รายงานวิจัยฉบับสมบูรณ์

ผลของสารไฟโตเอสโตรเจนต่อการเปลี่ยนแปลงระดับความกังวลในหนูขาวที่ถูกตัดรังไข่ กับการเปลี่ยนแปลงของระบบประสาทซีโรโตนิน

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จุฬาลงกรณ์มหาวิทยาลัย

ธันวาคม 2551

สัญญาเลขที่ MRG4980063

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง “ผลของสารไฟโตเอสโตรเจนต่อการเปลี่ยนแปลงระดับความกังวลในหนูขาวที่ถูกตัดรังไข่กับการเปลี่ยนแปลงของระบบประสาทซีโรโตนิน” ได้รับทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ ภายใต้การสนับสนุนจากสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย ประจำปีงบประมาณ 2549 (MRG4980063)

บทคัดย่อ

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ชื่อโครงการ: ผลของสารไฟโตเอสโตรเจนต่อการเปลี่ยนแปลงระดับความกังวลในหนูขาวที่ถูกตัดรังไข่ กับการเปลี่ยนแปลงของระบบประสาทซีโรโตนิน

ชื่อนักวิจัย: ผศ. สพ.ญ. ดร. สฤณี กลันทกานนท์ ทองทรง
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ภาวะวิตกกังวลเป็นปัญหาด้านสุขภาพจิตที่พบในเพศหญิงมากกว่าเพศชาย และจากรายงานทางคลินิกและการทดลองในสัตว์ พบว่าการขาดฮอร์โมนเอสโตรเจนเป็นปัจจัยที่สำคัญ อย่างไรก็ตามการให้ฮอร์โมนเอสโตรเจนนั้นมีข้อจำกัดในทางคลินิก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารไฟโตเอสโตรเจน ได้แก่ เจนิสเตอีน และไดด์ดีซีน ในการลดความกังวลเปรียบเทียบกับเอสโตรเจน และศึกษาถึงกลไกของสารดังกล่าวในการเปลี่ยนแปลงระดับสารสื่อประสาทในสมองที่สัมพันธ์กับพฤติกรรมความกังวล โดยการทดลองกระทำโดยการเหนี่ยวนำให้หนูเพศเมียขาดฮอร์โมนเอสโตรเจนโดยการตัดรังไข่ และให้สารต่างๆ ทดแทน ได้แก่ เอสโตรเจน เจนิสเตอีน หรือไดด์ดีซีนที่ขนาดต่างๆ เป็นเวลา 4 สัปดาห์ จากนั้นทำการทดสอบพฤติกรรมด้วยอุปกรณ์วัดความกังวล elevated T-maze และ open field เมื่อสิ้นสุดการทดลองทำการเก็บเลือด สมอง และมดลูก เพื่อวัดระดับเอสโตรเจน ระดับสารสื่อประสาทในสมอง และเพื่อดูผลต่อระบบสืบพันธุ์ตามลำดับ ผลการศึกษาพบว่า เจนิสเตอีนที่ระดับต่ำ (0.25 มก./กก.) และไดด์ดีซีน (0.25, 0.5 และ 1.00 มก./กก.) ให้ผลในการลดความกังวลไม่แตกต่างจากเอสโตรเจน (1-100 ไมโครกรัม/กก.) นอกจากนั้นเจนิสเตอีนและไดด์ดีซีนไม่มีผลต่อการเปลี่ยนแปลงระดับฮอร์โมนเอสโตรเจน น้ำหนักตัว หรือปริมาณอาหารที่กินเมื่อเทียบกับกลุ่มควบคุม สำหรับการเปลี่ยนแปลงน้ำหนักมดลูกพบว่าเจนิสเตอีน หรือไดด์ดีซีนในระดับที่ลดความกังวลไม่มีผลต่อการเปลี่ยนแปลงน้ำหนักมดลูกเมื่อเทียบกับกลุ่มควบคุม แต่การใช้เจนิสเตอีนหรือไดด์ดีซีนในขนาดที่เพิ่มขึ้นมีแนวโน้มที่จะทำให้มดลูกมีน้ำหนักเพิ่มขึ้น สำหรับเอสโตรเจนทุกขนาดที่ใช้ในการศึกษามีผลให้น้ำหนักมดลูกเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ และจากการวัดระดับสารสื่อประสาทในสมองด้วยวิธี HPLC พบว่ามีการเปลี่ยนแปลงของสารสื่อประสาทซีโรโตนิน หรือเมตาโบไลท์ ในส่วนต่างๆ ของสมองที่สัมพันธ์กับความกังวล โดยไม่มีผลต่อการเปลี่ยนแปลงของโปรตีนตัวขนส่งซีโรโตนินที่สมองส่วนกลาง ทั้งนี้การเปลี่ยนแปลงของสารสื่อประสาทดังกล่าวนั้นมีความแตกต่างกันระหว่างเอสโตรเจน เจนิสเตอีน หรือไดด์ดีซีน เป็นที่น่าสังเกตว่าสารดังกล่าวมีกลไกในการลดความกังวลที่แตกต่างกัน เนื่องจากมีการศึกษาว่าเจนิสเตอีนสามารถเพิ่มตัวรับเอสโตรเจน ชนิดเบต้าในไฮโปธาลามัสได้ จึงเป็นที่น่าสนใจว่าเจนิสเตอีน หรือไดด์ดีซีนทำงานโดยการเปลี่ยนการแสดงออกของตัวรับเอสโตรเจน และส่งผลให้เกิดการเปลี่ยนแปลงดังกล่าวหรือไม่ เนื่องจากตัวรับชนิดนี้มีผลในการลดความกังวล

คำหลัก: ความวิตกกังวล, ไดด์ดีซีน, เอสโตรเจน, เจนิสเตอีน, ซีโรโตนิน

Abstract

Project Code: MRG4980063

Project Title: The Effects Of Phytoestrogens On Anxiety Levels In Ovariectomized Rats and The Modulation Of Serotonergic System

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Project Period: July 1, 2006 – June 30, 2008

Anxiety is a mental disorder affecting women more than men. From both clinical reports and animal studies suggest that lacking of estrogen is one important factor; however, there are some limitations in given estrogens in clinic. Therefore, the aims of this study were to compare the anxiolytic effect of phytoestrogen i.e. genistein and daidzein in comparison to estrogen and to study the mechanism of these substances in modulation of neurotransmitters in differed breain areas related to anxiety. In the current study, female rats were ovariectomized and randomly designated into groups receiving vehicle, estrogen, genistein or daidzein at various doses for 4 weeks. Then the rats were tested with elevated T-maze and open field to measure the level of anxiety. Moreover, the blood, uterus and brain were collected for later analysis of serum estradiol, uterine weight and neurotransmiiter levels, respectively. The results demonstrated that genistein (0.25 mg/kg) and daidzein (0.25, 0.5 and 1 mg/kg) contained anxiolytic-like effects in comparable to estrogen (1-100 μg/kg). Further, genistein and daidzein had no effect on serum estradiol, body weight change or food intake compared to control group. For the uterine weight, genistein and daidzein at the effective doses in reducing anxiety had no uterotrophic effect; however, it should be noted that the higher the dose of genistein or daidzein can increase uterine weight in a dose dependent manner. On the contrary, estrogen at all doses increased uterine weight in a dose dependent manner. The HPLC analysis revealed that estrogen, genistein and daidzein modulated serotonin or its metabolite in various brain areas with no effect on serotonin transporter protein level in the midbrain. Interestingly, the modulatory effects of these substances on the neurotransmitters were differed suggesting that the mechanism in lowering anxiety in these rats were indeed differed. It had been reported that genistein could increase ERβ mRNA in the hypothalamus. It is then interesting to study further whether genistein or daidzein can alter ER and lead to change in behavioral or neurochemical responses as seen in this study.

Keywords: Anxiety, Daidzein, Estrogen, Genistein, Serotonin

Executive Summary

1. ความสำคัญ และที่มาของปัญหา

ภาวะสังคมและเศรษฐกิจในปัจจุบัน ส่งผลให้ประชากรมีภาวะความเครียดเกิดขึ้น ซึ่งภาวะเครียดที่คุกคามและไม่สามารถปรับตัวได้ มักก่อให้เกิดภาวะความวิตกกังวลขึ้น (anxiety disorders) จากรายงานของกรมสุขภาพจิต กระทรวงสาธารณสุข ในปี พ.ศ. 2546 (ค.ศ. 2003) พบว่าจากประชากรจำนวน 100,000 คน มีผู้ป่วยเป็นโรควิตกกังวล (anxiety disorders) 862.53 คน และเป็นที่น่าสังเกตว่าอัตราการป่วยด้วยโรควิตกกังวลนี้พบในเพศหญิงมากกว่าเพศชายสูงถึงสองเท่า โดยค่าสถิติดังกล่าวมีค่าใกล้เคียงกับรายงานในต่างประเทศเช่นกัน โดยเฉพาะอย่างยิ่ง ในเพศหญิงที่มีอายุมากกว่า 55 ปี หรืออยู่ในวัยหมดประจำเดือน (Seeman, 1997; Anxiety Disorders Association of America, 2003; Lim et al., 2005) นอกจากนั้นยังพบว่าเพศหญิงมักมีภาวะผันแปรทางอารมณ์ในระหว่างรอบประจำเดือนอีกด้วย (Seeman, 1997) ซึ่งการทดแทนฮอร์โมนในสตรีหลังหมดประจำเดือน ได้แก่ การทดแทนด้วยฮอร์โมนเอสโตรเจน (Estrogen Replacement Therapy; ERT) หรือฮอร์โมนเอสโตรเจนและโปรเจสเตอโรน (Hormone Replacement Therapy; HRT) สามารถช่วยลดอาการดังกล่าวลงได้ จึงเป็นที่น่าสังเกตว่าการลดลงหรือการเปลี่ยนแปลงของระดับฮอร์โมนเอสโตรเจนเป็นปัจจัยหนึ่งที่มีผลต่อความกังวลในเพศหญิง

ในปัจจุบัน ยาที่ใช้ในการรักษาโรควิตกกังวล ได้แก่ ยาในกลุ่ม benzodiazepine (BZ) และ ยากลุ่ม Selective Serotonergic Reuptake Inhibitors (SSRIs) โดยยาในกลุ่ม BZ ออกฤทธิ์โดยจับกับ BZ binding site ที่ตัวรับ gamma-aminobutyric acid ชนิด A ($GABA_A$) เป็นผลให้สารสื่อประสาท GABA ซึ่งเป็นสารสื่อประสาทประเภท inhibitory ออกฤทธิ์ได้ดียิ่งขึ้น สำหรับยากลุ่ม SSRI ออกฤทธิ์โดยการยับยั้งการดูดกลับของสารสื่อประสาทซีโรโตนิน เป็นผลให้ระดับของซีโรโตนินเพิ่มสูงขึ้นที่ synaptic cleft โดยเฉพาะที่สมองส่วน raphe nuclei และซีโรโตนินที่เพิ่มขึ้นจะไปจับกับตัวรับ $5\text{-}HT_{1A}$ ที่เป็นตัวรับประเภท autoreceptor และมีผลยับยั้งการทำงานของเซลล์ประสาทซีโรโตนิน ทำให้มีการหลั่งสารสื่อประสาทซีโรโตนินลดลงที่สมองส่วนหน้า คือ frontal cortex และ septohippocampal area และการทำงานที่ลดลงของประสาทซีโรโตนินที่สมองส่วนหน้านี้ทำให้ภาวะความวิตกกังวลลดลง ได้ผลเช่นเดียวกับยากลุ่ม BZ อย่างไรก็ตามการใช้ยากลุ่ม BZ เป็นระยะเวลานานจะส่งผลให้เกิดมีการแสดงออกของ BZ subunit ของตัวรับชนิด $GABA_A$ ลดลง และทำให้การใช้ยาดังกล่าวมีประสิทธิภาพลดลง จากสาเหตุดังกล่าวเป็นผลให้ยากลุ่ม SSRI เป็นยาหลักที่ได้รับอนุมัติให้ใช้รักษาโรควิตกกังวล จากองค์การอาหารและยาของประเทศสหรัฐอเมริกา เนื่องจากยากลุ่มนี้มีความปลอดภัย และสามารถใช้ติดต่อได้ในระยะเวลานาน (DeVane et al., 2005) นอกจากรายงานการใช้ยาทางคลินิกที่มีผลต่อระบบประสาทซีโรโตนินในการรักษาโรควิตกกังวลแล้ว ยังพบว่าในสัตว์ทดลองที่มีการปรับเปลี่ยนการทำงานของระบบประสาทซีโรโตนิน เช่นการใช้สารที่ออกฤทธิ์ต่อตัวรับซีโรโตนิน การปรับเปลี่ยนระดับสารซีโรโตนินในสมอง รวมถึงการศึกษาในหนูที่มีการทำงานของตัวรับซีโรโตนินที่ไม่สมบูรณ์ (knockout mice) ผลการทดลองเหล่านี้

ล้วนแต่ชี้ให้เห็นถึงความสำคัญของการทำงานที่ไม่สมดุลของระบบซีโรโตนินที่สัมพันธ์กับความกังวลทั้งในสัตว์ทดลอง และในคน (Graeff, 2002; Taylor and Stein, 2006)

นอกจากนั้น จากการรายงานการใช้ยาทางคลินิกพบว่า ระบบประสาทซีโรโตนินนี้สามารถปรับเปลี่ยนได้ด้วยฮอร์โมนเอสโตรเจน โดยพบว่าผู้ป่วยเพศหญิงที่อยู่ในภาวะหลังหมดประจำเดือน มีการตอบสนองต่อ SSRI ดีขึ้นเมื่อได้รับยาร่วมกับฮอร์โมนเอสโตรเจน (ERT) (Jacobs and Hyland, 2003; Nagata et al., 2005) การที่เอสโตรเจนมีผลต่อระบบประสาทซีโรโตนินนี้น่าจะเป็นผลมาจากการที่บริเวณสมองส่วนกลาง (midbrain) ซึ่งเป็นสมองส่วนที่สังเคราะห์และหลั่งสารสื่อประสาทซีโรโตนิน มีการแสดงออกของตัวรับเอสโตรเจน ชนิดเบต้า (estrogen receptor beta; ER_β) (Laflamme et al., 1998) และการทดแทนฮอร์โมนเอสโตรเจนในสัตว์ทดลองที่ถูกตัดรังไข่ สามารถเปลี่ยนแปลงระดับของ mRNA ของ SERT ตัวรับซีโรโตนิน และเอ็นไซม์ tryptophan hydroxylase ซึ่งมีหน้าที่ในการสังเคราะห์ซีโรโตนินได้ (reviewed by Bethea, 2002; 2003) ในขณะที่รายงานทางคลินิกและการทดลองแสดงให้เห็นความสัมพันธ์ระหว่างเอสโตรเจนและซีโรโตนิน การเปลี่ยนแปลงของระบบสารสื่อประสาท GABA ที่เกิดจากฮอร์โมนเอสโตรเจนที่สัมพันธ์กับความกังวลนั้นยังไม่พบว่ามีรายงาน แม้ว่าที่ $GABA_A$ จะสามารถปรับเปลี่ยนการทำงานได้ด้วยสารกลุ่ม neuroactive steroid แต่พบว่าสารที่สามารถจับกับ subunit ที่เป็น neuroactive binding site เป็นสารกลุ่ม allopregnanolone (ALLO) ซึ่งเป็นเมตาบอไลท์ของโปรเจสเตอโรน ซึ่งมีโครงสร้างที่แตกต่างไปจากเอสโตรเจน และสาร ALLO นี้เชื่อว่าสัมพันธ์กับการเกิดภาวะ premenstrual dysphoric disorder (PMDD) ในคน (Wihlback et al., 2005)

แม้ว่าความสำคัญของฮอร์โมนเอสโตรเจนต่อภาวะทางอารมณ์ในเพศหญิงจะเป็นที่ยอมรับ ทั้งจากกรณีศึกษาในผู้ป่วย (Seeman, 1997) และในสัตว์ทดลองก็ตาม (Marcondes et al., 2001; Koss et al., 2004) แต่อย่างไรก็ดีการใช้ฮอร์โมนเอสโตรเจนในการทดแทนนี้ยังมีข้อจำกัด โดยเฉพาะในผู้ป่วยที่มีประวัติการป่วยเป็นเนื้องอกของระบบสืบพันธุ์ เนื่องจากการได้รับฮอร์โมนเพศอาจทำให้เนื้อร้ายมีโอกาสกลับมาได้ ดังนั้นการใช้สารอื่นที่มีคุณสมบัติคล้ายกับฮอร์โมนเอสโตรเจน แต่ไม่มีคุณสมบัติในการกระตุ้นการทำงานของอวัยวะสืบพันธุ์จึงเป็นทางเลือกหนึ่งในผู้ป่วยกลุ่มนี้ สารกลุ่มที่ได้รับความสนใจกลุ่มหนึ่งคือ สารกลุ่มไฟโตเอสโตรเจน (phytoestrogen) จากการที่สารกลุ่มนี้มีลักษณะโครงสร้างที่คล้ายกับฮอร์โมนเอสโตรเจน สารไฟโตเอสโตรเจนสามารถแบ่งออกได้หลายกลุ่ม โดยกลุ่มที่ได้รับความสนใจได้แก่ กลุ่ม isoflavone เนื่องจากจากการศึกษาพบว่าสตรีที่อยู่ในแถบเอเชียมีอัตราการเกิดมะเร็งของระบบสืบพันธุ์น้อยกว่า และสตรีในกลุ่มนี้มีการบริโภคอาหารกลุ่มที่ได้จากถั่วเหลืองสูง (Peeters et al., 2003) ซึ่งสาร isoflavone ที่พบมากในถั่วเหลือง ได้แก่ เจนิสติน (genistein) และ ไดด์ซิน (daidzein) (Terreaux et al., 2003) โดยการทำงานของสารในกลุ่ม isoflavone เช่น เจนิสติน และ ไดด์ซิน นั้น มีคุณสมบัติแตกต่างจากฮอร์โมนเอสโตรเจน ได้แก่ ความสามารถในการจับกับตัวรับเอสโตรเจน (estrogen receptor; ER) โดย เจนิสติน และ ไดด์ซิน สามารถจับกับ ER_β ได้ดีกว่าชนิด alpha (ER_α) (Terreaux et al., 2003) ซึ่ง ER_α เป็นตัวรับที่พบในอวัยวะสืบพันธุ์ ในขณะที่ ER_β พบที่อวัยวะอื่นๆโดยเฉพาะที่สมองส่วนกลางที่มีความสัมพันธ์กับอารมณ์ความรู้สึก จึงเป็นไปได้ว่าการได้รับสารกลุ่มไฟโตเอสโตรเจนจะสามารถทดแทนฮอร์โมนเอสโตรเจนได้ โดยมีผลข้างเคียงต่ออวัยวะสืบพันธุ์ต่ำ

ดังนั้นจึงเป็นที่น่าสนใจว่าสารในกลุ่มไฟโตเอสโตรเจนที่ได้จากถั่วเหลือง เช่น เจนิสติน และไดด์ซิน ซึ่งมีคุณสมบัติโครงสร้างที่คล้ายคลึงกับฮอร์โมนเอสโตรเจน และสามารถจับกับตัวรับเอสโตรเจนชนิด ERβ สามารถให้ผลในการลดระดับความกังวลได้เทียบเคียงกับฮอร์โมนเอสโตรเจน เมื่อทำการทดสอบในหนูเพศเมียที่ถูกทำให้ขาดฮอร์โมนเพศโดยการตัดรังไข่ โดยใช้การวัดพฤติกรรมความกังวลด้วยอุปกรณ์ทดสอบ elevated T-maze (ETM) ซึ่งเป็นอุปกรณ์ทดสอบมาตรฐานที่เป็นที่ยอมรับว่าสามารถวัดระดับความกังวลในหนูได้ (Zangrossi and Graeff, 1997) และเพื่อศึกษาว่าผลในการเปลี่ยนแปลงระดับความกังวลดังกล่าว สัมพันธ์กับการเปลี่ยนแปลงการทำงานของระบบประสาทซีโรโตนิน ได้แก่ การเปลี่ยนแปลงระดับสารสื่อประสาทในสมอง หรือการเปลี่ยนแปลงของระดับโปรตีนที่ทำหน้าที่ในการขนส่งกลับของสารสื่อประสาทซีโรโตนิน (SERT) หรือไม่

2. วัตถุประสงค์

เพื่อศึกษาผลของไฟโตเอสโตรเจน (daidzein และ genistein) ในการเปลี่ยนแปลงระดับความกังวล เปรียบเทียบกับการใช้ฮอร์โมนเอสโตรเจนในหนูที่ถูกตัดรังไข่ และเพื่อศึกษาว่าผลในการเปลี่ยนแปลงความกังวลของสารดังกล่าว เป็นผลมาจากการปรับเปลี่ยนการทำงานของระบบสารสื่อประสาทซีโรโตนินหรือไม่

3. ระเบียบวิธีวิจัย

การวิจัยครั้งนี้แบ่งออกเป็น 2 ขั้นตอนดังนี้

ตอนที่ 1 การศึกษาถึงผลของไฟโตเอสโตรเจนในการเปลี่ยนแปลงระดับความกังวล เพื่อหาขนาดของสาร (dose) ที่เหมาะสมสำหรับการศึกษาต่อไป โดยทำการศึกษาในหนูขาวเพศเมียที่ทำการตัดรังไข่ และให้สารต่างๆทดแทน ได้แก่ ฮอร์โมนเอสโตรเจน (10 µg/kg), เจนิสติน (5-25 mg/kg) และไดด์ซิน (5-25 mg/kg) ในการศึกษาหนูจะได้รับฮอร์โมน หรือ ไฟโตเอสโตรเจนติดต่อกันเป็นเวลา 30 วัน ก่อนทำการทดสอบพฤติกรรมความกังวล ด้วย elevated T-maze ซึ่งเป็นอุปกรณ์ทดสอบมาตรฐานที่สามารถใช้วัดระดับความกังวลในหนู ผลจากการศึกษาในขั้นตอนนี้สามารถใช้ในการตอบคำถามงานวิจัยว่าสารไฟโตเอสโตรเจน เช่น เจนิสติน และ ไดด์ซิน มีผลต่อระดับความกังวลอย่างไร เมื่อจะนำมาใช้แทนฮอร์โมนเอสโตรเจน และเพื่อนำขนาดที่เหมาะสมไปใช้ในการศึกษาขั้นที่ 2 ต่อไป

ตอนที่ 2 การศึกษาถึงกลไกการทำงานของไฟโตเอสโตรเจนที่มีต่อระบบประสาทซีโรโตนิน ซึ่งมีความสัมพันธ์กับการเปลี่ยนแปลงระดับความกังวล โดยจากการศึกษาในตอนที่ 1 ขนาดที่เหมาะสมของสารไฟโตเอสโตรเจนแต่ละชนิด จะนำมาศึกษาผลที่มีต่อการทำงานของระบบประสาทซีโรโตนิน โดยการวัดระดับสารสื่อประสาทและการเปลี่ยนแปลงของตัวขนส่งสารสื่อประสาทซีโรโตนิน (serotonin reuptake transporter; SERT) ในสมองส่วนต่าง ๆ ได้แก่ สมองส่วน midbrain ซึ่งเป็นสมองส่วนที่ประกอบไปด้วยเซลล์ประสาทซีโรโตนินเป็นหลัก และสมองส่วนอื่น ๆ ได้แก่ frontal cortex amygdala hippocampus nucleus accumbens และ septum ซึ่งเป็นสมองส่วนต่าง ๆ เหล่านี้ ที่ทำหน้าที่ในการควบคุมการแสดงออกทางด้านอารมณ์ และพฤติกรรม

สำหรับขั้นตอนการทดลองในสัตว์ทดลองมีลักษณะเช่นเดียวกับขั้นตอนแรก แต่ไม่ทำการศึกษาด้านพฤติกรรมและการศึกษาในขั้นตอนนี้ แบ่งออกเป็น 2 วิธีดังนี้

วิธีที่ 1 ศึกษาการเปลี่ยนแปลงของสารสื่อประสาทซีโรโตนินในสมองส่วนต่าง ๆ ที่สัมพันธ์กับการควบคุมความกังวล ด้วยวิธี high performance liquid chromatography with electrochemical detection (HPLC-ED) โดยผลการศึกษาสามารถบอกได้ถึงการเปลี่ยนแปลงของระบบประสาทซีโรโตนิน ได้แก่ การสังเคราะห์สารสื่อประสาท การหลั่งสารสื่อประสาท ตลอดจนการทำงานของประสาทซีโรโตนินได้

วิธีที่ 2 ศึกษาการเปลี่ยนแปลงของตัวขนส่งสารสื่อประสาทซีโรโตนิน (serotonin reuptake transporter; SERT) ในสมองส่วนต่าง ๆ ที่สัมพันธ์กับการควบคุมความกังวล ด้วยวิธี semi-quantitative immunoblot analysis โดยผลการศึกษาสามารถบอกได้ถึงการเปลี่ยนแปลงของระบบประสาทซีโรโตนินในระดับที่เกี่ยวกับการขนส่งกลับของสารสื่อประสาท ซึ่ง SERT เป็นโมเลกุลที่มีความสำคัญที่มีผลต่อการทำงานของระบบประสาทซีโรโตนิน และเป็นโมเลกุลที่เป็นเป้าหมายในการรักษาทางคลินิก (SSRIs)

ผลการทดลองจากวิธีที่ 1 และ 2 เมื่อนำมาประกอบกัน สามารถบ่งบอกได้ว่าการเปลี่ยนแปลงของสารสื่อประสาทจากการวัดด้วยวิธี HPLC-ED นั้นเป็นผลมาจากการเปลี่ยนแปลงการทำงานในระดับการสังเคราะห์ การหลั่ง หรือการขนส่งกลับจากการมีปริมาณของ SERT ที่เปลี่ยนแปลง และผลการเปลี่ยนแปลงที่พบจากการศึกษาในขั้นตอนที่ 2 นี้ สามารถนำไปอธิบายผลของการเปลี่ยนแปลงระดับความกังวลจากการทดสอบพฤติกรรมในตอนที่ 1 ว่าสารทดสอบมีความสัมพันธ์ต่อการทำงานของระบบประสาทอย่างไร

4. แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วง 6 เดือน

ปีที่ 1 (ก.ค. 2549 – มิ.ย. 2550)

	2549						2550					
	ก.ค.	ส.ค.	ก.ย.	ต.ค.	พ.ย.	ธ.ค.	ม.ค.	ก.พ.	มี.ค.	เม.ย.	พ.ค.	มิ.ย.
<u>ขั้นตอนการทำงาน</u> - ตอนที่ 1 การศึกษาผลของสารไฟโตเอสโตรเจนที่มีต่อระดับความกังวล		←	—	—	—	—	—	—	—	→		
- การรวบรวมข้อมูล การวิเคราะห์ การเขียนรายงานสรุป ผลในตอนที่ 1 และ/หรือ การเผยแพร่บทความ									←	—	—	→

ปีที่ 2 (ก.ค. 2550 – มิ.ย. 2551)

	2550						2551					
	ก.ค.	ส.ค.	ก.ย.	ต.ค.	พ.ย.	ธ.ค.	ม.ค.	ก.พ.	มี.ค.	เม.ย.	พ.ค.	มิ.ย.
<u>ขั้นตอนการทำงาน</u> - ตอนที่ 2 การจัดซื้ออุปกรณ์และสารเคมีสำหรับการศึกษา ตอนที่ 2	←	—	→									
- การศึกษาผลของสารไฟโตเอสโตรเจนที่มีต่อการทำงานของระบบประสาท			←	—	—	—	—	—	—	→		
- การรวบรวมข้อมูล วิเคราะห์ การเขียนรายงาน และ/หรือเผยแพร่บทความ										←	—	→

INTRODUCTION

ANXIETY

Anxiety is a group of the most common psychiatric problems affected nearly 3-8 % of the population and the number is increasing (Anxiety Disorders Association of America, 2003). In human, anxiety represents internal emotional states, and is natural adaptive consequences of stress that helps to cope with the stressor. However, unlike the relatively mild, brief anxiety resulting from a stressful event such as an exam or public speaking, anxiety disorders are dysfunctional, chronic, persistent, and can grow progressively worse if not treated. According to the fourth edition of the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association, anxiety disorder can be classified into panic disorder (with or without agoraphobia), agoraphobia, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder, acute stress disorder and generalized anxiety disorder. In 2003, the Department of Mental Health, Ministry of Public Health, Thailand reported on the numbers and rates of mental illness that about 778.68 per a population of 100,000 in Thailand contracted anxiety disorder. Further, the result of National Mental Health Epidemiology Survey in 2003 revealed that generalized anxiety disorder (GAD) was the second most psychiatric problems in Thailand (the first, major depressive episode) that it affected about 1.85% of Thai population (Department of Mental Health, 2005). Anxiety has therefore become a very important area of research in psychopharmacology of this decade. Many factors determine the prevalence, onset, and course of anxiety disorders, including biochemistry, personality, life events, genetic, age and sex.

It is interesting as most reports have claimed that women were twice as much affected by anxiety disorders than men (Seeman, 1997; Department of Mental Health, 2003; 2005). It is then suggested that the fluctuation of sex steroid hormones may be partially involved; the incidence of mood swing, depression, irritability and anxiety was therefore higher during the low levels of circulating estrogen like in late luteal phase as well as in postmenopausal women (reviewed by Bloch et al., 2003). Beside, estrogen replacement therapy has been shown to improve these psychological symptoms (Ditkoff et al., 1991; Schmidt et al., 2000). Similarly, in animal models, the variation of anxiety levels across estrous cycle had been reported (Mora et al., 1996; Diaz-Veliz et al., 1997; Marcondes et al., 2001; Gouveia et al., 2004), and lacking of estrogen in ovariectomized rats produced higher level of anxiety than those replaced with estrogen (Pandaranandaka et al., 2006). Therefore, it is likely that estrogen is one factor affecting mood especially anxiety in both human and animals.

ESTROGEN AND ESTROGEN RECEPTORS

Estrogens are steroid hormone, mainly synthesizes in the ovaries using cholesterol as a precursor (Figure 1). In human, the most potent estrogen is 17β-estradiol. In addition to synthesize in ovaries, estrogens can be synthesized locally in the brain by converting androgens to estrogens by an aromatase enzyme and these local synthesis estrogens have been shown to play a major role in synaptogenesis and neurogenesis during development (Naftolin et al., 1988).

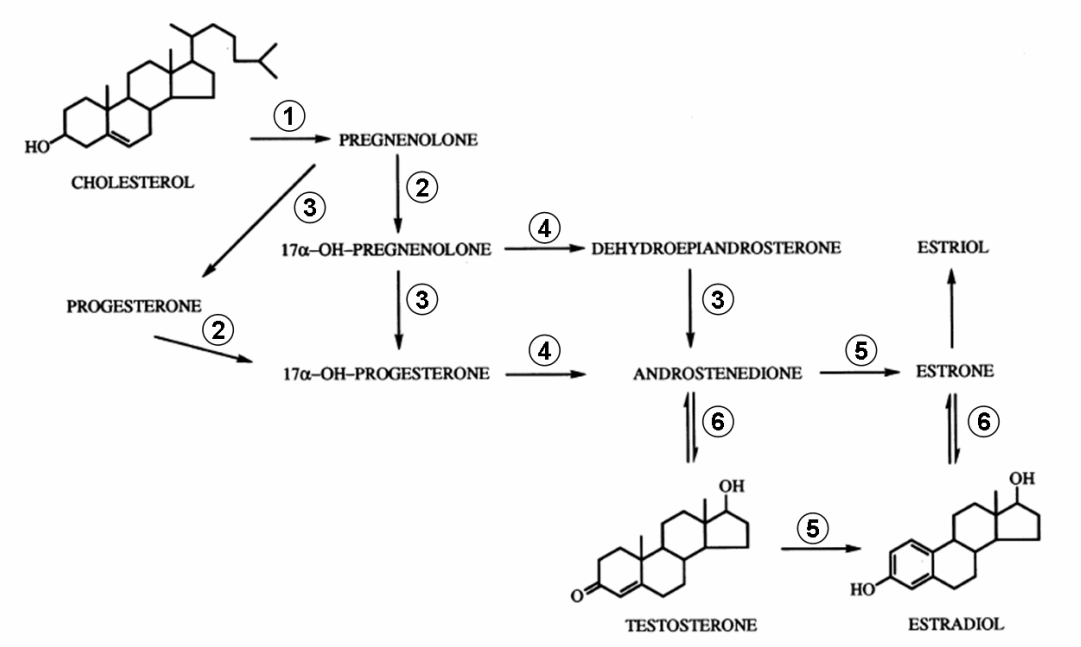


Figure 1 The biosynthetic pathway of estrogens. The enzymes responsible for each step are shown (①side-chain cleavage enzyme; ② 17α-hydroxylase; ③ 3β-hydroxy steroid dehydrogenase; ④ 17-20-desmolase; ⑤ aromatase; ⑥ 17β-hydroxy steroid dehydrogenase enzyme).

The physiological effects of estrogens are mediated through estrogen receptors. There are two types of estrogen receptor, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The ERα was expressed in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal; while ERβ was found expressed in prostrate, ovary, lung, bladder, brain, uterus and testis (Kuiper et al., 1997). The different distribution of these two receptors is believed to responsible for the diverse action of estrogen in various organs. Moreover, it is now well known that the ratio of ERα/ ERβ differed between normal and cancerous tissues and a higher ERα/ ERβ ratio was observed in breast and endometrial carcinoma (Ito, 2007).

Although estrogen is required to conserve normal activity including emotion, it is not always the case. It had been recently demonstrated that hormone replacement therapy (estrogen plus progesterone) increased the recipient's risk of developing breast cancer; while estrogen replacement therapy increased the risk of endometrial cancer (Ito, 2007). Therefore, estrogen may not be given to some patients with a risk of hormone dependent cancers.

ANXIETY AND ESTROGEN

As mentioned earlier that the incidence of anxiety disorder was higher in female than male, both from clinical and animal studies. The supplementation of estrogen can reduce anxiety in both postmenopausal women and ovariectomized animals; moreover, lacking of estrogen has been proved to be anxiogenic. Many studies have been done to elucidate the mechanism of estrogen in regulating anxiety. In 2007, Walf and Frye investigated whether estrogen reduced anxiety by action through ERα or ERβ. They implanted guide cannulae into the hippocampus of ovariectomized rats and each rat was received either vehicle, 17β-E2, SERMs with greater affinity for ERα than ERβ (17α-E2 or propyl pyrazole triol, PPT), or SERMs with greater affinity for ERβ than ERα (coumestrol or diarypropionitrie, DPN) 10 min before testing with anxiety models. Rats received 17β-E2 or ERβ SERMs entered central zone of an open field more frequent and spent more time on the open arms of the plus maze, suggesting that ERβ in the hippocampus may be involved in anti-anxiety effect of estrogen.

Previously, we have shown that in ovariectomized rat with or without estrogen supplementation, there were different in the levels of monoaminergic neurotransmitters (Pandaranandaka et al., 2006; 2008). These findings were also supported by other groups of researchers (Luine et al., 1998; Bowman et al., 2002; Heikkinen et al., 2002). One of the neurotransmitters that was rather interesting was serotonin as we found that the level of serotonin, its metabolite and the turnover rate were consistently different between ovariectomized with or without estrogen supplemented in brain areas that related to anxiety (Pandaranandaka et al., 2006; 2008). Further, Jacobs and Hyland (2003) reported that menopausal women taking Selective Serotonin Reuptake Inhibitor (SSRI) plus estrogen replacement therapy had fewer symptoms than whom taking SSRI alone, suggesting a relationship between serotonin and estrogen on anxiety.

SEROTONERGIC SYSTEM

Serotonin also called 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. The synthesis and primary metabolic pathway of 5-HT are shown in Figure 2. The initial step in the synthesis of 5-HT is the facilitated transport of the amino acid L-tryptophan from blood into brain. Serotonergic neurons contain the enzyme tryptophan hydroxylase (TPH), the rate limiting enzyme, which convert tryptophan to 5-hydroxytryptophan (5-HTP). Another enzyme involved in the 5-HT synthesis is aromatic L-amino acid decarboxylase (AADC) converting 5-HTP to 5-HT. Monoamine oxidase (MAO) converts 5-HT to 5-hydroxyindoleacetaldehyde, and this product is oxidized by an NAD+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA). In brain, 5-HIAA is the primary metabolite of 5-HT.

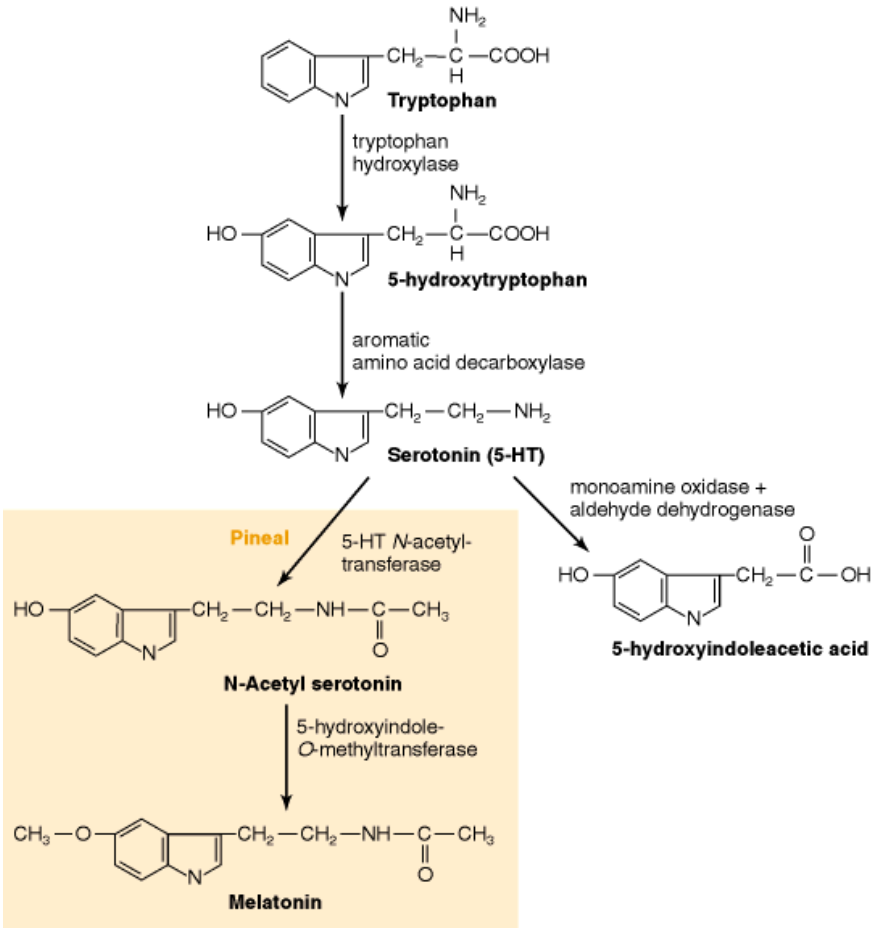


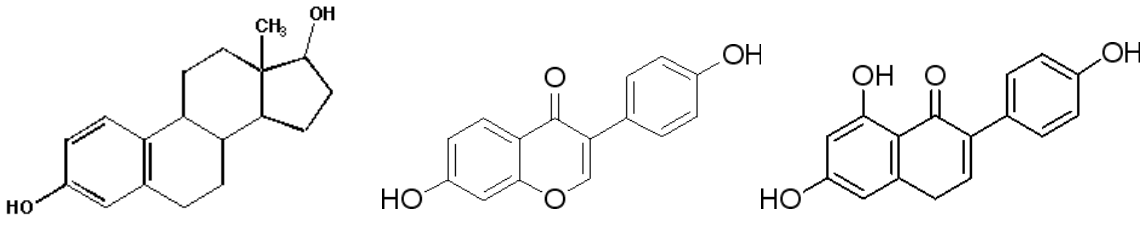
Figure 2 The biosynthesis and catabolism of 5-HT. Note that in the pineal gland, 5-HT is converted enzymatically to melatonin (Frazer and Hensler, 1999).

The activity of 5-HT in the synapse is terminated primarily by its re-uptake into serotonergic terminals. Synaptic effects of many amino acid and monoaminergic neurotransmitters, including 5-HT, are terminated by binding of these molecules to specific transporter proteins. The SERT is located on serotonergic neurons. Activity of the SERT regulates the concentration of 5-HT in the synapse, thereby influencing synaptic transmission.

Other than the clinical report of effect of estrogen in combination with SSRI, the interaction of estrogen and serotonergic neurotransmission is strengthened by the fact that estrogen receptor (ER) was found in other regions other than the reproductive organ, specifically in brain (Laflamme at al., 1998). Later, the immunohistochemistry data demonstrated the colocalization of SERT and ER in the serotonergic neuron in different species (reviewed by Bethea et al., 2002). By acting through nuclear estrogen receptors, it has been shown that estrogen increased gene and protein expression for tryptophan hydroxylase (TPH), decreased gene expression for the SERT and the 5-HT1A autoreceptor in the raphe nucleus of macaques (Gundlah et al., 2001).

PHYTOESTROGENS

Phytoestrogens are bioactive compounds present in plants with a chemical structure similar to 17β-estradiol, an endogenous estrogen as shown in figure 3. From this similarity, phytoestrogens can bind to estrogen receptor and bring about the estrogenic and anti-estrogenic effects. Phytoestrogens can be divided into three main classes, according to their chemical structures: isoflavones, lignan and coumestans (Usui, 2006). Of these three classes, isoflavones are the most interesting in research area since it can be found in soy and associated with lower rates of osteoporotic fractures, cardiovascular diseases, postmenopausal symptoms, and cancer in Asian than Western populations (Usui, 2006). Genistein and daidzein are the main compounds found in soy products.



17β-Estradiol **Genistein** **Daidzein**

Figure 3 Chemical structures of 17β-estradiol, genistein and daidzein.

Although phytoestrogens can bind to both ER subtypes. It has been shown that the phytoestroestrogens are preferable to ERβ than to ERα and causing a beneficial for osteoporosis, cardiovascular protection, lipid metabolism and breast cancer (Terreaux et al., 2003; Usui, 2006). The possible mechanisms of action of phytoestrogens are believed to be through both estrogen-dependent and estrogen-independent mechanisms. For the estrogen-dependent mechanisms, the phytoestrogens can either act through ERs or affect the estrogen endogenous synthesis pathway. Phytoestrogens can inhibit sulfotransferase, aromatase, 17β- and 3β- hydroxysteroid dehydrogenase which are enzymes involved in the metabolism and biosyntheis of estrogen; therefore, altering the metabolism and availability of endogenous estrogens (Magee and Rowland, 2004). For the estrogen-independent mechanisms, phytoestrogens like genistein was found to be a tyrosine kinase inhibitor, a free radical scavengers and an anti-metastatis (Magee and Rowland, 2004).

The effect of phytoestrogen on anxiety was reported by many researchers; however, the results were somewhat inconsistent. For instance, rats fed with phytoestrogen diet were either less anxious (Lund and Lephart, 2001; Lephart et al., 2002; 2004) or more anxious (Hartley et al., 2003) than the rats fed with phytoestrogen free diet. Moreover, Patisaul and Bateman (2008) demonstrated that injecting equol, the metabolite of phytoestrogen to neonatal male rats can induce anxiety in adulthood. The difference in these findings may be due to the length of treatment, age at exposure, route of administration, sex and strain of the animals. It is known that in rat phytoestrogen in diet are effectively converted to equol by the intestinal microflora (Setchell et al., 2003); however, in human, the converting to equol is limited. It is then interesting to see whether the parent compound of phyotestrogen like genistein and daidzein contain anxiogenic- or anxiolytic-like activity.

ANXIETY BEHAVIORAL MODEL

Animal models have been used to dissect the physiological basis of anxiety, these models base on animals' ethological conflict. The most common tests are open field test, elevated plus-maze, light-dark compartment test and social interaction test. These models base on the natural stimuli, fear of novel, open bright area and its exploratory nature.

Elevated plus maze (EPM) is widely accepted as a standard test for measuring anxiety in rats (Pellow et al., 1985). Rats with anxiety are unlikely to be in an opened elevated area of the maze and this anxiety behavior is improved upon receiving the anxiolytic agents, i.e. benzodiazepines. Open field test (OF) is set up so that rats's nature of avoiding the open area

would have less visit in the middle of the open field arena designated inner zone. Then rats with lower level of anxiety would spend more time in this area. From previously reports, it was shown that benzodiazepine increased number and time spent in open arm of the EPM (Pellow et al., 1985) and the time in inner zone of OF, and because of the use of this drug in clinically GAD; it may be implied that EPM and OF were suitable for GAD.

In 1993, Graeff and co-workers developed an animal model of anxiety, the elevated T-maze (ETM). This model can evaluate two types of anxiety in the same animal, i.e. learned (or conditioned) anxiety, represented by inhibitory avoidance behavior, and innate (or unconditioned) fear, represented by one-way escape (Graeff et al., 1993; Viana et al., 1994; Zangrossi and Graeff, 1997). This model was derived from the elevated plus-maze, composed of three elevated arms, one closed and two open. Inhibitory avoidance behavior was evaluated by placing the rat at the end of the closed arm and recording the time taken to withdraw from this arm in 3 consecutive trials. One-way escape was evaluated by placing the rat in the end of open arm and recording the time taken to withdraw from this arm. Pharmacological studies have revealed that inhibitory avoidance was impaired by drugs that were effective in treating generalized anxiety disorder (GAD) (Lu et al., 2003; Custódio Teixeira et al., 2000). Thus, the learned nature and the pharmacological sensitivity of this behavior suggest that the inhibitory avoidance task is related to GAD. On the other hand, one-way escape was increased by chronic treatments that were effective in treating panic disorder (PD) (Custódio Teixeira et al., 2000). Thus, based on the assumption that innate fear is related to PD and the pharmacological sensitivity of PD, one-way escape hypothetically represents panic anxiety (Graeff et al., 1993; Zangrossi and Graeff, 1997; Custódio Teixeira et al., 2000). Further, the extensive review on the effect of drugs that act upon the serotonergic system on the animal models of anxiety suggested that ETM is a promising animal model to represent GAD and PD, since the results are more consistent than those using the elevated-plus maze (Pinheiro et al., 2007).

The open field test is now one of the most popular procedures in animal psychology (reviewed by Prut and Belzung, 2003). Different versions are available, differing in shape of the environment, lighting, presence of objects within the arena. The procedure generally usually involves forced confrontation of a rodent with the situation. The animal was placed in the center or close to the walls of the apparatus and the following behavioral parameters were recorded for a period ranging from 2 to 20 min (usually 5 min): horizontal locomotion (number of crossings of the lines marked on the floor), frequency of rearing (sometimes termed vertical activity), grooming (protracted washing of the coat). In such a situation, rodents spontaneously prefer the periphery of the apparatus more than the central parts of the open field. Indeed, mice and rats

walk close to the walls, a behavior called thigmotaxis. Increases of time spent in the central part as well as of the ratio central/total locomotion or increase of the latency to enter the central part are indications of anxiolysis.

The open field has become a convenient procedure to measure not only anxiety-like behaviors, but also sedation or activity. In fact, anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal's breeding or natural environment). It is clear that these two factors may trigger anxiety behavior only in gregarious species and/or in species that show fear of open spaces into which they are forced. This is precisely the case with rodents that live in social groups and in small tunnels. This is of course not the case in species such as lambs or cows that live in large fields. The effects of many different drugs have been investigated in the open field, including compounds with effective or potential anxiolytic effects (BDZs, 5-HT ligands, neuropeptides) but also compounds with stimulant (amphetamine, cocaine), sedative (neuroleptic) or prostration-inducing (epileptogenic drugs) activity. An increase in central locomotion or in time spent in the central part of the device without modification of total locomotion and of vertical exploration can be interpreted as an anxiolytic-like effect while the contrary, that is a decrease of these variables, is associated with anxiogenic effects. Increased locomotion can be considered a stimulant effect while decreased vertical activity and locomotion are related to sedation.

The present investigation was therefore conducted to determine whether the phytoestrogens (genistein and daidzein) can produce anxiolytic effect like that of estrogen in ovariectomized rats, measured by the behavioral models of anxiety (ETM, OF). Further, the roles of phytoestrogens on the brain serotonergic system (i.e. 5-HT and its metabolite levels; and SERT protein levels) in brain regions involving anxiety was also investigated.

MATERIALS AND METHODS

Animals

Female Wistar rats weighing 180-200 gm at the beginning of the experiments were obtained from National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. All animals were housed in pair in shoebox cage under 12h light/dark cycle (lights on at 0600 h) at room temperature (25±2°C). Standard rat chow and water were supplied *ad libitum*. After 7-day adaptation period, all rats were bilaterally ovariectomized under anesthesia (Isoflurane; Terrell™, Minrad Inc., Bethlehem, PA, USA). Body weight and amount of food consumed were measured daily. The uterine weight, the indication of sex hormones deficiency was determined on the day of sacrifice. All procedures were done according to the National Institutes of Health Guide for care and used of Laboratory animals under the approval of Animal Used Committee, Faculty of Veterinary Science, Chulalongkorn University.

Surgery and hormone administration

The ovariectomized rats was randomly assigned into 4 groups, vehicle treated- (Ovx), estrogen treated- (E2), genistein treated- (Gen) and daidzein treated- (Dai) groups. For all rats, the chemical administration were started 2 days after ovariectomy by daily injection of either 17β-estradiol (Sigma, St. Louis, MO, USA; 1-100 μg/kg in 10% DMSO/propylene glycol), genistein (Sigma; 0.25 - 1 mg/kg) or daidzein (Sigma; 0.25 -1 mg/kg) subcutaneously into the dorsal region of the neck. Ovx groups, rats were injected by an equivalent volume of the vehicle (10% DMSO/propylene glycol).

Behavioral assessment

Elevated T Maze

The elevated T maze (ETM) is modified from EPM in order to test the different form of anxiety in the same rat. The ETM was made of wood and consisted of three arms with equal dimensions (50 × 10 cm) (Figure 4). One arm, enclosed by walls (40 cm high) was perpendicular to two opposed open arms. These three arms were connected by a square (10 × 10 cm). The apparatus was elevated 50 cm above the floor. To prevent rats from falling, the open arms were surrounded by a 1 cm high Plexiglas rim. Each test session consisted of three

inhibitory avoidance trials and one escape trial held at 30-s intervals according to the method of Graeff et al. (1993). Between the trials, the animals were placed in the Plexiglas cage. On the first three inhibitory avoidance trials, each animal was placed at the distal end of the enclosed arm facing the center of the maze. The baseline latency was defined as the time(s) required for the rat to leave this arm with all four paws. The same measurement was repeated in two subsequent trials (avoidance 1 and 2). Following avoidance training, the escape trial was conducted by placing the animal at the end of the right open arm facing the center of the maze. The time the animal took to exit this arm with four paws was recorded and designated as the escape time. For all tasks, a cutoff time of 300 s was established. The behavioral tests were conducted between 0900 am and 1200 pm.

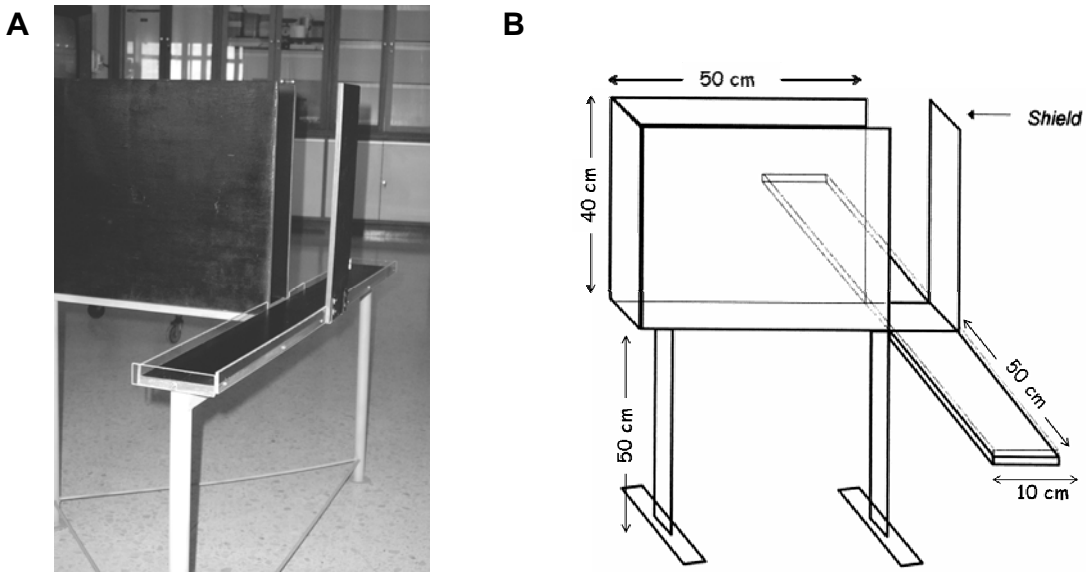


Figure 4 The elevated T-maze composed of two open- and one closed-arms of equal dimension (10 x 50 cm), connected by center platform (10 x 10 cm). The closed-arm was enclosed by a 50-cm wall, and the maze was elevated 50 cm above the floor.

Open field Test

After the ETM session, the animals were tested in the open field for 5 min to measure locomotor activity. The open field test was used in accordance with the methods described by McCarthy et al. (1995). The open field was a wooden box (76 cm long × 57 cm wide × 35 cm high) with a 48-square grid floor (6 × 8 squares, 9.5 cm per side) (Figure 5). All but the 24 peripheral squares were considered as inner zone. The numbers of total crosses that the rat made during the 5 min in this task were recorded as the locomotor activity. Times spent in the

inner zone were considered indicative of anti-anxiety behavior in the rodent. The experiments were recorded by a video camera for later analysis.

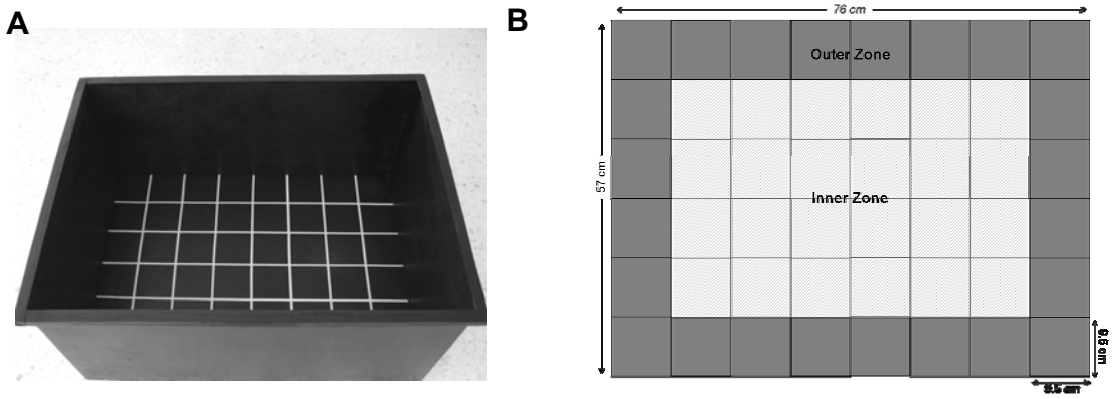


Figure 5 The open field (A) was a rectangular box of dimension (57 x 76 x 50 cm), in which the floor was divided in to 6 x 8 squares (9.5 x 9.5 cm). The perimeter squares closed to the wall was designated as outer zone, and the rest was designated as inner zone (B).

Brain dissection and Serotonin analysis

After the open field test, the rats were euthanized and their brains were rapidly removed, frozen in liquid nitrogen, and stored at -70^{o}C. Brains were isolated into each area (followed the instruction of Heffner et al., 1980) and placed in 1.5 ml Eppendorf tubes. Briefly, a frozen brain was placed on its dorsal surface in the trough of the brain cutting block (Figure 6). All of the following procedure was done on ice. Razor blades were carefully inserted through the cutting channels slicing the brain at right angles to the sagittal axis. This initial razor blade slices through the coronal plane of the brain at the level of the anterior commissure. The position of the initial razor blade served as a reference point from which brain sections were obtained. Total of eight razor blades were inserted anterior or posterior to the first blade as shown in Figure 6. The brain was thus divided into 8 sections (Figure 7). The razor blades were removed from the block with coronal brain slices adhering to their surfaces and were placed on a glass plate suspended on ice. Brain regions were then bilaterally dissected from these slices. The frontal cortex, nucleus accumbens, septum, amygdala, and hippocampus were used for HPLC and Western blot analysis.

The frontal cortex was consisted of the frontal poles, cortical tissue from section 1 (see Figure 7.1), as well as the cortical tissue superior to the rhinal sulcus from sections 2 and 3 (Figures 7.2 and 7.3). The nucleus accumbens was dissected from the rostral surface of the third brain section (Figure 7.3). The septum was dissected from the caudal surface of the fourth

brain section (Figure 7.4) based on their distinct morphological appearances. The amygdala included the tissue lateral to both portions of the hypothalamus (sections 5 and 6) and ventral to the rhinal sulcus. The hippocampus was separated from the midbrain and overlying cerebral cortex from sections 7 and 8 (Figures 7.7 and 7.8) based on its distinct morphological appearance. For the midbrain, the two most caudally placed razor blades were not inserted resulting in a total of seven brain sections. After the cerebellum is removed from the brainstem, the midbrain and pons-medulla are separated by a cut at the level of the inferior colliculi.

Neurochemical analysis by HPLC-ECD

The isolated brains were sonicated in the cold 0.1 M perchloric acid containing 3,4-dihydroxy-benzyl-amine hydrobromide (DHBA), as an internal standard. The brain homogenate were then centrifuged at 10,000x g (Universal 32/32R, Hettich zentrifugen GmbH & Co. KG, Germany) for 30 min at 4°C. The supernatants were collected and stored at -20°C for further analysis of serotonin and its metabolite (5-HIAA) using HPLC with electrochemical detector (HPLC-EC). The HPLC-EC was composed of a glassy carbon working electrode and amperometric control (Bioanalytical systems, West Lafayette, IN, U.S.A.). A Shimadzu Model LC-10 AD pump (Kyoto, Japan) was connected to a Rheodyne (Cotati, CA, U.S.A.) injector, equipped with a 20 μl fixed loop and a 15-cm phenomenex® column (Phenomenex, USA), packed with 5-μm particles. The mobile phase solution was composed of 1 mM Heptane sulfonate, 100 mM Sodium dihydrogen phosphate, 1 mM Na_2·EDTA and 5% Methanol, adjusted to pH 4.1 with saturated citric acid. The mobile phase was filtered through a 0.22-μm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 1.0 ml/min. The amperometer was set at a positive potential of 0.700 V with respect to the Ag/AgCl reference electrode, with a sensitivity of 2 nA. The supernatant (40 μl) from the brain was injected into the HPLC-EC system to detect the 5-HT and its metabolite 5-HIAA. Delta 5.0 software (Digital Solutions, Margate, QLD, Australia) was used to analyze the chromatography data. Standard solutions at different concentrations were injected into HPLC system. The retention time was evaluated by injecting the standard serotonin and its metabolite individually and by the injection of a standard mixture. The concentrations of transmitters and metabolites were calculated by reference to standards and internal standard using peak integration and expressed as ng/g of brain wet weight.

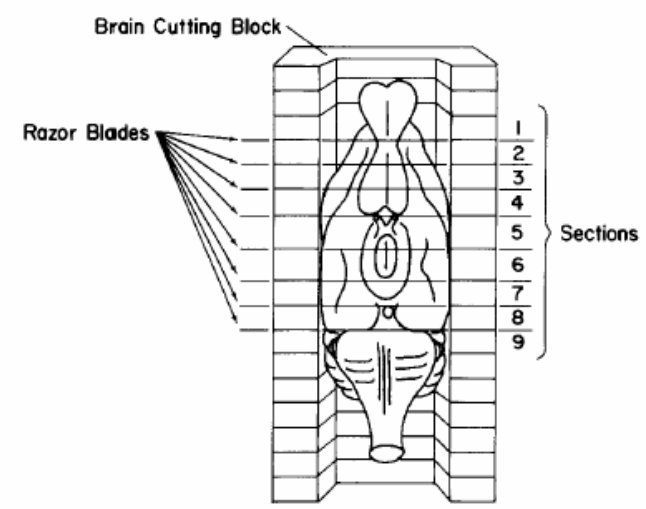


Figure 6 Diagrammatic representation of brain cutting block illustrating orientation of brain and placement of razor blades to obtain coronal brain sections. The numbers on the right refer to brain sections (Heffner et al., 1980).

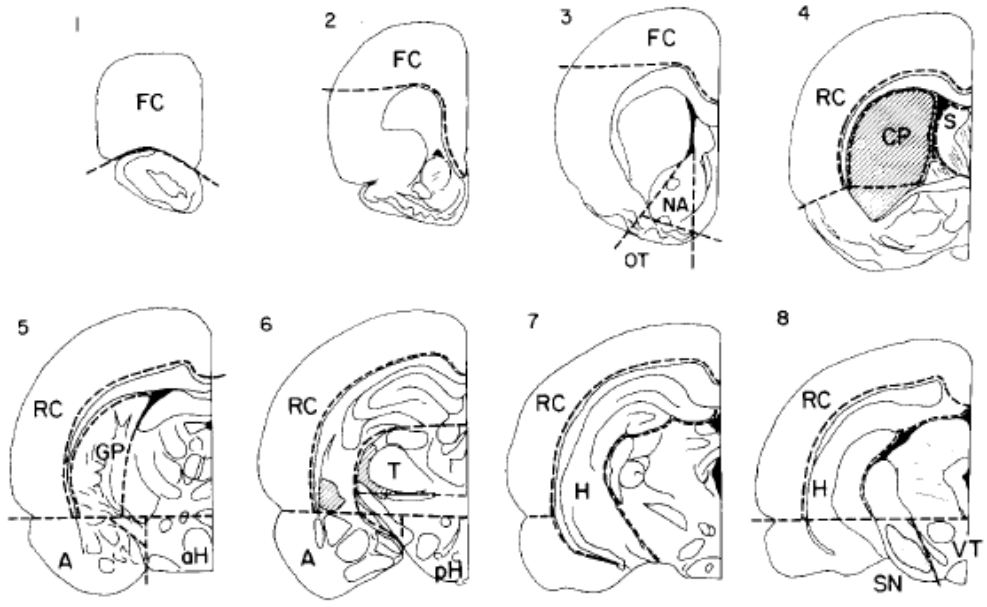


Figure 7 Diagrammatic representation of coronal brain sections from which brain regions are dissected. Dotted lines indicate borders of brain regions. FC, frontal cortex; NA, nucleus accumbens; OT, olfactory tubercle; S, septum; CP, caudate putamen; RC, remaining cortex; GP, globus pallidus; aH, anterior hypothalamus; pH, posterior hypothalamus; A, amygdala; T, thalamus; SN, substantia nigra; VT, ventral tegmentum; H, hippocampus. Numbers correspond to brain sections shown in Figure 10 (Heffner *et al.*, 1980).

Western blot analysis for SERT

The frontal cortex, nucleus accumbens, septum, amygdala, and hippocampus were dissected similar to previously described method modified from Heffner et al. (1980). All steps were performed on ice. Each 1 g of dissected brain was immediately homogenized in 200 μl of 50 mM Tris (pH 7.5) and 20 mM 2-β-mercaptoethanol and centrifuged at 12000x g (Universal 32/32R, Hettich zentrifugen GmbH & Co. KG, Germany) for 10 min. The pellet was resuspended in 10 mM Tris and 1 mM EDTA, pH 7.2, containing protease inhibitor cocktail and further homogenized with a handheld pestle and mortar. Sample protein concentrations were determined by the Lowry method (Lowry et al., 1951). Samples (75 μg protein/lane) were resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using vertical minigel system (Bio-Rad). Each sample was separated in triplicate. Subsequent to separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Arlington Heights, IL) in Tris-glycine transfer buffer. Blotted membranes were then blocked with 5% nonfat powdered milk in Tris-buffered saline for 4 hour at room temperature. For identification of proteins, membranes were washed (2 × 5 min) and incubated overnight at 4°C with the primary antibodies diluted in 1% milk. The primary antibodies was rabbit anti-rat SERT at the dilution of 1:1500. Following the primary antibody incubation, the membranes were washed and then incubated for 1 h in 1:4000 horseradish peroxidase-conjugated secondary antibodies at room temperature. This incubation step was terminated with several washes and the immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL Plus; Amersham Biosciences) according to manufacturer's instructions. Membranes were exposed to film (Hyperfilm-ECL; Amersham Biosciences) for times adequate to visualize chemiluminescent bands. Blots were reprobed with 1:100,000 monoclonal anti-β-actin (clone AC-15) and normalized to verify equivalent protein loading. Comparisons were made with known molecular weight standards. Differences in protein immunoreactivity between treatments were determined by scanning densitometry in proportion to β-actin immunoreactive bands (Scion Image; Scion Corporation, Frederick, MD).

Statistical analysis

All data were presented as mean and standard errors of mean (SEM). For comparison between groups, one way analysis of variance (ANOVA) followed by Duncan multiple-comparison test was used. Differences were considered statistically significant at $P < 0.05$, unless stated otherwise.

RESULTS

The results of this study were organized into two parts as follows:

Part 1: The effects of estrogen and phytoestrogens on the anxiety-like behaviors of the ovariectomized rats in the ETM

- The effects of estrogen on physiological parameters and anxiety- like behaviors
- The effects of phytoestrogen, genistein on physiological parameters and anxiety-like behaviors
- The effects of phystoestrogen, daidzein on physiological parameters and anxiety-like behaviors
- The comparison effects of estrogen, genistein and daidzein on physiological parameters and anxiety- like behaviors

Part 2: The effects of estrogen and phytoestrogens on the serotonergic neurotransmission:

- Levels of 5-HT and its metabolite, 5-HIAA
- Levels of SERT

Part 1: The effects of estrogen and phytoestrogens on the anxiety-like behaviors of the ovariectomized rats in the ETM

Physiological parameters

The effects of Estrogen on body weight, uterine weight and serum estradiol

At the beginning of the experiment, body weight of all rats were not different [F(7,58) = 1.58, *P* = 0.1606] (Figure 8). One month after ovariectomy, the body weight of 17β-estradiol treated rat (E_2, 1-100 μg/kg) were less than the vehicle treated- ovariectomized rats (Ovx) [F(7,58) = 8.37, *P* = 0.0001] (Figure 8). The percent change of body weight from the beginning of the experiment of all groups receiving various doses of rats was then lower than those of the Ovx [F(7,58) = 27.58, *P* = 0.0001] (Figure 9). The lowered percent change of body weight in estrogen treated rats was in a dose dependent manner (r^2 = -0.45, *P* < 0.0001, N = 66). There was no different in daily feed intake among groups [F(7,58) = 1.70, *P* = 0.1261].

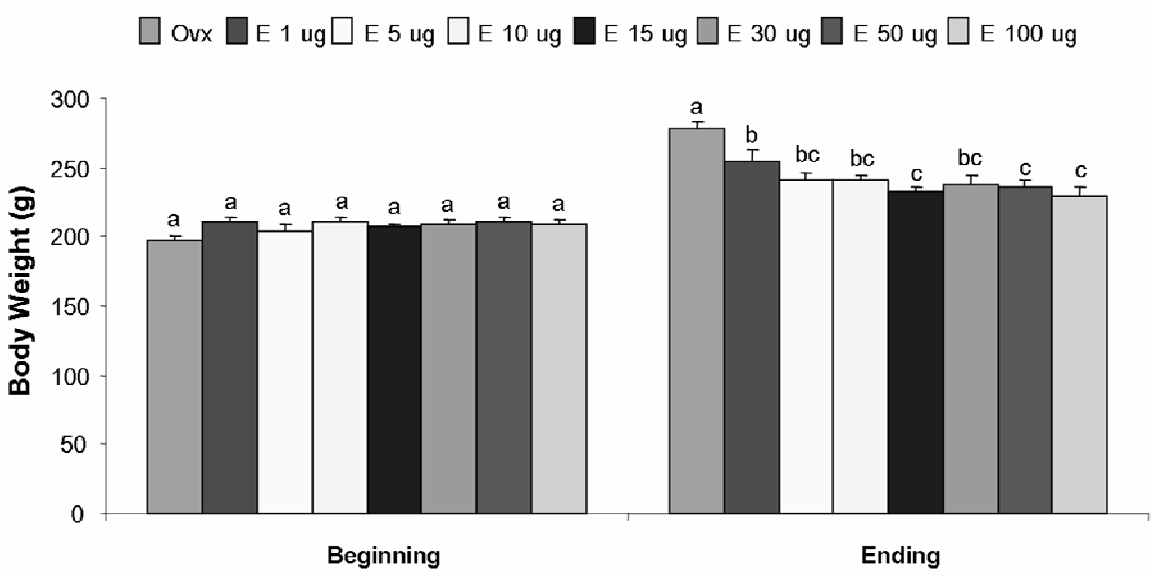


Figure 8 The body weight at the beginning and at the end of the experiment of the ovariectomized rat treated with vehicle (Ovx) or 17β-estradiol (E_2, 1-100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at *P* < 0.05, ANOVA followed by Duncan's multiple-range test, n= 6-10 to each group.

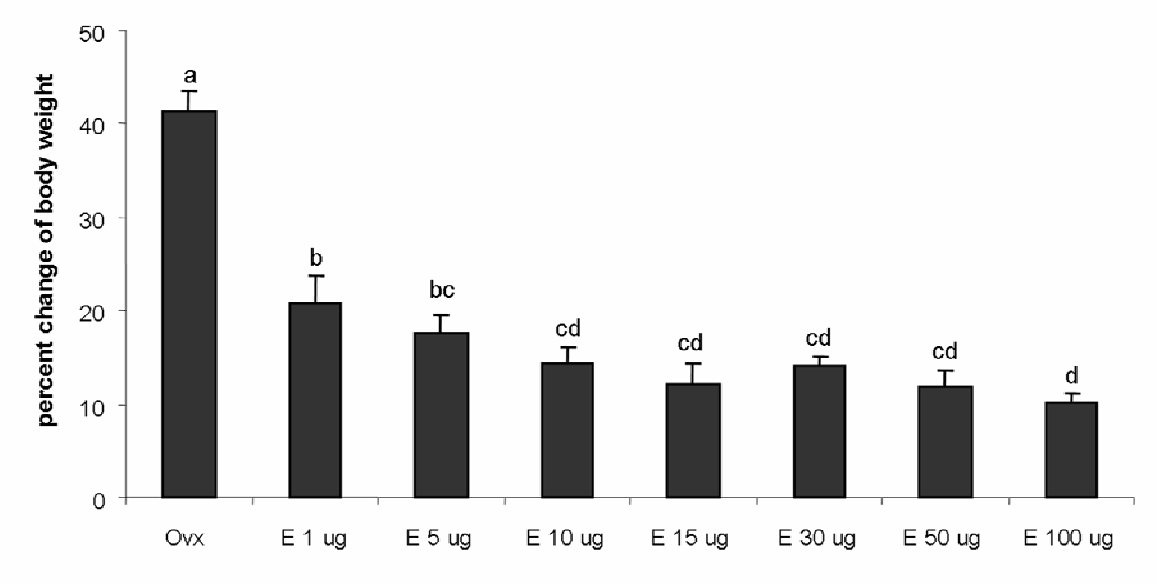


Figure 9 The percent change of body weight of the ovariectomized rat treated with vehicle (Ovx), 17β-estradiol or (E_2, 1-100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple-range test, n= 6-10 to each group.

The lacking of ovarian hormones was confirmed by the reduction in uterine weight in Ovx rats (Figure 10). The uterine weight was increased in estrogen treated rats in a dose dependent manner ($r^2 = +0.64$, $P < 0.0001$, N = 65).

The serum estradiol was increased as the dosage of exogenous estradiol was increased; however, the different in serum estradiol was significantly different at the highest dose used (100 μg/kg) as shown in Figure 11. The increased in serum estradiol was correlated to the doses of estrogen ($r^2 = +0.84$, $P < 0.0001$, N = 37).

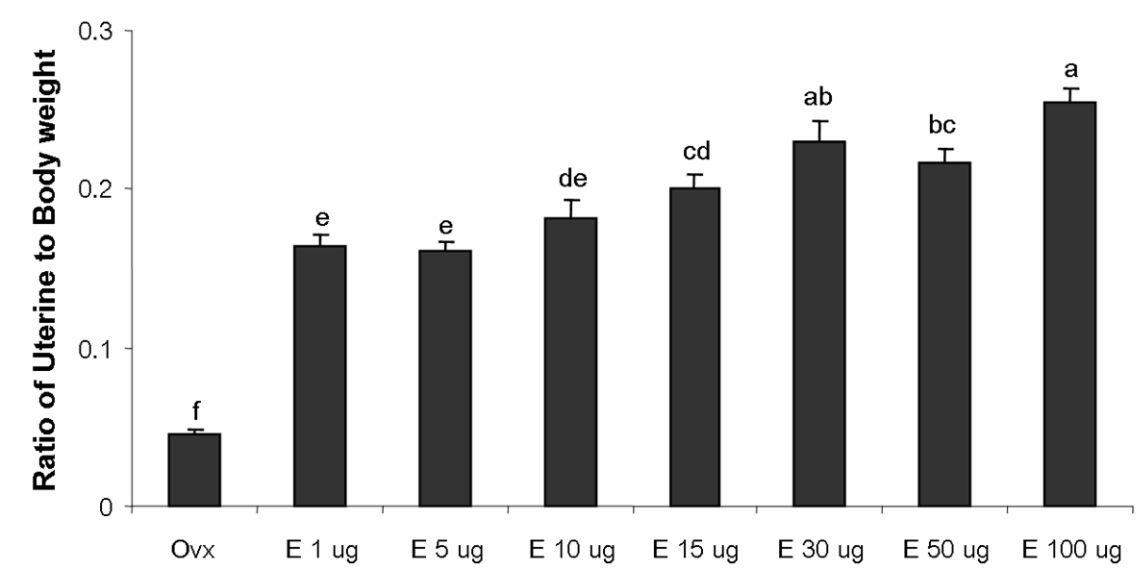


Figure 10 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle (Ovx) or 17β-estradiol (E_2, 1-100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple-range test, n= 6-10 to each group.

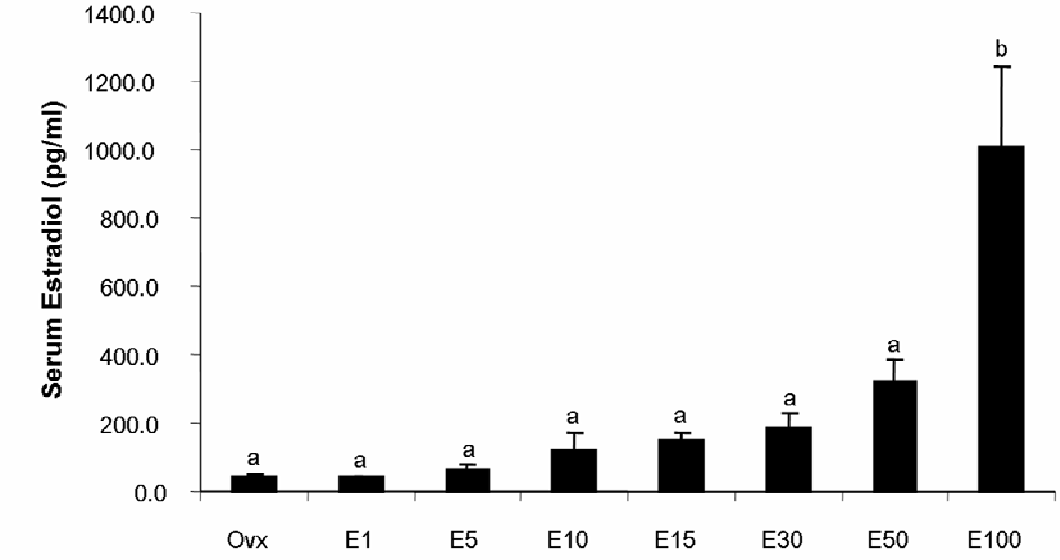


Figure 11 The serum estradiol of the ovariectomized rat treated with vehicle (Ovx), 17β-estradiol or (E_2, 1-100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple-range test, n= 4-5 to each group.

The effects of Phytoestrogen: Genistein on body weight and uterine weight

At the beginning of the experiment, body weight of all rats were not different [F(3,29) = 0.96, *P* = 0.4254] (Figure 12). One month after ovariectomy, the body weight of genistein treated rat (Gen, 0.25-1.00 mg/kg) were not different from the vehicle treated- ovariectomized rats (Ovx) [F(3,29) = 0.74, *P* = 0.5378] (Figure 12). There was no different in daily feed intake among groups [F(3,29) = 0.66, *P* = 0.5815].

Despite a small increased in the uterine weight in the genistein treated rats compared to Ovx rats, there was significantly different in the ratio of uterine weight to body weight [F(3,29) = 3.85, *P* = 0.0195] (Figure 13). Further, there was a positive correlation between doses of Gen and the uterine weight (r^2 = +0.53, *P* = 0.0014, N = 33).

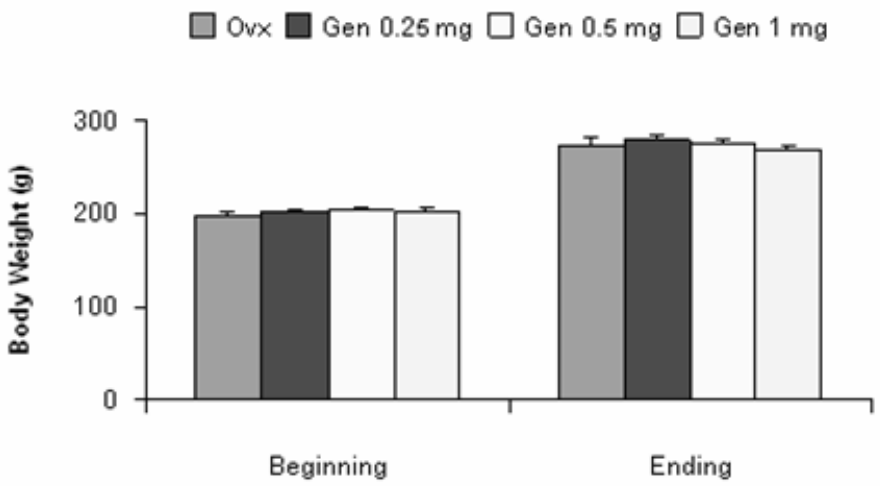


Figure 12 The body weight at the beginning and at the end of the experiment of the ovariectomized rat treated with vehicle (Ovx) or genistein (Gen, 0.25-1.00 mg/kg). Data presented as mean ± SEM, n= 8-9 to each group.

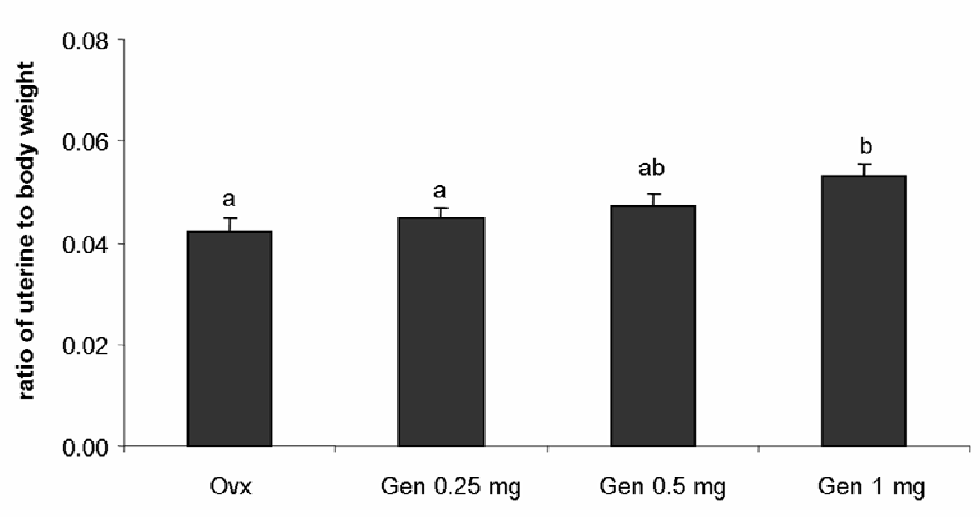


Figure 13 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle (Ovx) or genistein (Gen, 0.25-1.00 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple-range test, n= 8-9 to each group.

The effects of Phytoestrogen: Daidzein on body weight and uterine weight

At the beginning of the experiment, body weight of all rats were not different [F(3,30) = 1.65, $P = 0.1985$] (Figure 14). One month after ovariectomy, the body weight of daidzein treated rat (Dai, 0.25-1.00 mg/kg) were not different from the vehicle treated- ovariectomized rats (Ovx) [F(3,30) = 1.49, $P = 0.2384$] (Figure 14). However, the data revealed that the daily feed intake in the daidzein-treated group (1 mg/kg) trended to lower than the Ovx or other doses of daidzein [F(3,30) = 2.67, $P = 0.0657$].

The uterine weight in the daidzein-treated rats was not different from those of Ovx rats; however, there was a trend that daidzein at higher doses may increase the ratio of uterine weight to body weight [F(3,30) = 2.87, $P = 0.0528$] (Figure 15). Further, there was a positive correlation between doses of Dai and the uterine weight ($r^2 = 0.36$, $P = 0.0387$, N = 34).

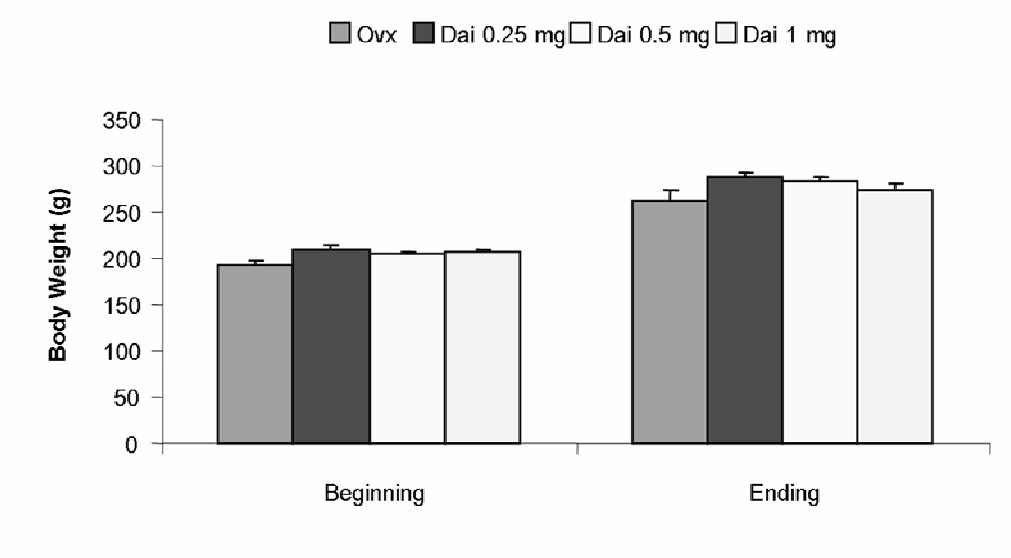


Figure 14 The body weight at the beginning and at the end of the experiment of the ovariectomized rat treated with vehicle (Ovx) or daidzein (Dai, 0.25-1.00 mg/kg). Data presented as mean ± SEM, n= 7-11 to each group.

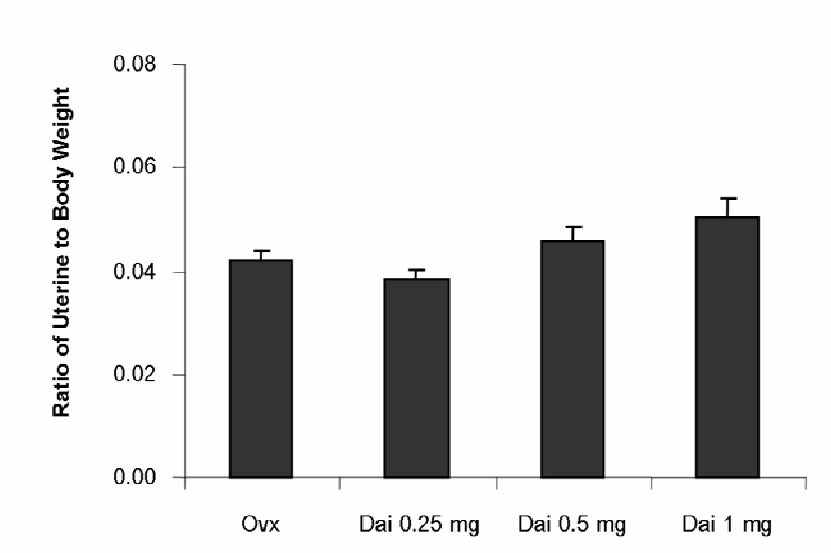


Figure 15 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle (Ovx) or daidzein (Dai, 0.25-1.00 mg/kg). Data presented as mean ± SEM, n= 7-11 to each group.

Behavioral assessment

The effects of Estrogen on anxiety and locomotor activity

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 16-17. For the inhibitory avoidance learning in the ETM, the baseline latency was not different among treatments [F(7,58) = 1.52, *P* = 0.1789]. Significant different was shown in the avoidance latencies in trial 2 [F(7,58) = 2.42, *P* = 0.0305] in that the inhibitory avoidance was impaired in the rats treated with estrogen (1-100 μg/kg) (Figure 16). The escape latency was not significantly different among treatment [F(7,58) = 1.08, *P* = 0.3883] (Figure 17). For the open field test, the total number of crosses in the open field was differed among groups [F(7,58) = 3.04, *P* = 0.0085] as shown in figure 18. The total number of crosses in the open field indicated that the locomotor activity in the Ovx group was less than the estrogen- treated group at the low dosages (1, 5, 10 and 15 μg/kg) but not different from the higher dosages of estrogen (30, 50 and 100 μg/kg).

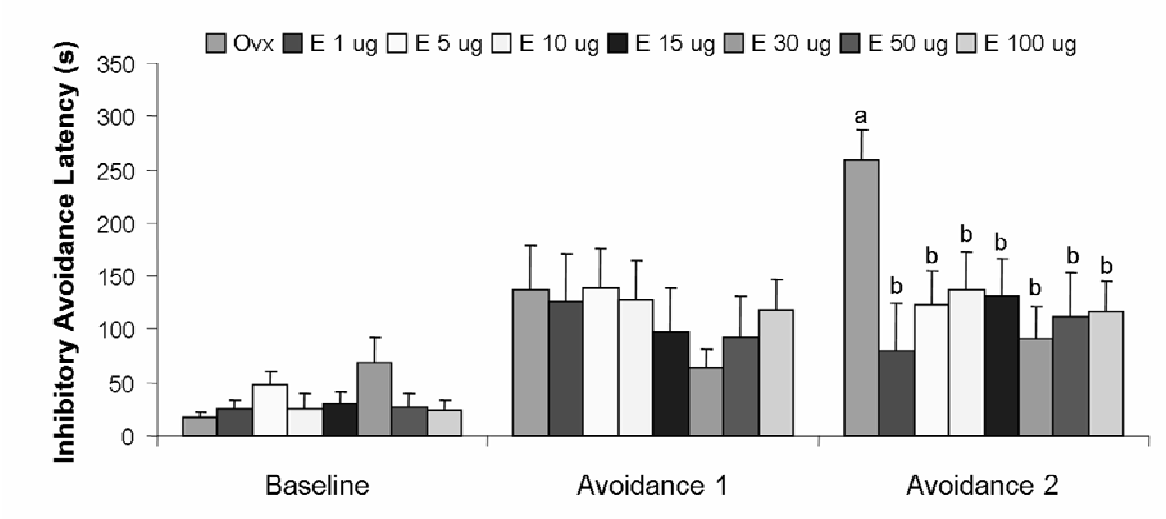


Figure 16 The inhibitory avoidance trial in the elevated T-maze of the ovariectomized rat treated with various doses of 17β-estradiol (0 -100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at *P* < 0.05, ANOVA followed by Duncan's multiple range test, n= 6-10 to each group.

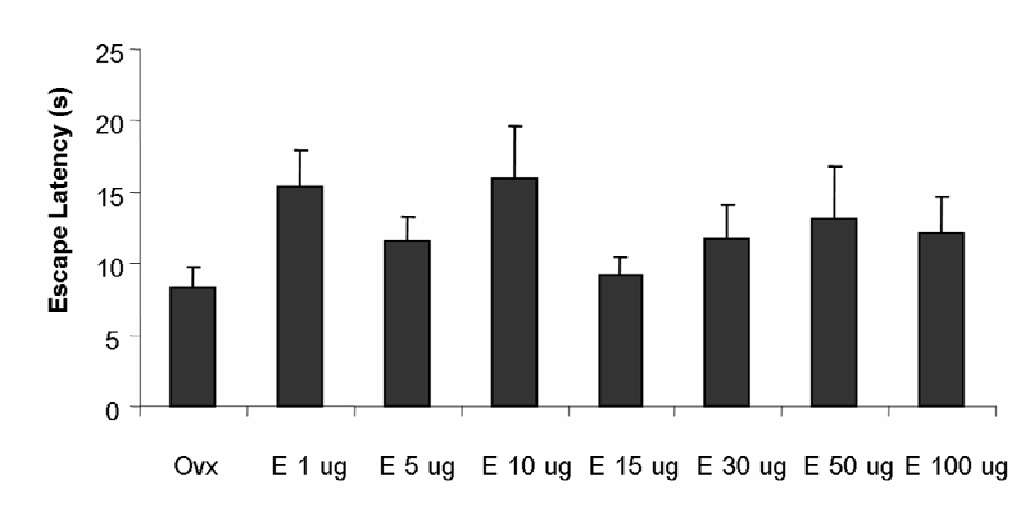


Figure 17 The escape latency in the elevated T-maze of the ovariectomized rat treated with various doses of 17β-estradiol (0 -100 μg/kg). Data presented as mean ± SEM, n= 6-10 to each group.

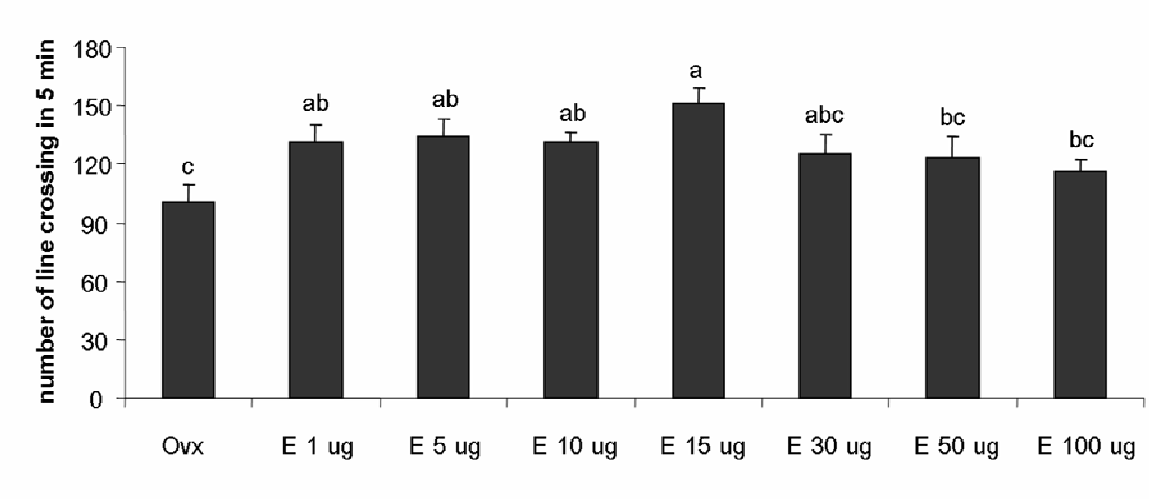


Figure 18 The number of 5 minute-line crossing in the open field of the ovariectomized rat treated with various doses of 17β-estradiol (0 -100 μg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 6-10 to each group.

The effects of Phytoestrogen: Genistein on anxiety and locomotor activity

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 19-20. For the inhibitory avoidance learning in the ETM, the baseline latency was not different among treatments [F(3,29) = 0.78, *P* = 0.5173]. Significant different was shown in the avoidance latencies in trial 2 [F(3,29) = 4.72, *P* = 0.0083] in that the inhibitory avoidance was impaired in the rats treated with the low dose genistein (0.25 mg/kg) (Figure 19). The higher doses of genistein (0.5 and 1.0 mg/kg) were not different from Ovx but different from the genistein low dose. The escape latency was not significantly different among treatment [F(3,29) = 1.41, *P* = 0.2606] (Figure 20). For the open field test, the total number of crosses in the open field was not different between treatments [F(3,29) = 0.13, *P* = 0.9399] as shown in figure 21.

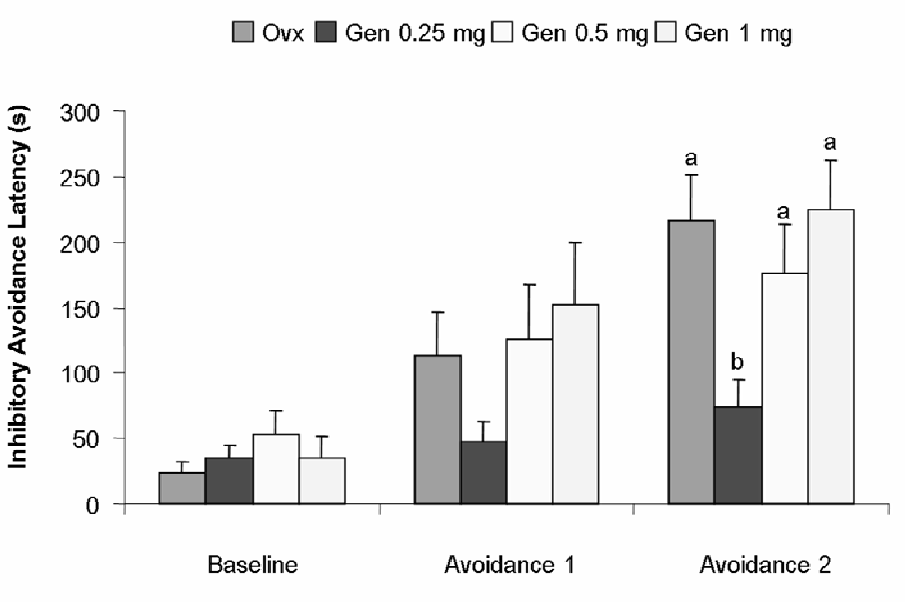


Figure 19 The inhibitory avoidance trial in the elevated T-maze of the ovariectomized rat treated with various doses of genistein (0 -1 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at *P* < 0.05, ANOVA followed by Duncan's multiple range test, n= 8-9 to each group.

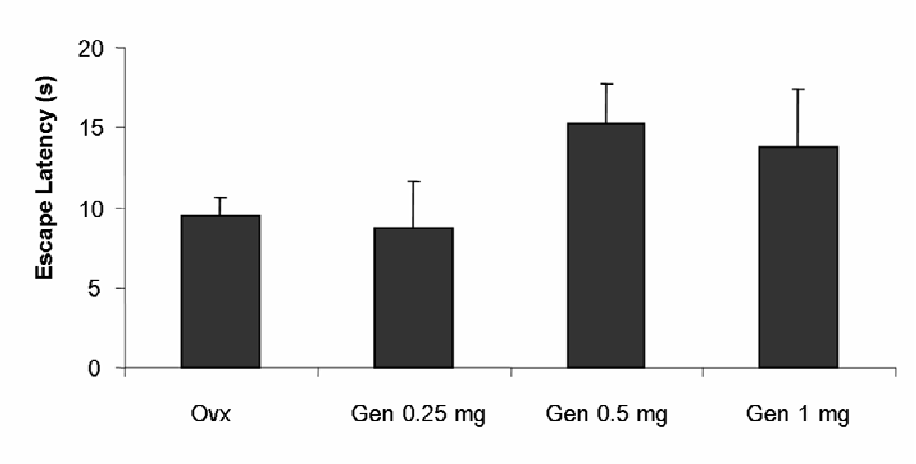


Figure 20 The escape latency in the elevated T-maze of the ovariectomized rat treated with various doses of genistein (0 -1 mg/kg). Data presented as mean ± SEM, n= 8-9 to each group.

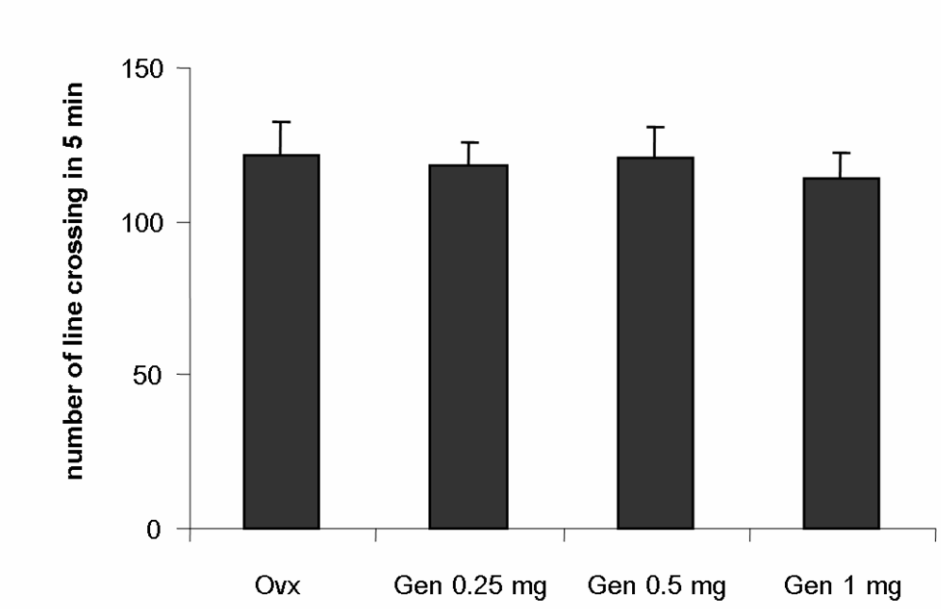


Figure 21 The number of 5 minute-line crossing in the open field of the ovariectomized rat treated with various doses of genistein (0 -1 mg/kg). Data presented as mean ± SEM, n= 8-9 to each group.

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The effects of Phytoestrogen: Daidzein on anxiety and locomotor activity

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 22-23. For the inhibitory avoidance learning in the ETM, the baseline latency was not different among treatments [F(3,30) = 1.13, *P* = 0.3529]. Significant different was shown in the avoidance latencies in trial 2 [F(3,30) = 3.34, *P* = 0.0322] in that the inhibitory avoidance was impaired in the rats treated with the daidzein (0.25-1.00 mg/kg) (Figure 22). The escape latency was not significantly different among treatment [F(3,30) = 0.29, *P* = 0.8293] (Figure 23). For the open field test, the total number of crosses in the open field was not different between treatments [F(3,30) = 0.52, *P* = 0.6734] as shown in figure 24.

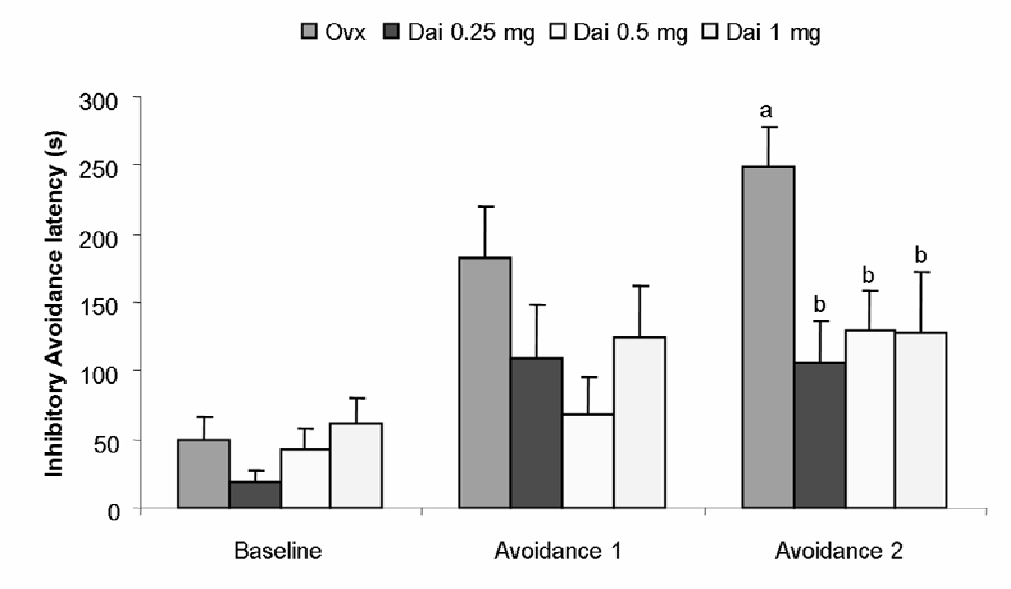


Figure 22 The inhibitory avoidance trial in the elevated T-maze of the ovariectomized rat treated with various doses of daidzein (0 -1 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at *P* < 0.05, ANOVA followed by Duncan's multiple range test, n= 7-11 to each group.

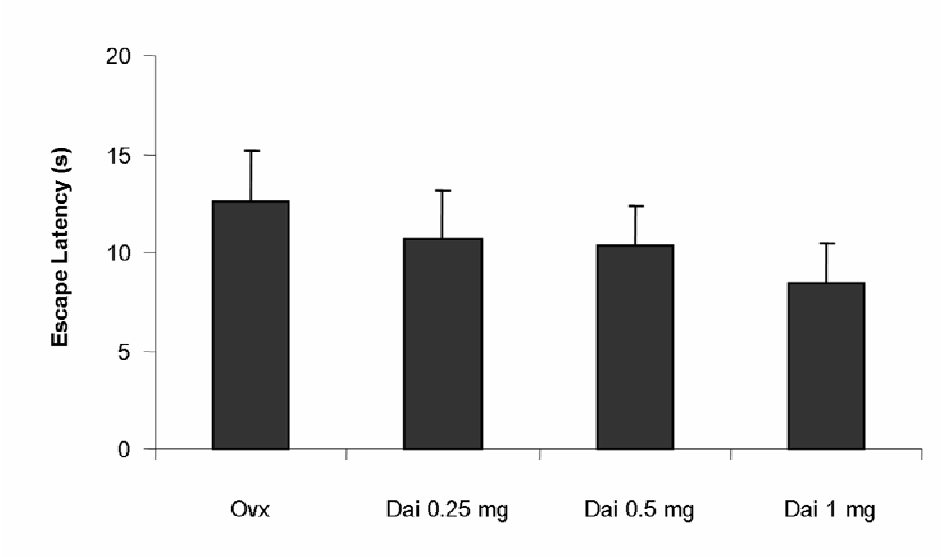


Figure 23 The escape latency in the elevated T-maze of the ovariectomized rat treated with various doses of daidzein (0 -1 mg/kg). Data presented as mean ± SEM, n= 7-11 to each group.

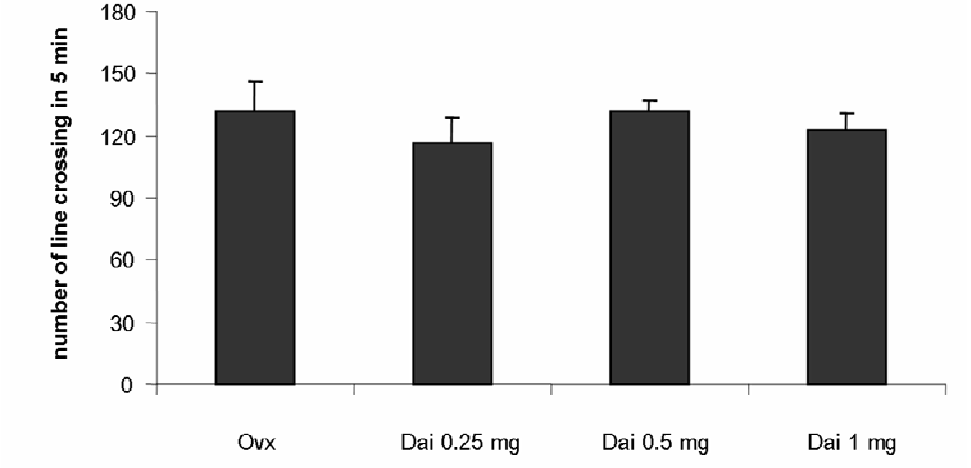


Figure 24 The number of 5 minute-line crossing in the open field of the ovariectomized rat treated with various doses of daidzein (0 -1 mg/kg). Data presented as mean ± SEM, n= 7-11 to each group.

The comparison effects of estrogen, genistein and daidzein on physiological parameters and anxiety- like behaviors

The low doses of estrogen, genistein and daidzein were selected since they had lesser effect on physiological parameters but contained anxiolytic-like effect.

At the beginning of the experiment, the body weight of vehicle-treated ovariectomized rats (Ovx) were slightly lower than estrogen-treated ovariectomized rat (E_2; 1 μg/kg) and daidzein-treated ovariectomized rat (Dai; 0.25 mg/kg) but not different from genistein-treated ovariectomized rat (Gen; 0.25 mg/kg) [$F(3,33) = 4.84$, $P = 0.0067$] (Figure 25). One month after ovariectomy, the body weight of E_2 group was lowered than Gen and Dai but not different from Ovx [$F(3,33) = 3.79$, $P = 0.0194$] (Figure 26). The percent changes in body weight in the E2 group was significantly lower than other groups [$F(3,33) = 7.25$, $P = 0.0007$] (Figure 27). However, this difference was not due to the different in feed intake, as the daily feed intake was not different among treatments [$F(3,33) = 0.31$, $P = 0.8210$].

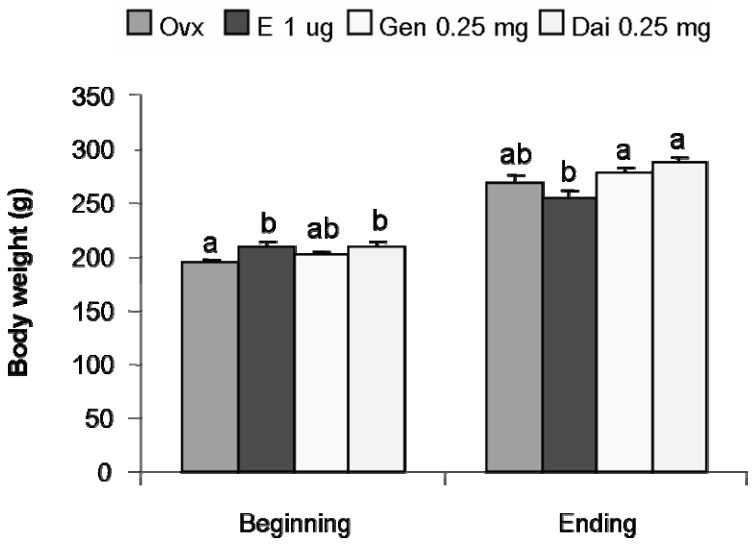


Figure 25 The body weight at the beginning and at the end of the experiment of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 6-14 to each group.

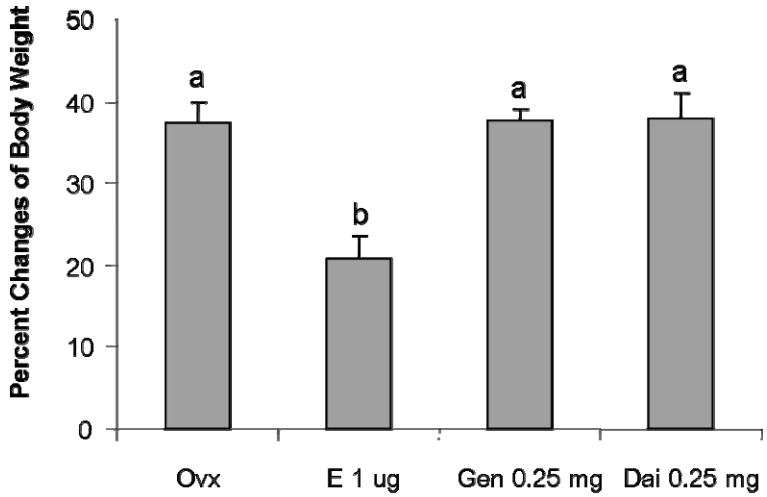


Figure 26 The percent changes of body weight of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 6-14 to each group.

The uterine weight in the estrogen-treated rats was higher than other groups as shown in figure 27 [$F(3,33) = 284.35$, $P = 0.0001$].

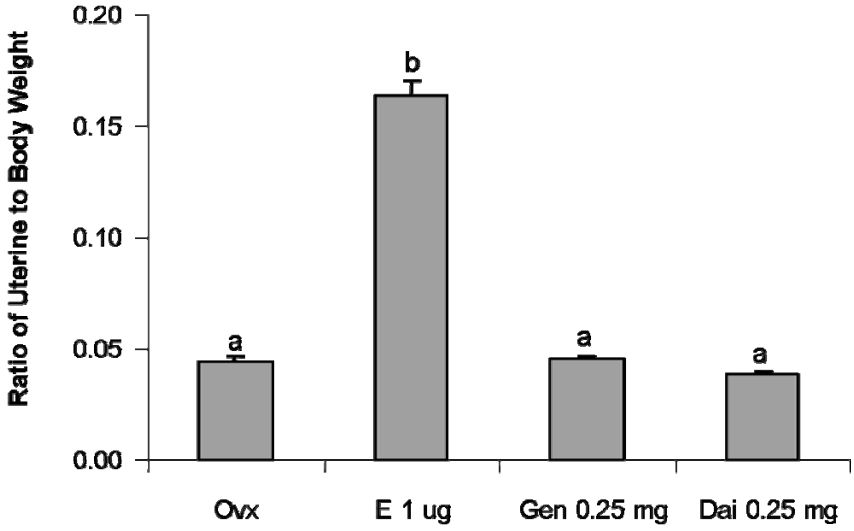


Figure 27 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 6-14 to each group.

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 28-29. For the inhibitory avoidance learning in the ETM, the baseline latency was not different among treatments [F(3,33) = 0.56, *P* = 0.6441]. In the inhibitory avoidance 1, there was a trend that the inhibitory avoidance was impaired in the genistein-treated group (Gen; 0.25 mg/kg) [F(3,33) = 2.34, *P* = 0.0910]. Significant different was shown in the avoidance latencies 2 [F(3,33) = 10.87, *P* = 0.0001] in that the inhibitory avoidance was impaired in the rats treated with the estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai; 0.25 mg/kg) (Figure 28). The escape latency was not significantly different among treatment [F(3,33) = 1.13, *P* = 0.3494] (Figure 29). For the open field test, the total number of crosses in the open field was not different between treatments [F(3,33) = 0.28, *P* = 0.8382] as shown in figure 30.

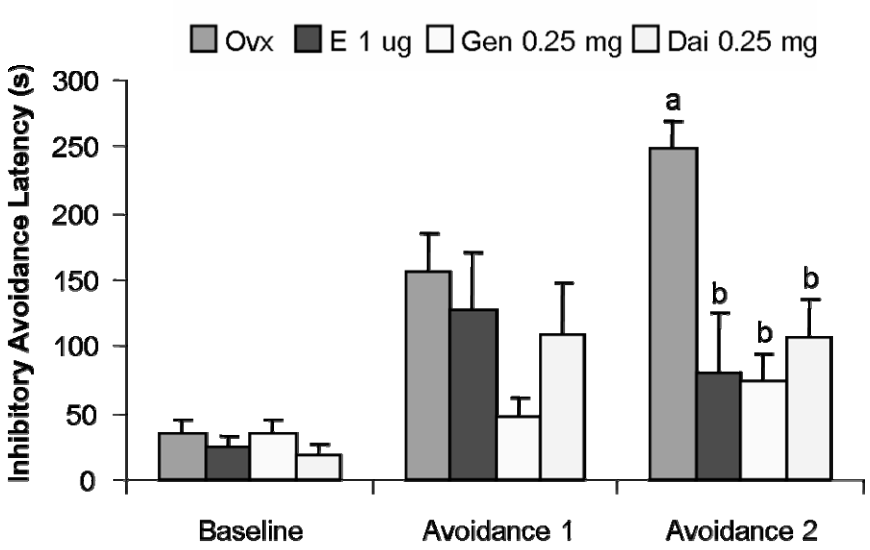


Figure 28 The inhibitory avoidance latency of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at *P* < 0.05, ANOVA followed by Duncan's multiple range test, n= 6-14 to each group.

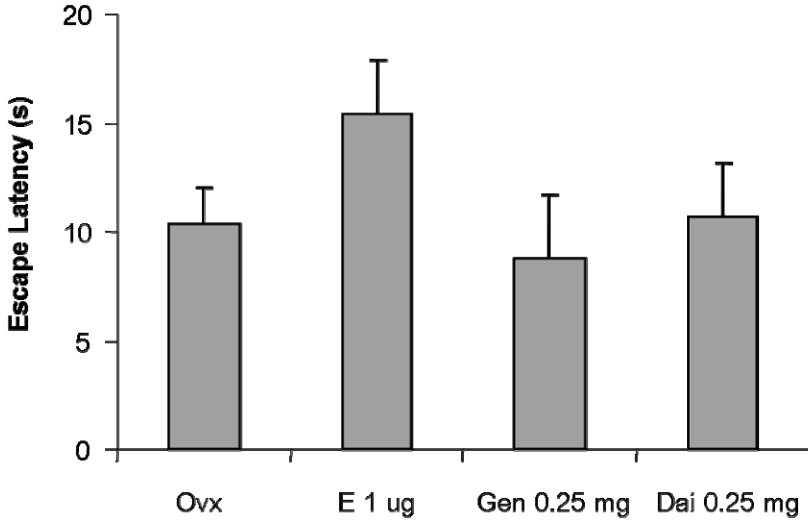


Figure 29 The escape latency of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, n= 6-14 to each group.

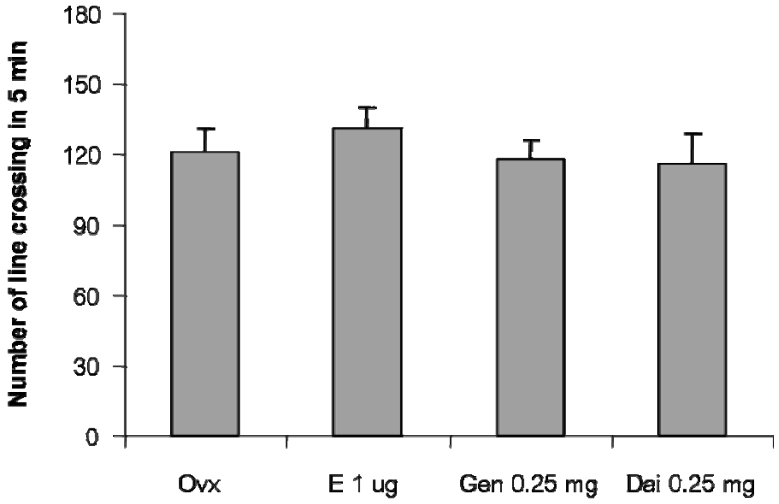


Figure 30 The number of line crossing in the open field of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, n= 6-14 to each group.

Part 2: The effects of estrogen and phytoestrogens on the serotonergic neurotransmission

The Ovx rats treated with estrogen (1 μg/kg), genistein (0.25 mg/kg) and daidzein (0.25 mg/kg) were selected for comparison the changes of serotonergic neurotransmission (levels of 5-HT and 5-HIAA, and the serotonin transporter (SERT) in order to correlate the behavior and the function of serotonergic system.

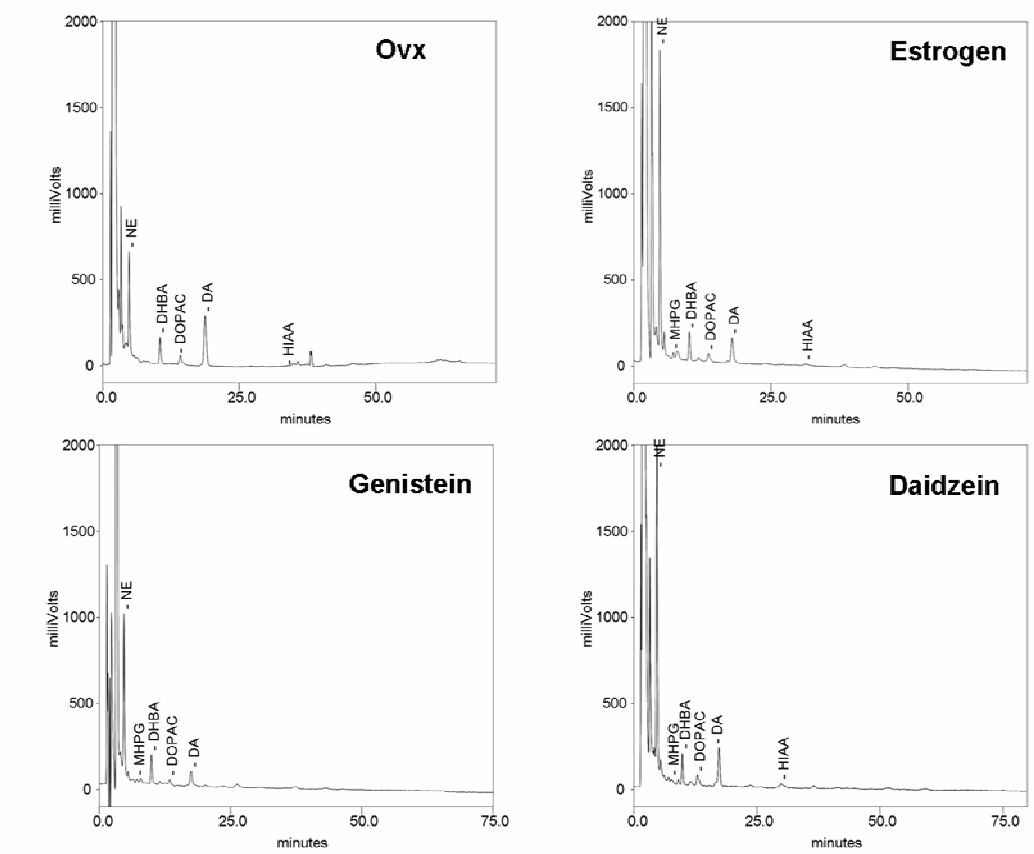


Figure 31 The chromatograms depicted the levels of monoaminergic neurotransmitters in the anterior hypothalamus of the ovariectomized rats treated with vehicle (Ovx), estrogen (1μg/kg; E2), genistein (0.25 mg/kg; Gen) or daidzein (0.25 mg/kg; Dai). The retention times of NE, MHPG, DHBA, DOPAC, DA, 5-HIAA, HVA, 5-HT is approximately at 4.74, 7.28, 10.12, 14.61, 18.61, 32.63, 43.82 and 69.76 min., respectively.

Serotonin and its metabolite levels in brain areas contributing to anxiety

Levels of brain 5-HT and its metabolite (5-HIAA) were not significantly different between groups (Figure 32, 33). The 5-HT turnover rate was significantly increased in hippocampus [$F(3, 14) = 4.79$; $P < 0.05$] of Dai-treated rats when compared to Ovx and Ovx+E_2 rats but not different from Gen-treated rats (Figure 34).

Figure 32 The level of 5-HT in the amygdala, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, septum, anterior hypothalamus, midbrain, thalamus and globus pallidus of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM.

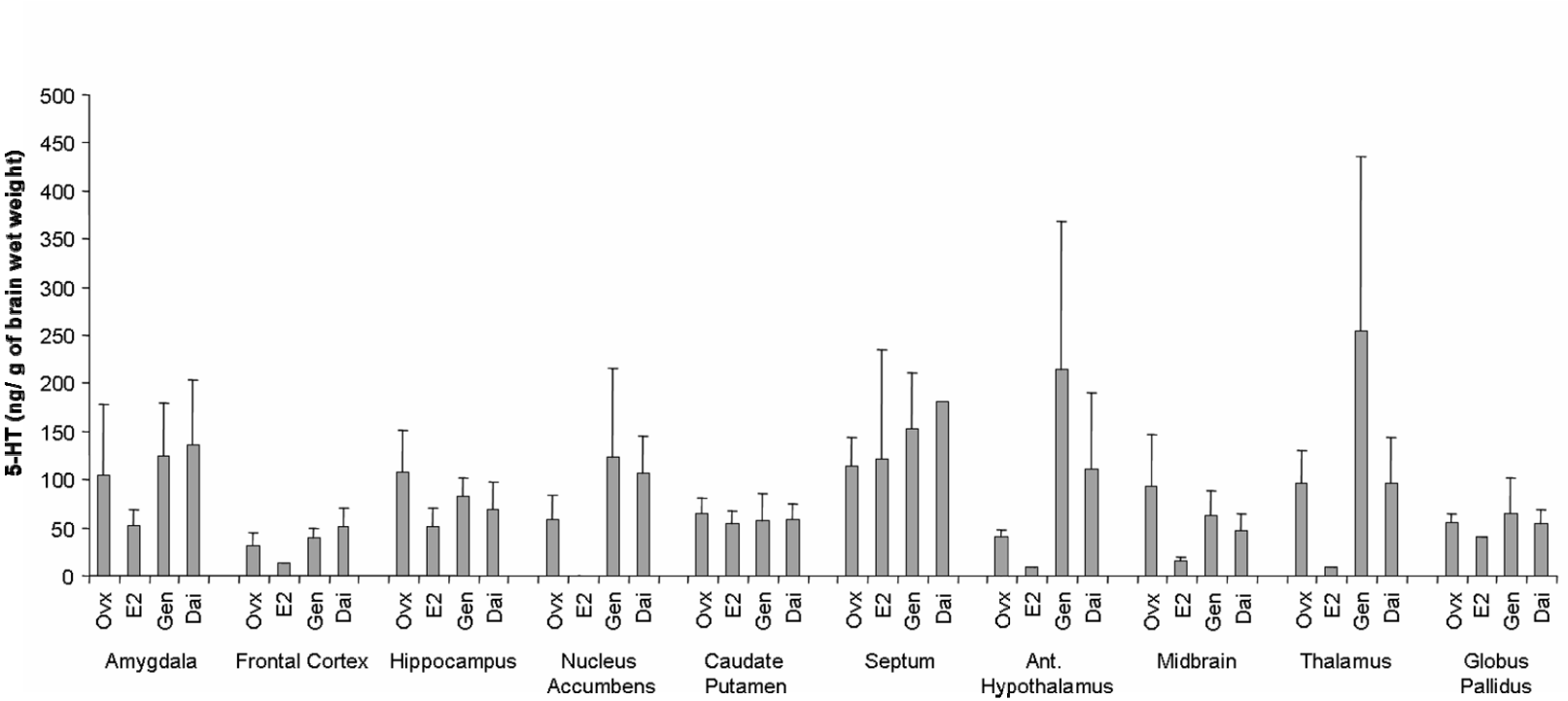


Figure 33 The level of 5-HIAA in the amygdala, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, septum, anterior hypothalamus, midbrain, thalamus and globus pallidus of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM.

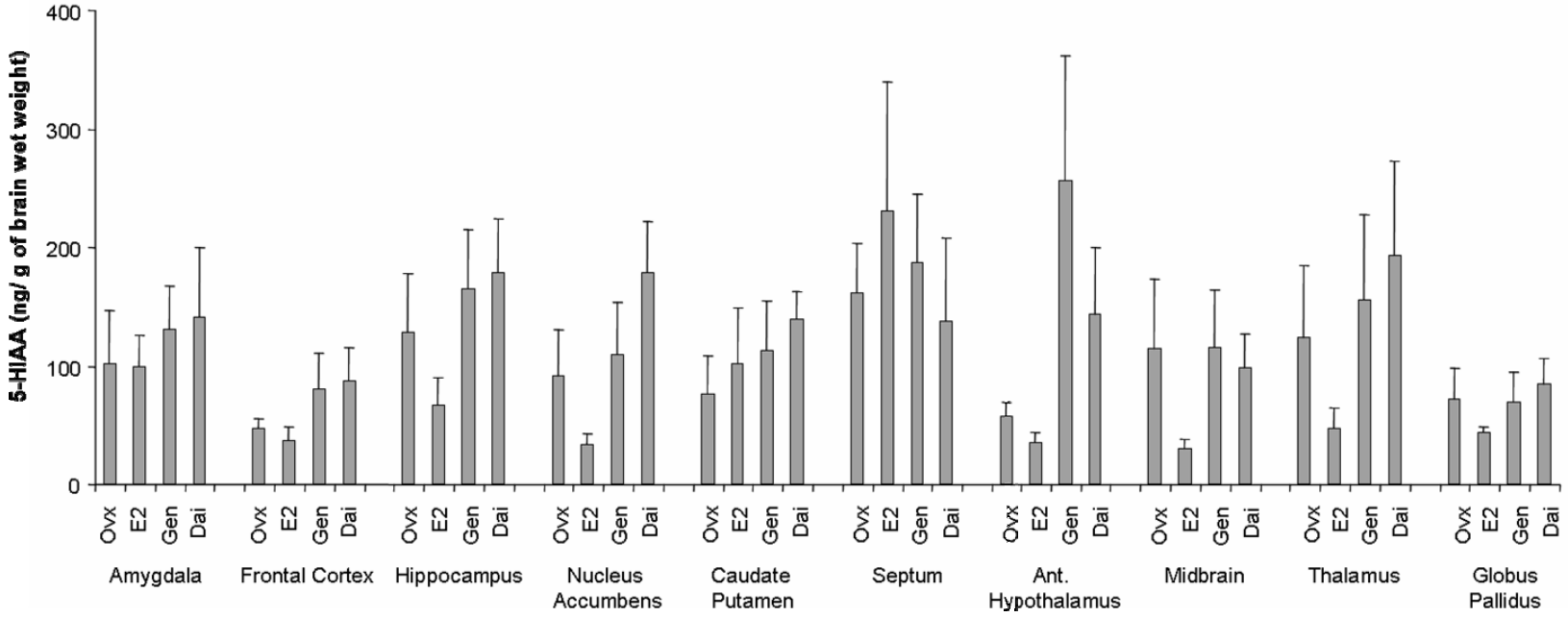
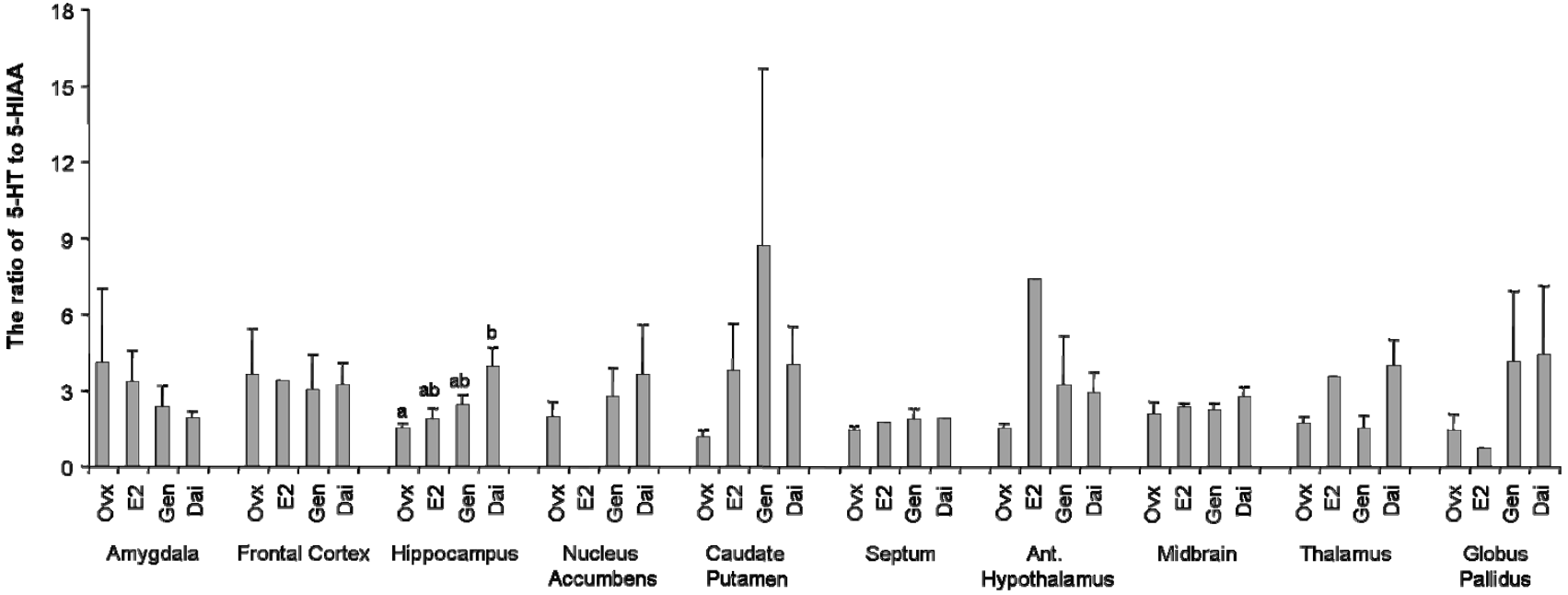


Figure 34 The ratio of 5-HT/5-HIAA in the amygdala, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, septum, anterior hypothalamus, midbrain, thalamus and globus pallidus of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test.



Serotonin transporter protein level in brain areas contributing to anxiety

Figure 35 demonstrates an example of serotonin transporter (SERT) and β-actin immunoreactive bands of ovariectomized rats treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai; 0.25 mg/kg) in the amygdala. The SERT protein levels did not show any significant difference between groups in all examined brain areas as shown in Figure 36.

Figure 35 Immunoreactive bands in Western blot probed with specific antibody to SERT and β-actin in amygdala.

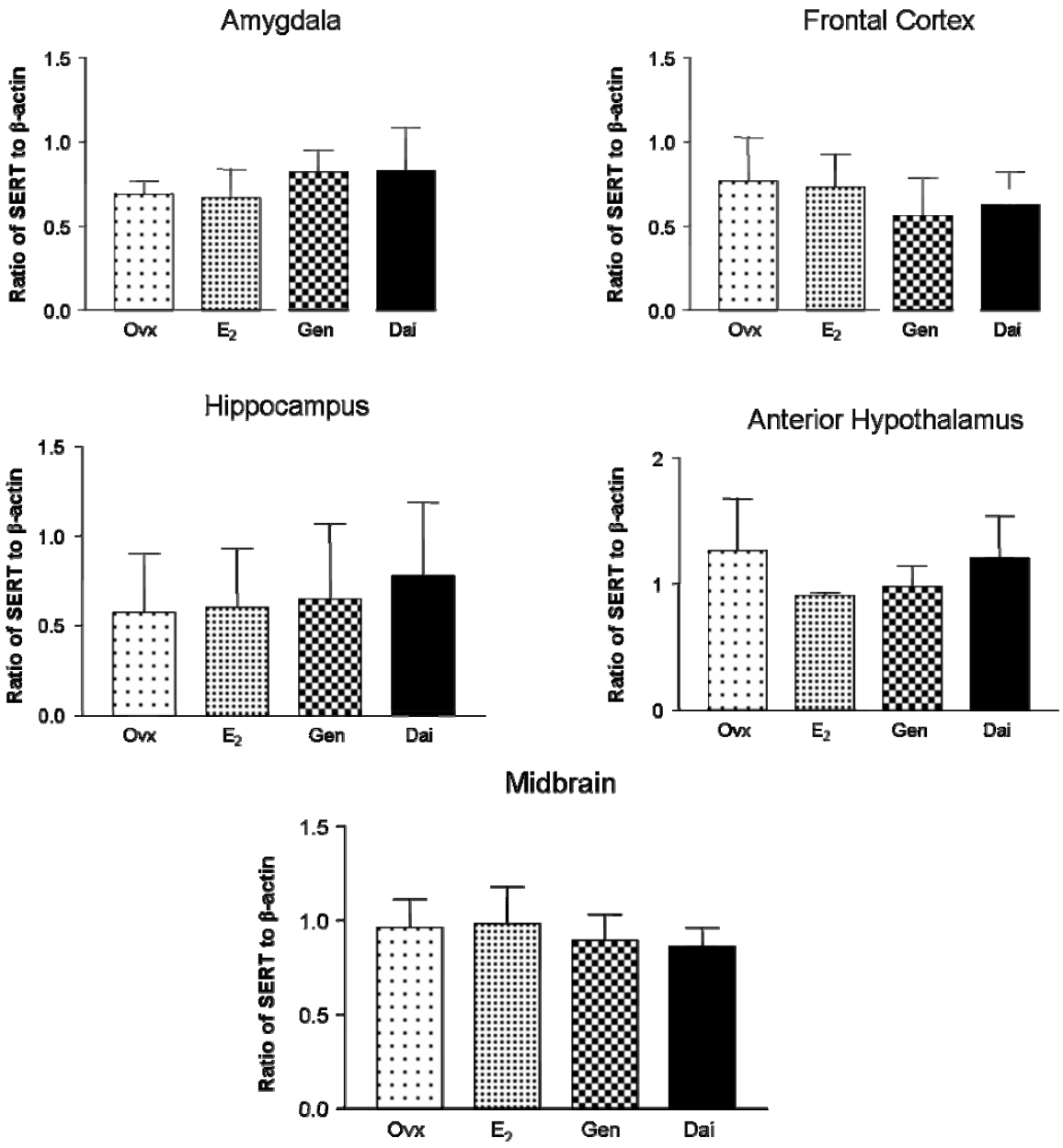


Figure 36 Histogram illustrates mean (± SEM) of SERT/β-actin protein levels in amygdala, frontal cortex, hippocampus, anterior hypothalamus and midbrain of the ovariectomized rats treated with vehicle (Ovx), estrogen (1μg/kg; E_2), genistein (0.25 mg/kg; Gen) or daidzein (0.25 mg/kg; Dai). n = 3 for each group.

The effects of estrogen and phytoestrogens on the noradrenergic and dopaminergic neurotransmission

The analysis of neurotransmitters from the brains of ovariectomized rats treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg) for 4 weeks demonstrated that there were changes in the neurotransmitters throughout the brain. The levels of norepinephrine (NE) were significantly different between groups in frontal cortex, hippocampus, anterior hypothalamus and midbrain, (Table 1; $P < 0.05$). In these areas, the highest level of NE was evident in Ovx and different from E_2 but not different from Gen and Dai. In the globus pallidus, the level of 3-Methoxy-4-hydroxyphenylglycol (MHPG) was high in the Ovx and significantly different from E_2 but not different from Gen and Dai. In the hippocampus, the MHPG was trended to different between treatments ($P = 0.0589$); in that the level was higher in the Ovx than the E_2 but not different from Gen and Dai. The NE turnover as determined by the ratio of NE to MHPG revealed that in the globus pallidus, the turnover rate was trended to be different between the Dai group and the E_2 and Gen but not from Ovx group ($P = 0.0698$).

The levels of dopamine (DA) were significant different between groups in septum, and trended to be different in nucleus accumbens, midbrain and thalamus (Table 2; $P = 0.0981$; 0.0642 and 0.0690, respectively). The DA levels in the septum of Ovx rats were significantly higher than E_2 and Gen rats but not different from Dai rats. Further, the DA in the Dai was significantly higher than E_2 but not different from Ovx or Gen rats. In the nucleus accumbens and the thalamus, the DA levels were high in Ovx and trended to be different from E_2 but not from Gen or Dai groups. In the midbrain, the DA levels were high in Gen and trended to be different from E_2 but not from Ovx or Dai groups. The levels of DA metabolites, 3-methoxytyramine (DOPAC) and homovanillic acid (HVA), revealed that there was a significant different of HVA in the caudate putamen and a trend of DOPAC in the amygdala ($P = 0.0975$). In the caudate putamen, the level of HVA was lowest in the Ovx and significantly different from others. The turnover rate of DA as determined by the ratio of DOPAC/DA and the ratio of HVA/DA demonstrated that there were different in the turnover rates of DA in the septum and the anterior hypothalamus with a trend in the caudate putamen and the globus pallidus. The ratio of DOPAC/DA and the HVA/DA in the caudate putamen and the ratio of DOPAC/DA in the globus pallidus was lower in E_2 and trended to be different from Ovx but not different from Gen or Dai ($P = 0.0536$, 0.0672 and 0.0742, respectively). The ratio of HVA/DA in the septum of E_2 was significantly higher than other groups as shown in Table 2.

Table 1 The levels of norepinephrine (NE), 3-Methoxy-4-hydroxyphenylglycol (MHPG) and the ratio of MHPG/NE in the amygdala, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, septum, anterior hypothalamus, midbrain, thalamus and globus pallidus of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg).

Brain area	Neurotransmiters	Ovx	E_2 (1 μg/kg)	Gen (0.25 mg/kg)	Dai (0.25 mg/kg)
Amygdala	**NE**	476.82 ± 119.25	136.07 ± 45.14	380.39 ± 95.47	370.34 ± 83.46
	MHPG	714.12 ± 371.86	48.72 ± 3.85	178.53 ± 58.86	152.19 ± 42.54
	MHPG/NE	1.29 ± 0.51	0.76 ± 0.38	0.51 ± 0.19	0.44 ± 0.11
Frontal cortex	**NE**	**274.15 ± 57.83**[a]	**73.15 ± 18.55**[b]	**191.67 ± 53.14**[ab]	**196.15 ± 45.34**[ab]
	MHPG	251.33 ±111.21	36.27 ± 5.02	118.62 ± 45.81	128.92 ± 34.17
	MHPG/NE	0.77 ± 0.29	0.87 ± 0.31	0.63 ± 0.19	0.64 ± 0.08
Hippocampus	**NE**	**455.76 ± 100.81**[a]	**188.06 ± 43.74**[b]	**424.25 ± 47.71**[a]	**337.21 ± 44.83**[ab]
	MHPG	324.74 ± 121.49	53.60 ± 11.84	166.84 ± 29.90	136.4 ± 42.46
	MHPG/NE	0.60 ± 0.18	0.37 ± 0.10	0.41 ± 0.06	0.40 ± 0.1
Nucleus	**NE**	249.51 ± 43.15	134.96 ± 44.96	214.74 ± 38.28	208.59 ± 48.60
Accumbens	**MHPG**	234.21 ± 66.17	45.54 ± 2.78	173.36 ± 38.56	278.34 ± 106.59
	MHPG/NE	0.84 ± 0.17	0.80 ± 0.26	0.85 ± 0.18	1.20 ± 0.25
Caudate Putamen	**NE**	139.61 ± 30.01	142.83 ± 40.72	195.66 ± 46.71	178.54 ± 40.10
	MHPG	236.95 ± 124.13	63.18 ± 11.82	208.09 ± 70.77	191.51 ± 69.89
	MHPG/NE	1.92 ± 0.83	0.93 ± 0.37	1.64 ± 0.80	1.48 ± 0.55
Septum	**NE**	1055.60 ± 219.89	838.21 ± 385.05	1333.31 ± 556.17	803.77 ± 163.96
	MHPG	238.02 ± 81.31	82.44 ± 22.88	238.78 ± 159.37	196.26 ± 69.29
	MHPG/NE	0.31 ± 0.15	0.25 ± 0.09	0.11 ± 0.03	0.39 ± 0.25
Anterior	**NE**	**1506.74 ± 242.56**[a]	**677.04 ± 270.98**[b]	**1601.70 ± 363.18**[a]	**1999.18 ± 199.16**[a]
Hypothalamus	**MHPG**	255.20 ± 75.16	109.71 ± 37.10	149.76 ± 36.53	132.55 ± 29.66
	MHPG/NE	0.20 ± 0.07	0.22 ± 0.08	0.12 ± 0.05	0.07 ± 0.02
Midbrain	**NE**	**458.34 ± 131.44**[a]	**90.37 ± 19.76**[b]	**298.40 ± 81.64**[ab]	**359.78 ± 70.65**[a]
	MHPG	128.51 ± 36.05	38.97 ± 5.37	127.43 ± 48.61	118.71 ± 29.51
	MHPG/NE	0.32 ± 0.05	0.57 ± 0.14	0.48 ± 0.17	0.33 ± 0.04
Thalamus	**NE**	541.46 ± 114.31	323.48 ± 85.27	564.59 ± 85.76	568.14 ± 64.15
	MHPG	180.27 ± 87.68	66.53 ± 25.23	204.48 ± 69.02	96.31 ± 25.62
	MHPG/NE	0.33 ± 0.12	0.27 ± 0.08	0.37 ± 0.10	0.17 ± 0.03
Globus pallidus	**NE**	145.64 ± 37.91	142.96 ± 40.90	120.67 ± 17.62	86.38 ± 21.75
	MHPG	**293.70 ± 98.09**[a]	**58.51 ± 4.53**[b]	**124.25 ± 34.76**[ab]	**181.34 ± 35.59**[ab]
	MHPG/NE	1.94 ± 0.49	0.72 ± 0.24	1.10 ± 0.31	3.68 ± 1.46

Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test.

Table 2 The levels of dopamine (DA), 3-methoxytyramine (DOPAC), homovanillic acid (HVA), the ratio of DOPAC/DA and HVA/DA in the amygdala, frontal cortex, hippocampus, nucleus accumbens, caudate putamen, septum, anterior hypothalamus, midbrain, thalamus and globus pallidus of the ovariectomized rat treated with vehicle (Ovx), estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg).

Brain area	Neurotransmiters	Ovx	E_2 (1 μg/kg)	Gen (0.25 mg/kg)	Dai (0.25 mg/kg)
Amygdala	**DA**	84.13 ± 26.84	18.95 ± 4.49	76.61 ± 35.06	83.91 ± 48.27
	DOPAC	33.90 ± 8.51	6.65 ± 1.27	25.54 ± 6.47	3.10 ± 10.55
	HVA	30.54 ± 3.78	31.58 ± 5.67	19.94 ± 7.06	36.69 ± 6.15
	DOPAC/DA	0.56 ± 0.20	0.37 ± 0.05	0.54 ± 0.16	0.67 ± 0.25
	HVA/DA	0.84 ± 0.56	2.28 ± 0.46	0.61 ± 0.26	1.16 ± 0.52
Frontal cortex	**DA**	31.43 ± 7.28	15.51 ± 3.42	27.01 ± 6.11	17.72 ± 4.15
	DOPAC	12.42 ± 2.88	4.74 ± 0.67	15.03 ± 4.52	10.01 ± 4.82
	HVA	30.35 ± 8.78	21.92 ± 1.33	25.12 ± 4.96	29.92 ± 4.35
	DOPAC/DA	0.45 ± 0.11	0.39 ± 0.06	0.56 ± 0.07	0.43 ± 0.11
	HVA/DA	1.05 ± 0.22	1.64 ± 0.23	1.17 ± 0.24	1.93 ± 0.44
Hippocampus	**DA**	24.16 ± 2.93	12.50 ± 2.24	20.74 ± 12.03	20.87 ± 8.48
	DOPAC	7.07 ± 2.24	5.05 ± 1.30	10.82 ± 6.04	9.93 ± 2.39
	HVA	33.04 ± 6.55	23.58 ± 7.53	22.37 ± 6.73	23.77 ± 7.96
	DOPAC/DA	0.34 ± 0.13	0.49 ± 0.17	0.49 ± 0.08	0.60 ± 0.10
	HVA/DA	1.39 ± 0.38	1.70 ± 0.58	1.78 ± 0.86	1.42 ± 0.71
Nucleus	**DA**	1048.38 ± 337.44	150.16 ± 81.50	517.12 ± 260.74	517.18 ± 198.48
Accumbens	**DOPAC**	388.97 ± 124.56	78.77 ± 41.39	278.1 ± 100.56	221.73 ± 80.14
	HVA	276.49 ± 52.02	228.16 ± 28.20	238.82 ± 39.76	244.09 ± 29.83
	DOPAC/DA	0.36 ± 0.07	0.64 ± 0.13	0.73 ± 0.23	0.49 ± 0.06
	HVA/DA	0.54 ± 0.23	3.99 ± 1.96	1.67 ± 1.10	2.91 ± 1.64
Caudate Putamen	**DA**	1792.5 ± 423.98	653.54 ± 389.49	1991.05 ± 524.46	2014.15 ± 733.27
	DOPAC	260.30 ± 87.15	218.61 ± 108.86	459.02 ± 176.77	385.47 ± 135.05
	HVA	**202.80 ± 60.96**[a]	**508.49 ± 43.93**[b]	**400.85 ± 58.60**[b]	**385.03 ± 70.12**[b]
	DOPAC/DA	0.14 ± 0.02	0.47 ± 0.13	0.24 ± 0.07	0.26 ± 0.06
	HVA/DA	0.39 ± 0.29	3.14 ± 1.24	0.32 ± 0.11	1.23 ± 0.92
Septum	**DA**	**655.01 ± 52.18**[a]	**225.37 ± 103.65**[c]	**311.66 ± 68.35**[bc]	**547.83 ± 146.04**[ab]
	DOPAC	144.91 ± 2.00	107.44 ± 55.24	107.09 ± 22.32	161.15 ± 32.97
	HVA	131.74 ± 23.23	154.26 ± 16.28	100.63 ± 16.46	149.16 ± 16.12
	DOPAC/DA	0.23 ± 0.04	0.52 ± 0.16	0.39 ± 0.09	0.33 ± 0.04
	HVA/DA	**0.21 ± 0.05**[a]	**3.06 ± 1.26**[b]	**0.44 ± 0.12**[a]	**0.42 ± 0.12**[a]

Table 2 (continue)

Brain area	Neurotransmiters	Ovx	E_2 (1 µg/kg)	Gen (0.25 mg/kg)	Dai (0.25 mg/kg)
Anterior	**DA**	204.04 ± 48.27	157.65 ± 105.53	127.12 ± 45.88	223.01 ± 57.68
Hypothalamus	**DOPAC**	49.28 ± 12.40	66.18 ± 39.90	42.49 ± 13.10	75.75 ± 29.00
	HVA	35.02 ± 9.60	55.36 ± 13.59	47.28 ± 26.29	53.68 ± 17.52
	DOPAC/DA	0.27 ± 0.06	0.59 ± 0.30	0.47 ± 0.16	0.34 ± 0.06
	HVA/DA	**0.27 ± 0.13**[a]	**1.51 ± 0.43**[b]	**0.24 ± 0.01**[a]	**0.40 ± 0.16**[a]
Midbrain	**DA**	30.48 ± 7.28	13.50 ± 1.60	37.27 ± 7.10	25.50 ± 5.99
	DOPAC	13.06 ± 2.35	2.89 ± 0.65	14.67 ± 6.05	11.51 ± 3.14
	HVA	22.58 ± 4.44	15.63 ± 2.36	20.67 ± 4.62	23.37 ± 2.77
	DOPAC/DA	0.49 ± 0.10	0.25 ± 0.05	0.37 ± 0.12	0.43 ± 0.09
	HVA/DA	0.83 ± 0.17	1.23 ± 0.25	0.61 ± 0.10	1.31 ± 0.51
Thalamus	**DA**	37.43 ± 6.76	17.92 ± 1.80	28.18 ± 5.35	40.20 ± 8.36
	DOPAC	10.11 ± 4.65	5.89 ± 1.54	16.70 ± 5.39	16.13 ± 8.07
	HVA	42.89 ± 7.35	202.08 ± 170.94	22.04 ± 11.22	36.35 ± 7.30
	DOPAC/DA	0.29 ± 0.19	0.34 ± 0.15	0.46 ± 0.07	0.38 ± 0.09
	HVA/DA	1.06 ± 0.40	9.56 ± 8.20	0.67 ± 0.25	1.32 ± 0.37
Globus pallidus	**DA**	1766.60 ± 578.16	347.32 ± 180.60	1195.87 ± 403.89	1544.04 ± 511.33
	DOPAC	205.29 ± 68.05	83.00 ± 43.58	175.47 ± 47.66	253.87 ± 102.15
	HVA	245.95± 48.56	304.73 ± 55.16	196.28 ± 31.58	525.62 ± 178.94
	DOPAC/DA	0.11 ± 0.01	0.27 ± 0.06	0.16 ± 0.02	0.19 ± 0.05
	HVA/DA	0.59 ± 0.45	2.47 ± 0.87	0.32 ± 0.15	1.44 ± 0.97

Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test.

DISCUSSION

The effects of estrogen and phytoestrogens on the body weight and food intake

In this study, the body weight of estrogen-treated rats was lowered than Ovx; while, the body weight of genistein- or daidzein- treated rats were not different from Ovx. The body weight gain of estrogen-treated rats was decreased in a dose dependent manner and this change was not due to the different in food intake. The reduced in body weight gain in estrogen-treated rats may be caused by the activation of ERα. As Heine et al. (2000) and Musatov et al. (2007) have shown that lacking ERα in knockout mice or silencing of ERα in the ventromedial hypothalamus in rats produced a decrease in basal metabolic rate and an increase in respiratory quotient reflecting a reduction in the efficiency of fatty acid oxidation. This was also evident by the increase in visceral fat accumulation in Ovx rats. Further, it had been shown that the anorexic effect can be induced by injection of ERα agonist (Santollo et al., 2007). In the current study, the food intake was not affected by either estrogen or phytoestrogens; therefore, it is likely that estrogen may activate ERα and resulting in higher basal metabolic rate. The reason that phytoestrogens like genistein and daidzein had no effect may probably due to the binding selectivity of these substances that it prefers ERβ over ERα. However, it was recently shown that phytoestrogen-rich diet could increase energy expenditure and decrease adiposity in mice (Cederroth et al., 2007) but it may require a higher dose than used in this study to induce this effect.

The effects of estrogen and phytoestrogens on the serum estradiol and uterine weight

In the current study, the levels of serum estradiol in estrogen-treated rat were significantly different only at the higher dosage used (100 μg/kg) and phytoestrogens had no effect on serum estradiol (data not shown). Despite the levels of serum estradiol from the dosages of 1 to 50 μg/kg was not different from vehicle treated Ovx rats; we did find a positive correlation between the doses and the levels of serum estradiol. This lack of significance could have due to the sensitivity of the method used in this current study. Although the levels of estradiol was not reflected the levels of estrogen replacement, the uterine weight was indeed increased in a dose dependent manner. It is likely that uterine weight is a better indicator of estrogen reaching target organ than the levels of serum estradiol. In the genistein group, the uterine weight was increased in corresponding to the doses of genistein; only at the lower two

doses that not different from Ovx group. The increase in uterine weight following genistein treatment has been shown by others although with a higher magnitude (Diel et al., 2006; Rimoldi et al., 2007). This difference may be due to the dosages (>10 mg/kg) and the length of administration (3 months). While genistein had effect on uterine weight, daidzein at the same dosage had no significant effect on the uterine weight. However, it should be noted that daidzein was tended to increased uterine weight in a dose dependent manner. And thus longer usage or the higher dosage of daidzein may also increase the uterine weight in these animals.

The uterotrophic effect of estrogen in the estrogen-treated Ovx rats may be signaling through Insulin-like growth factor 1 receptor -mediated pathway as shown by Zhu and Pollard (2007). From the ER localization study, the ERα was found mainly in the reproductive tissues (Usui, 2006); and from the binding efficacy of estrogen and phytoestrogen to ER, it has been shown that estrogen preferably binds to ERα while phytoestrogen prefers ERβ (Kuiper et al., 1998; Terreaux et al., 2003; Zierau et al., 2006). According to these findings, it could be that estrogen may activate ERα and resulting in uterine cell proliferation in the estrogen treated rats. In the genistein and daidzein groups, although uterine weight was increased in a very small magnitude, it was significantly different or tended to different from Ovx rats. This may be explained by the binding efficacy of these substances that although they preferred ERβ than ERα; however, at the higher dose it may bind to ERα and activate uterine cell proliferation similar to estrogen.

Therefore, it should keep in mind that longer or higher dosages of phytoestrogen may potentially activate estrogen receptor and resulting in cell proliferation.

The effects of estrogen and phytoestrogens on the anxiety-like behavior

The anxiety-like behavior was measured utilizing elevated-T maze, the decrease in avoidance 2 latency or the increase in escape latency explicit lowered level of anxiety in relating to GAD and PD, respectively. The vehicle treated Ovx rats demonstrated both types of anxiety, confirming that lacking ovarian hormones is anxiogenic which can be reversed by estrogen replacement. It has been shown previously that estrogen contained anxiolytic effect both in clinical trials and laboratory animal studies (Diaz-Veliz et al., 1997; Seeman, 1997; Shekhar et al., 2001; Pandaranandaka et al., 2006). In the current study, we found that all doses of estrogen contained anxiolytic effect when tested with ETM, and this effect was related to the availability of estrogen reaching target organs. As the uterine weight was increased indicating the activating effect of estrogen was higher; consequently, the anxiolytic like-effect was more

pronounce. This was confirmed through the negative correlation between uterine weight and the avoidance 2 latency in the ETM. On the other hand, there was no correlation between the dosage of estrogen and the avoidance 2 latency in the ETM (P = 0.2551), this finding was previous shown by Morgan and Pfaff (2002). This further confirms that uterine weight may be a better indicator of estrogen reaching its target organ and performing the associated physiological functions. Therefore, it is likely that the anxiolytic effect of estrogen, as indicated by the decrease in the avoidance 2 latency in the ETM found in this study, was related to the function of estrogen at the target organs.

As estrogen is able to reduce anxiety, it is then unsurprised that phytoestrogen like genistein and daidzein has similar effect. However, the effects were somewhat different. For genistein, there was no correlation between the dose and any the behavioral indices. Moreover, only the dosage of 0.25 mg/kg of genistein contained anxiolytic effect in the ETM, while the anxiety level was likely to increase as the higher doses of genistein were used. For daidzein, the effects were different; it contained anxiolytic effect in the ETM and the anxiety level was corresponded to the dose. The higher the dose of daidzein, the shortened avoidance 2 latency was achieved indicating that if daidzein levels were high then the rats would be less anxious. Previously, the effect of phytoestrogen on anxiety had been reported (Hartley et al.,2003; Lephart et al., 2002; 2004); both anxiogenic and anxiolytic were demonstrated. The differences between theirs and this study were the substance, route and length of administration. They used the phytoestrogen diet with varies level of phytoestrogen; however, amount of food consumption and the intestinal bacterial had to taken into account. On the other hand, we used pure substance with calculated given amount, so the effect was rather specific to each substance.

Beside it is now recognized that estrogen exert its anxiolytic effect through the ERβ (Krezel et al., 2001; Walf and Frye, 2005; 2007). However, from the immunohistochemical study, it was demonstrated that ERβ was indeed co-localized with ERα in the central nervous system (Shughrue et al., 1998). In which the ERα and ERβ were shown to signal in opposite ways (Paech et al., 1997); and it also showed that the estrogen can exert its effect through homodimerization or heterodimerization between two subtypes (Cowley et al., 1997). These may result in various outcomes. It is now known that phytoestrogen is more selective to ERβ than ERα while the estrogen prefers ERα than ERβ (Kuiper et al., 1998; Terreaux et al., 2003). However, different phytoestrogens also differed in its binding affinity; the relative binding affinity of ERβ to ERα was shown to be 20 and 5 fold for genistein and daidzein, respectively (Kuiper et al., 1998). Not only the difference in binding affinity that account for the action of phytoestrogen but estrogenic potency has to be taken too. Kuiper et al. (1998) reported the

ranking of estrogenic potency of phytoestrogens for both ER subtypes in the transactivation assay; that is E_2>>>genistein>daidzein for ERα and E_2>>>genistein>>daidzein. From all of above, we then postulated that genistein at the low dose (0.25 mg/kg) may activate ERβ and resulting in anxiolytic like effect. However, the higher dose of genistein may activate ERα and resulting in opposing effect. For daidzein, although the dose ranges were similar to that of genistein but it was shown to be anxiolytic in all three behavioral models. This may be explained by the fact that its estrogenic potency was less than genistein about 50%; therefore, the dosages used in this study may not activate the ERα.

The effects of estrogen and phytoestrogens on the monoaminergic neurotransmitter

The relationship between estrogen and the serotonergic system has been widely studied. It has been shown that estrogen may modulate this system by influencing synthesis, release, reuptake, or catabolism of the neurotransmitter (McQueen et al., 1997; McEwen et al., 2002; Lu et al., 2003). In this study, the levels of 5-HT, 5-HIAA and the ratio of 5-HIAA to 5-HT were changed in all examined area but significant different only in hippocampus. In the hippocampus the turnover rate as determined by the ratio of 5-HIAA/5-HT in the estrogen-, genistein- and daidzein-treated rats were higher than vehicle-treated rats. However, it is likely that the mechanism of action is differed. Previously, Pandaranandaka et al. (2006; 2008) demonstrated that estrogen at the dosage of 10 μg/kg contained anxiolytic effect in both EPM and ETM behavioral models by decreasing the level of 5-HT in specific brain areas which may responsible for the similar finding in this study. This finding was similar to some and different from some other studies; Zhang and co-workers (1999) reported that ovariectomy decreased the hippocampal 5-HIAA/5-HT ratio when compared with sham-operated rats while other studies reported that 5-HT turnover rate was not significantly affected by estrogen (Renner et al., 1986; Maswood et al., 1995). The reduction of 5-HT in the estrogen treated rat may be caused by the decrease in tryptophan hydroxylase enzyme, the rate limiting enzyme for the synthesis of 5-HT in the midbrain as shown by Pandaranandaka et al. (2008). This may play an important role in the anxiolytic effect of estrogen by decreasing the synthesis capacity for 5-HT in the midbrain and thereby reducing serotonergic input to specific forebrain projection areas. As Dos Santos and coworkers (2008) have showed that the anxiogenic effect mediated through 5-HT_{1A} receptors in the dorsal hippocampus was induced by increasing the activity of 5-HT neuron in the median raphe nuclei. However, for the genistein and daidzein groups, the higher turnover rate was not likely to be due to the reduction in 5-HT level but the increase in the activity of 5-

HT, as shown by the increase in 5-HIAA level in hippocampus. This finding is somewhat conflict with the study of Dos Santos et al. (2008). Interestingly, while the levels of 5-HT and its metabolite were changed throughout the brain, the level of serotonin transporter protein (SERT) was unaffected. Similarly, the previous study from our laboratory utilizing higher dose of estrogen demonstrated similar finding (Pandaranandaka et al., 2008) and in consistent with the SERT mRNA level which was unaffected (Zhou et al., 2002a); however, they reported that given estrogen for 21 days after ovariectomy can reduce SERT mRNA in midbrain (Zhou et al., 2002b). Then, it may be that the longer term of estrogen replacement (i.e. 28 days) may allow the body to adjust and thus the difference was no longer demonstrated.

The dopaminergic system has also been implicated in anxiety (Pitchot et al., 1992; Dazzi et al., 2001). The role of DA in anxiety has focused on DA mechanisms in amygdala, nucleus accumbens, septum, hippocampus, and frontal cortex, all of which are target structures of mesolimbic and mesocortical pathways of the dopaminergic system originating from the ventral tegmental area (Horvitz, 2000). Several studies have shown that anxiety-provoking situations caused a rise either in the release of DA or in its metabolites levels in the nucleus accumbens (Dunn et al., 1983; Imperato et al., 1992; McCullough et al., 1992; Ge et al., 1997). Further, inactivation of the dopaminergic system in the D3 knockout mice demonstrated anxiolytic effects in behavioral tests, including the open field and the EPM (Steiner et al., 1997). In addition to serotonergic and dopaminergic systems, the noradrenergic system has a role in modulating anxiety as evidenced in both clinical and animal studies. Patients who received a α2-adrenergic receptor antagonist produced higher levels of NE in the synapse, which may have been responsible for eliciting panic-like anxiety (Gurguis and Uhde, 1990). Similar effects were reproduced in rats (Tanaka et al., 2000). Conversely, the administration of a α2-adrenergic receptor agonist produced less anxiety and fear (Charney and Heninger, 1986; Uhde et al., 1989; Abelson et al., 1992). Further, in α2A knockout mice, higher levels of anxiety were found (Lahdesmaki et al., 2002). Therefore, it may be concluded that higher activity of dopaminergic and/or noradrenergic systems would lead to anxiety-like behavior. Previously, we have shown that the more anxious Ovx rat had higher levels of dopamine and norepinephrine in the nucleus accumbens and the caudate putamen, respectively when compared to estrogen treated rat (Pandaranandaka et al., 2006). In the current study, the levels of NE in the frontal cortex, hippocampus, ant. hypothalamus and the midbrain of E_2 supplemented rat were significant lower than Ovx counterparts; moreover, the DA level in the septum of E_2 treated rat was also lower with trend in other areas. These findings indicated that in addition to 5-HT, DA and NE also play a part in regulating anxiety in these animals.

For the phytoestrogens, we found that the modulatory action of genistein and daidzein were somewhat different from that of estrogen. These findings were rather interesting as from the binding property, both genistein and daidzein preferred ERβ than ERα, while estrogen preferred ERα than ERβ. However, this data indicated that genistein and daidzein had different action modulating neurotransmitters levels. One possible explanation is that the differences in binding affinity and the transactivation potency between genistein and daidzein. For the binding affinity, although both prefer ERβ than ERα but the binding affinity of genistein to ERβ and ERα is about 14 fold higher than those of daidzein; for the transactivation activity, genistein can activate both ERα and ERβ better than daidzein (Kuiper et al., 1998). Moreover, Patisaul et al. (2002) demonstrated that genistein increased ERβ mRNA expression in the hypothalamus. This modulatory effect of genistein in the brain may be not limited to the hypothalamus and this change in ER may lead to changes in behavior and neurotransmitter. Further studies are required to determine whether genistein and daidzein can modulate the ER expressions in specific brain areas related to anxiety-like behaviors.

From all above, we concluded that genistein and daidzein at a suitable dose can be used as an alternative for estrogen to preserve anxiolytic effect. They may be safer than estrogen as they are unlikely to activate the growth of cell in the reproductive organ; however, if the higher dose to be used it may then activate the uterine growth. Further, the mechanism of phytoestrogens on regulating anxiety is somewhat different from those of estrogen.

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The Modulation of Monoaminergic Neurotransmitters by Genistein and Daidzein in Ovariectomized Rat

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Introduction

A number of studies have revealed a role of hormone estrogen on the regulation of brain neurotransmitters. Previously, we have shown that monoaminergic neurotransmitters in various brain areas were changed following chronic treatment of estrogen (1). These changes were probably mediated through estrogen receptor; subtype alpha (ERα) and beta (ERβ). Recently, many researchers utilizing specific agonists and antagonists have shown that ERβ plays a major role in brain (2, 3). Further, from the localization studies, ERβ was more prominent in brain while ERα was expressed more in reproductive organs (4). Genistein and Daidzein are phytoestrogen found mainly in soy bean. They can bind to estrogen receptor like estrogen and thus exert their effects. However, they preferably bind to ERβ than ERα (4). Although estrogen can be clinically given as a replacement therapy in post-menopausal women; it has been shown that long term estrogen may be a predisposal cause of ovarian, uterine or breast cancers (5-7). Further, it may not be given to patients with a history of hormone dependent cancers.

We then hypothesized that the genistein and daidzein bind to ERβ in the brain and therefore change the levels of monoaminergic neurotransmitter.

Materials and Methods

Animals and Treatments: Female Wistar rats (180-200 g) were ovariectomized and randomly assigned into 4 groups receiving daily treatment of vehicle, estrogen (E_2; 1 μg/kg), genistein (Gen; 0.25 mg/kg) or Daidzein (Dai; 0.25 mg/kg). Four weeks following ovariectomy, all rats were sacrificed; their brains were rapidly removed, frozen in liquid nitrogen, and stored at -20°C.

Monoamine determinations: On the day of the assay, brains were isolated into regions known to contribute to anxiety, by following the instructions of Heffner et al. (8). These isolated brain regions were sonicated in cold 0.1 M perchloric acid, containing 3, 4-dihydroxybenzylamine hydrobromide as an internal standard. The sample was collected for analysis of dopamine (DA) and its metabolites DOPAC and HVA; norepinephrine (NE) and its metabolite MHPG; and serotonin (5-HT) and its metabolite 5-HIAA using HPLC-ECD. Chromatographic separations were performed using 15-cm phenomenex® (Torrance, CA) column packed with 5-μmparticles. The mobile phase solution was composed of 1 mM heptane sulfonate, 100 mM sodium dihydrogen phosphate, 1 mM Na2·-EDTA and 5% methanol, adjusted to pH 4.1 with saturated citric acid. The position and height of the peaks in tissue homogenates were measured and compared to an external calibrating standard solution containing DA, DOPAC, HVA, NE, MHPG, 5-HT, and 5-HIAA (Sigma, St. Louis, MO, USA). Concentrations of these substances in the samples were calculated and expressed as ng/g tissue. The activity (turnover) of the dopaminergic, noradrenergic, and serotonergic systems was expressed as ratios of DOPAC/DA, HVA/DA, MHPG/NE, and 5-HIAA/5-HT.

Statistical analysis: The data are expressed as mean ±SEM and evaluated with one-way ANOVA followed by Duncan's multiple comparison tests.

Results and Discussion

The chromatogram of monoaminergic neurotransmitters is shown in Fig. 1., the retention times of NE, MHPG, DHBA, DOPAC, DA, 5-HIAA, HVA, 5-HT are approximately at 4.74, 7.28, 10.12, 14.61, 18.61, 32.63, 43.82 and 69.76 min., respectively. The analysis of neurotransmitters from the brains of ovariectomized rats treated with vehicle control, estrogen, genistein or daidzein for 4 weeks demonstrated that there were changes in the neurotransmitters throughout the brain. In frontal cortex and midbrain, the level of NE was differed between groups ($p<0.05$) with the highest level in vehicle, followed by daidzein, genistein and estrogen groups. In the nucleus accumbens and globus pallidus, the MHPG levels were high in the vehicle group and significantly different from estrogen group. In the septum, the DA level in the daidzein group was highest and different from genistein and estrogen, but not different from vehicle group. In caudate putamen, the HVA level was higher in the estrogen and genistein and different from the vehicle group. Moreover, we also found trends of changes in other areas ($p<0.10$) such as NE and MHPG in hippocampus, NE in anterior hypothalamus, DA in thalamus and DA in globus

pallidus. From the HPLC data indicated that in estrogen treated rats, the levels of NE, MHPG, DA or HVA were either significant lower or higher than those of vehicle group. This data suggested that estrogen was able to modulate a process involved with neurotransmitters synthesis, release or degradation. For the phytoestrogens, we found that the modulatory action of genistein was somewhat similar to estrogen, while daidzein was somewhat different. These findings were rather interesting as from the binding property, both genistein and daidzein preferred ERβ than ERα, while estrogen preferred ERα than ERβ. However, this data indicated that genistein and daidzein had different action modulating neurotransmitters levels. One possible explanation is that the differences in binding affinity and the transactivation potency between genistein and daidzein. For the binding affinity, although both prefer ERβ than ERα but the binding affinity of genistein to ERβ and ERα is about 14 fold higher than those of daidzein; for the transactivation activity, genistein can activate both ERα and ERβ better than daidzein (9).

In conclusion, this study has shown that genistein and daidzein can modulate neurotransmitters in various brain regions; however, further studies need to be done to elucidate the mechanism of action and to correlate these changes to other physiological responses.

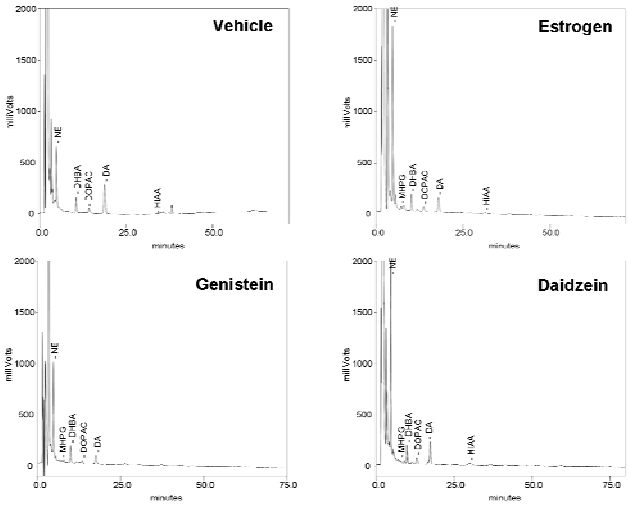

Fig. 1 The chromatogram of NE, MHPG, DA, DOPAC, HVA, 5-HT, 5-HIAA) and DHBA as an internal standard from the rat brains treated with vehicle, estrogen, genistein or daidzein. (Depicted from ant. Hypothalamus extraction).

Acknowledgement

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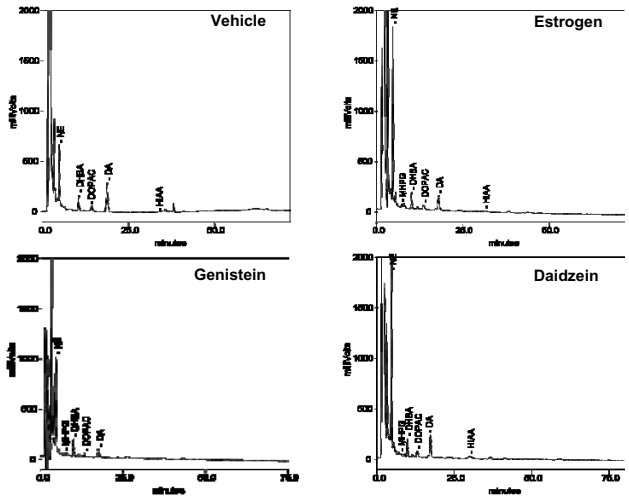

Figure 1: The chromatogram of NE, MHPG, DA, DOPAC, HVA, 5-HT, 5-HIAA) and DHBA as an internal standard from the rat brains treated with vehicle, estrogen, genistein or daidzein. (Depicted from ant. Hypothalamus extraction). The retention times of NE, MHPG, DHBA, DOPAC, DA, 5-HIAA, HVA, 5-HT are approximately at 4.74, 7.28, 10.12, 14.61, 18.61, 32.63, 43.82 and 69.76 min., respectively.

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