

Research Article

Phytoestrogen “Genistein”: Its Extraction and Isolation from Soybean Seeds

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ABSTRACT

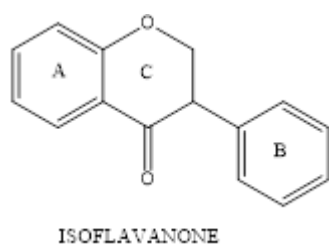
Phytoestrogens, structurally or functionally mimic of mammalian estrogens, are phenolic non-steroidal plant derived compounds. On the basis of their hormonal activity, they can bind to estrogen receptors and show potential benefits for human health. There are three main classes of phytoestrogens - isoflavones, lignans and coumestans out of which Isoflavones are most studied group of phytoestrogens and predominantly found in legumes such as soybeans. The soy isoflavones, genistein have most potent estrogenic activity. Estrogen level begin to decline with a woman's age and resulting in the end of menstrual cycle which results in menopausal symptoms, such as hot flashes, urogenital atrophy, incontinence, insomnia, heart problems and osteoporosis. Depending on their concentration and other factors, genistein can act like weak estrogens by binding to the estrogen receptor on cell membranes and as estrogen antagonists by preventing estrogens from binding to the receptors. Genistein come into focus of interest due to their positive effects in prevention of hormone dependent cancer, cardiovascular diseases by improving plasma lipid concentrations, osteoporosis and cognitive decline. In this study, our main focus is on the extraction and isolation of isoflavones-genistein from soybean seeds and optimization of extraction parameters to give a high yield of genistein. HPLC analysis revealed that the product thus obtained contained a high content of genistein.

Keywords:-Phytoestrogen, Estrogen receptor, Genistein, Extraction

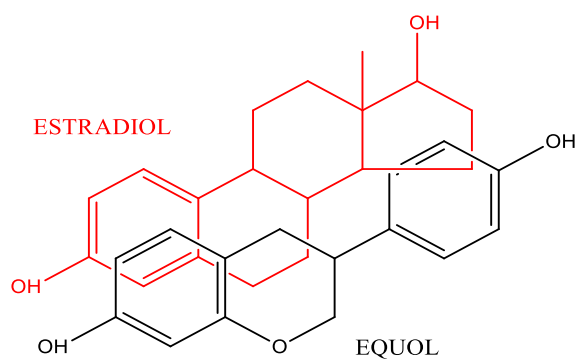
INTRODUCTION

Estrogen, a class of steroidal hormones are produced in mammals and responsible for many physiological activities that are important in homeostatic regulation of many cellular and biochemical events. In humans, the three most important estrogenic hormones are estradiol, estrone and estriol in which 17 β -estradiol is the most active. These are important for sexual and reproductive development, mainly in women. It is involved in development of mammary glands and the uterus, in maintaining pregnancy and bone density, in reducing the risk of cardiovascular disease and helps in relieving menopausal symptoms. Estrogen level begin to decline with a woman's age and resulting very low at the end of menstrual cycle. This shows menopausal symptoms such as hot flashes, Urinogenital atrophy, incontinence, insomnia, heart problems and a decrease in bone density which may lead to osteoporosis. Both deficiencies and excess of estrogen are associated with many health issues. Administration of estrogen (ERT) to post menopausal women is beneficial in reducing the risks associated with estrogen deficiency. Unfortunately this form of therapy is associated with health risks such as the development of estrogen-dependent tumors (breast and uterus cancer). Phytoestrogens are used as an alternative to the ERT because of the similarity in chemical structures with estradiol (Vaya & Tamir, 2004)¹. Many natural products are used in medicine have been accepted as important

source of cancer chemoprevention drug discovery and development (Mathewa et al, 2010.)². Plants occupy a good place for the treatment of many forms of cancer with no ill effect. The medicinal plants provide active chemopreventive molecules and their products have antioxidant activity helps in protecting cells from damage (Bachrach, 2012; Bhanot et al, 2011)^{3,4}. Legumes are major source of proteins and calories and in addition, these are excellent source of nutraceuticals constitutes such as fibre, polyphenols such as flavonoids, isoflavones and tannins. Phytoestrogens widely present in legumes such as soybeans that contain non-hormonal properties which may result in anticancer effects. Soybean (*Glycine max*) is a leguminous plant belongs to the fabaceae family. It is the most widely grown and utilised legume in the world. It has the high level of protein, essential fatty acids, vitamins and minerals, therefore constitute a useful source of food. It is reported as herbal for healthy functioning of heart, kidney, liver and stomach. Isoflavones are one of the major groups of phytoestrogens found in the human diet and legume is a good source of isoflavones and lignans, molecules with antioxidant properties that, among other effects, might help to fight and prevent several pathologies (Cederroth & Nef, 2009)⁵. Isoflavones are a class of plant produced steroidal compounds that play important role in prevention of menopausal symptoms, cancers, heart disease and osteoporosis (Vaya & Tamir, 2004). Isoflavone is also found naturally in the form of glucoside



Structure 1



Structure 2

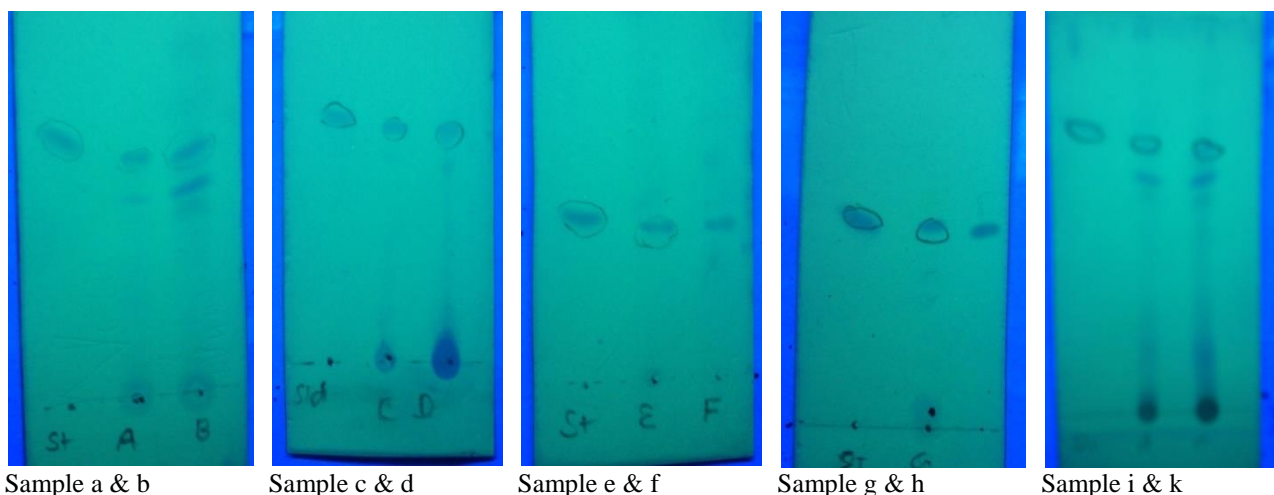


Figure: 1-TLCs of samples

and aglycone. There are 12 isomers of isoflavones have been found in soybeans in 4 different isoforms, aglycones (genistein, daidzein) and three glucoside conjugates, the acetyl- β - glucoside, the malonyl- β - glucoside and the β - glucoside (Xu et al, 2000; Luthria et al, 2007)^{6,7}. Genistein is a primary active component of soybean, is a major subject of discussion in the context of nutraceuticals and functional foods. It is mainly occur in glycosidic conjugate form and is rapidly converted into unconjugated genistein by intestinal bacteria. The genistein was originally identified as having a close similarity in structure to 17 β -estradiol, particularly the phenolic ring and the distance between its 4'- and 7- hydroxyl groups (Heisig, 2009; Phetnoo et al, 2013)^{8,9}. Genistein have estrogenic effects because they interact with estrogen receptors that are present in most tissues of the human body. (Setnikar; Gruber et al. 2002)^{10,11}. The affinity of genistein is comparable to that of estradiol. The affinity of other isoflavones is 100-500 times lower than that of estradiol (Pilsakova et al, 2010)¹². It has been reported that genistein is a potent inhibitor of cell proliferation, oncogenesis and clonogenic ability in animal and human cells (Fotsis et al, 1995; Barnes, 1995)^{13,14}.

RESULTS AND DISCUSSION

Extraction

A number of physical and chemical methods have been attempted, and the determined strategy for extracting and

purifying isoflavones, generally encompasses three steps: (1) extracting isoflavones from soybean flour with an organic solvents, methanol, 70% methanol, ethanol, 70% ethanol and isopropyl alcohol. These solvents were used for the extraction of isoflavones from soybean seeds on the basis of solubility of isoflavones in these five solvents. (2) The glucoside fractions of the extract hydrolysed into aglycones using acid (hydrochloric acid). It helps in increasing the concentration of aglycone form of isoflavones by converting the isoflavone glucosides (genistin) into aglycones (genistein). (3) Then by the addition of water to the hydrolyzed product, the isoflavones are crystallized from the solution. The solubility of Isoflavones aglycones is much lower in water than the methanol, 70% methanol, ethanol, 70% ethanol and isopropyl alcohol. Thus, water act as an antisolvent for the isoflavones which help in their crystallization from the alcoholic solution.

Identification of Genistein in Soybean seeds extract-(TLC)
TLC was carried out to separate and identify genistein from alcoholic extraction. The developing solution was Chloroform /methanol (10: 1,v/v) and Rf value for standard genistein was 0.50.

HPLC Analysis

HPLC chromatogram of genistein in our samples obtained under the different extraction conditions. The retention time of standard genistein was found to be 3.5 minutes.

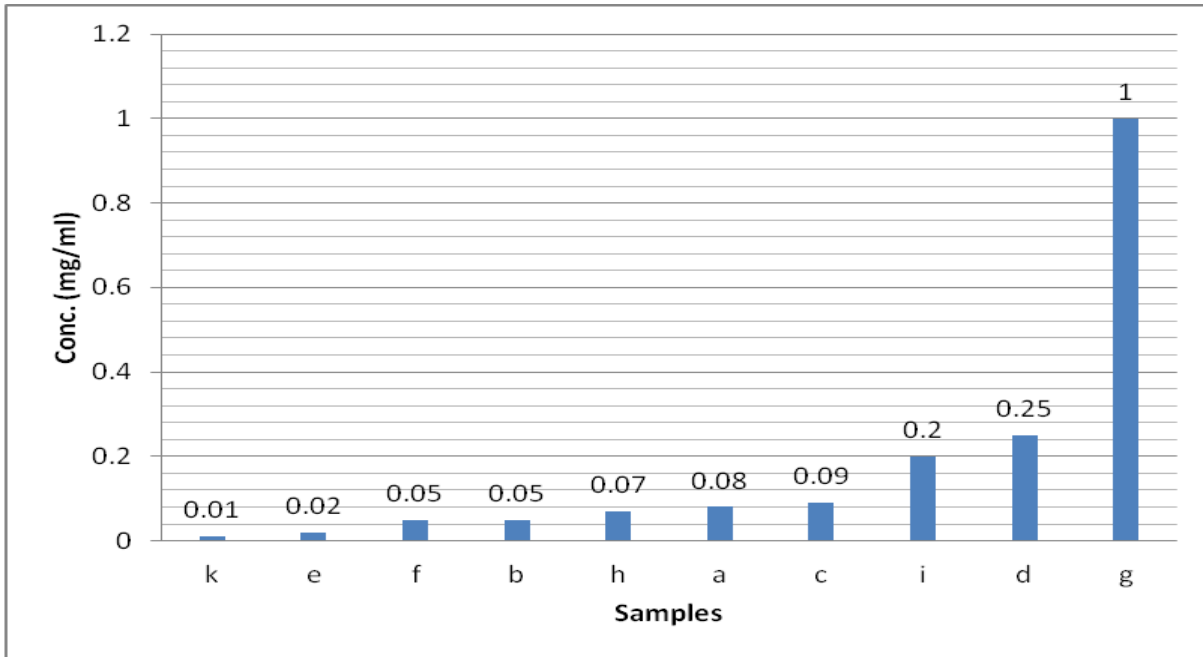


Figure: 2- Graph for concentration of genistein in different samples

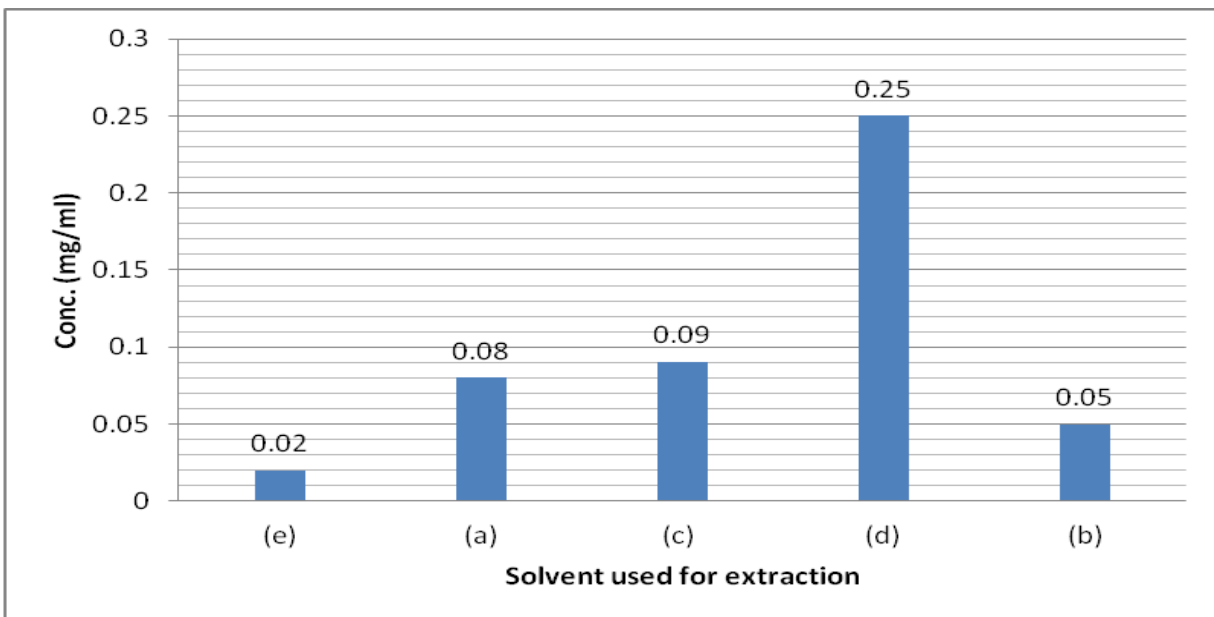


Figure: 3- Graph for concentration of genistein in samples extracted with different solvents

And the peak corresponding to genistein was eluted from the column at retention time of 3.5 minutes in our samples.

Optimization of solvents

Soybean seeds were extracted by using different solvents such as: **Sample a**: Solvent: methanol, Temperature: 60°C, Time: 4hr; **Sample b**: Solvent: 70% methanol, Temperature: 60°C, Time: 16hr.; **Sample c**: Solvent: Ethanol, Temperature: 75°C, Time: 4hr; **Sample d**: Solvent: 70% ethanol, Temperature: 75°C, Time: 4hr; **Sample e**: Solvent: Isopropyl alcohol, Temperature: 75°C, Time: 8hr. Among the solvents used, 70% ethanol extracted much higher amount of genistein than pure ethanol. And the 70% ethanol was determined as optimum solvent for the extraction of soybean seeds. The highest

concentration of genistein among the different solvent extraction was found in extraction with 70% ethanol at 75°C for 4hr, (d) i.e. 0.25mg/ml.

Optimization of time

To reduce the cost, a relatively cheaper solvent, 70% ethanol, was used. Soybean seeds were extracted by using 70% ethanol for different time of periods such as **Sample d**: Solvent: 70% ethanol, Temperature: 75°C, Time: 4hr; **Sample f**: Solvent: 70% ethanol, Temperature: 75°C, Time: 8hr; **Sample g**: Solvent: 70% ethanol, Temperature: 75°C, Time: 12hr; **Sample i**: Solvent: 70% ethanol, Temperature: 75°C, Time: 24hr. The yield of total isoflavones increased with increasing extraction time. Insufficient extraction duration yielded less isoflavones,

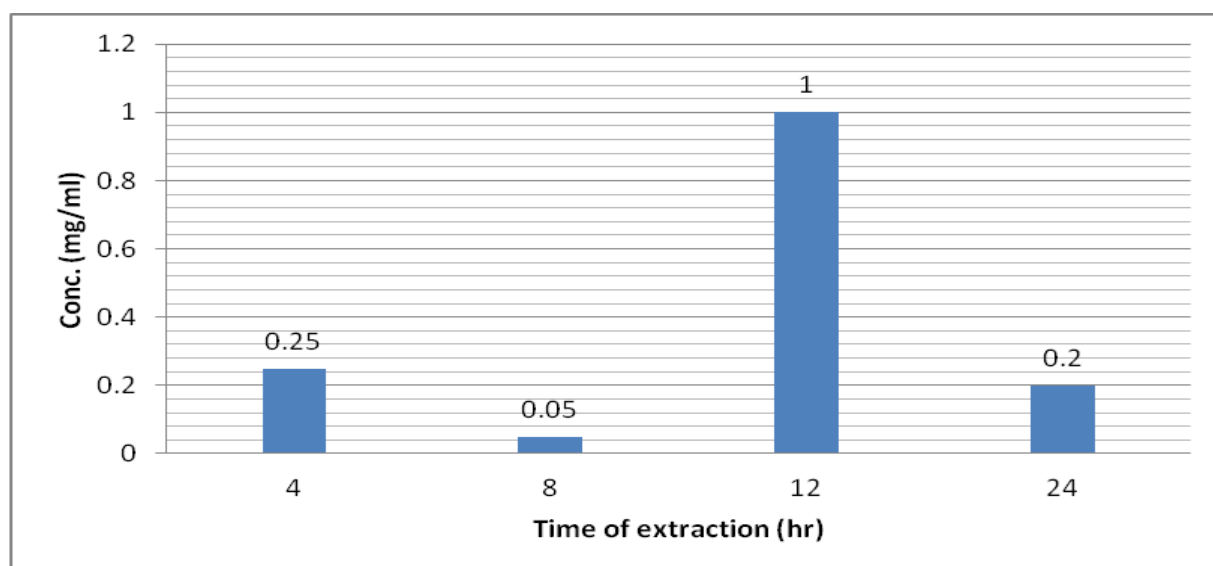


Figure:4- Graph for concentration of genistein in samples extracted with 70% ethanol for different durations.

Table 1: Concentration of genistein in different samples

Sample	Concentration (mg/ml)	Sample	Concentration (mg/ml)
a	0.08	f	0.05
b	0.05	g	1.0
c	0.09	h	0.07
d	0.25	i	0.20
e	0.02	k	0.01

and the yield reached a maximum after 7-8 h of extraction. Furthermore, a higher extraction time was preferred for maximizing the yield of isoflavone (genistein). And the extraction time of 12 hr was determined as optimum time for the extraction of soybean seeds. The highest concentration of genistein among the extraction of different time of periods was found in extraction with 70% ethanol at 75°C for 12hr, (g) i.e. 1.0mg/ml.

After germination

The germinated soybean seeds were extracted with 70% for 8 hr at 75°C (h) and this yield 0.07mg/ml of genistein. The yield obtained by this process is very low.

Maceration method

The soybean seeds were extracted with 70% ethanol for 24 hr at room temperature (k) by using maceration method and this yield 0.01mg/ml of genistein. The yield obtained by this process is very low.

Five solvents, methanol, 70% methanol, ethanol, 70% ethanol and isopropyl alcohol were chosen for the extraction. The highest concentration among the different solvent extraction was found in extraction with 70% ethanol at 75°C for 4hr, (d) i.e. 0.25mg/ml. The highest concentration among the extraction of different periods of time was found in extraction with 70% ethanol at 75°C for 12hr, (g) i.e. 1.0mg/ml.

CONCLUSION

Soybean are excellent source with a low content in saturated fat and a great amount of dietary fibre are

associated with a potential role in the prevention and treatment of different diseases. Isoflavones are natural phenolic compounds. The structure similarity to estrogens allows these compounds to bind to estrogen receptors. It is beneficial to treat many disease related to estrogen deficiency in females. This work includes the extraction of isoflavones and isolation of genistein to check the yield, purity to produce the valuable compound in a economic cost. Isoflavones were extracted and purified from soybean seeds using the different parameters followed by hydrolysis and crystallization to convert the isoflavone glycoside into aglycone. Crystallization and filtration are low cost steps which we used in extraction help in maximizing the yield and minimizing the production cost.

MATERIALS AND METHODS

Materials

Dry soybean (Family *Fabaceae* or *Leguminosae*) seeds were purchased from Shiv Flour Mill, Saket, New Delhi and authenticated by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi Ref. No. NISCAIR/RHMD/Consult/2014/2403-183. Standard Genistein was purchased from Sigma-Aldrich. HPLC grade methanol and water were purchased from Fisher Scientific. HPLC analysis was determined by SHIMADZU LAB SOLUTIONS.

Extraction

Sample preparation

The dry soybean seeds (50gm) were ground into powder using a mortar and pestle and extracted by using soxhlet apparatus. The powdered soybean (50gm) was packed and transferred to a thimble. The thimble was dropped into the extraction tube of soxhlet apparatus. Approximately 250 ml of solvent was poured through the sample in the tube into the flask. The bottom of the extraction tube was attached to a soxhlet flask and the top of the extraction tube was attached to the condenser.

Procedure

Isoflavones are extracted from soybean seeds using five different solvents (i.e. methanol, 70% methanol, ethanol,

70% ethanol and isopropyl alcohol) for different time of periods. The extract was separated from insoluble fractions by vacuum filtration through whatman paper. The filtrate was hydrolysed with 37% HCl and the pH is adjusted to 1-5. Then the mixture was heated and stirred constantly for different periods of time on magnetic stirrer. The hydrolysed product was mixed with distilled water at the volume ratio of 1:1 (ml/ml) by stirring constantly at room temperature. Then by vacuum filtration the precipitated crystals of isoflavones were separated. The solids retained on the membrane were dried and dissolved in pure methanol. The solution was stored at 4°C until analysis for genistein.

Optimization of solvent

Sample a: Solvent: methanol, Temperature: 60°C, Time: 4hr

Sample b: Solvent: 70% methanol, Temperature: 60°C, Time: 16hr

Sample c: Solvent: ethanol, Temperature: 75°C, Time: 4hr

Sample d: Solvent: 70% ethanol, Temperature: 75°C, Time: 4hr

Sample e: Solvent: Isopropyl alcohol, Temperature: 75°C, Time: 8hr

Optimization of time

Sample d: Solvent: 70% ethanol, Temperature: 75°C, Time: 4hr

Sample f: Solvent: 70% ethanol, Temperature: 75°C, Time: 8hr

Sample g: Solvent: 70% ethanol, Temperature: 75°C, Time: 12hr

Sample i: Solvent: 70% ethanol, Temperature: 75°C, Time: 24hr

After germination

Sample h: Solvent: 70% ethanol, Temperature: 75°C, Time: 8hr

Maceration method

Sample k: 70% ethanol, Temperature: RT, Time: 24hr

Identification of Genistein in Soyabean seeds extract-Thin layer chromatography

For detection of isoflavones, Thin layer chromatography (TLC) was developed by using Chloroform/methanol (10:1,v/v) as a solvent system. TLC is a simple and inexpensive procedure used to give a satisfactory separation of components in a mixture on TLC plates. And the sample to be analysed and the sample of standard genistein were spotted near the bottom of the TLC plate and placed inside the TLC chamber. The mobile phase was allowed to rise up the TLC plate by capillary action. TLC plates were dried and visualized as fluorescent spots under UV light (255 nm). The spots were marked and the R_f values were calculated. R_f value of compound is compared with the R_f value of standard genistein.

Purification of Genistein - Column Chromatography

The extracted compound was purified with the help of column chromatography on the basis of TLC. The compound was applied to silica gel column and eluted successively with the chloroform : methanol (10:1v/v) solvent system. Elutes were evaporated under reduced pressure and dissolved in pure methanol and stored at 4°C until analysis for genistein.

HPLC Analysis

Genistein in samples was analysed on C₁₈ column using (B) methanol : (A) water (80:20) as mobile phase at a flow rate of 1.0mL/min. The sample injection volume was 10µL and the temperature of the column was maintained at 40°C. The detection wavelength was set at 260nm, where absorbance peak areas were quantified. The identification of genistein was made by comparing the retention time with those of pure standards. Preliminary peak identification was based on a comparison of retention times of standard genistein and unknown peaks in the sample extracts.

Calibration Curve for Genistein

A result from a HPLC-analyzed sample is presented in area (i.e., the area under the relevant peak in the chromatogram). A calibration curve is necessary to transform the HPLC results from area to concentration. By analyzing samples with known concentrations and getting the results in area, a calibration curve can be made by plotting area vs. concentration. The trend line for the points makes the conversion factor between area and concentration. Calibration standards are also needed to make sure from one experiment to another that the results are comparable. This is confirmed by the results as the calibration standards were consistent over time.

Stock solution of genistein was prepared by dissolving 1mg of genistein into 1ml of methanol. And the five resulting genistein concentrations were 0.1, 0.2, 0.4, 0.5 and 1mg. A five point calibration curve was made by analyzing the five samples on the HPLC at 260 nm.

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