50 mg/Kg BW/day consisted of Genistein 6.78 μg plus unknown compound 1 (Un1) 31.59 μg. Also, Fraction E (FrE) (0.054% w/w yield) at the dose 150 mg/Kg BW/day consisted of Biochanin A 66.92 μg that was the main compound plus 9 unkown compounds was fed. All groups were treated for 14 consecutive days. Blood and sperms were collected for analysis before (pre-) and after (post-) treatment in all groups. Also, at the end of the experiments, the selected reproductive and vital organs were collected for comparative measurement. A relative change of body weight was also analysed.

The results showed that the relative body weights in all treated groups were not significant different from the control. Whereas, the spleen weight of fraction B, C and E treated groups were significantly heavier than those of the control (p<0.01). On the contrary, the stomach weight of fraction E treated group was lighter than sildenafil, crude extract, and the control groups (p<0.01). However, the testes weight of crude extract and sildenafil treated groups were significantly heavier than those of the fraction B and control groups (p<0.01). Also, the epididymis weight of fraction E and C were significantly heavier than others (p<0.01). Furthermore, the seminal vesicle weight of fraction B, C and E treated groups were significantly heavier than the remainder of these groups (p<0.01). Also, the prostate gland weights of control

and crude extract groups were significantly heavier than other groups (p<0.01). In addition, The results exhibited that there were significant increase in testosterone level of all post-treated groups compared to pre-treatment except for the control (p<0.01). In addition, the testosterone level of fraction C treated groups were significantly highest increase compared to other groups and both fraction C and E treated groups were significantly higher than those of other groups (p<0.01). Apart from this, The results exhibited that there were significant increase in sperm number of all post-treated groups compared to pre-treatment except for control (p<0.01). Also, fraction C showed the highest sperm number compared with others. Besides, the sperm number of crude extract, sildenafil, fraction B and E treated groups were significantly higher than control group (p<0.01). These results were confirmed by sperm morphology micrographs. In the same way, The sperm results exhibited that there were significant increase in sperm motility of all of post-treated groups compared to pre-treatment except for control (p<0.05). Apart from this, the highest motility level was found in the fraction C and E compared to others including sildenafil (p<0.01). These findings provide evidence that Genistein, Un1 and Biochanin A may play an important role in increasing of sperm number, motility and testosterone level. These results are supported previous finding that sildenafil-treated mice showed significant increased levels of total testosterone compared to control as well as Leydig cells presented noteworthy ultrastructural alterations, such as a vesicular smooth endoplasmic reticulum, large vacuoles, enlarged discontinue cristaes of mitochondria and vesicles of whole membranes at the periphery, which are typical characteristics of an activated steroid-secreting cell (Saraiva et al., 2009). Previous findings of Leydig cells were stimulated by sildenafil can be explained by theory that Leydig cells produce male sex hormone, such as testosterone. Testosterone functions to stimulate

spermatogenesis, promote the physical and functional maturation of spermatozoa, maintain the accessory organs of the male reproductive tract and etc (Martini, 2006). So, These results can be explained by assuming that the increase in testosterone often results in higher sperm number and motility because of spermatogenesis and sperm maturation are stimulated by this hormone. In fact, recent studies have also revealed that blood testosterone concentrations were lower in male patients suffering from Alzheimer's disease (AD) (Hogervorst et al., 2001; Moffat et al., 2004). Because of this, these BS extracts may useful to treat AD in men as a result of increase testosterone level in these patients. The blood analysis showed that the cholesterol level of fraction C, E and sildenafil were significantly higher difference from those of control (p<0.05). One reason could be that cholesterol, a precursor of testosterone, is prepared to synthesize testosterone (Maqdasy et al., 2013; Midzak et al., 2009). Whereas, hemoglobin level of sildenafil and crude extract post-treated groups were significantly lower than pre-treated at p<0.01 and 0.05 respectively. Although, the hemoglobin level of fraction E treated group was significantly higher than those of control and crude extract groups (p<0.05).

These findings provide evidence that *Butea superba* extract in fraction C and E can increase testosterone level, sperm number and motility of mice compared to those of the sildenafil and control groups. These results can be explained by assuming that Genistein, unknown compound 1, and Biochanin A may play the important role for these results. So, these findings may provide evidence that these BS extract could develop to increase testosterone level, sperm number, sperm motility and infertility or Alzheimer's disease treatment in men after safety level is investigated.

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APPENDICES

APPENDIX A

THE SPERM MOTILITY, SPERM COUNT AND TESTOSTERONE LEVEL

Table A.1 Effects of crude extract, Sildenafil, Fraction B, Fraction C and Fraction E on sperm motility (%) of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day, Group 4 = Fraction 2 40 mg/kg BW/day Group 5 = Fraction 3 50 mg/kg BW/day Group 6 = Fraction 5 150 mg/kg BW/day. All groups were treated for 14 days.

	Group1= Control (DW)		Group 2 = Crude extract		Group 3 = Sildenafil	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Sperm motility	59±6.6	63.6±2.3	59±5.8	74.6±10.1	59.2±4.2	68±4.9*

	Group4= Fraction B		Group 5 = Fraction C		Group 6 = Fraction E	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Sperm motility	61.2±1.3	77.2±6.6	60.2±3.3	97±1.6**	56.2±5.1	91.4±2.9

Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$

Table A.2 Effects of crude extract, sildenafil, fractionB, fractionC and fractionD on sperm number (x100,000 n/ml) of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day, Group 4 = Fraction2 40 mg/kg BW/day Group 5 = Fraction3 50 mg/kg BW/day Group 6 = Fraction5 150 mg/kg BW/day. All groups were treated for 14 days.

	Group1= Control (DW)		Group 2 = Crude extract		Group 3 = Sildenafil	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Sperm number	109.0±3.5	112.0±3.3	119.0±3.5	213.6±20.3**	117.2±6.2	212.8±8.1**

	Group4= Fraction B		Group 5 = Fraction C		Group 6 = Fraction E	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Sperm number	108±3.91	263.0±11.16**	113.2±4.27	331.2±18.30**	114.2±4.70	263.6±15.33**

Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$

Table A.3 Effects of crude extract, sildenafil,fractionB, fractionC and fractionE on testosterone level (ng/ml) of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day, Group 4 = Fraction2 40 mg/kg BW/day Group 5 = Fraction3 50 mg/kg BW/day Group 6 = Fraction5 150 mg/kg BW/day. All groups were treated for 14 days.

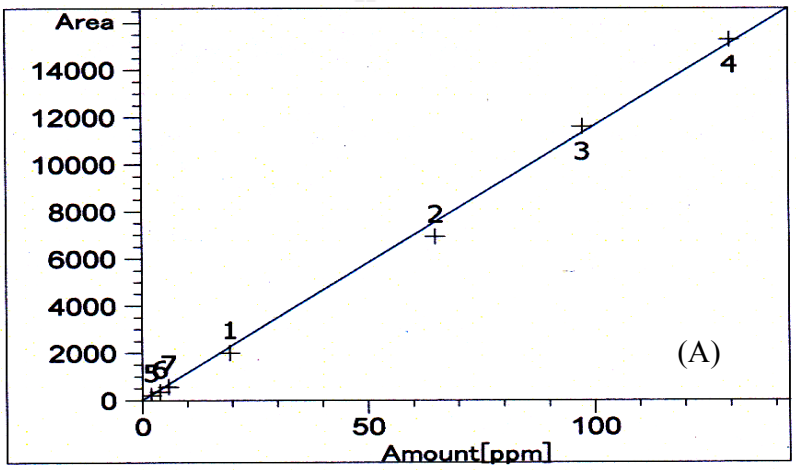
	Group1= Control (DW)		Group 2 = Crude extract		Group 3 = Sildenafil	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Testosterone	157.7±2.3	161.7±6.0	181.3±2.4	460.0±147.6**	148.0±21.8	670.0±104.1**

	Group4= Fraction B		Group 5 = Fraction C		Group 6 = Fraction E	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Testosterone	168±8.49	379.8±89.21**	166.0±11.11	1366±156.83**	180.0±14.57	735.0±282.4**

Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$, ** $p<0.01$

APPENDIX B

STANDARD CURVES



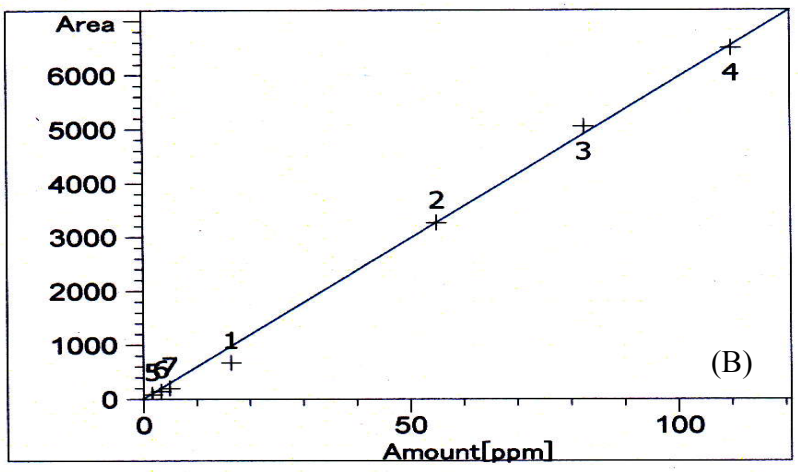


Figure 1B Standard curves of Amount (ppm) and peak area of genistein (A), biochanin A (B), determined using HPLC.

APPENDIX C

LIST OF PRESENTATIONS

Poster presentation

Naknarong, W., and Eumkeb, G. (2012). The effects of Red Kwao Kreu (*Butea superba* Roxb.) extract on reproductive system of male mice. **The 4th International Conference on Natural Products for Health and Beauty,** at Chiang Mai, Thailand.

Abstract No. P-A-084

NATPRO 4 Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), Chiang Mai University, Chiang Mai, 28-30 Nov. 2012

The effects of Red Kwao Kreu (*Butea superba* Roxb.) extract on reproductive system of male mice

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Introduction and Objective

Butea superba Roxb, a plant in the family Leguminosae has the domestic name of "Red Kwao Kreu", is a herb with Thai medicinal properties that has been used since ancient times. *Butea superba* Roxb has been popularly used among Thai men for rejuvenation because it contains Phytoandrogen that has structure and function like testosterone. The effect of *Butea superba* Roxb has been reported that it encouraged more black hair and stimulated more sperms, stimulated vascular growth in the body, decreased hypercholesterolaemia, relieved bone pain and hypertension and improved blood circulation. It has been claimed that *Butea superba* Roxb could improve sexual function, prevented prostate cancer and prostatic hyperplasia. However, no work has been done on the appropriate dose of this plant extract to use in sexual dysfunction. So, the objective of this study was to investigate the effects of the tuberous root of *Butea superba* Roxb extract on the reproductive system of male mice.

Methods

Group 2 of male mice was fed daily with the crude extract in distilled water by oral oute at dose 1250 mg/kg. Group 3 was intraperitoneal injected with sildenafil citrate 10 mg/kg BW. The control group (group 1) was fed with 0.5 ml of distilled water. All groups were treated for 14 consecutive days. Blood and sperm were collected for analysis before treatment. Also, at the end of the experiments, blood, sperm and selected vital organs were collected for comparative measurement. A relative change of body weight was also analysed.

Results:

The results showed that and relative body weight in all treated groups were not significant difference from the control. Most of selected vital weight organs of both crude extract and sildenafil treated groups were not significant difference from control group. However, the stomach of crude extract group was more significantly heavy than control. Similarly, the testis and prostate of crude extract and sildenafil treated groups were more significantly heavy than control. The cholesterol level of crude extract and sildenafil of post-treated groups were significantly increased, whereas hemoglobin and haematocrit were significantly decreased compared to pre-treatment. Testosterone level and sperm number of crude extract and sildenafil treated groups were also significantly sharp increased, while sperm motility was not significant difference compared to control.

Conclusion:

This investigation provides evidence that *Butea superba* extract could increase testosterone and sperm number of mice compared to control. This finding may provide evidence that this extract may use to treat erectile dysfunctions in men.

Keywords: *Butea superba, testosterone, sperm motility, sperm count, sildenafil, cholesterol*

The effects of Red Kwao Kreu (*Butea superba* Roxb.) extract on reproductive system of male mice

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Abstract

Introduction and Objective: *Butea superba* Roxb, a plant in the family Leguminosae has the domestic name of "Red Kwao Kreu", is a herb with Thai medicinal properties that has been used since ancient times. *Butea superba* Roxb has been popularly used among Thai men for rejuvenation because it contains Phytoandrogen that has structure and function like testosterone. The effect of *Butea superba* Roxb has been reported that it encouraged more black hair and stimulated more sperms, stimulated vascular growth in the body, decreased hypercholesterolaemia, relieved bone pain and hypertension, and improved blood circulation. It has been claimed that *Butea superba* Roxb could improve sexual function, and prevented prostate cancer and prostatic hyperplasia. The objective of this study was to investigate the effects of the tuberous root of *Butea superba* Roxb extract compared with sildenafil on the reproductive system of male mice.

Methods: Thirty male mice were divided in 3 groups. Each group contained 10 mice. The control group (group 1) was fed with 0.5 ml of distilled water. Group 2 was fed daily with the crude extract in distilled water by oral route at the dose of 1,250 mg/kg. Group 3 was *intraperitoneal injected* with sildenafil citrate at the dose of 10 mg/kg BW/day. All groups were treated for 14 consecutive days. Blood and sperm were collected for analysis before (pre-) and after (post-) treatment in all groups. Also, at the end of the experiments, the selected vital organs were collected for comparative measurement. A relative change of body weight was also analysed.

Results: The results showed that and relative body weights in all treated groups were not significant difference from the control. Most of selected vital weight organs of

both crude extract and sildenafil treated groups were not significant difference from control group. However, the stomach of crude extract group was significantly heavier than control. Similarly, the testis and prostate of both crude extract and sildenafil treated groups were significantly heavier than control. The cholesterol level of crude extract and sildenafil of post-treated groups were significantly increased, whereas hemoglobin and haematocrit were significantly decreased compared to pre-treatment. Testosterone level and sperm number of crude extract and sildenafil treated groups were also significantly sharp increased, while sperm motility was not significant difference compared to control.

Conclusion: This investigation provides evidence that *Butea superba* extract could increase testosterone and sperm number of mice compared to control. This finding may provide evidence that this extract may use to increase testosterone and sperm number in men.

Keywords: *Butea superba, Testosterone, Sperm motility, Sperm count, Sildenafil, Cholesterol*

1. Introduction

"Red Kwao Kreu" is of one of the four types of Kwao Krua such as, White Kwao Krua, Kwao Krua Mor and Black Kwao Krua. *Butea superba* Roxb. This herb has Thai medicinal properties that has been used since ancient times **(1)**. The crude extract of *Butea superba* Roxb had activity in the preservation of the capillary wall, increased strength and resistance to the capillary by the contraction of blood vessels which resulted in creasing urination. It was found that anthocyanin may expel carcinogenic, may also have activity to expand blood vessels and may reduce the risk of heart disease and paralysis **(2).** Flavonoids have been reported that it can prevent the degeneration of cells caused by oxidant that come from interactions oxidation **(3).** It has been claimed that *Butea superba* Roxb. could improve sexual function and prevented prostate cancer and prostatic hyperplasia. Flavonoid and flavonoid glycoside from *Butea superba* Roxb. have been reported to show high inhibitory effects on cyclic-adenosine 3',5'-monophosphate phosphodiesterase (cAMPphosphodiesterase) **(4)**. Sildenafil citrate (Viagra) is the most commonly prescribed therapy for the treatment of erectile dysfunction (ED) and has been prescribed to more than 20 million men in more than 110 countries **(5)**.

2.Objectives

The purpose of this study was to investigation the effects of tuberous root of *Butea superba* Roxb. extract compared with sildenafil on the selected vital organ, blood chemistry, haematology, testosterone, sperm number and motility of male mice.

3. Methods

3.1 Extract preparation: Fresh tuberous roots of *Butea superba* Roxb. were collected from Chiang Rai province, Thailand. The plant specimens were authenticated by the Forest Herbarium, National Park, Wildlife, and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand. The tuberous roots were washed thoroughly and dried in an oven at 50 °C. The dried samples were ground to a fine powder. The powder was extracted with ethanol by Soxhlet extraction apparatus. The solvent was removed by evaporation under reduced pressure evaporator **(6)**.

3.2 Experimental animals: Thirty adult male mice, aged about 130 days, weighing 30-40 g, were obtained from the Animal Care Building, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The experimental protocol was approved in accordance with guideline for the care and use of laboratory animal by animal care and use committee (ACUC), Suranaree University of Technology.

3.3 Experimental procedures: Mice were divided into 3 groups with 10 animals each. Before treatment (Pre-treatment), All mice in these groups were collected blood and sperm for comparison with after treatment (Post-treatment). During treatment period, the first group was fed with 0.5 ml of distilled water and used as negative control. The second group was fed with *Butea superba* Roxb crude extract at the dose of 1,250 mg/kg BW/day. The third group was intraperitoneally injected with sildenafil at the dose of 10 mg/kg BW/day. The experiment was performed throughout 14 consecutive days. At the end of treatment period, all mice in these groups were collected blood and sperm again for comparison with pre-treatment in each mouse.

3.4 Sperm motility and sperm count assay: Sperm motility was done

according to the method of Bavister and Andrews (1988) (7).

Motility (%) =Number of motility x 100
Number of all (%)

The cauda epididymis was cut and weighed. A cell suspension was prepared by macerating the cauda in 1.0 ml of 0.85% saline. The cell suspension was kept for 24 hrs at 4 ºC. The suspension was then filtered through a double gauze layer and an aliquot of the sample was used for sperm count in a Makler counting chamber. An aliquot of the epididymal sperm suspension was smeared and stained with hematoxylin and eosin and then examined under a light microscope (CH-2, Olympus, Japan) at magnification of 100X. The head and tail abnormalities (200 sperms per animal) were be recorded **(8)**.

3.5 Haematology and blood chemistry: At the end of the experiment, blood samples were collected by cardiac puncture under ether anesthesia from 9.00 to 10.00 A.M. and partly used for haematology. The remainder blood serum was prepared by centrifugation at 1000×*g* for 30 min and kept at −20 ºC for blood chemistry analysis, including cholesterol **(9)**.

3.6 Histology of testis and Testosterone level determination:

After 14 days, the mice were sacrificed under thiopental sodium anesthesia and subjected to necropsy. The heart, liver, spleen, kidney, and stomach were removed and weighed. Body weight measured on the day of necropsy was used to calculate the relative organ weight. All organs were preserved in 10% (w/v) neutral phosphate buffer formaldehyde. Heart, liver, spleen, kidney, stomach and the reproductive organs (testis, seminal vesicle and prostate glands) fixed-tissue were embedded in paraffin and prepared for microtome sectioning at 5 □m and haematoxylin and eosin were used for staining. The histopathology of the organ tissue slides were examined under light microscope **(9)**. Concentrations of mice testosterone were measured by radioimmunoassay techniques using reagents obtained from the National Hormone and Pituitary Program **(2).**

3.7 Body weight:

The animal's weight was recorded every day throughout experimental peroid.

3.8 Statistical analysis: All data are presented as the mean ± S.E.M. Significant differences between the relative selected organ weight and body weight of control and treatment groups were analyzed by ANOVA. The difference of haematology and blood chemistry between pre- and post-treatment groups were calculated by paired student's *t-test*. The *P* values at < 0.05 and <0.01 were considered statistically significant difference. Significant difference between different mean (ā) in each group was compared using ANOVA and Tukey HSD post hoc test at $p<0.05$ and<0.01.

4. Results and Discussion

4.1 Sperm motility

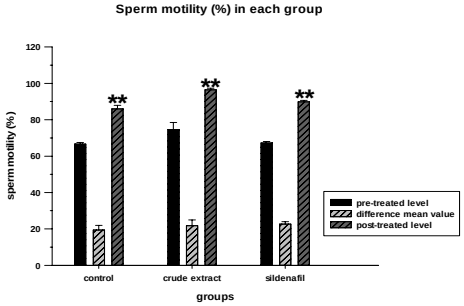

Figure 1 Effects of crude extract and sildenafil on sperm motility (%) of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day.

Figure 1 showed the effects of crude extract and sildenafil on sperm

motility (%) of mice. The results exhibited that there was significant increase in sperm motility of all of post-treated groups compared to pre-treatment (p<0.01). In contrast, the different means of sperm motility of crude extract and sildenafil treated groups were increased but not significant difference from control (p<0.01)

4.2 sperm count and morphology

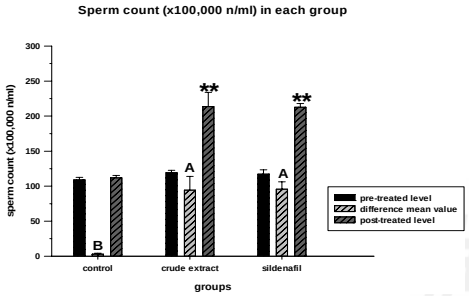

Figure 2 Effects of crude extract and sildenafil on sperm count (x100,000 n/ml) of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day.

(I) (II) (III)

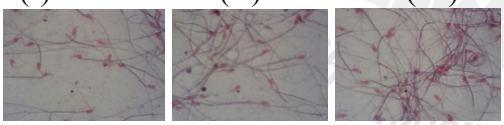

Figure 3 Micrographs of sperm morphology of mice; (I) control group; (II) crude extract treated group; (III) sildenafil treated group. All micrographs displayed at X100 magnifications.

Figure 2 showed the effects of crude extract and sildenafil on sperm number (x100,000 n/ml) of mice. The results exhibited that there was significant increase in sperm number of all post-treated groups compared to pre-treatment (p<0.01). In addition, the sperm number of crude extract and sildenafil treated groups were significantly higher than control (p<0.01). The morphology of these sperms seems normal appearance compared with control. This result is in substantial agreement with Manosroi et al., (2006) (10) that the sperm counts in male rats fed with 1,250 mg/kg BS crude drug group showed about 16% more than the control group.

4.3 Haematology and blood chemistry

The haematology and blood chemistry results showed that the cholesterol level of crude extract and sildenafil post-treated groups were significantly increased, whereas hemoglobin and haematocrit were significantly decreased compared to pre-treatment (p<0.05). Conversely, other parameters were not significant difference between pre- and post-treatment (Data not showed).

4.4 Histology of testis

(IV) (V) (VI)

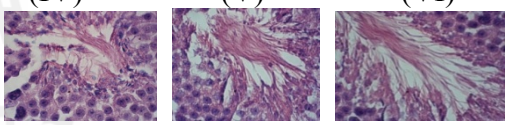

Figure 4 Micrographs of testicular (seminiferous tubules) section of mice; (IV) control group; (V) crude extract treated group; (VI) sildenafil treated group. All micrographs displayed at X100 magnification.

It can be seen from Figure 4 that the spermatogenesis of crude extract and sildenafil treated groups had more maturation process of spermatids than control. These spermatogenesis proceeds were higher in both spermatogenesis levels and spermatid numbers.

4.5 Testosterone Level

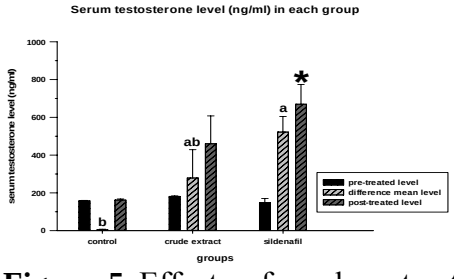

Figure 5 Effects of crude extract and sildenafil on testosterone level (ng/ml)

of mice. Group 1= control (DW) 0.5 ml/kg BW/day, Groups 2 = crude extract 1,250 mg/kg BW/day, Group 3 = sildenafil (IP) 10 mg/kg BW/day.

The effects of crude extract and sildenafil on testosterone level of mice is shown in figure 5. The results exhibited that there was significant increase in testosterone level of sildenafil post-treated group compared to pre-treatment (p<0.05). However, the testosterone level in pre-treated group was also raised in crude extract group but not significant difference (p<0.05) compared to post-treated. In addition, the testosterone level of sildenafil treated groups were significantly increased compared to control (p<0.01). The testosterone level of crude extract treated group was lower than sildenafil treated group but higher than control group without significant difference (p<0.05) (Fig. 5). These results are in substantial correspondence with Saraiva et al. (2009) (11) that sildenafil-treated mice showed significant increased levels of total testosterone compared to control.

4.6 Body weight

There was no significant difference in the relative growth rate measured by living body weight when compared to the control (Data not shown).

4.7 Selected vital organs

The results showed that most of selected vital organs weight of both crude extract and sildenafil treated groups were not significant difference from control group. However, the stomach weight of crude extract group was significantly heavier than control (p<0.05). Similarly, the testis and prostate weight of crude extract and sildenafil treated groups were significant heavier than control (p<0.01). These results seem consistent with Manosroi et al. (2006) (9) that *Butea superba* crude drug at the dose of 1,250 mg/kg showed significantly (p<0.05) higher percentage of testis/BW than the control.

The histopathology of the heart, liver, spleen, kidney, and stomach revealed normal appearance compared to the organs of control group (Data not shown).

5. Conclusion

This investigation provides evidence that *Butea superba* extract could increase testosterone and sperm number of mice compared to control, although this extract had effects on stomach, prostate gland, Hb and HCT when used in high dose. This finding may be applied that this extract may be used to increase testosterone and sperm number in men.

6. Acknowledgement

The author wish to thank the School of Biology and School of Pharmacology, Institute of Science, Suranaree University of Technology

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