RESEARCH ARTICLE

Effects of Pine Pollen Extract in Relieving Hot Flushes in Sex Hormone-Deficienct Rats

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Abstract

Hot flushes or hot flashes are one of the vasomotor symptoms frequently found in menopausal women and andropausal men. Pine pollen has been used in traditional Chinese medicine (TCM) for health restoration, anti-aging, and longevity tonic supplements. Since Scotch pine contains steroid hormones, it has been claimed for maintaining men's health. This study aimed to investigate the effects of pine pollen extract from *Pinus patula* ssp. *tecunumanii* in relieving hot flushes in rats with sex hormone depletion. Alpha-calcitonin gene-related peptide (αCGRP) was injected into female rats every other day for tail skin temperature (TST) elevation. Pine pollen extract significantly reduced TST in a concentration-dependent manner on the 3rd day of oral administration. Conjugated equine estrogen (CEE), used for estrogen replacement therapy, spent more time to reduce TST than pine pollen extract did (the 6th day of administration), and continuously decreased TST until the end of experiment. While TST in male rats was increased by daily injection of leuprorelin acetate, the pine pollen extract significantly reduced TST in a dose dependent manner on the 2nd day of oral administration. Testosterone undecanoate (TU), used for hormone replacement therapy in men, significantly reduced TST on every days of oral administration. The ELISA results showed that uterine estrogen concentrations of CEE and pine pollen extract treated groups were significantly higher than control while no significant difference in serum estrogen levels was found. Only serum testosterone concentration of TU treated group was significantly higher than control while testis weights of all groups were lower than sham-treated group. Pine pollen extract from *Pinus patula* ssp. *tecunumanii* could be classified as a phyto-androgen that might balance testosterone and estrogen levels, resulting in alleviating the symptoms of sex hormone deficiency such as hot flushes, night sweating, and sleep disturbance.

Keywords: Hot flushes, pine pollen extract, testosterone, estrogen, tail skin temperature (TST)

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ผลของสารสกัดเกสรสนในการลดอาการร้อนวูบวาบในหนูที่มีภาวะพร่องฮอร์โมนเพศ

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บทคัดย่อ

อาการร้อนวูบวาบจัดเป็นอาการของระบบหลอดเลือดที่พบบ่อยในผู้หญิงและผู้ชายวัยทอง เกสรสนถูกใช้ในแพทย์แผนจีนเพื่อบำรุงร่างกาย ชะลอวัย และเป็นยาอายุวัฒนะ เนื่องจากงานวิจัยพบว่าสนสก็อตที่ขายในท้องตลาดมีส่วนประกอบของสเตียรอยด์ฮอร์โมน ซึ่งใช้บำรุงสุขภาพเพศชาย การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดเกสรสนจาก *Pinus patula* ssp. *tecunumanii* ในการลดอาการร้อนวูบวาบในหนูที่มีภาวะพร่องฮอร์โมนเพศ จากการทดลองโดยฉีดแอลฟ่าแคลซิโตนินยีนรีเลเตดเปปไทด์ (แอลฟ่าซีจีอาร์พี) วันเว้นวันในหนูเพศเมียเพื่อเหนี่ยวนำให้อุณหภูมิที่หางหนูสูงขึ้น พบว่าอุณหภูมิหางหนูลดลงอย่างมีนัยสำคัญทางสถิติในวันที่ 3 ของการป้อนสารสกัดเกสรสน ซึ่งผลที่ได้สัมพันธ์กับขนาดของสารสกัดที่เพิ่มขึ้น และพบว่าคอนจูเกตเตดอีควายเอสโตรเจน (ซีอีอี) ซึ่งใช้ในการรักษาภาวะขาดฮอร์โมนเอสโตรเจน ใช้เวลาในการลดอุณหภูมิหางหนูนานกว่าสารสกัดเกสรสน (ในวันที่ 6 ของการป้อนสารสกัด) และอุณหภูมิหางหนูลดลงอย่างต่อเนื่องจนถึงวันสุดท้ายของการทดลอง ส่วนการทดลองในหนูเพศผู้ที่ฉีดลิวโปรรีลินอะซิเตดทุกวัน เพื่อเหนี่ยวนำให้อุณหภูมิที่หางหนูให้สูงขึ้น ผลการทดลองพบว่าอุณหภูมิหางหนูลดลงอย่างมีนัยสำคัญทางสถิติในวันที่ 2 ของการป้อนสารสกัดเกสรสน ซึ่งผลดังกล่าวสัมพันธ์กับขนาดของสารสกัดที่ได้รับ และการป้อนเทสโทสเตอโรนอันเดคคาโนเอต (ทียู) ซึ่งใช้ในการรักษาภาวะขาดฮอร์โมนเทสโทสเตอโรน สามารถลดอุณหภูมิหางหนูได้อย่างมีนัยสำคัญทางสถิติได้ทุกวัน ผล ELISA พบว่าระดับเอสโตรเจนในมดลูกของกลุ่มที่ได้รับซีอีอีและสารสกัดเกสรสนสูงกว่ากลุ่มควบคุม แต่ไม่พบความแตกต่างของระดับเอสโตรเจนในซีรัมเมื่อเทียบกับกลุ่มควบคุม และมีเฉพาะกลุ่มที่ได้รับทียูเท่านั้นที่มีระดับเทสโทสเตอโรนในซีรัมสูงกว่ากลุ่มควบคุม ในขณะที่น้ำหนักอัณฑะทุกกลุ่มไม่แตกต่างจากกลุ่มควบคุม โดยสรุป สารสกัดเกสรสนจาก *Pinus patula* ssp. *tecunumanii* อาจจัดเป็นไฟโตแอนโดรเจน ซึ่งมีผลต่อสมดุลระหว่างเทสโทสเตอโรนและเอสโตรเจนในร่างกาย ส่งผลช่วยลดอาการที่เกิดจากการขาดฮอร์โมนเพศได้เช่น อาการร้อนวูบวาบ เหงื่อออกตอนกลางคืน และนอนไม่หลับ

คำสำคัญ: อาการร้อนวูบวาบ, เกสรสน, เทสโทสเตอโรน, เอสโตรเจน, อุณหภูมิหางหนู

Introduction

Hot flusheses or hot flash are one of the vasomotor symptoms that is frequently found in menopausal women and andropausal men. The symptoms start from a suddenly warm sensation in the upper body to an outpouring of sweat, and an increase in heart rate and peripheral blood flow resulting in skin temperature elevation.[1] It is the reflection of a disorder of hypothalamic thermoregulatory mechanisms. The thermoneutral zone is narrow down in postmenopausal women caused by losing of thermoregulation of autonomic thermoeffectors such as vessels, muscle, and skin.[1] Onset of hot flushes is approximately one year after menopause, and the symptoms still remain for 6 months to 2 years. It occurs in up to 80% of menopausal women and can vary by culture and ethnicity.[2] In Thailand, the median age of menopausal onset is approximately at 50 years old, while the life expectancy of women is 75 years old.[3] Therefore, women are in postmenopausal phase about 25 years, which accounted for one-third of their lifetime. In men, although serum testosterone levels decline by approximately 1% per year after the age of 40, most of men still restore testosterone levels within the normal range to prevent hot flushes.[4] It is estimated that up to 80% of patients on androgen deprivation therapy (ADT) such as prostate cancer patients will experience hot flush.[5] Hot flushes impact on health-related quality of life because they involve poor sleep and depressed mood, leading to a major depressive episode.[6-7]

Hormone replacement therapy (HRT) is the most effective treatment for diminishing hot flushes and night sweats in menopause women by increasing the estrogen levels in the body.[8-11] The randomized controlled trial (RCT) studies showed that unopposed estrogen therapy (for hysterectomized women) or combined estrogen-progestin therapy (for intact uterus women) significantly decreased the symptoms of hot flushes compared to the placebo groups.[8-11] However, Women's Health Initiative (WHI) reported that HRT increased the risk of stroke, but there were no effects on coronary heart diseases (CHD),[12-14] and also increased the risk of breast cancer with duration of use.[15-17] Alternative treatments to HRT including phyto-estrogens (from plants with hormone-like effects e.g. black cohosh)[18], gabapentin 900 mg/day[19], evening primrose oil and vitamins e.g. vitamin E 800-1,000 IU/ day[20], anti-depressants; e.g. paroxetine 7.5 mg/day[21], venlafaxine SR 75 mg/day[22], α-2 agonists; clonidine 25-75 μg twice a day[23], are also used for relieving hot flushes. Yet, they still have side effects and/or interact with other drugs. Herbal remedies are other alternative treatments that people often use because they are obtained from natural sources, claimed to be safe with lower risk of side effects, lower cost, and widespread availability. However, it is necessary to determine the effectiveness, the appropriate use in patients with other complications e.g. liver or kidney disease, and the regulation of use of herbal remedies.[24]

Pine pollen refers to the pollen of the *pinus* genera trees, which is commonly used as dietary supplement. It has been used in traditional Chinese medicine (TCM) for more than 2,000 years according to "The Pandect of Materia Medica", the oldest classic pharmacology text in Han dynasty. Pine pollen from *Pinus massoniana* and *Pinus tabulaeformis* have been used as a folk Chinese medicine for health restorative, anti-aging, and longevity tonic supplements by traditional medicine physicians.[25]

Scotch pine (*Pinus sylvestris*) has been found to contain steroid hormones; testosterone (0.8 μg/10 g), epitestosterone (1.1 μg/10 g), and androstenedione (5.9 μg/10 g).[26] This pine is the most commonly used as a source of pollen in the market in the form of powder, tincture, and capsule for androgenic dietary supplement such as muscle growth and general male vitality. In Thailand, both wood and trunk of pine have been used in the paper industry, furniture, rosin or turpentine for musical cables instruments, color, watershed conservation, and line of fire on high ground. Two types of tropical pines that grow naturally in Thailand include *Pinus kesiya* Royal ex Gordon and *Pinus merkusii* Jungh. et de Vriers. Later in 1969, the Forest Department in collaboration with the Danish government led by DANIDA (Danish International Development Agency) conducted research about exotic pines. Exotic pines originated naturally from Central America e.g., *Pinus caribaea Morelet*, *Pinus oocarpa Schiede*, and *Pinus patula* ssp. *tecunumanii*[27], can thrive in environment of the north of Thailand. Unlike Scotch pine, the chemical constituents of pollens from these exotic pines, particularly natural sex steroids, have not yet been studied. Although some pine pollen products have been claimed for maintaining men's health such as boosting testosterone, balancing hormones, boosting energy, maintaining vitality during the aging process, increasing sperm count, promoting sexual libido, skin rejuvenation, improving immune system, anti-aging, and detoxification, this statement was not approved by FDA. Since there are no scientific evidences of hormonal effects of pine pollens, US FDA stated that "these products are not determined scientifically to treat, cure, or prevent any diseases.[28]

Therefore, this study aimed to evaluate the effects of pine pollen extract on relieving hot flushes in rats with sex hormone depletion.

Materials and Methods

Chemicals and reagents

Rat αCGRP was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in saline on the day of use to obtain a concentration of 10 μg/mL. Leuprorelin acetate 3.75 mg was purchased from Takeda Pharmaceutical Company Limited (Osaka, Japan) and dissolved in solvent supplied in the prefilled syringe to obtain a concentration 3.75 mg/mL. Conjugated equine estrogen (CEE) 0.625 mg/ tablet (Pfizer, Ringaskiddy County Cork, Ireland) was dissolved in 7% w/v acacia to obtain a concentration of 10 mg/mL. Testosterone undecanoate (TU) 40 mg/ capsule (MSD, Haarlem, Netherlands) was dissolved in soybean oil (Sigma-Aldrich, St. Louis, USA) to obtain a concentration of 10 mg/mL.

Pine pollen from *Pinus patula* spp. *tecunumanii* was collected at Huaybong Silvicultural Research Station, Tumbon Bo Luang, Amphoe Hod, Chiang Mai Province. It was extracted with 95% ethanol at room temperature for 2 weeks, and then was evaporated to obtain ethanolic pine pollen extract (% yield = 10%). Pine pollen extract solution was dissolved in 7% w/v acacia to obtain a concentration of 50 mg/mL.

Rat estradiol (E2) ELISA kit (Cat. No. MBS727445) and mouse/rat testosterone ELISA kit (Cat No. MBS580136) were purchased from MyBioSource, San Diego, USA.

Animals

Male Wistar rats weighing 300±20 g and female Wistar rats weighing 250±20 g were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The rats were maintained on a 12 h light/ dark schedule at a temperature of 23±1°C, with free access to food and water. Handling and testing in animals were in accordance with the "Guidelines for the care and use of laboratory animals". The study protocol was approved by the Thailand Institute of Scientific and Technological Research (TISTR) Animal Care and Use Committee, Pathum Thani, Thailand and Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Identification of vaginal cytology

Estrous cycle is a factor to determine the entirety of the hypothalamic-pituitary-ovarian reproductive axis or behavioral receptivity (also known as "heat") in animals. It is characterized by the morphological changes of cell lining in rat vagina that responds to the circulating sex hormones. The estrous cycle is divided into 4 phases as follows: proestrus, estrus, metestrus, and diestrus, which can be identified from different cell types in vaginal smear. The regular cycle is the classic cycle sequence (4-5 day/cycle) of smear that is successive pro-estrus smear with regular cycling. The irregular cycle consists of two contiguous intervals of 5 to 8 days of diestrus stages. While cyclic (constant estrus) smear shows repeated diestrus or no cycle within 9 days, persistent vaginal cornification lasts more than 9 days.

In this study, female rats were screened for irregular/ acyclic of estrous cycles for mimicking naturally senescence of ovarian function in humans by vaginal smear examination. The vaginal smears were collected every morning between 08.00 and 10.00 a.m. for 14 days in each month starting from 9 months old to 17 months old or until irregular/acyclic stage was found in all rats by swab smear technique modified from OECD Test Guideline (TG407).[29] Briefly, the rat was held around the thorax, ventral surface uppermost, whilst providing the lumbar as far as possible. The moistened cotton bud (dipped in saline) was inserted approximately 1.0 cm into the rat vagina, then gently and quickly removed and then transferred cells from vagina lumen on the glass slide. The risk of this method is cervical stimulation that causes pseudopregnancy. A stereoscopic microscope (N-180M, Boeco, Hamburg, Germany) with a magnification of between 80 and 100 used to identify vaginal cytology and smear reading.

Animal treatment and blood sample collection

Eighteen-month-old-female rats with irregular estrous cycles were divided into 6 groups (n=3). Five groups of rats were treated with subcutaneous (SC) injection of αCGRP (10 μg/kg) every other day for 15 days. On day 5, the αCGRP-treated rats were orally administered with 7% w/v acacia solution (control), CEE (10 mg/kg) or pine pollen extract (100, 300, and 500 mg/kg) every day for 11 days. For sham-treated control group, rats were treated every other day with SC injection of saline instead of αCGRP. Tail skin temperatures (TST) and core body temperature of all rats were recorded using digital thermometer with probe (TL8009, Keyan Technology Co. Ltd., Songjing, Shanghai, China) and mercury thermometer (CRW-

23A, Jianggsu Yuyue Medical Instruments Co. Ltd., Zhenjiang, Jiangsu, China), respectively, at baseline and 1 h after treatment for 15 days. The data were expressed as the change from baseline.

Two hours after administrating the final dose of CEE and pine pollen extract on day 15, all rats were sacrificed, and blood samples were collected from abdominal vein for determining estrogen concentration. The uteri were isolated, weighed and rapidly frozen in liquid nitrogen, and then stored at -80°C for estrogen receptor binding assay by ELISA technique.

Thirty-six male rats were divided into 6 groups (n=3). Five groups of rats were treated with SC injection of leuprorelin acetate (1 mg/kg) every other day for 11 days. On day 9, the leuprorelin acetate-treated rats were orally administered with soy bean oil, TU (10 mg/kg) or pine pollen extract (100, 300, and 500 mg/kg) daily for 3 days. For sham-treated control group, rats were treated every other day with SC injection of saline. TST and core body temperature of all rats were recorded using digital thermometer with probe (TL8009, Keyan Technology Co. Ltd., Songjing, Shanghai, China) and mercury thermometer (CRW-23A, Jianggsu Yuyue Medical Instruments Co. Ltd., Zhenjiang, Jiangsu, China), respectively, at baseline and 1 h after treatment for 11 days.

Two hours after administration the final dose of TU and pine pollen extract on day 11, all rats were sacrificed and then blood samples were collected from abdominal vein for determination of testosterone concentration. The testes were isolated, and immediately frozen in liquid, nitrogen and then stored at -80°C for androgen receptor binding assay by ELISA technique.

All procedures for determination of tail skin and core body temperatures were performed between 08.00-12.00 a.m. The room temperature was maintained at 23±1°C throughout the recording period.

Determination of estrogen and testosterone levels using ELISA technique

Rat estradiol (E2) ELISA kit is the competitive inhibition enzyme immuno-assay technique. The E2 from samples and estradiol conjugate with horse-radish peroxidase enzyme conjugate (E2-HRP) compete for the anti-E2 antibody binding site that is limited. The more binding sites are occupied by E2 from sample, the fewer sites are left to bind E2-HRP conjugate. The intensity of color measured via microplate reader is inversely proportional to the E2 concentration.

The mouse/rat testosterone ELISA kit is based on the competitive binding between testosterone from samples and testosterone-HRP conjugate for the anti-testosterone antibody binding site that is limited. The more binding sites are occupied by testosterone from sample, the fewer sites are left to bind testosterone-HRP conjugate. The intensity of color measured via microplate reader is inversely proportional to the testosterone concentration.

Statistical analysis

All data were expressed as the mean±SEM Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by LSD test, post-hoc analysis. Student t-test was also used. A value of $P<0.05$ was considered for statistical significance.

Results

Effect of pine pollen extract on αCGRP-induced elevation in female rats

The increasing in TST in female rats was induced by the administration of αCGRP on every other day. On the first day of oral administration of CEE or pine pollen extract (or day 5 of the experiment), all treatment groups had no significant difference in baseline TST when compared with the control group. On day 6 of the experiment, a significant reduction in TST was observed only in the group receiving 500 mg/kg pine pollen extract.

The pine pollen extract statistically reduced TST in a dose dependent manner on the 3rd day of treatment (day 7 of the experiment) while CEE reduced TST since the 6th day of treatment (day 10 of the experiment) and continuously decreased TST until the end of experiment (Figure 1). The summary of TST changes in each group was shown in Figure 2. Moreover, only the group receiving 10 mg/kg CEE significantly reduced core body temperature as shown in Figure 3.

Effect of pine pollen extract on uterine weight and estrogen levels in female rats

The weight of uteri in rats receiving 10 mg/kg CEE was significantly higher than the control group, while the extracts of pine pollen did not affect the uterine weight (Figure 4). However, pine pollen extract significantly increased estradiol (E2) levels in uterus, but not in serum (Figure 5).

Effect of pine pollen extract on leuprorelin-induced TST elevation in male rats

The increase in TST induced by administration of leuprorelin acetate for 11 days significantly reduced in rat receiving TU and 500 mg/kg pine pollen extracts on day 9 of the experiment (1st day of treatment).

The pine pollen extract significantly reduced TST in a dose dependent manner on the 2nd day of treatment (day 10 of the experiment) statistically. While, TU reduced TST since the 1st day of treatment (day 9 of the experiment) and continuously decreased TST until the end of experiment (Figure 6). TST changes in each group are summarized in Figure 7. No significant changes in core body temperature were observed (Figure 8).

Effect of pin pollen extract on testis weight and testosterone levels in male rats

The weight of testes in the control group was significantly lower than the sham group as shown in Figure 9. The serum testosterone levels in rat receiving 10 mg/kg TU group was significantly higher than the control group while no significant changes in serum testosterone levels were found in rats receiving the extract of pine pollen (Figure 10).

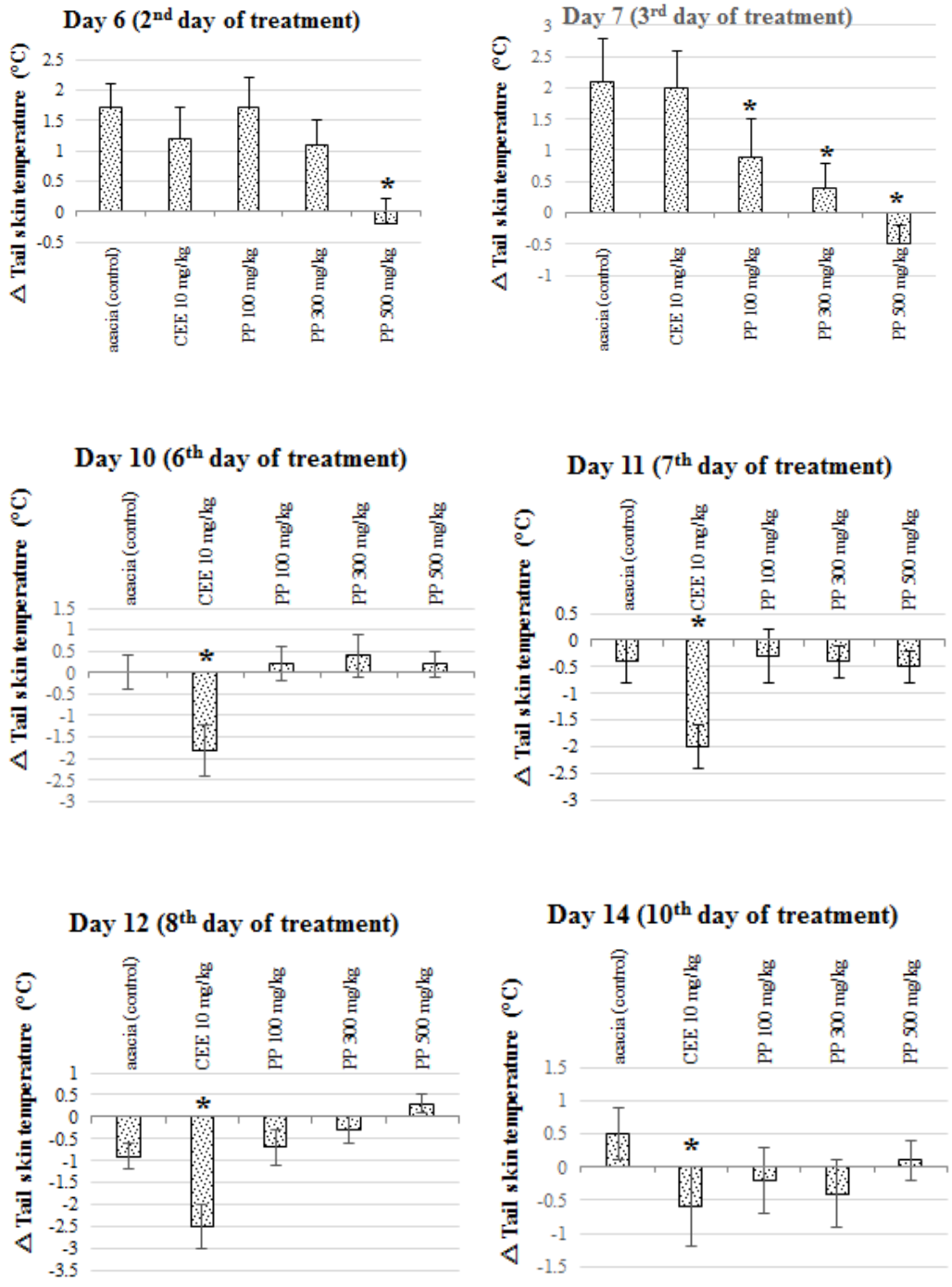

Figure 1. Changes in TST (mean±SEM) from baseline to 1 h after oral administration of CEE or pine pollen extract (PP) in female rats on days 6, 7, 10, 11, 12, and 14 of the experiment. Only the data on the day with significant differences in TST changes from baseline were shown. *statistically significant ($P<0.05$) when compared with control group (acacia).

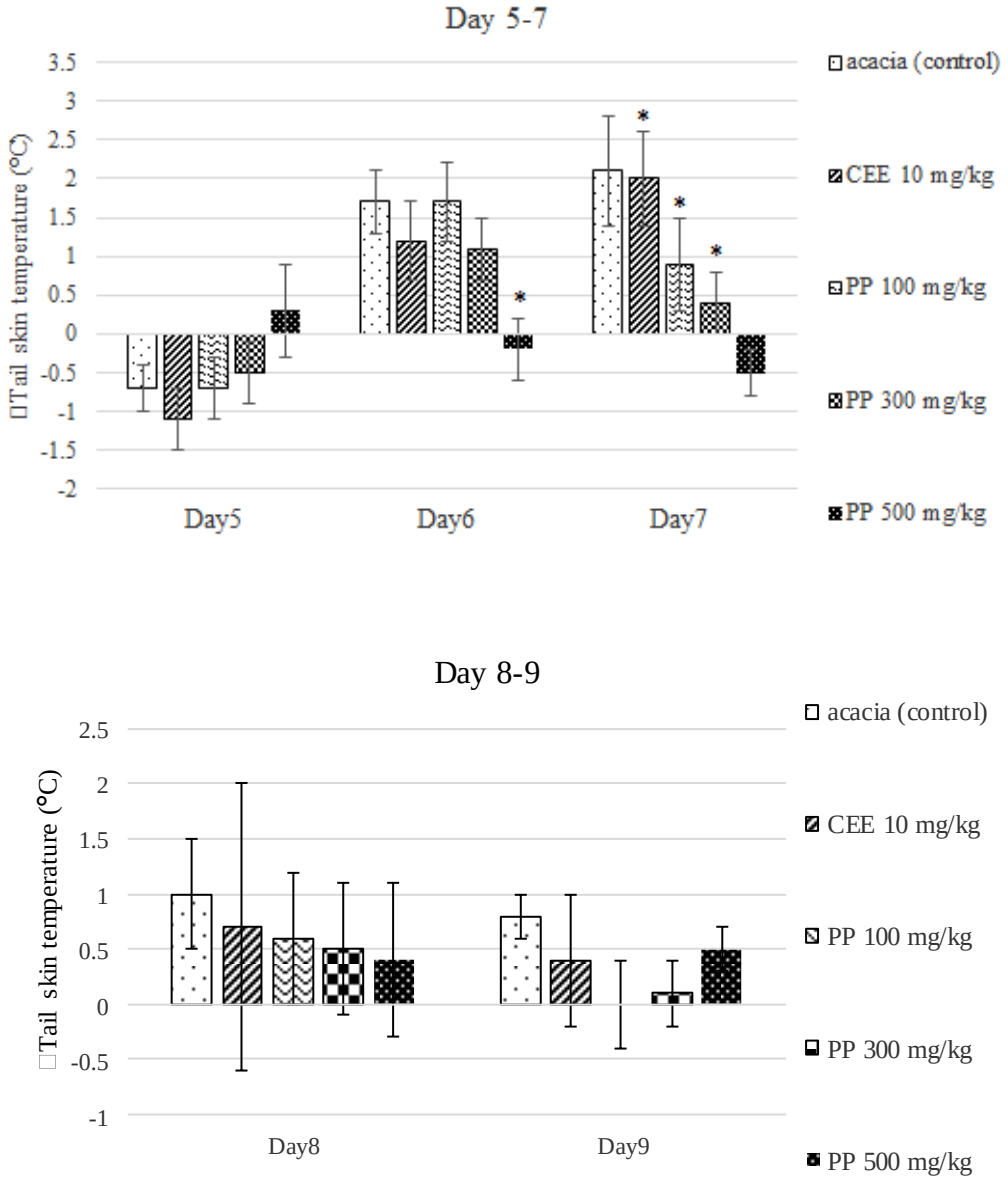

Figure 2. Changes in TST (mean±SEM) from baseline (day 5 or the 1st day of treatment) to day 15 (the 11th day of treatment) after oral administration of CEE or pine pollen extract (PP) in female rats. *statistically significant (P<0.05) when compared with control group (acacia).

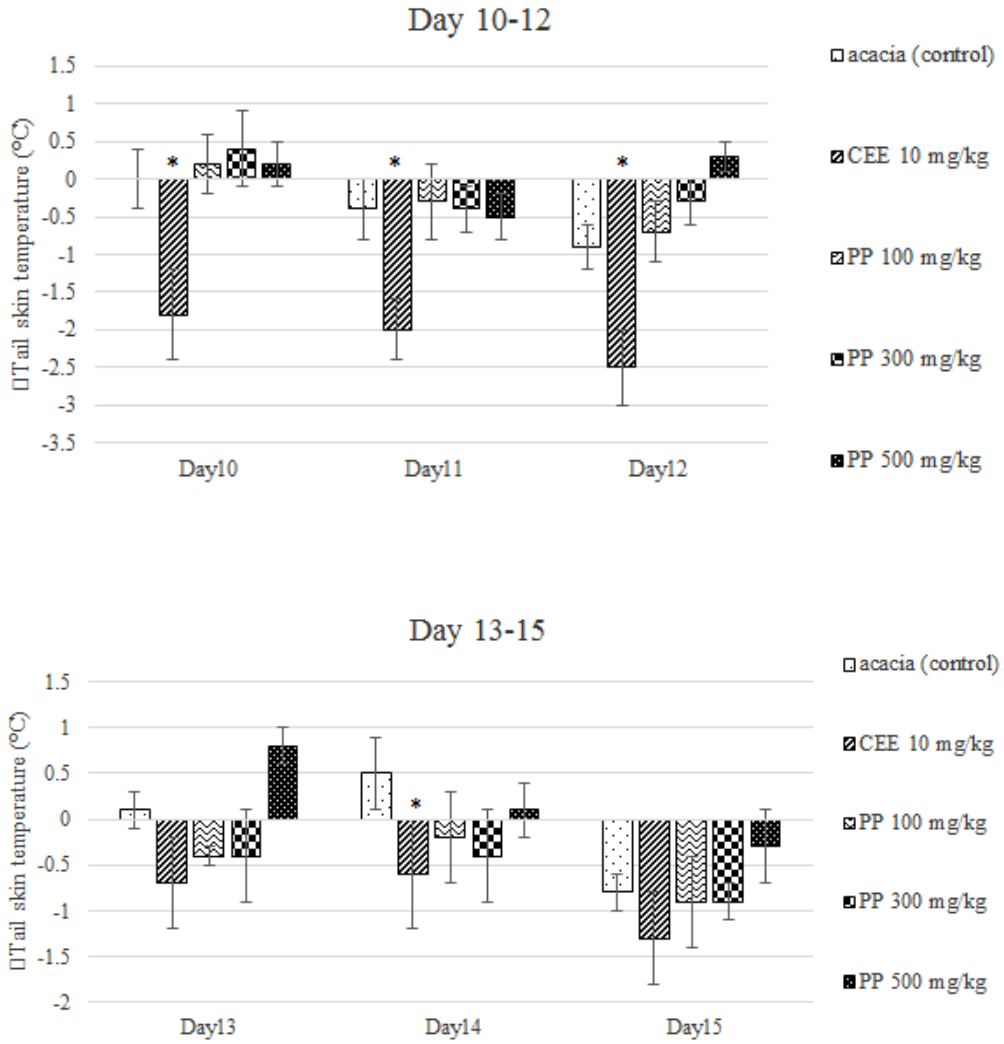

Figure 2 (Continued). Changes in TST (mean±SEM) from baseline (day 5 or the 1st day of treatment) to day 15 (the 11th day of treatment) after oral administration of CEE or pine pollen extract (PP) in female rats. *statistically significant (P<0.05) when compared with control group (acacia).

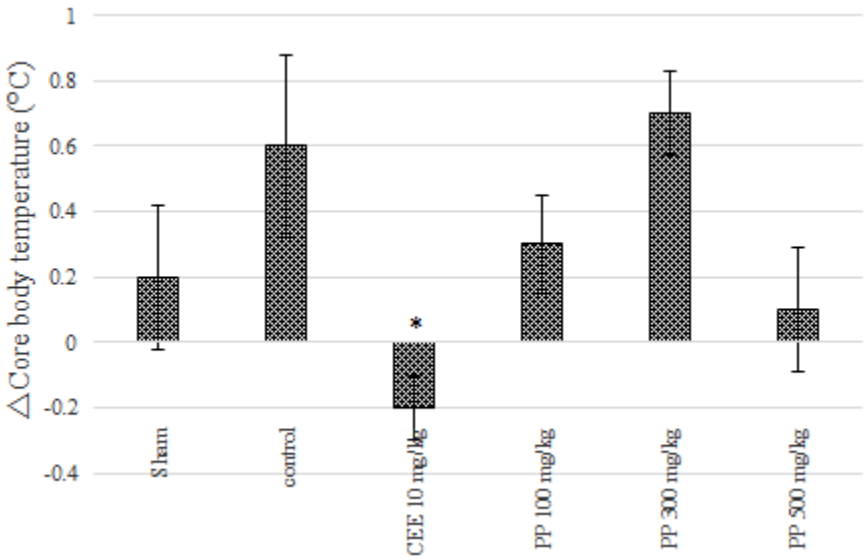

Figure 3. Changes in core body temperature (mean±SEM) of female rats on day 15 (the 11th day of the treatment) after oral administration of CEE or pine pollen extract (PP) in female rats.

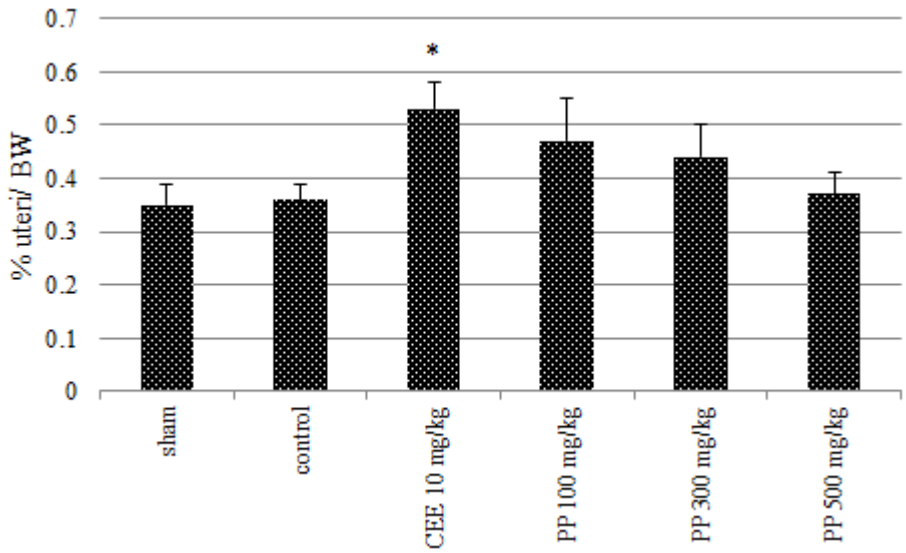

Figure 4. Weight of uteri (mean±SEM) in female rats after oral administration of CEE or pine pollen extract (PP). *statistically significant (P<0.05) when compared with control group (acacia).

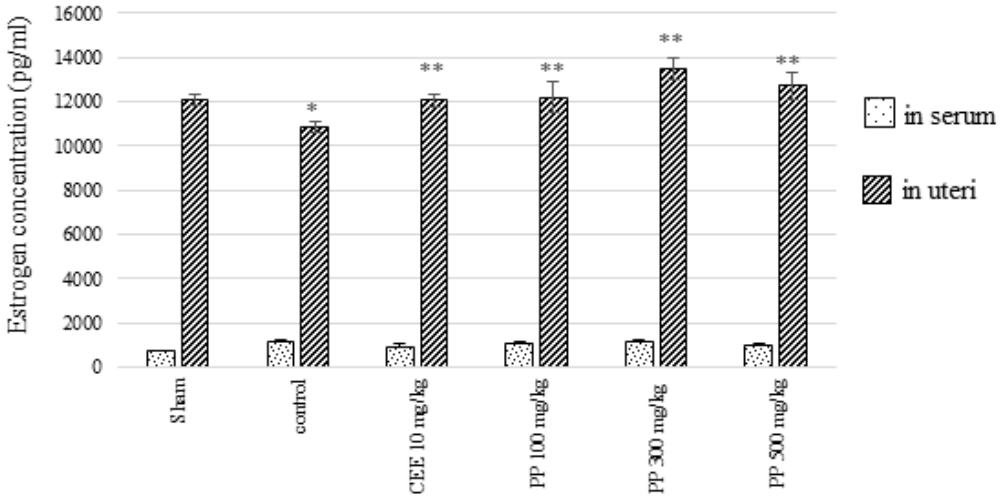

Figure 5. Estrogen concentration (mean±SEM) in serum and uteri in female rats after oral administration of CEE or pine pollen extract (PP). *statistically significant (P<0.05) when compared with sham, **compared with control.

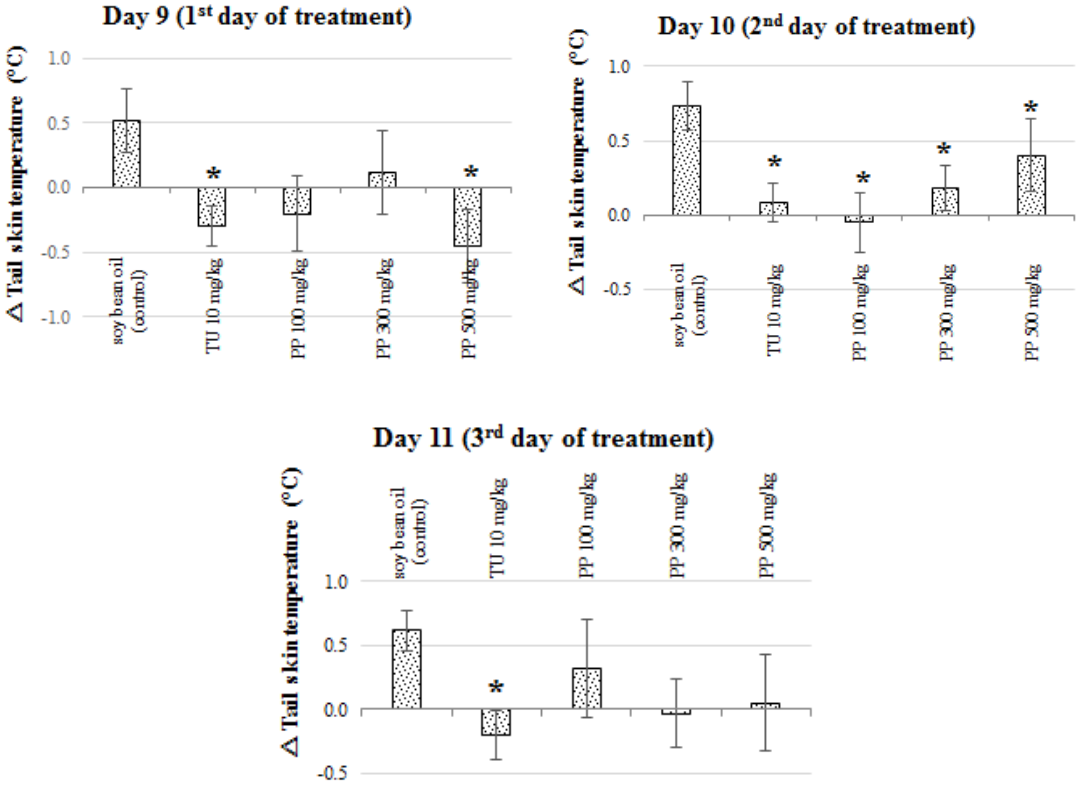

Figure 6. Changes in TST (mean±SEM) from baseline (day 9 or the 1st day of treatment) to 1 h after oral administration of TU and pine pollen extract (PP) in male rats (starting on day 9 of the experiment (the 1st day of treatment) until the end of experiment (day 11 or the 3rd day of treatment). *statistically significant (P<0.05) when compared with control group (soy bean oil).

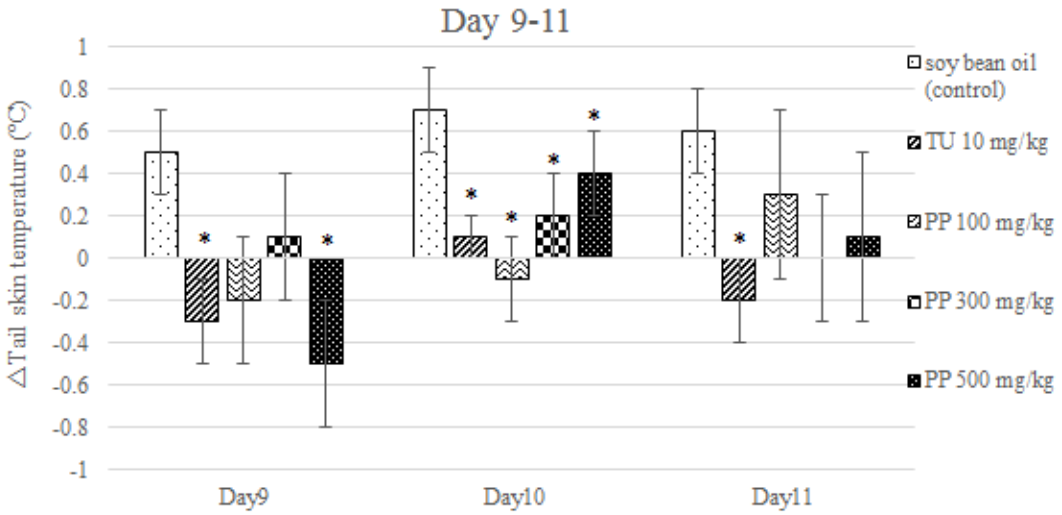

Figure 7. Changes in TST (mean±SEM) from baseline (day 9 or the 1st day of treatment) to day 11 (the 3rd day of treatment) after oral administration of TU and pine pollen extract (PP) in male rats. *statistically significant (P<0.05) when compared with control group (soy bean oil).

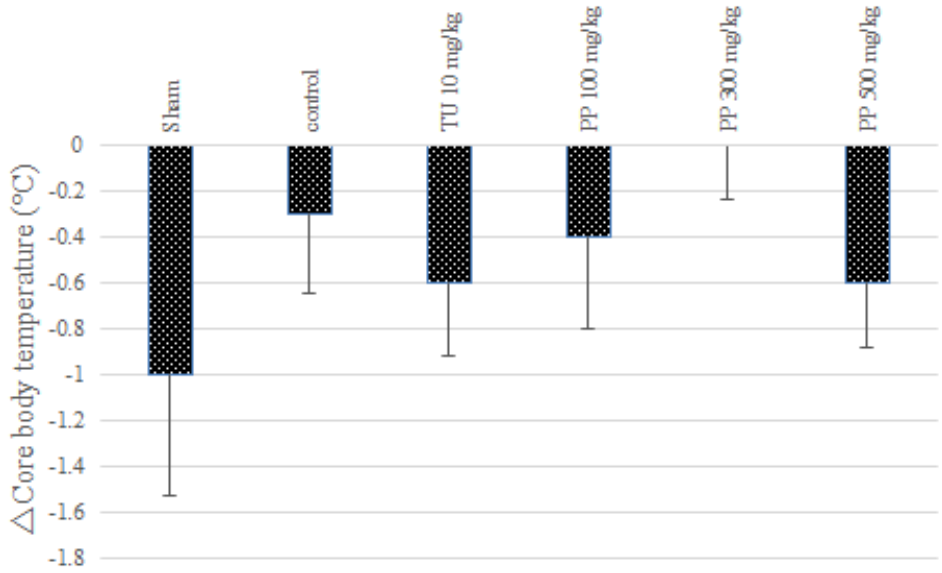

Figure 8. Changes in core body temperature (mean±SEM) of male rats on day 11 (the 3rd day of treatment) after oral administration of TU and pine pollen extract (PP) in male rats.

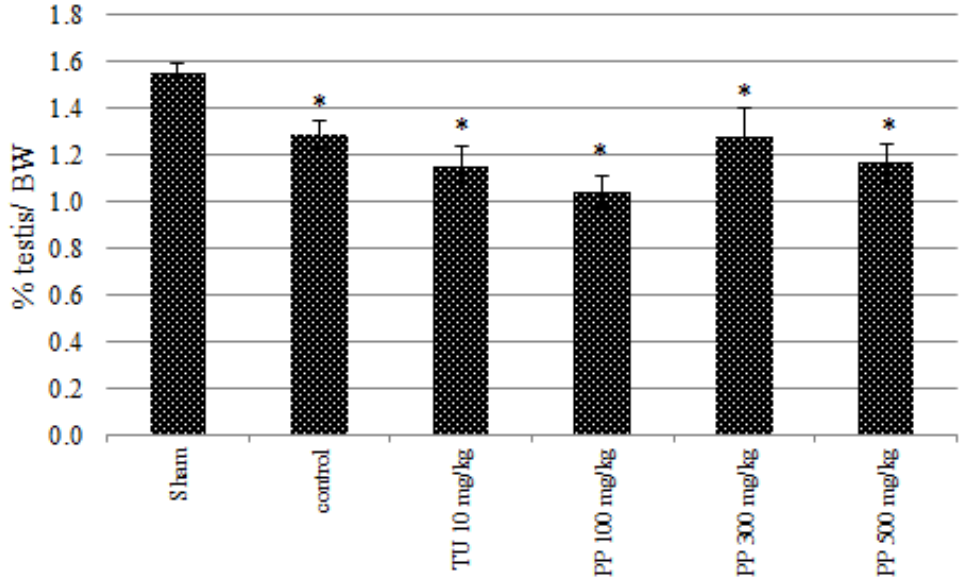

Figure 9. Weight of testes (mean±SEM) in leuprorelin-treated male rats after oral administration TU and pine pollen extract (PP). *statistically significant (P<0.05) when compared with sham-treated group.

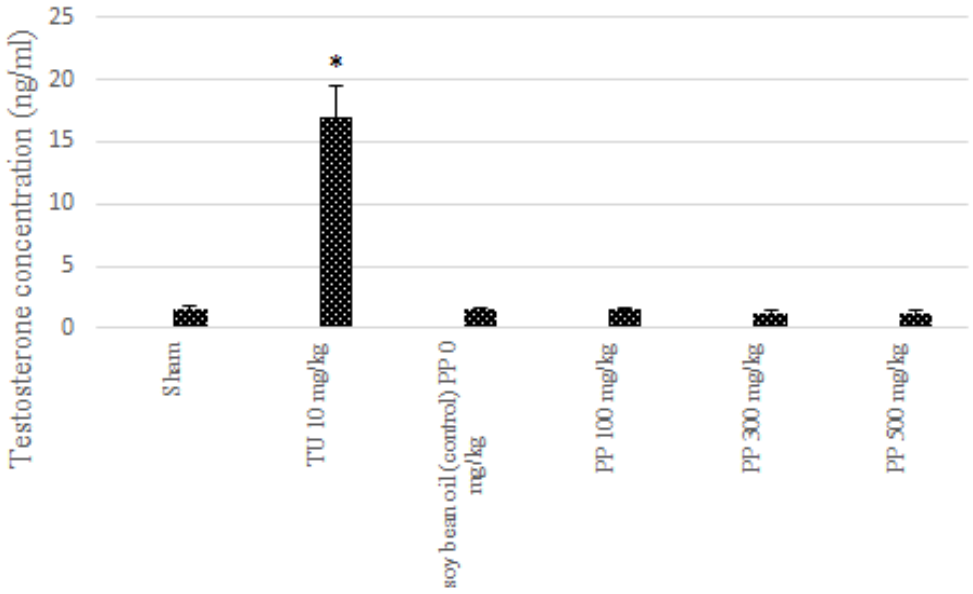

Figure 10. Testosterone concentration (mean±SEM) in serum of male rats after oral administration TU and pine pollen extract (PP). *statistically significant (P<0.05) when compared with control group (soy bean oil).

Discussion

In our previous studies[30], αCGRP and leuprorelin acetate were used to elevate TST for mimicking hot flush simulation in female and male rats. The estrogens play an important role in body's thermoregulation. Conjugated equine estrogen (CEE) is used for hormone replacement therapy (estrogen replacement therapy) to prevent osteoporosis, cardiovascular disease, and hot flushes, especially in women with severe menopausal or hormonal depletion. The key components of this drug are substance estrogens derived from horses (equine), which include at least 10 species of sulfated esters, for example, estrone (50.6%), equilin (23.6%), 17α-dihydroquilenin (15.3%), 17α-estradiol (3.8%), equilenin (3.3%), 17β-dihydroquilenin (2.3%), and 17β-estradiol (1.1%). These sulfated ester can bind to the estrogen receptor and exhibit estrogenic activity.[31]

Pharmacokinetics of CEE are quite complicated because because it comprises several components and has the first-pass metabolism. Troy *et al*.[32] found that after receiving a single dose of 0.625 mg CEE; the maximum concentration (C_{max}) of total serum estrone, serum estrone, total serum equilin, serum equilin were 5.7 ng/mL (at 7.7 h), 135 pg/mL (at 8.6 h), 4.1 ng/m> (at 5.8 h), and 70 pg/mL (at 7.3 h), respectively. No estradiol and 17β-dihydroquilenin were found.[32]

This study demonstrated that pine pollen extract and CEE significantly reduced TST in αCGRP-induced TST elevation in female rats, indicating that pine pollen extract could relieve hot flushes. An increase in uterine E2 levels induced by pine pollen extract was also observed while the changes in serum E2 levels and uterine weight were not significant.

Although pine pollen extract of *Pinus patula* ssp. *tecunumanii* had unknown components, it might have a similar composition like Scotch pine especially steroid hormones which could be altered to estrogen in the body. However, it did not reduced TST as much as CEE did. The results of uterine weight and ELISA showed that pine pollen extract probably bound to receptors same as CEE. Pine pollen might activate estrogen-controlled functions for thermoregulation activity similar to the way CEE did. CEE spent longer times for reducing TST than pine pollen extract did. It possibly bound to estrogen receptor in the nucleus that required time to show its effect. However, pine pollen extract had unknown key ingredients and mechanism of action on relieving hot flushes was not observed at the end of the experiment.

Pine pollen extract and TU also significantly reduced TST in leuprorelin acetate-induced TST elevation in male rats. Pine pollen might have the same mechanism of action as TU, *i.e.*, to activate receptors for thermoregulation activity. TU is used for testosterone replacement therapy in males with endogenous testosterone deficiency. In this study, only TU-treated rats showed a significant increase in serum testosterone level after 2 h administration. In correspondence to our findings, TU in castor oil had C_{max} in rat serum (6,408 pg/mL) at 1 h.[33] Probably, it reduced TST and significantly increased in serum levels within 2 h. TU and pine pollen extract could not restore the reduction of testis weight induced by chronic administration of leuprorelin.

The mechanism of action of pine pollen extract is probably through balancing hormones in patients with sex hormone deficiency by converting testosterone to estradiol[34] by aromatase enzyme (also called estrogen synthetase or estrogen synthase)[35], resulting in alleviating the symptoms of sex hormone deficiency such as hot flushes, night sweating, and sleep disturbance.

The core body temperature (Tc) was used as a marker of infection during the experiment. The results showed no significant differences in core body temperature changes, indicating no infection in animals.

Conclusion

We demonstrated that pine pollen extract could reduce TST-induced by αCGRP and leuprorelin acetate in female and male rats, respectively. Pine pollen extract and CEE significantly reduced TST in αCGRP-induced tail skin temperature elevation in female rat.

The results of uterine weight and ELISA showed that pine pollen extract probably bound to receptors and might activate estrogen-controlled functions for thermoregulation activity similar to CEE. Pine pollen extract might also activate receptors involved in thermoregulation, similar to TU, resulting in TST reduction. However, TU and pine pollen extract could not restore the reduction of testes weight induced by chronic administration of leuprorelin.

Pine pollen extract from *Pinus patula* ssp. *tecunumanii* could be classified as a phyto-androgen similar to Scotch pine (*Pinus sylvestris*). However, further studies are needed to confirm the results of this experiment in order to develop an animal model for screening the hot flush-alleviating substances in humans. In addition, acute and chronic toxicology of this plant should be tested.

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