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Bioavailability and metabolism of botanical constituents and enhancement of intestinal barrier function by caffeic acid derivatives in Caco-2 cells

by

Zhiyi Qiang

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Toxicology

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2011
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Public interest in botanical supplements has increased greatly in recent years as various plant materials might be used for anti-inflammatory, immunostimulatory, antioxidant and cancer preventive effects. Our long term goal is to improve our understanding of the characteristics of phytochemicals that contribute to human health benefits on gut functions, and thereby pave the way for optimizing herbal supplements for study in future clinical trials. In this dissertation, the overarching hypotheses were that major components of the ethanolic extracts of *Echinacea*, alkamides and ketones, and caffeic acid derivatives in the ethanolic extract of *Prunella vulgaris*, will be transferred by Caco-2 cell monolayers and caffeic acid derivatives will enhance the intestinal epithelial barrier function.

*Echinacea* has long been used as phytotherapy for wound healing, pain relief and treatment of the common cold. In the first study, Bauer alkamides, the key components contained in *Echinacea sanguinea* and *Echinacea pallida*, transferred across the Caco-2 cell monolayer via passive diffusion, independent of other constituents in plant extract. The apparent permeability coefficients ($P_{app}$) were $2.8 \pm 1.5 - 43.8 \pm 11.2$ cm/s $\times 10^{-6}$ for tested three alkamides and the order of the transfer of them across Caco-2 cells was increased paralleled with compound hydrophicity. Tested alkamides were seemingly N-glucuronidated and both *Echinacea* extracts stimulated apparent glucuronidation and basolateral efflux of alkamide metabolites. Bauer ketone 24 was totally metabolized to more hydrophilic metabolites as a pure compound, but not found in either *Echinacea* species. The addition of Bauer alkamides (175-230 µM) as well as the ethanolic extracts of *E. sanguinea* at 1 mg/mL (containing 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11) and *E. pallida* at 5 mg/mL (containing 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11) reduced the efflux of the P-glycoprotein transporter (P-gp) probe calcein-AM.
from Caco-2 cells. These results suggest that other constituents in the plant extract had a facilitating effect on the metabolism and efflux of alkamides and ketones from *Echinacea*, which would improve the therapeutic benefits of these extracts, and that alkamides and *Echinacea* extracts might be useful in potentiating some chemotherapeutics which are substrates for P-gp.

*Prunella vulgaris* is a perennial herb known as self-heal used to treat sore throat, fever, and wounds. Rosmarinic acid is a caffeic acid derivative found in various botanicals, especially in *P. vulgaris*. Ursolic acid, a pentacyclic triterpene acid, is also found in *P. vulgaris* but especially concentrated in *Salvia officinalis* (sage), which has been traditionally used to treat inflammation in the oral cavity, and may also be of interest in inhibiting gastrointestinal inflammation which is relevant to colitis and colon cancer. In the second study, $P_{app}$ for rosmarinic acid and rosmarinic acid in *P. vulgaris* extracts was $0.2 \pm 0.05 \times 10^{-6}$ cm/s, significantly increased to $0.9 \pm 0.2 \times 10^{-6}$ cm/s after β-glucuronidase/sulfatase treatment. $P_{app}$ for ursolic acid and ursolic acid in *S. officinalis* extract was $2.7 \pm 0.3 \times 10^{-6}$ cm/s and $2.3 \pm 0.5 \times 10^{-6}$ cm/s before and after β-glucuronidase/sulfatase treatment, respectively. Neither compound was affected in permeability by the herbal extract matrix. These results indicate that rosmarinic acid and ursolic acid in herbal extracts had similar uptake as that found using the pure compounds, which may simplify the prediction of compound efficacy, but the apparent lack of intestinal glucuronidation/sulfation of ursolic acid is likely to further enhance the bioavailability of that compound compared with rosmarinic acid.

In the third study, the effects of caffeic acid and related compounds on intestinal barrier function were investigated using Caco-2 cells as a model. Caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (mHPP, a microbial metabolite of
caffeic acid and rosmarinic acid) up-regulated the expression of zonula occludens (ZO-1), ZO-2, claudin-1 and occludin in Caco-2 cells. In addition, chlorogenic acid and mHPP were effective against adverse effects induced by inflammatory stimuli (LPS, interferon-γ, IL-1β, and TNF-α) on tight junction proteins in Caco-2 cells. Caffeic acid derivatives up-regulated claudin-4 in P-glycoprotein transporter MDR1- knockdown (KD) Caco-2 cells and only mHPP was effective against the changes in tight junction protein expression induced by inflammatory stimuli in MDR KD Caco-2 cells. Caffeic acid derivatives augmented TNF-α and IL-6 levels in Caco-2 cells under the stimulated condition, but significantly reduced both cytokines in MDR KD caco-2 cells plus the stimuli. These results indicate that caffeic acid derivatives enhanced barrier function in human intestinal Caco-2 cells and mHPP exhibited greater enhancement of intestinal barrier than the parent compounds. P-gp plays an essential role in the anti-inflammatory activities of caffeic acid derivatives.

In conclusion, these data confirmed the overarching hypotheses and suggest that the effect of plant matrix on bioavailability and metabolism of the constituent is compound specific, depending on the transfer mechanism; and caffeic acid derivatives could be gut health promoting as a dietary constituent, but these compounds might exacerbate damage under inflammatory stimuli. Moreover, intake of caffeic acid derivatives might speed up mucosal recovery or provide protection to the small-intestinal mucosa against the inflammatory mediators when P-gp inhibitors are co-administered, which may be attractive from a therapeutic point of view.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Echinacea* supplements were the most commonly used nonvitamin, nonmineral, natural products both for adults and children according to 2007 NHIS survey (National Health Interview Survey, 2007). Species of *Echinacea*, such as *E. angustifolia*, *E. pallida*, *E. sanguinea* and *E. purpurea*, have immune modulatory (Borchers et al., 2000), antiviral (Pleschka et al., 2009), and antibacterial activities (Sharma et al., 2010). Several alkamides from the roots of *E. angustifolia* and *E. pallida* have suppressed the expression of cyclooxygenase 2 (COX-2) and prostaglandin E 2 (PGE2) formation in H4 human neuroglioma cells (Hinz et al., 2007) and lipopolysaccharide (LPS) stimulated RAW264.7 macrophages. Bauer ketones 23 and 24 at 2.6 and 5 µM, respectively, were identified as significant contributors to the PGE (2) inhibition by *E. pallida* root extract at 25 µg/mL (LaLone et al., 2007). *E. pallida* root and aerial extracts at 2 mg/mL exhibited ~50% inhibition of 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical and the order of the antioxidant activities of the phenolics in *E. pallida* extract was echinacoside (EC_{50} = 6.6 µM) > cichoric acid (8.6 µM) > cynarin (11.0 µM) > chlorogenic acid (18.9 µM) > caffeic acid (19.1 µM) > caftaric acid (20.5 µM). The efficacy of *Echinacea* species may be related to the bioavailability of its constituents. In a randomized crossover single-dose study, 10 volunteers were orally administered standardized tincture of *E. purpurea* or twelve *E. purpurea* tablets, and both doses contained 0.07 mg of the major alkamides. Alkamides derived from the *Echinacea* tincture were detectable in serum within 30 min after administration and their concentration peaked at 0.4 ng/ml; alkamides in tablets reached a level of 0.12 ng/ml in serum 45 min after ingestion (Woelkart et al., 2006). However, caffeic acid conjugates (caftaric acid, cichoric acid and echinacoside) were not
detected in any plasma sample at any time after ingestion of *Echinacea* tablets manufactured from an ethanolic liquid extract in a human study (Matthias et al., 2005).

The genus *Prunella* (*Lamiaceae*), a perennial herb that is widely distributed throughout Asia and Europe, is used primarily as a traditional botanical medicine to reduce osteoarthritis, rheumatoid arthritis, and gingivitis (Song et al., 2007; Adámková et al., 2004; Jung et al., 2001). Rosmarinic acid (RA), one of the key constituent contained in *Prunella*, was found to exhibit anti-inflammatory and antioxidative properties (Huang et al., 2009; Psotova et al., 2003). Rosmarinic acid might be poorly absorbed in the small intestine because when *Perilla* extract containing 200 mg of RA was orally administered to six men, RA in both plasma and urine was present predominantly as glucuronide and/or sulfate conjugated forms, at 0.6 ± 0.2% and 1.5 ± 0.4% of the total intake, respectively, within 48 h after ingestion (Baba et al., 2005). Gut microbes may metabolize RA to give phenolics such as caffeic acid, *o*-coumaric acid and m-hydroxyphenylpropionic acid, which are then absorbed by monocarboxylic acid transporter (MCT)-mediated active processes (Konishi and Kobayashi, 2005).

Until now, studies have not been carried out to investigate the effects of plant extracts on the bioavailability and metabolism of alkamides, ketones and caffeic acid derivatives, nor have the mechanisms of the protection of caffeic acid derivatives on intestinal function been investigated. In this whole project, we focused our research interest in these less explored areas. The overarching hypotheses were that alkamides, ketones and caffeic acid derivatives will be transferred by Caco-2 cell monolayers and caffeic acid derivatives will enhance the intestinal barrier function. We conducted these studies to test three individual hypotheses within two main research fields: bioavailability and metabolism of botanical
constituents *in vitro* (Hypothesis and objective 1 and 2) and modulation of caffeic acid derivatives on intestinal function (Hypothesis and objective 3).

1. In the first study, we hypothesized that Bauer alkamides and ketones would be absorbed similarly as pure compounds or as part of complex plant extracts (*E. sanguinea* and *E. pallida*), and that these *Echinacea* extracts could inhibit P-glycoprotein (P-gp) transporter in Caco-2 human intestinal epithelial cells. Caco-2 cells were incubated with pure alkamides and ketone as well as with the two extracts that contain the specific alkamides to determine the permeability and metabolism of those key constituents both in pure solutions and in herbal extracts. Efflux of the P-gp probe calcein-AM across Caco-2 cells was assessed to study the inhibition of P-gp function by pure compounds and two extracts.

2. In the second study, our hypotheses were that the absorbability and metabolism of rosmarinic acid and ursolic acid was independent of the plant extract matrix. This study was also designed to investigate the permeability of rosmarinic acid and ursolic acid as pure compounds in comparison to the permeability of these compounds when added as a complex mixture in the form of *P. vulgaris* and *S. officinalis* ethanol extracts across Caco-2 cell monolayers. It is anticipated that the results of these studies will provide a foundation for future studies of to assess the efficacy of these herbs as therapeutic strategies to treat gastrointestinal inflammation.

3. In the third study, our hypothesis was that caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (mHPP), a microbial metabolite of caffeic acid and rosmarinic acid, will alter the intestinal barrier function in Caco-2 human intestinal epithelial cells and P-gp transporter, encoded by multidrug resistance (MDR1) gene, knockdown (KD) Caco-2 cells. Stimuli composed of interleukin (IL)-1β, tumor
necrosis factor (TNF)-α, interferon (IFN)-γ and lipopolysaccharides (LPS) were added to the cell system to represent acute inflammatory reactions and mimic inflammatory bowel disease. Tight junction protein expression and immune modulator excretion in both Caco-2 cell model and MDR knockdown (MDR KD) Caco-2 cells were measured.

**Dissertation Organization**

The contents of this thesis includes an introduction, which is comprised of a general introduction and a literature review that delves into the phytochemical profile, health benefits, bioavailability and metabolism of the key constituents in *Echinacea* and *Prunella vulgaris* ethanolic extracts. Chapter 3 consists of a manuscript that will be submitted to the *Journal of Planta Medica* entitled “Glucuronidation of alkamides and ketones in *Echinacea sanguinea* and *Echinacea pallida* extracts by Caco-2 cells and their modulation of P-glycoprotein transporter”. Co-authors on this manuscript include: Basil J. Nikolau, mentored Ludmila Rizhsky and provided the phytochemical profile in *Echinacea* ethanolic extracts using GC-MS; Patricia A. Murphy and Cathy Hauck worked on the extraction process for the *Echinacea* extracts; Mark P. Widrlechner and Joe-Ann McCoy provided all *Echinacea* plant material; Manju B. Reddy, mentored Zhiyi Qiang on Caco-2 cell culture; Suzanne Hendrich, mentored Zhiyi Qiang and provided guidance and edits to all research conducted and manuscript drafts. Chapter 4 consists of a manuscript submitted to the *Journal of Ethnopharmacology* entitled “Metabolism and permeability of rosmarinic acid in *Prunella vulgaris* and ursolic acid in *Salvia officinalis* extracts across Caco-2 cell monolayers”. Co-authors on this manuscript include: Patricia A. Murphy and Cathy Hauck worked on the extraction process for the *P. vulgaris* extracts; Mark P. Widrlechner and Joe-Ann McCoy provided all *P. vulgaris* plant material; Manju B. Reddy,
mented Zhiyi Qiang and Zhong Ye on Caco-2 cell culture; Suzanne Hendrich, mentored Zhiyi Qiang and Zhong Ye and provided guidance and edits to all research conducted and manuscript drafts. Chapter 5 consists of a manuscript that will be submitted to the *Journal of Nutrition* entitled “Caffeic acid derivatives enhanced epithelial barrier integrity and exhibited anti-inflammatory activities in P-glycoprotein transporter knockdown Caco-2 cells”. Co-authors on this manuscript include: Diane Birt, mentored Nan Huang and provided the guidance on Western Blot analysis of tight junction proteins and ELISA analysis of cytokines in Caco-2 cells; Kazuya Maeda, provided P-glycoprotein knockdown Caco-2 cells; Manju B. Reddy mentored Zhiyi Qiang on Caco-2 cell culture; Suzanne Hendrich, mentored Zhiyi Qiang and Li Li and provided guidance and edits to all research conducted and manuscript drafts. Finally, chapter 6 contains a general conclusion and recommendations for future research.
CHAPTER 2. LITERATURE REVIEW

1. GENERAL INTRODUCTION OF BOTANICAL MEDICINE

Botanical medicine, or phytomedicine, has become a promising area in US, an alternative to conventional medicine. Human use of botanical medicine has a long history, whose record can date back to about 60,000 years ago (Solecki, 1975). It is estimated that as much as 80% of the world population still depends on herbal medicines to meet primary healthcare, mainly for particular diseases or symptoms based on the properties of a particular botanical (van Wijk, 2000). Indeed, medicinal herbs play an essential role in drug development as ~30% of all current medical drugs sold worldwide contain compounds derived from plant material (FAO, 2004). In the US, herbal medicine is classified as dietary supplements, which are consumed without prescription. Notable examples of the botanical dietary supplements used widely in the US today include *Echinacea*, garlic, ginkgo biloba, ginseng, and St. John’s wort with the sales over 100 million annually.

2. OVERALL INTRODUCTION OF *ECHINACEA SANGUINEA* AND *PALLIDA*

The genus of *Echinacea* is a perennial herb that is native to North America and grouped within the *Asteraceae* family. Historically, *Echinacea* preparations have been used by Native Americans to relieve pain and to treat various conditions such as snakebites and wounds. Early European settlers learned of its medicinal effects, and expanded its uses in treatment of infectious diseases (Borchers et al., 2000). Today, *Echinacea* products are mainly used for reducing the duration and/or the symptoms associated with the common cold and other upper respiratory infections, although clinical trials have not established efficacy (Barrett et al., 2010; Turner et al., 2005). It is estimated that Americans spend over $100 million per year on *Echinacea* products and their sales make up 10% of the total
US herbal market (Islam and Carter, 2005). *Echinacea* supplement was the most commonly used nonvitamin, nonmineral, natural products both for adults and children according to 2007 NHIS survey (National Health Interview Survey, 2007). The nine species of *Echinacea* that have been characterized are *E. angustifolia*, *E. pallida*, *E. purpurea*, *E. sanguinea*, *E. simulata*, *E. tennesseensis*, *E. laevigata*, *E. atrorubens*, and *E. paradoxa*, with each possessing its own unique phytochemical components (Miller and Yu, 2005). The diversity, or in some cases similarity, of the constituents present in these *Echinacea* species contributes to the diverse bioactivities associated with the botanical *Echinacea*.

2.1 Phytochemistry of *E. sanguinea* and *E. pallida*

A wide range of phytochemicals have been found in *Echinacea*, some of which have been reported to have bioactivity. These include alkamides and ketones (Bauer, 2002; Woelkart et al., 2005), caffeic acid derivatives, such as caffeic acid, chlorogenic acid, caftaric acid, echinacoside, etc. (Cheminat et al., 1988, **Figure 1**), polysaccharides (Orinda et al., 1973), and glycoproteins (Egert and Beuscher, 1992).

![Figure 1](image1.jpg)

**Figure 1** Structure of common alkamides, ketones, and caffeic acid derivatives found in *Echinacea* species
E. angustifolia, E. sanguinea, E. purpurea, and E. tennesseensis ethanolic extracts (15 μg/mL of each) were analyzed by HPLC showing greater quantities of Bauer alkamides than ketones or caffeic acid derivatives. The concentration range of Bauer alkamides 3, 8-14 and 17 were 0.1-2.8 μM. Ketones were present around 0.1 μM. Cichoric acid and caftaric acid were present around 0.07-0.28 μM and 0.01-0.04 μM, respectively (LaLone et al., 2007). Another study investigated the metabolite distribution in E. pallida methanolic extract (15 μg/mL) and found that alkamide 1/2, 3 and 4 were contained at 1.5, 0.7 and 0.4 μM, but ketones, including ketone 19-28, were present at 30.5 -52.4 μM (Hou et al., 2010), 10 fold greater than the quantity of alkamides, but caffeic acid derivatives were not determined in this study. Pellati et al. (2004) report the total phenolic content for E. angustifolia, E. pallida, and E. purpurea to be 10.49 mg/g, 17.83 mg/g, and 23.23 mg/g, respectively. E. pallida methanolic extract (10 μg/mL) contained 2.5 μM, 20.3 μM and 1.7 μM of caftaric acid, echinacoside, and cichoric acid, respectively (Figure 1).

Although many of the active compounds of Echinacea have been identified, relative potency, mechanism of action, bioavailability and metabolism are less known.

2.2 Health benefits of E. sanguinea and E. pallida

E. pallida is one of the most medicinally used Echinacea species in the US and Europe and is being introduced into other regions due to the increasing popularity in alternative herbal remedies. E. sanguinea has been neglected in the scientific literature, however, Birt et al., (2008) reported that E. sanguinea ethanolic extract had greater anti-inflammatory activity in terms of the significant reduction of prostaglandin E 2 (PGE2) production in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages, than E. angustifolia, E. pallida, and E. simulata. In addition, our preliminary experiments showed that alkamide 8, 10, 11 and ketone 24 were only present in E. pallida and E. sanguinea ethanol extracts, but
not in *E. angustifolia* and *E. simulata* ethanolic assessments. Therefore, *E. pallida* and *E. sanguinea* were chosen in this study to investigate their absorption, metabolism and health benefits.

### 2.2.1 Anti-inflammatory effects

*E. angustifolia, E. pallida,* and *E. simulata* have been described to possess an anti-inflammatory activity which was differed from species, extracts, and fractionation and contributed to main constituents (LaLone et al., 2007; Birt et al., 2008). Treatment with echinacoside at 0.5 µM, a caffeic acid derivative from root extract of *E. pallida* at 100 mg/mL, inhibited the signs of inflammation injected by 1 ml of a gel (1% ethylcellulose) on the dorsal area of the rats for 48 and 72 h (Speroni et al., 2002). Bauer ketones 23 and 24 at 2.6 and 5 µM, respectively, were identified as significant contributors to the PGE (2) inhibition by *E. pallida* root extract at 25 µg/mL. Three different alkamides (alkamide 5, alkamide 7 and alkamide 8) from the root of *E. angustifolia* have suppressed the expression of cyclooxygenase 2 (COX-2) and PGE2 formation at sites of inflammation in H4 human neuroglioma cells (Hinz et al., 2007), but *E. pallida* root extract did not show any suppressive effect on COX-2 promoter activity at 50 µg/mL at both transcriptional and translational levels in 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated RAW 264.7 cells, human breast adenocarcinoma MCF-7 cell as well as mouse models (Hou et al., 2010). Combinations of different alkamides in *Echinacea* extracts might play a synergistic role in the inhibition of PGE2 production in LPS-stimulated RAW264.7 mouse macrophage cells. Active extracts containing < 2.8 µM of combined alkamides in 15 µg/mL of *E. angustifolia, E. pallida, E. simulata,* and *E. sanguinea* extracts significantly have inhibited PGE2 production (LaLone et al., 2007). In a summary, different species of
Echinacea had an effective anti-inflammatory action and the anti-inflammatory activity was mainly contributed to alkamides or caffeic acid derivatives.

2.2.2 Anti-oxidant effects

Assessing free radical scavenging was mainly used to evaluate the antioxidant capacity in medicinal plants and other nutritional antioxidant supplements. Echinacea extracts have been reported to be free radical scavengers and possess anti-oxidant properties. The roots of *E. purpurea*, *E. angustifolia* and *E. pallida* exhibited significant free radical scavenging capabilities by the inhibition of Fe$^{2+}$-induced lipid peroxidation (Sloley et al., 2001). *E. pallida* root and aerial extracts at 2 mg/mL exhibited ~50% inhibition of 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical and displayed ~55% of Fe$^{2+}$-chealating power (Orhan et al., 2009). Capacities to quench DPPH radicals of phenolic compounds in *Echinacea* species indicated that echinacoside (EC$_{50}$ = 6.6 μM) > cichoric acid (8.6 μM) > cynarin (11.0 μM) > chlorogenic acid (18.9 μM) > caffeic acid (19.1 μM) > caftaric acid (20.5 μM), which might be due to the number of hydroxyl groups on the aromatic ring as the more hydroxyl groups, the greater the radical scavenging activity (Wang et al., 2003). Alkamide 2 and 8 inhibited Cu (II)-catalyzed oxidation of human low-density lipoprotein *in vitro* and showed a synergistic effect with caffeic acid derivatives, and polysaccharides (Dalby-Brown et al., 2005). In a summary, *Echinacea* species had an effective antioxidant capacity across species and extracts; the antioxidant capacity was attributed to ability to scavenge free radical.

2.2.3 Anti-viral effects

Echinacea extracts and its metabolic constituents possessed antiviral activities (Cheminat et al., 1988), but the results are very controversial. Hydroalcoholic root extract of *E. pallida* (100 μg/mL) exhibited high levels of antiviral activity against both types of herpes
simplex virus types 1 and 2 (HSV-1, HSV-2) analyzed by plaque reduction assays (Schneider et al., 2009). Interleukin-2 is required for the clonal expansion and activation of T cells during viral infection and has also been linked to decreased symptoms and reduction of virus revival in experimental rhinovirus infection in human Jurkat T cells (Sasagawa et al., 2006). Aerial extract of *E. purpurea* at 50 µg/mL inhibited IL-2 production by ~65% and Bauer alkylamide 9 and Bauer alkylamide 11 at ~2 µM were both capable of inhibiting IL-2 production in Jurkat E6.1 T cells (Sasagawa et al., 2006).

In a human study, supercritical carbon dioxide, 60 % ethanol, or 20 % ethanol extracts of *E. angustifolia* root did not show any statistically significant effects on rhinovirus infection and severity of symptoms in 437 volunteers, measured by the volume of nasal secretions, polymorphonuclear leukocyte or interleukin-8 concentrations in nasal-lavage specimens, as well as quantitative-virus titer (Turner et al., 2005). The conflicting results might be because the dose of *Echinacea* extract was not be adequate to show protection against rhinovirus infection. However, aqueous-ethanolic *E. purpurea* extract induced a statistically significant increase in the survival rate, prolonged the mean survival time and reduced lung consolidation and virus titer after 6 days of intranasal infection with Influenza A virus in mice (Bodinet et al., 2002). Fusco et al. (2010) also showed that *E. purpurea* polysaccharide extract had lower systemic and pulmonary keratinocyte chemoattractant (KC) and interleukin (IL)-10 levels and lower systemic interferon-γ (IFN-γ) levels following influenza infection compared with controls in mice. Therefore, more and larger human studies are needed for the anti-viral effect of *Echinacea* extracts, and the dose, species and type of *Echinacea* extract, time to initiation of treatment, virus type as well as immune competence of volunteers should be considered. Meanwhile, more investigations
are critically required on the absorption and metabolism data regarding the various constituents of different *Echinacea* species.

### 2.3 Bioavailability of *E. sanguinea* and *E. pallida* chemical constituents

A major weakness with most *in vitro* studies dealing with botanicals and bioactivity is accounting for how much of the particular extract or compound actually enters the cells or the body of the model being studied. Therefore, it is essential to investigate the bioavailability of the diverse constituents contained in *Echinacea* species. Most of the published papers were focusing on the bioavailability and pharmacokinetics of different alkamides and caffeic acid derivatives in human and cell culture models.

Alkamides 8 ([Figure 1](#)) from *Echinacea* species has been reported to transport through human adenocarcinoma colonic cell line Caco-2 monolayer at 30 minutes after apical loading of 25 μg/ml and about 15% of these alkamides were detectable on the basolateral side, which was not affected by lipopolysaccharide or phorbol 12-myristate-13-acetate in order to mimic an inflammatory state (Jager et al., 2002). In another study, twelve alkylamides were shown to readily cross Caco-2 monolayers (apparent permeability >10⁻⁶ cm/s), and 2, 4-dienes were more readily transported than the equivalent 2-ene alkamides (Matthias et al., 2004). Among the tested caffeic acid derivatives, cinnamic acid, caftaric acid, cichoric acid, echinacoside, and tartaric acid exhibited poor uptake through the Caco-2 monolayers (less than 5% transported). Chicca et al. (2008) also used the Caco-2 system to study the permeability of ketones from *E. pallida* extract. Bauer ketone 22 and 24 were readily permeable through the cell system, with ketone 22 showing the highest apparent permeability at 32 ± 3 × 10⁻⁶ cm/s.
In a randomized crossover study, six healthy adults of both sexes consumed 4 g of *Echinacea* powder mixture which contained *E. angustifolia*, *E. purpurea*, and *E. pallida*. The 24 h urinary recovery of cichoric acid of *E. purpurea* and *E. pallida* were 0.36 ± 0.25 and 0.34 ± 0.31 % of ingested dose (Lee et al., 2006). However, another human study did not detect caffeic acid conjugates (caftaric acid, cichoric acid and echinacoside) in any plasma sample at any time after ingestion of *Echinacea* tablets manufactured from an ethanolic liquid extract, whereas alkamides were detected in plasma 20 minutes after tablet ingestion (Matthias et al., 2005). In a randomized crossover single-dose study, 10 volunteers were orally administered either 4 ml of the standardized *E. purpurea* tincture or twelve *E. purpurea* tablets, and both doses contained 0.07 mg of the major alkamides. Alkamides in *Echinacea* tincture in serum were present at 0.4 ng/ml 30 min after administration, whereas alkamides in tablets were 0.12 ng/ml serum after 45 min (Woelkart et al., 2006). Similar bioavailability of alkylamides was found from the liquids (200 and 300 mg/ml) and tablets (600 and 675 mg/tablet) *Echinacea* formulations in a two-way crossover study in humans (Matthias et al., 2007). Taken together, these results indicate that alkamides, as well as certain ketones, are likely to cross the intestinal barrier and be bioavailable in humans, and therefore, should be able to carry out their bioactivities in vivo, but caffeic acid derivatives are less likely to do so.

### 2.4 Metabolism of *E. sanguinea* and *E. pallida*

Due to potential interactions with other herbal supplements or pharmaceuticals, it is necessary to understand how bioactive constituents from *Echinacea* are metabolized in the liver or in other tissues. Cytochrome P450 (CYP) may affect the metabolism and further bioavailability of *Echinacea* constituents. Matthias et al. (2005) studied the metabolism of the alkamide constituents from *Echinacea* premium liquid, a mixture of 200 mg/mL of *E.*
angustifolia and 300 mg/mL of E. purpurea 60% ethanol/water root extracts, in human liver microsomes and provided evidence that NADPH dependent cytochrome P450 metabolized the 2-ene and 2, 4-dienes in the ethanolic extracts of Echinacea. Monohydroxylated, monoepoxidized, N-dealkylated metabolites were determined after incubation of parent Bauer alkamides with both NADPH and human liver microsomes. Further results from this study showed that 60% ethanol/water extract of E. purpurea containing 16 μM of Bauer alkylamides 8/9 significantly suppressed IL-2 secretion by 47% in the Jurkat T cells, but the suppression of IL-2 secretion was significantly lessened when NADH was added with the same treatment, indicating the importance of understanding the metabolism of constituents found in Echinacea and how it affects different bioactivities.

Echinacea preparations have been found to affect metabolizing enzyme activities and transporter functions in vitro, implying the importance regarding herb–drug interactions. E. purpurea extract inhibited CYP3A4 activity, tested by the inhibition of the metabolism of 7-benzyloxy-trifluoromethylcoumarin, 7- benzyloxyquinoline and testosterone, three substrates of CYP3A4, using fluorometric assays (Hansen et al., 2008). Another study showed that E. purpurea inhibited CYP 3A4 mediated metabolism in C-DNA baculovirus which expressed human CYP 3A4 supersomes based on the decreased formation of 6-OH-testosterone (Hellum et al., 2008). Several Echinacea ethanolic extracts (11.2 - 2447 µg/mL) have been shown to inhibit baculovirus expressed CYP 2C19, 2D6, and 3A4 by 20-100 %, and alkamides contributed to the inhibitory effects seen with Echinacea preparations (Modarai et al., 2007). The interaction between Echinacea and drugs has been investigated. E. purpurea root extract (400 mg) significantly increased the systemic clearance of intravenous midazolam by 34% but reduced clearance of caffeine and tolbutamide in twelve healthy subjects, indicating the inhibition on CYP3A at hepatic and
intestinal sites as well as improving of CYP 1A2 and CYP 2C9 activity (Gorski et al., 2004). Therefore, alkamides in *Echinacea* extracts not only can be enzymatically metabolized, but also may contribute to the inhibition of of these same enzymes as shown by extracts from different *Echinacea* species.

A previous study showed that n-hexane root extracts from *E. pallida*, *E. angustifolia* and *E. purpurea* at 30 µg/mL inhibited multidrug transporter P-glycoprotein (P-gp) activity in a human proximal tubular cell line (Romiti et al., 2008). P-gp transporter plays a key role in drug absorption and distribution because it limits the permeability across the GI tract by active efflux of potentially toxic substances back into the intestinal lumen. The transporter is also expressed in other tissues such as liver, kidney and blood–brain barrier where it also plays an important excretory role. On the other hand, P-gp confers resistance to anticancer chemotherapy because it is over-expressed in cancer cells (O'Connor 2007). The use of botanicals which have anticancer properties as ABC transporter inhibitors may allow a new paradigm of clinically useful drug resistance circumvention.

In summary, it is crucial to investigate the uptake and metabolism of key components found in various *Echinacea* species as well as the influences of *Echinacea* plant matrix on the bioavailability of these key constituents. Because *Echinacea* supplements are usually ingested on a chronic basis, their long term effects on metabolic enzymes and efflux transporters needs to be investigated and may be of importance regarding herb–drug interactions.
3. OVERALL INTRODUCTION OF *PRUNELLA VULGARIS*

*Prunella vulgaris*, a perennial herb known as self-heal, is widely distributed throughout Asia and Europe, is used primarily as a traditional botanical medicine to reduce osteoarthritis, rheumatoid arthritis, and gingivitis (Song et al., 2007; Adámková et al., 2004; Jung et al., 2001). In China, *P. vulgaris* is called ‘Xia Ku Cao’ and has a long history in therapeutic use as an antipyretic, and more recently for anti-keratitis purposes (Zheng et al., 1990). In Turkey, *P. vulgaris* is used primarily as an expectorant, tonic, antidiarrhoeic, against dysentery, for common cold, rheumatoid arthritis and cardiac arrhythmia (Pinkas et al., 1994; Markova et al., 1997). More recently, increasing evidence suggests that organic preparations of *P. vulgaris* exhibited anti-estrogenic, anti-inflammatory, and antioxidative properties *in vitro* cell models and in rats (Psotova et al., 2003).

3.1 Phytochemistry of *Prunella vulgaris*

It has been found that aqueous extract of *P. vulgaris* contains on an average alkaloid (1.1%), saponins (0.4%), phenolic acids (0.06%), tannins (0.06%), carbohydrates (0.4%), proteins (0.4%) and lipids (2.4%, Rasool et al., 2010). The amount of caffeic acid, chlorogenic acid and rosmarinic acid (*Figure 2*) in *P. vulgaris* was about 0.29, 0.32, and 9.46 mg/g, respectively, in dried plant (Sahin et al., 2011). Cheung and Zhang (2008) reported that the concentration range of oleanolic acid, ursolic acid, and betulinic acid (*Figure 2*) was 0.3-0.9, 1.5-4.0, and 4.7-5.5 mg/g, respectively, in *P. vulgaris* samples collected from different areas of China. Rutin, quercetin, hyperoside, isoquercitrin, luteolin, cinaroside, kaempferol, and kaempferol-3-O- glucoside, etc (*Figure 2*) were identified from the methanol extract of *P. vulgaris* and the total amount of flavonoids was in the range of 2.16-10.29 % (Liao et al., 2008).
3.2 Health benefits of *Prunella vulgaris*

3.2.1 Anti-oxidant effects

*P. vulgaris* ethanol extract, containing 6.1% of major hydroxycinnamic acids, showed significant anti-oxidative activities in terms of free radical scavenger effect on DPPH and the anti-oxidative activity was partly in relation to the content of rosmarinic acid in this plant (Lamaison et al., 1991). The aqueous extracts of *P. vulgaris* inhibited rat erythrocyte hemolysis by 81% and lipid peroxidation by 95% in rat kidney and brain homogenates at 200 µg/mL, as well as demonstrated strong superoxide- and hydroxyl radical-scavenging activity by 61% at 100 µg/mL, but the content of the major constituents of *P. vulgaris* was not determined in this study (Liu and NG, 2000).

3.2.2 Anti-inflammatory effects

Ethanol extracts of *P. vulgaris* at 30 µg/mL, rather than water extracts, significantly inhibited production of LPS-induced inflammatory mediators PGE2 and NO by RAW 264.7 mouse macrophages (Huang et al., 2009). In this study, rosmarinic acid in *P. vulgaris* extract at 2.67 µM was found to independently inhibit inflammatory response, but it only partially explained the extract’s activity. LPS-induced cyclooxygenase-2 (COX-2)
and nitric oxide synthase (iNOS) protein expression were both attenuated by *P. vulgaris* ethanol extracts, while rosmarinic acid only inhibited COX-2 expression. These authors suggest that the inhibition exists at the transcriptional level and there could be compounds other than rosmarinic acid in the extracts inhibiting the expression and/or the activity of the two critical enzymes in inflammation.

The anti-allergic and anti-inflammatory constituents in *P. vulgaris* have been identified to be triterpenes such as betulinic acid, ursolic acid, 2α, 3α-dihydroxy-ursoic acid, and 2α-hydroxyursolic acid. 2α, 3α-Dihydroxyursolic acid exhibited significant inhibition on the release of β-hexosaminidase, a marker molecule for examining the degranulation process in RBL-2H3 mast cells, in a dose-dependent manner and the IC₅₀ value was 57 µM. Ursolic acid and 2α-hydroxyursolic acid exhibited strong inhibition on the production of NO from RAW 264.7 cells and the IC₅₀ was 17 and 27 µM, respectively (Ryu et al., 2000). Therefore, major constituents contained in *P. vulgaris* contribute to the anti-inflammatory activities and NF-κB activation and COX-2 inhibition might be the underlying mechanism.

### 3.2.3 Anti-mutagenic effect

Several triterpenoids including oleanic acid, 2α, 3α, 19α-trihydroxyursolic acid, and maslinic acid inhibited A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells), using the sulforhodamin B bioassay to test the cytotoxicity, with the ED₅₀ range between 3.7 -28 µM (Lee et al., 2008). A water extracts of *P. vulgaris* at 50 µg/mL had a moderate antimitagenic activity against picrolonic acid and benzo[a]pyrene induced mutation using the *Salmonella* microsomal system (Lee and Lin, 1988). Polysaccharide P32 (5 and 10 g/kg) isolated from *P. vulgaris* extract, consisted of rhamnose, arabinose, xylose, mannose,
glucose and galactose in a molar ratio of 3.46:49.32:58.91:0.43:2.64:3.11, decreased the number of tumors and weight by 64% and 55%, respectively, and significantly increased the relative thymus weight to the body weight by 1.4 fold and the relative spleen weight to the body weight by 1.3 fold in tumor-bearing mice \((p < 0.05)\), indicating anti-tumor and immunomodulation effects (Feng et al., 2010). In addition, the protective activity of rosmarinic acid from *Perilla frutescens* on liver injury induced by LPS in D-GalN-sensitized mice was examined (Osakabe et al., 2002). In this study, *Perilla* extract (PE, 50-200 mg/kg in feed) and rosmarinic acid (135 mg/kg in feed) treatments significantly reduced the elevation of plasma asparatate aminotransferase levels by 75%, but PE and rosmarinic acid treatments did not significantly diminish iNOS mRNA expression or plasma nitrate/nitrite levels as well as TNF-\(\alpha\) mRNA expression both in liver and plasma, indicating that liver protection of RA is due to the scavenging or reducing activities-superoxide or peroxynitirite rather than to inhibition of TNF-\(\alpha\) production. Therefore, rosmarinic acid, triterpenoids and polysaccharides are responsible for the overall anti-tumor activites of *P. vulgaris* and the immunomodulatory and anti-oxidative effects may contribute to the anti-adenocarcinoma activity.

### 3.2.4 Anti-viral effects

The anti-HIV activity of *P. vulgaris* was reported by Tabba et al. (1989). The water extract of *P. vulgaris* (50 \(\mu\)g/mL) significantly inhibited HIV-1 replication by blocking cell to cell transmission of the virus, preventing syncytium formation, and interfering with the ability of the virus to bind to CD4 receptor, suggesting that *P. vulgaris* extract antagonized HIV-1 infection of susceptible cells by preventing viral attachment to the CD4 receptor (Tabba et al., 1989). Moreover, anionic polysaccharides and triterpenoids from *P. vulgaris* had anti-HSV activity *in vitro*. The antiviral activity (EC\(_{50}\)) was about 66 \(\mu\)M, 20
µM and 10 µg/mL for betulinic acid, 2α, 3α-dihydroxyursolic acid and polysaccharides, respectively, measured by plaque reduction assay (Xu et al., 1999). In summary, studies have shown that *P. vulgaris* possesses a variety of pharmacological properties, and these novel properties may be utilized for future potential therapeutic applications.

### 3.3 Bioavailability of *Prunella vulgaris* chemical constituents

Most of the published papers were focusing on the bioavailability and pharmacokinetics of caffeic acid derivatives, triterpenoids, and flavonoids in *P. vulgaris* in human and cell culture models. Konishi and Kobayashi (2005) investigated transepithelial transport of rosmarinic acid, the major bioactive constituent in *P. vulgaris*, in intestinal Caco-2 cell monolayers and found that rosmarinic acid transferred across the intestinal barrier mostly by paracellular diffusion, like other esterified phenolic acids, i.e. chlorogenic acid. The apparent permeability coefficient, $P_{app}$, for rosmarinic acid in *Salvia miltiorrhiza* ethanolic extract was $0.523 \pm 0.301 \times 10^{-6}$ cm/s (Lu et al., 2008). The effects of pH and the direction of permeation on the absorption of phenolic acids in Caco-2 cell system have been investigated, i.e. caffeic acid exhibited a pH-dependent directional transport from the apical to the basolateral side (Konishi et al., 2004). However, Lu et al. (2008) showed that there was no significant difference between $P_{app}$ of apical to basolateral and basolateral to apical side for rosmarinic acid, a caffeic acid derivative. Meanwhile, the permeation of rosmarinic acid from the apical to the basolateral side in the presence of a proton gradient (pH gradient 6.0/7.4) was nearly the same as that in the absence of a proton gradient (pH gradient 7.4/7.4), implying that proton coupled polarized transport was not involved for rosmarinic acid. In *in vivo* studies, Jirovsky et al. (2007) showed that after 91 days of feeding in pigs with methanol extract of *P. vulgaris*, rosmarinic acid and its metabolites were found in plasma at >0.08 µM after the hydrolysis by β-glucuronidase/arylsulfatase to
release the parent compound. Taken together, intact rosmarinic acid is poorly absorbed in the small intestine.

The uptake of triterpenoids such as oleanolic acid and betulinic acid has been studied both in vitro and in vivo. \( P_{\text{app}} \) value of oleanolic acid in Caco-2 cells was \( 1.1 - 1.3 \times 10^{-6} \text{ cm/s} \). The absolute oral bioavailability was 0.7% for oral doses of 25 and 50 mg/kg of oleanolic acid in rats, indicating that oleanolic acid is also poorly absorbed and might be extensively metabolized in vivo (Jeong et al., 2007). The uptake kinetics of 2,3-hydroxybetulinic acid in Caco-2 cells followed a one-phase exponential association process, reached near saturation after approximately 60 min and a maximal intracellular concentration of about 906.7 \( \mu \text{M/mg protein} \), indicating the facilitated absorption of this particular compound (Zheng et al., 2007).

The absorption of flavonoids has been studied. Walgren et al. (1998) found out that the reverse basolateral to apical flux of quercetin (\( P_{\text{app}} 11.1 \pm 1.2 \times 10^{-6} \text{ cm/s} \)) was almost 2-fold higher than the apical to basolateral flux (\( p < 0.001 \)). Quercetin 4-glucoside demonstrated no absorption but did demonstrate basal to apical flux (\( P_{\text{app}} 1.6 \pm 0.2 \times 10^{-6} \text{ cm/s} \)). Quercetin-3, 4'-diglucoside showed a low apical to basolateral transport (\( P_{\text{app}} 0.09 \pm 0.03 \times 10^{-6} \text{ cm/s} \)) and its reverse, basolateral to apical, transport was, however, 4-fold higher (\( p < 0.05 \)). These results suggest that facilitated absorption of quercetin through the human intestinal epithelium might be involved. Wang et al. (2005) also reported that quercetin, kaempferol and isorhamnetin displayed polarized transport, with the \( P_{\text{app}} \), basolateral-apical being higher than the \( P_{\text{app}} \) apical-basolateral in Caco-2 cells. Meanwhile, quercetin and kaempferol (50 \( \mu \text{M for each} \)) inhibited the ATPase activity, and isorhamnetin (50 \( \mu \text{M} \)) stimulated the ATPase activity (\( p < 0.05 \) and the concentrations of
these flavonoids were significantly increased when incubated with the P-gp inhibitor verapamil ($p < 0.05$). The results indicated that quercetin, kaempferol and isorhamnetin were substrates of P-gp and the P-gp type efflux pump might limit the bioavailability of these flavonoids.

### 3.4 Metabolism of *Prunella vulgaris*

A major limiting step in the utilization of bioactive compounds from *P. vulgaris* is their intestinal absorption and metabolism. Phase I and II metabolites are often, but not always, less bioactive than parent compounds. The absorption or metabolism of rosmarinic acid has been examined *in vivo* to a limited extent (Baba et al., 2004; Konishi et al., 2005; Baba et al., 2005). When *Perilla* extract containing 200 mg of rosmarinic acid was orally administered to six men, rosmarinic acid in both plasma and urine was present predominantly as glucuronide and/or sulfate conjugated forms, at 0.6 ± 0.2% and 1.5 ± 0.4% of the total intake, respectively, within 48 h after ingestion (Baba et al., 2005). Rosmarinic acid (1.5%), methylated rosmarinic acid (2.9%), caffeic acid (1.0%), ferulic acid (1.0%) and a trace of m-coumaric acid (0.02%) were detected in the urine after 48 h intake of the plant extract (Baba et al., 2004). Therefore, ingested rosmarinic acid was absorbed and metabolized into conjugated and/or methylated forms and degraded to other caffeic acid derivatives.

Gut microbes may metabolize rosmarinic acid to give phenolics such as caffeic acid, o-coumaric acid, 3, 4-dihydroxyphenylpropionic acid (DHPP) and m-hydroxyphenylpropionic acid (mHPP), in which m-coumaric acid and mHPP were absorbed by monocarboxylic acid transporter (MCT)-mediated active processes, whereas
DHPP was mainly permeated across Caco-2 cells via the paracellular pathway (Konishi and Kobayashi, 2005).

The metabolism of the flavonoids has been investigated both in vitro and in vivo. Quercetin was metabolized to several substances in human leukaemia (HL-60) cells, such as quercetin glucuronides, quercetin-sulfate, myricetin by hydroxylation, isorhamnetin by methylation, and quinone form via oxidation (Ludwig-Müller et al., 2005). Another study investigated the effect of quercetin and its metabolites on paraoxonase 2 levels in RAW264.7 macrophages (Boesch-Saadatmandi et al., 2009). Intracellular antioxidant enzyme paraoxonase 2 (PON2) may have a protective function in the prevention of atherogenesis. An enhancement of PON2 activity by dietary factors including flavonoids is therefore of interest. In this study, supplementation of RAW264.7 macrophages in culture with quercetin at 1, 10, and 20 µM resulted in a significant increase in PON2 mRNA and protein levels, as compared to untreated controls. Unlike quercetin, its glucuronidated metabolite quercetin-3-glucuronide did not affect PON2 gene expression in cultured macrophages. However the methylated quercetin derivative isorhamnetin enhanced PON2 gene expression in RAW264.7 cells to a similar extent as quercetin. These results indicate that circulating flavonoid metabolites possess biological properties different from their nonconjugated parent compounds, further proving the importance of understanding the metabolic fate of the bioactive constituents via Phase I and II biotransformation as well as gut microbial metabolism.
4. OVERALL INTRODUCTION OF CAFFEIC ACID DERIVATIVES

4.1 Source and intake of caffeic acid derivatives

Caffeic acid (3, 4-dihydroxycinnamic acid), one of the most common phenolic acids, frequently occurring in fruits, vegetables, grains, dietary supplements and traditional herbs as simple esters with quinic acid or saccharides (caffeic acid derivatives). Caffeic acid is the main phenolic acid aglycone in potatoes, with contents varying from 0.3 to 3.6 mg/100 g and 18.8 to 28 mg/100 g in tubers and peels, respectively (Radtke et al., 1998). Chlorogenic acid is an ester of caffeic and quinic acids in coffee and many types of fruits, coffee, tea and traditional herbs (Clifford, 1999). Coffee consumers ingest 0.5-1 g chlorogenic acid/d (Clifford et al., 1999). In another study, caffeic acid intake was found to be 206 mg/d, and the principal sources were coffee (92% of caffeic acid) and fruit and fruit juices combined (Radtke et al., 1998). Indeed, coffee consumption was shown to be positively correlated to caffeic acid and chlorogenic acid intake, indicating that they can be used as biomarkers for the estimation of dietary intake (Mennen et al., 2006).

Rosmarinic acid is a caffeic acid derivative found in many Lamiaceae herbs used commonly as culinary herbs such as lemon balm, rosemary, oregano, sage, thyme and peppermint (Lamaison et al., 1991). The first plant cell cultures found to be accumulating rosmarinic acid were derived from Coleus blumei (Razzaque and Ellis, 1977). Suspension cultures from this species were the first ones used for an attempted biotechnological production of rosmarinic acid since they were found to accumulate up to 21% rosmarinic acid in the dry weight. Basil, lemon thyme, mint, oregano, rosemary, sage, and thyme were investigated to contain 0.22-0.97% of rosmarinic acid (Park, 2011).
Caffeic acid derivatives were metabolized by both human and animal gut microbes. Rosmarinic acid (Konishi and Kobayashi, 2005; Yoshikawa et al., 2005), chlorogenic acid (Konishi and Kobayashi, 2005), and cichoric acid (Peppercorn et al., 1971) were cleaved by microbial esterase, hydrolyzed to caffeic acid and then reduced to dihydrocaffeic acid (3, 4-dihydroxyphenylpropionic acid); furthermore the later was dehydroxylated to m-hydroxyphenylpropionic acid (mHPP). After administration of chlorogenic acid at 250 μmol/day in rats for 8 days, total urinary excretion of caffeic, ferulic, and isoferulic acids was 28.1 % of intake (mol/mol); urinary mHPP was 4.0 %. Plasma metabolite concentrations in rats fed caffeic acid (250 μmol/day) for 8 d were caffeic acid (41.3 μM), ferulic acid (7.3 μM), isoferulic acid (4.5 μM), hippuric acid (54.2 μM), and mHPP (1.4 μM) (Gonthier et al., 2003). When Perilla extract containing 200 mg of rosmarinic acid (RA) was orally administered to six men, RA in both plasma and urine was present predominantly as glucuronide and/or sulfate conjugated forms, at 0.6 ± 0.2% and 1.5 ± 0.4% of the total intake, respectively, within 48 h after ingestion (Baba et al., 2005). Gut microbes metabolized RA to give phenolics such as caffeic acid, o-coumaric acid and m-hydroxyphenylpropionic acid, which are then absorbed by monocarboxylic acid transporter (MCT)-mediated active processes (Konishi and Kobayashi, 2005). In summary, caffeic acid derivatives are ingested by humans and metabolized to various metabolites, which may contribute to the health benefits.

4.2 Anti-inflammatory activities of caffeic acid derivatives

Caffeic acid phenethyl ester, at 25 μg/mL, has been shown to suppress NF-κB activation by suppressing the binding of the p50-p65 complex directly to DNA in human histiocytic U937 cells (Natarajan et al., 1996). Nuclear factor kappa-B (NF-κB) was a heterodimeric transcription factor with a pivotal role in orchestrating immune and inflammatory
processes. In this study, the activation of NF-κB by TNF-α was completely blocked by caffeic acid phenethyl ester in human histiocytic cell line U937 cells, which are used to study the behaviour and differentiation of monocytes (Natarajan et al., 1996). In another study, caffeic acid and the predominant isomer of chlorogenic acid, 5-caffeoylquinic acid, significantly inhibited the H₂O₂ or TNF-α induced interleukin (IL-8) secretion and its mRNA expression in a dose-dependent manner in the range of 0.25-2.00 mM in Caco-2 cells (Zhao et al., 2008). IL-8 is a central pro-inflammatory chemokine involved in the pathogenesis of IBD.

Chlorogenic acid (5-20 μM) also showed suppression of IL-1β-induced activation of NF-κB, degradation of inhibitor of κB (IκB)-α, as well as matrix metalloproteinases (MMP-1, MMP-3, MMP-13) expressions in chondrocytes isolated from rabbits (Chen et al., 2011). IL-1β is a pivotal catabolic factor in inflammatory cascades and induces inflammatory mediators and MMPs, leading to disruption of the balance between biosynthesis and degradation of the extracellular matrix (Chen et al., 2011). Chlorogenic acid (50 mg/kg, intraperitoneally) suppressed hepatic mRNA expression of toll-like receptor 4 (TLR4), TNF-α and phosphorylation of NF-κB p65 subunit in LPS-induced liver injury in mice (Xu et al., 2010). TLR4, one of the members of the TLR protein family, is essential for LPS-mediated signaling. After binding to TLR4 and CD14, LPS triggers several critical intracellular signaling pathways, including NF-κB signaling cascade, and leads to the production of inflammatory cytokines including TNF-α, INF-γ and nitric oxide (Takeuchi et al., 1999).

Rosmarinic acid, at 1μM, reduced serum levels of TNF-α, IL-6, high-mobility group box 1 protein, while increased serum level of IL-10 in rats induced by cecal ligation and puncture.
Amelioration of hemodynamics and decrease in myeloperoxidase in lung, liver, and small intestine were also observed after rosmarinic acid injection (Jiang et al., 2009). In another study, rosmarinic acid in *P. vulgaris* (2.67 µM) was found to independently inhibit LPS-induced inflammatory mediators PGE2 and NO by RAW 264.7 mouse macrophages, via COX-2 inhibition (Huang et al., 2009). These results suggest that caffeic acid derivatives, including caffeic acid, chlorogenic acid and rosmarinic acid, have the capacity to inactivate inflammatory response and the anti-inflammatory mechanism may inhibit activation of the NF-κB pathway by inhibiting IκB kinase activity.

5. INTESTINAL ABSORPTION, METABOLISM, INFLAMMATORY RESPONSES, AND RELEVANT METHODOLOGY

5.1 Intestinal function

5.1.1 Absorption mechanisms

Dietary bioactive compounds pass across the intestinal epithelium *via* passive and active mechanisms. In passive transport, the cells do not use any energy to move the molecules and the molecules move through the concentration gradient (higher concentration to lower concentration). Passive diffusion can be divided into either transcellular diffusion (through the cells) or paracellular diffusion (intercellular spaces). Transcellular passage includes the partition between the aqueous contents of intestinal lumen, the phospholipid bilayer of the apical cell membranes, the aqueous contents of the enterocytes, and again, the phospholipid bilayer of the basolateral cell membranes (Borchardt, 1998). The compound *via* paracellular pathway is usually polar or hydrophilic and small (Hidalgo, 1996) and the transcellular pathway is more common route, since the cellular absorbing surface is over 1000 times greater than the area of the paracellular spaces (Pappenheimer and Reiss, 1987). The chemical and physical properties of the compound are critical for the permeability
across the epithelial cells. For example, chlorogenic acid and rosmarinic acid are
considered to pass across the epithelial barrier via paracellular pathway, while alkamides
transport via transcellular pathway (Konishi and Kobayashi, 2005; Matthias et al., 2005;
Konishi and Kobayashi, 2004). Moreover, caffeic acid conjugates (caftaric acid, cichoric
acid and echinacoside) were not detected in any plasma sample at any time after ingestion
of *Echinacea* ethanolic extract, whereas alkamides were in the range of 0.1-120 ng/mL in
plasma 20 minutes after ingestion (Matthias et al., 2004), which might be because that
alkamides are more hydrophobic than caffeic acid derivatives, thus more bioavailable.

Membrane transporters located in the cell membranes are responsible for active transport
of nutrients. Active transport is the energy-demanding transfer of a nutrient across a cell
membrane against its concentration gradient (lower concentration to higher concentration,
Gropper et al., 2008). The energy for active transport comes from ATP generated by
respiration (in mitochondria). The active transport can be classified into facilitated
diffusion, primary active and secondary active transport, based on the energy requirement
(Amidon and Sadée, 1999). Facilitated diffusion does not require energy for its function
and specific molecules move down a concentration gradient, passing through the
membrane via a specific carrier protein. Thus, rather like enzymes, each carrier has its
own shape and only allows one molecule (or one group of closely related molecules) to
pass through. Primary active transport is an energy demanding process and the energy is
gained from hydrolysis of ATP to ADP from the high-energy phosphate bond. Secondary
active transport uses energy from ion gradients (mostly Ca^{2+}, Na^+, and H^+ -gradients)
across the cell membranes generated by primary active ion pumps (for example
Na^+/K^+-ATPase), and are also called symporters or antiporters (Amidon and Sadée, 1999).
Characteristics for active transport are the saturability of the carrier protein and
temperature dependency. Several absorptive transport systems with the primary function of transporting nutrients (amino acids, oligopeptides, monocarboxylic acids, monosaccharides, organic anions and cations, bile acids and several water soluble vitamins) are present in the small intestine (Steffansen et al. 2004). Caffeic acid and its colonic microbial metabolites, such as o-coumaric acid, ferulic acid and m-hydroxyphenylpropionic acid (mHPP) transport across the intestinal epithelial cells via monocarboxylic acid transporter (MCT, Konishi and Kobayashi, 2004), indicating that a monoanionic carboxyl group and a non-polar side chain or aromatic hydrophobic moiety are thought to be necessary components of a substrate for MCTs. Moreover, the major intestinal glucose transporters, such as Na\(^+\)-dependent glucose transporter SGLT1 (SLC5A1), the facilitative transporter GLUT2 (SLC2A2), and GLUT4 (SLC2A4), have been regarded as specific carriers for the transport of some flavonoids, such as quercetin, quercetin glycosides, myricetin, catechin-gallate, and genistein (Chen et al., 2007; Strobel, et al., 2005). Therefore, the membrane transfer of phytochemicals is a fundamental part of their bioavailability, and the uptake mechanisms of phytochemicals could be compound-specific, which depends on the compound structure and conjugated moieties.

5.1.2 Metabolism

Although the human small intestine serves primarily as an absorptive organ, it also has the ability to metabolize nutrients, drugs and other molecules. Many of the enzymes involved in Phase I and II reactions in the human liver have also been detected within intestinal epithelial cells. Cytochrome P450 (CYP) enzymes are located at the villous tip just beneath the brush border at the apex of the enterocyte, the main site of absorption for orally administered compounds and CYP content in human small intestine ranges from 20 to 210 pmol/mg, implying that significant variability in intestinal CYP expression: total CYP
content slightly increases from the duodenum to the jejunum and then decreases toward the ileum (Thelen and Dressman, 2009). The goblet cells and the epithelial cells of the crypts between the villi contain lower levels of drug metabolizing enzymes. As mentioned before, alkanides can be metabolized by CYPs to form epoxides, and N-dealkylated and hydroxylated products (Matthias et al., 2004). The most active hepatic CYP isoforms involved in the production of epoxides were CYP1A1, CYP1A2 and CYP2A13, whereas for the N-dealkylation only CYP1A1, CYP1A2 and CYP1B1 showed significant activity. Of these forms CYP 1A1 was detected in human intestinal samples, although at least at low levels (3.6–7.7 pmol/mg) while CYP1A2, is an almost exclusively hepatic CYP enzyme, being virtually undetectable in human small intestine (Paine et al., 2006; Shimadat et al., 1994). More interestingly, the 2, 4-diene alkanides were more rapidly metabolized when examined as the pure compound rather than in the *Echinacea* extract, suggesting that some of the other constituents present in the extract might have exerted an inhibitory effect towards their metabolism by CYPs.

Among Phase II enzymes, UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), which catalyze sulfation and glucuronidation, may play important roles in the conjugation and ultimately excretion and elimination of many phytochemicals containing hydroxyl functional group either present in the parent structure and/or after biotransformation by the phase I enzyme such as the CYPs (Rushmore and Kong, 2002). Some flavonoids, such as baicalein, quercetin, and methylated quercetin, are substrates of UGT1A3 and UGT 1A9 and the preferred substrates for UGT1A3 and UGT1A9 contain the hydroxyl group at the C7-position (Xie et al., 2010). Moreover, luteolin in *Artemisia afra* aqueous extract, regardless of its form (i.e. whether aglycone and 7-0-glucoside), was taken up better and more efficiently metabolized to luteolin monoglucuronide, sulfate and...
methylated luteolin sulfate conjugates than the aglycone and 7-0-glucoside forms administered as pure solutions in Caco-2 cells (Mukinda et al., 2010), implying the necessity to investigate the effect of the plant matrix on the uptake and metabolism of phytochemicals.

Therefore, the small intestine plays a key role in first-pass metabolism of orally ingested phytochemicals, which is supported by the position of the small intestine as the first site of exposure of phytochemicals to metabolic systems, by the large surface area available in the small intestine for absorption and subsequent metabolism, and by the transporters located both at apical and basolateral sides of the enterocytes involved in the transfer or efflux of the substrates.

Transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and organic anion transporting polypeptide (OATP) are expressed in intestine, where they provide a formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion (Xu et al., 2005, Figure 3). Because P-gp and MRP utilize ATP to transport substrate the cell membrane, they are called ATP binding cassette (ABC) transporters, which import or export a broad range of substrates, such as amino acids, ions, sugars, lipids, etc. In human, 46 ABC transporters have been identified (Chang, 2003). All ABC transporters are composed of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). The role of TMD is to recognize and mediate the passage of substrates across the cell membrane (Chang, 2003). First-pass metabolism can be affected by the interaction between CYP isoforms, specifically CYP 3A4, and P-gp in enterocytes (Patel and Mitra, 2001, Figure 3). Since CYP3A4 and P-gp are both localized to mature enterocytes on the villus tip, the extensive overlap among the
substrates for P-gp and CYP3A4, and pregnane X receptor (PXR) mediates co-induction of
CYP3A4 and P-glycoprotein, thus P-gp and CYP3A4 could be coordinately regulated, and
substrate for both P-gp and CYP3A4 may be absorbed directly into the systemic circulation,
metabolized by CYP3A4 in the enterocyte or secreted back into the intestinal lumen by
P-gp (Patel and Mitra, 2001). Therefore, P-gp-mediated efflux and CYP3A4 metabolism
may be functionally interrelated and act accordingly to limit the passage of the molecules
across the enterocyte.

However, some flavonoids, such as quercetin (Scambia et al., 1994), myricetin (Choi et al.,
2010) and genistein (Sergent et al., 2009) have been shown to inhibit P-gp-mediated efflux
and CYP3A4 both in Caco-2 cells and animal models. These results underline the
importance of intestinal studies to assess transporter activity linked to the ingestion of
phytochemical- enriched supplements.

Figure 3. Transporters in intestinal epithelial cells

5.2 Inflammatory responses of the intestine

Besides the absorptive and metabolic capabilities, the intestine mucosa membrane exercises a truly antitoxic and protective function with regard to the microbial poisons. Changes to this mucosal barrier can affect the absorption of both exogenous and endogenous nutrients. Many diseases, such as inflammatory bowel disease (IBD), ischemic disease, and graft-versus-host disease, are associated with the loss of intestinal mucosal barrier function (Clayburgh et al., 2004; Chen et al., 2003; Hill et al., 2000). Therefore, an intact intestinal mucosal barrier is of great importance for both ensuring adequate provision of dietary nutrients to the whole body and preventing gut-related diseases.

An intact mucosal barrier depends on intercellular junctions, which help to seal the space between adjacent epithelial cells (the paracellular space), and tight junctions, which are the key elements of the seal (Turner, 2006). Tight junctions play a key role in the intestinal physical barrier to the diffusion of pathogens, toxins, and allergens from the lumen into the circulatory system. Tight junctions are multi-protein complexes composed of transmembrane proteins (i.e. occludin, claudins and junctional adhesion molecule (JAM)), peripheral membrane (scaffolding) proteins (i.e. zonula occludens (ZO)), and regulatory molecules that include kinases (Figure 4).

The most important of the transmembrane proteins are members of the claudin family, which not only define tight junction permeability and affect barrier function, but also
enhance cell proliferation and regeneration, as might be necessary to compensate for cell loss in colitis. For example, increased claudin-1 and claudin-2 expression has been reported in intestinal epithelial cells of patients with IBD (Heller et al., 2005). Meanwhile, claudin-1 has also been shown to enhance neoplastic transformation, tumor growth and metastasis in experimental models (Dhawan et al., 2005). Weber et al. (2008) reported that claudin-1 and claudin-2 expression was elevated in active IBD, adenomas, and IBD-associated dysplasia, but not acute, self-limited colitis (ASLC). In contrast, claudin-4 expression was elevated in both active IBD and ASLC. Thus, increased claudin expression may contribute to carcinogenesis in IBD.

At least two routes allow transport across the tight junction: leaky pathway, which allows paracellular transport of large solutes, such as limited flux of proteins and bacterial LPS, and small pore pathway that is defined by tight junction-associated claudin proteins, which are primary determinants of charge selectivity and exclude molecules larger than 4 Å (Turner et al., 2009). Thus, tight junctions show both size selectivity and charge selectivity, and these properties may be regulated individually or jointly by physiological or pathophysiological stimuli.

Peripheral membrane proteins, such as ZO-1 and ZO-2, are crucial to tight junction assembly and maintenance, because recent work suggests that ZO-1, which interacts directly with actin, occludin, claudins and other proteins, may be an essential effector of perijunctional actomyosin ring-mediated tight junction regulation (Van et al., 2009). In deed, Ca\(^{2+}\)-calmodulin-dependent serine–threonine protein kinase myosin light chain kinase (MLCK), which phosphorylates myosin II regulatory light chain (MLC) within the perijunctional actomyosin ring to activate myosin ATPase activity, is essential for Na\(^{+}\)-nutrient co-transport-induced tight junction regulation (Scharl et al., 2009). Occludin,
a transmembrane tight junction protein also interacts directly with claudins and actin. However, although numerous in vitro studies demonstrated a role for occludin in tight junction function, occludin is an integral part of tight junction strands (Fujimoto, 1995), while not essential for strand assembly because intestinal barrier function is intact in occludin deficient embryonic stem cells and occludin-deficient mice (Saitou et al., 2000; Saitou et al., 1998).

![Intestinal epithelial intercellular junctions](image)

**Figure 4.** Intestinal epithelial intercellular junctions

Note: ZO: zonula occludens

In IBD, the paracellular space has increased permeability, and the regulation of tight junctions is defective (Wang et al., 2006; Bruewer et al., 2003). The hallmark of active IBD is a pronounced infiltration into the lamina propria of innate immune cells (neutrophils, macrophages, dendritic cells, and natural killer T cells) and adaptive immune cells (B cells and T cells). Increased numbers and activation of these cells in the intestinal mucosa elevate local levels of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and cytokines of the interleukin-23–T helper 17 pathway (Abraham and Cho, 2009). For example, in Crohn’s disease, there is increased production of the Th17
cytokine interleukin-17 and the Th1 cytokines interferon-γ and TNF-α in the intestinal mucosa. In ulcerative colitis, by contrast, there is usually an increase in interleukin-17 and Th2 cytokines, such as IL-4, 5 and 13 (Abraham and Cho, 2009).

The ability of cytokines, such as TNF-α and IFN-γ, to regulate the function of the tight junction barrier was first described 20 years ago (Madara et al., 1989). Myosin light chain kinase (MLCK) has been shown to have a central role in TNF-α induced epithelial and endothelial barrier dysregulation. Similar to Na⁺-nutrient co-transport, TNF-α-induced MLCK activation seems to increase paracellular flux through the leak pathway. IFN-γ-induced occludin internalization is mediated by myosin ATPase-dependent macropinocytosis and occludin cleavage may even modify the barrier to enhance transepithelial migration of inflammatory cells in the lung (Chun et al, 2009). Moreover, IL-1β levels are markedly elevated in intestinal tissues in patients with IBD (Turner et al., 2005). Recent studies have shown that IL-1β at physiologically relevant concentrations (1-10 ng/ml) causes an increase in intestinal epithelial tight junction permeability, which is modulated in part by regulation of MLCK (Al-Sadi et al., 2007). The IL-1β induced increase in Caco-2 tight junction permeability was preceded by an increase in MLCK mRNA and protein levels; and targeted inhibition of IL-1β induced MLCK mRNA or MLCK protein expression prevented the increase in tight junction permeability (Al-Sadi et al., 2008). The IL-1β induced increase in retinal pigment epithelial tight junction permeability was associated with a down-regulation of occludin protein and increase in claudin-1 expression (Abe et al., 2003). Other investigators have also shown that IL-1β causes an increase in claudin-1 and claudin-2 expressions and decrease in occludin expression in human astrocytes, Caco-2 cells and rat hepatocytes (Duffy et al., 2000; Yamamoto et al., 2004; Al-Sadi et al., 2008). Therefore, in vitro research on intestinal
epithelial cells has mainly focused on the involvement of aforementioned cytokines- IL-1β, TNF-α and IFN-γ, which activate intracellular cascades, increase transcriptional activity and the secretion of other cytokines and chemokines, as well as increase the paracellular permeability though defects in tight junctioning or assembly.

Therefore, it is critical to investigate the roles of the epithelial cell barrier in health and disease, with particular emphasis on the junctional complexes, which have a crucial role in barrier regulation. Previous studies showed that some polyphenols, such as epigallocatechin gallate, genistein, myricetin, kaempferol and quercetin, enhance intestinal tight junctions in human intestinal Caco-2 monolayers (Wells et al., 1999; Suzuki and Hara, 2010; Suzuki et al., 2011). Caffeic acid phenethyl ester inhibited the down-regulation of occludin, a transmembrane tight junction protein, induced by TNF-α in astrocytes, possibly via NF-κB pathway because NF-κB could act as a negative regulator of occludin expression in astrocytes (Wachtel et al., 2001). But very few studies have investigated the influences of phenolic acids on gut barrier functions, which might be the underlying mechanism of the protective effects of caffeic acid derivatives against colitis or colon cancer in animal models (Ye, et al., 2009; Tanaka, et al., 1990; Feng et al., 2010). Thus, our research will focus on the modulation of caffeic acid derivatives from traditional herbs on intestinal barrier function.

5.3 Relevant methodology to study intestinal function

It is important to determine the mechanism of the intestinal absorption of the ingested molecules and the interaction between the targeted compound and the intestinal epithelial cells, and hence it is vital to have good predictive models which offer information about the
fraction of compound absorbed across the intestinal wall, the amount of metabolism during absorption as well as the influence of the compound on the intestinal function.

5.3.1 Caco-2 cells

Caco-2 cell line was developed from a moderately well differentiated colon adenocarcinoma obtained from a 72-year-old male (Fogh et al. 1977). Caco-2 cells differentiate spontaneously and exhibit structural and functional differentiation patterns characteristic of mature enterocytes (Pinto et al. 1983). Caco-2 cells reach confluency within 4-6 days and reach fully differentiated status within 20 days (Pinto et al. 1983). The differentiated cells exhibit structural and functional differentiation patterns similar to mature human enterocytes, such as microvilli of the brush border membrane, tight intercellular junctions, and brush border associated enzymes, Phase I and II metabolizing enzymes as well as transporters. The structural and functional differentiation of the microvilli is associated with the polarization of the monolayer after confluency.

Some parameters, such as transepithelial electrical resistance (TEER) or transport of the hydrophilic molecule lucifer yellow or mannitol have been used as indicators of the epithelial integrity. Caco-2 monolayers have a TEER between 250 and 800 Ω•cm² which is similar to the TEER in the colon rather than that in the small intestine which is lower (Biganzoli et al., 1999). Indeed, fully differentiated Caco-2 cells form an epithelial membrane with a barrier function similar to the human colon (Artursson et al., 1993) but express carrier proteins similar to the small intestine (Baker and Baker 1992; Hidalgo et al. 1989). An increase in the transport of hydrophilic paracellular marker indicates increases in the cell monolayer permeability (Hubatsch et al., 2007). These parameters can be used to evaluate cytotoxicity of nutrients, drug and other compounds.
Bidirectional studies (apical to basolateral and basolateral to apical transport) in Caco-2 cells enable the investigation of the mechanisms of passive paracellular and passive transcellular permeability, carrier mediated absorptive transport and efflux, as well as active transport (Artursson et al., 1993). The apical compartment is used to mimic the intestinal lumen and the basal compartment mimicking the blood stream.

Caco-2 cells express phase I (i.e. CYPs) and phase II detoxification enzymes (i.e. gluthathione S-transferase (GST), UGT and ST). Because Caco-2 cells contain many of the metabolic enzymes found in the intestinal epithelium, they have been used to evaluate the metabolism of compounds during the transport across the intestinal barrier (Meunier et al., 1995). Meanwhile, Caco-2 monolayers also express several absorptive and efflux transporters, such as P-gp, MRP, OATP, monocarboxylic transporter (MCT), breast cancer resistance protein transporter (BCRP), etc (Laitinen, 2006). Thus the model can be useful to screen a large number of compounds liable to be substrates for the transporter, i.e. P-gp, and to define the role of the transporter in limiting the absorption of the applied compounds.

Caco-2 cells also have limitations. First, the high TEER due to the “tighter” monolayer compared with in vivo situation leads to very low permeability for hydrophilic, mostly paracellularly permeating compounds. Caco-2 cells are better to study transcellularly passively permeating drugs (Laitinen, 2006). Second, Caco-2 cells are a cancer-derived line and therefore may have properties different from normal cells. Third, Caco-2 cells lack the crypt-villus axis, which is important for fluid and ion transport in vivo, and lack the mucus producing goblet cells, which leads to the lack of a prominent mucus layer...
(Artursson 1996). More recently, mixed cultures of Caco-2 cells with mucin-producing cells, i.e. HT-29, have been used in attempts to overcome this limitation of the method.

In conclusion, the major advantage of cell culture models is that we don’t need to consider about the interspecies differences in the morphological and physiological characteristics, which are normal issues in animal models. So the cell culture model (such as Caco-2) has been adopted enthusiastically by the pharmaceutical industry, for the screening of compounds for intestinal absorption. It can provide comparative information, on the intestinal absorption, metabolism and the mucosal toxicity of applied molecules. Moreover, in vivo absorption studies performed with laboratory animals and humans are expensive and time consuming and can pose ethical challenge, thus cell model is a useful alternative to study uptake and transport of important compounds.

5.3.2 Paracellular permeability test
Paracellular permeability was measured by assessing transepithelial electrical resistance (TEER) and using the transfer of a paracellular marker, lucifer yellow (LY), across Caco-2 monolayers (Hubatsch et al., 2007). After 21 d post seeding the cells on the transwell, the transepithelial electrical resistance (TEER) was tested by Millicell ERS meter to reflect the tightness of intercellular junctions and only cells with TEER \( \geq 250 \, \Omega \cdot \text{cm}^2 \) were used for permeability study. As mentioned before, Caco-2 monolayers have a TEER between 250 and 800 \( \Omega \cdot \text{cm}^2 \) which is similar to the TEER in the colon, and greater than that in the small intestine (Biganzoli et al., 1999). The transfer of a paracellular transferred compound is correlated with the TEER change: the greater the TEER, the tighter the cell monolayer, the less transfer of the paracellularly transferred compound, i.e. rosmarinic acid and chlorogenic acid (Konishi and Kobayashi, 2004). Further, the paracellular marker, LY
dissolved in Hank's Buffered Salt Solution was added to the apical chamber. For LY quantification, the apical and basolateral solutions were collected after 1h, transferred to a 96 well plate and read spectrophotometrically at 450 nm. Previous studies used both TEER and LY transfer to investigate the enhancement of intestinal tight junctions in Caco-2 cells by some polyphenols, such as epigallocatechin gallate, genistein, myricetin, kaempferol and quercetin (Suzuki and Hara, 2010; Suzuki et al., 2011). Therefore, TEER and LY are often used together to reflect the tightness of the monolayer and the paracellular permeability.

5.3.3 Western blot, immunofluorescence and ELISA

Western blot is a widely used analytical technique for detecting specific proteins in the given sample of tissue homogenate or extract. Gel electrophoresis was used first to separate denatured proteins by the size of the polypeptide. The proteins were then transferred to a membrane (typically nitrocellulose or polyvinylidene fluoride, PVDF), where they were probed (detected) using antibodies specific to the target protein. Tight junction protein expression has been studied extensively using Western blotting (Al-Sadi et al., 2008; Liu et al., 2010; Mukinda, et al., 2010; Suzuki et al., 2011). Meanwhile, immunofluorescence uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. Suzuki et al. (2009) and Amasheh et al. (2008) performed Western blot and immunofluorescence to measure the enhancement of intestinal barrier function by quercetin through the assembly of zonula occluden (ZO)-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. Further, cytokine secretion, before and after treatment with stimuli to initiate the inflammatory response, was evaluated using a sandwich ELISA method and quantified using the standard provided with
the kit (Van De Walle et al., 2010). Results were subsequently expressed in relative terms to the negative control (untreated cells) to facilitate comparison between groups.

In conclusion, the overarching hypotheses in my study were that alkamides, ketones and caffeic acid derivatives were transferred by Caco-2 cell monolayers independent of plant matrix; and caffeic acid derivatives enhanced the intestinal barrier function. We used Caco-2 cell with HPLC detection to investigate the permeabilities and Phase II biotransformation of botanical constituents, i.e. alkamides, ketones, rosmarinic acid and ursolic acid, as pure compounds and in plant extracts. Western blot and immunofluorescence were used together to study the targeted tight junction protein expression and distribution, and ELISA was used to measure the cytokine excretion by Caco-2 cells with and without the stimuli treatment.
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CHAPTER 3. GLUCURONIDATION OF ALKAMIDES AND KETONES IN ECHINACEA SANGUINEA AND ECHINACEA PALLIDA EXTRACTS BY CACO-2 CELLS AND THEIR MODULATION OF P-GLYCOPROTEIN TRANSPORTER

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Abstract

The use of *Echinacea* as a medicinal herb is prominent in the United States and many studies have been done over the years to assess the effectiveness of *Echinacea* as an immunomodulator. In this study, Bauer alkamides and ketones were hypothesized to be absorbed similarly as pure compounds or from *Echinacea sanguinea* and *Echinacea pallid*
ethanol extracts, as well as that these *Echinacea* extracts could inhibit P-glycoprotein transporter in Caco-2 human intestinal epithelial cells. Using HPLC analysis, the order of the permeation rate of pure alkamides across Caco-2 cells was increased paralleled with compound hydrophobicity with passive diffusion, independent of the plant matrix. Tested alkamides were seemingly N-glucuronidated both as pure compounds and as found in two *Echinacea* species. Both *Echinacea* ethanol extracts stimulated apparent glucuronidation and basolateral efflux of alkamide metabolites. *E. pallida* ethanol extract inhibited the efflux of glucuronides of Bauer alkamide 8 and 11 apically (lumen) while inducing the transfer of the conjugates basolaterally (systemic). Bauer ketone 24 was totally metabolized to more hydrophilic metabolites when administered as a pure compound, but not as found in either *Echinacea* extracts. Bauer alkamide 8, 10 and 11 (175-230 µM) and *E. sanguinea* (1 mg/mL, containing 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11) and *E. pallida* (5 mg/mL, containing 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11) ethanol extracts reduced the efflux of the P-glycoprotein transporter probe calcein-AM from Caco-2 cells. These results suggest that other constituents in the plant extract had a facilitating effect on the metabolism and efflux of alkamides and ketones from *Echinacea*, which would improve the therapeutic benefits of these extracts, and that alkamides and *Echinacea* extracts might be useful in potentiating some chemotherapeutics which are substrates for P-gp.

**Key words:** *Echinacea*; Alkamides; Ketones; Permeability; P-glycoprotein; Caco-2;
Introduction

Plant materials from the genus *Echinacea* have been widely used for centuries in North America and later in Europe for the treatment and prevention of upper respiratory tract infections, such as the common cold and influenza (Barnes et al., 2005; Kligler, 2003). *Echinacea* supplements were the most commonly used nonvitamin, nonmineral, natural products both for adults and children according to 2007 NHIS survey (National Health Interview Survey, 2007). Species of *Echinacea*, such as *E. angustifolia*, *E. pallida*, *E. sanguinea* and *E. purpurea*, have immune modulatory, antiviral, and antibacterial activities (Borchers et al., 2000; Pleschka et al. 2009; Sharma et al., 2010).

*E. pallida* is one of the most medicinally used *Echinacea* species in the US and Europe and is being introduced into other regions due to the increasing popularity in alternative herbal remedies. *E. sanguinea* has been neglected in the scientific literature, however, Birt et al., (2008) reported that *E. sanguinea* ethanolic extract had greater anti-inflammatory activity in terms of the significant reduction of prostaglandin E 2 (PGE2) production in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages, than *E. angustifolia*, *E. pallida*, and *E. simulata*. Studies with pure compounds and plant extract fractions indicate that *Echinacea* species are rich in bioactive chemicals of which lipophilic alkamides and ketones, intermediately hydrophilic phenolic compounds (mainly caffeic acid derivatives) and polysaccharides are the most recognized for their immunomodulatory properties (Barnes et al., 2005). Bauer alkamides 8

((2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide), 10

((2E,4E,8Z)-N-isobutyldodeca-2,4,8-trienamide) and 11

((2E,4E)-N-isobutyldodeca-2,4-dienamide) at 50 µM and ketone 24
(pentadeca-8Z-ene-11,13-diyn-2-one) at 5 µM (Figure 1) possess anti-inflammatory properties because they have been shown to significantly reduce nitric oxide and prostaglandin E (2) production in lipopolysaccharide stimulated RAW264.7 macrophages (LaLone et al., 2007). These alkamides also inhibit Cu (II) - catalyzed oxidation of human low-density lipoprotein \textit{in vitro} (Dalby-Brown et al., 2005).

Despite many \textit{in vitro} studies ascribing biological activities to both the alkamides and ketones, these activities \textit{in vivo} are possible only if they are absorbed. Woelkart et al. (2005) found about 5% of the ingested dose of Bauer alkamide 8 and 1% of Bauer alkamide 10 (Figure 1) in human blood 3 h after oral administration of a 60% ethanolic extract of \textit{E. angustifolia} containing 0.4-2 mg of Bauer alkamide 8 and 10 (Figure 1). Jager et al. (2005) and Matthias et al (2005) showed the P_{app} (Apparent permeability coefficients), ranging from $3 \times 10^{-6}$ to $3 \times 10^{-4}$ cm/s, for various alkamides through Caco-2 monolayers, which was correlated to structural variations. But very few studies have focused on the metabolism of the bioavailable alkylamides (Toselli et al., 2010) and it is essential to understand the fate of alkylamides after ingestion because the metabolites may have lessened bioactivities compared with the parent compound (Cech et al., 2006). Caco-2 cells are immortalized human epithelial colorectal adenocarcinoma cells and offer a standard rapid, reliable, and low-cost model for \textit{in vitro} prediction of intestinal drug permeability and absorption (Hubatsch et al., 2007). Moreover, the plant material matrix may alter absorption or metabolism and, consequently, the bioavailability of phytochemicals (Manach et al., 2004). Therefore, it is crucial to investigate the effect that the plant matrix may have on the uptake of key components found in \textit{Echinacea} species. Neither \textit{in vitro} studies have been done in association with the uptake of Bauer alkamides and ketones in different species of \textit{Echinacea}, nor have the mechanism and metabolism
been investigated for their influence on pure compound uptake. In addition, our preliminary experiments showed that alkamide 8, 10, 11 and ketone 24 were only present in *E. pallida* and *E. sanguinea* ethanol extracts, but not in *E. angustifolia* and *E. simulata* ethanolic extracts (data not published). Therefore, *E. pallida* and *E. sanguinea* were chosen in this study to investigate their absorption and metabolism.

Previous study showed that n-hexane root extracts from *E. pallida*, *E. angustifolia* and *E. purpurea* (30 µg/mL) inhibited multidrug transporter P-glycoprotein (P-gp) activity in a human proximal tubular cell line (Romiti et al., 2008). P-gp transporter plays a key role in drug absorption and distribution because it limits the permeability across the GI (gastrointestinal) tract (Hunter and Hirst, 1997) by active efflux of potentially toxic substances back into the intestinal lumen. The transporter is also expressed in other tissues such as liver, kidney and blood–brain barrier where it also plays an important excretory role. On the other hand, P-gp confers resistance to anticancer chemotherapy because it is over-expressed in cancer cells (O'Connor 2007). The elucidation of the mechanisms by which botanicals mediate anticancer activities (i.e. inhibition of ABC transporters) may allow a new paradigm of clinically useful drug resistance circumvention.

Our hypotheses were that the absorbability of Bauer alkamides and ketones was independent of the plant extract matrix, alkamides and ketones were glucuronidated, and that ethanolic extracts of *E. pallida* and *E. sanguinea* extracts containing alkamides would inhibit P-gp activities in Caco-2 cells. This study was conducted to investigate not only the effect on epithelial barrier permeabilities and metabolism of Bauer alkamides and ketones as pure compounds and in *E. pallida* and *E. sanguinea* ethanol extracts across Caco-2 cell monolayers, but also the inhibitory effects of pure compounds and plant
extracts on P-gp, to facilitate future studies of the efficacy of these herbs against inflammation and herb-drug interactions.

2. Materials and methods

2.1. Plant extraction

Plant samples were provided by the North Central Regional Plant Introduction Station (NCRPIS, Ames, IA) of the Agricultural Research Service of the U.S. Department of Agriculture. Roots of *E. pallida* (PI 631293) and *E. sanguinea* (PI 633672) were obtained from the NCRPIS, harvested, dried and ground (Voucher records: Inventory sample: PI 631293 SD 97ncao01; PI 633672 SD 97ncao01). Dried *Echinacea* root per population (6 g) were extracted with 500 mL of 95% ethanol by Soxhlet percolation for 6 h, filtered, dried by rotary evaporation and lyophylized. Then the extracts were redissolved in 0.5 mL of ethanol and stored at -20 °C under nitrogen. Information about the specific provenance of both accessions obtained from the NCRPIS is available on the Germplasm Resources Information Network database at [http://www.ars-grin.gov/npgs/acc/acc_queries.html](http://www.ars-grin.gov/npgs/acc/acc_queries.html).

2.2. Bauer alkamide and ketone synthesis

Chemical synthesis of Bauer alkamides and ketones (Bauer et al., 1988) were conducted according to the procedures described by Wu et al. (2004) and Bae (2006). Alkamide and ketone concentrations were calculated after correcting for percent purity, yielding concentrations equivalent to 100 % pure synthetic constituent. Calculated percent purity before correction for Bauer alkylamide 8 was 70%, Bauer alkylamide 10 was 82 %, Bauer alkylamide 11 was 92 %, and Bauer ketone 24 was 99%. All synthetic Bauer alkamides and ketone were dissolved in DMSO and stored at -80 °C under argon gas.
2.3. HPLC analysis

Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). HPLC analysis was performed on a Beckman Coulter 126 HPLC, equipped with photodiode array detector model 168 and a model 508 autosampler (Beckman Coulter, Inc., Brea, CA). The mobile phase was CH$_3$CN/H$_2$O at a flow rate of 1.0 ml/min following a linear gradient of 40–80% CH$_3$CN in H$_2$O over 45 min. A reverse phase analytical YMC pack-ODS C$_{18}$ column (250 mm× 4.6 mm × 5 μm, Waters Corp., Milford, MA ) was used at room temperature. The wavelength was 260 nm and the retention times were 25.4 min for Bauer alkamide 8, 29.1 min for Bauer alkamide 10, 35.6 min for Bauer alkamide 11, and 17.6 min for Bauer ketone 24. The limit of detection (LOD) and limit of quantitation (LOQ), defined as a signal/noise ratio ≥ 3 and ≥ 6, was 0.04 μM and 0.08 μM for Bauer alkamide 8, 10 and 11, 0.5 μM and 1.0 μM for Bauer ketone 24. The linear regression equation for Bauer alkamide 8 was y = 27.234x - 0.3108 with R$^2$ of 0.9996, y = 75.084x + 0.3075 with R$^2$ of 0.9995 for Bauer alkamide 10, y = 36.758x - 0.2493 with R$^2$ of 0.9999 for Bauer alkamide 11 and y = 73.532x + 0.0716 with R$^2$ of 0.9994 for Bauer ketone 24, where y is the concentration of pure compound, and x is the ratio of peak area of the standard to peak area of the internal standard (2E,4E-hexadienoic acid isobutylamide, ChromaDex, Irvine, CA). The intraday and interday CVs were 6.1 ± 2.1 % and 2.1 ± 0.4 % for Bauer alkamide 8, and 8.4 ± 2.6 % and 5.4 ± 3.0 % for Bauer alkamide 10, 5.8 ± 1.4 % and 2.0 ± 0.4 % for Bauer alkamide 11, and 8.4 ± 2.5 % and 3.6 ± 0.4 % for Bauer ketone 24 across 0.08-100 μM.

2.4 Transepithelial transfer experiment
Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) at passage 18 and all experiments were performed from passages 30–35. The cells were cultured according to Hubatsch et al. (2007). Cytotoxicity of pure compounds and extracts was measured according to Nasser et al. (2008). Pure Bauer alkamides and ketone, at 10-1000 μM, and *E. pallida* and *E. sanguinea* ethanol extracts, at 0.1–50 mg/mL, were tested for cytotoxicity. DMSO in DMEM (Dulbecco's modified Eagle's medium, 0.3%, Gibco Invitrogen, Carlsbad, CA) was used as control.

After the cells in the flask grew to 90–100% confluency, cells were trypsinized and seeded on collagen-coated polytetrafluoroethylene membrane inserts (0.45 μm) fitted in bicameral chambers (Transwell-COL, 24 mm ID, Corning Inc., Corning, NY) at 1.2 × 10^5 cells/cm^2. The transepithelial electrical resistance (TEER) was tested by Millicell ERS meter (Fisher Sci., Pittsburgh, PA) to reflect the tightness of intercellular junctions and only cells with TEER ≥ 250 Ω·cm^2 were used for permeability study (Hubatsch et al., 2007). At 21 d post-seeding (90–100% confluence) onto the transwell membrane, pure Bauer alkamides and ketone and extracts at non-cytotoxic concentrations, dissolved in Hank's Buffered Salt Solution (HBSS, pH 7.4, Gibco Invitrogen, Carlsbad, CA), were added to the donor side with the receiving chamber containing 1.5 mL of HBSS. After 15, 30, 60, and 90 min, solutions were collected from the receiver side. Samples were collected from both sides at the end and membrane on the transwell insert was placed in 1.5 mL of ice-cold 0.5 mol sodium hydroxide/L and sonicated with a probe-type sonic dismembrator (Biologics Inc., Manassas, VA); pH was adjusted to 7.0 and all samples were injected directly to HPLC for analysis. The transport experiment was performed both at 37 °C and at 4 °C. Total cellular protein was determined by Coomassie (Bradford) assay (Pierce Laboratories, Rockford, IL).
2.5. Transepithelial transfer of pure compounds and extracts after treating with 
\(\beta\)-glucuronidase/sulfatase

Pure Bauer alkamides and ketone and plant extracts at non-cytotoxic concentrations were 
applied to Caco-2 cells. Twenty \(\mu\)L of \(\beta\)-glucuronidase (Type H-5 from \textit{Helix pomatia}, 40 
units/L, Sigma-Aldrich Co., St. Louis, MO) were added to the post-experimental apical and 
basolateral solutions and to cell homogenates and incubated overnight at 37 °C to release 
the parent compounds. These samples were then injected directly to HPLC.

Apparent permeability coefficients (\(P_{\text{app}}\)) were determined using the equation (Hubatsch et 
al., 2007): 
\[
P_{\text{app}} = \frac{dQ}{dt} \left( \frac{1}{A \times C_0} \right);
\]
\(dQ/dt\) was the permeability rate constant (\(\mu\)mol/s); \(A\) was the surface area of the membrane (cm\(^2\)); and \(C_0\) was the initial concentration of the 
compound (\(\mu\)M). Recovery relative to the initial amount (%) was calculated as the 
proportion of the original amount that permeated through the monolayer, which was 
calculated as the amount transported divided by the initial amount in the donor side. 
Permeation rate (\(\mu\)mol/min/mg protein) was calculated as the amount transported divided 
by incubation time and protein content.

2.6. P-gp assay

P-gp activity was evaluated with fluorimetric measurement of the intracellular 
accumulation of calcein produced by ester hydrolysis of the P-gp substrate calcein-AM 
using Vybrant™ Multidrug Resistance Assay Kit (Gibco Invitrogen, Carlsbad, CA). 
After the cells in the flask grew to 90–100% confluency, cells were trypsinized and seeded 
on 96 well plates at \(1.2 \times 10^5\) cells/cm\(^2\). Cells were preincubated for 15 min with pure 
compounds (Bauer alkamide 8, 10, 11 and ketone 24) or extracts at non-cytotoxic
concentrations, thereafter calcein-AM was added and the fluorescence measured after 1 hour using microtiter plate reader Bio-Tek ELX 808 (Bio-Tek instruments. Inc., Winooski, VT) at 490 nm. The control group received vehicle alone (DMSO in DMEM, 0.3% v/v). The known P-gp inhibitor verapamil (10 µg/mL) was used as a control.

2.7. Statistical analysis

Data are given as means ± S. D. Differences in cytotoxicity, $P_{\text{app}}$, transport kinetics, permeation rate, and absorbance representing calcein efflux by P-gp among treatments were evaluated statistically using two-sample t-test, ANOVA and Tukey’s multiple comparison tests by SAS 9.1 (SAS Institute Inc., Cary, NC). Differences were considered significant at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Determination of alkamides and ketone concentrations in E. sanguinea and E. pallida ethanol extracts

Bauer alkamide 8, 10 and 11 as well as ketone 24 were all present in *Echinacea* species studied, but with different profiles (Table 1). *E. sanguinea* ethanol extract had ~three fold greater Bauer alkamide 8 and ~six fold greater Bauer ketone 24 than *E. pallida* accession on mole basis. The amount of Bauer alkamide 11 in *E. pallida* was about six fold more than *E. sanguinea* and Bauer alkamide 10 content was similar in two species. Bauer alkamide 8 and ketone 24 were more concentrated in both *Echinacea* species compared with Bauer alkamide 10 and 11.

3.2 Cytotoxicity test
The concentrations of pure Bauer alkamide 8 greater than 350 μM, 950 μM for pure Bauer alkamide 10, 460 μM for pure Bauer alkamide 11, and 990 μM for pure Bauer ketone 24 were significantly cytotoxic compared with the control (0.3% DMSO in DMEM, \( p < 0.05 \)). Ethanol extracts of *E. sanguinea* and *E. pallida* up to 5 mg/mL (containing 425 μM of alkamide 8, 10 μM of alkamide 10, and 3.5 μM of alkamide 11) and 20 mg/mL (containing 860 μM of alkamide 8, 100 μM of alkamide 10, and 180 μM of alkamide 11) were significantly cytotoxic to the cells, respectively. Therefore, 10, 25, 50, 100 μM of pure Bauer alkamides, ketone 24, and *E. sanguinea* and *E. pallida* ethanol extracts containing the same concentrations of each compound were used for permeability studies.

3.3. Transepithelial transfer of pure compounds and extracts before and after treating with β-glucuronidase/sulfatase

Figure 2A shows the relationship between the initial permeation rate of Bauer alkamides and concentration. The rate of membrane permeation was calculated for Bauer alkamides as pure compounds. The uptake of Bauer alkamide 8, 10 and 11 increased linearly and exhibited non-saturable transport across the tested concentrations (10- 100 μM, Figure 2A). Bidirectional permeation of three Bauer alkamides across Caco-2 cell monolayers was examined. Transport rate of Bauer alkamide 8, 10 and 11 in the basolateral to apical (BL–AP) direction was nearly the same as that in the apical to basolateral (AP–BL) direction at each tested time point (Figure 2B, \( p > 0.05 \)). The transepithelial transport in the two directions was not saturated within 90 min as shown in Figure 2B. The transport of Bauer alkamide 8, 10 and 11 across Caco-2 cell monolayers was investigated at both 37 °C and 4 °C to evaluate the effect of temperature on the transport of three alkamides (AP-BL, Figure 2C). No significant differences were found in the permeation rates of three alkamides at 37 °C compared with that at 4 °C (\( p < 0.05 \)).
Further, there are differences in permeability that correlate with variations in alkamide structure and the rank of the permeation of three alkamides was Bauer alkamide 8 > 10 > 11 (Table 2, Figure 2A, B and C). No Bauer alkamide 11 was found in the basolateral side when 10 µM of the pure compound was applied to the apical side, which was due to the lesser permeability of Bauer alkamide 11 compared with Bauer alkamide 8 and 10. No significant differences were found between alkamides as pure compounds compared to alkamides present in both *E. sanguinea* or *E. pallida* extracts (*p* > 0.05) in apparent permeability coefficients before deconjugation with β-glucuronidase (*P*<sub>app</sub>, Table 2).

Formation of glucuronide conjugates during transcellular transport of the alkamides was investigated. After addition of the alkamides to the apical side of Caco-2 monolayers, glucuronide metabolites of three alkamides in the *E. sanguinea* extract were found in all compartments, including apical and basolateral sides as well as cell lysates, compared with only in apical side for pure Bauer alkamide 8, in apical side and cells for pure Bauer alkamide 10 as well as in basolateral side and cells for pure Bauer alkamide 11 (Figure 3A, B and C). Interestingly, glucuronide conjugates were only detected basolaterally for Bauer alkamide 8 and 10 from *E. pallida* extract (Figure 2 A and B), and only in cells for Bauer alkamide 11 from *E. pallida* extract (*p* < 0.05, Figure 3C).

The *P*<sub>app</sub> for Bauer ketone 24 in *E. sanguinea* and *E. pallida* ethanol extracts were 17.1 ± 7.8 and 12.2 ± 3.2 cm/s ×10<sup>-6</sup> with no significant difference (*p* > 0.05, Table 2), while Bauer ketone 24 was not found either apically or basolaterally nor in cell lysates at each time point (15-90 min) across tested concentrations (10-100 µM). Two unknown peaks were detected (retention time 13.3 and 15.4 min) in both apical and basolateral chambers and
cells after treating the Caco-2 cells with pure ketone 24, but not for the compound as found in the two plant extracts (Figure 4B, C and D), which implies that pure Bauer ketone 24 was metabolized to more hydrophilic metabolites but this biotransformation was inhibited by other constituents in *Echinacea* extracts. After β-glucuronidase treatment, Bauer ketone 24 recovery relative to the applied amount was significantly increased both basolaterally and in cells for the compound contained in *E. sanguinea* and *E. pallida* extracts (Figure 3D). P_app was significantly increased for three alkamides and Bauer ketone 24 in *E. sanguinea* extract and for Bauer alkamide 8, 10 and ketone 24 in *E. pallida* extract compared with pure compounds after deconjugation with β-glucuronidase (*p* < 0.05, Table 2).

3.4. Pgp inhibition by pure alkamides, *E. sanguinea* and *E. pallida* ethanol extracts

In the present study, the modulatory effects on P-gp activity of ethanol extracts of *E. sanguinea* and *E. pallida* as well as pure alkamides and ketone were investigated. As shown in Figure 5, After purity correction, pure Bauer alkamide 8 at 175 µM, Bauer alkamide 8 at 205 µM, Bauer alkamide 11 at 230 µM significantly inhibited P-gp activity (*p* < 0.05), but Bauer ketone 24 was not active across the tested concentrations (1-250 µM). The ethanolic extracts of *E. sanguinea* and *E. pallida* significantly inhibited P-gp at 1 mg/mL (containing 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11) and 5 mg/mL (containing 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11), respectively (Figure 5).
4. Discussion

This study investigated the uptake and metabolism of alkamides and ketone, when introduced as pure compounds or as components of the complex ethanolic extracts of *Echinacea* to cultures of the human intestinal epithelial cell line, Caco-2. The objective was to explore the effect that the compound structure and the plant matrix may have on the intestinal uptake and metabolism of alkamides and ketone from a traditional medicinal plant. We also used Caco-2 cells to study the interaction of alkamides and *Echinacea* ethanol extracts with P-gp, to identify compounds that could potentially inhibit P-gp.

The transport of pure alkamides and the accumulation from both the apical and basolateral sides of the cells were studied. Because Bauer alkamide 8 was considered to be absorbed *via* passive diffusion (Jager et al., 2002), it was expected that other alkamides would also be absorbed *via* similar pathway across Caco-2 cells. This is supported by the finding that the rate of membrane permeation of three alkamides increased linearly with concentration, was not saturable during the tested incubation period (15-90 min) and was not different between AP-BL and BL-AP direction (Figure 2A and B). Further, the transport of three alkamides was temperature independent (Figure 2C), which indicates passive diffusion through the Caco-2 cells. Because an active mechanism is involved, the transport of the molecule is almost completely abolished at 4°C compared with 37°C (Said et al., 2003).

The order of the uptake of three alkamides was Bauer alkamide 8 > 10 > 11, consistent with $P_{app}$ for Bauer alkamide 8, ~2.5 fold and ~15 fold greater than that of Bauer alkamide 10 and 11, respectively (Table 2). This trend for increases in hydrophilicity giving rise to
increases in the apparent permeability was also seen for the family of alkamides (Matthias et al., 2005).

Monohydroxylated, monoepoxidized, N-dealkylated metabolites were reported after incubation of parent Bauer alkamide 8 with both NADPH and human liver microsomes (Matthias et al., 2005b). But no studies have investigated Phase II biotransformation of alkamides. Various amides, including aliphatic amides, cyclic amides and sulphonamides, have been reported to undergo N-glucuronidation as a major elimination pathway (Sanna, 2010). In our study, after treating the post-experimental apical and basolateral solutions and cell lysates with β-glucuronidase, the amount of the parent compound increased by ~1.5 to 4.2 fold for Bauer alkamide 8, 10 and 11 in apical side, basolateral side or cell lysates, indicating that alkamides were seemingly N-glucuronidated to some extent based on the amide structure (O-glucuronide might be hard to form because of the steric hinderance, Figure 1), revealing another important metabolic pathway for alkamides besides cytochromes P450. E. sanguinea constituents increased total glucuronide formation for all alkamides, suggesting allosteric activation by plant constituents other than alkamides, given the short time frame for the observed apparent stimulation of UDP glucuronosyltransferases (UGTs). Caffeic acid (0.6 mmol), quercetin, and apigenin (2-38 nmol), which were all found in Echinacea species, induced UGTs expression both in animal models and in cell lines (Svehliková et al., 2004; Debersa et al., 2001; Galijatovic et al., 2000).

It is critical to investigate the effect of the plant matrix on the bioavailability of their bioactive compounds because the complex constituents may alter absorption or metabolism and, consequently, the bioavailability of phytochemicals by the influences on Phase I and II
enzymes and transporters. The plant extracts did not affect transport of pure alkanides before deconjugation with β-glucuronidase (Table 2), establishing that the uptake of alkanides was independent of the plant extract matrix for the two Echinacea species studied. This observation might be due to the transport mechanism of alkanides—passive diffusion across Caco-2 cell monolayers, without transporter mediated uptake involved for the parent compound. Our study is the first to report the effect of Echinacea species on the metabolism of alkanides and ketone. Interestingly, the recovery relative to the initial amount applied to the cells after β-glucuronidase hydrolysis was significantly increased in all compartments of the system, including apical and basolateral side as well as cell lysates, for all three alkanides studied in E. sanguinea extract. These results suggest that other constituents contained in Echinacea species might regulate the expression of MRP (multidrug resistance-associated protein) or OATP (organic anion transporter protein) transporters which in turn affect the permeation of glucuronidated alkanides, because anionic conjugates (glutathione, glucuronide or sulfate) cannot exit cells unless an MRP or OATP transporter is present (Peng et al. 1999). E. pallida ethanol extract inhibited the efflux of glucuronides of Bauer alkanide 8 and 10 apically (lumen) while enhancing the transfer of the conjugates basolaterally (systemic), which was not found for Bauer alkanide 11, indicating that alkanide conjugates may have different structural affinity to the efflux transporters (i.e. MRPs) and that greater amounts of Bauer alkanide 8 and 10 from plant sources may enter systemic circulation, compared with Bauer alkanide 11, after deconjugation by microbial β-glucuronidase leading to stronger immune modulatory effects.

The permeability and metabolism of Bauer ketone 24 were investigated as pure compound and the compound contained in Echinacea species. Pure Bauer ketone 24 might be
metabolized by CYP 450 based on the diene structure and ketone group. It has been shown that there is a strong overlap between substrates for gut metabolism by CYPs and gut active efflux by P-gp, suggesting that these two processes may work together to affect the intestinal absorption of a variety of drugs (Wacher et al., 1995). Therefore, P-gp transporter, located along the apical membrane of the enterocytes, might be involved in the efflux of Bauer ketone 24 metabolites. The transport of Bauer ketone 24 metabolites to the basolateral side might be due to passive diffusion along the concentration gradient. More interestingly, about 5% of the Bauer ketone 24 was recovered in the basolateral side for the compound contained in two *Echinacea* species, and the $P_{app}$ for Bauer ketone 24 was similar to that of Bauer alkamide 10 (Table 2). The reason for the difference in permeability of Bauer ketone 24 as pure compound or that in *Echinacea* species might be due to the effects of other constituents contained in the herb extract, because several *Echinacea* species (11.2 – 2447 µg/mL) have been shown to inhibit CYP 2C19, 2D6, and 3A4 by 20-100 %, and alkamides contributed to the inhibitory effects seen with *Echinacea* preparations (Modarai et al., 2007). Pure Bauer alkamide 8, 10 and 11 were incubated with ketone 24 across 10-100 µM, but ketone 24 was still totally metabolized as pure compound (data not shown). This implies that the CYP isoforms inhibited by those alkamides were not involved in the biotransformation of ketone 24, although we can not rule out the possibility that the doses of those alkamides were too low to exhibit inhibitory effects. Bioactivity of these metabolites should be examined to determine the influence of intestinal metabolism on bioavailability of the alkamides and ketones. How the plant constituents alter glucuronidation also needs to be understood as this has implications for herb/drug interactions.
P-gp actively effluxes a wide range of structurally diverse anticancer agents and P-gp-mediated multidrug resistance (MDR) has been associated with inhibition of caspase-dependent tumor cell apoptosis (Smyth et al., 1998). One strategy for reversal of MDR in cells expressing ABC transporters is combined use of anticancer drugs with modulators. Inhibitors of ABC transporters, like P-gp and MRP1, can be used to enhance effects of anticancer drugs in patients with MDR. In our study, *E. sanguinea* (1 mg/mL, containing 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11) and *E. pallida* (5 mg/mL, containing 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11) significantly inhibited P-gp-mediated efflux of calcein AM, a substrate for P-gp, indicating that alkamides were potentiated in the inhibition on P-gp by other plant extract constituents. For example, quercetin at 100 µM inhibited 30 % of P-gp mediated efflux of [³H]-taxol in Caco-2 cells (Hayeshi et al., 2006). There might be some interactions between glucuronidation and P-gp modulation, because coregulation of UGTs and ABC transporters by AhR (Ah-receptor) ligands has been studied in Caco-2 cells (Walle et al., 1999; Bock et al., 2000; Bock-Hennig et al., 2002) showing that AhR, PXR (pregnane X receptor), and CAR (constitutive androstane receptor) found in promoter/enhancer region of the UGT gene might also be involved in the regulation of ABC transporters (Bock and Bock-Hennig, 2010). Further, the concentrations of *Echinacea* plant extracts that inhibited of P-gp in our study were greater than in a published paper showing that extracts of roots of *Echinacea* species inhibited P-gp in human proximal tubular kidney cells, and *E. pallida* extract was the most active at 3 µg/mL compared with *E. angustifolia* and *E. purpurea* both at 30 µg/mL (Romiti et al. 2008). This might be due to the difference in the cell lines used or the preparation of the extracts (extraction ratio (raw material weight: final solution volume) was 12:1 in our study and 125:1 in Romiti’s study). The concentrations of *Echinacea* extracts showing inhibitory
activities of P-gp were greater than the concentrations of *E. sanguinea* and *E. pallida* containing 10-100 µM of Bauer ketone 24 (0.08-0.8 mg/mL and 0.3-3.4 mg/mL, respectively), thus P-gp might not be inhibited and was able to efflux pure Bauer ketone 24 metabolites in our permeability study (Table 2 and Figure 4). Romiti et al. (2008) found that the lipophilic ketones isolated from *E. pallida* roots, namely 8-hydroxypentadeca-(9E)-ene-11,13-diyn-2-one, 8-hydroxypentadeca-(9E,13Z)-diyn-11-yn-2-one, pentadeca-(9E)-ene-11,13-diyne-2,8-dione, pentadeca-(9E,13Z)-diyn-11-yne-2,8-dione and pentadeca-(8Z,13Z)-diyn-11-yn-2-one, in the range of 200-800 µM, inhibited P-gp. Our study is the first report of pure Bauer alkamide 8, 10 and 11 (greater than the amount contained in *E. sanguinea* at 1 mg/mL or *E. pallida* at 5 mg/mL) inhibiting P-gp mediated efflux in Caco-2 cells, which may have implications for anti-colitic or colon cancer drug interactions with P-gp. Because we have shown that alkamides are apparently glucuronidated, even though they are probably most absorbed in the small intestine, the glucuronides will be effluxed to the lumen to some extent, although not for the alkamides in *E. pallida*, and they would also be susceptible to biliary excretion into the intestine. Although N-glucuronides are more slowly hydrolyzed by β-glucuronidase, produced by gut bacteria, than O-glucuronides or S-glucuronides (Casarett and Doull, 2008), the parent alkamides would still be available to act in the large intestine. In the current study we analyze each chemically synthesized preparation prior to its use as an inhibitor of P-gp function, allowing for impurities in the alkamide preparations, and normalizing to a concentration at 100% purity; this normalization allowed for greater sensitivity in our screening of P-gp inhibition using the caco-2 intestinal cell line. In addition, alkamides were highly permeable, which permits their interaction with P-gp, since its binding sites are either inside the bilayer or at the inner leaflet of the cell membrane (C and Clarke, 2005). Although glucuronidation may prevent tissue uptake of
the alkamides, glucuronidase produced by liver and neutrophils (O'Leary et al., 2001; Bartholomé et al., 2010) may convert the glucuronide conjugates to parent compounds and alkamides may enter the hepatic portal vein at relative high concentrations, possibly permitting alkamide inhibitory activity of P-gp in tissues throughout the body.

In conclusion, Bauer alkamide 8, 10 and 11 were transferred across Caco-2 cells, independent of extract matrix, thus results on efficacy of the pure compounds may reasonably be extrapolated to results using plant materials containing these compounds. Alkamides were seemingly N-glucuronidated to some extent and Bauer ketone 24 was transferred and metabolized to glucuronide conjugates as the compound from plant extracts but not as pure compound, strongly suggesting that alkamides and Bauer ketone 24 in Echinacea species had a more extensive metabolism in the Caco-2 cells than pure compounds, and that plant matrix may have a facilitating effect on the metabolism of alkamides and ketones from Echinacea. Because the Caco-2 cell model is considered to be a good predictive model for human gastro-intestinal absorption, the results obtained should also closely reflect the in vivo intestinal uptake and metabolism of alkamides and ketones expected when Echinacea plant extracts and supplements are given to humans. Bauer alkamide 8, 10 and 11 as well as E. sanguinea and E. pallida extracts inhibited P-gp mediated efflux in Caco-2 cells. Considering the increasing knowledge about the role of P-gp in cancer resistance and the worldwide increasing use of Echinacea preparations, further studies in humans are required to specifically investigate the uptake and metabolism of alkamides and ketones derived from natural plant products in humans. The reason for the enhanced metabolism by other accompanying constituents in plant extracts should also be established. Because Echinacea supplements are usually ingested on a chronic basis,
their long term effects on the metabolizing enzymes and expression of efflux transporters needs to be investigated and may be of importance regarding herb–drug interactions.

Acknowledgements

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Figures

(2E,4E,8Z,10Z)-N-isobutyldec-2,4,8,10-tetraenamide (Bauer alkamide 8), m/z = 247

(2E,4E,8Z)-N-isobutyldec-2,4,8-trienamide (Bauer alkamide 10), m/z = 249

(2E,4E)-N-isobutyldec-2,4-dienamide (Bauer alkamide 11), m/z = 251

Pentadeca-(8Z,13Z)-dien-11-yn-2-one (Bauer ketone 24), m/z = 218

Figure 1. The chemical structures of Bauer alkamide 8, 10, 11 and ketone 24.
A

Permeation rate (μmol/minute × mg protein)

Concentration (μM)

B

Time (min)

C

Permeation rate (μmol/minute × mg protein)

Concentration (μM)
Figure 2. Transport of pure Bauer alkamide 8, 10 and 11 across Caco-2 cells. A. Concentration dependency of the transport of three alkamides. B. Non-saturable transport of three alkamides at 25 μM during 90 min incubation period with no difference between apical to basolateral (AP-BL) and basolateral to apical (BL-AP) direction at each time point. C. The effect of temperature on the transport of the three alkamides (37°C vs 4°C). Means bearing different letters were significantly different among three alkamides by ANOVA and Tukey’s multiple comparison ($p < 0.05$). Data are the mean ± S. D. (n=6).
Figure 3. Glucuronidation of Bauer alkamide 8, 10, 11 and ketone 24 for pure compounds or from plant extracts by Caco-2 cells. Before, prior to deconjugation with β-glucuronidase. After, subsequent to deconjugation with β-glucuronidase. The concentration of three Bauer alkamides and ketone 24 was 25 µM both for pure compounds or as found in the two *Echinacea* extracts. A. Bauer alkamide 8. B. Bauer alkamide 10. C. Bauer alkamide 11. D. Bauer ketone 24. * Significantly different compared with the recovery relative to the initial amount before the enzyme treatment by two-sample t-test (*p* < 0.05). Data are the mean ± S. D (n=6).
Figure 4. HPLC chromatograms of Bauer ketone 24 across Caco-2 cell monolayers as pure compound or from *Echinacea* species.  

A. Bauer ketone 24 standard at 100 µM (retention time 17.6 min).  

B. Bauer ketone 24 (100 µM) was not detected in the basolateral supernatant fluid, but two more hydrophilic metabolites were detected (retention time 13.3 and 15.4 min).  

C. Bauer ketone 24 was shown in the basolateral side after apically applied *E. sanguinea* containing 100 µM of ketone 24 (retention time 17.6 min).  

D. Bauer ketone 24 was shown in the basolateral side after apically applied *E. pallida* containing 100 µM of ketone 24 (retention time 17.6 min).
Figure 5. The effects of Bauer alkamide 8, 10, 11, ketone 24 on P-glycoprotein transporter activity. Control was 0.3% DMSO in PBS. Verapamil was used as positive control at 10 µg/mL. * Significantly different compared with control by two-sample t-test ($p < 0.05$). ** Significantly different compared with control by two-sample t-test ($p < 0.01$). Data are the mean ± S. D (n=6). The ethanolic extract of *E. sanguinea* at 1 mg/mL contained 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11, and 5 mg/mL of *E. pallida* extract contained 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11.
Table 1. The concentrations of alkamides and ketone in *Echinacea* ethanol extracts determined by HPLC\(^a\).

\(^a\) *E. sanguinea* and *E. pallida* ethanolic extracts were diluted by methanol for HPLC analysis (n=9). All values are means± SD.

<table>
<thead>
<tr>
<th></th>
<th>Bauer alkamide 8 (g/L (mM))</th>
<th>Bauer alkamide10 (g/L (mM))</th>
<th>Bauer alkamide11 (g/L (mM))</th>
<th>Bauer ketone 24 (g/L (mM))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. sanguinea</em></td>
<td>3.2 ± 0.2 (13.1 ± 0.7)</td>
<td>0.1 ± 0.01 (0.3 ± 0.02)</td>
<td>0.03 ± 0.01 (0.1 ± 0.04)</td>
<td>3.8 ± 0.5 (17.3 ± 2.5)</td>
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<tr>
<td>(PI 633672, 154.3 g/L(^b))</td>
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<tr>
<td><em>E. pallida</em></td>
<td>1.1 ± 0.1 (4.3 ± 0.3)</td>
<td>0.1 ± 0.01 (0.5 ± 0.02)</td>
<td>0.2 ± 0.03 (0.9 ± 0.1)</td>
<td>0.6 ± 0.04 (2.9 ± 0.2)</td>
</tr>
<tr>
<td>(PI 631293, 99.0 g/L)</td>
<td></td>
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</tbody>
</table>

\(^b\) PI, assesion number.
Table 2. Apparent permeability coefficients (P_{app}) for pure alkamides and extracts across the Caco-2 monolayer$^a$.

<table>
<thead>
<tr>
<th></th>
<th>Bauer alkamide 8</th>
<th>Bauer alkamide 10</th>
<th>Bauer alkamide 11</th>
<th>Bauer ketone 24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before $^d$</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Pure compound $^b$</td>
<td>43.8 ± 11.2 a</td>
<td>39.8 ± 13.4 b</td>
<td>17.7 ± 8.8 a</td>
<td>28.4 ± 5.9 b</td>
</tr>
<tr>
<td>E. sanguinea $^c$</td>
<td>54.6 ± 13.2 a</td>
<td>76.8 ± 11.7 *a</td>
<td>12.9 ± 5.6 a</td>
<td>28.4 ± 4.1 a</td>
</tr>
<tr>
<td>E. pallida</td>
<td>41.3 ± 7.4 a</td>
<td>65.2 ± 6.8 *a</td>
<td>14.7 ± 4.2 a</td>
<td>22.3 ± 7.9 a</td>
</tr>
</tbody>
</table>

$^a$ All values are means ± SD (n=9). TEER was 460-573 Ω·cm². Means bearing different letters were significantly different in each column ($p < 0.05$).

$^b$ Bauer alkamide 8, 10, 11 and ketone 24 were 10-100 μM for pure compounds.

$^c$ Two herb extracts were diluted to contain same concentrations of Bauer alkamide 8, 10, 11 or ketone 24 as the pure compounds.

$^d$ Before = before deconjugation; After = after deconjugation.

$^e$ Bauer ketone 24 was not detected in the receiver side for pure compound.

$^*$ Significantly different compared with the P_{app} before deconjugation with β-glucuronidase by ANOVA and Tukey’s multiple comparison.
References


Bock, K. W. & B. S. Bock-Hennig (2010) UDP-glucuronosyltransferases (UGTs): from purification of Ah-receptor-inducible UGT1A6 to coordinate regulation of subsets of CYPs, UGTs, and ABC transporters by nuclear receptors. Drug Metab Rev, 42, 6-13.


Evaluation of the cytotoxicity and genotoxicity of extracts of mussels originating from


CHAPTER 4. METABOLISM AND PERMEABILITY OF ROSMARINIC ACID IN PRUNELLA VULGARIS AND URSOLIC ACID IN SALVIA OFFICINALIS EXTRACTS ACROSS CACO-2 CELL MONOLAYERS

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Abstract

Ethnopharmacological relevance Rosmarinic acid (RA), a caffeic acid derivative found in high concentrations in Prunella vulgaris (self-heal), and ursolic acid (UA), a pentacyclic triterpene acid concentrated in Salvia officinalis (sage), have been traditionally used to treat inflammation in the oral cavity, and may also be of benefit to gastrointestinal health in general.
Aim of the study To investigate the permeability and metabolism of RA and UA as pure compounds and in *P. vulgaris* and *S. officinalis* ethanol extracts across human intestinal epithelial Caco-2 cell monolayers.

Materials and methods The permeability and Phase II biotransformation of RA and UA as pure compounds and in herbal extracts that contained same concentration as the pure compounds were compared using Caco-2 cells with HPLC detection.

Results The apparent permeability coefficient (P\text{app}) for RA and RA in *P. vulgaris* extracts was 0.2 ± 0.05 × 10^{-6} cm/s, significantly increased to 0.9 ± 0.2 × 10^{-6} cm/s after \(\beta\)-glucuronidase/sulfatase treatment. P\text{app} for UA and UA in *S. officinalis* extract was 2.7 ± 0.3 × 10^{-6} cm/s and 2.3 ± 0.5 × 10^{-6} cm/s before and after \(\beta\)-glucuronidase/sulfatase treatment, respectively. Neither compound was affected in permeability by the herbal extract matrix.

Conclusion RA and UA in herbal extracts had similar uptake as that found using the pure compounds, which may simplify the prediction of compound efficacy. The apparent lack of intestinal glucuronidation/sulfation of UA is likely to further enhance the bioavailability of that compound compared with RA.

Key words: *Prunella vulgaris*; *Salvia officinalis*; Rosmarinic acid; Ursolic acid; Permeability; Caco-2
Introduction

Rosmarinic acid (RA, Figure 1) is a caffeic acid (CA) derivative found in various botanicals, especially in *Prunella vulgaris*, a perennial herb known as self-heal used to treat sore throat, fever, and wounds (Psotová et al., 2003). RA and *P. vulgaris* limit liver damage derived from a model of bacterial inflammation (Osakabe et al., 2002) and inhibit nervous system inflammation in another model (Swarup et al., 2007). Ursolic acid (UA, Figure. 1), a pentacyclic triterpene acid, is also found in *P. vulgaris* but especially concentrated in sage leaves (*Salvia officinalis*), and inhibits inflammation-related changes in human gingival cells (Zdarilová et al., 2009) and in other models (Liu, 1995). This compound also has antimutagenic activities (Filipic et al., 1997). Both herbs have been used traditionally to treat inflammation in the mouth, and are of interest in inhibiting gastrointestinal inflammation which is relevant to colitis and colon cancer.

A major limiting step in the utilization of RA and UA is their intestinal absorption and metabolism. Both compounds contain hydroxyls and are likely to be glucuronidated or sulfated in intestinal cells, forms that are generally considered to be less bioactive than parent compounds. This metabolism has been demonstrated for RA but not for UA, and the plant matrix components might alter this metabolism but this has not been studied yet. The absorption or metabolism of RA has been examined *in vivo* to a limited extent (Baba et al., 2004; Konishi et al., 2005; Baba et al., 2005). When *Perilla* extract containing 200 mg of RA was orally administered to six men, RA in both plasma and urine was present predominantly as glucuronide and/or sulfate conjugated forms, at $0.6 \pm 0.2\%$ and $1.5 \pm 0.4\%$ of the total intake, respectively, within 48 h after ingestion (Baba et al., 2005). Gut microbes may metabolize RA to give phenolics such as caffeic acid, *o*-coumaric acid and
m-hydroxyphenylpropionic acid, which are then absorbed by monocarboxylic acid transporter (MCT)-mediated active processes (Konishi and Kobayashi, 2005). After the oral administration of *Sambucus chinensis* ethanol extract (40g/kg to rats, containing 80 mg UA/kg) about 0.6% of ingested UA was recovered in plasma based on estimated blood volume and plasma area under curve of this compound, suggesting poor absorption or extensive metabolism and distribution to other body tissues (Liao et al., 2005). Both RA and UA as constituents of *P. vulgaris* may contribute to its bioactivities, and the effects of plant matrix on uptake and metabolism of its key bioactive compounds are of interest.

Caco-2 cells are immortalized human epithelial colorectal adenocarcinoma cells and offer a standard rapid, reliable, and low-cost model for *in vitro* prediction of intestinal drug permeability and absorption (Hubatsch et al., 2007). Caffeic acid derivatives, such as RA and chlorogenic acid, have been studied using Caco-2 model and are proposed to transfer across the intestinal barrier by paracellular diffusion (Konishi and Kobayashi, 2005; Konishi and Kobayashi, 2004). The plant material matrix may alter absorption or metabolism and, consequently, the bioavailability of phytochemicals (Manach et al., 2004). *P. vulgaris* and *S. officinalis* contain sugars, steroids, alkaloids, essential oils, flavonoids, polyphenols, triterpenoids, and saponins (Rasool et al., 2010; Cheung and Zhang, 2008; Lu and Foo, 2000; Loizzo et al., 2008). Therefore, it is crucial to investigate the effect that the plant matrix may have on the uptake of RA and UA found in *P. vulgaris* and *S. officinalis*. *In vitro* studies have not been done in association with the permeation of RA in *P. vulgaris* extracts using Caco-2 cells nor have different sources of *P. vulgaris* plant material been compared for their influence on RA uptake. Uptake of UA in the Caco-2 cell model also has not been investigated, either as a pure compound or from plant extracts. Our hypotheses were that the absorbability and metabolism of RA and UA was independent of
the plant extract matrix and this study was conducted to investigate the permeabilities of RA and UA as pure compounds and in *P. vulgaris* and *S. officinalis* ethanol extracts across Caco-2 cell monolayers, to facilitate future studies of the efficacy of these herbs against gastrointestinal inflammation.

**Materials and methods**

*Plant extraction*

All *P. vulgaris* plant samples were provided by the U.S Department of Agriculture North Central Regional Plant Introduction Station in Ames, IA. *S. officinalis* extract was provided by Sabinsa Corporation (Payson, UT). Seeds from accessions *P. vulgaris* Ames 27664, 27665 (both originally collected in North Carolina), and 27748 (collected in Missouri) were germinated in Petri plates at 25 °C (Voucher records: Herbarium specimen. Taken by: McCoy, J., USDA, ARS. On: 08/24/2006. Located at: ISC. Inventory sample: Ames 27664, 27665 and 27748. SD 04ncao01). The resulting seedlings, segregated by accession, were transferred to flats in a greenhouse (held at 20–25 °C). Upper flowering portions of 14-month-old plants were harvested at the time of peak flowering, dried, and ground. Four g aliquots of the ground samples were extracted with 500 mL of 95% ethanol by Soxhlet percolation for 6 h, filtered, dried by rotary evaporation and lyophilized. Then the extracts were redissolved in 0.5 mL of ethanol and stored at -20 °C under nitrogen.

Information about the *P. vulgaris* accessions used for these experiments is available via the Germplasm Resources Information Network database at [http://www.ars-grin.gov/npgs/acc/acc_queries.html](http://www.ars-grin.gov/npgs/acc/acc_queries.html).
**HPLC analysis**

RA (90%) and UA (92%), both from Sigma-Aldrich Co. (St. Louis, MO) and 2, 4, 4'-trihydroxybenzoin (THB, internal standard), synthesized in Dr. Hendrich’s laboratory (Song et al., 1998) were dissolved in DMSO to use them as standards. Methanol, acetonitrile (HPLC grade) and phosphoric acid (AR grade) were obtained from Fisher Scientific (Pittsburgh, PA).

HPLC analysis was performed on a Beckman Coulter 126 HPLC, equipped with photodiode array detector model 168 and a model 508 autosampler (Beckman Coulter, Inc., Brea, CA). The mobile phase was 1.25% phosphoric acid: acetonitrile (15% acetonitrile at 0 min, then increased to 84% at 15 min and held for 40 min; decreased to 15% at 65 min) at a flow rate of 0.5 ml/min. A reverse phase analytical YMC pack-ODS C18 column (250 mm × 4.6 mm × 5 μm, Waters Corp., Milford, MA) was used at room temperature. The wavelength was 210 nm and the retention times were 21.5 min for RA and 26.5 min for UA. The limit of detection (LOD) and limit of quantitation (LOQ), defined as a signal/noise ratio \( \geq 3 \) and \( \geq 6 \), was 0.05 μM and 0.1 μM both for RA and UA. The linear regression equation for RA was \( y = 95.256x - 0.0188 \) with \( R^2 \) of 0.9995 and \( y = 236.07x + 0.0014 \) with \( R^2 \) of 0.9996 for UA, where \( y \) is the concentration of RA or UA, and \( x \) is the ratio of peak area of the standard to peak area of the internal standard (THB). The intraday and interday CVs were 7.3 ± 2.5 % and 3.4 ± 1.6 % for RA, and 9.1 ± 3.8 % and 5.7 ± 2.1 % for UA across 0.1-100 μM.

**Transepithelial transfer experiment**

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) at passage 18 and all experiments were performed from passages 25–30. The cells were
cultured according to Hubatsch et al. (2007). Cytotoxicity of RA, UA, and extracts was measured according to Nasser et al. (2008). RA, UA, *P. vulgaris* ethanol extracts containing the same concentrations of RA, and *S. officinalis* extract containing the same concentrations of UA, at 1, 10, 20, 50, and 100 μM in DMEM, were tested for cytotoxicity. DMSO in DMEM (Dulbecco's modified Eagle's medium, 0.3%, Gibco Invitrogen, Carlsbad, CA) was used as control.

After the cells in the flask grew to 90–100% confluency, cells were trypsinized and seeded on collagen-coated polytetrafluoroethylene membrane inserts (0.45 μm) fitted in bicameral chambers (Transwell-COL, 24 mm ID, Corning Inc., Corning, NY) at 1.2 × 10^5 cells/cm². The transepithelial electrical resistance (TEER) was tested by Millicell ERS meter (Fisher Sci., Pittsburgh, PA) to reflect the tightness of intercellular junctions and only cells with TEER ≥ 250 Ω·cm² were used for permeability study. At 20-21 d post seeding (90–100% confluence) on the transwell, RA, UA and extracts at non-cytotoxic concentrations or 100 μM of the paracellular marker, lucifer yellow (LY, Sigma-Aldrich Co., St. Louis, MO), dissolved in Hank's Buffered Salt Solution (HBSS, pH 7.4, Gibco Invitrogen, Carlsbad, CA), were added to the apical chamber, then basolateral solutions were collected after 0.5, 1, 2, and 4 h. After 4 h, apical solutions were collected and membrane on the transwell insert was placed in 1.5 mL of ice-cold 0.5 mol sodium hydroxide/L and sonicated with a probe-type sonic dismembrator (Biologics Inc., Manassas, VA); pH was adjusted to 7.0 and all samples were injected directly to HPLC for analysis. For LY quantification, the apical and basolateral solutions were transferred to a 96 well plate and read spectrophotometrically at 450 nm. Total cellular protein was determined by Coomassie (Bradford) assay (Pierce Laboratories, Rockford, IL).
Transepithelial transfer of single compounds and extracts after treating with
β-glucuronidase/sulfatase

Ten μM RA as a single compound and *P. vulgaris* ethanol extracts diluted to contain 10 μM RA or 20 μM UA and *S. officinalis* extract diluted to contain 20 μM UA were applied to Caco-2 cells. Twenty μL of β-glucuronidase/sulfatase (Type H-2 from *Helix pomatia*, 85 units/L of glucuronidase and 7.5 units/L of sulfatase, Sigma-Aldrich Co., St. Louis, MO) were added to the post-experimental apical and basolateral solutions and to cell homogenates and incubated overnight at 37 °C to release the parent compounds. These samples were then injected directly to HPLC.

Apparent permeability coefficients (P\textsubscript{app}) were determined using the equation (Hubatsch et al., 2007): P\textsubscript{app} = (dQ / dt) (1 / (A × C\textsubscript{0})); dQ/dt was the permeability rate constant (μmol/s); A was the surface area of the membrane (cm\textsuperscript{2}); and C\textsubscript{0} was the initial concentration of the compound (μM). Basolateral recoveries (%) were calculated as the proportion of the original amount that permeated through the monolayer, which was calculated as the amount transported divided by the initial amount in the apical chamber. Transport rate (μM/h/cm\textsuperscript{2}) was calculated as the amount transported divided by incubation time and the area of the membrane.

Statistical analysis

Data are given as means ± S. D. Differences in cytotoxicity, P\textsubscript{app}, transport kinetics, basolateral recoveries and transport rate of the pure compounds and extracts were evaluated statistically using ANOVA and Tukey’s multiple comparison tests by SAS 9.1 (SAS Institute Inc., Cary, NC). Differences were considered significant at *p* < 0.05.
Results

Determination of RA and UA concentrations in *P. vulgaris* and *S. officinalis* ethanol extracts

The amounts of RA in ethanol extracts varied ~sixfold across the three *P. vulgaris* accessions; UA content in *P. vulgaris* extract was ~four- to twenty-fold less on a molar basis than was UA in *S. officinalis* extract (Table 1). The ethanolic extract of *P. vulgaris* 27748 had threefold greater RA and tenfold more UA than the two other accessions studied. No RA was found in the *S. officinalis* extract.

Cytotoxicity test

For RA as a pure compound, concentrations greater than 50 μM were significantly cytotoxic compared with the control (0.3% DMSO in DMEM, *p* < 0.05). Ethanol extracts of *P. vulgaris* accessions containing ≥ 20 μM RA showed significant cytotoxicity. Concentrations ≥ 20 μM of UA as a pure compound and *S. officinalis* extract containing same amount of UA were toxic to the Caco-2 cells (data not shown). Therefore, 1, 2, 5, 10 μM of RA as a single compound or in *P. vulgaris* ethanol extracts, and 2, 5, 10, 20 μM of UA and *S. officinalis* extract containing these amounts of UA were used for permeability studies.

Transepithelial transfer of single compounds and extracts before and after treating with β-glucuronidase/sulfatase

The basolateral recovery of paracellular marker, LY, was 0.5 ± 0.1%. In the basal chamber after 4 h, 1.3 ± 0.3, 1.2 ± 0.2, 1.4 ± 0.6 and 0.9 ± 0.4 % of parent RA was transferred for pure compound, or for RA in *P. vulgaris* 27664, 27665 and 27748 extracts,
respectively (no significant differences, $p > 0.05$). The apical recoveries of RA were 86.7 ± 2.9, 85.8 ± 3.6, 81.2 ± 5.7 % and 79.1 ± 11.2 % for the pure compound, or for RA in *P. vulgaris* 27664, 27665 and 27748 extracts, respectively, with no significant differences ($p > 0.05$, data not shown).

The permeation rate was calculated for UA as a pure compound and UA in *S. officinalis* extract at the same doses. The uptake of UA increased linearly and significantly from 0.03 ± 0.01 to 0.2 ± 0.04 μM/h/cm² ($p < 0.01$) and was not saturable across the tested concentrations (5-20 μM, Figure 2). It was also not saturable across tested time-points (0.5-4 h) both for pure compounds and UA contained in *S. officinalis* extract (Figure 3A). Due to LOD of UA and the lesser amount of it in *P. vulgaris* extracts compared with RA, UA was not detected for apically-applied *P. vulgaris* extracts containing 1-10 μM RA.

Basolateral recoveries of RA as pure compound and RA in *P. vulgaris* 27664 increased over time (RA in *P. vulgaris* 27665 and 27748 are not shown), although RA was not detected basolaterally at 0.5 and 1h before deconjugation (Figure 3A). The basolateral transfer of UA was significantly greater than RA at each time point both for pure compounds or compounds contained in plant extracts ($p < 0.01$, Figure 3A). No significant differences were found between RA as a pure compound and RA in *P. vulgaris* extracts or UA as a pure compound and UA in *S. officinalis* extract ($p > 0.05$) in apparent permeability coefficients ($P_{app}$, Table 2).

After deconjugation using β-glucuronidase/sulfatase, RA recoveries significantly increased from 1.3 ± 0.3% to 6.1 ± 1.4%, 1.2 ± 0.2% to 6.5 ± 2.7 %, 1.2 ± 0.03% to 4.9 ± 0.6 %, 1.1 ± 0.2% to 5.5 ± 1.2 % ($p < 0.01$) in basal chamber for the single compound, or for RA in *P. vulgaris* 27664, 27665 and 27748 extracts, respectively (Figure 3C, RA in *P. vulgaris*
27665 and 27748 are not shown). The transfer of RA glucuronide/sulfate conjugates was not saturable during 4 h and increased linearly with the incubation time ($p < 0.01$, Figure 3B). Both RA as a single compound and RA in *P. vulgaris* extracts showed the same increase in $P_{app}$ after deconjugation (Table 2). To check the mass balance, the total recoveries (apical + basolateral) of RA increased from 80-88% before deconjugation to 84-93% after deconjugation for pure compound and RA in extracts, with no new peaks detected. The basolateral recoveries of UA were 19.0 ± 4.2% or 15.9 ± 3.2% for pure compound before or after incubation with β-glucuronidase/sulfatase, and 17.6 ± 2.5% or 14.5 ± 4.3% for *S. officinalis* extract, not statistically different ($p > 0.05$, Figure 3C). No significant differences were found in the apical recoveries of RA or UA ($p > 0.05$) and neither RA nor UA was found in cells before or after deconjugation. $P_{app}$ for UA or UA in *S. officinalis* extract did not change after deconjugation reaction (Table 2).

**Discussion**

Because *in vivo* absorption studies performed with laboratory animals and humans are expensive and time consuming and can pose ethical challenges, Caco-2 cells are a useful alternative to study uptake and transport of important compounds from plant materials (Walgren et al., 1998). Plant matrices may influence phytochemicals by altering transporters of some compounds or of compound metabolites (Mukinda et al., 2010). Phytochemical biotransformation may also be altered by other components of the plant matrix. To establish the effect that the plant matrix had on the *in vitro* uptake of RA and UA found in *P. vulgaris* and *S. officinalis*, two herbs that may be important for gut health, the uptake of pure solutions of the two compounds and extracts of two herbs that are major sources of these compounds were compared in Caco-2 cell system.
RA in *P. vulgaris* and UA in *S. officinalis* extracts had similar efficient absorption as that in pure solutions of each compound in Caco-2 cells (Figure 3A and Table 2, *p* > 0.05); this was not influenced by varied contents of RA across three accessions collected from different sources. The *P* app for RA in *Salvia miltiorrhiza* was 0.5 ± 0.3 × 10⁻⁶ cm/s in Caco-2 cells (Lu et al., 2008), consistent with our results. The results are also consistent with *in vivo* studies showing poor absorption of intact RA (Baba et al., 2004), suggesting that the absorption of RA is independent of other constituents contained in *P. vulgaris* ethanolic extracts. The impact of plant matrices on the bioavailability of phytochemicals is highly dependent on the compound of interest. For example, the absorption of epicatechin was not influenced by the ingredient composition of beverage food matrices *in vitro* (Neilson et al., 2009), but *Hypericum perforatum L.* product matrices affected the transport of rutin, hyperoside and isoquercitrin across Caco-2 cells (Gao et al., 2010), probably due to differences in matrix phytochemical composition and transport characteristics, i.e. paracellular transfer, carrier mediated or active transport. In our study, both RA and UA were major components of their respective plant material (~3% or greater by weight, and ~ 5-50 mM of ethanolic plant extract). It could be useful to compare RA or UA uptake from plant matrices in which these components were less concentrated.

Studies are also needed to investigate the influences of preservation or processing of plant extract on the bioavailability of RA and UA both *in vitro* and *in vivo*.

If the basolateral movement of the paracellular marker, LY, is less than 0.7% and TEER value is greater than 250 Ω·cm², the established monolayer is considered to be tight enough for permeability experiments (Ohashi et al., 2009). In our permeability studies, we tested the basolateral recoveries at time points and interval times chosen for a hydrophilic
paracellularly transferred compound (RA) (Hubatsch et al., 2007). RA uptake was not saturable during 4 h incubation period (Figure 3A), indicating that passive diffusion occurred for this parent compound. We only measured the uptake of RA at 10 µM due to its cytotoxicity; RA was not detected at 0.5 and 1 h because of its LOD and the low concentration applied to the apical chamber. Unidirectional transport (apical to basolateral transfer) was investigated for RA in our study and the pH of both apical and basolateral solutions was 7.4 (to simulate small intestine pH), because Konishi and Kobayashi (2005) showed that permeation of RA from the apical to the basolateral side is similar to that from basolateral to apical side; the uptake in the presence of a proton gradient (pH gradient 6.0/7.4) was nearly the same as that in the absence of a proton gradient (pH gradient 7.4/4.4), implying that proton coupled polarized transport was not involved for RA. The transepithelial flux of RA was inversely correlated with TEER in our experiment (data not shown), consistent with restriction of intestinal absorption of RA when the epithelial tight junction is tight enough, a finding similar to that shown for another caffeic acid derivative, chlorogenic acid (Konishi and Kobayashi, 2004).

Our finding of increased basolateral RA recoveries after deconjugation (Figure 3C) is the first direct report of this type of intestinal metabolism of RA; glucuronide/sulfate conjugates were transferred to the basolateral side (toward the circulation) rather than apical side (the intestinal lumen), but this finding is consistent with in vivo observations of RA glucuronide/sulfate conjugates as major forms of this compound in rat and human plasma (Konishi et al., 2005; Baba et al., 2005). These results suggest that RA is absorbed via both paracellular and transcellular diffusion and MRP (multidrug resistance protein) or OATP (organic anion transporter protein) transporters might be involved in the permeation of glucuronidated or sulfated RA, because anionic conjugates (glutathione, glucuronide or
sulfate) cannot exit cells unless an MRP transporter is present (Peng et al. 1999). Intestinal metabolism of RA seemingly differs from that of its possible metabolites, glucuronide/sulfate conjugates of caffeic acid, ferulic acid and \( o \)-coumaric acid which were found only apically (Kern et al., 2003). This might be due to the specificity of MRP transporters to the glucuronide/sulfate conjugates of different caffeic acid derivatives and the locations of various MRP in apical or basolateral sides of the enterocytes (Peng et al., 1999).

The plant extracts did not affect transfer of either UA or RA, and likewise the pure compounds and the compounds from the plant extracts behaved similarly in their apparent biotransformation as well (Figure 3A, B and C and Table 2), establishing that the transfer and metabolism of RA and UA was independent of the of the plant extract matrix for the two herbs studied. The transfer of UA seemed simpler than that observed for RA. The rate of membrane permeation of UA increased linearly with concentration and was not saturable at the tested time-points (Figure 2, 3A and 3B), indicating uptake by passive diffusion. Because the basolateral transfer of UA was greater than RA at each time point (Figure 3A), and the partition coefficient (Log P) of RA was 0.2, compared with Log P of UA of 6.4 (Konishi et al. 2005; Bérangère et al., 2004), more hydrophobic UA was more absorbable than RA. This result is consistent with an interpretation of previous \textit{in vivo} findings in rats that the low plasma concentration of UA was not due to poor absorption but to extensive uptake of UA by other tissues (Liao et al. 2005). UA was absorbed and transferred by Caco-2 cells apparently with little glucuronidation/sulfation (Figure 3C and Table 2), which is likely to further enhance the bioavailability of UA compared with RA. This lack of UA biotransformation might be due to the differences in glucuronidation and sulfation of aliphatic alcohol (UA) and phenol alcohol (RA) or because of the distinct
structures of the two compounds: triterpenoid (UA) vs. caffeic acid derivative (RA). But two studies showed that there was wide variability in affinity for UDP-glucuronosyltransferase (UGT) and sulfotransferase (ST) across both general classes of substrates (Chen et al., 1996; Ebner and Burchell, 1993). One related triterpenoid, glycyrrhetinic acid (50-300 µM), was found to inhibit UGT2B7 activity in human liver microsomes (Nakagawa et al., 2009), which might also occur for UA. To our knowledge, no other in vivo or in vitro study has been done on the metabolism of ursolic acid. It is possible that UA may undergo additional glucuronide or sulfate conjugation in the liver. Future studies are required to elucidate the structures of RA and UA metabolites, which affect compound transfer mechanism and efficacy.

In conclusion, RA was transferred across Caco-2 cells almost entirely in conjugated form, but UA was absorbed and transferred mostly intact, both independent of extract matrix. Our results predict no effect of plant matrix on the efficacy of either compound. It will be important to measure the uptake of gut microbial metabolites of RA and effects of RA on expression of efflux transporters and tight junction proteins which would particularly affect a compound transferred paracellularly such as RA. Cellular models of oral as well as intestinal uptake, metabolism and anti-inflammatory activity of RA and P. vulgaris and UA and S. officinalis will be of interest as well, given traditional uses of these herbs.

Acknowledgements

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9P50AT004155-06 from the National Center for Complementary and Alternative Medicine (NCCAM) and ODS, NIH.
Figures

Figure 1. The chemical structures of rosmarinic and ursolic acids.
Figure 2. The transport rate of ursolic acid for pure compound and *S. officinalis* extract across Caco-2 cell monolayer as a function of concentration at 5–20 μM after 4 h uptake study. *S. officinalis* extract was diluted to apply the same concentrations to the cells as were used for the pure compounds. Data are the mean ± S. D (n=9). Means bearing different letters were significantly different by ANOVA and Tukey’s multiple comparison (*p* < 0.01).
Figure 3. Characteristics of transfer of rosmarinic (RA) and ursolic acids (UA) for pure compounds or plant extracts across Caco-2 cells before and after deconjugation of post-experimental basolateral solutions. A. Before deconjugation. No RA was found at 0.5 or 1 h. Means bearing different letters were significantly different by ANOVA and Tukey’s multiple comparison ($p < 0.01$). B. After deconjugation with β-glucuronidase/sulfatase; for RA and RA in *P. vulgaris* 27664. C. Total basolateral recovery of RA and UA after deconjugation over 4 h incubation. * The basolateral recoveries of RA after β-glucuronidase/sulfatase incubation were significantly different from that before the enzyme treatment by two-sample t-test ($p < 0.01$). Basolateral recoveries were calculated as the amount transported divided by the initial amount in the apical chamber during 4 h uptake study. Data are the mean ± S. D (n=9).
Table 1. The contents of rosmarinic and ursolic acids in ethanol extracts of *P. vulgaris* and *S. officinalis* determined by HPLC.

<table>
<thead>
<tr>
<th></th>
<th>RA g/L (mM)</th>
<th>UA g/L (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgaris</em> 27664 (174.4 g/L)</td>
<td>8.4 ± 0.6 (23.3 ± 1.7)</td>
<td>0.1 ± 0.0 (0.2 ± 0.0)</td>
</tr>
<tr>
<td><em>P. vulgaris</em> 27665 (66.2 g/L)</td>
<td>3.2 ± 0.3 (9.0 ± 0.8)</td>
<td>0.1 ± 0.0 (0.1 ± 0.0)</td>
</tr>
<tr>
<td><em>P. vulgaris</em> 27748 (117.0 g/L)</td>
<td>19.9 ± 1.1 (55.2 ± 3.1)</td>
<td>0.6 ± 0.1 (1.0 ± 0.2)</td>
</tr>
<tr>
<td><em>S. officinalis</em> (10 g/L)</td>
<td>ND*</td>
<td>2.6 ± 0.4 (5.8 ± 0.9)</td>
</tr>
</tbody>
</table>

* Not detected.
Table 2. Apparent permeability coefficients ($P_{\text{app}}$) for lucifer yellow, rosmarinic acid, ursolic acid and extracts across the Caco-2 monolayer relative to deglucuronidation.

<table>
<thead>
<tr>
<th></th>
<th>Before deconjugation</th>
<th>After deconjugation $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY</td>
<td>0.3 ± 0.1</td>
<td>--</td>
</tr>
<tr>
<td>RA$^a$</td>
<td>0.2 ± 0.0</td>
<td>0.9 ± 0.2†</td>
</tr>
<tr>
<td>RA in <em>P. vulgaris</em> 27664</td>
<td>0.2 ± 0.0</td>
<td>1.0 ± 0.4†</td>
</tr>
<tr>
<td>RA in <em>P. vulgaris</em> 27665</td>
<td>0.2 ± 0.1</td>
<td>0.7 ± 0.1†</td>
</tr>
<tr>
<td>RA in <em>P. vulgaris</em> 27748</td>
<td>0.1± 0.1</td>
<td>0.9± 0.1†</td>
</tr>
<tr>
<td>UA$^b$</td>
<td>2.8 ± 0.1$^*$</td>
<td>2.4 ± 0.4$^*$</td>
</tr>
<tr>
<td>UA in <em>S. officinalis</em></td>
<td>2.5 ± 0.4$^*$</td>
<td>2.2 ± 0.6$^*$</td>
</tr>
</tbody>
</table>

$^a$ RA was 10 μM for pure compound and *P. vulgaris* extracts containing the same content of RA. $^b$ $P_{\text{app}}$ of UA as a pure compound and in *S. officinalis* extract was calculated using non-toxic concentrations (5, 10 and 20 μM).

$^c$ The $P_{\text{app}}$ of rosmarinic and ursolic acids after treating with $\beta$-glucuronidase/sulfatase.

$^*$ Significantly different compared with $P_{\text{app}}$ of LY and RA by two-sample t-test ($p < 0.01$).

$^†$ Significantly different before and after deconjugation by two-sample t-test ($p < 0.01$).

--: not determined.
References

Baba, S., Osakabe, N., Natsume, M., Terao, J., 2004. Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. Life Sciences 75, 165-178.


CHAPTER 5. CAFFEIC ACID DERIVATIVES ENHANCED EPITHELIAL BARRIER INTEGRITY AND EXHIBITED ANTI-INFLAMMATORY ACTIVITIES IN P-GLYCOPROTEIN TRANSPORTER KNOCKDOWN CACO-2 CELLS

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\textbf{Abstract}

Our hypothesis was that caffeic acid derivatives, namely caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (a microbial metabolite of caffeic acid and rosmarinic acid) altered the intestinal barrier and immune-modulators toward an anti-inflammatory profile in Caco-2 human intestinal epithelial cells and P-glycoprotein (P-gp) transporter, encoded by multidrug resistance (MDR1) gene, knockdown (KD) Caco-2 cells. Stimuli composed of interleukin (IL)-1\textbeta, tumor necrosis factor (TNF)-\alpha, interferon (IFN)-\gamma and lipopolysaccharides (LPS) were added to the cell system to represent the acute inflammatory reaction and mimic the inflammatory bowel disease. Transepithelial electrical resistance (TEER) across the monolayers increased rapidly during incubation with quercetin (positive control) and m-hydroxyphenylpropionic acid
(mHPP) after 12 h, and only chlorogenic acid and mHPP both at 50 µM showed protection against the reduction of TEER induced by the stimuli in Caco-2 cells. In MDR KD Caco-2 cells, all treatment groups at 50 µM significantly augmented the TEER at each time-point compared with the control both with and without the stimuli, which was correlated with Lucifer yellow flux, a paracellular marker, across the cell monolayer. Immunoblot analysis of tight junction proteins revealed that caffeic acid derivatives at 50 µM up-regulated ZO-1, ZO-2, claudin-1 and occludin in Caco-2 cells, and chlorogenic acid and mHPP were effective against the inflammatory stimuli induced reduction of ZO-2 and occludin, as well as induction of claudin-1 on tight junction proteins in Caco-2 cells. Caffeic acid derivatives up-regulated claudin-4 in MDR KD Caco-2 cells and only mHPP was effective against the inflammatory stimuli induced changes of tight junction protein expressions in MDR KD Caco-2 cells. Caffeic acid derivatives at 50 µM significantly decreased the concentration of IL-6 in Caco-2 cells, but augmented TNF-α and IL-6 levels under the stimulated condition. In MDR KD caco-2 cells plus the stimuli, all treatment groups significantly reduced both TNF-α and IL-6 concentrations. These results suggest that P-gp plays an essential role in the anti-inflammatory activities of caffeic acid derivatives and mHPP exhibited greater enhancement of intestinal barrier function than the parent compounds.
Introduction

Dietary polyphenols in fruit and vegetables are antioxidants and epidemiologic studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease and certain types of cancer (Bravo, 1998). Caffeic acid, one of the most common phenolic acids, frequently found in fruits, vegetables, grains and dietary supplements. Chlorogenic acid is combined from caffeic and quinic acids in coffee and many types of fruits. Rosmarinic acid is also a caffeic acid derivative found in many Lamiaceae herbs used commonly as culinary herbs such as rosemary, oregano and thyme. Phenolic acids such as caffeic acid, chlorogenic acid and rosmarinic acid (Figure 1) exhibit antioxidant, anti-inflammatory, anti-mutagenic and anti-carcinogenic effects in vitro (Rice-Evans et al., 1996; Scalbert, et al., 2002; Parnham and Kesselring, 1985), therefore are of interest in inhibiting gastrointestinal inflammation which is relevant to inflammatory bowel disease (IBD) and colon cancer.

Gastrointestinal epithelial cells provide a physical barrier to the diffusion of pathogens, toxins, and allergens from the lumen into the circulatory system. In healthy individuals, the intestinal barrier is constituted of an intact layer of epithelial cells, which are tightly connected by a surrounding system of tight junction strands. In IBD patients, epithelial barrier function is impaired (Gitter et al., 2001) with reduction of tight junction strands in ulcerative colitis and tight junction strand breaks in Crohn’s disease (Marin et al., 1983). Tight junction strands are formed by the assembly of a multiple-protein complex, which regulates the paracellular movement of ions, solutes, and water across the intestinal epithelium. Four integral transmembrane proteins, occludin, claudins, junctional adhesion molecule, and tricellulin, have been identified (Furuse et al., 1993; Furuse et al.,
The intracellular domains of these transmembrane proteins interact with scaffold proteins, such as zonula occludens (ZO) proteins, which in turn anchor the transmembrane proteins to the perijunctional actomyosin ring. Previous studies showed that some polyphenols, epigallocatechin gallate; genistein; myricetin; kaempferol and quercetin, enhance intestinal tight junctions in human intestinal Caco-2 monolayers (Suzuki and Hara, 2010; Suzuki et al., 2011). No studies have investigated the influences of phenolic acids on gut barrier functions, which might be the underlying mechanism of the protective effects of caffeic acid derivatives against colitis or colon cancer in animal models (Ye, et al., 2009; Tanaka, et al., 1990; Feng et al., 2010).

Several inflammatory mediators are thought to be implicated in the development of IBD. The in vitro effects of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and lipopolysaccharides (LPS) on intestinal epithelial cells have been shown to activate the intracellular cascades and increase the secretion of IL-6 (Vitkus et al., 1998), IL-8 (Schuerer-Maly et al., 1994), prostaglandin E2 (PGE-2, Wright et al., 2004) and nitric oxide (NO, Forsythe et al., 2002), as well as increase of the paracellular permeability through defects in tight junction assembly (Al-Sadi et al., 2007). IL-1β, TNF-α, and IFN-γ have been found in increased concentrations in the intestinal tissue of IBD patients (Ligumsky et al., 1990; Reimund et al., 1996) and LPS plays a key role in the initiation of IBD because microflora may cause an initial inappropriate inflammatory stimulus if the intestinal cells are dysregulated, leading to an exaggerated cytokine presence and IBD development (Caradonna et al., 2000).

P-gp transporter, encoded by multidrug resistance (MDR1) gene, plays a key role in drug absorption and distribution because it limits the permeability across the GI tract (Hunter
and Hirst, 1997) by active efflux of potentially toxic substances back into the intestinal lumen. A great insight into the physiological role of P-gp protein in the GI tract has derived from the phenotype of the MDR1 gene knockout mice. This model was prompted by the finding that the MDR1 gene is present in a region of the human genome (7q21.1) that may harbour a disease gene involved in susceptibility to IBD (Lathrop et al., 1996). Traditional herbs, such as *Prunella vulgaris* and *Hypericum gentinoides*, containing caffeic acid derivatives, have been found to act against the spontaneous colitis in MDR1 knockout mice in terms of increasing the survival percentage, elevating the anti-inflammatory cytokine and chemokines in serum and reducing typhlocolitis score (data not published), but the mechanism is unknown.

This study was conducted to investigate the regulation of caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (mHPP), a microbial metabolite of ingested caffeic acid or rosmarinic acid (Konishi and Kobayashi, 2004b; Konishi and Kobayashi, 2005), on tight junction proteins and immune modulators in both Caco-2 cell model and MDR knockdown (MDR KD) Caco-2 cells. Stimuli composed of IL-1β, TNF-α, IFN-γ and LPS were added to Caco-2 cell system to represent the acute inflammatory reaction as it might occur during the active phase of IBD. Our hypothesis was that caffeic acid derivatives altered the intestinal barrier and immune-modulators toward an anti-inflammatory profile and that the protection was stronger in MDR KD cells compared with Caco-2 cells.
Materials and methods

Chemicals. Caffeic acid (98%), rosmarinic acid (90%), chlorogenic acid (95%), and m-hydroxyphenylpropionic acid (mHPP, 92%), a metabolite of caffeic acid derivatives, were obtained from Sigma-Aldrich Co. (St. Louis, MO). Rabbit anti-claudin-1, claudin-4, ZO-1, ZO-2, and occludin were purchased from Zymed Laboratories (Gibco Invitrogen, Carlsbad, CA). Mouse anti-α-tubulin was purchased from Sigma (St. Louis, MO). HRP-conjugated anti-mouse and -rabbit IgG were purchased from Zymed Laboratories. IL-1β, TNF-α, and LPS were purchased from Sigma-Aldrich Co. (St. Louis, MO), and IFN-γ was obtained from Invitrogen (Carlsbad, CA).

Cell culture. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) at passage 18 and all experiments were performed from passages 35–45. MDR knockdown (MDR KD) Caco-2 cells (a gift from Dr. Kazuya Maeda, the University of Tokyo, constructed from Caco-2 cells from ATCC using siRNA technique, Watanabe et al., 2005) were used between passages 21 and 25. The cells were cultured according to Hubatsch et al. (2007). The cells were seeded on collagen-coated polytetrafluoro-ethylene membrane inserts (0.45 μm) fitted in bicameral chambers (Transwell-COL, 24 mm ID, Corning Inc., Corning, NY) at 1.2 × 10^5 cells/cm². All experiments were conducted on d 20-21 postseeding. Stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL) and IFN-γ (50 ng/mL) were applied at the basolateral side of the cells, and LPS (1 μg/mL) was applied at both sides, representing on the one hand the common luminal microflora and, on the other hand, the increased presence of bacterial components in serum during IBD (Walle et al., 2010).
**P-gp assay.** P-gp activity was evaluated with fluorimetric measurement of the intracellular accumulation of calcein produced by ester hydrolysis of the P-gp substrate calcein-AM using Vybrant™ Multidrug Resistance Assay Kit (Gibco Invitrogen, Carlsbad, CA) in Caco-2 and MDR KD Caco-2 cells. After the cells in the flask grew to 90–100% confluency, cells were trypsinized and seeded on 96 well plates at $1.2 \times 10^5$ cells/cm². Cells were preincubated for 15 min with or without the acknowledged P-gp modulator verapamil (10 µg/mL), thereafter calcein-AM was added and the fluorescence measured after 1 hour using microtiter plate reader Bio-Tek ELX 808 (Bio-Tek instruments. Inc., Winooski, VT) at 490 nm.

**Measurement of intestinal barrier function.** Intestinal barrier function was evaluated by measurement of transepithelial electrical resistance (TEER) and unidirectional flux of lucifer yellow (LY) across Caco-2 cell monolayers in transwells. Caffeic acid, rosmarinic acid, chlorogenic acid, mHPP (10, and 50 µM) and quercetin (100 µM) were added to the apical wells and the cells were incubated for 48 h. TEER was measured before and at 6, 12, 24 and 48 h after administration of the caffeic acid derivatives by Millicell ERS meter (Fisher Sci., Pittsburgh, PA). Lucifer yellow (LY, 100 µM), a paracellular marker, was added apically and the flux into the basolateral chamber was assessed before and after the experiment. For LY quantification, the apical and basolateral solutions were transferred to a 96 well plate and read spectrophotometrically at 450 nm. Whole cell extracts were prepared for immunoblot analysis of tight junction proteins after incubation for 48 h as described below. Cytotoxicity of caffeic acid, rosmarinic acid, chlorogenic acid and mHPP was measured according to Nasser et al. (2008).
Assessment of Protein Expression by Western Blot Analysis. At the end of the experimental period, Caco-2 monolayers on the transwell were immediately rinsed with ice-cold PBS, and cells were lysed with lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 2 mM sodium orthovanadate, 1 M phenylmethylsulfonyl fluoride, 3 mg/L leupeptin, 5 mg/L aprotinin, and 1% Triton X-100) and scraped, and the cell lysates were placed in microfuge tubes. Cell lysates were centrifuged to yield a clear lysate. Supernatant was collected, and total cellular protein was determined by Coomassie (Bradford) assay (Pierce Laboratories, Rockford, IL). Laemmli gel loading buffer (6% SDS, 30% glycerol, 15% 2-b-mercaptoethanol, and 0.02% bromophenol blue in 188 mM Tris, pH 6.8) was added to the lysate containing 20 µg of protein and boiled for 7 minutes, after which proteins were separated on SDS-PAGE gel. Proteins from the gel were transferred to polyvinylidene difluoride membranes. Membranes were blotted for ZO-1, ZO-2, occludin, claudin-1, claudin-4 and α-tubulin using specific antibodies in combination with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibodies. The blots were developed using the ECL chemiluminescence method (GE Healthcare, Piscataway, NJ). Quantification was performed by using densitometric analysis of specific bands on the immunoblots.

Determination of TNF-α and IL-6 secretion. At the end of the experimental period, the extracellular media in the transwell were collected and centrifuged at 10,000 Xg for 10 min. TNF-α and IL-6 secretions were evaluated using a sandwich ELISA method (BD Biosciences Pharmingen, San Diego, CA) and quantified in pg/ml using the standard provided with the kit, with sensitivity limits being of 7.8 and 4.7 pg/ml, respectively for the TNF-α and IL-6 assay.
Statistical analysis Data are given as means ± S. D. Differences in the percentage of the TEER relative to the initial value, lucifer yellow flux, tight junction protein density, cytokine concentrations and fluorimetric intracellular accumulation of calcein were evaluated statistically using ANOVA and Tukey’s multiple comparison tests by SAS 9.1 (SAS Institute Inc., Cary, NC). Differences were considered significant at \( p < 0.05 \).

Results

P-gp activity in Caco-2 cells and MDR KD Caco-2 cells. As shown in Figure 2, P-gp-mediated efflux of calcein-AM, a substrate for P-gp, was significantly less in MDR KD Caco-2 cells with or without the treatment of P-gp inhibitor, verapamil, and in Caco-2 cells incubated with verapamil, compared with Caco-2 cells \(( p < 0.05)\). This indicates that the P-gp function in the MDR KD Caco-2 cells was almost completely inhibited.

Caffeic acid derivatives enhance tight junction integrity in Caco-2 cells. In Caco-2 cells, 100 µM quercetin and 50 µM mHPP significantly increased TEER after 6 h and 12 h incubation, respectively, compared with control and caffeic acid, rosmarinic acid and chlorogenic acid \(( p < 0.05)\). The TEER in all treatment groups was higher than that of control after 48 h incubation period \(( p < 0.05, \text{ Figure 3A})\). This was correlated with the paracellular transport of LY, which was significantly reduced in all treatment groups compared with the control after 48 h incubation (data not shown). After adding the stimuli, the TEER diminished up to 35% (data not shown) in controls compared with that without adding the stimuli \((273 ± 15 \text{ vs } 420 ± 11 \text{ Ωcm}^2)\), and only chlorogenic acid and mHPP both at 50 µM showed protection against the adverse effects of the stimuli on the TEER (Figure 3B). Fifty µM of caffeic acid, rosmarinic acid or chlorogenic acid exacerbated the diminishment of the TEER induced by the stimuli (Figure 3B), which was
correlated with LY flux across the Caco-2 cells (Figure 4A), indicating that quercetin, caffeic acid, and rosmarinic acid increased the permeability of the Caco-2 cells when the stimuli were added to initiate the inflammatory responses.

In MDR KD Caco-2 cells, all treatment groups significantly augmented the TEER at each time-point compared with the control ($p < 0.05$, Figure 3C). Interestingly, the elevation of the TEER by caffeic acid derivatives-incubated cells was greater than quercetin in MDR KD cells compared with Caco-2 cells (Figure 3A and C), supported by the flux of LY in to the basolateral side after 48 h incubation (data not shown). After adding the stimuli in to MDR KD Caco-2 cells, the TEER was significantly reduced up to 29% (321 ± 16 vs 453 ± 23 Ω·cm²) in controls compared with that without adding the stimuli (data not shown), and all treatment groups significantly increased the TEER relative to the initial value compared with controls ($p < 0.05$, Figure 3D). Among the treatment groups, mHPP showed greatest protection against the stimuli in the elevation of the TEER ($p < 0.05$), correlated with the flux of LY across the cell monolayer (Figure 3D and 4B).

**Caffeic acid induces the up-regulation of tight junction proteins.** After adding the stimuli, the expression of ZO-1, ZO-2 and occludin was all significantly decreased, and that of claudin-1 and claudin-4 increased in controls compared with without adding the stimuli ($p < 0.05$, Table 1 and 2). Without the stimuli, the expression of ZO-1, ZO-2, claudin-1 and occludin in Caco-2 cells was increased by 50 µM of caffeic acid derivatives ($p < 0.05$, Table 1), but not at 10 µM (data not shown). After adding the stimuli, chlorogenic acid and mHPP exhibited reversal of the response to inflammation: up-regulated ZO-1 (not for mHPP), ZO-2 and occludin, as well as down-regulated claudin-1 expressions compared with controls. But this protection was not found for
claudin-4 in caffeic acid derivative treated Caco-2 cells, while quercetin at 100 µM attenuated the effect of the stimuli on the expression of claudin-4.

In MDR KD Caco-2 cells after adding the stimuli, similar trend of the changes of tight junction proteins in controls was found in MDR KD Caco-2 cells, compared with Caco-2 cells. No treatment effect was found for ZO-1, ZO-2 and occludin ($p > 0.05$). Quercetin, chlorogenic acid and mHPP increased claudin-1 expression and all treatment groups increased claudin-4 expression. Caffeic acid derivatives significantly increased the expression of ZO-1 (not for chlorogenic acid) and occludin, and reduced the expression of claudin-1 and claudin-4 induced by the stimuli. Only mHPP was effective in the elevation of the expression of ZO-2 in MDR KD cells with stimuli (Table 2).

**Caffeic acid derivatives reduced TNF-α and IL-6 in MDR KD Caco-2 cells.** TNF-α and IL-6 were not detected in Caco-2 cells both with or without treatment with caffeic acid or its derivatives (data not shown). After adding the stimuli, TNF-α and IL-6 increased to $357.6 \pm 14.1$ pg/mL and $6.3 \pm 1.8$ pg/mL in controls in Caco-2 cells. Rosmarinic acid and mHPP increased TNF-α and IL-6 concentrations compared with other treatments (Table 3).

In MDR KD Caco-2 cells, the level of TNF-α and IL-6 in control was greater than that in the Caco-2 cells (Table 4). After adding the stimuli, the level of TNF-α and IL-6 in controls further increased (Table 5). All treatment significantly reduced the concentration of TNF-α compared with control, while no treatment effect was found for IL-6 in MDR KD Caco-2 cells without stimuli (Table 4). After adding the stimuli, all treatments significantly decreased both cytokine concentrations (Table 5).
Transepithelial transfer of caffeic acid derivatives across Caco-2 and MDR KD Caco-2 cells. Basolateral recoveries of caffeic acid and mHPP were 4 fold and 3.3 fold greater in MDR KD Caco-2 cells than that in Caco-2 cells. The amount of caffeic acid and mHPP in MDR KD Caco-2 cell lysates was ~3 fold higher than that in Caco-2 cells. But no significant difference was found for the amount of quercetin, rosmarinic acid and chlorogenic acid in both basolateral side and cell lysates between Caco-2 and MDR KD Caco-2 cells (Table 6). These results suggest that caffeic acid and mHPP, but not quercetin, rosmarinic acid and chlorogenic acid, might be substrates for P-gp transporter.

Discussion

This study demonstrated that caffeic acid derivatives enhanced tight junction integrity through the expression of tight junction proteins in both Caco-2 cells, which are widely used to study the intestinal epithelium, and in MDR KD Caco-2 cells. The increment of TEER induced by caffeic acid derivatives was evidenced by the increase of the tight junction protein expression. Although the precise mechanisms underlying caffeic acid-mediated promotive effect on tight junction integrity remain to be clarified, P-gp seems important in the regulation of both tight junction proteins and immunomodulators. mHPP exhibited greater enhancement of intestinal barrier function than other caffeic acid derivatives, indicating the key role of this metabolite formed by microbes on the protection of gut function in vivo.

Tight junction proteins bound to the actin cytoskeleton have a pivotal role in tight junction integrity. Tight junction protein levels correlate with the permeability of tight junction in the epithelium (Betanzos et al., 2003; Basuroy, et al., 2006). The caffeic acid
derivatives-mediated increase of TEER was related to the increased ZO-1, ZO-2, occludin, and claudin-1, but not claudin-4 in Caco-2 cells. The flux of LY through paracellular pathway was reduced as expected, in conjunction with increased occludin which seemingly made the paracellular barrier tighter. These results indicate that the assemblies of these four proteins at the tight junction are responsible for the caffeic acid derivatives-mediated promotion of intestinal barrier function.

The intestinal inflammatory response requires the correct integration of the various signals that are released or received by the different cell types present in the gut (Van de Walle et al., 2010). The regulated expression of a specific array of proinflammatory cytokines in intestinal epithelial cell lines suggests that these cells are programmed to provide chemotactic and activating signals to adjacent and underlying immune and inflammatory cells in the initial period following microbial invasion of epithelial cells and further serve to amplify the mucosal inflammatory response (Jung et al., 1995). In our study, a cocktail composed of IL-1β, TNF-α, IFN-γ and LPS was utilized to represent the acute inflammatory reaction as it might occur during the active phase of IBD. After adding the cocktail, the expressions of claudin-1 and 4 were significantly increased, while those of ZO-1, ZO-2 and occludin were significantly reduced compared with that without stimuli treatment (Table 1). Previous studies in cultured monolayers and animal models have demonstrated that TNF-α which is central to Crohn disease pathogenesis, causes tight junction barrier dysfunction, such as induction of the expression of claudins via a process that requires myosin light chain kinase (MLCK) activation (Watterson et al., 2005; Zolotarevsky et al., 2002) and reduction of occludins by endocytosis of the specific protein (Wang et al., 2005), which in turn lead to the development of transient leaks and increases of the number of small pores (Weber et al., 2010). MLCK activation is observed in
intestinal epithelia of patients with active IBD (Blair et al., 2006) and increased claudin-1 and claudin-4 expression has also been reported in intestinal epithelial cells of patients with IBD (Heller et al., 2005). Therefore, the use of a cocktail of inflammatory stimuli at plausible concentrations on differentiated Caco-2 cells was aimed to better reflect the in vivo situation during ongoing IBD.

Among the tested caffeic acid derivatives, only chlorogenic acid and mHPP showed protection of the intestinal barrier function in terms of acting against the suppression of ZO-1, ZO-2 and occludin, as well as the induction of claudin-1 induced by the inflammatory stimuli in Caco-2 cells (Table 1). Our finding of the influence of caffeic acid derivatives on tight junction proteins is novel in showing this role of phenolic acid in gut barrier protection. Previous studies showed that two flavonoids, quercetin and kaempferol, promoted the assembly of tight junction proteins, claudin-1, occludin, and ZO-2, and the expression of claudin-4 through the inhibition of PKC. In our study, caffeic acid derivatives, belonging to phenolic acids, were also able to promote the gut barrier function both with and without inflammatory stimuli, and the mechanism might be similar to the flavonoids, because caffeic acid at 70 µM inhibited phosphorylase kinase (PhK), protein kinase A (PKA), and PKC by about 90, 59, 57% in vitro, similar to quercetin (Nardini et al., 2000). However, we can not rule out that caffeic acid derivatives may act differently compared with flavonoids as they are ionic in a neutral media (DMEM at pH 7.4 in our study), which might explain the different response of claudin-4 to phenolic acids vs. flavonoids, because claudin-4 creates an “electrostatic selectivity filter” controlling overall resistance and charge selectivity of the small pores (Anderson et al., 2009). Over expression of claudin-4 in Madin-Darby Canine Kidney (MDCK) epithelial cell lines decreased transmonolayer conductance by decreasing paracellular Na⁺ permeability.
without affecting permeability for Cl⁻ or flux for a noncharged solute. Therefore, overexpression of a claudin can confer paracellular ionic discrimination (Van Itallie et al., 2001). Further, in our study, over-expressing claudin-4 did not stimulate an overall increase in the levels of other tight-junction components in MDR KD Caco-2 cells (Table 2), consistent with previous studies showing that in epithelial cells claudin-1 and claudin-4 can assemble into tight junction strands without the participation of either ZO-1 or occludin in MDCK cells (McCarthy et al., 2000; Van Itallie et al., 2001). One possible role for the ZO proteins is to scaffold the integral membrane proteins and link them to the actin cytoskeleton (17). That levels of occludin and ZO measured do not diminish in the face of claudin-4 over-expression suggests that claudin-4 does not act to replace these integral membrane tight junction proteins, but adds to them (Van Itallie et al., 2001). Occludin is an integral part of tight junction strands (Fujimoto, 1995), but not essential for strand assembly because occludin deficient embryonic stem cells formed tight junctions (Saitou et al., 1998). Therefore, future studies should focus on the molecular mechanism of the regulation of caffeic acid derivatives on claudins because the claudin family not only define tight junction permeability and affect barrier function, but also enhance cell proliferation and regeneration, neoplastic transformation, tumor growth and metastasis in experimental models (Dhawan et al., 2005).

Of the several transport pumps investigated, MDR1 (i.e., P-gp) has been studied the most extensively in the context of overcoming chemo-resistance. Interestingly, P-gp was also proposed to function as a ‘gatekeeper’ at different tissue barriers. In our study, MDR KD Caco-2 cells responded differently compared with Caco-2 cells after exposure to caffeic acid derivatives in that only claudins were up-regulated but not for ZO and occludins. The interaction between P-gp transporter and tight junction proteins in intestinal cells is
unknown yet; but in blood testis barrier, P-gp co-localizes with several tight junction proteins such as occludin, claudin-11, JAM, ZO-1, N-cadherin and β-catenin. Meanwhile, P-gp co-localized and co-immunoprecipitated with actin, as well as with ezrin, radixin and moesin (ERM), a small family of adaptor proteins involved in linking tight junction proteins to actin filaments (Luciani et al., 2002). In our study, when MDR gene was knocked down, the up-regulation of caffeic acid derivatives on ZO and occludin proteins disappeared, while enhancement of claudin-4 occurred compared with Caco-2 cells with high expression of P-gp transporter (Table 2). This indicates that P-gp–actin interactions might be lost after the knockdown of MDR gene and that actin and ERM binding might be critical for caffeic acid derivatives to exhibit their protection, as well as that regulation of claudins, which are essential for forming tight junction strands, might be different with occludin and ZO proteins, which were indicated to be regulated by prolonged actin–myosin contraction produced contractile forces (Yao and Tsirka, 2011).

Intestinal epithelial cells produce various inflammatory mediators and participate in the development of intestinal inflammation. One of our key findings is that 50 µM of caffeic acid derivatives enhanced IL-6 and rosmarinic acid and mHPP augmented TNF-α secretion in intestinal epithelial cells after inflammatory stimulation (Table 1). Caffeic acid did not have significant inhibitory effects on stimulated cytokines in the present study, although caffeic acid phenethyl ester and methyl ester, in the range of 25-100 µM, inhibited the translocation of the transcription factor nuclear factor kappa B (NF-κB) and subsequent production and release of TNF-α in LPS induced RAW264.7 cells (Shin et al., 2004). This may be due to lower membrane permeability of caffeic acid compared with its more lipophilic esters. Previous studies showed that 3 µM rosmarinic acid down-regulated the levels of TNF-α, IL-6, and high-mobility group box 1 protein, inhibited the IκB kinase
pathway, and modulated NF-κB in LPS induced RAW264.7 cells or human gingival fibroblasts (Jiang et al., 2009; Zdarilová et al., 2009). In our study, rosmarinic acid and mHPP both at 50 µM exaggerated rather than attenuated the excretion of TNF-α and IL-6 under the stimulated condition (Table 3). This difference might be due to the tested cell lines or the stimuli applied to the model. We utilized the endogenous mediators IL-1β, TNF-α, and IFN-γ whose mucosal levels are increased during IBD as well as exogenous LPS (Fais et al., 1991; Reimund et al., 1996; Caradonna et al., 2000) concomitantly affect the inflammatory response in intestinal epithelial cells. The concentration of LPS used in our study (1 µg/mL) was greater than Jiang’s study (0.1 µg/mL alone, 2009). Thus, caffeic acid derivatives may exacerbate the inflammatory situation when stimuli were presented at the intestinal epithelial cells.

More interestingly, when P-gp transporter, encoded by MDR gene, was knocked down in Caco-2 cells, all treatments attenuated the inflammatory responses initiated by the stimuli in terms of the reduction of TNF-α and IL-6 levels (Table 4). There might be two reasons for the stronger inhibition of caffeic acid derivatives on the inflammatory responses. First, tested compounds might more easily enter the cells to exhibit their anti-inflammatory activities, which was proved by the finding that cellular content of caffeic acid and mHPP in the basolateral side and cell lysates of MDR KD Caco-2 system was ~3 fold greater than that in Caco-2 cells both with or without the stimuli (Table 6), but not for rosmarinic acid and chlorogenic acid, whose transport across the cell monolayer was mainly via paracellular pathway (Konishi and Kobayashi, 2004; Konishi and Kobayashi, 2005). Secondly, several transcription factors, such as pregnane X receptor, constitutive androstane receptor, NF-κB, activator protein 1, etc., function as regulators for most ABC transporter proteins, i.e. P-gp, multidrug resistance-associated protein, organic anion
transporter, etc. So knockdown of the MDR gene might affect transcriptional cascades that in turn affect inflammatory mediators.

In conclusion, caffeic acid derivatives enhanced barrier function in human intestinal Caco-2 cells and mHPP exhibited greater enhancement of intestinal barrier than the parent compounds. P-gp plays an essential role in the anti-inflammatory activities of caffeic acid derivatives. The present findings would suggest that the caffeic acid derivatives could be gut health promoting as a dietary constituent, but the activities under inflammatory situation might need further mechanistic or in vivo studies. Moreover, intake of caffeic acid derivatives might speed up mucosal recovery or provide protection to the small-intestinal mucosa against the inflammatory mediators when P-gp inhibitors are co-administered, which are attractive from a therapeutic point of view. In view of the abundance and possible medicinal properties of plant or food derived polyphenols, this field deserves continuing studies to ascertain any actual therapeutic benefits in humans.

Acknowledgements

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Figures

Figure 1. The chemical structures of caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (mHPP).
Figure 2. P-glycoprotein transporter activity in Caco-2 cells and multidrug resistance (MDR) gene knockdown (KD) Caco-2 cells. Verapamil was used as positive control at 10 µg/mL. Data are the mean ± S. D (n=6). Means bearing different letters were significantly different by ANOVA and Tukey’s multiple comparison ($p < 0.05$).
Figure 3. TEER in Caco-2 cell monolayers incubated with or without caffeic acid derivatives (50 μM) and quercetin (100 μM). (A) Caco-2 cells; (B) Caco-2 cells treated with the stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 μg/mL) for 24hs; (C) MDR KD Caco-2 cells; (D) MDR KD Caco-2 cells treated with the stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 μg/mL) for 24hs. Control was 0.3 % DMSO in DMEM. Values are expressed as
means ± SD, n = 4. Means bearing different letters at a time were significantly different by ANOVA and Tukey’s multiple comparison (p < 0.05).
Figure 4.  Lucifer yellow flux across cell monolayers incubated with or without caffeic acid derivatives (50 µM) and quercetin (100 µM) for 48 h incubation. (A) Caco-2 cells treated with the stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) for 24 hs; (C) MDR KD Caco-2 cells treated with the stimuli composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) for 24 hs.  Control was 0.3 % DMSO in DMEM.  Values are expressed as means ± SD, n
Means bearing different letters were significantly different by ANOVA and Tukey’s multiple comparison ($p < 0.05$). The dash line indicates the baseline of lucifer yellow flux across cell monolayers in each cell type.
Table 1. Immunoblot analysis of tight junction proteins in the whole extracts of Caco-2 cells incubated with caffeic acid derivatives (50 µM) and quercetin (100 µM) for 48 hs, quantitated by densitometric analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Quercetin</th>
<th>Caffeic acid</th>
<th>Rosmarinic acid</th>
<th>Cholorogenic acid</th>
<th>mHPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1</td>
<td>2.2 ± 0.1³</td>
<td>2.3 ± 0.2²</td>
<td>2.9 ± 0.1³</td>
<td>3.3 ± 0.2²</td>
<td>2.5 ± 0.2³</td>
<td>2.9 ± 0.1³</td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.1⁴</td>
<td>1.1 ± 0.1⁵</td>
<td>1.4 ± 0.2⁴</td>
<td>1.5 ± 0.2⁵</td>
<td>1.7 ± 0.3⁴</td>
<td>1.3 ± 0.1⁵</td>
</tr>
<tr>
<td>ZO-2</td>
<td>0.4 ± 0.01⁶</td>
<td>0.5 ± 0.01ª</td>
<td>0.5 ± 0.01ª</td>
<td>0.5 ± 0.02ª</td>
<td>0.5 ± 0.02ª</td>
<td>0.5 ± 0.02ª</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.01ª</td>
<td>0.3 ± 0.03ª</td>
<td>0.3 ± 0.02ª</td>
<td>0.4±0.03ª</td>
<td>0.4±0.02ª</td>
<td>0.5±0.01ª</td>
</tr>
<tr>
<td>Claudin -1</td>
<td>0.6 ± 0.01⁷</td>
<td>0.7 ± 0.01ª</td>
<td>0.7 ± 0.01ª</td>
<td>0.7 ± 0.02ª</td>
<td>0.7 ± 0.02ª</td>
<td>0.7 ± 0.02ª</td>
</tr>
<tr>
<td></td>
<td>0.7 ± 0.02ª</td>
<td>0.7 ± 0.01ª</td>
<td>0.7 ± 0.01ª</td>
<td>0.8 ± 0.04ª</td>
<td>0.6 ± 0.03ª</td>
<td>0.6 ± 0.03ª</td>
</tr>
<tr>
<td>Claudin -4</td>
<td>0.3 ± 0.02ª</td>
<td>0.4 ± 0.01ª</td>
<td>0.3 ± 0.01ª</td>
<td>0.3 ± 0.01ª</td>
<td>0.3 ± 0.01ª</td>
<td>0.3 ± 0.03ª</td>
</tr>
<tr>
<td></td>
<td>0.6 ± 0.01ª</td>
<td>0.4 ± 0.02ª</td>
<td>0.6 ± 0.01ª</td>
<td>0.7 ± 0.02ª</td>
<td>0.7 ± 0.04ª</td>
<td>0.6 ± 0.01ª</td>
</tr>
<tr>
<td>Occludin</td>
<td>11.7 ± 0.02ª</td>
<td>12.3 ± 0.02ª</td>
<td>12.9 ± 0.1²</td>
<td>14.6 ± 0.2³</td>
<td>12.6 ± 0.3²</td>
<td>14.8 ± 0.08ª</td>
</tr>
<tr>
<td></td>
<td>10.6 ± 0.1ª</td>
<td>11.7 ± 0.1ª</td>
<td>7.6 ± 0.1¹</td>
<td>9.4 ± 0.1¹</td>
<td>11.7 ± 0.2ª</td>
<td>14.6 ± 0.1ª</td>
</tr>
</tbody>
</table>

ª BOLD denotes cells treated with inflammatory cocktail, which was composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) and incubated for 24 hs. The density values were normalized by the internal standard, α-tubulin. Control was 0.3 % DMSO in DMEM. Values are means ± SD, n = 4. Significant changes within a protein are noted by letters compared with untreated cells by ANOVA and Tukey’s multiple comparison (p < 0.05).
Table 2. Immunoblot analysis of tight junction proteins in the whole extracts of MDR KD Caco-2 cells incubated with caffeic acid derivatives (50 µM) and quercetin (100 µM) for 48 hs, quantitated by densitometric analysis\(^a\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Quercetin</th>
<th>Caffeic acid</th>
<th>Rosmarinic acid</th>
<th>Cholorogenic acid</th>
<th>mHPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1</td>
<td>1.8 ± 0.03(^a)</td>
<td>1.7 ± 0.2(^a)</td>
<td>1.9 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^a)</td>
</tr>
<tr>
<td>ZO-2</td>
<td>1.4 ± 0.04(^b)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.7 ± 0.1(^b)</td>
<td>1.7 ± 0.2(^a)</td>
</tr>
<tr>
<td>Claudin -1</td>
<td>0.6 ± 0.01(^a)</td>
<td>0.6 ± 0.01(^b)</td>
<td>0.6 ± 0.01(^b)</td>
<td>0.6 ± 0.05(^b)</td>
<td>0.6 ± 0.07(^b)</td>
<td>0.6 ± 0.02(^a)</td>
</tr>
<tr>
<td>Claudin -4</td>
<td>0.4 ± 0.01(^b)</td>
<td>0.4±0.03(^b)</td>
<td>0.4±0.01(^b)</td>
<td>0.4±0.03(^b)</td>
<td>0.4±0.01(^b)</td>
<td>0.5±0.03(^b)</td>
</tr>
<tr>
<td>Occludin</td>
<td>1.0 ± 0.2(^a)</td>
<td>0.8 ± 0.1(^b)</td>
<td>0.7 ± 0.04(^c)</td>
<td>0.8 ± 0.03(^b)</td>
<td>0.7 ± 0.03(^c)</td>
<td>0.7 ± 0.01(^c)</td>
</tr>
<tr>
<td>Claudin -4</td>
<td>0.6 ± 0.04(^g)</td>
<td>0.7 ± 0.01(^c)</td>
<td>0.6 ± 0.01(^d)</td>
<td>0.7 ± 0.01(^b)</td>
<td>0.7 ± 0.02(^d)</td>
<td>0.7 ± 0.01(^bc)</td>
</tr>
<tr>
<td>Occludin</td>
<td>11.2 ± 0.08(^a)</td>
<td>11.2 ± 0.06(^a)</td>
<td>11.1 ± 0.5(^a)</td>
<td>11.2 ± 0.3(^a)</td>
<td>11.1 ± 0.3(^c)</td>
<td>11.0 ± 0.04(^a)</td>
</tr>
<tr>
<td>Occludin</td>
<td>4.8 ± 0.3(^g)</td>
<td>4.7 ± 0.1(^e)</td>
<td>7.3 ± 0.02(^b)</td>
<td>5.7 ± 0.1(^d)</td>
<td>6.3 ± 0.1(^c)</td>
<td>7.7 ± 0.1(^b)</td>
</tr>
</tbody>
</table>

\(^a\) BOLD denotes cells treated with inflammatory cocktail, which was composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) and incubated for 24 hs. The density values were normalized by the internal standard, α-tubulin. Control was 0.3 % DMSO in DMEM. Values are means ± SD, n = 4. Significant changes within a protein are noted by letters compared with untreated cells by ANOVA and Tukey’s multiple comparison (\(p < 0.05\)).
Table 3. Caffeic acid derivatives (50 µM, 48 h incubation) augmented the inflammatory responses after treating Caco-2 cells with the stimuli.

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>357.6 ± 14.1</td>
<td>6.3 ± 1.8</td>
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<tr>
<td>Quercetin</td>
<td>486.2 ± 95.4</td>
<td>16.8 ± 2.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>517.0 ± 193.5</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>665.1 ± 8.6</td>
<td>21.2 ± 6.6</td>
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<tr>
<td>Chlorogenic acid</td>
<td>338.6 ± 1.7</td>
<td>9.1 ± 5.2</td>
</tr>
<tr>
<td>mHPP</td>
<td>604.4 ± 27.3</td>
<td>18.3 ± 1.0</td>
</tr>
</tbody>
</table>

*Values are means ± SD, n = 4. Control was 0.3 % DMSO in DMEM. Means bearing different letters were significantly different within each column by ANOVA and Tukey’s multiple comparison (p < 0.05). Stimuli were composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) and incubated for 24 hs.
Table 4. Caffeic acid derivatives (50 µM, 48 h incubation) attenuated the inflammatory responses in MDR KD Caco-2 cells $^a$.

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.2 ± 28.3$^a$</td>
<td>15.2 ± 0.7$^{ab}$</td>
</tr>
<tr>
<td>Quercetin</td>
<td>ND</td>
<td>16.4 ± 0.2$^{ab}$</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>ND</td>
<td>18.1 ± 0.4$^a$</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>ND</td>
<td>20.7 ± 2.7$^a$</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>ND</td>
<td>11.4 ± 0.1$^b$</td>
</tr>
<tr>
<td>mHPP</td>
<td>ND</td>
<td>20.3 ±4.5$^a$</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± SD, n = 4. Control was 0.3 % DMSO in DMEM. Means bearing different letters were significantly different within each column by ANOVA and Tukey’s multiple comparison ($p < 0.05$). ND= Not detected. The limit of detection was 7.8 and 4.7 pg/ml for the TNF-α and IL-6 assay, respectively.
Table 5. Caffeic acid derivatives (50 µM, 48 h incubation) attenuated the inflammatory responses in MDR KD Caco-2 cells treated with the stimuli \(^a\).

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>122.2 ± 12.3(^a)</td>
<td>27.9 ± 4.6(^a)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>ND</td>
<td>11.3 ± 5.4(^b)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>ND</td>
<td>8.4 ± 0.2(^b)</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>ND</td>
<td>11.4 ± 1.2(^b)</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>ND</td>
<td>8.3 ± 2.1(^b)</td>
</tr>
<tr>
<td>mHPP</td>
<td>ND</td>
<td>8.4 ± 1.0(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Values are means ± SD, n = 4. Control was 0.3 % DMSO in DMEM. Means bearing different letters were significantly different within each column by ANOVA and Tukey’s multiple comparison (\(p < 0.05\)). ND = Not detected. Stimuli were composed of IL-1β (25 ng/mL), TNF-α (50 ng/mL), IFN-γ (50 ng/mL) and LPS (1 µg/mL) and incubated for 24 hs. The limit of detection was 7.8 and 4.7 pg/ml for the TNF-α and IL-6 assay, respectively.
Table 6. Transfer of quercetin (100 µM) and caffeic acid derivatives (50 µM) across Caco-2 and MDR KD Caco-2 cells.*

<table>
<thead>
<tr>
<th></th>
<th>Basolateral side</th>
<th>Cell lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caco-2 (%)</td>
<td>MDR KD (%)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.3 ± 0.7</td>
<td>4.2 ± 1.4</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>2.4 ± 0.2</td>
<td>9.6 ± 0.3*</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>1.3 ± 0.5</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>mHPP</td>
<td>2.5 ± 0.6</td>
<td>8.4 ± 1.4*</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD (n=4). Control was 0.3 % DMSO in DMEM. * indicates significant difference between Caco-2 and MDR KD Caco-2 cells by two-sample t-test (p < 0.05). ND= Not detected. % represents the recovery relative to the initial amount.
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CHAPTER 6. GENERAL CONCLUSIONS

General Discussion

A major limiting step in the utilization of herbal constituents is their intestinal absorption and metabolism. Compound passes across the intestinal epithelium via passive diffusion, both paracellularly and transcellularly, and by active transport. After entering the cells, herbal constituents may undergo Phase I and II biotransformation by metabolizing enzymes; the parent compounds and the metabolites are effluxed to the apical side (luminal) or transported to the basolateral side (systemic) by various transporters located at both sides of the intestinal epithelial cells. In addition, herbal constituents might also have influences on the metabolizing enzyme activities or the expression and function of transporters, which in turn affects the bioavailability of other herbal constituents or dietary nutrients, indicating possible herb-herb and herb-drug interactions. Furthermore, caffeic acid derivatives from diet or traditional herbs have been reported to have anti-inflammatory properties both in vitro and in vivo. Chapter 3, 4 and 5 are original projects which were performed on permeability and metabolism of herbal constituents and modulation of caffeic acid derivatives on intestinal function in Caco-2 cell line.

In Chapter 3, we have conducted the study of transport mechanism and metabolism of alkamides and ketone in Echinacea species and their inhibitory activities on P-glycoprotein transporter (P-gp) function. Tested alkamides transport the Caco-2 cell monolayer via passive diffusion, independent of the plant matrix, and were seemingly N-glucuronidated both as pure compounds and as found in E. saguinea and E. pallida extracts. Both Echinacea extracts stimulated apparent glucuronidation and basolateral efflux of alkamide metabolites. Interestingly, E. pallida ethanol extract inhibited the efflux of glucuronides of Bauer alkamides apically (luminal) while inducing the transfer of the conjugates
basolaterally (systemic), and the phytochemical profile might contribute to the difference between two *Echinacea* species. It was not expected that Bauer ketone 24 was totally metabolized to more hydrophilic metabolites as a pure compound, but not as found in either *Echinacea* species, indicating the influences of plant matrix on ketone metabolism in Caco-2 cells. We found that Bauer alkamide 8, 10 and 11 at 175-230 µM, *E. sanguinea* at 1 mg/mL (containing 85 µM of alkamide 8, 2 µM of alkamide 10, and 0.7 µM of alkamide 11) and *E. pallida* at 5 mg/mL (containing 215 µM of alkamide 8, 25 µM of alkamide 10, and 45 µM of alkamide 11) significantly inhibited P-gp function. Taken together, plant matrix had a facilitating effect on the metabolism and efflux of alkamides and ketones from *Echinacea*, and that alkamides and *Echinacea* extracts might be useful in potentiating some chemotherapeutics which are substrates for P-gp.

In Chapter 4, we have investigated the permeabilities and Phase II biotransformation of rosmarinic acid and ursolic acid as pure compounds and in herbal extracts using Caco-2 cells with HPLC detection. We found increased basolateral rosmarinic acid recoveries after deconjugation indicating that glucuronide/sulfate conjugates were excreted to the basolateral side (toward the circulation) rather than apical side (the intestinal lumen). In addition, the plant extracts, *P. vulgaris* and *S. officinalis*, did not affect transfer of either ursolic acid or rosmarinic acid, and likewise the pure compounds and the compounds from the plant extracts behaved similarly in their apparent biotransformation as well, which was different compared with alkamide metabolism in pure solutions vs. *Echinacea* species. This difference might be due to the transport mechanism (paracellular diffusion for rosmarinic acid vs. transcellular diffusion for alkamides) and phytochemical profile of *P. vulgaris* and *Echinacea* species. Meanwhile, ursolic acid was absorbed and transferred by
Caco-2 cells apparently with little glucuronidation/sulfation, which is likely to further enhance the bioavailability of ursolic acid compared with rosmarinic acid.

In Chapter 5, we performed the regulation of caffeic acid, rosmarinic acid, chlorogenic acid and m-hydroxyphenylpropionic acid (mHPP), a microbial metabolite of ingested caffeic acid, chlorogenic acid or rosmarinic acid, on tight junction proteins and immune modulators in both Caco-2 cell model and multidrug resistance (MDR) knockdown (MDR KD) Caco-2 cells. Stimuli composed of IL-1β, TNF-α, IFN-γ and LPS were added to Caco-2 cell system to represent the acute inflammatory reaction as it might occur during the active phase of IBD. We found that caffeic acid derivatives enhanced tight junction integrity through the expression of tight junction proteins in both Caco-2 cells and MDR KD Caco-2 cells. Among the tested caffeic acid derivatives, we reported for the first time that mHPP exhibited greater enhancement of intestinal barrier than the parent compounds, indicating that mHPP deserves more studies. One of our key findings was that 50 µM of caffeic acid derivatives were found to be enhancers of IL-6 and rosmarinic acid and mHPP augmented TNF-α secretion in intestinal epithelial cells after stimulation. More interestingly, when P-gp transporter, encoded by MDR gene, was knocked down in Caco-2 cells, all caffeic acid derivatives attenuated the inflammatory responses initiated by the stimuli in terms of the reduction of TNF-α and IL-6 levels, implying that knockdown of the MDR gene might affect transcriptional cascades that in turn affect inflammatory mediators.

As a conclusion, alkamides, ketone, RA and UA in herbal extracts was transported across Caco-2 cell monolayer with different permeability rates. The metabolism was a complex process, especially in different Echinacea species. Caffeic acid derivatives could be gut health promoting as dietary constituents, and mHPP was deserved the promising research
future in both anti-inflammatory and anti-oxidative areas, particularly in molecular mechanism related to revealing anti-colitic pathway.
**Recommendations for Future Research**

In Caco-2 transport study, glucuronide conjugates of herbal alkamides, ketones, and caffeic acid derivatives were found, and plant matrix had influences on the metabolism and efflux of the metabolites of alkamides based on the current preliminary result. As a next step, we need to further investigate the inducibility of glucuronidation/sulfation, by herbal components as well as the expression of Phase I and II enzymes in Caco-2 cells after treating different pure compounds or herbal extracts. The identification of the herbal constituent or combination of various components that have major effects on metabolizing enzymes and transporter functions are also required.

With respect to the enhancement of caffeic acid derivatives on intestinal epithelial function, more mechanistic studies need to be carried out in the future. More specifically, myosin light chain kinase (MLCK), protein kinase C (PKC), ezrin, radixin and moesin (ERM) and NF-κB pathways might be targeted to reveal the underlying mechanism of the protection of caffeic acid derivatives on intestinal barrier function.
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