รายงานการวิจัยฉบับสมบูรณ์

"การใช้สารสกัดจากถั่วเหลือง (เจนิสเตอีน และไดด์ดีซิน) ในการเปลี่ยนแปลงภาวะความวิตกกังวล โดยใช้หนูขาววิสตาร์เป็นต้นแบบศึกษา"

The Usage of Soy Bean Extracts (Genistein and Daidzein) to Modulate Anxiety Disorder: Using Wistar Rat as a Model

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ศ. นพ. อนันต์ ศรีเกียรติขจร	ที่ปรึกษาโครงการ
รศ. น.สพ. ดร. บุญฤทธิ์ ทองทรง	ผู้วิจัยร่วม
น.ส. ศิริเพ็ญ โกมลวานิช	ผู้วิจัยร่วม

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กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "การใช้สารสกัดจากถั่วเหลือง (เจนิสเตอีน และไดด์ดีซิน) ในการเปลี่ยนแปลงภาวะความวิตกกังวล โดยใช้หนูขาววิสตาร์เป็นต้นแบบศึกษา" The Usage of Soy Bean Extracts (Genistein and Daidzein) to Modulate Anxiety Disorder: Using Wistar Rat as a Model" ได้รับทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล ประจำปีงบประมาณ 2552

บทคัดย่อ

ชื่อโครงการ: การใช้สารสกัดจากถั่วเหลือง (เจนิสเตอีน และไดด์ดีซิน) ในการเปลี่ยนแปลงภาวะความวิตกกังวล โดยใช้หนูขาววิสตาร์เป็นต้นแบบศึกษา"

ไฟโตเอสโตรเจน เช่น เจนิสเตอีนและไดด์ดีซีน เป็นสารที่สามารถพบได้ในพืชตระกูลถั่ว ทั้งนี้สารดังกล่าวมีลักษณะโครงสร้างคล้ายเอสโตรเจน ซึ่งจากการศึกษาที่ผ่านมาพบว่าภาวะวิตกกังวลเป็นปัญหาด้านสุขภาพจิตที่พบในเพศหญิงมากกว่าเพศชาย และจากรายงานทางคลินิกและการทดลองในสัตว์ พบว่าการขาดฮอร์โมนเอสโตรเจนเป็นปัจจัยที่สำคัญ อย่างไรก็ตามการให้ฮอร์โมนเอสโตรเจนนั้นมีข้อจำกัดในทางคลินิก ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารไฟโตเอสโตรเจนได้แก่ เจนิสเตอีน และไดด์ดีซีน ในการลดความกังวลในหนูทดลองที่ถูกเหนี่ยวนำให้เกิดความกังวลด้วยการตัดรังไข่เปรียบเทียบกับเอสโตรเจน และเพื่อศึกษาผลของสารดังกล่าวในการเปลี่ยนแปลงระดับความกังวลในหนูเพศผู้ โดยการทดลองแบ่งออกเป็น 2 ตอน ตอนที่ 1 ทำการศึกษาในหนูเพศเมียโดยการเหนี่ยวนำให้ขาดฮอร์โมนเอสโตรเจนโดยการตัดรังไข่ หาระยะเวลาที่ทำให้หนูเกิดความกังวล และจากนั้นศึกษาว่า เอสโตรเจน เจนิสเตอีน หรือไดด์ดีซีนสามารถแก้ไขความผิดปกติที่เกิดขึ้นได้หรือไม่ ผลการศึกษาพบว่า การตัดรังไข่เป็นระยะเวลาอย่างน้อย 3 สัปดาห์สามารถเหนี่ยวนำให้หนูเกิดความกังวลเมื่อทำการวัดด้วยอุปกรณ์ทดสอบพฤติกรรม elevated T-maze ทั้งนี้หนูที่ตัดรังไข่และได้รับเอสโตรเจนทดแทนที่ระยะเวลาต่างๆ (7, 14, 21 และ 28 วัน) ไม่พบว่ามีความกังวลแตกต่างกัน เมื่อทำการทดสอบผลของเจนิสเตอีน (0.25 มก./กก.) และไดด์ดีซีน (0.25 มก./กก.) เทียบกับเอสโตรเจน (1 ไมโครกรัม/กก.) ในการลดความกังวลในหนูที่ถูกเหนี่ยวนำให้เกิดความกังวลโดยการให้สารต่างๆ เป็นเวลา 28 วัน ภายหลังจากการตัดรังไข่ 21 วัน พบว่าเจนิสเตอีนสามารถลดระดับความกังวลได้ไม่ต่างจากเอสโตรเจน ในขณะที่ไดด์ดีซีนสามารถลดความกังวลได้ไม่ต่างจากเจนิสเตอีนและเอสโตรเจน แต่พบว่าค่าความแตกต่างไม่มากพอที่จะทำให้เกิดความแตกต่างทางสถิติจากกลุ่มควบคุม โดยเจนิสเตอีนหรือไดด์ดีซีนไม่มีผลต่อน้ำหนักตัว หรือน้ำหนักมดลูกเมื่อเทียบกับกลุ่มควบคุม ตอนที่ 2 ทำการศึกษาถึงผลของเจนิสเตอีนหรือไดด์ดีซีนที่มีต่อระดับความกังวลในหนูเพศผู้ พบว่าการให้เจนิสเตอีน ขนาด 1 มก./กก. เป็นเวลา 5 สัปดาห์ มีแนวโน้มที่จะทำให้หนูมีความกังวลเพิ่มมากขึ้น และทำให้น้ำหนักของหนูเพิ่มขึ้นน้อยกว่าการได้รับเจนิสเตอีนขนาด 0, 0.25 และ 0.50 มก./กก โดยเจนิสเตอีนขนาดต่างๆ ไม่มีผลต่อการเปลี่ยนแปลงน้ำหนักอวัยวะสืบพันธุ์ ได้แก่ อัณฑะ อิพิดิไดมิส พรอสเตทแกลนด์ และเซมินอล เวสซิเคิล สำหรับผลของไดด์ดีซีนนั้นพบว่า การให้ไดด์ดีซีน ขนาด 0.25 มก./กก. เป็นเวลา 5 สัปดาห์ สามารถลดความกังวลในหนูกลุ่มนี้ได้ และถ้าให้ในขนาดที่สูงขึ้น (0.50 -1.00 มก./กก.) จะเป็นการเพิ่มระดับความกังวล ทั้งนี้ไดด์ดีซีนไม่มีผลต่อการเปลี่ยนแปลงน้ำหนักตัว หรือน้ำหนักอวัยวะสืบพันธุ์ แต่มีแนวโน้มว่าการใช้ได์ดีซีนในระดับสูง (1.00 มก./กก.) อาจมีผลต่อการทำงานของพรอสเตทแกลนด์เนื่องจากมีแนวโน้มที่จะมีน้ำหนักลดลง จากการทดลองสามารถสรุปได้ว่าการตัดรังไข่ทิ้งไว้เป็นเวลา 3 สัปดาห์ทำให้หนูเกิดความกังวลขึ้นได้ โดยเจนิสเตอีนสามารถแก้ไขความกังวลที่เกิดขึ้นดังกล่าวได้เมื่อให้ติดต่อกันเป็นเวลา อย่างน้อย 4 สัปดาห์ โดยไม่มีผลต่ออวัยวะสืบพันธุ์ ในขณะที่การให้ไดด์ดีซีนนั้นอาจจะต้องให้เป็นระยะเวลาที่นานขึ้น หรือให้ในระดับที่สูงขึ้น สำหรับในเพศผู้นั้นเป็นที่น่าสังเกตว่าสารดังกล่าวมีผลต่อความกังวลที่แตกต่างกัน โดยเจนิสเตอีนในระดับสูงทำให้มีความกังวลเพิ่มมากขึ้น ในขณะที่ได์ดีซินในระดับต่ำสามารถลดความกังวลได้ จึงเป็นที่น่าสนใจว่าเจนิสเตอีน หรือไดด์ดีซีนอาจมีการทำงานที่แตกต่างกัน เนื่องจากความสามารถในการจับกับตัวรับเอสโตรเจนแตกต่างกัน และการให้ในหนูเพศผู้ปกตินั้นเจนิสเตอีนอาจไปมีผลรบกวนการทำงานของฮอร์โมนเอสโตรเจน และ/หรือเทสโทสเตอโรน และทำให้เกิดความกังวลขึ้นได้

คำหลัก: ความวิตกกังวล, ไดด์ดีซีน, เอสโตรเจน, เจนิสเตอีน, หนูเพศผู้, หนูเพศเมียตัดรังไข่

Abbreviation

µg	microgram
Dai	daidzein
E2	estrogen
EPM	elevated plus-maze
ER	estrogen receptor
ETM	elevated T-maze
Gen	genistein
kg	kilogram
mg	milligram
min	minute
Ovx	ovariectomized
s	second

สารบัญตาราง

INTRODUCTION

ANXIETY

Anxiety is a group of the most common psychiatric problems which is developed from a complex set of risk factors; including genetics, brain chemistry, personality, and life events. This symptom may lead to physiological dysfunctions such as heart palpitations, nausea, stomach aches or headaches. In 2010, the Anxiety Disorders Association of America (ADAA) reported that 18% of the United States population contract anxiety. In addition, in 2008, department of Mental Health, Ministry of Public Health, Public Health reported that anxiety disorder can be found in 1% or 600,000 Thai populations. 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). According to the fourth edition of the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association, anxiety disorder can be classified into generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic disorder, posttraumatic stress disorder (PTSD), social anxiety disorder (also called social phobia), and specific phobias (American Psychiatric Association; 2000). Anxiety has therefore become a very important area of research in psychopharmacology of this decade.

ESTROGEN AND ESTROGEN RECEPTORS

Estrogens are sex steroid hormones found abundantly in female as they are called "the woman's hormone", it mainly synthesizes in the ovaries using cholesterol as a precursor (Figure 1-1). 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).

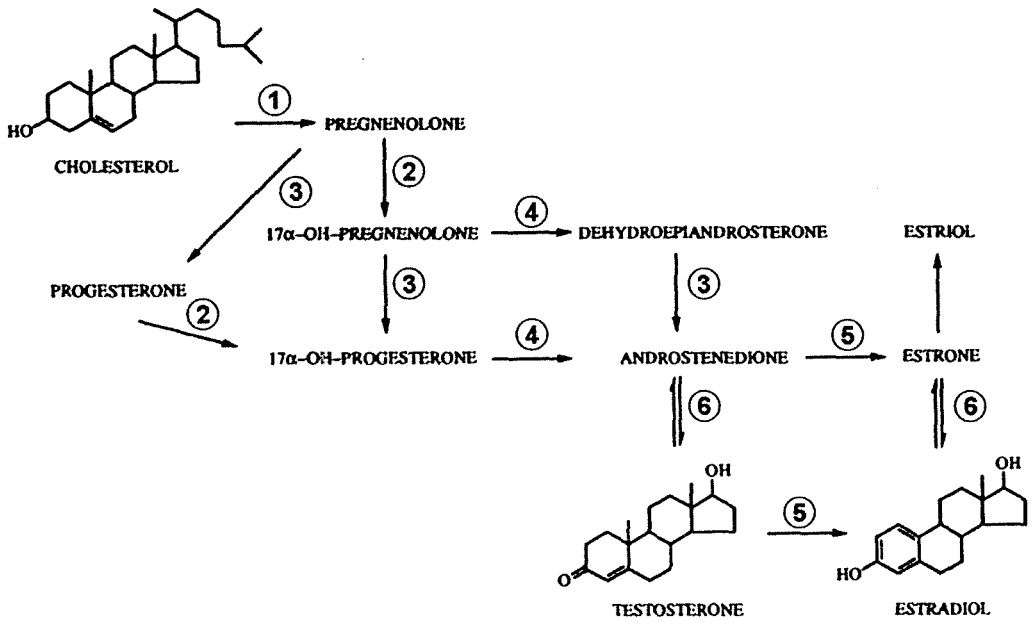


Figure 1-1 The biosynthetic pathway of estrogens. The enzymes responsible for each step are shown (①side-chain cleavage enzyme; ② 17α-hydroxylase; ③ 3β-hydroxy steroid dehydrogenase; ④ 17-20-desmolase; ⑤ aromatase; ⑥ 17β-hydroxy steroid dehydrogenase enzyme).

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). They differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain (Kuiper, et al, 1996). The ERα was expressed in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal; while ERβ was found expressed in prostate, 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). Further, ERs can also be classified into intracellular receptor and membrane estrogen receptor. Intracellular receptor or nuclear receptor acts as transcription factor and lead to genomic action such as protein synthesis. On the other hand, membrane ER contains non-genomic action; its action is rapid such as ion conductance. In the brain, estrogens can act via both receptors (Maggi et al, 2004; Gillies and Mcarthur, 2010).

Estrogens regulate many physiological effects in various tissues such as embryogenesis, development and reproduction, metabolism, hematopoiesis, cardiovascular

system, bone tissue and nervous system. In bone tissue, they can increase bone mass and lead to antiosteoporosis. In cardiovascular system, the lipid profile such as serum total, LDL, and VLDL cholesterol are increased when estrogen levels are decreased and in this case may lead to cardiovascular disease (Simpson and Davis, 2000; Yildiz, 2006). In central nervous system (CNS), estrogens modulate many functions including behavioral function such as feeding behavior, learning, memory, anxiety, arousal, aggression, emotion and sexual behavior, these functions in CNS are believed to be mediated through ERβ. In ERβ knockout mice (ERβKO), learning and memory were impaired while anxiety and aggression were increased (Simpson and Davis, 2000; Bodo and Rissman, 2006; Yildiz, 2006).

In menopause, estrogens deficiency leads to osteoporosis, cardiovascular disease, hot flushes, depression and anxiety (McEven, 2002) and these can be alleviated by estrogen replacement therapy. 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

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; 2009). Therefore, it is likely that estrogen is one factor affecting mood especially anxiety in both human and animals.

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.

Interestingly, Frye and Seliga (2001) demonstrated that in gonadectomized male rats, testosterone replacement could reduce anxiety when tested with elevated plus maze compared to control. Since testosterone can be metabolized to estrogen by aromatase, it cannot rule out whether anxiolytic effect is due to testosterone or estrogen.

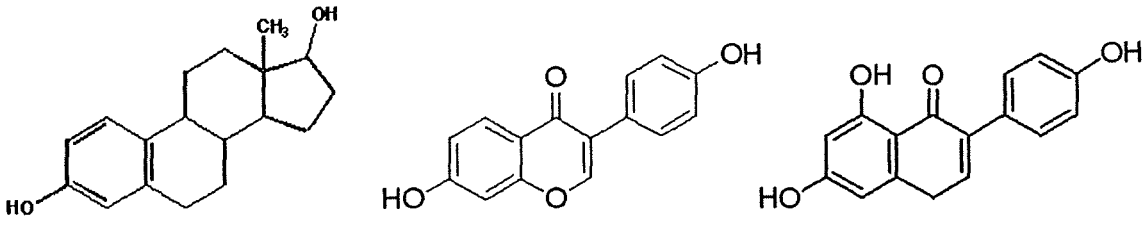
From all above, it is likely that estrogen by acting through ERβ is responsible for anxiety in female and possibly in male as well.

PHYTOESTROGENS

Phytoestrogens are bioactive compounds present in plants with a chemical structure similar to 17β-estradiol, an endogenous estrogen as shown in figure 1-2. They have hydroxyl group and phenolic group, and distance between groups similar to estrogens. 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.

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).



17β-Estradiol Genistein Daidzein

Figure 1-2 Chemical structures of 17β-estradiol, genistein and daidzein.

Phytoestrogens can exhibit both *in vitro* and *in vivo* weak estrogenic and antiestrogenic action (Murkies et al, 1998). Many data support that the cell proliferation can be induced by estrogen but not phytoestrogens. Moreover, both *in vitro* and *in vivo* studies have shown that genistein and daidzein can inhibit cancer development (Moore et al., 2007; Yavuz et al., 2007; Lavigne et al., 2008) and prevent osteoporosis in ovariectomized rats (Fanti et al., 1998; Picherit et al., 2000; Om and shim, 2007). However, some studies reported that low dose genistein and daidzein stimulated proliferation of breast cancer growth (Lemos, 2001; Helferich et al, 2008). These further support that phytoestrogens may act as estrogenic or antiestrogenic effects (Yildiz, 2006).

Phytoestrogens and anxiety

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 phytoestrogen like genistein and daidzein contain anxiogenic- or anxiolytic-like activity.

Phytoestrogen and reproductive system

In female, many data supported that PEs can affect uterine weight and cell proliferation which may lead to tumor like breast cancer (Diel et al, 2006; Jefferson et al, 2007; Helferich et al, 2008). Diel and co-workers (2006) found that genistein (10 mg/kg/d) increase uterine weight in ovariectomized rats but had no effect in intact female rats. Moreover, genistein enhanced proliferating cell nuclear antigen (PCNA; proliferation marker of epithelial cell in uterus) in ovariectomized rats compare with untreated ovariectomized rats but PCNA was decreased in genistein treatment in intact female or ovariectomized rats treated with estrogen. These suggested that genistein act as estrogenic effect in estrogen deficiency but act as antiestrogenic in estrogen sufficiency. Similarly, Jefferson and co-workers (2007) showed that given genistein (20 or 25 mg/kg) to neonatal mice increased uterine weight during adulthood. In addition, subcutaneous injection of genistein (50 mg/kg) can increase atrophic ovary while other concentrations (0.5, 5 or 25 mg/kg) can increase abnormal ovary with no atrophy. These suggest that high dose of genistein can induce infertility in adult female. However, perinatal female rat fed with diets containing 0 mg, 250 mg (low dose), and 1000 mg (high dose) daidzein/kg had no toxic effect in female reproductive tract (Lamartiniere et al., 2002).

The effect of phytoestrogens in male reproductive system is unclear. Vastag (2007) reported that soybean decreased prostate cancer. However, Pan and co-workers (2008) found that long-term treatment with high-dose daidzein (2-20 mg/kg, 90 days) induced erectile dysfunction. Moreover, both low and high levels phytoestrogens in diet can reduce germ cell and reproductive organs weight in male rodent (Weber et al., 2001; Robertson et al., 2002; Wisniewski et al., 2005; Assinder et al., 2007; Vicdan et al., 2007). However, many data reported that low dose phytoestrogens in diet had no influence on sperm quality or

male reproductive organs (Mitchell et al. 2001; Faqi et al., 2004; Wisniewski et al., 2005; Perry et al., 2007).

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 alleviated anxiety in ovariectomized rats compared to that of estrogen, measured by the behavioral models of anxiety (ETM, OF). Further, the effects of these phytoestrogens in the male rats on the anxiety level and on male reproductive organs were also investigated.

MATERIALS AND METHODS

The study was organized into two parts as follows:

Part 1: To study the effects of phytoestrogens i.e. genistein and daidzein on the anxiety-like behaviors in the ovariectomized-induced anxiety rats using behavioral model, the elevated T-maze

- To define the time required to induce anxiety in ovariectomized female rats
- To study the anxiolytic effects of phytoestrogens i.e. genistein and daidzein in ovariectomized-induced anxiety rats

Part 2: To study the effects of phytoestrogens on the anxiety-like behaviors in the male rats using behavioral model, the elevated T-maze

- To study the effects of genistein on the anxiety-like behaviors in the male rats
- To study the effects of daidzein on the anxiety-like behaviors in the male rats

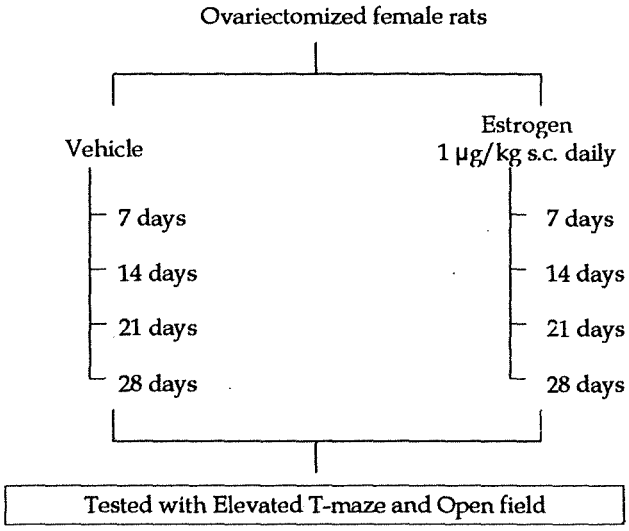
Animals

Male or 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 ($25\pm2^{\circ}C$). Standard rat chow and water were supplied *ad libitum*. After 7-day adaptation period, all female rats were bilaterally ovariectomized under anesthesia (Isoflurane; Terrell™, Minrad Inc., Bethlehem, PA, USA). Body weight and amount of food consumed were measured daily. In female rats, the uterine weight, the indication of sex hormones deficiency was determined on the day of sacrifice. In male rats, testes, epididymis, prostate gland and seminal vesicle were weighed on the day of sacrifice as indicators of hormone disruptions. 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.

Experimental protocol

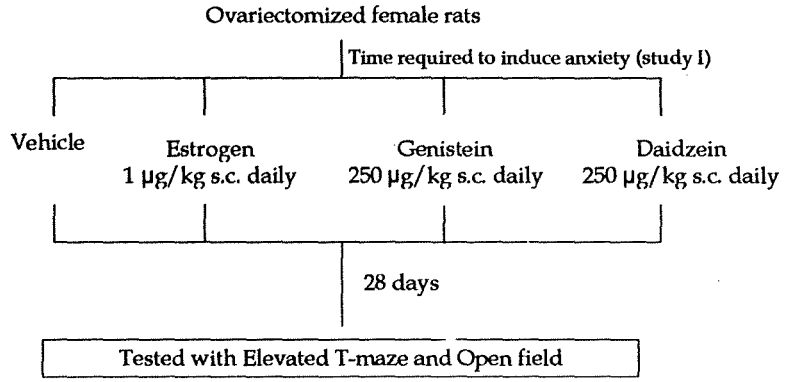
Part I To study the effects of phytoestrogens i.e. genistein and daidzein on the anxiety-like behaviors in the ovariectomized-induced anxiety rats using behavioral model, the elevated T-maze

Study 1 - To define the time required to induce anxiety in ovariectomized female rats



The ovariectomized rats were randomly assigned into 2 groups, vehicle treated- (Ovx) and estrogen treated- (E2) groups. For all rats, the chemical administration were started 2 days after ovariectomy by daily injection of 17β-estradiol (Sigma, St. Louis, MO, USA; 1 µg/kg in 10% DMSO/propylene glycol) subcutaneously into the dorsal region of the neck. Ovx groups, rats were injected by an equivalent volume of the vehicle (10% DMSO/propylene glycol). In each group, the rats were further assigned into 4 groups referring to time after ovariectomy i.e. 7, 14, 21 and 28 days in which the rats were tested with ETM and open field before sacrificed.

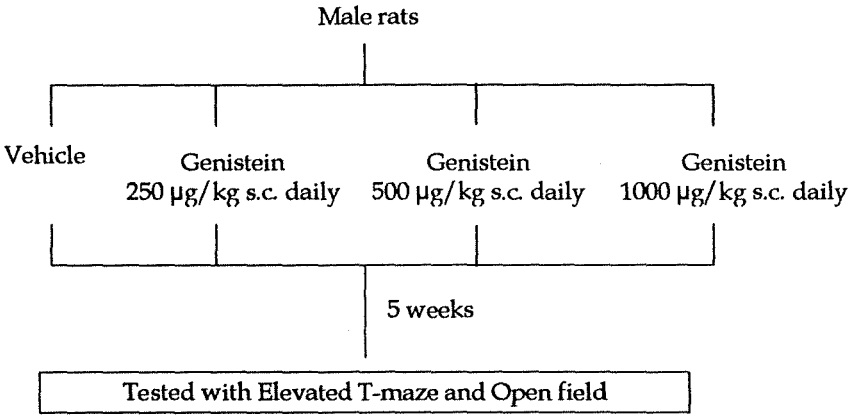
Study 2 - To study the anxiolytic effects of phytoestrogens i.e. genistein and daidzein in ovariectomized-induced anxiety rats



The rats were ovariectomized for time required to induce anxiety as determined from study 1 (21 days) then they were 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 was started 2 days after ovariectomy by daily injection of either 17β-estradiol (Sigma, St. Louis, MO, USA; 1 µg/kg), genistein (Sigma; 0.25 mg/kg) or daidzein (Sigma; 0.25 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). The chemical were given for 28 days before tested with ETM and open field.

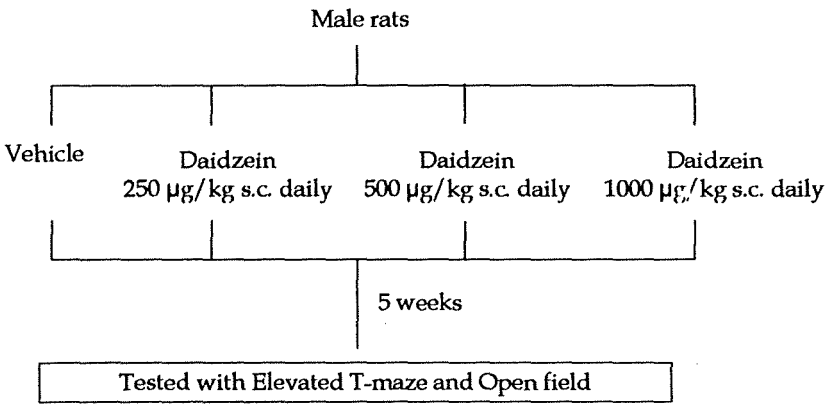
Part 2: To study the effects of phytoestrogens on the anxiety-like behaviors in the male rats using behavioral model, the elevated T-maze

Study 1 - To study the effects of genistein on the anxiety-like behaviors in the male rats



The male rats were randomly assigned into 4 groups, vehicle treated- (Veh) and genistein treated- (Gen) groups at the dosages of 0.25, 0.50 and 1.00 mg/kg. For all rats, the chemical administration was given by daily injection of vehicle (10% DMSO/propylene glycol) or genistein (Sigma; 0.25 -1 mg/kg) subcutaneously into the dorsal region of the neck. The chemical were given for 5 weeks before tested with ETM and open field.

Study 2 - To study the effects of daidzein on the anxiety-like behaviors in the male rats



The male rats were randomly assigned into 4 groups, vehicle treated- (Veh) and daidzein treated- groups at the dosages of 0.25, 0.50 and 1.00 mg/kg. For all rats, the chemical administration was given by daily injection of vehicle (10% DMSO/propylene glycol) or daidzein (Sigma; 0.25 -1 mg/kg) subcutaneously into the dorsal region of the neck. The chemical were given for 5 weeks before tested with ETM and open field.

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 x 10 cm) (Figure 2-1). One arm, enclosed by walls (40 cm high) was perpendicular to two opposed open arms. These three arms were connected by a square (10 x 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.

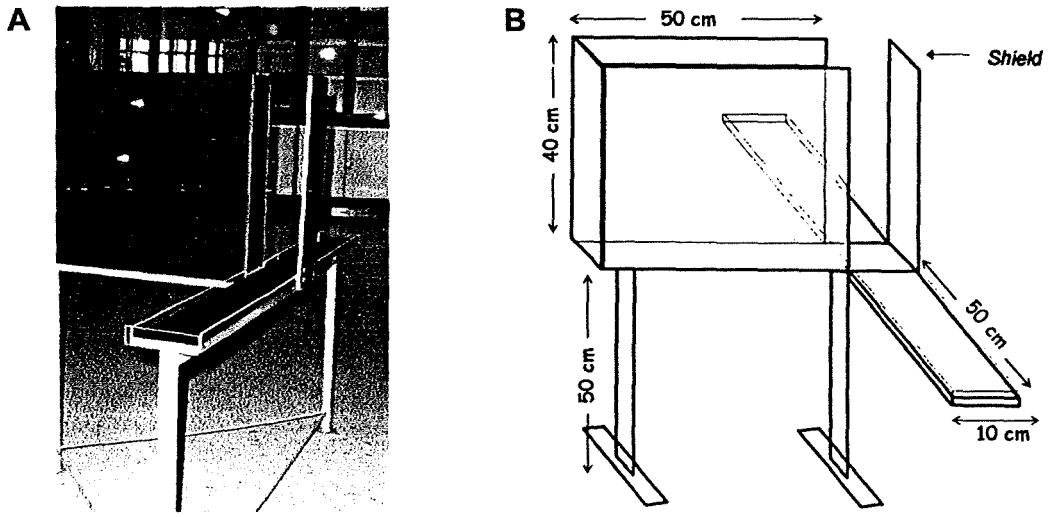


Figure 2-1 The elevated T-maze composed of two open- and one closed-arms of equal dimension (10 x 50 cm), connected by center platform (10 x 10 cm). The closed-arm was enclosed by a 50-cm wall, and the maze was elevated 50 cm above the floor.

Open field Test

After the ETM session, the animals were tested in the open field for 5 min and recorded with a video camera for later analysis. 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 x 57 cm wide x 35 cm high) with a 48-square grid floor (6 x 8 squares, 9.5 cm

per side) (Figure 2-2). The numbers of total crosses that the rat made during the 5 min in this task were recorded as the locomotor activity.

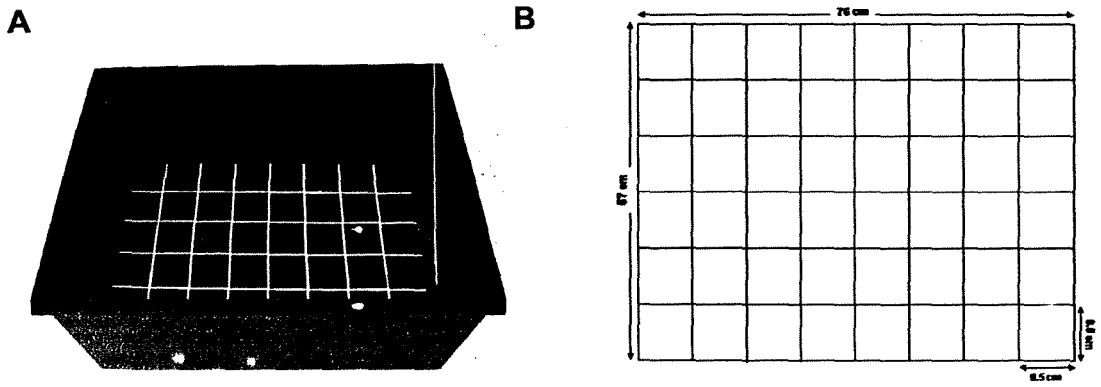


Figure 2-2 The open field (A) was a rectangular box of dimension (57 x 76 x 50 cm), in which the floor was divided in to 6 x 8 squares (9.5 x 9.5 cm) (B).

Statistical analysis

All data were presented as mean and standard errors of mean (SEM). For comparison between groups, student's T-test or one way analysis of variance (ANOVA) followed by Duncan multiple-comparison test was used as appropriate. 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 phytoestrogens i.e. genistein and daidzein on the anxiety-like behaviors in the ovariectomized-induced anxiety rats using behavioral model, the elevated T-maze

- The period of estrogen-deprivation on anxiety induction in ovariectomized female rats
- The anxiolytic effects of phytoestrogens i.e. genistein and daidzein in ovariectomized-induced anxiety rats

Part 2: The effects of phytoestrogens on the anxiety-like behaviors in the male rats using behavioral model, the elevated T-maze

- The effects of genistein on the anxiety-like behaviors in the male rats
- The effects of daidzein on the anxiety-like behaviors in the male rats

Part 1: The effects of phytoestrogens i.e. genistein and daidzein on the anxiety-like behaviors in the ovariectomized-induced anxiety rats using behavioral model, the elevated T-maze

Study 1 - The period of estrogen-deprivation on anxiety induction in ovariectomized female rats

The effects of Estrogen and estrogen deprivation at different time points on body weight and uterine weight

At the beginning of the experiment, body weights of all rats were not different. However after ovariectomy for 7, 14, 21 and 28 days, the percent change of body weight from the beginning of the experiment of 17β-estradiol treated rat (E_2, 1 μg/kg) were less than the vehicle treated- counterparts (Ovx) ($P < 0.0001$; Figure 3-1).

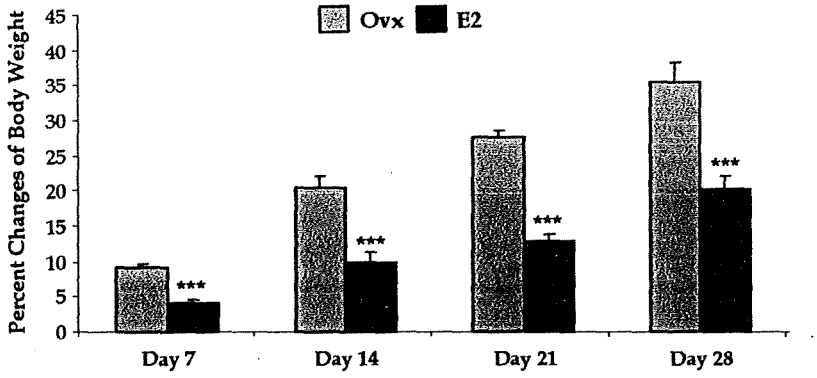


Figure 3-1 The percent change of body weight of the ovariectomized rat treated with vehicle (Ovx) or 17β-estradiol (E_2, 1 μg/kg). *** $P < 0.0001$ compared to Ovx on the same day by Student T-test, n= 12 per group.

The lacking of ovarian hormones was confirmed by the reduction in uterine weight in Ovx rats (Figure 3-2). The ratio of uterine weight to body weight was higher in estrogen treated rats compared to Ovx- counterparts on the same day ($P < 0.0001$; Figure 3-2). Moreover, we found that the reduction in uterine weight in the Ovx groups was in a time dependent manner. There was a negative correlation between day and the uterine weight ($r^2 = 0.7370$, $P < 0.0001$) in that the longer the time following ovariectomy, the lowered the uterine weight was found.

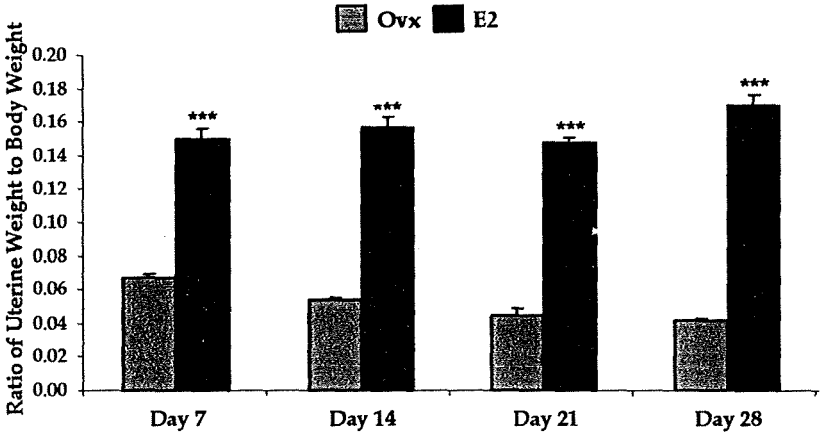


Figure 3-2 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle (Ovx) or 17β-estradiol (E_2, 1 μg/kg). *** $P < 0.0001$ compared to Ovx on the same day by Student T-test, n= 12 per group.

The effects of Estrogen and estrogen deprivation at different time points on anxiety

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 3-3, 3-4. For the inhibitory avoidance learning in the ETM of the Ovx groups, the baseline latency was not different among treatments. However, the inhibitory avoidance 1 and 2 were impaired in the rats that were ovariectomized for 14, 21 and 28 days (Figure 3-3A). The escape latency was not significantly different among rats that were ovariectomized at different time points (Figure 3-3B).

For the inhibitory avoidance learning in the ETM of the E2 groups, the baseline latency, the inhibitory avoidance 1 and 2 were not different among treatments (Figure 3-4A). Similarly, the escape latencies 1, 2 and 3 were not significantly different among ovariectomized rats treated with estrogen (E2) at different time points (Figure 3-4B).

For the open field test, the total number of line crossed in the open field was not differed among groups in both Ovx and E2 groups as shown in Figure 3-5. The total number of crosses in the open field, the indicator of the locomotor activity suggested that the effects seen in the ETM was not due to the changes of the rats' locomotor.

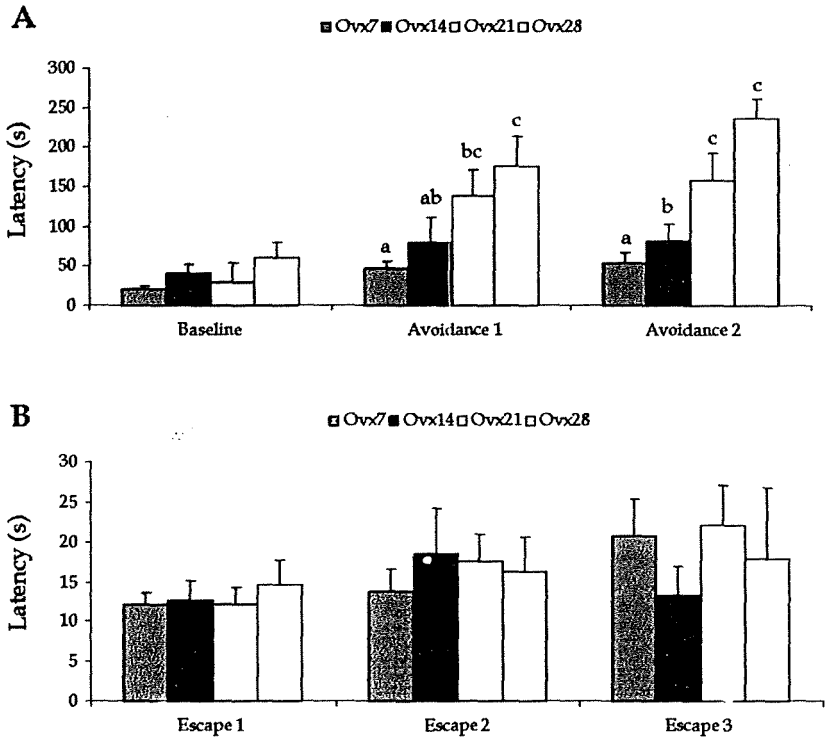


Figure 3-3A The inhibitory avoidance trials and **B** escape trials in the elevated T-maze of the ovariectomized rat at day 7, 14, 21 and 28. Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 12 to each group.

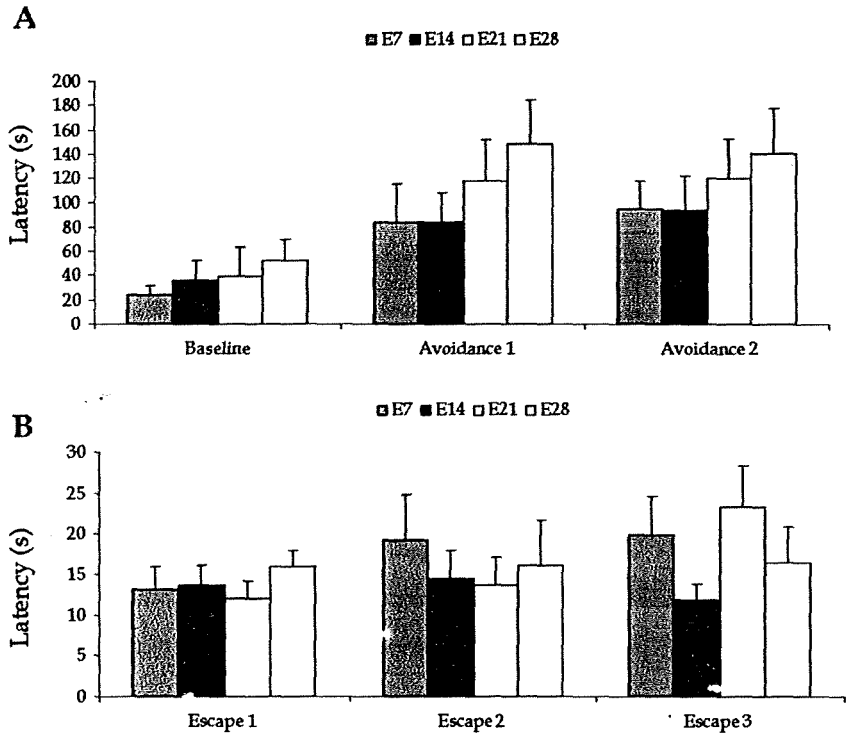


Figure 3-4A The inhibitory avoidance trials and B escape trials in the elevated T-maze of the ovariectomized rats treated with estrogen (E2) at day 7, 14, 21 and 28. Data presented as mean ± SEM, n= 12 to each group.

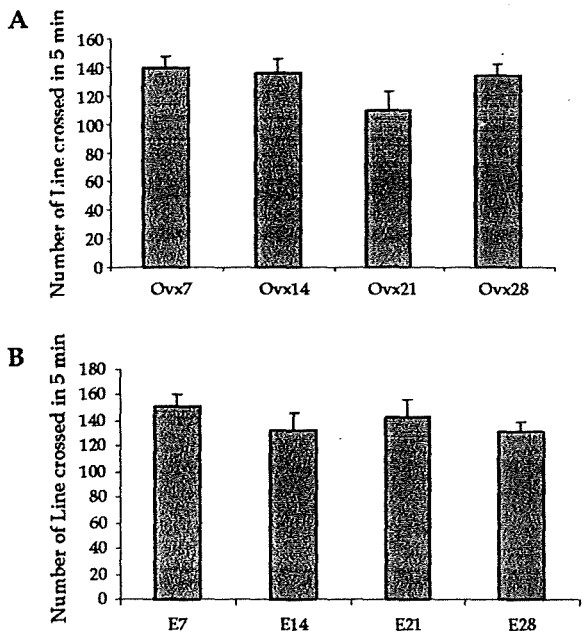


Figure 3-5 The number of 5 minute-line crossing in the open field of the ovariectomized rat **(A)** and the ovariectomized rat treated with estrogen (E2) **(B)**. Data presented as mean ± SEM, n= 12 to each group.

Study 2 - The anxiolytic effects of phytoestrogens i.e. genistein and daidzein in ovariectomized-induced anxiety rats

The results from the study 1 indicated that 21 days following ovariectomy could induce anxiety in female rats when tested with behavioral model, ETM. Therefore, this time point was used for further study.

The effects of phytoestrogens and estrogen on body weight and uterine weight

At the beginning of the experiment and 21 days after ovariectomy, the body weights of all rats were not different (Figure 3-6). However after treated with estrogen (1 μg/kg), genistein (0.25 mg/kg) or daidzein (0.25 mg/kg) for 28 days, the body weight of E2 group was lowered than Veh, Gen and Dai groups while the body weights of Gen and Dai groups were higher than Veh. Moreover, we found that the percent change of body weight at 28-day after treated with veh, E2, Gen or Dai from the body weight at day 21 after ovariectomy was lower in the E2 group compared to others (Figure 3-7).

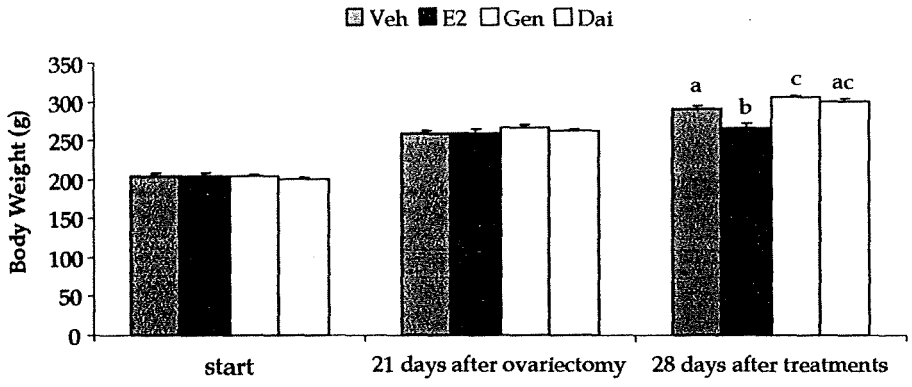


Figure 3-6 The body weight of the ovariectomized rat treated with vehicle (Veh), 17β-estradiol (E_2, 1 μg/kg), genistein (Gen, 0.25 mg/kg) or daidzein (Dai, 0.25 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-12 to each group.

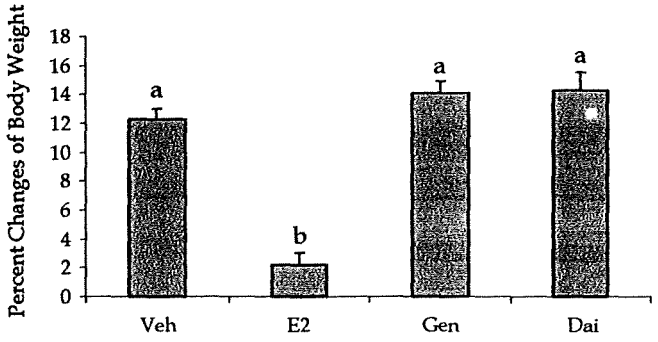


Figure 3-7 The percent change of body weight of the ovariectomized rat treated with vehicle, 17β-estradiol (E_2), genistein (Gen) or daidzein (Dai). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-12 to each group.

The uterine weight, an indicator of ovarian hormones deprivation and the estrogenic effects of hormone revealed that Gen and Dai had no uterotropic effect as shown by the ratio of uterine weight to body weight in these two groups was not different from vehicle but lower than estrogen treated rats ($P < 0.0001$; Figure 3-8).

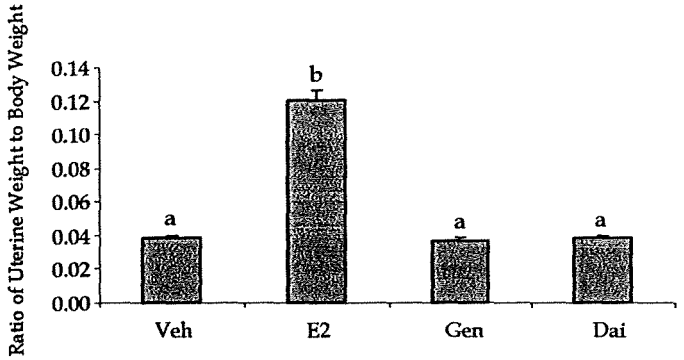


Figure 3-8 The ratio of uterine weight to body weight of the ovariectomized rat treated with vehicle, 17β-estradiol (E_2), genistein (Gen) or daidzein (Dai). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-12 to each group.

The effects of phytoestrogens and estrogen on anxiety

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 3-9. For the inhibitory avoidance learning in the ETM, the baseline latency was not different among treatments. However, the inhibitory avoidance 2 was impaired in the ovariectomized rats treated with vehicle (Figure 3-9A). Additionally, the data in the ETM revealed that in daidzein treated group, the avoidance 2 latency was not significantly different from vehicle, E2 or genistein treated groups. For the escape latency, there was no significantly different among groups (Figure 3-9B).

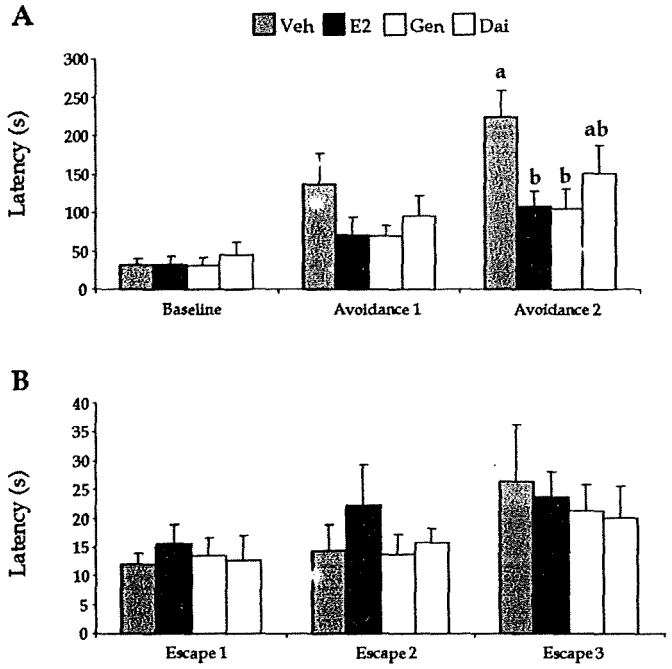


Figure 3-9A The inhibitory avoidance trials and **B** escape trials in the elevated T-maze of the ovariectomized rat treated with vehicle, 17β-estradiol (E_2), genistein (Gen) or daidzein (Dai). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 12 to each group.

For the open field test, the total number of line crossed in the open field was not differed among groups (Figure 3-10).

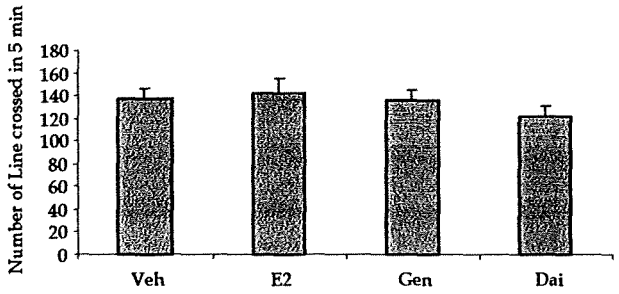


Figure 3-10 The number of 5 minute-line crossing in the open field of the ovariectomized rat treated with vehicle, 17β-estradiol (E_2), genistein (Gen) or daidzein (Dai). Data presented as mean ± SEM, n= 9-12 to each group.

Part 2: The effects of phytoestrogens on the anxiety-like behaviors in the male rats using behavioral model, the elevated T-maze

Study 1 - The effects of genistein on the anxiety-like behaviors in the male rats

The effects of phytoestrogen, genistein on body weight and reproductive organs weight

At the beginning of the experiment, the body weights of all rats were not different. However, the percent change of body weight at 35-day after treated with genistein at the dose of 1.00 mg/kg was lower than vehicle and genistein at the doses of 0.25 and 0.5 mg/kg (Figure 3-11).

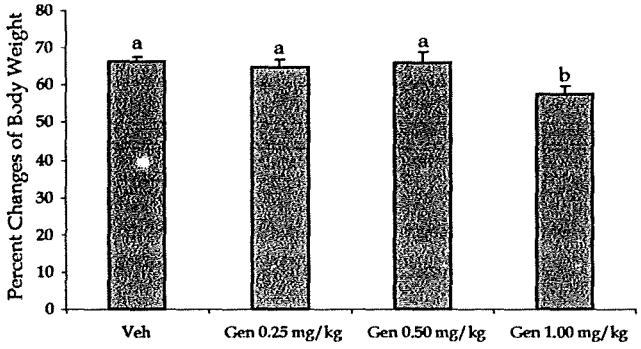


Figure 3-11 The percent change of body weight of the male rat treated with vehicle or genistein (Gen, 0.25-1.00 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-10 to each group.

For the male reproductive organs i.e. testis, epididymis, prostate gland and seminal vesicle, there was no statistically significant different between treatments (Table 1).

Table 3-1 The effects of genistein (0.25-1.00 mg/kg) on the ratio of reproductive organs to body weight.

	Testis[1]	Epididymis[1]	Seminal vesicle[1]	Prostate gland[1]
Vehicle	0.49 ± 0.01	0.13 ± 0.005	0.26 ±0.01	0.18 ± 0.01
Gen 0.25 mg/kg	0.49 ± 0.01	0.13 ± 0.002	0.24 ±0.02	0.17 ± 0.01
Gen 0.50 mg/kg	0.49 ± 0.01	0.14 ± 0.004	0.27 ±0.01	0.19 ± 0.01
Gen 1.00 mg/kg	0.50 ± 0.01	0.14 ± 0.004	0.27 ±0.01	0.18 ± 0.01

Data presented as mean ± SEM, n= 9-10 to each group.

[1] ratio of organ to body weight

The effects of phytoestrogen, genistein on anxiety

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 3-12. For the inhibitory avoidance learning in the ETM, there was no significantly different among groups (Figure 3-12A). For the escape latency, we found that the high dose of genistein (1 mg/kg) tended to increase anxiety as the escape 1 latency was lower than others (P = 0.0578; Figure 3-12B). The total number of line crossed during 5 min in the open field was not different between treatments (data not shown).

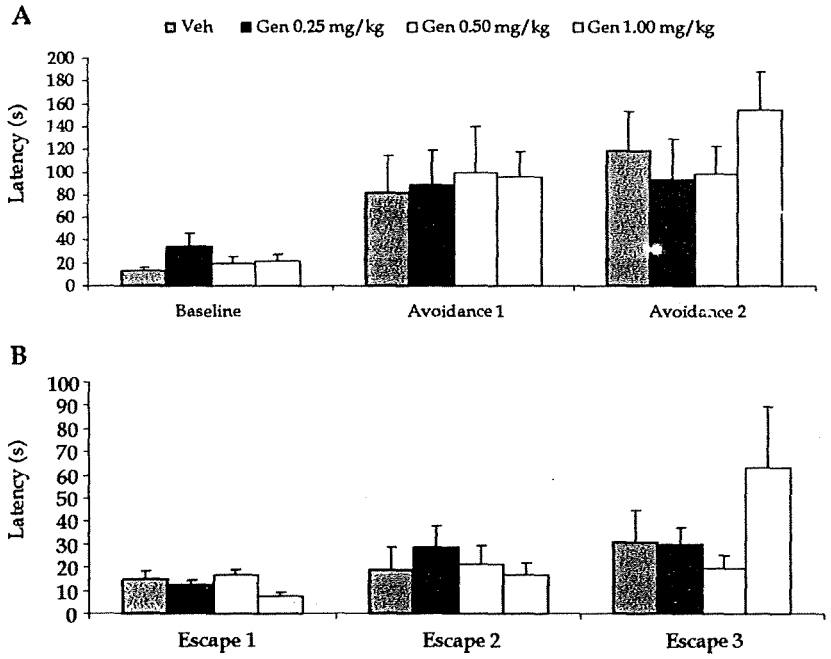


Figure 3-12A The inhibitory avoidance trials and **B** escape trials in the elevated T-maze of the male rat treated with vehicle or genistein (Gen, 0.25-1.00 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-10 to each group.

Study 2 - The effects of daidzein on the anxiety-like behaviors in the male rats

The effects of phytoestrogen, daidzein on body weight and reproductive organs weight

At the beginning of the experiment, the body weights of all rats were not different. Moreover, after treated with daidzein for 35 days, there was no significant different in body weight and percent change of body weight (Table 2).

Table 3-2 The effects of daidzein (0.25-1.00 mg/kg) on the body weight.

	Start	End	Percent change of body weight
Vehicle	270.00 ± 5.25	424.72 ± 7.04	57.53 ± 2.47
Dai 0.25 mg/kg	258.00 ± 6.77	408.25 ± 12.80	58.20 ± 2.59
Dai 0.50 mg/kg	262.25 ± 5.28	422.00 ± 2.38	61.44 ± 2.97
Dai 1.00 mg/kg	262.50 ± 6.65	427.75 ± 7.88	63.40 ± 2.75

Data presented as mean ± SEM, n= 9-10 to each group.

For the male reproductive organs i.e. testis, epididymis, prostate gland and seminal vesicle, there was no statistically significant different between treatments (Table 3). However, there was tended to be different in ratio of prostate gland to body weight (P = 0.0975) in that at high dose of daidzein (1 mg/kg), the prostate gland tended to be lower than others.

Table 3-3 The effects of daidzein (0.25-1.00 mg/kg) on the ratio of reproductive organs to body weight.

	Testis[1]	Epididymis[1]	Seminal vesicle[1]	Prostate gland[1]
Vehicle	0.47 ± 0.01	0.13 ± 0.004	0.26 ±0.01	0.160 ± 0.010
Dai 0.25 mg/kg	0.50 ± 0.02	0.14 ± 0.005	0.27 ±0.01	0.182 ± 0.006
Dai 0.50 mg/kg	0.47 ± 0.01	0.14 ± 0.003	0.28 ±0.01	0.175 ± 0.009
Dai 1.00 mg/kg	0.47 ± 0.02	0.14 ± 0.004	0.26 ±0.01	0.156 ± 0.007

Data presented as mean ± SEM, n= 9-10 to each group.

[1] ratio of organ to body weight

The effects of phytoestrogen, daidzein on anxiety

The level of anxiety as measured by the elevated T-maze (ETM) is shown in Figure 3-13. For the inhibitory avoidance learning in the ETM, the baseline latency was not different between groups. The inhibitory avoidance 2 latency was significant lower in daidzein treated rat but only at low dose (0.25 mg/kg) (Figure 3-13A). For the escape latency, there was no significant different between treatments (Figure 1-13B). The total number of line crossed during 5 min in the open field was not different between treatments (data not shown).

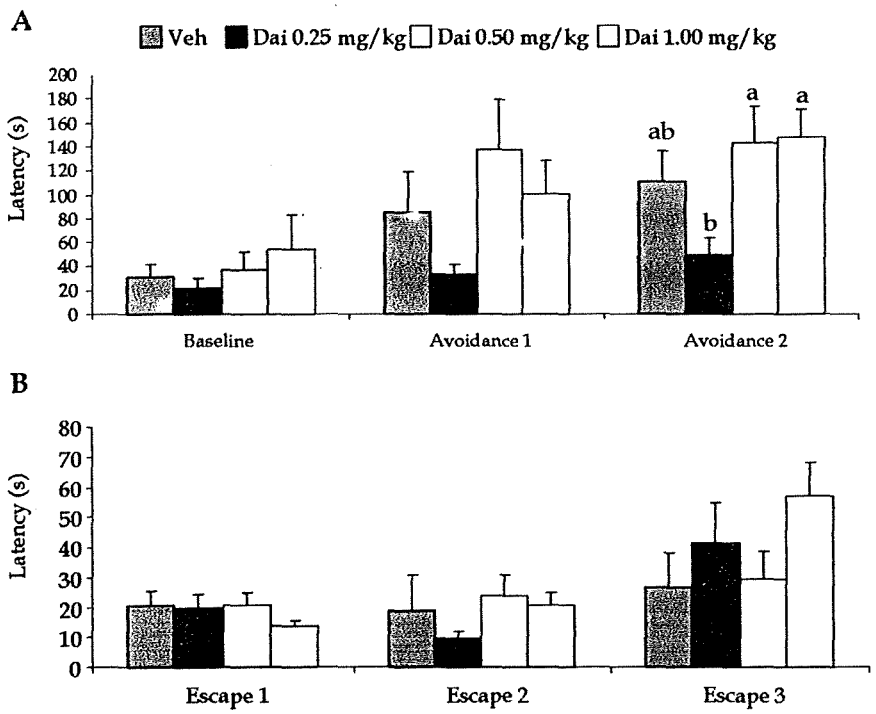


Figure 3-13A The inhibitory avoidance trials and **B** escape trials in the elevated T-maze of the male rat treated with vehicle or daidzein (Dai, 0.25-1.00 mg/kg). Data presented as mean ± SEM, different letters denoted significant different at $P < 0.05$, ANOVA followed by Duncan's multiple range test, n= 9-10 to each group.

DISCUSSION

The effects of phytoestrogens and estrogen on the body weight

In female rats that were ovariectomized and supplemented with or without estrogen for 7, 14, 21 and 28 days; the body weight of estrogen-treated rats was lowered than Ovx counterparts. In the next study, estrogen, genistein or daidzein were given to 21-day ovariectomized rats for 28 day, the results showed similar finding in that the body weight of estrogen-treated rats was lowered than Ovx, genistein and daidzein treated groups. 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). 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 on body weight 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.

In the intact male rats, the percent changes of body weight in genistein (0.25 and 0.50 mg/kg) and daidzein (0.25-1.00 mg/kg) treated groups were not different from control but was decreased in high dose genistein (1.00 mg/kg) treated group. Similarly Lund and Lephart (2001) fed diet containing phytoestrogen (600 μg/g diet) and found that the body weight of both male and fenale rats were lower than rats fed with phytoestrogen-free diet. In diet containing lower level of phytoestrogen (150 μg isoflavone /g diet) had no effect on body weight (Hartley et al., 2003). This data could be explained by that the high dose genistein may bind and activate ERα resulting in higher metabolic rate in this rat and thus weight gain was then less than others.

The effects of phytoestrogens and estrogen on the reproductive organs weight

In the current study, at the end of the experiment, the reproductive organs i.e. uterus in female; testis, epididymis, prostate gland and seminal vesicle in male were collected and used as indicators of hormone affecting target organs. In female that were ovariectomized, the ratio of uterine weight to body weight was lowered as the time after ovariectomized was lengthened. In the estrogen treated groups, the uterine weight was higher than vehicle group; while phytoestrogens, genistein and daidzein had no effect on uterine weight. These findings were somewhat different from others since many have shown that uterine weight was increased following genistein treatment (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).

In intact male rats, genistein and daidzein at all doses had no significant effect on male reproductive organs weight. However, it should be noted that daidzein at high dose (1 mg/kg) tended to reduce prostate gland weight. This reduction in prostate gland weight had been reported by Huang et al. (2008); however, in their study the daidzein were orally given at a higher dose (2, 20 and 100 mg/kg) for a longer time (90 days). Moreover, Hsu et al. (2009) had shown that genistein and daidzein can induce apoptosis in benign prostate hyperplasia (BPH-1) cells, suggesting that they may have cytotoxic effect.

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. In female that were ovariectomized and tested with ETM at day 7, 14, 21 and 28, we found that the avoidance 2 latency of Ovx rats at day 21 and 28 were significantly lowered than at day 7. This suggested that 21 days following ovariectomy was sufficient to induce anxiety in female rats. Moreover, in the current study we also demonstrated that in anxious rats, estrogen and genistein were able to alleviate anxiety in these rats. Although the avoidance 2 latency in daidzein treated group was lowered than vehicle but it was not statistically significant suggesting that higher dose or longer time may be required. These findings are consistent with previous reports 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; 2009). As estrogen is able to reduce anxiety, it is then unsurprised that phytoestrogen like genistein and daidzein has similar effect.

In intact male rats, the behavioral data showed that genistein had no effect on anxiety as measured by ETM. However, it should be noted that the escape latency of the rats treated with high dose genistein (1.00 mg/kg) tended to be lowered than others indicated that these rats were more anxious. For daidzein, it is likely that daidzein at low dose (0.25 mg/kg) contained anxiolytic effect in intact male rats as shown by lowered avoidance 2 latency in these rats.

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. As shown in this study, the sex of animal is one factor contributing to anxiety level. Lund and Lephart (2001) and Lephart et al. (2002) reported that given life-long phytoestrogen-riched diet (600 μg/g diet) can reduce anxiety in both male and female rats when tested with elevated plus-maze. Contrarily, Hartley et al. (2003) have shown that given phytoestrogen-riched diet (150 μg/g diet) for 14 days was indeed anxiogenic in male rats. 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. Nevertheless, in female that lacks of estrogen, it is quite clear that phytoestrogens i.e. genistein and daidzein can be used as an alternative for estrogen as they have anxiolytic effect with no adverse effect on reproductive organ. However, in intact male rats, the phytoestrogens were acting as anxiogenic, anxiolytic or no effect depending on the type of phytoestrogens (genistein v.s. daidzein) and doses of each substance.

It is now recognized that estrogen exerts 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 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 in intact male the high dose of genistein may activate ERα and resulting in anxiogenic effect. For daidzein, as it prefers to bind to ERβ than ERα , at the low dose it may activate ERβ and resulting in anxiolytic effect; while at the higher dose it may somehow bind and activate ERα and may counter the action of ERβ resulting in no effect on anxiety. Moreover, it had been shown that daidzein at higher dose (>2 mg/kg) can down regulate ERβ expression with no effect on ERα (Pan et al., 2008); this may be partially responsible for effect seen in this study.

In conclusion, the current study had demonstrated that lacking of ovarian hormones for at least 3 weeks was sufficient to induce anxiety in ovariectomized female rats when tested with the behavioral model, elevated T-maze. Further, after anxiety had been induced by ovariectomized, phytoestrogens i.e. genistein and daidzein (0.25 mg/kg, 28 days) can reverse this anxiogenic effect with no uterotrophic effect. This is of interested as it may be applicable for further clinical trials to be used as an alternative to estrogen in patients that the uses of estrogen were contraindicated. Moreover, the current study also demonstrated that phytoestrogen, genistein (1.00 mg.kg, 35 days) contained anxiogenic effect; while daidzein (0.25 mg/kg, 35 days) contained anxiolytic effect in intact male. Based on these findings, the usages of phytoestrogen as supplement food should be done with precaution in intact male. Further, the mechanism underlying behavioral changes is currently under investigated.

REFERENCES

Anxiety Disorders Association of America. 2003. Statistics and facts about anxiety disorders. Available: http://www.adaa.org.

Assinder, S., Davis, R., Fenwick, M. and Glover, A. 2007. Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells. Reproduction. 133: 11-19.

Bloch, M., Daly, R.C. and Rubinow, D.R. 2003. Endocrine factors in the etiology of postpartum depression. Compr. Psychiatry. 44(3): 234-246.

Bodo, C. and Rissman, E.F. 2006. New roles for estrogen receptor b in behavior and neuroendocrinology. Front. Neuroendocrinol. 27: 217-232.

Cederroth, C.R., Vinciguerra, M., Kühne, F., Madani, R., Doerge, D.R., Visser, T.J., Foti, M., Rohner-Jeanrenaud, F., Vassalli J.D. and Nef, S. 2007. A phytoestrogen-rich diet increases energy expenditure and decreases adiposity in mice. Environ. Health Perspect. 115(10): 1467-1473.

Cowley, S.M., Hoare, S., Mosselman, S. and Parker, M.G. 1997. Estrogen receptors alpha and beta form heterodimers on DNA. J. Biol. Chem. 272: 19858-19862.

Custódio Teixeira, R.C., Zangrossi, Jr. H. and Graeff, F.G. 2000. Behavioral effects of acute and chronic imipramine in the elevated T-maze model of anxiety. Pharmacol. Biochem. Behav. 65: 571-576.

Dazzi, L., Spiga, F., Pira, L., Ladu, S., Vacca, G., Rivano, A., Jentsch, J.D. and Biggio, G. 2001. Inhibition of stress- or anxiogenic-drug-induced increases in dopamine release in rat prefrontal cortex by long-term treatment with antidepressant drugs. J. Neurochem. 76: 1212-1220.

de Lemos, M.L. 2001. Effects of soy phytoestrogens genistein and daidzein on breast cancer growth. Ann. Pharmacother. 35(9): 1118-1121.

Department of Mental Health. Annual Report 2005. 2005. pp. 1-148. http://www.dmh.go.th/

Department of Mental Health. Mental Health in Thailand 2002-2003. 2003. The Express Transportation Organization of Thailand press. P. Boonyawongvirot Ed. pp. 1-138.

Diagnostic and Statistic Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR). 2000. Washington, DC: American Psychiatric Association;.

Diaz-Veliz, G., Alarcon, T., Espinoza, C., Dussaubat, N. and Mora, S. 1997. Ketanserin and anxiety levels: influence of gender, estrous cycle, ovariectomy and ovarian hormones in female rats. Pharmacol. Biochem. Behav. 58: 637-642.

Diel, P., Hertrampf, T., Seibel, J., Laudenbach-Leschowsky, U., Kolba, S. and Vollmer, G. 2006. Combinatorial effects of the phytoestrogen genistein and of estradiol in uterus and liver of female Wistar rats. J. Steroid Biochem. Mol. Biol. 102: 60-70.

Ditkoff, E.C., Crary, W.G., Cristo, M. and Lobo, R.A. 1991. Estrogen improves psychological function in asymptomatic postmenopausal women. Obstet. Gynecol. 78: 991-995.

Fanti, P., Monier-Faugere, M.C., Geng, Z., Schmidt, J., Morris, P.E., Cohen, D. and Malluche, H.H. 1998. The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. Osteoporos. Int. 8(3): 274-281.

Faqi, A.S., Johnson, W.D., Morrissey, R.L. and McCormick, D.L. 2004. Reproductive toxicity assessment of chronic dietary exposure to soy isoflavones in male rats. Reprod. Toxicol. 18: 605-611.

Frye, C.A. and Seliga, A.M. 2001. Testosterone increases analgesia, anxiolysis, and cognitive performance of male rats. Cogn. Affect Behav. Neurosci. 1(4): 371-381.

Ge, J., Barnes, N.M., Costall, B. and Naylor, R.J. 1997. Effect of aversive stimulation on 5-hydroxytryptamine and dopamine metabolism in the rat brain. Pharmacol. Biochem. Behav. 58(3): 775-783.

Gillies, G.E. and McArthur, S. 2010. Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Pharmacol. Rev. 62(2):155-198.

Gouveia, A. Jr., dos Santos, U.D., Felisbino, F.E., de Afonseca, T.L., Antunes, G. and Morato, S. 2004. Influence of the estrous cycle on the behavior of rats in the elevated T-maze. Behav. Processes. 67(2): 67-71.

Graeff, F.G., Netto, C.F. and Zangrossi, Jr. H. 1998. The elevated T-maze as an experimental model of anxiety. Neurosci. Biobehav. 23: 237-246.

Graeff, F.G., Viana, M.B. and Tomaz, C. 1993. The elevated T-maze, a new experimental model of anxiety and memory: effect of diazepam. Braz. J. Med. Biol. Res. 26: 67-70.

Hartley, D.E., Edwards, J.E., Spiller, C.E., Alom, N., Tucci, S., Seth, P., Forsling, M.L. and File, S.E. 2003. The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat. Psychophamacology 167: 46-53.

Heffner, T.G., Harman, J.A. and Seiden, L.S. 1980. A rapid method for the regional dissection of the rat brain. Pharmacol. Biochem. Behav. 13: 453-456.

Heikkinen, T., Puoliväli, J., Liu, L., Rissanen, A. and Tanila, H. 2002. Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice. Horm. Behav. 41: 22-32.

Heine, P.A., Taylor, J.A., Iwamoto, G.A., Lubahn, D.B. and Cooke, P.S. 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proc. Natl. Acad. Sci. U S A. 97(23), 12729-12734.

Helferich, W.G., Andrade, J.E. and Hoagland, M.S. 2008. Phytoestrogens and breast cancer: a complex story. Inflammopharmacology .16(5):219-226.

Hsu, A., Bray, T.M., Helferich, W.G., Doerge, D.R. and Ho, E. 2010. Differential effects of whole soy extract and soy isoflavones on apoptosis in prostate cancer cells. Exp. Biol. Med. 235: 90–97.

Huang, Y.F., Feng, Y., Pan, L.J. and Xia, X.Y. 2008. Preventive effect of daidzein on testosterone-induced prostatic hyperplasia in rats. Zhonghua Nan Ke Xue. 14(8): 713-718. (abstract)

Ito, K. 2007. Hormone replacement therapy and cancers: the biological roles of estrogen and progestin in tumorigenesis are different between the endometrium and breast. Tohoku J. Exp. Med. 212: 1-12.

Jeffersonv, W.N., Padilla-Banks, E. and Newbold, R.R. 2007. Disruption of the female reproductive system by the phytoestrogen genistein. Reprod. Toxicol. 23: 308–316.

Krezel, W., Dupont, S., Krust, A., Chambon, P. and Chepman, P.F. 2001. Increasesd anxiety and synaptic plasticity in estrogen receptor beta-deficient mice. Proc. Natl. Acad. Sci. 98: 12278-12282.

Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S. and Gustafsson, J.A. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 138(3): 863-870.

Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.Å. 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA. 93: 5925–5930.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139: 4252-4263.

Laflamme, N., Nappi, R.E., Drolet, G., Labrie, C. and Rivest, S. 1998. Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J. Neurobiol. 36(3): 357-378.

Lähdesmäki, J., Sallinen, J., MacDonald, E., Kobilka, B.K., Fagerholm, V. and Scheinin, M. 2002. Behavioral and neurochemical characterization of alpha(2A)-adrenergic receptor knockout mice. Neuroscience. 113(2): 289-99.

Lamartiniere, C.A., Wang, J., Smith-Johnson, M. and Eltoum, I.E. 2002. Daidzein: bioavailability, potential for reproductive toxicity, and breast cancer chemoprevention in female rats. Toxicol. Sci. 65(2): 228-238.

Lavigne, J.A., Takahashi, Y., Chandramouli, G.V., Liu, H., Perkins, S.N., Hursting, S.D. and Wang, T.T. 2008. Concentration-dependent effects of genistein on global gene expression in MCF-7 breast cancer cells: an oligo microarray study. Breast Cancer Res. Treat. 110(1): 85-98.

Lephart, E.D., Setchell, K.D., Handa, R.J. and Lund, T.D. 2004. Behavioral effects of endocrine-disrupting substances: phytoestrogens. ILAR J. 45: 443-454.

Lephart, E.D., West, T.W., Weber, K.S., Rhees, R.W., Setchell, K.D., Adlercreutz, H. and Lund, T.D. 2002. Neurobehavioral effects of dietary soy phytoestrogens. Neurotoxicol. Teratol. 24: 5-16.

Lu, N.Z., Eshleman, A.J., Janowsky, A. and Bethea, C.L. 2003. Ovarian steroid regulation of serotonin reuptake transporter (SERT) binding, distribution, and function in female macaques. Mol Psychiatry. 8: 353-360.

Lund, T.D., Rovis, T., Chung, W.C.J. and Handa, R. 2005. Novel actions of estrogen receptor-b on anxiety-related behaviours. Endocrinology 146: 797-807.

Lund, T.D. and Lephart, E.D. 2001. Dietary soy phytoestrogens produce anxiolytic effects in the elevated plus-maze. Brain Res. 913: 180-184.

Magee, P.J. and Rowland, I.R. 2004. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr. 91(4), 513-531.

Maggi, A., Ciana, P., Belcredito, S. and Vegeto, E. 2004. Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu. Rev. Physiol 66: 291–313.

Manas, E.S., Xu, Z.B., Unwalla, R.J and Somers, W.S. 2004. Understanding the selectivity of genistein for human estrogen receptor-beta using X-ray crystallography and computational methods. Structure. 12: 2197–2207.

Marcondes, F.K., Miguel, K.J., Melo, L.L. and Spadari-Bratfisch, R.C. 2001. Estrous cycle influences the response of female rats in the elevated plus-maze test. Physiol. Behav. 74(4-5): 435-440.

Maswood, S., Stewart, G. and Uphouse, L. 1995. Gender and estrous cycle effects of the 5-HT1A agonist, 8-OH-DPAT, on hypothalamic serotonin. Pharmacol. Biochem. Behav. 51(4): 807-813.

McCarthy, M.M., Felzenberg, E., Robbins, A., Pfaff, D.W. and Schwartz-Giblin, S. 1995. Infusions of diazepam and allopregnanolone into the midbrain central gray facilitate open-field behavior and sexual receptivity in female rats. Horm. Behav. 29, 279-295.

McCullough, L.D. and Salamone, J.D. 1992. Anxiogenic drugs beta-CCE and FG 7142 increase extracellular dopamine levels in nucleus accumbens. Psychopharmacology. 109: 379-382.

McEwen, B.S. 2002. Estrogen actions throughout the brain. Recent Prog. Horm. Res. 57: 357-384.

Mitchell, J.H., Cawood, E., Kinniburgh, D., Provan, A., Collins, A.R. and Irvine, D.S. 2001. Effect of a phytoestrogen food supplement on reproductive health in normal males. Clin. Sci. 100:613-618.

Moore, A.B., Castro, L., Yu, L., Zheng, X., Di, X., Sifre, M.I., Kissling, G.E., Newbold, R.R., Bortner, C.D. and Dixon, D. 2007. Stimulatory and inhibitory effects of genistein on human uterine leiomyoma cell proliferation are influenced by the concentration. Hum. Reprod. 22(10): 2623–2631.

Mora, S., Dussault, N. and Diaz-Veliz, G. 1996. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology. 21: 609-620.

Morgan, M.A. and Pfaff, D.W. 2002. Estrogen's effects on activity, anxiety, and fear in two mouse strains. Behav. Brain Res. 132: 85-93.

Murkies, A.L., Wilcox, G. and Davis, S.R. 1998. Phytoestrogens. J. Clin. Endocrinol. Metabo. 83(2): 297-303.

Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., Kaplitt, M.G. and Ogawa, S. 2007. Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. Proc. Natl. Acad. Sci. U S A. 104(7), 2501-2506.

Naftolin, F., MacLusky, N.J., Leranth, C.Z., Sakamoto, H.S. and Garcia-Segura, L.M. 1988. The cellular effects of estrogen on neuroendocrine tissues. J. Steroid Biochem. Mol. Biol. 30: 195-207.

Om, A.S. and Shim, J.Y. 2007. Effect of daidzein, a soy isoflavone, on bone metabolism in Cd-treated ovariectomized rats. Acta Biochim. Pol. 54(3): 641-646.

Paech, K., Webb, P., Kuiper, G.G.J.M., Nilsson, S., Gustafsson, J., Kushner, P.J., Scanlan, T.S. 1997. Differential Ligand Activation of Estrogen Receptors ERα and ERβ at AP1 Sites. Science. 277: 1508-1510.

Pan, L., Xia, X., Feng, Y., Jiang, C., Cui, Y. and Huang, Y. 2008. Exposure of juvenile rats to the phytoestrogen daidzein impairs erectile function in a dose-related manner in adulthood. J. Androl. 29: 55–62.

Pandaranandaka, J., Poonyachoti, S. and Kalandakanond-Thongsong, S. 2009. Differential effects of exogenous, endogenous estrogen on anxiety as measured by elevated T-maze in relation to the serotonergic system. Behav. Brain Res. 198: 142-148.

Pandaranandaka, J., Poonyachoti, S. and Kalandakanond-Thongsong, S. 2006. Anxiolytic property of estrogen related to the changes of the monoamine levels in various brain regions of ovariectomized rats. Physiol. Behav. 87: 828-35.

Patisaul, H.B., Blum, A., Luskin, J.R. and Wilson, M.E. 2005. Dietary soy supplements produce opposite effects on anxiety in intact male and female rats in the elevated plus-maze. Behav. Neuroscience. 119: 587-594.

Patisaul, H.B., Burke, K.T., Hinkle, R.E., Adewale, H.L. and Shea, D. 2009. Systemic administration of diarylpropionitrile (DPN) or phytoestrogens does not affect anxiety-related behaviors in gonadally intact male rats. Horm. Behav. 55(2): 319–328.

Patisaul, H.B. and Bateman, H.L. 2008. Neonatal exposure to endocrine active compounds or an ERbeta agonist increases adult anxiety and aggression in gonadally intact male rats. Horm. Behav. 53(4): 580-588.

Patisaul, H.B., Melby, M., Whitten, P.L. and Young, L.J. 2002. Genistein affects ER beta- but not ER alpha-dependent gene expression in the hypothalamus. Endocrinology. 143(6): 2189-2197.

Peeters, P.H., Keinan-Boker, L., van der Schouw, Y.T. and Grobbee, D.E. 2003. Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res. Treat. 77(2): 171-183.

Pellow, S., Chopin, P., File, S.E. and Briley, M. 1985. Validation of open, closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods. 14: 149-167.

Perry, D.L., Spedick, J.M., McCoy, T.P., Adams, M.R., Franke, A.A. and Cline, J.M. 2007. Dietary Soy Protein Containing Isoflavonoids Does Not Adversely Affect the Reproductive Tract of Male Cynomolgus Macaques (*Macaca fascicularis*). J. Nutr. 137: 1390-1394.

Picherit, C., Coxam, V., Bennetau-Pelissero, C., Kati-Coulibaly, S., Davicco, M.J., Lebecque, P. and Barlet, J.P. 2000. Daidzein is more efficient than genstein in preventing ovariectomy-induced bone loss in rats. J. Nutr. 130(7): 1675-1681.

Pinheiro, S.H., Zangrossi, H. Jr., Del-Ben, C.M. and Graeff, F.G. 2007. Elevated mazes as animal models of anxiety: effects of serotonergic agents. An. Acad. Bras. Cienc. 79(1): 71-85.

Pitchot, W., Ansseau, M., Gonzalez Moreno, A., Hansenne, M. and von Frenckell, R. 1992. Dopaminergic function in panic disorder: comparison with major and minor depression. Biol. Psychiatry. 32: 1004-1011.

Prut, L. and Belzung, C. 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463: 3-33.

Robertson, K.M., O'Donnell, L., Simpson, E.R. and Jones, M.E. 2002. The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. Endocrinology. 143(8): 2913-2921.

Santollo, J., Wiley, M.D. and Eckel, L.A. 2007. Acute activation of ER alpha decreases food intake, meal size, and body weight in ovariectomized rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293: R2194-R2201.

Schmidt, P.J., Nieman, L., Danaceau, M.A., Tobin, M.B., Roca, C.A., Murphy, J.H. and Rubinow, D.R. 2000. Estrogen replacement in perimenopause-related depression: a preliminary report. Am. J. Obstet. Gynecol. 183(2): 414-420.

Seeman, M.V. 1997. Psychopathology in women and men: focus on female hormones. Am. J. Psychiatry. 154: 1641-1647.

Setchell, K.D., Brown, N.M,, Desai, P.B., Zimmer-Nechimias, L., Wolfe, B., Jakate, A.S., Creutzinger, V. and Heubi, J.E. 2003. Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. J. Nutr. 133(4), 1027-1035.

Shughrue, P.J., Scrimo, P.J. and Merchenthaler, I. 1998. Evidence for the colocalization of estrogen receptor β mRNA and Estrogen Receptor α Immunoreactivity in neurons of the rat forebrain. Endocrinology. 139: 5267-5270.

Simpson, E. and Davis, S.R. 2000. Another role highlighted for estrogen in the male: sexual behavior. Proc. Natl. Acad. Sci. USA. 97:14737-14741.

Tanaka, M., Yoshida, M., Emoto, H. and Ishii, H. 2000. Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. Eur. J. Pharmacol. 405: 397-406.

Terreaux, C., Polasek, J. and Hostettmann, K. 2003. Plant constituents with hormonal effects. Curr. Org. Chem. 7: 1151-1161.

Usui, T. 2006. Pharmaceutical Prospects of Phytoestrogens. Endocr. J. 53: 7-20.

Vastag, B. 2007. Soy and Prostate Cancer Study Results Mixed. J. Natl. Cancer Inst. 99(18): 1364.

Viana, M.B., Tomaz, C. and Graeff, F.G. 1994. The elevated T-maze: a new animal model of anxiety and memory. Pharmacol. Biochem. Behav. 49: 549-554.

Vicdan, K., Erdogan, D., Ozogul, C. and Elmas, C. 2007. Effects of dietary phytoestrogens on mouse testis: evaluation by electron microscopy and caspase- 3 immunostaining. J. Turkish-German Gynecol. Assoc. 8(3): 290-296.

Walf, A.A. and Frye, C.A. 2006. A review and update of mechanisms of estrogen in the hippocampus and amygdale for anxiety and depression behavior. Neuropsycopharmacology. 31: 1097-1111.

Walf, A.A. and Frye, C.A. 2007. Administration of estrogen receptor beta-specific selective estrogen receptor modulators to the hippocampus decrease anxiety and depressive behavior of ovariectomized rats. Pharmacol. Biochem. Behav. 86: 407-414.

Walf, A.A., Koonce, C.J. and Frye, C.A. 2008. Estradiol or diarylpropionitrile decrease anxiety-like behavior of wildtype, but not estrogen receptor beta knockout, mice. Behav. Neurosci. 122(5): 974-981.

Walf, A.A. and Frye, C.A. 2005. ERbeta-selective estrogen receptor modulators produce antianxiety behavior when administered systemically to ovariectomized rats. Neuropsychopharmacology. 30: 1598-1609.

Weber, K.S., Setchell, K.D., Stocco, D.M. and Lephart, E.D. 2001. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J. Endocrinol. 170:591-599.

Wisniewski, A.B., Cernetich, A., Gearhart, J.P. and Klein, S.L. 2005. Perinatal exposure to genistein alters reproductive development and aggressive behavior in male mice. Physiol. Behav. 84(2): 327-334.

Yildiz, F. 2006. Phytoestrogens in functional foods. Boca Raton: CRC Press. 320p.

Zangrossi, Jr. H. and Graeff, F.G. A behavioral validation of the elevated T-maze: a new animal model of anxiety. Brain Res Bull, 1997, 44: 1-5.