Variable Isoflavone Content of Red Clover Products Affects Intestinal Disposition of Biochanin A, Formononetin, Genistein, and Daidzein

STEPHEN W.J. WANG, Ph.D.,† YAN CHEN, Ph.D.,‡ TIBY JOSEPH, B.Sc.,† and MING HU, Ph.D.†

ABSTRACT

Background: Marketed red clover (Trifolium pratense) products use a wide variety of labels, and the isoflavone content from the label is ambiguous.

Materials and methods: In the present study, we analyzed the content of various isoflavone products, and determined (1) the content and (2) how the sample matrix of red clover products affects the intestinal disposition of main isoflavones within it, using the human intestinal Caco-2 cell model.

Results: Analysis using high- and ultraperformance liquid chromatography indicates that the isoflavone content varied significantly (p < 0.05) between the chosen products. Consequently, rates of isoflavone absorption across the Caco-2 cell monolayers varied (p < 0.05) greatly. Unexpectedly, permeabilities of biochanin A and formononetin (two key biomarkers) were found to be significantly affected (p < 0.05) by the product matrix. As expected, biochanin A was the only isoflavone with noticeable metabolite peaks in both the apical and basolateral sides. Interestingly, rates of metabolism and the polarity of the glucuronidated biochanin A excretion were also significantly altered (p < 0.05) by the product matrix. Studies using the breast cancer resistance protein (BCRP) inhibitor, dipyridamole, showed that both the apical and basolateral excretion of biochanin A glucuronides were significantly (p < 0.05) reduced (7.5- and 9.4-fold, respectively) when dipyridamole is present. This provides evidence that BCRP is the main transporter responsible for the apical efflux of isoflavone glucuronides.

Conclusions: The isoflavone content of the marketed red clover products is highly variable, and the product matrix significantly affected the intestinal disposition of red clover isoflavones by altering their absorption rates, permeabilities, biochanin A glucuronide excretion rates, and the polarity of biochanin A glucuronide excretion. This research provides scientific evidence to support the standardization effort, so that consumers can make intelligent product choices.

INTRODUCTION

Isoflavones, a subclass of flavonoids, are phytochemicals that resemble the chemical structure of female hormone estrogens. Therefore, isoflavones are termed as phytoestrogens, and their purported effects range from prostate and breast cancer prevention in preclinical models,1,2 menopausal hormone replacement therapy,3 alleviation of various aging-related and hormone-dependent disorders (e.g., osteoporosis), and improvement of cardiovascular systems.1,4–7 Isoflavones have generated much interest in clinical nutrition and disease prevention,4,8–10 leading to various clinical trials for cancer prevention.11,12

The use of soy isoflavone supplements in large-scale clin-
ical trials for their chemopreventive effects has led to a recent trend, where the general public (especially women) is eagerly consuming natural isoflavone supplement products, even though their effects are not yet proven. This industry has spawned rapid growth (>18%), with annual sales exceeding $18 billion in 2002, according to the *Nutrition Business Journal*. Among the numerous isoflavone supplement products offered on the market today, isoflavones are usually derived from soybeans that contain isoflavones, such as daidzein, glycitein, and genistein, or from red clover (*Trifolium pratense*) that mainly contains biochanin A and formononetin.

This widespread use of self-administered isoflavones is somewhat unsettling, since much remains to be proven, including efficacy and possible side-effects. In order to further the preclinical and clinical research to show if isoflavone supplements are beneficial to human health, we must overcome several barriers, with the first being a lack of standardization in the marketed products.

In a previous study from our laboratory, a comparison among 13 different retailed soy isoflavone products showed that the exact compositions of these products varied significantly from product to product. Only 4 of 13 products contained 90% or more of the isoflavone content claimed on the label. Some products not only did not contain the claimed isoflavones, but also contained unknown impurities that exceeded 40%. Further, the major isoflavone components of a product changed significantly over time, making it highly variable between batches.

In the present study, we extended our investigation by analyzing the isoflavone content (as a measure of quality) of various marketed red clover products. We hypothesized that changes in isoflavone content will impact intestinal absorption and metabolism of the main isoflavones contained in the red clover. We also determined how an inhibitor of the breast cancer resistance protein (BCRP/ATP-binding cassette, subfamily G, member 2), dipyridamole, will affect the excretion (via efflux) of isoflavone glucuronides. As an important reference point, the red clover standard from the United States Pharmacopeia (USP, Rockville, MD) was used along with the purchased products. Developed under an important initiative, termed Dietary Supplement Verification Program (DSVP), this red clover standard can be used as a “mark of quality” for dietary supplement products.

### MATERIALS AND METHODS

#### Materials

Cloned Caco-2 TC7 cells were a kind gift from Dr. Monique Rouset of INSERM U178 (Villejui, France). Biochanin A, formononetin, daidzein, and genistein were purchased from Indofine Chemicals (Somerville, NJ). Hanks’ balanced salt solution (HBSS; powder form) and dipyridamole were purchased from Sigma-Aldrich (St Louis, MO). Powdered red clover standard extract was purchased from USP (catalog no. 1599500). Three red clover isoflavone supplement products were randomly obtained from health food and grocery outlets in the Houston, Texas, area, and two red clover isoflavone supplement products were purchased from the Internet* (Table 1). All other materials (typically analytical grade or better) were used as received.

#### Extraction procedures

Promensil® and Rimostil® tablets both manufactured by Novogen (U.S. Office in New Canaan, CT) were reduced to fine powder. Red clover products from the Eclectic Institute (Sandu, OR) and Solaray (manufactured by Nutraceutical Corp., Park City, UT) were in capsule form, and thus the content of each capsule was weighed after being

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**Table 1. Amounts (nmoles) of Genistein and Daidzein at (Donor) and 4 Hours (Receiver) in Caco-2 Transport Studies Using Six Red Clover (Trifolium pratense) Supplement Products**

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Daidzein amount (nmol)</th>
<th>Genistein amount (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP at 0 hours</td>
<td>BL at 4 hours</td>
</tr>
<tr>
<td>Eclectic institute, Inc.</td>
<td>1.63 ± 0.01</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Solaray</td>
<td>0.62 ± 0.06</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Gaia Herbs</td>
<td>6.95 ± 0.38</td>
<td>3.87 ± 0.58</td>
</tr>
<tr>
<td>Promensil</td>
<td>2.36 ± 0.05</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>Rimostil</td>
<td>3.62 ± 0.19</td>
<td>0.96 ± 0.16</td>
</tr>
<tr>
<td>USP</td>
<td>0.72 ± 0.04</td>
<td>0.18 ± 0.01</td>
</tr>
</tbody>
</table>

*www.drugstores.com

USP, United States Pharmacopeia; AP, apical; BL, basolateral.

*Manufactured by Nutraceutical Corp., Park City, UT.

*Novogen, U.S. office in New Canaan, CT.

*Rockville MD.
emitted. Red clover from Gaia Herbs (Brevard, NC) was in liquid form, while the USP red clover standard extract was in its original fine powder form. All of these products were extracted by adding a 6-mL mixture of acetonitrile and ethanol (50%:50%) to each 100-mg product (solid or liquid) and then sonicating the resulting suspension/solution for 30 minutes in a water bath at room temperature. The mixture was next centrifuged at 3500 rpm for 15 minutes. The supernatant was further diluted (1:50) with mobile phase A and analyzed for isoflavone content by using high-performance liquid chromatography (HPLC) and ultraperformance liquid chromatography (UPLC) after treatment (as described below). The remaining samples were stored at 4°C until use, and isoflavone content was stable during storage (not shown).

**HPLC analysis of isoflavones**

The conditions for analyzing isoflavones were as follows: system, Agilent 1090 (Agilent Technologies, Inc., headquartered in Santa Clara, CA) with diode array detector running the ChemStation software; column, Aqua (Phenomenex, Gilroy, CA), 5 μm, 150 × 0.45 cm; mobile phase A, 100% acetonitrile, mobile phase B, water (0.04% H₃PO₄ and 0.06% C₆H₁₅N; pH 3.0); gradient, 0–3 minutes, 80% B, 3–25, 80%–50% B, 25–26, 50%–80% B; wavelength, 254 nm; and injection volume, 200 μL. There was a 1-minute interval between the end of the run and the next injection to allow the column to be reequilibrated with 80% mobile phase B.

**UPLC analysis of isoflavones**

Selected samples were also run on UPLC that is much more efficient than HPLC. The conditions for analyzing isoflavones, using UPLC, were as follows: system, Waters Acquity UPLC (Waters, Milford, MA) with a photodiode array detector and Empower software (Waters Corporation, Milford, MA); column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm; mobile phase A, 100% acetonitrile, mobile phase B, water (0.06% C₆H₁₅N and 0.045% CH₂O₂; methanol 90:10); gradient, 0–3 minutes, 0% B, flow rate = 1 mL/min, 0.3–1.80, 0%–50% B, flow rate = 0.925 mL/min, 1.80–2.10, 50%–100% B, flow rate = 0.925 mL/min, 2.10–2.40, 100% B, flow rate = 0.925, 2.40–2.50, 100%–0% B, flow rate = 1 mL/min; wavelength, 254 nm; and injection volume, 10 μL.

**Caco-2 cell culture**

The culture conditions for growing Caco-2 cells have been described previously. The seeding density (100,000 cells/cm²), growth media (Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 10% fetal bovine serum [FBS]), and quality control criteria were all implemented in the present study, as described previously. Caco-2 TC7 cells were fed every other day, and the monolayers (4.2 cm² total area) were ready for experiments from 19 to 22 days post seeding.

**Transport experiments in the Caco-2 cell culture model**

Experiments in triplicate were performed in HBSS (pH 7.4). The protocol for performing cell-culture experiments was the same as described previously. Briefly, the cell monolayers were washed three times with HBSS (37°C, pH 7.4). The transepithelial electrical resistance values were measured, and those with transepithelial electrical resistance values less than 420 ohms × cm² were discarded. The monolayers were incubated with the buffer for 1 hour, and the incubation medium was then aspirated. Because of the intrinsic concentration differences that exist between the red clover compounds, all test solutions loaded onto the apical side of the cell monolayer contained the same concentration of biochanin A (10 μM). Additionally, transport experiments were also conducted by using dipyrindamole (10 μM) in the test solutions of USP red clover extract. Two donor (or apical) samples were taken at the beginning and at the end of an experiment, and four receiver (or basolateral) samples (400 μL) were taken every 60 minutes, followed by the addition of 400 μL of fresh buffer to keep a constant volume at the receiver side. Fifty microliters (50 μL) of 94% acetonitrile/6% glacial acetic acid containing 100 μM of testosterone were added to each sample as the internal standard. After, the mixture was centrifuged at 13,000 rpm for 8 minutes, and the supernatant was analyzed by HPLC and/or UPLC.

**Data analysis**

Rates of transport (Vt) were calculated based on the amounts of isoflavone transported as a function of time (Equation 1), where V is the volume of the receiver in units of mL or cm³ (typical volume is 2.5 mL) and dC/dt is the rate of concentration change in the receiver side at a unit of μM/min or μM/sec. In order to account for the concentration differences between products, experimental rates of absorption for all four isoflavones from the Caco-2 transport studies (fixed at 10 μM for biochanin A) were back-calculated to correspond to the actual concentration of each isoflavone normally found in 1 mg/mL of their respective product. The permeability (P) of isoflavones to cross a cellular membrane was calculated by dividing the rate of transport (Vt) by the surface area (A) of the monolayer and the initial concentration (Ci) of these compounds at the loading side, with the assumption that concentration on the other side of the membrane is negligible (Eq. 2).

\[ V_t = \frac{dC}{dt} \]

\[ P = \frac{V_t}{A} \]

**Statistical analysis**

One-way analysis of variance and the Student’s t test were used to analyze the data (NCSS 2001, Kaysville, UT). The prior level of significance was set at 5% (p < 0.05).
RESULTS

Determination of an optimal isoflavone extraction method from red clover products

Three extraction solvents were compared by using 50% acetonitrile in water, 100% pure ethanol, or an equal mix of acetonitrile and ethanol. The results showed a significant ($p < 0.05$) increase in extraction efficiency of the red clover extract when ethanol was used. The most pronounced difference was in the extraction of the isoflavone formononetin from Rimostil powder (Fig. 1), where the concentration of formononetin extracted, using either 100% ethanol or 50% acetonitrile and 50% pure ethanol, was 3.5-fold higher than using 50% acetonitrile in water. Thus, all red clover products analyzed in this study were extracted subsequently by using an equal mix of acetonitrile and ethanol. When using this solvent mixture, a second extraction of the 100-mg red clover insoluble matters (excluding Gaia), with a 6-mL equal mixture of acetonitrile and ethanol, did not yield significant amounts of additional isoflavones.

HPLC and UPLC profiles of various red clover products

Analysis of five chosen red clover products and the USP red clover standard extract showed substantial differences in their HPLC and UPLC chromatographic profiles (Fig. 2A and 2B). Even though chromatographs from both HPLC and UPLC both depicted accurate chromatographic profiles of red clover isoflavones, UPLC is more efficient in separating compounds with the run time decreased from 25 to 3 minutes per sample. As evident from the chromatographs, red clover consists of mainly formononetin and biochanin A, whereas daidzein and genistein are also present in minute quantities. Therefore, concentrations of formononetin and biochanin A should be the main determinants used to compare the isoflavone content differences between products. In our previous study of soy isoflavone supplement products, we noticed many peaks other than soy isoflavone peaks in 13 products that were analyzed and classified them as impurities. In our analysis of five red clover products and the USP red clover standard extract, peaks resulting from these impurities (i.e., peaks other than biochanin A, formononetin, genistein, and daidzein) are more prevalent in the chromatographic profiles of red clover products than from the standard USP red clover extract (Fig. 2A and 2B).

Isoflavones contents of red clover products

The concentration of formononetin found in Gaia, Solaray, Eclectic Institute, Rimostil, and Promensil red clover products in a 1/60 (W/V) solvent (i.e., an equal mix of acetonitrile and ethanol) extracts were 69.8 $\mu$M, 30.27 $\mu$M, 180.93 $\mu$M, 11.65 mM, and 1.20 mM, respectively. The same 1/60 (W/V) solvent extract of the USP red clover standard yielded a formononetin concentration of 2.26 mM. Among all the tested products, significant differences ($p < 0.05$) were found between their formononetin concentrations with a 385-fold difference between the lowest (Solaray) and the highest (Rimostil) concentration detected. Significant ($p < 0.05$) concentration differences were also noticed for the biochanin A found in Gaia, Solaray, Eclectic Institute, Rimostil, and Promensil, which had biochanin A concentrations of 112.7 $\mu$M, 80.52 $\mu$M, 384.7 $\mu$M, 2.48 mM, and 3.62 mM, respectively. The USP red clover standard extract had a biochanin A concentration of 7.15 mM. From this data, the biochanin A concentration difference between the least (Solaray) and the most (Rimostil) concentrated was 45-fold.

Transport rates of isoflavones in extracts of different red clover products

The amount of formononetin and biochanin A transported across the Caco-2 cell monolayer was measured by loading a diluted product extract that contains 10 $\mu$M of biochanin A (Fig. 3). The measured rates of transport of formononetin and biochanin A were normalized to a (donor) loading concentration of 1 mg of the product per ml of optimized solvent. The results indicated that intestinal transport rates (nmol/min) of formononetin and biochanin A in red clover compounds were dependent on isoflavone content, in that a lower content would translate into a slower rate of absorption across the Caco-2 cells (Fig. 4A). Surprisingly, permeabilities of biochanin A and formononetin were dependent on (extracted) product matrix, with the highest permeability (Solaray and USP standard) being twice as much as the lowest permeability (Eclectic Institute and Promensil) (Fig. 4B). Interestingly, in each product matrix, the permeability of formononetin was similar to that of biochanin A. For the other two isoflavones (genistein and daidzein) that were present in lower abundance, we also...
FIG. 2. High-performance liquid chromatography (HPLC) (A) and ultraperformance liquid chromatography (B) chromatographs that show both the quantitative and the compositional differences among five red clover (*Trifolium pratense*) natural isoflavone supplement products, as compared to the United States Pharmacopeia, Rockville, MD (USP) red clover standard. Eclectic Institute, Inc., Sandy, OR; Solaray, manufactured by Nutraceutical Corp., Park City, UT; Gaia Herbs, Brevard, NC; Rimostil and Promensil, Novogen, U.S. Office in New Canaan, CT.
observed a significant impact of product matrix on amounts of isoflavone transported at 4 hour, in that higher initial donor (apical) concentrations led to a higher 4-hour receiver (basolateral) concentration (Table 1). More interestingly, the largest difference in percent of transport (4 hour) for genistein and daidzein was not observed with the Solaray or USP standard (Table 1). Here, we did not calculate permeability because concentrations of genistein and daidzein at the receiver side were often very low at earlier time points, which often make it impossible to quantify. Therefore, this precluded them from being used as part of the multiple time points needed for calculating the transport rates and permeability via Equation 1.

**Metabolism and excretion of biochanin A in extracts of different red clover products**

The sample matrix was different for all six red clover products tested, as represented by different UPLC and HPLC profiles. We hypothesized that this composition difference may influence the metabolism of isoflavones in the Caco-2 transport studies. The results from these studies show that biochanin A was the only isoflavone metabolized with noticeable metabolite peaks in both the apical and basolateral sides (Table 2). The biggest difference in the total (apical + basolateral) amounts of metabolite excreted was more than three times (USP > Rimostil). For all product extract, the glucuronidated biochanin A was preferentially excreted to the apical side (p < 0.05 for all products). The biggest percent difference (140%) in polarity was displayed by Eclectic Institute, whereas the smallest was by Gaia and Solaray (27%). In the studies involving dipyridamole, results showed that the efflux of BCA glucuronides were significantly (p < 0.05) reduced when dipyridamole was introduced than without (Table 2). Apical and basolateral excretion of BCA glucuronides, when treated with dipyridamole, was reduced by 7.5- and 9.4-fold, respectively (Table 2).

**Lack of correlation between actual isoflavone content and product label values**

We examined whether the labels of the red clover products accurately report their isoflavone content, since each product claimed they contained a certain amount of isoflavones. We also listed or calculated recommended daily product dose, amount per dose, the actual isoflavone amounts present in each dose, and the actual amount required to reach the USP red clover standard (50 mg of USP red clover standard was extracted to yield 6.6 mg of isoflavones). As expected, the actual isoflavone contents found in each product varied tremendously and were not accurately reported in their respective labels (Table 3).

**DISCUSSION**

Analysis of isoflavone content and profiles, using the optimized extraction method, clearly showed that they varied greatly between products (Fig. 2). This analysis was enabled by an optimization of the solvent used in the extraction method that minimizes the possibility that isoflavones were not detected as the result of poor extraction. This difference between isoflavone contents affects their absorption rates across the Caco-2 cell monolayer (Fig. 3A, Table 1). It also affects the permeability of biochanin A and formononetin (Fig. 3B, Table 1) and the metabolism of biochanin A (Table
These results suggest that isoflavone content in each product will likely impact their bioavailabilities, which could potentially affect their biologic activity or efficacy. Our studies indicate that ethanol is a critical reagent for the optimal extraction of red clover isoflavones, where formononetin and biochanin A are the major components. This is somewhat different from the result of an earlier investigation, where acetonitrile or a solvent mixture of 50% acetonitrile in water was shown to be the best solvent for the extraction of soy isoflavones, which contain mostly glucoside and also smaller amounts of the aglycone forms of genistein and daidzein. This suggests the importance of optimizing the extraction procedure for each isoflavone in each different sample matrix.

HPLC and UPLC chromatographic profiles of extracts from different red clover products showed drastic differ-
Table 2. Concentration (µM) and Amount (nmoles) for Formononetin and Biochanin A Glucuronide at 4 Hours in Caco-2 Transport Studies Using Extracts Obtained from United States Pharmacopeia (USP) Red Clover (*Trifolium pratense*) Standard With and Without the Addition of Dipyridamole (10 µM)

<table>
<thead>
<tr>
<th>Caco-2 sides</th>
<th>Form dosed</th>
<th>Formo-Gluc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BCA dosed</th>
<th>BCA-Gluc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Form dosed</th>
<th>Formo-Gluc</th>
<th>BCA dosed</th>
<th>BCA-Gluc&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc (µM)</td>
<td>Apical</td>
<td>5.71 ± 0.17</td>
<td>0.53 ± 0.18</td>
<td>14.46 ± 0.52</td>
<td>9.58 ± 1.51*</td>
<td>12.11 ± 0.11</td>
<td>0.82 ± 0.05</td>
<td>19.60 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>Basolateral</td>
<td>6.09 ± 0.54</td>
<td>0.43 ± 0.03</td>
<td>16.28 ± 1.02</td>
<td>6.92 ± 0.77*</td>
<td>12.17 ± 0.20</td>
<td>0.32 ± 0.04</td>
<td>18.95 ± 1.43</td>
</tr>
<tr>
<td>Amount (nmoles)</td>
<td>Apical</td>
<td>14.30 ± 0.43</td>
<td>1.32 ± 0.45</td>
<td>36.21 ± 1.30</td>
<td>23.91 ± 3.78*</td>
<td>30.28 ± 0.28</td>
<td>2.05 ± 0.13</td>
<td>49.00 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>Basolateral</td>
<td>15.23 ± 1.35</td>
<td>1.08 ± 0.08</td>
<td>40.73 ± 2.04</td>
<td>17.30 ± 1.93*</td>
<td>30.43 ± 0.50</td>
<td>0.80 ± 0.10</td>
<td>47.38 ± 3.58</td>
</tr>
</tbody>
</table>

The asterisk indicates that there was a statistically significant (<i>p</i> < 0.05) difference between the concentration (and subsequent amount) of isoflavone glucuronides in either the apical and/or basolateral side when Caco-2 cells were treated either with or without dipyridamole.

<sup>a</sup>Rockville, MD.

<sup>b</sup>Metabolites produced in cell lines.

Concentration of biochanin A from extraction was targeted to be at approximately 10 µM, with actual dosed concentration (back-calculated from experimental data) to be slightly higher. BCA, biochanin A.
ences in isoflavone content (Fig. 2). As expected, for- 
memonetin and biochanin A were the main isoflavones pre- 
ent in red clover products. Daidzein and genistein, two 
isoflavones abundant in soybeans, were often present but 
only in minute quantities. In addition to differences in known 
isoflavones, other peaks in the chromatographic profiles that 
represent impurities were also very different between prod-
ucts and between the products and the USP standard.

We hypothesized that differences in isoflavone content 
would lead to different absorption rates. The results showed 
that normalized transport rates of biochanin A and for-
memonetin were directly influenced by isoflavone content 
(Figs. 3 and 4A). A correlating relationship could be ob-
served, since higher content usually results in higher rate of 
transport (not shown). Similar relationships were found for 

We hypothesized that the permeability of different 
isoflavones would stay the same in a different product ma-
rix, since these isoflavones are presumed to be absorbed via 
passive diffusion. However, this was not the case, as per-
meabilities of formononetin and biochanin A and the per-
cent of transport of genistein and daidzein were all affected 
by the sample matrix (Table 1).

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isoflavones would stay the same in a different product ma-
rix, since these isoflavones are presumed to be absorbed via 
passive diffusion. However, this was not the case, as per-
meabilities of formononetin and biochanin A and the per-
cent of transport of genistein and daidzein were all affected 
by the sample matrix (Table 1).

In addition to differences in permeability (e.g., percent ab-
sorption) and absorption rates, the extent of metabolism (as 
represented by biochanin A glucuronide formation and polar-
ity of biochanin A glucuronide excretion) was also affected 
by the product matrix, using the Caco-2 cell-culture model. This 
was unexpected, since we had used the same loading concen-
tration of biochanin A at the donor side. This difference was 
probably not owing to differences in the concentrations of for-
memonetin present in the various products, since higher for-
memonetin concentrations are not correlated with a faster or 
slower metabolism. Formononetin was rarely found to have 
metabolized at all in red clover matrix, as was observed ear-
er,

To further understand the mechanisms of the above ob-
served matrix effects, the BCRP inhibitor, dipyridamole, 
was used in an attempt to significantly reduce both the api-
cal and basolateral excretion of biochanin A metabolites 
(Table 2). This result is consistent with the work of Morris 
and coworkers, who have shown that isoflavones themselves 
can inhibit BCRP, MDR1, and MRP1.20–22 Therefore, our 
data suggest that BCRP might be predominant efflux trans-
porters involved in the apical efflux of isoflavone metabo-
lites. However, our data from previous research show that 
apical to basolateral transport of genistein was shown to be 
similar to its basolateral to apical transport.23 Nevertheless, 
these data clearly warrant further research into the role and 
capacity of BCRP in the efflux of isoflavone metabolites.

The ultimate cause of observed discrepancies in isoflavone 
content is likely owing to the lack of standardization among 
many red clover sources used by the manufacturers of these 
products. Based on product labels, red clover sources varied 
from red clover leaf extracts (Rimostil) to red clover tops 
(Gaia Herbs) to fresh-freeze-dried blossoms (Eclectic Insti-
tute). The variations in materials will certainly cause differ-
ces in isoflavone concentrations. Further, because of the

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Lot no.</th>
<th>Red clover source</th>
<th>Daily</th>
<th>Number of isoflavones per day</th>
<th>Amount of isoflavones required to reach 6.6 mg of USP standard per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eclectic Institute, Inc.</td>
<td>6997/4</td>
<td>Fresh-freeze-dried blossoms</td>
<td>600</td>
<td>45 caps</td>
<td>5.67</td>
</tr>
<tr>
<td>Solaray Red Clover Blossomsb</td>
<td>090109</td>
<td>Blossoms</td>
<td>1125</td>
<td>100 caps</td>
<td>4.14</td>
</tr>
<tr>
<td>Gaia Herbsc</td>
<td>59305-2004</td>
<td>Organic tops (liquid herbal extract)</td>
<td>2665</td>
<td>22.5 servings</td>
<td>6.08</td>
</tr>
<tr>
<td>Promensil by Novogenid</td>
<td>2L0449-R3106</td>
<td>Plant extract</td>
<td>40</td>
<td>30 tablets</td>
<td>3.24</td>
</tr>
<tr>
<td>Rimostil by Novogenid</td>
<td>28587-3113</td>
<td>Leaf extract</td>
<td>57</td>
<td>30 tablets</td>
<td>13.11</td>
</tr>
<tr>
<td>USP Standardd</td>
<td>FOC188</td>
<td>N/A</td>
<td>50</td>
<td>N/A</td>
<td>6.60</td>
</tr>
</tbody>
</table>

All red clover isoflavones were extracted by using the optimized method and quantified by using high-performance liquid chromato-
matography.

aSandy, OR.
bManufactured by Nutraceutical Corp., Park City, UT.
cBrevard, NC.
dU.S. office in New Canaan, CT.

fSpending calculated according to a 50-mg dose of the USP red clover standard, which contains 6.60 mg of isoflavones.
fact that botanicals can also vary in composition owing to geographical location (harvesting) as well as growing conditions, future studies will, and should, also investigate the comparison of content on intestinal absorption for individual products, perhaps in utilizing models such as the in situ intestinal perfusion model. Moreover, possible differences in extract production processes and inadequate quality control could be further attributed to content differences. We already described the biologic implication for the observed difference in isoflavone contents, and we will next describe the consequence for average consumers.

Our studies showed that actual amount of isoflavones present in each tablet/dose differed greatly from the respective labels of the commercial red clover products tested. This translates to larger differences in dosage amount required to reach a set USP red clover standard product (Table 3). Therefore, the product label is not a good predictor of quality and content, in that higher reported dosing strength does not guarantee larger amounts of isoflavones available for absorption or better quality. Taken together, these inaccuracies are highly detrimental to consumers and the marketplace because there is no scientific basis behind the product labeling.

Last, we would like to state that this paper aimed to determine the differences found in red clover products on the market, compared to the USP red clover standard. This paper did not attempt to endorse or demote a specific brand of red clover product, since we only sampled each marketed product once. The quality of these products could vary over time. The product that was shown in this study to be the “best” could be the “worst” in another sampling. To protect consumers, the Food and Drug Administration has just proposed new rules for dietary supplement manufacturers. If the manufacturers endorse and implement the upcoming new FDA regulation governing current good manufacturing practices, standardization efforts could produce better, more consistent products for consumers.

CONCLUSIONS

In conclusion, large differences in isoflavone content between the red clover products significantly impacted absorption rates, permeability, and metabolism of various isoflavones within these products. Therefore, our results support standardization processes, such as those provided by the DSVP program from USP. Because there is tremendous ambiguity in the labeling among the products, the labels are not reflective of the actual amount of pure red clover isoflavones. Consumers will be better served by the new FDA regulation that emphasizes standardization and current good manufacturing practices. Finally, this study further supports the necessity of an independent assay to precede any efficacy trials of dietary or herbal supplement in humans.

REFERENCES


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