GENETIC MODIFICATION OF YEAST CELLS TO DETECT PHYTOESTROGENS IN SOME MEDICINAL PLANTS AND TRADITIONAL MEDICINES

Palita Paewthaisong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

Academic Year 2020

การดัดแปลงพันธุกรรมเซลล์ยีสต์เพื่อใช้ตรวจหาสารไฟโตเอสโตรเจนในพืชสมุนไพรและยาแผนโบราณบางชนิด

นางสาวปาลิตา แปวไธสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2563

GENETIC MODIFICATION OF YEAST CELLS TO DETECT PHYTOESTROGENS IN SOME MEDICINAL PLANTS AND TRADITIONAL MEDICINES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Prof. Dr. Piyada Theerakulpisut)
Chairperson

(Assoc. Prof. Dr. Sineenat Siri)
Member (Thesis Advisor)

(Prof. Dr. Griangsak Eumkeb)
Member

(Asst. Prof. Dr. Santi Watthana)
Member

(Dr. Atcharaporn Thaeomor)
Member

(Assoc. Prof. Dr. Chatchai Jothityangkoon)
Vice Rector for Academic Affairs
and Quality Assurance

(Assoc. Prof. Dr. Worawat Meevasana)
Dean of Institute of Science

ปาลิตา แปวไธสง : การดัดแปลงพันธุกรรมเซลล์ยีสต์เพื่อใช้ตรวจหาสารไฟโตเอสโตรเจนในพืชสมุนไพรและยาแผนโบราณบางชนิด (GENETIC MODIFICATION OF YEAST CELLS TO DETECT PHYTOESTROGENS IN SOME MEDICINAL PLANTS AND TRADITIONAL MEDICINES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.สินีนาฏ ศิริ, 230 หน้า.

ประเทศไทยมีการใช้พืชสมุนไพรในตำรับยาแผนโบราณมาเป็นเวลานานเพื่อช่วยบรรเทาอาการจากประจำเดือนในสตรี อย่างไรก็ตาม ยังมีการตรวจวัดฤทธิ์เอสโตรเจนของพืชเหล่านี้น้อยมาก ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อสร้างระบบยีสต์ทูไฮบริดเพื่อใช้ในการตรวจวัดฤทธิ์เอสโตรเจนในพืชสมุนไพร 10 ชนิด และยาสมุนไพรที่มีการจำหน่าย 8 ชนิด โดยระบบยีสต์ทูไฮบริดที่สร้างขึ้นอาศัยหลักการปฏิสัมพันธ์ระหว่างบางส่วนของเอสโตรเจนรีเซฟเตอร์ชนิดแอลฟ่า (estrogen receptor, ERα) หรือเบต้า (estrogen receptor, ERβ) ลิแกนด์ (ligand) และโปรตีนโคแอคติเวเตอร์ (coactivator protein) คือ สเตียรอยด์รีเซฟเตอร์โคแอคติเวเตอร์ 1 (steroid receptor coactivator 1, SRC1) หรือ ทรานสคริปชันอินเตอร์มีเดียทแฟคเตอร์ 2 (transcription intermediated factor 2, TIF2) ระบบยีสต์ทูไฮบริดที่ดีที่สุด 2 ระบบที่ใช้ ERα และ ERβ ได้ถูกคัดเลือกเพื่อใช้เป็นระบบตรวจวัดฤทธิ์เอสโตรเจน ซึ่งเรียกว่าระบบ ERα-Y2H และ ERβ-Y2H โดยทั้งสองระบบมีประสิทธิภาพในการตรวจวัดสารมาตรฐาน 17 บีต้าเอสตราไดออล (17β-estradiol) และจีนิสทีน (genistein)

ระบบยีสต์ทูไฮบริดทั้งสองได้ใช้ในการตรวจวัดฤทธิ์เอสโตรเจนของสารสกัดเอทานอลและน้ำของพืชสมุนไพร 10 ชนิด คือ โกฐเชียง (*Angelica sinensis*) โกฐสอ (*Angelica dahurica*) คำฝอย (*Carthamus tinctorius*) ว่านชักมดลูก (*Curcuma xanthorrhiza*) กระดูกอึ่ง (*Dendrolobium lanceolatum*) ชะเอมเทศ (*Glycyrrhiza glabra*) ชะเอมจีน (*Glycyrrhiza uralensis*) ยอป่า (*Morinda coreia*) กวาวเครือขาว (*Pueraria mirifica*) และ ขิง (*Zingiber officinale*) ผลการศึกษาพบว่าสารสกัดเอทานอลของพืชมีฤทธิ์เอสโตรเจนสูงกว่าสารสกัดน้ำ นอกจากนี้สารสกัดเอทานอลมีฤทธิ์เอสโตรเจนผ่าน ERβ มากกว่า ในขณะที่สารสกัดน้ำมีฤทธิ์เอสโตรเจนผ่าน ERα มากกว่า จากสารสกัดทั้งหมดพบว่าสารสกัดเอทานอลของกระดูกอึ่งมีฤทธิ์เอสโตรเจนสูงที่สุดและผ่าน ERβ สารสกัดเอทานอลและน้ำจากพืชชนิดเดียวกันมีฤทธิ์เอสโตรเจนแตกต่างกัน ชี้ให้เห็นว่าการใช้ตัวทำละลายที่แตกต่างกันทำให้ได้สารออกฤทธิ์ที่แตกต่างกัน เมื่อนำสารสกัดเหล่านี้ไปย่อยแบบ *in vitro* พบว่ามีฤทธิ์เอสโตรเจนที่เปลี่ยนแปลงไป ซึ่งอาจเกิดจากสารเมแทบอไลต์ต่าง ๆ ที่ได้จากการย่อยด้วยเอนไซม์ สำหรับสารสกัดเอทานอลที่ผ่านการย่อย พบว่าส่วนใหญ่มีฤทธิ์เอสโตรเจนลดลงผ่านทั้ง ERα และ ERβ ในขณะที่มีสารสกัดเพียงบางชนิดเท่านั้นที่มีฤทธิ์เอสโตรเจนเพิ่มขึ้น ในทางตรงข้าม พบว่าสารสกัดด้วยน้ำที่ผ่านการย่อย ส่วนใหญ่มีฤทธิ์เอสโตรเจนผ่าน ERα เพิ่มขึ้น แต่มีฤทธิ์เอสโตรเจนผ่าน ERβ ลดลง

นอกจากนี้ได้ใช้ระบบ ERα-Y2H และ ERβ-Y2H ในการตรวจวัดฤทธิ์เอสโตรเจนของยาสมุนไพรทางการค้าจำนวน 8 ชนิด ซึ่งอ้างถือเป็น S1-S8 พบว่าสารสกัดเอทานอลมีฤทธิ์เอสโตรเจน

สูงกว่าสารสกัดน้ำ โดยส่วนใหญ่มีฤทธิ์เอสโตรเจนผ่าน ERα จากยาสมุนไพรทั้งแปดชนิด พบว่า S3 มีฤทธิ์เอสโตรเจนสูงที่สุด เมื่อสารสกัดนี้ผ่านการย่อยในหลอดทดลอง พบว่าฤทธิ์เอสโตรเจนเพิ่มขึ้นอย่างมีนัยสำคัญ โดยเฉพาะอย่างยิ่งผ่าน ERα นอกจากนี้พบว่าสารสกัดด้วยน้ำที่ผ่านการย่อยมีฤทธิ์เอสโตรเจนสูงกว่าสารสกัดจากเอทานอล ทั้งนี้สารสกัด S8 ที่ผ่านการย่อย มีฤทธิ์เอสโตรเจนสูงที่สุด ซึ่งมีค่า relative estrogenic activity ผ่าน ERα เท่ากับ 101.32% ทั้งนี้สันนิษฐานว่าฤทธิ์เอสโตรเจนของสารสกัด S8 นี้เป็นผลจากสารเมแทบอไลต์ของ ปักคี้ (*Astragalus membranaceus*) ชะเอมจีน (*Glycyrrhiza uralensis*) และแปะเจียก (*Paeonia lactiflora*) ที่เป็นองค์ประกอบหลัก

สาขาวิชาชีววิทยา
ปีการศึกษา 2563

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____

PALITA PAEWTHAISONG : GENETIC MODIFICATION OF YEAST CELLS TO DETECT PHYTOESTROGENS IN SOME MEDICINAL PLANTS AND TRADITIONAL MEDICINES THESIS ADVISOR :
ASSOC. PROF. SINEENAT SIRI, Ph.D. 230 PP.

ESTROGENIC ACTIVITY/MEDICINAL PLANTS/PHYTOESTROGEN/ TRADITIONAL MEDICINCES/YEAST TWO HYBRID

In Thailand, medicinal plants have long been used in traditional recipes for improving menstruation symptoms in females; however, their estrogenic activities have been poorly evaluated. Thus, this study aimed to construct the yeast two-hybrid system (Y2H) to determine estrogenic activities of ten medicinal plants and eight commercially traditional medicines. The produced Y2H systems were based on the specific interaction among the partial estrogen receptors alpha or beta, the ligands, and the coactivator proteins. Two best Y2H systems were chosen for determining estrogenic activity based on ERα and ERβ, referred to as ERα-Y2H and ERβ-Y2H systems. Both systems efficiently detected estrogenic activities of the standard 17β-estradiol and genistein. Both Y2H systems were used to detect the estrogenic activity of ethanolic and water extracts of 10 medicinal plants; *Angelica sinensis*, *Angelica dahurica*, *Carthamus tinctorius*, *Curcuma xanthorrhiza*, *Dendrolobium lanceolatum*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Morinda coreia*, *Pueraria mirifica*, and *Zingiber officinale*. The results revealed that the ethanolic extracts of these plants exhibited estrogenic activities higher than their water extracts. Also, the ethanolic extracts exhibited estrogenic activities preferentially via ERβ, whereas their water extracts were preferentially via ERα interaction. Among extracts, the ethanolic extract

of *D. lanceolatum* exhibited the highest estrogenic activity via ERβ. The ethanolic and water extracts of the same plant species exhibited different estrogenic activities, suggesting different active compounds obtained by different extraction solvents. When these extracts were *in vitro* digested, their estrogenic activities were changed, likely due to their different metabolites obtained by the enzymatic digestion. For the ethanolic extracts, their digested extracts mostly exhibited the reduction of estrogenic activity via both ERα and ERβ, whereas only a few digested extracts possessed increased estrogenic activity. In contrast, the digested water extracts mostly exhibited increased estrogenic activity via ERα, whereas possessed the reduction of estrogenic activity via ERβ.

ERα-Y2H and ERβ-Y2H systems were also used to detect the estrogenic activity of 8 commercially traditional medicines referred to as S1-S8. Their ethanolic extracts exhibited higher estrogenic activities than their water extracts. Also, their estrogenic activities were more potent via ERα. Among eight medicines, S3 exhibited the highest estrogenic activity. When these extracts were *in vitro* digested, their estrogenic activities significantly increased, particularly via the interaction with ERα. Also, the digested water extracts exhibited significantly higher estrogenic activities than those of ethanolic extracts. The digested S8 exhibited the highest estrogenic activity with the relative estrogenic activity of 101.32% via ERα. This estrogenic activity of the digested S8 was hypothesized to be resulted from the metabolites derived from *A. membranaceus*, *G. uralensis*, and *P. lactiflora*, which were the major ingredients.

School of Biology — Student's Signature P. Paeratthaisong

Academic Year 2020 — Advisor's Signature

ACKNOWLEDGEMENTS

The author would like to acknowledge the funding support from the OROG scholarship of the Suranaree University of Technology.

I would like to express my deepest and sincere gratitude to my advisor Assoc. Prof. Dr. Sineenat Siri for giving me the opportunity to be her advisee and allowing me to conduct the research for my thesis. I have also appreciated her valuable supervision, suggestion, encouragement, support, guidance and criticism throughout the course of my study.

My appreciation is also expressed to all staff in the School of Biology, Institute of Science, Suranaree University of Technology and all teachers, who sharpen my scientific knowledge and help me with the paperwork and instrument. I thank all SIRI lab members that give me helps and supports.

I would like to express my sincere gratitude to my deceased aunt, Mrs. Noowan Juntepa who collected the plant samples for my thesis.

I would like to express my sincere gratitude to my deceased father, Mr. Boonjun Paewthaisong who gave me love.

Finally, I would like to express my sincere gratitude and appreciation to my mother, Mrs. Nongkran Paewthaisong who give me a chance to study and all way support me.

Palita Paewthaisong

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

AD	Activation domain
BD	Binding domain
cDNA	Complementary DNA
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
E2-REP_{10}	10% relative potency as compared with E2
E3	Estriol
E4	Estetrol
ED	Endocrine disruptor
ERE	Estrogen response element
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERα-Y2H	Estrogen receptor alpha-yeast two hybrid system
ERβ	Estrogen receptor beta
ERβ-Y2H	Estrogen receptor beta-yeast two hybrid system
ER1	Estrogen receptor 1
ER2	Estrogen receptor 2

LIST OF ABBREVIATIONS (Continued)

FRET	Fluorescence resonance energy transfer
HA	Hyaluronic acid
HRT	Hormone replacement therapy
hSRC1	Human steroid receptor coactivator 1
HT-29	Human colorectal adenocarcinoma cell
hTIF2	Human transcriptional intermediary factor 2
LBD	Ligand binding domain
MCF-7	Breast cancer cell line
MDA-MB-231	Breast cancer cell line
NCBI	National Center for Biotechnology Information
NCOA2	Nuclear receptor coactivator 2
NTD	NH_2-terminal domain
PCR	Polymerase chain reaction
REC_{10}	10% relative effective concentration
RID	Receptor interaction domain
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-polymerase chain reaction technique
SD	Synthetic dropout medium
Y2H	Yeast two hybrid system

CHAPTER I

INTRODUCTION

1.1 General Introduction

Thailand has abundant natural resources, especially plant biodiversity, as well as the accumulated knowledge of how to use these plants for medicinal applications. In Thailand, at least 1,800 plant species are reported as the ingredients of traditional medicines (Schippmann et al., 2002). The learning experience from the ancient times in using various parts of each medicinal plant for healing and treating human illness has been transferred from generation to generation in Thai society. The knowledge of Thai traditionally herbal medicine is one of the most valuable heritages from Thai ancestors and many traditionally medicinal remedies have been commercially produced and promoted as the alternative medicine for primary health care.

In Thailand, the market of herbal products in 2020 reached 180,000 million baths, in which the majority of revenue came from cosmetics (10,000 million baths), supplement products (80,000 million baths), and traditional medicine (10,000 million baths), which the herbal market has been predicted to increase each year (Kwankhao et al., 2020). The surveys of medicinal plants and natural products in Thai markets revealed that traditional women remedies and postpartum drugs were the best sellers. Also, many women preferred to use traditional medicines for adjusting normal menstrual cycle, promoting blood circulation, lightening skin color and balancing women's health (S. Poomirat et al., 2020a; Wisodsongkram and Kitiyodom, 2020). In

general, these tradition remedies for women include at least one of medicinal plants containing phytoestrogenic compounds, for example Wan Chak Mot Luk (*Curcuma comosa* Roxb.), white Kwao Krua *(Pueraria mirifica* Airy Shaw & Suvat, Wan Sak Lek (*Molineria latifolia* (Dryand. ex W.T.Aiton) Herb. ex Kurz), safflower (*Carthamus tinctorius* L), and Szechwan lovage rhizome (*Conioselinum univitatum* Trucz.).

Phytoestrogens or phytoestrogenic compounds are naturally produced compounds produced by certain plant species that have chemical structure similar to human estrogen and possess either estrogenic or anti-estrogenic activities through the interaction with estrogen receptors (ERs) in animal and human cells (Glazier and Bowman, 2001). Four major classes of phytoestrogens found in typical human diets are isoflavones (concentrated in soybeans and soy products), stilbenes (distributed in red wine and peanuts), coumestans (found in broccoli and sprouts), and lignans (found in seeds, whole grains, berries, and vegetables) (Poluzzi et al., 2014). The physiological functions of phytoestrogens on animal and human cells are largely mediated through ERs; estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Paterni et al., 2014). In humans, each ER is differently expressed in tissues/organs at divergent levels to control physiological functions of various organ systems, such as reproductive, skeletal, cardiovascular, and central nervous systems. There are some common physiological roles for the two ERs, such as in the development and function of the ovaries, and in the protection of the cardiovascular system. In principle, ERα is present mainly in mammary gland, uterus, ovary, bone, prostate, liver, and adipose tissue. The alpha subtype has a more prominent role on the mammary gland and uterus, as well as on the preservation of skeletal homeostasis and the regulation of metabolism. By contrast, ERβ is found mainly in the prostate, bladder, ovary, colon, adipose tissue, and

immune system. The beta subtype seems to have a more profound effects on the central nervous and immune systems (Warner et al., 2017). In general, the ERα activation by estrogens is considered responsible for the enhanced proliferation of MCF-7 cell, whereas this is counteracted by the presence of ERβ, which exerts an anti-proliferative effect (B. Huang et al., 2014). From these informations, phytoestrogens that are capable to bind only certain ER subtype or both ER subtypes are very likely to exhibit similar or different effects on human. Nevertheless, the information regarding the binding capacity of each phytoestrogen to the ER subtypes is very limited and still in need, which is one of the interested purposes of this study. In addition, the information of whether the traditionally medicinal plants for women remedies really contains phytoestrogens is still questionable. Thus, the simple, easy, and fast method to determine phytoestrogens in medicinal plants is also in need and it is one of the interested questions in this study.

To determine whether the medicinal plants contain phytoestrogens, several in vitro techniques have been developed, which generally measure estrogenic and anti-estrogenic activities of the plant extracts. Examples of in vitro assays to determine estrogenic and anti-estrogenic activities are listed below. The E-screen assay is the classic estrogenic activity assay, which is based on the use of established estrogen-responsive cell lines, such as ERα

positive MCF-7 cell line, to determine the estrogenic activities of the tested compounds via the evaluation of the increase of cell proliferation in response to the tested compounds. The limitation of this assay is the long assay duration of approximately 5-7 days to determine the cell proliferative effect (Soto et al., 1995). Alternatively, the two hybrid assay offers a shorter assay time (few hours) and a higher sensitivity of

detection. This assay relies on the use of yeast or mammalian cells that genetically modified for determining the ligand-ER interaction.

Based on the advantages of the two-hybrid assay, this work is interested on the production of the yeast two hybrid (Y2H) system to determine phytoestrogens in medicinal plant extracts and commercial traditional medicines. In this system, the genetically modified yeast cells can produce two fusion proteins; the DNA binding domain (BD) of GAL4 fused ligand binding domain (LBD) of ERs (referred to as BD-ER) and the activation domain (AD) of GAL4 fused receptor interaction domains (RID) of co-activation (CO) proteins (referred to as AD-CO). Two CO proteins are used in this work; transcriptional intermediary factor 2 (TIF2) and steroid receptor coactivator-1 (SRC1). The interaction between the tested compounds (phytoestrogens) and the BD-ER protein recruits the AD-CO proteins, thus reconstituting a functional transcription factor. As the result, RNA polymerase II is recruited to activate the expression of the reportor gene, the beta-galactosidase gene. With the substrate, 2-nitrophenyl-D-galactoside (ONPG), the enzyme beta-galactosidase can change the substrate to the purple product, which can be measured by a spectrophotometer. The color intensity reflects the phytoestrogenic activity of the tested compounds. Thus, the Y2H system is the simple and fast assay is not only to dissect the functions of the tested compounds through each ER subtype, but also to identify its effective co-activation proteins, which differently localizes at different tissues/organs in order to understand their molecular actions (Bruckner et al., 2009; Mueller, 2002).

1.2 Objectives of the Thesis

1) To construct four Y2H systems and compare their capabilities to measure estrogenic activity of the standard 17β-estradiol (E2)

- pGBKT7-hERα and pGADT7-hTIF2 system
- pGBKT7-hERα and pGADT7-hSRC1 system
- pGBKT7-hERβ and pGADT7-hTIF2 system
- pGBKT7-hERβ and pGADT7-hSRC1 system

2) To use the suitable Y2H systems to determine estrogenic activities of ethanolic and water extracts of 10 medicinal plants via the interaction of ERα and ERβ and to compare the estrogenic activities of these ethanolic and water extracts under the digested and undigested conditions.

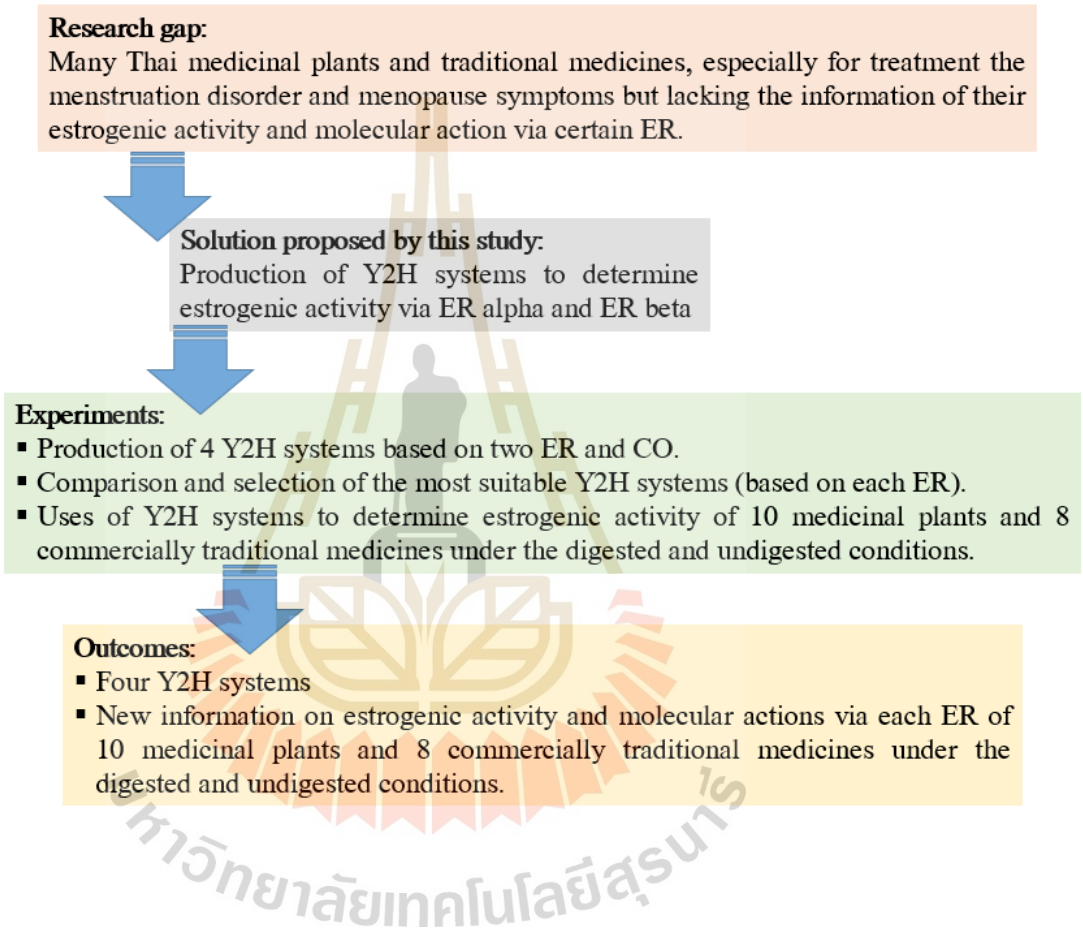
3) To use the suitable Y2H systems to determine estrogenic activities of ethanolic and water extracts of 8 commercial traditional medicines via the interaction of ERα and ERβ and to compare the estrogenic activities of these ethanolic and water extracts under the digested and undigested conditions.

1.3 Scopes of the Thesis

The scopes of this study are shown below, which cover the following experiments.

- The production of four Y2H systems
- The efficacies of four Y2H systems to determine the estrogenic activity of the standard E2.

- Uses of the selected Y2H systems (based on ERα and ERβ) to determine the estrogenic activities of ethanolic and water extracts of 10 medicinal plants and 8 commercial traditional medicines at the digested and undigested conditions.



CHAPTER II

LITERATURE REVIEW

2.1 Estrogens and Estrogen Receptors

2.1.1 Estrogens

Estrogens are steroid hormones primarily known for their roles in the promotion of female sex characteristics and reproductive capability. In humans, estrogens are produced from cholesterol in the ovaries and adrenal glands and circulate throughout the body where they have effects on most organs and systems, including brain, breast, bladder, skin, bone, cardiovascular system, immune system, and reproductive system (Figure 2.1–2.2) (Mancinelli et al., 2010). There are 4 types of estrogens in the female body: estrone (E1), estradiol (E2), and estriol (E3), and estetrol (E4). During a woman's reproductive years, the principal circulating estrogen is 17β-estradiol (E2), which is also the most potent form of estrogen (Fuentes and Silveyra, 2019) The cellular effects of estrogens are mediated by two estrogen receptors, ERα and ERβ. The level of E2 synthesis is a critical biochemical change for females and the sharp reduction in E2 levels during menopause is commonly associated with various diseases observed in post-menopausal women (Amenyogbe et al., 2020). Postmenopausal women appears to have the climacteric, hot flashes, vasomotor symptoms, cardiovascular disease, and high risk of osteoporosis (Edwards et al., 2019). Hormone replacement therapy (HRT) was shown to be effective in reducing some symptoms relating with this menopausal transition; such as hot flashes. Also, Women's

Health Initiative (WHI) study suggested that the HRT was efficient to prevent fractures associated with osteoporosis in postmenopausal females (Rossouw et al., 2002). Nevertheless, there has been a controversial question of the association between HRT and breast cancer as some researches showed an increased risk of invasive breast cancer associated with the use of exogenous estrogens (Ringa et al., 2005). Therefore, there have been many global efforts to develop the suitable preparations that yield the benefits of HRT with minimal health risks for postmenopausal females (Brzezinski and Debi, 1999). Among these practices, estrogens from foods have been suggested due to their low side effects, thus food supplements derived from plants have received increasing interests in the markets.

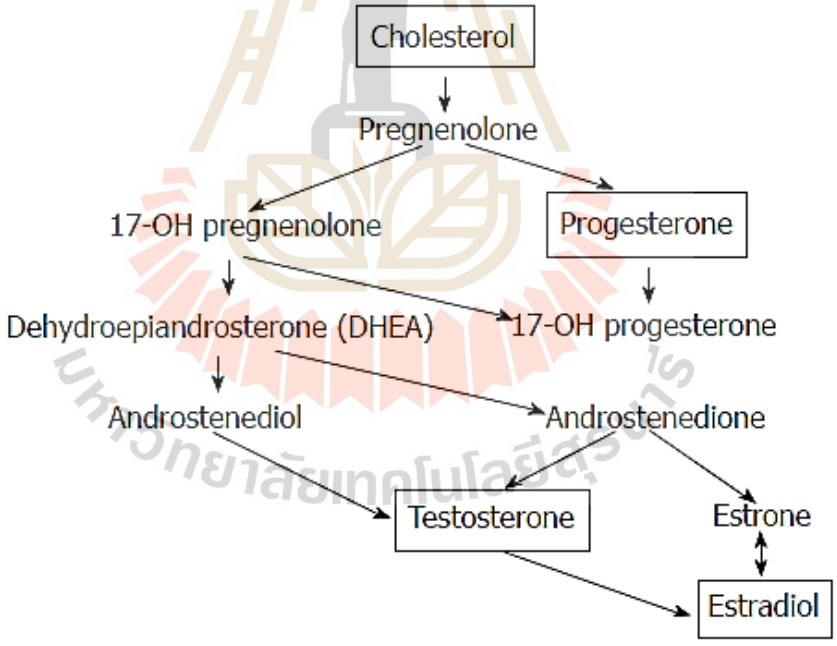


Figure 2.1 The synthesis pathway of estrogen from cholesterol (Mancinelli et al., 2010).

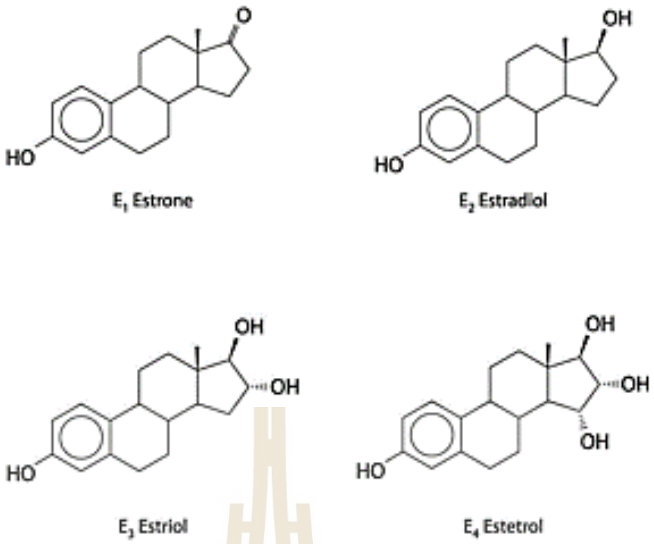


Figure 2.2 Four types of estrogens and their synthesis pathway from cholesterol (Mancinelli et al., 2010).

2.1.2 Mechanisms of estrogen action

Estrogens can penetrate through the phospholipid bilayer of cells and specifically bind to the estrogen receptors (ERs) in the cytosol and move into the nucleus (Macgregor and Jordan, 1998). With estrogen, the receptors form a dimer and bind to specific response elements (estrogen response elements; EREs) located in the promoters of the target genes. Hormone binding also induces a conformational change within the ligand binding domain of the receptors, thus allowing co-activator proteins to interact with (Bjornstrom and Sjoberg, 2005). These ligand-protein complex can induce the transcription by interacting with and activating necessary components of the transcriptional apparatus (Figure 2.3).

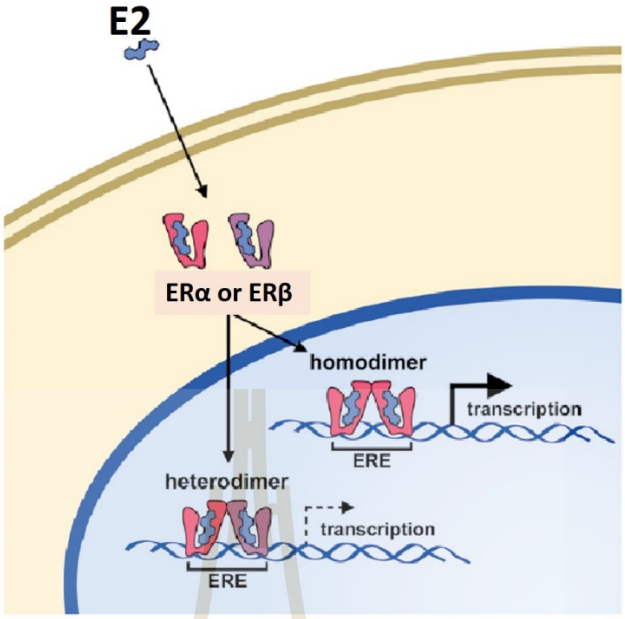


Figure 2.3 Mechanism of estradiol to activate the gene expression in the cell (Bean et al., 2014).

2.1.3 Estrogen receptors

The biological effects of estrogens are mediated by ERs. The mammalians have two ligand-activated transcription factors that bind estrogens, encoded by separate genes, estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ). Structure of ERs can be divided into 6 domains (A-F), which are important for their functions (Figure 2.4).

- The NH_2-terminal domain (NTD) of the A/B domain
- The C domain (DNA-binding domain; DBD)
- The D domain (a hinge region)
- The E domain (ligand biding domain; LBD)
- The F domain (C-terminal extension region of the LBD)

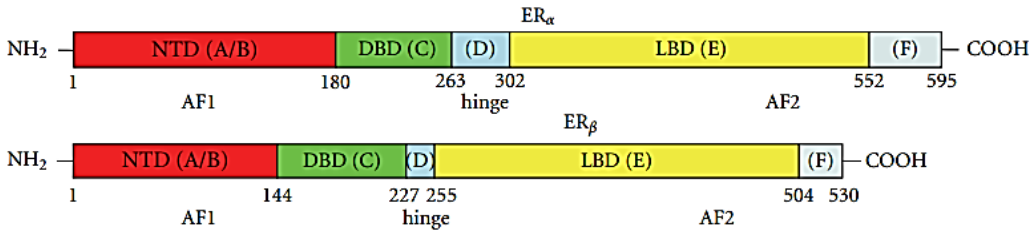


Figure 2.4 Schematic representation of the functional domain organization of ERα and ERβ (Kumar et al., 2011).

Both ERs share structural characteristics that are responsible for similar functional features. Estradiol binding induces a major structural re-organization of LBD that converts the inactive ERs to the functionally active form by generating surfaces for enhancing stability of the ER dimer and of the interacting co-regulatory protein (Thakur et al., 2009).

Interestingly, ERα and ERβ have different distributions throughout the body (Figure 2.5). ERα is highly expressed in the uterus, prostate stroma, ovarian theca cells, leydig cells in testis, epididymis, breast, and liver. ERβ is highly expressed in prostate epithelium, testis, ovarian granulosa cells, bone marrow, and brain. Since ERα and ERβ have different downstream transcriptional activities, they have different tissue-specific biological actions (Warner et al., 2017).

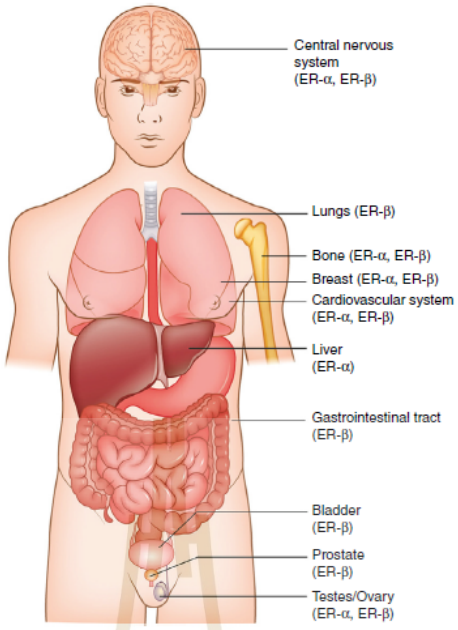


Figure 2.5 Different distribution of ERα and ERβ throughout the body (Warner et al., 2017).

2.2 Phytoestrogen

2.2.1 Classification of phytoestrogens

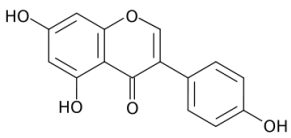
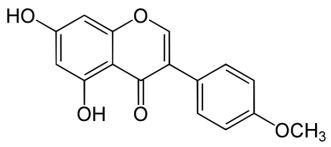
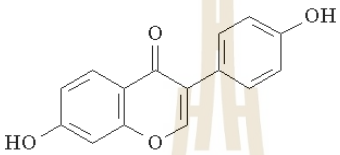
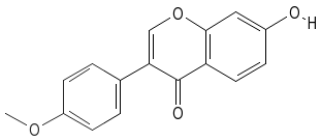
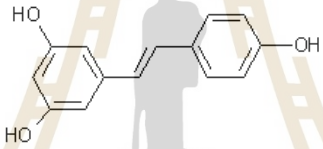
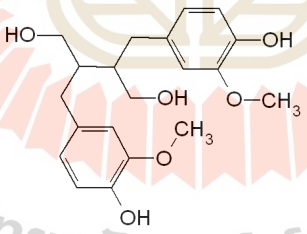
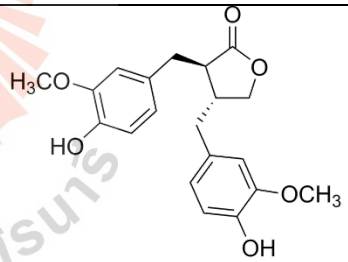
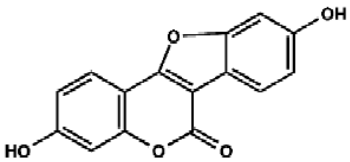
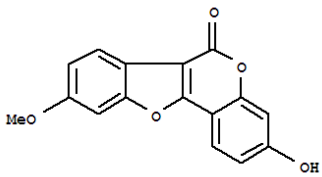
Phytoestrogens are plant-derived dietary compounds, found in a wide variety of foods, especially in soy. They represent a diverse group of naturally occurring chemicals with structural similarity to 17β-estradiol (E2), the primary female sex hormone. Their structural similarity to E2 enables them to cause estrogenic/anti-estrogenic effects by binding to ERs (Rietjens et al., 2017). It is thought that phytoestrogens can replace estrogens during hormone replacement therapy (Gupta et al., 2016). Phytoestrogens are classified in four main distinct classes: isoflavones, lignans, coumestans, and stilbenes (Moreira et al., 2014). The chemical structures of major phytoestrogens are shown in Table 2.1.

Isoflavones are the most well-known phytoestrogens and found in the Fabaceae family. The major sources of isoflavone are food legumes such as soy, peanut, and clover. The members of isoflavones are such as biochanin A, daidzein, formononetin, and genistein. Among isoflavones, genistein has received the most attention due to a controversy on its activity in relation to breast cancer. The cell proliferation activity of genistein have been tested with several breast cancer cell lines and the results indicated that its activity depended on the concentration. At low concentration it could promote the proliferation of cancer cells, but showed anti-proliferation activity at high concentration (Sirotkin and Harrath, 2014; Shigeyuki Kawai et al., 2010; Cornwell et al., 2004).

Stilbenes are secondary metabolites that play key roles in plant protection against environmental stresses and resistance to pathogens. The main dietary source of phytoestrogenic stilbenes is resveratrol from red wine and peanuts (Chong et al., 2009; Cornwell et al., 2004).

Lignans were first identified in plants and later in biological fluids of mammals. The most well-known phytoestrogenic lignans are secoisolariciresinol and matairesinol, which are converted by bacterial action in the gut into enterodiol and enterolactone. Lignans are commonly found in rye bread and oilseeds such as flaxseed (Kiyama, 2016).

Table 2.1 Chemical structures of some major phytoestrogens.

phytoestrogen	Structure	
Isofavones	 Genistein	 Biochanin A
	 Daidzein	 Formononetin
Stilbenes	 Resveratrol	
Lignans	 Secoisolariciresinal	 Metairesinal
Coumestans	 Coumestrol	 4-Methoxycoumestrol

Sources: (Sirotkin and Harrath, 2014; Cornwell et al., 2004).

Coumestans are another group of plant phenols that show estrogenic activity. The main coumestans with phytoestrogenic effects are coumestrol and 4′-methoxycoumestrol. They are especially high in clover and soy sprouts (Sirotkin and Harrath, 2014).

2.2.2 Mechanisms of phytoestrogen action

Phytoestrogens are proposed to interact with both ERs and display estradiol-like effects (Figure 2.6). Although phytoestrogens have similar molecular structure to estradiol (Figure 2.7), they have lower affinity for ERs than estradiol and most of them exhibit a higher affinity for ERβ than ERα (Turner et al., 2007). After binding to ERs, the ER-phytoestogen complexes can translocate into the nucleus to activate the expression of the responsive genes.

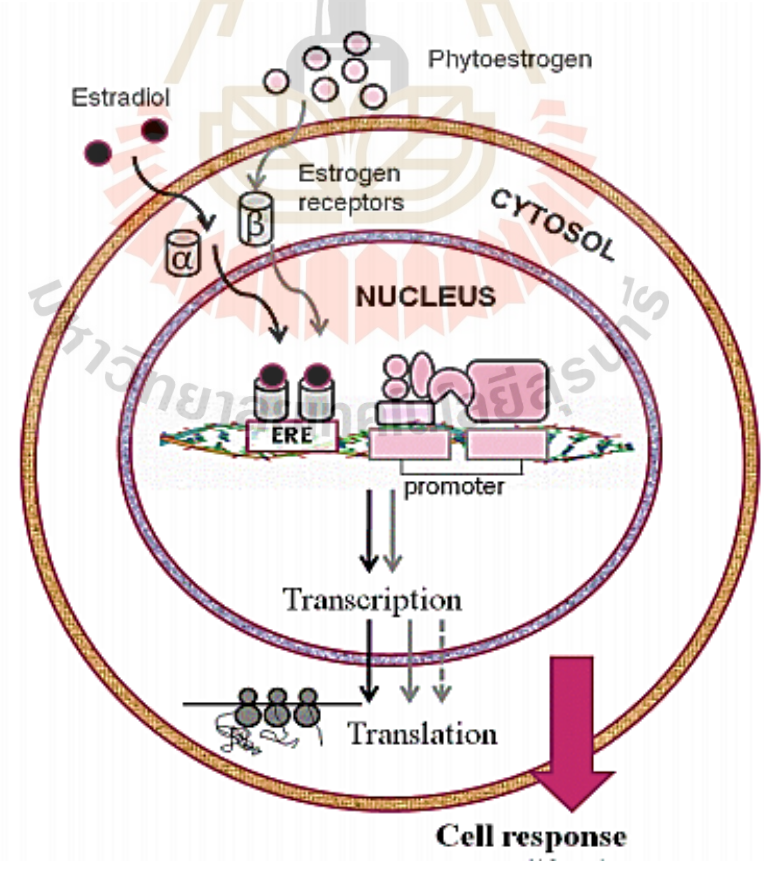
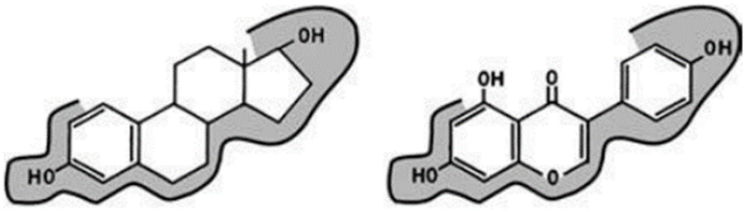


Figure 2.6 Phytoestrogen mechanism of action (Ruff et al., 2000).



Estradiol **Phytoestrogen Genistein**

Figure 2.7 Structure of the human steroid hormone estradiol and the phytoestrogenic genistein (Demmig and Mcuauley, 2005).

2.3 Traditional Medicinal Plants Used for Female

In many countries, including Thailand, medicinal plants and traditional medicines have very high impacts on local women's healthcare, especially in rural areas. Effective traditional medicines have been commercially produced as traditional medicines in modern packages and some have been developed as new pharmaceutical products. Some common medicinal plants used for female fertility are listed in Table 2.2.

In this work, ten plants were chosen to investigate their estrogenic activities according to Thai traditional medicines (Table 2.3, Figure 2.8). Among these plants, *Angelica dahurica* (Piao et al., 2006), *Carthamus tinctorius* (Young Seok Lee et al., 2009), *Curcuma xanthorrhiza* (Anggakusuma et al., 2009), *Glycyrrhiza glabra* (Dong et al., 2007), *Glycyrrhiza uralensis* (K. U. Kim et al., 2020), *Pueraria mirifica* (Cherdshewasart and Sriwatcharakul, 2008), and *Zingiber officinale* (In Gyu Kim et al., 2008) were previously reported for their estrogenic activities, whereas *Angelica sinensis*, *Dendrolobium lanceolatum*, and *Morinda coreia* had not reported yet. Nevertheless, the estrogenic activities of these plants through ERα or ERβ were not clear, leading to the investigation in this study.

Table 2.2 Medicinal plants used for female.

Plant species	Effects
Angelica sinensis (Oliv.) Diels	Promote blood circulation, treat blood deficiency pattern, and menstrual disorders (Wei et al., 2016)
Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav.	Exhibit strong reducing power, superoxide dismutase activity, catalase activity, DNA damage prevention, and antiproliferative activity against HT-29 and CMT-93 cell lines (Pervin et al., 2014).
Achyranthes aspera L.	Exhibit anti-fertility effects. Use as an abortifacient and contraceptive. Use to treat menstrual disorders (De Bore and Cotingting, 2014)
Ammannia baccifera L.	The p-hydroxybenzoic acid of this plant: estrogen-like effects, inducing vaginal cornification and uterotrofic activity in both immature female mice and ovary ectomize mice (Setchell et al., 1999).
Arachis hypogaea L.	High levels of phytoestrogens that structurally or functionally mimic mammalian estrogens (Al-Snafi, 2015; Ibarreta et al., 2003).
Artemisia vulgaris L.	Traditionally use in Southeast Asia: treat menstrual conditions (amenorrhea, dysmenorrhea and oligomenorrhea, and pregnancy disorders) (Kumar et al., 2011).

Table 2.2 Medicinal plants used for female (Continued).

Plant species	Effects
Aquilaria crassna Pierre ex Lecomte.	Crude extracts and some isolated compounds: exhibit anti-allergic, anti-inflammatory, anti-diabetic, anti-oxidant, anti-ischemic, and anti-microbial activities (Shigeyuki. Kawai et al., 2010).
Asplenium trichomanes L.	Use for expectorant, anti-cough remedy, laxative, emmenagogue, abortifacient, and irregular menstrual cycle (Dallacqua et al., 2009).
Caesalpinia crista L.	Decrease the duration of estrous cycle and average ovarian weight (Amudha et al., 2016).
Carica papaya L.	Flavonoid quercetin in leaves: exhibit estrogenic effects (Sugiyanto et al., 2012)
Cassia siamea Lam.	Exhibit antimicrobial, antimalarial, antidiabetic, anticancer, hypotensive, diuretic, antioxidant, laxative, anti-inflammatory, analgesic, antipyretic, anxiolytic, antidepressant, and sedative activities (Mamadou et al., 2014)
Cicer arietinum L.	Isolated isoflavones: exhibit estrogenic activity (Hairong et al., 2013).
Citrus medica L.	Extract of *Citrus medica* L. Seeds: exhibit potent antiovulatory activity (Sang Jun Lee et al., 1998).
Curcuma longa L.	Treat menstrual disorders (Thakur et al., 2009).

Table 2.2 Medicinal plants used for female (Continued).

Plant species	Effects
Carthamus tinctorius L.	Treat menstrual abnormal symptom, cardiovascular disease, pain, and swelling associated with trauma. The active compounds: exhibit anti-coagulantion, vasodilation, anti-hypertension, anti-oxidation, neuroprotection, melanin production inhibition, immunosuppression, and antitumor activity with trauma complications (Delshad et al., 2018; Asgarpanah and Kazemivash, 2013; Hao et al., 2010).
Curcuma xanthorrhiza Roxb.	Isolated sesquiterpenoid from rhizome: exhibit the estrogenic activity through ERs (De Bore and Cotingting, 2014)
Glycyrrhiza uralensis Fisch. ex DC.	Alleviate menopausal symptoms via estrogen receptors (K. U. Kim et al., 2020).
Picrorhiza kurroa Royle ex Benth.	Treat gastrointestinal, urinary disorders, and inflammatory (Shetty et al., 2010)
Pueraria mirifica Airy Shaw & Suvat.	Treat symptoms related to estrogen deficiency in menopausal women (Suchinda Malaivijitnond, 2012).
Saussurea lappa (Decne.) C.B.Clarke	Treat irregular menstruation, tenesmus, and abdominal pain (Zahara et al., 2014)

Table 2.3 List of ten plants and their parts used in this study.

Plant species	Common name	Used part
Angelica sinensis (Oliv.) Diels.	Dong-quai	Root (Wei et al., 2016)
Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav.	Bai-zhi	Root (Kyungjin Lee et al., 2015)
Carthamus tinctorius L.	Safflower	Flower (Delshad et al., 2018)
Curcuma xanthorrhiza Roxb.	Java ginger	Rhizome (Anggakusuma et al., 2009)
Dendrolobium lanceolatum (Dunn) Schindl.	-	Root (Albertazzi and Purdie, 2002)
Glycyrrhiza glabra L.	Licorice	Root (Jafari et al., 2019)
Glycyrrhiza uralensis Fisch.	Chinese liquorice	Root (Yang et al., 2017)
Morinda coreia Buch.-Ham.	Hairy noni	Bark (Avasthi et al., 2020)
Pueraria mirifica Airy Shaw & Suvat.	Kwao-krua	Tuberous root (Suchinda Malaivijitnond, 2012)

Remark: Species names according to The Plant List (TPL).

Figure 2.8 Images of ten plants used in this study.

2.4 Thai Medicinal Plants and Traditional Medicines for Woman

Based on Thai traditional medicines, several parts of diverse plant species have been used as the major ingredients for relieve symptoms relating to female reproductive system. Currently, some traditional medicines and plant extracts have been commercialized as capsule and liquid products for treating symptoms relating to menstruation disorder and for dietary supplements. The examples of these medicinal plants and concentrations that often found in traditional medicines are shown in Table 2.4.

2.5 *In Vitro* Assays to Determine Estrogenic and Anti-estrogenic Compounds.

Several *in vitro* assays to determine estrogenic and anti-estrogenic compounds have been developed. These assays are summarized in Table 2.5.

Table 2.4 Medicinal plants and other natural components used as ingredients in 10 brands of Thai traditional medicines for women.

	Commercial products	Types	Medical uses	Main ingredient	
				Phytoestrogens	Unknown activity
1	Porkhuneia-bor	Liquid	Menstruation disorders, hematonic	*Codonopsis pilosula* (Chiu et al., 2014)	*Angelica sinensis*, *Leonurus heterophyllus*, *Ligusticum sinense*
2	Flora	Liquid	Menstruation disorders, hematonic	*Carthamus tinctorius* (Young Seok Lee et al., 2009), *Cassia siamea* (Mamadou et al., 2014), *Dalbergia candenatensis* (Ali M El-Halawany et al., 2011), *Panax ginseng* (Joonwoo Park et al., 2017), *Curcuma zanthorrhiza* (Demmig and Mcuauley, 2005)	*Artemisia annua*, *Curcuma zedoaria,* *Leonurus heterophyllus*, *Ligusticum sinense*, *Ligusticum acutilobum*, *Picrorhiza kurroa*, *Plumbago indica*
3	Tankwe	Pill	Menstruation disorders, haematinic, postnatal	*Astragalus propinguus* (C. Z. Zhang et al., 2005), *Codonopsis pilosula*.	*Angelica sinensis*, *Albizia myriophylla*, *Ligusticum sinense*, *Paeonia lactiflora*, *Poria cocos*, *Rehmannia glutinosa*

Table 2.4 Medicinal plants and other natural components used as ingredients in 10 brands of Thai traditional medicines for women (Continued).

	Commercial products	Types	Medical uses	Main ingredient	
				Phytoestrogens	Unknown activity
4	Chom-tong	Capsule	Irregular bleeding, dysmenor-rhea	*Angelica dahurica* (Piao et al., 2006), *Carthamus tinctorius*, *Curcuma zanthorrhiza*, *Glycyrrhiza uralensis*, *Panax ginseng*.	*Amomum xanthioides*, *Angelica sinensis, Asclepias curassavica*, *Mimusops elengi*, *Molineria latifolia*, *Leonurus heterophyllus* *Ligusticum sinense*, *Zingiber cassumunar*
5	Penpark	Capsule	Irregular menstruation, haematinic	*Carthamus tinctorius*	*Angelica sinensis*, *Aquilaria crassna*, *Caesalpinia sappan*
6	Leng-khun	Capsule	Irregular menstruation, haematinic	*Codonopsis pilosula*, *Glycyrrhiza glabra* (Dong et al., 2007), *Curcuma zanthorrhiza*, *Panax ginseng*, *Pueraria mirifica* (Cherdshewasart and Sriwatcharakul, 2008)	*Aucklandia lappa*, *Angelica sinensis, Atractylodes lancea*, *Corydalis yanhusuo*, *Cyperus rotundus*, *Leonurus heterophyllus*, *Poria cocos*, *Rehmannia glutinosa*

Table 2.4 Medicinal plants and other natural components used as ingredients in 10 brands of Thai traditional medicines for women (Continued).

Commercial products		Types	Medical uses	Main ingredient	
				Phytoestrogens	Unknown activity
7	Chomjai	Capsule	Irregular menstruation, haematinic	*Carthamus tinctorius*, *Curcuma zanthorrhiza*, *Panax ginseng*, *Panax notoginseng* (Chan et al., 2002), *Zingiber officinale* (In Gyu Kim et al., 2008)	*Asclepias curassavica*, *Asprella hexandra*, *Caesalpinia sappan*, *Citrus hystrix*, *Ligusticum acutilobum*, *Molineria latifolia*, *Picrorhiza kurroa*, *Piper nigrum*, *Piper retrofractum*, *Piper ribesioides*, *Piper sarmentosum*, *Plumbago indica*, *Zingiber cassumunar*
8	Meree	Capsule	Menstrual disorder, blood tonic	*Curcuma zanthorrhiza*, *Panax ginseng*	*Ligusticum acutilobum*, *Ligusticum sinense*, *Poria cocos*
9	Ladina	Capsule	Irregular menstruation, blood tonic	*Carthamus tinctorius*, *Curcuma zanthorrhiza*	*Caesalpinia sappan*, *Piper nigrum*, *Leonurus heterophyllus*, *Ligusticum acutilobum*, *Senna garrettiana*
10	Kwao-Krua	Capsule	Tonic	*Panax ginseng*, *Pueraria mirifica*	*Ligusticum acutilobum*

Table 2.5 Comparison of *in vitro* assays to evaluate the estrogenic and anti-estrogenic compounds.

***In vitro* assay**	**Measured endpoint**	**Advantages**	**Limitation**
E-screen assay	Proliferation of ERα-positive cells	Measure physiological endpoint of estrogen action, measure estrogenic and anti-estrogenic effects	No defined ER expression (Mueller, 2002)
Ligand-binding assay	Binding affinity to ERα and ERβ	Simple and high throughput	Dose not measure ER activation (Mueller, 2002)
Fluorescence resonance energy Transfer (FRET) assay	Ligand-dependent association of ERα or ERβ with co-activators	Analysis of molecular interaction, versatile: defined ER subtypes or ER domains as well as co-activators can be used, measures estrogens and anti-estrogens	Artificial system, does not measure ER activation, low throughput (Mueller, 2002)
Transactivation assay in yeast or mammalian cells	ERα, ERβ mediated activation of reporter	Simple and high-throughput, measures estrogens and anti-estrogens	Artificial system (Mueller, 2002)

Table 2.5 Comparison of in vitro assays to evaluate the estrogenic and anti-estrogenic compounds (Continued).

***In vitro* assay**	**Measured endpoint**	**Advantages**	**Limitation**
Analysis of gene and protein expression	Expression of ER-regulated genes and proteins	Analysis of physiological response, versatile, measures estrogens and antiestrogen	Low-throughput (Mueller, 2002)
Analysis of enzyme activity and steroid biosynthesis	Activity of stereoidogenic enzymes, ER regulated enzymes and analysis of estrogen biosynthesis	Analysis of physiological response, measures estrogens and antiestrogens	Only cell lines with active steroidogenesis and marker enzymes suitable (Mueller, 2002)
Yeast two hybrid assay	Protein-protein interaction associated of ERα or ERβ with ligand and co-activator	Defined ER subtypes or ER domains as well as co-activators can be used, measures estrogens and anti-estrogens	Some natural protein-protein interaction cannot be detected by Y2H system (such as glycosylated, oxidatively modified proteins) (Koegl and Uetz, 2007).

2.6 Review of Related Studies

The endocrine disruptors (EDs) is referred to exogenous substances that have adverse effects in hormonal system to human health and wildlife. The examples of EDs in environment are phytoestrogens (chemicals synthesized by plants), mycoestrogens (chemicals synthesized by fungi), and xenoestrogen (chemicals synthesized in industries) (Darbre, 2019). Among several *in vitro* methods, the Y2H system is one of the effective assays to evaluate endocrine disruptors (EDs), which this method is based on ligand-dependent interaction between nuclear hormone receptor and co-activator that is developed in a yeast cell.

In 1999, Nishikawa and colleagues were the first group reporting the development of yeast two hybrid (Y2H) system to detect the estrogen (17β-estradiol or E2) based on the interaction between the ligand binding domain (LBD) of rat estrogen receptor (rERα; amino acid residues 252 to 600) and the receptor interaction domain (RID) of several co-activators. Among co-activators, RID of human transcriptional intermediary factor 2 (hTIF2; amino acid residues 624 to 1287) exhibited the greatest activity followed by RID of human steroid receptor coactivator 1 (hSRC1; amino acid residues 570 to 782). The developed Y2H systems were also effective to other natural estrogen (estriol, E3), phytoestrogen (genistein), and xenoestrogens (diethystilbesterol (DES), *p*-nonylphenal, and bisphenol A) (Nishikawa et al., 1999).

In general, environmental estrogens are known as the endocrine disruptors (ED), which their potential effects to animal and human have been concerned. In 2000, this research team, thus, reported the use of the rERα-hTIF2 Y2H system to evaluate the estrogenic activity of 517 chemicals derived from natural substances, medicines, pesticides, and industrial chemicals. The results reported as the 10% relative effective

concentration (REC_{10}), the concentration of the test chemical having 10% agonist activity of 10^{-7} M E2. The positive result was determined when the test substance within the concentration tested, had the higher activity than REC_{10}. Out of 517 tested chemicals, 64 chemicals were positive, suggesting that this assay was efficient to detect xenoestrogens and phytoestrogens (Nishihara et al., 2000).

As there are two receptors (ERα and ERβ) for estrogens, Lee and colleagues (2002) developed the Y2H systems based on the LBDs of hERα and hERβ (amino acid residues 311 to 595 and 213 to 477, respectively) and the nuclear receptor binding domain (NRBD) of co-activators (hSRCl and hTIF2; amino acid residues 231 to 1094 and 670 to 1750, respectively). They reported that LBD-hERβ and NRBD-hSRCl was excellent for evaluating the estrogenic activity of xenoestrogens and phytoestrogens than the previous yeast system using rat ERα that was described by Nishikawa and colleagues (Haeng. Seog Lee et al., 2002).

In 2004, Ahn and colleagues were used the developed Y2H system of Nishihara and colleagues to evaluate the estrogenic activity of the extract derived from the Chinese medicinal plant, *Moghania philippinensi*, used for reliving symptoms of menopausal syndrome. Also, the estrogen-dependent proliferation of MCF-7 (human breast cancer) cells was used to compare with the Y2H system. The methanol-extract of this plant was purified into five fractions, in which two fraction exhibited the estrogenic activity as determined by both Y2H system and MCF-7 cell proliferation assay. Interesting, one fraction exhibited the estrognic activity as determined by MCF-7 proliferation assay but not Y2H system. Nevertheless, the Y2H system was efficient and required less time to operate as compared with MCF-7 cell proliferation assay (Ahn et al., 2004).

Later, several research teams reported the uses of Y2H systems to detect estrogenic and anti-estrogenic activities of crude and purified plant extracts as follows. In 2007, El-Halawany and colleagues studied the estrogenic activity of isolated compounds of *Cassia tora*. This Y2H system was based on the Nishikawa's report (amino acid residues of hERα at 247 to 595 and hERβ at 213 to 503) (Ali Mahmoud El-Halawany et al., 2007). Later, this research team also reported the anti-estrogenic activity of the methanol extract of the heartwood of *Mansonia gagei*, which is folkloric medicine in Thailand and it can be used as a cardiac stimulant, anti-emetic, anti-depressant, and refreshing agent. Three new coumarins (mansorins I, II, III) and naphthoquinones (mansonone) were isolated from this plant extract. These compounds had the higher binding affinities to hERβ than to hERα (Ali Mahmoud El-Halawany et al., 2007; Kanayama et al., 2003). In 2010, this research team reported the use of Y2H system to detect the estrogenic activity of the *Sophora japonica* L. extract, which this Egypt plant was chosen due to its high flavonoid content. Also, the estrogenic activities of the isolated compounds under naringinase-treated and untreated conditions were investigated. Naringinase is a debittering enzyme complex containing β-glucosidase and α-rhamnosidase enzyme, which can simulate a metabolism process in a gastrointestinal tract (GIT). Interestingly, the nariginase-treated extracts contained the aglycones genistein and jaempferol that exhibited the estrogenic activity similar to genistein. However, the compounds detected in the untreated extract showed weak estrogenic activity and bind to ERβ only (A. M. El-Halawany et al., 2010). In 2011, this research team reported the use of Y2H system to screen the estrogenic and anti-estrogenic activities of 40 medicinal plants growing in Egypt and Thailand. It was noted that no Thai researcher was in this research team. Both hERα and hERβ were used as

well as the naringinase-treated and untreated samples were studied. The results showed that the extracts of *Derris reticulata* and *Dracaena lourieri* had the strong potent estrogenic activity for both estrogen receptor subtypes. Only *Butea monosperma*, *Erythrina fusca* and *Dalbergia candenatensis* extracts were specific to hERβ. The *Nigella sativa*, *Sophora japonica*, *Artabotrys harmandii* and *Clitorea hanceana* extracts exhibited the estrogenic effect only after naringinase-treatment. Among 40 species, the extracts derived from *Aframomum melegueta*, *Dalbergia candenatensis*, *Dracena loureiri* and *Mansonia gagei* showed the most potent anti-estrogenic activity via the hERβ interaction (Ali M El-Halawany et al., 2011).

In 2010, Cherdshewasart and colleagues studied estrogenic and anti-estrogenic activities of the Thai plant, *Butea superba* Roxb, under the native and *in vitro* hepatic digestion by the use of Y2H systems (hERα-hTIF2 and hERβ-hSRC1) as described by Nishikawa's report. This work reported that *Butea superba* extracts could function via the stronger interaction to hERα than hERβ (Cherdshewasart et al., 2010).

In 2010, Boonchird and colleagues also reported the use of Y2H systems (hERα-hTIF2 and hERβ-hSRC1) based on the Nishikawa's report to determine the estrogenic activity of the other Thai plant, *Pueraria mirifica* (Boonchird et al., 2010). This plant was collected from 27 provinces in Thailand to determine the variation of estrogenic activity. Plant samples showed different estrogenic activity and some samples showed no estrogenic activity. Based on the hERα-hTIF2 system, the top five samples had 0.006-0.011% of the activity of E2, while on the hERβ-hSRC1 system, those were 0.027-0.091% of the activity of E2. These results suggested that the action of phytoestrogens of *Pueraria mirifica* was more potent via the interaction to hERβ (8.3 folds) than hERα.

In 2018, Wang and colleagues reported the use of Y2H system based on the Nishihara's report (hERα-hTIF2 and hERβ-hTIF2) to examine the estrogenic activity of crude drugs derived from five widely prescribed Japanese herbal medicines for menopausal syndrome. The samples were under the metabolic activation using the rat liver S9 fraction and the un-activation conditions. The results showed that only three herbal drugs exhibited estrogenic activity and their metabolites exhibited the stronger activities. One of the herbal drugs, Unkeito, was further purified, which four chemicals were detected. Interestingly, the synergistic effects were detected in some combinations of these chemicals (Zeyun Wang et al., 2018).

In 2020, Basu and colleagues (Basu et al., 2020) reported the use of Y2H system (hERα) to determine the estrogenic and antiestrogenic activities of latex extract of *Euphorbia bicolor*, a plant native to south-central USA. The isolated genistein and coumestrol from the plant latex exhibited biphasic effects on Y2H system. At 1-5 μM, both compounds stimulated estrogenic activities; however, at 5 μM both compound exhibited both estrogenic and antiestrogenic activities.

Based on these publications, the Y2H system is efficient to examine the estrogenic activity of crudes and purified phytochemicals. Although the Y2H systems were used for determine the estrogenic activity of some Thai medicinal plants, due to diverse plant species in Thailand, especially ones used for herbal medicines relating to female treatment, there are still many medicinal plants and traditional medicines lacking the information about their estrogenic activities and their actions via hERα and hERβ. Therefore, this work is interesting to investigate the activity of 10 medicinal plants and 8 traditional medicines that have not report on their estrogenic/anti-estrogenic activities via both estrogen receptors.

CHAPTER III

METHODOLOGY

3.1 Construction of Recombinant Plasmids

Four recombinant plasmids were constructed; pGADT7-hSRC1, pGADT7-hTIF2, pGBKT7-hERα, and pGBKT7-hERβ. The DNAs encoding hSRC1, hTIF2, hERα, and hERβ were obtained by the reverse transcription-polymerase chain reaction (RT-PCR) technique. Total RNA of MCF-7 cells was extracted by using *TRIzol® reagent* (Thermo Fisher Scientific, Waltham, MA, USA) and purified by phenol-chloroform. The quantity and quality of the RNA were determined by measuring the absorption at 260 and 280 nm. The cDNA was generated by the reverse transcription of 2 μg of total RNA using SuperScript III first-strand synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR reactions were performed using the cDNA as a template and all primer pairs were shown in Table 3.1. The PCR products were analyzed by electrophoresis and the DNA bands stained with ethidium bromide were visualized under an UV trans-illuminator.

Table 3.1 List of PCR primers.

Gene	Accession No.	Primer name	Sequence
Homo sapiens nuclear receptor coactivator 1 (NCOA1, SRC1)	NM_147223.2	hSRC1-upper	5′TCAATTCAAGAGGATGGAGAAG3′
		hSRC1-lower	5′CATGCCTGATCTCATATTGATG3′
		hSRC1-NdeI upper	5′CCAATTCCATATGTCAATTCAAGAGGATGG3'
		hSRC1-SmaI-lower	5′TCTCCCGGGTTCATGCCCTGATCTCATATTG3′
Homo sapiens mRNA for transcriptionintermediary factor 2	X97674.1	hTIF2-upper	5′AGAAGAGCTGACGGGCAGG 3′
		hTIF2-lower	5′GCTCATAGTTGCTGGCATACC 3′
		hTIF2-NdeI-upper	5′CCAATTCCATATGGAGAGAGCTGACGGGC 3′
		hTIF2-SmaI-lower	5′TCTCCCGGGTTGCTCATAGTTGCTGGC 3′
Homo sapiens estrogen receptor 1 (ERα, ESR1)	NM_000125.1	hERα-upper	5′AAAGGTGGGATACGAAAAGAC 3′
		hERα-lower	5′TCAGACTGTGGCAGGGAAAC 3′
		hERα-NdeI-upper	5′CCAATTCCATATGAAAGGTGGGATACGAAAAG3′
		hERα-BamHI-lower	5′CGGGATCCGTCAGACTGTGGCAGGGAAACC 3′
Homo sapiens estrogen receptor 2	NM_001437.2	hERβ-upper	5′ACAAGGAGTTGGTACACATGATCAG 3′
		hERβ-lower	5′TTACATCTACAGCAGCAGGTCATA3′
		hERβ-NdeI-upper	5′GGGTTTCATATGGACAAGGAGTTGGTACAC3′
		hERβ-BamHI-lower	5′CGGATCCTTACATCTCCAGCAGCAGGTC 3′

To construct pGADT7-hSRC1, the RT-PCR reaction was performed, which the hSRC1-upper and hSRC1-lower primers were used as the initial primer set in the first PCR. Then, the hSRC1-NdeI-upper and hSRC1-SmaI-lower primers were used in the second PCR, in which the first PCR product was used as the template. The amplified SRC1 fragment was ligated to pTG19T cloning vector (Vivan Technologies, Selangor, Malaysia). After the recombinant pTG19T-hSRC1 plasmid was obtained, the DNA fragment of hSRC1 was cut off using NdeI and SmaI restriction enzymes. The excised DNA was subcloned into the pre-cut pGADT7-vector using the same restriction enzymes (Takara Bio, Mountain View, CA, USA). After the recombinant pGADT7-hSRC1 plasmid was obtained, it was sequenced by the Bio Basic DNA Sequencing Service (Bio Basic, Markham, Canada) to confirm the correct open reading frame before using.

To construct pGADT7-hTIF2, the hTIF2 DNA were obtained by RT-PCR. The hTIF2 cDNA was amplified using the hTIF2-upper and hTIF2-lower primers in the first PCR. In the second PCR, the hTIF2-NdeI-upper and hTIF2-SmaI-lower primers were used and the PCR product from the first PCR was used as the template. The amplified hTIF2 fragment was ligated to pTG19T cloning vector. After the recombinant pTG19T-hTIF2 plasmid was obtained, the DNA fragment of hTIF2 was cut by NdeI and SmaI restriction enzymes. The excised DNA was subcloned into the pre-cut pGADT7 vector using the same restriction enzymes. After the recombinant pGADT7- hTIF2 plasmid was obtained, it was sequenced to confirm the correct open reading frame before using.

To construct pGBKT7-hERβ, the hERβ DNA was obtained by RT-PCR reaction, which two PCR steps were performed. The first PCR was used the hERβ-upper and hERβ-lower primers and the second PCR was used the hERβ-NdeI-upper and hERβ-

BamHI-lower primers. The amplified hERβ fragment was ligated to pTG19T cloning vector. After the recombinant pTG19T-hERβ plasmid was obtained, the hERβ DNA was excised using NdeI and BamHI and subcloned into the pre-cut pGBKT7-vector. After the recombinant pGBKT7-hERβ plasmid was obtained, it was sequenced to confirm the correct open reading frame before using.

To construct pGBKT7-hERα, the hERα DNA was synthesized by RT-PCR technique. Two PCR steps were performed, which the first PCR was used the hERα-upper and hERα-lower primers, while in the second PCR, the first PCR product was amplified using the hERα-NdeI-upper and hERα-BamHI-lower primers. The amplified hERα fragment was ligated to pTG19T cloning vector. After the recombinant pTG19T-hERα plasmid was obtained, the hERα DNA fragment was excised using NdeI and BamHI restriction enzymes. The excised DNA was subcloned into the pre-cut pGBKT7-vector before transforming into *E. coli*. After the recombinant pGBKT7-hERα plasmid was obtained, it was sequenced to confirm the correct open reading frame before using.

3.2 Transformation

3.2.1 Bacteria transformation

E. coli strain DH5α was used as the host cell for pGADT7-hTIF2, pGADT7-hSRC1, pGBKT7-hERα, and pGBKT7-hERβ. To prepare the competent cells, the bacteria were grown on a Luria-Bertani (LB) agar plate. Single colony was suspended in 5 ml LB broth medium and grown at 37 °C for 6 h. Cells were diluted in LB broth to obtain an OD600 of 0.15 prior to cultivating overnight for 150 rpm at 18 °C. The cell culture was grown at 18 °C until reached an OD600 of 0.75. The bacterial culture was

chilled on ice for 10 min, harvested by centrifugation at 2,000 ×g for 10 min, and washed with cold TB buffer (10 mM Pipes, 55 mM $MnCI_2$, 15 mM $CaC1_2$, 250 mM KCI). The cells were dispersed in TB buffer and 7% DMSO, frozen, and stored at -80 °C. When used, the frozen stock of competent cells was thawed on ice before adding 10 ng plasmid DNA. After chilling on ice for 30 min, the mixture was transferred to a water-bath at 42 °C for 30 sec and then hold on an ice-bath for 1 min. Then, 0.8 ml of LB broth was added to the mixture and subsequently cultivated for 1 h at 37 °C for 180 rpm. The cells were plated on LB agar plates containing the appropriated antibiotic and the transformation efficiency was calculated as the colony forming unit (cfu) per µg plasmid DNA (Inoue et al., 1990).

3.2.2 Yeast transformation

The yeast used in this experiment is *Saccharomyces cerevisiae* strain AH109 (Clontech Laboratories, Mountain View, CA, USA). Yeast cells were co-transformed with each pair of recombinant plasmids using a lithium acetate transformation method (Shigeyuki. Kawai et al., 2010); 1) pGADT7-hSRC1 and pGBKT7-hERα, 2) pGADT7-hSRC1 and pGBKT7-hERβ, 3) pGADT7-hTIF2 and pGBKT7-hERα, and 4) pGADT7-hTIF2 and pGBKT7-hERβ. The transformed yeast cells were selected on the agar plates containing synthetic dropout (SD) medium lacking tryptophan and leucine according to the Clontech yeast protocol handbook. The clones grown on the selective agar media were selected and subsequently cultured in the selective liquid media.

3.3 Purification of Plasmid DNA

Single bacterial colony was cultured for 12-16 h in 3 ml LB broth with the appropriate antibiotics in an incubator shaker at 37 °C. The bacterial culture was

collected by centrifugation at 6,000g ×g for 5 min at 4 °C. The cell pellet was resuspend in 100 µl cold solution I (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 µg/ml Rnase A). Then, the solution II (200 mM NaOH, 1% SDS) of 200 µl was added to lyse the bacterial cells and the tube was inverted several times to well mix. The solution was incubated at room temperature for 5 min. Once the cells were lysed, the cold solution III (3.0 M potassium acetate, pH 5.5) of 150 µl was added and gently mixed by inversion several times. The solution mixture was incubated on ice for 20 min before centrifuging at 12,000 ×g for 20 min at 4 °C. The supernatant containing the plasmid DNA was removed to a new tube before RNaseA (2 mg/ml) of 5 µl was added. It was incubated at 37 °C for 5 min. The equal volume of TE-saturated phenol-chloroform was added to the plasmid DNA sample before centrifuging at 12,000 ×g for 5 min at 4 °C. The aqueous phase containing plasmid DNA was collected before the equal volume of chloroform was added. The mixture was centrifuged again at 12,000 ×g for 20 min at 4 °C. The aqueous phase containing plasmid DNA was removed to a new tube and precipitated by adding 0.1 volume of 3 M sodium acetate (NaOAc) pH 5.2 and 2 volumes of ethanol (EtOH). The solution was mixed and incubated at -20 °C for 30 min. The mixture was centrifuged at 12,000 ×g for 20 min at 4 °C. The pellet was washed with 70% EtOH and air-dried for 30 min. The DNA pellet was re-dissolved with 50 µl TE buffer and stored at -20 °C until use.

3.4 Extraction of Medicinal Plants and Traditional Medicines

In this work, 10 medicinal plants and 8 commercially traditional medicines were analyzed as listed in Table 3.2-3.3, respectively.

Table 3.2 Medicinal plants used in this work.

	Medicinal plants		
	Scientific name	Common name	Used parts
1	*Angelica sinensis*	dong quai	Root (Wei et al., 2016)
2	*Angelica dahurica*	bai zhi	Root (Kyungjin Lee et al., 2015)
3	*Carthamus tinctorius*	safflower	Flower (Delshad et al., 2018)
4	*Curcuma xanthorrhiza*	java ginger	Rhizome (Anggakusuma et al., 2009)
5	*Dendrolobium lanceolatum*	-	Root (Albertazzi and Purdie, 2002)
6	*Glycyrrhiza glabra*	licorice	Root (Jafari et al., 2019)
7	*Glycyrrhiza uralensis*	chinese liquorice	Root (Yang et al., 2017).
8	*Morinda coreia*	-	Bark (Avasthi et al., 2020).
9	*Pueraria mirifica*	kwao krua	Tuberous root (Suchinda Malaivijitnond, 2012).
10	*Zingiber officinale*	ginger	Rhizome (Chen X. Chen et al., 2016).

Table 3.3 Commercially traditional medicines used in this work.

S1	Chomjai	Capsule	*Curcuma xanthorrhiza* 7.63%, *Carthamus tinctorius* 5.1%, *Zingiber officinale* 2.54%, *Panax ginseng* 1.27%, *Panax notoginseng* 1.27%
S2	Chomtong	Capsule	*Carthamus tinctorius* 3.24%, *Glycyrrhiza uralensis* 3.24%, *Curcuma xanthorrhiza* 3.24%, *Angelica dahurica* 1.94%, *Panax ginseng* 0.97%, *Codonopsis pilosula* 0.97%
S3	Kwaokrua	Capsule	*Panax ginseng* 22%, *Pueraria mirifica* 20%
S4	Ledina	Capsule	*Carthamus tinctorius* 14%, *Curcuma xanthorrhiza* 14%
S5	Lengkhun	Capsule	*Curcuma xanthorrhiza* 12.5%, *Panax ginseng* 12.5%, *Codonopsis pilosula* 7.5%, *Pueraria mirifica* 2.5%, *Glycyrrhiza glabra* 2.5%
S6	Meree	Capsule	*Curcuma xanthorrhiza* 20%, *Panax ginseng* 3.33%
S7	Penpark	Capsule	*Carthamus tinctorius* 2.92%
S8	Tankwe	Pill	*Codonopsis pilosula* 7.34%, *Astragalus propinquus* 7.34%

Note: Composition as indicated by the commercial products

3.4.1 Plant extraction

The medicinal plants were collected in the areas of Burirum and Nakhonratchasima, Thailand. Voucher specimens were preserved in the Klong Phai plant conservation center. The plants were cleaned, sliced, and completely dried in a hot-air oven at 50 °C before grinding to powder. The extraction method is based on the method of Zhang and colleagues (C. Z. Zhang et al., 2005). The powder was extracted by 70% ethanol at the 1:8 (w/v) ratio for 24 h with shaking at room temperature. The ethanol extraction was evaporated under vacuum and the obtained gummy-solids were freeze-dehydrated in a freeze-dryer. Each crude plant extract was kept in a tightly-capped tube in a light protected container and stored at -20 °C

3.4.2 Extraction of traditional medicines

The commercially traditional medicines for treating female-hormone imbalance symptoms were purchased from local pharmaceutical shops. The extraction method was based on Zhang and colleagues (C. Z. Zhang et al., 2005). The powder medicines were extracted with 70% ethanol (1:8, (w/v)) for 24 h with shaking at room temperature. The ethanol extraction was evaporated under vacuum and the obtained gummy-solid was freeze-dried. Each crude plant extract was kept in a tightly-capped tube in a light protected container and stored at -20 °C.

3.4.3 *In vitro* simulation of digestion process

In vitro simulation of digestion process was described by Sommerfeld and colleagues (Sommerfeld et al., 2017; Tarko et al., 2009; Zyla et al., 2007). The extraction was hydrated in double-distilled water and 1.5 M HCl was added to adjust the pH to 5.8. The sample was placed into a gently moving water-bath at 37 °C for 30 min. Following incubation, 1.5 M HCl was added to adjust the pH to 2.0 before adding

3000 U pepsin (Sigma-Aldrich, Louis, MO, USA). The tube was mixed and further incubated for 2 h. Then, 1 M $NaHCO_3$ containing 3.7 mg pancreatin/ml (Sigma-Aldrich, Louis, MO, USA) was added to adjust pH to 7. The mixture was transferred into a dialysis tube (molecular weight cut-off 12,000-14,000) (Membrane filtration products, Seguin, TX, USA) before placing in an Erlenmeyer flask containing 50 ml phosphate buffer and incubating at 37 °C for 4 h with stirring. The PBS buffer together with the compounds that passed through the membrane (dialysate) were treated as an equivalent of the raw material absorbed in the intestine after digestion. The digestion product was freeze-dehydrated in a freeze-dryer and stored at -20 °C.

3.5 Determination of Estrogenic Activity

To examine the estrogenic activity of the extracts, the induction of β-galactosidase activity in the Y2H systems expressing ERα and ERβ couple with the co-activation proteins (SRC1 and TIF2) was carried out. The genetically-engineered yeast cells were cultured in 5 ml liquid SD medium without tryptophan and leucine at 30 °C overnight in a shaker incubator for 180 rpm. The overnight culture was vortexed for 0.5-1 min to disperse cell clumps before transferring (2 ml) to 8 ml yeast extract peptone dextrose (YPD) medium. The fresh culture was incubated at 30 °C for 3-5 h by shaking (180 rpm) until the cells were in a mid-log phase (OD_{600} of 1 ml at 0.5-0.8). The exact OD_{600} was recorded. The culture of 1.5 ml was transferred to a glass tube and the 19-µl tested chemical in dimethyl sulfoxide (DMSO) or 19-µl of DMSO (as control was added before incubating at 30 °C for 5 h. The treated yeasts were collected by centrifugation and washed with 1.5 ml Z buffer before resuspending in 0.3 ml Z buffer. The cell suspension (0.1 ml) were transferred to a new micro-centrifuge tube and broken down

by freeze-thawing. Then, Z buffer of 700 µl (containing 0.27% β-mercaptoethanol) and 2-nitrophenyl-β-D-galactoside of 160 µl (ONPG, 4 mg/ml stock) was added to the cell lysate to start the reaction. When the yellow color developed, 1 M Na_2CO_3 (100 µl) was added to stop the reaction. The β-galactosidase activity was calculated by the following equation (Miller, 1972; Miller, 1992).

$$\beta\text{–galactosidase unit} = \frac{[1000 \times OD420]}{[t \times V \times OD600]}$$

where: t is the elapsed time (in min) of incubation

V is 0.1 ml × concentration factor

OD_{420} nm is the absorbance by o-nitrophenol

OD_{600} nm is the cell density at the start of assay

The estrogenic activity of the samples was also calculated for the 10% relative effective concentration (REC_{10}), which was the concentration of the sample exhibiting 10% the highest estrogenic of E2 in each Y2H system. The samples that exhibited the REC10 values within the tested concentrations referred to as the positive estrogenic samples. Also, the estrogenic activity of the samples was determined by the 10% relative potency (E2-REP_{10}), which was the percent of the REC_{10} of E2 to the REC_{10} of the tested sample. The E2-REP_{10} of E2 was considered 100% (Yoshinouchi et al., 2019)

3.6 Statistical Analysis

Mean and standard deviation (SD) of at least three replicates were calculated for the β-galactosidase activity. One-way ANOVA and Duncan's multiple analysis were

used to analyze the statistical significance at $p<0.05$ using the SPSS Version 11 statistical software program (IBM, Armonk, NY, USA).

CHAPTER IV

RESULT AND DISCUSION PART I

Construction of yeast two hybrid systems

In this chapter, four yeast two hybrid (Y2H) systems were constructed; 1) Y2H containing pGBKT7-hERα and pGADT7-hSRC1, 2) Y2H containing pGBKT7-hERα and pGADT7-hTIF2, 3) Y2H containing pGBKT7-hERβ and pGADT7-hSRC1, and 4) Y2H containing pGBKT7-hERβ and pGADT7-hTIF2.

4.1 Construction of Recombinant Plasmids

In this part, four recombinant plasmids were constructed: pGADT7-hSRC1, pGADT7-hTIF2, pGBKT7-hERα, and pGBKT7-hERβ. The DNAs encoding the receptor interaction domain (RID) of coactivators (SRC1 and TIF2) and the ligand binding domain (LBD) of ERs (ERα and ERβ) were obtained by the reverse transcription-polymerase chain reaction technique (RT-PCR). Each cDNA was synthesized using the total RNA extracted from MCF-7 cells. The extracted RNA appeared as three band on a 0.8% agarose gel electrophoresis, which were 28S rRNA, 18S rRNA and 5S rRNA (Figure 4.1). The extraction yield was 53.40 μg per a T25 flask of cultured cells. The absorption ratio of A260/280 was 2.0, which was in a range of good RNA quality (1.80–2.20) (Villa-Rodriguez et al., 2018)

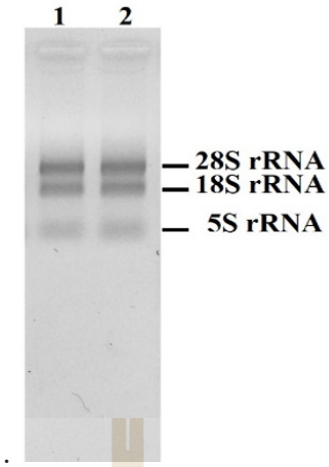


Figure 4.1 Total RNA of MCF-7 cells visualized on 0.8% agarose gel. Lane 1-2: the extracted RNA samples.

4.1.1 Construction of pGADT7-hSRC1 and pGADT7-hTIF

To construct pGADT7-hSRC1 and pGADT7-hTIF, the DNA fragments of RID-hSRC1 and RID-hTIF2 were synthesized by RT-PCR. The amplified DNA fragments were visualized on a 0.8% agarose gel, which their sizes were well corresponded to the predicted sizes at 2,592 and 1,992 bp of RID-hSRC1 and RID-hTIF2 fragments, respectively (Figure 4.2).

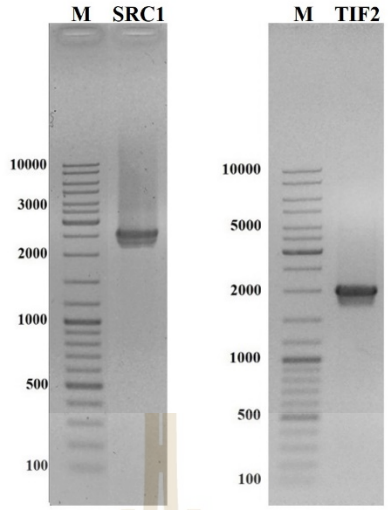


Figure 4.2 The amplified DNA fragment of RID-hSRC1 and RID-hTIF2 visualized on 0.8% agarose gel electrophoresis, the specific bands were showed the correct sizes of 2,592 and 1,992 bp respectively. M: DNA marker.

The DNA fragment of RID-hSRC1 and RID-hTIF2 were ligated to the pTG19T cloning vector (2,880 bp) and the positive clones were selected using PCR screening method. After the recombinant pTG19T-hSRC1 and pTG19T-hTIF2 plasmids were obtained, the DNA fragments of RID-hSRC1 and RID-hTIF2 were excised by NdeI and SmaI restriction enzymes. The obtained RID-hSRC1 and RID-hTIF2 fragments were 2,592 and 1,992 bp as visualized on the agarose gel (Figure 4.3). Each fragment was subcloned into the pre-cut pGADT7-AD vector using the same restriction enzymes. The obtained recombinant plasmids referred to as pGADT7-hSRC1 and pGADT7-hTIF2 plasmids, which could produce the fusion proteins that contained the activation domain of GAL4 transcription factor.

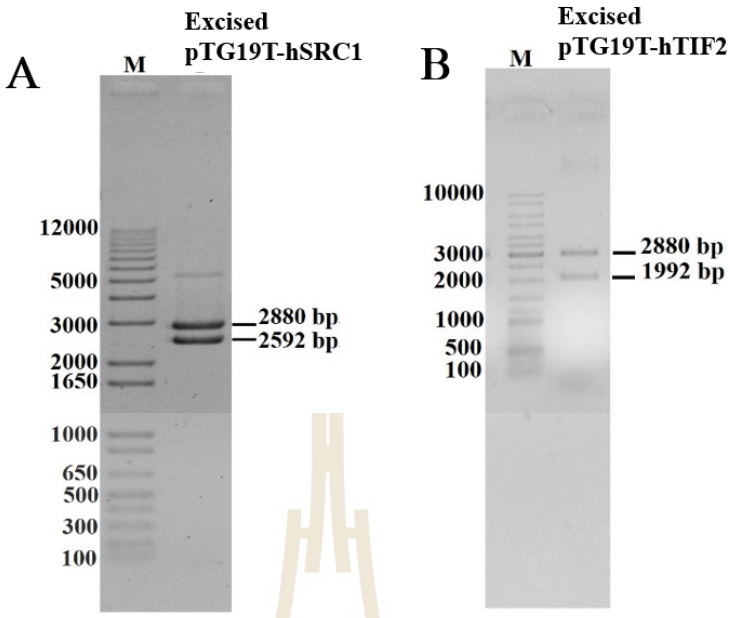


Figure 4.3 The excised pTG19T-hSRC1 plasmid (A) and the excised pTG19T-hTIF2 plasmid (B) visualized on 0.8% agarose gels. M: DNA marker.

The positive clones containing the recombinant pGADT7-hSRC1 and pGADT7-hTIF2 plasmids were identified by two methods; PCR screening method and the digestion method. Figure 4.4 shows the results of the PCR screening method, which the specific primers were used to amplify the target DNA fragments. The amplified DNA fragments were 2,592 and 1,992 bp, well corresponding to the sizes of RID-hSRC1 and RID-hTIF2, respectively. Figure 4.5 shows the results of the digestion method. In this method, NdeI and SmaI restriction enzymes were used to excise the insert fragment from the recombinant plasmids; pGADT7-hSRC1 and pGADT7-hTIF2s. The results showed the sizes of the plasmid vector of 8,000 bp, RID-hSRC1 of 2,592 bp, and RID-hTIF2 of 1,992 bp, suggesting the positive clones contained the correct recombinant plasmids. After that, all recombinant plasmids were isolated from the positive clones and their DNA sequences were analyzed by DNA-sequencing

analysis. The correct open reading frame (ORF) of each recombinant plasmid was confirmed before using in the next experiment.

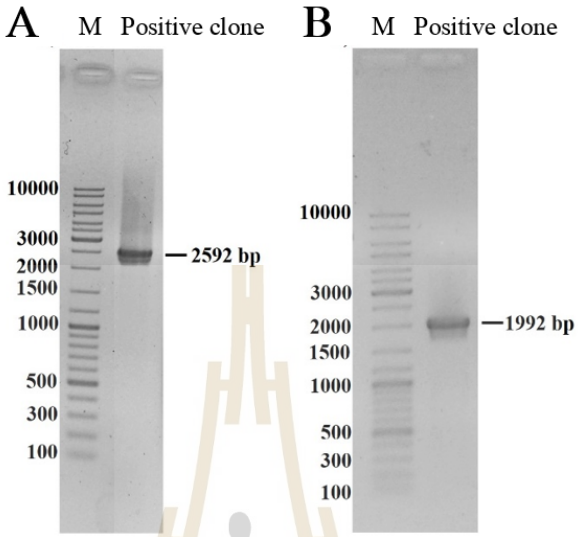


Figure 4.4 The positive clones containing pGADT7-hSRC1 (A) and pGADT7-hTIF2 (B) as analyzed by the PCR screening method. M: DNA marker.

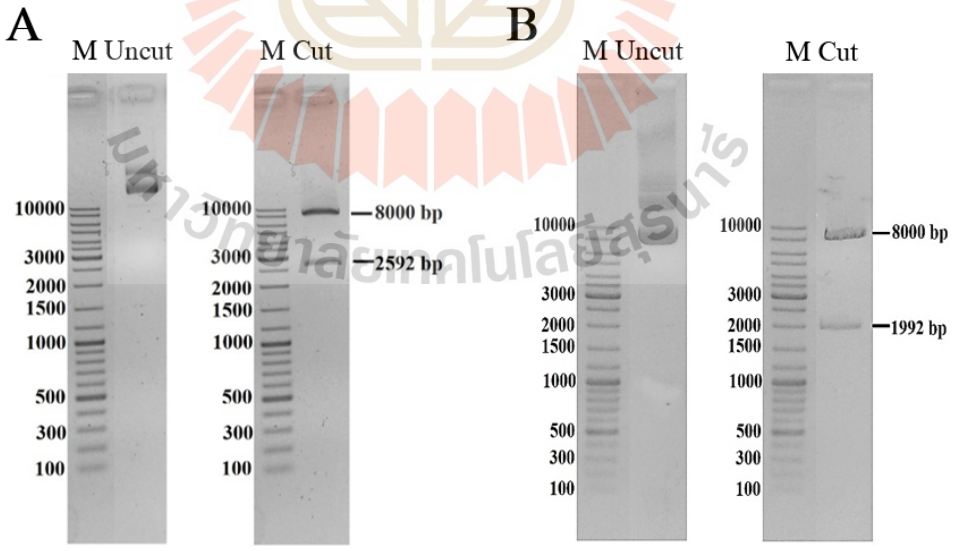
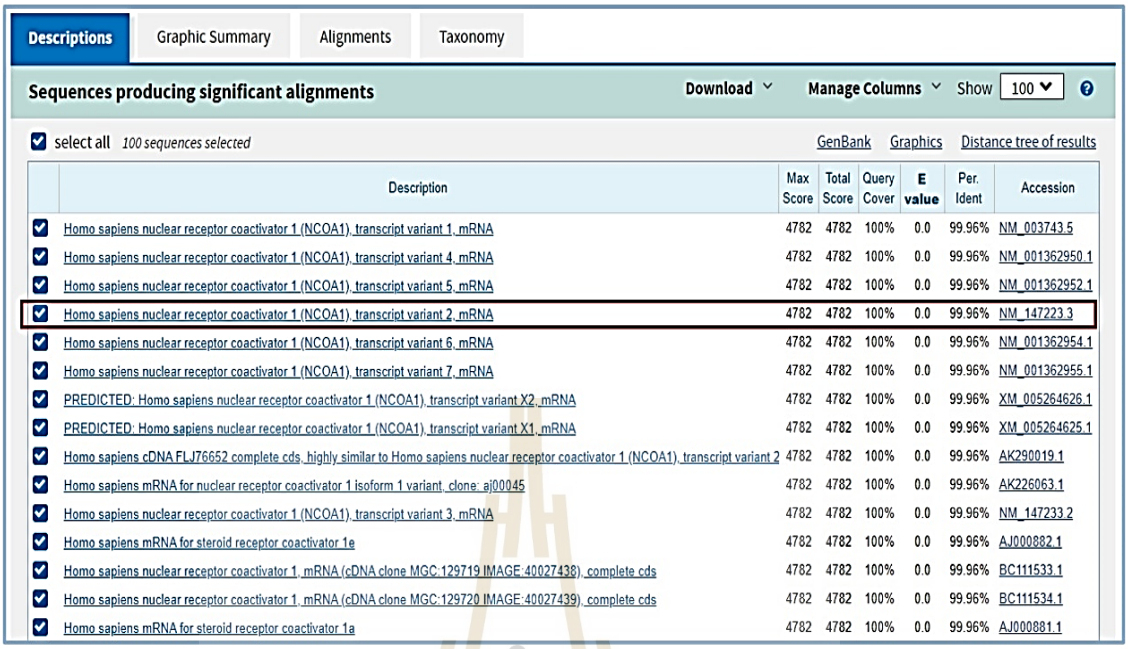


Figure 4.5 The positive clones containing pGADT7-hSRC1 (A) and pGADT7-hTIF2 (B) as analyzed by the digestion method. M: DNA marker.

The DNA sequences of both plasmids were also analyzed by Expasy translation and NCBI Blastn programs. Figure 4.6 shows the result of the translational sequence of the RID-hSRC1 insert in the pGADT7-hSRC1 plasmid. The RID-hSRC1fragment contained 2,592 bp, which encoded for 865 amino acid residues. Figure 4.7 shows the result of the NCBI Blastn analysis. The obtained RID-hSRC1 sequence showed the highest similarity to the *Homo sapiens* nuclear receptor coactivator 1 or steroid receptor coactivator 1 (NCOA1 or SRC1) with 99.96% identity. It was noted that this sequence was detected in all seven transcript variants of SRC1.

For pGADT7-hTIF2, it contained 1,992 bp of the RID-hTIF2 insert as analyzed by Expasy translation program (Figure 4.8). The RID-hTIF2 sequence encoded for 666 amino acid residues. For NCBI Blastn analysis, it showed high similarity to *Homo sapiens* nuclear receptor coactivator 2 or transcriptional intermediary factor 2 (NCOA2 or TIF2) with 99.75% identity (Figure 4.9).

Figure 4.6 The translational sequence of the RID-hSRC1 insert of pGADT7-hSRC1 as analyzed by Expasy translation program.



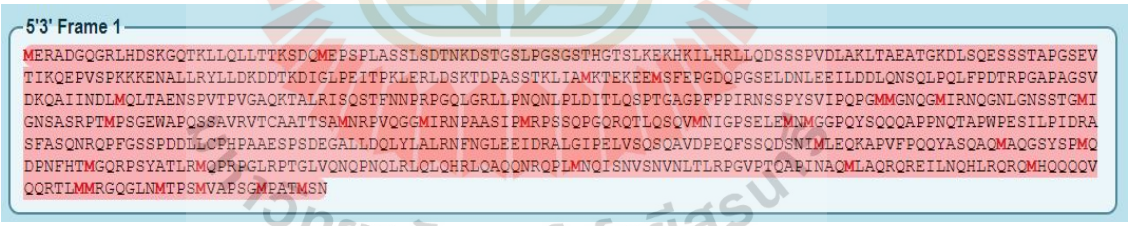
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 1, mRNA	4782	4782	100%	0.0	99.96%	NM_003743.5
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 4, mRNA	4782	4782	100%	0.0	99.96%	NM_001362950.1
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 5, mRNA	4782	4782	100%	0.0	99.96%	NM_001362952.1
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 2, mRNA	4782	4782	100%	0.0	99.96%	NM_147223.3
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 6, mRNA	4782	4782	100%	0.0	99.96%	NM_001362954.1
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 7, mRNA	4782	4782	100%	0.0	99.96%	NM_001362955.1
PREDICTED: Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant X2, mRNA	4782	4782	100%	0.0	99.96%	XM_005264626.1
PREDICTED: Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant X1, mRNA	4782	4782	100%	0.0	99.96%	XM_005264625.1
Homo sapiens cDNA FLJ76652 complete cds, highly similar to Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 2	4782	4782	100%	0.0	99.96%	AK290019.1
Homo sapiens mRNA for nuclear receptor coactivator 1 isoform 1 variant, clone: aj00045	4782	4782	100%	0.0	99.96%	AK226063.1
Homo sapiens nuclear receptor coactivator 1 (NCOA1), transcript variant 3, mRNA	4782	4782	100%	0.0	99.96%	NM_147233.2
Homo sapiens mRNA for steroid receptor coactivator 1e	4782	4782	100%	0.0	99.96%	AJ000882.1
Homo sapiens nuclear receptor coactivator 1, mRNA (cDNA clone MGC:129719 IMAGE:40027438), complete cds	4782	4782	100%	0.0	99.96%	BC111533.1
Homo sapiens nuclear receptor coactivator 1, mRNA (cDNA clone MGC:129720 IMAGE:40027439), complete cds	4782	4782	100%	0.0	99.96%	BC111534.1
Homo sapiens mRNA for steroid receptor coactivator 1a	4782	4782	100%	0.0	99.96%	AJ000881.1

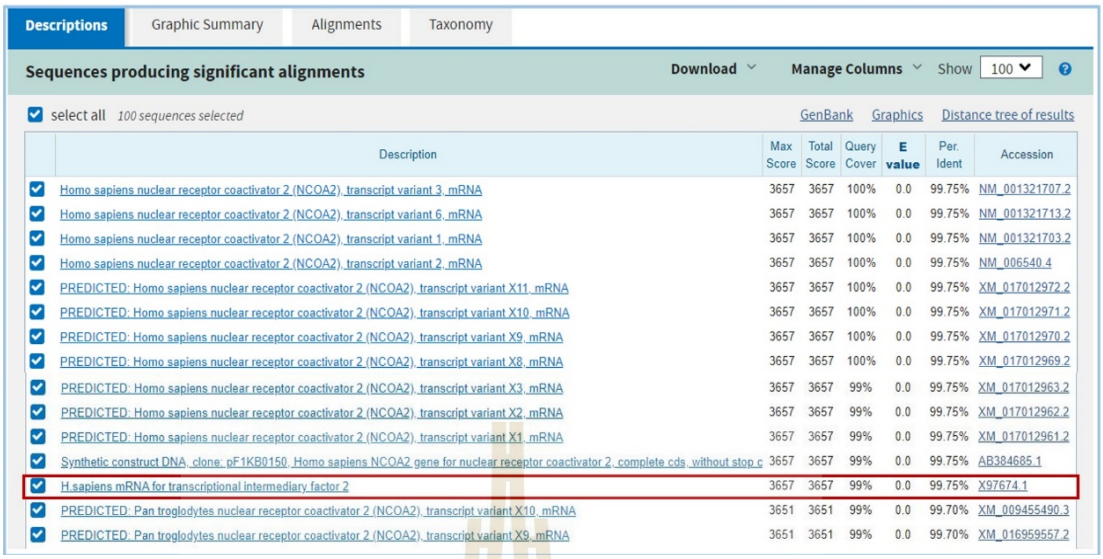
Figure 4.7 The identity of the RID-hSRC1 insert of pGADT7-hSRC1 as analyzed by NCBI Blastn program. Red box indicated the gene used to design primers in this



5'3' Frame 1

MERADGQGRLHDSKGQTKLLQLLTTKSDQMEPSPLASSLSDTNKDSTGSLPGSGSTHGTSLKEKHKILHRLLQDSSSPVDLAKLTAEATGKDLSQESSSTAPGSEV
TIKQEPVSPKKKENALLRYLLDKDDTKDIGLPEITPKLERLDSKTDPASSTKLIAMKTEKEEMSFEPGDQPGSELDNLEEILDDLQNSQLPQLFPDTRPGAPAGSV
DKQAIINDLMQLTAENSPVTPVGAQKTALRISQSTFNNPRPGQLGRLLPNQNLPLDITLQSPTGAGPFPPIRNSSPYSVIPQPGMMGNQGMIRNQGNLGNSSTGMI
GNSASRPTMPSGEWAPQSSAVRVTCAATTSAMNRPVQGGMIRNPAASIPMRPSSQPGQRQTLQSQVMNIGPSELEMNMGGPQYSQQQAPPNQTAPWPESILPIDRA
SFASQNRQPFGSSPDDLLCPPAAESPSDEGALLDQLYLALRNFNGLEEIDRALGIPELVSQSQAVDPEQFSSQDSNIMLEQKAPVFPQQYASQAQMAQGSYSPMQ
DPNFHTMGQRPSYATLRMQPRPGLRPTGLVQNQPNQLRLQLQHRLQAQQNRQPLMNQISNVSNVNLTLRPGVPTQAPINAQMLAQRQREILNQHLRQRQMHQQQQV
QQRTLMMRGQGLNMTPSMVAPSGMPATMSN

Figure 4.8 The translational sequence of the RID-hTIF2 insert of pGADT7-hTIF2 as analyzed by Expasy translation program.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant 3, mRNA	3657	3657	100%	0.0	99.75%	NM_001321707.2
Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant 6, mRNA	3657	3657	100%	0.0	99.75%	NM_001321713.2
Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant 1, mRNA	3657	3657	100%	0.0	99.75%	NM_001321703.2
Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant 2, mRNA	3657	3657	100%	0.0	99.75%	NM_006540.4
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X11, mRNA	3657	3657	100%	0.0	99.75%	XM_017012972.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X10, mRNA	3657	3657	100%	0.0	99.75%	XM_017012971.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X9, mRNA	3657	3657	100%	0.0	99.75%	XM_017012970.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X8, mRNA	3657	3657	100%	0.0	99.75%	XM_017012969.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X3, mRNA	3657	3657	99%	0.0	99.75%	XM_017012963.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X2, mRNA	3657	3657	99%	0.0	99.75%	XM_017012962.2
PREDICTED: Homo sapiens nuclear receptor coactivator 2 (NCOA2), transcript variant X1, mRNA	3657	3657	99%	0.0	99.75%	XM_017012961.2
Synthetic construct DNA, clone: pF1KB0150, Homo sapiens NCOA2 gene for nuclear receptor coactivator 2, complete cds, without stop c	3657	3657	99%	0.0	99.75%	AB384685.1
H.sapiens mRNA for transcriptional intermediary factor 2	3657	3657	99%	0.0	99.75%	X97674.1
PREDICTED: Pan troglodytes nuclear receptor coactivator 2 (NCOA2), transcript variant X10, mRNA	3651	3651	99%	0.0	99.70%	XM_009455490.3
PREDICTED: Pan troglodytes nuclear receptor coactivator 2 (NCOA2), transcript variant X9, mRNA	3651	3651	99%	0.0	99.70%	XM_016959557.2

Figure 4.9 The identity of the RID-TIF2 insert of pGADT7-hTIF2 as analyzed by NCBI Blastn program. Red box indicated the gene used to design primers in this work.

4.1.2 Construction of pGBKT7-hERα and pGBKT7-hERβ

The pGBKT7-hERα and pGBKT7-hERβ plasmids were constructed, which the LBD-hERα and LBD-hERβ inserts were synthesized by RT-PCR. The amplified DNA fragments of LBD-hERα and LBD-hERβ were visualized on 0.8% agarose gel, which their sizes were 1,035 and 576 bp as expected, respectively.

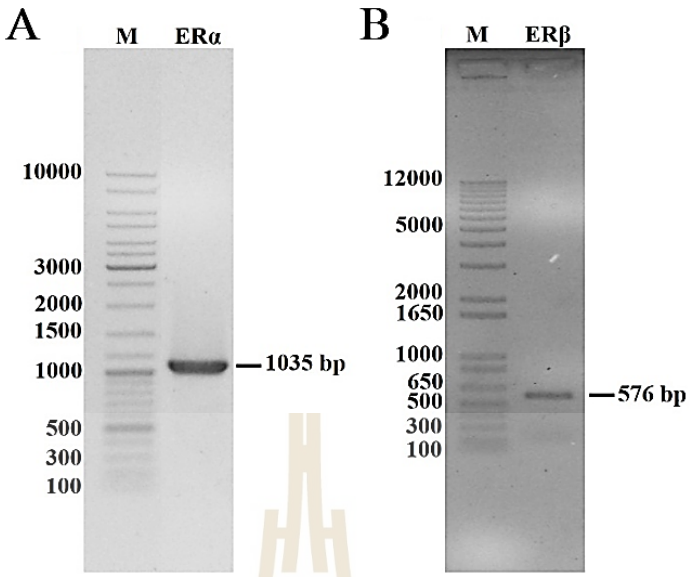


Figure 4.10 The amplified DNA fragments of LBD-hERα (A) and LBD-hERβ (B) visualized on 0.8% agarose gel. M: DNA marker.

Each amplified DNA fragment was ligated to the pTG19T cloning vector (2,880 bp) and positive clones were selected, which they referred to as pTG19T-hERα and pTG19T-hERβ. Then, the LBD-hERα and LBD-hERβ inserts were excised from these plasmids using NdeI and BamHI restriction enzymes, which their sizes remained the same; 1,035 and 576 bp, respectively (Figure 4.11). Each DNA fragment was subcloned into the pre-cut pGBKT7-BD vector using the above mentioned restriction enzymes. The pGBKT7-BD vector was used for a production of fusion proteins that contained the DNA-binding domain of the GAL4 transcription factor. The obtained recombinant plasmids were referred to as pGBKT7-hERα and pGBKT7-hERβ.

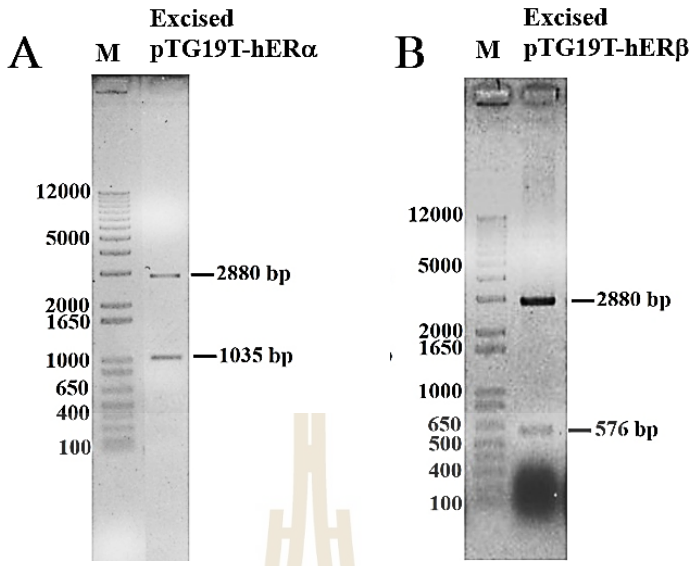


Figure 4.11 The excised pTG19T-hERα plasmid (A) and the excised pTG19T-hERβ plasmid (B) visualized on 0.8% agarose gels. M: DNA marker.

The positive clones containing the recombinant plasmids, pGBKT7-hERα and pGBKT7-hERβ, were screened by two methods; PCR screening and digestion methods. In the PCR screening method, the bacterial clones were checked by amplifying the insert DNA using the specific primers in PCR reaction. Figure 4.12 shows the examples of the positive clones that contained pGBKT7-hERα and pGBKT7-hERβ. The amplified DNA fragments of 1,035 and 576 bp indicated that the bacterial cloned contained pGBKT7-hERα and pGBKT7-hERβ, respectively. Also, the digestion method was used to confirm the positive clones, in which the recombinant plasmids were cut by NdeI and BamHI restriction enzymes (Figure 4.13). The obtained cut DNA fragments of 1,035 and 576 bp also indicated that the positive clones contained pGBKT7-hERα and pGBKT7-hERβ, respectively. The recombinant plasmid were confirmed by sequencing analysis to prove the correct open reading frame (ORFs).

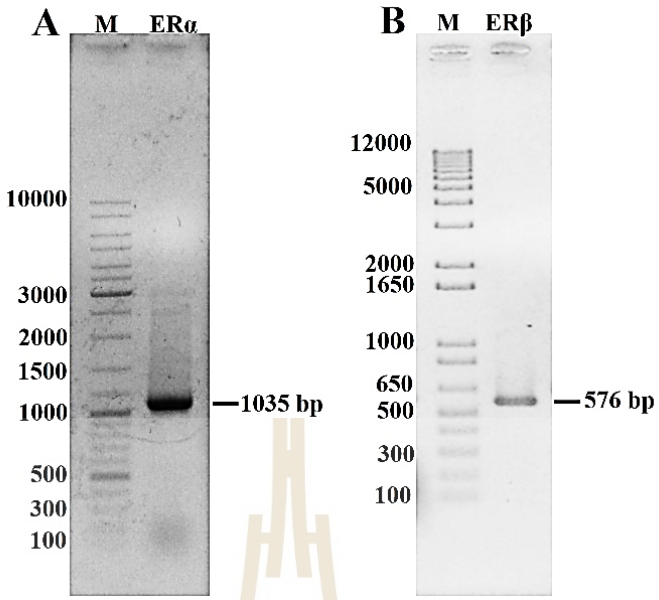


Figure 4.12 The positive clones containing pGBKT7-ERα (A) and pGBKT7-ERβ (B) as analyzed by the PCR screening method. M: DNA marker.

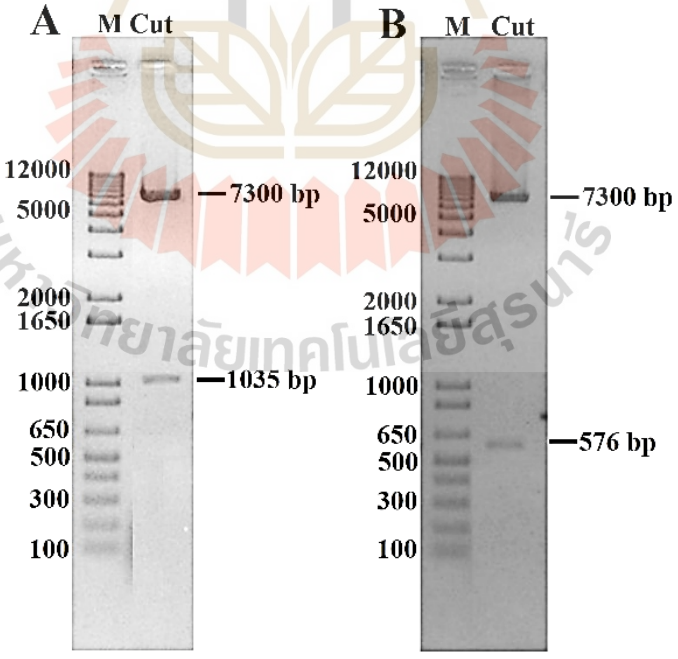


Figure 4.13 The positive clones containing pGBKT7-hERα (A) and pGBKT7-hERβ (B) as analyzed by the digestion method. M: DNA marker.

For pGBKT7-hERα, it consisted of 1,035 bp of LBD-hERα insert as analyzed by Expasy translation program (Figure 4.14). The LBD-hERα sequence encoded for 345 amino acid residues. For NCBI Blastn analysis, it showed high similarity to *Homo sapiens* estrogen receptor 1 (ESR1 or ERα) with 99.71% identity (Figure 4.15).

For pGBKT7-hERβ, it contained the 576-bp LBD-hERβ insert as analyzed by Expasy translation program (Figure 4.16). This DNA sequence encoded for 193 amino acid residues, which had high homology to *Homo sapiens* estrogen receptor 2 (ER beta, ESR2) with 100% identity as determined by NCBI Blastn analysis (Figure 4.17).

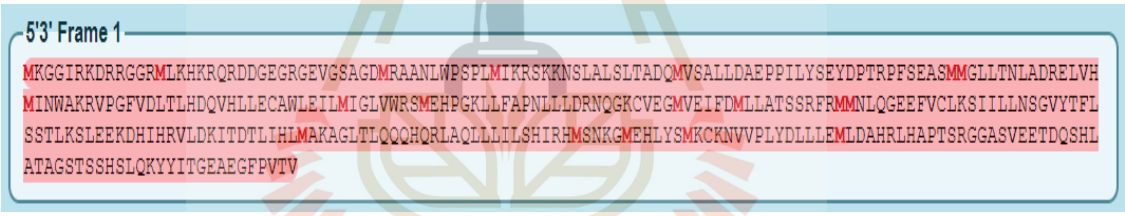


Figure 4.14 The translation sequence of the LBD-hERα insert of pGBKT7-hERα as analyzed by Expasy translation program.

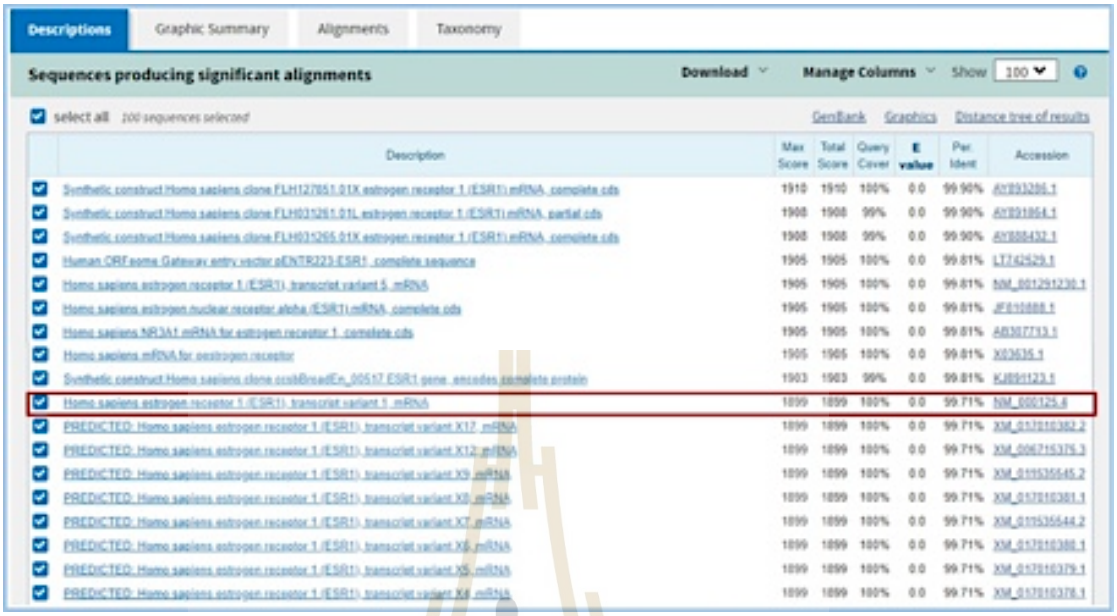


Figure 4.15 The identity of the LBD-hERα insert of pGBKT7-hERα as analyzed by NCBI Blastn program. Red box indicated the gene used to design primers in this work.

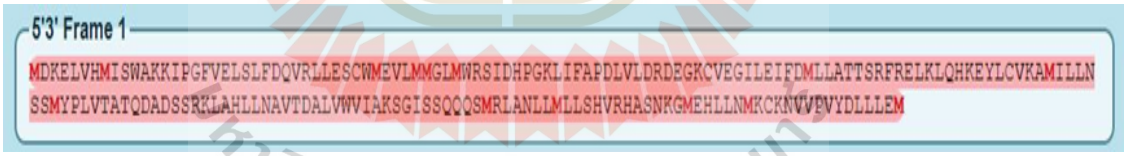
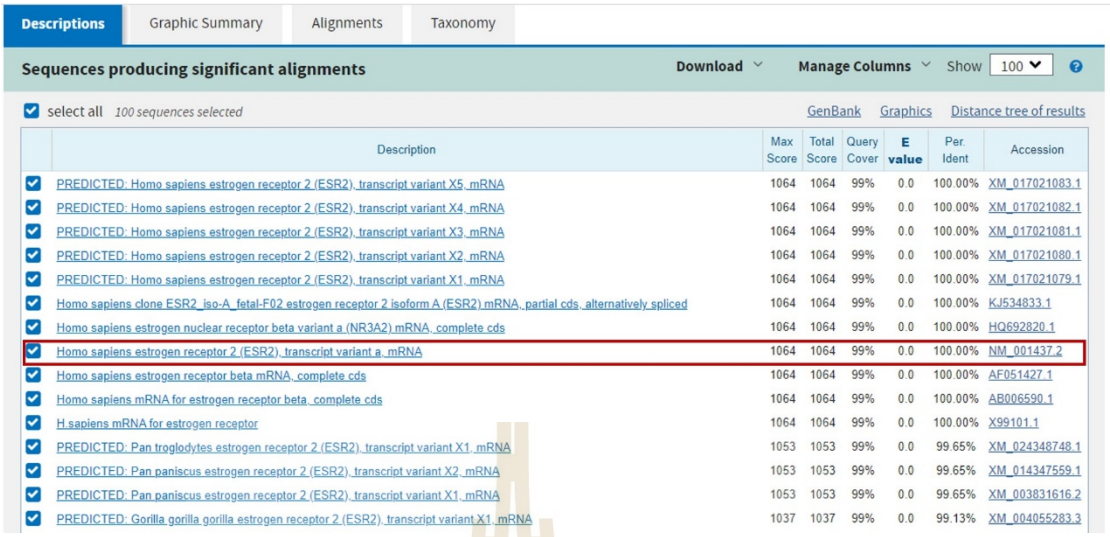


Figure 4.16 The translation sequence of the LBD-hERβ insert of pGBKT7-hERβ as analyzed by Expasy translation program.



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Homo sapiens estrogen receptor 2 (ESR2), transcript variant X5, mRNA	1064	1064	99%	0.0	100.00%	XM_017021083.1
PREDICTED: Homo sapiens estrogen receptor 2 (ESR2), transcript variant X4, mRNA	1064	1064	99%	0.0	100.00%	XM_017021082.1
PREDICTED: Homo sapiens estrogen receptor 2 (ESR2), transcript variant X3, mRNA	1064	1064	99%	0.0	100.00%	XM_017021081.1
PREDICTED: Homo sapiens estrogen receptor 2 (ESR2), transcript variant X2, mRNA	1064	1064	99%	0.0	100.00%	XM_017021080.1
PREDICTED: Homo sapiens estrogen receptor 2 (ESR2), transcript variant X1, mRNA	1064	1064	99%	0.0	100.00%	XM_017021079.1
Homo sapiens clone ESR2_iso-A_fetal-F02 estrogen receptor 2 isoform A (ESR2) mRNA, partial cds, alternatively spliced	1064	1064	99%	0.0	100.00%	KJ534833.1
Homo sapiens estrogen nuclear receptor beta variant a (NR3A2) mRNA, complete cds	1064	1064	99%	0.0	100.00%	HQ692820.1
Homo sapiens estrogen receptor 2 (ESR2), transcript variant a, mRNA	1064	1064	99%	0.0	100.00%	NM_001437.2
Homo sapiens estrogen receptor beta mRNA, complete cds	1064	1064	99%	0.0	100.00%	AF051427.1
Homo sapiens mRNA for estrogen receptor beta, complete cds	1064	1064	99%	0.0	100.00%	AB006590.1
H.sapiens mRNA for estrogen receptor	1064	1064	99%	0.0	100.00%	X99101.1
PREDICTED: Pan troglodytes estrogen receptor 2 (ESR2), transcript variant X1, mRNA	1053	1053	99%	0.0	99.65%	XM_024348748.1
PREDICTED: Pan paniscus estrogen receptor 2 (ESR2), transcript variant X2, mRNA	1053	1053	99%	0.0	99.65%	XM_014347559.1
PREDICTED: Pan paniscus estrogen receptor 2 (ESR2), transcript variant X1, mRNA	1053	1053	99%	0.0	99.65%	XM_003831616.2
PREDICTED: Gorilla gorilla gorilla estrogen receptor 2 (ESR2), transcript variant X1, mRNA	1037	1037	99%	0.0	99.13%	XM_004055283.3

Figure 4.17 The identity of the LBD-hERβ insert of pGBKT7-hERβ as analyzed by NCBI Blastn program. Red box indicated the gene used to design primers in this work.

4.2 Construction of Yeast Two Hybrid Systems

In this work, four Y2H systems were constructed, which each system contained two recombinant plasmids (bait and prey plasmids). One recombinant plasmid (bait plasmid) produced the fusion protein of ER and DNA binding domain (BD). The other one (prey plasmid) produced the fusion protein of coactivator and activation domain (AD). The bait plasmids in this work were pGBKT7-hERα and pGBKT7-hERβ. The prey plasmids were pGADT7-hSRC1 and pGADT7-hTIF2. The yeast *Saccharomyces cerevisiae* stain AH109 were transformed with the first plasmid (pGADT7-hSRC1 or pGADT7-hTIF2). The grown colonies on a selective medium lacking leucine were the transformants, which were subsequently co-transformed with the second plasmid (pGBKT7-hERα or pGBKT7-hERβ). The grown colonies in a selective medium lacking both leucine and tryptophan were considered the putative Y2H colonies. After

these colonies were confirmed to obtain the correct plasmids inside, they were considered as the Y2H system. Four Y2H systems in this work were the Y2H (LBD-ERα and RID-hSRC1), the Y2H (LBD-hERα and RID-hTIF2), the Y2H (LBD-ERβ and RID-hSRC1) and the Y2H (LBD-hERβ and RID-hTIF2). PCR analysis was used to detect the presence of both target plasmids in each Y2H system as follows.

For the Y2H (LBD-ERα and RID-hSRC1), the amplified DNA fragments of 2,592 and 1,035 bp suggested that the Y2H clone contained pGADT7-hSRC1 and pGBKT7-hERα, respectively (Figure 4.18).

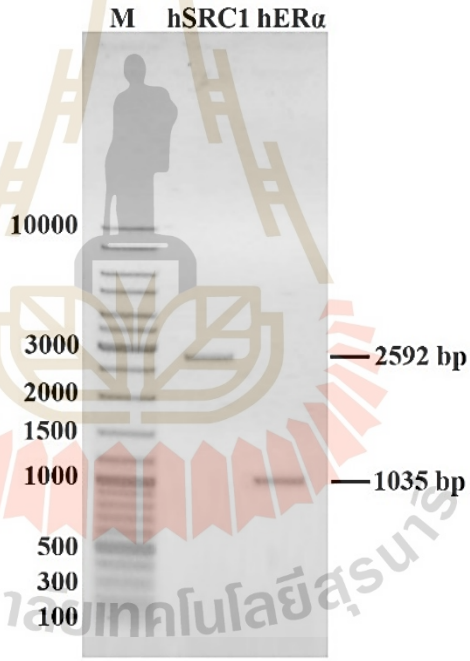


Figure 4.18 The Y2H (LBD-ERα and RID-hSRC1) positive clone analyzed by the PCR screening method. M: DNA marker.

For the Y2H (LBD-ERα and RID-hTIF2), the amplified DNA fragments of 1,992 and 1,035 bp indicated that this Y2H clone contained pGADT7-hTIF2 and pGBKT7-hERα, respectively (Figure 4.19).

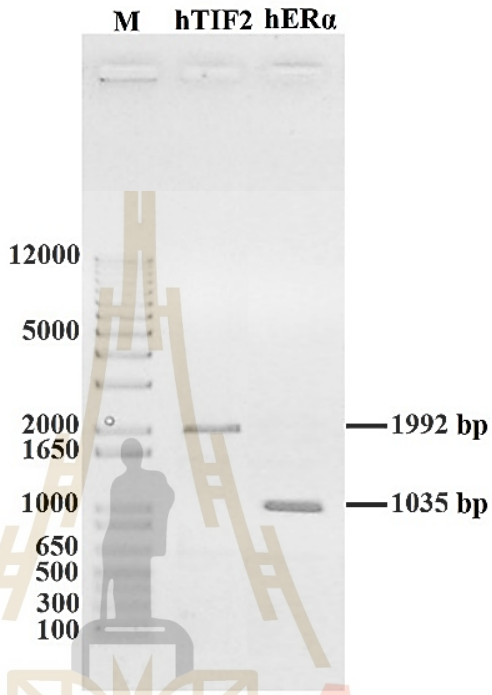


Figure 4.19 The Y2H (LBD-ERα and RID-hTIF2) positive clone as analyzed by the PCR screening method. M: DNA marker.

For the Y2H (LBD-ERβ and RID-hSRC1), the amplified DNA fragments of 2,592 and 576 bp indicated that this Y2H clone contained pGADT7-hSRC1 and pGBKT7-hERβ, respectively (Figure 4.20).

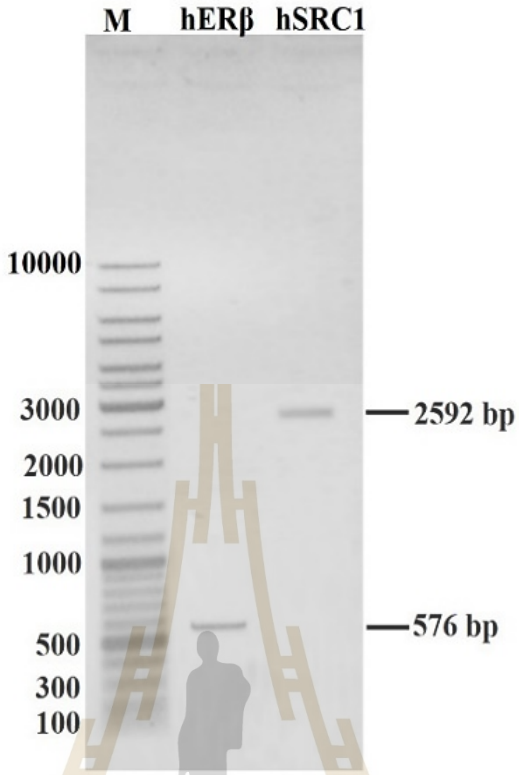


Figure 4.20 The Y2H (LBD-ERβ and RID-hSRC1) positive clone as analyzed by the PCR screening method. M: DNA marker

For the Y2H (LBD-ERβ and RID-hTIF2), the amplified DNA fragments of 1,992 and 576 bp indicated that this Y2H clone contained pGADT7-hTIF2 and pGBKT7-hERβ, respectively (Figure 4.21).

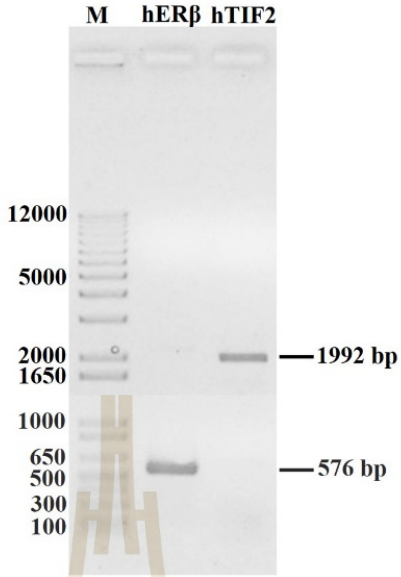


Figure 4.21 The Y2H (LBD-ERβ and RID-hTIF2) positive clone as analyzed by the PCR screening method. M: DNA marker.

4.3 Efficiency of Y2H Systems to Detect the Standard 17β-Estradiol

The efficiencies of four Y2H systems were compared by determining the estrogenic activity of 17β-estradiol (E2) at various concentrations (10^{-11}-10^{-7} M), which the results were shown in Figure 4.22.

Via the action through ERα, the Y2H (LBD-hERα and RID-hRC1) and Y2H-(LBD-ERα and RID-TIF2) systems demonstrated similar efficiencies to determine the estrogenic activity of E2 in a linear range at 10^{-11}-10^{-9} M. At 10^{-8}-10^{-7} M E2, both systems could detect the estrogenic activity but in a non-linear range (plateau and reduction phases), indicating that the upper detection limit of these Y2H systems in a linear range was at 10^{-9} M.

Via the action through ERβ, only the Y2H (LBD-hERβ and RID-hSRC1) system was efficiently detect the estrogenic activity of E2 in a linear range at the concentrations

of 10^{-11}-10^{-7} M, but not the Y2H-(LBD-hERβ and RID-hTIF2) system. It was hypothesized the production of either protein in the yeast cells might be ineffective, causing no interaction between LBD-ERβ and RID-TIF2 upon the activation of E2 to induce the expression of the reporter gene.

Thus, the Y2H (LBD-hERα and RID-hTIF2) and Y2H (LBD-hERβ and RID-hSRC1) systems were selected as the suitable Y2H systems to determine the estrogenic activity of the tested chemicals that functioned via ERα and ERβ, respectively.

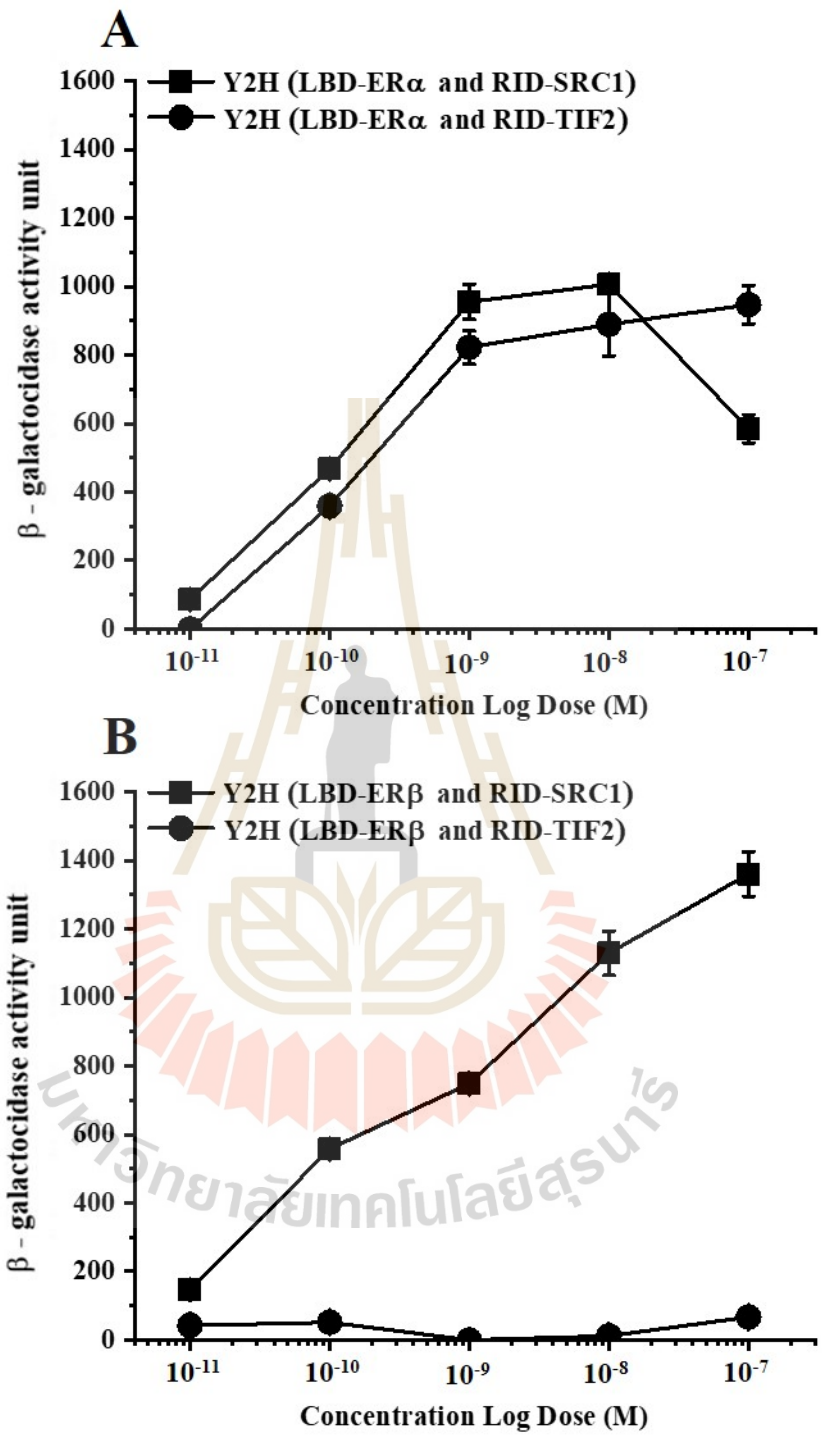


Figure 4.22 The efficiencies of four Y2H systems to determine the estrogenic activity of the standard 17β-estradiol (10^{-11}-10^{-7} M).

4.4 Efficiency of Y2H Systems to Detect the Standard Genistein

As the Y2H systems were also used to determine estrogenic activity of phytoestrogens, their efficiencies to detect the estrogenic activity of the standard genistein (10^{-11}-10^{-7} M), one of the common phytoestrogen compounds, were determined.

The efficiencies of the Y2H (LBD-hERα and RID-hTIF2) and Y2H-(LBD-hERβ and RID-hSRC1) systems to determine estrogenic activity of genistein were shown in Figure 4.23. As compared with the standard E2, the maximum estrogenic activities of genistein via ERα and ERβ were approximately 2.0 and 1.4 folds lower, respectively. Similar results were reported by Lee and colleagues (Haeng. Seog Lee et al., 2002), in which the estrogenic activities of genistein via both receptors were lower than those of E2. The estrogenic activity of genistein via ERα could be detected in a range of 10^{-10}–10^{-7} M, whereas its activity via ERβ were in a range of 10^{-9}–10^{-7} M. Genistein was reported to have lower affinity to ERβ than ERα, therefore, it required higher concentration to efficiently bind to ERβ (An et al., 2001).

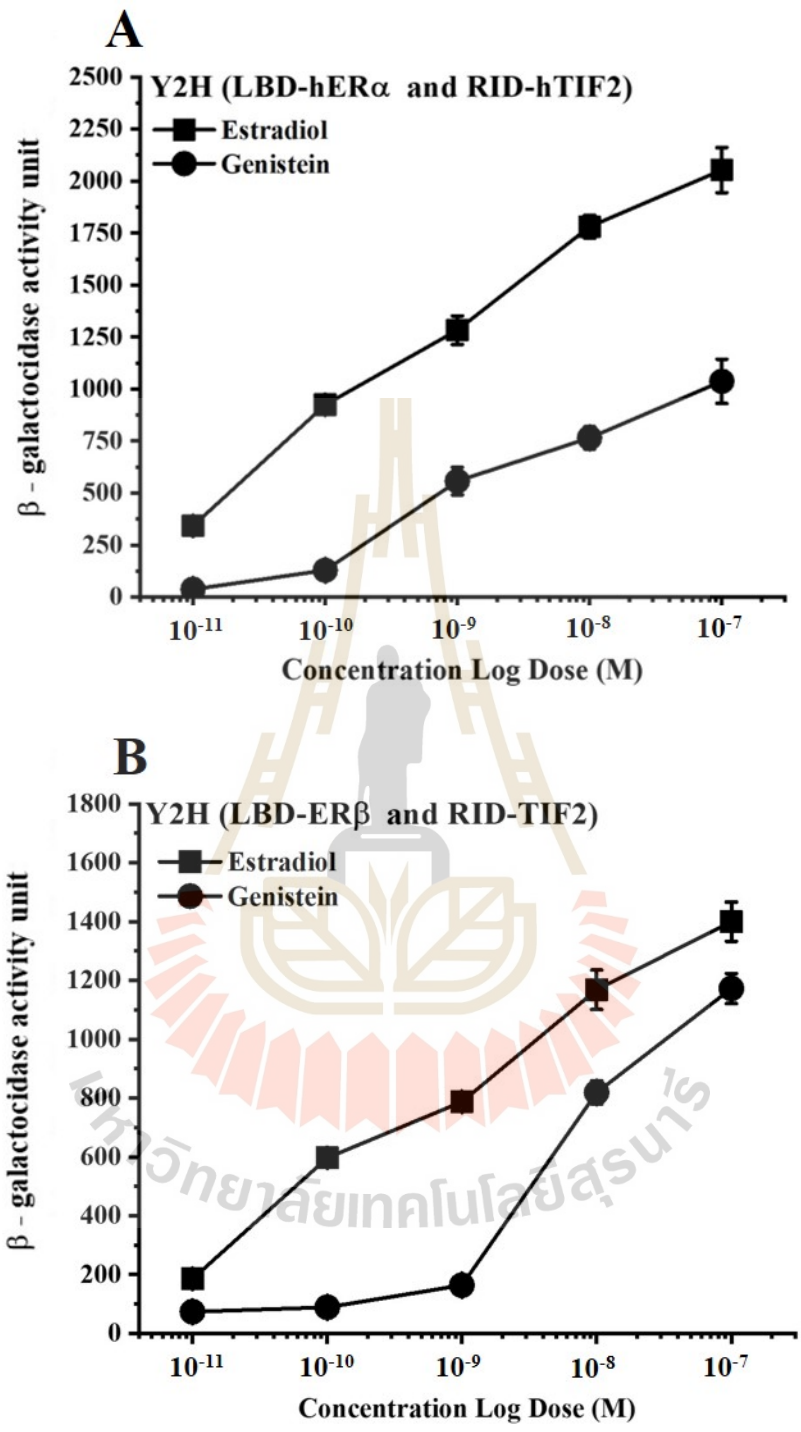


Figure 4.23 The efficiencies of four Y2H systems to determine the estrogenic activity of the standard genistein (10^{-11}-10^{-7} M) as compared with E2 (10^{-11}-10^{-7} M).

CHAPTER V

RESULT AND DISCUSION PART II

Evaluation of estrogenic activity of ten plant extracts by yeast two hybrid system

In this chapter, yeast-two-hybrid (Y2H) systems were used to detect the estrogenic activity of 10 plant extracts to better understand their actions via both estrogen receptors; ERα and ERβ. To study the action via ERα, the Y2H system containing pGBKT7-hERα and pGADT7-hTIF2 was used and referred to as the ERα-Y2H system. To study the action via ERβ, the Y2H system containing pGBKT7-hERβ and pGADT7-hSRC1 was used and referred to as the ERβ-Y2H system.

5.1 Preparation of Plant Extracts

In this work, ten medicinal plants were selected to evaluate estrogenic activity. They were selected according to their folkloric uses for treating symptoms relating to female hormone imbalance (Table 5.1). The medicinal plants were either purchased from the traditional pharmacies or collected in the areas of Buriram and Nakhon Ratchasima provinces.

Table 5.1 Medicinal plants used as the ingredients of Thai traditional medicines for female treatments relating to female-hormone imbalance

	Scientific name	Common name	Parts	Folkloric uses
1	*Angelica sinensis*	Dong-quai	Root	Blood nourishment and production (Wei et al., 2016).
2	*Angelica dahurica*	Bai-zhi	Root	Blood nourishment (Kyungjin Lee et al., 2015).
3	*Carthamus tinctorius*	Safflower	Petal	Dysmenorrhea relief (Delshad et al., 2018)
4	*Curcuma xanthorrhiza*	Java ginger	Rhizome	Menstruation regularity and dysmenorrhea relief (Anggakusuma et al., 2009)
5	*Dendrolobium lanceolatum*	-	Root	Menstruation regularity (Albertazzi and Purdie, 2002).
6	*Glycyrrhiza glabra*	Licorice	Root	Menstruation regularity and dysmenorrhea relief (Jafari et al., 2019)
7	*Glycyrrhiza uralensis*	Chinese liquorice	Root	Menstruation regularity (Yang et al., 2017)
8	*Morinda coreia*	Hairy noni	Bark	Uterus involution (Avasthi et al., 2020).
9	*Pueraria mirifica*	Kwao-krua	Tuberous	Menopausal symptom relief (Suchinda Malaivijitnond, 2012).

Roots, rhizomes, barks, or petals of these plants were used according to their traditional uses. They were extracted in 70% ethanol for 1 day before the solvent was evaporated by a rotary evaporator. The yield, maximal solubility in DMSO, pH, and color of the crude ethanolic extracts were in Table 5.2. Their yields were in a range of 4.3–27.5 g per 100 g plant. The dried extracts could solubilize in DMSO with maximal solubility at 0.1 g/mL. The colors of the extracts were either yellow or brown. Their pH after solubilizing in the medium was 6.5.

Table 5.2 The yields of the crude ethanolic extracts.

	Plants	**Yield (g / 100 g plant)**	**Max. solubility (g/mL)**	**pH**	**Color**
1	*Angelica sinensis*	10.1	0.1	6.5	Yellow
2	*Angelica dahurica*	27.5	0.1	6.5	Brown
3	*Carthamus tinctorius*	17.8	0.1	6.5	Brown
4	*Curcuma xanthorrhiza*	13.3	0.1	6.5	Brown
5	*Dendrolobium lanceolatum*	6.4	0.1	6.5	Brown
6	*Glycyrrhiza glabra*	7.3	0.1	6.5	Yellow
7	*Glycyrrhiza uralensis*	4.3	0.1	6.5	Brown
8	*Morinda coreia*	4.4	0.1	6.5	Brown
9	*Pueraria mirifica*	12.8	0.1	6.5	Brown
10	*Zingiber officinale*	5.5	0.1	6.5	Brown

In general, the indigenous peoples of Thailand were prepared these medicinal plants by the decoction method. Therefore, this work used a similar approach to prepare the crude-hot water extracts, which were subsequently compared their activities with the crude ethanolic extracts. The crude-hot water extracts were obtained via 1-h extraction in water at 95 °C before freeze-drying. The yield, maximal solubility in DMSO, pH, and color of the crude-hot water extracts were in Table 5.3. Their yields were in a range of 0.56–7.96 g per 100 g plant. The dried extracts could solubilize in DMSO with maximal solubility at 0.1 g/mL. The colors of the extracts were either yellow or brown. Their pH after solubilizing in the medium was 6.5.

Table 5.3 The yields of the crude-hot water extracts.

	Plants	**Yield (g / 100 g plant)**	**Max. solubility (g/mL)**	**pH**	**Color**
1	*Angelica sinensis*	7.84	0.1	6.5	Yellow
2	*Angelica dahurica*	7.96	0.1	6.5	Brown
3	*Carthamus tinctorius*	3.76	0.1	6.5	Brown
4	*Curcuma xanthorrhiza*	2.32	0.1	6.5	Brown
5	*Dendrolobium lanceolatum*	1.56	0.1	6.5	Brown
6	*Glycyrrhiza glabra*	2.00	0.1	6.5	Yellow
7	*Glycyrrhiza uralensis*	5.24	0.1	6.5	Brown
8	*Morinda coreia*	0.56	0.1	6.5	Brown
9	*Pueraria mirifica*	3.24	0.1	6.5	Brown
10	*Zingiber officinale*	1.40	0.1	6.5	Brown

5.2 Estrogenic Activity of Crude Ethanolic Extracts

5.2.1 Evaluation by ERα-Y2H system

In this part, the estrogenic activities of crude ethanolic extracts (10^{-7}–10^{-3} g/mL) were determined by the ERα-Y2H system with the reference to the estrogenic activity of the standard E2 (2.7×10^{-11}–2.7×10^{-4} g/mL). The results showed that five out of ten plant extracts exhibited estrogenic activity in a dose-dependent response via the interaction of ERα (Figure 5.1). The extract of *Dendrolobium lanceolatum* exhibited the highest estrogenic activity, followed by the extracts of *Morinda coreia*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, and *Pueraria mirifica*, respectively.

The REC_{10} (10% relative effective concentration) and E2-REP_{10} (10% relative potency) were also determined to compare the estrogenic activity of the plant extracts with the standard E2. Five out of ten plant extracts were positive for estrogenic activity as their REC_{10} values could be determined (Table 5.4). Among these extracts, the extract of *Dendrolobium lanceolatum* exhibited the highest activity, followed by the extracts of *Morinda coreia*, *Puraria mirifica*, *Glycyrrhiza glabra*, and *Glycyrrhiza uralensis*, respectively. Nevertheless, their estrogenic activities were very low as compared with the standard E2 as indicated by the calculated E2-REP_{10} values; 0.009, 0.0002, 0.00006, 0.00004, and 0.00002%, respectively.

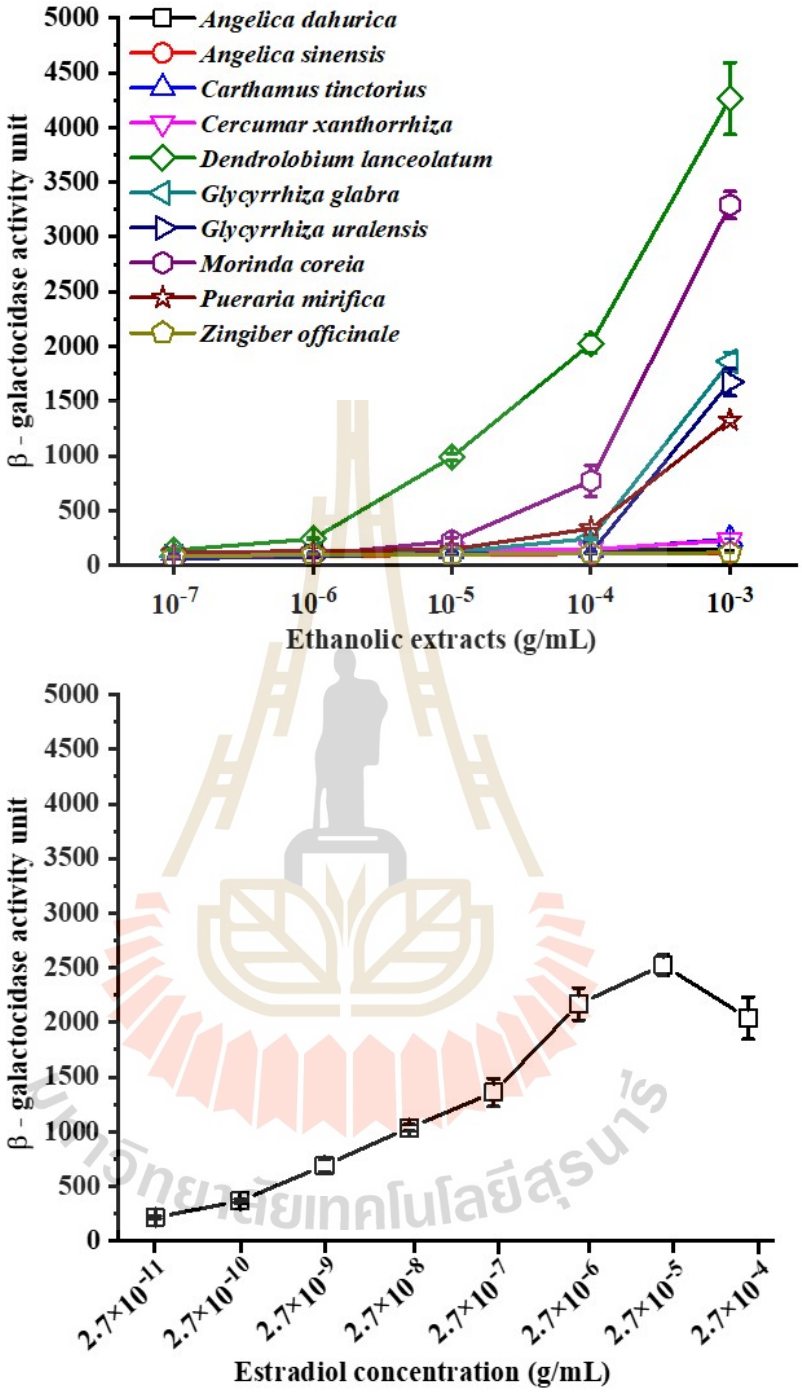


Figure 5.1 The estrogenic activities of 10 crude ethanolic extracts (A) and standard E2 (B) as evaluated by the ERα-Y2H system.

Table 5.4 Estrogenic activity of 10 crude ethanolic extracts evaluated by ERα-Y2H system.

No.	Plant species	ERα-Y2H system	
		REC_{10} (g/mL)	**E2-REP_{10} (%)**
1	*Angelica sinensis*	N	N
2	*Angelica dahurica*	N	N
3	*Carthamus tinctorius*	N	N
4	*Curcuma xanthorrhiza*	N	N
5	*Dendrolobium lanceolatum*	4.53×10^{-7}	0.009
6	*Glycyrrhiza glabra*	9.20×10^{-5}	0.00004
7	*Glycyrrhiza uralensis*	2.39×10^{-4}	0.00002
8	*Morinda coreia*	2.48×10^{-5}	0.0002
9	*Pueraria mirifica*	6.96×10^{-5}	0.00006
10	*Zingiber officinale*	N	N
11	E2	4.01×10^{-11}	100

Noted: N, negative.

5.2.2 Evaluation by ERβ-Y2H system

The estrogenic activities of ten crude ethanolic extracts were also evaluated by the ERβ-Y2H system as compared with the standard E2. The results showed that nine out of ten extracts exhibited estrogenic activity in a dose-dependent response via the interaction of ERβ (Figure 5.2). The extract of *Dendrolobium lanceolatum* exhibited

the highest estrogenic activity, followed by the extracts of *Morinda coreia*, *Glycyrrhiza uralensis*, *Curcumar xanthorrhiza*, *Angelica dahurica*, *Pueraria mirifica*, *Glycyrrhiza glabra*, *Angelica sinensis*, and *Carthamus tinctorius*, respectively.

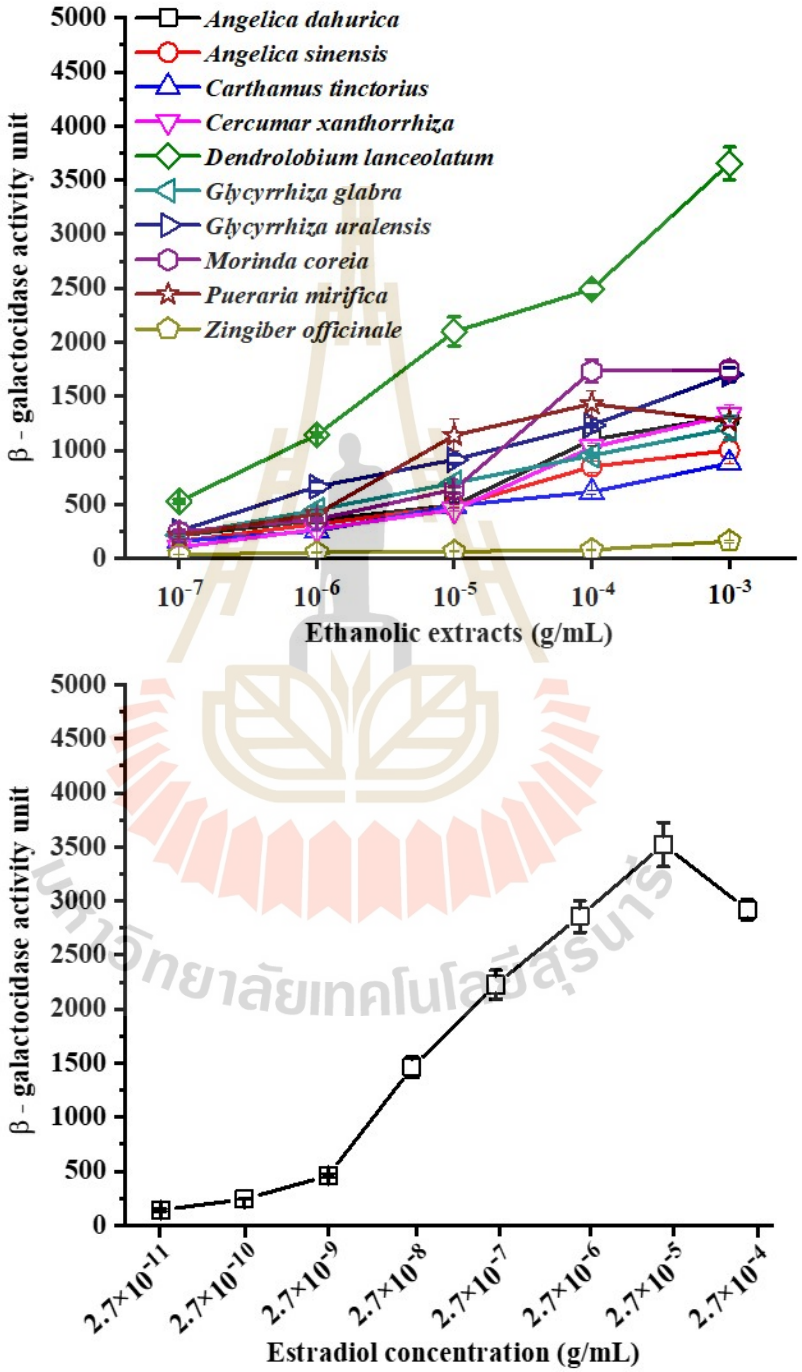


Figure 5.2 The estrogenic activities of 10 crude ethanolic extracts (A) and standard E2 (B) as evaluated by the ERβ-Y2H system.

Via the ERβ-Y2H system, the REC_{10} and E2-REP_{10} were also determined to compare the estrogenic activity of the crude ethanolic extracts with the standard E2. Based on the REC_{10} values, nine out of ten plant extracts were positive for estrogenic activity (Table 5.5).

Table 5.5 ERβ-estrogenic activity of 10 crude ethanolic extracts evaluated by the ERβ-Y2H system.

No.	Plant species	ERβ-Y2H system	
		REC_{10} (g/mL)	**E2-REP_{10} (%)**
1	*Angelica sinensis*	5.62×10^{-6}	0.02
2	*Angelica dahurica*	1.72×10^{-6}	0.05
3	*Carthamus tinctorius*	3.30×10^{-6}	0.03
4	*Curcuma xanthorrhiza*	3.03×10^{-6}	0.03
5	*Dendrolobium lanceolatum*	5.87×10^{-8}	1.53
6	*Glycyrrhiza glabra*	3.03×10^{-6}	0.03
7	*Glycyrrhiza uralensis*	1.75×10^{-7}	0.51
8	*Morinda coreia*	3.96×10^{-6}	0.02
9	*Pueraria mirifica*	5.10×10^{-6}	0.02
10	*Zingiber officinale*	N	N
11	E2	8.98×10^{-10}	100

Noted: N, negative.

The extract of *Dendrolobium lanceolatum* demonstrated the highest activity, followed by the extracts of *Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, *Puraria mirifica*, *Morinda coreia*, *Curcuma xanthorrhiza*, *Angelica dahurica*, *Carthamus tinctorius*, *and Angelica sinensis*, respectively. Their estrogenic activities were also compared with the standard E2, which their E2-REP_{10} values were 1.53, 0.51, 0.05, 0.03, 0.03, 0.03, 0.02, 0.02, and 0.02%, respectively. Interestingly, the extract of *Dendrolobium lanceolatum* exhibited very high estrogenic activity, even higher than the standard E2, and it could function via both ER. It was also noted that the estrogenic activity of this plant was firstly reported here according to the literature review. Therefore, it was interesting for the further investigation of the potential applications.

5.2.3 Comparison of estrogenic activity of ethanolic plant extracts via ERα- and ERβ

The results of this work clearly showed that the phytoestrogens in these estrogenic-positive plants preferentially functioned via the interaction with ERβ since nine plant extracts were estrogenic-positive via the evaluation by the ERβ-system (Table 5.6). It was also noted that the extracts of *Dendrolobium lanceolatum*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Morinda coreia*, and *Pueraria mirifica* were estrogenic-positive as evaluated by both ERα- and ERβ-systems but demonstrated more potent activity as determined by the ERβ-system. These results suggested that phytoestrogens in these plants could function via both forms of ER but at different efficacies. However, four other plant extracts likely functioned via only ERβ; *Angelica sinensis*, *Angelica dahurica*, *Carthamus tinctorius*, and *Curcuma xanthorrhiza*.

Table 5.6 Comparison of estrogenic activity of ethanolic plant extracts via ERα- and ERβ

Plant species	ERα-Y2H system		ERβ-Y2H system	
	REC_{10} (g/mL)	E2-REP_{10} (%)	REC_{10} (g/mL)	E2-REP_{10} (%)
Angelica dahurica	N	N	5.62×10^{-6}	0.02
Angelica sinensis	N	N	1.72×10^{-6}	0.05
Carthamus tinctorius	N	N	3.30×10^{-6}	0.03
Curcuma xanthorrhiza	N	N	3.03×10^{-6}	0.03
Dendrolobium lanceolatum	4.53×10^{-7}	0.009	5.87×10^{-8}	1.53
Glycyrrhiza glabra	9.20×10^{-5}	0.00004	3.03×10^{-6}	0.03
Glycyrrhiza uralensis	2.39×10^{-4}	0.00002	1.75×10^{-7}	0.51
Morinda coreia	2.48×10^{-5}	0.0002	3.96×10^{-6}	0.02
Pueraria mirifica	6.96×10^{-5}	0.00006	5.10×10^{-6}	0.02
Zingiber officinale	N	N	N	N
E2	4.01×10^{-11}	100	8.98×10^{-10}	100

Several novel information on the estrogenic activities of these plants was reported in this work. First, two plant extracts were never studied for their estrogenic activity, which this work was the first to report their estrogenic activities; *Dendrolobium lanceolatum* and *Morinda coreia*. Also, this work reported their actions via the interaction with both ERα and ERβ, but more preference to ERβ. Also, *Angelica dahurica* was firstly reported for its estrogenic activity via the interaction with

ERβ but not ERα in this work. There was one report by Kim and colleagues indicating that *Angelica dahurica* did not process estrogenic activity via the Y2H system based on ERα (I. G. Kim et al., 2008). Thus, this work clarified that *Angelica dahurica* possessed estrogenic activity via ERβ but not ERα. For *Angelica sinensis*, it was reported to possess estrogenic activity as determined by MCF-7 cell proliferation assay (Amato et al., 2002), however, many other publications were reported that it did not exhibit the estrogenic activity as determined by the ERα-based assays (C. Z. Zhang et al., 2005; Jianghua Liu et al., 2001). This work clarified these controversies as we found that *Angelica sinensis* extract (0.0001−1 mg/mL) exhibited estrogenic activity via the ERβ but not ERα. For *Carthamus tinctorius*, it was reported to possess no estrogenic activity as determined by the ERα-Y2H system (I. G. Kim et al., 2008). In this work, it was clarified that *Carthamus tinctorius* exhibited estrogenic activity via the ERβ, but not the ERα. For the *Curcuma xanthorrhiza*, it was reported to no estrogenic activity as determined by the MCF-7 cell proliferation assay (Haryanti et al., 2020). Herein, this work clarified that *Curcuma xanthorrhiza* exhibited estrogenic activity via ERβ but not ERα. For *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*, this work reported that both extracts possessed estrogenic activity through both ERα and ERβ, but more estrogenic activity via ERβ. Also, *Glycyrrhiza uralensis* exhibited estrogenic activity greater than *Glycyrrhiza glabra*. These results well corresponded to the report by Hajirahimkhan (Hajirahimkhan et al., 2018), in which the estrogenic activity was evaluated by the alkaline phosphatase activity via ERα in Ishikawa cells and the ERβ-ERE-luciferase induction via ERβ in MDA-MB-231 cells. For *Pueraria mirifica*, its estrogenic activity was more potent via ERβ than ERα, which was similar to the report by Boonchird

(Boonchird et al., 2010) as evaluated by a yeast-based estrogenic assay. For *Zingiber officinale*, this work reported no detectable estrogenic activity via ERα nor ERβ. This result well corresponded to the report by Elkady (Elkady et al., 2012), which indicated the anti-proliferative effect of *Zingiber officinale* extract in MCF-7 cells (no function through ERα nor ERβ). Also, Abdullah (Abdullah et al., 2010) reported the anti-proliferation effect of the extract of *Zingiber officinale* in positive ERβ cell lines, HCT 116 colon cancer cell. (Fiorelli et al., 1999). This result also suggested no estrogenic activity of this plant extract.

5.3 Estrogenic Activity of Crude-Water Extracts

5.3.1 Evaluation by ERα-Y2H system

The estrogenic activity of crude-water extracts derived from 10 species of traditional medicinal plants was evaluated by the ERα-Y2H system as compared with the standard E2 (2.7×10^{-11} – 2.7×10^{-4} M) as shown in Figure 5.3. The results showed that seven out of ten plant extracts exhibited estrogenic activity in a dose-dependent response via the interaction of ERα. The extract of *Glycyrrhiza ularensis* showed the highest estrogenic activity, followed by the extracts of *Morinda coreia*, and *Pueraria mirifica,* respectively. The crude-water extracts of *Glycyrrhiza glabra, Dendrolobium lanceolatum, Curcumar xanthorrhiza*, and *Zingiber officinale* had very low estrogenic activity at the high concentrations.

The REC_{10} and E2-REP_{10} values of these crude-water extracts were reported in Table 5.7. Six out of ten crude-water extracts exhibited estrogenic activity as determined by REC_{10}. The crude-water extract of *Glycyrrhiza uralensis* had the strongest activity, followed by the extracts of *Pueraria mirifica*, *Dendrolobium*

lanceolatum, *Curcuma xanthorrhiza*, *Morinda coreia*, and *Glycyrrhiza glabra*, respectively. Their REC_{10} values were 4.35×10^{-7}, 3.05×10^{-5}, 2.57×10^{-5}, 1.22×10^{-5}, 1.19×10^{-5}, and 1.16×10^{-4} g/mL, respectively. The extract of *Glycyrrhiza uralensi* had the highest E2-REP_{10} value, followed by *Morinda coreia*, *Curcuma xanthorrhiza*, *Dendrolobium lanceolatum*, *Pueraria mirifica*, *and Glycyrrhiza glabra*: 0.194, 0.016, 0.07, 0.003, 0.003, and 0.007%, respectively.

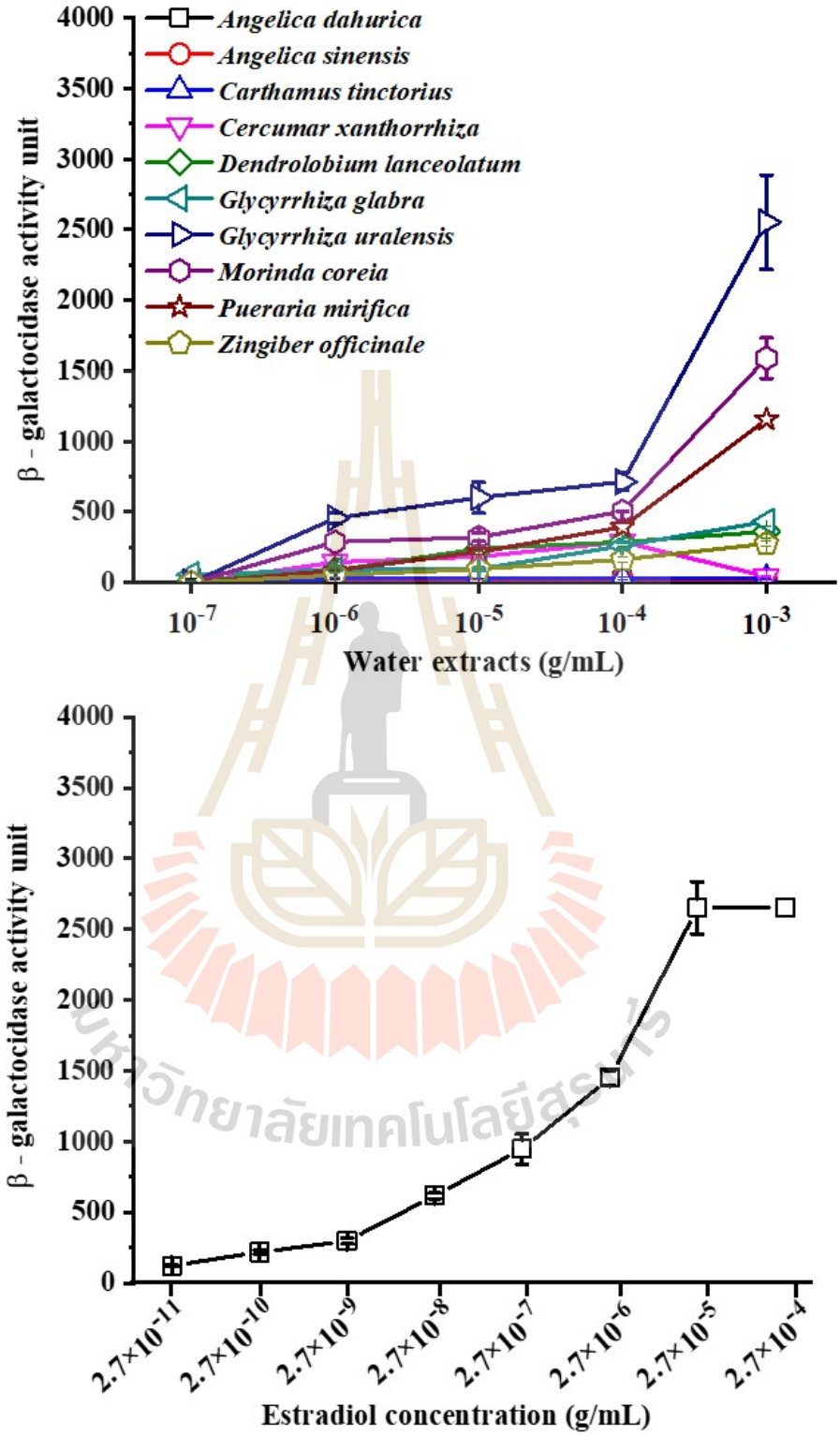


Figure 5.3 The estrogenic activities of 10 crude-water extracts (A) and standard E2 (B) as evaluated by the ERα-Y2H system.

5.7 Estrogenic activity of crude-water extracts detected by the ERα-Y2H system.

No.	Plant species	ERα-Y2H system	
		REC_{10} (g/mL)	**E2-REP_{10} (%)**
1	*Angelica dahurica*	N	N
2	*Angelica sinensis*	N	N
3	*Carthamus tinctorius*	N	N
4	*Curcuma xanthorrhiza*	1.22×10^{-5}	0.006
5	*Dendrolobium lanceolatum*	2.57×10^{-5}	0.003
6	*Glycyrrhiza glabra*	1.16×10^{-4}	0.0007
7	*Glycyrrhiza uralensis*	4.35×10^{-7}	0.194
8	*Morinda coreia*	1.19×10^{-5}	0.07
9	*Pueraria mirifica*	3.05×10^{-5}	0.003
10	*Zingiber officinale*	N	N
11	E2	8.44×10^{-10}	100

Noted: N, negative.

5.3.2 Evaluation by ERβ-Y2H system

The estrogenic activity of 10 crude-water extracts was also evaluated by the ERβ-Y2H system as compared with the standard E2 (2.7×10^{-11} – 2.7×10^{-4} M) as shown in Figure 5.4. The results showed that two out of ten plant extracts had estrogenic activity via the interaction with ERβ. The extract of *Morinda coreia* exhibited the highest estrogenic activity at the concentration of 10^{-4} mg/mL, while the extract of *Dendrolobium lanceolatum* had the estrogenic activity at 10^{-3} mg/mL. The other eight

water extracts exhibited no estrogenic activity via ERβ: *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius, Curcuma xanthorrhiza*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Pueraria mirifica*, and *Zingiber officinale*.

The REC_{10} and E2-REP_{10} values of these crude-water extracts were calculated as displayed in Table 5.8. Only two extracts exhibited the estrogenic activity as determined by the REC_{10} values; *Morinda coreia* and *Dendrolobium lanceolatum* (1.87×10^{-5} and 8.63×10^{-4} g/mL, respectively). As compared with the standard E2, the E2-REP_{10} values of the extracts were 0.067 and 0.002%, respectively.

Table 5.8 Estrogenic activity of crude-water extracts detected by ERβ-Y2H system.

No.	**Plant species**	**ERβ-Y2H system**	
		REC_{10} (g/mL)	**E2-REP_{10} (%)**
1	*Angelica dahurica*	N	N
2	*Angelica sinensis*	N	N
3	*Carthamus tinctorius*	N	N
4	*Curcuma xanthorrhiza*	N	N
5	*Dendrolobium lanceolatum*	8.63×10^{-4}	0.002
6	*Glycyrrhiza glabra*	N	N
7	*Glycyrrhiza uralensis*	N	N
8	*Morinda coreia*	1.87×10^{-5}	0.067
9	*Pueraria mirifica*	N	N
10	*Zingiber officinale*	N	N
11	E2	1.26×10^{-8}	100

Noted: N, negative.

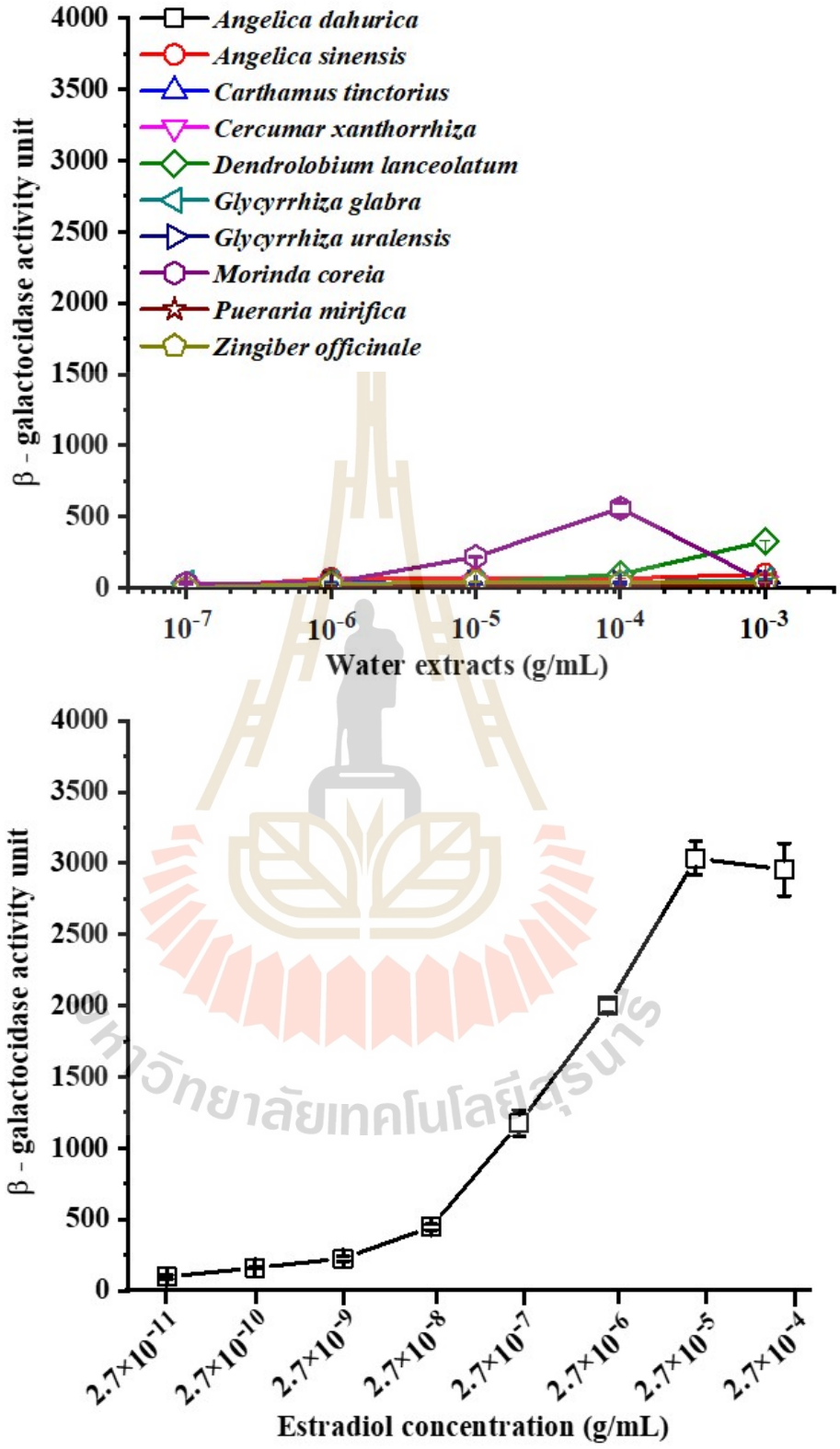


Figure 5.4 The estrogenic activities of 10 crude-water extracts (A) and standard E2 (B) as evaluated by the ERβ-Y2H system.

5.3.3 Comparison of estrogenic activity of water extracts via ERα and ERβ

As some medicinal recipes used boiling water to prepare the medicines from these plants, this work also included the investigation of estrogenic activity of the water extracts of these plant samples via ERα and ERβ (Table 5.9).

Table 5.9 Comparison of estrogenic activity of plant water extracts via ERα and ERβ.

No.	Plant species	ERα-Y2H system		ERβ-Y2H system	
		REC_{10} (g/mL)	E2-REP_{10} (%)	REC_{10} (g/mL)	E2-REP_{10} (%)
1	*Angelica dahurica*	N	N	N	N
2	*Angelica sinensis*	N	N	N	N
3	*Carthamus tinctorius*	N	N	N	N
4	*Curcuma xanthorrhiza*	1.22×10^{-5}	0.006	N	N
5	*Dendrolobium lanceolatum*	2.57×10^{-5}	0.003	8.63×10^{-4}	0.002
6	*Glycyrrhiza glabra*	1.16×10^{-4}	0.0007	N	N
7	*Glycyrrhiza uralensis*	4.35×10^{-7}	0.194	N	N
8	*Morinda coreia*	1.19×10^{-5}	0.07	1.87×10^{-5}	0.067
9	*Pueraria mirifica*	3.05×10^{-5}	0.003	N	N
10	*Zingiber officinale*	N	N	N	N
11	E2	8.44×10^{-10}	100	1.26×10^{-8}	100

Noted: N, negative.

Among ten crude water extracts, only two plant extracts exhibited estrogenic activity via the action of both ERα and ERβ; *Morinda coreia* and *Dendrolobium lanceolatum*. Four plant extracts could exhibit estrogenic activity only via ERα; *Glycyrrhiza uralensis*, *Curcuma xanthorrhiza*, *Pueraria mirifica*, *and Glycyrrhiza glabra*. From these results, the active phytoestrogens in water extracts dominantly functioned via ERα, which differed from the active phytoestrogens in ethanolic extracts that mainly worked through ERβ.

In this work, the crude-water extracts of *Morinda coreia* and *Dendrolobium lanceolatum* exhibited estrogenic activity via both ERα and ERβ, which from the literature search these two plant species have not yet reported about their estrogenic activities. Therefore, this work was the first to report their estrogenic activity.

Four crude-water extracts exhibited estrogenic activity through the action of ERα in this report; *Glycyrrhiza uralensis*, *Curcuma xanthorrhiza*, *Pueraria mirifica*, and *Glycyrrhiza glabra*. The estrogenic activity of water extract of *Glycyrrhiza uralensis* via ERα was well supported by the previous reports of Zang (Yong-Jiao Zhang et al., 2018; C. Hu et al., 2009). The crude-water extract of *Glycyrrhiza uralensis* was reported to exhibit estrogenic activity via ERα as evaluated by MCF-7 cell proliferation assay(C. Hu et al., 2009), but exhibit no estrogenic activity via ERβ as determined by the B16-F10 melanoma cell proliferation test (Yunfeng Zheng et al., 2018). In this work, the low estrogenic activity of water extract of *Curcuma xanthorrhiza* was detected via ERα but not ERβ. However, based on the literature search, there is no study of the estrogenic evaluation of its water extract yet. For *Pueraria mirifica,* this work reported the estrogenic activity of its crude-water extract

via ERα but not ERβ. Based on the literature review, the study of the estrogenic activity of water extract of *Pueraria mirifica* is not reported yet. For *Glycyrrhiza glabra*, this work reported its very low estrogenic activity via ERα, but not via ERβ. However, Nazmi (Nazmi et al., 2018) reported that the crude-water extract of *Glycyrrhiza glabra* had no estrogenic activity via both receptors as determined by the cell-proliferation assays using MCF-7 (ERα) and HT-29 (ERβ). It was likely that the Y2H assay is more sensitive for estrogenic activity detection, therefore low estrogenic detection of this crude-water extract of *Glycyrrhiza glabra* was detected in this work.

In this work, four water extracts had no estrogenic activity as determined by both systems; *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius*, *and Zingiber officinale.* Similar results of water extract of *Angelica dahurica* were reported by Tuy-on and Pervin (Tuy-on et al., 2020), in which it had no estrogenic activity as determined by the proliferation assays of MCF-7 (ERα) and HT-29 (ERβ). For *Carthamus tinctorius*, the polysaccharides isolated from water extract of this plant exhibited no estrogenic activity via the cell proliferation assay of MCF-7 cell (ERα) (Z. Luo et al., 2015). Also, Liang (Liang et al., 2017) reported the water extract of this plant had no estrogenic activity as evaluated by the proliferation effect in HT-29 (ERβ). For *Zingiber officinale,* Elkady reported that its crude water extract had no estrogenic activity as determined by the proliferation assay of MCF-7 (ERα) cell (Elkady et al., 2012). Also, Tahir (Tahir et al., 2015) reported that crude-water extract of *Zingiber officinale* exhibited no estrogenic activity as determined by the proliferation assay of HT29 (ERβ) cell.

5.4 Comparison of Estrogenic Activity of Ethanolic and Water Extracts of Ten Plant Species via the Actions of ERα and ERβ

The estrogenic activity of the ethanolic and water extracts derived from 10 plant species was compared in Table 5.10, which showed both REC_{10} and $E2\text{-}REP_{10}$ values. As $E2\text{-}REP_{10}$ values were calculated by normalizing the data with the standard E2, they were more suitable to use for the comparison of the estrogenic activity between the ethanolic and water extracts derived from each plant species.

Based on $E2\text{-}REP_{10}$, the extraction via ethanol was more efficient to extract phytoestrogenic compounds than the extraction by water. The ethanolic extracts of these plants were more efficient to function via ERβ than ERα as evaluated by the higher values of $E2\text{-}REP_{10}$. Nine out of ten ethanolic extracts exhibited estrogenic activity via ERβ, and five out of ten ethanolic extracts functioned via ERα. Among 10 plant species, the ethanolic extract of *Dendrolobium lanceolatum* exhibited the greatest estrogenic activity with the $E2\text{-}REP_{10}$ of 1.53%. As compared between ethanolic and water extracts of the same plant species, their estrogenic activities via both receptors were different, suggesting that different estrogenic compounds were obtained by both methods. Some plant extracts exhibited estrogenic activity via both ERα and ERβ, whereas some exhibited estrogenic activity via only ERα or ERβ. In this work, only one plant species (*Zingiber officinale*) showed no estrogenic activity of both ethanolic and water extracts via both receptors.

In general, the action of E2 via ERα involves in regulation of the female reproductive system, preservation of skeletal homeostasis, and regulation of metabolism (Paterni et al., 2014). ERα is present in the liver, kidney (Amenyogbe et

al., 2020), mammary gland, uterus, ovary (thecal cells), bone, testis, epididymis, prostate (stroma), adipose tissue, and heart cells (Paterni et al., 2014). While ERβ is mainly founded in the ovary, central nervous system, cardiovascular system, lung, male reproductive organs, prostate, colon, and immune system (Xue Ling Xu et al., 2021). In this study, the estrogenic activities of all ethanolic extracts (except *Zingiber officinale* extract) mainly functioned via ERβ. According to the report of Nanashima and colleagues, a possible mechanism of the phytoestrogenic compounds in herbal plant extracts act as estradiol agonists via selective binding to ERβ. Therefore, these phytoestrogens are used to treat senile-stage disorders associated with ERβ such as menopausal disorder. These phytoestrogens are preferentially used as hormone alternatives for hormone therapy (Nanashima et al., 2017).

Phytoestrogens were reported for improving skin health, which might be related to the dominant presence of ERβ in skin cells (Lephart and Naftolin, 2021). The functions of phytoestrogens via ERβ involve the increased contents of collagen, water, and elastin fiber in the skin (Rzepecki et al., 2019). Several phytoestrogens were reported to exhibit positive effects on human skin. Genistein, the phytoestrogen extracted from soybeans, was added to cosmetic products to improve skin dryness and wrinkle. Genistein was reported to increase skin collagen by inducing vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) in the skin (Tao Liu et al., 2020). Soy isoflavone was reported to stimulate the production of collagen and hyaluronic acid (HA) by human fibroblasts (Tao Liu et al., 2020). HA is a member of GAGs that functions to increase moisture content and support the proliferation of fibroblasts. Resveratrol, a phytoestrogen naturally found in grapes, was reported to increase elastin fibers (Lephart and Naftolin, 2021). As elastin supports the structure of the skin and

reinstitutes skin shape after stretching or contracting, it was added in cosmetics for anti-aging and anti-wrinkle functions (Tronnier, 2018).

Phytoestrogens that function via the interaction to ERα have been applied for treating an osteoporosis problem in menopausal women since the ERα activation involves the regulation of skeleton growth and bone remodeling (Gustafsson et al., 2016). Wu and colleagues reported that genistein could improve bone healing via triggering ERα-mediated osteogenesis-associated gene expressions and subsequent osteoblast maturation (Wu et al., 2020). Moreover, Li and colleagues (Li et al., 2015) reported that the seed extract of bakuchiol, a meroterpene from *Psoralea corylifolia*, had a binding affinity to ERα more than ERβ. It could improve bone health and reduce the risk of osteoporosis in aging persons (Bharathi and Baby, 2017).

Moreover, phytoestrogens that exhibited the activity via the binding to ERα were important for female fertility and postpartum uterine involution (W. Winuthayanon et al., 2014). Postpartum uterine involution is a regenerative process for the uterus to return to non-pregnant size (Zi. Huan Zheng et al., 2019). Zheng and colleagues (Zi. Huan Zheng et al., 2019) reported that *Apios americana* extract could improve uterine involution in female rats. These plant extracts contained high levels of isoflavone and saponin. Kaneta and colleagues (Kaneta et al., 2016) reported four types of isoflavone in the *Apios americana* extract exhibiting estrogenic activity via ERα. These researches suggested that phytoestrogens with preferential binding to ERα had potential applications in postpartum care.

Table 5.10 Comparison of estrogenic activity of ethanolic and water extracts of 10 plant species via the actions of ERα and ERβ

No.	Plant species	ERα-Y2H system				ERβ-Y2H system			
		Ethanolic extracts		Water extract		Ethanolic extracts		Water extract	
		REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)
1	*Angelica dahurica*	N	N	N	N	5.62×10^{-6}	0.02	N	N
2	*Angelica sinensis*	N	N	N	N	1.72×10^{-6}	0.05	N	N
3	*Carthamus tinctorius*	N	N	N	N	3.30×10^{-6}	0.03	N	N
4	*Curcuma xanthorrhiza*	N	N	1.22×10^{-5}	0.006	3.03×10^{-6}	0.03	N	N
5	*Dendrolobium lanceolatum*	4.53×10^{-7}	0.009	2.57×10^{-5}	0.003	5.87×10^{-8}	1.53	8.63×10^{-4}	0.002
6	*Glycyrrhiza glabra*	9.20×10^{-5}	0.00004	1.16×10^{-4}	0.0007	3.03×10^{-6}	0.03	N	N
7	*Glycyrrhiza uralensis*	2.39×10^{-4}	0.00002	4.35×10^{-7}	0.194	1.75×10^{-7}	0.51	N	N
8	*Morinda coreia*	2.48×10^{-5}	0.0002	1.19×10^{-5}	0.07	3.96×10^{-6}	0.02	1.87×10^{-5}	0.067
9	*Pueraria mirifica*	6.96×10^{-5}	0.00006	3.05×10^{-5}	0.003	5.10×10^{-6}	0.02	N	N
10	*Zingiber officinale*	N	N	N	N	N	N	N	N
11	E2	4.01×10^{-11}	100	8.44×10^{-10}	100	8.98×10^{-10}	100	1.26×10^{-8}	100

5.5 Estrogenic Activity of *In Vitro* Simulated Digestion of Ethanolic Extracts of 10 Plant Species

5.5.1 Estrogenic activity of the digested extracts (derived from ethanolc extracts) evaluated by ERα-Y2H system

In this part, *in vitro*, simulated digestion method was used to mimic the gastrointestinal tract digestion of crude ethanolic medicinal plants. The estrogenic activities of *in vitro* digested plant extracts (derived from ethanolic extracts, 10^{-4} g/mL) and standard E2 (2.7×10^{-4} g/mL or 10^{-7} M) were determined by the ERα-Y2H system as shown in Figure 5.5 and Table 5.11. For the standard E2, the digested E2 exhibited the decreased estrogenic activity to 92.82±1.02%. Similarly, six digested plant extracts showed reduced estrogenic activity; the extracts of *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius*, *Glycyrrhiza glabra*, *Morinda coreia*, and *Pueraria mirifica*. In contrast, four out of ten digested plant extracts demonstrated the increased estrogenic activity; the extracts of *Curcuma xanthorrhiza, Dendrolobium lanceolatum, Glycyrrhiza glabra*, and *Zingiber officinale*. Among all digested extracts, the digested extract of *Dendrolobium lanceolatum* exhibited the highest estrogenic activity (92.82±4.01%), which was close to the activity of the digested E2.

The increased estrogenic activity found in digested plant extracts was hypothesized to involve the structural changes of plant compounds from inactive into active forms, such as a change of glycosides to active aglycones (Basu et al., 2016). For E2 and some other plant extracts, the digested forms showed reduced estrogenic activity. The activity reduction might be due to the digestion condition damaging their active forms or changing their structures to exhibit antiestrogenic activity (Sirotkin and Harrath, 2014).

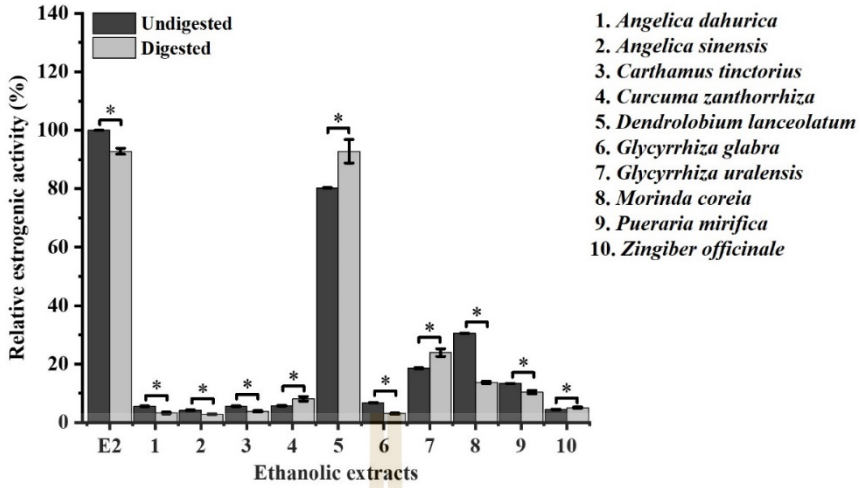


Figure 5.5 The relative estrogenic activities of undigested and digested plant extracts (ethanolic extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) by ERα-Y2H system. Independent sample T-test was analyzed at $p < 0.05$, n=3.

Table 5.11 The relative estrogenic activity of 10 ethanolic extracts under the digested and undigested conditions evaluated by ERα-Y2H system.

	Ethanolic plant extracts	**Relative estrogenic activity (%)**	
		Undigested condition	**Digested condition**
1	*Angelica dahurica*	5.50±0.13	3.26±0.27*
2	*Angelica sinensis*	4.20±0.12	2.79±0.13*
3	*Carthamus tinctorius*	5.52±0.11	3.80±0.23*
4	*Curcuma xanthorrhiza*	5.72±0.13	8.10±0.77*
5	*Dendrolobium lanceolatum*	80.27±0.06	92.82±4.01*
6	*Glycyrrhiza glabra*	6.68±0.03	3.02±0.17*
7	*Glycyrrhiza uralensis*	18.56±0.11	23.91±1.30*
8	*Morinda coreia*	30.53±0.05	13.72±0.34*
9	*Pueraria mirifica*	13.33±0.05	10.39±0.58*
10	*Zingiber officinale*	4.41±0.07	5.07±0.18*
11	E2	100.00	92.82±1.02*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3.

5.5.2 Estrogenic activity of the digested extracts (derived from ethanolic extracts) evaluated by ERβ-Y2H system

The digested plant extracts derived from ethanolic extracts of 10 plant species (10^{-4} g/mL) and the standard E2 (2.7×10^{-7} g/mL) were also evaluated for their estrogenic activities by the ERβ-Y2H system (Figure 5.6 and Table 5.12). The digested E2 exhibited reduced estrogenic activity to 94.23±1.53%. Similarly, eight out of ten digested plant extracts exhibited the reduction of estrogenic activity; *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius*, *Curcuma xanthorrhiza*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Morinda coreia*, and *Pueraria mirifica*. In contrast, only two digested plant extracts exhibited the increased estrogenic activity; *Dendrolobium lanceolatum* and *Zingiber officinale*. Interestingly, the digested extract of *Dendrolobium lanceolatum* exhibited the highest estrogenic activity (160.06±8.20%), which was much higher than the standard E2. The results of this work suggested that *Dendrolobium lanceolatum* might be the source of potent phytoestrogens for medicinal and cosmetic applications due to its strong estrogenic activity as compared with other known phytoestrogenic plants in Thailand. Also, the estrogenic activity of this plant species was first reported here. So, its potential applications should be further studied for commercial products.

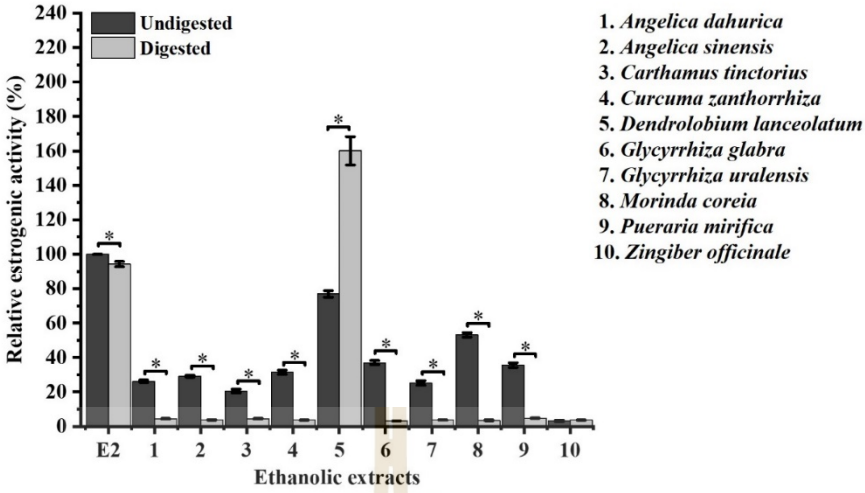


Figure 5.6 The relative estrogenic activities of undigested and digested plant extracts (ethanolic extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) evaluated by ERβ-Y2H system. Independent sample T-test was analyzed at $p < 0.05$, n=3.

Table 5.12 The relative estrogenic activity of 10 ethanolic extracts under the digested and undigested conditions evaluated by ERβ-Y2H system

Ethanolic plant extracts	Relative estrogenic activity (%)	
	Undigested condition	Digested condition
Angelica dahurica	26.27±0.70	4.48±0.04*
Angelica sinensis	29.18±0.55	3.69±0.09*
Carthamus tinctorius	20.53±1.02	4.47±0.19*
Curcuma xanthorrhiza	31.43±1.18	3.67±0.08*
Dendrolobium lanceolatum	76.96±1.92	160.06±8.20*
Glycyrrhiza glabra	37.01±1.20	3.12±0.00*
Glycyrrhiza uralensis	25.19±1.27	3.76±0.00*
Morinda coreia	53.15±1.28	3.52±0.20*
Pueraria mirifica	35.58±1.32	4.80±0.05*
Zingiber officinale	3.18±0.39	3.70±0.10
E2	100.00	94.23±1.53*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3

5.5.3 Comparison of estrogenic activities of the digested ethanolic extracts evaluated by ERα-Y2H and ERβ-Y2H systems

The estrogenic activities of the digested ethanolic extracts derived from 10 plant species that were evaluated by ERα-Y2H and ERβ-Y2H systems were compared in Table 5.13. The results revealed that ethanolic extracts of these plant after in vitro digestion mostly exhibited the reduction of estrogenic activity through the interaction of both ERα and ERβ. However, few digested extracts could possess increased estrogenic activity. Via ERα, four digested extracts derived from *Curcuma xanthorrhiza*, *Glycyrrhiza uralensis*, *Dendrolobium lanceolatum*, and *Zingiber officinale* exhibited escalated estrogenic activity; 146.73, 128.83, 115.63, and 114.97% increase, respectively. Via ERβ, only digested extracts of *Dendrolobium lanceolatum* exhibited the rise of estrogenic activity; 207.98% increase.

For *Dendrolobium lanceolatum*, this work was the first report to compare its estrogenic activity in the conditions with and without simulated digestion via the interaction with ERα and ERβ. Since its digested extract exhibited significant increase of estrogenic activity via both receptors, its phytoestrogens were potential for pharmaceutical applications that required an oral uptake. Also, an in vitro digestion of this plant extract likely generated new effective compounds exhibiting estrogenic activity, which should further study. Based on the report of Kanokmedhakul and colleagues, the major isolated compounds of *Dendrolobium lanceolatum* were flavanones, flavan, and dibenzocycloheptene derivative (Kanokmedhakul et al., 2004). Previous report revealed that plant flavanone and flavan, belonging to a flavonoid group, were considered as phytoestrogens due to their exhibition of estrogenic activity

(Durazzo et al., 2019). Nevertheless, these isolated compounds from *Dendrolobium lanceolatum* have never been tested for their estrogenic activity.

For *Curcuma xanthorrhiza*, this work was the first report of the estrogenic activity of its in vitro digested extract. Its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. Its estrogenic activity was 146.73% increase under digested condition. Based on the reported of Aznam and Atun (Aznam and Atun, 2017), the major isolated compounds of *Curcuma xanthorrhiza* was curcuminoids. They exhibited estrogenic activity via ERα as determined by positive proliferation effect in ERα-positive MCF-7 cells. However, they did not stimulate the proliferation of ERβ-positive MDA-MB-231 cells (Bachmeier et al., 2010). Moore and colleagues (Moore et al., 2014) reported that the in vitro digested UBS109 (curcumin analogue) exhibited moderate estrogenic activity via a proliferation assay of MDA-MB-231 cells. Winuthayanon and colleagues reported about that the estrogenic activity of diarylheptanoids, the main active compounds in the extract of Curcuma comosa, in the conditions with and without digestion with a rat liver S9-fraction. They found that the metabolic activation of diarylheptanoids significantly increased their estrogenic activity (Wipawee Winuthayanon et al., 2009).

For *Glycyrrhiza uralensis*, its digested extract also exhibited increased estrogenic activity via ERα but not ERβ. Its estrogenic activity was 128.83% increase under digested condition. Previously reported, the major compounds of *Glycyrrhiza uralensis* were glycyrrhizic acid (10.8%), liquiritin (2.9%), and isoliquiritigenin (2.2%) (Barbosa et al., 2017). In the digested system, liquiritin was metabolized to liquiritigenin by intestinal flora (Meng et al., 2012). Liquiritigenin exhibited estrogenic activity as it could stimulate proliferation of ERα-positive MCF-7

cells. However, it did not function via ERβ since it exhibited no proliferation effect in ERβ-positive MDA-MB-231 cells (Lattrich et al., 2013). Although, glycyrrhizic acid is a major compound of *Glycyrrhiza uralensis*, it exhibited no estrogenic activity as evaluated by the alkaline phosphatase activity via ERα in Ishikawa cell assay (Poh and Yin, 2013).

For *Zingiber officinale*, its digested extract showed increased estrogenic activity via ERα but not ERβ. Its estrogenic activity was 114.97% increase under digested condition. Previously reported, the major bioactive compounds of *Zingiber officinale* was gingerols (Asamenew et al., 2019). Among gingerols, 6-gingerol was the predominant bioactive compound that reported for its estrogenic activity as determined by cell proliferation assay (Mao et al., 2019). It induced a proliferation of ERα-positive osteoblast MG-63 cells (Fan et al., 2015), but not of ERβ-positive MDA-MB-231 cells (Luna et al., 2018). Also, 6-gingerol was reported to stable in a gastrointestinal condition and be absorbed in an intestinal tract (Annunziato et al., 2018).

For the rest of the plant extracts, their estrogenic activities were reduced under a simulated digestion condition. These findings were supported by several publication reports as follows.

For *Angelica dahurica*, one of its major compounds is imperatorin, which its digested metabolite, heraclenin, exhibited no estrogenic activity as evaluated by ERα-positive MCF-7 cells (Mottaghipisheh et al., 2018) and positive ERβ-positive DU145 cells (Y. Xu et al., 2017).

For *Angelica sinensis*, its major compound is ligustilide, which could be metabolized into senkyunolide I in a condition with a rat liver S9 extract (Xie et al.,

2020). Hsieh and colleagues (Hsieh et al., 2015) reported that senkyunolide I showed no estrogenic activity in ERβ-positive HT-29 cells. However, based on a literature search, this digested extract of *Angelica sinensis* has not yet been reported on its estrogenic activity via ERα.

For *Carthamus tinctorius*, its major active compounds were flavonoids (Mani et al., 2020), which could be hydrolyzed into diosmetin and luteolin by intestinal enzymes (Rice, 2004). Diosmetin was reported to exhibit no estrogenic activity via ERα-positive MCF-7 cells (Bin Liu et al., 2016), nor ERβ-positive DU145 cells (Soares et al., 2019). Luteolin was also reported to exhibit no estrogenic activity as determined in both ERα-positive T47D cells and ERβ-positive MDA-MB 231 cells (Liming Huang et al., 2019).

For *Morinda coreia*, its major active compound was anthraquinone (Kannan et al., 2020). In the intestine, anthraquinone was metabolized into anthraquinone aglycone based on the functions of enzymes in the intestinal flora (Dongpeng Wang et al., 2021). The anthraquinone aglycone of the other plant species, *Rubia philippinensis*, was reported to exhibit no estrogenic activity in both ERα-positive MCF-7 cells and ERβ-positive MDA-MB-231 cells. (Bajpai et al., 2018) However, based on a literature search, there is not any report on the digested extract of *Morinda coreia* and its estrogenic activity via ERα and ERβ.

For *Pueraria mirifica*, its bioactive compounds were in the groups of isoflavones, lignans, and coumestans (Lin et al., 2017). Among these compounds, isoflavones were a major group of phytoestrogens in this plant (S. Malaivijitnond et al., 2004). Puerarin, genistin, and daidzin were the dominant compounds in an isoflavone group, which could be metabolized into daidzein and genistein by intestinal microflora

(Suchinda Malaivijitnond, 2012). Both genistein and daidzein were reported to exhibit no estrogenic activity in ERα-positive MCF-7 cells and ERβ-positive MDA-MB-231 cells (Zhu et al., 2018).

Table 5.13 Comparison of estrogenic activities of the digested ethanolic extracts derived from 10 plant species evaluated by ERα-Y2H and ERβ-Y2H systems

	Digested ethanolic extracts derived from plants	Evaluation by ERα-Y2H		Evaluation by ERβ-Y2H	
		Relative estrogenic activity (%)		Relative estrogenic activity (%)	
		Undigested condition	Digested condition	Undigested condition	Digested condition
1	*Angelica dahurica*	5.50±0.13	3.26±0.27*	26.27±0.70	4.48±0.04*
2	*Angelica sinensis*	4.20±0.12	2.79±0.13*	29.18±0.55	3.69±0.09*
3	*Carthamus tinctorius*	5.52±0.11	3.80±0.23*	20.53±1.02	4.47±0.19*
4	*Curcuma xanthorrhiza*	5.72±0.13	8.10±0.77*	31.43±1.18	3.67±0.08*
5	*Dendrolobium lanceolatum*	80.27±0.06	92.82±4.01*	76.96±1.92	160.06±8.20*
6	*Glycyrrhiza glabra*	6.68±0.03	3.02±0.17*	37.01±1.20	3.12±0.00*
7	*Glycyrrhiza uralensis*	18.56±0.11	23.91±1.30*	25.19±1.27	3.76±0.00*
8	*Morinda coreia*	30.53±0.05	13.72±0.34*	53.15±1.28	3.52±0.20*
9	*Pueraria mirifica*	13.33±0.05	10.39±0.58*	35.58±1.32	4.80±0.05*
10	*Zingiber officinale*	4.41±0.07	5.07±0.18*	3.18±0.39	3.70±0.10
11	E2	100.00	92.82±1.02*	100.00	94.23±1.53*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3

5.6 Estrogenic Activity of *in vitro* Simulated Digestion of Water Extracts of 10 Plant Species

5.6.1 Estrogenic activity of the digested extracts (derived from water extracts) evaluated by ERα-Y2H system

The water extracts of 10 plant species under undigested and digested conditions were also evaluated for their estrogenic activities by ERα-Y2H and ERβ-Y2H systems.

In this part, their estrogenic activities were determined by the ERα-Y2H system as shown in Figure 5.7 and Table 5.14. Similar to the previous study, the digested E2 exhibited reduced estrogenic activity to 94.14±1.56%. In this work, only one extract showed reduced estrogenic activity; *Glycyrrhiza glabra*. Two extracts showed no significantly different estrogenic activities of undigested and digested extracts; *Curcuma xanthorrhiza* and *Zingiber officinale*. In contrast, seven digested extracts demonstrated the increased estrogenic activities; *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius*, *Dendrolobium lanceolatum*, *Glycyrrhiza uralensis*, *Morinda coreia, and Pueraria mirifica* potent estrogenic activity than undigested extracts. Interestingly, the digested extract of *Glycyrrhiza uralensis* exhibited the highest estrogenic activity 82.73±1.90%, which was a 3.65-fold increase as compared with its undigested extract. As compared with ethanolic extracts of these plant species, their estrogenic activities evaluated by the ERα-Y2H system greatly differed from those of water extracts. These results suggested that different phytoestrogens were likely obtained from ethanolic and water extracts. Therefore, the digested extracts of these plant extracts from water and ethanolic extracts exhibited significantly different estrogenic activities via the interaction of the same receptor, ERα.

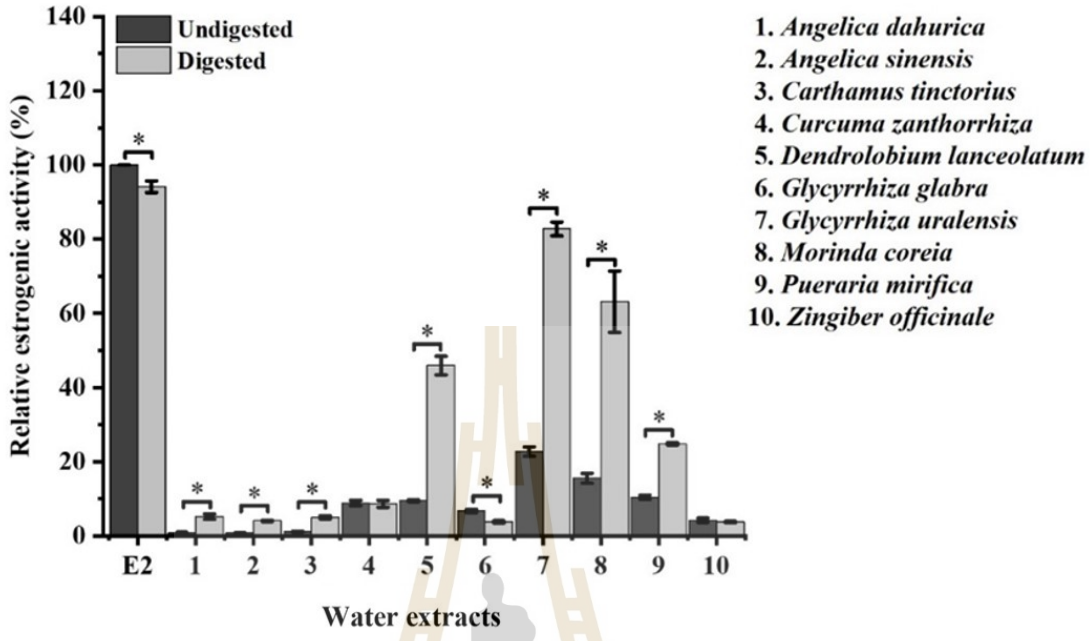


Figure 5.7 The relative estrogenic activities of undigested and digested plant extracts (water extracts, 1×10^{-4} g/mL) and standard E2 (1×10^{-7} g/mL) evaluated by ERα-Y2H system. Independent sample T-test was used at $p < 0.05$, n=3.

Table 5.14 The relative estrogenic activity of water extracts derived from 10 plant species under the digested and undigested conditions evaluated by ERα-Y2H system.

Water plant extracts	Relative estrogenic activity (%)	
	Undigested condition	Digested condition
Angelica dahurica	0.84±0.06	5.17±0.73*
Angelica sinensis	0.81±0.02	4.02±0.18*
Carthamus tinctorius	1.14±0.08	4.96±0.41*
Curcuma xanthorrhiza	8.86±0.71	8.63±0.95
Dendrolobium lanceolatum	9.48±0.27	45.91±2.51*
Glycyrrhiza glabra	6.67±0.42	3.73±0.30*
Glycyrrhiza uralensis	22.69±1.28	82.73±1.90*
Morinda coreia	15.51±1.32	63.10±8.28*
Pueraria mirifica	10.39±0.58	24.82±0.32*
Zingiber officinale	4.21±0.57	3.73±0.15
E2	100.00	94.14±1.56*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3.

5.6.2 Estrogenic activity of the digested extracts (derived from water extracts) evaluated by ERβ-Y2H system

The estrogenic activities of *in vitro* digested plant extracts (derived from water extracts) were also evaluated by the ERβ-Y2H system as shown in Figure 5.8 and Table 5.15. The digested E2 exhibited reduced estrogenic activity to 93.46±1.78%. Similarly, 7 digested plant extracts exhibited reduced estrogenic activities, which were

the extracts of *Angelica dahurica*, *Angelica sinensis*, *Carthamus tinctorius*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Morinda coreia*, *and Zingiber officinale*. Two digested extracts of *Curcuma xanthorrhiza* and *Pueraria mirifica* showed no significant difference between the estrogenic activities of undigested and digested samples. In contrast, one digested extract showed the induced estrogenic activities, which was the extract of *Dendrolobium lanceolatum*. Among these plant extracts, the digested extract of *Dendrolobium lanceolatum* exhibited the highest estrogenic activity (43.52±1.01%).

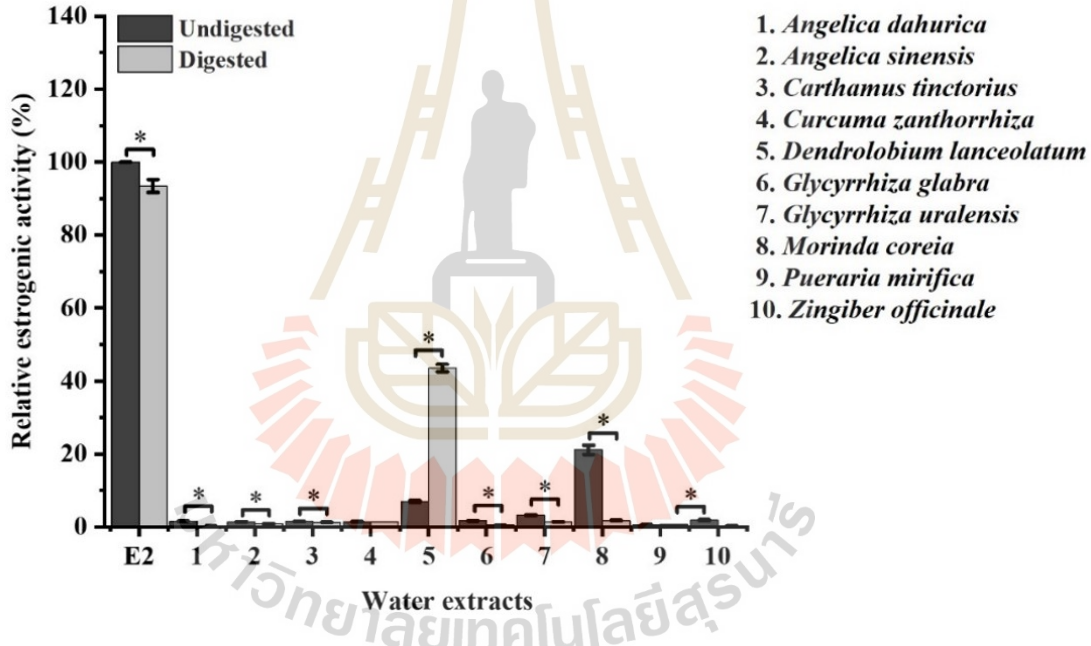


Figure 5.8 The relative estrogenic activities of undigested and digested plant extracts (water extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) evaluated by ERβ-Y2H system. Independent sample T-test was analyzed at $p < 0.05$, n=3.

Table 5.15 The relative estrogenic activity of water extracts of 10 plant species under the digested and undigested conditions evaluated by ERβ-Y2H system.

Water plant extracts	Relative estrogenic activity (%)	
	Undigested condition	Digested condition
Angelica dahurica	1.55±0.06	0.24±0.01*
Angelica sinensis	1.35±0.07	0.83±0.01*
Carthamus tinctorius	1.49±0.04	1.23±0.03*
Curcuma xanthorrhiza	1.44±0.08	1.40±0.00
Dendrolobium lanceolatum	6.98±0.30	43.52±1.01*
Glycyrrhiza glabra	1.66±0.08	0.44±0.05*
Glycyrrhiza uralensis	3.19±0.11	1.37±0.05*
Morinda coreia	21.09±1.27	1.73±0.09*
Pueraria mirifica	0.49±0.09	0.63±0.00
Zingiber officinale	1.83±0.14	0.25±0.00*
E2	100.00	93.46±1.78*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3.

5.6.3 Comparison of estrogenic activities of the digested water extracts evaluated by ERα-Y2H and ERβ-Y2H systems

The estrogenic activities of the digested water extracts derived from 10 plant species that were evaluated by ERα-Y2H and ERβ-Y2H systems were compared in Table 5.16. The results revealed that water extracts of these plants after in vitro digestion mostly exhibited the increases of estrogenic activity via ERα, whereas possessed the reduction of estrogenic activity via ERβ. Based on the function via ERα,

seven out of ten plant extracts under an in vitro digestion exhibited the increases in estrogenic activity, including the extracts derived from *Angelica dahurica*, *Angelica sinensis*, *Dendrolobium lanceolatum*, *Carthamus tinctorius*, *Morinda coreia*, *Glycyrrhiza uralensis*, and *Pueraria mirifica* exhibited escalated estrogenic activity. Their estrogenic activities increased 6.2, 5.0, 4.4, 4.8, 3.6, 4.1, and 2.4 folds, respectively. Based on the action via ERβ, only digested extract of *Dendrolobium lanceolatum* exhibited the rise of estrogenic activity with a 6.2-fold increase. The rest of the extracts exhibited a reduction of estrogenic activity.

For *Dendrolobium lanceolatum*, this work was the first report of the estrogenic activity of its in vitro digested extract, which exhibited increased estrogenic activity via both ERα and ERβ. Based on the literature search, the water extract of this plant has not yet been reported, nor its estrogenic activity via ERα and ERβ.

For *Angelica dahurica*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. The major bioactive compounds of a water extract of *Angelica dahurica* was kaempferol (Pervin et al., 2014). These compounds were metabolized into kaempferol derivatives by gut microbiota (Vollmer et al., 2018). Guo and colleagues (Guo et al., 2012) reported that kaempferol derivatives induced ERα phosphorylation assay in cultured rat osteoblasts, suggesting its action via ERα activation.

For *Angelica sinensis*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. The major bioactive compound of *Angelica sinensis* water extract was ferulic acid (Wu et al., 2020). Ferulic acid could be absorbed in the intestine without digested by intestinal enzyme, according to studies carried out in situ in the rat stomach (Paiva et al., 2013). Hao and colleagues (Hao et al., 2010) reported

that ferulic acid increased the level of ERα protein expression in T47D cells. However, it exhibited no estrogenic activity via interaction with ERβ in MDA-MB-231 cells.

For *Carthamus tinctorius*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. The major bioactive compound of *Carthamus tinctorius* water extract was phenylethanoid glycoside (Delshad et al., 2018). Via digestion, the phenylethanoid glycoside was metabolized into caffeic acid and hydroxytyrosol by intestinal bacteria (Xiaoming Wang et al., 2019). Liu and colleagues (Lili Liu et al., 2021) reported that caffeic acid exhibited estrogenic activity in ERα-positive MG-63 cells. However, caffeic acid did not function via ERβ since it exhibited no proliferation effect in ERβ-positive MDA-MB-231 cells (Hernandes et al., 2020). Moreover, Garcia-Martinez and colleagues (Garcia et al., 2016) reported that hydroxytyrosol could increase cell proliferation of ERα-positive MG-63 cells. However, hydroxytyrosol exhibited no estrogenic activity in ERβ-positive Du145 cells (Zubair et al., 2017).

For *Morinda coreia*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. The major bioactive compound of *Morinda coreia* water extract was iridoid glycoside (Kanchanapoom et al., 2002). Via a digestion, the iridoid glycoside of *Rehmannia glutinosa* extract was metabolized into catalpol (Tao et al., 2016). Chen and colleagues (Qi Chen et al., 2021) reported that catalpol could increase ERα expression in J774A.1 mouse macrophage cells. However, catalpol did not exhibit estrogenic activity in ERβ-positive Du145 cells (Hwang et al., 2012).

For *Glycyrrhiza uralensis*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. Kim and colleagues (K. U. Kim et al., 2020) reported that *Glycyrrhiza uralensis* water extract after fermenting with *Monascus albidulus*

resulted in bioconverting glycosides into their corresponding aglycones. Its fermented extracts exhibited increased estrogenic activities of 7.3 and 1.52 folds as determined by ERα- and ERβ-yeast estrogen binding assays, respectively. Later, the study showed that a major bioactive compound in the fermented *Glycyrrhiza uralensis* water extract was liquiritigenin. Based on literature search, liquiritigenin exhibited estrogenic activity in ERα-positive MCF-7 cells but not in ERβ-positive MDA-MB-231 cells (Lattrich et al., 2013). This result clarified that digested *Glycyrrhiza uralensis* water extract did not function via ERβ.

For *Pueraria mirifica*, its digested extract exhibited increased estrogenic activity only via ERα but not ERβ. The major bioactive compound of *Pueraria mirifica* water extracts was daidzin, puerarin and genistin (S. Malaivijitnond et al., 2004). Puerarin and genistin were metabolized to equol by human intestinal flora (Jin et al., 2008). Tsuji and colleagues (Tsuji et al., 2018) reported that equol exhibited estrogenic activity in ERα-positive MCF-7 cells (Tsuji et al., 2018). However, equol did not exhibit estrogenic activity in ERβ-positive LnCaP, DU145, and PC3 human prostate cancer cells (Lu et al., 2016).

For *Curcuma xanthorrhiza*, in this work its undigested and digested extracts exhibited no significant difference of estrogenic activity. Its estrogenic activity was quite low, but its function was ERα-preference. Nevertheless, based on literature search, its major compound was curmarin (Panigoro et al., 2013), which its digested metabolites, tetrahydrocurcumin and octahydrocurcumin, exhibited no estrogenic activity as evaluated by ERα-positive MCF-7 cells (Pandey et al., 2020) and positive ERβ-positive MDA-MB-231 (Mock et al., 2015). It was likely that the active phytoestrogens in *Curcuma xanthorrhiza* was not curmarin.

For *Glycyrrhiza glabra*, its estrogenic activities were reduced after digestion via both ERα- and ERβ-assays in this work. The major active compound of *Glycyrrhiza glabra* extract is glycyrrhizin belonging to the group of triterpenoid saponin (Varsha Sharma et al., 2017). Glycyrrhizin was hydrolyzed by intestinal microflora to glycyrrhetinic acid (He et al., 2019), which exhibited no estrogenic activity in ERα-positive MCF-7 cells (Zhen Zhang et al., 2019) and ERβ-positive MDA-MB-231 (Y. Cai et al., 2017)

For *Zingiber officinale*, this work showed very low estrogenic activity of this plant extract. Also, its digested extract showed significant reduced estrogenic activity via ERβ-assay. From literature, the major compound of *Zingiber officinale* extract was zingerone (Ahmad et al., 2015), which stable in a gastrointestinal system and absorbed rapidly into the circulatory system (Ling Ling Li et al., 2019). However, zingerone did not exhibit estrogenic activity as evaluated by ERα-positive MCF-7 cells (Gan et al., 2019) and ERβ-positive MDA-MB-231 (Chenxu Wang et al., 2020). Instead, it was quite toxic to MDA-MB-231 cells and induced their apoptosis. So its active phytoestrogens might not be zingerone.

Table 5.16 Comparison of estrogenic activities of the digested water extracts derived from 10 plant species evaluated by ERα-Y2H and ERβ-Y2H systems.

	Digested water extracts derived from plants	Evaluation by ERα-Y2H		Evaluation by ERβ-Y2H	
		Relative estrogenic activity (%)		Relative estrogenic activity (%)	
		Undigested condition	Digested condition	Undigested condition	Digested condition
1	*Angelica sinensis*	0.84±0.06	5.17±0.73*	1.55±0.06	0.24±0.01*
2	*Angelica dahurica*	0.81±0.02	4.02±0.18*	1.35±0.07	0.83±0.01*
3	*Carthamus tinctorius*	1.14±0.08	4.96±0.41*	1.49±0.04	1.23±0.03*
4	*Curcuma xanthorrhiza*	8.86±0.71	8.63±0.95	1.44±0.08	1.40±0.00
5	*Dendrolobium lanceolatum*	9.48±0.27	45.91±2.51*	6.98±0.30	43.52±1.01*
6	*Glycyrrhiza glabra*	6.67±0.42	3.73±0.30*	1.66±0.08	0.44±0.05*
7	*Glycyrrhiza uralensis*	22.69±1.28	82.73±1.90*	3.19±0.11	1.37±0.05*
8	*Morinda coreia*	15.51±1.32	63.10±8.28*	21.09±1.27	1.73±0.09*
9	*Pueraria mirifica*	10.39±0.58	24.82±0.32*	0.49±0.09	0.63±0.00
10	*Zingiber officinale*	4.21±0.57	3.73±0.15	1.83±0.14	0.25±0.00*
11	E2	100.00	94.14±1.56*	100.00	93.46±1.78*

Remark: Comparison between undigested and digested samples, * $p < 0.05$, n=3.

CHAPTER VI

RESULTS AND DISCUSSION PART III

Evaluation of estrogenic activity of 8 commercially traditional medicines by yeast two hybrid system

In this chapter, the estrogenic activity of 8 commercially traditional medicines were evaluated by ERα-Y2H and ERβ-Y2H systems to understand their actions via both estrogen receptor types. Also, their estrogenic activities after *in vitro* simulated digestion were investigated.

6.1 Preparation of 8 Commercially Traditional Medicines Extracts

Eight commercially traditional medicines were selected in this study according to their applications to treat symptoms relating to menstruation disorder and female reproductive system. These medicines were referred to as S1, S2, S3, S4, S5, S6, S7, and S8 (Table 6.1). They were bought from the locally traditional pharmacies as capsules and pills (Figure 6.1). The retrieved powder from each capsule or pill is shown in Figure 6.2. The composition of medicinal plants (% mass) of each medicine as indicated by its label is shown in Table 6.2.

6.1.1 Ethanolic extraction

Dried powder of these traditional medicines were extracted in 70% ethanol for 1 day before evaporating by rotary evaporator. The yield, maximal solubility in DMSO, pH, and color of the crude-ethanolic extracts are shown in Table 6.3. Their yields were in a range of 5.2–26.5 g per 100 g powder. The dried extracts were solubilized in DMSO with the maximal solubility at 0.1 g/mL. The extracts were yellow or brown colors. Their pH after solubilizing in YPD culture medium was 6.5. The recommended dosed based on the manufactures and the obtained ethanolic-extracts were listed in Table 6.4.

Table 6.1 Commercially traditional medicines used in this study.

Commercial name	Referred to as	Indication uses
1 Chomjai	S1	Induction of lochia, uterus involution, menstrual regularity, and blood production.
2 Chomtong	S2	Relief of dysmenorrhea, menstrual disorder, and hematinic tonic.
3 Kwaokrua	S3	Increased blood production.
4 Ledina	S4	Relief of menstrual disorder and increase of blood production.
5 Lengkhun	S5	Relief of menstrual disorder and increase of blood production.

Table 6.1 Commercially traditional medicines used in this study (Continued).

Commercial name	Referred to as	Indication uses
6 Meree	S6	Induction of lochia, uterus involution, menstrual regularity, and blood production.
7 Penpark	S7	Increased blood production and circulation, and stimulation of regular menstruation.
8 Tankwe	S8	Relief of menstrual disorder and increase of blood production.

Figure 6.1 The commercially traditional medicines (S1-S8) were used in this study.

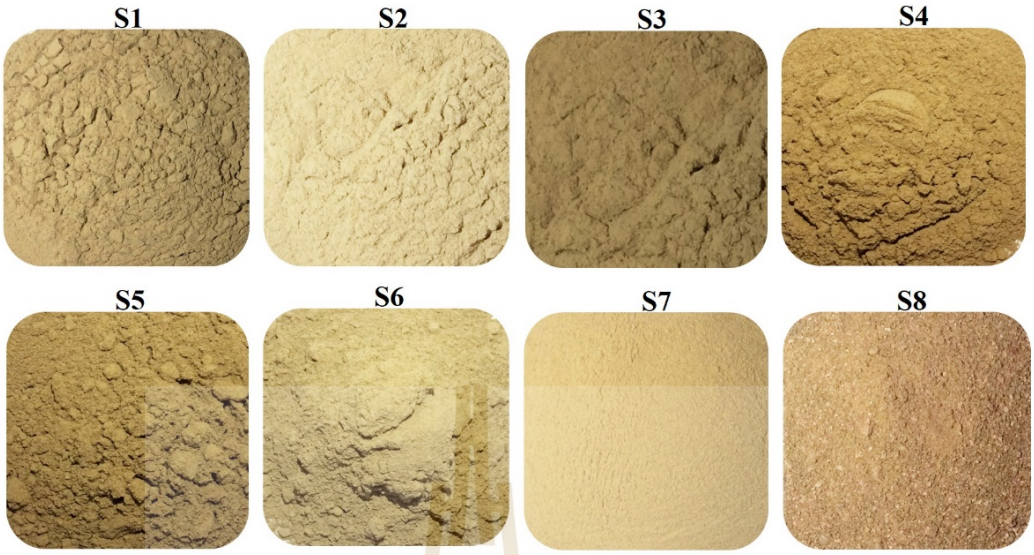


Figure 6.2 Dried powder of eight traditional medicines (S1-S8) as retrieved from their capsules and pill.

Table 6.2 The composition of medicinal plants in (% mass) as indicated on the medicines' labels.

	Plant species	Composition of medicinal plant (% mass) in							
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
1	*Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch.		1.94						
2	*Angelica sinensis* (Oliv.) Diels	2.54	1.94	22.00	14.00	7.50	25.00	3.45	
3	*Aquilaria crassna* Pierre ex Lecomte							2.46	17.48
4	*Astragalus membranaceus* (Fisch.) Bunge								7.34
5	*Atractylodes lancea* (Thunb.) DC.					6.25			
6	*Caesalpinia sappan* L.	5.09			14.00			9.73	
7	*Capsicum frutescens* L.	2.54							
8	*Carthamus tinctorius* L.	5.09	3.24		14.00			2.92	
9	*Citrus hystrix* DC.	2.54							
10	*Corydalis yanhusuo* (Y.H.Chou & Chun C.Hsu) W.T.Wang extract					6.25			
11	*Curcuma xanthorrhiza* Roxb.	7.63	3.24		14.00	12.50	20.00		
12	*Cyperus rotundus* L.					6.25			
13	*Glycyrrhiza grabra* L.					2.50			
14	*Glycyrrhiza uralensis* Fisch.		3.24						4.20
15	*Leersia hexandra* Sw.	2.54							

Table 6.2 The composition of medicinal plants in (% mass) as indicated on the medicines' labels (Continued).

	Plant species	Composition of medicinal plant (% mass) in							
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
16	*Leonurus sibiricus* L.		3.24		14.00	7.50			
17	*Ligusticum sinense* Oliv.		3.24			5.00	2.50	3.45	7.34
18	*Mimusops elengi* L.		3.24						
19	*Molineria latifolia* (Dryand. ex W.T.Aiton) Herb. ex Kurz	2.54	3.24						
20	*Neopicrorhiza scrophulariiflora* (Pennell) D.Y.Hong	2.54							
21	*Paeonia lactiflora* Pall.					5.00			7.34
22	*Panax ginseng* C.A.Mey.	1.27	0.97	22.00		12.50	3.33		
23	*Panax pseudoginseng* Wall.	1.27							
24	*Piper interruptum* Opiz	1.27							
25	*Piper nigrum* L.	2.54			14.00				
26	*Piper sarmentosum* Roxb.	1.27							
27	*Plumbago indica* L.	1.27							
28	*Poria cocos*					6.25	3.33		7.34
29	*Pueraria mirifica* Airy Shaw & Suvat.			20.00		2.50			
30	*Rehmannia glutinosa* (Gaertn.) DC.					6.25			7.34
31	*Salvia miltiorrhiza* Bunge		0.97			7.50			7.34

Table 6.2 The composition of medicinal plants in (% mass) as indicated on the medicines' labels (Continued).

	Plant species	Composition of medicinal plant (% mass) in							
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
32	*Saussurea lappa* (Decne.) Sch.Bip.					6.25			
33	Scoparia dulcis L.	2.54	3.24						
34	*Senna garrettiana* (Craib) H.S.Irwin & Barneby				14.00				
35	*Zingiber cassumunar* Roxb.	2.54	3.24						
36	*Zingiber nigrum* Gaertn.		3.24						
37	*Zingiber officinale* Roscoe	2.54							

Table 6.3 Properties of crude-ethanolic extracts of 8 commercially traditional medicines.

Traditional medicines		Yield (g / 100 g dried powder)	Maximal solubility (g/mL)	Soluble pH	Color
1	S1	17.6	0.1	6.5	Brown
2	S2	10.4	0.1	6.5	Yellow
3	S3	15.0	0.1	6.5	Yellow
4	S4	12.7	0.1	6.5	Brown
5	S5	5.2	0.1	6.5	Brown
6	S6	8.6	0.1	6.5	Yellow
7	S7	19.0	0.1	6.5	Brown
8	S8	26.5	0.1	6.5	Yellow

Table 6.4 The recommended doses and the equivalences to crude ethanolic extracts of the commercial traditional medicines.

Traditional medicine	Recommended dose/Day	Equivalent to crude extract/Day (g)
S1	6 capsules	0.47
S2	6 capsules	0.34
S3	1 capsule	0.08
S4	2 capsules	0.10
S5	6 capsules	0.16
S6	12 capsules	0.51
S7	4 capsules	0.32
S8	30 pills	2.60

6.1.2 Water extraction

Dried powder of these traditional medicines were also extracted in water at 95 °C for 1 h before freeze-drying. The yield, maximal solubility in DMSO, pH, and color of the crude-water extracts are shown in Table 6.5. Their yields were in a range of 4.49–75.30 g per 100 g powder. The dried extracts were solubilized in DMSO with the maximal solubility at 0.1 g/mL. The extracts were yellow or brown colors. Their pH after solubilizing in YPD culture medium was 6.5. The recommended doses of each traditional medicine were listed and the obtained extract from each dose was given is listed in Table 6.6.

Table 6.5 Properties of crude water-extracts of 8 commercially traditional medicines.

Traditional medicines	**Yield (g / 100 g dried powder)**	**Maximal solubility (g/mL)**	**Soluble pH**	**Color**
S1	4.49	0.10	6.50	Yellow
S2	3.81	0.10	6.50	Brown
S3	35.03	0.10	6.50	Brown
S4	11.41	0.10	6.50	Yellow
S5	20.24	0.10	6.50	Brown
S6	19.51	0.10	6.50	Brown
S7	75.30	0.10	6.50	Yellow
S8	27.93	0.10	6.50	Brown

Table 6.6 The recommended doses and the obtained water-extracts of the commercially traditional medicines.

Traditional medicine	Recommended dose/Day	Crude extract/Day (g)
S1	6 capsules	0.12
S2	6 capsules	0.13
S3	1 capsule	0.18
S4	2 capsules	0.09
S5	6 capsules	0.61
S6	12 capsules	1.15
S7	4 capsules	1.28
S8	30 pills	2.74

6.2 Estrogenic Activities of Crude-Ethanolic Extracts

6.2.1 Evaluation by ERα-Y2H system

In this part, the estrogenic activities of crude ethanolic-extracts (10^{-7}–10^{-3} g/mL) were evaluated by ERα-Y2H system with the reference to the estrogenic activity of standard E2 (2.7×10^{-11}–2.7×10^{-4} g/mL). The results were showed eight traditional medicine extracts exhibited the estrogenic activity in a dose-dependent response via the interaction of ERα (Figure 6.3). The extracts of S4 exhibited the highest estrogenic activity, followed by the extracts of S3, S8, S1, S5, S6, S7, and S2, respectively.

The REC_{10} (10% relative effective concentration) and E2-REP_{10} (10% relative potency) were determined to compare the estrogenic activity of the traditional

medicine extracts with the standard E2. Although all eight extracts exhibited the estrogenic activity via ERα-Y2H analysis in Figure 6.3, only seven extracts could calculated for REC_{10} and $E2\text{-}REP_{10}$ (Table 6.7). So only seven extracts were confirmed to exhibit estrogenic activity based on REC_{10} and $E2\text{-}REP_{10}$ evaluations. Among these extracts, the extract of S3 exhibited the highest activity, followed by S8, S4, S6, S1, S5, and S7. Their estrogenic activities were compared with the standard E2, which their $E2\text{-}REP_{10}$ values were 0.4496, 0.0135, 0.0073, 0.0031, 0.0026,0.0012, and 0.0003%, respectively.

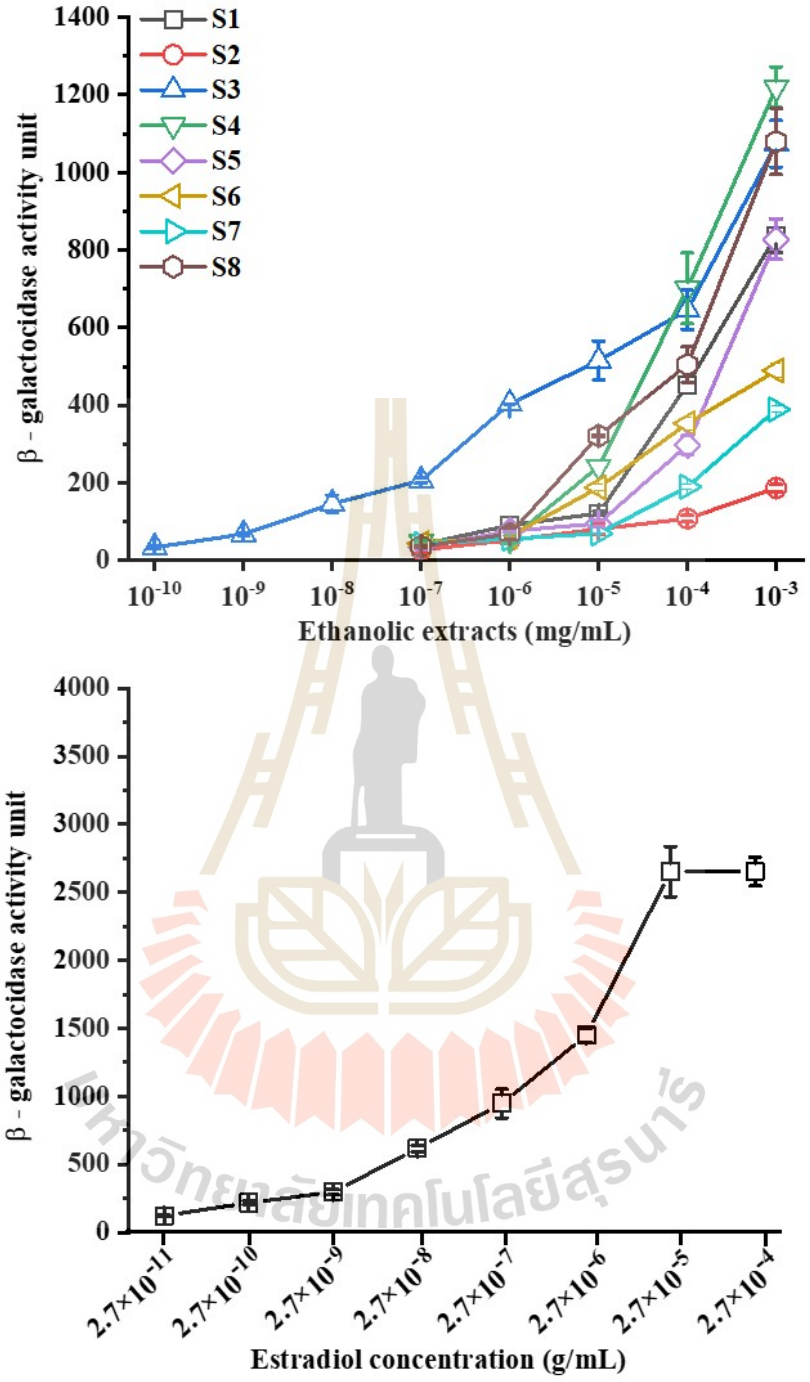


Figure 6.3 The estrogenic activities of ethanolic-extracts derived from 8 commercially traditional medicine extracts (A) and standard E2 (B) as evaluated by the ERα-Y2H system.

Table 6.7 Estrogenic activities of crude ethanolic-extracts derived from 8 commercially traditional medicines evaluated by ERα-Y2H system.

Commercial traditional medicines	ERα-Y2H system	
	REC_{10} (g/mL)	**E2-REP_{10} (%)**
S1	3.36×10^{-5}	0.00260
S2	N	N
S3	1.96×10^{-7}	0.44960
S4	1.21×10^{-5}	0.00730
S5	7.34×10^{-5}	0.00120
S6	2.83×10^{-5}	0.00310
S7	2.97×10^{-4}	0.00030
S8	6.55×10^{-6}	0.01350
E2	8.82×10^{-10}	100.00

6.2.2 Evaluation by ERβ-Y2H system

The estrogenic activity of eight crude-ethanolic extracts were also determined by ERβ-Y2H system as compared with the standard E2. The results showed that seven out of eight traditional medicine extracts exhibited the estrogenic activity in a dose-dependent response via the interaction of ERβ (Figure 6.4). The extract of S5 exhibited the highest estrogenic activity, followed by the extracts of S4, S1, S3, S2, S7 and S8.

Via ERβ-Y2H system, the REC_{10} and E2-REP_{10} were determined to compare the estrogenic activity of the crude-ethanolic extracts with the standard E2. Based on the REC10 values, four out of eight traditional medicine extracts were positive for

estrogenic activity (Table 6.8). The extract of S5 exhibited highest estrogenic activity, followed by S4, S1, and S3. Their estrogenic activities were compared standard E2, presenting via E2-REP_{10} values of 0.00166, 0.00020, 0.00004, and 0.00004%, respectively,

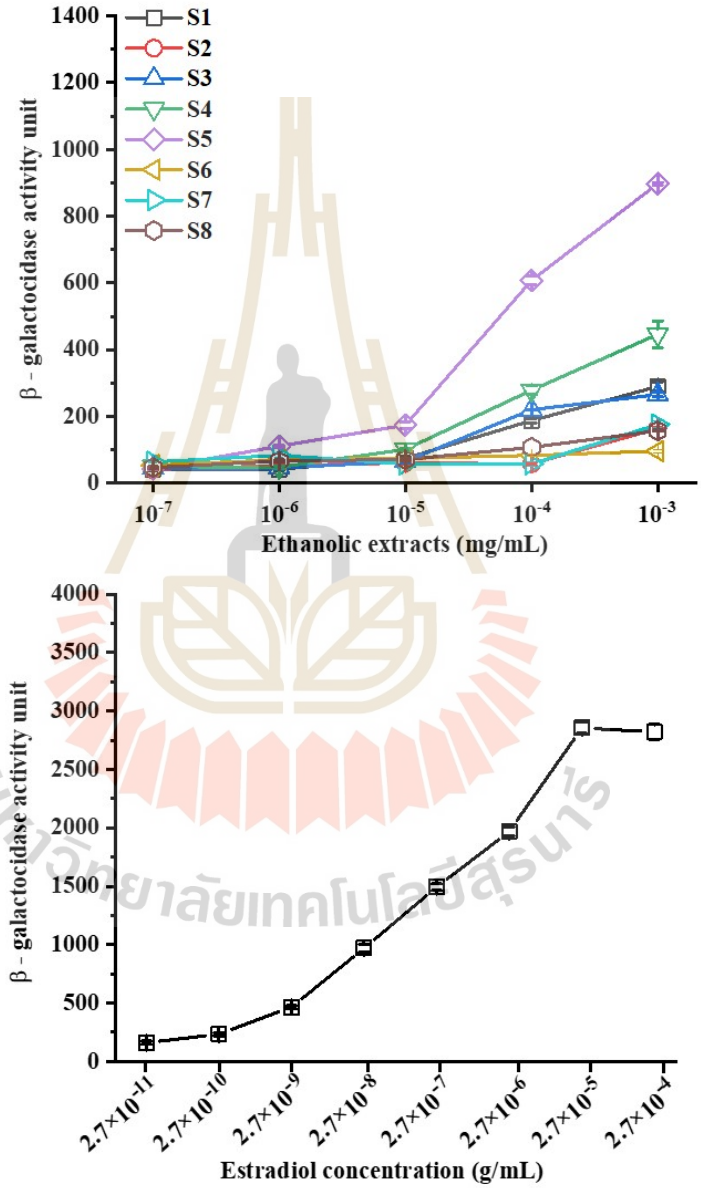


Figure 6.4 The estrogenic activities of ethanolic-extracts derived from 8 commercially traditional medicine extracts (A) and standard E2 (B) as evaluated by the ERβ-Y2H system.

Table 6.8 Estrogenic activities of crude-ethanolic extracts derived from 8 commercially traditional medicines evaluated by ERβ-Y2H system.

Commercial traditional medicines	ERβ-Y2H system	
	REC_{10} (g/mL)	**E2-REP_{10} (%)**
S1	7.31×10^{-4}	0.00004
S2	N	N
S3	7.67×10^{-4}	0.00004
S4	1.39×10^{-4}	0.00020
S5	1.69×10^{-5}	0.00166
S6	N	N
S7	N	N
S8	N	N
E2	2.82×10^{-10}	100.00

6.2.3 Comparison of estrogenic activity via ERα and ERβ actions of the ethanolic extracts derived from commercially traditional medicines

The estrogenic activities of eight ethanolic extracts derived from commercially traditional medicines were compared via the evaluations of their actions through ERα and ERβ: S1, S2, S3, S4, S5, S6, S7, and S8 (Table 6.9). Among eight extracts, seven extracts exhibited estrogenic activity via both receptors. Only one extract derived from S2 exhibited no estrogenic activity via both receptor interactions. Among seven extracts exhibiting estrogenic activity, four extracts exhibited the activity via the actions of both receptors; S1, S3, S4, and S5. The rest of three extracts exhibited estrogenic activity via only ERα interaction: S6, S7, and S8. In an overall picture, the

ethanolic extracts of these commercially traditional medicines preferentially functioned via the interaction with ERα, except S5 that exhibited the greater estrogenic activity via ERβ.

In general, the action of E2 via ERα involves in regulation of the female reproductive system, preservation of skeletal homeostasis, and regulation of metabolism (Paterni et al., 2014). ERα was controlled normal menstrual and estrous cycle acts through the homeostasis of various sex hormones (Gouw et al., 2017). ERα increases during the endometrium proliferative phase in response to estrogen for a menstrual cycle (Dorostghoal et al., 2018). According to the manufacturers, S1-S8 traditional medicines involve the treatment of irregular menstrual cycles and ovary ovulation. As this work reported for the actions of seven out of eight traditional medicines mainly functioned via ERα, these results suggest their possible medicinal functions possibly relating to the interaction of their phytoestrogens to ERα.

Both ER subtypes have some common physiological roles in the development and function of the ovaries, and in the protection of the cardiovascular system. The alpha subtype has significant roles on the mammary gland and uterus, the preservation of skeletal homeostasis, and the regulation of metabolism. The beta subtype has major effects on the central nervous and immune systems. Also, ERβ generally counteracts the ERα-promoted cell hyperproliferation in tissues such as the breast and uterus, thus it was reported to reduce a risk of breast cancer in postmenopausal women. Although both estrogen receptors have significant roles in the development and function of the ovaries, their expressions were dominant in different cells. During the child to menopausal stages of women, ERα dominantly localizes in thecal cells and the ovarian stroma in the corpus luteum and surface epithelium of the ovary. In postmenopausal

women, ERα is in the stroma, the epithelial inclusion cyst, and the ovarian surface epithelium. The main locations of ERβ are granulosa cells (Lecce et al., 2001). According to the manufacturers, S1-S8 traditional medicines involve the treatment of irregular menstrual cycles and ovary ovulation. In this work, four out of eight traditional medicines could interact with ERβ, thus their medicinal functions possibly related to the functions of phytoestrogen to stimulate the functions of the ovaries via ERβ.

Table 6.9 Comparison of estrogenic activity of ethanolic-extracts derived from traditional medicines evaluated by ERα-Y2H and ERβ-Y2H systems.

Commercially traditional medicines	ERα-Y2H system		ERβ-Y2H system	
	REC_{10} (g/mL)	E2-REP_{10} (%)	REC_{10} (g/mL)	E2-REP_{10} (%)
S1	3.36×10^{-5}	0.00260	7.31×10^{-4}	0.00004
S2	N	N	N	N
S3	1.96×10^{-7}	0.44960	7.67×10^{-4}	0.00004
S4	1.21×10^{-5}	0.00730	1.39×10^{-4}	0.00020
S5	7.34×10^{-5}	0.00120	1.69×10^{-5}	0.00166
S6	2.83×10^{-5}	0.00310	N	N
S7	2.97×10^{-4}	0.00030	N	N
S8	6.55×10^{-6}	0.01350	N	N
E2	8.82×10^{-10}	100.00	2.82×10^{-10}	100.00

This work was the first report of estrogenic activities of these commercially traditional medicines and their possible interactions with ERα and ERβ. Among all samples, S3 exhibited the most potent estrogenic activity, which was 0.45% as compared with the standard E2. The discussion about the estrogenic activities of all tested samples were following.

In this study, S1 exhibited the estrogenic activity via both receptors, but highly via ERα. In S1, its reported components are from 18 plant species. Among which, five species were previously reported to exhibit estrogenic activity via ERα, including *Angelica sinensis* (C. Z. Zhang et al., 2005), *Carthamus tinctorius* (in this work), *Curcuma xanthorrhiza* (in this work), *Panax ginseng* (Shim and Lee, 2012), and *Panax pseudoginseng* (Becker et al., 2015). Among five species, the extract from *Panax ginseng* also exhibited estrogenic activity via ERβ (Shim and Lee, 2012), where the other four species have not yet reported their estrogenic activity via ERβ. These five plants accounted for 17.80% mass of the S1 traditional medicines. However, the rest of the nine plant species listed as the ingredients in S1 were reported with no estrogenic activity.

In S2, its components are from 14 plant species. Among which, 7 species were previously reported to exhibit estrogenic activity, including *Angelica dahurica* (in this work), *Angelica sinensis* (C. Z. Zhang et al., 2005), *Carthamus tinctorius* (in this work), *Curcuma xanthorrhiza* (in this work), and *Glycyrrhiza uralensis* (Hajirahimkhan et al., 2018). These seven plants accounted for 13.60% mass of the S2 traditional medicines. In this study, S2 exhibited no estrogenic activity via both receptors. So it was hypothesized that other ingredients such as *Salvia miltiorrhiza* (Sung et al., 2015) and *Zingiber cassumunar* (Saovapak Poomirat et al., 2020b) might suppress estrogenic activity via both ERα and ERβ as both plant extracts were reported to exhibit anti-estrogenic activity.

In S3, its reported components are from 3 plant species, which were previously reported to exhibit estrogenic activity; *Angelica sinensis* (C. Z. Zhang et al., 2005), *Panax ginseng* (Shim and Lee, 2012), and *Pueraria mirifica* (Boonchird et al.,

2010). These three plants accounted for 64.00% mass of the S3 traditional medicines. In this study, S3 exhibited estrogenic activity via both receptors, but more preferred via ERα. Among these plant species, *Panax ginseng* (Shim and Lee, 2012) and *Pueraria mirifica* (Boonchird et al., 2010) were reported to exhibit estrogenic activities via both ERs.

In S4, its reported components are from 7 plant species. Three out of seven plant species were previously reported to exhibit estrogenic activity, *Angelica sinensis* (C. Z. Zhang et al., 2005), *Carthamus tinctorius* (in this work), and *Curcuma xanthorrhiza* (in this work). These three plants accounted for 42.00% mass of the S4 traditional medicines. As S4 exhibited estrogenic activity, it was likely due to phytoestrogens in these three plant species. For the rest of the four plant species, two species (*Caesalpinia sappan* and *Piper nigrum*) were reported to have no estrogenic activity (Saovapak Poomirat et al., 2020b; Raj et al., 2014). The other two species have not yet been reported for their estrogenic activity; *Leonurus sibiricus* and *Senna garrettiana*.

In S5, its reported components are from 15 plant species. Among which, five species were previously reported to exhibit estrogenic activity, including *Angelica sinensis* (C. Z. Zhang et al., 2005), *Curcuma xanthorrhiza* (Chapter 5), *Glycyrrhiza grabra* (Hajirahimkhan et al., 2018), *Panax ginseng* (Shim and Lee, 2012), and *Pueraria mirifica* (Boonchird et al., 2010). These five plants accounted for 37.50% mass of the S5 traditional medicines. In this study, S5 exhibited estrogenic activity via both receptors, but more preferred via ERβ. Among these plant species, *Glycyrrhiza grabra* (Shim and Lee, 2012), *Panax ginseng* (Shim and Lee, 2012), and *Pueraria mirifica* (Boonchird et al., 2010) exhibited estrogenic activities via both ERs. From the

ingredients of 15 plant species, seven plant species were reported with no estrogenic activity, whereas the other three species have not yet reported for estrogenic activity.

In S6, its reported components are from 5 plant species, among which three species were previously reported to exhibit estrogenic activity, including *Angelica sinensis* (C. Z. Zhang et al., 2005), *Curcuma xanthorrhiza* (Chapter 5), and *Panax ginseng* (Shim and Lee, 2012). These three plants accounted for 48.33% mass of the S6 traditional medicines. Therefore, the estrogenic activity of S6 is likely attributed to phytoestrogens of these plant species. Among ingredients derived from five plant species, one species (*Poria cocos*) was reported to exhibit no estrogenic activity (Nie et al., 2020), and the other one (*Ligusticum sinense*) has not been studied for its estrogenic activity.

In S7, its reported components are from 5 plant species, among which two species were previously reported to exhibit estrogenic activity, including *Angelica sinensis* (C. Z. Zhang et al., 2005), and *Carthamus tinctorius* (in this work). These two plants accounted for 6.37% mass of the S7 traditional medicines. For the rest of the three plant species, two species (*Aquilaria crassna* and *Caesalpinia sappan*) exhibited no estrogenic activity (Abbas et al., 2018; Raj et al., 2014), whereas the other species (*Ligusticum sinense*) has not been studied for its estrogenic activity yet. In S8, its reported components are from eight plant species, among which only two species were previously reported to exhibit estrogenic activity, including *Glycyrrhiza uralensis* (Hajirahimkhan et al., 2018), and *Paeonia lactiflora* (Jieqiong Wang et al., 2020). These two plants accounted for 11.54% mass of the S8 traditional medicines. For the other six plant ingredients, four plant species exhibited no estrogenic activity; *Aquilaria crassna* (Abbas et al., 2018), *Astragalus membranaceus* (Chaudhry et al., 2021), *Poria*

cocos (Nie et al., 2020), and *Salvia miltiorrhiza* (Sung et al., 2015). The other two plant species have not yet been reported for their estrogenic activity; *Ligusticum sinense* and *Rehmannia glutinosa*.

6.3 Estrogenic Activities of Crude-Water Extracts

The water-extracts derived from eight commercially traditional medicines (S1-S8) were evaluated their estrogenic activity via their interactions with ERα and ERβ.

6.3.1 Evaluation by ERα-Y2H system

In this part, the estrogenic activities of water-extracts derived from 8 commercially traditional medicines (10^{-10}–10^{-3} g/mL) were determined by ERα-Y2H system with the comparison with the standard E2 (2.7×10^{-11}–2.7×10^{-4} g/mL, equivalent to 1×10^{-13}–1×10^{-7}). The results are shown in Figure 6.5, in which four out of eight extracts exhibited the estrogenic activity in a dose-dependent response via the interaction with ERα; S1, S2, S3, and S8. The water-extract of S3 exhibited the highest estrogenic activity, followed by those of S1, S2, and S8, respectively.

The REC_{10} (10% relative effective concentration) and E2-REP_{10} (10% relative potency) were calculated to compare the estrogenic activities of water-extracts derived from commercially traditional medicines with the standard E2. Four out of eight commercial traditional medicine extracts were positive, indicated by their REC_{10} values (Table 6.10). These were S1, S2, S3, and S8, which had the REC_{10} values of 2.51×10^{-5}, 2.67×10^{-4}, 4.01×10^{-7}, and 5.17×10^{-4} g/ML respectively. Compared with the standard E2, the water-extract of S3 exhibited the highest estrogenic activity among these medicinal extracts with the E2-REP_{10} of 0.10423%. The E2-REP_{10} values of S1,

S2, and S8 extracts were 0.00166, 0.00016, and 0.00008%, respectively. The estrogenic activity was not detected in the water-extracts of S4, S5, S6, and S7.

In this work, water-extract of S3 exhibited the highest estrogenic activity via the interaction with ERα. The S3 contains *Angelica sinensis* 22%, *Panax ginseng* 22%, and *Pueraria mirifica* 20% as the major ingredients. The earlier experiment (Chapter 5) showed that the water-extract of *Angelica sinensis* exhibited no estrogenic activity via ERα. A similar negative result was reported by Zhang and colleagues (Wendy L. Zhang et al., 2013). For *Panax ginseng*, Tian and colleagues (Tian et al., 2020) reported its water-extract exhibited estrogenic activity via an MCF-7 cell proliferation assay. For *Pueraria mirifica,* its water extract exhibited estrogenic activity via ERα in Chapter 5. Similar positive results were reported by Siangcham and colleagues (Siangcham et al., 2010) that showed its estrogenic activity via the induced vaginal cell cornification and increased uterine weight in immature female mice (*Mus musculus*), which likely functioned via ERα (Ting Li et al., 2016). Therefore, the estrogenic activity of S3 was likely from the estrogenic activities of *Angelica sinensis* and *Pueraria mirifica,* which mainly functioned through ERα.

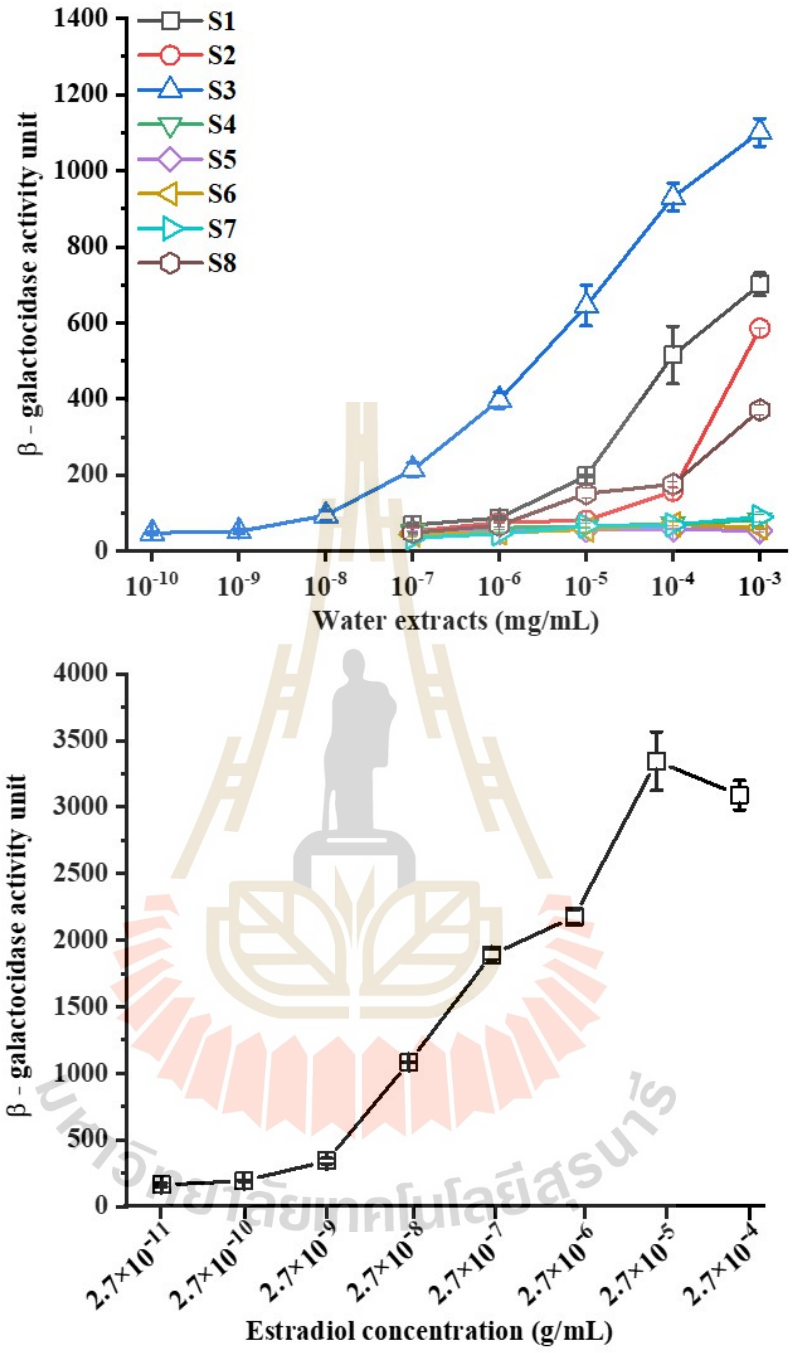


Figure 6.5 The estrogenic activities of water-extracts derived from 8 commercially traditional (A) and the standard E2 (B) as evaluated by the ERα-Y2H system.

Table 6.10 Estrogenic activities of water-extracts derived from 8 commercially traditional medicines evaluated by ERα-Y2H system.

Commercial traditional medicines	ERα-Y2H system	
	REC_{10} (g/mL)	**E2-REP_{10} (%)**
S1	2.51×10^{-5}	0.00166
S2	2.67×10^{-4}	0.00016
S3	4.01×10^{-7}	0.10423
S4	N	N
S5	N	N
S6	N	N
S7	N	N
S8	5.17×10^{-4}	0.00008
E2	4.18×10^{-10}	100.00

Noted: N, negative

6.3.2 Evaluation by ERβ-Y2H system

The estrogenic activities of water-extracts derived from 8 commercially traditional medicines were also evaluated by the ERβ-Y2H system. As seen in Figure 6.6, only S4 extract showed a slight estrogenic activity at the high concentration (10^{-3} mg/mL), whereas the rest of the extracts exhibited no estrogenic activity via the interaction with ERβ. Nevertheless, to confirm this estrogenic activity of S4 extract, the REC_{10} and E2-REP_{10} analyses were required. When the REC_{10} and E2-REP_{10} values of these extracts were calculated, the data suggested that all extracts exhibited no estrogenic activity via ERβ (Table 6.11).

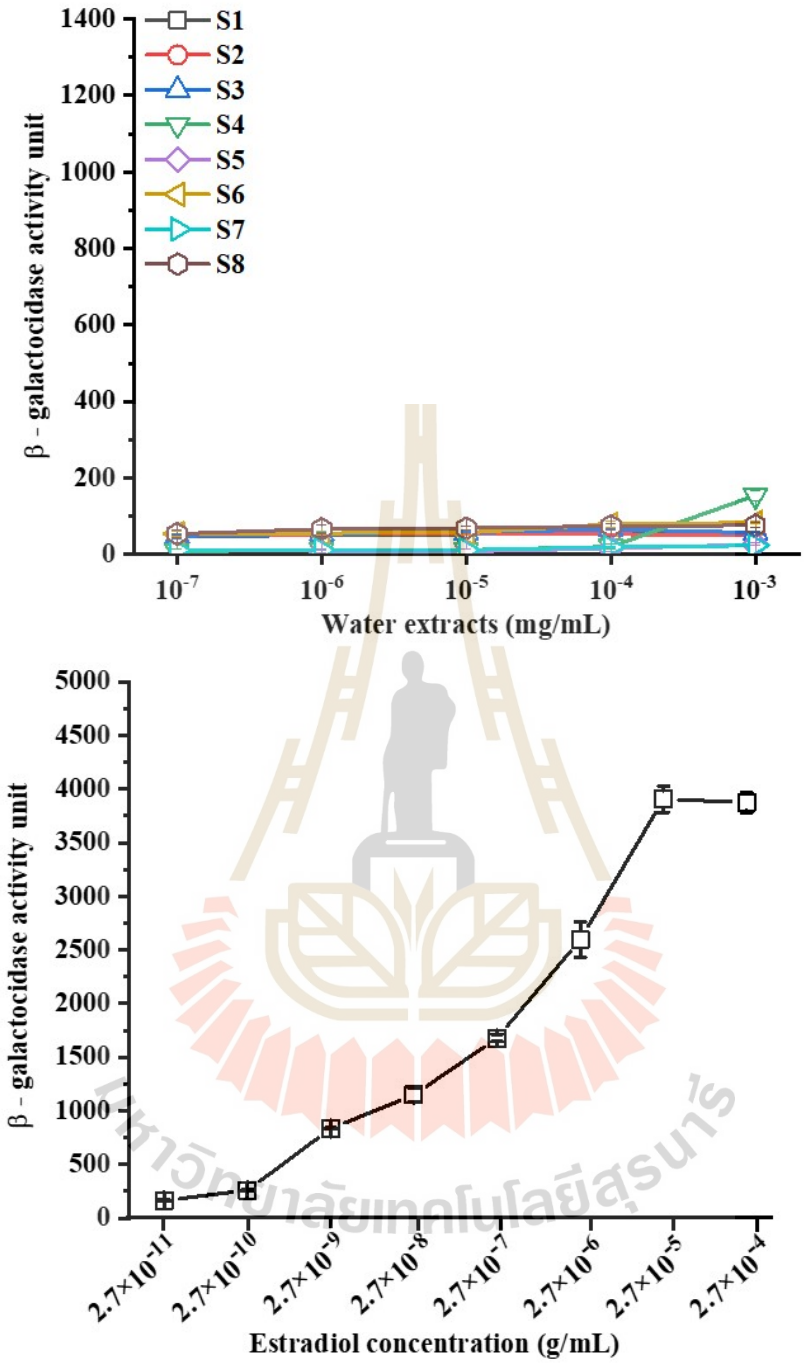


Figure 6.6 The estrogenic activities of water-extracts derived from 8 commercially traditional medicine extracts (A) and the standard E2 (B) as evaluated by the ERβ-Y2H system.

Table 6.11 Estrogenic activity of 8 commercial traditional medicine extracts evaluated by ERβ-Y2H system

Commercial traditional medicines	ERβ-Y2H system	
	REC_{10} (g/mL)	**E2-REP_{10} (%)**
S1	N	N
S2	N	N
S3	N	N
S4	N	N
S5	N	N
S6	N	N
S7	N	N
S8	N	N
E2	3.16×10^{-10}	100.00

6.3.3 Comparison of estrogenic activities via ERα and ERβ of water-extracts

This work was the first report of estrogenic activities of these commercially traditional medicines and their possible interactions with ERα and ERβ. The estrogenic activities of water-extracts derived from eight commercially traditional medicines evaluated by ERα-Y2H and ERβ-Y2H systems were compared; S1, S2, S3, S4, S5, S6, S7, and S8 (Table 6.12). Among eight extracts, four extracts exhibited estrogenic activity via ERα; S1, S2, S3, and S8. Interestingly, all eight extracts exhibit no estrogenic activity via ERβ. Among all samples, S3 exhibited the most potent estrogenic activity, which was 0.10% as compared with the standard E2 (1×10^{-7} M). The discussion about the estrogenic activities of all tested samples was following, which related to only water extracts of these plants.

Table 6.12 The estrogenic activities of water-extracts derived from 8 commercially traditional medicines through the interactions with ERα and ERβ.

Commercial traditional medicines	ERα-Y2H system		ERβ-Y2H system	
	REC_{10} (g/mL)	E2-REP_{10} (%)	REC_{10} (g/mL)	E2-REP_{10} (%)
S1	2.51×10^{-5}	0.00166	N	N
S2	2.67×10^{-4}	0.00016	N	N
S3	4.01×10^{-7}	0.10423	N	N
S4	N	N	N	N
S5	N	N	N	N
S6	N	N	N	N
S7	N	N	N	N
S8	5.17×10^{-4}	0.00008	N	N
E2	4.18×10^{-10}	100.00	3.16×10^{-10}	100.00

The S1 medicine is composed of 18 plant species, among which the water extracts only from 2 species were previously reported to exhibit estrogenic activity via ERα; *Curcuma xanthorrhiza* (in this work) and *Panax ginseng* (Ying Xu et al., 2016). These three plants accounted for 8.90% mass of the S1 traditional medicines. The estrogenic activity of S1 was likely due to phytoestrogens from these two plant species. From 18 plant species, ten plant species listed as the ingredients in S1 were reported with no estrogenic activity; *Angelica dahurica* (Tuy-On et al., 2020), *Caesalpinia sappan* (Bukke et al., 2018), *Capsicum frutescens* (Keser et al., 2018), *Citrus hystrix* (Sakpakdeejaroen and Itharat, 2009), *Carthamus tinctorius* (in this work), *Piper interruptum* (Sakpakdeejaroen and Itharat, 2009), *Piper nigrum* (Saovapak Poomirat et al., 2020b), *Plumbago indica* (Sakpakdeejaroen and Itharat, 2009), *Zingiber cassumunar* (Sakpakdeejaroen and Itharat, 2009) and *Zingiber officinale*

(Sakpakdeejaroen and Itharat, 2009). The rest of the six plant species have not yet been reported for their estrogenic activity; *Leersia hexandra*, *Molineria latifolia*, *Neopicrorhiza scrophulariiflora*, *Panax pseudoginseng*, *Scoparia dulcis*, and *Zingiber nigrum.*

In S2, its reported components are from 14 plant species, among which the water extracts of three plant species were reported to exhibit estrogenic activity, including *Curcuma xanthorrhiza* (in this work), *Glycyrrhiza uralensis* (C. Hu et al., 2009), and *Panax ginseng* (Ying Xu et al., 2016). These three plants accounted for 7.45% mass of the S2 traditional medicine, which probably contributed to the estrogenic activity of this medicine. For the other eleven plant ingredients, five plant species exhibited no estrogenic activity; *Angelica dahurica* (TuyOn et al., 2020), *Angelica sinensis* (Wendy L. Zhang et al., 2013), *Carthamus tinctorius* (Z Luo et al., 2015), *Mimusops elengi* (Vonna et al., 2014), and *Zingiber cassumunar* (Saovapak Poomirat et al., 2020b). The other six plant species have not yet been reported for their estrogenic activity; *Leonurus sibiricus*, *Ligusticum sinense*, *Molineria latifolia*, *Salvia miltiorrhiza*, *Scoparia dulcis,* and *Zingiber nigrum*.

In S3, their reported components are from 3 plant species, among which the water extract of *Panax ginseng* was previously reported to exhibit estrogenic activity (Ying Xu et al., 2016). This plant accounted for 22.00% mass of the S3 traditional medicine, which mainly contributed to the estrogenic activity of the medicine. For the other two plant species, *Pueraria mirifica* has not yet reported for the estrogenic activity of its water-extract and *Angelica sinensis* (Wendy L. Zhang et al., 2013) was reported with no estrogenic activity.

In S4, its reported components are from 7 plant species, among which only water extract of *Curcuma xanthorrhiza* (in this work) exhibited estrogenic activity. The content of *Curcuma xanthorrhiza* was 14.00% of the total mass of S4. It was hypothesized that the negative estrogenic activity of S4 might due to the very low estrogenic activity of the water extract *Curcuma xanthorrhiza* and the interference from the rest ingredients from six plant species. For the other six plant species, the water extracts of four plant species exhibited no estrogenic activity; *Angelica sinensis* (Wendy L. Zhang et al., 2013), *Caesalpinia sappan* (Bukke et al., 2018), *Carthamus tinctorius* ((in this work), *and Piper nigrum* (Saovapak Poomirat et al., 2020b). The other two plant species have not yet been reported for their activity from the water extracts; *Leonurus sibiricus and Senna garrettiana.*

In S5, its reported components are from 15 plant species, among which the water extracts of *Curcuma xanthorrhiza* (in this work) and *Panax ginseng* (Ying Xu et al., 2016) were reported to exhibit estrogenic activities. Although the contents of *Curcuma xanthorrhiza* and *Panax ginseng* were 25.00% mass of the total of S5, no estrogenic activity of S5 was detected. It was hypothesized that the rest of the plant ingredients (derived from 13 plant species) might inhibit the estrogenic activity of these two active ingredients. For other thirteen plant species, the water extracts of five plant species exhibited no estrogenic activity; *Angelica sinensis* (Zhang et al., 2013), *Astragalus membranaceus* (Zhang et al., 2013), *Cyperus rotundus* (Memariani et al., 2016), *Glycyrrhiza grabra* (Yong-Jiao Zhang et al., 2018), and *Poria cocos* (K. Hu et al., 2019). The other eight plant species have not yet been reported for their activity from the water extracts; *Corydalis yanhusuo*, *Leonurus sibiricus*, *Ligusticum*

sinense, *Paeonia lactiflora*, *Pueraria mirifica*, *Rehmannia glutinosa*, *Salvia miltiorrhiza*, and *Saussurea lappa.*

In S6, its reported components are from 5 plant species, among which the water extracts of *Curcuma xanthorrhiza* (in this work) and *Panax ginseng* (Ying Xu et al., 2016) were previously reported to exhibit estrogenic activity. Although the contents of *Curcuma xanthorrhiza* and *Panax ginseng* were 23.33% mass of the total of S6, there was not detected estrogenic activity of S6 in this work. It might be possible that the other ingredients derived from the other three plant species might suppress the estrogenic activity of these two active plant species. For the rest of the three plant species, the water extracts of 2 plant species exhibited no estrogenic activity; *Angelica sinensis* (Wendy L. Zhang et al., 2013) and *Poria cocos* (K. Hu et al., 2019). The other, *Ligusticum sinense*, has not yet been reported for the estrogenic activity of its water extract.

In S7, its reported components are from five plant species, among which the water extracts of *Angelica sinensis* (Wendy L. Zhang et al., 2013), *Aquilaria crassna* (Dahham et al., 2016), *Caesalpinia sappan* (Bukke et al., 2018), and *Carthamus tinctorius* (Z Luo et al., 2015) were reported no estrogenic activity. The other, *Ligusticum sinense*, has not yet been studied for the estrogenic activity of its water extract. Therefore, no detected estrogenic activity of S7 was probably due to the negative estrogenic activity of these five major ingredients in S7.

In S8, its reported components are from 8 plant species, among which the water extract of only one plant species was previously reported to exhibit estrogenic activity; *Glycyrrhiza uralensis* (C. Hu et al., 2009). This plant species accounted for 4.20% mass of S8 traditional medicine, which likely contributed to the estrogenic

activity of this medicine. For the other seven plant ingredients, the water extracts of three plant species exhibited no estrogenic activity; *Aquilaria crassna* (Dahham et al., 2016), *Astragalus membranaceus* (Leong et al., 2016), and *Poria cocos* (K. Hu et al., 2019). The water extracts of the other four plant species have not yet been reported for their estrogenic activity; *Ligusticum sinense*, *Paeonia lactiflora*, *Rehmannia glutinosa*, and *Salvia miltiorrhiza.*

6.4 Comparison of Estrogenic Activity of Ethanolic- and Water-extracts of Eight Commercial Traditional Medicines via the Action of ERα and ERβ

The estrogenic activity of the ethanolic and water extracts derived from 8 commercial traditional medicine was compared in Table 6.13, which showed both REC_{10} and E2-REP_{10} values. As E2-REP_{10} values were calculated by normalizing the data with the standard E2, they were more suitable to use for the comparison of estrogenic activity between the ethanolic and water extracts derived from each plant species as compared with the standard E2.

Based on E2-REP_{10}, the ethanol extraction method was more efficient to extract phytoestrogenic compounds than the water extraction method since the estrogenic activities of these traditional medicines were mostly detected in the ethanolic extracts. As these traditional medicines were orally uptake, it raised the question of whether these active compounds can well dissolve in water in the digestive system of the body and whether the digestive fluid in the digestive system facilitates the release of these active compounds in the body. Therefore, these questions lead to the experiments in the

next part to investigate the estrogenic activity of these traditional medicines via in vitro digestion.

Nevertheless, as compared between the ethanolic and water extracts of these eight traditional medicines, most active compounds were extracted by ethanol but very few were extracted by water. Also, these ethanolic extracts of most traditional medicines, except only S2, exhibited estrogenic activity via the interaction with ERα. Among them, S3 exhibited the highest estrogenic activity of 0.45% as compared with the standard E2. In general, the action of E2 via ERα involves in regulation of the female reproductive system (especially ovaries), preservation of skeletal homeostasis, and regulation of metabolism (Paterni et al., 2014). As these traditional medicines were used for regulating normal menstrual cycles, the detection of their estrogenic activity via ERα gave the support evidence for their possible action to stimulate the action of phytoestrogens via ERα to regulate the ovary functions. Based on the literature review, there was a report of some phytoestrogenic extracts exhibiting the function to regulate ovary functions. For example, phytoestrogenic extract of *Cyclea barbata* exhibited E2-agonist functions via selective binding to ERα to induce the maturation effect of oocytes to secondary follicles (Noviyanti et al., 2020). Also, phytoestrogens that exhibited estrogenic activity via the binding to ERα were important for female fertility and postpartum uterine involution (W. Winuthayanon et al., 2014). It was noted that postpartum uterine involution is a regenerative process for the uterus to return to non-pregnant size (Zi. Huan Zheng et al., 2019). Lee and colleagues (K. H. Lee et al., 2013) reported that Sheng Hua Tang Chinese traditional medicine improved uterine involution in postpartum cows. Also, taking Sheng Hua Tang in the first month of the postpartum period and followed by Si Wu Tang could speed the recovery rate of the

mother after baby delivery (Chang et al., 2013). Chiu and colleagues (Chiu et al., 2014) reported that Si Wu Tang extracts exhibited estrogenic activity via ERα. These researches suggested that phytoestrogens with preferential binding to ERα had potential applications in postpartum care. Another Chinese traditional medicine, Qingyan extract functioned via both ERα and ERβ could promote vaginal cornification, prolong the estrus status of the immature mice, promote the growth and development of uterus and vagina, and upregulate the ERα and ERβ expression at a protein level in the uterus and vagina (Zhao et al., 2019).

It was also noted that the estrogenic activities of S1, S3, S4, and S5 traditional medicines via ERβ were also contributed to the applications for menopausal syndrome. Some Japanese herbal medicines, Unkeito, Kamishoyosan, and Nyoshinsan are widely used as alternative estrogen therapy for a menopausal syndrome, which exerts estrogenic actions based on the interaction with ERβ (Zeyun Wang et al., 2018).

Table 6.13 Comparison of estrogenic activity of ethanolic and water extracts of 8 commercial traditional medicines via actions of ERα and Erβ.

Commercial traditional medicines	ERα-Y2H system				ERβ-Y2H system			
	Ethanolic extracts		Water extract		Ethanolic extracts		Water extract	
	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)	REC_{10} (g/mL)	$E2\text{-}REP_{10}$ (%)
S1	3.36×10^{-5}	0.00260	2.51×10^{-5}	0.00166	7.31×10^{-4}	0.00004	N	N
S2	N	N	2.67×10^{-4}	0.00016	N	N	N	N
S3	1.96×10^{-7}	0.44960	4.01×10^{-7}	0.10423	7.67×10^{-4}	0.00004	N	N
S4	1.21×10^{-5}	0.00730	N	N	1.39×10^{-4}	0.00020	N	N
S5	7.34×10^{-5}	0.00120	N	N	1.69×10^{-5}	0.00166	N	N
S6	2.83×10^{-5}	0.00310	N	N	N	N	N	N
S7	2.97×10^{-4}	0.00030	N	N	N	N	N	N
S8	6.55×10^{-6}	0.01350	5.17×10^{-4}	0.00008	N	N	N	N
E2	8.82×10^{-10}	100.00	4.18×10^{-10}	100.00	2.82×10^{-10}	100.00	3.16×10^{-10}	100.00

6.5 Estrogenic activities of *In Vitro* Simulated Digestion of Ethanolic Extracts of 8 Commercial Traditional Medicines

6.5.1 Estrogenic activity of the digested extracts evaluation by ERα-Y2H system

In this part, a simulated digestion method was used to mimic the gastrointestinal tract digestion. The estrogenic activities of *in vitro* digested extracts derived from ethanolic extracts of traditional medicines (10^{-4} g/mL) and the standard E2 (2.7×10^{-4} g/mL or 10^{-7} M) were determined by ERα-Y2H system as seen in Figure 6.7 and Table 6.14. For the standard E2, the digested E2 exhibited the decreased estrogenic activity to 96.85±0.25%. However, all digested extracts of traditional medicines (S1-S8) exhibited the increased estrogenic activity. Among all digested extracts, the digested S5 extract exhibited the highest increase of estrogenic activity, which was 2.79 folds increase as compared with its undigested extract. The increased estrogenic activity found in the digested ethanolic extracts of traditional medicines was hypothesized to involve the structural changes of plant ingredient compounds from inactive into active forms by enzymatic digestion, such as a change of glycosides to active aglycones (Basu et al., 2016). As a result, the digested compounds could exhibit estrogenic activity higher than the undigested ones.

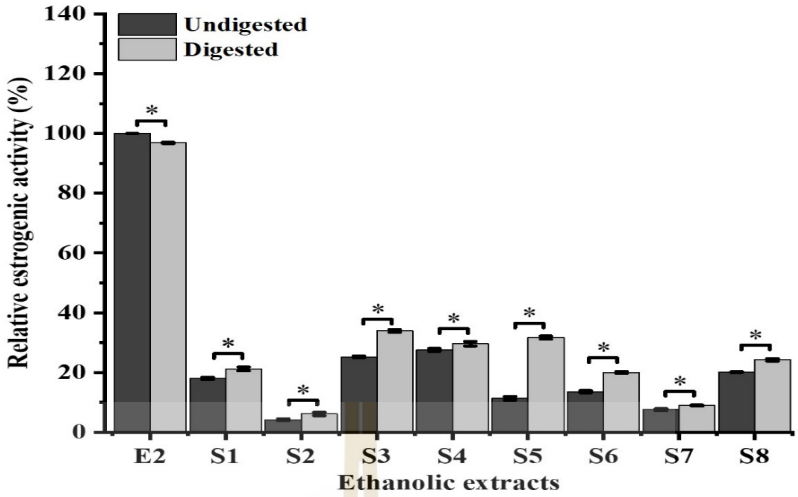


Figure 6.7 Relative estrogenic activities of undigested and digested ethanolic extracts of S1-S8 (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) determined by ERα-Y2H system. Independent sample T-test, $p < 0.05$, n=3.

Table 6.14 Relative estrogenic activities of digested and undigested ethanolic extracts of S1-S8 evaluated by ERα-Y2H system.

Commercial medicines	Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]
S1	18.03±0.42	21.18±0.62*	1.17
S2	4.21±0.33	6.13±0.61*	1.46
S3	25.24±0.25	33.91±0.47*	1.34
S4	27.57±0.48	29.60±0.77*	1.07
S5	11.36±0.61	31.68±0.54*	2.79
S6	13.59±0.37	20.01±0.31*	1.47
S7	7.57±0.39	8.97±0.18*	1.19
S8	20.13±0.15	24.25±0.48*	1.20
E2	100.00	96.85±0.25*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$, n=3.

6.5.2 Estrogenic activity of the digested extracts evaluation by ERβ-Y2H system

The digested ethanolic extracts of traditional medicines (10^{-4} g/mL) and the standard E2 (2.7×10^{-7} g/mL) were also evaluated for their estrogenic activities by the ERβ-Y2H system (Figure 6.8 and Table 6.15). The digested E2 exhibited reduced estrogenic activity to 98.22±0.38%. In contrast, seven out of eight digested extracts of traditional medicines exhibited the increased estrogenic activity; S1, S2, S3, S4, S5, S6, and S8. Among these, the digested S3 extract exhibited the highest increase of estrogenic activity, which was 1.80 folds increase as compared with the undigested one. These results suggested that S5 exhibited the greatest estrogenic activity among tested traditional medicines, especially in the digested condition, which would be benefit for estrogen-deficient treatment in the body.

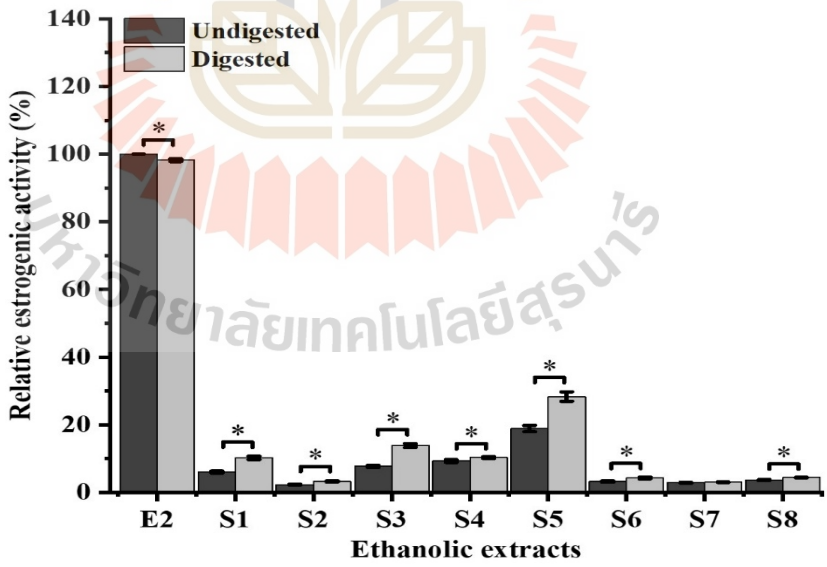


Figure 6.8 Relative estrogenic activities of undigested and digested ethanolic extracts of traditional medicines (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) by ERβ-Y2H system. Independent sample T-test, $p < 0.05$, n=3.

Table 6.15 Relative estrogenic activities of 8 ethanolic extracts under the digested and undigested conditions evaluated by ERβ-Y2H system.

Traditional medicines	Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]
S1	6.00±0.30	10.21±0.51*	1.70
S2	2.30±0.13	3.23±0.19*	1.40
S3	7.71±0.31	13.87±0.55*	1.80
S4	9.25±0.39	10.29±0.23*	1.11
S5	18.87±0.92	28.30±1.38*	1.50
S6	3.22±0.23	4.25±0.30*	1.32
S7	2.85±0.11	3.03±0.07	-
S8	3.65±0.09	4.38±0.11*	1.20
E2	100.00	98.22±0.38*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$, n=3.

6.5.3 Comparison of estrogenic activities of the digested ethanolic extracts evaluated by ERα-Y2H and ERβ-Y2H system

Table 6.16 shows the comparison of estrogenic activities of the undigested and digested ethanolic extracts of 8 commercial traditional medicines (S1-S8) evaluated by ERα-Y2H and ERβ-Y2H systems. The results revealed that ethanolic extracts of these commercial traditional medicines after *in vitro* digestion mostly exhibited increased estrogenic activity as evaluated by ERα-Y2H and ERβ-Y2H systems. The increases of estrogenic activity of the digested samples were in a range of 1.17–2.79 and 1.11–1.80 folds as determined by ERα-Y2H and ERβ-Y2H systems, respectively.

In this study, the estrogenic activities of S1 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.17-fold and 1.70-fold increases as compared with those of the undigested samples, respectively. In S1, its components are from 18 plant species. Some phytoestrogens derived from two plant species were reported to exhibit increased estrogenic activity after being digested. Diarylheptanoid, one of the curcuminoids isolated from *Curcuma xanthorrhiza*, that was metabolized with S9 rat liver fraction exhibited an increase of estrogenic activity via ERα evaluated by recombinant yeast system (Wipawee Winuthayanon et al., 2009). The 6-gingerol, the major bioactive metabolite of *Zingiber officinale* extract, also exhibited high estrogenic activity (Annunziato et al., 2018). Out of 18 plant species, the other three plant species, *Piper interruptum, Piper nigrum, and Piper sarmentosum*, also exhibited estrogenic activity (Shaheer et al., 2020); however, there was no report about their digestion and estrogenic activity thereof. Out of 18 plant species, seven plant extracts were reported no estrogenic activity; *Angelica sinensis* (Hsieh et al., 2015), *Caesalpinia sappan* (Bukke et al., 2018), *Carthamus tinctorius* (Soares et al., 2019), *Panax ginseng* (Anshul Sharma and Lee, 2020), *Panax pseudoginseng* (Wen-Jin Zhang et al., 2020), *Plumbago indica* (Shukla et al., 2021), and *Zingiber cassumunar* (Su and Ho, 2013). Their digestions have not been studied. So far, the rest species have not yet been studied for estrogenic activity; *Capsicum frutescens*, *Citrus hystrix*, *Leersia hexandra*, *Molineria latifolia*, *Neopicrorhiza scrophulariiflora*, and *Scoparia dulcis*. In this work, the increased estrogenic activity of the digested S1 was hypothesized to involve the metabolites derived from *Curcuma xanthorrhiza* and *Zingiber officinale*. Nevertheless, it could not rule out the possible active metabolites from *Piper interruptum, Piper nigrum*, *Piper*

sarmentosum, and *Zingiber officinal*e, which their digested extract are needed to investigate.

The estrogenic activities of S2 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.46-fold and 1.40-fold increases as compared with those of the undigested samples, respectively. In S2, its reported components are from 14 plant species. Two plant species were reported about the estrogenic activities of their metabolites; *Curcuma xanthorrhiza* (Diarylheptanoid) (Wipawee Winuthayanon et al., 2009) and *Glycyrrhiza uralensis* (liquiritigenin) (Lattrich et al., 2013). Six out of 14 plant species were reported about no estrogenic activity; *Angelica dahurica* (Koziol and Skalicka-Wozniak, 2016), *Angelica sinensis* (Hsieh et al., 2015), *Carthamus tinctorius* (Soares et al., 2019), *Panax ginseng* (Anshul Sharma and Lee, 2020), *Salvia miltiorrhiza* (Sun et al., 2018), and *Zingiber cassumunar* (Su and Ho, 2013). The other six plant species have not yet been reported their estrogenic activity; *Leonurus sibiricus*, *Ligusticum sinense*, *Mimusops elengi*, *Molineria latifolia*, *Scoparia dulcis*, and *Zingiber nigrum*. So, the increased estrogenic activity of the digested S2 was hypothesized to involve the metabolites derived from *Curcuma xanthorrhiza* and *Glycyrrhiza uralensis*.

The estrogenic activities of S3 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.34-fold and 1.80-fold increases as compared with those of the undigested samples, respectively. The digested extracts derived from these three plant species were reported about their estrogenic activities (Sa Yoon Park et al., 2018; Zhu et al., 2018; Boonchird et al., 2010). Therefore, the active metabolites from these three plant species were likely contributed to the increases in estrogenic activity of the digested S3.

The estrogenic activities of S4 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.07-fold and 1.11-fold increases as compared with those of the undigested samples, respectively. In S4, its reported components are from 7 plant species. The active metabolites contributed to the increased estrogenic activity of the digested S4 was likely from *Curcuma xanthorrhiza* extract (Wipawee Winuthayanon et al., 2009) as mentioned before. Although *Piper nigrum* extract exhibited estrogenic activity, its digested extract has not yet been investigated for estrogenic activity. Four plant species were reported no estrogenic activity; *Angelica sinensis* (Hsieh et al., 2015), *Caesalpinia sappan* (Bukke et al., 2018), *Carthamus tinctorius* (Soares et al., 2019), and *Senna garrettiana* (Mashwani et al., 2017). One plant species, *Leonurus sibiricus*, has not yet been studied for estrogenic activity.

The estrogenic activities of S5 evaluated by ERα-Y2H and ERβ-Y2H systems were 2.79-fold and 1.50-fold increases as compared with those of the undigested samples, respectively. The active metabolites contributed to the increased estrogenic activity of the digested S5 was likely from *Curcuma xanthorrhiza* extract (Wipawee Winuthayanon et al., 2009) and *Pueraria mirifica* (Boonchird et al., 2010) as mentioned before. Seven out of fifteen digested plant extracts were reported no estrogenic activity; *Angelica sinensis* (Hsieh et al., 2015), *Glycyrrhiza grabra* (Dong Cai et al., 2019), *Paeonia lactiflora* (Hsiu et al., 2004), *Panax ginseng* (Anshul Sharma and Lee, 2020), *Poria cocos* (Jiang and Fan, 2020), *Salvia miltiorrhiza* (Sun et al., 2018), *Saussurea lappa (D. Liu et al., 2021)*. The other six plant species have not yet reported for the estrogenic activity; *Atractylodes lancea*, *Corydalis yanhusuo*, *Cyperus rotundus*, *Leonurus sibiricus*, *Ligusticum sinense*, and *Rehmannia glutinosa.*

The estrogenic activities of S5 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.45-fold and 1.32-fold increases as compared with those of the undigested samples, respectively. In S6, its reported components are from 5 plant species. It was likely that the active metabolites of S6 were from the extract of *Curcuma xanthorrhiza*, which previously mentioned about its digested extract exhibiting estrogenic activity (Anggarani et al., 2018); (Wipawee Winuthayanon et al., 2009). Among five plant species as the components in S6, three plant species reported no estrogenic activity; *Angelica sinensis* (Hsieh et al., 2015), *Panax ginseng* (Anshul Sharma and Lee, 2020), and *Poria cocos* (Jiang and Fan, 2020). One plant species, *Ligusticum sinense* has not yet been reported for estrogenic activity. Nevertheless, these four plant species have not yet studied their digestion and estrogenic activity thereof.

The estrogenic activities of S7 evaluated by ERα-Y2H system was 1.19-fold increase as compared with those of the undigested sample. S7 has the ingredients derived from 5 plant species, In S7, its reported components are from 5 plant species. Among the ethanolic extracts of these five plant species, two species were previously reported to exhibit estrogenic activity, including *Angelica sinensis* (C. Z. Zhang et al., 2005), and *Carthamus tinctorius* (in this work). Two other species (*Aquilaria crassna* and *Caesalpinia sappan*) exhibited no estrogenic activity (Abbas et al., 2018; Raj et al., 2014), and one species (*Ligusticum sinense*) has not been studied for its estrogenic activity yet. There is no report on the digested extracts of these plants and estrogenic activity thereof.

The estrogenic activities of S8 evaluated by ERα-Y2H and ERβ-Y2H systems were 1.20-fold increases in both systems as compared with those of the undigested samples. In S8, its reported components are from 8 plant species. One

metabolite derived from *Glycyrrhiza uralensis* extract, liquiritigenin, was previously reported for estrogenic activity in ERα-positive MCF-7, but not in ERβ-positive MDA-MB-231 cells (Lattrich et al., 2013). Five other species were reported no estrogenic activity; *Aquilaria crassna* (Wisutthathum et al., 2018), *Astragalus membranaceus* (El-Kott et al., 2019), *Paeonia lactiflora*(Hsiu et al., 2004), *Poria cocos* (Jiang and Fan, 2020), and *Salvia miltiorrhiza* (Sun et al., 2018). The rest of the two species, *Ligusticum sinense* and *Rehmannia glutinosa* have not yet been reported estrogenic activity of their digested extracts.

Table 6.16 Comparison of estrogenic activities of undigested and digested ethanolic extracts of 8 commercial traditional medicines evaluated by ERα-Y2H and ERβ-Y2H systems.

Traditional medicines	Evaluation by ERα-Y2H			Evaluation by ERβ-Y2H		
	Relative estrogenic activity (%)			Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]	Undigested	Digested	Fold increase[#]
S1	18.03±0.42	21.18±0.62*	1.17	6.00±0.30	10.21±0.51*	1.70
S2	4.21±0.33	6.13±0.61*	1.46	2.30±0.13	3.23±0.19*	1.40
S3	25.24±0.25	33.91±047*	1.34	7.71±0.31	13.87±0.55*	1.80
S4	27.57±0.48	29.60±0.77*	1.07	9.25±0.39	10.29±0.23*	1.11
S5	11.36±0.61	31.68±0.54*	2.79	18.87±0.92	28.30±1.38*	1.50
S6	13.59±0.37	20.01±0.31*	1.47	3.22±0.23	4.25±0.30*	1.32
S7	7.57±0.39	8.97±0.18*	1.19	2.85±0.11	3.03±0.07	-
S8	20.13±0.15	24.25±0.48*	1.20	3.65±0.09	4.38±0.11*	1.20
E2	100.00	96.85±0.25*	-	100.00	98.22±0.38*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$,

6.6 Estrogenic activities of *In Vitro* Simulated Digestion of Water Extracts of 8 Commercial Traditional Medicines

6.6.1 Estrogenic activity of the digested extracts evaluation by ERα-Y2H system

In this part, the water extracts of 8 commercial traditional medicine were digested using a mimic gastrointestinal digestion condition. These undigested and digested extracts were evaluated for their estrogenic activity by ERα and ERβ-Y2H systems.

Figure 6.9 and Table 6.17 show the results of the estrogenic activities of undigested and digested extracts derived from the water extracts of traditional medicines (10^{-4} g/mL) and the standard E2 (2.7×10^{-4} g/mL or 10^{-7} M) as determined by ERα-Y2H system. Similar to the previous study, the digested E2 exhibited the decreased estrogenic activity to 96.31±0.97%. In this work, only one digested S3 extract exhibited reduced estrogenic activity, whereas the rest extracts exhibited had increased estrogenic activity (S1, S2, S4, S5, S6, S7, and S8). The digested S8 extract exhibited the highest increase of estrogenic activity, which was 29.60 folds increase as compared with its undigested extract. Its estrogenic activity was very high, which almost equivalent to that of the standard E2 at 10^{-7} M. The increased estrogenic activity of the digested extracts derived from water extracts of S1-S8 traditional medicines was hypothesized to involve the structural changes of phytoestrogens by enzymatic digestion. For instance, 8-prenylnaringenin (8PN), the metabolite of xanthohumol, exhibited more potent estrogenic activity than its original plant compound (Cady et al., 2020).

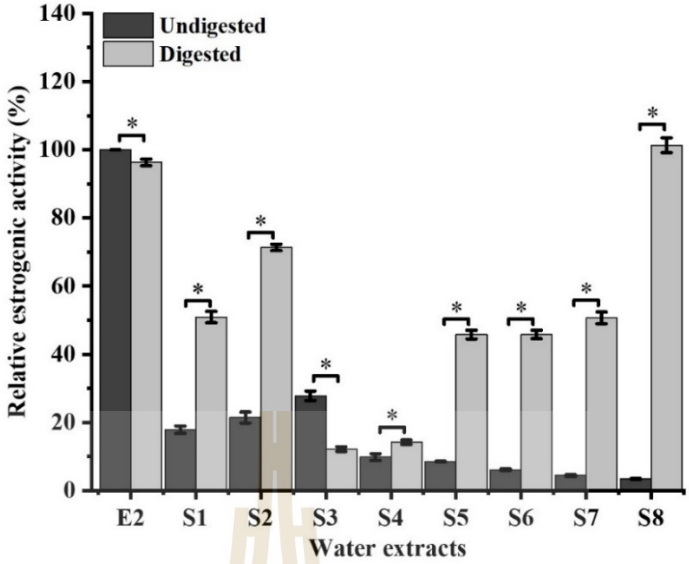


Figure 6.9 Relative estrogenic activities of undigested and digested water extracts of traditional medicines (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) by ERα-Y2H system. Independent sample T-test, $p < 0.05$, n=3.

Table 6.17 Relative estrogenic activities of 8 water extracts under the digested and undigested conditions evaluated by ERα-Y2H system.

Traditional medicines	Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]
S1	17.87±1.07	50.87±1.67*	2.85
S2	21.42±1.59	71.34±0.95*	3.33
S3	27.81±1.41	12.18±0.71*	-
S4	9.82±1	14.20±0.66*	1.45
S5	8.49±0.09	45.76±1.27*	5.39
S6	6.10±0.26	45.78±1.26*	7.50
S7	4.40±0.28	50.65±1.72*	11.52
S8	3.42±0.14	101.32±2.18*	29.60
E2	100.00	96.31±0.97*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$, n=3.

6.6.2 Estrogenic activity of the digested extracts evaluation by ERβ-Y2H system

The estrogenic activity of *in vitro* digested 8 traditional medicines (derived from water extracts) were evaluated by ERβ-Y2H system as shown in Figure 6.10 and Table 6.18. The digested E2 exhibited reduced estrogenic activity to 96.08±0.53%. The undigested extracts exhibited very low estrogenic activity via ERβ, which were only 0.47–2.11% as compared with the standard E2. Nevertheless, most digested extracts exhibited increased estrogenic activity, but still at low activity as compared with the standard E2 (1.03–3.80%). Therefore, these results indicated that the estrogenic activities of S1-S8 were dominantly from the interaction with ERα, not ERβ.

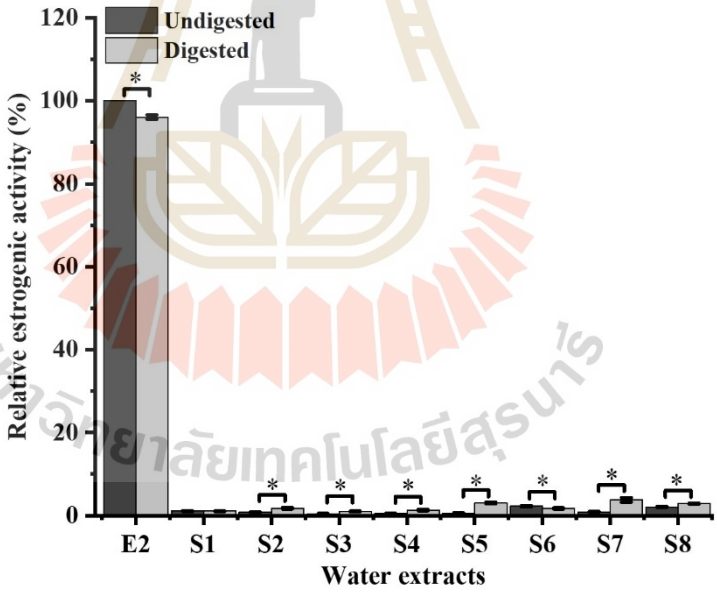


Figure 6.10 Relative estrogenic activities of undigested and digested water extracts of traditional medicines (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) by ERβ-Y2H system. Independent sample T-test, $p < 0.05$, n=3.

Table 6.18 Relative estrogenic activities of 8 water extracts under the digested and undigested conditions evaluated by ERβ-Y2H system.

Traditional medicines	Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]
S1	1.11±0.11	1.07±0.06	-
S2	0.83±0.06	1.79±0.15*	2.17
S3	0.47±0.03	1.03±0.09*	2.18
S4	0.53±0.02	1.33±0.13*	2.48
S5	0.59±0.08	3.10±0.17*	5.25
S6	2.28±0.14	1.75±0.08*	-
S7	0.92±0.07	3.80±0.54*	4.14
S8	2.11±0.09	2.91±0.09*	1.38
E2	100.00	96.08±0.53*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$, n=3.

6.6.3 Comparison of estrogenic activities of the undigested and digested water extracts evaluated by ERα-Y2H and ERβ-Y2H

In this part, the research data were compared between the estrogenic activities of the undigested and digested water extracts of 8 commercial traditional medicines (S1-S8) evaluated by ERα-Y2H and ERβ-Y2H systems. The results demonstrated that the water extracts of S1-S8 exhibited estrogenic activities via ERα higher than ERβ. Also, after digesting, the water extracts of S1-S8 exhibited increased estrogenic activities via both estrogen receptors. Via ERα-Y2H system, the estrogenic activities of S1-S8 under non-digested condition were in a range of 3.42–27.42% as compared with the standard E2 (1×10^{-7} g/mL). After digesting, their estrogenic activities were increased to 12.18–101.32% as compared with the standard E2. Via ERβ-Y2H system, the

estrogenic activities of S1-S8 under non-digested condition were in a range of 0.47–2.11% as compared with the standard E2. After digesting, their estrogenic activities were increased to 1.03–3.80% as compared with the standard E2.

For S1, it was possible that the active compounds exhibiting estrogenic activity were derived from *Angelica sinensis*, *Carthamus tinctorius*, and *Panax ginseng.* Ferulic acid, the estrogenic compound in *Angelica sinensis*, was reported to be absorbed in the intestine (Paiva et al., 2013) and exhibited estrogenic activity via ERα (Hao et al., 2010). The caffeic acid, the major bioactive metabolite derived from *Carthamus tinctorius*, exhibited estrogenic activity in ERα positive MG-63 cells (Hernandes et al., 2020). The metabolized ginsenosides Rb1 derived from *Panax ginseng* exhibited estrogenic activity via ERα positive in MCF-7 cell (Y. J. Lee et al., 2003). The rest ingredients of S1 derived from the plant species that have not yet investigated for their digested compounds and estrogenic activity thereof.

For S2, its reported components are from 14 plant species. Five plant species were reported about the estrogenic activities of their metabolites; *Angelica dahurica* (kaemferal) (Guo et al., 2012), *Angelica sinensis* (Ferulic acid) (Wu et al., 2020), *Carthamus tinctorius* (caffeic acid) (Xiaoming Wang et al., 2019), *Glycyrrhiza uralensis* (liquiritigenin) (Lattrich et al., 2013), and *Panax ginseng* (ginsenosides Rb1) (Y. J. Lee et al., 2003). The rest ingredients of S2 derived from the plant species that have not yet investigated for their digested compounds and estrogenic activity thereof.

For S3, three plant species were reported about the estrogenic activities of their metabolites; *Angelica sinensis* (Ferulic acid) (Wu et al., 2020), *Panax ginseng* (ginsenosides Rb1) (Y. J. Lee et al., 2003), and *Pueraria mirifica* (equol) (Tsuji et al.,

2018). Therefore, the active metabolites from these three plant species were likely contributed to the increases in estrogenic activity.

For S4, its reported components are from 7 plant species. The active metabolites of S4 were likely from *Angelica sinensis* and *Carthamus tinctorius*, which previously mentioned about their digested extracts exhibiting estrogenic activities (Wu et al., 2020; Xiaoming Wang et al., 2019). The rest ingredients of S4 derived from the plant species that have not yet investigated for their digested compounds and estrogenic activity thereof.

For S5, its reported components are from 15 plant species. Among the water extracts of these fifteen plant species, four species were previously reported to exhibit estrogenic activity, including *Angelica sinensis*, *Paeonia lactiflora*, *Panax ginseng*, and *Pueraria mirifica* (Tsuji et al., 2018; Li Liu et al., 2012; Hao et al., 2010; Y. J. Lee et al., 2003). Four other species *Atractylodes lancea*, *Curcuma xanthorrhiza*, *Glycyrrhiza grabra*, and *Salvia miltiorrhiza* exhibited no estrogenic activity (Abotaleb et al., 2020; Dae Yong Kim and Choi, 2019; Zhen Zhang et al., 2019; Jinhee Kim et al., 2013), and seven species (*Corydalis yanhusuo*, *Cyperus rotundus*, *Leonurus sibiricus*, *Ligusticum sinense*, *Poria cocos*, *Rehmannia glutinosa*, and *Saussurea lappa*) have not been studied for its estrogenic activity yet. So, the increased estrogenic activity of the digested S5 was hypothesized to involve the metabolites derived from *Angelica dahurica*, *Paeonia lactiflora*, *Panax ginseng,* and *Pueraria mirifica.*

For S6, its reported components are from 5 plant species. Among the water extracts of these two plant species were previously reported to exhibit estrogenic activity, including *Angelica sinensis* (Hao et al., 2010), and *Panax ginseng* (Y. J. Lee et al., 2003). One species (*Curcuma xanthorrhiza*) reported no estrogenic activity

(Pandey et al., 2020) and two species (*Ligusticum sinense* and *Poria cocos*) have not been studied for its estrogenic activity yet. So, the increased estrogenic activity of the digested S6 was hypothesized to involve the metabolites derived from *Angelica dahurica* and *Panax ginseng,*

For S7, its reported components are from 5 plant species. It was likely that the active metabolites of S7 were from the extract of *Angelica sinensis* and *Carthamus tinctorius* (Lili Liu et al., 2021; Hao et al., 2010). The rest ingredients of S7 derived from the plant species that have not yet investigated for their digested compounds and estrogenic activity thereof.

For S8, its reported components are from 8 plant species. Three plant species were reported about the estrogenic activities of their metabolites; *Astragalus membranaceus* (daidzein) (Subcharoen and Chthaputti, 2018), *Glycyrrhiza uralensis* (liquiritigenin) (Lattrich et al., 2013), and *Paeonia lactiflora* (Albiflorin) (Li Liu et al., 2012). The rest ingredients of S8 derived from the plant species that have not yet investigated for their digested compounds and estrogenic activity thereof. So, the increased estrogenic activity of the digested S8 was hypothesized to involve the metabolites derived from *Astragalus membranaceus*, *Glycyrrhiza uralensis*, and *Paeonia lactiflora*. Five other species were reported no estrogenic activity; *Aquilaria crassna* (Wisutthathum et al., 2018), *Astragalus membranaceus* (El-Kott et al., 2019), *Paeonia lactiflora*(Hsiu et al., 2004), *Poria cocos* (Jiang and Fan, 2020), and *Salvia miltiorrhiza* (Sun et al., 2018). The rest of the two species, *Ligusticum sinense* and *Rehmannia glutinosa* have not yet been reported estrogenic activity of their digested extracts.

Table 6.19 Comparison of estrogenic activities of undigested and digested water extracts of 8 commercial traditional medicines evaluated by ERα-Y2H and ERβ-Y2H systems.

Traditional medicines	Evaluation by ERα-Y2H			Evaluation by ERβ-Y2H		
	Relative estrogenic activity (%)			Relative estrogenic activity (%)		
	Undigested	Digested	Fold increase[#]	Undigested	Digested	Fold increase[#]
S1	17.87±1.07	50.87±1.67*	2.85	1.11±0.11	1.07±0.06	-
S2	21.42±1.59	71.34±0.95*	3.33	0.83±0.06	1.79±0.15*	2.18
S3	27.81±1.41	12.18±0.71*	-	0.47±0.03	1.03±0.09*	2.18
S4	9.82±1.00	14.20±0.66*	1.45	0.53±0.02	1.33±0.13*	2.48
S5	8.49±0.09	45.76±1.27*	5.39	0.59±0.08	3.10±0.17*	5.25
S6	6.10±0.26	45.78±1.26*	7.50	2.28±0.14	1.75±0.08*	-
S7	4.40±0.28	50.65±1.72*	11.52	0.92±0.07	3.80±0.54*	4.14
S8	3.42±0.14	101.32±2.18*	29.60	2.11±0.09	2.91±0.09*	1.38
E2	100.00	96.31±0.97*	-	100.00	96.08±0.53*	-

Remark: [#]Comparison between undigested and digested samples, * $p < 0.05$, n=3.

6.7 Comparison of Estrogenic Activities of Digested and Undigested Ethanolic and Water Extracts of 8 Commercial Traditional Medicines

The estrogenic activity of the digested and undigested ethanolic and water extracts of 8 commercial traditional medicines that were evaluated by ERα-Y2H and ERβ-Y2H system were compared in Table 6.20, which showed relative estrogenic activity (%) values.

The results indicated that the digested ethanolic and water extracts of all tested medicines (S1-S8) exhibited increased estrogenic activities via the interaction with both ERα and ERβ. These results suggested the potential functions of these tested medicines after ingestion in the body. Interestingly, all tested medicines preferentially functioned via the interaction with ERα more than ERβ. These results were well related to the property of these medications to relieve the symptoms relating to the ovary, which highly expressed ERα (Paterni et al., 2014). Compared between ethanolic and water extracts of these medicines, the *in vitro* digested traditional medicines derived from water extracts exhibited significantly higher estrogenic activities. The ethanolic extracts of these medicinal samples exhibited the maximal estrogenic activity via ERα of 27.57%, and those of digested samples exhibited increased estrogenic activity to the maximal value of 33.91%. Interestingly, their water extracts exhibited the maximal estrogenic activity via ERα of 27.81%, and those of digested samples exhibited increased estrogenic activity to the maximal value of 101.32%. These results suggested that the active compounds of these medicines were water-soluble, which were suitable for their oral ingestions in an aqueous system in the body. Among these eight traditional

medicines, the digested S8 sample exhibited the highest estrogenic activity, which was 101.32% as compared with the estrogenic activity of the standard E2 (1×10^{-7} g/mL). The estrogenic activity of the digested S8 was hypothesized to involve the metabolites derived from *Astragalus membranaceus* (daidzein) (Subcharoen and Chthaputti, 2018), *Glycyrrhiza uralensis* (liquiritigenin) (Lattrich et al., 2013), and *Paeonia lactiflora* (Albiflorin). Nevertheless, the actions of metabolites derived from other ingredients (six plant species) in the S8 sample still cannot rule out as their digestions and estrogenic activity thereof have not been studied.

Table 6.20 Comparison of Estrogenic Activities of Digested and Undigested Ethanolic and Water Extracts of 8 Commercial Traditional Medicines

Samples	Ethanol extract				Water extract			
	Relative estrogenic activity (%) by ERα-Y2H		Relative estrogenic activity (%) by ERβ-Y2H		Relative estrogenic activity (%) by ERα-Y2H		Relative estrogenic activity (%) by ERβ-Y2H	
	Undigested	Digested	Undigested	Digested	Undigested	Digested	Undigested	Digested
S1	18.03±0.42	21.18±0.62*	6.00±0.30	10.21±0.51*	17.87±1.07	50.87±1.67*	1.11±0.11	1.07±0.06
S2	4.21±0.33	6.13±0.61*	2.30±0.13	3.23±0.19*	21.42±1.59	71.34±0.95*	0.83±0.06	1.79±0.15*
S3	25.24±0.25	33.91±047*	7.71±0.31	13.87±0.55*	27.81±1.41	12.18±0.71*	0.47±0.03	1.03±0.09*
S4	27.57±0.48	29.60±0.77*	9.25±0.39	10.29±0.23*	9.82±1.00	14.20±0.66*	0.53±0.02	1.33±0.13*
S5	11.36±0.61	31.68±0.54*	18.87±0.92	28.30±1.38*	8.49±0.09	45.76±1.27*	0.59±0.08	3.10±0.17*
S6	13.59±0.37	20.01±0.31*	3.22±0.23	4.25±0.30*	6.10±0.26	45.78±1.26*	2.28±0.14	1.75±0.08*
S7	7.57±0.39	8.97±0.18*	2.85±0.11	3.03±0.07	4.40±0.28	50.65±1.72*	0.92±0.07	3.80±0.54*
S8	20.13±0.15	24.25±0.48*	3.65±0.09	4.38±0.11*	3.42±0.14	101.32±2.18*	2.11±0.09	2.91±0.09*
E2	100.00	96.85±0.25*	100.00	98.22±0.38*	100.00	96.31±0.97*	100.00	96.08±0.53*

Remark: #Comparison between undigested and digested samples, * $p < 0.05$,

CHAPTER VII

CONCLUSION

In this study, the yeast two-hybrid (Y2H) systems were developed to determine phytoestrogens and employed to study the estrogenic activities of ten crude medicinal plant extracts and eight commercially traditional medicines.

In the first part, four Y2H systems were constructed, which each system contained two recombinant plasmids (bait and prey plasmids). The bait plasmids in this work were pGBKT7-hERα and pGBKT7-hERβ. The prey plasmids were pGADT7-hSRC1 and pGADT7-hTIF2. The yeast *Saccharomyces cerevisiae* strain AH109 was co-transformed with the bait- prey plasmids. Four Y2H systems were constructed, which each system contained two recombinant plasmids; 1) pGBKT7-hERα and pGADT7-hSRC1, 2) pGBKT7-hERα and pGADT7-hTIF2, 3) pGBKT7-hERβ and pGADT7-hSRC1, and 4) pGBKT7-hERβ and pGADT7-hTIF2. The most suitable Y2H systems to evaluate the estrogenic activity via the interactions to ERα and ERβ were the systems containing pGBKT7-hERα and pGADT7-hTIF2, and pGBKT7-hERβ and pGADT7-hSRC1, respectively. These Y2H systems were referred to as ERα-Y2H and ERβ-Y2H systems, respectively. These systems efficiently detected the estrogenic activities of the standard E2 and genistein. In the second part, the ERα-Y2H and ERβ-Y2H systems were used to detect the estrogenic activity of 10 medicinal plant extracts via the interactions of both estrogen receptors (ERα and ERβ). The ethanolic and water

extracts of these plant species included *Angelica sinensis*, *Angelica dahurica*, *Carthamus tinctorius*, *Curcuma xanthorrhiza*, *Dendrolobium lanceolatum*, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Morinda coreia*, *Pueraria mirifica*, and *Zingiber officinale*. Based on E2-REP_{10} values, the ethanolic extracts of these plants exhibited higher estrogenic activities than their water extracts (Table 7.1). Interestingly, the ethanolic plant extracts exhibited more estrogenic activities via ERβ than ERα, whereas the water extracts exhibited more estrogenic activities via ERα. Among ethanolic extracts, the ethanolic extract of *Dendrolobium lanceolatum* exhibited the highest estrogenic activity via ERβ with the E2-REP_{10} of 1.53%. Among water extracts, the water extract of *Glycyrrhiza uralensis* exhibited the highest estrogenic activity via ERα with the E2-REP_{10} of 0.194%. The ethanolic and water extracts of the same plant species exhibited different estrogenic activities via both receptors, suggesting different active compounds obtained by different extraction solvents (ethanol and water).

When the ethanolic and water extracts derived from these plant species were *in vitro* digested, their estrogenic activities were changed, likely due to their different metabolites obtained by the enzymatic digestion. For the ethanolic extracts, their digested extracts mostly exhibited the reduction of estrogenic activity via the interaction of both ERα and ERβ (Table 7.2). Only a few digested extracts possessed increased estrogenic activity. Via ERα, four digested extracts derived from *Curcuma xanthorrhiza*, *Glycyrrhiza uralensis*, *Dendrolobium lanceolatum*, and *Zingiber officinale* exhibited escalated estrogenic activity. Via ERβ, only digested extracts of *Dendrolobium lanceolatum* exhibited the rise of estrogenic activity. In contrast, the water extracts of these plants after in vitro digestion mostly exhibited the increases of estrogenic activity via ERα, whereas possessed the reduction of estrogenic

activity via ERβ. Via ERα, seven plant extracts under an in vitro digestion exhibited increased estrogenic activity; *Angelica dahurica*, *Angelica sinensis*, *Dendrolobium lanceolatum*, *Carthamus tinctorius*, *Morinda coreia*, *Glycyrrhiza uralensis*, and *Pueraria mirifica*. Via ERβ, only digested extract of *Dendrolobium lanceolatum* exhibited the rise of estrogenic activity.

In the third part, the ERα-Y2H and ERβ-Y2H systems were used to detect the estrogenic activity of 8 commercially traditional medicines via the interactions of both estrogen receptors (ERα and ERβ). These medicines were referred to as S1-S8. The ethanolic extracts of these medicines exhibited higher estrogenic activities than their water extracts. Also, their estrogenic activities were more potent via ERα (Table 7.3). Among ethanolic extracts, the S3 ethanolic extract exhibited the highest estrogenic activity via ERα with the E2-REP_{10} of approximately 0.45%. Among water extracts, the S3 water extract also exhibited the highest estrogenic activity via ERα with the E2-REP_{10} of about 0.10%.

When the ethanolic and water extracts derived from these medicines were *in vitro* digested, their estrogenic activities significantly increased, particularly via the interaction with ERα. These results suggested the potential functions of these tested medicines after ingestion in the body. Also, these results were well related to the property of these medications to relieve the symptoms relating to the ovary, which highly expressed ERα. Compared between ethanolic and water extracts of these medicines, the *in vitro* digested medicines derived from water extracts exhibited significantly higher estrogenic activities (Table 7.4). The ethanolic extracts of these medicinal samples exhibited the maximal estrogenic activity via ERα of 27.57%, and those of digested samples exhibited increased estrogenic activity to the maximal

relative estrogenic activity of 33.91%. Interestingly, their water extracts exhibited the maximal relative estrogenic activity via ERα of 27.81%, and those of digested samples exhibited increased estrogenic activity to the maximal relative estrogenic activity of 101.32%. These results suggested that the active compounds of these medicines were water-soluble, which were suitable for their oral ingestions in an aqueous system in the body. Among these eight traditional medicines, the digested S8 sample exhibited the highest estrogenic activity (relative estrogenic activity of 101.32%) via ERα as compared with the estrogenic activity of the standard E2 (1×10^{-7} g/mL). The estrogenic activity of the digested S8 was hypothesized to involve the metabolites derived from *Astragalus membranaceus*, *Glycyrrhiza uralensis*, and *Paeonia lactiflora*. Nevertheless, the actions of metabolites derived from other ingredients (from other six plant species) in the S8 sample still cannot rule out as their digestions and estrogenic activity thereof have not been studied.

Table 7.1 Comparison of estrogenic activities of ethanolic and water extracts of 10 medicinal plants evaluated by ERα-Y2H and ERβ-Y2H systems evaluated by E2-REP_{10} values.

Extracts derived from	Estrogenic activities evaluated by E2-REP_{10}			
	ERα-Y2H		ERβ-Y2H	
	Ethanolic extracts	Water extracts	Ethanolic extracts	Water extracts
Angelica dahurica	N	N	0.02	N
Angelica sinensis	N	N	0.05	N
Carthamus tinctorius	N	N	0.03	N
Curcuma xanthorrhiza	N	0.006	0.03	N
Dendrolobium lanceolatum	0.009	0.003	1.53	0.002
Glycyrrhiza glabra	0.00004	0.0007	0.03	N
Glycyrrhiza uralensis	0.00002	0.194	0.51	N
Morinda coreia	0.0002	0.07	0.02	0.067
Pueraria mirifica	0.00006	0.003	0.02	N
Zingiber officinale	N	N	N	N

Remark: N, Negative.

Table 7.2 Estrogenic activities of ethanolic and water extracts of 10 medicinal plants as compared between digested and undigested extracts that were evaluated by ERα-Y2H and ERβ-Y2H systems.

Extracts derived from	Comparison of estrogenic activities of the digested and undigested extracts			
	ERα-Y2H		ERβ-Y2H	
	Ethanolic extracts	Water extracts	Ethanolic extracts	Water extracts
Angelica dahurica	0.59 (↓)	6.15 (↑)	0.17 (↓)	0.15 (↓)
Angelica sinensis	0.66 (↓)	4.96 (↑)	0.13 (↓)	0.61 (↓)
Carthamus tinctorius	0.69 (↓)	4.35 (↑)	0.22 (↓)	0.83 (↓)
Curcuma xanthorrhiza	1.42 (↑)	0.97 (↓)	0.12 (↓)	0.97 (↓)
Dendrolobium lanceolatum	1.16 (↑)	4.84 (↑)	2.08 (↑)	6.23 (↑)
Glycyrrhiza glabra	0.45 (↓)	0.56 (↓)	0.08 (↓)	0.27 (↓)
Glycyrrhiza uralensis	1.29 (↑)	3.65 (↑)	0.15 (↓)	0.43 (↓)
Morinda coreia	0.45 (↓)	4.07 (↑)	0.07 (↓)	0.08 (↓)
Pueraria mirifica	0.78 (↓)	2.39 (↑)	0.13 (↓)	1.29 (↓)
Zingiber officinale	1.15 (↑)	0.89 (↓)	1.16 (↑)	0.14 (↑)

Remarks:

- Estrogenic activity of digested extract / Estrogenic activity of undigested extract
- ↓ Reduced estrogenic activity
- ↑ Increased estrogenic activity

Table 7.3 Comparison of estrogenic activities of ethanolic and water extracts of 8 traditional medicines evaluated by ERα-Y2H and ERβ-Y2H systems evaluated by E2-REP_{10} values.

Commercial traditional medicines	Estrogenic activities evaluated by E2-REP_{10}			
	ERα-Y2H		ERβ-Y2H	
	Ethanolic extracts	Water extracts	Ethanolic extracts	Water extracts
S1	0.00260	0.00166	0.00004	N
S2	N	0.00016	N	N
S3	0.44960	0.10423	0.00004	N
S4	0.00730	N	0.00020	N
S5	0.00120	N	0.00166	N
S6	0.00310	N	N	N
S7	0.00030	N	N	N
S8	0.01350	0.00008	N	N

Remark: N, Negative.

Table 7.4 Estrogenic activities of ethanolic and water extracts of 8 traditional medicines as compared between digested and undigested extracts that were evaluated by ERα-Y2H and ERβ-Y2H systems.

Commercial traditional medicines	Comparison of estrogenic activities of the digested and undigested extracts			
	ERα-Y2H		ERβ-Y2H	
	Ethanolic extracts	Water extracts	Ethanolic extracts	Water extracts
S1	1.17 (↑)	2.85 (↑)	1.70 (↑)	0.96 (↓)
S2	1.46 (↑)	3.33 (↑)	1.40 (↑)	2.16 (↑)
S3	1.34 (↑)	0.44 (↓)	1.80 (↑)	2.19 (↑)
S4	1.07 (↑)	1.45 (↑)	1.11 (↑)	2.51 (↑)
S5	2.79 (↑)	5.39 (↑)	1.50 (↑)	5.25 (↑)
S6	1.47 (↑)	7.50 (↑)	1.32 (↑)	0.77 (↓)
S7	1.18 (↑)	11.51 (↑)	1.06 (↑)	4.13 (↑)
S8	1.20 (↑)	29.63 (↑)	1.20 (↑)	1.38 (↑)

Remarks:

- Estrogenic activity of digested extract / Estrogenic activity of undigested extract
- ↓ Reduced estrogenic activity
- ↑ Increased estrogenic activity

REFERENCES

REFERENCES

Abbas, P., Hashim, Y., and Salleh, H.M. (2018). Uninfected agarwood branch extract possess cytotoxic and inhibitory effects on MCF-7 breast cancer cells. **Journal of Research in Pharmacy**. 23: 120-129.

Abdullah, S., Abidin, S.A.Z., Morad, N., Makpol, S., Ngah, W.Z.W., and Mohd Yusof, Y. (2010). Ginger extract *(Zingiber officinale)* triggers apoptosis and G0/G1 cells arrest in HCT 116 and HT 29 colon cancer cell lines. **African Journal of Biochemistry Research**. 4: 134-142.

Abotaleb, M., Liskova, A., Kubatka, P., and Busselberg, D. (2020). Therapeutic potential of plant phenolic acids in the treatment of cancer. **Biomolecules**. 10(2): 1-23.

Ahmad, B., Rehman, M.U., Amin, I., Arif, A., Rasool, S., Bhat, S.A., Afzal, I., Hussain, I., Bilal, S., and Mir, M.u.R. (2015). A review on pharmacological properties of Zingerone (4-(4-Hydroxy-3-methoxyphenyl)-2-butanone). **Science**. 2015: 1-6.

Ahn, E.M., Nakamura, N., Akao, T., and Nishihara, T. (2004). Estrogenic and antiestrogenic activities of the roots of *Moghania philippinensis* and their constituents. **Biological and Pharmaceutical Bulletin**. 27: 548-553.

Al-Snafi, A. (2015). Therapeutic properties of medicinal plants: A review of plants with anticancer activity (part 1). **International Journal of Pharmacy and Biological Sciences Research**. 5(4): 240-248.

Albertazzi, P., and Purdie, D. (2002). The nature and utility of the phytoestrogens: A review of the evidence. **Maturitas**. 42: 173-185.

Amato, P., Christophe, S., and Mellon, P.L. (2002). Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. **Menopause**. 9(2): 145-150.

Amenyogbe, E., Chen, G., Wang, Z., Lu, X., Lin, M., and Lin, A.Y. (2020). A review on sex steroid hormone estrogen receptors in mammals and fish. **Journal of Endocrinology**. 2020: 1-9.

Amudha, P., Pushpa, B.N., and Varadharaj, V. (2016). *Caesalpinia bonducella* – A review on pharmacological and phytochemical activity of seeds. **International Journal of Pharmacy and Biological Sciences**. 4(7): 674-680.

An, J., Tzagarakis-Foster, C., Scharschmidt, T.C., Lomri, N., and Leitman, D.C. (2001). Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. **Journal of Biological Chemistry**. 276(21): 17808-17814.

Anggakusuma, Yanti, Lee, M., and Hwang, J.K. (2009). Estrogenic activity of xanthorrhizol isolated from curcuma xanthorrhiza ROXB. **Biological and Pharmaceutical Bulletin**. 32(11): 1892-7897.

Anggarani, M.A., Rusijono, and Maulana, D.A. (2018). Optimizing the drying temperature of Temulawak simplicia (*Curcuma xanthorrhiza* roxb.) based on water and ash content and functional compound. **Journal of Physics: Conference Series**. 1108(2018): 012099.

Annunziato, G., Falavigna, C., Pieroni, M., Faccini, A., Micheli, F., and Costantino, G. (2018). *In vitro* digestion of *Zingiber officinale* extract and evaluation of

stability as a first step to determine its bioaccesibility. **Natural Product Communications**. 13(9): 1143-1146.

Asamenew, G., Kim, H., Lee, M.K., Lee, S.H., Kim, Y.J., Cha, Y.-S., Yoo, S.M., and Kim, J.-B. (2019). Characterization of phenolic compounds from normal ginger (*Zingiber officinale* Rosc.) and black ginger (*Kaempferia parviflora* Wall.) using UPLC–DAD–QToF–MS. **European Food Research and Technology**. 245(3): 653-665.

Asgarpanah, J., and Kazemivash, N. (2013). Phytochemistry, pharmacology and medicinal properties of *Carthamus tinctorius* L. **Chinese Journal of Integrative Medicine**. 19: 153-159.

Avasthi, A., Bhatnagar, M., Thirumoorthy, A., Selvamurthy, W., Sinha, N., and Ghosal, S. (2020). Methods of extraction and fractionationof bio-active components of *Morinda coreia* for anti-tubercular and anticancer activities. **International Journal of Pharmaceutical Sciences and Research**. 11(8): 4062-4069.

Aznam, N., and Atun, S. (2017). The development of temulawak (*Curcuma Xanthorrhiza*) herb production and pharmacological test as anti-hypercholesterolemia. **International Journal of Green Pharmacy**. 11: S809-S813.

Bachmeier, B.E., Mirisola, V., Romeo, F., Generoso, L., Esposito, A., Dell'Eva, R., Blengio, F., Killian, P.H., Albini, A., and Pfeffer, U. (2010). Reference profile correlation reveals estrogen-like trancriptional activity of curcumin. **Cellular Physiology and Biochemistry**. 26(3): 471-482.

Bajpai, V.K., Alam, M.B., Quan, K.T., Choi, H.-J., An, H., Ju, M.-K., Lee, S.-H., Huh, Y.S., Han, Y.-K., and Na, M. (2018). Cytotoxic properties of the anthraquinone

derivatives isolated from the roots of *Rubia philippinensis*. **BMC Complementary Medicine and Therapies**. 18(1): 1-6.

Barbosa, R.N., Leong, S.L., Vinnere, P.O., Chen, A.J., Souza, M.C.M., Frisvad, J.C., Samson, R.A., Oliveira, N.T., and Houbraken, J. (2017). Phylogenetic analysis of *Monascus* and new species from honey, pollen and nests of stingless bees. **Studies in Mycology**. 86: 29-51.

Basu, P., Meza, E., Bergel, M., and Maier, C. (2020). Estrogenic, antiestrogenic and antiproliferative activities of *Euphorbia bicolor* (Euphorbiaceae) latex extracts and its phytochemicals. **Nutrients**. 12(1): 59.

Basu, P., Sunny, S., and Maier, C. (2016). Estrogenic and antiestrogenic activities of commercial dietary supplements containing herbal ingredients and isoflavones. **International Journal of Pharmacy and Pharmaceutical Sciences**. 8: 307-312.

Bean, L., Ianov, L., and Foster, T. (2014). Estrogen receptors, the hippocampus, and memory. **The Neuroscientist**. 20: 534 - 545.

Becker, L.C., Bergfeld, W.F., Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D.C., Marks, J.G., Jr., Shank, R.C., Slaga, T.J., Snyder, P.W., and Andersen, F.A. (2015). Safety assessment of *Panax* spp root-derived ingredients as used in cosmetics. **Journal of Toxicology**. 34(3): 5-42.

Bjornstrom, L., and Sjoberg, M. (2005). Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. **Molecular Endocrinology**. 19(4): 833-842.

Boonchird, C., Mahapanichkul, T., and Cherdshewasart, W. (2010). Differential binding with ERα and ERβ of the phytoestrogen-rich plant *Pueraria mirifica*. **Brazilian Journal of Medical and Biological Research**. 43(2): 195-200.

Bruckner, A., Polge, C., Lentze, N., Auerbach, D., and Schlattner, U. (2009). Yeast two-hybrid, a powerful tool for systems biology. **International Journal of Molecular Sciences**. 10(6): 2763-2788.

Brzezinski, A., and Debi, A. (1999). Phytoestrogens: The "natural" selective estrogen receptor modulators? **European Journal of Obstetrics & Gynecology and Reproductive Biology**. 85(1): 47-51.

Bukke, A.N., Nazneen Hadi, F., Babu, K.S., and shankar, P.C. (2018). In vitro studies data on anticancer activity of *Caesalpinia sappan* L. heartwood and leaf extracts on MCF7 and A549 cell lines. **Data in Brief**. 19: 868-877.

Cady, N., Peterson, S.R., Freedman, S.N., and Mangalam, A.K. (2020). Beyond metabolism: The complex interplay between dietary phytoestrogens, gut bacteria, and cells of nervous and immune systems. **Frontiers in Neurology**. 11(150): 1-15.

Cai, D., Zhang, Z., Chen, Y., Zhang, Y., Sun, Y., and Gong, Y. (2019). Exploring new structural features of the 18β-Glycyrrhetinic acid scaffold for the inhibition of anaplastic lymphoma kinase. **Molecules.** 24(19): 3631.

Cai, Y., Zhao, B., Liang, Q., Zhang, Y., Cai, J., and Li, G. (2017). The selective effect of glycyrrhizin and glycyrrhetinic acid on topoisomerase IIα and apoptosis in combination with etoposide on triple negative breast cancer MDA-MB-231 cells. **European Journal of Pharmacology**. 809: 87-97.

Chan, R.Y.K., Chen, W.F., Dong, A., Guo, D., and Wong, M.S. (2002). Estrogen-like activity of Ginsenoside Rg1 derived from *Panax notoginseng*. **The Journal of Clinical Endocrinology & Metabolism**. 87(8): 3691-3695.

Chang, P.J., Lin, C.C., Chen, Y.C., Chuang, C.H., Tseng, Y.C., Hsieh, W.-S., Lin, S.J., and Chen, P.C. (2013). Use of herbal dietary supplement si-wu-tang and health-related quality of life in postpartum women: a population-based correlational study. **Evidence-Based Complementary and Alternative Medicine**. 2013: 790474-790474.

Chaudhry, G., Jan, R., Akim, A., Zafar, M.N., Sung, Y.Y., and Muhammad, T.S.T. (2021). Breast cancer: A global concern, diagnostic and therapeutic perspectives, mechanistic targets in drug development. **Advanced Pharmaceutical Bulletin**: 1-24.

Chen, C.X., Barrett, B., and Kwekkeboom, K.L. (2016). Efficacy of oral ginger (*Zingiber officinale*) for dysmenorrhea: A systematic review and meta-analysis. **Evidence-Based Complementary and Alternative Medicine**. 2016: 1-10.

Chen, Q., Qi, X., Zhang, W., Zhang, Y., Bi, Y., Meng, Q., Bian, H., and Li, Y. (2021). Catalpol inhibits macrophage polarization and prevents postmenopausal atherosclerosis through regulating estrogen receptor alpha. **Frontiers in Pharmacology**. 12(1073): 1-16.

Cherdshewasart, W., and Sriwatcharakul, S. (2008). Metabolic activation promotes estrogenic activity of the phytoestrogen-rich plant. **Maturitas**. 59(2): 128-136.

Chiu, J.H., Chang, C.J., Wu, J.C., Liu, H.J., Wen, C.S., Hsu, C.H., Chen, J.L., Tseng, L.M., Chen, W.S., and Shyr, Y.M. (2014). Screening to identify commonly used Chinese herbs that affect *ERBB2* and *ESR1* gene expression using the human

breast cancer MCF-7 cell line. **Journal of Evidence-Based Integrative Medicine**. 2014: 1-11.

Chong, J., Poutaraud, A., and Hugueney, P. (2009). Metabolism and roles of stilbenes in plants. **Plant Science**. 177(3): 143-155.

Cornwell, T., Cohick, W., and Raskin, I. (2004). Dietary phytoestrogens and health. **Phytochemistry**. 65(8): 995-1016.

Dahham, S., Tabana, Y., Sandai, D., Ahmed, M., and Abdul Majid, A.M.S. (2016). *In vitro* anti-cancer and anti-angiogenic activity of essential oils extracts from agarwood (*Aquilaria Crassna*). **Journal of Applied Research on Medicinal and Aromatic Plants**. 5: 2167-0412.

Dallacqua, S., Tome, F., Vitalini, S., Agradi, E., and Innocenti, G. (2009). *In vitro* estrogenic activity of *Asplenium trichomanes* L. extracts and isolated compounds. **Journal of Ethnopharmacology**. 122(3): 424-429.

Darbre, P.D. (2019). The history of endocrine-disrupting chemicals. **Current Opinion in Endocrine and Metabolic Research**. 7: 26-33.

De Bore, H.J., and Cotingting, C. (2014). Medicinal plants for women's healthcare in southeast Asia: A meta-analysis of their traditional use, chemical constituents, and pharmacology. **Journal of Ethnopharmacology**. 151(2): 747-767.

Delshad, E., Yousefi, M., Sasannezhad, P., Rakhshandeh, H., and Ayati, Z. (2018). Medical uses of *Carthamus tinctorius* L. (Safflower): A comprehensive review from traditional medicine to modern medicine. **Electronic Physician Journal**. 10(4): 6672-6681.

Demmig, A.B., and Mcuauley, L. (2005). Breast cancer, estrogen, soy genistein, and other dietary factors. **Nutrition & Food Science**. 35(1): 35-42.

Dong, S., Inoue, A., Zhu, Y., Tanji, M., and Kiyama, R. (2007). Activation of rapid signaling pathways and the subsequent transcriptional regulation for the proliferation of breast cancer MCF-7 cells by the treatment with an extract of *Glycyrrhiza glabra* root. **Food and Chemical Toxicology**. 45(12): 2470-2478.

Dorostghoal, M., Ghaffari, H.O., Marmazi, F., and Keikhah, N. (2018). Overexpression of Endometrial Estrogen Receptor-Alpha in The Window of Implantation in Women with Unexplained Infertility. **International Journal of Fertility and Sterility**. 12(1): 37-42.

Durazzo, A., Lucarini, M., Souto, E.B., Cicala, C., Caiazzo, E., Izzo, A.A., Novellino, E., and Santini, A. (2019). Polyphenols: A concise overview on the chemistry, occurrence, and human health. **Phytotherapy Research**. 33(9): 2221-2243.

Edwards, H., Duchesne, A., Au, A.S., and Einstein, G. (2019). The many menopauses: Searching the cognitive research literature for menopause types. **Menopause** 26(1): 45-65.

El-Halawany, A.M., Chung, M.H., Abdallah, H.M., Nishihara, T., and Hattori, M. (2010). Estrogenic activity of a naringinase-treated extract of *Sophora japonica* cultivated in Egypt. **Pharm Biol**. 48(2): 177-181.

El-Halawany, A.M., Chung, M.H., Nakamura, N., Ma, C.-M., Nishihara, T., and Hattori, M. (2007). Estrogenic and anti-estrogenic activities of *Cassia tora* phenolic constituents. **Chemical and Pharmaceutical Bulletin**. 55(10): 1476-1482.

El-Halawany, A.M., El-Dine, R.S., Chung, M.H., Nishihara, T., and Hattori, M. (2011). Screening for estrogenic and antiestrogenic activities of plants growing in Egypt and Thailand. **Pharmacognosy Research**. 3(2): 107-113.

El-Kott, A.F., Al-Kahtani, M.A., and Shati, A.A. (2019). Calycosin induces apoptosis in adenocarcinoma HT29 cells by inducing cytotoxic autophagy mediated by SIRT1/AMPK-induced inhibition of Akt/mTOR. **Clinical and Experimental Pharmacology and Physiology**. 46(10): 944-954.

Elkady, A.I., Abuzinadah, O.A., Baeshen, N.A., and Rahmy, T.R. (2012). Differential control of growth, apoptotic activity, and gene expression in human breast cancer cells by extracts derived from medicinal herbs *Zingiber officinale*. **Journal of Biomedicine and Biotechnology**. 2012: 614356.

Fan, J.Z., Yang, X., and Bi, Z.G. (2015). The effects of 6-gingerol on proliferation, differentiation, and maturation of osteoblast-like MG-63 cells. **Brazilian Journal of Medical and Biological Research**. 48(7): 637-643.

Fiorelli, G., Picariello, L., Martineti, V., Tonelli, F., and Brandi, M.L. (1999). Functional estrogen receptor beta in colon cancer cells. **Biochem Biophys Res Commun**. 261(2): 521-527.

Fuentes, N., and Silveyra, P. (2019). Estrogen receptor signaling mechanisms. **Advances in Protein Chemistry and Structural Biology**. 116: 135-170.

Gan, H., Zhang, Y., Zhou, Q., Zheng, L., Xie, X., Veeraraghavan, V.P., and Mohan, S.K. (2019). Zingerone induced caspase-dependent apoptosis in MCF-7 cells and prevents 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in experimental rats. **Journal of Biochemical and Molecular Toxicology**. 33(10): e22387.

Garcia, M., O., De Luna, B., E., Ramos, T., J., Ruiz, C., Milia, E., Lorenzo, M.L., Jimenez, B., Sanchez, O., A., and Rivas, A. (2016). Phenolic compounds in

extra virgin olive oil stimulate human osteoblastic cell proliferation. **PLoS One**. 11(3): e0150045.

Glazier, M., and Bowman, M.A. (2001). A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. **Archives of Internal Medicine**. 161(9): 1161-1172.

Gouw, A.M., Efe., G., Barakat, R., Preecha, A., Mehdizadeh, M., Garan, S.A., and Brooks, G.A. (2017). Roles of estrogen receptor-alpha in mediating life span: the hypothalamic deregulation hypothesis. **Physiological Genomics**. 49(2): 88-95.

Guo, A.J., Choi, R.C., Zheng, K.Y., Chen, V.P., Dong, T.T., Wang, Z.T., Vollmer, G., Lau, D.T., and Tsim, K.W. (2012). Kaempferol as a flavonoid induces osteoblastic differentiation via estrogen receptor signaling. **Chinese Medicine**. 7: 10.

Gupta, C., Prakash, D., and Gupta, S. (2016). Phytoestrogens as pharma foods. **Advances In Food Technology and Nutritional Sciences**. 2(1): 19-31.

Hairong, M., Huabo, W., Zhen, C., Yi, Y., Zhenghua, W., Madina, H., Xu, C., and Akber, A.H. (2013). The estrogenic activity of isoflavones extracted from chickpea *Cicer arietinum* L sprouts *in vitro*. **Phytotherapy Research**. 27(8): 1237-1242.

Hajirahimkhan, A., Mbachu, O., Simmler, C., Ellis, S.G., Dong, H., Nikolic, D., Lankin, D.C., van Breemen, R.B., Chen, S.N., Pauli, G.F., Dietz, B.M., and Bolton, J.L. (2018). Estrogen receptor (ER) subtype selectivity identifies 8-Prenylapigenin as an ERβ agonist from *glycyrrhiza inflata* and highlights the

importance of chemical and biological authentication. **J Nat Prod**. 81(4): 966-975.

Hao, Q., Zhao, P., Niu, J., Wang, J., Yu, J., and Xue, X. (2010). Effect of ferulic acid on proliferation and mechanism in human breast cancer cells. **Zhongguo Zhong Yao Za Zhi**. 35(20): 2752-2755.

Haryanti, S., Galuh, R., and Nuning, R. (2020). *Curcuma zanthorrhiza extracts induce G2/M Cell cycle arrest and apoptosis in 4T1 and MCF-7 human breast cancer cells*. In: the 4th International Symposium on Health Research (ISHR 2019). pp. 415-419.

He, Y., Hu, Z., Li, A., Zhu, Z., Yang, N., Ying, Z., He, J., Wang, C., Yin, S., and Cheng, S. (2019). Recent advances in biotransformation of saponins. **Molecules** 24(13): 2365.

Hernandes, L.C., Machado, A.R.T., Tuttis, K., Ribeiro, D.L., Aissa, A.F., Dévoz, P.P., and Antunes, L.M.G. (2020). Caffeic acid and chlorogenic acid cytotoxicity, genotoxicity and impact on global DNA methylation in human leukemic cell lines. **Genetics and Molecular Biology**. 43: e20190347.

Hsieh, S.L., Chen, C.T., Wang, J.J., Kuo, Y.H., Li, C.C., Hsieh, L.C., and Wu, C.C. (2015). Sedanolide induces autophagy through the PI3K, p53 and NF-κB signaling pathways in human liver cancer cells. **Journal of Oncology**. 47(6): 2240-2246.

Hsiu, S.L., Lin, Y.T., Hou, Y.C., and Chao, P.D. (2004). A deglucosylated metabolite of paeoniflorin of the root of *Paeonia lactiflora* and its pharmacokinetics in rats. **Planta Medica**. 69: 1113-1118.

Hu, C., Liu, H., Du, J., Mo, B., Qi, H., Wang, X., Ye, S., and Li, Z. (2009). Estrogenic activities of extracts of Chinese licorice (*Glycyrrhiza uralensis*) root in MCF-7 breast cancer cells. **The Journal of Steroid Biochemistry and Molecular Biology**. 113(3-5): 209-216.

Hu, K., Luo, Q., Zhu, X.F., Sun, S.H., Feng, G.L., Liu, Z.Y., and Chen, X.M. (2019). Isolation of homogeneous polysaccharide from *Poria cocos* and effect of its sulfated derivatives on migration of human breast cancer MDA-MB-231 cells. **Zhongguo Zhong Yao Za Zhi**. 44(13): 2835-2840.

Huang, B., Omoto, Y., Iwase, H., Yamashita, H., Toyama, T., Coombes, R.C., Filipovic, A., Warner, M., and Gustafsson, J.A. (2014). Differential expression of estrogen receptor alpha, beta1, and beta2 in lobular and ductal breast cancer. **Proceedings of the National Academy of Sciences of the United States of America**. 111(5): 1933-1938.

Huang, L., Jin, K., and Lan, H. (2019). Luteolin inhibits cell cycle progression and induces apoptosis of breast cancer cells through downregulation of human telomerase reverse transcriptase. **Oncology Letters**. 17(4): 3842-3850.

Hwang, H., Kim, C., Kim, S.M., Kim, W.S., Choi, S.H., Chang, I.M., and Ahn, K.S. (2012). The hydrolyzed products of iridoid glycoside with β-glucosidase treatment exert anti-proliferative effects through suppression of STAT3 activation and STAT3-regulated gene products in several human cancer cells. **Pharm Biol**. 50(1): 8-17.

Ibarreta, D., Daxenberger, A., and Meyer Heinrich, H.D. (2003). Possible health impact of phytoestrogens and xenoestrogens in food. **Acta Pathologica, Microbiologica, Et Immunologica Scandinavica**. 109(3): 161-184.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. **Gene**. 96(1): 23-28.

Jafari, Z., Emtiazy, M., Sohrabvand, F., Talei, D., Oveidzadeh, L., Abrishamkar, M., Meyssami, M., and Kamalinejad, M. (2019). The effect of *Glycyrrhiza glabra* L. on primary dysmenorrhea compared with ibuprofen: A randomized, triple-blind controlled trial. **nternational Journal of Pharmacy and Biological Sciences**. 18(Suppl1): 291-301.

Jiang, Y., and Fan, L. (2020). Evaluation of anticancer activities of *Poria cocos* ethanol extract in breast cancer: *In vivo* and *in vitro*, identification and mechanism. **Journal of Ethnopharmacology**. 257(2020): 112851.

Jin, J.S., Nishihata, T., Kakiuchi, N., and Hattori, M. (2008). Biotransformation of C-glucosylisoflavone puerarin to estrogenic (3S)-equol in co-culture of two human intestinal bacteria. **Biological and Pharmaceutical Bulletin**. 31(8): 1621-1625.

Kanayama, T., Mamiya, S., Nishihara, T., and Nishikawa, J. (2003). Basis of a high-throughput method for nuclear receptor ligands. **The Journal of Biochemistry**. 133(6): 791-797.

Kanchanapoom, T., Kasai, R., and Yamasaki, K. (2002). Iridoid and phenolic glycosides from *Morinda coreia*. **Phytochemistry**. 59(5): 551-556.

Kaneta, H., Koda, M., Saito, S., Imoto, M., Kawada, M., Yamazaki, Y., Momose, I., and Shindo, K. (2016). Biological activities of unique isoflavones prepared from *Apios americana* Medik. **Bioscience Biotechnology Biochemistry**. 80(4): 774-778.

Kannan, N., Manokari, M., and Shekhawat, M.S. (2020). Enhanced production of anthraquinones and phenolic compounds using chitosan from the adventitious roots of *Morinda coreia* Buck. and Ham. **Industrial Crops and Products**. 148: 112321.

Kanokmedhakul, S., Kanokmedhakul, K., Nambuddee, K., and Kongsaeree, P. (2004). New bioactive prenylflavonoids and dibenzocycloheptene derivative from roots of *Dendrolobium lanceolatum*. **J Nat Prod**. 67(6): 968-972.

Kawai, S., Hashimoto, W., and Murata, K. (2010). Transformation of *Saccharomyces cerevisiae* and other fungi: Methods and possible underlying mechanism. **Bioeng Bugs**. 1(6): 395-403.

Kawai, S., Hashimoto, W., and Murata, K. (2010). Transformation of *Saccharomyces cerevisiae* and other fungi: Methods and possible underlying mechanism. **Bioengineered Bugs**. 1(6): 395-403.

Keser, S., Kaygili, O., Keser, F., Tekin, S., Yilmaz, O., Demir, E., Kirbag, S., and Sandal, S. (2018). Phytochemical composition, antiradical, antiproliferative and antimicrobial activities of *Capsicum frutescens* L. **Analytical Chemistry Letters**. 8(5): 642-652.

Kim, D.Y., and Choi, B.Y. (2019). Costunolide-A bioactive sesquiterpene lactone with diverse therapeutic potential. **International Journal of Molecular Sciences**. 20(12): 2926.

Kim, I.G., Kang, S.C., Kim, K.C., Choung, E.S., and Zee, O.P. (2008). Screening of estrogenic and antiestrogenic activities from medicinal plants. **Environ Toxicol Pharmacol**. 25(1): 75-82.

Kim, I.G., Kang, S.C., Kim, K.C., Choung, E.S., and Zee, O.P. (2008). Screening of estrogenic and antiestrogenic activities from medicinal plants. **Environmental Toxicology Pharmacology**. 25(1): 75-82.

Kim, J., Jayaprakasha, G.K., and Patil, B.S. (2013). Limonoids and their anti-proliferative and anti-aromatase properties in human breast cancer cells. **Food & Function journal**. 4(2): 258-265.

Kim, K.U., Lee, S.J., and Lee, I. (2020). Development of an improved menopausal symptom-alleviating licorice (*Glycyrrhiza uralensis*) by biotransformation using *Monascus albidulus*. **Journal of Microbiology and Biotechnology**. 30(2): 178-186.

Kiyama, R. (2016). Biological effects induced by estrogenic activity of lignans. **Trends in Food Science & Technology**. 54: 186-196.

Koegl, M., and Uetz, P. (2007). Improving yeast two-hybrid screening systems. **Briefings in Functional Genomics**. 6(4): 302-312.

Koziol, E., and Skalicka-Wozniak, K. (2016). Imperatorin-pharmacological meaning and analytical clues: Profound investigation. **Phytochemistry Reviews**. 15: 627-649.

Kumar, R., Zakharov, M.N., Khan, S.H., Miki, R., Jang, H., Toraldo, G., Singh, R., Bhasin, S., and Jasuja, R. (2011). The dynamic structure of the estrogen receptor. **Journal of Amino Acids**. 2011: 1-7.

Kwankhao, P., Chuthaputti, A., Tantipidok, Y., Pathomwichaiwat, T., Theantawee, W., Buabao, S., Chantraket, R., Puttarak, P., Petrakart, P., Chinsoi, P., Chungsiriporn, D., Bongcheewin, B., and Sermsinsiri, V. (2020). The current

situation ofthe herbal medicinal product system in Thailand. **Journal of Medicine and Health Sciences**. 29(Special Issue): 82-93.

Lattrich, C., Stegerer, A., Häring, J., Schüler, S., Ortmann, O., and Treeck, O. (2013). Estrogen receptor β agonists affect growth and gene expression of human breast cancer cell lines. **Steroids**. 78(2): 195-202.

Lecce, G., Meduri, G., Ancelin, M., Bergeron, C., and Perrot, A.M. (2001). Presence of estrogen receptor beta in the human endometrium through the cycle: Expression in glandular, stromal, and vascular cells. **The Journal of Clinical Endocrinology & Metabolism**. 86(3): 1379-1386.

Lee, H.S., Miyauchi, K., Nagata, Y., Fukuda, R., Sasagawa, S.-i., Endoh, H., Kato, S., Horiuchi, H., Takagi, M., and Ohta, A. (2002). Employment of the human estrogen receptor β ligand-binding domain and co-activator SRC1 nuclear receptor-binding domain for the construction of a yeast two-hybrid detection system for endocrine disrupters1. **The Journal of Biochemistry**. 131(3): 399-405.

Lee, K., Shin, M.S., Ham, I., and Choi, H.-Y. (2015). Investigation of the mechanisms of *Angelica dahurica* root extract-induced vasorelaxation in isolated rat aortic rings. **BMC Complementary Medicine and Therapies**. 15: 395-395.

Lee, K.H., Lee, Y.T., Chen, T.C., Yeh, C.C., Chen, J.Y., Liu, L.Y., and Chi, C.H. (2013). Effects of Sheng Hua Tang on uterine involution and ovarian activity in postpartum dairy cows. **Journal of Animal Science**. 26(9): 1247-1254.

Lee, S.J., Chung, H.Y., Maier, C.G.A., Wood, A.R., Dixon, R.A., and Mabry, T.J. (1998). Estrogenic flavonoids from *Artemisia vulgaris* L. **Journal of Agricultural and Food Chemistry**. 46(8): 3325-3329.

Lee, Y.J., Jin, Y.R., Lim, W.C., Park, W.K., Cho, J.Y., Jang, S., and Lee, S.K. (2003). Ginsenoside-Rb1 acts as a weak phytoestrogen in MCF-7 human breast cancer cells. **Arch Pharm Res**. 26(1): 58-63.

Lee, Y.S., Choi, C.W., Kim, J.J., Ganapathi, A., Udayakumar, R., and Kim, S.C. (2009). Determination of mineral content in methanolic safflower (*Carthamus tinctorius* L.) seed extract and its effect on osteoblast markers. **International Journal of Molecular Sciences**. 10(1): 292-305.

Leong, L.M., Chan, K.M., Hamid, A., Latip, J., and Rajab, N.F. (2016). Herbal formulation C168 attenuates proliferation and induces apoptosis in HCT 116 human colorectal carcinoma cells: Role of oxidative stress and DNA damage. **Evidence-Based Complementary and Alternative Medicine**. 2016: 2091085.

Lephart, E.D., and Naftolin, F. (2021). Menopause and the skin: Old favorites and new innovations in cosmeceuticals for estrogen-deficient skin. **Dermatology Therapy**. 11(1): 53-69.

Li, L.L., Cui, Y., Guo, X.H., Ma, K., Tian, P., Feng, J., and Wang, J.M. (2019). Pharmacokinetics and tissue distribution of gingerols and shogaols from ginger (*Zingiber officinale Rosc*.) in rats by UPLC-Q-Exactive-HRMS. **Molecules** 24(3): 512.

Li, T., Ma, Y., Zhang, H., Yan, P., Huo, L., Hu, Y., Chen, X., Li, T., Zhang, M., and Liu, Z. (2016). Differential regulation of morphology and estrogen receptor-alpha expression in the vagina of ovariectomized adult virgin rats by estrogen replacement: A histological study. **Journal of Toxicology**. 2016: 1093512-1093512.

Liang, A., Jianghong, Z., Taijun, Z., Xiaoqing, L., Qiong, Z., and Jun, C. (2017). Analysis of the inhibitory effect of safflower polysaccharide on HT29 colorectal cancer cell proliferation and its relevant mechanism. **Biomedical Research**. 28: 2966-2970.

Lin, T.-C., Wang, K.-H., Kao, A.-P., Chuang, K.-H., and Kuo, T.-C. (2017). *Pueraria mirifica* inhibits 17β-estradiol-induced cell proliferation of human endometrial mesenchymal stem cells. **Taiwanese Journal of Obstetrics & Gynecology**. 56(6): 765-769.

Liu, B., Shi, Y., Peng, W., Zhang, Q., Liu, J., Chen, N., and Zhu, R. (2016). Diosmetin induces apoptosis by upregulating p53 via the TGF-β signal pathway in HepG2 hepatoma cells. **Molecular Medicine Reports**. 14(1): 159-164.

Liu, D., Zeng, M., Pi, J.W., Liu, M.J., Ding, W.Z., Mei, X.Y., Liu, J.L., and Cao, X.Y. (2021). Exploring the potential mechanism of costunolide-induced mcf-7 cells apoptosis by multi-spectroscopy, molecular docking and cell experiments. **Chemistry & Biodiversity**. 18(5): e2001069.

Liu, J., Burdette, J.E., Xu, H., Gu, C., van Breemen, R.B., Bhat, K.P.L., Booth, N., Constantinou, A.I., Pezzuto, J.M., Fong, H.H.S., Farnsworth, N.R., and Bolton, J.L. (2001). Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. **Journal of Agricultural and Food Chemistry**. 49(5): 2472-2479.

Liu, L., Ma, H., Tang, Y., Chen, W., Lu, Y., Guo, J., and Duan, J.-a. (2012). Discovery of estrogen receptor α modulators from natural compounds in Si-Wu-Tang series decoctions using estrogen-responsive MCF-7 breast cancer cells. **Bioorganic & Medicinal Chemistry Letters**. 22(1): 154-163.

Liu, L., Mu, H., and Pang, Y. (2021). Caffeic acid treatment augments the cell proliferation, differentiation, and calcium mineralization in the human osteoblast-like MG-63 cells. **Pharmacognosy Magazine**. 17(73): 38-44.

Liu, T., Li, N., Yan, Y., Liu, Y., Xiong, K., Liu, Y., Xia, Q., Zhang, H., and Liu, Z. (2020). Recent advances in the anti-aging effects of phytoestrogens on collagen, water content, and oxidative stress. **Phytotherapy Research**. 34(3): 435-447.

Lu, Z., Zhou, R., Kong, Y., Wang, J., Xia, W., Guo, J., Liu, J., Sun, H., Liu, K., Yang, J., Mi, M., and Xu, H. (2016). S-equol, a secondary metabolite of natural anticancer isoflavone daidzein, inhibits prostate cancer growth *in vitro* and *in vivo*, though activating the Akt/FOXO3a pathway. **Current Cancer Drug Targets**. 16(5): 455-465.

Luna, D.L., Tomasin, R., Naves, M.A., Dasilva, J.A., and Cominetti, M.R. (2018). Autophagy-dependent apoptosis is triggered by a semi-synthetic [6]-gingerol analogue in triple negative breast cancer cells. **Oncotarget**. 9(56): 30787-30804.

Luo, Z., Zeng, H., Ye, Y., Liu, L., Li, S., Zhang, J., and Luo, R. (2015). Safflower polysaccharide inhibits the proliferation and metastasis of MCF-7 breast cancer cell. **Mol Med Rep**. 11(6): 4611-4616.

Luo, Z., Zeng, H., Ye, Y., Liu, L., Li, S., Zhang, J., and Luo, R. (2015). Safflower polysaccharide inhibits the proliferation and metastasis of MCF-7 breast cancer cell. **Molecular Medicine Reports**. 11(6): 4611-4616.

Macgregor, J.I., and Jordan, V.C. (1998). Basic guide to the mechanisms of antiestrogen action. **Pharmacognosy Review**. 50(2): 151-196.

Malaivijitnond, S. (2012). Medical applications of phytoestrogens from the Thai herb *Pueraria mirifica*. **Frontiers in Medicine**. 6(1): 8-21.

Malaivijitnond, S., Kiatthaipipat, P., Cherdshewasart, W., Watanabe, G., and Taya, K. (2004). Different effects of Pueraria mirifica, a herb containing phytoestrogens, on LH and FSH secretion in gonadectomized female and male rats. **Journal of Pharmaceutical Sciences**. 96(4): 428-435.

Mamadou, K., Camille, K., Ngoran, M.K., Aminata, A., Nguessan, A.R.Y., and Henri, M.D.K. (2014). Ethnobotany, phytochemistry, pharmacology and toxicology profiles of *Cassia siamea* Lam. **The Journal of Phytopharmacology**. 3: 57-76.

Mancinelli, R., Onori, P., Demorrow, S., Francis, H., Glaser, S., Franchitto, A., Carpino, G., Alpini, G., and Gaudio, E. (2010). Role of sex hormones in the modulation of cholangiocyte function. **World Journal of Gastrointestinal Pathophysiology**. 1(2): 50-62.

Mani, V., Lee, S.K., Yeo, Y., and Hahn, B.S. (2020). A metabolic perspective and opportunities in pharmacologically important safflower. **Metabolites**. 10(6): 253.

Mao, Q.Q., Xu, X.Y., Cao, S.Y., Gan, R.Y., Corke, H., Beta, T., and Li, H.B. (2019). Bioactive compounds and bioactivities of ginger (zingiber officinale Roscoe). **Foods** 8(6): 185.

Mashwani, Z.R., Mashwani, R., Khan, A., Laraib, S., Wali, R., Sarwar, U., Ain, Q., Shakeel, S., Rahimullah, and Sohail. (2017). Conservation status and therapeutic potential of *Saussurea lappa*: An overview. **American Journal of Plant Sciences**. 8: 602-614.

Memariani, T., Hosseini, T., Kamali, H., Mohammadi, A., Ghorbani, M., Shakeri, A., Spandidos, D.A., Tsatsakis, A.M., and Shahsavand, S. (2016). Evaluation of the cytotoxic effects of *Cyperus longus* extract, fractions and its essential oil on the PC3 and MCF7 cancer cell lines. **Oncology Letters**. 11(2): 1353-1360.

Meng, X., Shibin, Y., Pi, Z.-F., Song, F.-R., and Liu, Z. (2012). An investigation of the metabolism of liquiritin and the immunological effects of its metabolites. **Journal of Liquid Chromatography & Related Technologies**. 35(11): 1538-1549.

Mock, C.D., Jordan, B.C., and Selvam, C. (2015). Recent advances of curcumin and its analogues in breast cancer prevention and treatment. **RSC Advances**. 5(92): 75575-75588.

Moore, T.W., Zhu, S., Randolph, R., Shoji, M., and Snyder, J.P. (2014). Liver S9 fraction-derived metabolites of curcumin analogue UBS109. **ACS Medicinal Chemistry Letters**. 5(4): 288-292.

Moreira, A.C., Silva, A.M., Santos, M.S., and Sardao, V.A. (2014). Phytoestrogens as alternative hormone replacement therapy in menopause: What is real, what is unknown. **The Journal of Steroid Biochemistry and Molecular Biology**. 143: 61-71.

Mottaghipisheh, J., Nové, M., Spengler, G., Kúsz, N., Hohmann, J., and Csupor, D. (2018). Antiproliferative and cytotoxic activities of furocoumarins of *Ducrosia anethifolia*. **Pharm Biol**. 56(1): 658-664.

Mueller, S.O. (2002). Overview of *in vitro* tools to assess the estrogenic and antiestrogenic activity of phytoestrogens. **Journal of Chromatography B**. 777(1): 155-165.

Nanashima, N., Horie, K., and Maeda, H. (2017). Phytoestrogenic activity of blackcurrant anthocyanins is partially mediated through estrogen receptor beta. **Molecules**. 23(1): 1-11.

Nazmi, S.A., Nourazarian, A., Bahhaj, R., and Khaki, K.F. (2018). The anticancer effect of *Arctium lappa* and *Glycyrrhiza glabra* on HT-29 colon cancer and MCF-7 Breast cancer cell lines. **Crescent Journal of Medical and Biological Sciences**. 5: 133-137.

Nie, A., Chao, Y., Zhang, X., Jia, W., Zhou, Z., and Zhu, C. (2020). Phytochemistry and pharmacological activities of *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb. **Frontiers in Pharmacology**. 11(505249): 1-17.

Nishihara, T., Nishikawa, J.-i., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., and Utsumi, H. (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. **Journal of Health Science and Medical Research**. 46(4): 282-298.

Nishikawa, J.i., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., and Nishihara, T. (1999). New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. **Toxicology and Applied Pharmacology**. 154(1): 76-83.

Noviyanti, N., Yueniwati, Y., Ali, M., Rahardjo, B., and Permatasari, G.W. (2020). *Cyclea barbata* Miers ethanol extract and coclaurine induce estrogen receptor α in the development of follicle pre-ovulation. **Open Access Macedonian Journal of Medical Sciences**. 8(A): 434-440.

Paiva, L.B.d., Goldbeck, R., Santos, W.D.d., and Squina, F.M. (2013). Ferulic acid and derivatives: molecules with potential application in the pharmaceutical field. **Brazilian Journal of Pharmaceutical Sciences**. 49: 395-411.

Pandey, A., Chaturvedi, M., Mishra, S., Kumar, P., Somvanshi, P., and Chaturvedi, R. (2020). Reductive metabolites of curcumin and their therapeutic effects. **Heliyon**. 6(11): e05469.

Panigoro, R., Surialaga, S., and Dhianawaty, D. (2013). Comparison of curcumin level in fresh and decoction of dried *Curcuma xan-thorrhiza* Roxb. rhizome. **International Journal of Pharmacy and Biological Sciences Research**. 4: 256-259.

Park, J., Song, H., Kim, S., Lee, M.S., Rhee, D.K., and Lee, Y.J. (2017). Effects of ginseng on two main sex steroid hormone receptors: Estrogen and androgen receptors. **Journal of Ginseng Research**. 41(2): 215-221.

Park, S.Y., Park, J.H., Kim, H.S., Lee, C.Y., Lee, H.J., Kang, K.S., and Kim, C.E. (2018). Systems-level mechanisms of action of *Panax ginseng*: a network pharmacological approach. **Journal of Ginseng Research**. 42(1): 98-106.

Paterni, I., Granchi, C., Katzenellenbogen, J.A., and Minutolo, F. (2014). Estrogen receptors alpha (ERα) and beta (ERβ): Subtype-selective ligands and clinical potential. **Steroids**. 90: 13-29.

Pervin, M., Hasnat, M.A., Debnath, T., Park, S.R., Kim, D.H., and Lim, B.O. (2014). Antioxidant, anti-inflammatory and antiproliferative activity of *Angelica Dahurica* Root extracts. **Journal of Food Biochemistry**. 38(3): 281-292.

Piao, X.L., Yoo, H.H., Kim, H.Y., Kang, T.L., Hwang, G.S., and Park, J.H. (2006). Estrogenic activity of furanocoumarins isolated from *Angelica dahurica*. **Arch Pharm Res**. 29(9): 741-745.

Poh, M., and Yin, C.Y. (2013). Estrogenic and anti-proliferative activity of water extract of *Glycyrhizzic uralensis* (licorice root). **International Journal of Phytomedicine**. 5(1): 119.

Poluzzi, E., Piccinni, C., Raschi, E., Rampa, A., Recanatini, M., and Ponti, F.D. (2014). Phytoestrogens in postmenopause: The state of the art from a chemical, pharmacological and regulatory perspective. **Current Medicinal Chemistry**. 21(4): 417-436.

Poomirat, S., Jaiarree, N., Itharat, A., and Ruangnoo, S. (2020b). Cytotoxicity against cervical and breast cancer cells of Leard-Ngam remedy and its plant compositions. **Science and Technology Asia Journal**. 25(3): 38-50.

Poomirat, S., Jaiarree, N., Itharat, A., and Threrapanithan, C. (2020a). Phytochemical analysis of Leard-Ngam remedy and its composition. **Thammasat Medical Journal**. 20(1): 60-73.

Raj, C.D., Dhinesh, M.G., Lavanya, R., and Brindha, P. (2014). Studies on antiproliferative and antioxidant efficacy of *caesalpinia sappan* L. heartwood. **Asian Journal of Chemistry**. 26: 3683-3686.

Rice, E.C. (2004). Flavonoids and isoflavones: absorption, metabolism, and bioactivity. **Free Radical Biology & Medicine**. 36(7): 827-828.

Rietjens, I., Louisse, J., and Beekmann, K. (2017). The potential health effects of dietary phytoestrogens. **British Journal of Pharmacology**. 174(11): 1263-1280.

Ringa, V., Varnoux, N., Piault, S., and Breart, G. (2005). Hormone therapy use among postmenopausal French women before the publication of the women's health initiative study: Duration of use and factors associated with discontinuation. **Fertility and Sterility**. 83(6): 1771-1779.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., and Ockene, J. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial. **Journal of the American Medical Association**. 288(3): 321-333.

Ruff, M., Gangloff, M., Marie Wurtz, J., and Moras, D. (2000). Estrogen receptor transcription and transactivation: Structure-function relationship in DNA-and ligand-binding domains of estrogen receptors. **Breast Cancer Research**. 2(5): 353-359.

Rzepecki, A.K., Murase, J.E., Juran, R., Fabi, S.G., and McLellan, B.N. (2019). Estrogen-deficient skin: The role of topical therapy. **International Journal of Women's Dermatology**. 5(2): 85-90.

Sakpakdeejaroen, I., and Itharat, A. (2009). Cytotoxic compounds against breast adenocarcinoma cell (MCF-7) form Pikutbenjakul. **Journal of Health Research**. 23: 70-77.

Schippmann, U., Leaman, D., and B Cunningham, A. (2002). *Impact of cultivation and gathering of medicinal plants on biodiversity: Global trends and issues.* In: the biodiversity and the ecosystem approach in agriculture, forestry and fisheries.

Satellite event on the occasion of the ninth regular session of the commission on genetic resources for food and agriculture, Rome. pp. 142-167.

Setchell, D.K., Nechemias, Z.L., Cai, J., and Heubi, E.J. (1999). Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. **The American Journal of Clinical Nutrition**. 68: 1453S-1461S.

Sharma, A., and Lee, H.-J. (2020). Ginsenoside compound K: Insights into recent studies on pharmacokinetics and health-promoting activities. **Biomolecules**. 10(7): 1028.

Sharma, V., Katiyar, A., and Agrawal, R.C. (2017). *Glycyrrhiza glabra*: Chemistry and pharmacological activity. **Sweeteners**: 87-100.

Shetty, S.N., Mengi, S., Vaidya, R., and Vaidya, A.D. (2010). A study of standardized extracts of *Picrorhiza kurroa* Royle ex Benth in experimental nonalcoholic fatty liver disease. **Journal of Ayurveda and Integrative Medicine**. 1(3): 203-210.

Shim, M.K., and Lee, Y.J. (2012). Estrogen receptor is activated by korean red ginseng *in vitro* but not in *vivo*. **Journal of Ginseng Research**. 36(2): 169-175.

Shukla, B., Saxena, S., Usmani, S., and Kushwaha, P. (2021). Phytochemistry and pharmacological studies of *Plumbago zeylanica* L.: A medicinal plant review. **Clinical Phytoscience**. 7(1): 1-11.

Siangcham, T., Saenphet, S., and Saenphet, K. (2010). Estrogen bioassy of *Pueraria mirifica* Airy Shaw & Suvatabandhu. **Journal of Medicinal Plants Research**. 4: 741-744.

Sirotkin, A.V., and Harrath, A.H. (2014). Phytoestrogens and their effects. **European Journal of Pharmacology**. 741: 230-236.

Soares, J.M., Faria, B.M., Ascari, L.M., Souza, J.M., Soares, A.G., Cordeiro, Y., and Romao, L.F. (2019). Diosmin induces caspase-dependent apoptosis in human glioblastoma cells. **Anais da Academia Brasileira de Ciencias**. 91(4): e20191031.

Sommerfeld, V., Schollenberger, M., Hemberle, L., and Rodehutscord, M. (2017). Modification and application of an *in vitro* assay to examine inositol phosphate degradation in the digestive tract of poultry. **Journal of the Science of Food and Agriculture**. 97(12): 4219-4226.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., and Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. **Environmental Health Perspectives**. 103: 113-122.

Su, Y.-C., and Ho, C.-L. (2013). Composition and *in-vitro* cytotoxic activities of the leaf essential oil of *Beilschmiedia erythrophloia* from Taiwan. **Natural Product Communications**. 8(1): 143-144.

Subcharoen, P., and Chthaputti, A. (2018). *Puraria mirifica*. **Thai traditional medicine kingdom of Thailand.**: 1-16.

Sugiyanto, R., Khamsita, R., Lambertus, M., Utomo, R., and Susidarti, R. (2012). Ethanolic extract of papaya (*Carica papaya*) leaf exhibits estrogenic effects *in vivo* and in *silico*. **Indonesian Journal of Cancer Chemoprevention**. 3: 384.

Sun, J., Song, J., Zhang, W., Jing, F., Xu, W., Leng, P., Quan, X., Du, G., and Sui, Z. (2018). Some pharmacokinetic parameters of salvianolic acid A following single-dose oral administration to rats. **Pharm Biol**. 56(1): 399-406.

Sung, B., Chung, H., Kim, M., Kang, Y., Kim, D., Seong Yeon, H., Kim, M., Kim, C., Chung, H.-Y., and Kim, N.D. (2015). Cytotoxic effects of solvent-extracted active components of *Salvia miltiorrhiza* Bunge on human cancer cell lines. **Experimental and Therapeutic Medicine**. 9: 1421-1428.

Tahir, A.A., Sani, N.F.A., Murad, N.A., Makpol, S., Ngah, W.Z.W., and Yusof, Y.A.M. (2015). Combined ginger extract & Gelam honey modulate Ras/ERK and PI3K/AKT pathway genes in colon cancer HT29 cells. **Nutrition Journal**. 14(1): 31.

Tao, J.H., Zhao, M., Wang, D.G., Yang, C., Du, L.Y., Qiu, W.Q., and Jiang, S. (2016). Biotransformation and metabolic profile of catalpol with human intestinal microflora by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. **Journal of Chromatography B**. 1009-1010: 163-169.

Tarko, T., Duda Chodak, A., and Tuszyński, T. (2009). Simulation of phenolic compounds transformations and interactions in an *in vitro* model of the human alimentary tract. **Food Science and Technology International**. 15(3): 235-241.

Thakur, S., Bawara, B., Dubey, A., Nandini, D., Chauhan, N.S., and Saraf, D.K. (2009). Effect of *Carum carvi* and *Curcuma longa* on hormonal and reproductive parameter of female rats. **International Journal of Phytomedicine**. 1(1): 31-38.

Tian, M., Li, L.-N., Zheng, R.-R., Yang, L., and Wang, Z.-T. (2020). Advances on hormone-like activity of *Panax ginseng* and ginsenosides. **Chinese Journal of Natural Medicines**. 18(7): 526-535.

Tronnier, M. (2018). Cutaneous disorders characterized by elastolysis or loss of elastic tissue. **Journal der Deutschen Dermatologischen Gesellschaft**. 16(2): 183-191.

Tsuji, M., Tanaka, T., Nagashima, R., Sagisaka, Y., Tousen, Y., Nishide, Y., Ishimi, Y., and Ishimi, Y. (2018). Effect of daidzein and equol on DNA replication in MCF-7 cells. **The Journal of Biochemistry**. 163(5): 371-380.

Turner, J.V., Agatonovic, K.S., and Glass, B.D. (2007). Molecular aspects of phytoestrogen selective binding at estrogen receptors. **Journal of Pharmaceutical Sciences**. 96(8): 1879-1885.

TuyOn, T., Itharat, A., Maki, P., Thongdeeying, P., Pipatrattanaseree, W., and Ooraikul, B. (2020). *In vitro* cytotoxic activity against breast, cervical, and ovarian cancer cells and flavonoid content of plant ingredients used in a selected thai traditional cancer remedy: Correlation and hierarchical cluster analysis. **Evidence-Based Complementary and Alternative Medicine**. 2020: 8884529.

Villa-Rodriguez, E., Ibarra-Gamez, C., and de los Santos-Villalobos, S. (2018). Extraction of high-quality RNA from *Bacillus subtilis* with a lysozyme pre-treatment followed by the Trizol method. **Journal of Microbiological Methods**. 147: 14-16.

Vollmer, M., Esders, S., Farquharson, F.M., Neugart, S., Duncan, S.H., Schreiner, M., Louis, P., Maul, R., and Rohn, S. (2018). Mutual Interaction of phenolic compounds and microbiota: Metabolism of complex phenolic apigenin-C and kaempferol-O-derivatives by human fecal samples. **Journal of Agricultural and Food Chemistry**. 66(2): 485-497.

Vonna, A., Urip, H., and Rosidah, G. (2014). Cytotoxic activity and phase G2-M cell cycle inhibition active fraction of *Mimusops elengi* stem bark on T47D cells. **International Journal of Pharmacy and Biological Sciences Research**. 6(2): 868.

Wang, C., Wang, L., Li, C., Hu, C., and Zhao, S. (2020). Anti-proliferation activities of three bioactive components purified by high-speed counter-current chromatography in essential oil from ginger. **European Food Research and Technology**. 246(4): 795-805.

Wang, D., Wang, X.-H., Yu, X., Cao, F., Cai, X., Chen, P., Li, M., Feng, Y., Li, H., and Wang, X. (2021). Pharmacokinetics of anthraquinones from medicinal plants. **Frontiers in Pharmacology**. 12(306): 1-19.

Wang, J., Song, C., Gao, D., Wei, S., Sun, W., Guo, Y., Sun, S., Tian, X., Li, H., and Qiao, M. (2020). Effects of *Paeonia lactiflora* extract on estrogen receptor β, TPH2, and SERT in rats with PMS anxiety. **BioMed Research International**. 2020: 1-8.

Wang, X., Chang, X., Luo, X., Su, M., Xu, R., Chen, J., Ding, Y., and Shi, Y. (2019). An integrated approach to characterize intestinal metabolites of four phenylethanoid glycosides and intestinal microbe-mediated antioxidant activity evaluation *in vitro* using UHPLC-Q-Exactive high-resolution mass spectrometry and a 1,1-diphenyl-2-picrylhydrazyl-based assay. **Frontiers in Pharmacology**. 10: 826.

Wang, Z., Kanda, S., Shimono, T., Enkh-Undraa, D., and Nishiyama, T. (2018). The *in vitro* estrogenic activity of the crude drugs found in Japanese herbal medicines

prescribed for menopausal syndrome was enhanced by combining them. **BMC Complementary Medicine and Therapies**. 18(1): 107-107.

Warner, M., Huang, B., and Gustafsson, J.A. (2017). Estrogen receptor beta as a pharmaceutical target. **Trends in Pharmacological Sciences**. 38(1): 92-99.

Wei, W.-L., Zeng, R., Gu, C.-M., Qu, Y., and Huang, L.-F. (2016). *Angelica sinensis* in China-a review of botanical profile, ethnopharmacology, phytochemistry and chemical analysis. **Journal of Ethnopharmacology**. 190: 116-141.

Winuthayanon, W., Hewitt, S.C., and Korach, K.S. (2014). Uterine epithelial cell estrogen receptor alpha-dependent and -independent genomic profiles that underlie estrogen responses in mice. **Biology of Reproduction**. 91(5): 110.

Winuthayanon, W., Suksen, K., Boonchird, C., Chuncharunee, A., Ponglikitmongkol, M., Suksamrarn, A., and Piyachaturawat, P. (2009). Estrogenic activity of diarylheptanoids from *Curcuma comosa* Roxb. requires metabolic activation. **Journal of Agricultural and Food Chemistry**. 57(3): 840-845.

Wisodsongkram, P., and Kitiyodom, S. (2020). The association between herbal substances and endometrial neoplasia in Thai women with postmenopausal bleeding: A case-control study at maharat Nakhon ratchasima hospital. **Thai Journal of Obstetrics and Gynaecology**. 28(2): 71-79.

Wisutthathum, S., Kamkaew, N., Inchan, A., Chatturong, U., Paracha, T.U., Ingkaninan, K., Wongwad, E., and Chootip, K. (2018). Extract of *Aquilaria crassna* leaves and mangiferin are vasodilators while showing no cytotoxicity. **Journal of Traditional and Complementary Medicine**. 9(4): 237-242.

Wu, H., Huang, Q., Chao, S., Yu, J., Xu, S., Wang, F., Shang, X., and Zhu, Y. (2020). Determination of ferulic acid in *Angelica sinensis* by temperature-controlled

hydrophobic ionic liquids-based ultrasound/heating-assisted extraction coupled with high performance liquid chromatography. **Molecules**. 25(15): 3356.

Xie, Q., Zhang, L., Xie, L., Zheng, Y., Liu, K., Tang, H., Liao, Y., and Li, X. (2020). Z-ligustilide: A review of its pharmacokinetics and pharmacology. **Phytotherapy Research**. 34(8): 1966-1991.

Xu, X.L., Deng, S.L., Lian, Z.X., and Yu, K. (2021). Estrogen receptors in polycystic ovary syndrome. **Cells**. 10(2): 459.

Xu, Y., Ding, J., An, J., Qu, Y., Li, X., Ma, X., Zhang, Y., Dai, G., and Lin, N. (2016). Effect of the interaction of *Veratrum Nigrum* with *Panax Ginseng* on estrogenic activity *in Vivo* and *in vitro*. **Science Report**. 6(1): 26924.

Xu, Y., Zhu, J.Y., Lei, Z.M., Wan, L.J., Zhu, X.W., Ye, F., and Tong, Y.Y. (2017). Anti-proliferative effects of paeonol on human prostate cancer cell lines DU145 and PC-3. **Journal of Physiology and Biochemistry**. 73(2): 157-165.

Yang, L., Chai, C.Z., Yan, Y., Duan, Y.D., Henz, A., Zhang, B.L., Backlund, A., and Yu, B.Y. (2017). Spasmolytic mechanism of aqueous licorice extract on oxytocin-induced uterine contraction through inhibiting the phosphorylation of heat shock protein 27. **Molecules** 22(9): 1392.

Yoshinouchi, Y., Shimizu, S., Lee, J.S., Hirano, M., Suzuki, K.T., Kim, E.Y., and Iwata, H. (2019). *In vitro* assessment of effects of persistent organic pollutants on the transactivation of estrogen receptor α and β (ERα and ERβ) from the Baikal seal (*Pusa sibirica*). **Ecotoxicology and Environmental Safety**. 181: 463-471.

Zahara, K., Tabassum, S., Sabir, S., Arshad, M., Qureshi, R., Amjad, M.S., and Chaudhari, S.K. (2014). A review of therapeutic potential of *Saussurea lappa*-

an endangered plant from Himalaya. **Asian Pacific Journal of Cancer Prevention**. 7: S60-S69.

Zhang, C.Z., Wang, S.X., Zhang, Y., Chen, J.P., and Liang, X.M. (2005). *In vitro* estrogenic activities of Chinese medicinal plants traditionally used for the management of menopausal symptoms. **Journal of Ethnopharmacology**. 98(3): 295-300.

Zhang, W.-J., Wang, S., Kang, C.-Z., Lv, C.-G., Zhou, L., Huang, L.-Q., and Guo, L.-P. (2020). Pharmacodynamic material basis of traditional Chinese medicine based on biomacromolecules: a review. **Plant methods**. 16: 26-26.

Zhang, W.L., Zheng, K.Y.Z., Zhu, K.Y., Zhan, J.Y.X., Bi, C.W.C., Chen, J.P., Dong, T.T.X., Choi, R.C.Y., Lau, D.T.W., and Tsim, K.W.K. (2013). Chemical and biological assessment of *Angelica* roots from different cultivated regions in a Chinese herbal decoction Danggui Buxue Tang. **Evidence-Based Complementary and Alternative Medicine**. 2013: 483286.

Zhang, Y.-J., Yang, Y.-a., Gong, H.-b., and Zhu, H. (2018). A systematic review of the comparison of three medicinal licorices, based on differences of the types and contents about their bioactive components. **Journal of Chemical Biology and Pharmaceutical Chemistry**. 1(1): 1-6.

Zhang, Z., Feng, Y., Li, Z.Y., and Cao, X.Z. (2019). Antiproliferative and apoptotic activity of glycyrrhizinic acid in MCF-7 human breast cancer cells and evaluation of its effect on cell cycle, cell migration and m-TOR/PI3K/Akt signalling pathway. **Archives of Medical Science**. 15(1): 174-182.

Zhao, Y., Zheng, H.-X., Xu, Y., and Lin, N. (2019). Estrogenic effect of the extract of Qingyan formula on reproductive tissues in immature mice. **Evidence-Based Complementary and Alternative Medicine**. 2019: 1-8.

Zheng, Y., Wang, H., Yang, M., Peng, G., Dong, T.T., Xu, M.L., and Tsim, K.W. (2018). Prenylated flavonoids from roots of *Glycyrrhiza uralensi*s Induce differentiation of B16-F10 melanoma cells. **International Journal of Molecular Sciences**. 19(8): 8.

Zheng, Z.H., Han, Y., You, S.Y., Chen, Z., and Zheng, X.D. (2019). Improvement in post-partum uterine involution in rats treated with *Apios americana*. **Journal of Zhejiang University-SCIENCE B**. 20(7): 576-587.

Zhu, Y., Yao, Y., Shi, Z., Everaert, N., and Ren, G. (2018). Synergistic effect of bioactive anticarcinogens from soybean on anti-proliferative activity in MDA-MB-231 and MCF-7 human breast cancer cells *in vitro*. **Molecules**. 23(7): 1557.

Zubair, H., Bhardwaj, A., Ahmad, A., Srivastava, S.K., Khan, M.A., Patel, G.K., Singh, S., and Singh, A.P. (2017). Hydroxytyrosol induces apoptosis and cell cycle arrest and suppresses multiple oncogenic signaling pathways in prostate cancer cells. **Nutrition and Cancer**. 69(6): 932-942.

Zyla, K., Ledoux, D.R., Garcia, A., and Veum, T. (2007). An *in vitro* procedure for studying enzymic dephosphorylation of phytate in maize-soyabean feeds for turkey poults. **BMC Complementary Medicine and Therapies**. 74(1): 3-17.

APPENDICES

APPENDIX A

CHEMICALS AND MEDIA

1. General chemicals and materials

2-Mercaptoethanal	Biobasic, Ontario, Canada
Agarose	vivantis, Selangor Darul Ehsan, Malaysia.
Disodium salt, dihydrate	Biobasic, Ontario, Canada
Leu/Trp DO supplement	Clonetech, Mountain view, Canada
ortho-Nitrophenyl-β-galactoside	Biobasic, Ontario, Canada
Pancreatin	Panreac Applichem, Barcelona, Spain
Pepsin	Sigma, Missouri, USA
TRIzol™ Reagent	Thermofisher, Carlsbad, Canada
Yeast nitrogen base w/o amino acids	Becton Dickinson, Newjersey, USA
β-Estradiol	Sigma, Sigma, Missouri, USA

2. Media and Antibiotics

2.1 Bacterial Media (per L)

2.1.1 LB (Luria-Bertani) medium

Bactotryptone	10	g
Bacto yeast extract	5	g
NaCl	10	g

Adjust to pH 7.0 with 5 N NaOH. Add H_2O to a final volume of 1 L and autoclave.

2.1.2 LB agar

Bactotryptone	10	g
NaCl	10	g
Yeast extract	5	g

2.2 Yeast media (per L)

2.2.1 YPD (Yeast extract peptone dextrose) medium

Peptone	20	g
Yeast extract	10	g
Adjust to pH 6.5 with 5 N NaOH. Add H_2O to a final volume of 950 mL and autoclave.		
Add 40% sterile glucose	50	mL

2.2.2 YPD agar

Peptone	20	g
Yeast extract	10	g
Agar	20	g
Adjust to pH 6.5 with 5 N NaOH. Add H_2O to a final volume of 950 mL and autoclave.		
Add 40% sterile glucose	50	mL

3. Antibiotics

Antibiotics	Stock solution (mg/mL)	Solvent
Ampicilin	25	Water
Kanamycin	50	Water

APPENDIX B

REAGENT PREPERATION

1. For Transformation of Yeast

1.1 Polyethylene glycol/Lithium acetate (10 ml)

Prepare fresh just prior to use.

50% PEG 4000	8	mL
10X TE	1	mL
10X LiAc	1	mL

1.2 10X TE buffer (per L)

1 M Tris-HCl, pH 7.5	100	mL
0.5 M EDTA, pH 7.5	20	mL

Adjust to pH 7.5 with 5 N NaOH. Add H_2O to a final volume of 1 L and autoclave

1.3 10X LiAc (per L)

lithium acetate	102	g

Adjust to pH 7.5 with dilute acetic acid and autoclave.

1.4 1 M Tris-HCl, pH 7.5 (per L)

Tris Base	121.14	g

Adjust to pH 7.5 with concentrated HCl. Add H_2O to a final volume of 1 L and autoclave

1.5 0.5 M EDTA, pH 7.5 (per L)

EDTA disodium salt	186.1	g

Adjust to pH 7.5 with NaOH. Add H_2O to a final volume of 1 L and autoclave

2. For β-galactosidase Assay

2.1 Z buffer (per L)

Prepare fresh just prior to use.

Sodium phosphate dibasic heptahydrate	16.10	g
Sodium phosphate monobasic monohydrate	5.50	g
Potassium chloride	0.75	g
Magnesium sulfate heptahydrate	0.246	g

Adjust to pH 7.0 with 5 N NaOH. Add H_2O to a final volume of 1 L and autoclave.

2.2 Z buffer with β-mercaptoethanol (100 mL)

Z buffer	100	mL
β-mercaptoethanol	0.27	mL

2.3 ONPG (o-nitrophenyl β-D-galactopyranoside)

Z buffer	1	mL
ONPG	4	mg

Adjust to pH 7.5 with dilute acetic acid and autoclave.

3. For Gel Electrophoresis

3.1 Tris-acetate (50× TAE)

Tris base	242	g
Glacial acetic acid	57.1	mL
0.5 M EDTA, pH 8.0	100	mL

Adjust the volume to 1 L of dH_2O

3.2 0.8% Agarose gel (100 mL)

1X TAE	100	mL
Agarose	0.8	g

Heat until the solution is clear and agarose appears to be fully dissolved.

3.3 Gel-loading buffers (6×)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30%

Adjust to pH 7.5 with dilute acetic acid and autoclave.

4 Alkaline Lysis Buffers for A Minipreparation of Plasmid DNA

4.1 Solution I

Glucose	50	mM
Tris-Cl (pH 8.0)	25	mM
EDTA (pH 8.0)	10	mM

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 min at 121 ºC, and stored at 4 ºC.

4.2 Solution II

Sodium hydroxide (NaOH)	0.2	N
Sodium dodecyl sulfate (SDS)	1%	

4.3 Solution III

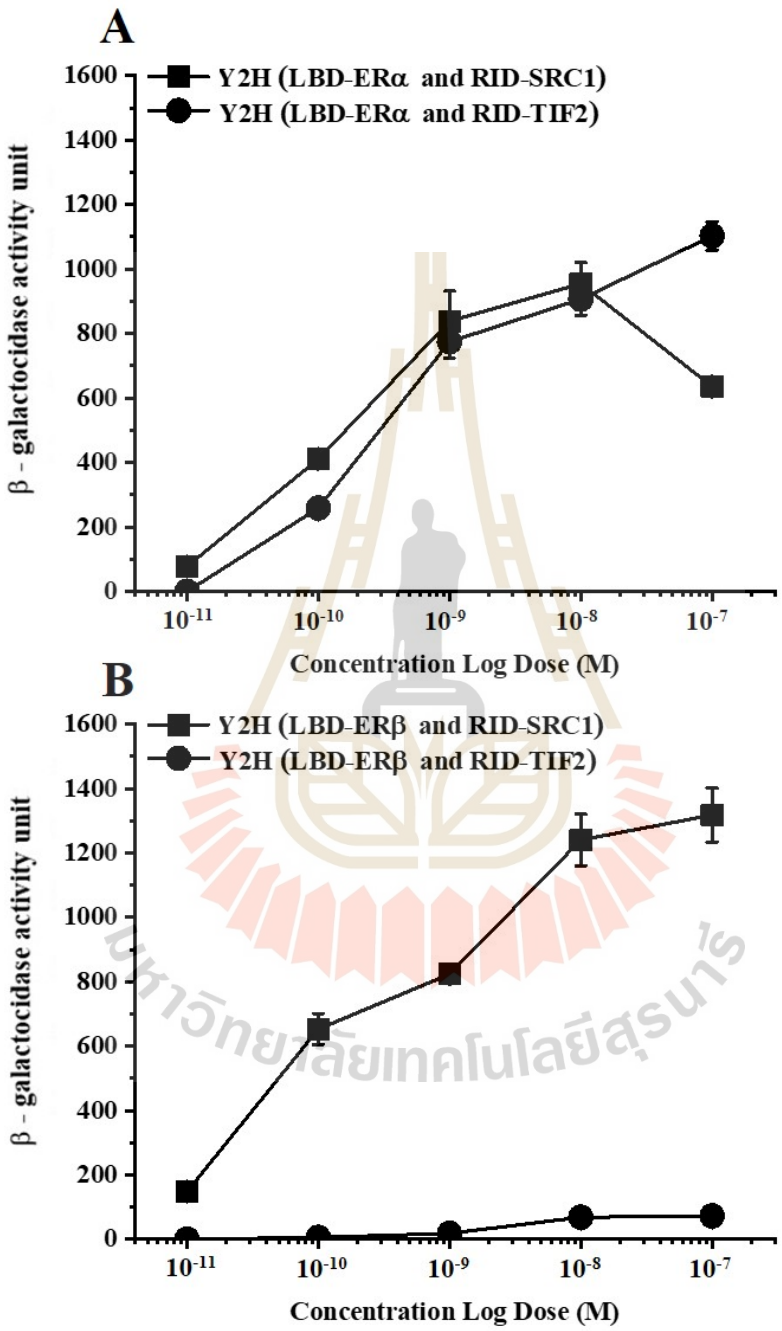
5M potassium acetate	60	mL
Glacial acetic acid	11.5	mL
H_2O	28.5	mL

APPENDIX C

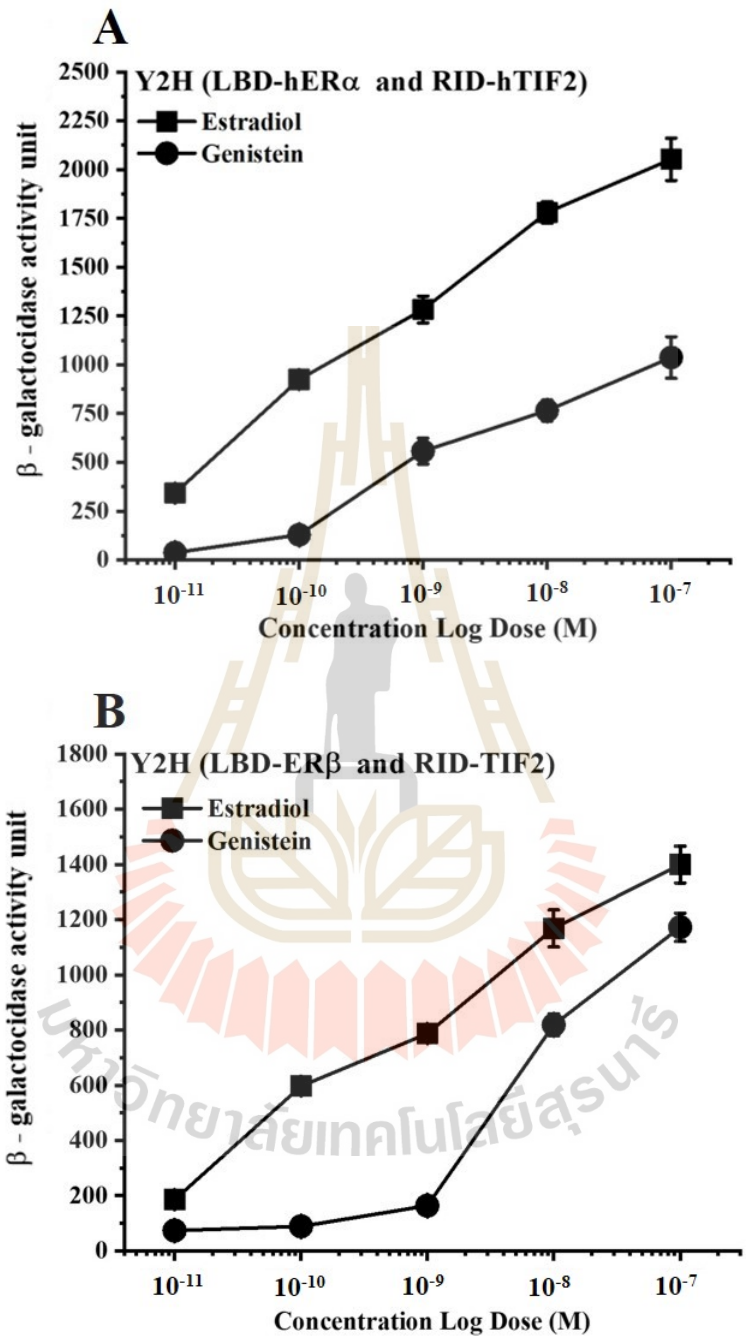
SUPPLEMENTARY DATA

Data of The Independently Repeated Experiments

1. **Y2H systems to evaluate the standard E2**

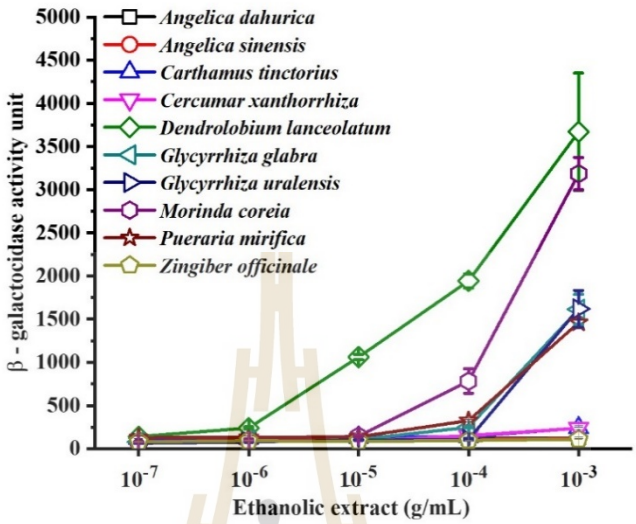


2. Y2H systems to evaluate genistein

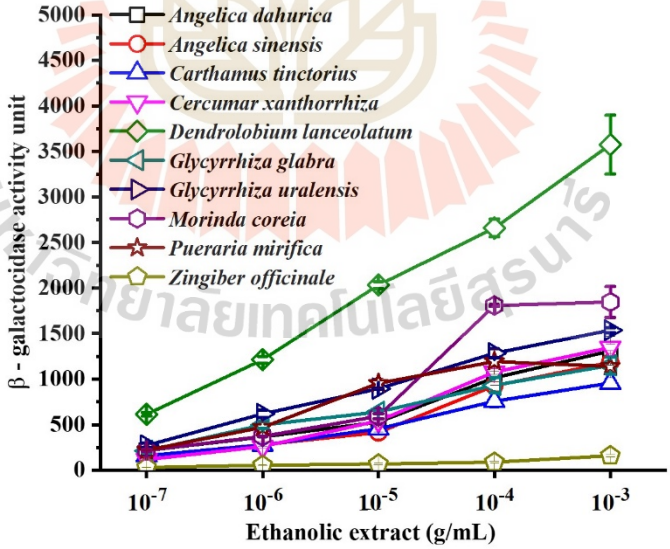


3. Y2H systems to evaluate 10 plant extracts (undigested and digested conditions)

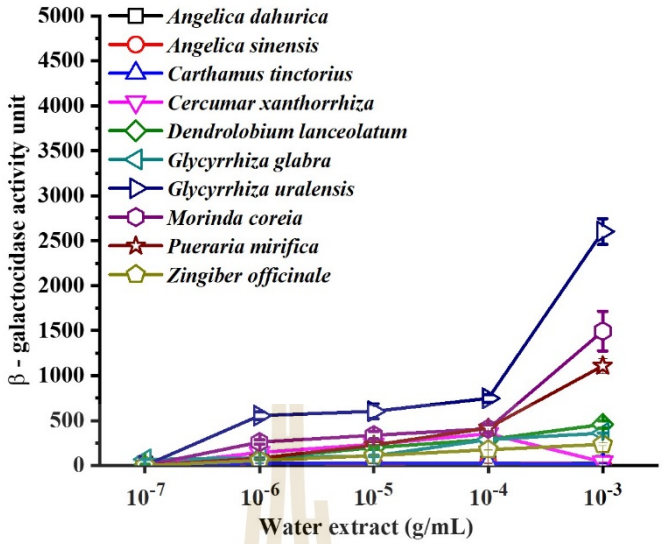
3.1 The estrogenic activities of 10 crude ethanolic extracts as evaluated by the ERα-Y2H system.



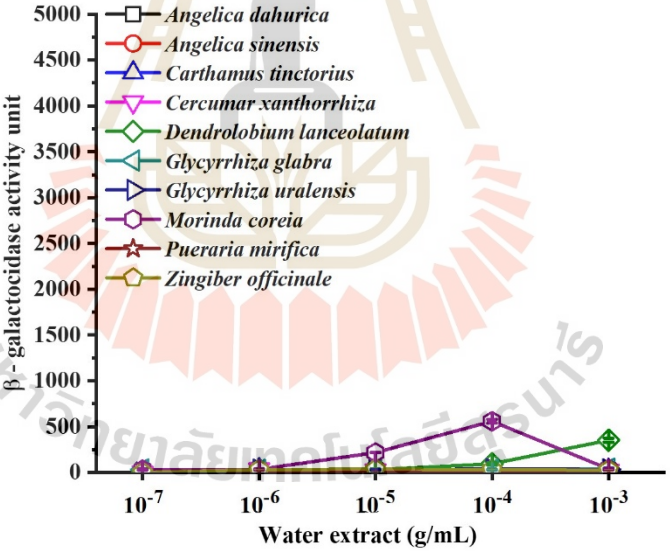
3.2 The estrogenic activities of 10 crude ethanolic extracts as evaluated by the ERβ-Y2H system



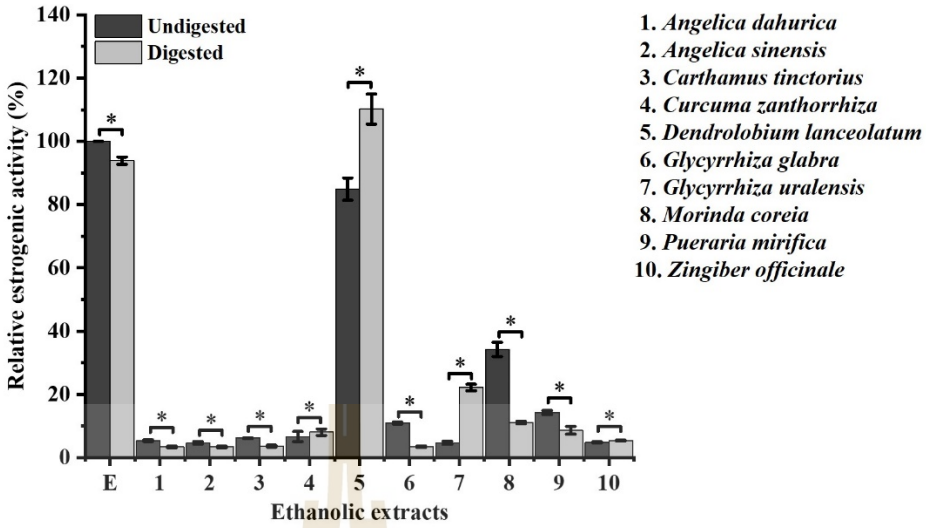
3.3 The estrogenic activities of 10 crude water extracts as evaluated by the ERα-Y2H system



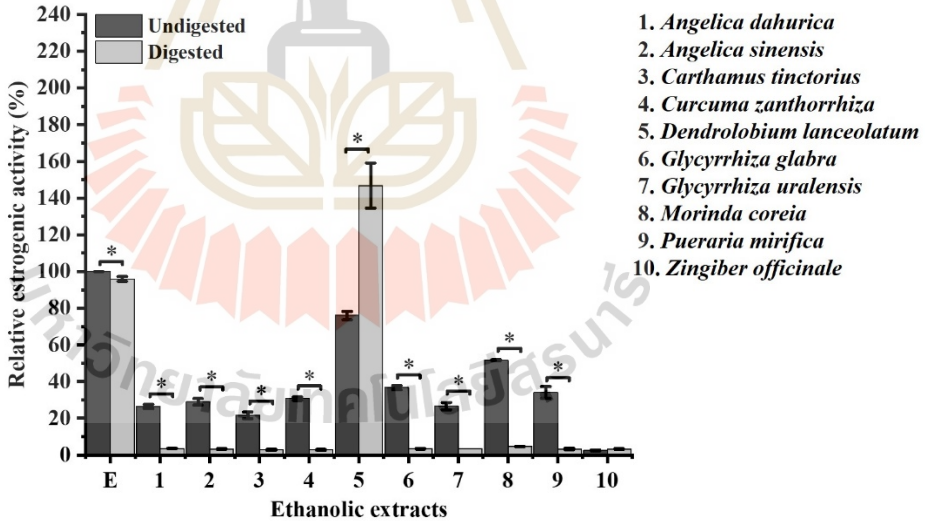
3.4 The estrogenic activities of 10 crude water extracts as evaluated by the ERβ-Y2H system



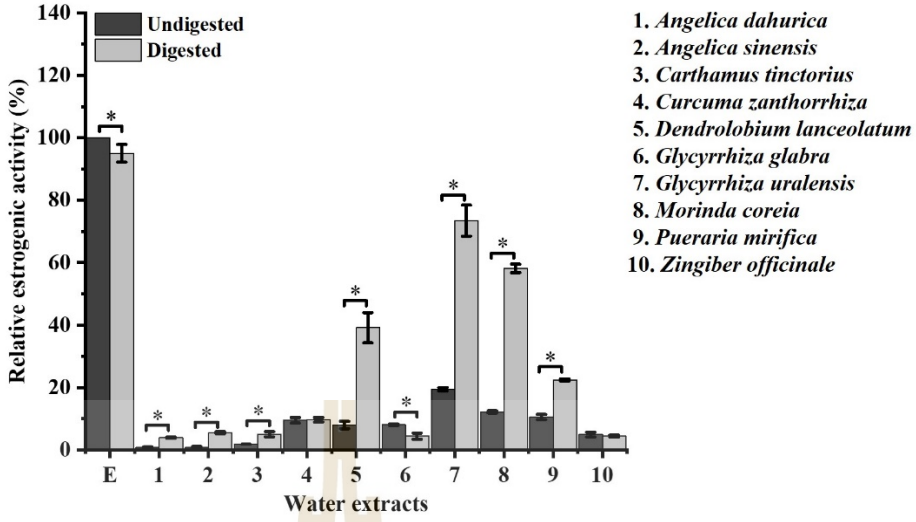
3.5 The relative estrogenic activities of undigested and digested plant extracts (ethanolic extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) by ERα-Y2H system.



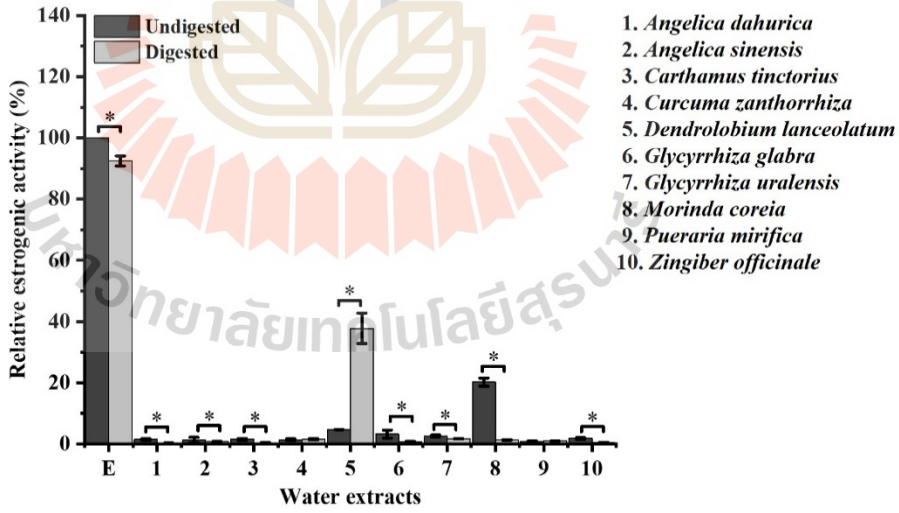
3.6 The relative estrogenic activities of undigested and digested plant extracts (ethanolic extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) by ERβ-Y2H system.



3.7 The relative estrogenic activities of undigested and digested plant extracts (water extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) by ERβ-Y2H system.

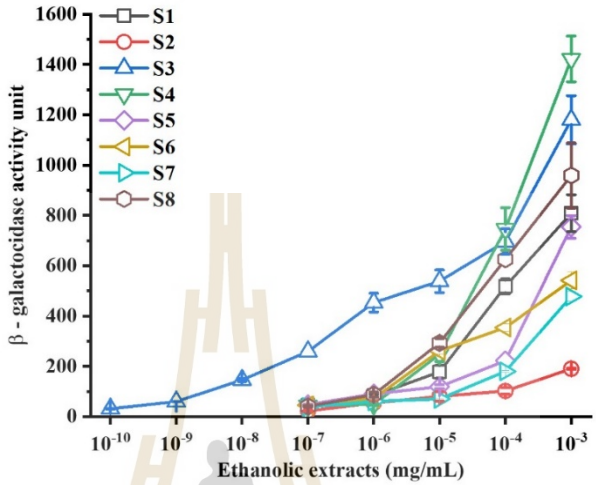


3.8 The relative estrogenic activities of undigested and digested plant extracts (water extracts, 1×10^{-4} g/mL) and E2 (1×10^{-7} g/mL) by ERβ-Y2H system.

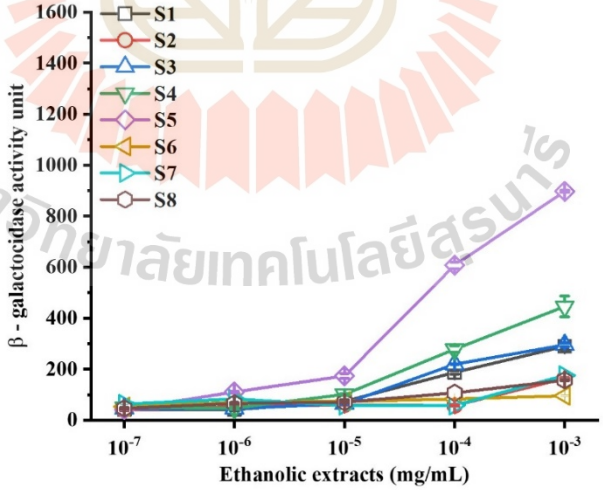


4. Y2H systems to evaluate medicinal plant extracts (undigested and digested conditions)

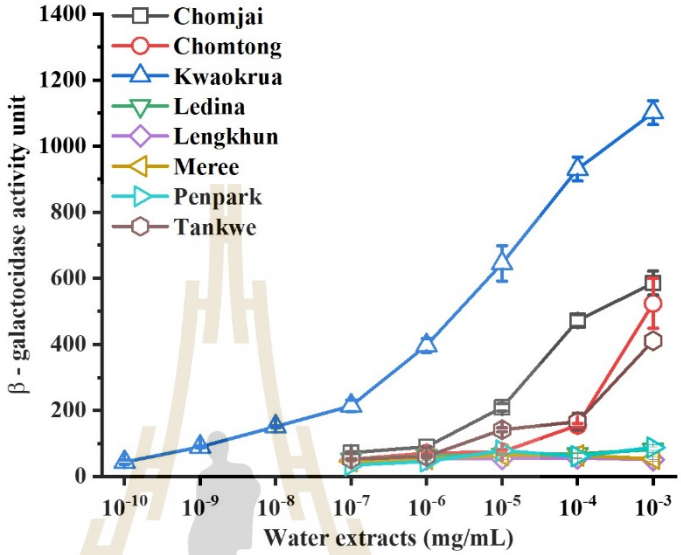
4.1 The estrogenic activities of ethanolic-extracts derived from 8 commercially traditional medicine extracts as evaluated by the ERα-Y2H system.



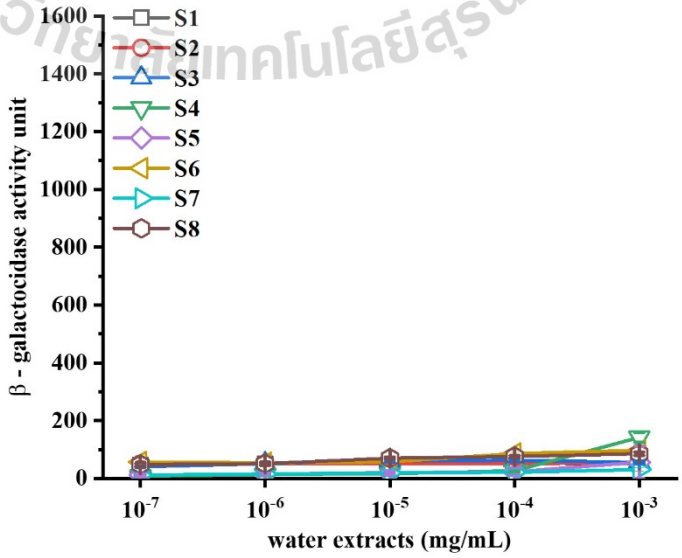
4.2 The estrogenic activities of ethanolic-extracts derived from 8 commercially traditional medicine extracts as evaluated by the ERβ-Y2H system.



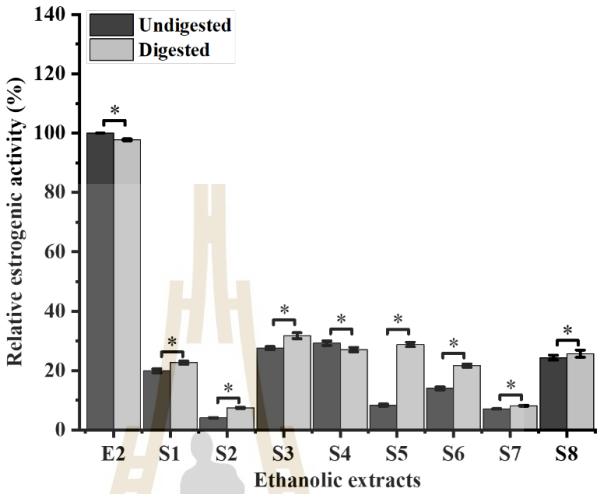
4.3 The estrogenic activities of water-extracts derived from 8 commercially traditional medicine extracts as evaluated by the ERβ-Y2H system.



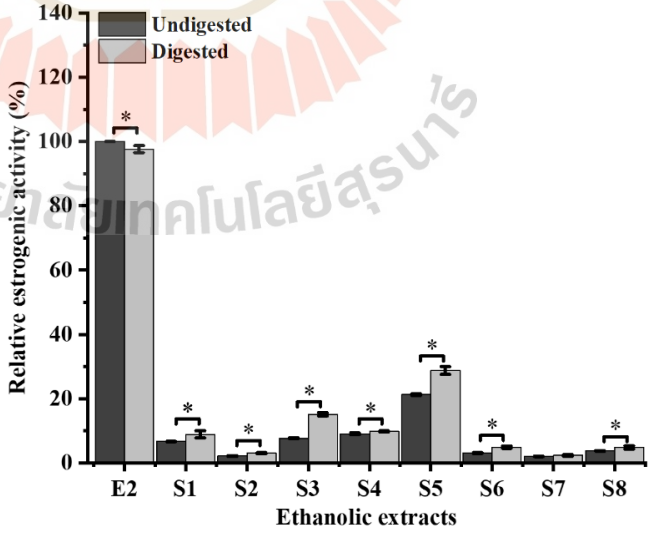
4.4 The estrogenic activities of water-extracts derived from 8 commercially traditional medicine extracts as evaluated by the ERβ-Y2H system.



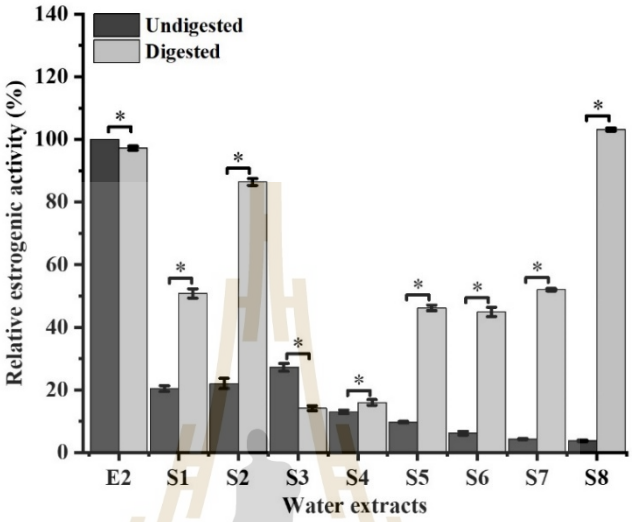
4.5 Relative estrogenic activities of undigested and digested ethanolic extracts of S1-S8 (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) determined by ERα-Y2H system.



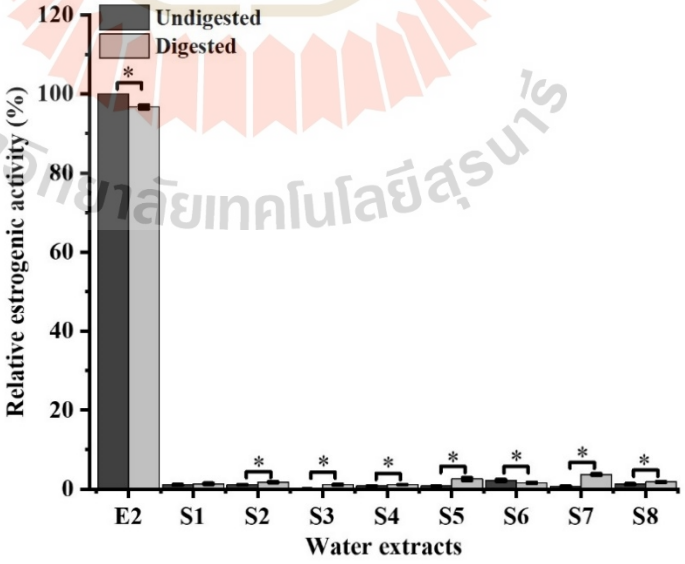
4.6 Relative estrogenic activities of undigested and digested ethanolic extracts of S1-S8 (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) determined by ERβ-Y2H system.



4.7 Relative estrogenic activities of undigested and digested water extracts of S1-S8 (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) determined by ERα-Y2H system.



4.8 Relative estrogenic activities of undigested and digested water extracts of S1-S8 (1×10^{-4} g/mL) and the standard E2 (1×10^{-7} g/mL) determined by ERβ-Y2H system.



CURRICULUM VITAE

Miss Palita Paewthaisong was born in Burirum Province, Thailand. She received the Bachelor's degree in Biology from Mahasarakham University in 2002 and the Master's degree in Genetic from Chulalongkorn University in 2007. She has pursued the Ph.D. degree in Environmental Biology at Suranaree University of Technology in 2015. Her research expertise is in the field of Molecular Biology and Medicinal plants. During her study, she attended the national and international conferences for oral and poster presentations.

Conferences:

1. 2016. Paewthaisong P. and Siri S. Screening of estrogenic activity of 10 Thai medicinal plants by yeast two hybrid system. International Conference on Advances in Science, Engineering, Technology and Natural Resources (ICASETNR-16). May 11-12, 2016. Bayveiw Hotel, Singapore. (Oral Presentation).
2. 2019. Paewthaisong P. and Siri S. Estrogenic activity of eight commercial traditional women remedies analyzed by yeast two hybrid system. The 45th Congress on Science and Technology of Thailand (STT45). October 7-9, 2019. Mae Fah Luang University, Chiang Rai, Thailand (Oral Presentation)