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Spermatogenic Inhibition Properties of a Phenolic Phytoestrogen Isolated from *Momordica charantia* (Bitter Guard) Seeds

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Abstract:

Mechanisms and potencies of phytoestrogens are not completely clarified and they may be considered potential endocrine disruptors, and therefore caution should be exercised while taking them. Eating very high levels of some phytoestrogens may pose some health risks. Reproductive problems have been documented in laboratory animals, farm animals and wildlife that ate very high (up to 100% of their diet) amounts of phytoestrogen-rich plants. Sheep consuming large amounts of clover showed infertility and reproductive disorders. Cheetahs in captivity also had reduced fertility rates when consuming a feline diet composed of a soybean product, which was reversed when it was removed from the diet. Toxicities associated with herbal medicines that include phytoestrogens have also been reported in the literature.

Phenolphthalein a phenolic phytoestrogen has been isolated from the crude ethanol extract of *Momordica charantia* Linn. seeds. After preparative HPLC whitish amorphous compound was obtained. Its structural elucidation using IR, NMR and Mass spectral data revealed that the molecule isolated from the ethanol extract of *M. charantia* seeds was surprisingly, phenolphthalein. In order to clarify testicular influence of ethanol extract, fractions and isolated phenolphthalein were treated for sixty days to adult male albino rats. All the treated groups showed statistically significant reduction in testis weight. On histological examinations of testis showed spermatogenic inhibition effect, as the number of spermatogonia, spermatocytes, spermatids and spermatozoa were significantly decreased.

1. INTRODUCTION

Hormonally active chemicals that are capable of inducing adverse effects on reproduction and/or carcinogenesis in wildlife as well as human beings are featured as “endocrine disruptors” (ED). The National Academy of Science [1] and the U.S. Environmental Protection Agency recommend various animal studies to clarify the characteristics of those hormonal active compounds. As for adverse health effects, the most likely risks are associated with infertility and developmental problems [2]. Humans have used plants for medicinal and contraceptive purposes for a long period of time. Australian sheep suffered from reproductive problems and infertility after grazing in pastures with the phytoestrogen-containing clover *Trifolium subterraneum* [3]. Two phytoestrogen compounds, equol and coumestrol, were identified as the culprits. Rat pups, exposed to high doses of the plant estrogen coumestrol (found in sunflower seeds and oil and alfalfa spouts) through their mother's milk, suffered permanent reproductive problems, female pups when grown did not ovulate, and males had altered mounting behavior and fewer ejaculations [4].

Momardica charantia Linn. (Bitter guard) belongs to the family Cucurbitaceae growing in Asian countries like India, China, Japan, parts of the Amazon, East Africa and throughout South America [5]. The plant has been used as vegetable food as well as the indigenous system of medicine for the treatment of various diseases. It has wide range of actions like antimutagenic, antioxidant, antiulcers, antidiabetic, antitumour, purgative, immunomodulatory, antibiotic, emmenogague etc. Two proteins known as α and β - monorcharin which are present in the seeds, fruits and leaves have shown to inhibit the AIDS virus *in vitro* [6, 7]. An ethanol extract of whole plant of *M. charantia* causes infertility in dogs [8]. Our earlier studies of seeds had exhibited antiovulatory activities in albino rats [9]. In the present study we carried out isolation and identification of molecule from the seeds and their effect on morphological alteration of spermatogenic elements in rats. We conducted a 60-day toxicity study of an ethanol extract, Fractions and phenolphthalein in male rats and found testicular toxicity characterized by atrophy of seminiferous tubules and in Leydig cells. Germ cell maturation in the testis is maintained by complex hormonal regulation and local effecter cell.

2. MATERIALS AND METHODS

2.1. Plant Material

The ripe fruits were collected from the agricultural fields of Gulbarga (Hyderabad – Karnataka region) in the month of December and authenticated in Department of Botany Gulbarga University, Gulbarga. The voucher specimen (HGUG-905) has been deposited in the herbarium. The seeds were removed from the fruits and shade dried.

2.2. Extraction

The seeds of *M. charantia* were powdered and subjected to soxhlet extraction with ethanol. Extract was concentrated to dryness in flash evaporator (Buchi) and reduced pressure and controlled temperature (40-50°C)

2.3. Isolation

Ethanol extract was subjected for fractionation to isolate the compound. To isolate the compound from ethanol extract (80g), water was added to it and extracted repeatedly with n-butanol. The butanol layer was separated and concentrated to dryness in flash evaporator. The n-butanol fraction on concentration yielded a brownish semisolid which was further purified by repeated preparative thin layer chromatography over silica gel ‘G’ as absorbent with benzene: methanol (7:3). The compounds having high Rf value was designated as Fraction I, and compounds having low Rf values were designated as Fraction II. The Fraction I yielded brownish semisolid (48g) and Fraction II yielded dark brown semisolid (28g) when silica gel was washed with methanol. The Fraction I was then subjected to column chromatography with silica gel (60–120 mesh) on a glass of length 52cm and diameter 6cm mobile phase as the adsorbent using the mobile phase (chloroform: ethyl acetate 80:20 v/v). The four major fractions were collected, evaporated to dryness and designated as S1, S2, S3 & S4 fractions. These fractions were rechromatographed separately on column length of 52cm and diameter of 2cm to purify the fractions. The elution was carried out on solvent mixtures of increasing polarities. All the four fractions were yielded whitish amorphous powder. HPLC analysis of these four fractions was conducted on spherisorb ODS 1 column (25 x 4.6mm i.d) at ambient temperature, using mobile phase (methanol–water 50:50 v/v) at a flow rate of 2ml/min with injection of 20µl, UV detection was at 215nm. Three of the fractions exhibited very similar profiles showing a prominent early-eluting peak (*k* 0.9) and accordingly these were combined for the subsequent preparative HPLC carried out a view to isolating a pure form of the compound giving rise to this peak. The preparative HPLC of the combined semi purified fractions was

conducted under broadly equivalent conditions to the analytical work using a 25 x 22mm i.d sperisorb ODS 1 column at ambient temperature with the same (methanol–water 50:50 v/v) mobile phase but at a flow rate of 8ml/min. to allow for the scale up and an injection volume of 2ml to allow for scale up and increase sample loading and thereby throughput. Sample concentrations were 7mg/ml and UV detection was at 215nm. Compound obtained as a white amorphous powder and subjected for IR, NMR and Mass spectral analysis.

2.4. Animals

Colony bred adult male albino rats of Wistar strain (140-160g) were selected for the experiment (Animal ethics Reg. No. 34800/2001/CPAEA/ dated 1/9-08-2001). A maximum of six animals were housed in polycarbonate cages with soft rice husk bedding in a room controlled for light-dark cycle, ventilation (air exchange rate of 18 time/hour), temperature (23-25°C) and relative humidity (50-60%) during the study. The cages and husk bedding was exchanged twice a week. The animal had given balanced food as prescribed by Central Food and Technological Research Institution (CFTRI) Mysore, India and water *ad libitum*.

2.5. Drug Preparation and Treatment

The doses containing ethanol extract (25 and 50mg/100g), Fraction I (15 and 25mg/100g) Fraction II (15 and 25mg/100g) and phenolphthalein (10, 15 & 20mg/100g) were prepared in Tween-80 (1%) as suspension. All the groups received their respective doses orally.

2.6. Experimental Designs

In the first experiment animals were divided into three groups, the groups II and III were treated with 25 and 50mg/100g oral doses of ethanol extract. Group I animals were maintained as control received 1% tween-80 as a vehicle. In the second experiment animals were divided into five groups, the groups II-V were treated with 15 and 25mg/100g oral doses of Fraction I and Fraction II. Group I animals were maintained as control and received 1% tween-80 as a vehicle. In the third experiment animals had four groups, the groups II-IV were treated with 10, 15 & 20mg/100g isolated phenolphthalein. Group I animals were maintained as control receiving 1% Tween-80 as a vehicle. Six animals were maintained in control and drug treated groups. The above treatment was given for 60 days, on the 61st day the rats were sacrificed by cervical decapitation. The testis, epididymis (caput and cauda), prostate gland, seminal vesicles, vas deferens and Levator Ani muscle were dissected out immediately after the sacrifice, trimmed off from fat and connective tissue and weighed up to the nearest mg on an electronic balance. The organs from one side of each animal were fixed in Bouin's fluid for histological studies. They were embedded in paraffin wax sectioned at 5µ, stained with Ehrlich's haematoxylin and eosin. The morphometric measurements like diameter of testis and seminiferous tubules were made from randomly choosen 20 sections from each group by using ocular and stage micrometer. The spermatogenic elements like spermatogonia, spermatocytes and spermatids were counted from 20 round sections of each group. The number of spermatozoa from cauda epididymis was determined by using haemocytometer [10].

2.7. Statistical Analysis of Data

Results are expressed as Mean ± S. E. Difference between controls and the treatments were tested by the Student's *t-test* for unpaired data.

3. RESULTS

3.1. Characterization of Isolated Compound by Spectral Studies

Phenolphthalein was found to be present in the seeds of *M. charantia*. The seeds extract container when taken for a wash pink colour started appearing, as the laboratory tap water has got pH more than 7 i.e. alkaline pH. This gave an indication that extract may be containing an organic molecule which can give colour in alkaline pH. The variation in the colour caused due to change in pH is the property of the indicators. When this is repeated with the double distilled water the colour did not appear. This became a strong point to assume that the extract contains suitable indicator which gives no colour at pH 7, but pink colour when pH is more than 7. The crude ethanol extract of *M. charantia* seeds after preparative TLC over silica gel 'G', repeated column chromatography and preparative HPLC afforded phenolphthalein molecule. The compound so obtained from chromatographic separation was amorphous in nature. Compound phenolphthalein was isolated as a white amorphous powder, m.p. 261- 263 °C, with a molecular formula $C_{20}H_{14}O_4$. The IR spectrum of amorphous powder exhibited δ C=O peak at 1735 cm-1. This may be due

Table 1: Gravimetric, Morphometric and Spermatogenic Changes in the Testis Due to Administration of Ethanol Extract of *M. charantia* Seeds in Adult Rats

Treatment	Dose (mg/100-g body wt.)	Testis weight (mg/100g body wt.)	Diameter of testis (μm)	Diameter of seminiferous tubule (μm)	Spermatogo - nia	Spermatoc -ytes	Spermatids	sperm count (millions/ cauda)
Control	Tween-80 (1%)	1251.32 ± 10.51	3590.81 ± 20.31	185.54 ± 3.01	90.13 ± 2.52	175.32 ± 4.32	280.11 ± 5.80	2.15 ± 0.08
Ethanol extract	25	1121.75* ± 10.21	3443.80* ± 12.80	166.13* ± 2.03	70.23* ± 2.90	83.13** ± 2.01	43.03** ± 2.10	0.67** ± 0.03
Ethanol extract	50	1103.00* ± 13.53	3256.00* ± 15.03	153.03* ± 2.23	53.08** ± 1.90	78.83** ± 2.30	40.13** ± 1.83	0.44** ± 0.02

Duration: 60 days.
Six animals were maintained in each group.
Values are mean ± S.E.
*P<0.01, **P<0.001 when compared to control.

to either C=O of carboxylic ester or C=O of lactone. The two sharp peaks were observed at 3378 cm-1 and 3290 cm-1 corresponding phenolic hydroxyl, groups. In the NMR spectrum of this compound the aromatic cluster is seen from 6.352 – 7.8 δ corresponding to the resonance of aromatic protons. A sharp singlet is seen at 9.7 δ which can be accounted for proton of phenolic-OH. In the mass spectrum of compound the molecular ion peak M+ is appeared at 318 corresponding to the molecular weight of the phenolphthalein molecule. This molecular ion peak has lost CO_2 to give a base peak at m/z 274. These spectral data suggest that the compound isolated from the ethanol extract of seeds of *M. charantia* contains a phenolphthalein molecule.

3.2. Gravimetric, Morphometric and Spermatogenic Changes in Testis

Experiment I: (Table 1, Figure 1b)

Both the groups treated with different doses of ethanol extract showed significant decrease in weight of the testis, when compared to that of the control group (Figure **1a**). Morphometric examination of the diameter of testis was decreased significantly (P<0.01) with the treatment of both the doses of ethanol extract. Similarly the diameter of seminiferous tubules was decreased and the process of spermatogenesis was impaired in extract treated groups. The number of spermatogonia was decreased with slight significance (P<0.01) with low dose; and highly significantly decreased (P<0.001) with high dose of ethanol extract treatment. Highly significant (P<0.001) reduction was observed in the number of spermatocytes and spermatids with the treatment of both doses of ethanol extract. The cauda epididymal sperm count was decreased significantly (P<0.01) and highly significantly (P<0.001) with low and high doses of ethanol extract, respectively.

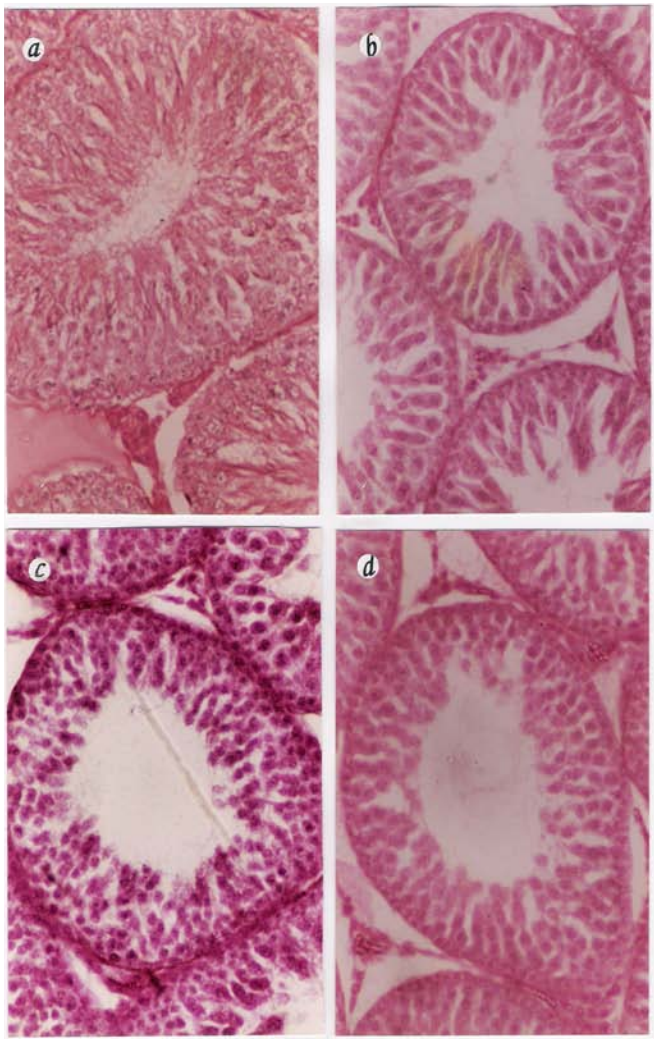

Figure 1: a. Section of control rat testis showing normal spermatogenic activity. Seminiferous tubules, Leydig cells and Sertoli cells are normal. **b**, **c** & **d**. Sections of rat testis treated with 50mg ethanol extract, 25mg Fraction I&II of *M. charantia* seeds showing significant decrease in the spermatogenic elements. Leydig cells and Sertoli cells are degenerative. Haematoxylene-Eosine stains X magnification 400.

Table 2: Gravimetric, Morphometric and Spermatogenic Changes in the Testis Due to Administration of Fraction I and II in Adult Rats

Treatment	Dose (mg/100-g body wt.)	Testis weight (mg/100g body wt.)	Diameter of testis (μm)	Diameter of seminiferous tubule (μm)	Spermatogo - nia	Spermatoc -ytes	Spermatids	Sperm count (millions/ cauda)
Control	Tween-80 (1%)	1245.00 ±11.22	3670.00 ±14.66	179.50 ±2.82	94.50 ±2.41	183.60 ±2.67	253.60 ±3.85	2.05 ±0.07
Fraction I	15	1109.16* ±11.97	3180.50** ±8.76	141.83** ±2.64	68.83** ±2.35	106.50** ±3.43	70.83** ±2.24	0.53** ±0.04
Fraction I	25	1032.16* ±10.99	2983.50** ±8.67	139.00** ±2.48	57.66** ±1.84	85.00** ±3.20	47.33** ±2.13	0.18** ±0.01
Fraction II	15	1210.00* ± 8.08	3566.30* ±17.32	165.50* ±1.61	86.57 ±1.60	148.83** ±3.97	200.80* ±5.85	1.26* ±0.07
Fraction II	25	1200.50* ±11.12	3476.30* ±15.63	160.60* ±3.30	83.00 ±2.37	143.80** ±3.66	180.83* ±3.83	1.05* ±0.06

Duration: 60 days.
Six animals were maintained in each group.
Values are mean ± S.E.
* P < 0.01, ** P < 0.001 when compared to control.

Experiment II: (Table 2, Figure 1c & d)

Weight of the testis was changed significantly with the administration of both the doses of fraction I and II. The diameter of testis and seminiferous tubules was decreased with the administration of both the doses of fraction I (P<0.001) and fraction II (P<0.01). Reduction in the number of spermatogonial elements was observed with both the doses of fraction I and II. The number of spermatogonia, spematocytes, spermatids and cauda epididymal sperm count was reduced significantly (P<0.001) with both the doses of fraction I treatment. The parallel results with less significance were obtained with both the doses of fraction II treatment, as the number of spermatocytes (P<0.001), spermatids (P<0.01) and cauda epididymal sperm count (P<0.01) were reduced.

Experiment III: (Table 3, Figure 2a, b & c)

A significant decrease in the weight of testis was seen with the administration of 10, 15 and 20mg isolated compound treatment to the albino rats in comparison with that of control. The morphometric measurements like diameter of testis and seminiferous tubule show significant (P<0.01) decrease with all the doses of isolated compound. The number of spermatogonia reduced significantly with 10mg (P<0.01), 15mg and 20mg (P<0.001) treatment. The number of spermatocytes, spermatids and cauda epididymal

Table 3: Gravimetric, Morphometric and Spermatogenic Changes in the Testis Due to Administration of Different Doses of Phenolphthalein in Adult Rats

Treatment	Dose (mg/100-g body wt.)	Testis weight (mg/100g body wt.)	Diameter of testis (μm)	Diameter of seminiferous tubule (μm)	Spermato-gonia	Spermato-ytes	Spermatids	Sperm count (millions/ cauda)
Control	Tween-80 (1%)	1242.5 ± 10.97	3650.03 ± 23.71	182.02 ± 8.52	87.66 ± 3.94	170.00 ± 5.79	256.66 ± 9.92	2.25 ± 0.10
Phenolphth-alein	10	1032.53* ± 11.71	3166.62* ± 13.38	152.56* ± 4.44	67.33* ± 2.50	108.07** ± 2.30	65.50** ± 2.41	0.43** ± 0.12
Phenolphth-alein	15	1004.16* ± 11.32	3108.34* ± 17.46	138.83* ± 4.18	57.00** ± 2.31	84.00** ± 1.24	50.83** ± 2.04	0.31** ± 0.04
Phenolphth-alein	20	1003.88* ± 10.23	3080.83** ± 17.60	124.66* ± 3.21	46.33** ± 1.88	71.00** ± 2.31	44.83** ± 1.80	0.18** ± 0.04

Duration: 60 days.
Six animals were maintained in each group.
Values are mean ± S.E.
*P<0.01, **P<0.001 when compared to control.

sperm count decreased highly significantly (P<0.001), in all the compound treated groups.

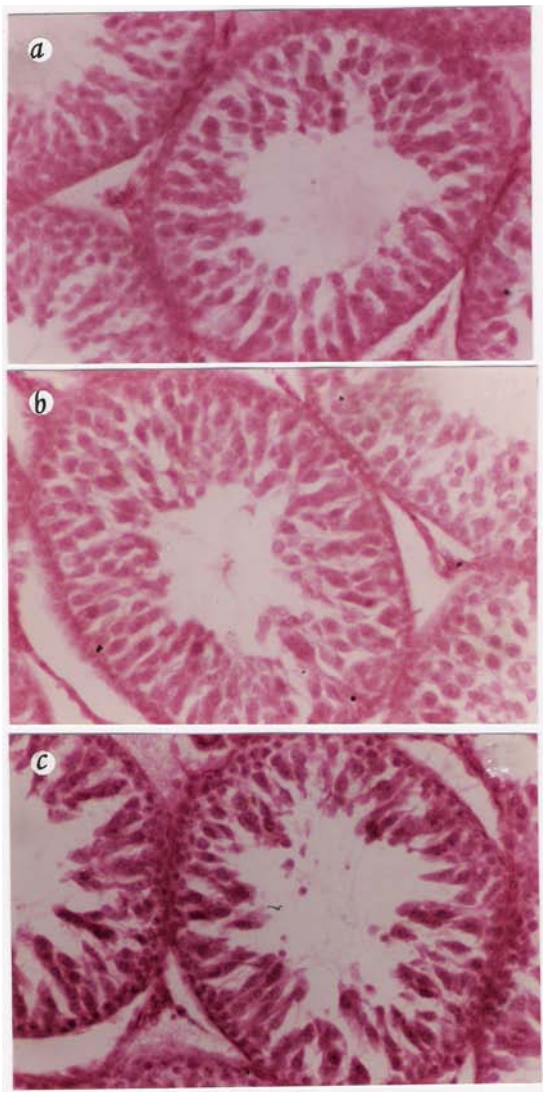

Figure 2: a, **b** & **c**. Sections of rat testis treated with 10, 15, 20mg phenolphthalein of *M. charantia* seeds showing absence of spermatids and spermatozoa. Leydig cells and Sertoli cells are degenerative and enlarged interstitial space. Haematoxylene-Eosine stains X magnification 400.

4. DISCUSSION

The study of the *Momordica charantia* was focused on its phytoestrogenic activity. In an earlier experiment conducted on screening of seeds of *M .charantia* it has exhibited very well estrogenic and antiovulatory activities at very low concentration [11, 9]. The preliminary phytochemical studies of extracts of *M. charantia* showed phenolic compounds. The key structural elements crucial for estradiol like effects are the phenolic ring that are indispensable for binding to estrogen receptors and low molecular weight similar to estrogens. Diet rich in certain phytoestrogens also adversely affects the fertility of experimental and domestic animals. For instance, phytoestrogen in dry, summertime grasses reduced the number of offspring in wild populations of California quail [12] and deer mice [13]. The use of phytoestrogen in fast meals and other processed foods as a low-cost substitute for meat products may lead to consumption of isoflavonoids by fast food eaters. A research team at the Queen's University in Belfast, in a review article, claims that such intake may lead to a slight decrease in male fertility, including a decrease in reproductive capability in isoflavones are taken in excess during childhood [14]. This prompted us to proceed to isolate and identify the phytoestrogenic molecule from the extract of *M. charantia* seeds. The objective of the present study was to investigate the effect of ethanol extract, Fraction I, II and phenolphthalein isolated from *M. charantia* seeds on spermatogenesis in male rats.

Spermatogenesis in the testis is maintained by complex hormonal control through the hypothalamic-pituitary-testicular axis and the local somatic cells, i.e., Leydig, Sertoli and peritubular cells [15, 16]. This complex regulatory process provides a variety of targets for chemical-induced disruption of spermatogenesis, each associated with characteristic morphological lesions of the testis at an early phase [17]. Therefore, the characterizations of early structural alterations are useful to ascertain cellular targets and define the underlying mechanisms. Exposure to high levels of phytoestrogens in men could alter their hypothalamic-pituitary-gonadal axis.

However, our studies on administration of ethanol extracts, fraction I and II and isolated Phenolphthalein of *M.charantia* have reduced the weight of testis. This reduction in the testicular weight may be attributed to the altered production of seminiferous tubular fluid [18]. According to Lostroch [19], FSH stimulates the development of spermatogonia and both FSH and LH are necessary for meiosis and development of spermatids [20, 21]. In the present study, the reduction in the number spermatogonia, spermatocytes and spermatids could be attributed to the decreased availability of pituitary FSH and LH due to the treatment of extracts. The degenerated Leydig cells observed in the testis of rats administered with various extracts of *M. charantia* seeds also supports the above fact as the development and function of Leydig cells depend on pituitary LH / ICSH [21].

The spermatozoa produced in the testis attain further development, motility and physiological maturation in

the microenvironment of the epididymis [22, 23]. The decrease in the cauda epididymis sperm count may not only be due to decrease in the testicular spermatogenic process, but also due to altered microenvironment of the epididymis.

5. CONCLUSION

The spermatogenic inhibition effects of *M. charantia* seeds seem to be mediated by disturbances in testicular somatic cell functions (Leydig and Sertoli cells) involved in the histophysiological events of spermatogenesis. The overall findings of this study enrich the present pool of studies on phytoestrogen content in food plants, consumption of phytoestrogen rich foods, effects of phytoestrogens on health and the establishment of a common information basis for consumers, industry and researchers.

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CONFLICT OF INTEREST DISCLOSURE

There is no conflict of interest.

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