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Germination Dramatically Increases Isoflavonoid Content and Diversity in Chickpea (*Cicer arietinum* L.) Seeds

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ABSTRACT: The effect of germination on bioactive components in legume seeds was investigated in terms of the antioxidant capacity and total phenolic contents. Germination increased the total phenolic content and antioxidant capacity of most seeds. Particularly in chickpea seeds, the isoflavone contents increased by over 100 fold, mainly due to the increase of formononetin and biochanin A level. As a result, these two compounds were conveniently isolated from the germinated seeds in preparative scale and structurally confirmed by UV−vis, ESI-MS, and ^{1}H NMR spectroscopies. Isoflavonoid fingerprints analyzed by HPLC-PDA and LC-ESI-MS demonstrated that germination could significantly increase isoflavonoids diversity. Twenty-five isoflavonoids were detected and identified tentatively. These include 20 isoflavones, 2 isoflavanones, and 3 pterocarpan phytoalexins. Total isoflavonoid content of germinated chickpea was approximately 5-fold of that of germinated soybean. Our findings suggest that the germinated chickpea seeds could serve as a promising functional food rich in isoflavonoids.

KEYWORDS: *chickpea, germination, isoflavones, pterocarpan phytoalexins, antioxidant capacity*

INTRODUCTION

Legumes have high nutritional value and play an important role in traditional diets throughout the world. Consumption of legumes has been linked to reduced risk of diabetes and obesity, coronary heart disease, colon cancer, prostate cancer, and gastrointestinal disorders.[1] Legume seed sprouts are also popular globally. During germination, some components of seed are degraded and used for respiration and synthesis of new cell constituents for plant development, thus causing significant changes in the biochemical characteristics.[2] Numerous investigations have shown that germination is an inexpensive and effective way to improve the nutritional quality of legumes as it increases amino acids content, total dietary fibers, and total soluble sugars while reducing antinutrient levels such as α-galactosides.[2−4] Germination is also a convenient natural process to enrich polyphenolic contents and related antioxidant activity.[5−8]

Chickpea (*Cicer arietinum* L.) is an important grain legume crop in the world. Due to the high protein content (25.3−28.9% after dehulling), they are consumed as a meat substitute, particularly by vegetarians in developing countries.[9] Studies of germinated chickpea seeds are mostly focused on protein content, amino acid composition, polysaccharides, and mineral composition.[4,5,9,10] Isoflavonoids have been reported as the main bioactive components of chickpea plant. The major compounds in chickpea seed are formononetin (4′-*O*-methyl ether of daidzein), biochanin A (4′-*O*-methyl ether of genistein), ononin (formononetin glucoside), and sissotrin (biochanin A glucoside) (Figure 1).[11] In a study on sprouted chickpea seeds, seven isoflavonoids were isolated and identified, i.e., biochanin A, calycosin, formononetin, genistein, trifolirhizin, ononin, and sissotrin.[12]

Isoflavonoids are a large group of plant secondary metabolites; they play important roles in plant defense as antimicrobial phytoalexins. Over the past decades, isoflavonoids have received considerable attention for their diverse biological activities, including antiestrogenic, anticancer, antioxidative, antimicrobial, anticardiovascular diseases, and antiosteoporosis properties.[13] To date, there are more than 1600 known isoflavonoids, and the subclass of isoflavonoid includes isoflavan, isoflav-3-ene, isoflavone, isoflavanone, isoflavanquinone, pterocarpan, pterocarpene, rotenoid, etc.[14,15] Interestingly, the majority of isoflavonoids are isolated from Faboideae, a subfamily of the flowering plant family Leguminosae, and are synthesized through the central phenylpropanoid pathway and the specific isoflavonoid branch pathways in legumes.[16,17]

Our previous work has shown that germination of black soybean results in an increase of total isoflavone and phenolic contents, and food-grade microbial-stressed germination of soybeans leads to generation of a few glyceollin isomers, a type of isoflavonoid (prenylated pterocarpan) phytoalexins.[7,18] We have shown that germination of peanut seeds, with or without

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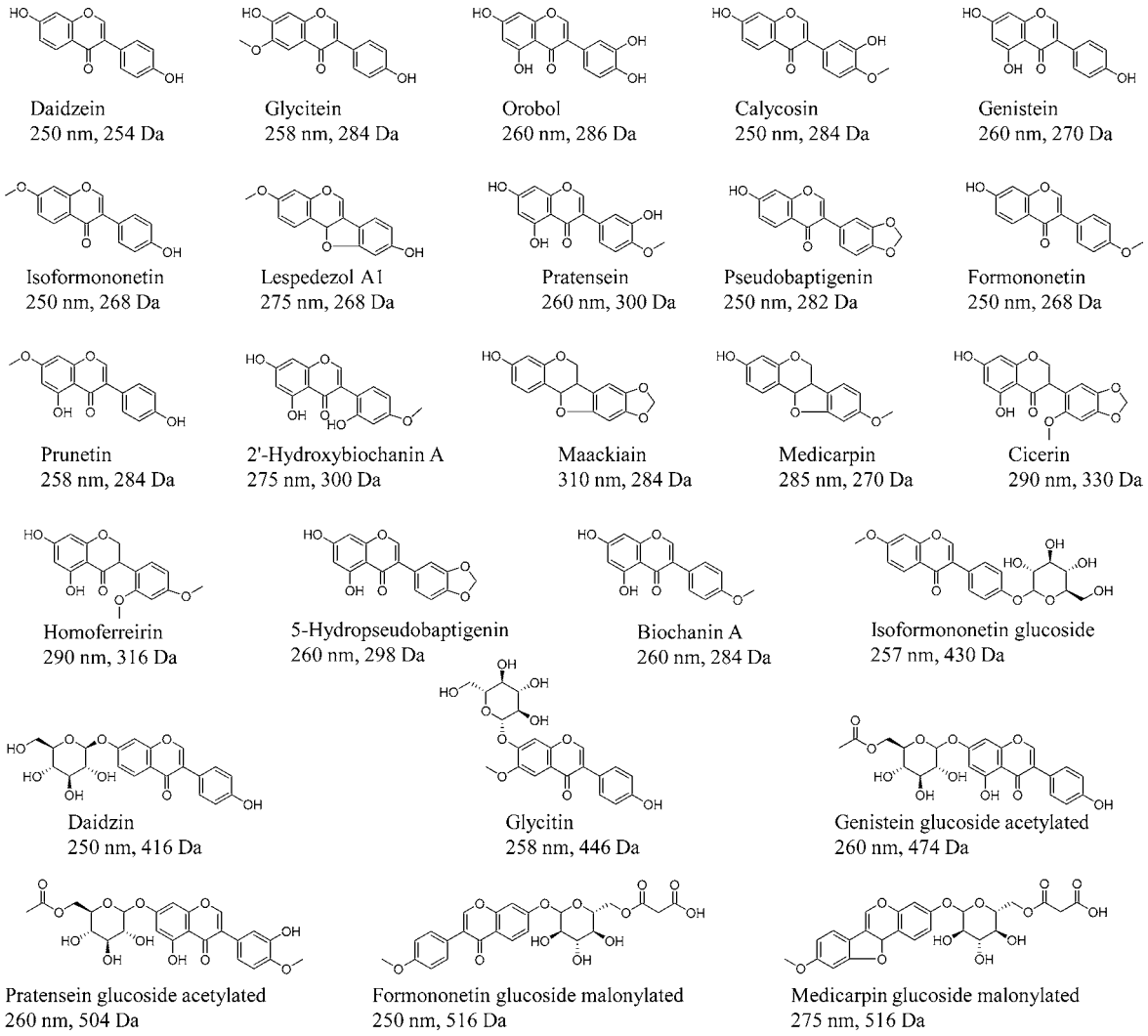

Figure 1. Structures, maximum UV absorptions, and molecular weights of major isoflavonoid aglycones and a few representatives of their glycoside conjugates identified from germinated chickpea and soybean in this study.

fungal stress, can enhance polyphenolic antioxidants and induce a number of phytoalexins (resveratrol derivatives).[8] We extended the germination treatment on nine legume seeds to investigate the change of antioxidant capacity and isoflavonoid content before and after germination. Specifically, the isoflavonoid profiles of chickpea were characterized.

MATERIALS AND METHODS

Materials. Chickpea (*Cicer arietinum* L., Turkey), black soybean (*Glycine max* (L.) Merr., China), hyacinth bean (*Lablab purpureus* (L.) Sweet, India), azuki bean (*Vigna angulariz* (Willd.) Ohwi & H. Ohashi, China), mung bean (*Vigna radiata* (L.) R. Wilczek, Thailand), kidney bean (*Phaseolus vulgaris* L., China), black-eyed pea (*Vigna unguiculata* subsp. *unguiculata*, Myanmar), yardlong bean/cowpea (*Vigna unguiculata* subsp. *sesquipedalis*, China), and sword bean (*Canavalia gladiata* (Jacq.) DC., Indonesia) were obtained from a supermarket in Singapore, and the origins of the product were obtained from Singapore Trade Statistics.

Reagents. Trolox (97%), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) fluorescein, Folin-Ciocalteau reagent (FCR), and gallic acid were purchased from Sigma Aldrich Chemical Co. (Singapore). Daidzein (99%), genistein (99%), and glycitein (99%) were purchased from Tauto Biotech (Shanghai, China). The 96-well polystyrene microplates with a flat bottom were purchased from Fisher Scientific (Nunc, Rochester, NY, USA). Acetone, acetonitrile, *n*-hexane, methanol, and other solvents were of spectroscopic grade or HPLC grade from commercial sources.

Instruments. A Synergy HT microplate reader (Biotek, Winooski, VT, USA) was used in antioxidant capacity assays. HPLC analysis was carried out on a Waters HPLC system (Milford, MA, USA) with an Alliance 2659 separation module and a 2996 photodiode array (PDA) detector, and the column used was a 250 mm × 4.6 mm i.d., 5 μm, Atlantis T3 C_{18} with a 20 mm × 4.6 mm i.d. guard column of the same materials (Waters, Ireland). LC-MS spectra were acquired using a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a TSP 4000 HPLC system and an electrospray ionization (ESI) source, which consisted of a P4000 quaternary pump, a UV6000LP PDA detector, and an AS3000 autosampler. The capillary temperature and spray voltage were maintained at 250 °C and 4.5 kV, respectively. ^{1}H NMR spectra were recorded in CD_3OD with a Bruker AC300 spectrometer (Karlsruhe, Germany) operating at 300 MHz.

Seed Germination. Germination of the nine legume seeds was carried out according to the method described previously.[8,18] In brief, seeds were surface sterilized with 70% ethanol for 3 min and then rinsed with water before they were soaked for 24 h at room temperature (25 °C). The seeds were placed on sterile plastic Petri dishes (90 mm × 13 mm) lined with two autoclaved filter papers moistened with 5 mL of sterile water. The Petri dishes were sealed with parafilm and incubated for 4 days at 25 °C in the dark. Two milliliters of sterile water was added into the Petri dishes each day. Four replicates were conducted for each sample.

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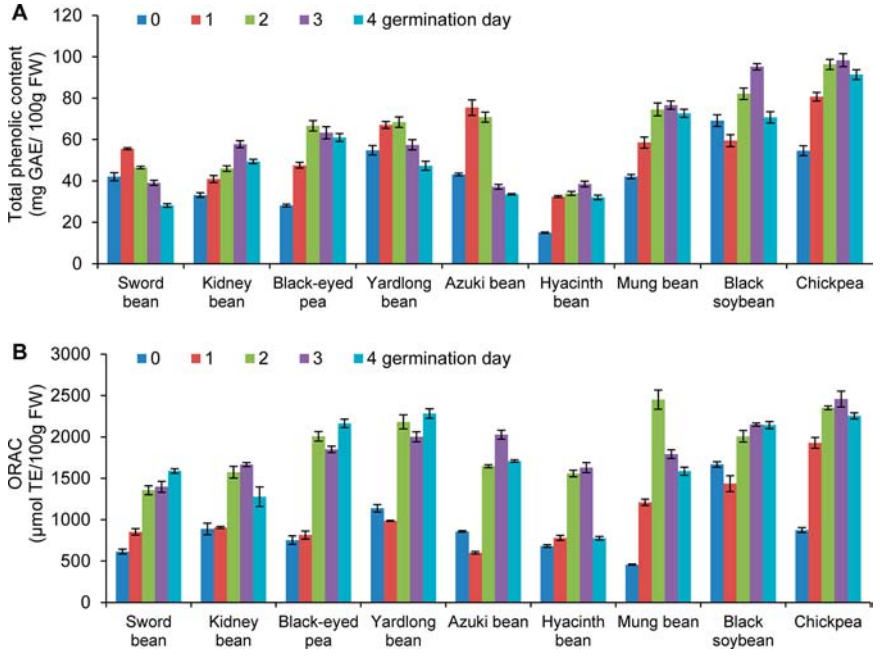

Figure 2. Total phenolic content and oxygen radical absorbing capacity (ORAC) values of ungerminated seed (0 day) and germinated seed (1−4 days) in nine legumes. Total phenolic content expressed as milligrams of gallic acid equivalents per 100 g of fresh weight basis (mg GAE/100 g FW); ORAC value expressed as micromoles of Trolox equivalents per 100 g of fresh weight basis (μmol TE/100 g FW); data is presented as mean, $n = 4$.

Sample Preparation Procedures. From 2 to 10 germinated seeds of each sample were collected from day 1 to day 4 , weighed, homogenized in 5.0 mL of acetone/ethanol/water (2:2:1; v/v) containing 0.1% acetic acid in a 15 mL screw-cap tube, and placed on a shaking incubator at 200 rpm and room temperature for 12 h. The mixture was centrifuged at 4000*g* for 10 min. The supernatant was collected and stored at −20 °C before analysis of antioxidant capacity and total phenolic contents. The supernatant was filtered through a Sartorius Minisart polytetrafluoroethylene (PTFE) membrane (0.45 μm) before phytochemical HPLC and LC-ESI-MS analysis.

Quantification of Antioxidant Capacity and Total Phenolic Contents. Assays for hydrophilic antioxidant capacity were carried out using the automated oxygen radical absorbing capacity (ORAC) procedure based on previous reports.[19] AAPH was used as the peroxyl generator and Trolox as the antioxidant standard with concentration ranging from 100 to 6.25 μM. Fluorescein solution (160 μL) (9.57×10^{-5} mM), 20 μL of AAPH (81 mM), and 20 μL of sample were mixed in each well, fluorescence (excitation at 485 nm and emission at 525 nm) readings were taken every 2 min for 2 h, and the area under the curve was calculated. Results were expressed as Trolox equivalents (μmol TE/100 g fresh weight sample). Total phenolic contents were measured based on the Folin−Ciocalteau method according to previous reports.[20] Gallic acid (50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/L, correlation coefficient $r = 0.999$) was used to establish the standard curve. FCR (100 μL) was diluted 10 times from the original reagent and mixed with 80 μL of Na_2CO_3 (75 g/L) and 20 μL of sample in each well. Absorbance was measured at 765 nm after standing for 30 min at 37 °C. These results were expressed as gallic acid equivalents (mg GAE/100 g fresh weight sample).

Detection of Isoflavonoids by PDA and MS. HPLC analysis was performed on a Waters apparatus equipped with PDA detector. The detection wavelength was set from 210 to 800 nm. The column used was a 250 mm × 4.6 mm i.d., 5 μm, Atlantis T3 C_{18} with a 20 mm × 4.6 mm i.d. guard column with water (A), acetonitrile (B), and 2% acetic acid in water (C) as mobile phase. The column temperature was 35 °C. The injection volume was 20 μL. Solvent C proportion was maintained at 5% for the entire run. Solvent A and B gradient was as follows: 0−1 min, A 95%; 1−8 min, A from 95% to 85%; 8−24 min, A from 85% to 70%; 24−34 min, A from 70% to 40%; 34−50 min, A from 40% to 20%; 50−55 min, A from 20% to 5%; 55−58 min, A from 5% to 95%; 58−60 min, A 95%. The flow rate was 1.0 mL/min. The LC conditions for LC-MS analysis used solvent A (water with 0.05% acetic acid) and B (acetonitrile with 0.05% acetic acid) as mobile phase. The gradient was identical to those used for HPLC analysis above. The injection volume of each sample was 20 μL. For ESI-MS, both the positive and the negative ion modes were used for further characterization of the phytochemicals. The capillary temperature and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was supplied at 80 psi as sheath gas and at 20 psi as auxiliary gas. Full scan mass spectra from m/z 50 to 2000 were recorded with a scan speed of one scan per second.

Isolation and Identification of Biochanin A and Formononetin. Chickpea seeds (1.0 kg) were germinated at 25 °C in the dark for 3 days. The resulting germinated seeds were homogenized in methanol and then extracted three times on a shaking incubator at 200 rpm and room temperature for 6 h each time. Extraction solutions were concentrated in a rotary evaporator at 50 °C. The concentrated residue was transferred to a silica gel column (35 × 6 cm, silica gel 60 (0.040−0.063 mm)) pre-equilibrated with hexane. Successive elution with hexane/ethyl acetate (9:1, 9:2, 8:2, 8:3, 7:3) mixture at a flow rate of 5 mL/min gave many fractions (each fraction volume was 50 mL). After HPLC analysis, the fractions containing biochanin A and formononetin were combined and the two compounds were obtained and their identities confirmed by UV−vis, ESI-MS, and ^{1}H NMR spectra. ^{1}H NMR spectra were recorded in deuterated methanol with a Bruker AC300 spectrometer operating at 300 MHz.

Identification and Quantification of Isoflavonoids from Chickpea and Soybean. Identification of isoflavones was achieved by comparing their retention times and UV−vis and MS spectra with those of the standards. For those compounds without commercially available standards, the compounds were tentatively identified using HPLC retention times and UV−vis and MS fragments information and assigned by matching with compounds reported in the literature and existing metabolite databases, such as PubChem, Kegg Ligand database, Massbank, and Scifinder Scholar. Five isoflavone standards including biochanin A, daidzein, formononetin, genistein, and glycitein were used to obtain the standard curves of major isoflavones. Quantifications of individual and total isoflavones in chickpea and soybean were performed. The concentrations of those isoflavones without standards were calculated using the standard curve of genistein at 260 nm.

Statistical Analysis. Analysis of variance (ANOVA) was carried out with the SAS statistical program (version 9.00, SAS Institute Inc., Cary, NC), and differences between multiple means of treatments were determined by Duncan's multiple range test at $P < 0.05$.

RESULTS AND DISCUSSION

Antioxidant Capacity and Total Phenolic Content. In this study, we examined total phenolic content and antioxidant capacity in nine legume seeds that are most commonly consumed and available at Singapore food markets.

Data presented in Figure 2A show that germination could significantly increase total phenolic content in the nine legume seeds. Furthermore, ungerminated (0 day) black soybean has the highest total phenolic content (69.2 mg GAE/100 g FW) among the 9 selected legume seeds, but after germination, its value is less than that of chickpea (76.9 vs 91.7 mg GAE/100 g FW). Meanwhile, chickpea has the highest total phenolic content among the geminated seeds.

Antioxidant capacity was evaluated by oxygen radical absorbing capacity (ORAC) assay, and the ORAC values of ungerminated and germinated legume seeds are shown in Figure 2B. Overall, the results are similar to that of total phenolic content in Figure 2A. The nine legumes undergo a significant increase in antioxidant activity after germination. For ungerminated seeds, black soybean possesses the highest ORAC value (1667 μmol TE/100 g FW); however, the germination process of the soybean only slightly improves the ORAC value. In contrast, germination leads to an almost three times increase in the ORAC of chickpea (874 in 0 day vs 2457 in 3 day, μmol TE/100 g FW). Our results are similar to previous investigations on phenolic contents and antioxidant capacity of some germinated edible seeds[6] and selected legumes.[21] In addition, our data clearly indicate that chickpea not only produces great change in phenolic contents and antioxidant capacity during germination but also processes the highest values among the nine germinated legume seeds.

It should be noted that the total phenolic content measured by the Folin−Ciocalteau method and radical scavenging activity quantified by ORAC assay are nonspecific to certain phenolic compounds. Instead, these values give a preliminary indication of reducing activity and peroxyl radical scavenging capacity, and total phenolic contents and ORAC values have good linear correlations as shown in many fruit and vegetable samples. For Folin−Ciocalteau assay, some nonphenolic reductants such as vitamin C will give positive responses. It is essential to further characterize the specific polyphenolic compounds founds in germinated chickpea seeds.

Profiles of Isoflavonoids in Germinated Chickpea Seeds. To elucidate the identities of phytochemicals that produce the significant increase in phenolic antioxidants, HPLC analysis was carried out to detect the phytochemical change during germination of chickpea. HPLC chromatograms (260 nm) of acetone/ethanol/water (2:2:1) extracts of chickpea seeds during germination are given in Figure 3. The HPLC-PDA detector provided UV−vis spectra in the range of 210−800 nm for the peaks. The chromatograms at 260 nm are presented in this study since most compounds have absorbance at this wavelength. As shown in Figure 3, there are a number of new peaks produced during germination; in addition, there are increased concentrations of several compounds, especially those at retention times between 30 and 40 min.

Previous studies on chickpea seeds and sprouts suggested that the major constituents are isoflavonoids including biochanin A, formononetin, genistein, and their glycoside conjugates

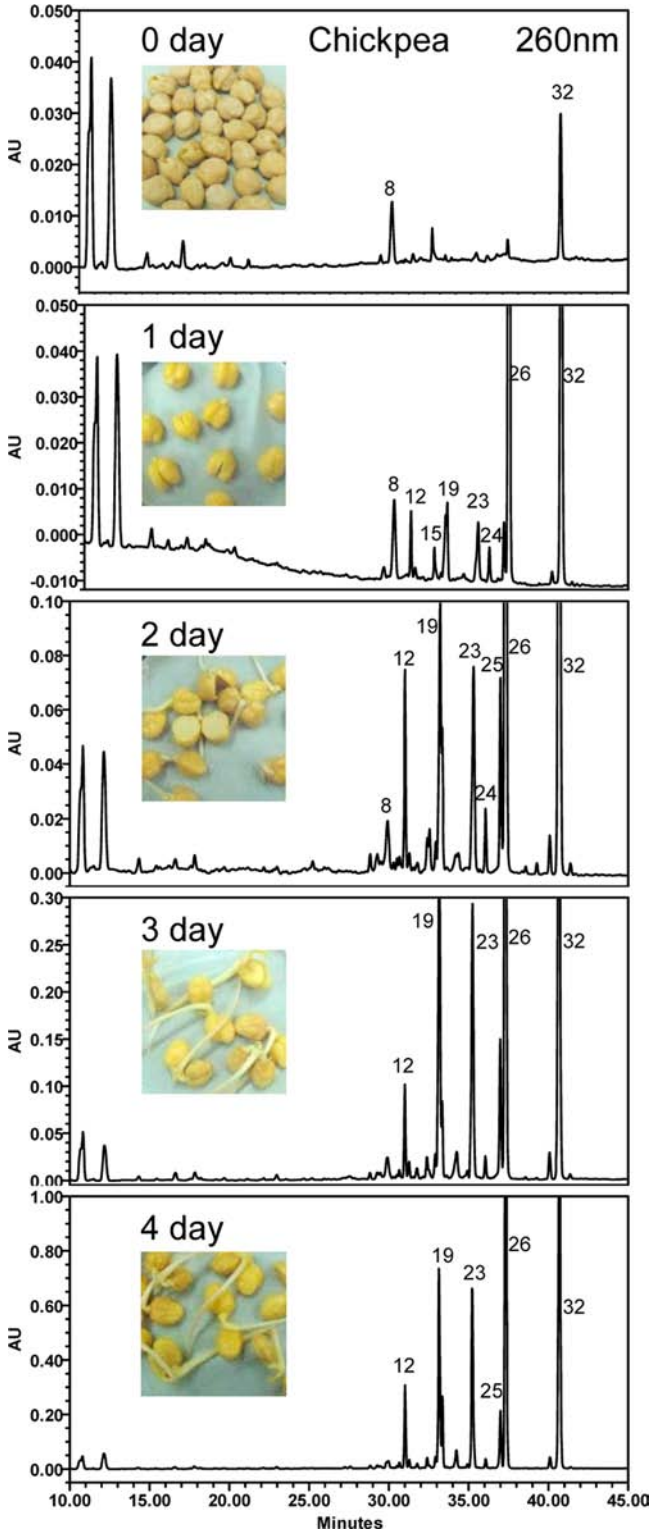

Figure 3. Effect of germination time on phytochemical production in chickpea. Germinated chickpea seeds are extracted by acetone/methanol/water (2:2:1), and HPLC chromatograms are shown at 260 nm. Tentative identification of compounds is listed in Table 1.

(Figure 1).[11,12] In agreement with this finding, we isolated two major compounds (peaks 26 and 32) by silica column chromatography and confirmed their structures by ^{1}H NMR, UV−vis, and MS spectra (Table 1, Figure 1) to be formononetin (peak 26) and biochanin A (peak 32).

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Table 1. Peak Assignments of Isoflavonoids in Germinated Chickpea and Soybean Presented According to Retention Time, Maximum UV Absorption, and Molecular Ions

peak	LC-RT (min)	compounds	UV_{max} (nm)	ESI+ $[M + H]^+$	ESI− $[M + H]^-$
1	22.65	daidzin	250	417	475, 253
2	23.31	glycitin	258	285	505, 283
3	25.72	glycitein glucoside malonylated	256	447, 285	445
4	27.00	daidzein glucoside malonylated	249	503, 417, 255	415, 253
5	27.22	genistin	261	433	431, 269
6	28.27	genistein glucoside malonylated	262	473, 271	269
7	28.79	formononetin glucoside	251	593, 269	591, 447, 267
8	29.22	garbanzol	277	273	271
9	29.94	glycitein glucoside acetylated	256	285	547, 283
10	30.25	genistein glucoside malonylated	261	519	517, 271
11	30.73	orobol glucoside malonylated	259	535, 287	533
12	30.99	isoformononetin glucoside malonylated	258	553, 431, 269	489, 267
13	31.55	lespedezol A1 glucoside malonylated	276	269, 255	267, 253
14	31.84	prunetin glucoside	259	285	283
15	32.45	formononetin glucoside malonylated	249	269	267
16	32.49	daidzein	249	255	253
17	32.85	glycitein	258	285	283
18	32.89	maackiain glucoside	286	285	445, 283
19	33.33	biochanin A glucoside	260	447, 285	283
20	34.40	biochanin A glucoside malonylated	261	285	533, 283
21	34.73	maackiain/medicarpin glucoside acetylated	275	475, 285, 271	487, 283, 269
22	35.06	5-hydroxypseudobaptigenin glucoside acetylated	261	503, 299	297
23	35.36	genistein	262	271	269
24	36.00	pratensein	261	301	299
25	36.98	pseudobaptigenin	249	283	281
26	37.31	formononetin	250	269	267
27	38.36	2′-hydroxybiochanin A	275	301	299
28	38.58	maackiain	310	285	283
29	39.10	medicarpin	287	271	269
30	39.81	cicerin/homoferreirin	291	331/317	329/315
31	40.10	5-hydroxypseudobaptigenin	263	299	297
32	40.69	biochanin A	261	285	283

In order to identify the other compounds, LC-ESI-MS^n analysis was carried out to obtain the molecular masses or their fragmentation information of those peaks. The m/z values of molecular masses or fragments in chickpea were matched with known phytochemicals reported in the literature[22−25] and supported by UV absorption maxima, HPLC relative retention time, and standards, including biochanin A, formononetin, daidzein, genistein, and glycitein, as well as online metabolite databases including KNApSAcK, PubChem, Kegg Ligand database, Massbank, and Scifinder ScholarTM. For instance, there are mainly two steps to identify maackiain (peak 28), medicarpin (peak 29), cicerin (peak 30), and homoferreirin (peak 30) in this study. On the basis of literature data, the elution times for the four compounds were likely to be between that of formononetin and biochanin A in the reversed-phase HPLC system (column 250 × 4.6 mm, RP-C_{18}, 5 μm).[24] We then checked the MS and UV absorption of the four compounds in the literature and matched with those of peaks in between formononetin (peak 26) to biochanin A (peak 32). Overall, 25 isoflavonoids and a flavanonol (garbanzol, peak 8) were identified in the germinated chickpea seeds (Figure 4). HPLC retention time, maximum UV absorption, and fragment ion masses in positive ion ($[M + H]^+$) and negative ion ($[M - H]^-$) modes of compounds assigned are listed in Table 1.

The UV absorption maximum of the individual compound was presented in Figure 1 and Table 1, as well as the UV spectra of major isoflavonoids in germinated chickpea (Figure 4). UV absorption spectra have been used as a complementary method in identifying isoflavonoids, especially daidzein, genistein, glycitein, and their conjugates.[26] Isoflavonoids have a characteristic UV absorption band II with maxima in the 240−280 nm range due to absorption of the A-ring benzoyl system. The UV absorption maxima of individual isoflavonoids mainly depend on the number of present aglycone hydroxyl groups, their relative positions, the glycosidic substitution pattern, and aromatic acyl groups.[27] A recent study suggested that glucuronidation on the 5-hydroxyl group resulted in a UV absorption maxima blue shift of 5−10 nm. In contrast, glucuronidation on the 7-hydroxyl group did not cause any change in UV absorption, whereas glucuronidation on the 6-hydroxyl group did not cause predictable changes in UV maxima values.[28]

As shown in Figures 3 and 4, the major compounds in germinated chickpea seed are biochanin A (peak 32), formononetin (peak 26), pseudobaptigenin (peak 25), pratensein (peak 24), genistein (peak 23), biochanin A glucoside (peak 19), formononetin glucoside malonylate (peak 15), and isoformononetin glucoside malonylate (peak 12). Remarkably, these compounds belong to isoflavones, the largest subgroup of isoflavonoids. Biochanin A and formononetin were the first phytochemicals found in the sprouted germs of *Cicer arietinum* in 1945.[25] Subsequently daidzein, pratensein, sissotrin (biochanin A 7-*O*-glucoside), genistein, genistin (genistein-7-*O*-glucoside), ononin (formononetin-7-*O*-glucoside),

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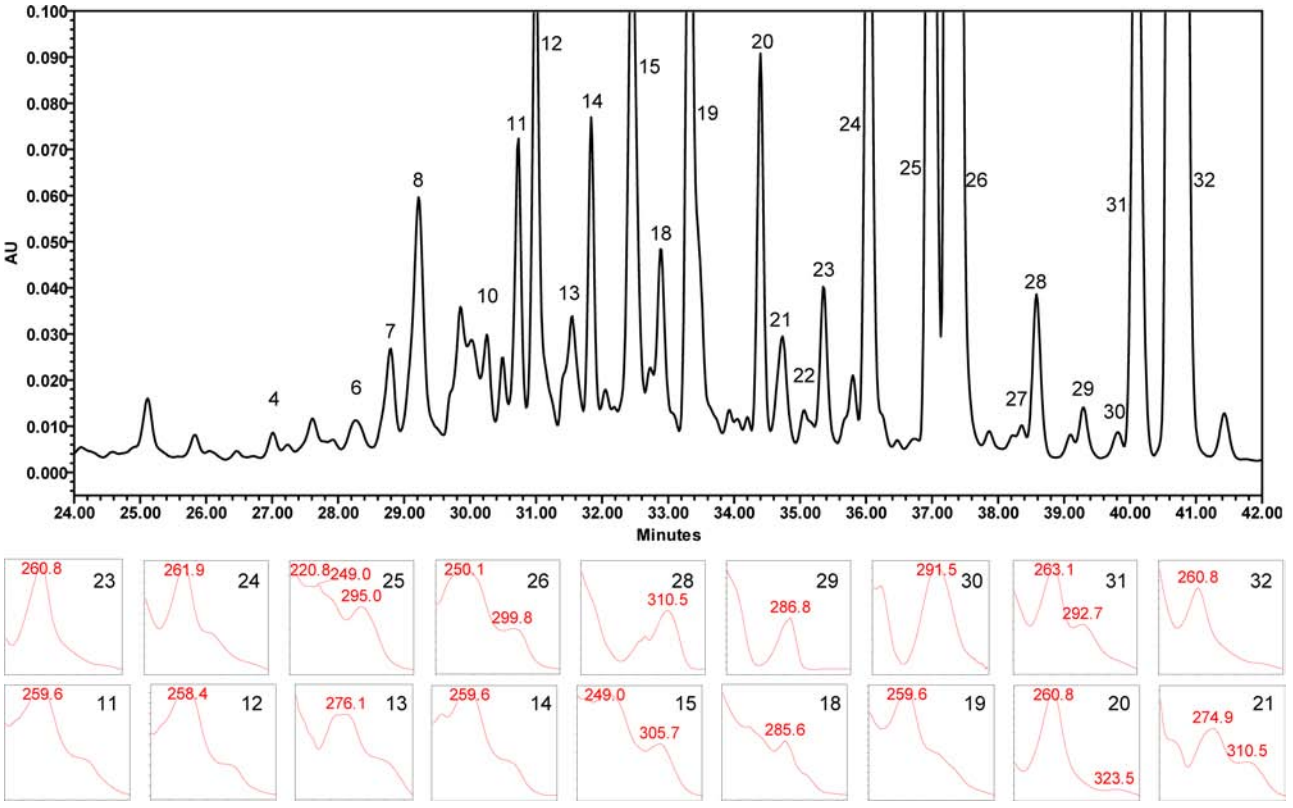

Figure 4. HPLC chromatogram (260 nm) and UV absorption spectra of isoflavonoids in germinated chickpea. Samples are concentrated from raw acetone/methanol/water (2:2:1) extracts in order to detect the lower concentration isoflavonoids. Tentative identification of compounds is listed in Table 1.

2′-hydroxyformononetin, calycosin, pseudobaptigenin, and other isoflavones and their glycoside conjugates were identified in seedlings, germinated cotyledons, roots, or other parts of *Cicer* spp.[25]

In this study, 20 isoflavones and conjugates were detected and identified in germinated chickpea and the majority of them are 4′-*O*-methylated isoflavones. In addition, two pterocarpans maackiain and medicarpin as well as two isoflavanones, cicerin and homoferreirin, were detected. Maackiain and medicarpin were proposed as phytoalexins produced in response to elicitor's induction and infection in chickpea roots, seedlings, and cell cultures.[29] They may be important compounds in chickpea defense of microbial infection, since it was reported that their potent antifungal activity could fight against several pathogens causing Ascochyta blight in chickpea.[25] However, their bioactivity for animals and humans is less studied than the other pterocarpan phytoalexin glyceollins, primarily from soybean.

Quantification of Isoflavonoids in Chickpea and Soybean. Soybeans are a major source of dietary isoflavones since they are rich in isoflavones. We compared the HPLC chromatogram (Figure 5) of isoflavonoids between black soybean, chickpea, and their germinated seeds at day 4 in acetone/methanol/water (2:2:1) extracts. It illustrated clearly that the changes of isoflavonoids production after germination was more significant in chickpea than that in black soybean. We also examined the two major isoflavones biochanin A and formononetin productions during germination in chickpea seed (Figure 6A). It was remarkable that the content of biochanin A and formononetin changed from 19 and 1.0 (μg/g FW) of ungerminated seeds (0 day) to 702 and 1484 (μg/g FW) of germinated seeds at day 4 (G4d), respectively, an increase of approximately 40 and 1400 times. Soybean germination has much less impact on the total flavonoids, with an increase of only 43.6% (1132 μg/g FW in ungerminated seeds to 1626 μg/g FW after germination). In comparison, approximately 90 times increase in total flavonoids was found in chickpea (83 μg/g FW in ungerminated seeds to 7568 μg/g FW in germinated seeds) (Figure 6B).

The antioxidant capacity of germinated chickpea (2253 μmol TE/100 g FW) and germinated soybean (2142 μmol TE/100 g FW) is comparable, while the total isoflavonoids content for germinated chickpea was approximately 4-fold higher than for germinated soybean. This may be due to the weaker antioxidant activity of 4′-*O*-methylated isoflavones biochanin A and formononetin than that of genistein and daidzein.[30] It should be pointed out that there may be other unknown compounds or the interactions among the components in the extracts contribute to the overall antioxidant activity of the extracts.

The health benefits of chickpea seed received much less attention. It has been reported that these 4′-*O*-methylated isoflavones can be converted by 4′-*O*-demethylation to the more potent phytoestrogens daidzein and genistein in human,[31] and the resulting daidzein and genistein would be further metabolized to other metabolites including equol.[32] Moreover, germination of chickpea could produce 4-fold higher total isoflavonoids content than soybean in addition to a few of pterocarpan phytoalexins, such as maackiain and medicarpin. Recent investigation has shown that red clover, another leguminous plant not used as a food source, also contains significantly higher concentrations of biochanin A, formononetin, and other isoflavones;[22,23,33] a total isoflavone content of more than 7500 μg/g FW was measured in this study, and this amount could be comparable to that of

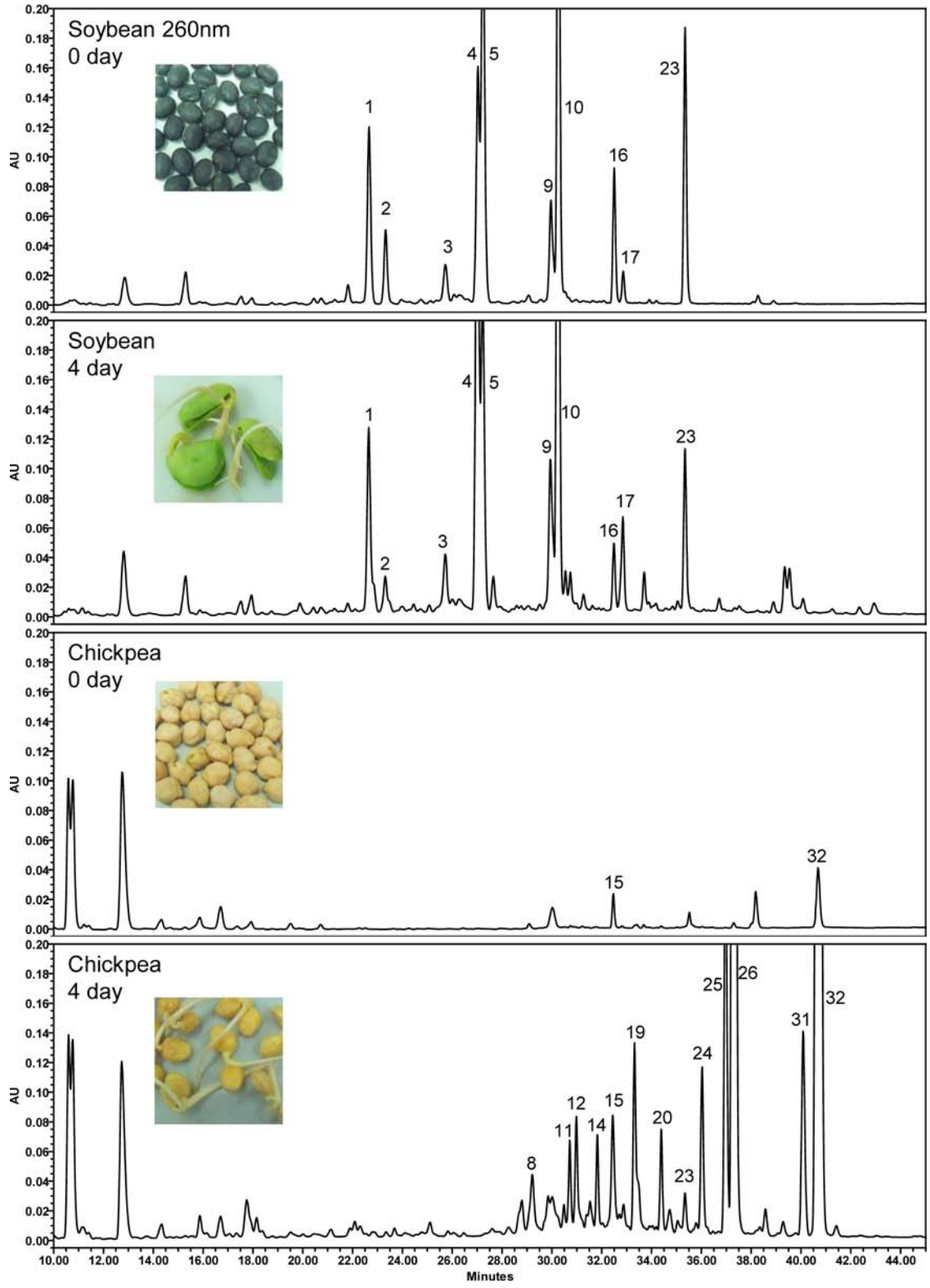

Figure 5. Comparative HPLC chromatogram (260 nm) of isoflavonoids between black soybean, chickpea, and their germinated seeds at day 4 in acetone/methanol/water (2:2:1) extracts. Tentative identification of compounds is listed in Table 1.

10 red clover cultivars ranging between 8.92 and 12.75 mg/g of dry matter.[34] Germination also increases phytic acid, ascorbic acid, folic acid, β-carotene content, protein solubility, and in vitro protein digestibility of chickpea seeds.[4,5,9] Although most of these compounds have positive health effects, phytic acid may pose some problem for absorbpion of minerals. The overall impact of such composition changes on the nutritional outcome in human on consuming germinated chickpea seeds needs to be further understood. It should be noted that isoflavones also have some controversy in the health benefits and adverse effects on consumption of isoflavones.

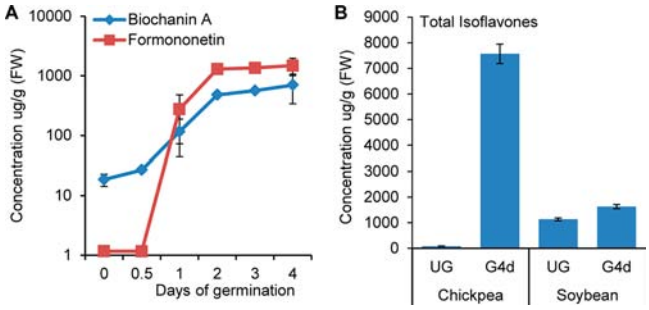

Figure 6. Quantitative analysis of isoflavonoids in chickpea and black soybean. (A) Production of the two major isoflavones biochanin A and formononetin during germination of chickpea seed. (B) Total isoflavonoid content in ungerminated (UG) black soybean, chickpea, and their germinated seeds at day 4 (G4d).

Isoflavonoids are synthesized by the central phenylpropanoid pathway and the specific isoflavonoid branch pathways in legumes (Figure 7). The central phenylpropanoid pathway is common to all plant species, and it produces lignin, coumarins, benzoic acids, stilbenes, and flavonoids/isoflavonoids.[8,35]

Chalcone is the first step in the production of flavonoids and isoflavonoids that requires the enzyme chalcone synthase (CHS). In the isoflavonoid biosynthetic pathway, the branch-point enzyme of isoflavonoid specific branch is introduced by 2-hydroxyisoflavanone synthase (isoflavone synthase, IFS) and yields an immediate product 2-hydroxyisoflavanone. The immediate product is then dehydrated to daidzein and genistein through catalysis by 2-hydroxyisoflavanone dehydratase (HID).[16] In soybean, the daidzein is a precursor to the major phytoalexin glyceollins. In chickpea, 2-hydroxyisoflavanone is methylated by 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyl transferase (HI4′OMT) to form methoxyisoflavanones, which are further dehydrated by HID to form biochanin A and formononetin.[16] Chickpea constitutively accumulates biochanin A and formononetin, mainly as 7-*O*-glucoside-6″-*O*-malonate esters stored in cell vacuoles.[17] Under environmental stress, the two pterocarpan phytoalexins medicarpin and maackiain are induced.[36] It is known that several subgroups of isoflavonoids are represented in chickpea. Generally, aglycones are represented more frequently than glycosides and substituted most frequently by glucose, which is consistent with our finding that the major compounds are

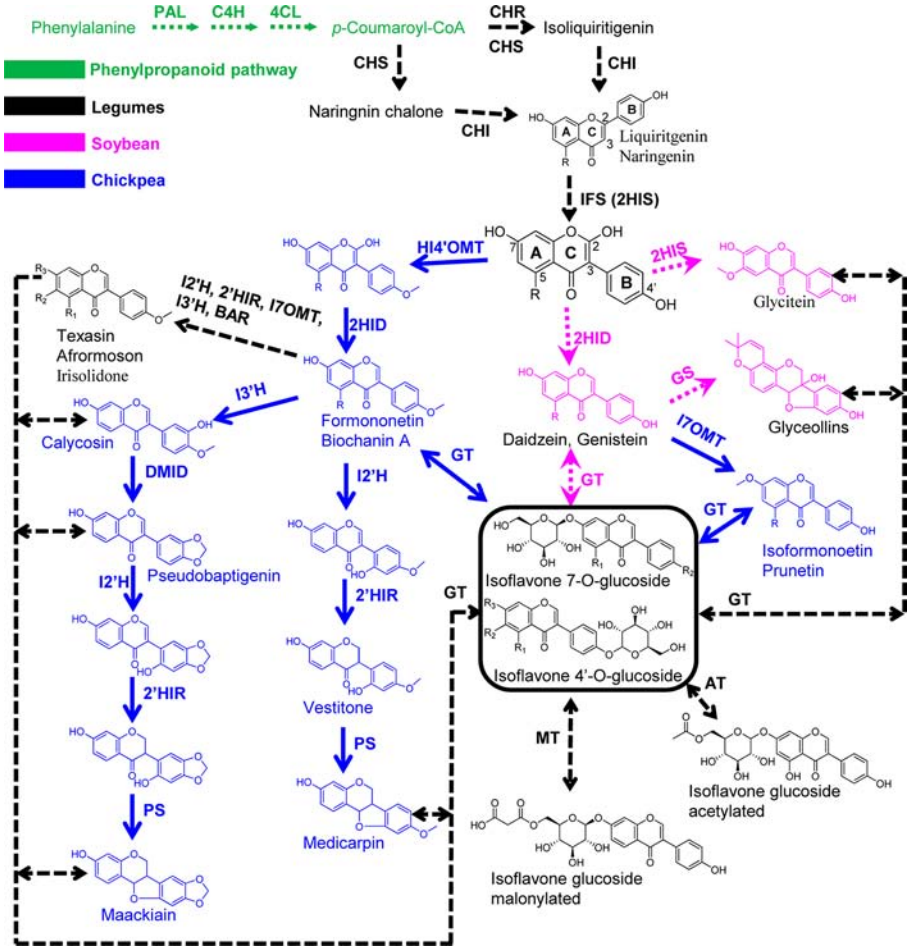

Figure 7. Proposed isoflavonoid biosynthesis pathway in legumes. PAL, phenylalanine ammonialyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; 2HIS, 2-hydroxyisoflavanone synthase; HI4′OMT, 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyl transferase; I7OMT, isoflavone 7-*O*-methyltransferase; 2HID, 2-hydroxyisoflavanone dehydratase; GS, glyceollin synthase; I2′H, isoflavone 2′-hydroxylase; 2′HIR, 2′-hydroxyisoflavone reductase; VR, vestitone reductase; DMID, 7,2′-dihydroxy,4′-methoxyisoflavanol dehydratase; PS, pterocarpin synthase; I3′H, isoflavone 3′-hydroxylase; BAR, biochanin A reductase; GT, uridine diphosphoglucose-isoflavone 7-*O*-glucosyltransferase; MT, isoflavone-7-*O*-beta-glucoside 6″-*O*-malonyltransferase; AT, isoflavone-7-*O*-beta-glucoside 6″-*O*-acetyltransferase.

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aglycone isoflavones biochanin A (peak 32), 5-hydroxypseudobaptigenin (peak 31), formononetin (peak 26), pseudobaptigenin (peak 25), pratensein (peak 24), and genistein (peak 23) in germinated chickpea seeds (Figures 3−5). Many isoflavonoids in *Cicer* occur as glycosides and often in larger quantities than the aglycones, which is probably for storage purposes since the glycosides appear to occur most abundantly in the roots and stems but only the aglycones occur in other parts of the plant tissues.[25]

In conclusion, germinated chickpea seed has a higher total phenolic content and antioxidant capacity than the other eight germinated seeds. In total, 31 isoflavonoids in chickpea and soybean are tentatively identified, based on their chromatographic retention time, UV spectra, and positive and negative MS fragments patterns in comparison to known compounds and the literature. Remarkably, germination greatly increases isoflavonoid diversity and content in germinated chickpea, which has more than 4-fold higher total isoflavonoid content than soybean. Our findings in this study provide important information for further studies on utilization of germinated chickpea seeds as a source for nutraceuticals and functional foods.

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Notes

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