EGYPTIAN PHARMACEUTICAL JOURNAL EPJ
Original article 10

# Effect of soy protein isolate supplementation on the estradiol biosynthesis pathway in a female rat model

Noha A. Abd El-Latif[a], Asmaa M. Elfiky[b], Sherif A. Abdelmottaleb Moussa[b], Samir W. Aziz[b]

[a]Department of Biochemistry, Biotechnology Research Institute, Dokki, Giza, Egypt, [b]Department of Environment and Occupational Medicine, Environmental and Climate Change Research Institute, National Research Center, Dokki, Giza, Egypt

Correspondence to Noha A. Abd El-Latif, PhD, Department of Biochemistry, Biotechnology Research Institute, National Research Centre, 12622, Egypt. Tel: +20 109 905 8357; e-mail: nohaahmed_80@hotmail.com

**Received:** 23 April 2024
**Revised:** 14 June 2024
**Accepted:** 14 June 2024
**Published:** 14 November 2024

**Egyptian Pharmaceutical Journal**
2025,24:10–16

**Background**
Soy protein isolate (SPI) has gained popularity as an alternative to animal proteins. Soy consumption may have a link to rising serum estrogen in humans. This concern is attributed to phytoestrogens, specifically isoflavones present in soy protein, acting as estrogen substitutes and modulators.
**Objective**
The objective of this research was to assess the influence of SPI supplementation on estradiol hormone levels and the expression of genes associated with estrogen synthesis in female rats.
**Materials and methods**
Female Wistar rats (*n*=18) were evenly divided into three groups: group 1 (normal control) received oral administration of a saline vehicle. Group 2 (low dose) received 450 mg/kg body weight of SPI for 30 days. Group 3 (high dose) received 900 mg/kg body weight of SPI for 30 days. All administrations were conducted intragastrically.
**Results and conclusion**
The results of our study indicate that there was a significant increase in the levels of estradiol hormone in groups receiving low and high doses of SPI when compared with the control group. There was an upregulation in the messenger ribonucleic acid expression of the *Cyp19* gene in low-dose and high-dose groups. Moreover, the low-dose group showed upregulation in the expression of the HSD3B gene. These genes are involved in the biosynthesis pathway of estrogen hormone in females. Therefore, the use of SPI should be cautious because it contains phytoestrogens (isoflavones), which have structural similarities to endogenous female estrogen and may cause hormonal disturbance in females.

**Keywords:**
biosynthesis pathway, estradiol hormone, soy protein isolate

Egypt Pharmaceut J 24:10–16

1687-4315

## Introduction

A crucial component of human nutrition is soy. It is a common ingredient in traditional eastern cuisine, and in recent years, vegetarian and self-conscious diets have begun to incorporate it. According to Rizzo *et al.* [1], the success of soy is primarily dependent on its adaptability and the purported health benefits of soy-based foods and components. Soybeans, one of the main crops grown across the world, are consumed all over the world because of their high nutritional value and possible health benefits [2]. Within the food industry, soy proteins pertain to dehydrated and processed soy items intended for human and animal consumption. These primarily consist of soy protein isolate (SPI), full-fat or defatted soy flour, and soy protein concentrate. SPI, which has been used extensively in processed meat, plant-based beverages, and meat substitutes, is thought to be the purest form of soy protein with a protein content of more than 90% [3].

Phytoestrogens are plant-based compounds that resemble 17β-estradiol E2 in structure and have an estrogenic effect. Isoflavones, stilbene, coumestan, and lignan are four phenolic compounds that are categorized as phytoestrogens [4,5]. Isoflavones derived from plants are naturally occurring nonsteroidal substances that have an estrogenic appearance and are often categorized as phytoestrogens [6]. Isoflavones can attach to estrogen receptors and, in certain experimental scenarios, replicate the functions of estradiol because of their structural resemblance [7]. The estrogenic and nonhormonal qualities of soy isoflavones have garnered significant interest in recent years [8].

DOI: 10.21608/epj.2025.402105

It has also been documented that isoflavone influences sex hormones through estrogen receptor-independent mechanisms, like changing the levels of hormone-binding globulin (SHBG). Only a small portion of circulating estrogens and androgens is free; the majority is bound to albumin and SHBG. Because androgens and estrogens are only biologically active in their free form, SHBG has an impact on steroidal activity. Preclinical research has indicated that phytoestrogens affect sexual function as well as the risk of cancers related to the reproductive system, including breast and ovarian cancers [9].

The steroid hormone estrogen is connected to the female reproductive system and is in charge of the development of female sexual characteristics. The terms estrone, estradiol, and estriol are frequently used to refer to estrogen. Estrone (E1), estradiol (E2), and estriol (E3) are the three primary endogenous forms of physiological estrogens in women. As adipose tissue forms E1 from adrenal dehydroepiandrosterone, it plays a significant role after menopause. However, the primary and most powerful byproduct of the estrogen biosynthesis process is E2, commonly known as estradiol. The E3 form of estrogen, which is produced from E1 or E2, is the least common type [10].

Aromatase cytochrome P450 family 19 (CYP19), an enzyme complex also known as estrogen synthetase, is encoded by the CYP19 gene. It is located in the endoplasmic reticulum and regulates the amount of estrogen in the body. It catalyzes the final stages of the biosynthesis of estrogen from androgens, converting testosterone to 17β-estradiol (E2) and androstenedione to estrone [11,12]. 3-beta-hydroxysteroid dehydrogenase (HSD3B) is an enzyme that catalyzes the biosynthesis of the steroid progesterone from pregnenolone, 17α-hydroxyprogesterone from 17α-hydroxypregnenolone, and androstenedione from dehydroepiandrosterone in the adrenal gland [13]. It is also present in other steroid-producing tissues, including the ovary, testis, and placenta. HSD3B is encoded by the *Hsd3b* gene. The production of progesterone, glucocorticoids, mineralocorticoids, androgens, and estrogens, all classes of steroid hormones, requires the activity of this enzyme [14].

This research aims to assess the influence of SPI supplementation on female rats. This investigation involved a comprehensive examination encompassing estradiol (E2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels. Moreover, the study evaluated the expression profiles of genes implicated in estrogen biosynthesis and conducted a histopathological assessment of ovarian tissues among distinct groups of female rats. A comparative analysis was conducted between cohorts administered varying doses of SPI (low and high) and a control group to elucidate any discernible effects.

## Materials and methods

### Chemicals and dose preparation

SPI used was insulated from the market by Imtenan Company (Sp19, Code: F050079, Obour City, Egypt). Its protein content is 88%. According to O'Flynn *et al.* [15], the dosage was prepared by stirring water (20°C) with SPI powder. The total isoflavones content in SPI is 120 mg/100 g (genistein, daidzein, and glycitein) [16].

### Animals

Eighteen female Wistar rats (8 weeks old) weighing 180±10 g were given to the Animal House of the National Research Centre in Giza, Egypt. Before the experiments, the rats were housed in polypropylene cages and allowed 3 days to acclimate to a specific environment devoid of pathogens. The rats were maintained at 24±1°C and between 55 and 65% humidity with a 12-h light/dark cycle. The rats were given an unlimited supply of water and a standard rodent diet that included 17.48% protein, 6.85% fat, 62.99% carbohydrates, 4.08% ash, and 2.16% minerals and vitamins. The process was authorized under the number "09420923" after the National Research Center's Medical Research Ethical Committee's ethical guidelines for animal handling were adhered to National Research Centre in Giza, Egypt.

### Experimental setting

After the time for acclimatization, the rats ($n$=18) were weighed and divided equally into three groups.

Group 1: normal control group ($n$=6), administered saline orally.

Group 2: low-dose SPI group ($n$=6) was orally administered with 450 mg/kg body weight SPI (0.09 g/2.5 ml water) for 30 days intragastrically [17,18].

Group3: high-dose SPI group ($n$=6) was orally administered with 900 mg/kg body weight SPI (0.18 g/2.5 ml water) for 30 days intragastrically [17,18].

### Samples collection

Blood samples were taken from the retro-orbital plexus of female rats and allowed to clot to separate the sera

using dry and clean centrifuge tubes. Serum samples were separated by centrifugation at 4000 r/min for 10 min at 4°C. Serum aliquots were frozen and stored at −20°C for further hormonal assay (estradiol E2, FSH, and LH) determination. The ovaries were removed, and the left ovary was immediately removed, blotted, and stored at −80°C until it was required for the analysis of *HSD3B* and *CYP19* gene expression. The right ovary was preserved for histological examination in 10% phosphate-buffered formalin (pH 7.4).

## Methods

### Hormonal assays

The levels of estradiol E2 hormone, FSH, and LH in rats were measured using enzyme-linked immunosorbent assay kits [Shanghai Korain Company (Shanghai, China), bioassay technology laboratory (BT-Lab), Cat. No. E0174Ra, EA0015Ra, and EA0013Ra, respectively], in accordance with the manufacturer's instructions using the mini VIDAS immunoassay system based on the enzyme-linked fluorescent assay principles.

### Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis

Test tissue samples from all animals were stored, and ovarian specimens weighing about 30 mg were homogenized using a handheld homogenizer. Total ribonucleic acid (RNA) was isolated from tissues using the Thermo Scientific GeneJET RNA Purification Kit (Waltham, Massachusetts, USA). A spectrophotometer (ND-1000; NanoDrop) was used to measure RNA using spectrophotometry. A 1 mg sample of tissue-derived RNA was used to extract complementary deoxyribonucleic acid (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), and the gradient thermal cycler (Bio-Rad) was utilized for the incubation.

### Quantitative real-time gene expression analysis (PCR)

Gene expression analysis was carried out quantitatively in real-time using a Gilent Technologies Stratagene Mx3000P real-time PCR system. The quantitative real-time-PCR was carried out in duplicate for each sample using Thermo Scientific's Maxima SYBR Green qPCR Master Mix. With a total volume of 25 µl, the reaction mixture consisted of 10 µl of SYBR Green Master Mix, 4 µl of cDNA (100 ng/ml), and 20 µl of nuclease-free water. Each gene was adjusted for expression using the housekeeping gene GAPDH. Table 1 displays the primer sequences for the genes *Cyp19* and *Hsd3b*. The authors designed the primers for target genes according to the primer blast tool standards. qPCR amplification was carried out as follows: 95°C for 10 min; 40 cycles in three steps: 94°C for 15 s, 60°C for 30 s, and 70°C for 30 s. The relative quantification method of messenger ribonucleic acid expression (mRNA), known as $2^{-\Delta\Delta CT}$, was used to compute the fold difference in gene expression [19].

### Histological procedures

Ovaries from female rats were kept in a 10% neutral-buffered formalin solution for at least 12 h. Each specimen was soaked in tap water for 30 min, dehydrated using progressively higher alcohol grades, cleaned in xylene, and then embedded in paraffin. For histopathological analysis, serial sections of 3 µm thickness were cut and stained with hematoxylin and eosin [20]. The pathology laboratory at the National Research Centre used a picture analysis system with an Olympus CX41 light microscope and a SC100 video camera connected to a PC to take pictures. Using Adobe Photoshop, version 8.0, photomicrographs captured at different magnifications were processed.

### Statistical analysis

The analysis was carried out using Prism 8.0.1 on Windows. The data that was shown was the mean ±SEM. The differences between the low-dose and high-dose groups in comparison to the control group were analyzed for statistical significance using one-way analysis of variance and Tukey's test. Every data analysis considered $P$ value less than 0.05 to be significant.

## Results and discussions

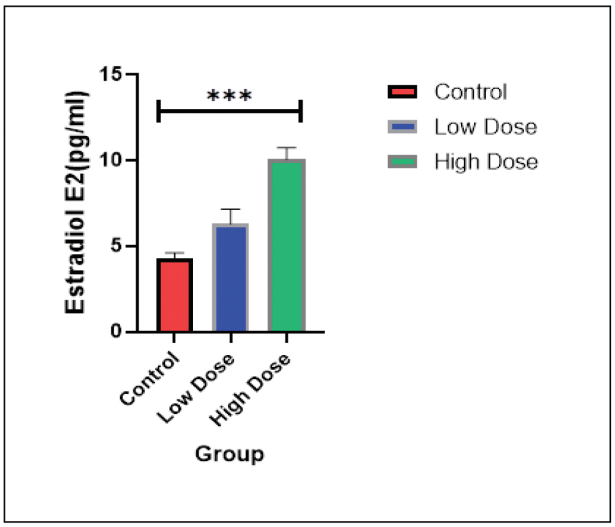
### Effects of soy protein isolate on estradiol, follicle-stimulating hormone, and luteinizing hormone concentrations

Figure 1 illustrates the significant increase in serum estradiol E2 levels for both the low-dose and high-dose groups of SPI when compared with the control group ($P<0.0001$). However, Figs 2 and 3 do not demonstrate any significant differences in serum

**Table 1 Specific primer sequences**

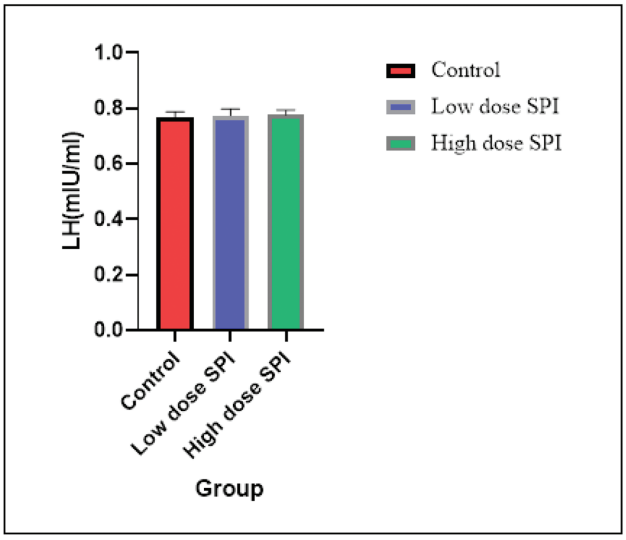
| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| *Hsd3b* | ACTCAGCTCCTGTTGGATGC | CAGAGGAAGGCTCCAACTGG |
| *Cyp19* | TCATGGTCCCGGAAACTGTG | CCGAGTTGTCAGTGACGTCA |

**Figure 1**




Effect of high and low doses of soy protein isolate on the concentration of serum estradiol E2 in the female rats compared with the control group. *n*=6. Data are shown as mean±SEM. **P* value less than 0.05, ****P* value less than 0.0001.

**Figure 3**

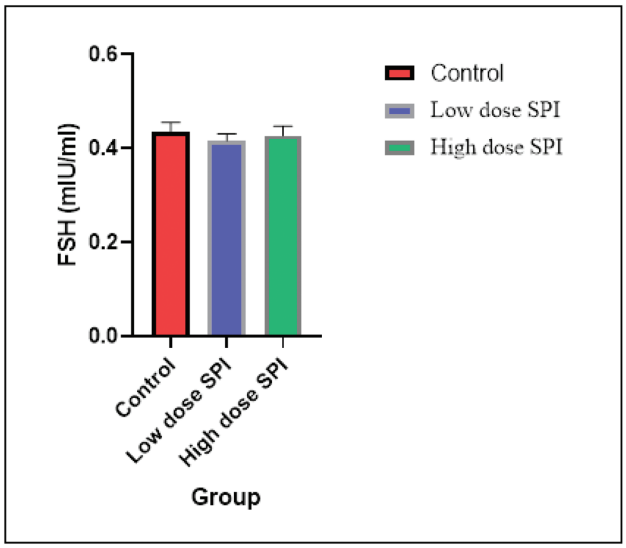



Effect of high and low doses of soy protein isolate on the level of serum-luteinizing hormone (LH) in female rats compared with the control group. *n*=6. The data are shown as mean±SEM.

FSH and LH levels between the groups receiving low and high doses of SPI when compared with the control group.

The increasing use of soy products for human consumption and as cattle feed has resulted in an increase in the intake of phytoestrogens [21]. Red clover and other legumes, such as soybeans, contain isoflavones. Genistein, daidzein, glycitein, formononetin, and biochanin A are the primary phytoestrogens found in soybeans as isoflavones

**Figure 2**



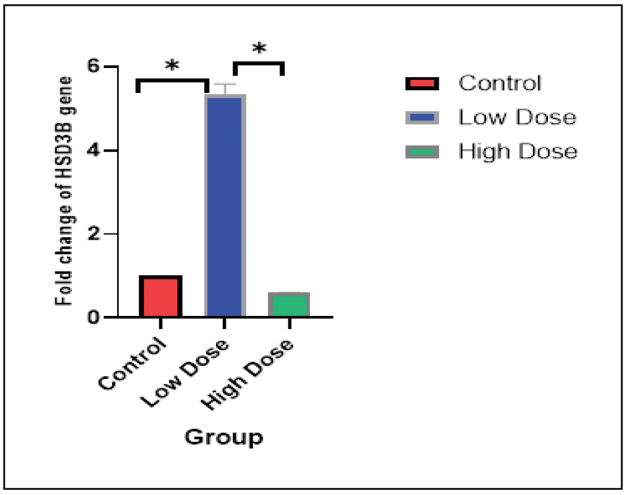

Effect of high and low doses of soy protein isolate on the level of serum follicle-stimulating hormone (FSH) in female rats compared with the control group. *n*=6, the data are shown as mean±SEM.

[22,23]. Because of their structural resemblance to the primary female sex hormone, 17-β estradiol (E2), phytoestrogens have antiestrogenic properties [24]. As a soy product, SPI has drawn a lot of interest because of its many functional qualities (emulsification and gelling), abundance, low cost, and high nutritional value [25].

According to numerous scientific studies [26,27], isoflavones are frequently categorized as endocrine disruptors. It is commonly known that there may be a health risk associated with exposure to endocrine disruptors [28]. These compounds alter metabolism like that of endogenous hormones. Because of their highly estrogenic qualities that affect the reproductive tract among other organs and because they are naturally present in plants like soybeans, isoflavones are regarded as endocrine disruptors due to their structure, which is similar to estradiol E2 [29]. These results are consistent with our research findings, where our study showed an increase in the estradiol levels in the animals exposed to the SPI, the same as other authors who have shown similar results and showed that long-term treatment with commercial isoflavones was involved with the increase in estradiol levels [30,31].

Our study showed no significant changes in the levels of FSH and LH after SPI supplementation. This outcome is in line with many previous studies [32–35], which indicate that the hypothalamic–pituitary–adrenal axis is not negatively impacted by SPI.

**Figure 4**

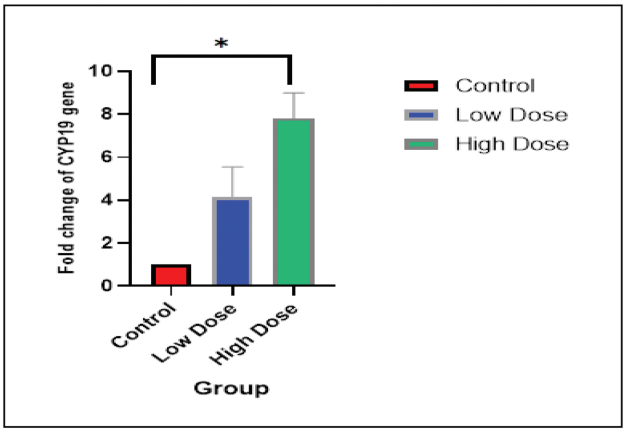



Effect of high and low doses of soy protein isolate on messenger ribonucleic acid expression levels of 3β-hydroxysteroid dehydrogenase (*HSD3B*) gene. *n*=6. Data are shown as mean±SEM. **P* value less than 0.05.

### Effect of soy protein isolate on aromatase cytochrome P450 family 19 (*Cyp19*) and 3β-hydroxysteroid dehydrogenase (*Hsd3b*) gene expression

Figure 4 illustrates that mRNA expression of the *Hsd3b* gene was significantly ($P<0.05$) downregulated in the group receiving high doses of SPI compared with the low doses group, whereas this gene was upregulated in groups receiving low doses of SPI as compared with the normal control. Figure 5 shows a significant ($P<0.05$) upregulation of the *Cyp19* gene's mRNA expression in the group receiving high doses of SPI when compared with the control group. In the androgen pathway, 3β- or 3 beta-hydroxysteroid dehydrogenase catalyzes multiple reactions that result in the production of testosterone and androstenedione and also acts as a catalyst to convert pregnenolone into progesterone. According to Thomas *et al.* [36], the HSD3B enzyme is expressed in the testis, ovary, and adrenal glands, and uterine expression has been documented [37,38]. Androstenedione is the direct precursor of estrogens and powerful androgens, and its synthesis requires the HSD3B enzyme [39]. In all vertebrate species, aromatase (CYP19), a cytochrome P450 enzyme (P450arom), is essential for the biosynthesis of estrogens from androgens [40]. On chromosome 15, the *Cyp19* gene is crucial for the process of aromatization, which converts androgens into estrogen [41]. In the study of Wang *et al.* [42], the mRNA expression of the *Hsd3b* gene was downregulated in the high-dose group of isoflavone when compared with the control group, where the low-dose group showed upregulation of the same gene. This may be due to the increase in the methylation rate of DNA.

**Figure 5**




Effect of high and low doses of soy protein isolate on messenger ribonucleic acid expression levels of aromatase cytochrome P450 family 19 (*CYP19*) gene. *n*=6. Data are shown as mean±SEM. **P* value less than 0.05.

We investigated the mRNA expression of two genes involved in the biosynthesis of estrogen hormones (*Hsd3b* and *Cyp19*). Interestingly, we found that the group receiving a low dose of SPI showed a significant upregulation of the *Hsd3b* gene with downregulation in the high-dose group and a notable upregulation of the *Cyp19* gene's mRNA expression in groups receiving low and high doses of SPI when compared with the control group. This result is consistent with the previous results as the SPI caused an increase in the level of estrogen hormones in the rat groups that ingested soy supplements compared with the control group. This means that the presence of phytoestrogen in SPI is a cause of increased estrogen levels, causing hormonal disturbance.

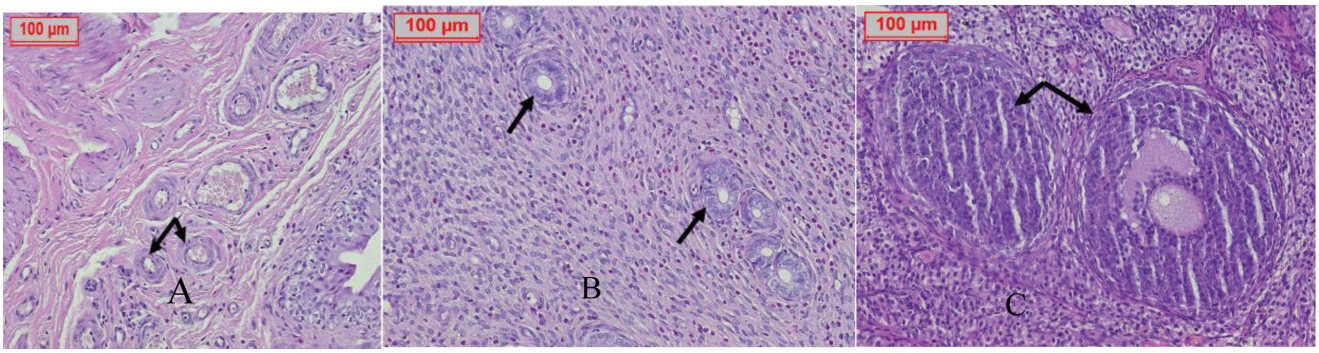
### Effect of soy protein isolate on ovarian histology in all groups of female rats

Figure 6 shows the histological cross-sections (photomicrography) of ovarian tissue of various groups to investigate overall morphological changes. The control group without SPI (Fig. 6a), the group with a low dose of SPI (Fig. 6b), and the group with a high dose of SPI (Fig. 6c). All groups show normal vascularity, compact stroma, and intact germinal epithelium. This result shows that there is no effect of SPI supplementation on the ovarian tissues of female rats that received low or high doses.

## Conclusion

Our findings show that administering SPI has a deleterious effect on the genetic expression of the*CYP19* gene in low and high doses and of the *HSD3B* gene in the low-dose group, which are

**Figure 6**




(a) Photomicrography of ovarian tissue of the control group. (b) Photomicrography of ovarian tissue of the low-dose group. (c) Photomicrography of ovarian tissue of the high-dose group.

linked to estrogen synthesis in female rats, resulting in elevated estrogen levels. Consequently, prolonged and high-dose use of the SPI may pose potential risks to female health. It is crucial to exercise caution in its utilization due to phytoestrogens, notably isoflavones, which bear structural resemblances to endogenous female estrogen. Further investigation and vigilant monitoring are necessary to comprehensively elucidate the potential health ramifications of SPI consumption, particularly concerning estrogen regulation in females.

**Acknowledgment**

The authors acknowledge all the participants who generously shared their time and experiences for this research.

Authors' contributions: Noha A. Abd El-Latif conceived the study, designed the model, planned the experiments, developed the theoretical framework, performed the biochemical experiments, and wrote the manuscript with input from all authors. Asmaa M. Elfiky performed the molecular experiments, calculations, analyzed the data, and contributed to the interpretation of the results. Sherif A. Abdelmottaleb Moussa and Samir W. Aziz provided critical feedback, helped shape the research, and contributed to the final version of the manuscript.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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