RESEARCH ARTICLE

The effects of different extracts of *Glycine max* (L.) Merr. on primordial, primary and secondary follicles

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Abstract

Aim: The effects of plant soya (*Glycine max L.*) on the female genital system have been investigated.

Materials and Methods: *n*-hexane, ethyl acetate, ethanol extracts of soya plant were administered to the rats by oral route at 100 and 200 mg/kg doses (SID) for 1 month. By monitoring the menstrual cycle with vaginal smear method, di-estrus stage of the rats were determined. At the end of expe-rimental period the animals were euthanized. Genital system organs taken of rats were analyzed by stereological methods. Follicles were counted with optical disector method in the ovaryum stereologically, the weights of the ovaries, uterus were also evaluated.

Results: Some changes occured in genital system of the rats which were given at 200 mg/kg dose of soy ethanol extract. In test groups vaginal aperture increased, uterus appeared likehyperemia while significant weight increase did not oc-cur. The weight of relative ovarium, uterus weight increased, coarsening of epithelial cells was observed. No significant difference was observed in all groups regarding the numbers of primordial, primary follicles. Diameter of secondaryfollicle reduced while the number of secondary follicule incr-eased in 200 mg/kg ethanol extract administered group, when compared to control group. The amount of isoflavone content was determined by using high pressure liquid chro-matography technique on the active extract. The amount of daidzein, genistein were found to be 3.851 µg/g and 3.127 µg/g, respectively in the ethanol extract.

Conclusion: It could be stated that phytoestrogens in soya plant dose-dependently affect the female genital system.

Keywords: Phytoestrogen, *Glycine max* L., reproductive system, stereology
Introduction

Glycine max (L.) Merr. (soy, soybean, soya bean), belongs to the Fabaceae family, is cultivated to produce oil and flour especially in Asian countries (Modaresi et al 2011). Soy is rich in protein content, approximately 40%, including mainly essential amino acids (El Din et al 2011, Modaresi et al 2011). This plant is very important due to its phytoestrogen content (Modaresi et al 2011). Phytoestrogens are plant-derived nonsteroidal secondary metabolites have the ability to cause estrogenic or and antiestrogenic activities due to their structural similarity with estradiol (17-β-estradiol) (Jefferson 2010, Modaresi et al 2011).

Many studies have indicated positive effects of isoflavons on hormone-dependent disorders, cardiovascular diseases, cancer and osteoporosis. Their negative effects on menstrual cycle were also reported in several researches (Sethell et al 1998, Lee et al 2004). Isoflavons accumulate mainly in uterus, ovary and vagina rather than the other peripheral organs. Soy consumption of women is related with the morphology and function of the ovary (Brasil et al 2009, Modaresi et al 2011).

Abnormal estrus cycle and uterotrophic effects have been reported due to the consumption of soya during the neonatal and mature period (Brasil et al 2009). Gallo et al (1999) reported that the average period of the menstrual cycle increased and vaginal opening occurred earlier in the animals receiving soy supplemented feed. In high-dose females, effects on the uterine including increases in weight, oedema, endothelial hyperplasia and vaginal inflammation were observed (Gallo et al 1999). Contraceptive effects of soya were also demonstrated (Jefferson 2010).

Although many researches were conducted about the effects of soya on the female genital system, to the best of our knowledge, no data has been found regarding the effects of soya on the primordial, primary and secondary follicles (Goncharova et al 1997, Delclos et al 2001). According to the considerations of the previously published data regarding the effects of soya on the female genital system, it has been hypothesized that soya may effect primordial, primary and secondary follicles progression and these changes in the ovary may be determined by stereologic methods (Awoniyi et al 1998, Dinsdale and Ward 2010).

The present study was designed to investigate the potential effects of the different extracts of Glycine max on primordial, primary and secondary follicles by using stereologic methods. Due to the lack of information about the effects of soya on female genital system, as well as in terms of its methodology in which optical dissector method is used, the present study will be highly important for the further researches.

Material and Methods

Material

The plant material was provided from Çukurova Agricultural Research Institute, Turkey, in May 2014.

 Extraction of the material for activity assessment

Plant material was shade dried and powdered. The extracts were prepared according to previously reported method (Carrao-Panizzi et al 2002). Briefly, n-hexane extract: Dried plant material (100 g) was extracted with n-Hexane at room temperature three times (x1000 mL). The combined n-hexane phases were evaporated to dryness in vacuo to give crude n-hexane extract (Yield: 17.2%). Ethyl acetate extract: Remaining plant materials were extracted with ethyl acetate at room temperature three times (x1000 mL). The combined ethyl acetate (EtOAc) phases were lyophilized to give the crude ethyl acetate extract (Yield: 21.8%). Ethanol extract (EtOH): Remaining plant materials were extracted with 0.1% acetic acid in 70% ethanol at room temperature four times (x1000 mL). The combined ethanol phases were lyophilized to give the crude ethanol extract (Yield: 48.9%). In thin layer chromatography (TLC) analysis, n-hexane and ethyl acetate extracts were found to be devoid of isoflavon contents, while ethanol extract was found to be rich in isoflavons. Therefore, HPLC analysis was performed on ethanol extract. For this purpose, powdered material was macerated with 0.1% acetic acid in 70% ethanol for two days. After filtration the extract was evaporated to dryness under reduced pressure and at 40°C. Isoflavon stock solutions were prepared in 1 mg/mL concentration.

Chemicals

Standard daidzein (EC 207–635–4, 25 mg, St. Louis, USA) and genistein (EC Number 207-174-9, 25 mg, St. Louis, USA) were purchased from Sigma Aldrich Chemicals. All the solvents used were of analytical grade (Merck).

HPLC conditions

The analysis was performed with a LC system consisting of an Varian Modular Analytical HPLC System quaternary pump with degasser and photodiode array detector. Samples were injected with an HP Agilent 1100 autosamplers with a thermostatted column compartment on an ACE-5 C18 column (5 μm, 250 mm; 4.6 mm) at 40°C. The system was controlled and data analyses were performed with Agilent ChemStation. All the calculations for quantitative analysis were performed with external standardization by measurement of peak areas. A mobile phase consisting of formic acid (0.2% v/v)Solvent A) Acetonitrile (Solvent B) was chosen to achieve maximum separation and sensitivity (Kledjus et al...
2004). Standard stock solutions contained 808 μg/mL daidzein and 1000 μg/mL genistein in HPLC grade methanol. To establish the linear detection range for each standard, individual standard stock solutions were prepared in mobile phase in 10 mL measuring flasks. Aliquots of these solutions were diluted and analyzed to determine method linearity. Calibration ranges were 0.1263–202 μg/mL for daidzein and 0.07813–125 μg/mL for genistein. Triplicates of 10 μL injections were made for each standard solution. The limit of detection (LOD) was established at a signal-to-noise ratio (S/N) of 3. The limit of quantification (LOQ) was established at S/N 10.

**Pharmacological procedures**

**Animals**

Eight-week-old female, intact, 64 Sprague Dawley rats weighing 180-220 g were purchased from the animal breeding laboratories of Afyon Kocatepe University (Afyonkarahisar, Turkey). The animals were housed in polusulfone cages at 21-24°C, at 40-45% humidity, and light-controlled (12 h light/12 h dark) conditions. A minimum of eight animals was used in each group. All animals were maintained in accordance with the directions of Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Experimental Animal Ethics Committee of Afyon Kocatepe University (AKÜHADYEK-83-12).

**Preparation of test samples and dose estimation for bioassays**

Since no ethnomedical data was available on the amount for treatment in traditional medicine, all extracts in the activity testing were administered in 100 and 200 mg/kg/day doses after suspending in 0.5% carboxymethyl cellulose in distilled H2O for 1 month. The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. On the 30th day of the experiment, diestrus animals were sacrificed under general anesthesia and the organs were removed. Other animals were followed 12 hour-intervals for diestrus and then euthanized (Myers et al 2004).

**Determination of estrus cycle by vaginal lavage method**

By using a plastic pipette filled with 10 µL of 0.9% NaCl, vaginal secretion was collected. Afterwards, vaginal fluid was placed on glass slides and the material was observed under a light microscope (Olympus CX21FS1), with 10 and 40 x objective lenses. The phases of the estrous cycle were determined according to the proportion among leukocytes, epithelial cells, cornified cells (Marcondes et al 2002).

**Dissection of the rats**

All rats in diestrus cycle were weighed and anesthetized by intramuscular injection of 50 mg/kg Ketamin HCl and 10 mg/kg Ksilazin HCl (Tapısız and Özat 2009, Erdem et al 2014). Animals were sacrificed by cervical dislocation and bilateral uterus and ovaries were removed and weighed.

**Histological analysis**

All ovaries were fixed in the neutral buffered formalin solution during 24 hours and embedded in paraffin after histological processing.

**Evaluation of the uterus**

All uterine tissues were sliced into 1 mm³ pieces and fixed in the 1/15 M phospat buffered glutaraldehyde solution (2.5%, 7.4 pH) at 40°C.

The tissue pieces were washed four times with phospat buffer and fixed in the 1/15 M phospat buffered glutaraldehyde solution (1%, 7.4 pH) at 40°C. After histological processing
the tissues were dried during two hours at the room temperature and two hours at the incubator adjusted to 400°C. All uterine tissues embedded into gelatine. All uteri and ovaries were sliced by microtome (Leica RM 2155) and slices were stained by Giemsa. The slices were observed with Olympus BH2 microscope under X40 magnification. The diameter of the follicles, shape of the thecal cells and corpus luteum were evaluated by Image Pro-Plus 5.1 cell analysis software.

Estimation of the primordial, primary, and secondary follicle numbers

The follicle types were determined according to the size of the follicles, and shape and size of the granular cells surrounding the oocyte (Myers et al 2004). The counting was performed with follicles as counting units. The number of the follicles was estimated using the computer software loaded StereoIm 12, Olympus BH2 light microscope with motorized stage (Lang MS 316) (for the step lengths on the X, Y axis) and 14 MP MShot camera (China), under x40 lens objective.

The thickness of the tissue was measured and the movements in the Z axis were controlled using a microcator (Heidenhain, Germany).

All follicle counts were performed by one of the stereological methods; optical disector (Figure 1). For counting nearly 100-200 follicles per ovary were made according to disector principle (Gundersen et al 1999). Area of the counting frame was 4225 μm² and the step lengths for the X and Y axis was 110 μm. The height of the disector was 11.2 μm (Figure 2).

Statistical analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA) and Tukey test (SPSS 10.00, Chicago, IL, USA). Values are presented as means ± SE. P<0.05 level was accepted statistically significant.

Results

Ingredients of the extracts

According to TLC analyses, isoflavons were not detected in the n-hexane and EtOAc extracts, while they were rich in the ethanol extract. Therefore, HPLC analyses were conducted on the ethanol extract. In this method, the retention times of daidzein and genistein were detected as 22.3 and 25.8 minutes. The concentrations of daidzein and genistein in the sample were 3.851 µg/g and 3.127 µg/g, respectively.

Live weight, relative ovary and uterus weight

The relative ovary and uterus weights increased in 200 mg/kg ethanol extract administered group (P<0.05), whereas, there was no significant difference (P>0.05) between the live weights (Table 1).

Histological changes in the ovary and the uterus diameters and counts of primordial, primary and secondary follicles

There was no significant difference between the groups regarding the histological analysis on the uterus. The uterus and the ovary were found as hyperemic and the vessels were apparent. No abnormality was seen for the primordial and primary follicles. Corpus luteum and interstitial tissue of the ovaries were normal.

There was no significant difference between primordial and primary follicles count. On the other hand, secondary follicle count increased in the 100 and 200 mg/kg ethanol extract administered groups and higher in 200 mg/kg ethanol extract group (P<0.05, Table 2).

Moreover, no significant difference was detected between the diameters of primordial and primary follicles (P>0.05). Secondary follicle diameter decreased in the 200 mg/kg ethanol extract administered group (P<0.05, Table 3).

Table 1. Live weights of the rats before and after the experiment (g) and relative ovary and uterus weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>LWInitial (g)</th>
<th>LWFinal (g)</th>
<th>Relative ovary+uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>172±3.66</td>
<td>190±3.48</td>
<td>0.327±0.01*</td>
</tr>
<tr>
<td>CMC</td>
<td>--</td>
<td>174±2.87</td>
<td>193±3.41</td>
<td>0.332±0.022b</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>100</td>
<td>174±2.93</td>
<td>190±5.66</td>
<td>0.390±0.039b</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>173±2.01</td>
<td>194±3.78</td>
<td>0.348±0.038b</td>
</tr>
<tr>
<td>EtOAc</td>
<td>100</td>
<td>171±2.08</td>
<td>194±4.83</td>
<td>0.302±0.031b</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>174±2.44</td>
<td>192±4.78</td>
<td>0.400±0.037em</td>
</tr>
<tr>
<td>EtOH</td>
<td>100</td>
<td>173±1.58</td>
<td>192±4.78</td>
<td>0.385±0.036em</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>174±2.39</td>
<td>194±5.04</td>
<td>0.474±0.023*</td>
</tr>
</tbody>
</table>

LW: Live weight, CMC: Carboxymethyl cellulose
Glycine max is rich in phytoestrogens, however the types and amounts of phytoestrogens change according to the extraction method and the solvent used for the extraction (Anthony et al. 1996, Delclos et al. 2001, Jung et al. 2004, Medigovic et al. 2012). Luthria and Natarajan (2009) used seven different solvent mixture and various extraction methods for the extraction of isoflavons from soy. The method in which dimethyl sulfoxide: ethanol: water (5:70:25, v/v/v) mixture was used, was found to be the most efficient method (Luthria and Natarajan 2009). In the present study, different extracts were prepared from the powdered plant material. TLC analysis showed the presence of isoflavons in the ethanol extract, therefore, HPLC analysis was conducted on this extract for quantification.

As it is known, phytoestrogens bind estrogen receptors and exert estrogen-like activity resulting in secretion and proliferation in the uterus and cause increase in the uterus weight (Erlandsson et al. 2005, Dinsdale and Ward 2010). Similarly, uterus weights of the animals, which were fed phytoestrogen rich diet, were detected to be increased. Genistein found in the soy caused proliferation of the epithelium cells and enlarged uterus when administered to the rats at high doses (Erlandsson et al. 2005, Dinsdale and Ward 2010). The results of the present study, in which relative ovary and uterus weights increased in 200 mg/kg ethanol extract administered animals (P<0.05, Table 1), supported the previous findings (Whitten et al. 1992, Erlandsson et al. 2005, Dinsdale and Ward 2010, Jefferson et al. 2012). On the other hand, no statistical significant difference was detected in 100 mg/kg ethanol extract administered animals, which could be related with the low concentration of the isoflavons (Table 2).

In the present study, soy extracts did not cause any difference in the uterus histology, however, hiperemia was detected in 200 mg/kg ethanol extract administered group (P>0.05). A previous study revealed that isoflavons affect endometrium histology. Soy isoflavons did not cause changes in uterus morphology at normal doses, however, caused abnormal ovarian follicles and abnormal cell maturation in vagina at higher doses (Delclos et al. 2001). Awoniyi et al. (1998) showed that genistein caused suppression of birth weight and decrease in the body weight in female rats.

### Table 2. Primordial, primary and secondary follicle count.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>PMFC</th>
<th>PFC</th>
<th>SFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>13954±1447</td>
<td>3219±349.7</td>
<td>3.33±0.55</td>
</tr>
<tr>
<td>CMC</td>
<td>--</td>
<td>14917±806</td>
<td>3444±216.3</td>
<td>4.00±0.37</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>100</td>
<td>13589±1233</td>
<td>3908±441.0</td>
<td>5.00±0.36</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13322±1533</td>
<td>3105±302.0</td>
<td>4.66±0.55</td>
</tr>
<tr>
<td>EtOAc</td>
<td>100</td>
<td>15179±1068</td>
<td>3789±672.0</td>
<td>3.50±0.50</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13125±1278</td>
<td>3570±563.9</td>
<td>5.66±0.91</td>
</tr>
<tr>
<td>EtOH</td>
<td>100</td>
<td>14123±2142</td>
<td>4161±559.5</td>
<td>11.66±0.76</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>15320±2023</td>
<td>4789±442.4</td>
<td>17.00±1.09</td>
</tr>
</tbody>
</table>

PMFS: Primordial follicle count; PFS: Primary follicle count; SFS: Secondary follicle count; CMC: Carboxymethyl cellulose.

### Table 3. Primordial, primary and secondary follicle diameters.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>PMFD</th>
<th>PFD</th>
<th>SFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>16.42±1.52</td>
<td>38.61±2.46</td>
<td>154.27±10.32</td>
</tr>
<tr>
<td>CMC</td>
<td>--</td>
<td>15.54±2.06</td>
<td>44.22±5.37</td>
<td>166.04±7.32</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>100</td>
<td>18.40±1.53</td>
<td>58.64±3.61</td>
<td>155.84±15.45</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.30±2.97</td>
<td>60.42±6.08</td>
<td>164.16±6.01</td>
</tr>
<tr>
<td>EtOAc</td>
<td>100</td>
<td>16.90±2.35</td>
<td>70.56±5.57</td>
<td>154.46±13.59</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>16.02±1.78</td>
<td>57.39±7.77</td>
<td>166.40±10.40</td>
</tr>
<tr>
<td>EtOH</td>
<td>100</td>
<td>19.72±3.14</td>
<td>59.20±3.96</td>
<td>124.84±5.03</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>18.88±3.07</td>
<td>57.60±4.57</td>
<td>94.55±10.40</td>
</tr>
</tbody>
</table>

PMFD: Primordial follicle diameters; PFD: Primary follicle diameters; SFD: Secondary follicle diameters; CMC: Carboxymethyl cellulose.

### Discussion

Glycine max is rich in phytoestrogens, however the types and amounts of phytoestrogens change according to the extraction method and the solvent used for the extraction (Anthony et al. 1996, Delclos et al. 2001, Jung et al. 2004, Medigovic et al. 2012). Luthria and Natarajan (2009) used seven different solvent mixture and various extraction methods for the extraction of isoflavons from soy. The method in which dimethyl sulfoxide: ethanol: water (5:70:25, v/v/v) mixture was used, was found to be the most efficient method (Luthria and Natarajan 2009). In the present study, different extracts were prepared from the powdered plant material. TLC analysis showed the presence of isoflavons in the ethanol extract, therefore, HPLC analysis was conducted on this extract for quantification.

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Due to the fact that a follicle starts from primordial phase and goes through the preantral, antral, graaf and preovulatory
follicle phases, we focused on folliculogenesis in the present study. Primordial follicle originates from germ cell and primitive cell pool begins to expand. Primordial follicles occur the third day of the birth and develops to antral follicle in three weeks. The mature secondary follicles appear in the 7th day. Apoptosis of the minimal ovarian cell starts in the 18th day. Follicles develop until ovulation or atresia. In adults at further ages, follicle resource diminishes because primordial pool runs out (Tingen et al 2009). In a previous study, primordial and primary follicle count decreased in organic and transgenic soy administered female rats (Brasil et al 2009). The effects of the estrogen on the follicle development were assessed and estrogen was found to control the dimensions of the follicles (Britt et al 2004). According to a study by Medigovic et al (2012), genistein decreased primordial, primary, secondary follicle count but increased atretic follicle count, revealing its estrogen antagonist effect. On the other hand, estrogen agonist effect of genistein was also demonstrated by its capacity to enhance the number of the follicles which develop from preantral phase to antral phase (Medigovic et al 2012). In another study, the toxicity of genistein was investigated and it was reported that there was no significant difference in the primordial follicle number (Lamartiniere et al 1998). In the present study, no significant difference was shown in the primordial follicle number (P>0.05, Table 2), similarly. Therefore, it was suggested that soy did not cause primordial follicle to change primary follicle and did not change the speeding process and mother follicle pool. In the ethanol extract administered group, primordial, primary and secondary follicles increased when compared to control and other test groups, but the difference was not statistically significant for the primordial and primary follicles. Similarly in literature reports, decrease in primary and secondary follicles, and reduction in the diameter of secondary follicle were detected in the present study. As the size of the follicle increased, the number of the follicle progressively decreased. In the ethanol extract administered group, increase of secondary follicle count indicated the inhibition of the follicle development and reduction the tertiary follicle growth. It was suggested that, due to the apoptotic effect of the estrogen, secondary follicle diameter decreased in 200 mg/kg ethanol extract administered group owing to high genistein and daidzein concentration. High levels of these compounds in the ethanol extract could be also associated with the increase in primordial follicle count. On the other hand, primordial follicles were reported to be the smallest and the most abundant ones among the other follicles. Similar to a previous report by Britt et al (2004), primary follicle count decreased when compared to the primordial follicle count (Britt et al 2004).

Conclusion

In conclusion, the ethanol extract of Glycine max was found to change the follicle development in a dose-dependent manner. Future works are necessary to clarify the relationship between these effects and hormonal mechanisms as well as apoptosis.

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