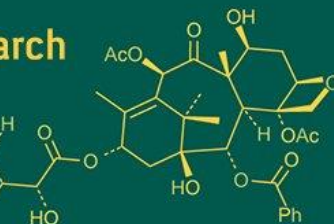
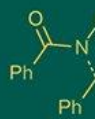


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DB Kale

Department of Veterinary
Pathology, Post Graduate
Institute of Veterinary and
Animal Sciences, Krishnagar,
Akola, Maharashtra, India

RS Ingle

Professor & Head, Department
of Veterinary Pathology, Post
Graduate Institute of
Veterinary and Animal
Sciences, Krishnagar, Akola,
Maharashtra, India

NV Kurkure

Department of Veterinary
Pathology, Nagpur Veterinary
College, Nagpur, Maharashtra,
India

SW Hajare

Department of Veterinary
Pharmacology and Toxicology,
Post Graduate Institute of
Veterinary and Animal
Sciences, Krishnagar, Akola,
Maharashtra, India

MV Ingawale

Department of Animal
Reproduction and Gynecology,
Post Graduate Institute of
Veterinary and Animal
Sciences, Krishnagar, Akola,
Maharashtra, India

BP Kamdi

Department of Veterinary
Pathology, Post Graduate
Institute of Veterinary and
Animal Sciences, Krishnagar,
Akola, Maharashtra, India

Corresponding Author:**Dr. RS Ingle**

Professor & Head, Department
of Veterinary Pathology, Post
Graduate Institute of
Veterinary and Animal
Sciences, Krishnagar, Akola,
Maharashtra, India

Qualitative and quantitative evaluation of isoflavones from *Glycine max* using chromatographic techniques

DB Kale, RS Ingle, NV Kurkure, SW Hajare, MV Ingawale and BP Kamdi

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Abstract

Soybean is an edible legume used for edible oil production and fulfilling protein requirements in humans and animals all over the world. Though soybeans contain many nutritional components, but it also contains many anti-nutritional phytoconstituents, of which phytoestrogenic isoflavones are one of them. In the present study, ethanolic (80%) extracts were prepared from 10 different soybean samples collected from in and around Akola district of Maharashtra and the phytoconstituents were analyzed. Further, the ethanolic extract samples were processed for phytoestrogenic isoflavone quantitative analysis for genistin, daidzin, and glycitin by using HPLC. Soy seed flour organoleptic properties revealed mild yellow color, mild sweet bitter taste and characteristic beany smell. Soy flour was soluble in ethanol, acetone, methanol, water, hexane and acetonitrile. The quantitative phytochemical analysis of all soy samples revealed presence of flavonoids, alkaloids, saponins, phenols, glycosides, tannins, steroids and terpenoids. The HPLC analysis of soy isoflavones for genistin, daidzin, and glycitin showed average mean values of 159.180 ug/g, 145.762 ug/g, and 28.532 ug/g, respectively. The findings thus demonstrated a significant variation in isoflavone content among soybean samples from the region, which may influence their nutritional and functional properties. The results provide a basis for further exploration of soy isoflavones in dietary formulations and highlighted the importance of selecting soybean varieties with optimal phytochemical profiles.

Keywords: Soybean, soy isoflavone, phytoestrogens, genistin, daidzin, glycitin

Introduction

Phenolic phytoconstituents, especially isoflavones, are abundantly found in legumes and are known for their diverse biological activities, including phytoestrogenic, anticancer, and antibacterial effects (Chuankhayan *et al.*, 2007) ^[1]. Among legumes, soybeans (*Glycine max*) stand out as a rich source of biologically active isoflavonoids such as daidzein, genistein, and glycitein. These compounds have been extensively studied for their antioxidant and anticancer properties (Zhu *et al.*, 2005; Sinha *et al.*, 2013) ^[2, 3]. Structurally, soy isoflavones are plant secondary metabolites that share similarity with 17 β -estradiol due to the presence of a phenolic ring within their non-steroidal framework. This structural resemblance allows them to bind to estrogen receptors (ERs), thereby exhibiting either estrogenic or anti-estrogenic activities depending on the physiological context (Dixon, 2004) ^[4]. Baird *et al.*, (1995) ^[5] suggested that a diet rich in phytoestrogens may provide protective effects against estrogen-related conditions which includes alleviation of menopausal symptoms, reduction in the risk or severity of estrogen-dependent cancers such as prostate (Denis *et al.*, 1999) ^[6] and breast cancer (Lee *et al.*, 1991) ^[7] as well as osteoporosis (Potter *et al.*, 1998) ^[8]. In contrast to these potential benefits, animal studies have revealed possible adverse effects associated with phytoestrogen exposure. For instance, soy isoflavones have been reported to negatively impact the reproductive organs in laboratory animals (Glover, 2006) ^[9]. High doses of genistein, a predominant soy isoflavone, administered to adult rodents have been shown to cross the placental barrier and cause harmful effects in offspring, affecting multiple systems including reproductive, skeletal, central nervous, and cardiovascular systems (Todaka *et al.*, 2005) ^[10].

However, despite these promising findings, the protective effects attributed to phytoestrogens are often modest (Cooke, 2006) ^[11], and current evidence is insufficient to firmly establish their role in improving bone mineral density (Liu *et al.*, 2009) ^[12].

Moreover, some critiques point out that the health benefits of phytoestrogens may be exaggerated (Jacob *et al.*, 2009) [13].

Because of its nutritional value and affordability compared to animal proteins, soy flour is widely incorporated in various foods and beverages. Due to the growing scientific interest in soy isoflavones, particularly their potential role in managing non-communicable diseases such as obesity, type 2 diabetes, hypertension, menopausal symptoms, and cardiovascular diseases, it is important to characterize their phytochemical profiles. Emerging evidence indicates that soy isoflavones may influence lipid and glucose metabolism through several molecular pathways. However, the isoflavone content in soybeans can vary considerably based on factors such as cultivar genotype, climatic conditions, and geographical origin (Siddiqui and Hakin, 1995) [14].

In light of these factors, the present study focuses on the phytochemical characterization of selected soybean flour samples, with particular emphasis on the identification and quantification of soy isoflavones known for their phytoestrogenic activity using High Performance Liquid Chromatography (HPLC).

Materials and Methods

Collection of soy samples

Ten distinct soybean seed samples were obtained from multiple locations within and around the Akola district of Maharashtra, India. The sampling was conducted irrespective of variety, crop age, or cultivation season of soybean.

Preparation of ethanolic extract from soy flour

Soybean seeds were finely ground using an electronic grinder to obtain uniform flour. For extraction, a solvent mixture comprising 80% ethanol and 20% distilled water was prepared and added to the soy flour in a ratio of 5 mL solvent per gram of flour. The resulting mixtures were subjected to continuous agitation on a rotary shaker for two hours to facilitate efficient extraction of bioactive compounds. Following extraction, the samples were centrifuged at 2000 rpm for 20 minutes to separate the solid residues. The supernatant was carefully collected and concentrated by evaporation in a hot air oven maintained at 50 °C until the volume was reduced to one-twentieth of the initial extract volume. This concentrated extract was then used for subsequent phytochemical analyses.

Preliminary evaluation

Extracts obtained from soy flour were subjected to initial sensory evaluations which include color, odor, taste, and texture. The solubility behavior of these extracts was examined in a range of solvents such as ethanol, acetone, methanol, distilled water, boiling water, and acetonitrile using established standard protocols as outlined earlier (Caldwell *et al.*, 2005) [15].

Qualitative phytochemical estimations

Phytochemical screening of ethanolic extracts of soybean samples was carried out to identify the presence of various bioactive compounds, including flavonoids, alkaloids, saponins, phenols, tannins, glycosides, steroids, terpenoids, cardenolides, phlobatannins, and carbohydrates by following established standard protocols (Harborne, 1988) [16] at the Department of Veterinary Pharmacology and Toxicology, PGIVAS, Akola.

Analysis and quantification of soy isoflavones by HPLC

The quantification of isoflavones in ethanolic extracts from collected soy samples was performed using high-performance liquid chromatography (HPLC), following the standardized procedure (Ebaid *et al.*, (2016) [17]. Standard reference compounds for genistin, daidzin, and glycitin were procured from Carbosynth Ltd. (Compton, Berkshire, RG20 6NE, UK) to facilitate the quantification of these isoflavones. Ten samples of dried soy ethanolic extracts were analyzed using high-performance liquid chromatography (HPLC) using Shimadzu UFLC system (SPD-M20A) equipped with a photodiode array (PDA) detector for precise measurement of the target compounds at the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur.

Chromatographic conditions for HPLC analysis

The chromatographic separation was conducted using a C18 reverse-phase analytical column (Spinchotech Pvt. Ltd., Enable). Mobile phases specific to the analysis of soy isoflavones were individually prepared in appropriate solvents and subjected to sonication for 20 minutes to ensure complete dissolution. A mixture of HPLC-grade acetonitrile and water in a 70:30 (v/v) ratio was selected as the mobile phase, providing effective resolution and shorter retention times for the target isoflavones *i.e.* genistin, daidzin, and glycitin. The system was operated at a constant flow rate of 1.0 mL/min and detection was performed at 260 nm, corresponding to the maximum absorbance wavelength (λ_{max}) identified for these compounds, which facilitated their accurate quantification (Table 1).

Standard solutions and sample preparation for HPLC analysis

Accurately weighed 1.0 mg each of genistin, daidzin, and glycitin standards were separately dissolved in HPLC-grade methanol and diluted to a final volume of 1.0 mL to prepare stock solutions at a concentration of 1000 µg/mL (1000 ppm). Working standard solutions were subsequently prepared by diluting the stock solutions to concentrations of 10, 50, 100, and 500 µg/mL. These solutions were stored at 20 °C until further analysis. Calibration curves for each isoflavone standard were generated by plotting the peak area obtained from HPLC analysis against the respective concentrations of the working standards. These calibration curves were employed to quantify isoflavone content in soy ethanolic extracts based on the area under the curve (AUC) of sample chromatograms.

Extract sample preparations for HPLC analysis

Solutions at a concentration of 100 ppm (0.1 mg/mL) were prepared from each of the ten dried soy ethanolic extracts using HPLC-grade methanol. These solutions were subjected to sonicator treatment for 30 minutes to ensure thorough dissolution, followed by filtration through 0.22 µm membrane filters. The resulting filtrates were then used for subsequent analysis.

Procedure for HPLC analysis

Chromatographic analysis was performed under the specified conditions. The mobile phase was allowed to equilibrate with the stationary phase until a stable baseline was achieved, indicating system equilibrium. Subsequently, 20 µL aliquots of the standard solution and soy ethanolic

extract samples were injected using a Hamilton syringe. Chromatograms for both the standard and sample injections were then recorded for further analysis.

Quantification of soy flour extracts

The calibration curve was plotted and obtained the 'Y' value for each standard solution by using area under curve (AUC) from standard peak against different working standard concentrations (ug/ml). All ten soy samples were calculated for genistin, daidzin and glycitin isoflavones from their area under curve (AUC).

Results and Discussion

Soybean is a widely utilized food crop, especially among vegans, due to its affordability and high protein content. Additionally, deoiled soybean cakes serve as a significant protein source in animal feed formulations.

Organoleptic properties

The organoleptic evaluation of soy seed flour revealed a mild yellow to brown coloration, a characteristic beany odor, and a sweet-bitter taste (Table 2). These sensory attributes are consistent with previous reports for commercial soy flours, concentrates, and isolates (Kalbrener *et al.*, 1971) [18]. Similarly, Afifah *et al.*, (2005) [19] documented mild brown coloration in soy flour, which they attributed to the presence of phenols and tannins. Studies on soy plant seed revealed that tannins get oxidized or enzymatic reaction takes place in phenols during flour making process of seed cause yellow, brown and dark coloration of seed flour (Wignoyanto and Kumalaningsih, 2013; Harrison and Dake, 2005) [20, 21] which was further confirmed by the presence of phenols and tannin during phytochemicals estimations in the present study. Furthermore, the bitter, astringent, and chalky taste of soybean flour is likely associated with unsaturated lipid degradation and the presence of carbonyl compounds (Sessa & Rackis, 1977) [22], and this might be also due to phenolic constituents present in oilseed as identified and can produce effect on amino acids, odors, flavors, and colors (Sosulski, 1979) [23].

Solubility properties

Solubility tests revealed that soy seed flour is soluble in a range of polar and non-polar solvents including ethanol, methanol, acetone, hexane, water, and acetonitrile (Table 3). These results align with previous findings by Alnokari *et al.*, (2016) [24], who used ethanol and acetonitrile to extract isoflavones such as genistin, daidzin, and glycitin. Similarly, Kanetro *et al.*, (2018) [25] reported solubility of soy flour in hexane, ethanol, and water, while Yoshiara *et al.*, (2012) [26] found mixtures of acetone, ethanol, acetonitrile, and water to be effective for glycosidic isoflavone extraction. Uwen *et al.*, (2017) [27] further confirmed the suitability of methanol and water for phytochemical extraction from soy flour.

Phytochemical Screening

Qualitative phytochemical analysis indicated the presence of multiple bioactive compounds including flavonoids, alkaloids, saponins, phenols, glycosides, tannins, steroids, and terpenoids in all the soy extract samples (Table 4). The appearance of an orange coloration in flavonoid assays and froth formation in saponin tests supported their presence. These phytoconstituents not only contribute to the

nutritional value of soy but may also influence its sensory characteristics and antioxidant potential. These observations are in agreement with Uwen *et al.*, (2017) and Lisanti & Arwin (2019) [27, 28] that analysed the phytochemical composition of methanolic and aqueous extracts of soybean seeds, confirming the presence of similar constituents. The detection of tannins in the present study supports earlier findings of Schofield *et al.*, (2001) [29], who identified tannins in ethanolic extracts of the wild soybean species *Glycine soja*. Saponins and sterols, which are bioactive compounds commonly found in numerous plant-based foods were also identified in the soy extracts. In soybeans, these compounds predominantly occur as soya saponins and their aglycone forms, known as soya sapogenols. These molecules are widely recognized as natural constituents of both human and animal feed (Mateljian, 2015) [30]. The presence of phenolic compounds is of particular interest due to their well-documented antioxidant potential. Soybeans, like many legumes, contain phenolic acids such as phytic, coumaric, caffeic, sinapic, and ferulic acids. These compounds have been associated with protective effects against oxidative stress-related conditions, including cancer, metabolic disorders like diabetes, and various inflammatory diseases (Ajay & Bhatiya, 2009) [31].

HPLC Quantification of Isoflavones

High-Performance Liquid Chromatography (HPLC) was employed to quantify the biologically active isoflavones present in ten different ethanolic soy extract samples. Calibration curves were constructed for each of the three isoflavone standards genistin, daidzin, and glycitin and are presented in Figures 1, 2, and 3. The calibration results demonstrated excellent linearity over a broad concentration range, with correlation coefficients (R^2) exceeding 0.999, indicating the reliability and accuracy of the HPLC method for quantitative analysis.

The results obtained from the soy isoflavone standards revealed the retention times for genistin, daidzin, and glycitin. The standard chromatographic peaks corresponding to these compounds, along with those from ethanolic soy extracts, are illustrated in Figures 4, 5, 6 and 7. The retention times (in minutes) for the soy isoflavone standards were as follows: daidzin-4.070 min, glycitin-4.270 min, and genistin-5.145 min, as summarized in Table 5. Quantitative analysis showed a wide range of isoflavone concentrations across samples (Table 6).

- Genistin: 62.172-447.470 µg/g (mean: 159.180 µg/g)
- Daidzin: 4.243-385.980 µg/g (mean: 145.762 µg/g)
- Glycitin: 5.047-54.037 µg/g (mean: 28.532 µg/g)

The total isoflavone content (sum of genistin, daidzin, and glycitin) varied between 59.189-744.713 µg/g, indicating significant variability, potentially due to differences in seed origin, environmental factors, and processing conditions. In one of the previous studies, extraction of crushed soy with 80% ethanol and 80% acetonitrile revealed the presence of glycones between 539.9-661.14 µg/g for genistin, 298.72-390.8 µg/g for daidzin and 78.1-130.08 µg/g for glycitin by HPLC analysis (Alnokari *et al.*, 2016) [24]. Their observed concentration ranges for genistin, daidzin, and glycitin fall within similar limits to those found in the present study. This consistency across studies reinforces the reliability of ethanol based extraction as an efficient method for isolating soy isoflavones in their glycosidic form. The current study

investigated the quantification of major glycosidic isoflavones genistin, daidzin, and glycitin in soybean flour extracts using 80% ethanol as the solvent. The findings indicated that genistin was present in the highest concentration among the three isoflavones, followed by daidzin and glycitin. These results are consistent with previous studies, suggesting that glycosidic isoflavones are the predominant forms in non-fermented soy products, whereas aglycones are more prevalent in fermented products due to enzymatic hydrolysis as reported by Puri and Panda (2015) [32]. The dominance of genistin in the extract reflects not only its natural abundance in soybeans but also the solvent's effectiveness in selectively extracting polar compounds. The low concentration of aglycones observed prior to hydrolysis further supports existing knowledge that glycosidic forms are more soluble in aqueous ethanol and represent the major storage forms in raw soybeans. Environmental and agronomic factors appear to have a significant influence on isoflavone content. Choi *et al.*, (1996 and Lozovaya *et al.*, (2005) [33, 34] suggested that elevated temperatures can negatively impact isoflavone synthesis, while soil moisture, genotype, and cultivation practices all contribute to natural variability in isoflavone levels. The fluctuations in isoflavone concentration between samples may be attributed to differences in irrigation levels during soybean cultivation. Previous research has demonstrated that controlled irrigation can enhance isoflavone yields, especially when compared to plants grown under rain-fed conditions as suggested by Al-Tawaha *et al.*, (2007) [35]. Furthermore, the influence of genotype and seasonal variation, as discussed by Murphy *et al.*,

(2009) [36], may also play a critical role in determining the final isoflavone profile of soybean seeds. These complex interactions between genetic and environmental factors highlight the importance of standardized cultivation and processing practices to ensure consistent bioactive compound content in soy-based nutraceuticals.

In summary, the current study confirms that 80% ethanolic extraction followed by HPLC analysis is a reliable approach for quantifying glycosidic isoflavones in soy flour. The findings emphasize the need for careful consideration of environmental and agronomic conditions to maximize isoflavone yield, especially for industrial applications where consistency and potency are critical.

Table 1: Chromatographic conditions for HPLC analysis of soy ethanolic extract

Particulars	Remarks
HPLC with PDA Detector:	UFLC Shimadzu.
Serial No. of HPLC:	L2024491148.
Column used:	Princeton C18 250 X 4.5 mm 5 μ .
Software of HPLC:	LCSolution.
Mobile Phase (Genistin, Daidzin and Glycitin):	Acetonitrile: water (70:30).
Flow Rate used:	1 ml/min.
Volume of sample injection:	20 μ l.
Temperature:	Ambient/Room Temperature
Pressure used:	10.2-12.3psi.
Wavelength detection of genistin:	260 nm.
Wavelength detection of daidzin:	260 nm.
Wavelength detection of glycitin:	260 nm.

Table 2: Organoleptic properties of soy seed

Sample	Color	Odour	Taste	Texture
Soy seed flour	Mild yellow to brown	Characteristic beany smell	Mild sweet and bitter	Fine powder

Table 3: Solubility of soy seed

Sample	Ethanol	Acetone	Methanol	Water	Boiling water	Hexane	Acetonitrile
Soy seed flour	+	+	+	+	+	+	+
Note: (+) Soluble and (-) Insoluble							

Table 4: Phytochemical components present in soy ethanolic extract

Sr. No	Phytochemical constituents	Name of the test	Result
1	Flavonoids	Shinoda test	+
2	Alkaloids	Mayer's test	+
3	Saponins	Foam test	+
4	Phenols	Ferric chloride test	+
5	Tannins	Ferric chloride test	+
6	Glycosides	Legal's test	+
7	Steroids and Terpenoids	Liebermann-Burchard test	+
8	Cardenolides	-----	-
9	Phlobatannins	Precipitate test	-
10	Carbohydrates	Molish's test	-

Note: (+): Present and (-): Absent

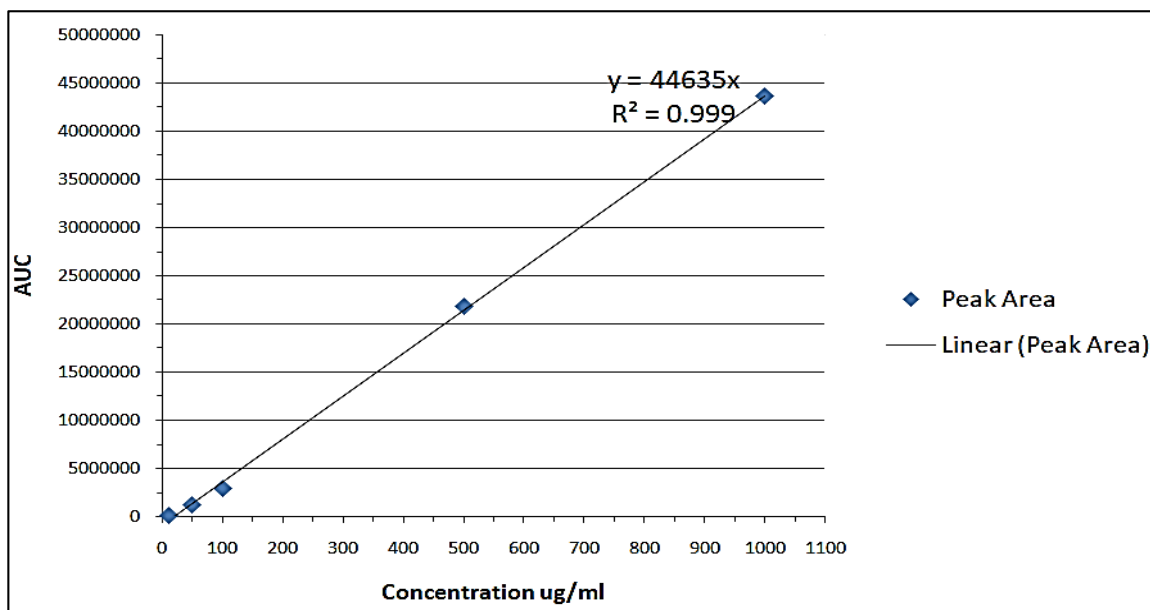
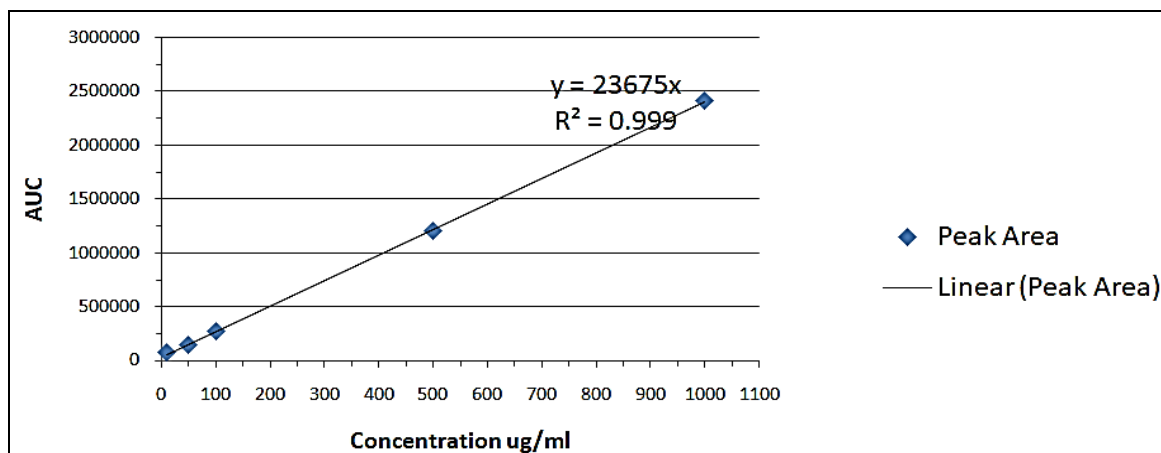
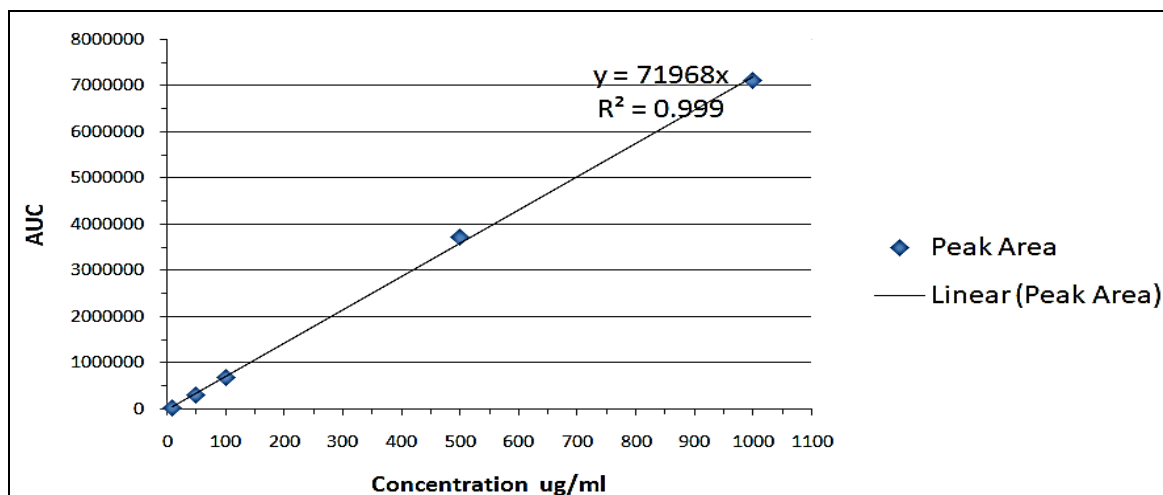
Table 5: Retention time values for soy isoflavone standards

Soy isoflavone	Daidzin	Glycitin	Genistin
Rt(min)	4.079	4.270	5.145

Table 6: Quantification of isoflavones present in soy flour ethanolic extract

Name of isoflavone	Average	Range quantified in ethanolic extract (ng/g)
Genistin	159.180 ± 47.47	62.172-447.472
Daidzin	145.762 ± 43.09	4.243-385.981
Glycitin	28.532 ± 6.63	5.047-54.037
Total isoflavones	333.474 ± 83.799	59.189-744.713

$n = 10$; mean $\pm$ se

**Fig 1:** Soy genistin standard calibration curve**Fig 2:** Soy Daidzin standard calibration curve**Fig 3:** Soy Glycitin standard calibration curve

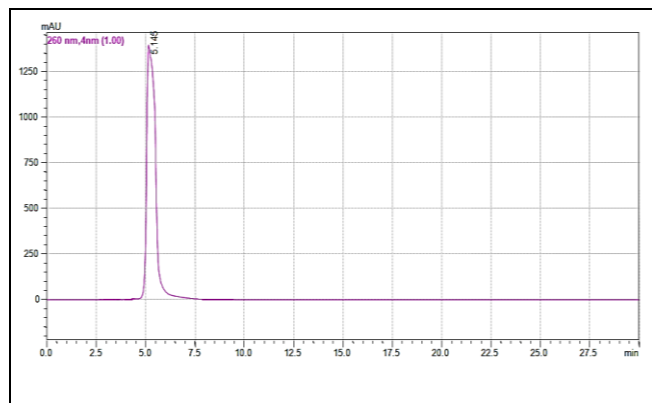


Fig 4: HPLC chromatogram of genistin standard peak by using a solvent system of acetonitrile and water (70:30)

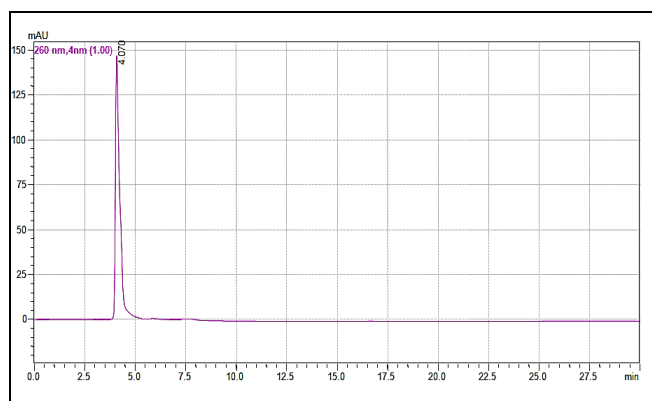


Fig 5: HPLC chromatogram of daidzin standard peak by using a solvent system of acetonitrile and water (70:30)

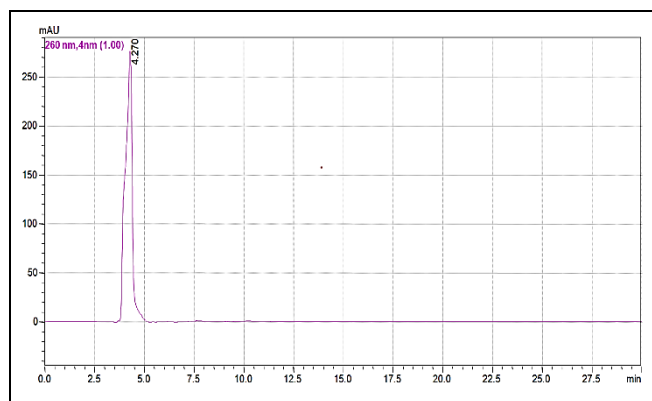


Fig 6: HPLC chromatogram of glycitin standard peak by using a solvent system of acetonitrile and water (70:30)

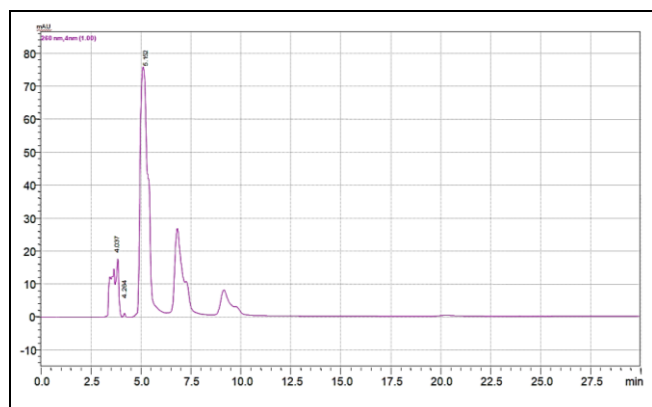


Fig 7: HPLC chromatogram of soy flour ethanolic extract peak by using solvent system of acetonitrile and water (70:30).

Conclusion

Thus, from the observations, it is concluded that Soy seed flour is yellow to brown, sweet, bitter in taste and characteristic beany smell having solubility in ethanol, acetone, methanol, water, hexane and acetonitrile. The qualitative phytoconstituent examination revealed the presence of flavonoids, alkaloids, saponins, phenols, glycosides, tannins, steroids and terpenoids. The phytoestrogen genistin, daidzin and glycitin showed wide variation in their quantification.

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Conflict of interests

The authors declared no conflict of interest.

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