DOI:10.1021/jf904459u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Highly Variable Contents of Phenolics in St. John's Wort Products Affect Their Transport in the Human Intestinal Caco-2 Cell Model: Pharmaceutical and Biopharmaceutical Rationale for Product Standardization

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The purposes of this study were to determine content uniformity of phenolics in the St. John's wort (SJW) supplements and to demonstrate how variations in the product matrices affect their absorption and efflux. LC and LC-MS/MS methods were used to determine the phenolic contents of 12 different products purchased locally or from the Internet. Three representative extracts were further submitted to Caco-2 cell transport experiment, and transport of rutin, hyperoside, and isoquercitrin was evaluated. The results indicated that the 12 different products displayed 12 different HPLC fingerprints, but all products contained the following major compounds: rutin, hyperoside, isoquercitrin, quercitrin, quercetin, and amentoflavone. The content uniformity of these major compounds was poor across products, with the smallest difference in the amounts of amentoflavone (3.6-fold) and largest difference in that of isoquercitrin (28.8-fold). The Caco-2 experiments indicated transport of rutin in products was vectorial, with the permeabilities varied about 3.6-fold in both directions of transport. The vectorial permeabilities of hyperoside and isoquercitrin were similarly different. Use of efflux transporter inhibitor studies suggested that MRP2 was involved in isoquercitrin's efflux and the product matrix affected the extent of its efflux. In conclusion, different SJW supplements had highly variable contents of phenolics, and the variability in product matrix and phytochemical compositions affected the permeabilities of key phenolics across the Caco-2 monolayers, which may further affect their bioavailabilities. Therefore, standardization will be necessary to ensure safe and efficacious using of supplements such as SJW.

KEYWORDS: St. John's wort; dietary supplement; standardization; Caco-2 cell model; bioavailability; MRP

INTRODUCTION

Depression is a common but serious medical condition that physically and emotionally affects millions worldwide, resulting in loss of motivation, disability, physical or emotional impairment, and increasing risk of death from suicide (*1*−*3*). Although pharmaceutical industries offer several approved drugs, many depression patients' experience were only slight improvement. They were suffering from negative side effects, or refusing to take medicinal treatment (*4*, *5*). As a consequence, an increasing number of patients are relying on alternative medicinal therapies.

The extract of St. John's wort (SJW, *Hypericum perforatum* L.) is a popular herbal dietary supplement intended to treat mild to moderate symptoms of depression (*6*). Clinical trials have shown SJW to be superior to placebo when treating major depression and equally effective as conventional antidepressants but with fewer side effects (*7*, *8*). Although the mechanism of SJW's efficacy remains unclear, its pharmacological effects are attributed to its phytochemical content. Recent studies suggested that its apparent antidepressant effects were due to the presence of phenolics (*8*, *9*). At any rate, SJW extract has become one of the top 10 selling herbal supplement and is produced by over 15 different manufacturers (*10*).

Due to its apparent commercial successes, many manufacturers have rushed to produce, sell, and market SJW products. Because SJW is defined as a dietary supplement, these products are sold without first demonstrating safety and efficacy as normally required for FDA-approved drugs. This practice may be inevitably associated with inconsistent quality standards. Therefore, it is entirely possible that one brand of SJW supplement may contain very different amounts and proportions of phytochemical compounds as opposed to another. This could be a serious problem because, despite its supposed benefits, SJW may cause adverse side effects as well as interact with other drugs. For example, SJW was shown to reduce the effectiveness of oral contraceptives and other important drugs (e.g., cyclosporine and digoxin), with narrow therapeutic indices (*11*). Therefore, without an established standard or a set of standards, it would be difficult to determine which product is safe and effective to use and which product will cause serious side effects and should be pulled out of the marketplace.

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 Published on Web 05/07/2010

Table 1. St. John's Wort Product Information

product	manufacture	vendor	claimed hypericin content (mg/daily dose)	daily dose (capsules or tablets per day)	lot
1	Sundown	Walgreens	2.7	6	4503101A
2	GNC Nature's Fingerprint	GNC	nc[a]	3	2297EH1942
3	Now	iherb.com	2.7	3	nf[b]
4	CVS Pharmacy	CVS	2.7	3	6671020
5	Kroger	Kroger	2.7	3	7CBO669
6	Whole Health	wholehealth product.com	2.7	1	7H719-0
7	Nature Made	Walgreens	2.7	6	RD10334
8	Nature's Resource	CVS	2.7	2	QF10003
9	GNC herbal plus standardized	GNC	2.7	3	1926EH1945
10	Safe way Select	Randalls	2.7	3	6HA0900
11	Nature's Way	Randalls	nc	4	nf
12	Puritan's Pride	puritan.com	2.7	3	168323-08

[a] nc, no claim. [b] nf, not found.

Establishment of quality control criteria based on phytochemical components of a SJW extract can be done with relative ease. The more difficult task is to establish a universal standard that every manufacturer can agree upon, as was done routinely for FDA-regulated drug products. This is because there is a lack of scientific evidence that will demonstrate one set of standards will produce a product superior to another. Moreover, it is difficult to determine which ingredients in an herbal supplement are responsible for the reported beneficial or side effects. This lack of direct scientific evidence was partially responsible for the lack of government regulation as regulatory agencies cannot determine the best criteria to be used to regulate a product.

The current investigation represents a new approach to the problem. Conventionally, a product is often defined by its phytochemical composition, but it is difficult to define how one may be different from another biologically. In this paper, we showed not only how products differed phytochemically but also biopharmaceutically with respect to absorption and efflux, the first steps in the bioavailability continuum.

We have chosen LC fingerprinting combined with LC-MS/MS to define products phytochemically because more than 12 compounds with documented biological activities have been identified in SJW (*12*). The LC fingerprinting technique is a state-of-the-art and powerful approach to identify and quantify phytochemicals in an herbal product, and LC-MS/MS is very helpful in confirming a compound's identity. We have chosen the Caco-2 model system to determine the absorption of phytochemicals present in SJW because the model system is recognized by FDA as a validated model to predict drug absorption in humans (*13*).

MATERIALS AND METHODS

SJW Products and Chemical Reagents. Twelve herbal supplements were bought from national chain stores located in Houston, TX, or from the Internet (**Table 1**). Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, and amentoflavone were obtained from ChromaDex (Irvine, CA). Hank's balanced salt solution was purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were HPLC grade and purchased from VWR (Suwanee, GA). Other chemicals were used as received.

HPLC Conditions for Fingerprint Analysis. The HPLC conditions were as follows: an Agilent 1050 running ChemStation software with a 759 A absorbance detector (Applied Biosystem, Foster city, CA); column, Dikma Diamonsil C_{18}, 5 μm, 250 × 4.6 mm (Dikma Technologies, Beijing, China); mobile phase A (MPA), acetonitrile; mobile phase B, 0.1% formic acid in water; elution gradient, 0−15 min, 10% MPA, 10−40 min, 10−30% MPA, 40−50 min, 30−60% MPA, 50−60 min, 60−90% MPA, 60−65 min, 90−10% MPA, 65−70 min, 10% MPA; detect wavelength, 254 nm; flow rate, 1 mL/min; and injection volume, 200 μL. The retention times for rutin, hyperoside, isoquercitrin, quercitrin, quercetin, and amentoflavone were 21.00, 23.95, 25.28, 36.48, 46.48, and 48.33 min, respectively.

Table 2. Concentrations of the Marker Compounds in Caco-2 Experiments

product	rutin (μM)	hyperoside (μM)	isoquercitrin (μM)
1	9.70	5.00	6.97
2	3.32	3.20	0.30
4	4.30	3.35	2.64

Identification of Selected Compounds Present in SJW Extracts with UPLC-MS/MS. The UPLC conditions were as follows: system, Waters Acquity with PDA detector; column, Acquity UPLC BEH C_{18} column (50 × 2.1 mm i.d. 1.7 μm; Waters, Milford, MA); mobile phase A (MPA), 0.1% formic acid in water; mobile phase B (MPB), 100% acetonitrile; elution gradient, 0−0.5 min, 0% MPB, 0.5−0.7 min, 0−10% MPB, 0.7−3.0 min, 10% MPB, 3.0−4.0 min, 10−90% MPB, 4.0−4.5 min, 90% MPB, 4.5−5.0 min, 90−0% MPB, 5.0−5.5 min, 0% MPB. An ABI 3200 Q-TRAP triple-quadruple mass spectrometer (Applied Biosystems/MDS SCIEX) equipped with a turbo ion spray source operating in negative ion mode was used to perform the MS/MS analysis.

Sample Preparation from Extracts and Analysis with HPLC. An amount equal to the daily dose of each of the 12 products was suspended in equivalent volumes (60 mL) of MeOH/H_2O = 1:1 and sonicated three times at room temperature (22 °C), each time for a period of 1 h. The supernatant was removed following each sonication, and the supernatants were combined to afford 12 extracts. The original extracts (1 mL of each) were diluted 8 times by 20% MeOH in water, and 200 μL of each of the diluted samples was injected for the HPLC analysis; 10 μL of some diluted samples was injected in UPLC-MS/MS for phytochemical identification and measurements. Prunetin was used as internal standard.

HPLC Standard Curves and Quality Control Sample Preparation. The standard curve for HPLC analysis was prepared in 20% MeOH from the stock solution (10 mg/mL, in DMSO/EtOH = 1:4) of each of the individual standard compounds. Predetermined amounts of the stock solutions were mixed in 20% MeOH, which was further diluted by the same solvent to afford the standard curve samples. The standard curves ranged from 16.6 to 133.3 μg/mL for both rutin and quercetin, from 5.0 to 40.0 μg/mL for hyperoside, from 6.6 to 53.0 μg/mL for isoquercitrin, and from 1.6 to 13.3 μg/mL for amentoflavone, and they produced correlation coefficient values of >0.99 for all six compounds. Prunetin was also selected as internal standard. Ten microliters of 0.25 mM prunetin in acetonitrile was added to 300 μL of samples. The injection volume was 200 μL. The quality control samples were prepared by mixing rutin, hyperoside, and isoquercitrin in 20% MeOH from stock solution at low, medium, and high concentration for method validation study.

Extract Sample Stability. Portions of the diluted extracts (200 μL) from products 1, 2, and 4 extracts were injected in HPLC. Additional portions of the same extracts (200 μL) were then kept at room temperature for 24 h and injected again at day 2. The relative peak areas of the six identified compounds were compared to evaluate sample stability.

Table 3. Compound-Dependent Parameters in UPLC-MS Analysis

compound	Q1 (*m*/*z*)	Q3 (*m*/*z*)	DP (V)	CEP (V)	CE (V)	CXP (V)	dwell time (ms)
rutin	609.0	300.8	−73	−31	−49	−2	100
hyperoside	463.0	300.8	−58	−31	−33	−3	100
isoquercitrin	463.3	300.3	−61	−57	−35	−2	100
kaempferol-3-rutinoside	593.3	284.6	−50	−27	−49	−2	100
formononetin	267.1	252.1	−47	−23	−26	−2	100

Table 4. Intraday and Interday Precision and Accuracy for Rutin, Hyperoside, and Isoquercitrin

	HPLC					UPLC-MS/MS				
		interday (n = 5)		intraday (n = 5)			interday (n = 5)		intraday (n = 5)	
analyte	concn (μg/mL)	accuracy (bias, %)	precision (CV, %)	accuracy (bias, %)	precision (CV, %)	concn (μM)	accuracy (bias, %)	precision (CV, %)	accuracy (bias, %)	precision (CV, %)
rutin	66.6	106.2	0.7	104.0	1.2	10	101.6	3.4	93.0	9.6
	40.0	101.2	1.1	100.7	0.6	0.3125	97.4	2.6	103.3	9.5
	16.6	107.8	0.7	110.5	4.3	0.00488	97.1	4.4	98.9	9.8
hyperoside	32.0	109.1	0.4	106.7	1.3	10	99.7	4.1	90.2	7.5
	12.0	103.0	1.1	102.1	0.6	0.3125	94.9	1.5	91.7	8.1
	6.0	109.8	0.8	115.2	7.5	0.00488	97.3	9.8	102.3	9.3
isoquercitrin	40.0	102.8	0.9	104.9	1.3	10	103.3	9.5	92.7	6.0
	20.0	103.3	1.1	102.0	0.8	0.3125	98.8	7.3	95.1	13.3
	8.0	103.0	0.8	95.4	4.1	0.00488	107.6	3.5	97.6	9.4

The result indicated that there were no statistical differences ($p > 0.05$) between these two injections for all of six compounds.

Extraction Reproducibility. Three daily doses of product 1 were extracted five times according to the normal extraction procedure described previously. Samples were analyzed by HPLC. The relative peak areas of the six compounds did not change significantly ($p > 0.05$), suggesting the extraction procedure was reproducible.

Selection of Marker Compounds for Transport Study. Three key components of SJW, rutin, hyperoside, and isoquercitrin, were selected as transport markers due to their high contents and potential biological benefits (e.g., antidepressant) (*14*, *15*). Moreover, they are homologous compounds, and the transport result may reveal structure transport relationships. Products 1, 2, and 4 were selected as the study products because they are the most popular brands on the market. The concentrations of these three compounds used in the current study ranged from 0.3 to 9.7 μM (**Table 2**).

UPLC-MS/MS Analysis of Rutin, Hyperoside, and Isoquercitrin. The UPLC conditions were described previously under the UPLC-MS/MS compounds identification section. The MS quantitative analysis for rutin, hyperoside, and isoquercitrin in Caco-2 transport study conditions were as follows: negative mode, ion spray voltage, −4.5 kV; ion source temperature, 650 °C; nebulizer gas (gas 1), nitrogen, 40 psi; turbo gas (gas 2), nitrogen 60 psi; curtain gas. The quantification was performed by using a multiple-reaction monitoring (MRM) method with ion pair transition to monitor each analyte. Unit mass resolution was set in both mass-resolving quadruples Q1 and Q3. Compound-dependent parameters are in **Table 3**.

Transport Study Sample Preparation and Stability. A 4 mL portion of the original extract, as prepared above, was dried under N_2. The dried residues were dissolved in 60 μL of DMSO/EtOH = 1: 4, and the resulting solution was diluted 1000 times by HBSS (pH 6.8) to afford the donor solution. Pure rutin, hyperoside, isoquercitrin, and kaempferol-3-rutinoside were prepared as 10 mM (50 mM for kaempferol-3-rutinoside) stock solutions in DMSO/EtOH = 1: 4 and then diluted 1000 times by HBSS for use as the donor solution in a Caco-2 experiment. The experimental solutions were kept in a 37 °C water bath, and samples were taken at 0, 1, 2, 4, and 6 h for UPLC-MS/MS analysis. The relative peak areas of rutin, hyperoside, and isoquercitrin were compared, and there was no statistical difference as a function of time, indicating that these three compounds in samples were stable.

Standard Curves and Quality Control Samples for Transport Study. The standard curves for UPLC-MS/MS analysis of rutin, hyperoside, and isoquercitrin were prepared in HBSS (pH 6.8) from 10 mM stock solution (DMSO/EtOH = 1:4) at 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0.0195, 0.00975, 0.00488, and 0.00244 μM. Formononetin was used as the internal standard, and 125 μL of 10 μM formononetin in acetonitrile was added to 500 μL of samples for UPLC-MS/MS analysis (injection volume = 10 μL). The quality control samples were prepared by mixing rutin, hyperoside, and isoquercitrin from stock solution in HBSS (pH 6.8).

Caco-2 Cell Culture. Caco-2 TC7 cells were originally a kind gift of Prof. Monique Rousset of INSERM U178 (Villejuif, France). The Caco-2 cells were routinely performed in our laboratory for more than a decade. Briefly, a cell monolayer was prepared by seeding 400,000 cells per insert (Nunc, surface area = 4.2 cm^2, 3 μm pore size). Cells were maintained at 37 °C under 90% humidity and 5% CO_2. Monolayers were used between 19 and 22 days after seeding. The integrity of each monolayer was checked by measuring the transepithelial electrical resistance (Millicell ERS) before the experiment. The normal TEER values obtained were between 500 and 750 $\Omega \cdot cm^2$. Cell monolayers with TEER values of $< 400\ \Omega \cdot cm^2$ were not used. HBSS (9.8 g/mL) supplemented with $NaHCO_3$ (0.37 g/L), HEPES (5.96 g/L), and glucose (3.5 g/L) was used for all experiments after the pH had been adjusted to a desired value. Cells were used at passages 41−49.

Caco-2 Experiment. The experiment protocol was from that described previously (*16*). Briefly, the testing solution with appropriate concentrations of product extract or pure compound was loaded onto the apical or basolateral (donor) side. Five donor samples (500 μL) and five receiver samples (from basolateral or apical side, respectively) (500 μL) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 500 μL of fresh donor solution to the donor side or 500 μL of fresh buffer to the receiver side. The samples were then analyzed by UPLC-MS/MS. Inhibitors were added only at the apical side. The apparent permeability coefficient (P) was determined by the equation

$$P = (dQ/dt)/(A \times C_0)$$

where dQ/dt is the drug permeation rate (mmol/s), A is the surface area of the epithelium (cm^2), and C_0 is the initial concentration in the donor compartment at time 0 (mMol).

Statistical Analysis. One-way ANOVA or a Student's t test (Microsoft Excel) was used to analyze the data. The prior level of significance was set at 5%, or $p < 0.05$. Values are expressed as mean ± SD.

Method Validation for HPLC and UPLC-MS/MS Quantitative Analysis. The interday and intraday precisions for HPLC and UPLC-MS/MS analyses were determined by injecting the quality control samples at low, medium, and high concentrations for rutin, hyperoside, and

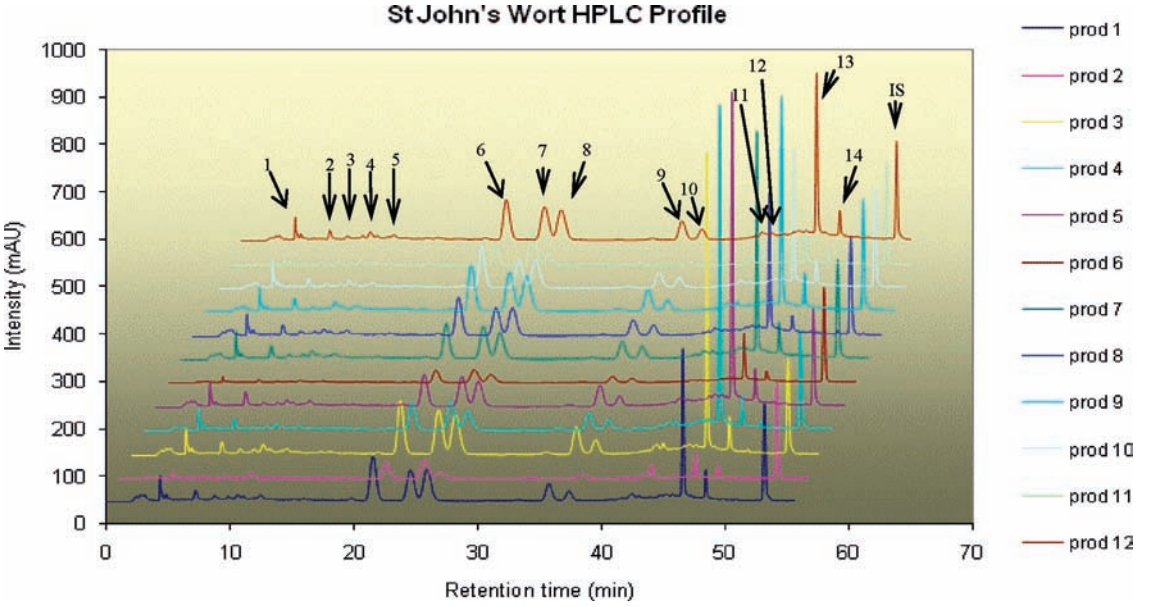

Figure 1. HPLC chromatograms of extracts of 12 St. John's wort supplement products.

isoquercitrin on the first day and on each of the following two days. The quality control samples were prepared as described previously. The validation results, which are summarized in **Table 4**, revealed that the precision and accuracy values for HPLC and UPLC-MS/MS quantitative analysis were well within the acceptance range (<15%).

RESULTS

HPLC Fingerprints and Identification of Major Compounds. The 12 different SJW products displayed distinctive HPLC fingerprint chromatographs (**Figure 1**). Fourteen common peaks were observed in these chromatographs including five minor peaks eluted before 20 min and six major and three minor peaks eluted after 20 min. The HPLC profiles were similar among these 12 products, indicating that the UV−visible components contained in SJW products were essentially identical. However, the relative peak areas of the major components were highly variable. Six major peaks were unambiguously assigned as rutin (6), hyperoside (7), isoquercitrin (8), quercitrin (10), quercetin (13), and amentoflavone (14) (**Figure 2**), respectively, by HPLC co-injection with authentic compounds, which were further confirmed by UV spectra and MS/MS data (*12*) (**Table 5**). The possible MS/MS spectra of these identified compounds are also shown in **Figure 3**.

Lack of Content Uniformity among the 12 Products. The content uniformity of these major compounds was poor across the 12 products (**Table 6**). The amount per daily dose varied from 3.51 to 18.93 mg (5.4-fold) for rutin, from 2.00 to 9.27 mg (4.6-fold) for hyperoside, from 0.37 to 10.66 mg (28.8-fold) for isoquercitrin, from 2.86 to 18.39 mg (6.4-fold) for quercitrin, from 1.31 to 13.70 mg (10.5 times) for quercetin, and from 0.47 to 1.71 mg (3.6-fold) for amentoflavone. The total amount of these phenolics in one product ranged from 70.33 to 12.35 mg, nearly 6-fold. Statistical analysis showed that all of the compound contents in product 2 were statistically different from those in product 3, indicating that product 2 was significantly different from product 3 (**Table 7**). Similarly, product 2 was significantly different from product 12, whereas products 3 and 5 were different from product 6. Statistical analysis also revealed that there was no difference between products 1 and 10 as well as no difference between products 8, 9, 10, and 12. However, except for products 1, 8, 9, 10, and 12, at least one compound was different among all of the tested products. Therefore, poor content uniformity among the SJW products was obvious, which is disconcerting because they all have the same product name.

Transepithelial and Vectorial Transport of Marker Compounds in Different Product Matrices. Transepithelial transport of three key components in SJW products across the Caco-2 cell monolayer was found to be different from those of control experiments employing pure compounds (10 μM, **Figure 4**). For rutin, $P_{a\text{-}b}$ in the three products was not statistically altered when compared with that of control. However, rutin's $P_{b\text{-}a}$ values in products 1 and 4 were 7.3-fold higher and in product 2 was 3.7-fold higher than that of control. Hyperoside's $P_{a\text{-}b}$ in product 1 was not altered, but in products 2 and 4 these values were only 54 and 46% of the control's. Moreover, hyperoside's $P_{b\text{-}a}$ values in products 1 and 4 were increased 1.9- and 3.4-fold, respectively. For isoquercitrin, when compared to control, its $P_{a\text{-}b}$ values were 3.5-, 2.3-, and 2.0-fold higher in products 1, 2, and 4, respectively. On the other hand, its $P_{b\text{-}a}$ values in products 1 and 2 were statistically lower (78% in product 1 and 42% in product 2) than that of control. The results clearly revealed that product matrices affected permeabilities of key components.

Vectorial transport, as measured by permeability ratios between basolateral to apical permeability and apical to basolateral permeability, was affected differently across tested products (**Figure 5**). For rutin, efflux ratios (P_{ba}/P_{ab}) were 1.63 and 3.64 in products 1and 4, respectively. In product 2, the ratio was approximately 1. When transport of hyperoside was performed, efflux ratios were 1.39 and 4.25 in products 2 and 4 but 1 in product 1. When transport of isoquercitrin was conducted, efflux ratios were 1.56 and 3.59 in products 1 and 4, whereas the ratio was 1 in product 2. For controls, only isoquercitrin showed significant efflux with a ratio of 7.13. These results suggested that transport behaviors of key compounds were significantly affected by product matrices.

Effects of Matrix on Transport of Kaempferol-3-rutinoside. To confirm the presence of matrix effect, transport of kaempferol-3-rutinoside (**Figure 2**) was studied because kaempferol-3-rutinoside is not present in SJW and is structurally similar to other key components present in SJW. The results showed that when kaempferol-3-rutinoside was used as a pure compound in the Caco-2 cell transport experiment, its vectorial transport

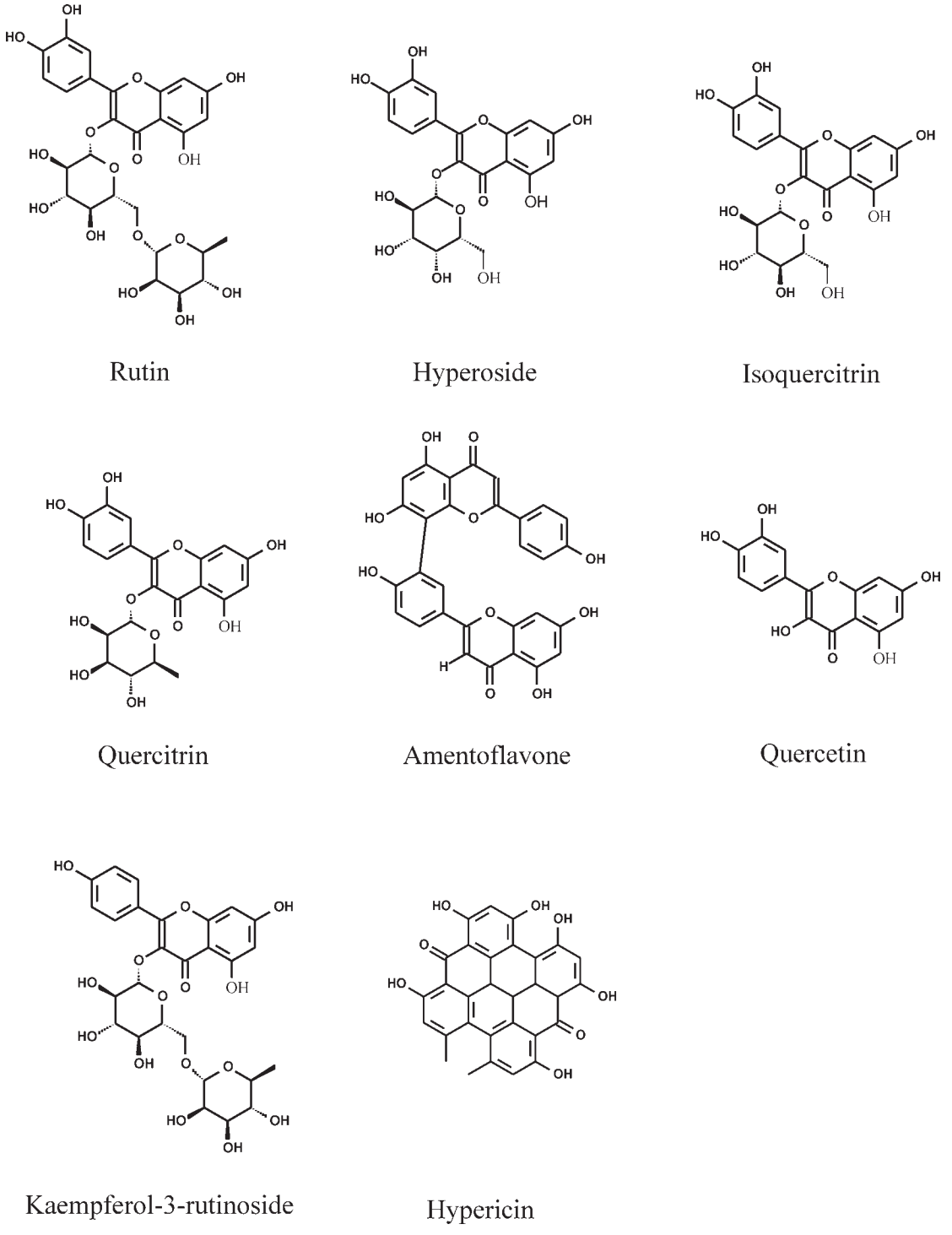

Figure 2. Chemical structures of compounds studied in this paper.

ratio was 0.16. However, in product 4, its efflux ratio was 3.5 (**Figure 6**). The result clearly revealed that the absorptive transport was inhibited and the efflux was accelerated in product 4.

Effect of MRP2 Inhibitor on Isoquercitrin Transport. When transport of pure isoquercitrin was studied, its vectorial transport ratio was 7.13, suggesting the involvement of an efflux transporter. To determine which efflux transporters might be involved, studies were conducted in the presence of 50 μM MK 571 (**Figure 7a**,**b**). The results showed that $P_{a\text{-}b}$ increased 3.2 times, whereas $P_{b\text{-}a}$ decreased 5.2 times compared to control, resulting in a vectorial transport ratio of 1, suggesting efflux was inhibited completely or nearly completely. When isoquercitrin was transported in the presence of 50 μM MK 571 in product (**Figure 7c**,**d**), its $P_{b\text{-}a}$ did not change but $P_{a\text{-}b}$ was significantly increased (2.9 times) in product 4. The $P_{b\text{-}a}$ was not statistically different from $P_{a\text{-}b}$. In product 2, $P_{b\text{-}a}$ was reduced 1.8 times, whereas $P_{a\text{-}b}$ did not increase significantly. The efflux ratio was 0.4 in product 2.

Phloridzin, a substrate of both SGLT1 and MRP1/2 (*17*), was used to study the uptake transporter of isoquercitrin (**Figure 7a**,**b**). Interestingly, in the presence of 50 μM phloridzin, isoquercitrin $P_{a\text{-}b}$ was increased 2.9 times (compared to the control), and its $P_{b\text{-}a}$ was decreased 2.1 times. The difference between its $P_{a\text{-}b}$ and $P_{b\text{-}a}$ was diminished.

Effects of Concentration on Rutin Transport. Because it was not possible to determine the transport of the same flavonoid glucoside at one uniform concentration across different products, effects of concentration on transport of rutin were determined at 5 and 10 μM concentrations. The results showed that concentration change did not significantly affect transepithelial transport of rutin (**Figure 8**).

Marker Compound Hydrolysis and Metabolism. Marker compounds rutin, hyperoside, and isoquercitrin used in transport studies were not hydrolyzed because their aglycone was not detected by UPLC-MS/MS. Glucuronidation and sulfation metabolites of these three marker compounds and their aglycone were not detected either.

Table 5. Identification of Key Phenolic Compounds by HPLC-UV and UPLC-MS

	retention time (min)			
compound	HPLC	UPLC	UV λ_{max} (nm)	ESI-MS (−) *m/z*
rutin	21.0	2.80	254.7, 354.4	609.3 $[M-H]^-$
hyperoside	23.95	2.60	254.7, 354.4	463.1 $[M-H]^-$
isoquercitrin	25.28	2.85	254.7, 354.4	463.2 $[M-H]^-$
quercitrin	36.48	3.56	254.7, 354.4	447.3 $[M-H]^-$
quercetin	46.48	3.71	254.7, 367.3	301.1 $[M-H]^-$
amentoflavone	48.33	3.84	268.9, 335.3	537.1 $[M-H]^-$

DISCUSSION

Our study showed clearly that different SJW products have different HPLC fingerprints and that phenolic contents of different products were highly variable. Furthermore, our study demonstrated for the first time that the transport of phenolic compounds

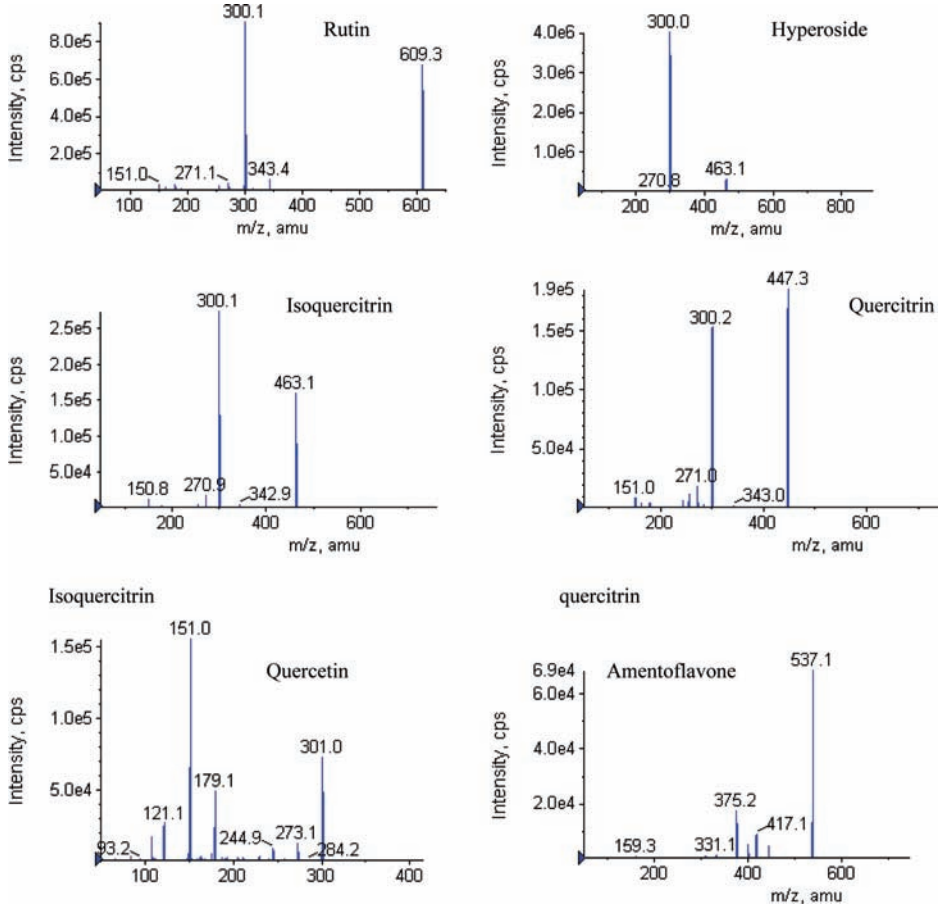

Figure 3. MS/MS profiles of six identified phenolics derived from SJW.

Table 6. Contents of Six Major Phenolic Compounds per Daily Dose[a]

product	rutin (mg)	hyperoside (mg)	isoquercitrin (mg)	quercitrin (mg)	quercetin (mg)	amentoflavone (mg)	total phenolics (mg)
1	16.07 ± 0.76	6.32 ± 0.78	8.81 ± 1.11	12.57 ± 1.75	6.02 ± 0.55	1.46 ± 0.05	51.25
2	5.47 ± 0.13	4.05 ± 0.19	0.37 ± 0.06	3.75 ± 1.33	1.31 ± 0.06	0.67 ± 0.15	15.62
3	18.93 ± 0.67	9.27 ± 0.78	10.66 ± 0.84	18.39 ± 0.54	11.50 ± 0.21	1.58 ± 0.29	70.33
4	7.08 ± 0.15	4.36 ± 0.34	3.34 ± 0.17	8.42 ± 0.68	10.16 ± 0.34	1.21 ± 0.15	34.57
5	10.26 ± 0.50	5.67 ± 0.55	5.78 ± 0.46	11.52 ± 1.62	13.70 ± 0.43	1.71 ± 0.19	48.64
6	3.51 ± 0.07	2.00 ± 0.16	0.97 ± 0.07	3.29 ± 0.15	2.11 ± 0.03	0.47 ± 0.02	12.35
7	10.69 ± 0.37	6.36 ± 0.84	6.68 ± 1.12	14.19 ± 1.55	8.43 ± 0.12	1.42 ± 0.18	47.77
8	13.65 ± 1.25	5.78 ± 0.84	7.68 ± 1.27	12.65 ± 1.91	6.79 ± 0.11	0.95 ± 0.09	47.68
9	14.97 ± 0.18	7.25 ± 1.06	9.00 ± 1.35	11.67 ± 2.84	7.19 ± 0.73	1.51 ± 0.19	51.59
10	15.84 ± 0.97	6.94 ± 1.02	9.36 ± 1.36	15.07 ± 2.84	6.57 ± 0.22	1.25 ± 0.12	55.03
11	5.69 ± 0.34	7.91 ± 0.23	1.56 ± 0.37	2.86 ± 0.49	1.56 ± 0.15	0.56 ± 0.15	20.14
12	13.89 ± 1.24	7.46 ± 0.49	8.61 ± 0.46	11.25 ± 1.37	6.77 ± 0.19	1.22 ± 0.12	49.20

[a] Data were based on three individual experiments.

Table 7. Statistical Analysis of Six Major Compounds in Different Products per Daily Dose[a]

product	1	2	3	4	5	6	7	8	9	10	11	12
1		+§#∗$	+#∗	+§∗	+§∗	+§#∗$	+∗	+	∗		+§#∗$	+
2	+§#∗$		+Δ§#∗$	§∗$	+§#∗$	Δ	+§#∗$	+§#∗$	+§#∗$	+§#∗$	Δ	+Δ§#∗$
3	+#∗	+Δ§#∗$		+Δ§#∗	+§#∗	+Δ§#∗$	+§∗	+§#∗	+#∗	+#∗	+§#∗$	+#∗
4	+§∗*	§∗$	+Δ§#∗		+∗	+Δ#∗$	+§#∗	+§∗	+§∗	+§#∗	Δ#∗$	+Δ§∗
5	+§∗	+§#∗$	+§#∗	+∗		+Δ§#∗$	∗	+∗$	+§∗	+§∗	+§#∗$	+§∗
6	+§#∗$	Δ	+Δ§#∗$	+Δ#∗$	+Δ§#∗$		+§#∗$	+§#∗	+§#∗$	+§#∗$	+Δ	+Δ§#∗$
7	+∗	+§#∗$	+§∗	+§#∗		+§#∗$		+§∗	+∗	+§∗	+§#∗$	+∗
8	+	+§#∗	+§#∗$	+§∗	+∗$	+§#∗	+∗			+∗	+§#∗	
9	∗	+§#∗$	+#∗	+§∗	+§∗	+§#∗$	+∗				+§#∗$	
10		+§#∗$	+#∗	+§#∗	+§∗	+§#∗$	+§∗	+			+§#∗$	
11	+§#∗$	Δ	+§#∗$	Δ#∗$	+§#∗$	+Δ	+§#∗$	+§#∗	+§#∗$	+§#∗$		+#∗$
12	+	+Δ§#∗$	+#∗	+Δ§∗	+§∗	+Δ§#∗$	+∗				+#∗$	

[a] One-way ANOVA was used to analyze the data shown in **Table 6**. The symbols indicate statistically significant difference ($p < 0.05$): + for rutin; Δ for hyperoside; § for isoquercitrin; # for quercitrin; * for quercetin; $ for amentoflavone. For example, data in this table showed that among the six major compounds, products 1 and 2 were statistically different in the contents of rutin (+), isoquercitrin (§), quercitrin (#), quercetin (∗), and amentoflavone ($). There was no difference in the content of hyperoside (no symbol).

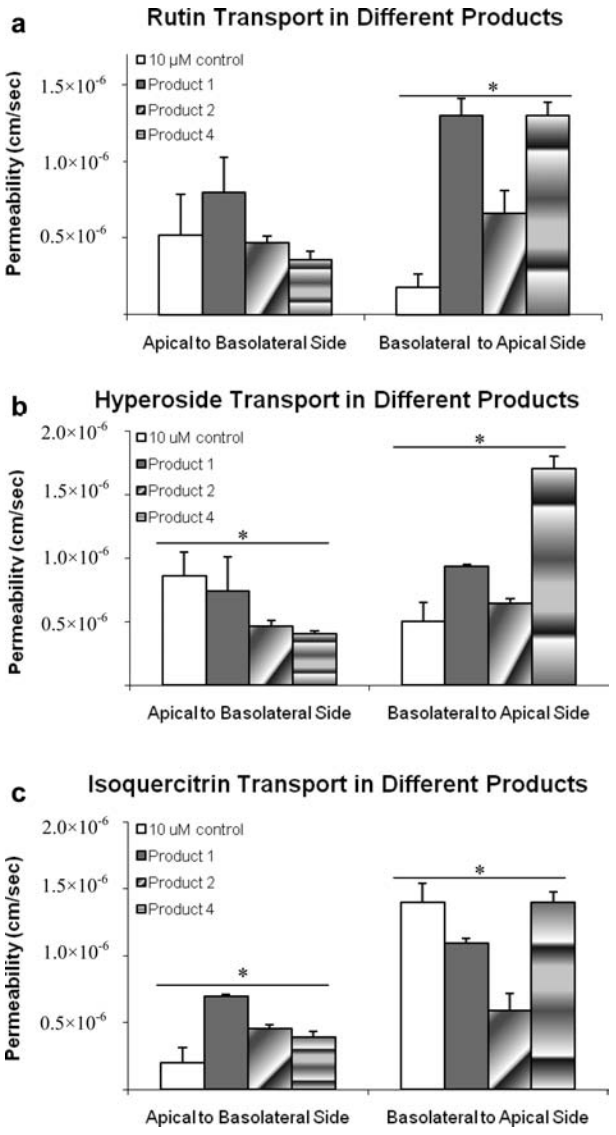

Figure 4. Transport of three marker compounds in pure form or in one of the three product matrices. The buffer used in both donor and receiver sides was pH 6.8 HBSS. The experiments were performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean. The asterisk (∗) indicates a statistically significant difference between product and control ($p < 0.05$, one-way ANOVA).

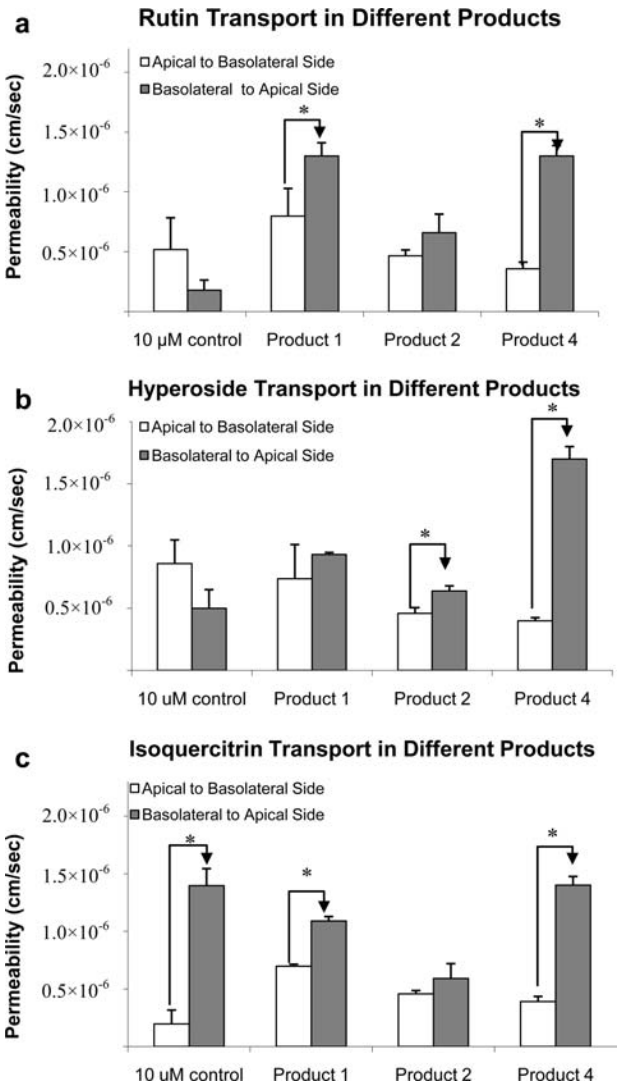

Figure 5. Effect of product matrices on the basolateral to apical over apical to basolateral permeability ratios. The buffer used in both donor and receiver sides was pH 6.8 HBSS. The experiments were performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean. The asterisk (∗) indicates a statistically significant difference between apical to basolateral and basolateral to apical transport ($p < 0.05$, unpaired Student *t* test).

across the Caco-2 cell monolayers was significantly altered by the product matrices. Use of MRP2 inhibitor MK-571 suggests that some of the matrix effects observed in the transport of key phenolic products were possibly the results of interaction of other components present in these products with MRP efflux transporters.

Contents of major phenolics were highly variable across 12 products, even though all of the products contained these phenolics on the basis of UV and MS chromatograms (**Figure 1**; **Tables 6** and **7**). The high variability means that consumers cannot obtain the same amount of these compounds by taking different SJW products, even if they correctly follow the manufacturers' recommendations. For example, by taking product 1, a consumer will take 51.25 mg of major phenolics, including 16.07 mg of rutin, 6.32 mg of hyperoside, 8.81 mg of isoquercitrin, 12.57 mg of quercitrin, 6.02 mg of quercetin, and 1.46 mg of amentoflavone. On the contrary, he/she will take almost equal amounts (48.64 mg, 0.95-fold) of total phenolics by consuming product 5, but the compounds were quite different, being 10.26 mg (0.64-fold) of

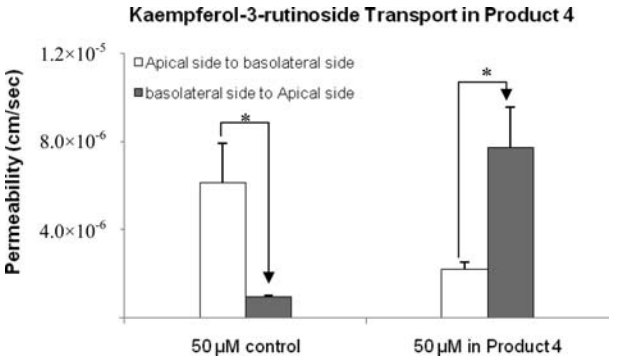

Figure 6. Matrix effect on kaempferol-3-rutinoside transport. The buffer used in both donor and receiver sides was pH 6.8 HBSS. The experiments were performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean. The asterisk (∗) indicates a statistically significant difference between apical to basolateral and basolateral to apical transport ($p < 0.05$, unpaired Student *t* test).

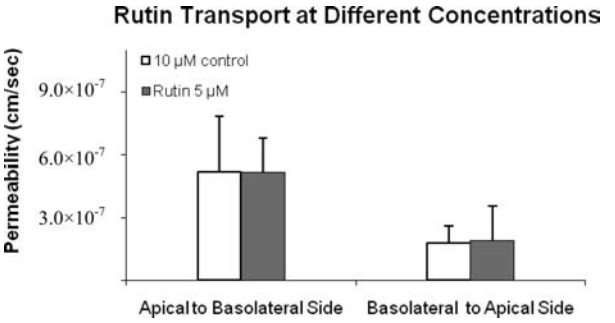

Figure 8. Effects of concentration on rutin transport. The buffer used in both donor and receiver was pH 6.8 HBSS. The experiments were performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean.

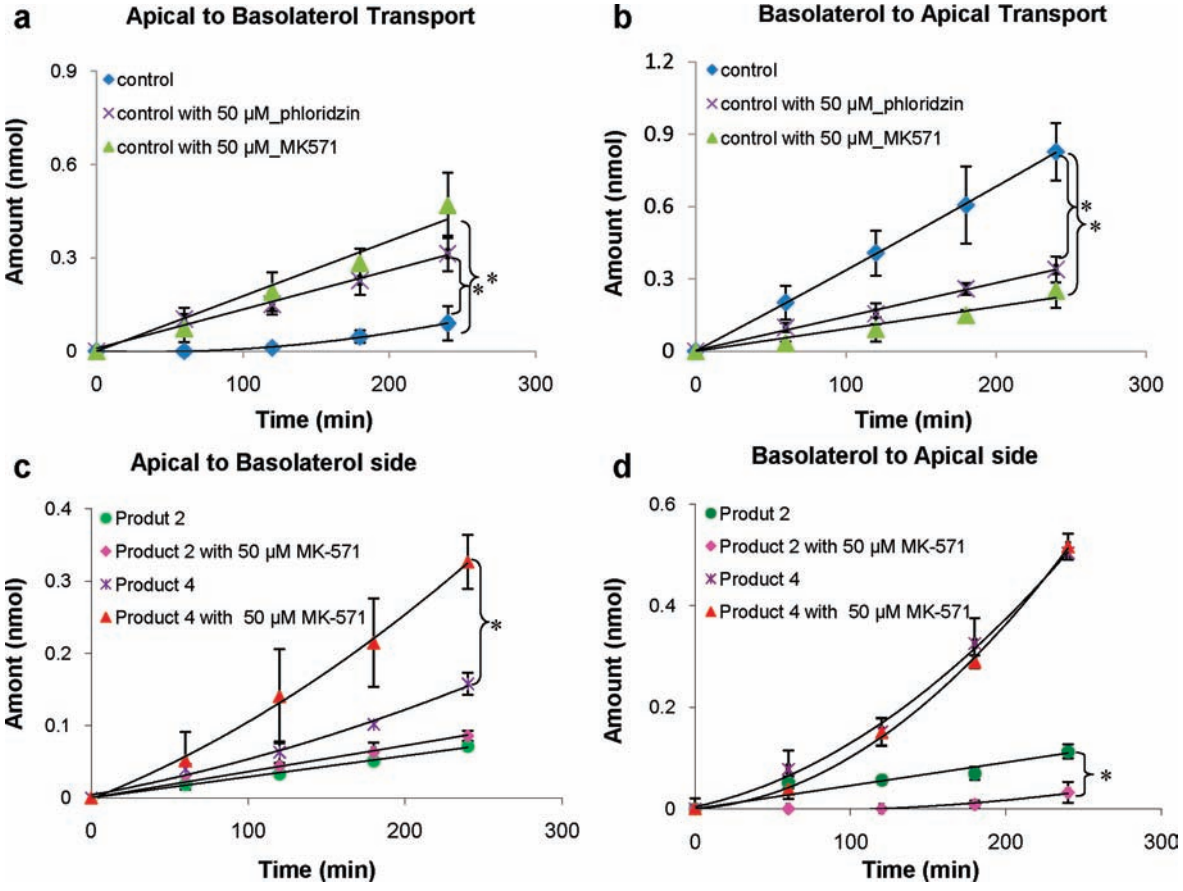

Figure 7. Effect of MK-571 and phloridzin on transport of isoquercitrin in pure form (upper two panels) or in product matrices (lower two panels). Both apical to basolateral (**a**, **c**) and basolateral to apical (**b**, **d**) transports were monitored. The concentration of control is 10 μM. The buffer used in both donor and receiver sides was pH 6.8 HBSS. The experiments were performed at 37 °C. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean. The asterisk (∗) indicates a statistically significant difference between with and without MK-571 ($p < 0.05$, unpaired Student *t* test).

rutin, 5.67 mg (0.90-fold) of hyperoside, 5.78 mg (0.66-fold) of isoquercitrin, 11.52 mg (0.9-fold) of quercitrin, 13.70 mg (2.3-fold) of quercetin, and 1.71 mg (1.2-fold) of amentoflavone. In product 5, the amounts of quercitrin and amentoflavone were higher than in product 1, whereas the other key components were less. If products 3 and 6 were taken, 70.33 and 12.35 mg of total phenolics will be administered, respectively, and the latter is significantly less than those of products 1 and 5. The lack of quality control criteria for SJW dietary supplement means that consumers will get different amounts of components by taking different products with the same product name.

In addition to the lack of uniformity in major phenolic contents, product differences also significantly affected the transport of these phenolics, making the differences in exposure to phenolics even greater. The study clearly revealed that product matrices, most likely due to the differences in phytochemical compositions, significantly affected the transport of phenolics across the Caco-2 cell monolayers (**Figures 4**−**7**). The transport was affected at multiple levels, although a higher permeability displayed by a phenolic was not correlated with a higher content in the product (**Table 6**; **Figure 4**). Surprisingly, the effects of matrices on transepithelial transport of all three phenolics had similar patterns (**Figure 4**), in that all three phenolics from the same product displayed the highest transepithelial permeabilities in either the apical to basolateral or basolateral to apical direction, although the extent of the differences was variable. The more dramatic differences were apparent when the permeability ratios were compared (**Figures 5** and **6**). The transport ratios often changed from favoring apical to basolateral absorption to favoring basolateral to apical efflux (**Figures 5** and **6**), except for isoquercitrin, which always favored efflux (**Figure 5c**). Another way to examine the effects of matrix on transport is to determine how different product matrices affect the permeability ratios of the phenolics. Product 4 always increased efflux ratios (**Figures 5** and **6**), including that of kaempferol-3-rutinoside, which is not present in SJW.

To determine the possible reasons why different products have influenced transport of phenolics differently, we first examined the known mechanisms of transport for flavonoid glycosides. Earlier works have shown that SGLT1 was involved in the apical uptake of flavonoid glycoside (e.g., quercetin-3-glucoside, quercetin 4′-glucoside, isorhamnetin-3-*O*-rutinoside), and MRP2 was involved in its apical efflux (*17*−*20*). Later studies showed that flavonoid glycosides presented in plants could inhibit the functions of MRPs (*21*, *22*). The results of the present studies are consistent with these published conclusions such that three of the four pure flavonoid glycosides (other than isoquercitrin) had higher (although not always statistically significant) apical to basolateral permeability than basolateral to apical permeability, suggesting that SGLT1 may be involved in the uptake of these flavonoid glycosides (*17*, *18*). However, evidence accumulated here did not support the involvement of SGLT1 in isoquercitrin uptake as its apical to basolateral transport was always lower than the basolateral to apical transport in the absence of an inhibitor (**Figures 5c** and **7**). At any rate, this role of SGLT1 could not be excluded completely because this compound is heavily effluxed and possibly poorly transported by SGLT1, making the detection of its contribution more difficult. It was quite surprising that in product 4, basolateral to apical transport of all four flavonoid glycosides became substantially higher than their apical to basolateral transports, suggesting this product may contain ingredients that enhance the functions of MRP2 (**Figure 5**). If more detailed in vivo studies can confirm this observation, it would represent the first known case that short-term treatment of herbal supplement could enhance the function of an efflux transporter. Earlier study has shown that the transporter function was inhibited by the flavonoid and other phytochemical glycosides (*21*, *22*).

One of the interesting findings of our study was that three marker compounds, all glycosides, were not found to be hydrolyzed in the study. Although it is generally recognized that flavonoid glycosides can be hydrolyzed by microflora β-glycosidases to the aglycone in the gastrointestinal tract (*23*, *24*). Caco-2 cells usually do not possess β-glycosidase activities (*19*, *25*). This is different from the in vivo situation, where microflora bacteria are abundantly present in the lower small intestine (i.e., ileum) and colon. Another interesting finding is that all three flavonoid glycosides were shown to be permeable, albeit poorly. Previously, Serra et al. reported that diosmin, hesperidin, and naringin were not permeable (*26*), but the difference may be due to the lower sensitivity of analysis method used in that paper (HPLC-UV) and shorter duration of the transport study (120 min). In our study, a more sensitive UPLC-MS analysis method was used and the transport duration was 240 min. In any case, our reported permeabilities were close to two previously reported permeability values of flavonoids by Zou et al. (*25*) and Tian et al. (*22*), both less than 1.0×10^{-6} cm/s, but higher than the permeability of naringin reported by Tourniaire et al., which was 0.8×10^{-7} cm/s at 90 μM (*27*). The reason for this discrepancy is unclear. Taken together, all of the flavonoid glycosides have permeability that is lower than 1×10^{-6} cm/s, indicating poor absorption with a predicted percent absorption in humans of $< 15\%$ (*28*).

One of the unexpected findings of this study was that hypericin was not detected in any of the product extracts. Hypericin (**Figure 2**), a potentially beneficial naphthodianthrone standardized by the manufacturer, was not detected by HPLC in this experiment. We believed that this is because hypericin is highly unstable in aqueous solutions, especially under light exposure as used in our experiments (*29*). We tried to extract hypericin by adding 0.1% Vc and 0.01% of EDTA without light exposure, which is considered to be a common way to prevent oxidation via free radicals (*30*, *31*), but hypericin was not found using our UPLC-MS method. Draves and co-workers showed that most labels overestimate hypericin content in commercial SJW products (*32*). Only two products were proven to have a total naphthodianthrone concentration within 10% of their labeled claims, and no naphthodianthrone was detected in other products (*32*).

In conclusion, 12 St. John's wort products used in the present study contain highly variable amounts of phenolics. Because different products possessed highly variable matrices, the transepithelial transport of three key phenolics and one model phenolic (kaempferol-3-glycoside) across the Caco-2 cell monolayers was substantially affected by differences in the matrix composition. Because permeation across the intestinal membrane (i.e., Caco-2 cells) is the first step in the bioavailability continuum, the bioavailabilities of these phenolics will most likely be highly variable. Therefore, we conclude that standardization of herbal supplement is very important from pharmaceutical and biopharmaceutical points of view, as long as the ultimate goal is to make alternative herbal medicine such as SJW an attractive alternative to conventional western medical care.

ABBREVIATIONS USED

SJW, St. John's wort; LC, liquid chromatography; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; HPLC, high-performance liquid chromatography; UPLC, ultraperformance liquid chromatography; UPLC-MS/MS, ultraperformance liquid chromatography coupled with tandem mass spectrometry; MRP1/2, multidrug resistance-associated protein 1 or 2; SGLT1, sodium−glucose transport protein 1; TEER, transendothelial electrical resistance; HBSS, Hank's balanced salt solution.

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Received for review December 17, 2009. Revised manuscript received February 25, 2010. Accepted March 29, 2010. Funding for the study was provided by The Gustavus and Louise Pfeiffer Research Foundation and NIH GM070737, both to M.H.