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ORIGINAL ARTICLE

# Determination of isoflavones from soy-milk, *masoor* and *mung dal* soups in Bangladeshi postmenopausal women

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## ABSTRACT

**Background:** Isoflavones daidzein and genistein generate estrogenic compounds in human without any side-effect. **Aims**: To measure the determinants of two isoflavones daidzein and genistein in Bangladeshi postmenopausal women consuming soy-milk and soups prepared from *mung* and *masoor dal*. **Subjects and Methods**: Sixteen healthy postmenopausal women (age, mean ± SD, 52.5 ± 5.8 years) were included. After an overnight fast, each participant was given freshly-prepared soy-milk (~350-mL) and soups subsequently. Soy-milk and soups were prepared from 100 g powders of soybeans, *masoor* and *mung dal* respectively. Blood samples (5 mL) were collected before (baseline) and at an interval of 2, 4, 6, 8, 24, 36, and 48 hours after ingestion of milk and soups. Blood samples were centrifuged at 1200 rpm and serum (~2 mL) was immediately frozen at -20°C until analysis. Isoflavones were extracted from the defrosted serum, and the sample was cleaned using solid-phase extraction (SPE C18 Cartridge). Levels of isoflavones, in the serum, were quantified using liquid chromatographic (LC)-PDA analysis. **Results**: The area under the curve (AUC) of serum genistein in soy-milk, *masoor,* and *mung dal* soups, was 0.82 ± 0.22, 1.01 ± 0.32, and 1.12 ± 0.31 µg/mL respectively. A significant (p = 0.03) association was found between the Cmax of serum isoflavones genistein of soy-milk and *mung dal* soup. **Conclusions**: The findings indicate that the determinants of isoflavones was found in non-soy foods among Bangladeshi postmenopausal women.

**Keywords**: Isoflavones, soy-milk, Masoor dal, Mung dal, determinants, Bangladeshi menopausal women.

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## 1 Introduction

Lack of estrogen production, during menopause, is associated with an increased risk of hormone-dependent disorders [1]. Hormone replacement therapy (HRT) is being used for the treatment of these disorders however, the usage of HRT is associated with an increased risk of endometrial [2] and breast [3] cancer. Globally, alternatives such as isoflavones from foods are becoming increasingly popular as they offer the same beneficial effects of HRT without any side-effect [4,5]. Results of epidemiological [6-8] and small-scale human clinical trials [9,10] have shown that isoflavones prevent hormone-dependent disorders.

Postmenopausal women in Bangladesh do not get enough support due to their poor socioeconomic condition, illiteracy, and ignorance and due to the inadequate healthcare system too. During this period, many women are not well-accepted in the family and society. Consequently, they consider themselves as a burden. It is paradoxical that HRT is more focused in poorer countries, where economic consideration itself represents a great obstacle to achieve the goal of wellbeing. Furthermore, it is difficult for them to bear the high cost of HRT therapy. It would therefore be

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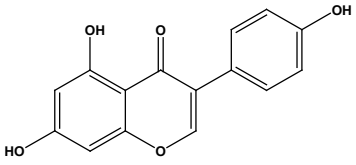
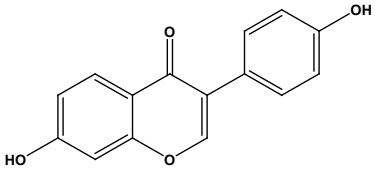
necessary to assess easily-accessible food materials containing high amount of isoflavones for the menopausal women.

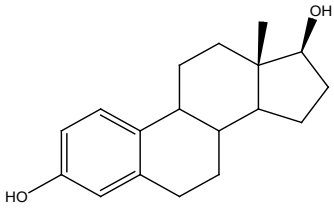
Isoflavones, a group of non-steroidal plant-derived compounds, are structurally similar to estrogen (Figure 1) and can exert weak estrogenic effects [11-13]. The major isoflavones, such as genistein and daidzein, have several features in common with estradiol-17β [14]. Soy and its products, and legume seeds (lentils, beans, and peas) constitute the richest sources of isoflavones, including genistein and daidzein [15]. Soybean and lentils, particularly *mung* and *masoor dal*, contain isoflavones. In Bangladesh, a considerable amount of soybean is produced, although its consumption is limited while *mung* and *masoor dals* are commonly consumed. Despite the positive effects of isoflavones, data on the bioavailability of dietary isoflavones, in postmenopausal women, is lacking. Only three studies covered the bioavailability of daidzein or genistein among postmenopausal women [16-18]. To the best of our knowledge, no studies have been conducted in Bangladeshi postmenopausal women, which prompted us to undertake the present pilot study. The aim of the present study was to measure the determinants of isoflavones daidzein and genistein in Bangladeshi postmenopausal women consuming soy-milk and soups prepared from *mung* and *masoor dal*.




Genistein




Daidzein




Estradiol-17β

**Figure 1.** Structure of genistein, daidzein and estradiol

## 2 Subjects and Methods

### 2.1 Selection of subjects

Eighteen healthy postmenopausal women were included in the 36-day study and 16 completed this. Two women dropped out, due to their difficulties to be involved in such a long period of study. The participants were screened at the Bangladesh Institute of Health Sciences (BIHS) hospital and the following parameters were assessed: age, blood pressure, pulses, body weight and body mass index (BMI). Women aged between 45 to 60 years and who had natural menopause or due to surgery had menopause for last 2 years, were considered for the study.

Women with chronic renal, liver, pulmonary or cardiovascular diseases were excluded. Besides, those who had been administrated antibiotics, within the preceding three months and were taking oral contraceptives or HRT, were excluded.

### 2.2 Study design

A non-randomized, single-dose sequence, crossover study design with a two-weeks washout period.

Postmenopausal women were divided into four groups and each group consisted of four women to facilitate the study procedure performance. On day 1, they were hospitalized in the evening and were served an isoflavones-free meal. On day 2, i.e., after an overnight fast, they consumed freshly-prepared soy-milk (~350 mL) as a single bolus. It was ensured that soy-milk was ingested by our participants in front of principal investigator (PI). Blood samples (5 mL) were collected before (baseline) and at an interval of 2, 4, 6, 8, 24, 36, and 48 hours after ingestion. Isoflavones-free meals were given at dinner on day 1, at lunch and dinner on day 2, at breakfast, lunch, and dinner on day 3, and at breakfast on day 4. A physician as a co-investigator helped PI in screening and in monitoring women from the start and during the study.

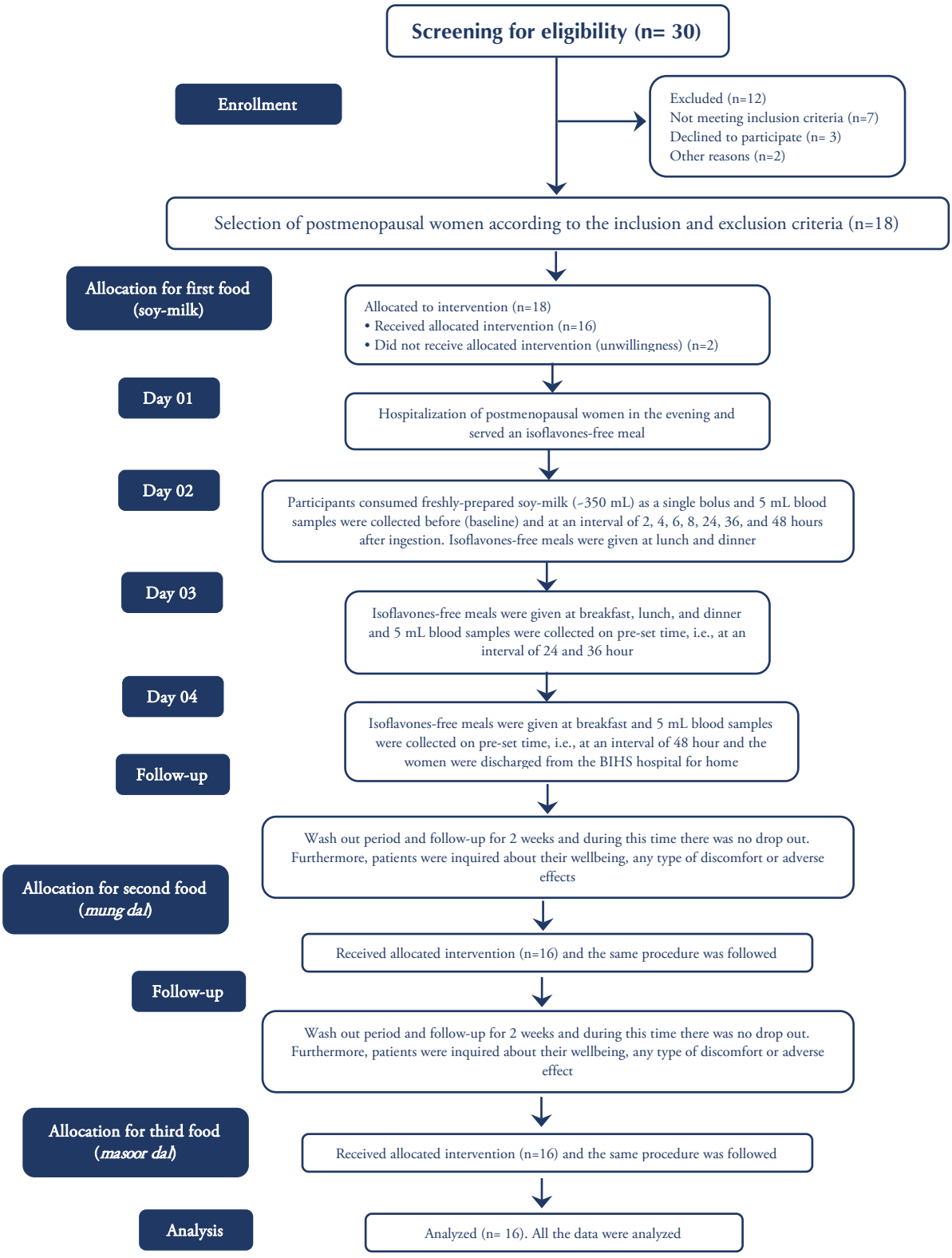



**Figure 2.** Flow chart of the study

Later, the participants were discharged from the BIHS hospital for home. After a two-week washout period, the experiment was reconducted with *masoor dal* soup under the same conditions. Subsequently a two-week washout period, under the same conditions and for the third time the experiment was reproduced with *mung dal* soup. The flowchart of the study is illustrated on Figure 2.

## 2.3 Determination of isoflavones in soy-milk, masoor and mung dal

Soy-milk, *masoor* and *mung dal* soups were prepared from 100 g of soybean, powders of *masoor* and *mung dal*. The same batch of soybean, *masoor* and *mung dal* was utilized throughout the study period, and the identification and quantification of daidzein and genistein, in the three different foods, were performed through liquid chromatographic (LC)-PDA analysis [19]. As *masoor* and *mung* are consumed after cooking, to follow the similar food habit condition, soups were prepared from the *dal*, and the determinants of isoflavones were assessed in *masoor* and *mung dal* soups in women. Soy-milk (350 mL) contained 36.25 µg daidzein and 43.81 µg genistein. *Masoor* and *mung dal* soups (350 mL) contained daidzein (37.66 and 27.66 µg) and genistein (37.33 and 44.00 µg).

A food-chart was provided to all participants who were informed to avoid food containing isoflavones (such as *mung dal*, *masoor dal*, soybean, raw garlic, green beans, potatoes, sweet potatoes, chickpeas, wheat flour, grapefruits, dates, eggs, and nuts) at least for one week before and during the study.

## 2.4 Preparation of soy-milk, mung and masoor dal soups

The whole soybean amount (100- g) was immersed in drinking-water in a pot for 4-5 hours. The soft beans (water socked) were washed with water, blended into mould using a kitchen blender. Water (500- mL) was added to the mould, boiled for three minutes, and stirred with a wooden kitchen stirrer. The milk was collected by squeezing through a pre-cleaned cloth filter and was boiled with medium temperature again for 20 minutes and stirred to reduce its volume up to ~350 mL.

Both *mung* and masoor *dal* amounts (100 g) were washed with clean water, and 500 mL of water with a small amount of salt (NaCl) and turmeric powder were added to it then boiled with medium temperature for 25 minutes, and stirred with a wooden kitchen stirrer to reduce the volume up to ~350 mL.

## 2.5 Standard of Isoflavones

For measuring the determinants of isoflavones in human serum, authentic standards of daidzein and genistein, purchased from Sigma-Aldrich, were preserved at 4°C and at -20 °C respectively.

## 2.6 Isoflavones extraction from human serum

In overall, 384 (8x16x3) serum samples were collected from 16 postmenopausal women during the three test periods, after serving soy-milk and soups, prepared from *masoor* and *mung dal*. The blood samples were centrifuged at 1200 rpm, and the serum (~2 mL) was separated and immediately frozen at -20 °C until analysis, to avoid any target compounds biodegradation.

Isoflavones were extracted from the defrosted serum, and the sample was cleaned up using solid-phase extraction (SPE C18 Cartridge). The cartridge was conditioned with water (1 mL x 3) followed methanol (1 mL x 3) and then water again. The serum sample was thawed and then passed through the conditioned SPE cartridge, then aqueous 5% methanol (800 µL) was added. The isoflavones were eluted in ethyl acetate-acetonitrile mixture (1:1; 400 µL x 2). Sixty-four serum samples of 16 women from each test foods were cleaned. Thus, the total number of cleaned serum samples was 192 (64 x 3) from 384 serum samples. The cleaned serum samples were filtered through the LC samples filter having a pore size of 0.22 µm [PTFE (polytetrafluoro ethylene)]-syringe filter cartridge and transferred to a sample vial (2-mL) and analysis was done using LC-PDA.

For the chromatographic analyses, a Shimadzu SCL 10A vp LC system (Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD-10A vp), a Supelco discovery reversed phase C18 column (25 cm×4.6 mm i.d. particle size: 5 µm) and a Rheodyne injector (loop size 20 µL) was used. Standards and cleaned extracts (100 µL) were injected through a Rheodyne injector.

Separations were carried out at 268 nm, using an isocratic mobile phase of acetonitrile and water (ACN: H2O) (75: 25), with a flow rate of 0.5 mL/min, and the running time was 10 minutes. The LC system was conditioned by passing mobile phase until the smooth baseline was obtained.

## 2.7 Determination of daidzein and genistein in human serum samples

The standard solutions of daidzein and genistein at the concentrations of 5, 10, 15, 20, and 25 µg/mL were injected into LC-PDA. Two calibration curves were established from above five solutions of the daidzein and genistein by plotting peak area vs concentration (µg/g).

The certified standards of daidzein and genistein (10 µg/mL) were injected separately. The retention time of both certified standard isoflavones were found to be 5.52 and 6.03 minutes respectively, before serum extracts analysis using LC-PDA. The serum samples were injected into the LC system with one injection of standard after each two or three injections of the samples, to control whether there was a deviation in the retention times of standards or not. By comparing the retention times of standard peaks with that of the sample peaks; the possible daidzein and genistein peaks in chromatograms of the samples were determined.

The amount of daidzein and genistein, in human serum samples, were calculated from the external calibration curve of certified standard of daidzein and genistein, taking into consideration that the peak area is in the midpoint of the curve (considering linearity of the curve). A number of unknown analytes in the respective samples were identified using formula (1):

Amount of unknown sample

$$= \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Area}_{\text{Std}} \times \text{Conc.}_{\text{Matrix}}} \quad \ldots (1)$$

Peak Area sample = Peak Area of the sample
Conc. Std = Concentration of the standard
Peak Area std = Peak Area of the standard
Conc. matrix = Concentration of the matrix

The daidzein and genistein serum concentration-time profiles, for each individual, and the mean concentrations at each dose, were determined employing a non-parametric estimation of AUC (area under the curve) and $C_{max}$ (maximum concentration). Trapezoidal formula was used for calculating AUC.

## 2.8 Statistical Analysis

Statistical analysis was performed using SPSS (Statistical package for social Science) software for Windows version 22 (SPSS Inc, Chicago, Illinois, USA). For analysis, log-transformation of the data was done. The data was expressed as geometric mean ±SD (Standard deviation). The statistical significance of differences between the values was analyzed by ANOVA (Analysis of variance). A *P* value of <0.05 was considered statistically significant.

# 3 Results

The present study concerned the determination of two isoflavones daidzein and genistein in the serum of 16 middle socioeconomic class postmenopausal women, whose mean age was 52.5 ± 5.8 years. Other clinical parameters selected were: pulse (mean ± SD, beats/min, 68.2 ± 6.4), systolic blood pressure (SBP) [mean ±SD, mmHg, 116.5 ± 6.7], diastolic blood pressure (DBP) [mean ± SD, mmHg, 76.5 ± 5.1] and body mass index (BMI) [mean ± SD, kg/m2 25.7 ± 5.3] (Table 1).

**Table 1. Demographic and clinical characteristics of postmenopausal women (n=16)**

| Variables | |
|---|---|
| **Age (years)** | 52.5 ± 5.8 |
| **Pulse (beats/min)** | 68.2 ± 6.4 |
| **SBP (mmHg)** | 116.5 ± 6.7 |
| **DBP (mmHg)** | 76.5 ± 5.1 |
| **BMI (kg/m2)** | 25.7 ± 5.3 |

Results are expressed as mean ±SD; BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure

An excellent symmetrical elution pattern was obtained in the chromatograms during 4-8 hours of duration (Figures 3-5). The chromatograms showed efficient separation and correct integration of genistein in the blood samples. After four hours, degradation might have occurred because several small peaks were found earlier than the retention time of genistein. At an interval of 2, 8, 24, 36, and 48 hours, no elution pattern of genistein was observed. The determinant of genistein were responded in 8, 15, and 5 postmenopausal women following a single dose of orally-administered soy-milk, *masoor* and *mung dal* soups during 4-6 hours respectively (Figure 6). The determinations of the serum isoflavones genistein was found 2.6 % in soy-milk, 3.8 % in *masoor dal* soup and 3.7% in *mung dal* soup, respectively.

No peak was observed at the retention time of daidzein which indicates its non-availability in the blood samples. From the area in the chromatogram, the maximum concentration amount of each sample was calculated. The results are expressed as $C_{max}$, AUC and $T_{max}$ (at time of the maximum concentration) [16,17,20-22].

Table 2 presents the geometric mean AUC and $C_{max}$ of serum isoflavones genistein of soy-milk, *masoor* and *mung dal* soups. In soy-milk, the geometric mean AUC and the geometric mean $C_{max}$ of serum genistein was 0.82 ± 0.22 µg/mL and 0.17 ± 0.19 µg/mL. The geometric mean AUC and the geometric mean $C_{max}$ of serum isoflavones genistein in the *masoor* and *mung dal* soup was 1.01 ± 0.32 µg, 0.45 ± 0.32 µg, 1.12 ± 0.31 µg, 0.63 ± 0.31 µg per mL, respectively. The basis of the calculation of AUC (Trapezoidal formula) is the supposition that there exists an AUC during 4 – 6 hours.

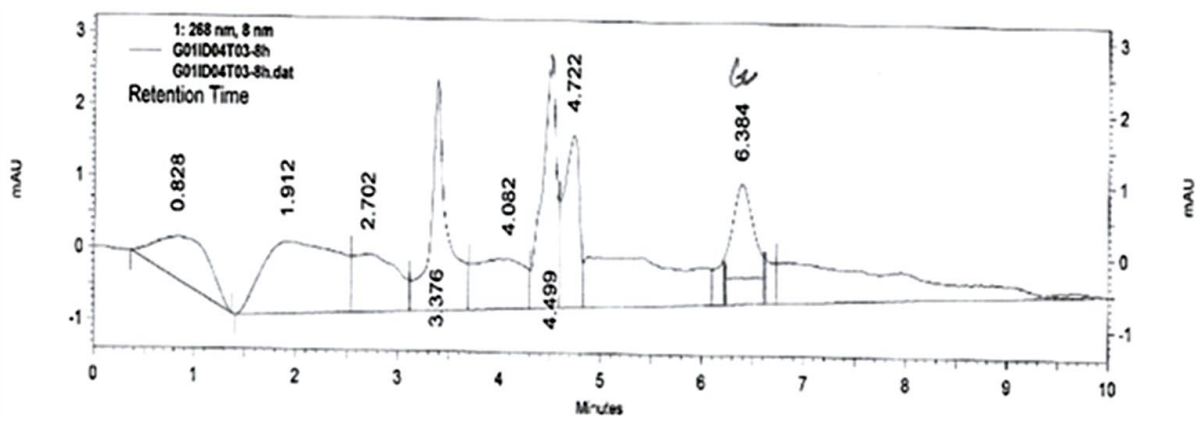



**Figure 3.** Chromatogram of serum sample of one subject at 8 hours after consuming soy-milk

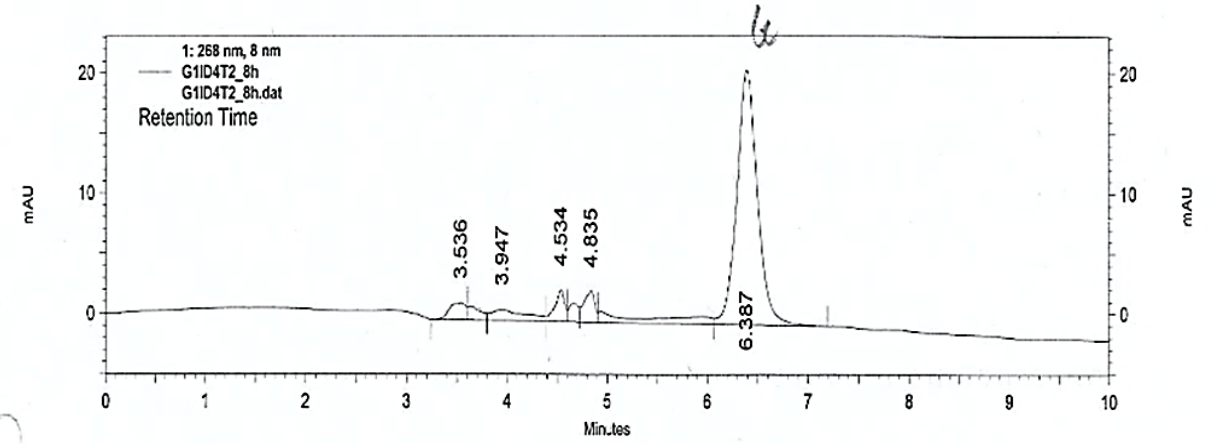



**Figure 4.** Chromatogram of serum sample of one subject at 8 hours after consuming *masoor dal* soup

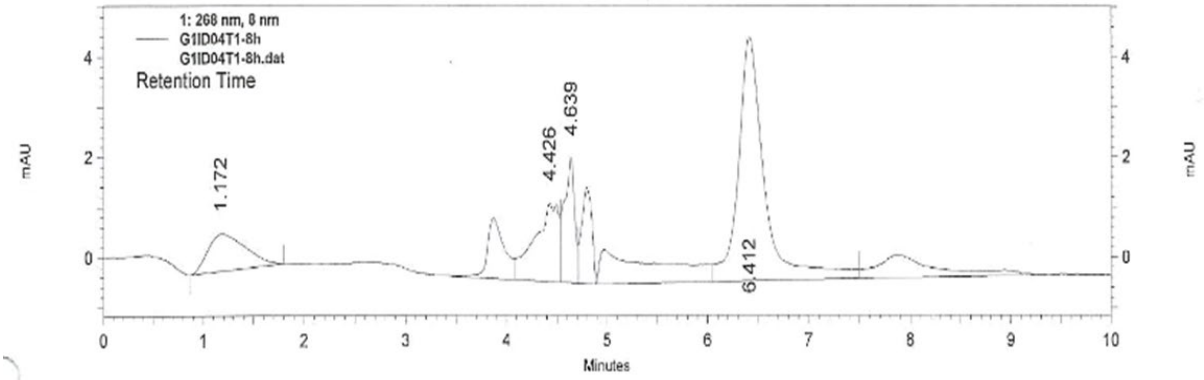



**Figure 5.** Chromatogram of serum sample of one subject at 8 hours after consuming *mung dal* soup

The pharmacokinetics data (AUC and $C_{max}$) were compared according to food items, using ANOVA. No significant differences, in serum genistein AUC, were observed within the three food items (soy-milk vs *masoor dal* vs *mung dal*) though a significant (p = 0.03) association, was found between the $C_{max}$ of serum isoflavones genistein of soy-milk and *mung dal* soup.

**Table 2.** AUC and Cmax of genistein in serum following a single dose of orally-administered soy-milk, *masoor* and *mung dal* soups

| Food Items | AUC (µg/mL) | Cmax (µg/mL) |
|---|---|---|
| Soy-milk | 0.82 ±0.22 | 0.17 ±0.19 |
| *Masoor dal* soup | 1.01 ±0.32 | 0.45 ±0.32 |
| *Mung dal* soup | 1.12 ±0.31 | 0.63 ±0.31 |
| F/p | 2.36/0.11 | 4.38/0.02 |
| *P*-value | | |
| Soy-milk vs *Masoor dal* soup | ns | ns |
| Soy-milk vs *Mung dal* soup | ns | 0.03 |
| *Masoor dal* soup vs *Mung dal* soup | ns | ns |

Results are expressed as Geometric mean ±SD. One-way ANOVA (Post Hoc Bonferroni) is performed as the test of significance. *p < 0.05 is taken as level of significance; AUC= Area Under the Curve; $C_{max}$= Maximum Concentration; ns = not significant.

## 4 Discussion

The determination of serum daidzein and genistein were assessed in Bangladeshi postmenopausal women, after a single dose of orally administered soy-milk, soups of *masoor* and *mung dals* respectively. Results of two studies showed that that the AUC of soy genistein was greater than that of daidzein [21,23]. In the present study, the mean AUC and the $C_{max}$ of serum isoflavone genistein of soy-milk were 0.82 µg/mL and 0.17 µg/mL, respectively.

Cassidy *et al.* reported that the AUC of genistein was 54.06 µmol/L after the ingestion of soy-milk in postmenopausal women [20] whereas the AUC of soy-milk genistein was less in the study subjects. The $C_{max}$ of soy-milk genistein, among our participants, was higher than that was found in the study of Cassidy *et al.* [20]. The availability of genistein in serum was 2.6 % after the ingestion of orally administrated single dose soy-milk and the amount was less compared to the study of Okabe *et al.* [16]

Variations in results might be due to the physiological differences (intestinal condition) between races, due to the ingestion form of isoflavones, and for the location of cultivation too. Other factors, such as differences in the food matrix (liquid vs solid), composition of habitual diets (fiber, fat, protein), low content of isoflavones in foods, and study design might also play a role in the determination of serum daidzein and genistein among the postmenopausal women [24].

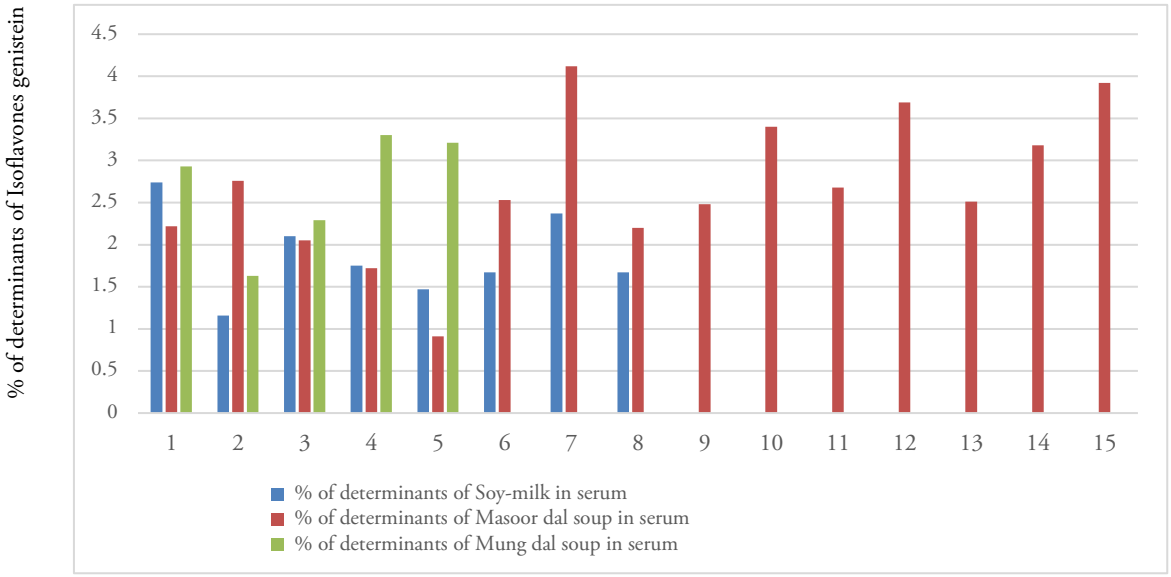



**Figure 6.** Percentage of availability of genistein in serum following a single dose of orally-administered soy-milk, *masoor* and *mung dal* soups

Generally, 1 – 6 hours are needed to obtain maximum plasma concentrations for free genistein and 4 – 6 hours for total genistein (aglycone + conjugates) [25]. In the current study, the average time to obtain maximum concentration ($T_{max}$) of soy-milk was 4 – 6 hours. Another study showed that liquid matrix yields a faster absorption rate, higher peak serum concentration, and maximum time concentration than a solid matrix [20].

Blood sampling frequency and timing were fixed in the present study following the studies among Thai [17], the UK [21], and the USA [22] menopausal women. No literature was found on the bioavailability or the determination of isoflavones in South-East Asian postmenopausal women. The absorption patterns of isoflavones among USA, UK, and other Asian menopausal women were not similar to those we found.

To the best of our knowledge, there is no study on the determination of isoflavones in non-soy food, such as *masoor* and *mung dal*. In the present study, the AUC and $C_{max}$ of genistein was 1.01 µg/mL and 0.45 µg/mL respectively in the *masoor dal* soup and, the AUC and $C_{max}$ of genistein was 1.12 µg/mL and 0.63 µg/mL respectively In *mung dal* soup that were similar to the bioavailability of isoflavones in soy-foods done by other investigators [16,20,21,23,26].

After consuming soy-milk, the mean AUC of serum genistein was 0.82 µg/mL, which was lower compared to *masoor* (1.01 µg/mL) and *mung dal* soups (1.12 µg/mL), however, the differences were not significant. The mean serum genistein concentrations ($C_{max}$) of *masoor* (0.45 µg/mL) and *mung* (0.63 µg/mL) *dal* soups were higher too compared to that of soy-milk (0.17 µg/mL) and there was a significant ($P$=0.03) difference between soy-milk and *mung dal* soup. This finding is valuable and promising for general population, especially for menopausal women living in developing country such as Bangladesh, in which, soybean or such food are not widely known to the general population but pulses (*dal*) called the poor men's protein, constitutes the most common foodstuff consumed by Bangladeshi population almost every day. Consequently, the determinant of isoflavones genistein of *masoor* (3.8 %) and *mung dal* (3.7 %) soups (Figure 6) was also found high in postmenopausal women. It was not possible to compare the determination of isoflavones of non-soy foods in serum to other countries due to the lack of data. However, in the current study, determinant of isoflavones genistein of non-soy food was found more than soy milk.

The preset study had a couple of limitations. The sample size was small. Daidzein from the *masoor* and *mung dal* soups and soy-milk did not show any peak at their retention time, which suggests the non-availability of daidzein in blood samples, although genistein from the above three foods gave some peaks at the allocated time but not full profile. It might happen due to following the literature-based fixed frequency and timing of blood collection, which was not appropriate for the present study. Therefore, it was not possible to get the full profile and as such the serum concentration curve could be produced. Another limitation was the failure to collect urine samples from the study subjects because of their unwillingness and lack of fund and time.

It may be assumed that different ethnicity, selection of appropriate time for blood collection, after the ingestion of food, quantity of individual and total isoflavones in foods, played an important role in the determination of isoflavones in the Bangladeshi postmenopausal women.

## 5 Conclusion

In conclusion, the findings of this pilot study suggest that the determination of isoflavones in non-soy foods (*mung* and *masoor dals*) is comparatively better than soy foods in Bangladeshi postmenopausal women, and only isoflavones genistein show considerable availability in women. It seems to be beneficial for menopausal women in our country as an alternative of prescribing HRT.

Based on our findings, it is strongly recommended that before the ingestion of isoflavones-rich foods, the form of isoflavones (aglycones vs glycosides) must be known, food matrix shall be considered, and a careful study design is necessary to know the complete pharmacokinetic characteristics of daidzein and genistein in Bangladeshi postmenopausal women.

### Ethical approval

All procedures performed in our protocol study, involving human participants, were in accordance with the ethical standards of the Ethical Review Committee of the Diabetic Association of Bangladesh (BADAS) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written consent was obtained from each postmenopausal participant woman after the full explanation of the nature of test, purpose, and potential risks of all the procedures to be used in the study. Their personal information was kept confidential.

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