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Facet of isoflavone, phenol and flavonoid content in Soybean (*Glycine max* Merrill) varieties under dissimilar processing conditions

Shivani Chaturvedi, Suaib Luqman* and Sunil Kumar Khare

Enzyme and Microbial Biochemistry Laboratory, Department of Chemistry, Indian Institute of Technology, Delhi, New Delhi-110006, India

**Academy of Scientific and Innovative Research (AcSIR), Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, Uttar Pradesh, India*

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Abstract

Effect of different processing conditions on isoflavone, phenol and flavonoid content of Indian soybean varieties 'Punjab long', 'Hardee' and 'Kalitur' were appraised employing a variety of commonly prevalent processing practices such as boiling, soaking, sprouting and drying. The isoflavone contents were reduced on boiling, soaking, roasting and defatting, however, exposure of flour to ultra-violet radiation, soaking and sprouting under 50°C increased the amount. The examined varieties contained 1020.0 g, 611.8 g and 659.0 g of isoflavone per kg, respectively in soyflour. The black variety 'Kalitur' showed a superior phenol and flavonoid content, compared to other two varieties. The investigation revealed that process of soaking and sprouting contributes towards increasing the isoflavone content in soybean varieties. The phenol and flavonoid contents were found to be significantly higher in Hardee and Kalitur varieties as compared to Punjab Long.

Key words : Soybeans, isoflavone, ultraviolet radiation, sprouting, phenol, flavonoid

1. Introduction

Isoflavones are natural bioflavonoids synthesized predominantly by the plants of Leguminaceae family (Fritsche and Steinhart, 1999). Crop up as a secondary metabolite in large amounts in soybeans, chickpeas, beans, clover, toothed medick and bluegrass, its structure is similar to that of oestrogen and, therefore, it can demonstrate weak oestrogenic effects (Reinli and Block, 1996). Prior researches revealed that consumption of soybean and their products provides protective and beneficial effect against hormone-dependent cancer (breast and prostate), cardiovascular diseases, osteoporosis and menopausal symptoms (Lichtenstein, 1998; Messina *et al*., 1994; Potter *et al*., 1998). In soybeans, isoflavones are present both as aglycones as well as glycosides (β-glycosides, acetyl and malonyl glycosides). The principal isoflavones present in soybeans are daidzein (42 ,7-dihydroxyisoflavone) and genistein (42 ,5,7-trihydroxyisoflavone). The total concentration of isoflavone in soybeans was reported to be between 0.2-0.4% inclined by the genotype, location and crop year (Wang and Murphy, 1994; Eldridge and Kwolek, 1983), whereas in processed soy products, the isoflavone content depends on the variety as well as the type of processing used (Murphy *et al*., 2002). The glycosides gets hydrolysed in the human gut and converted to their respective aglycones, metabolised and excreted from the body (Kulling *et al*., 2002). So, it is preferred to determine the aglycones formed after acid or enzymatic hydrolysis as described previously (Mullner and Sontag, 2000; Wang *et al*., 1990; Franke *et al*., 1994).

It has been noted that during various processing conditions, the isoflavone may either be degraded/lost or gets converted into the different chemical forms (Chien *et al*., 2005; Kao *et al*., 2004; Wang *et al*., 1998). Rinaldi *et al*. (2000) also found that during soymilk or tofu preparation, the waste residue (Okara) contained 1241 mg/g isoflavone, signifying that a large amount of isoflavone content leached out during different processing conditions. In prior study, it was revealed that defatted soybean contained a high amount (2121.9 mg/g) of isoflavones (Kao and Chen, 2002). Soy cake, a by-product of soybean oil, is often used as animal feed contain high amount of isoflavones which could be beneficial for the animals. Thus, if recovered as bioactive constituent in large amount it may help in bracing animal as well as human health. It was also found that among varieties, the isoflavone content of black colored soybean is higher than normal soybean. Black colored soybean also showed three times more antioxidant activity than the control (wild) variety (Kim *et al*., 2004).

High oxidative stress and generation of reactive species leads to a number of diseases and disorders like atherosclerosis, cancer, cirrhosis, ageing *etc*. Consumption of antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyl anisole (BHA) limits, the generation of reactive species and combat the damage caused by the oxidative stress. However, due to their safety concern, researchers are now-a-days focussing on the use of natural antioxidants for protection against oxidative damage. Soybean and its products being a great source of compounds like polyphenols,

Author for correspondence: Professor Sunil Kumar Khare
Enzyme and Microbial Biochemistry Laboratory, Department of Chemistry, Indian Institute of Technology, Delhi, New Delhi-110006, India
E-mail: skkhare@chemistry.iitd.ac.in; skhare@rocketmail.com
Tel.: +91-11-26596533
Cell.: +91-09350408194, **Fax.:** +91-11-26581102

isoflavones, flavonoids (Fritz *et al*., 2003), masquerade greater concern as functional food because of its extraordinary desirable characteristics (Tripathi and Misra, 2005). It is also pertinent to mention that India holds fifth position as producer of soybean. Thus, it is important to estimate and know the concentrations of isoflavones, phenols and flavonoid content of Indian soybean varieties 'Punjab long', 'Hardee' and 'Kalitur'. Earlier studies revealed soybean and soy foods content available in the USA (Setchell, *et al*., 1998), Korea and China (Lee *et al*., 2007), Austria and Germany (Beatrix and Sontag, 2004) but to the best of our knowledge and information, there is no such report on Indian variety. Therefore, the present study is vital to examine Indian soybean varieties 'Punjab long', 'Hardee' and 'Kalitur'for their isoflavone, phenol and flavonoid content and their prolific potential.

2. Materials and Methods

2.1 Materials

Three cultivars of soybean (*Glycine max* Merrill) namely; Punjab long (available from local market), Hardee and Kalitur (obtained from National Research Centre for Soybean, NRCS, Indore, India) were taken up for the study.

2.1.1 *Preparation of defatted soybean meal and other processing treatments*

The soybean flour was prepared by milling the beans at 'in-house' facility. The flour was subjected to different processing treatments. (i) defatting for 48 h with hexane, (ii) boiling of flour in an open vessel without pressure for 5 min at 100°C, (iii) boiling of the flour in a closed cooker with pressure (1.4 psi) for 5 min, (iv) soaking of seeds for 6 h at 30°C followed by sprouting for 72 h at 30°C, (v) soaking of seeds for 6 h at 30°C, (vi) ultra-violet irradiation of the flour for 20 min, (vii) soaking of seed for 6 h at 50°C followed by sprouting of seeds for 72 h at 50°C after, and (viii) microwave based processing of the flour for 5 min and roasting of the flour for 5 min.

Distilled water (pH 6.9) was used for all processing except defatting process. The wet samples were air-dried at room temperature (29°C) inside a laminar hood for 48 h, grounded properly into powder, sieved through a sieve 8-mesh size and then powdered samples were stored in dark brown, air-tight bottles at-80°C for further extraction process.

2.1.2 *Extraction of soy saponin*

10 g of dry powdered sample was mixed with 50 ml of HPLC grade methanol in Erlenmeyer flask and covered with cork lid and paraffin. The mixture in the Erlenmeyer flask was stirred for 48 h, followed by centrifugation at 8000 rpm for 10 min and concentrated up to 10 ml. 1 ml out of 10 ml (HPLC grade methanol) was analysed, using HPLC technique (Zhang *et al*., 2009; Lin and Wang, 2004).

2.1.3 *Instruments*

Soy isoflavone analysis were performed using HPLC (Agilent Technologies, 1200 Series, USA) equipped with UV detector (Agilent G1365B MWD). A C_{18} reverse phase column (5µm, 4.6 X 250mm i.d. Thermo Electron Corporation Massachusetts, MA, USA) was used.

2.1.4 *Procedure used for* HPLC

The method of Zhang *et al*. (2008) originally described by Jin *et al*. (2006) was used for the HPLC analysis. 0.025% acetic acid in water (solvent A) and HPLC grade 0.025% acetic acid in acetonitrile (solvent B) were used as solvents. The mobile phase flow rate was 1ml/min at 35°C column temperature. The separation of isoflavone was done at elution gradient condition and UV absorbance was monitored at 250 nm. The solvent program was as follows: Time 0 (13% B), 12.5 min (30% B), 17.5 min (40% B), 23.5 min (40% B), 27.5 min (60% B), 30-35 min (100% B), and 40 min (13% B). All the experiments were done in triplicate and the variation was within ± 4% (Lin and Wang, 2004).

2.1.5 *Method used for total phenolics and flavonoid content*

Total phenolics were estimated spectrophotometrically, using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). To the 100 µL of the sample extract (80% ethanol), 2.9 ml of deionized water, 0.5 ml of Folin-Ciocalteu reagent and 2.0 ml of 20% Na_2CO_3 solutions were added. The mixture was allowed to stand for 90 min and absorption was measured at 760 nm against a reagent blank in UV-Vis Spectrophotometer (VARIAN Cary 50). Results were expressed as gallic acid equivalent (mg of GAE /100g). Total flavonoids were analyzed using Aluminum chloride method (Zhishen *et al*., 1999). An aliquot (1 ml) of extract in 10 ml of volumetric flask containing 4 ml of distilled water, 0.3 ml of 5% $NaNO_2$ and 0.3 ml of 10% $AlCl_3.6H_2O$ was added. At 6^{th} min, 2 ml of 1M NaOH was added and volume was raised to 10 ml with double distilled water. The absorbance of the solution versus a blank at 510 nm was measured immediately. The results were expressed as catechin equivalent (mg CE/100 g).

3. Results and Discussion

3.1 Chromatographic interpretation of isoflavone peak area

Isoflavone contents in de-oiled soybean cake were quantified before processing treatments. These were extracted by methanol and analysed by HPLC. The quantification was done comparable to an unprocessed seed cake soy isoflavone which appeared from 13.2 to 36.9 min, the peak of standards (Daidzein and Genistein) appeared between at 36.38 and 36.68 min. Soybean is reported to have twelve types of isoflavone or its derivatives-3 aglycones (daidzein, genistein, and glycitein and nine glucosides genistin, daidzin, glycitin, and their malonyl forms and aglucons, Wang and Murphy, 1996). We observed seven major peaks, the first appeared between 15.4-15.8 min, second to seventh peak appeared between 16.1-17.4, 20.2-21.1, 23.9-24.8, 26.1-28.5, 29.2-32.7 and 35.5-37.5 min, respectively. Lin and Giusti (2005) and Song *et al*. (2012) found these peaks and identified them as different isoflavone derivatives like daidzein, genistein, glycetein, malonyl daidzin *etc*. (Table 1). The area under the individual peaks varied according to different processing condition applied on soybean cake. The comparison of each individual peak is given in Tables 2 and 3.

Table 1: Reported retention time of isoflavone and its derivatives

S.No.	Constituents	RT (min)
1	Genistein	15.51
2	Malonyl Daidzein	16.16
3	Malonyl Genistein	20.63
4	Daidzein	24.38
5	Acetyl Genistein	26.72
6	Genistein	29.44
7	Uncharacterized	36.90

Table 2: Reported peak area of isoflavone and its derivatives in different varieties of soybean

Constituents	"Punjab long" Soy Flour	"Punjab long" Defatted Soy Flour	"Hardee" Soy Flour	"Kalitur" Soy Flour
	Area (mAU*s)			
Genistin	53077.1	49037.1	34392.0	^
Malonyl Daidzin	31429.7	29363.9	39320.7	36703.1
Malonyl Genistin	25998.5	21852.9	26668.3	^
Daidzein	33989.5	26067.6	25844.4	17033.8
Acetyl Genistin	25104.8	16141.0	9767.7	36061.7
Genistein	21106.7	7866.3	9020.7	31993.5
Uncharacterized	51028.3	4709.7	^	34403.2

*Peak present, ^Peak absent

Table 3: Effect of varieties in isoflavone, phenol and flavonoid content of soyflour

Varieties	Isoflavone content	Phenol content	Flavonoid content
	g/kg	µg/100g	µg/100g
Punjab long	1020.0 ±1.96	148.10 ±4.57	11.64 ±0.19
Hardee	611.8 ±1.01	181.30 ±5.24	7.49 ±0.25
Kalitur	659.0 ±1.18	247.73 ±4.83	25.14 ±0.72

Table 4: Effect of processing conditions on full fat soy flour (FFSF) of Punjab long variety

Process followed	Soy isoflavone content (µg per g sample)	Phenol content (µg/100g)	Flavonoid content (µg/100g)
Unprocessed soy flour	1020.0 ±1.96	148.10 ±4.16	11.64 ±0.28
Defatting	920.2 ±2.35	155.37 ±2.97	10.66 ±0.27
Boiling of flour (without pressure) in a open vessel	834.4 ±1.42	209.75 ±5.38	7.06 ±0.12
Boiling of flour (with pressure) in a closed vessel like cooker	927.8 ±1.91	143.63 ±4.25	3.06 ±0.05
Soaking of seed (6 hrs) and then sprouting (72 hrs) at 30°C	499.1 ±0.82	177.65 ±3.33	21.13 ±0.58
Soaking of seed (6 hrs) at 30°C	700.7 ±1.23	188.50 ±4.72	8.09 ±0.12
Flour exposed to Ultra violet rays for 20 min	1135.8 ±2.14	209.73 ±3.86	15.04 ±0.37
Soaking of seed (6 hrs) followed by sprouting (72 hrs) at 50°C	1027.1 ±1.37	234.78 ±6.39	21.49 ±0.35
Exposed flour to microwave for 5 min	1003.0 ±1.81	238.63 ±4.26	19.16 ±0.48
Roasting of flour	654.2 ±1.11	249.25 ±4.48	19.69 ±0.37

3.2 Quantitative comparison of isoflavone content with unprocessed soy flour

From our results, it is evident that there was a substantial reduction in the isoflavone content of soybean flour upon defatting soaking, sprouting (30°C and 50°C), roasting and boiling (Table 4). Table 4 depicts the absolute values of soy isoflavone. The process of defatting of soy flour lead to reduction of isoflavone content by 9.8% compared to unprocessed soybean flour which may perhaps be due to the removal of oil from the soy cake. The reduction of isoflavone content was 18.2 % on boiling without pressure (open vessel) indicating loss of some heat labile isoflavones as reported previously (Booth *et al*., 1960). Tsukamoto *et al*. (1995) also noticed reduction of isoflavone content during high temperature seed development of soybean. In closed vessel boiling (with pressure), the content recorded was 9.03 % lower than that of unprocessed seed cake. Xu and Chang (2009) also found reduction in saponin content after cooking the legumes whereas Iorgyer *et al*. (2009) reported 17.97% reduction in pigeon pea due to increase in temperature (*i.e*., during boiling).

An increase in isoflavone content was also recorded in other processing method. Ultra-violet radiation recorded an increase of 11.4% in isoflavone content supporting the report of Dixit *et al*. (2010) where gamma radiation was induced to increase the total isoflavone content in soybean. One of the possible reasons for the increased value of isoflavone content may be an enhanced defence mechanism due to stress. However, when the sprouting was carried out at 50°C, the increase in isoflavone content was only marginal (0.7%). This observation regarding impact of germination and soaking temperature on the quality in terms of bioactive components is very much similar to the findings of Yen and Kao (2002). In addition, an increase in isoflavone content on raising the temperature from 30°C to 50°C was also recorded. Claudio *et al*. (2010) also found an improvement in nutritional values including isoflavone status when soybean was process through germination. Exposure to microwave decreases the isoflavone content by 1.7% which supports earlier findings of Claudio *et al*. (2010) in some legumes. Simple soaking and roasting reduces 51% and 35% of isoflavone content, respectively concurring the earlier findings where saponin content gets reduced by 17% after soaking for 18h due to leaching in water (Khokhar and Chauhan, 1986; Jood *et al*., 1986; Kataria *et al*., 1988). However, Ruiz *et al*. (1997) reported that soaking did not modify the saponin content.

A significant reduction in daidzein content of soybean flour was also observed when processed through boiling, simple soaking, soaking with sprouting at different temperature and roasting whereas enhancement occurred in microwave, ultra-violet radiation and defatting processes. Genistein content was found increased in all processing conditions, highest in microwave, followed by defatting. Dixit *et al*. (2010) revealed that gamma radiation on black soybean (Kalitur) showed significant increase in genistein content comparatively.

3.3 Varietal comparison of isoflavone in soybean cake

Punjab long, a yellow variety showed significantly higher value of isoflavone content compared with other varieties (Kalitur and Hardee). The increase in isoflavone content is supported by previous findings of Dixit *et al*. (2010) where total isoflavone content was reported highest in yellow variety (NRC37), followed by black soybean (Kalitur) variety. The daidzein and genistein contents are depicted in Figure 1 and were found highest in Punjab long, followed by Kalitur and Hardee.

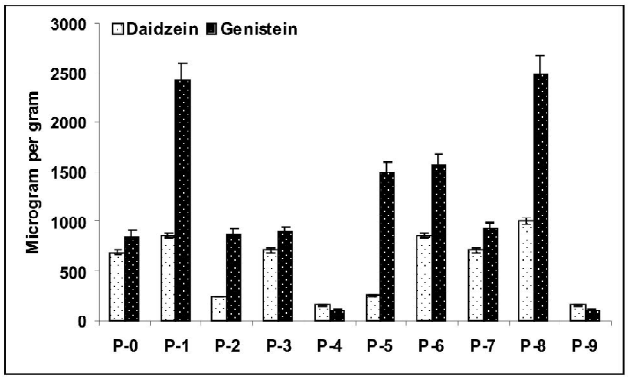

Figure 1: Effect of processing conditions on Daidzein and Genistein content of Punjab long variety

3.4 Quantitative comparison of antioxidant activity (phenol and flavonoid) with unprocessed soy flour

There was a substantial reduction in the phenol content of soybean flour processed through boiling in a closed vessel (Table 4). The percentage (3.3%) reduction in phenol content was observed when soy flour was boiled with pressure (in a closed vessel) compared to unprocessed flour. The reduction in the content might be due to the loss of phenol during processing as described previously (Lee *et al*., 2007; Devi *et al*., 2009). Xu and Chang (2009) also found reduction in total phenolic content by boiling lentil under pressure. The phenol value was found higher in boiling without pressure, ultra-violet radiation, soaking and sprouting at 30°C and 50°C, microwave exposed and roasted soybean. Earlier studies support our findings related to increase in phenolic content caused by spurt in the activities of key enzymes of phenylpropanoid metabolic pathway at a low dose of irradiation (Pendharkar and Nair, 1995; Oufedjikh *et al*., 2000). This increase in total phenolic content was also observed by Variyar *et al*. (2004), which was due to the radiation-induced breakdown of glycosides and release of free isoflavones. Jeong *et al*. (2004) also found increase of phenol content in citrus peel due to heat treatment. The black soybean 'Kalitur' showed high phenol compared to yellow soybean 'Punjab long' and 'Hardee'. Earlier findings suggest high phenol content in 'Kalitur' followed by 'Punjab long' and 'Hardee' (Dixit *et al*., 2010; Xu *et al*., 2007) and demonstrated the health benefits of black variety of soybean over yellow variety in terms of strong free radical scavenging activity (Xu and Chang, 2008; Takahashi *et al*., 2005).

The flavonoid content was the highest in sprouting after soaking at 50°C (45.8%), followed by soaking at 30°C (44.9%). The roasting also showed significant higher values (40.8%) but boiling in open and closed vessel, ultra-violet radiation showed reduction in flavonoids. Kalitur, variety showed 53% increase compared to Punjab long and Hardee display poor flavonoid content among the tested varieties.

4. Conclusion

Processing of soybean flour is a critical determinant of its isoflavone, phenol and flavonoid content. Soaking followed by sprouting at 50°C and exposure to ultra-violet rays is the most efficient method

for isoflavone retaining. Daidzein content of soybean flour was significantly increased through microwave, ultra-violet radiation and defatting processes. Genistein content increased in all processing conditions, highest in microwave, followed by defatting process. The process of sprouting at 50°C, microwave and roasting increases the phenol and flavonoid content. The black variety 'Kalitur was found superior in terms of both phenolic and flavonoid content compared to yellow varieties of soybean (Punjab long and Hardee).

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Conflict of interest

We declare that we have no conflict of interest.

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