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**Metabolism of herbal phenolics in gut/oral microbiota or Caco-2 cells and
bioavailability associated efficacy of caffeic acid in mouse colitis**

by

Zhong Ye

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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2009

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LIST OF ABBREVIATIONS

BHI-----	Brain-heart infusion
BMI-----	Body mass index
CA-----	Caffeic acid
CAPE-----	Caffeic acid phenethyl ester
CB2-----	Cannabinoid receptor 2
COX-2-----	Cyclooxygenase-2
CREB-1-----	cAMP response element binding protein
CTAB-----	Cetyltrimethylammonium bromide
CYP-----	Cytochrome P450
CYP4B1-----	Cytochromes P450, family 4B 1
DGGE-----	Denaturing gradient gel electrophoresis
DMEM-----	Dulbecco's modified Eagle's medium
DMSO-----	Dimethyl sulfoxide
DPPH-----	2,2-diphenyl-1-picrylhydrazyl
DSS-----	Dextran sulphate sodium
EP-----	Echinacea purpurea
ER-----	Endoplasmic reticulum
H&E-----	Hematoxylin and eosin
HBSS-----	Hank's balanced salt solution
Hp-----	Hypericum perforatum
HPLC-----	High performance liquid chromatography
IBD-----	Inflammatory bowel diseases
ICAM-1-----	Intercellular adhesion molecule-1
IFN- γ -----	Interferon-gamma
IL-1-----	Interleukin-1 beta
iNOS-----	Inducible nitric-oxide synthase
JNK-----	Jun N-terminal
LPS-----	Lipopolysaccharide
MAPK-----	Mitogen-activated protein kinase

MPO	Myeloperoxidase
NF- κ B	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE ₂	Prostaglandin E ₂
PMSF	Phenylmethylsulfonyl fluoride
PPO	Polyphenol oxidases
RA	Rosmarinic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SOD	Superoxide dismutase
TER	Transepithelial Electrical Resistance
THB	2, 4, 4'-trihydroxydeoxybenzoin
TMB	3, 3', 5, 5'-tetramethylbenzidine hydrochloride
TNF- α	Tumor necrosis factor alpha

ABSTRACT

Phenolics, one category of micronutrients abundant in human diet, are believed to prevent some degenerative diseases such as cardiovascular diseases, digestive system diseases, and cancer. Numerous researchers have become increasingly interested in phenolics because of the recognition of the anti-inflammatory, antioxidant, anti-antiviral, and immune-stimulating properties of phenolics. In this dissertation, the overarching hypotheses were that plant phenolics were degraded by gut or oral microorganisms, influencing the absorption of these components; phenolics were transported by Caco-2 cell monolayers; and phenolic efficacy of colitic prevention depended on bioavailability. Our long-term goal is to elucidate the metabolism pattern of phenolics that may benefit colon health to prevent colitis. The first study, with *in vitro* anaerobic incubations for human fecal or mouse cecal samples, the degradation rates of major phenolics from *Echinacea purpurea* and *Hypericum perforatum* extracts were significantly different in both incubation methods. Caffeic acid was produced and one metabolite was generated during the metabolism of *Echinacea* extract. The second study with salivary bacterial incubation *in vitro*, 7- mixture compound oral degradation rates were statistically significant differences. Cluster analysis showed that significantly different groups of high and low degraders of caffeic acid and rutin were evident. In the third study with Caco-2 cell line, *Hypericum perforatum* components, chlorogenic acid, an ester of caffeic acid and quinic acid, quercetin, amentoflavone, and pseudohypericin were compared to test the apparent permeabilities from apical to basolateral transfer, the results demonstrated that the components had a low permeable ability after 4 h incubation with the monolayer. The fourth study in dextran sulfate sodium (DSS)-induced C3H/HeOuJ mouse colitis model, treated with caffeic acid compared with rutin (both of 1.0 mmol/kg in diet) and hypoxoside extract, only caffeic acid protected against DSS-induced colitic histopathological damage, in association with normalization of CYP4B1 expression. Finally, to examine interindividual variability in the efficacy of caffeic acid, the other strain, a CD-1/*IGS* mouse model was used with DSS-induced colitis. The expression of CYP4B1 was also increased by caffeic acid/DSS treatment. The main other findings showed that two significant different subgroups of caffeic acid and rutin were identified based upon cluster analysis of cecal histopathological score in mice fed caffeic acid/DSS. Caffeic acid-fed mice with severe cecal damage had significantly

greater colonic MPO activity than did mice with mild cecal damage. Furthermore, the severe cecal damage subgroup was significantly associated with a lower plasma concentration of caffeic acid. These effects in mice fed caffeic acid/DSS were associated with the variety of caffeic acid bioavailability, probably due to gut microbial ecology, which is an important controllable variable in the effects of caffeic acid on colitis.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Medicinal plants and herbs may be used to prevent or treat diseases. Different herbs act on different systems of the body. Some herbs have been scientifically studied and found to be effective. *Echinacea purpurea* was used as preparations by the Plain Indians for the treatment of upper respiratory infections, burns, snakebites, and cancers. It has been demonstrated that plant extracts stimulated the immune system to combat bacterial and viral infections (Bany et al., 2003; Cohen et al., 2004; Schoop et al., 2006; Birt et al., 2008). Flavonoids, caffeic acid derivatives (caftaric acid, caffeic acid, cichoric acid) are the main chemical constituents in *Echinacea* (Roesler et al., 1991; Barnes et al., 2005). Superoxide dismutase (SOD) activity in peripheral blood was increased because of echinacoside and caffeic acid in *E. purpurea* which eliminated superoxide (O_2^-) by a free radical scavenging effect in mice (Mishima et al., 2004). Also fourteen-day 30 and 100 mg/kg *E. purpurea* daily were shown to significantly induce apoptosis; and decreased Fas-Ag expression or increased in Bcl-2 expression from the splenic lymphocytes in mice compared to mice treated only with the vehicle (Di Carlo et al., 2003). The efficacy of *E. purpurea* may be related to the better bioavailability of its constituents. Caftaric acid, chlorogenic acid, and caffeic acid (1 μ mol) were inoculated with human fecal slurry (10 ml) in fermentation bottles and all phenolics were degraded quickly after 2 hours of incubation (Gonthier et al., 2006). Two main metabolites of m-hydroxyphenylpropionic acid (m-HPP) and 4-ethylcatechol were detected from caffeic acid fecal incubation (Olthof et al., 2001). Cichoric acid was degraded by polyphenol oxidases (PPO) into caftaric acid and caffeic acid during the preparation of *Echinacea purpurea* products (Nüsslein et al., 2000). Caffeic acid phenethyl ester (CAPE) decreased colonic NF- κ B and prevented colitis in peptidoglycan-polysaccharide (PG-PS)-treated rats injected with 30 mg CAPE /kg for seven days (Fitzpatrick et al., 2001).

Hypericum perforatum (Hp), as known *St. John's Wort*, has been used for centuries to treat anxiety, stress, and mental disorders. American Indians treated tuberculosis, wounds and severe pain with a tea made from its flowers. Hypericin, a main compound, had a value in the treatment of mild depression, strongly antiviral property and the treatment of HIV/AIDS (Chatterjee et al, 1998; Muller et al, 2001; Wurglics et al, 2006). Oral twice daily 100 mg/kg BW of *Hypericum perforatum* inhibited carrageenan-induced paw edema in mice and the same treatment with 100 mg/kg Hp inhibited both inducible nitric-oxide synthase (iNOS) and COX-2

expression modulated by lipopolysaccharide (LPS) and interferon in peritoneal macrophages (Raso et al,2002). Hp had significant radical-scavenging activity in cell-free and human vascular systems (Hunt et al, 2001; Cakir et al, 2003). Hp contains flavonoids (hyperoside, rutin, and quercetin) and naphthodianthrone (pseudohypericin and hypericin) (Butterweck et al, 2007). The efficacy of Hp may depend on the better absorption of its components and metabolites. Rutin was absorbed as quercetin because it was hydrolysed by the cecal microflora (Manach et al., 1997). Rutin (quercetin-3-rutinoside) was transformed into hyperoside (quercetin-3-glucoside) by splitting off a rhamnose molecule. Then sugar moiety in quercetin glycoside (hyperoside) was deglycosylated by microbial glucosidase to quercetin (Baba et al., 1983). *Eubacterium ramulus* was a quercetin-3-glucoside-degrading anaerobic microorganism in *in vitro* (Schneider et al., 2000). Glycosides were almost completely metabolized by the intestinal microbiota within 20 min to 4 h depending on the sugar moiety and the type of glycosidic bond (Hein et al., 2008). Dietary rutin (feeding 0.1% rutin diet for 2 weeks) prevented DSS-induced colitis and possible colorectal carcinogenesis by suppressing pro-inflammatory cytokines (Kwon et al., 2005). In a summary, *Echinacea purpurea* and *Hypericum perforatum* still have broad research areas for further investigation such as bioavailability, metabolism, anti-inflammatory action, especially related to molecular mechanism in anti-colitic pathway.

Until now, no plant extracts have been studied in fecal or oral bacterial microbiota. Metabolism is more complex and may interact with each other. *Hypericum perforatum* extract and phenolic mixture bioavailability studies in Caco-2 cell monolayers have not been reported. No studies have been done previously with caffeic acid to prevent colitis in DSS-induced animal model; and this compound seems to be an important component of some *Echinacea* species that may contribute a health effect. Furthermore, the study of bioavailability related to efficacy of caffeic acid preventive effect in colitis had not been studied before. In this whole project, we focused our research interest in these less explored areas. The overarching hypotheses were that plant phenolics were degraded by gut/oral microorganisms and absorbable in the human intestinal Caco-2 cells; and phenolic preventive efficacy of colitis depended on bioavailability. We conducted these studies into five individual hypotheses which contained two main research fields: microbial metabolism of herbal phenolics *in vitro* (Hypothesis and objective 1, 2, and 3)

and bioavailability related to anti-colitic effect of caffeic acid *in vivo* (Hypothesis and objective 4 and 5).

1. In the first study, we hypothesized that the degradation rates were similar for major phenolics from *Echinacea purpurea* and *Hypericum perforatum* extracts between human fecal and mouse cecal bacteria. Human fecal and mouse cecal samples were incubated with two extracts to determine which phenolics were least degraded, and hence predicted to be most bioavailable to the mouse gastrointestinal mucosa.
2. In the second study, we hypothesized that human oral bacteria had significant but highly variable phenolic degradation capability, which may influence ability of these compounds to prevent periodontal disease. The objective was to assess oral phenolic degradations and to identify microbes which were present in the human oral cavity.
3. In the third study, we hypothesized that these *Hypericum perforatum* components were absorbable in a human intestinal cell model (Caco-2). Such absorption would be a necessary precondition for Hp component activity *in vivo*.
4. In the fourth study, we hypothesized that administration of dietary phenolics, caffeic acid and rutin, would suppress expression of inflammatory markers and thus, intestinal damage in a mouse model of colitis.
5. In the fifth study, we hypothesized that anti-colitis effect of caffeic acid depended on its bioavailability. The different subgroups were identified based on the histopathological score. Higher plasma caffeic acid concentration was related to the greater efficacy of colitis prevention.

Dissertation Organization

This dissertation is organized in eight chapters including general introduction, literature review of *Echinacea purpurea*, *Hypericum perforatum* and caffeic acid, three *in vitro* original research papers, two *in vivo* caffeic acid related papers, and general conclusion. Chapter 1 is a general introduction to the dissertation. Chapter 2 is a review of *Echinacea purpurea*, *Hypericum perforatum* and caffeic acid. It provides background information related to our research in metabolism pathway of herbal phenolics *in vitro* including gut or oral microflora, discussion of cell culture models, and methodologies. It also provides background information related to

caffeic acid metabolism pathway *in vivo* and *in vitro*; and related mechanisms of colitis.

Three original research papers on *in vitro* studies are arranged in chapter 3-5 and two caffeic acid related papers on *in vivo* studies are arranged in chapter 6-7. Each paper was designed to address a specific research interest. One paper (chapter 6) was accepted by Experimental Biology and Medicine; and papers were also presented at the Experimental Biology Annual Meeting and Central States Society of Toxicology Meeting or some other scientific research meeting during the recent years. This dissertation ends in chapter 8 with a general discussion on the five original experimental papers and certain recommendations for future research.

CHAPTER 2. LITERATURE REVIEW

I. GENERAL INTRODUCTION AND HEALTH EFFECTS OF MEDICINAL PLANTS

Botanical medicine, or phytomedicine, has become a promising area, an alternative to conventional medicine. Numerous researchers are focusing on herbal medicine all over the world. The use of various herbal remedies and supplements were found throughout human history from the origin of modern medicine. Many current drugs originated from plant sources and some of the most effective drugs contained the basic components of plants. The development of drugs from plants was required for detailed and large scale pharmacological screening of herbs. Chinese traditional herbal medicine was the most dominant of the ancient herbalism which was only based on the whole plant preparations and general effects. Modern Western herbalism focused on the effects of herbs on individual body systems and specific effects on cellular or molecular pathway. Herbs might be used for anti-inflammatory, immunostimulatory, antioxidant, antitumor, hemostatic and expectorant properties. It was estimated that around 80% of the worldwide population used herbal medicines, mainly for particular disease or symptom based on the property of a particular herb (Duke et al., 1999; van Wyk et al., 2004). The possibility of efficiency of plant-derived medicine has brought the growing scientific exploration in medicinal plant field. Based on current research, medical plants have shown to play important roles in human health. Seeds, roots and flowers were used as plant parts. Use of medicinal plants included culinary use or consumption of an herbal tea or supplements (Tapsell et al., 2006).

Herbal medicines were commonly used to treat some specific illness. Artichoke may reduce cholesterol levels according to a small clinical study (Bundy et al., 2008). Peppermint oil may have benefits for individuals with irritable bowel syndrome (Cappello et al., 2007). Thymus vulgaris has been shown to slow down the ageing process by maintaining the vigour of human body cells and as a treatment of epilepsy and depression (Andreatini et al., 2002; Tapsell et al., 2006). Garlic was used to reduce the risk of heart disease by lowering blood fats and cholesterol levels; and was used to fight colds, sinusitis, and other respiratory infections based on antibiotic and antiviral properties (Singh et al., 2001; Yeh et al., 2001). Ginseng was generally used for

debility and weakness during recovery from illness; and was used to improve immunity and mental functioning, and promoted the healing processes (Chang et al., 2003).

II. OVERALL INTRODUCTION OF *ECHINACEA PURPUREA*

Echinacea is a perennial herb growing as a wildflower in the prairies of the Great Plains states of USA and Canada. There are three main *Echinacea* species which are used medicinally, including *Echinacea purpurea* (purple coneflower), *Echinacea pallida* (pale purple coneflower) and *Echinacea augustifolia* (narrow-leaved purple coneflower) (Cheminat et al., 1988). The extracts of roots, leaves and flowers are used for bacterial and viral infections, especially for upper respiratory infections such as tonsillitis, bronchitis and laryngitis and the treatment of fevers, colds and flu. It is reported to be insecticidal (Melchart et al., 1998; Borchers et al., 2000).

There are many different chemical constituents within each variety, such as flavonoids, caffeic acid derivatives (caftaric acid, caffeic acid, cichoric acid), alkylamides, alkaloids, polysaccharides (Roesler et al., 1991; Pellati et al., 2004; Barnes et al., 2005; LaLone et al., 2007; Figure 2.1.). Although many of the active compounds of echinacea have been identified, mechanism of action, bioavailability, and relative potency are less known.

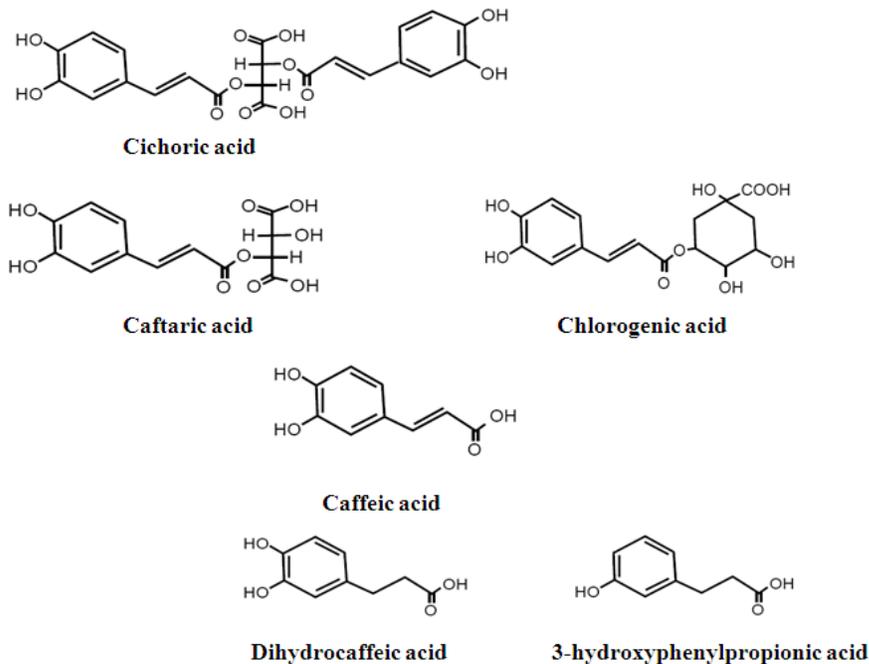


Figure 2.1. Structure of *Echinacea purpurea* phenolic compounds

Composition of Echinacea purpurea

Using 80% methanol for extraction and analyzing by RP-LC method with diode-array detector (DAD) to quantify caffeic acid derivatives in various 3-year-old *Echinacea* roots in Italy, the total phenolic contents were detected as 10.49 mg/g for *E. angustifolia*, 17.83 mg/g for *E. pallida* and 23.23 mg/g for *E. purpurea*. Ranges of cichoric acid (0.83-19.27 mg/g), caftaric acid (0.81-3.97 mg/g), and echinacoside (1.08-16.28 mg/g) were found in these three plants. Cichoric acid and caftaric acid were the main phenolic compounds of *E. purpurea*. Caffeic acid and chlorogenic acid were not detected in these root samples (Pellati et al., 2004). Different *Echinacea* extracts which were obtained from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the USDA were analyzed by HPLC at 15 μ g/mL of extract to identify the concentrations of known constituents. The different accessions and harvest years of species included *E. angustifolia*, *E. sanguinea*, *E. purpurea*, and *E. tennesseensis* which contained greater quantities of Bauer alkaloids than of ketones or caffeic acid derivatives. The concentration range of Bauer alkaloids 3, 8–14 and 17 were around 0.1 μ M to 2.8 μ M. Ketones were present around 0.1 μ M. Cichoric acid was present in one extract of each accession from the 2003 harvest of *E. purpurea* at 0.07 μ M to 0.28 μ M. Caftaric acids were present at 0.01 μ M to 0.04 μ M. Caffeic acid concentrations were not reported at this study (LaLone et al., 2007). However, based on earlier NCRPIS Annual Report (2004), the concentrations of caffeic acid extracted from *Echinacea* roots including *E. angustifolia*, *E. purpurea*, and *E. pallida* were from 0.10 to 0.21 mg/g dry root. Many factors affected the level of these phenolic compounds including seasonal variations, drying methods, extraction methods, and growing location of the plant. Calculated by individual phenolic compound/dried plant material, the percentage of caffeic acid in *E. purpurea* extracts was 0.6-1.1% and cichoric acid (3.5-5.7 %) and caftaric acid (3.1-4.5 %) were also the main phenolic compound of total polyphenols at 7.9-10.9% (Iranshahi et al., 2008). Using 60% ethanol at 60°C was found to be suitable for the extraction of the active ingredient yields of phenolics (53.4 mg/g dry weight (DW)), caftaric acid (3.6 mg/g DW), and cichoric acid (28.8 mg/g DW) from dried adventitious roots of *Echinacea purpurea* obtained in bioreactor cultures in South Korea (Wu et al., 2008).

Caffeic acid derivatives of Echinacea purpurea

One study was done in China to analyze natural changes of active components of *E. purpurea* by measuring content of cichoric acid. The results were shown that the maximum

content of cichoric acid above ground occurred in the blooming stage of mid July (Wang et al., 2002). A method was validated in Denmark to determine the content of cichoric acid and alkamides 2 and 8/9 in *Echinacea purpurea*, in which these two groups of compounds were measured by the same procedure. A reversed-phase high-performance liquid chromatography eluents were A = MeCN (acetonitrile)/water (5:95), B = MeCN/water (95:5), and C = MeCN/water (5:95) containing 0.1% v/v Trifluoroacetic acid (TFA). The products were shown a great variation in the content of two groups of compounds. The concentrations of cichoric acid and alkamides 2 and 8/9 were 24.3, 0.77, 1.20 mg/g in root of Danish-grown *Echinacea purpurea* (Mølgaard et al., 2003). Cichoric acid was also detected by capillary electrophoresis method as an appropriate marker of the quality of *E. purpurea* in dried press juice (Mancek et al., 2005). Cichoric acid (19.21 mg/g dry plant biomass), caftaric acid (3.56 mg/g dry biomass), and chlorogenic acid (0.93 mg/g dry biomass) were found in inoculation of leaf explants of *Echinacea purpurea* (Moench) with *Agrobacterium rhizogenes*. These results demonstrated that the biosynthetic pathway might be feasible to produce biologically active caffeic acid derivatives (Liu et al., 2006). In a summary, caffeic acid derivatives compositions of *Echinacea purpurea* were different depending on the preparation methods and herbal locations.

A. Health effect of of *Echinacea purpurea*

a. Studies of *Echinacea purpurea* on common colds or upper respiratory tract

Extracts of *Echinacea* were widely used by consumers and practitioners for preventing and treating common colds and the second top-selling herbal product currently in the USA. Some controlled clinical trials have investigated *E. purpurea* might be effective for the early treatment of upper respiratory tract in adults (Barrett 2003; Blumenthal 2005). A randomized, double-blind, placebo-controlled study to evaluate the effectiveness and safety of a preparation containing *Echinacea* in four hundred thirty children (aged 1 to 5 years), herbal preparation (Chizukit) containing 50 mg/mL of echinacea prevented the incidence of respiratory tract infection during 12 weeks (Cohen et al., 2004). To determine whether *Echinacea* prevented *rhinovirus*-induced colds or not in placebo-controlled trials, Schoop et al (2006) performed a systematic search of English- and German-language literature using the MEDLINE, EMBASE, and many other databases with total of 234 articles through the literature search. The meta-analysis reported that extracts of *Echinacea* were effective in the prevention of symptoms of the

common cold after clinical inoculation with rhinovirus (Schoop et al., 2006). However, in another randomized, double-blind, placebo-controlled trial, using Kaplan-Meier curves and Wilcoxon rank sum test, patients who received 100 mg of *E. purpurea* 3 times daily for 14 days did not relieve symptoms (sneezing, nasal discharge, nasal congestion, headache, sore or scratchy throat, hoarseness, muscle aches, and cough) during the common cold (Yale et al., 2004). Also in two review articles, there was not enough evidence or not fully consistent evidence to recommend *Echinacea* preparations for the treatment or prevention of upper respiratory tract infections (Melchart et al., 2000; Linde et al., 2006). In a summary, *Echinacea* preparations were not recommended for treating common colds and upper respiratory tract because of the controversial investigation result.

b. Anti-inflammatory effects

Many studies have reported that *E. angustifolia*, *E. pallida*, and *E. simulata* possessed an antiinflammatory activity which was differed from species, extracts, and fractionation and contributed to main constituents, including alkamides or caffeic acid derivatives (LaLone et al., 2007; Birt et al., 2008). Mouse paw edema which was induced by subplantar injections of 50 μ L of a 1% w/v carrageenan solution into one hind paw (2 to 72 h) was inhibited at 100 mg/kg concentration of *E. purpurea* by gavage. Treatment with *Echinacea* down-regulated cyclooxygenase-2 (COX-2) expression induced by lipopolysaccharide (LPS) and interferon- γ (Raso et al., 2002). Three different alkamides (A5, A7, A8) from a CO₂ extract of the roots of *E. angustifolia* have suppressed the expression of COX-2 and prostaglandin (PG) E₂ formation at sites of inflammation in H4 human neuroglioma cells (Hinz et al., 2007). Another caffeic acid derivative, Echinacoside, was from root extract of *E. pallida* and 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). Combination of different alkamides in *Echinacea* extracts might play a synergistic role in inhibition of PGE₂ production in lipopolysaccharide-stimulated RAW264.7 mouse macrophage cells. Active extracts contained <2.8 μ M of specific alkamide in 15 μ g/mL of extract *E. angustifolia*, *E. pallida*, *E. simulata*, and *E. sanguinea* significantly have inhibited PGE₂ production. However, 10 μ M was needed as a minimum concentration of the synthetic alkamides to inhibit PGE₂ (LaLone et al., 2007). In a summary, different species of *Echinacea* had an effective antiinflammatory action which was differed from species, extracts,

and fractionation; the antiinflammatory activity was contributed to alkamides or caffeic acid derivatives.

c. Antioxidant effects

Assessing free radical scavenging was mainly used to evaluate the antioxidant capacity in medicinal plants and other nutritional antioxidant supplements. Antioxidant measurements could be based on the evaluation of lipid peroxidation or the test of free radical scavenging ability (Sanchez-Moreno, 2002). The radical scavenging activity of phenolic compounds was largely influenced by the number of hydroxyl groups on the aromatic ring. The higher the number of hydroxyl groups, the greater the radical scavenging activity (Wang et al., 2003). Capacities to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals of phenolic compounds in *Echinacea spp* were shown that echinacoside ($EC_{50} = 6.6 \mu M$) > cichoric acid ($8.6 \mu M$) > cynarin ($11.0 \mu M$) > chlorogenic acid ($18.9 \mu M$) > caffeic acid ($19.1 \mu M$) > caftaric acid ($20.5 \mu M$) because of cichoric acid and echinacoside possessing two adjacent hydroxyl groups on each of their phenolic rings. The average EC_{50} value for *E. purpurea* ($134 \mu g/ml$) was lower than *E. pallida* and *E. angustifolia* (167 and $231 \mu g/ml$, respectively) (Pellati et al., 2004). The enzyme superoxide dismutase (SOD) was an important antioxidant defense in nearly all cells exposed to free radical. Mishima et al (2004) reported SOD activity in peripheral blood was increased because of antioxidants such as echinacoside and caffeic acid in *E. purpurea* which eliminated superoxide (O_2^-) by a free radical scavenging effect in male ICR mice (Mishima et al., 2004). Recently, Ali et al. (2008) indicated that *E. purpurea* decreased liver SOD activity (156.7 ± 10.3 and 226.1 ± 7.5 at two and four weeks, respectively) in rats treated with Cyproterone Acetateto induce toxicity (Ali et al., 2008). 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.

d. Antiantiviral effects

Echinacea extracts and its metabolic constituents possessed some antiviral and antibacterial activities (Cheminat et al., 1988). An earlier study did not report *E. angustifolia* root extracts had an effect against *rhinovirus* infection in 437 volunteers (Turner et al., 2005). By comparison with placebo, Schoop et al (2006) performed a systematic search. The meta-analysis was reported that extracts of *Echinacea* were effective in the prevention of rhinovirus-induced

colds (Schoop et al., 2006). And recent studies within *Echinacea* against HIV in vitro, extracts of *E. purpurea* consistently provided the most robust inhibition of HIV without cellular cytotoxicity. When *E. purpurea* was fractionated, one fraction which contained cichoric acid had significant anti-HIV activity in the HeLa37 cell line (Birt et al., 2008).

e. Immunomodulatory properties of Echinacea

Echinacea was also commonly used as an immunostimulant for a cold and flu remedy. Stimulation of tumor necrosis factor alpha (TNF- α), IL-10 and nitric oxide (NO) by *Echinacea* extract (5 μ g/mL) was dose-dependent and was statistically significant compared with placebo control in RAW264.7 macrophage cells with LPS. And TNF- α and IL-10 levels produced by *Echinacea* increased at approximately 30 h poststimulation and then declined sharply by 24-48 h, whereas LPS-induced TNF- α and IL-10 continued to increase over the 48-h time period (Rininger et al., 2000). Purified cichoric acid has been shown to decrease NF- κ B, TNF-alpha and NO levels in LPS-stimulated macrophage (Stevenson et al., 2005). Alkylamides, main constituents of *Echinacea* plant preparations, were showed to inhibit TNF-alpha via cannabinoid receptor 2 (CB2) receptors, increased cAMP, Jun N-terminal (JNK) and mitogen-activated protein kinase (MAPK)/p38 signaling pathways as well as NF- κ B, ATF-2 and cAMP response element binding protein (CREB-1) activation in primary human monocytes/macrophages with LPS (Gertsch et al., 2004). And this signaling cascade within macrophages was confirmed by infection with *Listeria monocytogenes* recently (Sullivan et al., 2008). *E. purpurea* activated macrophages to stimulate IFN-gamma production which was association with the secondary activation of T lymphocytes, resulting in a decrease in IgG and IgM production. Also cytokines released from macrophages in mouse peripheral blood after *E. purpurea* administration and CD 4 and CD 8 subsets were more immunologically enhanced by *E. purpurea* (Mishima et al., 2004). In a recent study, *Echinacea* preparations were reported to modulate both innate and adaptive immune responses in mice which were gavaged with 130 mg/kg above *Echinacea* extract daily for seven days and immunized with sheep red blood cells. The percentages of CD49+ and CD19+ lymphocytes in spleen and natural killer cell cytotoxicity were increased and antibody response to sRBC was significantly augmented. In mitogen-stimulated splenic cells, cytokine production of interferon-alpha was increased, however, tumor necrosis factor-gamma and interleukin (IL)-1beta were inhibited and more interestingly, IL-4 and IL-10 were presented on higher level only in *E. angustifolia* and *E. pallida*-treated mice (Zhai et

al., 2007a). Furthermore, in another study, *E. pallida* (EPA) and *E. purpurea* (EP) reported to inhibit nitric oxide (NO) production and TNF-alpha release as a dose-dependent manner in LPS-stimulated RAW 264.7 macrophages *in vitro* (Zhai et al., 2007b). In a summary, *Echinacea* species possessed an effective immunomodulatory property with different species and extracts; especially, the activity was contributed by the change of cytokine expression in macrophage cells, lymphocytes and natural killer cell.

f. Echinacea immune-stimulating properties and tumor cells

Based on the research in cancer therapy for *Echinacea* immune-stimulating properties, an animal study demonstrated *Echinacea* had enhanced cellular immunity in leukemic AKR/J mice, resulting in a suppressive effect on leukemia, via increased production of endogenous interferon-gamma (Hayashi et al., 2001). *E. purpurea* immunized mice had significantly prolonged life spans compared with non-immunized mice. Natural killer (NK) cells were demonstrated as mediators of tumor cytolysis and significantly elevated in immunized leukemic mice treated with *E. purpurea* (Currier et al., 2002). Also fourteen-day 30 and 100 mg/kg *E. purpurea* daily were shown to significantly induce apoptosis; and decreased Fas-Ag expression or increased in Bcl-2 expression from the splenic lymphocytes in mice compared to mice treated only with the vehicle (Di Carlo et al., 2003). *Echinacea* preparation have inhibited angiogenesis response induced by human lung and kidney cancer cells in mice skin evaluated 3 days intracutaneous grafting; also the incidence of CD16+ and CD56+ NK cells and the stimulation of granulocytes metabolic activity have been increased in human volunteer blood tested by chemiluminescence assay (Rogala et al., 2008). In a summary, *Echinacea* species did not clearly protect from cancer based on the present studies. More research will be needed to investigate these interesting areas related to preventing cancer or cancer treatment.

g. Cytotoxic activity of Echinacea

Different concentrations of *Echinacea* have been tested for cytotoxicity study. Ranging from 240 to 1102 $\mu\text{g/mL}$ of all *Echinacea* extracts (including each species, accession and yearly harvest) showed significant cytotoxicity with 25–72% survival compared with vehicle control in RAW264.7 macrophage cells using the Celltiter96 Aqueous One Solution Cell Proliferation Assay, whereas all extracts diluted from 1% concentration to 0.1% (concentrations ranging from 24 to 110 $\mu\text{g/mL}$ of extract) had no significant cytotoxicity remained after a 24 h

incubation. However, only one extract of *E. pallida* from the 2003 harvest still showed that cytotoxic at the 0.1% dilution (LaLone et al., 2007). On two human cancer cell lines, polyacetylenes and polyenes isolated from roots of *Echinacea pallida* were assessed on human pancreatic MIA PaCa-2 and colonic COLO320 cancer cell as concentration-dependent cytotoxicity manner, with a greater potency in the colonic cancer cells (Chicca et al., 2008).

B. Bioavailability of *Echinacea purpurea*

Bioavailability of caffeic acid conjugates (caftaric acid, cichoric acid and echinacoside) has not yet been well studied. Most of the present research was focused on the bioavailability and pharmacokinetics of different alkamides in human and some cell culture models. An earlier study showed that alkamides from *Echinacea* species, dodeca-2 E,4 E,8 Z,10 E/ Z-tetraenoic acid isobutylamides (1/ 2) transported 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 (Jager et al., 2002). In a randomized crossover study, six healthy adults of both genders consumed 4 g of each *Echinacea* root powder in a single dose which contained *E. angustifolia* (mainly 49.6 µmol echinacoside), *E. purpurea* (mainly 41.2 µmol cichoric acid), and *E. pallid* (mainly 41.0 µmol echinacoside and cichoric acid). The 24h urinary recovery of cichoric acid of *E. purpurea* and *E. pallida* were 0.36 ± 0.25 and 0.34 ± 0.31 % of ingested dose. The bioavailability of cichoric acid in these two *Echinacea* species was very low (Lee et al., 2006). However, another healthy volunteer 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 alkylamides were detected in plasma 20 minutes after tablet ingestion (Matthias et al., 2004; Matthias et al., 2005). In *in vitro* Caco-2 assays, some caffeic acid conjugates were not found to diffuse across the monolayers and suggested that these compounds did not cross the intestinal barrier. In contrast to this, alkylamides were found to diffuse rapidly through Caco-2 monolayers (Matthias et al., 2005). One alkamide pharmacokinetics study in *Echinacea angustifolia* as a randomized crossover design for oral *E. angustifolia* extract in 11 healthy subjects, the maximum concentration of main alkamides, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides, appeared after 30 minutes at 10.88 ng/mL for the 2.5 mL dose analyzed by liquid chromatography/mass spectrometry (LC/MS) (Woelkart et al., 2005). In another randomized

crossover single-dose study for *E. purpurea*, 10 volunteers had received either 4 ml of the standardized *E. purpurea* (Echinaforce) tincture or 12 *E. purpurea* (Echinaforce) tablets orally which contained the same 0.07 mg of the major alkamides (dodeca-2E,4E, 8Z,10E/Z-tetraenoic acid isobutylamides). Alkamides in *Echinaforce tincture* appeared 0.40 ng/ml serum at 30 min after application, whereas alkamides in tablets were 0.12 ng/ml serum at 45 min (Woelkart et al., 2006). Similar bioavailability of alkylamides was found from the liquid (200, 300 mg/ml) and tablet (600, 675 mg/tablet) *Echinacea* formulations in a two-way crossover study in humans (Matthias et al., 2007). Woelkart et al. (2008) performed similar study *E. purpurea*, the maximum concentrations of dodeca-2 E,4 E,8 Z, 10 E/ Z-tetraenoic acid isobutylamides in plasma were 0.22 ng/mL after administration of Echinaforce tablets, 0.22 ng/mL after taking Echinaforce Junior tablets and 0.23 ng/mL after administration of an *Echinacea* sore throat spray (Woelkart et al., 2008). Three different dose levels (0.07, 0.21 and 0.9 mg) of the major alkamides, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides, from *Echinacea purpurea* phytotherapeutic lozenges were consumed by six healthy volunteers of both genders. Alkamides were found to be rapidly absorbed and measurable in plasma 10 min after administration of 0.21 and 0.9 mg lozenges and remained detectable for 3 h; 0.07 mg lozenges were measurable 20 min after administration and remained 2 h (Guiotto et al., 2008). In a summary, caffeic acid derivatives (caftaric acid, cichoric acid and echinacoside) in *Echinacea* species were not detectable in both cell culture models and human studies whereas the alkamides were measured in two kind studies. That implied the caffeic acid derivatives were not absorbable or metabolized and formed to new products which triggered our interesting in further research.

C. Degradation and metabolism of *Echinacea purpurea*

Degradation of cichoric acid in Echinacea purpurea products

During the preparation of *Echinacea purpurea* products, cichoric acid (2R, 3R-O-dicaffeoyltartaric acid) was degraded by polyphenol oxidases (PPO) into caftaric acid (2-O-caffeoyltartaric acid; monocaffeoyltartaric acid) and caffeic acid. In the meantime, initial esterase activity was involved to catalyze the hydrolysis of cichoric acid. Caftaric acid was degraded as well but more slowly than cichoric acid. Both antioxidant ascorbic acid and ethanol inhibited oxidative degradation and hydrolysis as synergistic effect (Nüsslein et al., 2000). The other three natural antioxidants (citric acid, malic acid, and hibiscus extract) also stabilized the

major caffeic acid derivatives (caftaric acid, caffeic acid, cichoric acid), whereas caffeic acid derivatives were subject to degradation in the control sample. Stability was dependent upon the concentration of antioxidant added (Bergeron et al., 2002). With respect to alkamide stability, comparing with phenolic-depleted and phenolic-rich dry *E. purpurea* extracts and phenolic-depleted and phenolic-rich DMSO *E. purpurea* extracts, alkamides degradation was faster in dry films than in DMSO solution resulted from greater surface exposure to oxygen in dry films. The phenolic acids played an important role in inhibiting the loss of the alkamides as antioxidants in dry *E. purpurea* extracts, whereas phenolics accelerated to degrade alkamides in DMSO *E. purpurea* extracts (Liu et al., 2007). In a summary, caffeic acid derivatives (caftaric acid, cichoric acid and echinacoside) in *Echinacea* species were not stable during preparation of *Echinacea* products and hydrolyzed by polyphenol oxidases.

Cytochrome P450 activity in metabolism of Echinacea purpurea

Cytochrome P450 (CYP) may affect the metabolism and further bioavailability of *Echinacea*. Some studies have shown *Echinacea* preparation was able to inhibit CYP in vitro. Using CYP probe drugs caffeine for CYP1A2, tolbutamide for CYP2C9, dextromethorphan for CYP2D6, and midazolam for hepatic and intestinal CYP3A to assess the effect of *Echinacea purpurea* root (1600 mg x 8 days) in twelve healthy subjects, *Echinacea* administration significantly increased the systemic clearance of intravenous midazolam by 34% and the oral availability of midazolam was significantly increased. In the meantime, the oral clearance of caffeine and tolbutamide were significantly reduced. These results were due to the inhibition of CYP3A at hepatic and intestinal sites as well as improving of CYP1A2 and CYP2C9 activity during interaction with *Echinacea* (Gorski et al., 2004). Recently, an *in vitro* study showed that *E. purpurea* extract had inhibited CYP3A4 activity at specific site which was associated with higher inhibition for metabolism of 7-benzyloxy-trifluoromethylcoumarin (BFC) and 7-benzyloxyquinoline (BQ) compared with that of testosterone in fluorometric assays (Hansen et al., 2008). Although *Echinacea purpurea* extract possessed the weakest inhibition ability with an IC₅₀ value (5.03 mg/ml) which was an inhibitory potential only 0.3% of that of St. John's wort among six commercially available herbal products. The study reported *Echinacea purpurea* inhibited CYP3A4 mediated metabolism in C-DNA baculovirus which expressed human

Cytochrome P450 3A4 supersomes based on the decreased formation of 6-OH-testosterone (Hellum et al., 2008).

III. OVERALL INTRODUCTION OF *HYPERICUM PERFORATUM*

Hypericum perforatum (Hp), also called St. John's wort, is a perennial plant with yellow-flower common to the western United States, Europe, and Asia. St. John's wort has a complex and diverse chemical component system. Constituents contain flavonoids (including hyperoside, rutin, and quercetin), naphthodianthrones (pseudohypericin and hypericin), anthraquinones, carotenoids, coumarins, carboxylic acids, phloroglucins (hyperforin), xanthones, proanthocyanidins, and volatile oils (Lawvere et al., 2005 & Butterweck et al., 2007). The constituents which were presented in the extracts depended on different extraction procedures and different accessions (Hammer et al., 2007). Chlorogenic acid was the highest level concentration observed in Soxhlet ethanol Common and Elixir extracts. Rutin was the most abundant flavonoid detected in all accessions. However, very few constituents were detected in the Soxhlet chloroform extracts (Hammer et al., 2007). The main active components of St. John's wort were thought to be hypericin and hyperforin. The recent interests are other plant constituents (e.g., flavonoids and flavonoid derivatives, pseudohypericin, amentoflavone) that may have antidepressant and anti-inflammatory effects (Wurglics et al., 2006 & Hammer et al., 2008).

Extracts of *Hypericum perforatum* has been recommended traditionally for a wide range of medical conditions. The most common use of Hp is the treatment of depression. Hp also has anti-inflammatory, antibacterial, antiviral properties, and has been used to help heal wounds and burns (Herold et al., 2003; Raso et al., 2002). Because Hp can cause some serious interactions with prescription drugs, herbs, or supplements; safety concerns exist as with most conventional and complementary therapies. So more research is needed in this area to understand how Hp will affect human health.

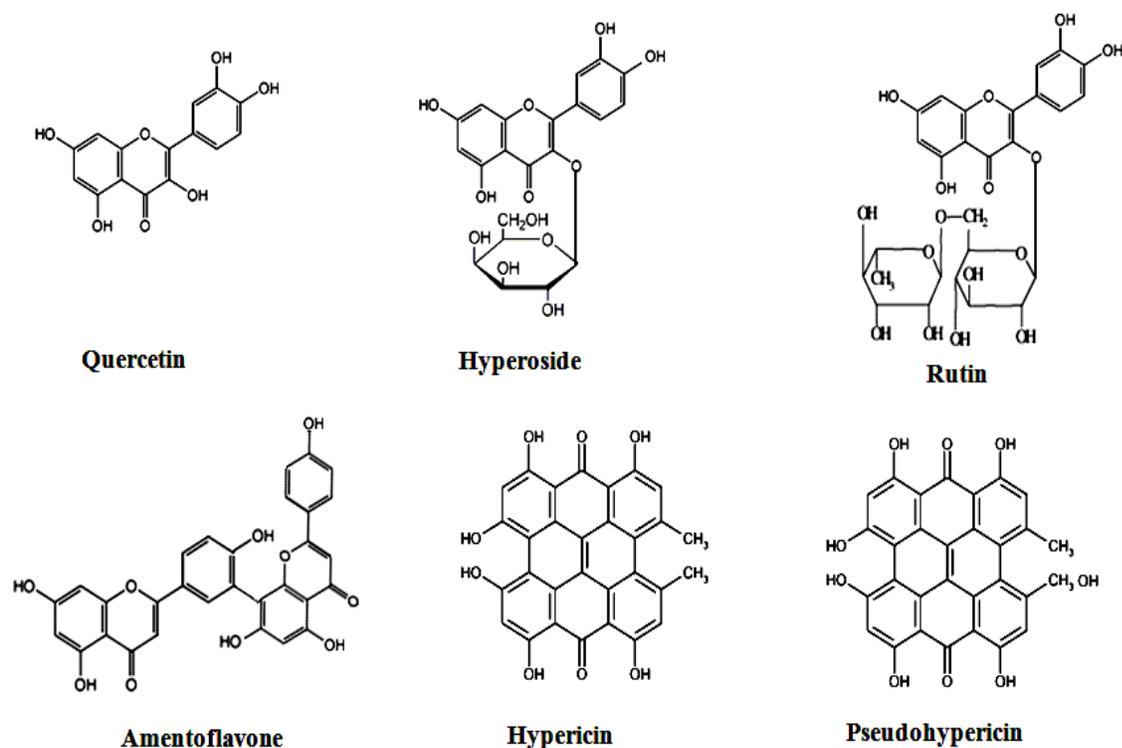


Figure 2.2. Structure of *Hypericum perforatum* phenolic compounds

Constituents of Hypericum perforatum extract

Many different classes of chemicals were detected from select plant species (Lavie et al., 1995). The four classes and main active constituents which are related to our present studies will be discussed below including: flavonoids (such as hyperoside, quercetin and rutin), and biflavonoids (amentoflavone), caffeic acid derivatives (chlorogenic acid), and naphthodianthrone (pseudohypericin). From a total of 30 *H. perforatum* individuals which were collected by full flowering at 10 sites in Northern Turkey, the concentrations of pseudohypericin among populations were up to 2.94 mg/g dry weight and the variability of populations was significant difference and the concentrations of hyperforin ranged from traces to 2.94 mg/g dry weight (Cirak et al., 2008). The total contents of hypericin and pseudohypericin were much lower from 31.34% to 80.18% for tablets than product label claims in commercially available *Hp* herbal preparations (Draves et al., 2003). The 10 constituent concentrations were quantified in 10 µg/ml of *Hp* extracts, fractions and subfractions (Hammer et al., 2008). The most abundant constituents in the original *Hp* extract were hyperforin (12.5 µM), chlorogenic acid (6.1 µM), rutin (2.7 µM), hyperoside (1.6 µM), isoquercitrin (0.3 µM), quercitrin (0.03 µM), quercetin (0.2

μM), amentoflavone (0.2 μM), pseudohypericin (0.2 μM), and hypericin (0.1 μM). After the third round of fractionation, the concentrations of six constituents were not detected. Only four bioactive components in this fraction were found as below: greatest amount of chlorogenic acid, followed by roughly equal amounts of quercetin and amentoflavone, and the least amount of pseudohypericin (0.1: 0.07: 0.08: 0.03 μM).

A. Health effect of of *Hypericum perforatum*

a. Antidepressant properties

Although the plasma concentration of the hypericin could not be measured or very low in the brain after *H. perforatum* extracts or pure hypericin in oral administration compared with hyperforin which was detected as 10-fold level of hypericin in humans (Wurglics et al., 2006). The constituents such as pseudohypericin, hypericin, hyperforin, and flavonoid compounds may contribute to the antidepressant actions for Hp (Chatterjee et al., 1998 & Muller et al., 2001; Schulz et al., 2001). A meta-analysis analysis showed the significantly positive responses to Hp involving over 1,500 individuals based on the Hamilton Depression Scale (HAMD) before and after treatment (Linde et al., 1996). The later meta-analysis covering clinical trials also supported this observation on Hp therapies for depression and dysthymia (a chronic but milder form of depression) (Kasper et al., 2001).

Two double-blind, randomized trials reported 800 mg (Harrer et al., 1999) or 500 mg (Schrader et al., 2000) Hp daily was as effective as 20 mg/day of fluoxetine (Prozac®), a drug for antidepressant. Both of two trials took six weeks and only fewer side effects were found in patients taking Hp. The other two similar trials showed *Hypericum* extract had 83% and 100% of fluoxetine therapy efficacy (Friede et al., 2001 & Behnke et al., 2002). In an animal study, *H. perforatum* extract displayed dose-dependent antidepressant effect (doses 7, 35 and 70 mg/kg BW) in mild to moderate depression induced by the forced-swimming and tail-suspension methods (Bach-Rojecky et al., 2004). However, a recent randomized 8-week double-blind trial in a Brazilian study was less efficacious using Hp 900 mg/day compared with 20 mg/day fluoxetine and placebo in seventy-two outpatients (Moreno et al., 2006). For a pilot study to examine the effectiveness, safety, tolerability, and pharmacodynamics of *Hypericum perforatum*, 150 to 900 mg/day St. John's wort three times daily had an effective treatment for thirty-three youths who were diagnosed with major depressive disorder for four weeks (Findling et al., 2003). More

recently, from the trials which were searched in computerised databases and bibliographies of relevant articles, including a total of 29 trials (5489 patients) compared with placebo and synthetic standard antidepressants, *Hypericum* extracts were still superior to placebo in patients with major depression to close standard antidepressants and had fewer side effects than standard antidepressants (Linde et al., 2008). In a summary, *Hypericum perforatum* possessed the pharmacological effects, which were contributed by pseudohypericin, hypericin, hyperforin, and flavonoid compounds with antidepressant property.

b. Antidepressant mechanism of Hypericum perforatum

The action mechanism of *H. perforatum* was complicated and studied by several aspects, including a non-selective blockade of the reuptake of serotonin, noradrenaline and dopamine; an increase in density of serotonergic and dopaminergic receptors; an increased affinity for GABAergic receptors; and the inhibition of monoaminoxidase enzyme activity (Rodríguez-Landa et al., 2003). The other in vitro studies reported that the mechanism could involve the actions of hyperforin on non-specific presynaptic effects which resulted in the non-selective inhibition of the uptake of many neurotransmitters and the interaction with dopamine or opioid receptors. In an animal studies, *Hypericum* extract might indirectly activate sigma receptors or produce endogenous ligand in vivo (Mennini et al., 2004). Hp was given for 7 days to twenty healthy males to measure evening salivary cortisol. Salivary cortisol was increased and suggested that HP may enhance salivary cortisol and via 5-HT₂ mechanism (Franklin et al., 2006). Furthermore, amentoflavone was found to inhibit brain benzodiazepine binding sites of the GABA_A-receptor in vitro (Baureithel et al., 1997). Amentoflavone had also remarkable affinity for the δ -opioid receptor subtype. Binding at serotonin (5-HT), D(3)-dopamine, and delta-opiate receptors were also decreased by amentoflavone (Butterweck et al., 2003 & Butterweck et al., 2007).

c. Anti-inflammatory activity of Hypericum perforatum

An earlier animal model in rats induced by injection of caraginan and prostaglandin E₁, *Hypericum perforatum* was found to suppress both the inflammatory effect and the leukocyte infiltration (Shipochliev et al., 1981). Oral twice daily 100 mg/kg BW of *Hypericum perforatum* inhibited carrageenan-induced paw edema in mice and the same treatment with 100 mg/kg Hp

inhibited both inducible nitric-oxide synthase (iNOS) and COX-2 expression modulated by lipopolysaccharide (LPS) and interferon in peritoneal macrophages (Raso et al., 2002). Also *Hypericum perforatum* was reported to inhibit 5-lipoxygenase (5-LO) activity in cell-free systems, a key enzyme in the formation of proinflammatory eicosanoids from arachidonic acid (Herold et al., 2003).

In the other two different human epithelial cell lines (alveolar A549/8 and colon DLD-1 cells), iNOS expression was also inhibited by dose dependently. Furthermore, the DNA binding activity of the transcription factor signal transducer and activator of transcription-1alpha (STAT-1alpha) was down-regulated by Hp extract, although nuclear factor-kappaB was not affected (Tedeschi et al., 2003). Hyperoside and quercetin inhibited nitric oxide synthase (NOS) in rat cerebral homogenate and blood at 63.06 and 57.54 μM , and those of 56.23 and 158.49 μM , respectively. These results suggested that the galactose moiety in hyperoside may be associated with the selectivity of the NOS inhibition (Luo et al., 2004).

To test Hp extracts for inhibiting the production of prostaglandin E₂ (PGE₂) in RAW264.7 Mouse Macrophage Cells, pseudohypericin at 1 and 2 μM significantly reduced LPS induced PGE₂ levels in light-activated but not dark treatments. Hyperforin significantly decreased PGE₂ levels at 40 and 80 μM . These two constituent concentration ranges were present in the Hp extracts from different *Hypericum* accessions, and this work demonstrated that pseudohypericin and hyperforin might be the primary anti-inflammatory constituents along with the flavonoids. However, quercetin significantly reduced PGE₂ at 5-40 μM . Quercitrin and isoquercitrin reduced PGE₂ levels at 5-20 μM . Amentoflavone significantly reduced PGE₂ levels at 10 μM . These four compound concentrations were much higher than all Hp extract accessions. Rutin was the only flavonoid that did not significantly reduce LPS-induced PGE₂ levels at the used doses (Hammer et al., 2007). Furthermore, in a 4 component system simulating one *Hypericum* fraction, these combined constituents (0.1 μM chlorogenic acid, 0.08 μM amentoflavone, 0.07 μM quercetin, and 0.03 μM pseudohypericin) inhibited lipopolysaccharide (LPS)-induced prostaglandin E₂ level, the production of the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α), and the anti-inflammatory cytokine interleukin-10 (IL-10) (Hammer et al., 2008).

d. Anti -microbial activity of Hypericum perforatum

To investigate the antimicrobial activities of *H. perforatum*, different extracts (MeOH; petroleum ether; CHCl₃ and EtOAc) were tested against selected microorganisms. Gram-positive bacteria, *B. subtilis* and *B. cereus* were the most susceptible to being inhibited by *Hypericum* extract. Hyperforin and hypericin were most active chemicals for antimicrobial property (Avato et al., 2004). In two earlier studies, hypericin had also been shown to have antimicrobial activity against Gram-positive bacteria and numerous viruses. This property was also attributed to the essential oils, phloroglucinols, and flavonoid constituents, and might involve a photoactivation process that disrupted certain components of the *Golgi* apparatus or endoplasmic reticulum (ER) (Lenard et al., 1993 & Weber et al., 1994). In a later study, a petrolether extract of *Hypericum perforatum* was found to be antimicrobially effective against Gram-positive bacteria with methicillin-resistant strains of *Staphylococcus aureus* (MIC values of 1.0 µg/ml). A butanol fraction of St. John's Wort had anti-*Helicobacter pylori* activity with MIC values ranging between 15.6 and 31.2µg/ml (Reichling et al., 2001). Also several bacterial species of *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis* and *Escherichia coli* were inhibited by the degradation products of hyperforin, including furohyperforin, furohyperforin A, pyranohyperforin (Vajs et al., 2003). Recently, the two *H. perforatum* subspecies in central Italy were particularly active against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and the yeast *Candida albicans* via the Kirby-Bauer agar diffusion method (Cecchini et al., 2007).

e. Antioxidant activity of Hypericum perforatum

Hypericum perforatum has been shown to possess a certain antioxidant activity in vitro (Couladis et al., 2002 & Benedí et al., 2004). Also hyperforin and hypericin have been reported to protect against oxidative damage in neuronal cells (Park et al., 2002). However, in another study, fractions containing flavonoids and/or caffeoylquinic acids were found to be the main contributors to the free radical-scavenging activity of ethanolic extract of *Hypericum perforatum*, in which lipid peroxidation-induced by ascorbate/Fe²⁺ was significantly reduced in Hp (EC₅₀=26 µg dwb/ml). Hypericins and hyperforins had no significant contributions to the antioxidant properties of Hp (Silva et al., 2005). Hp had significant DPPH radical-scavenging activity (Cakir et al., 2003) and free radical scavenging properties in cell-free and human vascular systems (Hunt et al., 2001).

To test the free radical scavenging and antioxidant activities of *Hypericum perforatum*, 1 and 50 µg/ml of Hp effectively inhibited lipid peroxidation of rat brain cortex mitochondria induced by Fe²⁺/ascorbate or NADPH system. 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH) scavenging for Hp was shown with a dose-dependent manner. However, the hydroxyl radical scavenging was occurred at high doses. In a pheochromocytoma cell line PC 12 using 8-h cell exposure to H₂O₂ (300 µM), 0.1-100 µg/ml of Hp decreased the caspase-3 activity and suppressed the H₂O₂-induced reactive oxygen species generation (Benedí et al., 2004).

f. *Hypericum perforatum* and Cancer cells

In plant-derived antitumor agents, St. John's wort was used in some trials because of its significant antitumor activity without many side effects. Some studies have shown that the antitumor properties of Hp were attributed to naphthodianthrone, hypericin, which is a powerful photosensitizer. Its use in photodynamic therapy for cancer patients had gained researcher attention (Agostinis et al., 2002). Once taken up by tumor cells, hypericin reacted in the presence of oxygen and activated multiple apoptosis pathways that resulted in malignant cell death (Thomas et al., 1992). Inhibition of mitochondrial succinoxidase was occurred by hypericin via singlet-oxygen generation and was found to be drug-dose, light-dose, and wavelength dependent (Hadjur et al., 1995). In *in vitro* study, hypericin-induced phototoxicity was dependent on oxygen in EMT6 mouse mammary carcinoma cells (Thomas et al., 1992). Hyperforin has also been investigated to possess antitumor effects in animals and *in vitro* studies (Schempp et al., 2002). Rat and human mammary cancer, squamous cell carcinoma, malignant melanoma, as well as lymphoma cells were found to be inhibited by hyperforin as dose dependent manner. This study had revealed that hyperforin might induce tumor cell apoptosis via activation of mitochondria, release of cytochrome C, and caspase activation to trigger cell death pathways. Another type cell, prostate cancer cell, was also inhibited by *Hypericum perforatum*. Hp components (containing 0.3% hypericin and 3.8% hyperforin) acted as serotonin-reuptake inhibitors and exerted cytotoxic effects in Caucasian prostate adenocarcinoma cell and athymic male nude mice. This study highlighted a significant reduction of tumor growth and number of metastasis for Hp in prostate cancer (Martarelli et al., 2004). Commercial *Hypericum perforatum* preparation had potential to inhibit CYP1A1-catalyzed benzo (a) pyrene epoxidation, which produced the terminal reaction to the ultimate carcinogenic product (diolepoxide 2). The

inhibitory potencies were shown at IC (50) values of 0.5 μ M (hypericin), 1.2 μ M (hyperforin), 1.5 μ M (quercetin), and 8 μ M (pseudohypericin) (Schwarz et al., 2003).

B. Bioavailability of *Hypericum perforatum*

Bioavailability of Hypericum perforatum extracts study

Bioavailability of *Hypericum perforatum* extracts with their constituents were well studied compared with *Echinacea purpurea*. Most of these were oral administration trials. In an earlier animal study, hyperforin in *Hypericum perforatum* extracts was measured in plasma when rats were administrated orally 300 mg alcoholic Hp (5% hyperforin), the maximum plasma level was 370 ng/ml which was reached after 3 h. If using film coated tablets containing 300 mg *Hypericum* extracts (14.8 mg hyperforin), the maximum plasma level of approximately 150ng/ml was reached 3.5 h after administration (Biber et al., 1998). To determinate hyperforin and hypericin plasma concentration in human, alcoholic *Hypericum* extracts (300 mg, containing 5% hyperforin and 0.3 % hypericin) were administered by 12 healthy people in the morning after 12 hours fasting as soft gel and hard gelatin capsules. Cmax value of hyperforin was 168.35ng/ml (soft gel) and 84.25ng/ml (hard gelatin). The Tmax (time to reach Cmax) value for hyperforin was 2.50 h (soft gel) and the total AUC was 1482.7 h x ng/ml, whereas for hypericin, plasma levels were just detectable in half of the subjects (Agrosi et al., 2000). However, another trial with oral 612 mg *Hypericum* extracts in 18 healthy male volunteers for 14 days, hypericin was measurable as Cmax (3.14 ng/ml), Tmax (8.1 h), AUC (75.96 h x ng/ml). And similar results were shown that hyperforin Cmax was 83.5 ng/ml, Tmax was 4.4 h, and AUC (1009.0 h x ng/ml). Furthermore, the other three constituents were also reported as pseudohypericin Cmax (8.50 ng/ml), Tmax (3.0 h), and AUC (93.03 h x ng/ml); Quercetin Cmax (47.7 ng/ml) and Tmax (1.17 h); isorhamnetin Cmax (9.0 ng/ml) and Tmax (6.42 h) (Schulz et al., 2005a). Schulz et al. at same year also reported the five compounds for pharmacokinetic study in 18 healthy male volunteers with different dose of 900 mg dry St John's wort extract. The data corresponded well with values first published (Schulz et al., 2005b).

Bioavailability of individual compound in Hypericum perforatum

The other compounds, rutin and quercetin, in *Hypericum perforatum* were also studied for bioavailability issue in animal models. Two groups of 0.4% rutin and 0.2% quercetin semi-

purified diets were fed in ninety male Wistar rats for 10 days. The plasma concentrations at 24 h were 35 μM rutin (0.4% rutin group) and 51 μM quercetin (0.2% quercetin group) after the first meal. The quercetin plasma concentration at 24 h in 0.4% rutin feeding group was 36 μM . Rutin was absorbed more slowly than quercetin because it must be hydrolysed by the cecal microflora, whereas quercetin was absorbed from the small intestine. Further, conjugated derivatives of quercetin, and its methylated forms isorhamnetin and tamarixetin, were also recovered in plasma from rats receiving the two kinds of experimental diets after the first meal (Manach et al., 1997). Also in another rat study, the bioavailability of quercetin and rutin (quercetin-3-rutinoside) was assessed in *in vivo* with single-meal experiments and *in vitro* method with ligated loops of rat small intestine. Rutin was more slowly absorbed than quercetin. Absorption of both quercetin and rutin from the small intestine of rat was evident. Experiments with [^{14}C] quercetin showed that only 1.5% quercetin crossed the gut wall *in vitro* and more than half of the total quercetin was bound to the small intestinal tissue (Carbonaro et al., 2005).

In human studies, from a double blind, diet-controlled and cross-over trial, 16 healthy volunteers received orally for three different quercetin and rutin doses which corresponded to 8 mg, 20 mg and 50 mg quercetin aglycone. No rutin was detected. Both quercetin and rutin treatments were found in plasma as glucuronides/sulfates of quercetin and as unconjugated quercetin aglycone. The maximum plasma concentration (C_{max}) values of quercetin in two treatments for three doses were similar (Erlund et al., 2000). The similar results were found in another human study. To determine the influence of the sugar moiety or matrix on the quercetin absorption, four groups of quercetin-4'-O-glucoside and onion supplement (both equivalent to 100 mg quercetin), as well as quercetin-3-O-rutinoside (rutin) and buckwheat tea (both equivalent to 200 mg quercetin) were administered to 12 healthy volunteers in a four-way crossover study. Only plasma quercetin glucuronides, but no free quercetin was detected. There was no significant difference in the quercetin (QU) plasma concentrations between the quercetin-4'-O-glucoside (2.1 $\mu\text{g}/\text{ml}$ QU) and onion (2.3 $\mu\text{g}/\text{ml}$ QU), as well as between rutin (0.3 $\mu\text{g}/\text{ml}$ QU) and buckwheat tea (0.6 $\mu\text{g}/\text{ml}$ QU). The different plant sugar moiety influenced quercetin bioavailability because the site of absorption might be different for quercetin-4'-O-glucoside and quercetin-3-O-rutinoside (Graefe et al., 2001).

C. Metabolism of *Hypericum perforatum*

Cytochrome P450 induction or inhibition activities of of Hypericum perforatum

Hypericum perforatum has been reported to interact with several cytochrome P450 (CYP) families which are hepatic drug-metabolism enzymes. Using a probe drug cocktail in 12 healthy subjects with 3-period open-label study, tolbutamide (CYP2C9), caffeine (CYP1A2), dextromethorphan (CYP2D6), oral midazolam (intestinal wall and hepatic CYP3A) were administered with 2 weeks of intake (Hp 900 mg daily) to determine CYP activities. St John's wort administration increased oral clearance of midazolam and declined oral bioavailability, as well as AUC was decreased at >50% when midazolam was administered orally. There was no change in CYP1A2, CYP2C9, or CYP2D6 activities as a result of St John's wort administration. St John's wort administration only resulted in a significant and selective induction of CYP3A activity in the intestinal wall (Wang et al., 2001). On the other hand, *Hypericum perforatum* compound metabolism were also changed when added cytochrome P-450 inhibitor or inducer. In a placebo-controlled, double blind study, 33 healthy volunteers were randomized into three treatment groups that received Hp extract with different groups (placebo, cimetidine, and carbamazepine) for 7 days. Hypericin AUC was increased from 119 $\mu\text{g h/l}$ to 149 $\mu\text{g h/l}$ with cimetidine because cytochrome P-450 was inhibited, whereas pseudohypericin AUC was decreased from 51.0 $\mu\text{g h/l}$ to 36.4 $\mu\text{g h/l}$ with carbamazepine because cytochrome P-450 was activated compared to the baseline pharmacokinetics in each group (Johns et al., 2004). In another human study, St John's wort also showed to activate cytochrome CYP3A and MDR1. Twelve days pretreatment with St John's wort on the disposition of probe drugs in 21 young healthy subjects, oral administration of midazolam was used to assess CYP3A activity in both the intestinal epithelium and the liver; oral fexofenadine was assumed to be a measure of MDR1 function. St John's wort significantly increased both of the drugs with associated their clearance (Dresser et al., 2003).

In a cell culture model, the main constituents of *Hypericum perforatum* extract, hyperforin and hypericin, were also identified if affecting the drug-metabolizing enzymes or not. For the induction or inhibition effects of the constituents on CYP3A4, CYP1A2, CYP2C9, and CYP2D6 in the human hepatocyte model, hyperforin resulted in significant increases in mRNA and activity of CYP3A4 and CYP2C9; whereas, hypericin had no effect on any of the enzymes

used. These results demonstrated that some compounds might play an important role in inductive effect of Hp extracts on drug-metabolizing enzymes (Komoroski et al., 2004).

In a summary, from the studies selected throughout *Hypericum perforatum* review, the compositions of *Hypericum perforatum* were different depending on the preparation methods and herbal locations. The main compounds included hyperoside, quercetin, rutin, biflavonoids (amentoflavone), chlorogenic acid, and pseudohypericin. *Hypericum perforatum* were recommended for treating depression. Different species of Hp possessed an effective anti-inflammatory activity, anti-microbial activity and antioxidant capacity. Also *Hypericum perforatum* was believed to inhibit the cancer cells in many different cell lines, which was contributed by the different active compounds. Hp was detected in both culture models and human studies as different individual compound as well as their metabolites. *Hypericum perforatum* also interacted with cytochrome P450 as results in both induction and inhibition activities. However, the mechanism of metabolism, anti-inflammatory activity and anti-microbial activity remain unclear. Further study related to these areas will be needed.

IV. OVERALL INTRODUCTION OF CAFFEIC ACID

A. Analysis of caffeic acid in fruits, vegetables and *Echinacea purpurea*

Caffeic acid (3,4-dihydroxycinnamic acid), one of the most common phenolic acids, frequently occurs in fruits, grains and dietary supplements for human consumption as simple esters with quinic acid or saccharides, and are also found in traditional herbs. Phenolic acids include derivatives of hydroxybenzoic and hydroxycinnamic acids with hydroxycinnamic acids being the more common. Hydroxycinnamic acids mainly consist of *p*-coumaric, caffeic, ferulic, and sinapic acids when caffeic acid, both free and esterified (Figure 2.3), is generally the most abundant phenolic acid and contains from 75% to 100% of the total hydroxycinnamic acid content of most fruit. Chlorogenic acid is combined from caffeic and quinic acids in coffee and many types of fruit, such as blueberries, kiwis, plums, cherries, apples (Clifford, 1999).

Some studies have shown that 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; Mattila et al., 2002). A recent published study showed the chlorogenic acid was widely distributed and present in 175 apple varieties and determined the

mean chlorogenic acid content was 70.7 μ g/g and the maximum value for chlorogenic acid was 396.9 μ g/g in apple juice collected from approximately 12 countries and several USA geographical areas (Eisele et al., 2005). Another investigated study showed the distribution and contents of phenolic acids in a wide range of vegetables consumed in Finland. The data indicated that caffeic acid was the most dominant phenolic acid aglycone in the samples studied. Highest contents of soluble phenolic acids were found in raw and cooked potato peels: 23-45 mg/100 g fresh weight calculated as aglycones (Mattila et al., 2007). Total caffeic acid contents in potato samples (*Solanum tuberosum*) as aglycones for potato/timo cooked and potato/siikli cooked were 14 \pm 1.2 and 12 \pm 0.90 mg/100 g fresh weight, separately (Mattila et al., 2007).

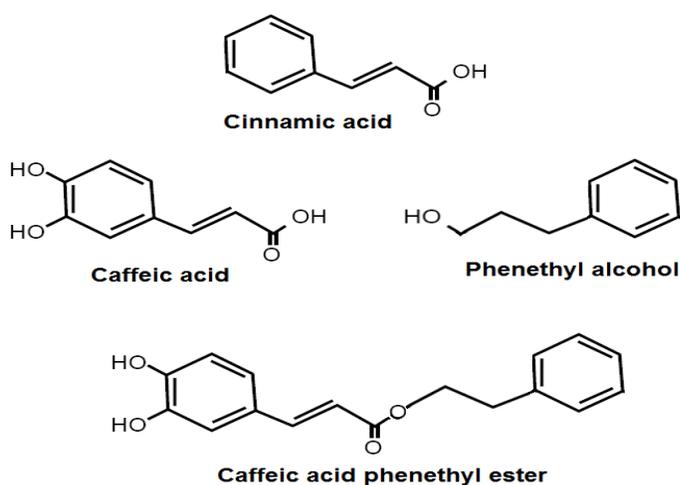


Figure 2.3. The structure of Cinnamic acid, Caffeic acid and Caffeic acid phenethyl ester (CAPE)

One study was done in China to analyze the natural change of active components of *E. purpurea* by measuring content of cichoric acid. The results were shown that the maximum content of cichoric acid above ground occurred in the blooming stage of mid July (Wang et al., 2002). Cichoric acid (19.21 mg/g dry biomass), caftaric acid (3.56 mg/g dry biomass), and chlorogenic acid (0.93 mg/g dry biomass) were found in inoculation of leaf explants of *Echinacea purpurea* (Moench) with *Agrobacterium rhizogenes*. These results demonstrated that the biosynthetic pathway might be feasible to produce biologically active caffeic acid derivatives in heterotrophic culture (Liu et al., 2006). Different *Echinacea* extracts which were obtained from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the USDA were analyzed by HPLC to identify the concentrations of known constituents. The different accessions

and harvest years of species included *E. angustifolia*, *E. sanguinea*, *E. purpurea*, and *E. tennesseensis* which contained greater quantities of Bauer alkaloids than of ketones or caffeic acid derivatives (LaLone et al., 2007). From the earlier NCRPIS Annual Report (2004), the concentrations of caffeic acid extracted from *Echinacea* roots including *E. angustifolia*, *E. purpurea*, *E. pallida* were from 0.10 to 0.21 mg/g dry root. Many factors affected the level of these phenolic compounds including seasonal variations, drying methods, extraction methods, and growing location of the plant. Recently, in some other reports, by calculating individual phenolic compound/dried plant material, the percentage of caffeic acid in *E. purpurea* extracts was 0.6-1.1%; and cichoric acid (3.5-5.7 %) and caftaric acid (3.1-4.5 %) were also the main phenolic compound of total polyphenols of 7.9-10.9% (Iranshahi et al., 2008). 60% ethanol at 60°C was found to be suitable for the extraction of the active ingredient yields of phenolics (53.4 mg/g dry weight), caftaric acid (3.6 mg/g DW), and cichoric acid (28.8 mg/g DW) from dried adventitious roots of *Echinacea purpurea* obtained in bioreactor cultures in South Korea (Wu et al., 2008).

B. Daily intake of caffeic acid

Regarding the daily intake of caffeic acid, some studies have reported on the general population. Estimation of dietary intake is particularly difficult because of partial availability of food composition data and dietary assessment methods. Regular coffee consumers generally ingest 0.5–1 g chlorogenic acid/d and are equal to about 250–500 mg caffeic acid/d (Clifford et al., 1999). A German study estimated daily consumption of hydroxycinnamic acids and hydroxybenzoic acids at 211 and 11 mg/d, respectively. For certain polyphenols consumed daily including flavonols and flavones, catechins, and isoflavones, have been found on the food analysis (Radtke et al., 1998). The results showed that the daily consumption of caffeic acid intake was 206 mg/d, and the principal sources were coffee (92% of caffeic acid) and fruit and fruit juices combined (Radtke et al., 1998). The useful biomarkers of urinary flavonoids for intake may help to estimate dietary intake and showed the coffee consumption was positively correlated to caffeic and chlorogenic acids (Mennen et al., 2006).

C. Bioavailability of caffeic acid

Many studies have been done about the bioavailability of chlorogenic acid and caffeic acid in gut. Based on a human study, only one third of chlorogenic acid was absorbed in the small intestine of humans when almost all of the caffeic acid was absorbed in the ileostomy subjects ingested 2.8 mmol chlorogenic acid and 2.8 mmol caffeic acid for 24 h (Olthof et al., 2001). In contrast with intake of pure caffeic acid, better absorption in the small intestine, was associated with a higher plasma concentration and urinary excretion of intact caffeic acid and its tissular metabolites in humans (Olthof et al., 2001).

In another human study, five healthy males consumed 100, 200, and 300 mL of red wine corresponding 0.9, 1.8, and 2.7 mg of caffeic acid, respectively. Plasma concentration were measured at different times (0–300 min) for evaluating the antioxidant effect of caffeic acid. Plasma samples were prepared by HCl-hydrolysis method through Sep-Pak C18 cartridge and analyzed by HPLC. The method of plasma total radical-trapping antioxidant parameter (TRAP) was determined for antioxidant potential of caffeic acid. Plasma concentrations of caffeic acid and antioxidant property were dose-dependent and the C_{max} was reached at about 60 min after red wine intake. At this time point, plasma caffeic acid concentrations were 1.19, 3.23, and 4.90 ng/mL for each group. And the antioxidant parameters were 6.0, 19.6, and 25.4 % variation of TRAP. Caffeic acid was bioavailable and was correlated with the antioxidant potential of red wine intake (Simonetti et al., 2001).

Coffee was one of the most popular sources to investigate the bioavailability and metabolism of caffeic acid derivative studies. Five nonsmoking healthy male volunteers were administered two cups of coffee containing 4 g of instant coffee powder. The results showed a highly significant increase in the urine cumulative excretion of isoferulic, ferulic, and dihydroferulic acid ranging from 1.9 to 15.1 mg. 3-Hydroxyhippuric acid was increased as 102.9 mg in postsupplementation (Rechner et al., 2001).

Most of caffeic acid was present in plasma as the glucuronate/sulfate forms. Plasma caffeic acid was derived from hydrolysis of chlorogenic acid in the gastrointestinal tract when drinking the no free caffeic acid coffee (Nardini et al., 2002). Ten healthy male nonsmoker

moderate-coffee drinkers were asked to administer a standard 200 mL brewed coffee (corresponding 166mg caffeic acid) which only contained 478.9 $\mu\text{g/mL}$ chlorogenic acid in original nonhydrolyzed coffee when caffeic acid was undetectable. If hydrolyzed, no chlorogenic acid and only 830.0 $\mu\text{g/mL}$ caffeic acid and small amount of *p*-coumaric acid and ferulic acid were found in hydrolysis solution. Plasma samples were collected 1 and 2 h after coffee administration for analyzing free and total phenolic acid content. Caffeic acid was the only phenolic acid found in plasma samples after coffee administration, while chlorogenic acid was undetectable. One hour after coffee consumption, free plasma caffeic acid level was 20.9 ng/mL, whereas 91.1 and 91.3 ng/mL were found with β -glucuronidase treatment and alkaline hydrolysis (Nardini et al., 2002).

However, in another human study, chlorogenic acid was detectable in human plasma and urine after acute coffee consumption. To determine the plasma distribution of the main chlorogenic acid isomers and metabolites, six healthy adults consumed a standard amount (190 mL) of brewed coffee which contained 2928 $\mu\text{mol}/190$ mL caffeoylquinic acid (CQA), a main composition of chlorogenic acid isomers. Pharmacokinetic parameters of CQA and caffeic acid were identified in plasma for 4 h after coffee consumption. The CQA C_{max} was 4.89 $\mu\text{mol/L}$ and T_{max} was 2.25 h; caffeic acid C_{max} was 1.56 $\mu\text{mol/L}$ and T_{max} was 1.42 h. The urinary concentrations of caffeic acid were identified in each subject during 2 h after coffee consumption and the ranging was 0.37 to 1.57 mmol/mmol creatinine (Monteiro et al., 2007).

In animal studies, the bioavailability of chlorogenic acid and caffeic acid were studied with different dosages to obtain plasma pharmacokinetic profiles of their metabolites. One earlier rat study, using 700 $\mu\text{mol/kg}$ body weight of chlorogenic or caffeic acid and collecting blood from the tail for 6 h after administration, the results were reported that no chlorogenic acid was absorbed from the alimentary tract, only traces of caffeic and ferulic acids conjugates were detected in rat plasma for 6 h after chlorogenic acid administration. On the other hand, after caffeic acid administration, not only free caffeic acid and ferulic acid were detected as 1.2 and 1.6 $\mu\text{mol/L}$, respectively; caffeic acid glucuronides was main plasma metabolites 2 h after administration with a concentration of 26.1 $\mu\text{mol/L}$; caffeic acid sulfate/glucuronide conjugates was 12 $\mu\text{mol/L}$ (Azuma et al., 2000). However, in another animal model, chlorogenic acid was absorbed in the rat stomach with its intact form. With infusing chlorogenic acid in the ligated

stomach of food-deprived rat model to test gastric absorption of chlorogenic acid, intact chlorogenic acid was found in the gastric vein and aorta after 30 min of infusion (Lafay et al., 2006a). When feeding rat as a diet supplemented with chlorogenic acid (0.25%, wt: wt), chlorogenic acid and its metabolites were estimated in the stomach, small intestine and cecal contents as well as in bladder urine and plasma by HPLC. The results were indicated that small amount of hydrolysis of chlorogenic acid (<1%) was occurred in the stomach and small intestine contents, whereas 15-32% of ingested chlorogenic acid was hydrolyzed into caffeic acid in the cecum (Lafay et al., 2006a).

One another animal study in rats (250 μ mol/d chlorogenic acid for 8 d) have reported that the urinary recovery of chlorogenic acid was low (0.8%, mol/mol); and the total urinary excretion of caffeic acid released from chlorogenic acid was less 0.5% (mol/mol) of the dose ingested. Most parts were microbial metabolites in both urine and plasma including m-coumaric acid and derivatives of phenylpropionic, benzoic and hippuric acids (57.4% mol/mol) in rats. That was concluded high abundance of microbial metabolites resulted from gut microflora metabolism of chlorogenic acid (Gonthier et al., 2003). The same rat study with 250 μ mol/d caffeic acid for 8 d, total urinary excretion of caffeic, ferulic, and isoferulic acids was 28.1 % of intake (mol/mol); urinary 3-hydroxyphenylpropionic acids (3-HPP) was 4.0 %. Plasma metabolite concentrations in rats fed caffeic acids for 8 d were caffeic acid as 41.3 μ mol/L, ferulic acid as 7.3 μ mol/L, and 3-hydroxyphenylpropionic acid as 1.4 μ mol/L (Gonthier et al., 2003).

One study was compared to rosmarinic acid in animal model. Both of 100 μ mol/kg BW of caffeic acid (CA) and rosmarinic acid (RA) were fed to male Wistar rats by gastric intubation. The serum concentration of intact CA and RA in the portal vein peaked at 10 min after administration were quantified by a coulometric detection method using HPLC-ECD, with a C(max) of 11.24 μ mol/L for CA and 1.36 μ mol/L for RA. The area under the curve (AUC) for intact CA and RA was calculated from the serum concentration-time profile as 585.0 and 60.4 μ mol min /L. The absorption efficiency of CA was about 9.7-fold higher than that of RA. And the concentrations of CA and RA glucuronide were 30 and 0.8 μ mol /L, respectively (Konishi et al., 2005).

To determine whether chlorogenic acid was directly absorbed or hydrolysed in the small intestine. Individual chlorogenic and caffeic acids were perfused into a segment of ileum plus jejunum during 45 min using an *in situ* intestinal perfusion rat model. The net perfused absorption accounted for 8 % chlorogenic acids and 19.5 % caffeic acid. Part of the chlorogenic acid (1.2 % of the perfused flux) was recovered in the gut effluent as caffeic acid because of trace esterase activity in the gut mucosa. No chlorogenic acid was detected in either plasma or bile. The results showed that chlorogenic acid was absorbed and hydrolysed in the small intestine (Lafay et al., 2006b).

D. Metabolism of caffeic acid

Metabolism of caffeic acid by Phase I & II enzymes

In an earlier study with isolated perfused rat liver to investigate the mode of biotransformation of caffeic acid, the main results were shown that the first-pass effect was small due to 93.3% of unchanged caffeic acid after one liver passage. Phase I products of caffeic acid oxidation (cycloignan) and cyclization product (esculetin) as well as Phase II methylation products (ferulic and isoferulic acid) were found in the perfusion medium. Also the other Phase II conjugation products (mainly glucuronides/sulfates of caffeic acid) were determined in bile (Gumbinger et al., 1993). To compare the *Phase I* products of caffeic (CA), dihydrocaffeic (DHCA), and chlorogenic (CGA) acids, using rat hepatocyte microsomes and dihydrocinnamic acids oxidation assay, the results showed that dihydroesculetin, *p*-quinone, and hydroxylated dihydrocaffeic acid were main products oxidized by peroxidase/H₂O₂ or tyrosinase/O₂ in rat liver where microsome catalyzed CA-, CGA-, and DHCA-glutathione conjugate formation (Moridani et al., 2001). Caffeicins-like structures, dimers, and trimeric derivatives of caffeic acid were also formed by the tyrosinase-catalyzed oxidation method using high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) (Pati et al., 2006). Another *Phase I* products of caffeic acid oxidation study, the biotransformation of caffeic acid was catalyzed by peroxidase in H₂O₂/*Momordica charantia* assay. The isolation of nine caffeic acid trimers, dimers, and monomer was found *in vitro* and confirmed by 2D NMR measurement (Wan et al., 2008).

To investigate the metabolism of caffeic acid in three enzymes including cytochrome P450 enzymes, catechol-O-methyltransferase (COMT), and beta-oxidation enzymes, caffeic

(CA), chlorogenic (CGA), and dihydrocaffeic (DHCA) acids were incubated with either hepatocytes or rat liver microsomes. The main results were shown that ferulic (FA) or dihydroferulic (DHFA) acids were formed as the result of CA- or DHCA-O-methylation by COMT and also O-demethylated by CYP1A1/2. The CA- and DHCA-o-quinones were formed by NADPH/P450 (Moridani et al., 2002).

Metabolism of caffeic acid in cell cultures (HepG2 and Caco-2 cells)

To investigate the bioavailability and metabolism of caffeic acid in cell culture, the most studies have used the HepG2 cell or Caco-2 as biotransformation models. One study was done to test the hepatic uptake and metabolism in human hepatoma HepG2 cells which were incubated for 2 and 18 h with caffeic, ferulic, and chlorogenic acids. The results were shown that the caffeic acid was moderate uptake and methylation/glucuronidation/sulfation were the main pathway for caffeic acid metabolism; ferulic acid underwent glucuronides as the only metabolites and more slowly metabolized by HepG2 cells; and chlorogenic acid had the lowest absorption due to the esterification of the caffeic acid moiety with quinic acid (Mateos et al., 2006).

Some cell culture studies were shown that chlorogenic acid (CGA) and caffeic acid (CA) were absorbed by paracellular diffusion in human intestinal Caco-2 cells as well as CA had low affinity for monocarboxylic acid transporter (MCT). This resulted in the greater absorption efficiency of caffeic acid compared to chlorogenic acid (Konishi et al., 2004a). Caffeic acid was absorbed as 0.20 % and 1.57 % of initial CA in the basolateral phase with or without apical to basolateral proton gradient. More than 98% of apically loaded caffeic acid was retained on the apical side, suggesting CA was restricted by the tight junctions (Konishi et al., 2004a). Furthermore, the major metabolites of caffeic acid formed by gut microflora including m-coumaric acid, m-hydroxyphenylpropionic acid (mHPP), and 3,4-dihydroxyphenylpropionic acid (DHPP) were transported by MCT via proton-coupled direction, in which the transport of m-coumaric acid, mHPP, and DHPP was inhibited by an MCT substrate, whereas DHPP was mainly permeated across Caco-2 cells via the paracellular pathway (Konishi et al., 2004b).

E. Antioxidative efficacy of caffeic acid

Reactive oxygen species (ROS), various forms of activated oxygen, are formed continuously in cells as a consequence of oxidative biochemical reactions. ROS include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH\cdot$) and non-free radical

species such as H_2O_2 and singlet oxygen (O_2). These molecules exacerbate factors in cellular injury and aging process (Halliwell and Gutteridge, 1989). ROS are harmful because of producing in excess under certain abnormal conditions including inflammation, ischemia and in the presence of catalytic iron ions. ROS may cause cellular damage via peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown (Serarslan et al., 2007; Lee et al., 2008). Caffeic acid was found to significantly reduce tert-butyl hydroperoxide (t-BHP)-induced hepatotoxicity in a cultured HepG2 cell line as determined by cell cytotoxicity, and lipid peroxidation and reactive oxygen species (ROS) levels in a dose-dependent manner (Lee et al., 2008). The free radical scavenging activity of these natural compounds was evaluated through their ability to quench the synthetic DPPH radical. This assay provided information on the reactivity of tested compounds with a stable free radical, independently of any enzymatic activity. Caffeic acid was shown to possess the DPPH radical scavenging action with a higher quenching efficiency (Kroon and Williamson, 1999; Gülçin et al., 2006).

In common with several other dietary polyphenols, caffeic acid and its esters chlorogenic and caftaric acids have free hydroxyl groups and acted as antioxidants in vitro (Foley et al., 1999; Baderschneider et al., 2001). Caffeic acid is a natural ingredient not only in coffee beans but also in apples, bell peppers, pears, and some herbs such as different species of *Echinacea*. Caffeic acid has a variety of biological activities including antioxidant, anti-ischemia reperfusion, anti-thrombosis, anti-hypertension, anti-fibrosis, antiviral and antitumor properties (Jiang et al., 2005). One study showed the antioxidant properties of the caffeic acid were evaluated by using different in vitro antioxidant assays. 10 and 30 $\mu\text{g/mL}$ of caffeic acid showed 68.2 and 75.8% inhibition on lipid peroxidation of linoleic acid emulsion. Caffeic acid was an effective 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging, superoxide anion radical scavenging, total reducing power and metal chelating on ferrous ions activities (Gülçin et al., 2006). To investigate the chain-breaking antioxidation mechanism of caffeic acid, the antioxidant reaction conditions were designed by using freshly purified ethyl linoleate (50 mM) as the oxidation substrate. The result was shown that a quinone derivative of methyl caffeate was produced as an antioxidation product during the antioxidation reaction, which was identified by

(13)C NMR. Methyl caffeate showed a very strong antioxidant activity for the initial stage (Masuda et al., 2008).

In animal studies, the antioxidant properties of caffeic acid have been also established. Oral administration of caffeic acid (12 mg/kg/BW) in rats for 45 days significantly reduced the severe oxidative stress in alcohol toxicity as evidenced by the decrease in the levels of lipid peroxidation with a simultaneous increase in the level of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), and glutathione S-transferase (GST) in liver and kidney (Karthikesan and Pari, 2007). CAPE was shown to significantly attenuate the intravenous LPS-induced TNF- α and IL-1 β concentration and inhibit LPS-induced nuclear transcription factor- κ B (NF- κ B) activation in mice. It also down-regulated matrix metalloproteinase-9 (MMP-9) activity triggered by LPS in mouse lung (Jung et al., 2008). To examine the effects of CAPE on antioxidant parameters and colitis induced by trinitrobenzene sulfonic acid (TNBS) in bilateral ovariectomized Wistar Albino rats. Treatment with CAPE 10 and 30 mg/kg significantly reduced in colon injury in rats of the TNBS-colitis. The levels of malondialdehyde (MDA), catalase and reduced glutathione (GSH) were increased from the level 10 mg/kg in colitis rats (Ek et al., 2008).

F. Antiinflammatory effects of caffeic acid

Caffeic acid phenethyl ester (CAPE) is known to have antimitogenic, anticarcinogenic, antiinflammatory, and immunomodulatory properties (Michaluart et al., 1999; Orban et al., 2000), and a specific inhibitor of the transcription factor nuclear factor- κ B (NF- κ B) (Natarajan et al., 1996; Fitzpatrick et al., 2001; Abdel-Latif et al., 2005).

Nuclear factor kappa-B (NF- κ B) was a heterodimeric transcription factor with a pivotal role in orchestrating immune and inflammatory processes. An earlier cell culture model, solutions of CAPE and analogues were made at 25 μ g/mL to treat the human histiocytic cell line U937 cells. The result was shown that the activation of NF- κ B by tumor necrosis factor (TNF) was completely blocked by CAPE in a dose and time dependent manner. CAPE also inhibited NF- κ B activation induced by other inflammatory agents including phorbol ester, ceramide, hydrogen peroxide, and okadaic acid (Natarajan et al., 1996). CAPE induced apoptotic cell death in a dose-dependent fashion and to a similar extent in glucocorticoid-sensitive and -resistant cell line of lymphoid origin. CAPE decreased expression of cytosolic NF-kappaB and increased

nuclear translocation of NF- κ B p65 subunit (Orban et al., 2000). In a rat model of carrageenin-induced subcutaneous inflammation, local administration of CAPE resulted in increased leukocyte apoptosis and marked reduction in exudate leukocyte, neutrophil and monocyte concentrations at the inflammatory site (Orban et al., 2000). CAPE (6 μ g/ml) had caused significant cytotoxicity and increased apoptosis in lung cancer cells (Chen et al., 2004). And no significant cytotoxicity was found in normal lung fibroblast cells compared with lung cancer cells (Chen et al., 2005) by treating exponentially growing cells for 1 h prior irradiation. CAPE treatment also significantly decreased the nuclear binding of NF- κ B in lung cancer cells as compared with normal lung cells after 4 hour 9 Gy irradiation. For *in vivo* study, the mice were injected intraperitoneally with CAPE (10 mg/kg, solubilized in saline) 30 min before irradiation and once a day for 10 days after irradiation in 24 mice with 20 Gy irradiation. CAPE treatment decreased the expression of inflammatory cytokines including IL-1 alpha and beta, IL-6, TNF-alpha and TGF- beta after irradiation (Chen et al., 2005). Severe sepsis induced with a cecal ligation and puncture (CLP) in forty Sprague Dawley rats was inhibited by CAPE (10 μ mol/kg) injected intraperitoneally. CAPE was reported to reduce mortality in sepsis and to improve histopathologic variables best when it was administered after the onset of sepsis. Apoptosis, inducible nitric oxide synthase, and heat shock protein 70 evaluation were significantly decreased (Fidan et al., 2007). In another same sepsis model study, the CAPE treatment resulted in a significant decrease in serum leukocytes, glucose, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN) and plasma malondialdehyde (MDA) levels in the sepsis treated with CAPE group (Tekin et al., 2008).

G. Caffeic acid and cancer cell biology

Some studies were done with cancer cell lines to investigate the biological activity of tumor growth inhibition. The synthesized caffeic acid phenethyl ester-like compounds were tested and reported the significant cytotoxicity on oral submucosus fibroblast (OSF), neck metastasis of Gingiva carcinoma (GNM), and tongue squamous cell carcinoma (TSCCa) cells. The results suggested that CAPE-like compounds may be potential chemotherapy agents against oral cancer (Lee et al., 2000). Caffeic acid phenethyl ester (CAPE) inhibited NF κ B activity, activated Fas and induced apoptosis in human breast cancer MCF-7 cells, then induced p53-regulated Bax protein and activated caspases (Watabe et al., 2004). CAPE was a potent

antimetastatic agent which markedly inhibited the metastatic and invasive capacity of malignant cells. In human HT1080 fibrosarcoma cells, caffeic acid phenethyl ester (CAPE) had the effect on tumor invasion and metastasis by determining the regulation of matrix metalloproteinases (MMPs) as decreasing gene expression of MMPs (MMP-2, MMP-9, MT1-MMP), tissue inhibitor of metalloproteinase-2 (TIMP-2) and in vitro invasiveness of human fibrosarcoma cells (Hwang et al., 2005). Caffeic acid phenethyl ester inhibited cell growth, and induced G1 phase arrest and apoptosis in a dose-dependent manner in both HCT116 and SW480 cells (Xiang et al., 2006). To investigate the mitogenic, cytoprotective, and antiapoptotic activities on PC12-AC cells, a clonal derivative of the PC12 rat adrenal pheochromocytoma cell line (ATCC), the concentration of 54 naturally occurring phenolics including caffeic acid around 0.6 to 7.3 $\mu\text{mol/L}$ were used in this assay because of the physiologically relevant levels of plasma phenolic concentration detected in individuals on high phenolic diets (Lotito and Frei, 2006). Caffeic acid was found to possess the antiapoptotic capacity at 5.6 $\mu\text{mol/L}$. These findings demonstrated substantive mitogenic, cytoprotective, and antiapoptotic biological activities of plant phenolics on neoplastic cells at physiologically relevant dietary concentrations that should be considered in chemopreventive and chemotherapeutic strategies (Harris et al., 2007).

Recently, in another cell line, human pancreatic cancer cells were chosen to investigate the activity of inducing apoptosis for caffeic acid by using a trypan blue dye exclusion test, observation of morphology, sub-G1 DNA content, annexin-V/PI staining, caspase-3 and caspase-7 assay, and DNA agarose gel electrophoresis. CAPE (10 $\mu\text{g/mL}$) resulted in marked inhibition of human pancreatic cancer cells as different evidence. And the data suggested that CAPE was a potent apoptosis-inducing agent together with mitochondrial dysfunction and activation of caspase (Chen et al., 2008). The modified resistant hepatocyte model was used to investigate the anticarcinogenic properties of CAPE in Fischer-344 rats with diethylnitrosamine (DEN) administration. CAPE treatment decreased gamma-glutamyl transpeptidase-positive (GGT+) staining of hepatocyte foci by 59% on day 25. The result suggested that CAPE modified the enzymatic activity of CYP isoforms which were involved in the activation of DEN, such as CYP1A1/2 and CYP2B1/2 (Beltrán-Ramírez et al., 2008).

H. Cardioprotective effects of caffeic acid

Caffeic acid contained wine consumption may have a preventive role in cardiovascular disease by moderate chronic wine consumption (Bertelli et al., 2007). In rat models, administration of CAPE reduced malondialdehyde (MDA) production and prevented depletion of GSH content in oxygen free radical-mediated injury of the ischemic-reperfused (I/R) myocardium in rats (Ozer et al., 2005). Chronic moderate red or white wine intake substantially improved the LDL/HDL cholesterol ratio and enhanced LDL clearance rate from the blood on fasted and postprandial lipemia (Daher et al., 2006). Rabbits were injected with a solution of CAPE 60 min before (3 mg/kg ip) or 30 min after (15 mg/kg ip) to acute myocardial ischemia/reperfusion (I/R) injury. Infarct dimensions in the area at risk were reduced by 2-fold with CAPE treatment. The levels of cytosolic enzymes lactate dehydrogenase, creatine kinase (CK), and cardiac-specific troponin were significantly reduced in CAPE treatment. CAPE-treated tissues displayed significantly less cell death due to inhibition of p38 mitogen-activated protein kinase activation and reduced DNA fragmentation associated with caspase activation (Tan et al., 2005). Also CAPE was shown to attenuate NO production, reduced apoptosis, and diminished serum CK activities as same ischemia/reperfusion (I/R) model in rat heart (Ince et al., 2006; Ozyurt et al., 2006).

I. Mechanisms of DSS-induced animal colitis

Inflammatory bowel diseases (IBDs; e.g., Crohn's disease, ulcerative colitis) models induced by enteric bacteria were well established (Hutto et al., 1998; Jergens et al., 2007). Colitis in mice was also induced by dextran sodium sulfate (DSS) in drinking water (Dieleman et al., 1998) causing weight loss, diarrhea with blood and/or mucus, shortening of the colon, erosion of the mucosal epithelium, and acute neutrophilic infiltration (Stevceva et al., 1999).

Some studies were conducted to elucidate the mechanisms of DSS-induced animal colitis models. Aberrant or exaggerated immune responses to bacterial antigens derived from the intestinal lumen have been thought to serve as key initiating event leading to the development of colitis (Mayer, 2000; Shanahan, 2002). Shortening of the large intestine was thought to be induced by the thickening of colon caused by edema and muscular hypertrophy, in which this phenomenon was usually observed in ulcerative colitis. Then diarrhea was caused due to shortening of the colon (Ciancio et al., 1992). DSS-induced colitis was characterized by multi-

focal areas of mucosal erosion, colonic epithelial cell injury, and significant mucosal infiltration of neutrophils (Cooper et al., 1993). On the other hand, some studies showed the acquired immune response plays a central role in the chronic colitis induced by DSS. Epithelial restitution has been shown to be impaired by chronic immune activation (Murthy et al., 1993), while damage to the epithelium was a key feature of acute DSS-induced colitis, chronic lesions may be the result of exaggerated and prolonged immune activation within the mucosa (Dieleman et al., 1998).

Recent studies showed the mechanism of DSS-induced colitis was related to the level of NF- κ B expression. These results were determined by the temporal relationship between the activation of NF- κ B and messenger RNA expression of upstream activators and downstream mediators of NF- κ B action in rats administered DSS (Marrero et al., 2000). These studies involved to elucidate the expression of NK-1R, SP, TNF- α , IL-1 β , VCAM-1, ICAM-1, E-selectin, CINC-1, MIP-1 α , and iNOS. Furthermore, the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules were increased in some investigation (Breider et al., 1997; Sasaki et al., 2000). These results demonstrated that enhanced colonic mucosal endothelial cell ICAM- expression was an early event in the inflammatory cascade of DSS-induced colitis. IL-1beta and GRO/CINC-1 mRNA expression were increased while TNF-alpha mRNA expression was significantly decreased. Similar results were also indicated in human studies about NF- κ B expression related to inflammatory bowel disease (IBD) (Neurath et al., 1998; Schottelius et al., 2006). Crohn's disease (CD) and ulcerative colitis (UC) displayed high levels of NF- κ B DNA-binding activity accompanied by an increased production of IL-1, IL-6, and TNF α in patient macrophages. NF- κ B stimulated the proliferation of tumor cells and enhanced their survival through the regulation of anti-apoptotic genes. Another study showed caffeic acid phenethyl ester (CAPE) might possess a potent multiple immunomodulatory and antiinflammatory role due to the inhibiting of NF- κ B activated by TNF, phorbol ester, ceramide, hydrogen peroxide, and okadaic acid in a dose- and time-dependent manner (Natarajan et al., 1996). The preventive effect of CAPE in colitis was due to decreasing the NF- κ B level (Fitzpatrick et al., 2001). The other mechanism may be related to cytokine expression in DSS colitis, a range dose of dietary rutin which prevented DSS-induced colitis and possible colorectal carcinogenesis was resulted from attenuation of pro-inflammatory production (TNF- α , IL-1 β) (Kwon et al., 2005).

In a summary, from the studies selected throughout caffeic acid review, caffeic acid, one of the most common phenolic acids, frequently occurs in fruits, grains, herbs and dietary supplements. Regular coffee consumers generally ingest about 250–500 mg caffeic acid/d. Plasma C_{max} of caffeic acid was reached at about 60 min after caffeic acid diet intake. Metabolites included isoferulic, ferulic, dihydroferulic acid, and 3-hydroxyhippuric acid in urine or plasma in postsupplementation. The total urinary excretion of caffeic, ferulic, and isoferulic acids was 28.1 % of intake. Most of caffeic acid was present in plasma as the glucuronate/sulfate forms. Chlorogenic acid was hydrolyzed to caffeic acid and chlorogenic acid was undetectable in the gastrointestinal tract when taking the chlorogenic acid supplementation. Caffeic acid was metabolized by Phase I & II enzymes. Some cell culture showed that chlorogenic acid (CGA) and caffeic acid (CA) were absorbed by paracellular diffusion in human intestinal Caco-2 cells and CA had low affinity for monocarboxylic acid transporter (MCT). Caffeic acid possessed antioxidative efficacy, anti-inflammatory, and cardioprotective effects. Some studies were shown that caffeic acid affected cancer cell lines with the biological activity of tumor growth inhibition. However, the mechanism of anti-inflammatory activity and bioavailability related efficacy remain unclear. The further study related to these areas will be needed.

V. MICROBIAL METABOLISM OF PHENOLICS AND HEALTH SIGNIFICANCE

A. *Bioavailability of phenolics and gut microbial metabolism*

Bioavailability is defined as the proportion of a compound that appears in plasma over time and the proportion excreted in the urine and feces compared to the amount ingested, when the compound is administered orally. From a toxicological perspective then, bioavailability is a measure of the potential for entry of a chemical into sites of action and implies movement of a chemical into the systemic circulation because this is a good indication of the biologically effective dose. Casarett and Doull (2001) define bioavailability as the fraction of the oral dose that is absorbed. That means that bioavailability equals to mass of chemical absorbed compared with mass of chemical administered.

Over the past ten years, Hendrich and co-workers indicated that a variety of factors affected bioavailability of isoflavones, the phenolics in soybean, in which the gut microbiota was an important controllable variable for bioavailability studied as a broaden range. The

bioavailability of isoflavone is complex and widened variability in response to dietary isoflavones. Many human studies and animal models have been established by Hendrich and co-workers to study bioavailability of isoflavones. Some focused on understanding how isoflavones are metabolized in the body including plasma kinetics as well as urinary and fecal excretion.

Xu et al. (1994) showed that daidzein is a more bioavailable soymilk isoflavone than genistein in adult women while glycitein was not measured. Xu et al. (1994) fed 12 women 3 doses of isoflavones (2.5, 4.8 and 7.4 $\mu\text{mol/kg BW}$ from soymilk powder; 44% genistein and 56% daidzein). Plasma levels of daidzein and genistein were similar to each other at time 6.5 and 24h following ingestion at all 3 doses. Urinary recovery of daidzein and genistein were 21% and 9% of the ingested dose, respectively, and fecal excretion was 1-2% ingested dose. Based on urinary excretion, Xu et al. (1994) concluded that daidzein was more bioavailable than genistein. Soybean milk isoflavones seemed to be 85% degraded in the intestine. Daidzein may be sufficient to exert some health-protective effects.

Xu et al. (1995) found that the efficiency of absorption of soymilk isoflavones varied from 13 to 35%, depending on individual gut microflora. Xu et al. (1995) performed a similar study to that of Xu et al. (1994) with three doses of isoflavones fed (3.4, 6.9 and 10.3 $\mu\text{mol isoflavones/kg body weight}$; n=7 women), but looked at the individual results and found that 2 subjects had significantly higher fecal excretion of isoflavones compare to the 5 others (about 6% vs. 0.6% of the ingested dose, respectively and regardless of the dose fed). 48 h urinary recovery was $16\pm 4\%$ and $10\pm 4\%$ of the ingested dose for daidzein and genistein, respectively in the 5 subjects with low fecal excretion. The two subjects with high fecal excretion had $32\pm 5\%$ and $37\pm 6\%$ urinary recovery expressed as a % ingested dose. As for plasma, subjects with high fecal and urinary excretion had plasma isoflavone level 2.5-fold higher compare to subjects who were low isoflavone excreters. This study established the principles of phenotypes of isoflavone bioavailability, in which people can be grouped as high apparent absorbers (high urinary, plasma and fecal isoflavone contents) or low apparent absorbers. Moreover, high apparent absorbers do not seem to have as much gut microbial activity degrading isoflavones, because they are excreted intact and in greater level in the feces those subjects. The role of the gut microflora in determining the extent of isoflavone bioavailability became then a factor that could not be overlooked. These two studies performed by Xu et al. (1994 & 1995) did not consider bioavailability of glycitein, a minor, but still important soy isoflavones.

Zhang et al. (1999; erratum, 2001) indicated urinary daidzein excretion was the greatest among that of three isoflavones in moderate fecal degraders and glycitein bioavailability was similar to daidzein in humans. On the other hand, comparing with this present study with two other studies in our laboratory, glycitein bioavailability was greater than daidzein and genistein excretion in hamsters.

Hendrich et al. (2001) found that plasma daidzein and genistein concentration was negatively correlated with in vitro fecal daidzein and genistein disappearance rate constant ($r = -0.74$, $P = 0.04$; $r = -0.88$, $P = 0.01$, respectively), supporting an important role for gut microbial activity in isoflavone bioavailability. Recently Zheng et al. (2003) demonstrated a relationship between isoflavone disappearance phenotypes and GTT and suggested that gut microorganisms may affect GTT. Among 35 Chinese vs. 33 Caucasian women, Chinese subjects who were low degraders of genistein had threefold greater bioavailability of genistein than Chinese high degraders. The Chinese who were low isoflavone degraders had the average GTT of 40 h vs. 65 h for Chinese high degraders. Caucasian subjects, regardless of isoflavone degradation phenotypes, had GTT > 80 h, and less apparent isoflavone absorption than did the Chinese subjects who were low degraders of isoflavones. GTT may be a crucial determinant of human differences in isoflavone bioavailability.

In an animal study, Lee et al. (2005) fed pure synthetic daidzein, genistein, or glycitein to female Golden Syrian hamsters (11-12 weeks of age, 10 hamsters/treatment) for 4 weeks and reported the urinary isoflavone excretion was glycitein > daidzein > genistein (32.2% > 4.6% > 2.2%). Meanwhile, Renouf et al. (2006) showed similar data in feeding either 1.18 or 1.77 mmol total isoflavones/kg diet to 19 one-year old hamsters for 10 d in both males and females. These results indicated that the microbes in hamsters seem to differ from those in humans. They reported similar isoflavone urinary excretion and gut microbial degradation patterns compare to humans in Golden Syrian hamster fecal and cecal microbial degradation of isoflavones. Daidzein excretion was significantly greater than glycitein and genistein excretion in urine and female urinary excretion was significantly greater than male. Therefore, this study established Golden Syrian hamsters as a potential animal model to be used instead of humans to study some chronic diseases which could not be induced in humans, such as cancer.

Renouf et al. (2005) conducted two separate studies, one focusing on fecal (study #1, n=20/sex) and the other on cecal (study #2, n=10/sex) microbial degradation of isoflavones in

Golden Syrian hamsters. They reported that urinary excretion was significantly lower by 2-4 fold in males compared to females in both studies. In addition, females from study #1 had significantly greater urinary excretion levels of daidzein ($44.2 \pm 13.7\%$ vs. $29.6 \pm 13.4\%$), glycitein ($31.4 \pm 11.2\%$ vs. $18.2 \pm 8.0\%$) and genistein ($26.7 \pm 11.5\%$ vs. $15.8 \pm 9.4\%$ ingested dose) compared to cecal study #2, respectively. Fecal isoflavone excretion was not significantly different between sexes or isoflavones (study #1) and showed extremely low levels of excretion ($<0.5\%$ ingested dose). *In vitro* fecal degradation rates from study #1 showed low degradation levels and no significant correlation between urinary and fecal isoflavone excretion. The most importance finding was that cecal isoflavone degradation rates (study #2) were much higher than fecal isoflavone degradation rates (study #1) and were statistical correlated with urinary excretion of daidzein ($R = 0.90$; $p = 0.01$) and genistein ($R = 0.93$; $p = 0.004$). They concluded that Golden Syrian hamsters displayed similar patterns of bioavailability of isoflavones compared to humans in terms of apparent absorption, urinary excretion and gut microbial degradation. Renouf et al. (2005) showed *Bacteroides ovatus*, *Bacteroides acidifaciens*, *Eubacterium ramulus*, *Clostridium orbiscindens* and *Tannerella forsythensis* were the major human gut microbial species that degraded isoflavones and established that Golden Syrian hamsters may be good models to study bioavailability of isoflavones and their possible health promoting effects. Renouf et al. (2005) identified high fecal isoflavone degradation rate to coincide with distinct fecal bacterial species. Fresh feces from 33 healthy adult subjects (20 men, 13 women) were incubated anaerobically with isoflavones to assess degradation rates using HPLC. Fecal DNA was extracted, bacterial 16S rDNA sequences amplified by polymerase chain reaction (PCR) and separated by denaturing gradient gel electrophoresis (DGGE). Cluster analysis identified high and low degraders of daidzein, genistein and glycitein. DGGE analysis showed that high genistein degraders ($n = 4$; fecal degradation rate $1.47 \pm 0.14\text{h}^{-1}$) shared 5 bands of greater intensity than found in feces of low genistein degraders ($n = 4$; fecal degradation rate $0.146 \pm 0.034 \text{h}^{-1}$) high glycitein degraders ($n = 4$; $0.574 \pm 0.299\text{h}^{-1}$) also shared 5 bands of greater intensity than found in feces of low glycitein degraders ($n = 4$; $0.146 \pm 0.034 \text{h}^{-1}$). They also showed concordance with known species from the *Bacteroides* and *Prevotella* genus as well as the *Clostridiales* order using sequencing of 16S rDNA from the bands of interest. After developing two in vitro systems, one rich (rumen fluid based brain heart infusion media) and one poor in nutrients (feces incubated overnight in brain heart infusion media), they identified

Bacteroides ovatus, *Bacteroides acidifaciens*, *Eubacterium ramulus*, *Clostridium orbiscindens* and *Tannerella forsythensis* as the major human gut microbial species that degraded isoflavones under both nutrient rich and poor conditions, thus these species may be the most significant ones in degrading isoflavone in the human gut. They also concluded that bacterial species shared by both high and low degraders with greater amounts in high degraders may be predictors of gut microbial degradation and overall bioavailability of isoflavones. Secondary species that may be specific to each individual fecal isoflavone degradation rate may be of importance for assessing microbial activity and will deserve further attention.

Ye et al. (2006) reported that apparent absorption of isoflavones varied greatly among individuals and was relatively stable within an individual. Fifty Golden Syrian hamsters were fed a high fat/casein diet (n = 10) or a high fat/soy protein diet (n = 40) for 4 wk. Two distinct urinary isoflavone excretion phenotypes were identified using a pairwise correlation plots analysis, or using a hierarchical cluster test. High isoflavone excreters showed significantly greater urinary isoflavones ($p < 0.05$) than did low isoflavone excreters. High urinary isoflavone excreters had significantly less non-HDL cholesterol than did the low isoflavone excreters or casein-fed controls ($p < 0.05$). Urinary isoflavone excretion phenotypes predicted the cholesterol-lowering efficacy of soy protein. Isoflavone absorbability, probably due to gut microbial ecology, was an important controllable variable in studies of effects of soy protein on blood lipids.

In a summary, our long-term goals are to establish a screening assay for dietary component microbial metabolism and to understand the relationship between the role of gut microorganisms and beneficial dietary compounds such as antioxidant phenolics that may be found in commonly used herbs and plant foods. Bioavailability of phenolics on plasma kinetics, urinary and fecal excretion is now well understood. For instance, isoflavone absorption is a fast process with a maximum plasma peak occurring within 12 h after ingestion. Only traces of isoflavone are excreted in the urine after 48 h; most of the isoflavone ingested are being excreted within 24-48h. From animal and human studies obtained until now, we believed that potential animal models could be used to study phenolic metabolism. At meantime, both humans and animals suggested that a great amount of phenolic disappear in metabolism. There is now good evidence that gut microbiota plays an important role in the process of metabolism. We now know that phenolic bioavailability varies greatly among individuals in terms of the amount of phenolic

degradation microbial species which are relatively stable within an individual. Inter-individual variability in phenolic bioavailability needs more investigation.

B. *Echinacea purpurea* and Bacteria

Echinacea purpurea was widely used in prophylaxis and therapy of various infections in animals and humans. *E.* extracts were thought to make immune system more efficient at attacking bacteria. To evaluate the cellular immunity related effect of *Echinacea purpurea* extract on the development of *Pseudomonas aeruginosa* infection in various strains of mice. Bany et al. (2003) reported that *E.* extracts feeding decreased bacteria number in liver of C57Bl/6 (susceptible strain) as well as B6C3F1 (relative resistant strain) mice. *Echinacea* feeding of the second relative resistant strain (BALB/c x C3H) F1 resulted in stimulation of granulocytes chemiluminescent and lymphocytes proliferative response (Bany et al., 2003). However, using plate culture microbiological methods, *E. purpurea* significantly increased total aerobic bacteria, *Bacteroides* group and *Bacteroides fragilis* in human gastrointestinal (GI) tract after fifteen human subjects consumed 1000 mg of standardized *E. purpurea* for 10 days (Hill et al., 2006), whereas *Bacteroides* was shown to associate with diarrhea, inflammatory bowel disease and increased risk of colon cancer (Basset et al., 2004; Malinen et al., 2005). Recently, various *E.* extracts were shown to inhibit selective five bacteria (*Streptococcus pyogenes*, *Haemophilus influenzae*, *Legionella pneumophila*, *Clostridium difficile*, and *Propionibacterium acne*) and two pathogenic fungi which were related to upper and lower respiratory infections including sinusitis, bronchitis, pharyngitis, tonsillitis, and pneumonia, as well as cutaneous infections (Sharma et al., 2008). In a summary, *E.* extract inhibition of bacteria still is controversial issue because some bacteria which were related to upper and lower respiratory infections were inhibited in animal models and cell culture, whereas total aerobic bacteria, *Bacteroides* group and *Bacteroides fragilis* in human gastrointestinal (GI) tract were grown well using plate culture microbiological methods,

C. *Hypericum perforatum* and Bacteria

Rutin was absorbed as quercetin because it was hydrolysed by the cecal microflora from rats receiving the different experimental diets (Manach et al., 1997). Within 24-48 h of incubation, using a new in vitro model system the deglycosylation of rutin and the degradation of

its aglycone quercetin were investigated by fresh pig caecal inocula and 6 wk/5 months frozen inocula. The pattern of quercetin and rutin degradation products was similar in both approaches. And reported that the preservation of the microbial vitality and the metabolic efficiency by fresh or freeze-preparation were independent in time and locality (Keppler et al., 2006). For studying the metabolism of flavonoids by the intestinal microbiota, in vitro model system of intestinal microbiota was developed from the cecum of freshly slaughtered pigs to investigate the microbial deconjugation and degradation of several flavonols and flavonol glycosides (Keppler et al., 2005). In this model system, the microbiota was directly isolated from the cecal lumen of pigs which was identified by fluorescence in situ hybridization (*FISH*) with 16S rRNA-based oligonucleotide probes and confirmed the suitability for studying metabolism by the human microbiota (Hein et al., 2008). The microbial degradations of quercetin with different aglycones, sugar moieties, and types of glycosidic bonds were investigated. The main results were concluded that the glycosides were almost completely metabolized by the intestinal microbiota within 20 min and 4 h depending on the sugar moiety and the type of glycosidic bond. The structure of the aglycone had not influenced the intestinal metabolism. The liberated aglycones were completely metabolized within 8 h (Hein et al., 2008). In a summary, the bioavailability of flavonoids in Hp was influenced by the metabolism of intestinal microflora in culture models.

D. Metabolism of caffeic acid in bacterial models

Caffeic acid was shown to be metabolized by the intestinal microbiota of human and experimental animals and some bacteria isolated from human feces (Peppercorn et al., 1971; Olthof et al., 2001; Gonthier et al., 2003). In earlier study, the basic growth media of both Thiol Broth (Difco) and Beef (Difco) were used at 37⁰C under anaerobic conditions for transformation of caffeic acid in mixed cultures of feces or individual bacteria. Two main metabolites of 3-hydroxyphenylpropionic acid (3-HPP) and 4-ethylcatechol were detected in mixed cultures of fecal incubation. In some organisms with pure culture or mixed cultures, *Peptostreptococcus sp.* and *Clostridium perfringens* were capable of reducing caffeic acid. In mixed culture, *Escherichia coli* and *Streptococcus fecalis* were required for the dehydroxylation of dihydroxyphenylpropionic acid (dihydrocaffeic acid) (Peppercorn et al., 1971). To study the microbial conversion of phenolics in an in vitro fermentation model, individual caftaric acid, chlorogenic acid, and caffeic acid (1 μmol) were inoculated with either active or inactive human

faecal slurry (10 ml) in fermentation bottles and incubated with stirring for 0, 2, 4, 6, 8 or 24 h in anaerobic conditions at 37 °C. All phenolics were degraded quickly and none of the free acids (caffeic, quinic or tartaric acids) were detected after 2 hours of incubation. Two major microbial metabolites were identified as 3-hydroxyphenylpropionic (3-HPP) and benzoic acids (BA). Maximal levels of 3-HPP were reached after 2 h of fermentation and accounted for 9-24% of the dose of caffeic acid and its esters. The similarities in the metabolic patterns observed for caffeic, chlorogenic and caftaric acids suggested that esterification did not affect the metabolism of caffeic acid by the gut microbiota (Gonthier et al., 2006).

With respect to metabolism of chlorogenic acid by esterase of colonic microflora, several studies have done with bacteria-based models. Comparing with extracts of human small intestine epithelium, liver, plasma and colonic microflora (as a faecal sample) by incubation with chlorogenic acid, only esterase activity in colonic microflora played an important role in esterified acid (chlorogenic acid) ingested by humans (Plumb et al., 1999). Chlorogenic acid was hydrolysed by esterase produced by the indigenous microflora. *Bifidobacterium lactis*, *Lactobacillus gasseri*, and *Escherichia coli* were identified by genotypic characterization (16S rRNA sequencing) in culture incubation of human faecal bacteria in a chlorogenic acid-based medium (Couteau et al., 2001). In a summary, the bioavailability of caffeic acid was influenced by the metabolism of intestinal microflora in culture models. Some specific bacteria may play an important role for some metabolites related to phenolics.

VI. RELEVANT METHODOLOGY

A. BHI microbial incubations VS Caco-2 monolayers

With regard to metabolism of phenolics, the early bacteria-based model, a pig caecum anaerobic method was developed to conduct the metabolism of flavonoids using intestinal microbes (Labib et al., 2004). Another method was performed by using the inoculum of caecum which was isolated from freshly slaughtered pigs (Keppler et al., 2005).

The other anaerobic fermentation method was developed with hamster cecal content or human gut microflora in Brain-heart infusion (BHI) broth media to investigate the metabolism of phenolic compounds (Renouf, 2005; Simons et al., 2005). Fourteen flavonoids were degraded by anaerobic fermentation with human gut microflora and showed the 5, 7, 4'-trihydroxyl flavonoids (apigenin, genistein, naringenin, and kaempferol) disappeared quickly compared to the other

structural motifs (Simons et al., 2005). This method is suitable for the metabolism studies incubated with *Echinacea purpurea* and *Hypericum perforatum* extracts phenolics using human fecal and mouse cecal samples as well as the other plant phenolic compound using salivary bacterial fermentation.

On the other hand, Caco-2 cell model was used for studying the absorption and transport of a variety of compounds found in botanicals. Group B soyasaponins was studied in Caco-2 cell absorption by comparing with human apparent absorption (Hu et al., 2004). Also genistein was shown with efficient absorption by Caco-2 cells (Oitate et al., 2001). Limited uptake of caffeic and chlorogenic acids (1.5 and 0.1% transfer, respectively) was found in Caco-2 cells (Konishi and Kobayashi, 2004a). In this model, to test botanical compound absorbability, Caco-2 cell was used to investigate if the extract matrix and interactions within a simulated botanical fraction affect compound absorbability or not. It is suitable for using Caco-2 cells model for human intestinal uptake and metabolism of herbal compounds.

Using isolated rat small intestine as an *ex-vivo* animal model, Andlauer et al. (2000b) studied the absorption rate and biotransformation of isoflavones daidzin and genistin derived from pre-digested tofu. Tofu contained small amounts of malonyl-isoflavone and isoflavone aglycone. 8% genistein and 8.9% daidzein appeared at the vascular side, either as aglycone, glucuronide and glucoside. A 3 and 2-fold increase in the aglycone genistein and daidzein, respectively was found in the luminal side. This method should also be thought as an alternative approach. However, the apparent permeability was not measured in this method.

B. Colitis models with bacteria VS dextran sodium sulfate

Inflammatory bowel diseases (IBDs; e.g., Crohn's disease, ulcerative colitis) models induced by enteric bacteria were well established (Hutto et al., 1998; Jergens et al., 2007). Recently, the *Helicobacter bilis*-induced colitis model also used to investigate the mucosal gene expression profiles (Liu et al., 2009). Also caffeic acid phenethyl ester (CAPE) has been showed to decrease the level of colonic NF- κ B and prevented colitis in peptidoglycan-polysaccharide (PG-PS)-induced rat model (Fitzpatrick et al., 2001).

On the another aspect, the colitis in mice was also induced by dextran sodium sulfate (DSS) in drinking water (Dieleman et al., 1998) causing weight loss, diarrhea with blood and/or mucus, shortening of the colon, erosion of the mucosal epithelium, and acute neutrophilic

infiltration (Stevceva et al., 1999). The colitis have been developed to investigate the molecular and cellular mechanisms of inflammation and showed that IBDs were characterized by up-regulated nuclear factor kappa B (NF- κ B) and pro-inflammatory cytokines and dysregulated immune responses resulting in tissue damage (Elson et al., 1995; Reed et al., 2005). Despite of the less beneficial action, glucocorticosteroids was still used to treat IBD (Podolsky et al., 1991; Podolsky et al., 2002). Immunosuppressive and immunoregulatory agents have also been used to control severe disease, however, the serious complications and toxic side effects were associated with these agents (Shanahan et al., 2001). DSS induced colitis model were used for screening dietary phenolics to treat this disease in animal. In this model, a range dose of dietary rutin prevented DSS-induced colitis and possible colorectal carcinogenesis via attenuation of pro-inflammatory cytokine production (TNF- α , IL-1 β) (Kwon et al., 2005). Thus, DSS induced colitis model is reasonable choice for screening caffeic acid to treat this disease.

C. P CR/DGGE and DNA sequencing on microbial analysis VS traditional microbiological methods

Based on our previous work, several human gut microbial species were identified to degrade isoflavones, and would therefore be highly likely to degrade flavonoids in general using microbial DNA extracted from human fecal samples and from high and low flavonoids degraders with amplification of microbial sequences by PCR of 16S rRNA gene variable regions (Muyzer et al., 1993). Renouf et al. (2005) showed *Bacteroides ovatus*, *Bacteroides acidifaciens*, *Eubacterium ramulus*, *Clostridium orbiscindens* and *Tannerella forsythensis* were the major human gut microbial species that degraded isoflavones and established that Golden Syrian hamsters may be good models to study bioavailability of isoflavones and their possible health promoting effects. Renouf et al. (2005) identified high fecal isoflavone degradation rate to coincide with distinct fecal microbial species. They concluded that bacterial species shared by both high and low degraders with greater amounts in high degraders may be predictors of gut microbial degradation and overall bioavailability of isoflavones. Secondary species that may be specific to each individual fecal isoflavone degradation rate may be of importance for assessing microbial activity and will deserve further attention. This method is suitable for the metabolism study incubated with plant phenolic compound using salivary bacterial fermentation and identifying the higher and lower degrader for caffeic acid and rutin.

Compared with using plate culture microbiological methods or traditional microbiological methods, the main measurement is for plating and counting microbes. For example, *E. purpurea* significantly increased total *aerobic bacteria*, *Bacteroides* group and *Bacteroides fragilis* in human gastrointestinal (GI) tract after fifteen human subjects consumed 1000 mg of standardized *E. purpurea* for 10 days (Hill et al., 2006). However, we will not identify the bacterial species related the high or low degrader phenotypes.

D. HPLC Analysis VS LC-MS-UV analysis

High-performance liquid chromatography (HPLC) has high resolution, speed, and sensitivity advantages for measuring compounds and was used as a broad range in research areas. Renouf et al. (2005) conducted two separate studies, one focusing on fecal and the other on cecal microbial degradation of isoflavones in Golden Syrian hamsters. HPLC was used in testing the compound level to calculate the degradation rate. Also Simons et al. (2005) used this method to measure the fourteen flavonoids which were degraded by anaerobic fermentation with human gut microflora (Simons et al., 2005). Ye et al. (2006) also performed HPLC in fifty Golden Syrian hamsters which were fed a high fat/casein diet or a high fat/soy protein diet study. In this study, urinary isoflavone excretion amounts were detected by HPLC. Two incubation studies in this dissertation have used enough amount compounds which were detectable for HPLC.

However, in this dissertation, the second caffeic acid (CA) study which used 0.67mmol/kg CA in diet had lower plasma concentration. Caffeic acid was not detectable in such a method. Extraction plasma sample concentrations were detected by high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) with a UV absorption detector. One animal study, the bioavailability of caffeic acid was studied with different dosages to obtain plasma pharmacokinetic profiles of their metabolites. The rat with 250 μ mol/d caffeic acid for 8 d, total urinary excretion of caffeic, ferulic, and isoferulic acids was 12.8 % of intake (mol/mol) and urinary 3-hydroxyphenylpropionic acids (3-HPP) was 4.0 %. Using HPLC-electrospray ionization-tandem mass spectrometry, plasma metabolite concentrations in rats fed caffeic acids for 8 d were caffeic acid as 41.3 μ mol/L, ferulic acid as 7.3 μ mol/L, and 3-hydroxyphenylpropionic acid as 1.4 μ mol/L (Gonthier et al., 2003). The

present caffeic acid concentration was detectable for LC-MS-UV analysis whereas HPLC was not suitable for measuring the lower caffeic acid concentration in plasma.

VII. REFERENCES

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CHAPTER 3. METABOLISM OF *ECHINACEA* AND *HYPERICUM* EXTRACT PHENOLICS IN *IN VITRO* BY HUMAN FECAL OR MOUSE CECAL MICROFLORA^{1,2}

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Abstract

Different phenolics show a range of apparent degradability, as we have seen previously with isoflavones and flavonoids in *in vitro* anaerobic incubations with human fecal or animal cecal samples. We hypothesize that human fecal and mouse cecal degradation rates will be similar for major phenolics from *Echinacea Purpurea* and *Hypericum perforatum* extracts. Human fecal and mouse cecal samples were incubated with these two botanical ethanolic extracts to determine which phenolics were least degraded, and hence predicted to be most bioavailable to the mouse gastrointestinal mucosa. These studies compared *Echinacea* with *Hypericum* phenolics in BHI media incubation; and at different time points, the phenolic concentrations were measured by HPLC. All of the phenolic compounds tested were degraded by human fecal and mouse cecal content and some specific metabolites were produced during the incubation period. With twenty human fecal incubations, the degradation rate of rutin ($k = 0.57 \pm 0.13 \text{ h}^{-1}$) was significantly greater than that of hyperoside ($k = 0.31 \pm 0.10 \text{ h}^{-1}$, $p < 0.01$) in *Hypericum*. Of the 3 major compounds in *Echinacea*, the degradation rate of caffeic acid ($k = 0.40 \pm 0.23 \text{ h}^{-1}$) was less compared with caftaric acid ($k = 0.63 \pm 0.17 \text{ h}^{-1}$, $p < 0.01$) and there was no difference when compared with cichoric acid ($k = 0.49 \pm 0.16 \text{ h}^{-1}$, $p > 0.05$). Similar degradation patterns were found in mouse cecal content incubation except that phenolic degradation rates were somewhat slower than in human fecal incubations. Briefly, in mouse cecal content incubation with *Echinacea*, the degradation rate of caffeic acid ($k = 0.36 \pm 0.16 \text{ h}^{-1}$) was less compared with both caftaric acid ($k = 0.59 \pm 0.10 \text{ h}^{-1}$, $p < 0.01$) and cichoric acid ($k = 0.53 \pm 0.24 \text{ h}^{-1}$, $p < 0.01$). Feces from six human subjects out of 20 and cecal samples from one

of six mice were found to produce caffeic acid after 3 hour incubation with *E. purpurea* extract. The caffeic acid degradation rate in caffeic acid producers was significant lower than that of non caffeic acid producer in humans. A metabolite was identified as m-hydroxyphenylpropionic acid (*mHPP*), which was reduced and dehydroxylated from caffeic acid. Rutin and caffeic acid were, therefore, predicted to be least degradable from *Hypericum* or *Echinacea* extracts *in vivo* as the major phenolics from each botanical in the human or mouse gut.

Introduction

Our long-term goals are to establish a screening assay for dietary components that may benefit colon health, to prevent colitis, possibly to prevent colon cancer and to understand the relationship between the role of gut microorganisms and beneficial dietary compounds such as antioxidant phenolics that may be found in commonly used herbs and plant foods. Isoflavone bioavailability has been studied using *in vitro* anaerobic BHI incubations with human fecal or hamster cecal samples according to our previous work (Zheng et al., 2003). Therefore, *in vitro* cecal/fecal incubations offer a reasonable *in vitro* model to evaluate the gut metabolism of phenolics.

Echinacea purpurea, *Echinacea pallida*, and *Echinacea augustifolia* are perennial herbs growing as wildflowers on the prairies of the Great Plains (Cheminat et al., 1988). Caffeic acid derivatives (caftaric acid, caffeic acid, and cichoric acid) are found in the highest concentration in each variety (Barnes et al., 2005; LaLone et al., 2007). *Echinacea* was widely used by consumers and practitioners for preventing and treating common colds and is the second top-selling herbal product currently in the USA. Some controlled clinical trials have investigated beneficial effects of *E. purpurea* for the early treatment of upper respiratory tract in adults (Barrett, 2003). Caffeic acid phenethyl ester was indicated to decrease bacterial peptidoglycan polysaccharide-induced colitis in rats as an inhibitor of nuclear factor kappa B (NF- κ B) (Fitzpatrick et al., 2001). *Echinacea* was shown to possess antioxidant activity by assessing free radical scavenging ability and evaluation of lipid peroxidation level (Sanchez-Moreno, 2002). Recently, *E. purpurea* was indicated to decrease liver superoxide dismutase (SOD) activity in rats treated with cyproterone acetate resulting in toxicity (Ali et al., 2008). *Hypericum perforatum* is an herb with yellow-flower which includes a diverse chemical composition,

including rutin, hyperoside, quercetin, pseudohypericin, and hypericin (Lawvere et al., 2005). Four components in fraction, these combined constituents (0.1 μ M chlorogenic acid, 0.08 μ M amentoflavone, 0.07 μ M quercetin, and 0.03 μ M pseudohypericin) inhibited lipopolysaccharide (LPS)-induced prostaglandin E₂ level, the production of the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α), and the anti-inflammatory cytokine interleukin-10 (IL-10) (Hammer et al., 2008). A study showed that dietary rutin improved dextran sulfate sodium-induced experimental colitis in mice through attenuation of pro-inflammatory gene expression (Kwon et al., 2005).

The fecal and cecal phenolic disappearances which were observed in the present study will need to compare with compound efficacy in future studies. The aim of this study was to further characterize whole extract phenolic metabolism in human and mice by elucidating the variability of the apparent microbial degradation and to detect possible bioactive metabolites of caffeic acid derivatives or rutin using anaerobic BHI fermentation systems. Caffeic acid derivatives in *Echinacea purpurea* extract were metabolized by gut microbes. Cichoric acid was hydrolyzed to caftaric acid and caffeic acid by microbial esterase (Peppercorn et al., 1971). Then caftaric acid was also hydrolyzed to caffeic acid which was reduced to dihydrocaffeic acid (3, 4-dihydroxyphenylpropionic acid); furthermore the later was dehydroxylated to m-hydroxyphenylpropionic acid (mHPP) (Figure 3.1).

Materials and Methods

Plant materials and extracts

Echinacea purpurea and *Hypericum perforatum* plant material were obtained from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture and processed as described previously (Schmitt et al., 2006). Two extracts of plant were provided by the NCRPIS and Dr. Murphy's Laboratory: *Echinacea purpurea* Soxhlet ethanolic extract (E194), Plant Introduction (PI) 631307 (77 mg/mL); and *Hypericum perforatum* Soxhlet ethanolic extract (H165) 'Common' (286 mg/mL). *Hypericum* 'common' was grown from seeds supplied by Johnny's Selected Seeds (Winslow, ME).

Chemicals

Cichoric acid, caftaric acid, caffeic acid, and 3-hydroxyphenylpropionic acid were purchased from Chroma Dex™, Inc. Santa Ana, CA. Rutin, chlorogenic acid, quercetin, and

hyperoside were purchased from Fisher Scientific (Hanover Park, IL). 2, 4, 4'-Trihydroxydeoxybenzoin (THB) were synthesized using the method (Song et al., 1998). High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, acetic acid, dimethyl sulfoxide (DMSO), and all other chemicals were from Fisher Scientific (Fairlawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Study protocol for human fecal and mouse cecal sample preparation

The first human fecal incubation study was performed with twenty young adult humans. The ages ranged from 22 to 55 years (mean age = 32.9 ± 4.2 years). Ten men (8 Asian and 2 Caucasian) and 10 women (5 Asian and 5 Caucasian) from Iowa State University and the surrounding Ames area were required to avoid dietary phenolics for 5 days before providing a fecal sample. A list of foods and herbs that might be eaten and those that were to be avoided was provided. The other selection criteria required that the subjects be in good health and not taking any medication. The body mass index (BMI) was 19.5-25.1 kg/m² (mean BMI = 22.8 ± 1.1 kg/m²). All subjects provided one fresh fecal sample in sealed sterile containers (Sage Products Inc., Crystal Lake, IL) in the morning before the incubation study. An aliquot of the homogenized fecal sample was immediately put into Brain-heart infusion (BHI) media for anaerobic incubation. The fecal sample storage time (T) was 1.5-3.5 h (mean T = 2.8 ± 1.2 h). Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee.

A second study using mouse cecal contents was conducted with 24 C3H/HeOuJ mice at 6 weeks of age (12 male, 12 female) which were fed a semi-purified AIN-93G diet (Harlan Teklad, Madison, WI) for one week and sacrificed by CO₂. Cecal contents were removed under sterile conditions into BHI media for anaerobic incubation. All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.

Phenolic incubation

Brain-heart infusion (BHI) broth medium (Difco Laboratories, Detroit, MI) was used as previously described (Zheng Y., et al., 2003) and prepared with the addition of 4 g sodium bicarbonate/L medium as a buffer and 20 mL cysteine sulfide (Sigma, St Louis) as an oxygen indicator. All plant extracts were dissolved in 100% DMSO. One and a half grams of freshly voided human feces or the cecal content of individual mice were transferred to incubation test

tubes (Fisher Scientific) containing 30 mL of BHI. *Echinacea purpurea* and *Hypericum perforatum* ethanolic extracts were added to the incubation test tubes for a final concentration of 1.83mg/mL and 0.65 mg/mL of BHI solution based on HPLC analysis, separately. Twenty fecal samples and six cecal contents were prepared for each extracts. The fermentations for fecal samples were performed in duplicate. The incubation test tubes were flushed with CO₂, sealed with rubber stoppers and autoclave tape, and then vortexed for 10 s. One and a half milliliters were taken anaerobically from each test tube immediately for time 0 and frozen on dry ice. The tubes were placed in a 37 °C incubator. One and a half milliliters aliquots were sampled from the incubation test tubes at 3, 6, 9, 12, and 24 h. All the samples taken were put at -60°C until extraction and HPLC analysis. Incubation tubes were maintained anaerobically at all times during this process to preserve bacterial quality. Negative controls consisted of the fecal suspension without extracts. Microbial degradation by the fecal suspension was confirmed by positive controls, which consisted of BHI media and extracts with sterilized feces or cecal contents and positive controls without the fecal suspension.

Phenolic extraction

THB was added at 100 µM to the thawed fermentation samples as an internal standard and slowly loaded onto preconditioned C18 solid phase extraction cartridges (Waters Corporation, Milford, MA). The cartridge was washed twice with 2 mL of Milli-Q system water. The phenolics were eluted with 1 mL of 80% methanol, filtered through 0.45 µm filters, and analyzed by HPLC.

HPLC analysis

Both phenolic compounds from *E. purpurea* and *H. perforatum* were analyzed by HPLC. The HPLC system consisted of a Hewlett-Packard 1050 Series. Twenty microliters of sample was injected onto a reversed-phase, 5 µm, C18 AM 303 column (250 mm × 4.6 mm) (YMC Co. Ltd., Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and 100% acetonitrile (B). Solvent B increased from 25 to 48% in 10 min, increased to 90% in 7 min, and was held for 5 min. The gradient was recycled back to 25% in 2 min for the next run. The flow rate was 1 mL/min. The wavelengths used for the preparation of standard curves, detection, and quantification of phenolic peaks were 254 and 292 nm. Chem station^{3D} software (Hewlett-Packard Company, Scientific Instruments Division, Palo Alto, CA) was used to integrate the peak area responses and to evaluate the ultraviolet absorbance spectra.

Data analysis

The ratio of peak area of a phenolic to THB (100 μM) vs the phenolic concentration was used as an internal standard curve to estimate the concentration of phenolics in the fecal or cecal fermentations. The rate of disappearance or appearance of phenolics or metabolites in fecal or cecal fermentation mixtures was estimated by plotting \ln (% remaining phenolic) vs time. The negative slope of these lines was the apparent first-order degradation rate constant. The positive slope of these lines was the metabolite appearance/generated rate constant. Statistical evaluation of disappearance or appearance rate was performed using the SAS Institute (2003, Cary, NC) and rates were reported as means \pm SEM. Statistical significance was set at $P < 0.05$. Differences between the overall and the individual degradation rates of phenolics as well as caffeic acid producer /non caffeic acid producer were estimated using one-way analysis of variance (ANOVA) followed by Tukey method as a Post Hoc test. The equal variance and normality of residuals assumptions were verified by a residual vs. predicted values plot and a histogram of residuals.

Results

Metabolism of *Echinacea purpurea* phenolics in human fecal and mouse cecal incubation

HPLC chromatogram of different times (0, 3, and 6 h) of caffeic acid derivatives in *E. purpurea* extract also showed that cichoric acid and caftaric acid were decreased at 3 h of incubation, whereas caffeic acid was increased and new peak was shown in this time point. At 6 h of incubation, caffeic acid was decreased and new peak was shown to further increase which was identified as m-hydroxyphenylpropionic acid (mHPP) by HPLC (Figure 3.3) and also decreased at 12 h incubation (data not shown).

For the disappearance rates of caffeic acid derivatives in *E. purpurea* extract during the whole time period, among the 3 major compounds in human fecal incubation, the degradation rate of caffeic acid ($k=0.40 \pm 0.23 \text{ h}^{-1}$) was lower compared with caftaric acid ($k= 0.63 \pm 0.17 \text{ h}^{-1}$, $p < 0.01$) and not different from cichoric acid ($k= 0.49 \pm 0.16 \text{ h}^{-1}$, $p > 0.05$). In mouse cecal content incubation, caftaric acid and caffeic acid degradation rates were somewhat lower and cichoric acid degradation rate was higher than in human fecal incubations. Appearance rate of the metabolite *mHPP* was not different between the human fecal and mouse cecal incubations (Table 3.1; Figure 3.5).

The peak area of *E. purpurea* extraction phenolics after different time points of incubation with human feces showed that six human subjects of 20 peoples and one of six mice were found to generate caffeic acid between 3 to 6 hour microflora incubation. A metabolite (*mHPP*) was increased after 3 h to 9 h, which was also reduced after 12 h incubation (Figure 3.6). Finally caffeic acid (CA) producers (n=6) and non CA producers (n=14) from *Echinacea purpurea* extract after incubation with human feces were identified. A significant difference was found between with the two phenotypes in fecal CA degradation rates (Figure 3.8).

Metabolism of *Hypericum perforatum* in human fecal and mouse cecal incubation

HPLC chromatogram of two times (0 and 6 h) of rutin and hyperoside in *Hypericum perforatum* extract in the human fecal incubation showed that rutin decreased quickly, whereas hyperoside was decreased slowly compared with rutin, however, no increase in hyperoside nor new peaks were found in 20 humans or in mice at any time point (Figure 3.4). With degradation rates of rutin /hyperoside in *Hypericum* extract in whole time period incubation, in twenty human fecal incubations, the degradation rate of rutin ($k = 0.57 \pm 0.13 \text{ h}^{-1}$) was significantly greater than that of hyperoside ($k = 0.31 \pm 0.10 \text{ h}^{-1}$, $p < 0.01$) in *Hypericum* (Figure 3.7; Table 1). Similar degradation patterns were found in mouse cecal content incubation except that phenolic degradation rates were somewhat lower than in human fecal incubations.

Discussion

The adult human gut microbiota is composed of 30 – 40 major species which are anaerobes (97%) or aerobes (3%), and apparently thousands of minor species. The most common anaerobic genera in terms of concentration within gastrointestinal tract (GI) are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium* and *Lactobacillus*. Among these genera, the aerobes are the Gram-negative enteric bacteria (*Escherichia coli* and *Salmonella*), the Gram-positive cocci (*Enterococcus*, *Staphylococcus* and *Streptococcus*) and aerobic fungal species (*Candida albicans*) (Marteau et al., 2004; Andoh et al., 2006; Guerrero Hernández et al., 2008).

With respect to metabolism of phenolics, which presumably involves esterases derived from colonic microbiota, methods were developed by *in vitro* studies. Several studies have performed using bacteria-based models. A pig caecum anaerobic method was developed to conduct the metabolism of flavonoids using intestinal microbes. In this approach, quercetin was

metabolized to 3, 4-dihydroxyphenylacetic acid, 3, 4-dihydroxytoluene, and phloroglucinol (Labib et al., 2004). Another method was performed using the inoculum of caecum which was isolated from freshly slaughtered pigs and placed in an anaerobic jar to retain the anaerobic atmosphere. All anthocyanidin glycosides (including phloroglucinol acid gallic acid) were hydrolysed by the microflora between 20 min and 2 h of incubation depending on the sugar moiety being measured (Keppler et al., 2005).

The anaerobic incubation of flavonoids in hamster cecal content and human gut microflora with brain-heart infusion (BHI) broth medium was developed to investigate the bioavailability of phenolic compounds (Renouf, 2005; Simons et al., 2005). An *in vitro* cecal isoflavone degradation rate cluster analysis revealed that bioavailability phenotypes were due to gut microbial degradation which differed from individual hamsters (Renouf, 2005). Fourteen flavonoids were degraded by anaerobic fermentation with human gut microflora and showed the 5,7,4'-trihydroxyl flavonoids (apigenin, genistein, naringenin, and kaempferol) disappeared quickly compared to the other compounds (Simons et al., 2005).

With respect to metabolism of caffeic acid derivatives by esterases of colonic microflora, although chlorogenic acid was not found in *Echinacea* extract in present study; one study that compared incubation of chlorogenic acid in human small intestine epithelium, liver, plasma and colonic microflora (as a fecal sample), only esterase activity in colonic microflora played an important role in chlorogenic acid deesterification (Plumb et al., 1999). Chlorogenic acid was hydrolysed by esterase produced by member of the indigenous microbiota. *Bifidobacterium lactis*, *Lactobacillus gasseri*, and *Escherichia coli* were identified by genotypic characterization (16S rRNA sequencing) in culture incubation of human faecal bacteria in a chlorogenic acid-based medium (Couteau et al., 2001).

Caffeic acid was shown to be metabolized by the intestinal microbiota of human and experimental animals and some bacteria isolated from human feces (Peppercorn et al., 1971; Olthof et al., 2001; Gonthier et al., 2003). In an earlier study, the basic growth media of both Thiol Broth (Difco) and Beef (Difco) were used at 37°C under anaerobic conditions for transformation of caffeic acid in mixed cultures of feces or individual bacteria. Two main metabolites, m-hydroxyphenylpropionic acid (*m-HPP*) and 4-ethylcatechol were detected in mixed cultures of fecal incubation. In some organisms with pure culture or mixed cultures, *Peptostreptococcus sp.* and *Clostridium perfringens* were capable of metabolizing caffeic acid.

In mixed culture, *E. coli* and *Streptococcus fecalis* were required for the dehydroxylation of dihydroxyphenylpropionic acid (dihydrocaffeic acid) (Peppercorn et al., 1971). The same result was found in present study. To study the microbial conversion of phenolics *in vitro* incubation model, individual caftaric acid, chlorogenic acid, and caffeic acid (1 μmol) were inoculated with either active or inactive human faecal slurry (10 ml) in fermentation bottles and incubated with stirring for 0, 2, 4, 6, 8 or 24 h in anaerobic conditions at 37 °C. All phenolics were degraded quickly and none of the free acids (caffeic, quinic or tartaric acids) were detected after 2 hours of incubation. Two major microbial metabolites were identified as 3-hydroxyphenylpropionic (3-HPP) and benzoic acids (BA). Maximal levels of 3-HPP were reached after 2 h of fermentation and accounted for 9-24% of the dose of caffeic acid and its esters. The similarities in the metabolic patterns observed for caffeic, chlorogenic and caftaric acids suggested that esterification did not affect the metabolism of caffeic acid by the gut microbiota (Gonthier et al., 2006).

No previous studies have been conducted to evaluate the metabolism of cichoric acid by gut microbiota *in vitro* or *in vivo*. One study showed that cichoric acid (2R, 3R-O-dicaffeoyltartaric acid) was degraded by polyphenol oxidases (PPO) into caftaric acid (2-O-caffeoyltartaric acid; monocaffeoyltartaric acid) and caffeic acid during the preparation of *E. purpurea* products. Caftaric acid was degraded as well but more slowly than cichoric acid. Both ascorbic acid and ethanol inhibited oxidative degradation and hydrolysis of caftaric acid as synergistic effect (Nüsslein et al., 2000). However, the present study was the first study showing that cichoric acid was apparently degraded by gut microflora *in vitro*. During human fecal fermentations with *E. purpurea* extract, caffeic acid production was found after incubation. A significantly lower disappearance rate of caffeic acid was identified in fecal samples from caffeic acid producers than in feces from CA non-producers. The increased caffeic acid was produced by the hydrolysis of caftaric acid because the cichoric acid was not increased.

With respect to metabolism of *H. perforatum* phenolics by cleaving off rhamnose with a microbial glucosidase, some studies were done in microbial metabolism of rutin, hyperoside, and quercetin *in vitro*. Rutin (quercetin-3-rutinoside) was transformed into hyperoside (quercetin-3-glucoside) by splitting off a rhamnose molecule. Then the sugar moiety in quercetin glycoside (hyperoside) was deglycosylated by microbial glucosidase to quercetin which was degraded by intestinal microbes to 3, 4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid,

and m-hydroxyphenylpropionic acid (mHPP) (Baba et al., 1983; Figure 3.2). In present study, although the hyperoside was not increased, the amount of hyperoside at 3 to 6 h incubation seemed to be partly a product of rutin metabolism because hyperoside degradation was significantly slower than degradation of rutin (Figure 3.4).

To investigate the biotransformation of flavonoid metabolites by member of the microbiota, *Eubacterium ramulus*, a strictly anaerobic bacterium in the gastrointestinal tract and a quercetin-3-glucoside-degrading anaerobic microorganism, was isolated from human feces and incubated with flavonoids. *E. ramulus* cleaved the ring structure of flavonols and flavones generating hydroxyphenylacetic and hydroxyphenylpropionic acids, as well as acetate and butyrate (Schneider et al., 2000; Blaut et al., 2003).

The bioavailability of flavonoids was influenced by the metabolism of the microbiota in the intestine. Rutin was absorbed as quercetin because it was hydrolysed by the cecal microbiota from rats receiving dietary rutin (Manach et al., 1997). Within 24-48 h of incubation, using a new in vitro model system the deglycosylation of rutin and the degradation of its aglycone quercetin were investigated by fresh pig caecal inocula and 6 wk/5 months frozen inocula. The pattern of quercetin and rutin degradation products was similar in both approaches. The preservation of the microbial vitality and the metabolic efficiency of fresh or frozen-preparations were independent of time and locality (Keppler et al., 2006). In this model system, the microbiota were directly recovered from the cecal lumen of pigs which was identified by fluorescence in situ hybridization (FISH) using 16S rRNA-based oligonucleotide probes and confirmed the suitability for studying metabolism by the human microbiota (Hein et al., 2008). The microbial degradation of quercetin with different aglycones, sugar moieties, and types of glycosidic bonds were investigated. It was concluded that various quercetin glucosides were almost completely metabolized by the intestinal microbiota within 20 min to 4 h depending on the sugar moiety and the type of glucosidic bond. The liberated aglucones completely disappeared within 8 h during anaerobic fecal incubations (Hein et al., 2008).

In conclusion, caffeic acid derivatives in *Echinacea purpurea* extracts as well as rutin/hyperoside in *Hypericum perforatum* extracts were degraded by human fecal and mouse cecal microbiota. The compound m-hydroxyphenylpropionic acid was generated by microbial fermentation. Caffeic acid was produced during the metabolism of *E. purpurea* extract. Caffeic

acid and rutin would be more bioavailable based on lesser degradation compared with other phenolic compounds in *E. purpurea* and *H. perforatum* extracts.

Footnotes

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Table 3.1. Phenolic appearance rates of *Echinacea purpurea*/*Hypericum perforatum* extracts incubated with human feces and mouse cecal contents

Herb/Plant	Phenolic compound	Appearance rate (h ⁻¹)	
		Human fecal incubation (n=20)	Mouse cecal incubation (n=24)
<i>Echinacea purpurea</i>	Caftaric acid	-0.63±0.17 ^a	-0.59±0.10 ^a
	Cichoric acid	-0.49±0.16 ^b	-0.53±0.24 ^{ab}
	Caffeic acid	-0.40±0.23 ^{bc}	-0.36±0.16 ^c
	mHPP	0.32±0.13 [#]	0.25±0.12 [#]
<i>Hypericum perforatum</i>	Rutin	-0.57±0.13 ^a	-0.49±0.11 ^a
	Hyperoside	-0.31±0.10 ^b	-0.29±0.11 ^b

Letters 'a', 'b' and 'c' indicated $p < 0.01$ significant difference compared each compound in column with human fecal incubation or mouse cecal incubation in each Herb/Plant; [#]mHPP (m-hydroxyphenylpropionic acid) was found from *E. purpurea* in human fecal incubation/mouse cecal incubation.

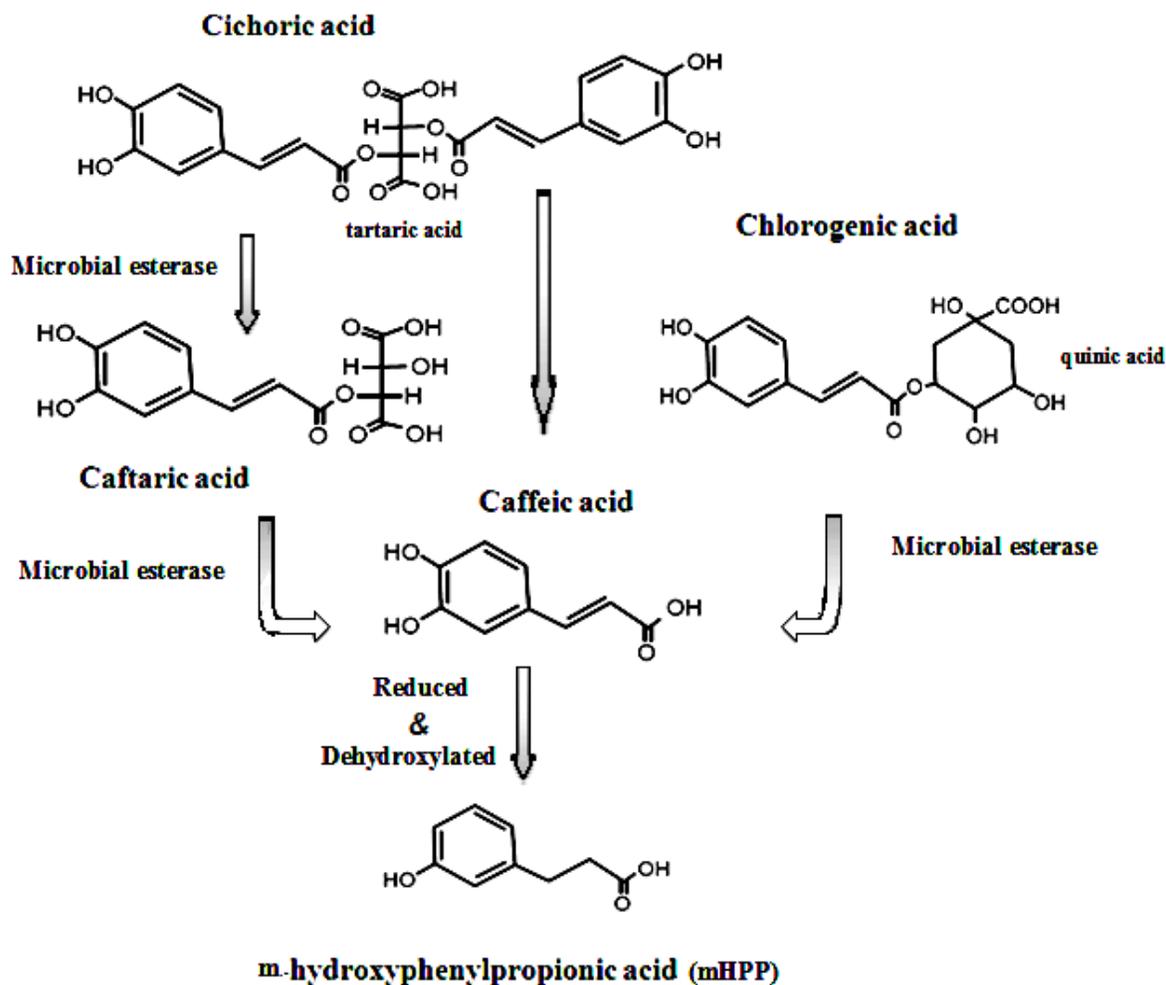


Figure 3.1. Putative microbial metabolism of caffeic acid derivatives in *E. purpurea* extract. Cichoric acid was hydrolyzed to caftaric acid and caffeic acid by microbial esterase. Then caftaric acid was also hydrolyzed to caffeic acid which was reduced to dihydrocaffeic acid (3,4-dihydroxyphenylpropionic acid); furthermore the later was dehydroxylated to m-hydroxyphenylpropionic acid (mHPP) (Peppercorn et al., 1971). In another pathway, chlorogenic acid might be split to caffeic acid by microbial esterase.

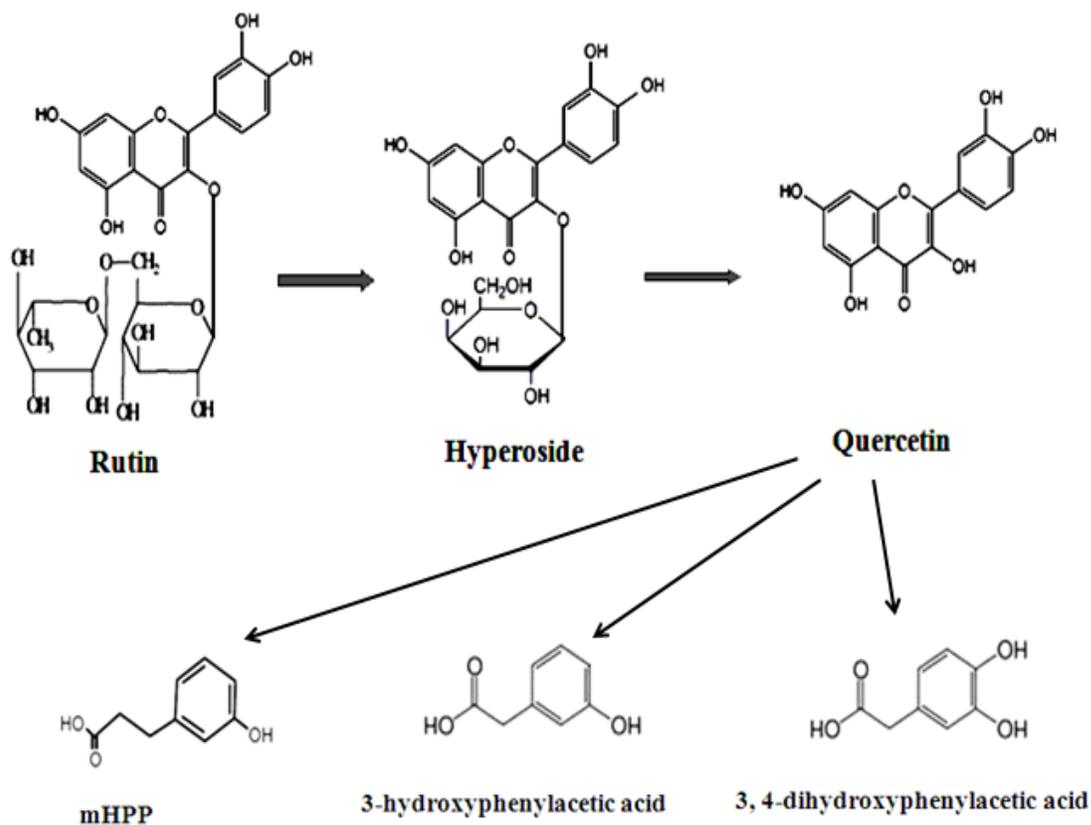


Figure 3.2. Microbial metabolism of rutin and hyperoside in *Hypericum perforatum*.

Rutin (Quercetin-3-rutinoside) was transformed into hyperoside (quercetin-3-glucoside) by splitting off a rhamnose molecule. Then sugar moiety in quercetin glycoside (hyperoside) was deglycosylated by microbial glucosidase to quercetin which was degraded by intestinal microbes to 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and m-hydroxyphenylpropionic acid (mHPP) (Baba et al., 1983).

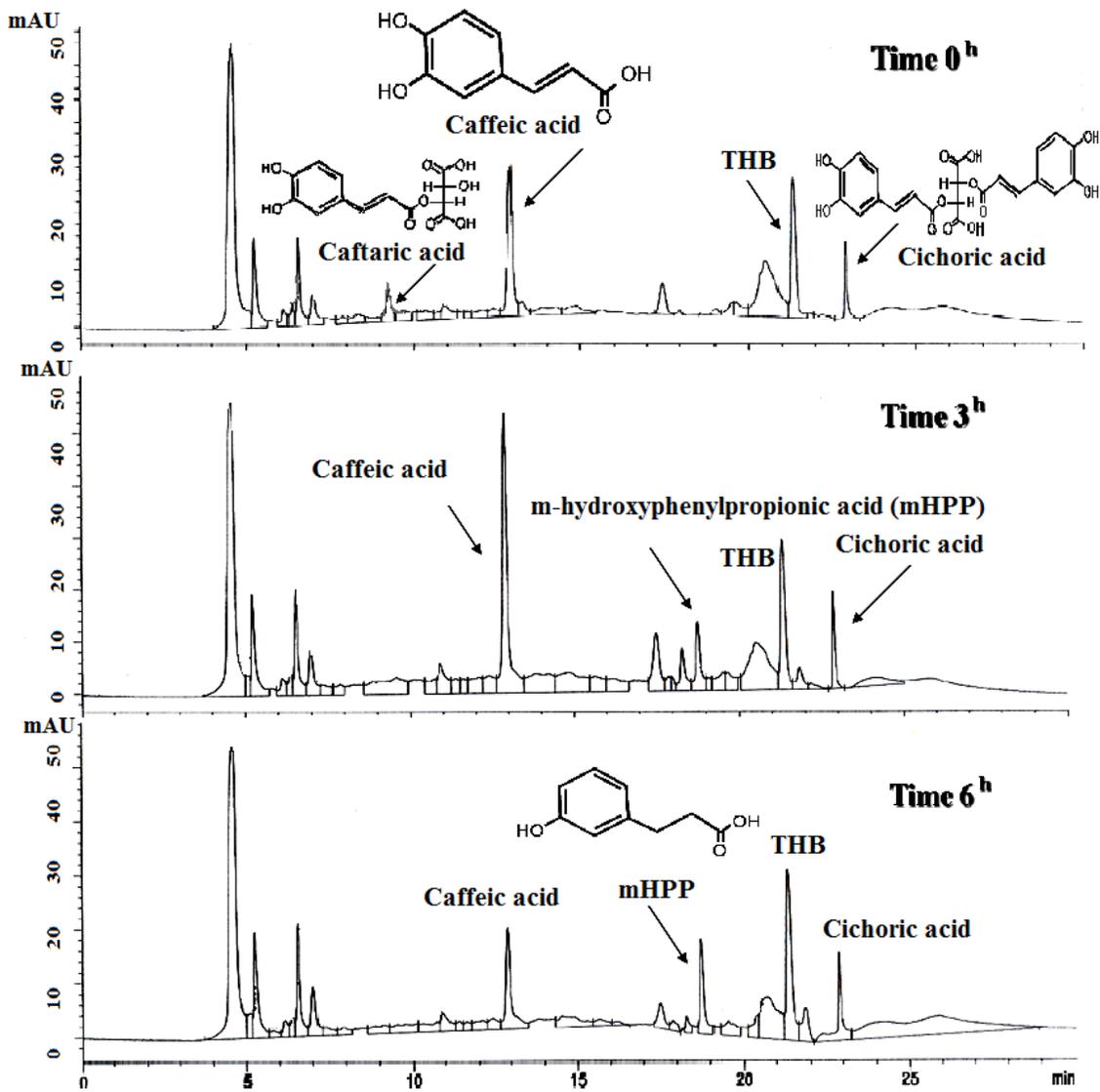


Figure 3.3. HPLC chromatogram of different times (0, 3, and 6 h) of caffeic acid derivatives in *Echinacea purpurea* extract during the human fecal incubation. At 3 h of incubation, cichoric acid and caftaric acid were decreased, whereas caffeic acid was increased and new peak was shown in this time point. At 6 h of incubation, caffeic acid was decreased and new peak was shown to further increase which was identified as m-hydroxyphenylpropionic acid (*mHPP*) by HPLC and also decreased at 12 h incubation.

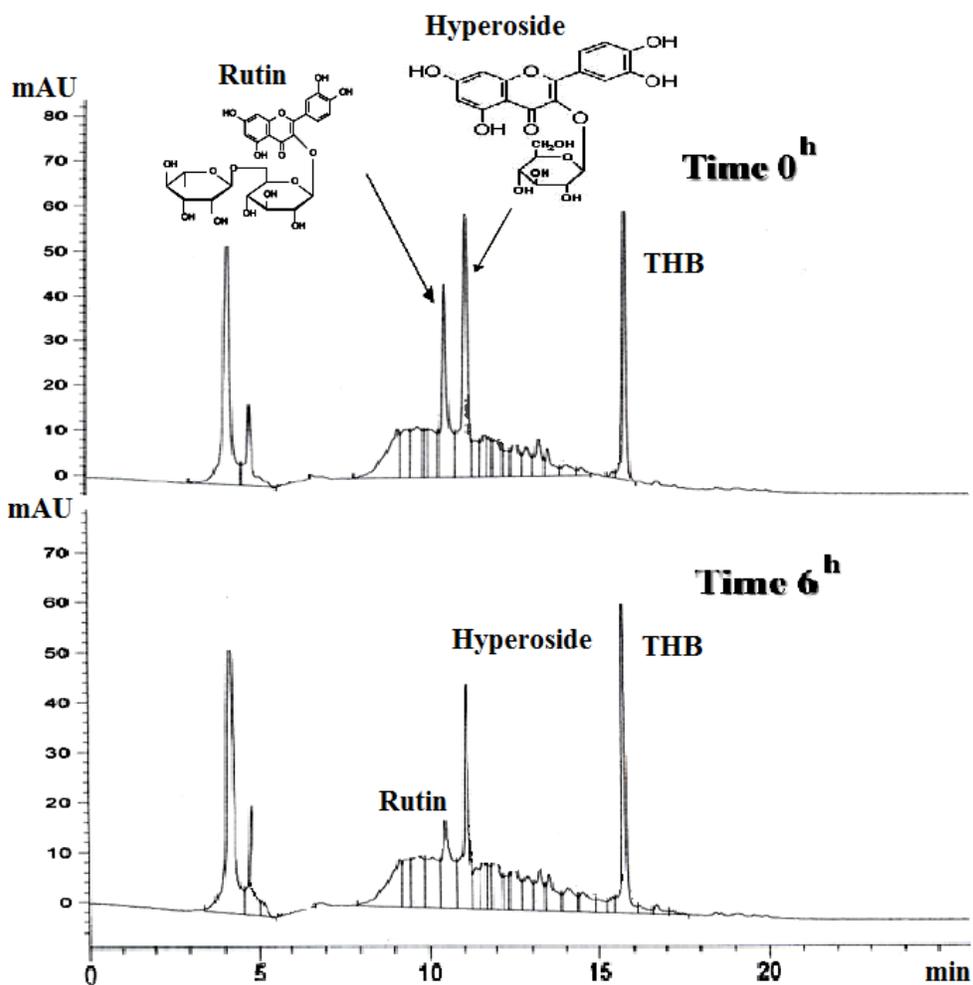


Figure 3.4. HPLC chromatogram depicting the degradation of rutin and hyperoside at two times (0 and 6 h) in *Hypericum perforatum* extract during the human fecal incubation period. After 6 h incubation, rutin was decreased quickly, whereas hyperoside was decreased slowly compared with rutin; no increased hyperoside and new peaks were found in 20 peoples and mice at any time point.

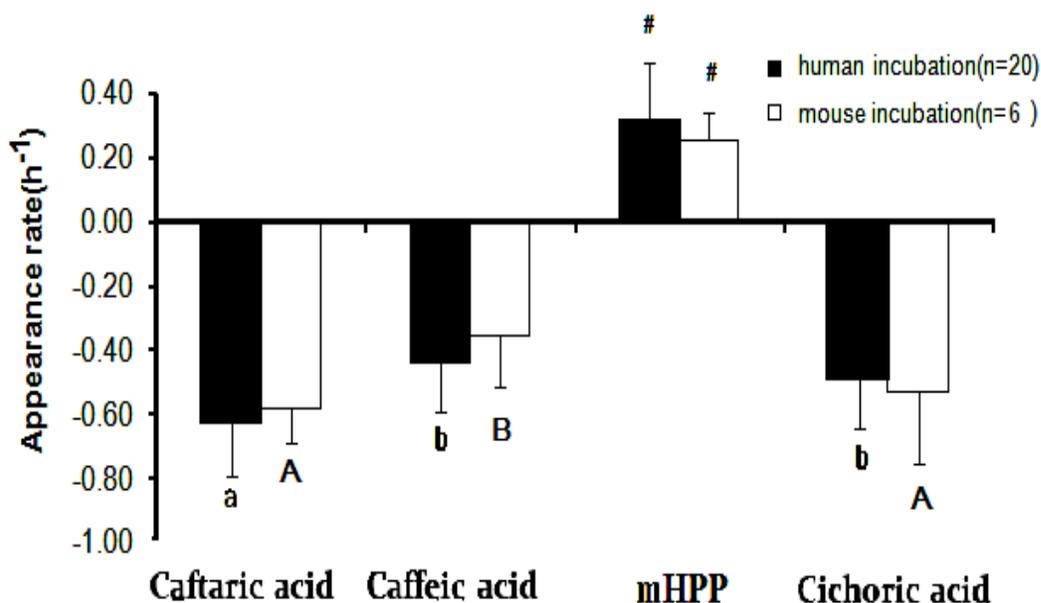


Figure 3.5. Disappearance rates of caffeic acid derivatives in *Echinacea purpurea* extract during the whole time period. Among the 3 major compounds of *Echinacea* in human fecal incubation, the degradation rate of caffeic acid was lower compared with caftaric acid and no different with cichoric acid. In mouse cecal content incubation, caftaric acid and caffeic acid degradation rates were somewhat lower and cichoric acid degradation rate was higher than in human fecal incubations. Letters 'a', 'b' indicated $p < 0.01$ significant difference compared each compound in human fecal incubations and 'A' 'B' indicated $p < 0.01$ significant difference compared each compound in mouse cecal incubations. #Appearance rate of metabolite (mHPP) was not different between the human fecal and mouse cecal incubations.

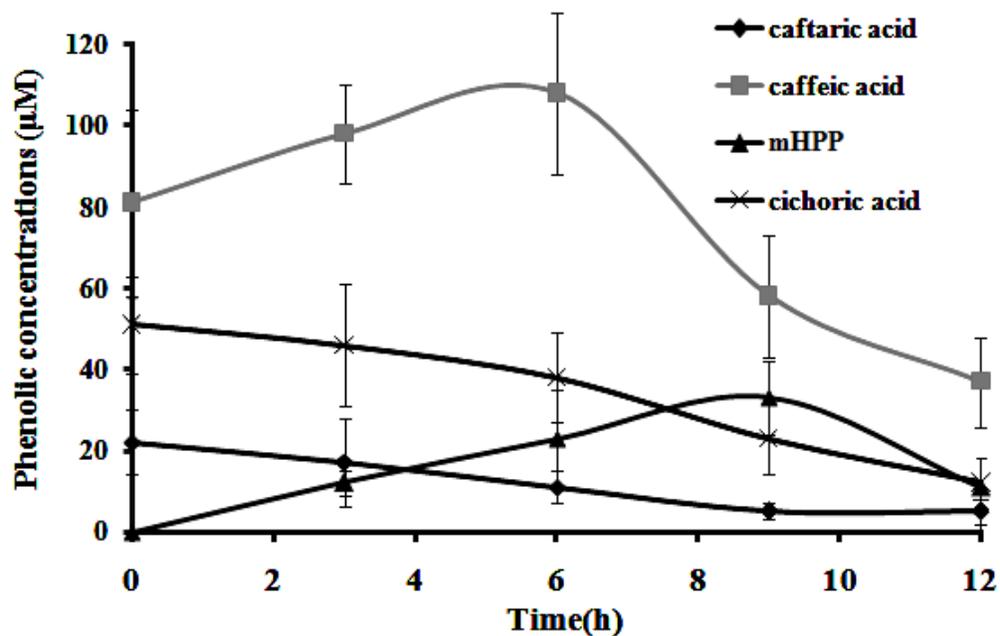


Figure 3.6. Chronological evaluation of phenolic peak areas in *E. purpurea* extract after different time points of incubation with human feces (n=6). Feces from six of 20 human subjects were found to generate caffeic acid from 3 to 6 hour of incubation. A metabolite (mHPP) was increased after 3 h to 9 h, which was also decreased after 12 h incubation.

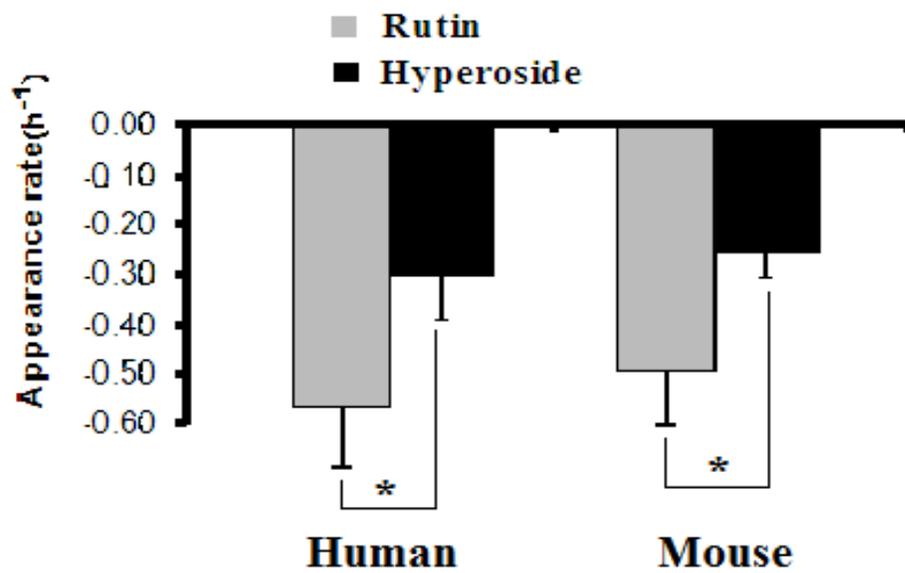


Figure 3.7. Disappearance rates of rutin /hyperoside from anaerobic incubations of *Hypericum perforatum* extract. With twenty human fecal and mouse cecal content incubations, the degradation rate of rutin was significantly greater than that of hyperoside from *Hypericum*. In mouse cecal content incubations, the degradation rates were similar pattern but slightly lower. * indicated $p < 0.01$ significant difference compared between compounds.

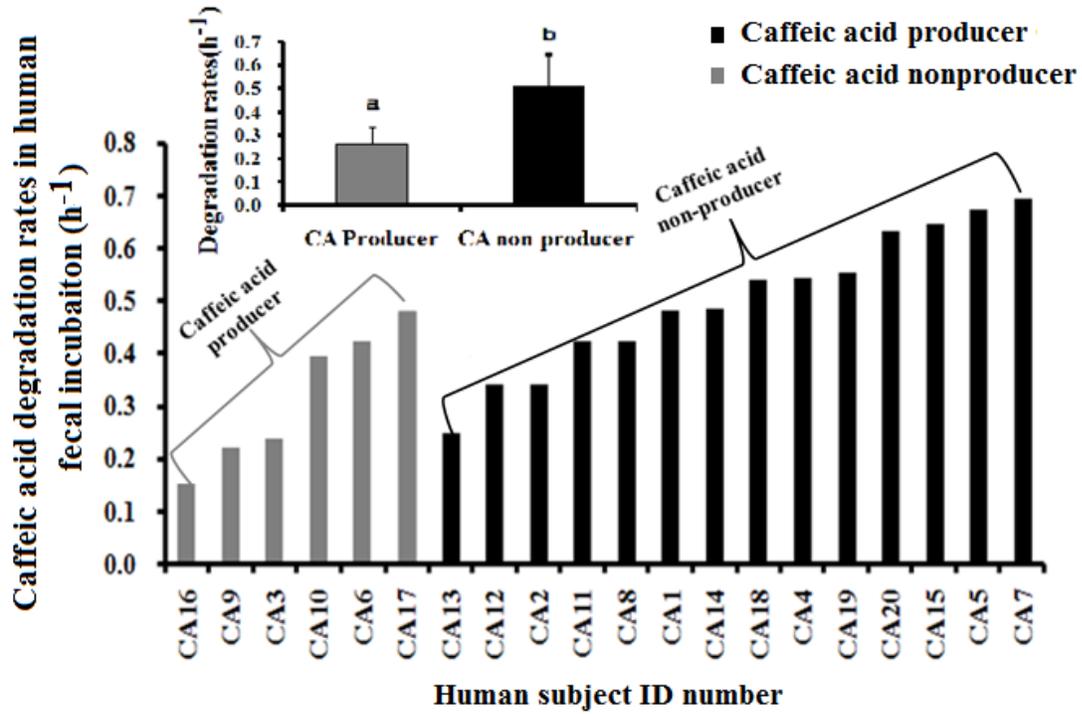


Figure 3.8. Caffeic acid (CA) producer (n = 6) and non CA producer (n = 14) in human fecal samples incubated with *Echinacea purpurea* extract. Letters 'a' and 'b' indicated $p < 0.01$ significant difference in CA degradation rate compared between the two CA producer phenotypes.

CHAPTER 4. DETERMINATION OF HUMAN ORAL MICROBIAL PHENOLIC DEGRADATION CAPABILITY AND IDENTIFICATION OF BACTERIAL SPECIES ASSOCIATED WITH PHENOLIC-DEGRADING IN HUMAN ORAL CAVITY¹

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Abstract

Certain phenolics in foods are less feasible candidate protectors against periodontal disease than are other phenolics because their antibacterial and anti-inflammatory efficacies may be inhibited by oral phenolic-degrading microbes. We hypothesized that human oral bacteria had significant but highly variable phenolic degradation capability and the phenolic degradation rates were related to structure modification. To assess oral phenolic degradation and identify microbes which were present in the human oral cavity, Caucasian (2 men, 7 women) and Asian (3 men, 8 women) subjects who had no dental disease and were not taking dietary supplements provided 5-7mL saliva before toothbrushing. An aqueous ethanolic 7-compound mixture of rutin, caffeic acid, daidzein, quercetin, naringenin, luteolin and myricetin (each 100 μ M) was incubated with saliva in anaerobic BHI media. Incubation tubes were placed at 37°C and concentrations of phenolics were assessed by taking duplicate samples from each tube at 0, 3, 6, 9, 12 and 24h. Saliva DNA was extracted, bacterial 16S rDNA sequences amplified by PCR and separated by denaturing gradient gel electrophoresis (DGGE). HPLC analysis showed that oral degradation rates of the compounds differed as follows: caffeic acid = rutin > quercetin = myricetin = naringenin > luteolin > daidzein ($p < 0.05$). Cluster analysis showed statistically significant differences in compound degradation rates between high and low degraders of rutin, caffeic acid and naringenin ($p < 0.05$). A significant positive correlation between rutin and naringenin degradation rates with higher caffeic acid degradation rate was found ($r = 0.61$, $p < 0.05$; $r = 0.55$, $p < 0.05$, respectively). Two human saliva microbial DNA bands were associated with greater caffeic acid degradation rate. Two different bands were associated with greater rutin degradation rate. Sequencing of 16S rDNA from bands of interest showed concordance with species of the *Actinomycetales* Order (*Streptomyces coelicolor* and *Streptomyces avermitilis*) for

greater caffeic acid degradation rate and *Lactobacillus brevis*/*Lactobacillus reuteri* with greater rutin degradation rate, thus these species may affect human oral degradation of these phenolics and prevention of gum disease by phenolic treatments.

Introduction

Dietary factors that might prevent periodontal disease have not been well explored, and both microbial pathogens and inflammatory reactions in gum tissue might be targets for dietary phytochemicals. Flavonoids are commonly occurring and diverse dietary phytochemicals that may have antibacterial activity (Hatano et al., 2005). The exposure of the oral cavity to flavonoids may occur throughout a meal or over longer time periods in use of flavonoids that would fit around the teeth and gums. In this study, we assess human oral microbial flavonoid degradation capability while identifying which bacterial species associated with flavonoid degradation are present in the human oral cavity. Based on our previous work, several human gut microbial species were identified to degrade isoflavones, and would therefore be highly likely to degrade flavonoids in general using microbial DNA extracted from human fecal samples and from high and low flavonoids degraders with amplification of microbial sequences by PCR of 16S rRNA gene variable regions (Muyzer et al., 1993). These DNA sequences were separated by denaturing gradient gel electrophoresis and microbial species tentatively identified by match with existing databases (Renouf, 2005). Several *Prevotella* spp. (*pallens*, *oralis*, *Bacteroides acidifaciens* and *uniformis*, *Bacillus fragilis* and *eggerthii*, *Tannerella forsythensis*) significantly increased isoflavone disappearance. *Prevotella intermedia* and *Tannerella forsythensis* were shown to be human oral microbes associated with periodontal pathogenesis (Sakamoto et al., 2005). Thus some of the same species responsible for flavonoid degradation are associated with periodontal disease. Our main hypotheses was that humans with different oral salivary phenolic degradation phenotypes had different oral microbial ecologies and that individuals sharing similar in vitro oral salivary degradation rates also possessed similar microorganisms that could be involved in degrading phenolics as reflected by PCR-DGGE analysis. The long term goal is to determine how to optimize dietary habits and constituents, oral microbial ecology and other factors in the human oral epithelium to achieve the greatest protection against periodontal disease. This research may help to improve the quality of life, especially for older populations,

and may result in novel products, such as flavonoid-containing chewing gum or biofilms for overnight use for prophylaxis against periodontitis.

Materials and Methods

Chemicals and reagents

Purified rutin, caffeic acid, daizein, quercetin, naringenin, luteolin and myricetin were purchased from Chroma Dex™, Inc. Santa Ana, CA. 2,4,4'-Trihydroxydeoxybenzoic acid (THB) were synthesized using the method (Song et al., 1998). High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, acetic acid, dimethyl sulfoxide (DMSO), and all other chemicals were from Fisher Scientific (Fairlawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Human subjects

This project was approved by the Institutional Review Board of Iowa State University. Caucasian (2 men, 7 women) and Asian (3 men, 8 women) subjects were recruited at Iowa State University and surrounding areas for this study. Eligibility criteria included age of 19 to 53 years, no use of antibiotics in the past three months, no dental disease, and no current use of dietary supplements or herbs. Users of oral contraceptives or smokers were not excluded.

Phenolic incubation

The anaerobic fermentation of flavonoids in hamster cecal content and human gut microflora with Brain-heart infusion (BHI) broth media was developed to investigate the bioavailability of phenolic compounds (Renouf, 2005; Simons et al., 2005). In this study, saliva (5-7mL) was collected in the morning without tooth brushing from the previous evening. Saliva was put into individual anaerobic BHI media tube with an ethanol 7-compound mixture of rutin, caffeic acid, daizein, quercetin, naringenin, luteolin and myricetin (each 100μM). Incubation tubes were placed at 37°C and over time concentrations of phenolics were assessed by taking duplicate samples from each tube at 0, 3, 6, 9, 12 and 24h. All the samples taken were put at -60°C until extraction and HPLC analysis. Time 0 and 24 h salivary samples were collected for identifying microbial profile using PCR-Denaturing Gradient Gel Electrophoresis (DGGE) method. Incubation tubes were maintained anaerobically at all times. Negative controls consisted of the salivary suspension without 7-compound mixture. Microbial degradation by the salivary suspension was confirmed by positive controls, which consisted of BHI media and 7-compound

mixture without the salivary suspension, as well as another control which consisted of BHI media and 7-compound mixture with the sterile-filtered salivary suspension using 22mm PFTE filters (Alltech, Deerfield, IL).

Phenolic extraction from salivary incubation samples and HPLC analysis

Phenolic extraction from salivary samples in vitro incubations was based on the protocol from Zheng et al. (2003). Fifty μL of 2mg/mL THB in 80% methanol, as an internal standard, was added to the thawed salivary samples and slowly loaded onto preconditioned C18 solid phase extraction cartridges (Waters Corporation, Milford, MA). The cartridge was washed twice with 2 mL of Milli-Q system water. The phenolics were eluted with 1 mL of 80% methanol, filtered through 0.45 μm filters, and analyzed directly by HPLC. The HPLC system consisted of a Hewlett-Packard 1050 Series. Fifteen microliters of sample was injected onto a reversed-phase, 5 μm , C18 AM 303 Column (250 mm \times 4.6 mm) (YMC Co. Ltd., Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and 100% acetonitrile (B). Solvent B increased from 25 to 48% in 10 min, increased to 90% in 7 min, and was held for 5 min. The gradient was recycled back to 25% in 2 min for the next run. The flow rate was 1 mL/min. The wavelengths used for the preparation of standard curves, detection, and quantification of phenolic peaks were 254 and 292 nm. Chem station^{3D} software (Hewlett-Packard Company, Scientific Instruments Division, Palo Alto, CA) was used to integrate the peak area responses and to evaluate the ultraviolet absorbance spectra. Degradation rates were established by calculating the natural logarithm of the percentage remaining phenolics over time. All 4 values were considered (2 tubes/sample and 2 samples/time point) at each time point for each subject to establish the phenolic degradation rate. To quantify phenolics, standard curves for each compound were analyzed by reverse-phase HPLC using the same protocol as above.

Genomic DNA extraction from salivary samples

At baseline of the salivary incubation, 2 samples from each tube for each individual were taken and immediately frozen at -60°C . To extract DNA from salivary bacteria, a Wizard genomic DNA kit from Promega (Madison, WI) was used to extract salivary microbial DNA according to the associated protocol. DNA was quantified with NanoDrop ND-1000 UV-Vis spectrophotometer (Wilmington, DE).

Bacterial 16S rDNA amplification

Bacterial DNA was amplified using a touchdown PCR method, which decreased the annealing temperature over time (1°C every other cycle) in order to target non-specifically all bacterial 16S rRNA gene variable region sequences. Primers were synthesized to target non-specifically the constant region of the variable region of the rRNA gene. PCR was performed as described by Muyzer et al. (1993). Three conserved region primers (reverse primer 5'-ATTACCGCGGCTGCTGG-3', forward primer 5'-CCTACGGGAGGCAGCAG-3' and forward primer added with a 5' GC clamp 5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGGCACGGGGGG-3') were used to amplify the V3 region of 16S rRNA gene (positions 341–534 in the *E. coli* gene). Each PCR mixture contained (final concentration): 1µl each primer (1pMol), 1µl dNTP mixture (50pMol each dNTP), 5µl of nuclease-free PCR buffer (500mMol KCl, 100mMol Tris HCl pH=9 @ 25°C, 1.0% Triton X-100 and 15mMol MgCl₂), 28µl water and target DNA (8ng/µl PCR mixture). Primers were synthesized by Sigma Genosis (The Woodlands, TX) and PCR components obtained as a kit from Promega (Madison, WI). After the first step of the PCR program (4min 94°C), 1µl nuclease-free PCR buffer, 0.5µl water and 0.5µl Taq polymerase at 5 units /µl were added. The PCR program was established as followed: 4min 94°C, 20 cycles with touchdown: 1min 94°C, 1min 65°C (decrease 1°C every other cycle), 3min 72°C; 10 regular cycles: 1min 94°C, 1min 55°C, 3min 72°C; 7min 72°C; cool down at 4°C. PCR products should be around 200bp and were checked using a 0.1% agarose gel with ethidium bromide and a PCR ladder used as a standard (Sigma). A UV lamp was used to visualize DNA and compare it to a standard DNA PCR ladder (mixture of 50, 150, 300, 500, 750 and 1,000 base pair DNA fragments) commercially available (Sigma, St. Louis, MO). DNA concentration was measured using a Beckman DU® 640 spectrophotometer (Beckman, Schaumburg, IL) at 260 and 280nm wavelength.

Denaturing gradient gel electrophoresis

Two acrylamide stock solutions were used to prepare the gel: 100% urea denaturing solution (42 g urea, 16.9 mL acrylamide, 2 mL 50×Tris Acetate EDTA (TAE), 40 mL of 100% formamide and made to 100mL with distilled deionized (dd) water) and 0% urea denaturing solution (16.9 mL acrylamide, 2mL 50×TAE and made to 100mL with dd water). The 233 bp PCR product was purified with Promega kit and loaded to the 20-80% gradient (urea and formamide) acrylamide gel running at 60°C and 120V for 4.5hr on DCode™ Universal Mutation

Detection System (Biorad, Hercules, CA). DNA fragments were separated according to the GC content of the DNA sequence. DGGE gels were stained with nitrate silver solution and scanned by the GS-800 calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA). The bands pattern was analyzed with the Quantity One Software (Biorad, Hercules, CA).

Sequence of PCR-amplified product

The bands of interested were exercised from DGGE gel with scalpel and recovered with elution and re-amplification with the same PCR program described above. DNA eluted from acrylamide was amplified with a slightly different PCR mixture (15 μ L TE buffer containing the target DNA eluted as above, 1 μ L each primer (5 pMol), 1 μ L dNTPs mixture (20 pMol each dNTP), 5 μ L buffer with 15mMol MgCl and 24 μ L dd water. After 4min 94°C, the TAQ mixture was added as described before. After PCR, the samples were checked for length of the products (200bp, using a standard DNA ladder as above) using a 0.1% agarose gel, purified using a PCR purification kit from Promega (Madison, WI) and quantified as described before. For sequencing, PCR samples concentrations were 50 μ g/ μ L and the primer with the lowest T_m or %GC was at 10pmol/L. Sequencing was performed by the Iowa State University DNA sequencing facility and results checked to confirm sequence identity. Sequences were searched against the Ribosomal Database Project II (<http://rdp.cme.msu.edu>) to get the candidate bacteria information. 100 possible matches were provided for each sequence and the possible matches that corresponded to known microorganisms were considered as possible species that degraded phenolics.

Statistical analysis

All statistical analyses were performed using the SAS Institute (2003, Cary, NC). One-way ANOVA followed by Tukey's method as a Post Hoc test and multiple comparisons were used to compare band intensity, degradation rates for each compound and between clustered phenolic degradation phenotypes. Data were reported as means \pm SEM. Statistical significance was set at $P < 0.05$.

Results and Discussions

In vitro screening of oral salivary degradation rates

Twenty subjects were screened for their oral salivary phenolic degradation over 24h with a mixture including rutin, caffeic acid, daizein, quercetin, naringenin, luteolin and

myricetin. A representative HPLC chromatogram showed retention times with UV detection of the seven compounds (each 4mM) in saliva incubation mixtures (Figure 4.2). Daidzein, luteolin, quercetin, and myricetin showed relatively similar retention times, but were separable. Rutin, caffeic acid and naringenin were more easily separated from the other compounds (Figure 1). The saliva degradation rates of each phenolic compound showed high variability. The degradation rate of caffeic acid ($k = 0.11 \pm 0.07 \text{ h}^{-1}$) was similar to that of rutin ($k = 0.10 \pm 0.04 \text{ h}^{-1}$) ($p > 0.05$); both rates were greater ($p < 0.05$) than that of quercetin, myricetin or naringenin (average $k = 0.07 \pm 0.05 \text{ h}^{-1}$); the three degradation rate of quercetin or myricetin or naringenin were not different; whereas they were greater than the degradation rate of luteolin ($k = 0.04 \pm 0.02 \text{ h}^{-1}$) which was significantly greater than that of daidzein ($k = 0.01 \pm 0.02 \text{ h}^{-1}$) ($p < 0.05$) (Figure 4.3.A). The degradation rate of monophenol and glucoside (caffeic acid & rutin) were the fastest; both 5-OH and 3-OH (quercetin & myricetin) were faster than 5-OH only (luteolin); the non-5-OH was the slowest (daidzein) (Figure 4.3.B). In our previous study, the anaerobic fermentation with 14 flavonoids in human gut microflora ($n = 11$ subjects) showed the 5,7,4'-trihydroxyl flavonoids (apigenin, genistein, naringenin, and kaempferol) disappeared quickly compared to the other structural motifs. The degradation rate of naringenin ($k=0.3 \pm 0.02 \text{ h}^{-1}$) was significantly greater than that of daidzein ($0.1 \pm 0.02 \text{ h}^{-1}$) (Simons et al., 2005). In the present study, the degradation rates of quercetin, myricetin, naringenin and luteolin, all of which belonged to the 5,7,4'-trihydroxyl flavonoids category, were also faster than daidzein in salivary degradation level except for caffeic acid and rutin. Compared with our previous twenty human fecal incubation study, the degradation rate of rutin was $k = 0.57 \pm 0.13 \text{ h}^{-1}$ in *Hypericum* ethanolic extract. In *Echinacea*, the degradation rates of caffeic acid was $k = 0.40 \pm 0.23 \text{ h}^{-1}$. Because of the much higher amount of bacteria in the fecal incubations than that of salivary sample, the comparison was not reasonable between these two studies. However, the relative degradation rates of caffeic acid and rutin in this study were also higher than other compounds including 5,7,4'-trihydroxyl flavonoids category and isoflavones.

DGGE of oral salivary microbial DNA in high vs. low phenolic degraders

Cluster analysis of caffeic acid, naringenin and rutin degradation rates was shown that high and low degraders in 7-compound in mixture. Two subjects (#1 and #4) were stable as higher degraders of rutin, caffeic acid and naringenin; six subjects including #2, #5, #8, #11, #12, #19 were stable low degraders of these three compounds (Figure 4.4). Higher and lower

degraders subgroups showed significantly different degradation rates for caffeic acid, rutin and naringenin ($p < 0.05$); whereas the higher and lower degradation rate clusters for naringenin were not significantly different ($p > 0.05$) (Figure 4.5). Degradation rates of caffeic acid compared with rutin or naringenin degradation rates showed significant positive correlations ($r = 0.61$, $p < 0.05$; $r = 0.55$, $p < 0.05$, respectively) (Figure 4.6).

Sequencing and identification of microorganisms from the bands of interest

Sequencing and matching of the sequences using BLAST Assembled Genomes and Ribosomal Database Project reported for each band 100 possible matches to known and unknown bacteria. The overall results from all bands associated with caffeic acid and rutin degradation showed strong similarities in microorganism identification. Saliva microbial profiles of high and low degraders of rutin showed two DNA bands in five higher rutin degraders as well as one DNA band associated with five lower rutin degraders. Sequencing of 16S rDNA from “Band 1” of interest showed concordance with known species, *Lactobacillus brevis*/*Lactobacillus reuteri* in higher rutin degraders (Figure 4.7). Oral microbial profile of high and low degraders of caffeic acid showed two DNA bands in common among the five high caffeic acid degraders as well as one DNA band in common among with the five lower caffeic acid degraders. Sequencing of 16S rDNA from “Band 2” of interest showed concordance with known species of the *Actinomycetales* Order (*Streptomyces coelicolor* /*Streptomyces avermitilis*) in higher caffeic acid degraders (Figure 4.8). Gel densitometry showed that the intensity of bands of interest in higher degraders of rutin was significantly greater than that of those bands in the lower degraders of rutin ($P < 0.05$) (Figure 4.9); similar band density differences were seen between high and low degraders of caffeic acid ($P < 0.05$) (Figure 4.10).

We have confirmed the phenolic degrading ability of several human oral microbial species associated with high oral phenolic degradation rates. Species within the *Actinomycetales* Order (*Streptomyces coelicolor* /*Streptomyces avermitilis*) in higher caffeic acid degrader may be related to some periodontal diseases (Guthmiller et al., 1993; Sakamoto et al., 2005). However, the association of *Lactobacillus brevis*/*Lactobacillus reuteri* in higher rutin degrader with periodontal pathogenesis is unclear. The complexity of oral microflora and how oral microbial ecology may be associated with oral disease is not well elucidated. For future studies, we propose a model system to study effects of phenolics using oral epithelial cells, oral pathogenic bacteria, and oral bacteria that degrade potentially protective plant phenolics, to simulate

interactions in the human oral cavity as a “proof of concept” before further animal model or human clinical trials with plant phenolics of interest.

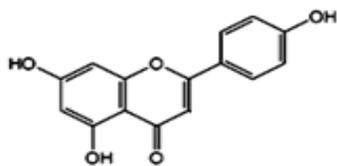
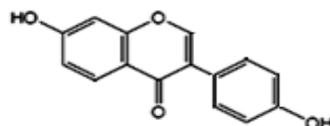
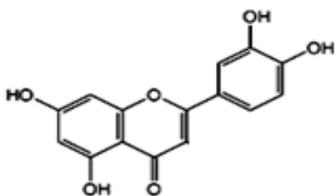
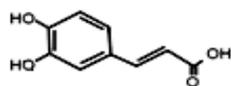
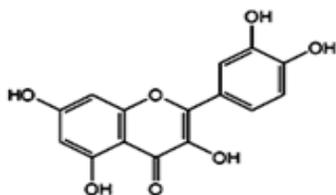
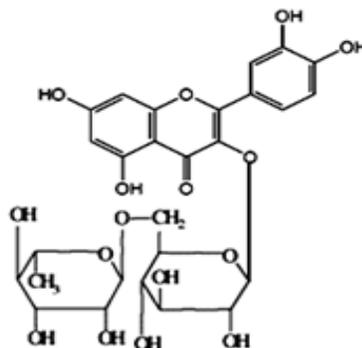
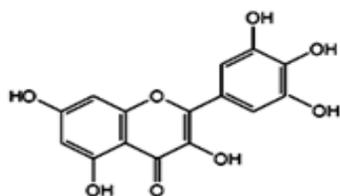
Footnotes

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**Naringenin****Daidzein****luteolin****Caffeic acid****Quercetin****Rutin****Myricetin****Figure 4.1.** Structure of seven phenolic compounds

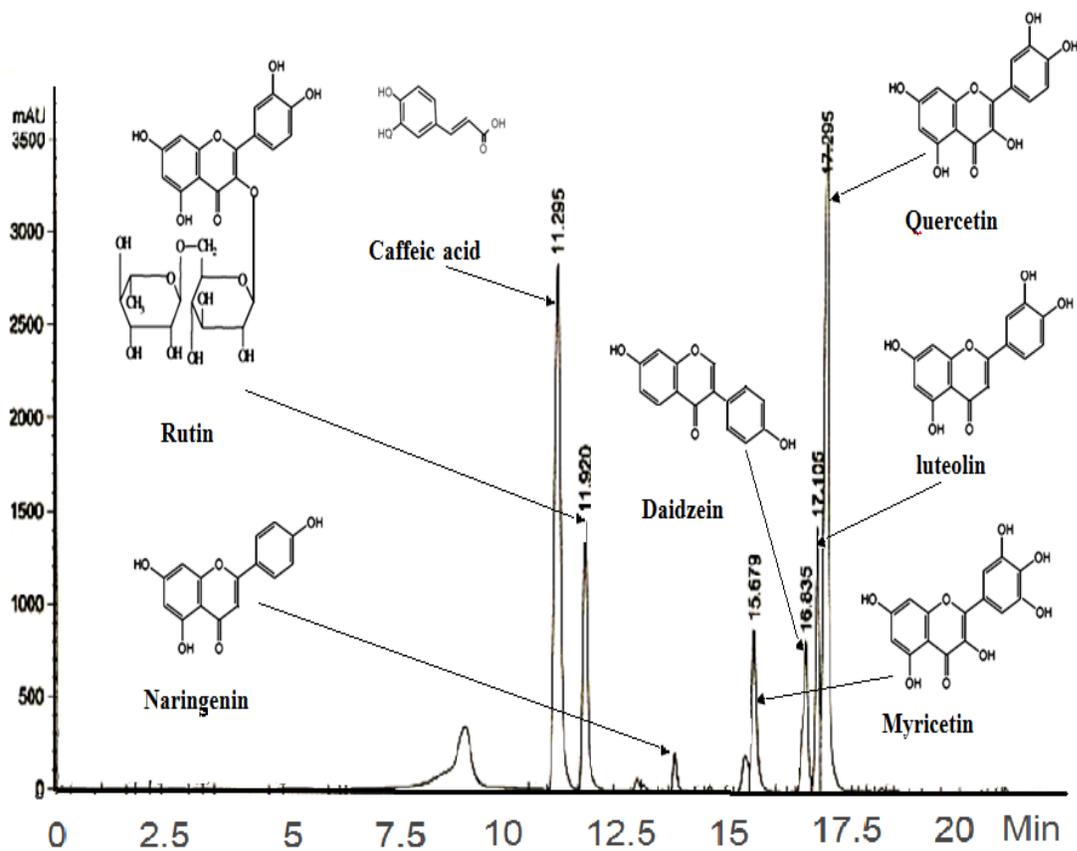


Figure 4.2. HPLC chromatogram was shown that the retention time and UV absorbance of the seven compounds (each 4mM) in salivary incubation at baseline.

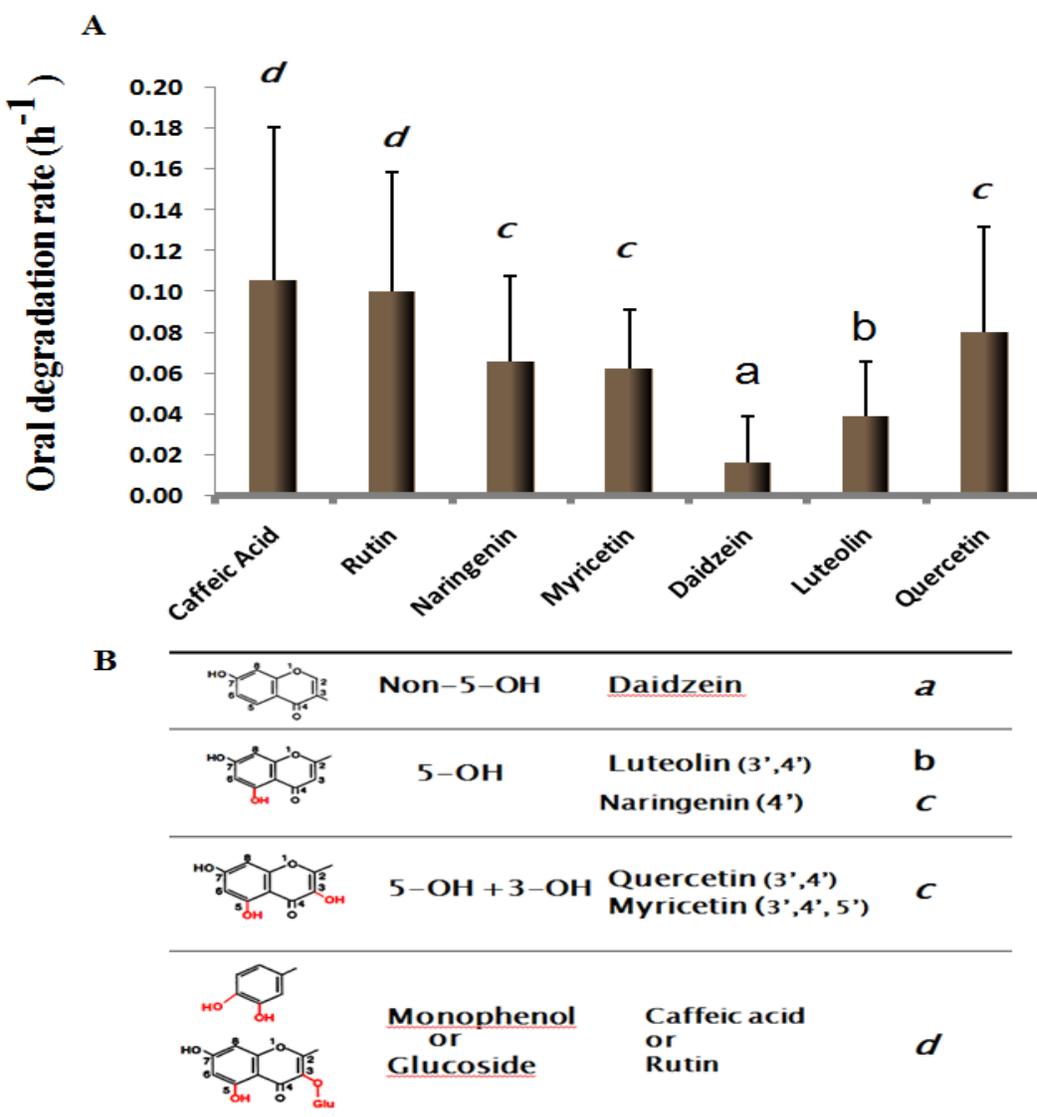


Figure 4.3A. Oral salivary degradation rates of each phenolic compound in salivary incubation showed that the degradation rate of caffeic acid was similar to that of rutin ($p > 0.05$); the both rates were greater ($p < 0.05$) than that of quercetin or myricetin or naringenin which of the later three were no difference; whereas they were greater than that of luteolin and luteolin was greater than that of daizein significantly ($p < 0.05$). **Figure 4.3B.** The degradation rate of monophenol and glucoside (caffeic acid & rutin) were the fastest; both 5-OH and 3-OH (quercetin & myricetin) were faster than 5-OH only (luteolin); the non-5-OH was the slowest (daidzein). 'a, b, c, and d': $p < 0.05$ indicated the significant difference compared with each other.

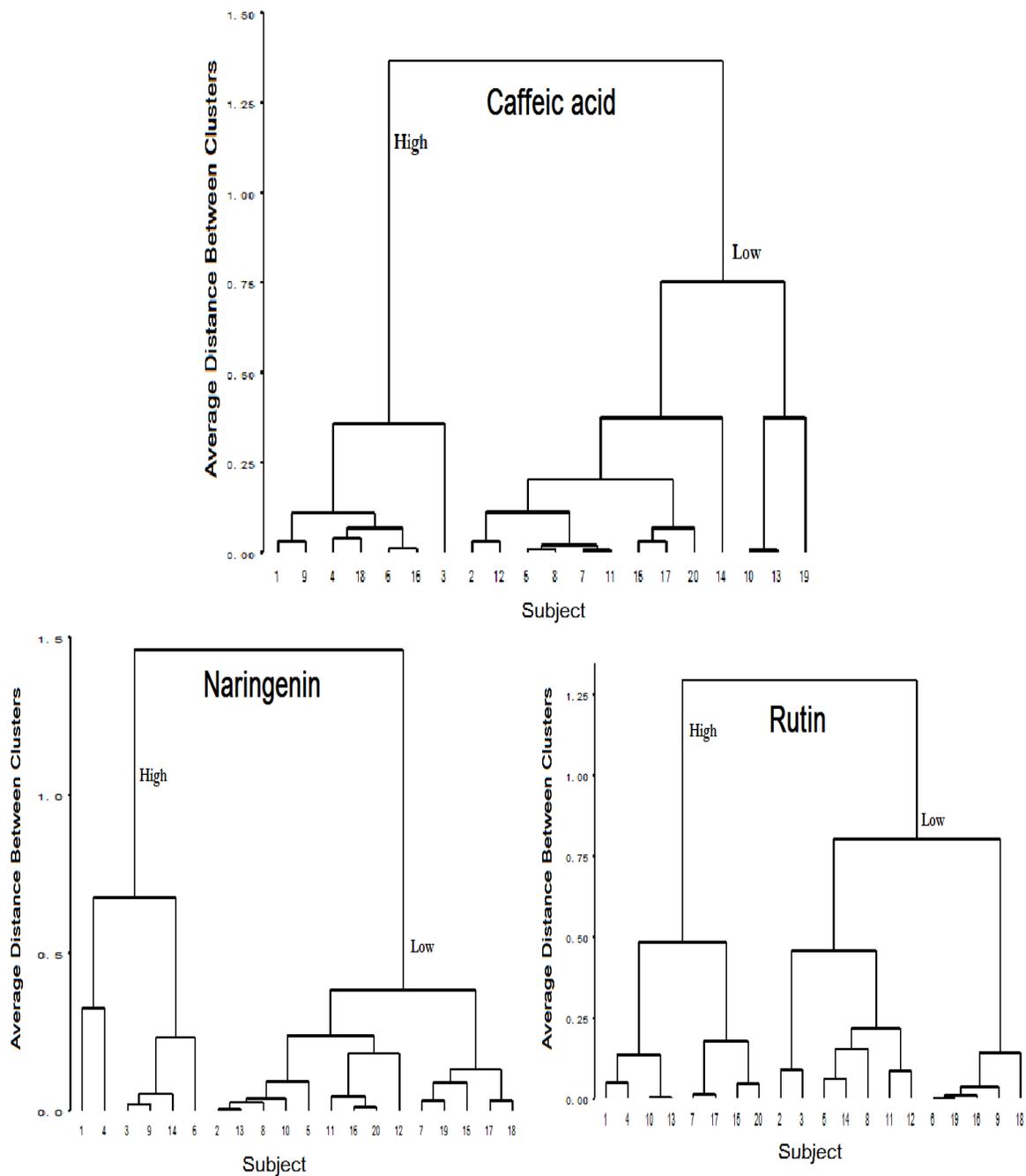


Figure 4.4. Cluster analysis of caffeic acid, naringenin and rutin degradation rates was shown that high and low degraders in 7-compound in mixture. Two subjects (#1 and #4) were stable higher degraders of rutin, caffeic acid and naringenin; six subjects including #2, #5, #8, #11, #12, #19, were stable lower degraders of the three compounds.

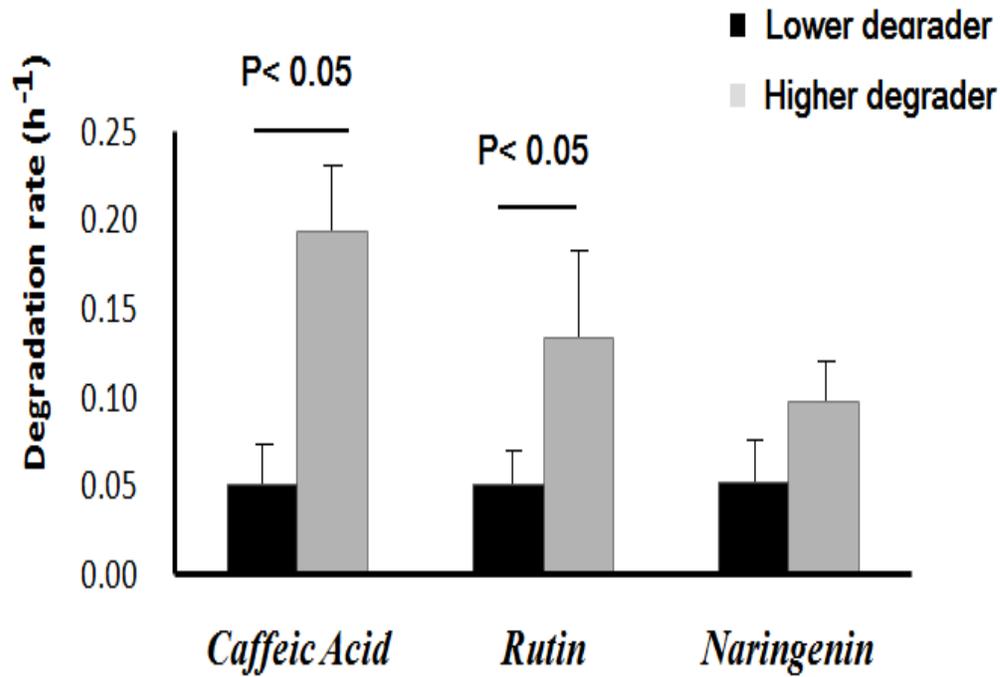


Figure 4.5. Higher and lower degraders of caffeic acid, rutin and naringenin were significantly different ($p < 0.05$); whereas the higher and lower degraders of naringenin were not significantly different ($p > 0.05$) based on the two clustered subgroups.

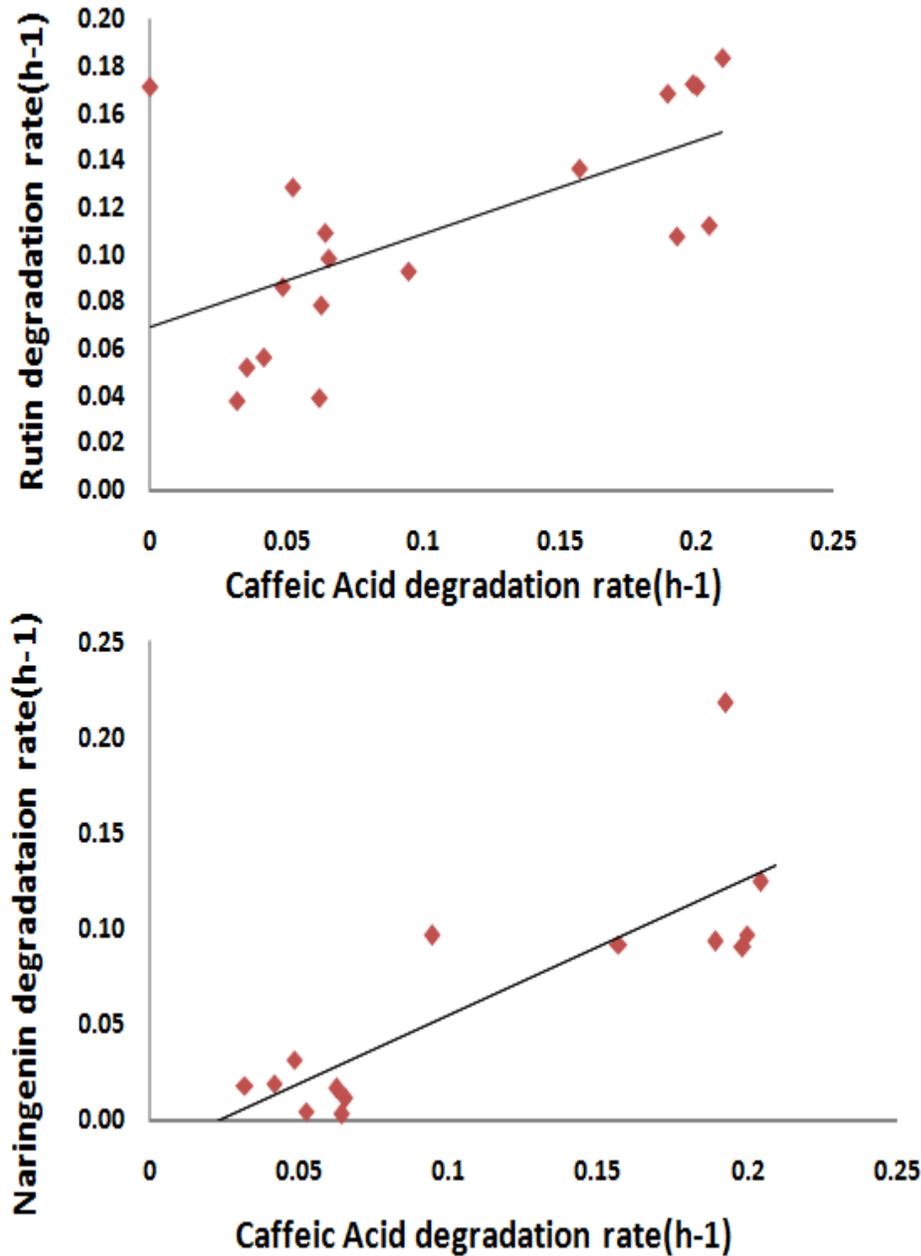
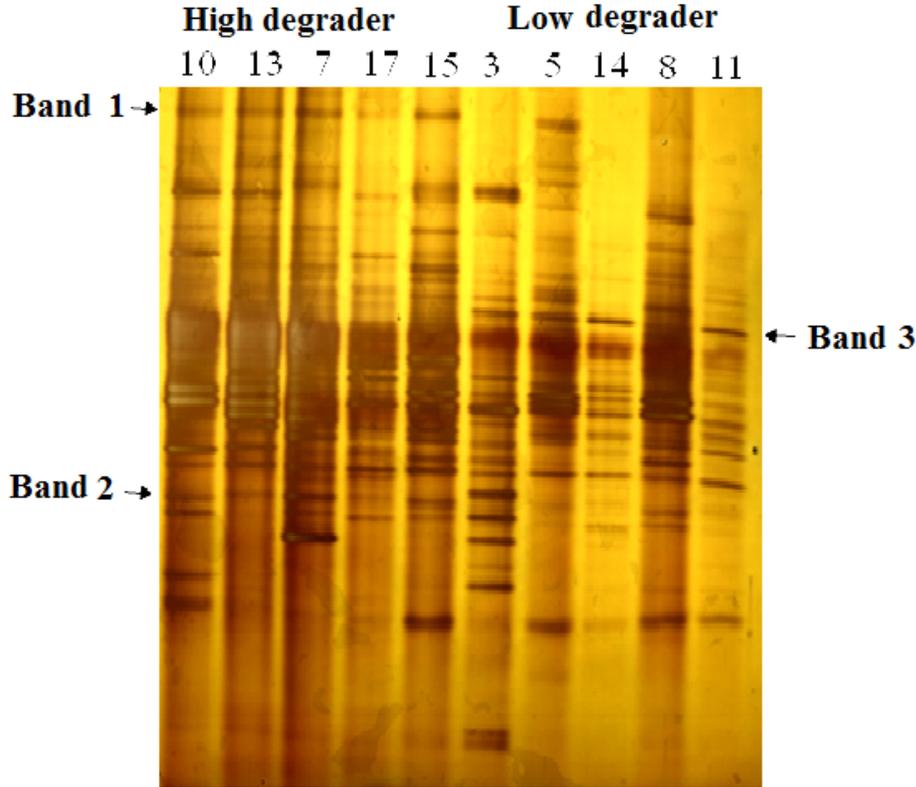


Figure 4.6. Correlation of caffeic acid with rutin or naringenin was shown that a significant positive correlation between caffeic acid and rutin or naringenin with higher caffeic acid degradation rate associated with greater rutin or naringenin ($r = 0.61$, $p < 0.05$; $r = 0.55$, $p < 0.05$, respectively).



Lactobacillus brevis / *Lactobacillus reuteri*

Figure 4.7. Saliva microbial profile of high and low degraders in rutin cluster was displayed by the DGGE gel. Two DNA bands were associated with five higher rutin degraders as well as one DNA band was associated with five lower rutin degraders. Sequencing of 16S rDNA from “Band 1” of interest showed concordance with known species, *Lactobacillus brevis*/*Lactobacillus reuteri* in higher rutin degrader using BLAST Assembled Genomes and Ribosomal Database Project.

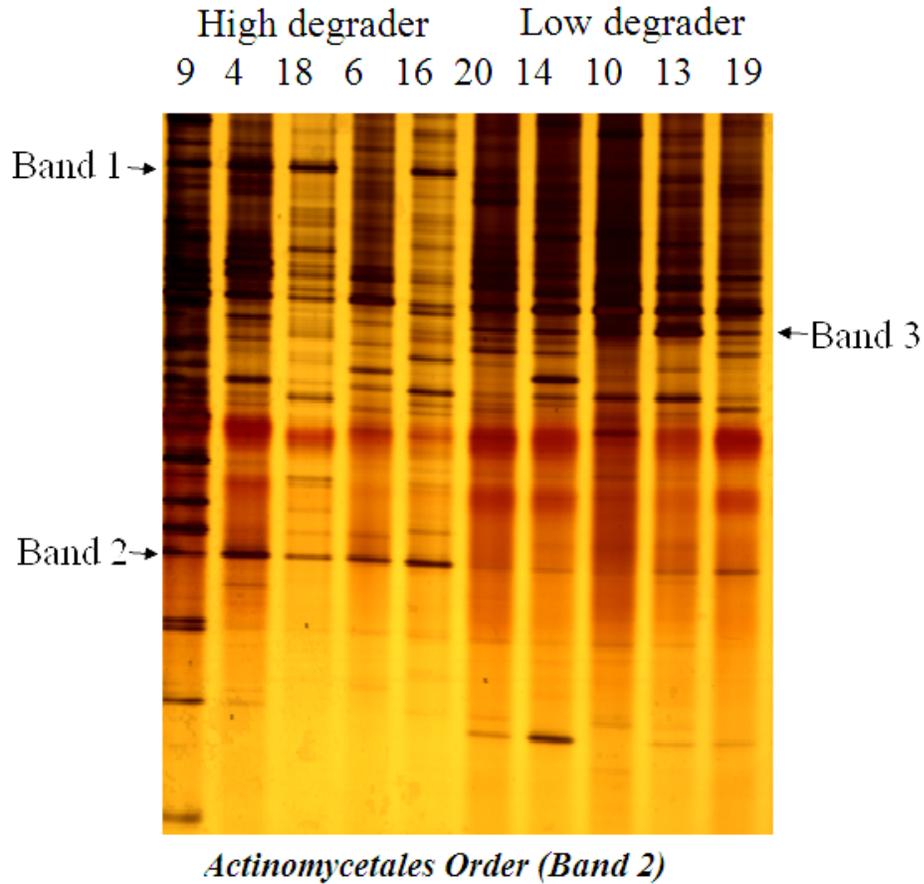


Figure 4.8. Saliva microbial profiles of high and low degraders of caffeic acid were displayed by the DGGE gel. Two DNA bands were associated with five higher caffeic acid degraders as well as one DNA band was associated with five lower caffeic acid degraders. Sequencing of 16S rDNA from “Band 2” of interest showed concordance with known species as the *Actinomycetales* Order (*Streptomyces coelicolor* /*Streptomyces avermitilis*) in higher caffeic acid degraders using BLAST Assembled Genomes and Ribosomal Database Project.

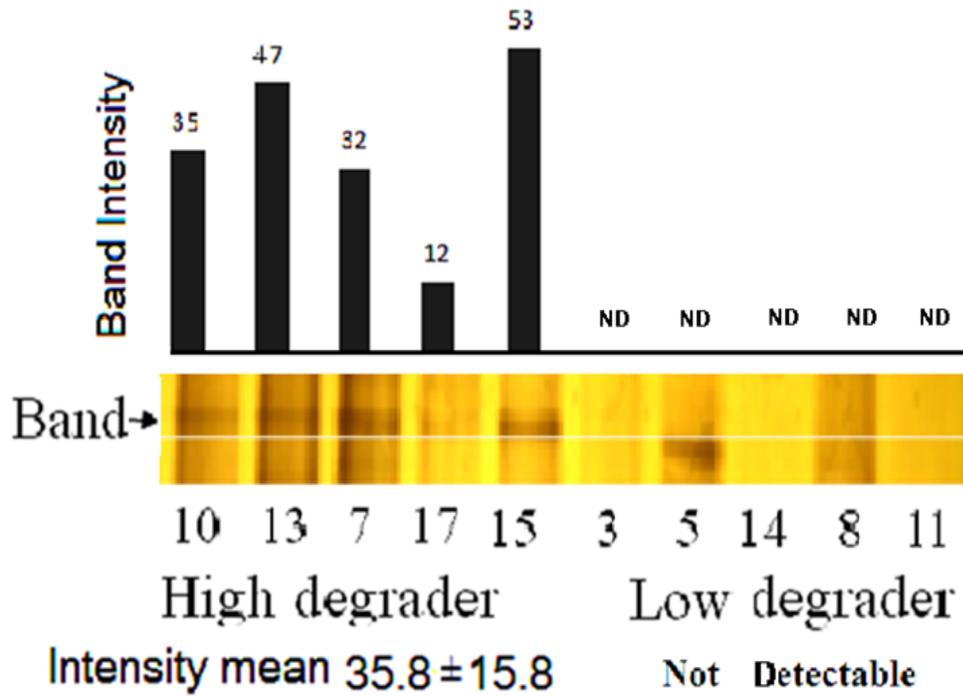


Figure 4.9. Band intensity was shown in each subject between the high and low degrader in rutin degradation; the intensity test showed that the intensity of higher degrader of rutin was significant greater than that of lower degrader of rutin ($P < 0.05$).

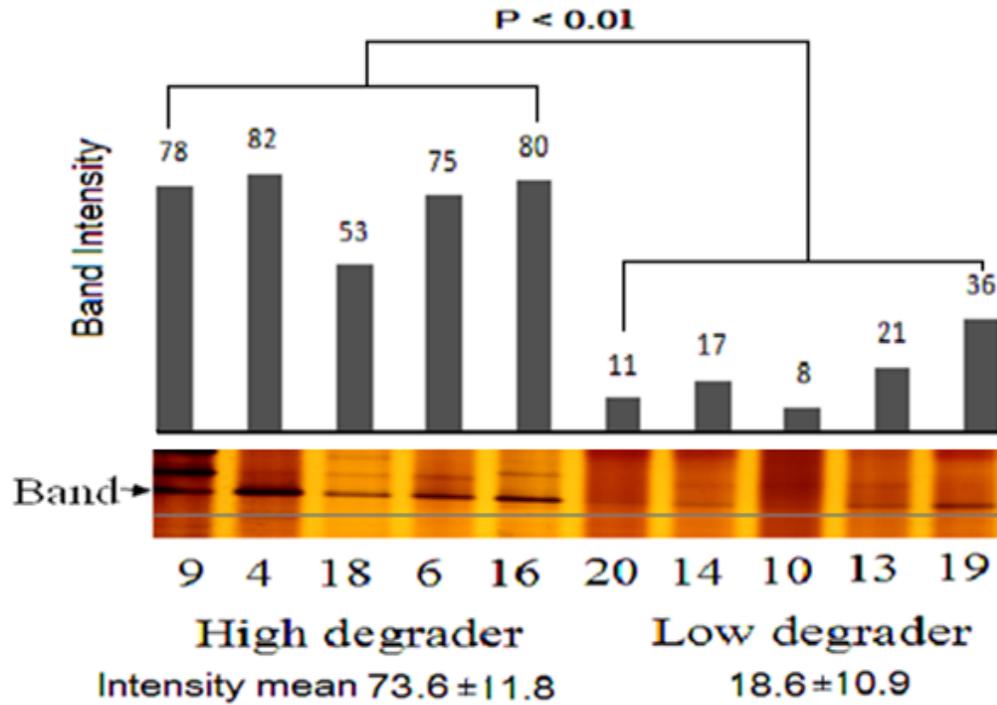


Figure 4.10. Band intensity was shown in each subject between the high and low caffeic acid degraders; the band intensity of higher degrader of caffeic acid was significant greater than that of lower degrader of caffeic acid ($P < 0.05$).

CHAPTER 5. CYTOTOXICITY OF *HYPERICUM PERFORATUM* EXTRACT/COMPONENT AND BIOAVAILABILITY OF CHLOROGENIC ACID, QUERCETIN, AMENTOFLAVONE AND PSEUDOHYPERICIN IN INTESTINAL CACO-2 CELL MONOLAYERS¹

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Abstract

Hypericum perforatum (Hp) ethanolic extract components were shown to have antiinflammatory ability by inhibiting prostaglandins in a macrophage cell line. We hypothesized that these Hp components were absorbable in a human intestinal cell model (Caco-2). Such absorption would be a necessary precondition for Hp component activity *in vivo*. Also botanical compound absorbability was independent of extract matrix, and interactions within a simulated botanical fraction would not affect compound absorbability. To determine the bioavailability of extracts, mixtures and individual components, Caco-2 cell line was used as a model for human intestinal uptake and metabolism of herbal compounds. Caco-2 cell cytotoxicity of Hp (Accession Elixir) components, chlorogenic acid, quercetin, amentoflavone and pseudohypericin, individually or as a 4-compound mixture at 5 different concentrations (100:75:50:25:10 μ M) and Hp extract (50:25:10:5 μ M) based on chlorogenic acid were assayed using MTS reagent. 100 μ M concentrations of chlorogenic acid, quercetin, and pseudohypericin and 75 μ M of amentoflavone or 75 μ M of each of the 4 components combined were significantly cytotoxic, whereas Hp (Elixir) extract containing 50 μ M chlorogenic acid and less amounts of the other 3 compounds was cytotoxic. Cytotoxicity of the 4-compound mixture was explained by the toxicity of amentoflavone, unidentified components of the Hp extract must have contributed to its greater cytotoxicity than seen for the 4-compound mixture. Caco-2 cell monolayers were used to investigate the cellular uptake and metabolism of Hp compounds/mixture/extract. Three concentrations (50, 20, and 5 μ M) of chlorogenic acid, quercetin, amentoflavone, pseudohypericin, and 4-component mixture and Hp extract based on 20 μ M and 5 μ M chlorogenic acid were studied. 4 compounds individually or together showed detectable transfer across Caco-2 cells at 50 and 20 μ M. The apparent permeabilities (P_{app}) from apical to

basolateral transfer were $9.02E-06$ cm/s for chlorogenic acid, $3.60E-06$ cm/s for quercetin, $8.00E-07$ cm/s for amentoflavone, $5.20E-07$ cm/s for pseudohypericin after 4h incubation with $50\mu\text{M}$ concentrations. The individual compound *Papp* showed chlorogenic acid > quercetin > amentoflavone > pseudohypericin in incubation separately, and 4 compound mixture *Papp* showed chlorogenic acid = quercetin > amentoflavone > pseudohypericin after 4h incubation. Quercetin, amentoflavone and pseudohypericin showed similar *Papp* between individual and mixture, whereas individual *Papp* of $20\mu\text{M}$ chlorogenic acid was greater than that of incubation in mixture and Hp extract. The hypothesis was partially confirmed. The effects of the mixture and extract on compound metabolism and transfer require further study.

Introduction

Ethanollic extracts of *Hypericum perforatum* has been recommended traditionally for a wide range of medical conditions. The most common use of Hp is the treatment of depression. Hp also has anti-inflammatory, antibacterial, antiviral properties, and has been used to help heal wounds and burns (Schulz et al., 2001; Raso et al., 2002; Hammer et al., 2007). An earlier animal model in rats induced by injection of carageenan and prostaglandin E_1 , *Hypericum perforatum* was found to suppress both the inflammatory effect and the leukocyte infiltration (Shipochliev et al., 1981). Recently, two *H. perforatum* subspecies found in central Italy were shown to be active against Gram-positive (Staphylococcus aureus and Enterococcus faecalis), two Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa) and the yeast Candida albicans via the Kirby-Bauer agar diffusion method (Cecchini et al., 2007). Furthermore, a 4 component system ($0.1\mu\text{M}$ chlorogenic acid, $0.08\mu\text{M}$ amentoflavone, $0.07\mu\text{M}$ quercetin, and $0.03\mu\text{M}$ pseudohypericin found in a fraction of *H. perforatum*) inhibited lipopolysaccharide (LPS)-induced prostaglandin E_2 level, the production of the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α), and the anti-inflammatory cytokine interleukin-10 (IL-10) ((Figure 5.1; Hammer et al., 2008).

Many studies have shown the validity of using Caco-2 cells in studying the absorption of a variety of compounds found in botanicals. Group B soyasaponins was studied in Caco-2 cell absorption by comparing with human apparent absorption (Hu et al., 2004). As an example of flavonoid uptake, genistein was also shown with efficient absorption by Caco-2 cells (Oitate et al., 2001). Sfakianos et al. (1997) showed that genistein was ~100% absorbed in rats, with

70% biliary excretion in a rapid first-pass effect, with most of the genistein being excreted as glucuronide conjugate (Sfakianos et al., 1997). These uptake data were in concordance with the absorption of genistein in Caco-2 cells (Oitate et al., 2001). Limited uptake of caffeic and chlorogenic acids (1.5 and 0.1% transfer, respectively) was found in Caco-2 cells (Konishi and Kobayashi, 2004a). In a rat study, only a small fraction of the caffeic-acid derivative, rosmarinic acid, was absorbed (0.5% of ingested dose), whereas a ~10 fold greater amount of caffeic acid itself was absorbed (Konishi et al., 2005). Gut microbial deglycosylation may be required before significant absorption of these compounds can occur, but mammalian glucose transporters, cytosolic β -glucosidase and lactase phlorizin hydrolase (which may hydrolyze some glucosides) were thought to be involved in the absorption of at least some such compounds (Scalbert and Williamson, 2000). In this study, we hypothesized that botanical compound absorbability was independent of extract matrix, and interactions within a simulated botanical fraction would not affect compound absorbability. To determine the bioavailability of extracts, mixtures and individual components, Caco-2 cells was used as a model for human intestinal uptake and metabolism of herbal compounds.

Materials and Methods

Plant extracts and cell culture materials

Hypericum perforatum plant material was obtained from the North Central Regional Plant Introduction Station (NCRPIS) (Ames, IA) of the U.S. Department of Agriculture and processed as described previously (Schmitt et al., 2006). The ethanolic extract of plant was provided by the NCRPIS and Dr. Murphy's laboratory: 1.79g Dried *Hypericum perforatum* (Hp) Soxhlet ethanolic extract 250, Ames, 27452 'Elixir'. The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum, glutamine, nonessential amino acids, penicillin, and streptomycin (10000 units/mL and 10 mg/mL in 0.9% sodium chloride, respectively), phosphate-buffered saline, and Hank's balanced salt solution (HBSS) were all purchased from Invitrogen Corp. (Carlsbad, CA). Type I collagen was purchased from Nitta Gelatin Inc. (Osaka, Japan). Plastic dishes, plates, and Transwell inserts with 0.4- μ m polycarbonate membranes (12 mm in diameter) were obtained

from Corning (Corning, NY). Chlorogenic and caffeic acids were from Wako Pure Chemicals Inc., Ltd. (Osaka, Japan). All other chemicals used in this study were of analytical grade.

Chemicals

Pseudohypericin and amentoflavone were purchased from Chroma Dex™, Inc. Santa Ana, CA; quercetin and chlorogenic acid were purchased from Fisher Scientific (Hanover Park, IL). 2, 4, 4'-Trihydroxydeoxybenzoin (THB) were synthesized for an internal standard using the method (Song et al., 1998). High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, acetic acid, dimethyl sulfoxide (DMSO), and all other chemicals were from Fisher Scientific (Fairlawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Experimental Caco-2 study protocol

Hypericum perforatum ethanolic extracts, 4 compounds from Hp (amentoflavone, pseudohypericin, chlorogenic acid and quercetin) and four compound mixture at three concentrations were used in duplicate assays. *Hypericum perforatum* ethanolic extract concentrations were based on Chlorogenic acid as 20 and 5 µM. Concentrations of chlorogenic acid, quercetin, amentoflavone and pseudohypericin were 50:20:5 µM. The mixture concentrations were based on the same concentration with each compound at 50:20:5 µM. Caco-2 cells were cultured in ethanol extracts (dissolved in DMSO) from *Hypericum perforatum* extracts, 4 compounds from Hp and four compound mixtures.

General protocol for use of CellTiter 96® AQueous Assay reagents

Hypericum perforatum extracts, 4 compounds from Hp (Amentoflavone, Pseudohypericin, Chlorogenic acid and Quercetin) and four compound mixture at five concentrations were used for the CellTiter 96® AQueous Assay (MTS Assay) and the measurement of the absorbance of the formazan was carried out using 96 well microplates. Cell viability of Caco-2 cells was detected using MTS cell viability assay kit according to the manufacturer protocol. The following recommendations were for the preparation of reagents sufficient for one 96-well plate containing cells cultured in a 100µl volume. Cells were grown in 48 well plates (day 1), then were treated cells with compounds at 60% confluence (day2), and added 20µl MTS for incubating 1.5hrs and transferred 100 µl into 96 well plate (reading at 562 nm, day3).

Transepithelial kinetics of materials in the Caco-2 cell model

Caco-2 cells were purchased at passage 18 from American Type Culture Collection and experiments were conducted at passages 35–45. The cells were grown in DMEM (Sigma) with 16% fetal bovine serum (Sigma), 1% nonessential amino acids (Gibco BRL), and 1% antibiotic-antimycotic solution (Gibco BRL) at 37°C in an incubator with 5% CO₂/95% air. At 80–100% 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 Costar) at 5.5 x 10⁴ cells /cm². At 14-16 d postseeding (90-100% confluence), A serum-free medium (1% antibiotic-antimycotic solution, 4 mg hydrocortisone/L, 10 mmol Pipes/L, 5 µg selenium/L, and 34 µg triiodothyronine/L in DMEM medium) was used to perform the transport assay. Occasionally, cells were tested for mycoplasma contamination using the DNA-based assay kit purchased from Gen-Probe (San Diego).

Epithelial uptake was measured for each compound, mixture, and extract at three concentrations in duplicate assays. All materials were suspended in the transport buffer by sonication for 30 s (Sonic Dremembrator, Fisher Scientific). Caco-2 monolayers grown on the membrane inserts were first rinsed with 2 mL of Earle's balanced salt solution (EBSS) and then bathed in 2 mL transport buffer, 37°C, 15 min before treatment. Then the apical solution was replaced with 1.5 mL transport buffer containing soyasaponin I or soyasapogenol B. A total of 1.0 mL transport buffer was added to the basal chamber. The system was incubated at 37°C for 4 h and samples were taken from the basal chamber at 30 min and 1, 2, and 4 h. The basal chamber buffer was replenished with transport buffer at each time point. Cumulative transport rates were the sum of the amount transported from all time points (Cogburn et al., 1991). At the end of the experiment, the buffer in the apical chamber was collected to determine untransported test compound. Samples were stored at –20°C until analysis.

The contents of compounds in the samples were determined as follows. The sample from the basal chamber was directly loaded at RT onto a preconditioned Sep-Pak cartridge (light short-body C₁₈, Waters), washed with 3 mL of 5% methanol and eluted with 0.8 mL HPLC-grade methanol. The sample from the apical chamber was loaded onto a larger size preconditioned Sep-Pak cartridge (classic short-body C₁₈, Waters) and eluted with 2.0 mL HPLC-grade methanol. Then all samples were analyzed by HPLC. Permeability coefficient (P_{app}) was determined using

the following equations (Artursson, 1991; Oitate et al., 2001). The apparent permeability coefficient (P_{app}) equation:

$$P_{app} = \frac{dC}{dt} \frac{V_r}{AC_0}$$

V_r : volume of the basal chamber (ml),

A : surface area of the membrane (cm^2),

C_0 : the initial concentration in the apical chamber (μM),

dC/dt : the flux ($\mu\text{M/s}$) determined by the linear slope of basal concentration versus time.

Measurement of transepithelial electrical resistance (TER)

Cells were grown for TER measurement in Transwell inserts with the semipermeable membrane first coated with type I collagen (12-mm diameter and 0.4- μm pore size, Corning Costar, Corning, NY). The cells were seeded at a density of $1 \times 10^5/\text{cm}^2$, and the medium was changed every 1 or 2 days. Monolayers were formed after culturing for 2 weeks. The integrity of the cell layer was evaluated by measurement of TER with Millicell-ERS equipment (Millipore, MA). Monolayers with TER of $>250 \Omega \cdot \text{cm}^2$ were used for the experiments. The TER of the monolayer was measured before and after an assay sample was added to the insert.

Statistical analysis

All data are expressed as means \pm SD. Statistical analyses were performed with SAS Institute (2003, Cary, NC). The transport kinetics of compounds across the Caco-2 cell monolayer was analyzed by general linear regression. Differences in cell uptake, transport kinetics, and cytotoxicity of each material compound at different concentrations were compared with ANOVA and Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$.

Results and Discussions

The Caco-2 cell cytotoxicity of Hp (Accession Elixir) components, chlorogenic acid, quercetin, amentoflavone and pseudohypericin, individually or as a 4-compound mixture at 5 different concentrations (100:75:50:25:10 μM) and Hp extract (50:25:10:5 μM) were shown 100 μM concentrations of chlorogenic acid, quercetin, and pseudohypericin and 75 μM of amentoflavone or 75 μM of each of the 4 components combined were significantly cytotoxic,

whereas Hp (Elixir) extract containing 50 μM chlorogenic acid and lesser amounts of the other 3 compounds was cytotoxic (Figure 5.2).

In Caco-2 cells, 4 compounds individually or together showed detectable transfer across Caco-2 cells at 50 and 20 μM (Figure 4). Chlorogenic acid and quercetin in were transferred by the Caco-2 monolayer from Hp extract containing both 20 and 5 μM chlorogenic acid (Figure 5.3), whereas transfer of amentoflavone and pseudohypericin was detectable only from the Hp extract containing the greatest amount of the compounds (extract containing 20 μM chlorogenic acid). The apparent permeabilities from apical to basolateral transfer of 4 individual components were chlorogenic acid ($9.02\text{E-}06$ cm/s), quercetin ($3.60\text{E-}06$ cm/s), amentoflavone ($8.00\text{E-}07$ cm/s), pseudohypericin ($5.20\text{E-}07$ cm/s) after 4 h incubation on the monolayer with 50 μM concentrations of each compound, respectively (Figure 5.4). The individual compound *Papp* showed chlorogenic acid > quercetin > amentoflavone > pseudohypericin in incubation separately, and 4 compound mixture *Papp* showed chlorogenic acid = quercetin > amentoflavone > pseudohypericin after 4h incubation with both 50 and 20 μM . Quercetin, amentoflavone and pseudohypericin showed similar *Papp* between individual and mixture with both 50 and 20 μM , whereas individual *Papp* of 20 μM chlorogenic acid was greater than that of incubation in mixture and Hp extract. Most compounds showed concentration-dependent absorption with a higher concentration having greater apparent permeability than did the lower concentration in individual compound and mixture incubations with Caco-2 monolayer except for chlorogenic acid and pseudohypericin in the mixture (Figure 5.4).

To investigate the bioavailability and metabolism of chlorogenic acid (CGA) and caffeic acid in cell culture, most studies have used HepG2 cell or Caco-2 as biotransformation models. One study has tested the hepatic uptake and metabolism in human hepatoma HepG2 cells which were incubated for 2 and 18 h with chlorogenic acid, caffeic and ferulic acids. The results showed that caffeic acid had been transported and methylation/glucuronidation/sulfation were the main pathways for its metabolism; ferulic acid underwent glucuronidation as its only metabolite and was more slowly metabolized by HepG2 cells; and chlorogenic acid had the lowest absorption due to the esterification of the caffeic acid moiety with quinic acid (Mateos et al., 2006).

Some cell culture studies showed that chlorogenic acid (CGA) and caffeic acid (CA) were absorbed by paracellular diffusion in human intestinal Caco-2 cells. CA had some although

low affinity for monocarboxylic acid transporter (MCT). This resulted in the greater absorption of caffeic acid compared to chlorogenic acid (Konishi and Kobayashi, 2004a). Caffeic acid was absorbed as 0.20 % and 1.57 % of initial CA in the basolateral phase with or without apical to basolateral proton gradient. More than 98% of apically loaded caffeic acid was retained on the apical side, suggesting CA was restricted by the tight junctions (Konishi and Kobayashi, 2004a). Furthermore, the major metabolites of caffeic acid formed by gut microflora including m-coumaric acid, m-hydroxyphenylpropionic acid (mHPP), and 3,4-dihydroxyphenylpropionic acid (DHPP) were transported by MCT via proton-coupled direction, in which the transport of m-coumaric acid, mHPP, and DHPP was inhibited by an MCT substrate, whereas DHPP was mainly permeated across Caco-2 cells via the paracellular pathway (Konishi and Kobayashi, 2004b).

Conclusions

Chlorogenic acid, quercetin, amentoflavone, pseudohypericin, as individual compounds and from a mixture of the 4 compounds or from *Hypericum perforatum* extract were taken up and transferred by Caco-2 cells after incubation. The apical to basolateral transport of chlorogenic acid and quercetin were higher than amentoflavone and pseudohypericin after 4-h incubation on this monolayer. Quercetin, amentoflavone and pseudohypericin showed similar P_{app} between individual and mixture with both 50 and 20 μ M. Most compounds showed concentration-dependent absorption in individual compound and mixture incubations with Caco-2 monolayer.

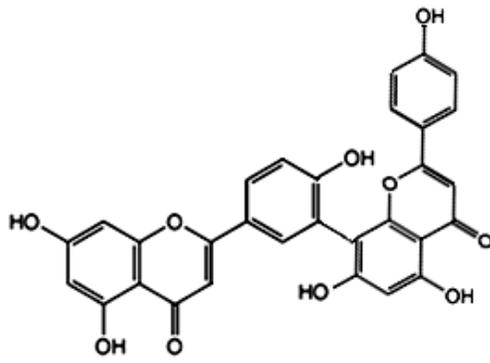
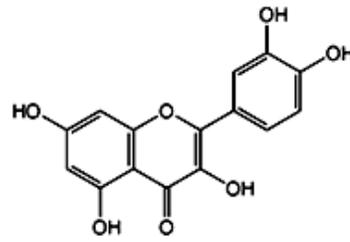
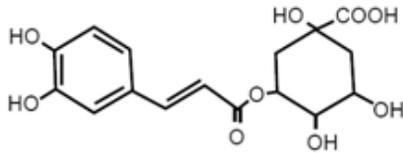
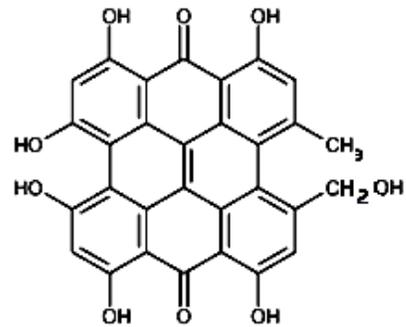
Footnotes

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**Amentoflavone****Quercetin****Chlorogenic acid****Pseudohypericin****Figure 5.1.** Structure of 4 components in *Hypericum perforatum* (Elixir)

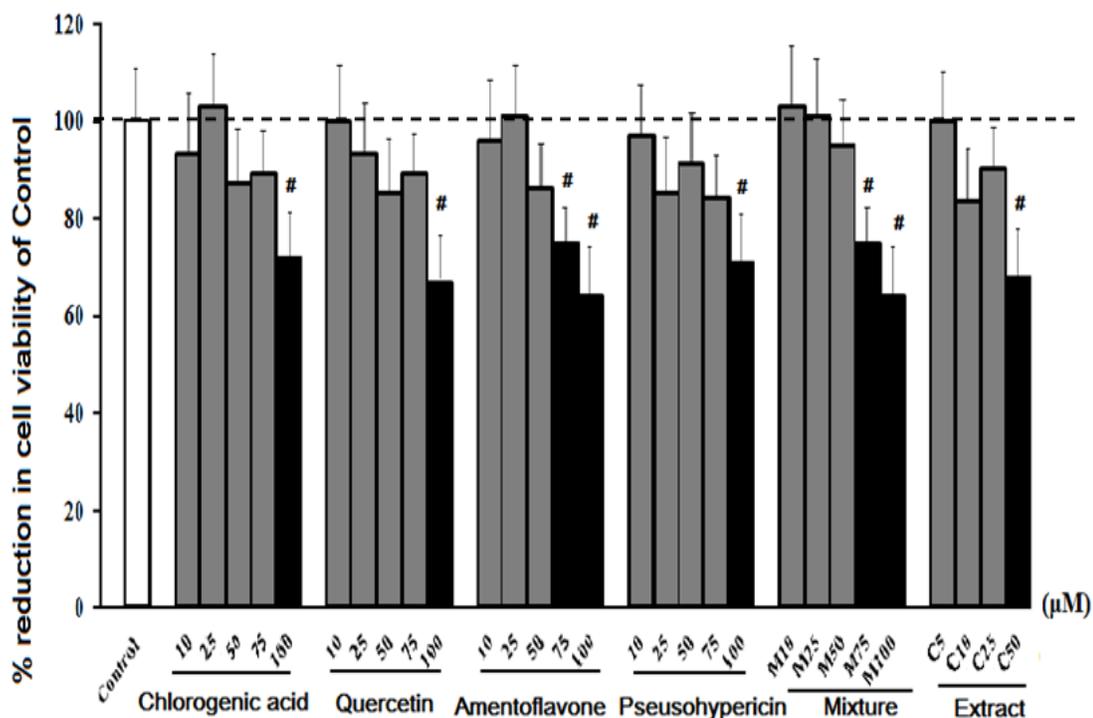


Figure 5.2. Cytotoxicity of 4 component system, mixture, and *Hypericum perforatum* extract were measured as mean percent reduction in cell viability compared with media + DMSO control-treated cells \pm standard error (n=6). 100 μ M concentrations of chlorogenic acid, quercetin, and pseudohypericin and 75 μ M of amentoflavone or 75 μ M of each of the 4 components combined were significantly cytotoxic, whereas HP (Elixir) extract containing 50 μ M chlorogenic acid and lesser amounts of the other 3 compounds were cytotoxic. # $P < 0.05$ indicated that the % cell viability was significantly less than control.

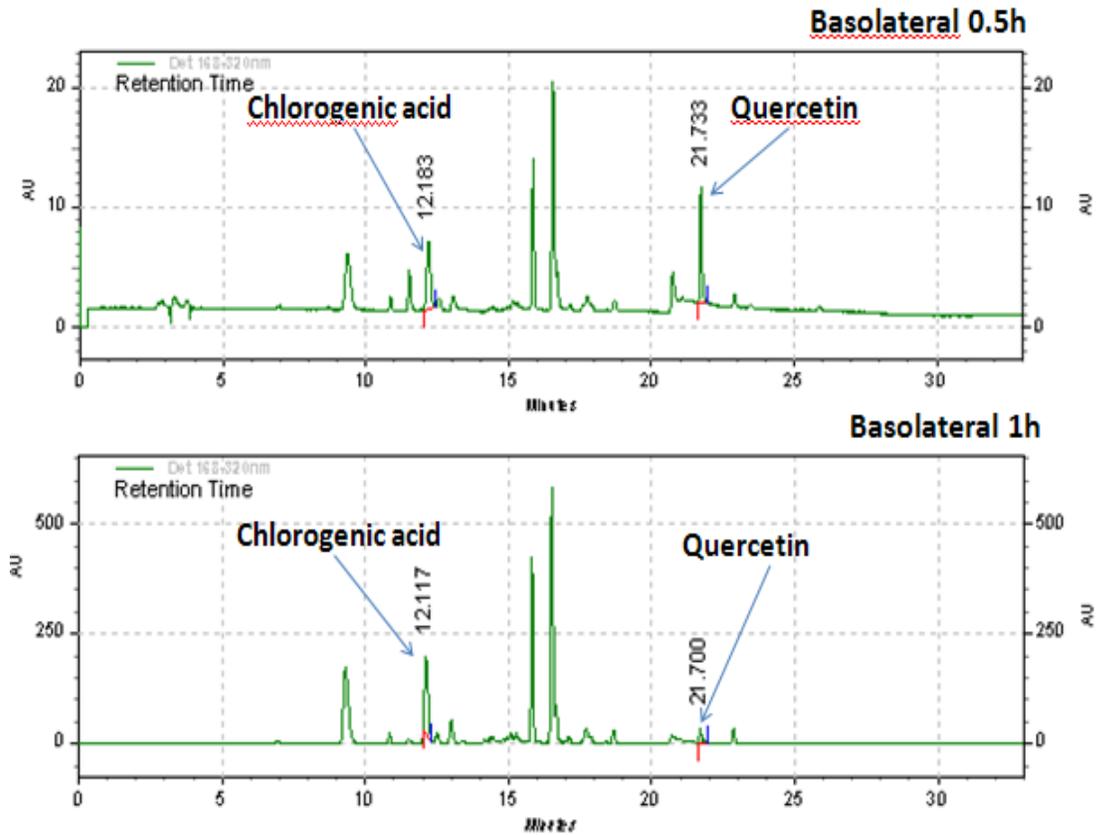


Figure 5.3. *Hypericum perforatum* extract chromatogram during 0.5 and 1h period. Extract concentration was based on chlorogenic acid concentration of 5 μ M.

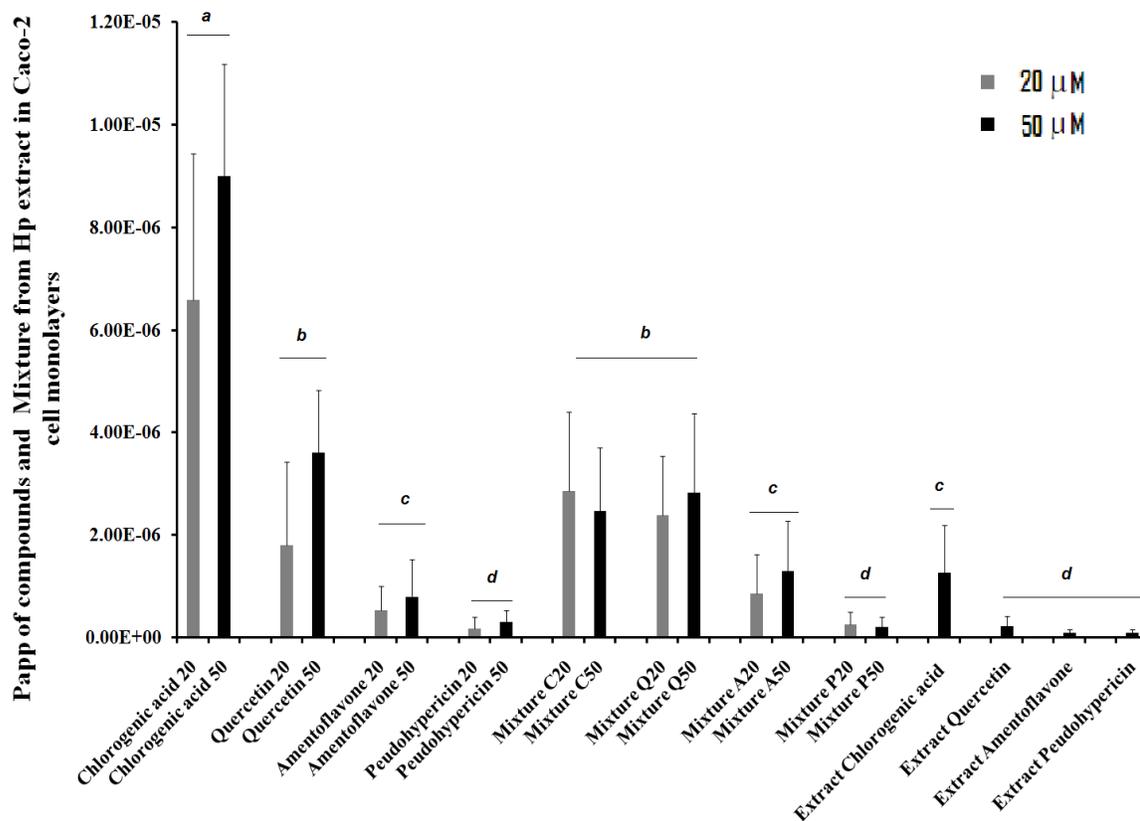


Figure 5.4. Apparent permeability of four individual components from *Hypericum perforatum* during 4h period, each individual component was used at 20 and 50 μ M. The individual compound P_{app} showed chlorogenic acid > quercetin > amentoflavone > pseudohypericin in incubation separately, and 4 compound mixture P_{app} showed chlorogenic acid = quercetin > amentoflavone > pseudohypericin after 4h incubation with both 50 and 20 μ M. Most compounds showed concentration-dependent absorption with a higher concentration having greater apparent permeability than did the lower concentration in individual compound and mixture incubations with Caco-2 monolayer except for chlorogenic acid and pseudohypericin in mixture. Letters 'a', 'b', 'c' and 'd' indicated $p < 0.05$ significant difference compared between compounds.

CHAPTER 6. INCREASED CYP4B1 MRNA IS ASSOCIATED WITH THE INHIBITION OF DEXTRAN SULFATE SODIUM-INDUCED COLITIS BY CAFFEIC ACID IN MICE^{1,2}

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² A paper was accepted by Experimental Biology and Medicine.

Abstract

Susceptibility to inflammatory bowel diseases depends upon interactions between the genetics of the individual and induction of chronic mucosal inflammation. We hypothesized that administration of dietary phenolics, caffeic acid and rutin, would suppress upregulation of inflammatory markers and intestinal damage in a mouse model of colitis. Colitis was induced in C3H/HeOuj mice (8 wk old, 6 male/6 female per treatment) with 1.25% dextran sulfate sodium (DSS) for 6 d in their drinking water. Rutin (1.0 mmol (524 mg)/kg in diet), caffeic acid (1.0 mmol (179 mg)/kg in diet), and hypoxoside extract (15 mg/d, an anticolitic phenolic control) were fed for 7 d before and during DSS treatment, as well as without DSS treatment. Body weight loss was prevented by rutin and caffeic acid during DSS treatment. Colon lengths in mice fed caffeic acid and hypoxoside during DSS treatment were similar to DSS-negative control. Food intake was improved and myeloperoxidase (MPO) was decreased with each phenolic treatment in DSS-treated mice compared with DSS treatment alone. Colonic mRNA expression of IL-17 and iNOS were inhibited when IL-4 was increased by each phenolic treatment combined with DSS, whereas CYP4B1 mRNA was increased only by caffeic acid in DSS-treated mice, compared with DSS treatment alone. Colonic and cecal histopathology scores of DSS-treated mice were significantly more severe ($P < 0.01$) than in mice fed caffeic acid before and

during DSS treatment based on mucosal height, necrosis, edema, erosion, and inflammatory cell infiltration. Although both rutin and caffeic acid suppressed the expression of selected inflammatory markers, only caffeic acid protected against DSS induced colitis, in association with normalization of CYP4B1 expression. The inhibition of DSS-induced colitic pathology by caffeic acid was mediated by mechanisms in addition to anti-inflammatory effects that deserve further study.

Key words: caffeic acid, rutin, colitis, CYP4B1, inflammation, mouse

Introduction

The pathogenesis of inflammatory bowel diseases (IBDs; e.g., Crohn's disease, ulcerative colitis) is not fully understood. Genes, environment, enteric microbiota, and other factors alter disease risk (1). Nutritional factors (e.g., elemental diets, sucrose or other specific carbohydrate diets, other dietary components that alter gut microbial populations) modulate these diseases, and play a significant role in the treatment of IBD and influence the disease course and prognosis (2). Increased colon cancer risk is a key concern associated with colitis, as evident in a Danish epidemiological study of more than 5,500 ulcerative colitis patients who showed greater risk for colon cancer than controls (3). IBD models induced by enteric bacteria are well established (4, 5). Colitis in mice also may be induced by dextran sodium sulfate (DSS) in drinking water (6) causing weight loss, diarrhea with blood and/or mucus, shortening of the colon, erosion of the mucosal epithelium, and acute neutrophilic infiltration (7). Experimental colitis models are characterized by up-regulated nuclear factor kappa B (NF- κ B) and pro-inflammatory cytokines (e.g., interleukin-1 β (IL-1 β), tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ)) resulting in tissue damage (8, 9). Despite adverse side effects, glucocorticosteroids are used to treat IBD (1, 10). Immunosuppressive and immunoregulatory agents (cyclosporine, aminosaliclates and azathioprine) have also been used to control severe disease, however, serious complications and toxic side effects were associated with them (11). Thus, dietary phenolics may be an alternative to control this disease. Caffeic acid phenethyl ester (CAPE) decreased colonic NF- κ B and prevented colitis in peptidoglycan-polysaccharide (PG-PS)-treated rats injected with 30 mg CAPE /kg for seven days (12). Dietary rutin (feeding 0.1% rutin diet for 2 weeks) prevented DSS-induced colitis and possible colorectal carcinogenesis by suppressing pro-inflammatory cytokines (TNF- α , IL-1 β , 13). Using DSS- and *Brachyspira*

hyodysenteriae-induced colitis, hypoxoside extract showed protection (Wannemuehler laboratory, unpublished). Hypoxoside Extract, a known anti-inflammatory component of African potato (14, 15), decreased inflammatory damage, was possibly related to the down-regulation of NF- κ B pathways in mucosa. Dietary caffeic acid, a major phenolic acid widely distributed in plant foods and herbs, has not been studied for its effect on DSS-induced mouse colitis and the relationship between specific inflammation-related cytokine mRNA expression, myeloperoxidase and mucosal histopathology. In the present study, commonly occurring dietary phenolics, rutin and caffeic acid, were compared with hypoxoside extract, for protective efficacy in a C3H/HeOuJ mouse model of colitis. This work may lead to improvement of the therapeutic and prophylactic benefits of plant foods and botanical supplements. Our long term goals are to identify novel anti-inflammatory phenolics that may be used to prevent or treat human colitis and to establish and validate a screening assay for dietary components that may prevent colitis and colon cancer.

Materials and Methods

Chemicals and Reagents

Purified caffeic acid and rutin were purchased from Chroma Dex™, Inc. Santa Ana, CA. Hypoxoside extract was a gift from Allison AC, Dawa Corp., Belmont, CA (Figure 1). Dextran sulphate sodium was purchased from Fisher Scientific (Pittsburgh, PA). RNeasy® Tissue Collection solution was purchased from Applied Biosystems Business (Foster City, CA). Working solutions of 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (TMB, Sigma; 2.5 mM in water) and hydrogen peroxide (5 mM in water) were prepared immediately before use. Sulfuric acid (Fisher Scientific; 2 M) was used as a reaction stop solution. The detergent cetyltrimethylammonium bromide (CTAB, Sigma; 0.02% in water) was used as a lysing agent for determining total myeloperoxidase content of neutrophils. Phenylmethylsulfonyl fluoride (PMSF, Sigma), dimethyl sulfoxide (DMSO, Sigma) and phosphate buffered saline (PBS, pH 7.4) were used for tissue preparation.

Diets

In the experimental period, C3H/HeOuJ mice were fed AIN 93 G (Harlan Teklad, Madison, WI) diet with or without DSS or treatment diets based on AIN 93 G containing rutin (1.0 mmol/kg or 524 mg/kg in diet) or caffeic acid (1.0 mmol/kg or 179 mg/kg in diet). For the

remaining treatment, mice were fed AIN 93 G diet and gavaged with hypoxoside extract (15 mg/d, 60 mg solid crude hypoxoside extract/mL sterile saline, each mouse given 0.25 mL daily using a 22 gauge feeding needle) with or without DSS. Experimental diets were prepared and stored at 4°C.

Experimental Design and Animals

Forty eight male and 48 female mice at 6 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were acclimated for 2 week before starting the experiment and were randomly assigned to eight treatment groups in order to achieve similar mean body weight/group. Mice were individually housed in microisolater cages with wood chip bedding and consumed standard rodent lab chow and tap water ad libitum during the acclimation period. The animal room was maintained at 23°C with a 12-h light/dark cycle during the experimental period. The experiment was a 4 x 2 factorial design: 8 treatment groups (control, caffeic acid, rutin and hypoxoside) and 2 disease statuses (with and without DSS, Figure 2). Colitis was induced in groups of 12 C3H/HeOuJ mice (6 males and 6 females) with DSS in their drinking water, a method previously reported with some modifications ([6](#), [16](#), [17](#)), namely 1.25% DSS was used, and only for 6 days rather than repeated DSS administration over longer time periods. The various phenolic treatments were fed to the mice for 7 days prior to DSS exposure and continually during DSS treatment (Figure 2). Food intake was measured weekly over 2-3 consecutive days per week. Body weights were measured twice a week. Signs of disease (weight loss, diarrhea, dehydration) were observed daily. The supplemented diets and the DSS-containing drinking water were provided to the mice continually until the experiment was terminated. All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.

Tissue Sample Collection and Preparation

The colon and cecum were removed after euthanasia. After washing in phosphate-buffered saline (PBS), they were placed on filter papers to measure colonic lengths, score macroscopic cecal lesions and obtain photographs of each tissue. The colonic and cecal contents were removed, and the colon and cecum from each animal were fixed in formalin and six of the 12 tissue samples were randomly selected from each group for histopathological analyses. Gross cecal lesions were scored using published criteria ([18](#)). Macroscopic cecal lesions were scored 0-4 as follows: no gross lesions (grade 0, normal); evidence of atrophy (grade 1, mild); excess

intraluminal mucus with atrophy localized to the cecal apex (grade 2, moderate); generalized cecal atrophy with increased intraluminal mucus and no cecal contents (grade 3, severe); score 3 plus bloody cecal content (grade 4, most severe). Colonic tissues for myeloperoxidase activity were put in 15% DMSO and 0.1 mM PMSF in cryovials (Corning Company, Corning, New York). A portion of colon (approximate 1.5 cm) for each sample was placed into *RNA Later* (1.2 mL) for subsequent RT-PCR analysis of cytokine-specific mRNA expression. All above samples were stored at -85°C until analysis.

Colonic Mucosal Histopathological Analysis

Cecum and proximal colon in 10% neutral buffered formalin were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Sections of the cecum and proximal colon were scored by a pathologist (Dr. J. Hostetter) who was blinded to the treatment group (19). Briefly, histological scores were evaluated based on the severity of mucosal epithelial damage, architectural/glandular alterations, and the magnitude/character of lamina propria cellular infiltration. Scoring system for the histopathological evaluation of gastrointestinal inflammation included mucosal height, erosions, inflammatory score, edema score and inflammatory cells. Parameters were scored 1-5, resulting in a maximal total histological score of 20 based on the four evaluation parameters.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Colonic Inflammation-Related Cytokine Gene Expression

Primers for IL-1 β , IL-4, IL-6, IL-10, IL-17, GAPDH, IFN- γ , TNF- α , iNOS (inducible nitric oxide synthase), ICAM-1 (intercellular adhesion molecule-1), CYP4B1 (cytochromes P450, family 4B1) were analyzed in pooled colonic samples from each treatment group as a preliminary screen of gene expression (Table 6.1). Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA in each pooled sample was performed as previously described with some modifications (20, 21). 20 mg samples were prepared from each colonic tissues and total mRNA was extracted using a Qiagen RNeasy mini Kit (Ambion, Austin, TX) for dissected tissue homogenization. The mRNA concentrations were detected by NanoDrop[®] ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) in 2.0 μl mRNA samples using RNA program. Then the mRNA extractions were treated with TURBO DNA-free DNase (Ambion, Austin, TX) to remove genomic DNA. SYBR green (Invitrogen, San

Diego, CA) *real-time* polymerase chain reaction (the same method as described below) was used for testing DNA contamination in mRNA samples to determine the mRNA sample purity. The mRNA extraction was reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, San Diego, CA). The cDNA converted from 20ng mRNA was amplified using Platinum® SYBR® Green *qPCR* SuperMix-UDG (Invitrogen, San Diego, CA). *Real time PCR* was used to provide quantitative assessment of mucosal cytokine expression. The PCR condition was 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 15 seconds) run in *Rotor-Gene 3000* (Corbett Research, Mortlake, Australia). Then the specific primers were used to evaluate the gene expression of IL-17, IL-4, iNOS and CYP4B1 in six individual colon tissue samples from eight treatment groups (3 males and 3 females). Standard curves of specific genes and housekeeping gene GAPDH were made by two-fold serial dilutions of cDNA using *real-time PCR*. The relative mRNA quantity was normalized to GAPDH.

Myeloperoxidase Assay Method in Colonic Tissue

Myeloperoxidase (MPO) activity which was used to quantify neutrophil accumulation in tissues was assessed using 96-well flat bottom microtiter plates (Linbro/Titertek, USA) and was previously described with some modifications (22, 23). Colonic tissues were thawed and blotted on paper towel at room temperature to absorb as much as moisture as possible. Tissue samples were trimmed to approximately 35mg. Each sample was homogenized in 1 mL PBS and PMSF (0.1mM) and the probe was washed 5 times with PBS. Each sample was sonicated on pulse (output control set to 2; 50% duty cycle setting; 5-10 pulse per sample, Sonicator 3000, Misonix, Inc. Farmingdale, NY). Samples were centrifuged 15 minutes at 1200 rpm (Eppendorf® Micro Centrifuge Model, USA). At least 150 µl supernatant was collected. Each sample was measured by NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) to measure total protein concentrations. 150 µl of each sample supernatant was plated in triplicate wells (per sample). TMB (50 µL) was added, followed immediately with 50 µL 10 µM H₂O₂. The color change reaction was allowed to proceed for 2 min, and 50 µL of 2 M sulfuric acid was added to stop the reaction. The optical density (OD) in each well was determined at 405 nM using a microtiter plate spectrophotometer (V-Max, Molecular Devices, USA) with SOFTmax PRO 4.0 software. The total myeloperoxidase content was calculated from OD of lysed neutrophils using a standard curve. Standard suspensions of mouse neutrophils

(3×10^6 cells mL^{-1}) with PMSF (1 mM in absolute ethanol) were prepared and stored at -85°C . Two-fold serial dilutions of a standard neutrophil suspension were lysed with CTAB. The MPO content of these known concentrations of neutrophils was compared to the OD values and a standard curve was calculated. Total myeloperoxidase content in mouse colonic tissue were normalized for number of neutrophils. The MPO activity was expressed as the units of enzyme per gram of wet weight of tissue.

Statistical Analysis

Data were analyzed by the difference between means and statistical significance was calculated using three-way ANOVA followed by Tukey method as a Post Hoc test (SAS Institute, 2003, Cary, NC). The equal variance and normality of residuals assumptions are verified by a residual vs. predicted values plot and a histogram of residuals. Both group and male/female of colon length, food intake, body weight change, MPO activity, total histopathology scores, and cytokine/enzyme RT-PCR products were reported as means \pm SEM. Statistical significance was set at $P < 0.05$. Pearson correlation analysis was used for relationship between MPO activity/cytokine gene expression and colonic histopathology score.

Results

General Effects of Phenolic Treatments on DSS-Induced Colitis

Regardless of the treatment group, food intakes measured on the day before drinking DSS-supplemented water and second day of the DSS treatment period did not differ (Table 6.2). Food intake was decreased at final day after DSS-treated alone compared with control not treated with DSS. Food intakes in the phenolic-treated groups not treated with DSS did not differ from control not treated with DSS (Table 6.2). Mice given rutin, hypoxoside, and caffeic acid had normalized food intake at the sixth day DSS-treated compared to animals not given DSS control (Figure 3). Body weight change (the percentage of body weight gained in DSS period over the body weight before DSS-treated) of 0.8% in DSS-positive control was significantly less than in DSS- negative control by fourfold ($P < 0.01$). DSS-treated mice given diets supplemented with either rutin or caffeic acid showed improved body weight whereas mice administered hypoxoside with DSS did not significantly increase their body weight (Figure 6.3). The mice fed rutin, hypoxoside, or caffeic acid not treated DSS were similar to controls without DSS in body weight

increase (Figure 6.3). The cecal macroscopic scores did not differ among treatments with or without DSS. No diarrhea and rectal bleeding were observed during the period of DSS treatment. The colon length of the mice given DSS or DSS plus rutin was significantly shortened by ~8% ($P < 0.01$) as compared with all treatment groups not given DSS (Table 6.2). The colon lengths of DSS-treated mice fed caffeic acid or hypoxoside were significantly longer than those from the DSS-only controls ($P < 0.01$) (Table 6.2). Within treatment groups, no differences were found between males and females for colon length, food intake or body weight change (data not shown).

Colonic Myeloperoxidase (MPO) Activity of Phenolic Treatments and DSS Controls

Colonic MPO activity in DSS-only controls was significantly greater than in non-treated control mice ($P < 0.01$). MPO activities were significantly decreased in the colonic extracts from DSS-treated mice fed each of the supplemented diets (rutin, hypoxoside, or caffeic acid) compared with the colonic extracts from the DSS-only control mice ($P < 0.01$). MPO activities in mice fed phenolics without DSS exposure did not differ from untreated control mice (Figure 6.4). Within treatments, no difference was found between males and females for MPO activity (data not shown).

Colonic and Cecal Histopathology Changes in C3H Mice

Colonic and cecal histopathology scores of the DSS-treated mice were significantly more severe compared with control mice not given DSS, respectively ($P < 0.01$, Table 6.3). The group fed caffeic acid with DSS had significantly less severe colonic and cecal microscopic lesion scores than mice treated with DSS alone. Representative histological images from each group are shown (Figure 6.7). The mean colonic and cecal histopathology scores were both significantly decreased by 37% in the group fed caffeic acid with DSS compared to DSS-only controls ($P < 0.01$, Figure 6.7), whereas the histopathology scores in mice given rutin or hypoxoside along with DSS were not significantly attenuated in comparison to control mice not given DSS ($P < 0.01$, Table 6.3). Controls fed the non-supplemented AIN 93 G alone or diets containing rutin, caffeic acid or hypoxoside had significantly lesser histopathology scores than the DSS treated controls ($P < 0.01$) (Table 6.3). Within treatments, no difference was found between males and females for histopathology score (data not shown).

Reverse Transcription–Polymerase Chain Reaction Analysis of Cytokine mRNA

Some genes have been implicated in the pathogenesis of colitis, including IL-1 β , IL-4, IL-6, IL-10, IL-17, iNOS, IFN- γ , TNF- α , ICAM and CYP4B1 (9, 13, 40, 41, 51, Table 1). To delve into the molecular mechanism underlying the suppression of colitis by phenolics in DSS-induced treatments, mRNA expression levels of possible pro-inflammatory mediators in colonic tissue were measured by RT-PCR. Preliminary evaluations demonstrated that the expression levels of IL-17-, iNOS-, IL-4-, and CYP4B1-specific mRNA differed among the various treatments. There was significant up-regulation of colonic tissue mRNA expression of IL-17 and iNOS ($P < 0.01$, Figure 6.5A), and significant down-regulation of IL-4 and CYP4B1 mRNA expression in DSS-treated controls compared to controls not given DSS ($P < 0.01$, Figure 6.5B). Colonic tissue mRNA expression of IL-17 and iNOS were significantly inhibited by each phenolic treatment (rutin, hypoxoside, and caffeic acid) when given with DSS, compared with DSS-only controls ($P < 0.05$, Figure 6.5A) whereas IL-4 –specific mRNA was significantly increased by each phenolic treatment (rutin, hypoxoside and caffeic acid) in groups treated with DSS compared with DSS-only controls ($P < 0.05$, Figure 6.5B). However, only mice fed caffeic acid and treated with DSS had significantly increased CYP4B1-specific mRNA levels compared with DSS-only controls ($P < 0.05$).

Relationship between the Colonic Myeloperoxidase Activity/Cytokine Gene Expression and Colonic Histopathology Score Evaluation

Relationships between colonic MPO activity/IL-17 gene expression and histopathology score in DSS-treated control and caffeic acid with DSS were shown (Figure 6.6). There was a significant association with colonic histopathology score on colonic level of IL-17 gene expression and colonic MPO activity in DSS-induced with or without caffeic acid treated mice. In both DSS-treated control and caffeic acid with DSS mice, colonic MPO activity (Figure 6.6A) and colonic level of IL-17 gene expression (Figure 6.6B) were significantly related to colonic histopathology score, with less MPO activity and greater decrease in colonic IL-17 gene expression associated with decreased histopathology score.

Discussions

The study of dietary phenolics that may prevent diseases such as colitis is made more relevant to humans by considering the likelihood of ingestion of effective doses of these phenolics from common foods or dietary supplements. Regular coffee consumers generally ingest 0.5–1 g chlorogenic acid/d, which may be converted to 250–500 mg caffeic acid/d (24).

From a German survey, daily intake of caffeic acid was 206 mg/d, and the principal sources were coffee (92% of caffeic acid) and fruit and fruit juices combined (25). In our study, based on mouse body weight and food intake, the daily caffeic acid intake was 45 mg/kg BW/day, which was approximately 7-fold higher than the dose of 500 mg/d (7 mg/kg BW). Thus, the dose used in these studies was relevant to that typically consumed by humans, because mice have a tenfold greater surface area than do humans proportionate to body weight. The human equivalent dose of a compound given to mice would therefore be about 10-fold greater than the human dose per kg BW. This assumes similar absorbability of caffeic acid in mice and humans. From an estimate of flavonol intake in Finland of ~ 20 mg/person per day or ~0.3 mg/kg BW (26), the current dose of the flavonol glucoside, rutin (~45 mg/kg BW), was approximately an order of magnitude greater than a human dietary equivalent dose (by the same logic as above), but this might be a feasible human intake if supplements were included. Additional studies of human bioavailability of phenolics are needed for the development of colitis preventive diets.

DSS-induced colitis is partially triggered by aberrant or exaggerated immune responses to bacterial antigens derived from the intestinal lumen (27, 28). Shortening of the large intestine is thought to be induced by the thickening of colon caused by edema and muscular hypertrophy, as observed in ulcerative colitis. Diarrhea may be due to loss of absorptive epithelium that results in the shortening of the colon (29). Damage to the epithelium is a key feature of acute DSS-induced colitis (30), characterized by multi-focal areas of mucosal erosion, colonic epithelial cell injury, and significant mucosal infiltration of neutrophils, key immune cells during inflammatory responses. Increased IL-17, a pro-inflammatory cytokine and decreased IL-4, an anti-inflammatory cytokine accompany DSS-induced colitis, with these changes hypothesized to be prevented by proposed anti-inflammatory dietary components, caffeic acid and rutin. In our study, expression of several other genes possibly associated with inflammatory responses including cytochrome p450 (CYP4B1) were evaluated in response to prophylactic treatment with phenolic compounds during DSS-induced colitis.

Herbs and plant foods contain a variety of phenolic compounds that may modulate immune function. Several caffeic acid derivatives, major phenolic acids in plants, have been identified from herbs such as *Echinacea* (e.g., caftaric acid, echinacoside, cichoric acid). The flavonoid, rutin is found in many herbs including St. John's wort (*Hypericum perforatum*). *In vivo* administration of extracts from *H. perforatum* and *E. purpurea* in carrageenan-induced paw

edema in mice showed that *H. perforatum* at 100 mg/kg inhibited both iNOS and COX-2 expression, two pro-inflammatory genes, whereas treatment with 100 mg/kg *E. purpurea* decreased COX-2 expression only (31). In the present study, colonic gene expression of iNOS was inhibited by rutin, hypoxoside, and caffeic acid in DSS-treated mice in association with decreased MPO (neutrophil activity). Caffeic acid has antioxidant, anti-inflammatory, and antibacterial properties. Ovariectomized rats subjected to trinitrobenzene sulfonic acid (TNBS)-induced colitis and treated with a caffeic acid derivative (CAPE, 30 mg/kg) had decreased malondialdehyde (MDA), increased superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) associated with decreased colitis (32). *E. coli*-induced pyelonephritis was also decreased in rats given CAPE, along with decreased MDA and nitric oxide and increased GSH and SOD activity (33) suggesting that anti-inflammatory effects of caffeic acid might occur through antioxidant mechanisms (32, 33). Rutin had protective effects in a Wistar rat model of gastric lesions induced by 50% ethanol (34). The gastroprotection of 200 mg rutin/kg diet given before ethanol treatment was thought to be due to anti-lipoperoxidative and antioxidant enzyme activity based on decreased MDA and increased glutathione peroxidase, compared with ethanol-treated controls. However, the anti-inflammatory marker, ethanol-induced neutrophil infiltration expressed as myeloperoxidase activity was not decreased by rutin in gastric lesions, compared with our study in which rutin diminished MPO in DSS-induced colitis (34). Anti-inflammatory and antioxidant effects of dietary phenolics generally occur together, but which effects or mechanisms are most crucial are not clear as of yet. Additional factors in inflammatory responses are under investigation as well.

Following DSS treatment, colonic mRNA expression of IL-17 was inhibited by each phenolic treatment compared with DSS-only controls. IL-17⁺ cells, IL-17 mRNA expression, and IL-17 protein levels were detectable and significantly elevated in inflamed mucosa and in the serum of patients with IBD (35). IL-17 family members (IL-17A/F) have been associated with inflammatory diseases, autoimmune diseases and cancer. Induction of IL-17A/F induced chemokines (CXCL8, CXCL6, CXCL1), growth factors (G-CSF, GM-CSF, IL-6), and adhesion molecules (ICAM-1) have been shown to augment neutrophil accumulation (36). Increased IL-17 gene expression was detected in a mouse colitis model in the acute phase at day 7 after exposure to 5% DSS in drinking water, but the DSS concentration was much higher than our study (37). Upregulation of IL-17, IL-1 β , and IL-12 p70 was found during chronic colitis in

C57BL/6 mice whereas production of IL-1 β , IL-6, IL-18, and G-CSF was elevated in BALB/c mice. Chronic production of IL-17 and IL-12 p70 has been correlated with extensive inflammatory cell infiltration as DSS-induced colitis progressed from the acute to chronic stages of inflammation in C57BL/6 mice (37). In the present studies, IL-17 gene expression was elevated in DSS-induced colitis in C3H/HeOuJ mice (Figure 6.5A). Colonic IL-17 gene expression was also associated with colonic histopathological score in DSS-induced with or without caffeic acid treated mice (Figure 6.6B). IL-17 gene expression may be related to histopathological change to evaluate the anti-inflammatory mechanisms. In contrast, dietary supplementation with each of the three phenolic compounds attenuated IL-17 gene expression that was also associated with other anti-inflammatory effects (decreased iNOS, increased IL-4) but diminished histopathological scores were only associated with caffeic acid treatment.

Colonic mRNA expression of IL-4 was increased by each phenolic treatment compared with DSS-only controls. In a previously reported study, CD4⁺ T cells, IL-4, IL-2, and IFN- γ mRNA expression were significantly increased in female BALB/c mice by supplementation with 20 mg/kg CAPE (38). In contrast, Ansorge et al. (39) showed that propolis as well as its constituent (CAPE) suppressed Th2 type (IL-4) and Th1 type (IFN- γ) lymphocytes. Based on the results of the present study, upregulation of IL-4 gene expression may not be strongly associated with attenuated colitic lesions because only caffeic acid suppressed the severity of histopathological lesion scores, even though all three phenolics increased IL-4 (Figure 6.5B).

A key finding in the present study was that the colonic tissue mRNA expression of CYP4B1 was increased in DSS-treated mice fed caffeic acid compared with DSS treated controls. CYP4B1 has attracted interest due to its possible ability to oxygenate fatty acids and form some eicosanoids (40) that suggest a function in inflammatory processes. In our study, increased CYP4B1 occurred in association with decreased colitic pathology after caffeic acid ingestion by DSS-treated mice. The CYP4B1 gene encodes a cytochrome P450 monooxygenase that catalyzes many xenobiotic and endogenous reactions, both detoxifying and activating (41). P450 gene expression is altered according to gender, microsomal enzyme inducers, age, diet, and hormones (42). Species-specific CYP4B1 mRNA have been reported to be mostly distributed in heart, brain, spleen, testis, lung, liver, skeletal muscle, and kidney in mice and humans (43). CYP4B1 mRNA has also been measured throughout small intestine and colon in rabbit and at low levels in human colon using in situ hybridization (44). Although induced further by 2-aminofluorene,

CYP4B1 was an abundantly expressed isoform of P450 in rabbit gastrointestinal tract. Our finding is the first evidence that CYP4B1 is found in mouse colon, and may be metabolically important in colitis. In addition to the intestinal tract, P450 expression and activity has been previously reported to be decreased in lung, liver and extrahepatic tissues during inflammation and infection (42, 45). The mRNA level of CYP4B1 was increased after resolution of the allergic pulmonary inflammation (45). Although CYP4B1 is responsible for bioactivation of many toxicants, a CYP4B1 transgene may provide some benefits of gene therapy for cancer or replacement studies using 2-aminoanthracene/4-ipomeanol (46, 47). The CYP4B1/4-1M system efficiently and rapidly killed hepatocellular carcinoma cells (48). Thus, the role of CYP4B1 in colitis and its putative regulation by caffeic acid are interesting targets for further study.

Some mechanisms reported to regulate certain members of P450 may be related to elucidate CYP4B1 gene regulation. CYP2C11 gene contains a binding site for the transcriptional factor NF- κ B (49). The inhibition of NF- κ B binding also improved the CYP2C11 promoter-reporter gene. Down-regulation of CYP2C11 and CYP3A mediated by IL-2 in combination with cytokine-induced activation of NF- κ B was reported in rat hepatocytes and may relate to IL-2 induction of the proto-oncogene transcription factor *c-myc* (50). However, little is known about the relationship between the CYP2C11 and CYP4B1 gene. It was recently reported that CYP3A4 expression was suppressed following NF- κ B activation by lipopolysaccharide (LPS) and TNF- α through interactions between NF- κ B and pregnane X receptor (PXR) and retinoid X receptor (RXR) complex. NF- κ B p65 disassociated the PXR/RXR complex from DNA sequences as determined by electrophoretic mobility shift assay and chromatin immunoprecipitation assays (51). Based on the above evidence, the effect of caffeic acid phenethyl ester (CAPE) to decrease NF- κ B shown in bacterial peptidoglycan polysaccharide-induced colitis in rats (12) suggests that caffeic acid might modify CYP4B1-specific mRNA expression through effects on NF- κ B as well, which deserves further study.

Another mechanism related to P450 regulation was associated with the role of nitric oxide. Khatsenko et al. (52) indicated that down-regulation of CYP2B1/2 mRNA and protein induced by LPS was blocked with phenobarbital in rats, mediated at least in part by nitric oxide. Takemura et al. (53) reported that LPS-induced suppression of CYP2C11 and CYP3A2 gene expression was prevented by an inhibitor of iNOS, but they did not study regulation of CYP4B1. Expression of iNOS-specific mRNA was decreased in adult male Wistar rats treated with 10

μmol CAPE/kg prior to torsion/detorsion injury in the testis (54). iNOS expression and NF- κ B binding activity were inhibited by CAPE in RAW 264.7 cells induced by LPS. The suppression of iNOS gene expression by CAPE may exert anti-inflammatory effects through NF- κ B inactivation (55). Nitric oxide (NO) levels were reduced in CAPE-treated Wistar rats during *Escherichia coli*-induced pyelonephritis (33). Decreased iNOS mRNA expression might play a role in normalizing CYP4B1 mRNA expression in the present study, but iNOS was decreased by all 3 phenolic treatments compared with DSS-only controls, and CYP4B1 was only normalized by caffeic acid compared with the no DSS group in the present study (Figure 6.5B). Thus the relation between the ability of phenolics to alter iNOS and CYP4B1 needs more study.

In conclusion, caffeic acid, rutin, and hypoxoside, decreased the gene expression of pro-inflammatory genes IL-17, iNOS, and increased IL-4 gene expression partially protecting from DSS-induced colitis, but CYP4B1 upregulated by caffeic acid was a key correlate of attenuated DSS-induced colitis in mice. Caffeic acid is a common phytochemical found widely in plant foods that may protect against IBD. Future studies should examine the extent to which various caffeic acid derivatives in plant foods and herbs might be metabolized to caffeic acid or other bioavailable metabolites related to caffeic acid (e.g., ferulic acid), and the function of CYP4B1 in colonic health and disease.

Footnotes

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Table 6.1. Sequences of Primers Were Used for Reverse Transcription Polymerase Chain Reaction Amplification

Gene— Object	Primer	Sequence (5'–3')	Amplification product size (bp)	Accession
GAPDH	Forward	TCACCACCATGGAGAAGGC	168	
	Reverse	GCTAAGCAGTTGGTGGTGCA		
IL-1-beta	Forward	CAACCAACAAGTGATATTCTCCATG	152	M15131
	Reverse	GATCCACACTCTCCAGCTGCA		X04964
IL-4	Forward	ACAGGAGAAGGGACGCCAT	95	M25892
	Reverse	GAAGCCCTACAGACGAGCTCA		X05253
IL-6	Forward	GAGGATACCACTCCCAACAGACC	141	X54542
	Reverse	AAGTGCATCATCGTTGTTTCATACA		M20572
IL-10	Forward	GGTTGCCAAGCCTTATCGGA	191	M37897
	Reverse	ACCTGCTCCACTGCCTTGCT		M84340
IL-17	Forward	GCTCCAGAAGGCCCTCAGA	142	NM_010552
	Reverse	AGCTTTCCTCCGCATTGA		U35108
IFN-gamma	Forward	TCAAGTGGCATAGATGTGGAAGAA	92	K00083
	Reverse	TGGCTCTGCAGGATTTTCATG		M74466
TNF-alpha	Forward	CATCTTCTCAAATTCGAGTGACAA	175	M13049
	Reverse	TGGGAGTAGACAAGGTACAACCC		Y00467
iNOS	Forward	CAGCTGGGCTGTACAAACCTT	95	U43428
	Reverse	CATTGGAAGTGAAGCGTTTCG		L23806
ICAM-1	Forward	CCGCAGGTCCAATTCACACT	143	X52264
	Reverse	TCCAGCCGAGGACCATACAG		X15372
CYP4B1	Forward	CACCTGGACTTCCTCGACAT	233	
	Reverse	TCATCCCACTGGAAGGAGTC		

Table 6.2. Colon Lengths Were Increased by Caffeic Acid or Hypoxoside and Food Intakes Were Improved by Each Phenolic in C3H/HeOuJ Mouse Model of Colitis¹

	Colon length (mm)	Food intake (g/d)		
		First day before DSS	Second day after DSS	Final day after DSS
DSS-only	52.5±5.0*	5.7±1.2 [#]	5.8±0.9 [#]	4.5±0.7 [‡]
Rutin and DSS	54.8±5.7*	5.3±0.9	5.3±0.7	5.1±0.6
Hypoxoside and DSS	57.1±5.5	5.2±1.3	5.1±1.7	4.9±1.4
Caffeic acid and DSS	58.8±3.2	5.5±0.7	5.6±1.3	5.1±1.1
Control diet (no DSS)	57.2±5.6	5.8±0.8	6.0±1.4	5.8±0.9
Rutin (no DSS)	57.6±4.4	5.3±1.1	5.5±0.9	5.7±0.8
Hypoxoside (no DSS)	59.6±4.9	5.2±1.3	5.1±1.3	5.3±1.2
Caffeic acid (no DSS)	60.0±4.3	5.6±0.8	5.3±0.6	5.5±0.9

* $P < 0.01$ significantly shorter compared to the control diet (no DSS) with other treatment groups in colon length; [#] $P < 0.01$ significantly greater compared with food intake on final day after DSS, within DSS-only group; [‡] $P < 0.05$ significantly less than the control diet (no DSS) in final day food intake after DSS. ¹In the experimental period, C3H/HeOuJ mice were fed AIN 93G supplemented with rutin, caffeic acid, or hypoxoside.

Table 6.3. Histopathology Scores Were Decreased by Caffeic Acid treatment in C3H/HeOuJ Mouse Model of Colitis

Treatment	Colon histopathology score	Cecal histopathology score
DSS-only	10.0±1.8*	10.9±2.5 [#]
Rutin and DSS	8.0±2.0*	9.2±4.7 [#]
Hypoxoside and DSS	8.8±1.7*	9.0±2.8 [#]
Caffeic acid and DSS	6.3±1.2	6.9±2.8
Control (no DSS)	4.3±1.4	7.1±4.8
Rutin (no DSS)	4.8±1.0	5.1±1.4
Hypoxoside (no DSS)	5.0±0.9	5.7±1.6
Caffeic acid (no DSS)	4.8±1.5	4.8±1.2

Scoring for the colonic and cecal histopathology evaluation of gastrointestinal inflammation included mucosal height, inflammatory cells, erosions, inflammatory score, and edema score in C3H/HeOuJ mouse treated with rutin, hypoxoside and caffeic acid with or without DSS. * $P < 0.01$ significantly greater than control (no DSS) in colon histopathology score; [#] $P < 0.01$ significantly greater than control (no DSS) in cecal histopathology score.

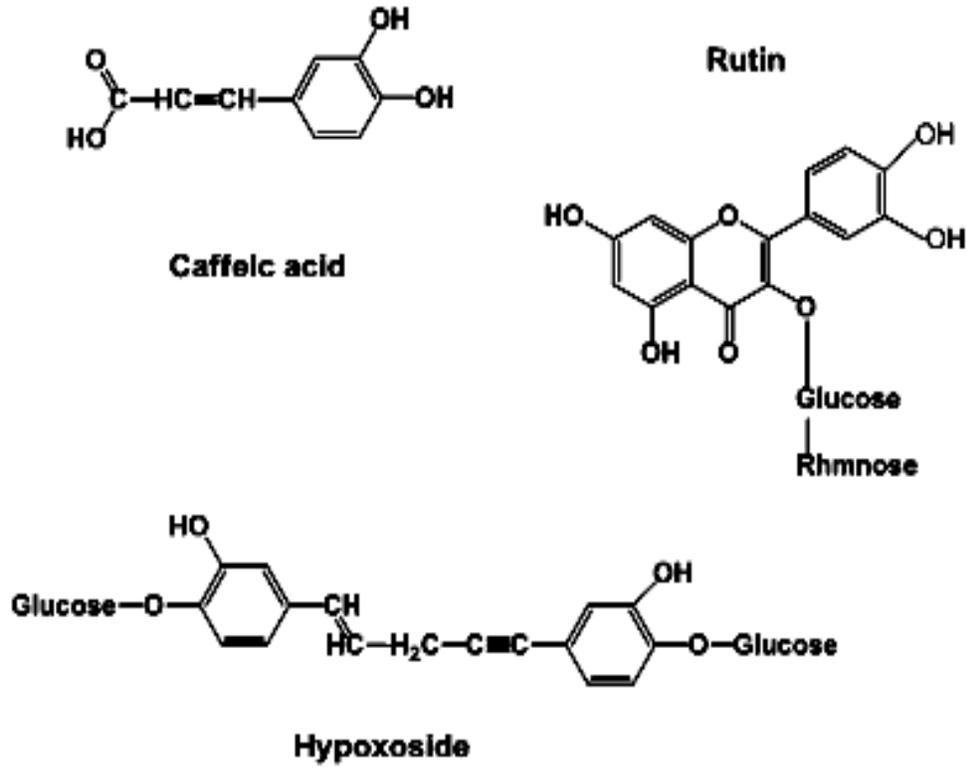


Figure 6.1. Structures of caffeic acid, rutin and hypoxoside.

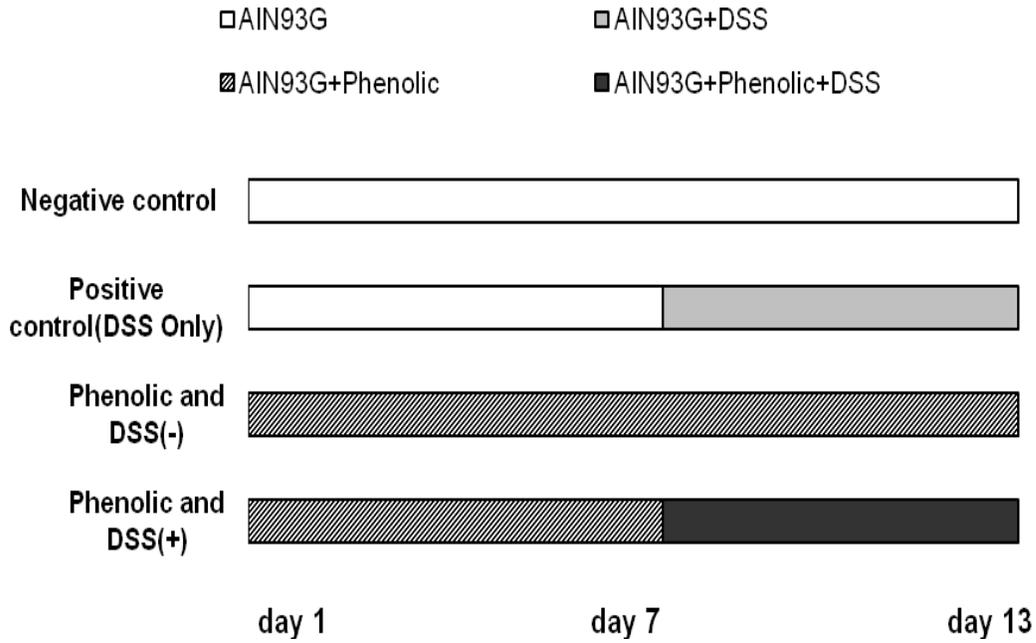


Figure 6.2. Experimental design. Phenolics fed were rutin (1.0 mmol/kg diet), hypoxoside extract (15 mg/d by gavage) and caffeic acid (1.0 mmol/kg diet). The study included eight groups of C3H/HeOuJ mice: DSS (-) without and with each of the 3 phenolics; DSS-only control: DSS (+); Rutin/DSS (+); Caffeic acid/DSS (+); Hypoxoside/DSS (+).

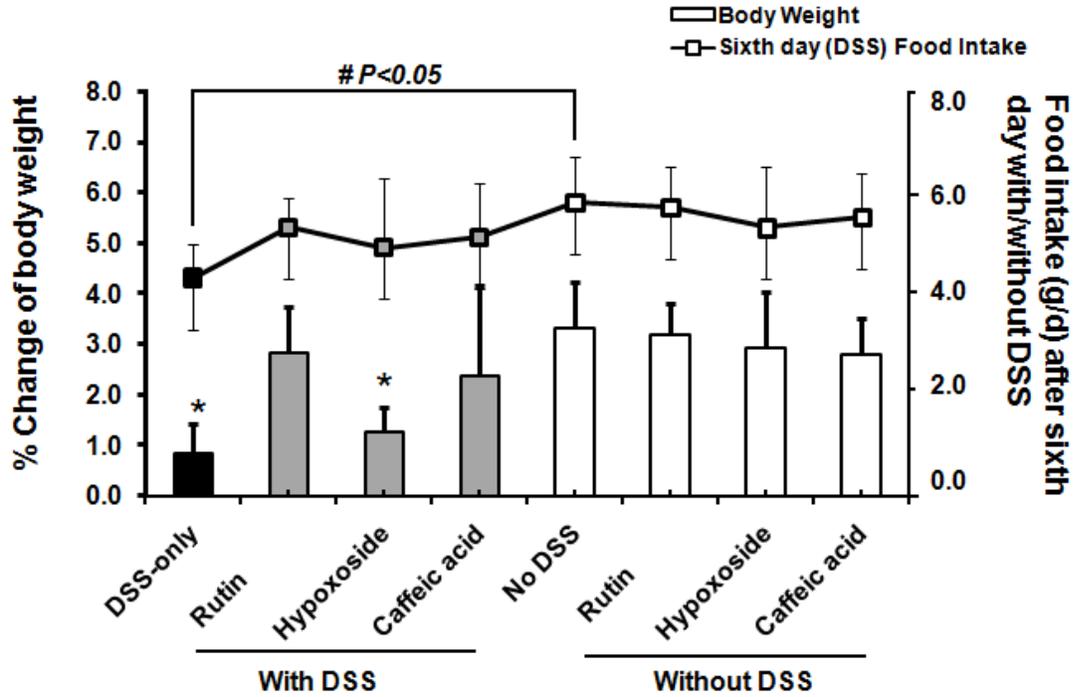


Figure 6.3. Rutin and caffeic acid normalized body weight in DSS-treated mice. $*P < 0.01$ significantly less compared with control (no DSS) group; mice given rutin, hypoxoside, and caffeic acid had normalized food intake at the sixth day DSS-treated compared with animals not given DSS control; $\#P < 0.05$ significantly less in DSS-only group on food intake after the sixth day DSS-treated than the control diet (no DSS).

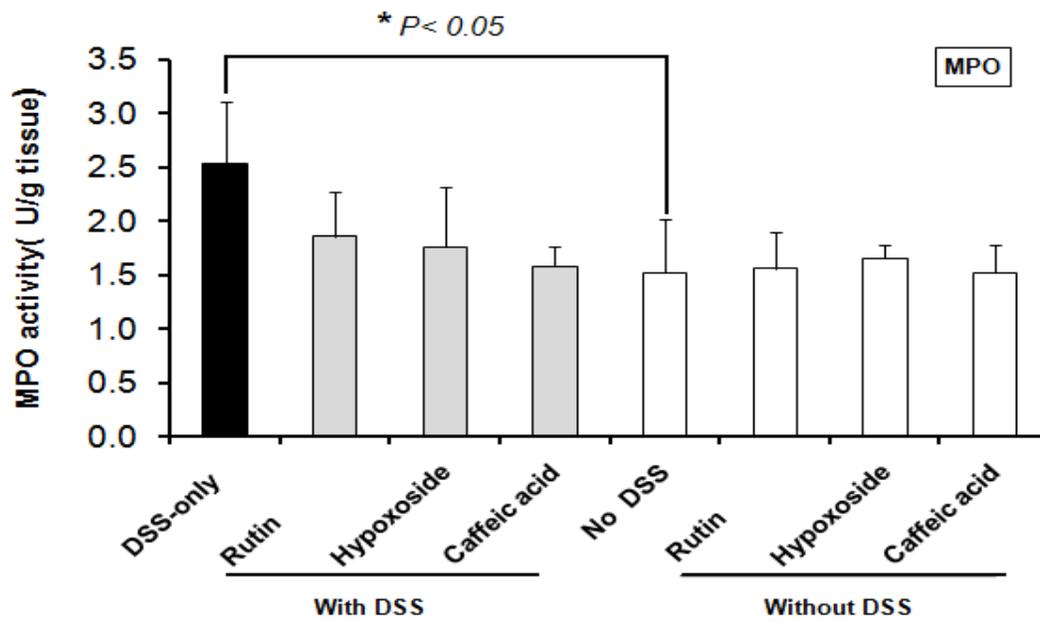
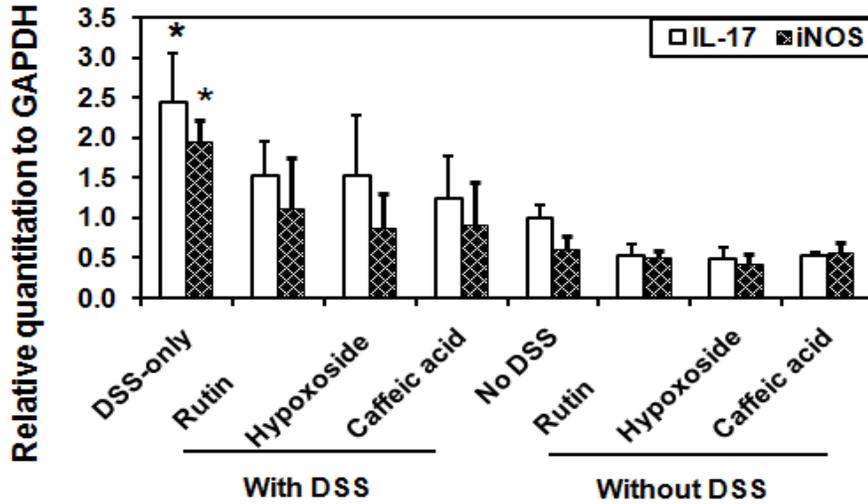


Figure 6.4. Rutin, hypoxoside and caffeic acid normalized colonic myeloperoxidase (MPO) activity in DSS-treated mice. * $P < 0.05$ greater compared with control (no DSS) group.

A



B

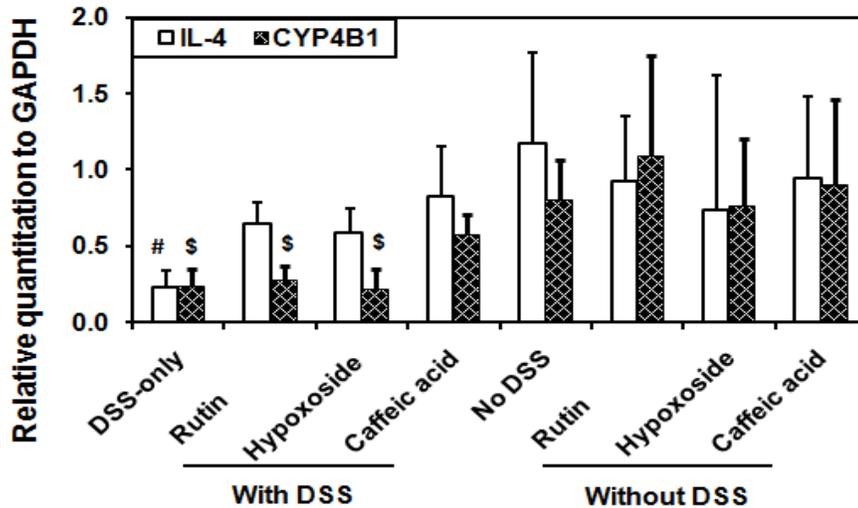


Figure 6.5. Pro-inflammatory gene expressions in rutin, hypoxoside and caffeic acid with or without DSS-treated mice ($n=6$). (A) Rutin, hypoxoside and caffeic acid normalized IL-17 and iNOS gene expressions in DSS-treated mice. $*P < 0.05$ greater in DSS-only group compared with control (no DSS) group. (B) Rutin, hypoxoside or caffeic acid normalized IL-4 gene expressions and caffeic acid normalized CYP4B1 gene expression in DSS-treated mice. $\#P < 0.05$ less in DSS-only group compared with control (no DSS) group; $\$P < 0.05$ less compared with control (no DSS) group.

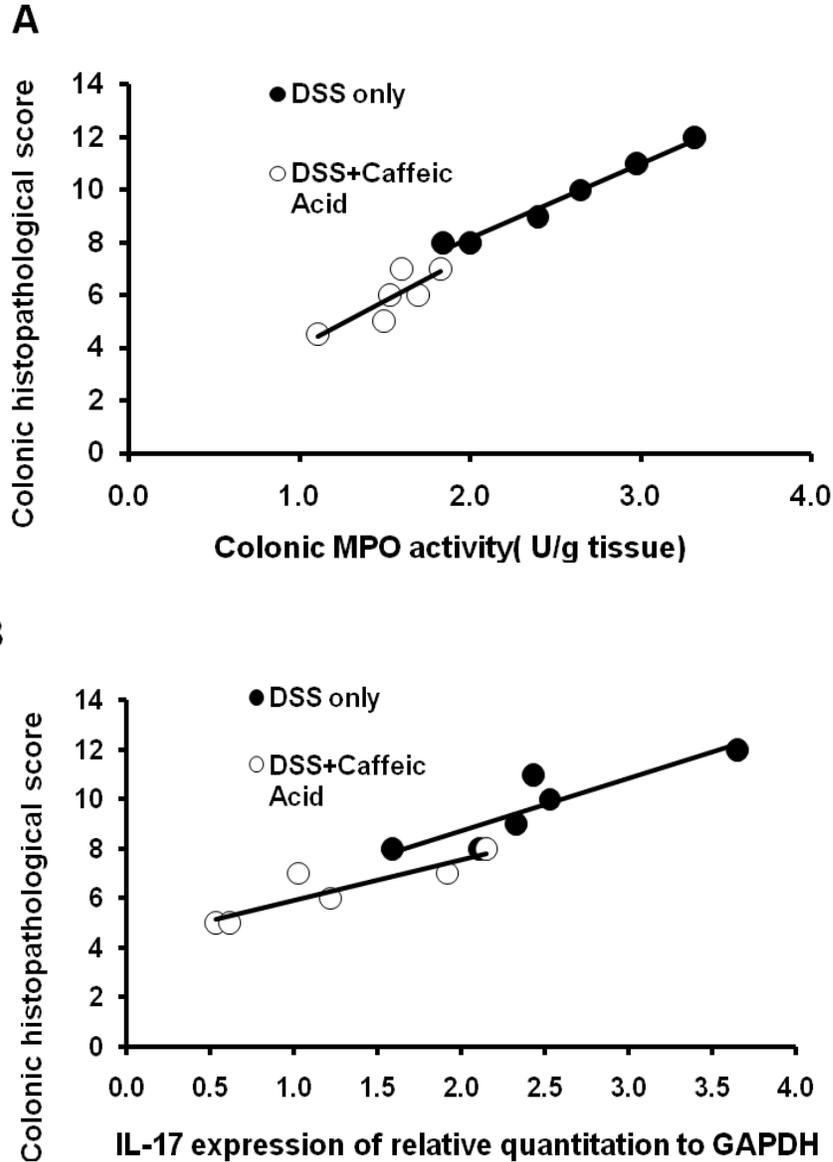


Figure 6.6. Relationship between colonic MPO activity/ IL-17 expression and histopathology score in DSS-only control and caffeic acid-treated with DSS mice. (A) Colonic histopathological score was significantly associated with colonic MPO activity in DSS-only control ($r = 0.83$, $P = 0.04$); and in caffeic acid-treated with DSS mice ($r = 0.92$, $P = 0.009$). (B) Colonic histopathological score was significantly associated with IL-17 expression in DSS-only control ($r = 0.88$, $P = 0.02$); and in caffeic acid-treated with DSS mice ($r = 0.89$, $P = 0.02$).

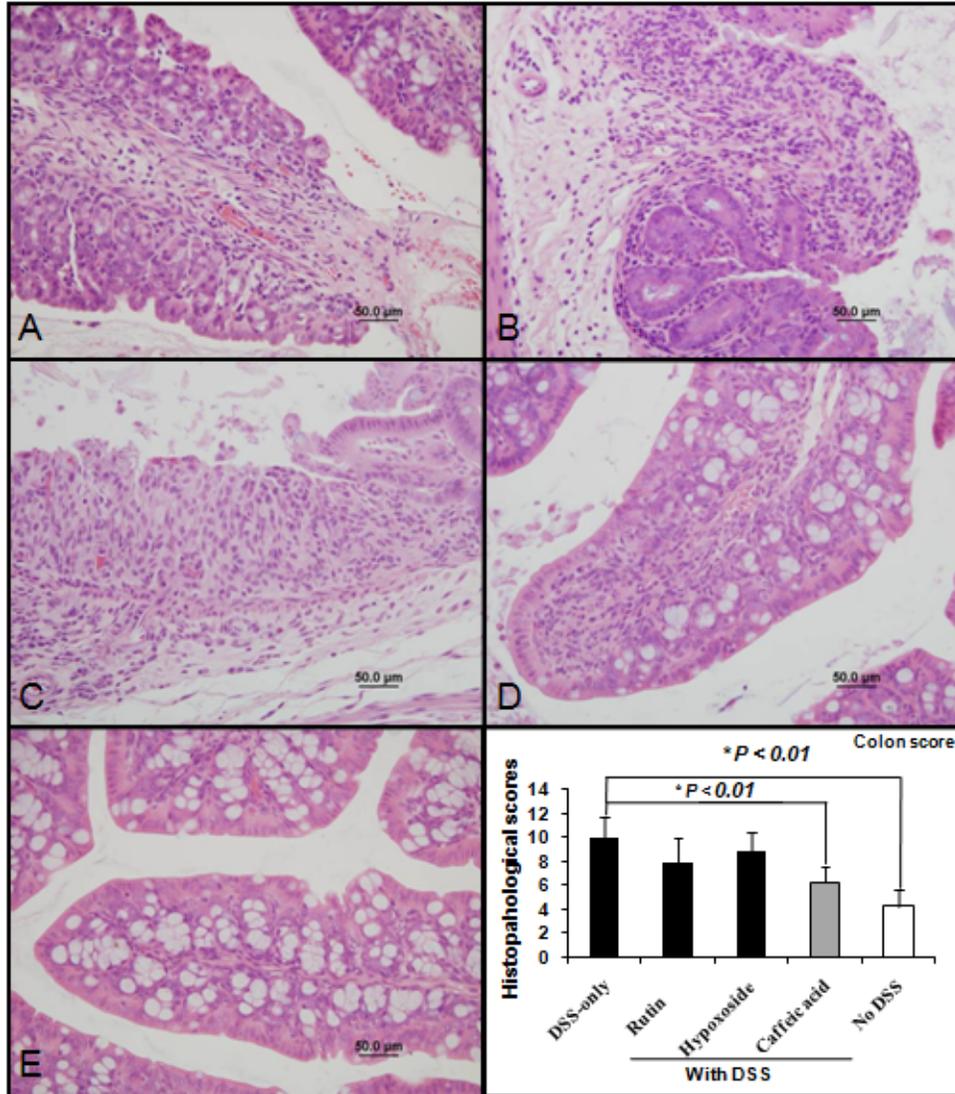


Figure 6.7. Colon histopathology (A-E, magnification = 400X, Scale bar = 50um). Colon from mouse given DSS only (A), Rutin + DSS (B), Hypoxicoide + DSS (C), Caffeic acid + DSS (D), and control mouse given neither DSS or dietary supplement (E). Note that caffeic acid treatment leads to reduced inflammatory cell infiltration within the lamina propria and prevents epithelial ulceration. * $P < 0.01$ less in caffeic acid + DSS and no DSS as compared with DSS-only control.

CHAPTER 7. VARIABILITY IN CAFFEIC ACID PROTECTION FROM DEXTRAN SULFATE SODIUM-INDUCED COLITIS IN MICE^{1,2}

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Abstract

Dietary caffeic acid protected against dextran sulfate sodium (DSS) induced colitis in C3H/HeO_J mice. To examine interindividual variability in the efficacy of caffeic acid, thirty six 10-wk-old CD-1/IGS female mice were fed 120 mg caffeic acid/kg diet, with 12 controls, both groups fed for 7 days. After this, half of the mice in each treatment were given 1.25% DSS in drinking water for 5 days. Caffeic acid treatment prevented mouse body weight loss by DSS ($p < 0.05$). Food intake suppression was prevented by caffeic acid/DSS compared with DSS-treated controls. Colon lengths in mice fed caffeic acid/DSS were longer than in DSS-treated control. Myeloperoxidase (MPO) was inhibited in mice given caffeic acid/DSS compared with DSS-treated controls. Cecal histopathological score (necrosis, edema, erosion and neutrophil infiltration) of DSS-fed mice was significantly more severe compared with mice fed caffeic acid before and during DSS treatment. Also expression of CYP4B1 was increased by caffeic acid/DSS treatment compared with DSS-treated control. Two subgroups were identified based upon cluster analysis of cecal histopathological score in mice fed caffeic acid/DSS. Mice with “severe” cecal damage showed mean cecal histopathological score of 8.5 ($n = 11$, $p < 0.05$) than did mice showing “mild” cecal damage (mean score = 4.5, $n = 4$). Caffeic acid-fed mice with severe cecal damage had significantly greater colonic MPO activity than did mice with mild cecal damage ($p < 0.05$). These effects in mice fed caffeic acid/DSS were also related to differences in caffeic acid bioavailability. In contrast, the subgroup of caffeic acid-fed mice with severe cecal damage showed a lower mean plasma concentration of caffeic acid. Relationships between cecal histopathological score and colonic MPO activity or colon length or caffeic acid plasma

concentration in caffeic acid/DSS treatment indicated a significant positive association with cecal score for colonic MPO activity ($r = 0.72, P < 0.01$) and a significant negative association with cecal score on colon length ($r = -0.41, P < 0.05$) or plasma concentration of caffeic acid ($r = -0.56, P < 0.01$).

Introduction

Caffeic acid is an antioxidant monophenol common in the human diet; and its potential health benefit depends on its bioavailability. Our long-term goals are to establish a screening assay for dietary components that may benefit colon health, to prevent colitis and possibly prevent colon cancer. Our previous study showed dietary caffeic acid protected against dextran sulphate sodium (DSS) induced colitis in C3H/HeO u J mice treated with different phenolics. The mechanism may involve upregulated CYP4B1 in intestinal expression. However, the antiinflammatory pattern related to the colonic myeloperoxidase activity or colon length and cecal histopathological score were not evaluated. And the caffeic acid bioavailability which related to gut microbial degradation remains unknown. In vitro cecal/fecal bacterial incubations with phenolics are a reasonable method to study to gut metabolism of phenolics. Caffeic acid has antioxidant, anti-inflammatory and antibacterial properties, including decreased malondialdehyde (MDA) and increased superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) in bilateral ovariectomized rats subjected to trinitrobenzene sulfonic acid (TNBS)-induced colitis (Ek RO et al., 2007). Better absorption of pure caffeic acid in the small intestine was associated with a higher plasma concentration and urinary excretion of intact caffeic acid and its tissular metabolites (Olthof et al., 2001). We hypothesize that variability in efficacy of caffeic acid against DSS-induced colitis is strongly linked with variability in caffeic acid bioavailability. Thus caffeic acid may be a useful model compound for studying the role of gut microbial degradation and metabolism of phenolics in compound efficacy.

Materials and Methods

Chemicals and Reagents

Purified caffeic acid was purchased from Chroma DexTM, Inc. Santa Ana, CA. Dextran sulphate sodium was purchased from Fisher Scientific (Pittsburgh, PA). RNAlater® Tissue

Collection solution was purchased from Applied Biosystems Business (Foster City, CA). Working solutions of 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (TMB, Sigma; 2.5 mM in water) and hydrogen peroxide (5 mM in water) were prepared immediately before use. Sulfuric acid (Fisher Scientific; 2 M) was used as a reaction stop solution. The detergent cetyltrimethylammonium bromide (CTAB, Sigma; 0.02% in water) was used as a lysing agent for determining total myeloperoxidase content of neutrophils. Phenylmethylsulfonyl fluoride (PMSF, Sigma), dimethyl sulfoxide (DMSO, Sigma) and phosphate buffered saline (PBS, pH 7.4) were used for tissue preparation.

Diets

In the experimental period, CD-1/*IGS* female mice were fed AIN 93 G (Harlan Teklad, Madison, WI) diet with or without DSS or treatment diets based on AIN 93 G containing caffeic acid (120 mg/kg in diet). Experimental diets were prepared and stored at 4°C.

Experimental Design and Animals

Forty eight 10-wk-old CD-1/*IGS* female mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were acclimated for 2 week before starting the experiment and were randomly assigned to four treatment groups in order to achieve similar mean body weight/group. Mice were individually housed in microisolater cages with wood chip bedding and consumed standard rodent lab chow and tap water ad libitum during the acclimation period. The animal room was maintained at 23°C with a 12-h light/dark cycle during the experimental period. Thirty six 10-wk-old CD-1/*IGS* female mice were fed 120 mg caffeic acid/kg diet, with 12 controls, both groups fed for 7 days. After this, half of the mice in each treatment were given 1.25% DSS in water for 5 days. Colitis was induced in two groups with DSS in their drinking water, a method previously reported with some modifications (Kitajima et al., 1999; Murakami et al., 2003). The various caffeic acid treatments were fed to the mice for 7 days prior to DSS exposure and continually during DSS treatment. Food intake was measured weekly over 2-3 consecutive days per week. Body weights were measured twice a week. Signs of disease (weight loss, diarrhea, dehydration) were observed daily. The supplemented diets and the DSS-containing drinking water were provided to the mice continually until the experiment was terminated. At the end of the feeding period, diets were not withdrawn from hamsters before they were killed by exposure to CO₂. Blood samples were collected by cardiac puncture in EDTA tubes and centrifuged at 5000 x g; 10 min, 4°C. Plasma samples were then frozen at -20°C until analysis.

All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.

Tissue Sample Collection and Preparation

The colon and cecum were removed after euthanasia. After washing in phosphate-buffered saline (PBS), they were placed on filter papers to measure colonic lengths, score macroscopic cecal lesions and obtain photographs of each tissue. The colonic and cecal contents were removed, and the colon and cecum from each animal were fixed in formalin and all tissue samples were evaluated from each group for histopathological analyses. Gross cecal lesions were scored using published criteria (Nibbelink et al., 1992). Macroscopic cecal lesions were scored 0-4 as follows: no gross lesions (grade 0, normal); evidence of atrophy (grade 1, mild); excess intraluminal mucus with atrophy localized to the cecal apex (grade 2, moderate); generalized cecal atrophy with increased intraluminal mucus and no cecal contents (grade 3, severe); score 3 plus bloody cecal content (grade 4, most severe). Colonic tissues for myeloperoxidase activity were put in 15% DMSO and 0.1 mM PMSF in cryovials (Corning Company, Corning, New York). A portion of colon (approximate 1.5 cm) for each sample was placed into *RNA Later* (1.2 mL) for subsequent RT-PCR analysis of cytokine-specific mRNA expression. All above samples were stored at -85°C until analysis.

Colonic Mucosal Histopathological Analysis

Cecum and proximal colon in 10% neutral buffered formalin were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Sections of the cecum and proximal colon were scored by a pathologist (Dr. J. Hostetter) who was blinded to the treatment group as described previously (Jergens et al., 2006).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Colonic Inflammation-Related Cytokine Gene Expression

Based on our previous study, primers for CYP4B1 (cytochromes P450, family 4B1), IL-1 β , IL-4, IL-17, GAPDH were analyzed in pooled colonic samples from each treatment group. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA in each pooled sample was performed as previously described with some modifications (Overbergh et al., 2003)

Myeloperoxidase Assay Method in Colonic Tissue

Myeloperoxidase (MPO) activity which was used to quantify neutrophil accumulation in tissues was assessed using 96-well flat bottom microtiter plates (Linbro/Titertek, USA) and was previously described with some modifications (Xia et al., 1997)

Statistical Analysis

Data were analyzed by the difference between means and statistical significance was calculated using two-way ANOVA followed by Tukey method as a Post Hoc test (SAS Institute, 2003, Cary, NC). The equal variance and normality of residuals assumptions are verified by a residual vs. predicted values plot and a histogram of residuals. Colon length, food intake, body weight change, MPO activity, cecal histopathology scores, and cytokine/enzyme RT-PCR products were reported as means \pm SEM. Statistical significance was set at $P < 0.05$. Pearson correlation analysis was used for relationship between MPO activity/cytokine gene expression, colon length and cecal histopathology score.

Plasma Extraction for Detecting Caffeic Acid Concentration

To 1.0 mL of thaw plasma sample was added 1.0 mL of 0.1M sodium acetate buffer (pH 5.5), 50 μ l of o-coumaric acid (Conc. 100 μ g/mL in 100% MeOH), and 30 μ L β -glucuronidase (4493units)/ sulfatase (133units) (H₂ type). The mixture was vortexed and incubated for 2 hours at 37 °C on a shaker, and adjusted pH 3.0 with 4N HCl. Then added 600 mg of NaCl and 4 volume of ethyl acetate; and centrifuged 10000 x g for 10min at 4°C to collect supernatant; repeated three time above procedure (add 4 volume of ethyl acetate and centrifuge) to collect whole supernatant. Pooled supernatants were dried under N₂ (organic phases), then dissolved with 200 μ l of 80% MeOH to filter into HPLC vial for analysis.

Caffeic Acid Identification and Quantification Using LC-MS-UV Analysis

Caffeic acid concentration was detected by high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) after extraction from the blood plasma. An Agilent Technologies 100 Ion Trap Liquid Chromatography-Electron Spray Ionization-Mass Spectrometer, with a coupled UV absorption detector (LC-MS-UV) was used for quantification of caffeic acid. The instrument was tuned and calibrated in the ESI mode. The mass spectrometer scanned from 50 to 450 M/Z. Heat block temperature for the analysis was 450 °C. The nebulizer gas flow was 2.5 L/min; the detector voltage, 1.6 V. 10 μ L of sample was injected onto a reversed-phase, C18 ODS-AM 5 μ m, 120 A column (150 mm \times 2.0 mm) (YMC

Co. Ltd., Wilmington, NC). Standard was run on the LC-MS. The peaks from the extract samples were confirmed by evaluating the retention time and mass spectra of peak with the standard.

Results

General Effects of Treatments on DSS-Induced Colitis

Regardless of the treatment group, food intakes measured on the day before drinking DSS-supplemented water and second day of the DSS treatment period did not differ (data not shown). Food intake was decreased at final day after DSS-treated alone compared with control not treated with DSS (Table 7.1). Food intakes in the caffeic acid-only group did not differ from control not treated with DSS (Figure 7.2). Mice given caffeic acid had normalized food intake at the final day DSS-treated compared to animals not given DSS control (Figure 7.2). Body weight change (the percentage of body weight gained in DSS period over the body weight before DSS-treated) in DSS-positive control was significantly less than in DSS-negative control ($P < 0.01$). DSS-treated mice given diets supplemented with caffeic acid showed improved body weight (Figure 2). The mice fed caffeic acid not treated DSS were similar to controls without DSS in body weight increase (Figure 7.2). No diarrhea and rectal bleeding were observed during the period of DSS treatment. The colon lengths of DSS-treated mice fed caffeic acid was significantly longer than those from the DSS-only control ($P < 0.01$) (Table 7.1).

Colonic Myeloperoxidase (MPO) Activity of Caffeic acid Treatments and DSS Controls

Colonic MPO activity in DSS-only control was significantly greater than in non-treated control mice ($P < 0.01$). MPO activities were significantly decreased in the colonic extracts from DSS-treated mice fed caffeic acid compared with the colonic extracts from the DSS-only control mice (Figure 7.3; $P < 0.05$). MPO activities in mice fed caffeic acid without DSS exposure did not differ from untreated control mice (Figure 7.3).

Cecal Histopathology Changes in Mice

Cecal histopathology scores of the DSS-treated mice were significantly more severe compared with control mice not given DSS ($P < 0.01$, Table 7.1). The group fed caffeic acid with DSS had significantly less severe cecal microscopic lesion scores than mice treated with DSS alone. Representative histological images from each group are shown (Figure 7.4). The mean cecal histopathology scores were both significantly decreased in the group fed caffeic acid

with DSS compared to DSS-only control ($P < 0.05$, Figure 7.4). In the group fed caffeic acid with DSS, hierarchical clustering on cecal pathological score was showed that mice with “severe” cecal damage had higher mean cecal histopathological score of 8.5 ($n = 11$, $p < 0.05$) than mice showing “mild” cecal damage (mean score = 4.5, $n = 4$) (Figure 7.1).

Reverse Transcription–Polymerase Chain Reaction Analysis of Cytokine mRNA

Some genes have been implicated in the pathogenesis of colitis, including CYP4B, IL-1 β , IL-4, IL-17, GAPDH (Ding et al., 2003; Reed et al., 2005; Baer et al., 2006; Ye et al., 2009). CYP4B1-specific mRNA differed among the various treatments. There was significant down-regulation of CYP4B1 mRNA expression in DSS-treated control compared to control not given DSS ($P < 0.01$, Figure 5). Mice fed caffeic acid and treated with DSS had significantly increased CYP4B1-specific mRNA levels compared with DSS-only control ($P < 0.05$, Figure 7.5).

Plasma Caffeic acid Concentration and Histological Changes

In the group fed caffeic acid with DSS, caffeic acid concentration was measured by high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) (Figure 7.6). The range of caffeic acid concentration was 0.8 to 3.2 $\mu\text{mol/L}$ and average concentration was $1.9 \pm 0.6 \mu\text{mol/L}$. Based on the hierarchical clustering on cecal pathological score in this treatment, the higher cecal histopathological score subgroup with “severe” cecal damage had a lower caffeic acid concentration at $1.7 \mu\text{mol/L}$; whereas the lower cecal histopathological score subgroup with “mild” cecal damage had a higher caffeic acid concentration at $2.8 \mu\text{mol/L}$ ($p < 0.05$). Furthermore, the higher cecal histopathological score subgroup with “severe” cecal damage had significantly greater colonic MPO activity than did mice with mild cecal damage ($p < 0.05$). These effects in mice fed caffeic acid/DSS were related to differences in caffeic acid bioavailability. In contrast, the subgroup of caffeic acid-fed mice with severe cecal damage was significantly associated with a lower plasma concentration of caffeic acid (Figure 7.7).

Relationship between the Colonic Histopathology Score Evaluation and Colonic Myeloperoxidase Activity/Colon Length

Relationships between cecal histopathological score and colonic MPO activity or colon length or caffeic acid plasma concentration in caffeic acid/DSS treatment were indicated that a significant positive association with cecal score on colonic MPO activity and a significant negative association with cecal score on colon length or plasma concentration of caffeic acid,

with greater caffeic acid plasma concentration ($r = -0.56$, $P < 0.01$) or longer colon length ($r = -0.41$, $P < 0.05$, data not shown) and less MPO activity ($r = 0.72$, $P < 0.01$) associated with decreased cecal histopathological score (Figure 8). Relationships between caffeic acid plasma concentration and colonic MPO activity in caffeic acid/DSS treatment were indicated that a significant negative association with plasma concentration of caffeic acid on colonic MPO activity ($r = -0.49$, $P < 0.01$) (Figure 7.8).

Discussions

Epidemiological studies have also highlighted the association between the consumption of caffeic acid-rich food and beverages and the prevention of colitis and cancer (Watabe et al., 2004). Caffeic acid is the major representative of the hydroxycinnamic acids and occurs in foods mainly as chlorogenic acid (5-caffeoylquinic acid), an ester of quinic acid. Coffee is a major source of chlorogenic acid in the diet, daily intake of chlorogenic acid in coffee drinkers up to 1 g. Dietary caffeic acid have been widely assumed to be beneficial to human health by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity (Baderschneider et al., 2001). Chlorogenic acid and caffeic acid have vicinal hydroxyl groups on an aromatic residue and exhibit antioxidant activities, antimutagenic and anticarcinogenic effects in vitro (Rice-Evans et al., 1996; Scalbert et al., 2002). Indeed, this is consistent with the reported inverse correlation between coffee intake and colon cancer in some epidemiologic studies (Tavani et al., 1997).

In humans, the absorption and metabolism of chlorogenic acid and caffeic acid have been studied in human gut and related microflora. Based on a human study, only one third of chlorogenic acid was absorbed in the small intestine of humans whereas almost all of the caffeic acid was absorbed in the ileostomy subjects who ingested both 2.8 mmol of chlorogenic acid and caffeic acid during 24 h (Olthof et al., 2001). In contrast, intake of pure caffeic acid was better absorbed in the small intestine than chlorogenic acid and associated with a higher plasma concentration and urinary excretion of intact caffeic acid and its tissue metabolites in humans (Olthof et al., 2001).

In another human study, five healthy male consumed 100, 200, and 300 mL of red wine corresponding 0.9, 1.8, and 2.7 mg of caffeic acid, respectively. Plasma concentration were measured at different times (0-300 min) for evaluating the antioxidant effect of caffeic acid.

Plasma samples were prepared by HCl-hydrolysis method and analyzed by HPLC. The method of plasma total radical-trapping antioxidant parameter (TRAP) was determined for antioxidant potential of caffeic acid. Plasma concentrations of caffeic acid and antioxidant property were dose-dependent and the C_{max} was reached at about 60 min after red wine intake. At this time point, plasma caffeic acid concentrations were 1.19, 3.23, and 4.90 ng/mL for each group, associated with 6.0, 19.6, and 25.4 % increases in TRAP. Caffeic acid was bioavailable and was correlated with the antioxidant potential of red wine intake (Simonetti et al., 2001).

Coffee was one of the most popular sources to investigate the bioavailability and metabolism of caffeic acid derivatives. Five nonsmoking healthy male volunteers were administered two cups of coffee containing 4 g of instant coffee powder (corresponding to total caffeic acid intake of 441.4 mg, or 6.3 mg/kg BW). The results showed a highly significant increase in cumulative urinary excretion of isoferulic, ferulic, and dihydroferulic acid ranging from 1.9 to 15.1 mg. Urinary 3-hydroxyhippuric acid was increased to 102.9 mg over 24 h urine for postsupplementation (Rechner et al., 2001). In present study, we did not find the isoferulic, ferulic, and 3-Hydroxyhippuric acid in plasma. The reason may be that this strain of mice did not form these metabolites. The concentration of caffeic acid was less in mouse diet (120mg/kg diet, 24 mg/kg BW, equivalent to a human dose of ~2.4 mgk/kg), which might result in less ability to detect these metabolites.

Most of the caffeic acid was present in plasma as glucuronate/sulfate forms. Plasma caffeic acid was derived from hydrolysis of chlorogenic acid in the gastrointestinal tract when drinking the coffee containing no free caffeic acid (Nardini et al., 2002). Ten healthy male nonsmoker moderate-coffee drinkers were asked to ingest a standard 200 mL brewed coffee (corresponding 166mg caffeic acid) which contained 478.9 $\mu\text{g/mL}$ chlorogenic acid and undetectable caffeic acid. If the coffee was hydrolyzed by HCl-hydrolysis method, no chlorogenic acid and only 830.0 $\mu\text{g/mL}$ caffeic acid and small amount of *p*-coumaric acid and ferulic acid were found in hydrolysis solution. Plasma samples were collected 1 and 2 h after coffee administration for analyzing free and total phenolic acid content. Caffeic acid was the only phenolic acid found in plasma samples after coffee administration, whereas chlorogenic acid was undetectable. One hour after coffee consumption, free plasma caffeic acid level was 20.9ng/mL, whereas 91.1 and 91.3 ng/mL were found with β -glucuronidase treatment and alkaline hydrolysis (Nardini et al., 2002). In present study, caffeic acid was also present in

plasma as glucuronate/sulfate forms. Plasma caffeic acid concentration was also measured after β -glucuronidase/sulfatase treatment; free plasma caffeic acid was not detected in mice.

It is much better to compare with animal models for present study than human trials because some factors may be different from humans (dosage, duration, gut microbes). The absorption and metabolism of caffeic acid were studied with different dosages to test plasma profiles of their metabolites. One rat study, using 700 $\mu\text{mol/kg}$ body weight of caffeic acid and collecting blood from the tail for 6 h after administration, free caffeic acid and ferulic acid were detected at 1.2 and 1.6 $\mu\text{mol/L}$, respectively; caffeic acid glucuronides were the main plasma metabolites 2 h after administration with a concentration of $26.1 \pm 3.5 \mu\text{mol/L}$, corresponding to 41% of all of the metabolites determined at this time; caffeic acid sulfate/glucuronide conjugates was $12 \mu\text{mol/L}$ (Azuma et al., 2000). Comparing with our present study, mice were fed 120 mg caffeic acid/kg diet for 7 days before DSS treatment and 5 days during DSS in drinking water and food intake was around 5 g daily. The intake of caffeic acid was $3.35 \mu\text{mol/day}$ and total intake was $40.2 \mu\text{mol}$ (x12 day). In rat study, using 700 $\mu\text{mol/kg}$ body weight of caffeic acid and based on the body weight of rats (around 200g), the total amount was 140 μmol which was close to 40-fold that of the present study as a daily dose. In a rat study, caffeic acid glucuronides were $26.1 \mu\text{mol/L}$, corresponding to 41% of all of the metabolites which included free caffeic acid, ferulic acid, and caffeic acid sulfate/glucuronide conjugates. The total concentration was $63.7 \mu\text{mol/L}$ which was 34-fold of the present study (average of CA concentration: $1.9 \mu\text{mol/L}$). Also in another rat study, giving $250 \mu\text{mol/day}$ caffeic acid for 8 d, total urinary excretion of caffeic, ferulic, and isoferulic acids was 28.1 % of intake (mol/mol); urinary m-hydroxyphenylpropionic acids (*mHPP*) was 4.0 %. Plasma metabolite concentrations in rats fed caffeic acids for 8 d were caffeic acid ($41.3 \mu\text{mol/L}$), ferulic acid ($7.3 \mu\text{mol/L}$), isoferulic acid ($4.5 \mu\text{mol/L}$), hippuric acid ($54.2 \mu\text{mol/L}$), and m-hydroxyphenylpropionic acid ($1.4 \mu\text{mol/L}$) (Gonthier et al., 2003). The total intake for 8d was 2mmol ($250 \mu\text{mol/day} \times 8 \text{ d}$) which was 50-fold our present total amount of $40.2 \mu\text{mol}$ ($3.35 \mu\text{mol/day} \times 12 \text{ day}$) and the total absorbed caffeic acid equivalent concentration was $108.73 \mu\text{mol/L}$ which was also close to 50-fold that of plasma CA concentration in our present study ($1.9 \mu\text{mol/L}$). Furthermore, a later study in which the dose was much lower than previous studies was more relevant to our present study. The dose in the male Wistar rats was 100 $\mu\text{mol/kg}$ body weight of caffeic acid by gastric intubation and the average BW was 150g. The total amount consumed was 15 μmol which was ~5-fold greater

than the present dosage (3.35 μ mol/day). The serum concentration of intact CA in the portal vein peaked at 10 min after administration as quantified by a coulometric detection method using HPLC-ECD, with a C max of 11.24 μ mol/L for caffeic acid (Konishi et al., 2005) which was also 5-fold that of CA concentration in the present study (1.9 μ mol/L). Thus, the plasma concentration detected in the present study relative to dose seemed proportionately similar to previous findings.

Across these three animal studies, the mean and standard deviation of plasma caffeic acid concentration were 26.1 \pm 3.5 μ mol/L (n = 3, Azuma et al. (2000)), 41.3 \pm 6.1 μ mol/L (n= 8, Gonthier et al. (2003)) and 11.2 \pm 2.3 μ mol/L (n = 3, Konishi et al. (2005)), respectively, whereas the individual range and variable difference were not reported. The percentages of standard deviation of mean (CV) were 13.4%, 14.8%, 20.5%, respectively. Our present study showed 1.9 \pm 0.6 μ mol/L (CV=31.5%, n = 15) which indicated more variability, from 0.8 to 3.2 μ mol/L at a lower dose of CA diet fed to mice compared with the other three animal study, possibly associated with greater interindividual differences in gut microbial degradation of caffeic acid in the present study than in previous studies, but this remains to be determined.

Myeloperoxidase (MPO) is a mammalian enzyme stored in neutrophils and macrophages. During inflammatory process, these leukocytes infiltrated to the site of inflammation and increased the local releasing of non-specific inflammatory mediators, such as cytokines, chemokines, nitric oxide (NO), and including MPO, enhancing tissue destruction (Podolsk, 2002; Jackson et al., 2006). Tyrosyl radical was generated by myeloperoxidase and activated phagocytes to damage both proteins and lipids. Increased tyrosyl had been detected in inflammatory disease and many degenerative disorders (Heinecke et al., 2002).

High MPO level was found in DSS-induced experimental colitis in which MPO activity was decreased in DSS plus curcumin-treated BALB/c mice (Deguchi et al., 2007). In the other acetic acid-induced rat colitis, tropisetron, a 5-HT receptor antagonist, significantly decreased colonic MPO activity, lipid peroxidation, and inflammatory cytokines interleukin-1beta, interleukin-6 and tumour necrosis factor-alpha levels (Mousavizadeh et al., 2009). On the other hand, 10 μ mol/kg caffeic acid phenethyl ester attenuated the tissue levels of MPO and the testicular injury induced by testicular torsion in rats (Atik et al., 2006). On acute necrotizing pancreatitis rat model which induced by glycodeoxycholic acid, caffeic acid phenethyl ester (CAPE) significantly reduced the activity of MPO in pancreatitis plus CAPE infusion treatment

(Turkyilmaz et al., 2008). In our present study, colonic MPO activity was significantly decreased in DSS-treated mice fed caffeic acid compared with the DSS-only control mice. Furthermore, a significant negative association with plasma concentration of caffeic acid on colonic MPO activity was indicated with greater caffeic acid plasma concentration related to lower colonic MPO activity.

One important result showed that CYP4B1-specific mRNA was also upregulated by caffeic acid treated DSS-induced mice, compared with DSS treated controls which confirmed the previous study in C3H/HeOJ colitis (Ye et al., 2009). We did not find a correlation of CYP4B1 with plasma caffeic acid concentrations and clustering difference on cecal pathological score in this treatment, but technical problems only permitted analysis of CYP4B1 in 6 of 18 mice fed caffeic acid + DSS. The relation between caffeic acid concentration and CYP4B1 deserves further investigation given the previous finding of the importance of CYP4B1 in caffeic acid protection from colitis.

Furthermore, in the group fed caffeic acid with DSS, based on the hierarchical clustering on cecal pathological score in this treatment, higher caffeic acid concentration was associated with decreased cecal histopathological score. This study was in concordance with our previous isoflavone bioavailability study which reported that high urinary isoflavone excreters had significantly decreased non-HDL cholesterol compared with low isoflavone excreters among 38 Golden Syrian hamsters fed soy protein diet for 4 wk. Urinary isoflavone excretion phenotypes predicted the cholesterol-lowering efficacy of soy protein (Ye et al., 2006). In our present study, we investigated that the caffeic acid plasma concentration was an important, potentially controllable variable in studies of effects of caffeic acid on colitis. In a conclusion, dietary caffeic acid protected against DSS induced colitic pathology in mice. This protective effect may have been partly mediated by increasing CYP4B1. Also these effects in mice treated with caffeic acid and DSS were influenced strongly by caffeic acid bioavailability, possibly related to interindividual differences in gut microbial degradation of caffeic acid, which is under study currently.

Footnotes

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Table 7.1. Colon length short /food intake suppression were prevented and cecal histopathology score was significantly decreased in mice fed caffeic acid after DSS treatment¹

	Colon length (mm)	Cecal histopathology score	Food intake (g/d)	
			First day before DSS	Final day after DSS
No DSS	71.1±9.2	4.5±1.0	4.1±1.1	3.9±1.5
DSS only	61.5±3.6*	9.2±1.7 [#]	4.5±0.9	2.5±1.7 ^{\$}
Caffeic acid and DSS	68.5±6.6	6.9±2.7	4.1±1.2	3.9±0.9
Caffeic acid only	79.6±1.9	5.1±1.9	4.2±0.8	4.3±0.9

* $P < 0.01$ significantly shorter compared to the control diet (no DSS) with other treatment groups in colon length; [#] $P < 0.01$ significantly greater compared with cecal histopathology score within DSS-only group; ^{\$} $P < 0.05$ significantly less than the control diet (no DSS) in final day food intake after DSS. ¹In the experimental period, 48 CD1/*IGS* female mice were fed AIN 93G supplemented with/without caffeic acid.

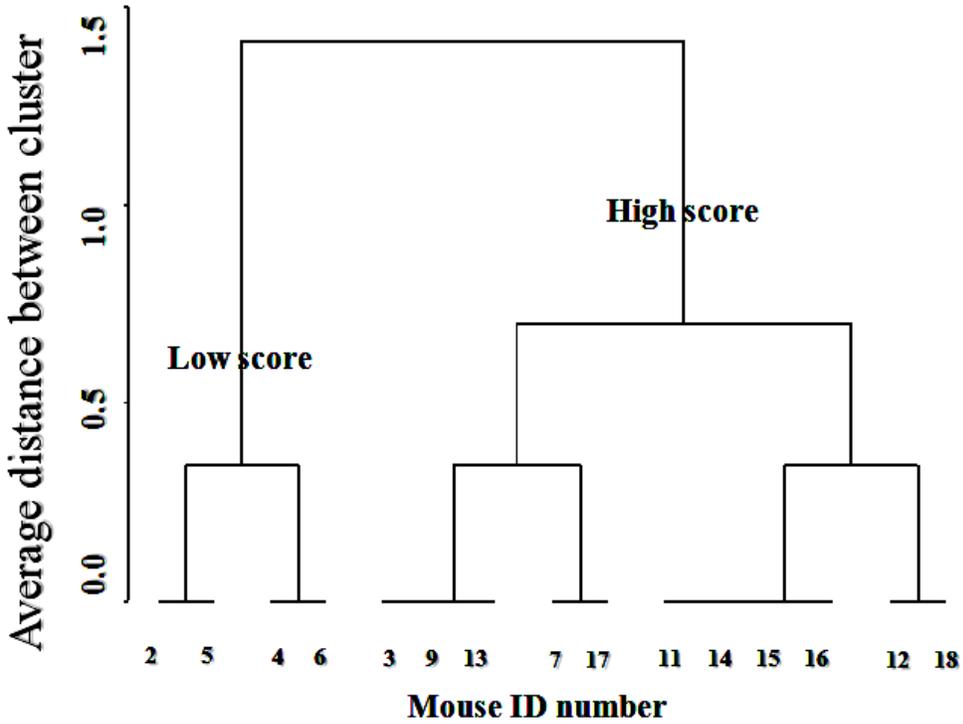


Figure 7.1. Hierarchical clustering on cecal pathological score with caffeic acid treated in DSS-induced colitis. Mice with “severe” cecal damage showed mean cecal histopathological score of 8.5 ($p < 0.05$) than did mice showing “mild” cecal damage (mean score = 4.5).

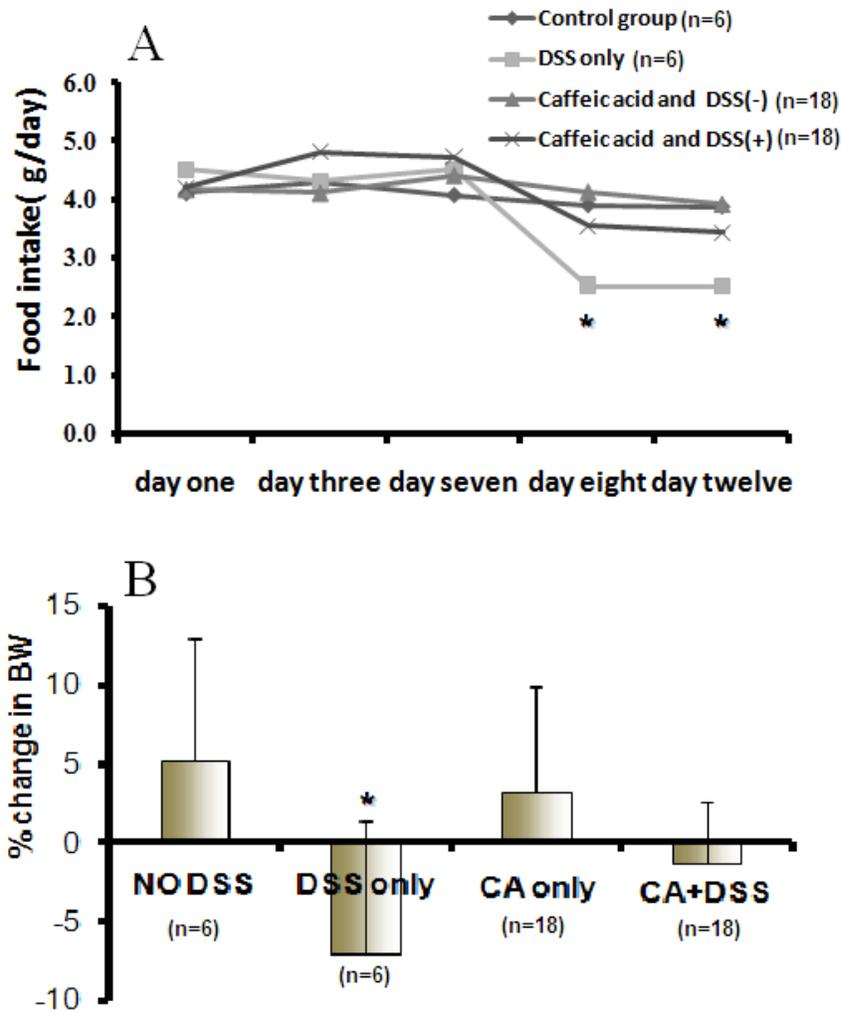


Figure 7.2. Food intake and body weight for DSS-induced colitis in CD1/IGS female mice. (A) Food intake suppression was prevented by caffeic acid/DSS compared with DSS-treated controls; $*P < 0.05$ significantly less than the control diet (no DSS) in day 8 and 12 food intake after DSS. (B) Caffeic acid treatment prevented mouse body weight loss by DSS; $*p < 0.05$ indicated that caffeic acid/DSS treatment had a less body loss compared to DSS-only positive control.

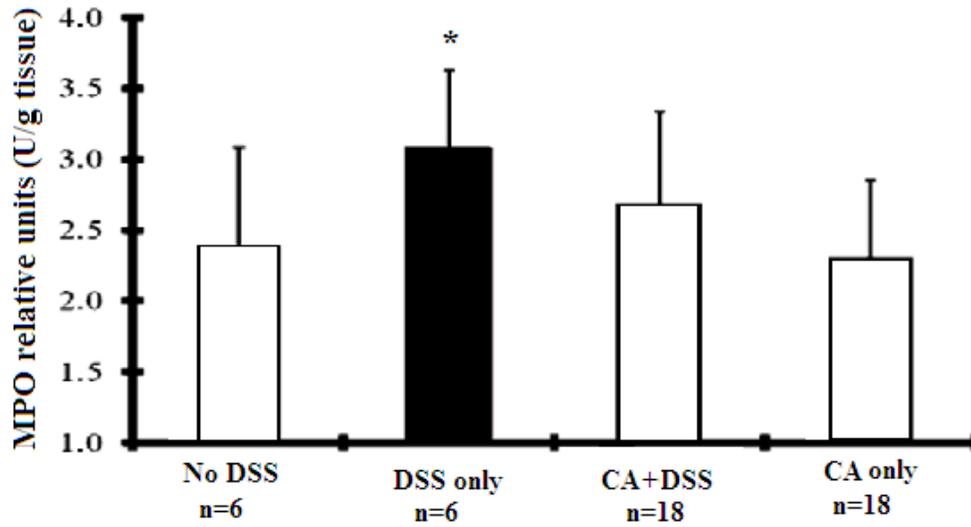


Figure 7.3. MPO activity was decreased by caffeic acid treated in DSS treatment group; $*p < 0.05$ indicated myeloperoxidase (MPO) was inhibited in mice given caffeic acid/DSS compared with DSS-treated controls.

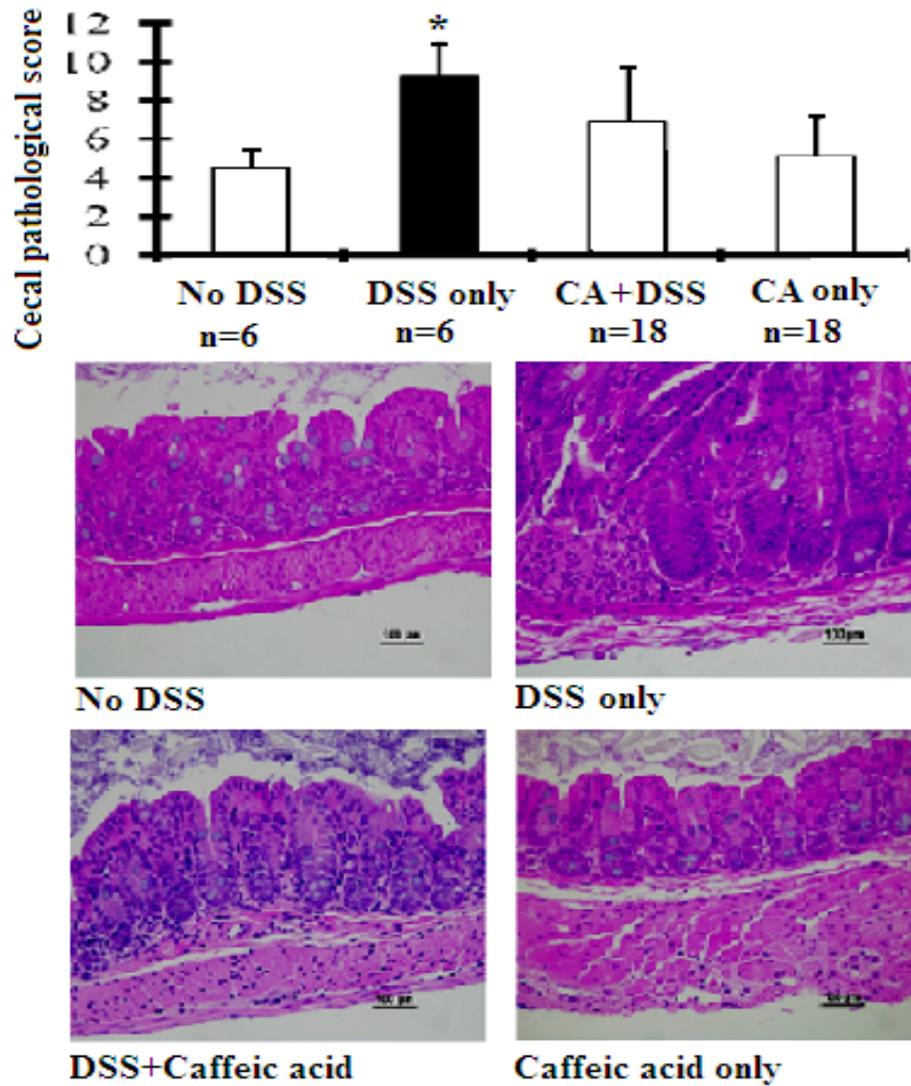


Figure 7.4. Cecal histopathology magnifications (400X, Scale bar = 100µm) of cecal mucosa from mouse were given as No DSS, DSS only, Caffeic acid + DSS, and Caffeic acid only treatments; * $p < 0.05$ indicated that caffeic acid treatment reduced inflammatory cell infiltration within the lamina propria and prevented epithelial ulceration compared to DSS only control.

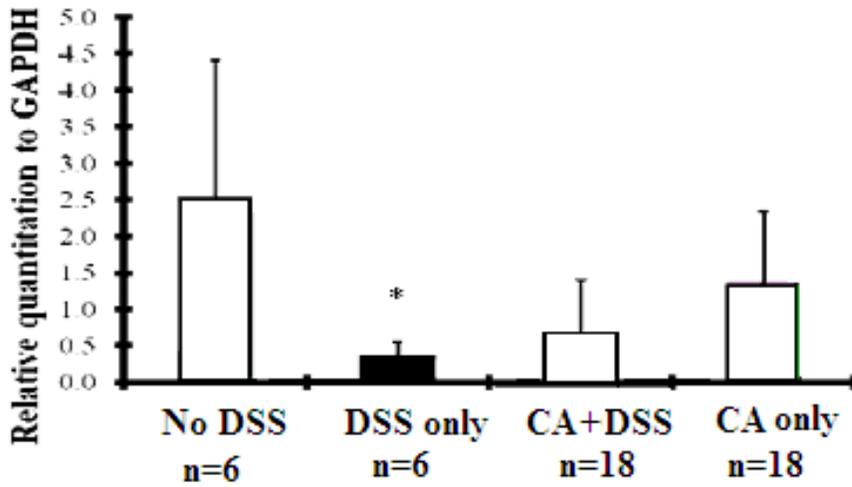


Figure 7.5. CYP4B1 gene expression was improved in caffeic acid/DSS treatment; $*p < 0.05$ indicated expression of CYP4B1 was increased by caffeic acid/DSS treatment compared with DSS-treated control.

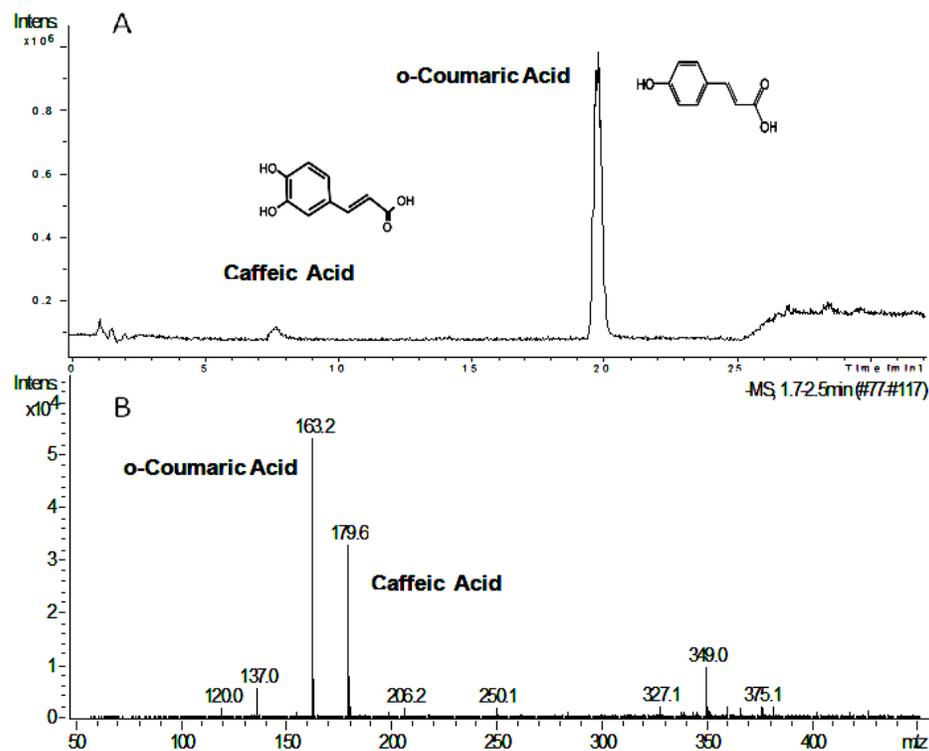


Figure 7.6. (A) Caffeic acid and o-coumaric acid (standard) LC-MS chromatogram profile and (B) ESI+TIC (Negative mode) mass spectrum. The expected molecular weight for caffeic acid is 180 g/mol. The [M]-peak for caffeic acid with m/z of 179 was observed on ESI negative ion mass spectra based on the loss of -H from the -COOH group. The molecular weight for standard o-coumaric acid is 164 g/mol. The [M]-peak for o-coumaric acid with m/z of 163 was observed on ESI negative ion mass spectra.

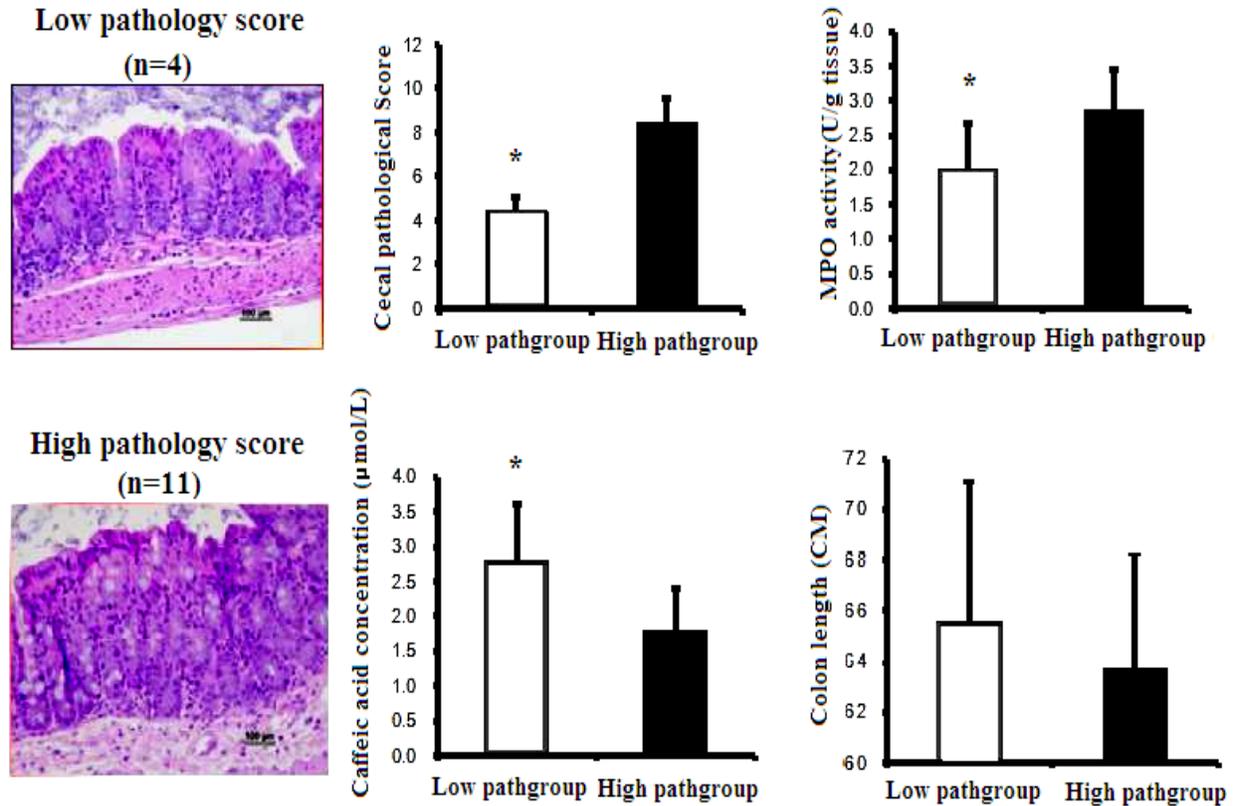


Figure 7.7. Histological changes, MPO activity, colon length and plasma concentration of caffeic acid in low and high subgroups. * $p < 0.05$ indicated caffeic acid-fed mice with severe cecal damage had significantly greater colonic MPO activity and lower caffeic acid plasma concentration than did mice with mild cecal damage. No difference was found between the high/low pathological score and short/long colon length.

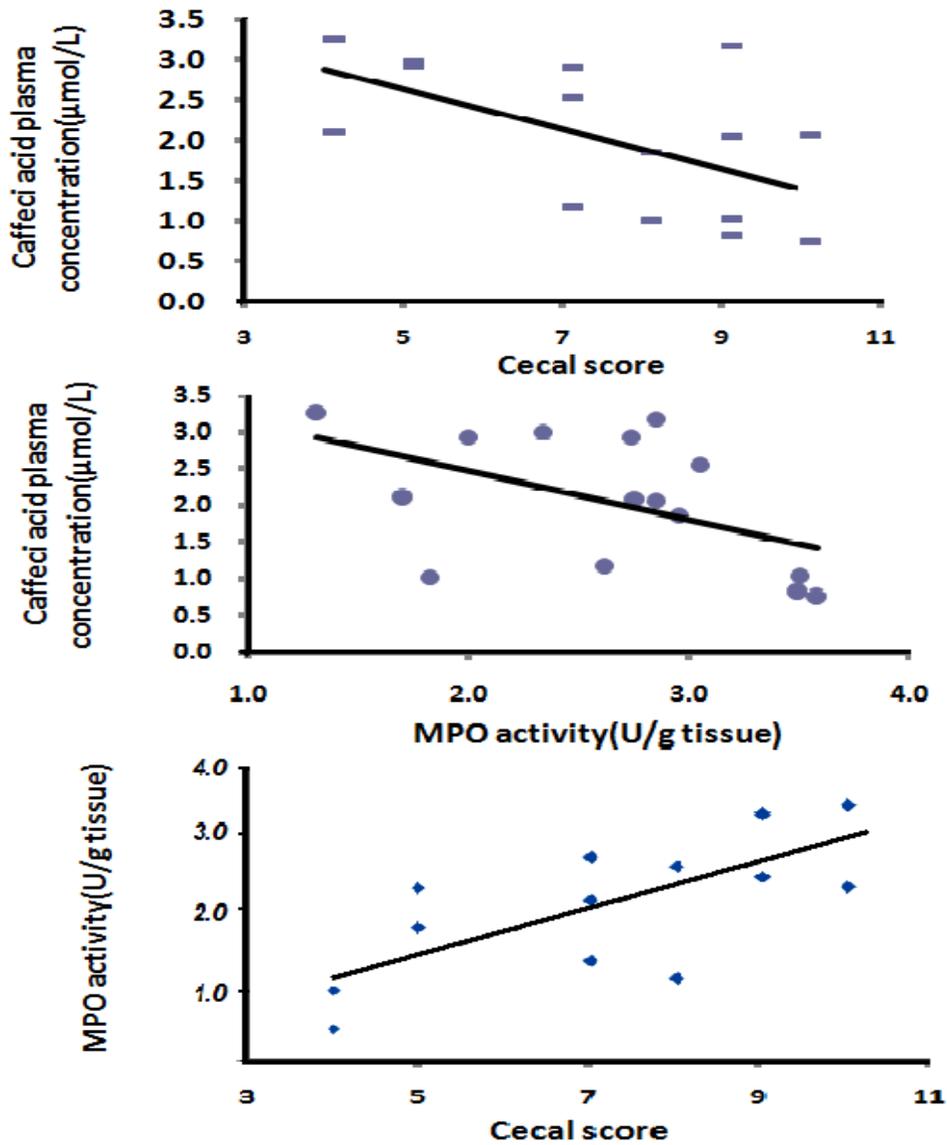


Figure 7.8. Relationships between caffeic acid plasma concentration and cecal histopathological score or colonic MPO activity in caffeic acid/DSS treatment were indicated that a significant a significant negative association with plasma concentration of caffeic acid on cecal score ($r = -0.56$, $P < 0.01$) and colonic MPO activity ($r = -0.49$, $P < 0.01$); a positive association with MPO activity on cecal score ($r = 0.72$, $P < 0.01$).

CHAPTER 8. GENERAL CONCLUSIONS

General Discussion

Phenolics that are not absorbed in the upper small intestine reach the large intestine. The gut microflora hydrolyzes these glycosides or esters into aglycones and further metabolizes the aglycones into various aromatic acids. Specific active metabolites are produced by the colonic microflora. After absorption, original phenolics or microbial metabolites are mainly conjugated with glucuronic acid, sulfate, or glycine in intestinal cell or liver. Gut microflora play an important role in the metabolism of these compounds. Moreover, the identification of microbial metabolites becomes a new field of research because microbial metabolites may have a physiologic effect and may be used as biomarkers for phenolic intake. Based on above essential principles, Chapter 3, 4 and 5 are original projects which were performed on metabolism or transport of phenolics in different microbial models or Caco-2 cell line *in vitro*.

In Chapter 3, we have conducted with the two whole plant extract phenolic mixtures in human fecal or mouse cecal bacterial *in vitro* anaerobic incubations, in which *E. purpurea* extract consists of three main phenolics- cichoric acid, caftaric acid, and caffeic acid when the *H. perforatum* extract consists of two main phenolics- rutin and hyperoside. We found that all phenolic compounds were degraded by human fecal and mouse cecal content with similar patterns. Of the 3 major compounds in *Echinacea* were the degradation rate of caffeic acid = cichoric acid < caftaric acid. In mouse cecal content incubation with *Echinacea*, the degradations rate were caffeic acid < caftaric acid = cichoric acid. More interestedly, we found one specific metabolite, m-hydroxyphenylpropionic acid (mHPP), which was reduced and dehydroxylated from caffeic acid by gut bacteria during *E. purpurea* incubation. Six of 20 human subjects' fecal samples produced caffeic acid, resulting in significantly lower caffeic acid degradation rate than that of caffeic acid nonproducers. Although no hyperoside productions were found in fecal incubations of *Hypericum* extracts, the degradation of hyperoside was significantly slower than that of rutin. We may predict that some hyperoside was metabolized from rutin according its metabolic pathway.

In Chapter 4, we have performed oral phenolic degradation and identified some specific microbes which were presented in the human oral cavity. A 7-compound mixture of caffeic acid,

rutin, daizein, quercetin, naringenin, luteolin and myricetin was incubated with saliva in anaerobic BHI media. In this study, we were interested in the interindividual variations and the influence of oral microflora composition on phenolic metabolism. Definitely, we found all phenolic compounds were degraded in oral incubation at different rates. Oral degradation rates of the compounds differed as follows: caffeic acid = rutin > quercetin = myricetin = naringenin > luteolin > daidzein ($p < 0.05$). The cluster analysis was showed that higher and lower subgroups of caffeic acid, rutin and naringenin degradation rate were significantly different. Moreover, sequencing of 16S rDNA from the higher intensity band of interest showed concordance with known species as the *Actinomycetales* Order with higher caffeic acid degrader and *Lactobacillus brevis/Lactobacillus reuteri* with higher rutin degrader which may affect human oral degradation of these phenolics and prevention of gum disease.

H. perforatum extract and the phenolic mixture were conducted within Caco-2 cell monolayers in Chapter 5. We used chlorogenic acid, compared with quercetin, amentoflavone, and pseudohypericin as individual compound, mixture, or Hp extract. Although the partial results were reported in this biotransformation study of phenolic compounds, these particular compounds, especially pseudohypericin was unstable or light sensitive in environment, were found to transport the Caco-2 cell monolayers with at least some different permeable activities.

The other major aspect investigated in the present project was the bioavailability study related to anti-colitic effect of caffeic acid in *in vivo*, including two separate animal designs which were discussed in Chapter 6 and 7. Firstly, C3H/HeOuJ mice were used to induce colitis by DSS. Three dietary phenolics, caffeic acid, rutin, and hypoxoside were fed and only caffeic acid protected against DSS induced colitis, in association with normalization of CYP4B1 expression. Although the mechanism of regulation of CYP4B1 related to inflammatory disease was unclear, this is a promising research area for future study.

One of most important studies in this dissertation was how the bioavailability issue affected the efficacy of phenolic compounds in animal model (Chapter 7). We chose caffeic acid because of its protective effect on DSS- induced colitis in C3H/HeOuJ mice. In this second caffeic acid study, the main idea was to examine interindividual variability in the efficacy of caffeic acid in CD-1/IGS female mice which was used for carcinogen-induced cancer model; and

the female alone mice selected was due to the more absorbability of caffeic acid than male mice based on our previous isoflavone bioavailability study. The main finding was that two subgroups were identified based upon cluster analysis of cecal histopathological score in mice fed caffeic acid/DSS. The effect in mice was related to differences in caffeic acid bioavailability. The relationship analysis indicated that greater caffeic acid plasma concentration and less MPO activity were associated with decreased cecal histopathological score.

As a conclusion, phenolic compounds were degraded by oral or gut microflora. The metabolism pathway was a complex process, especially in *E. purpurea* and *H. perforatum* extract. The novel phenolics in *H. perforatum* extract were absorbable by carcinoma colon cell monolayers. Caffeic acid was deserved the promising research future in anti-inflammatory area, particularly in molecular mechanism related to revealing anti-colitic pathway.

Recommendations for Future Research

With respect to the bacterial incubation screening, much about the microbial metabolite mechanisms of hydrolyzing of phenolics remains unknown, including which species release esterase to split the sugar moiety and what bacterial enzyme cleave the aglycones into various aromatic acid metabolites. It is necessary to determine which species produce caffeic acid during the whole extract microbial metabolism and are responsible for generating metabolites. In future study, using genomic PCR-DGGE and DNA sequence technique, identification of bacterial species may be a valuable objective to further investigate as well as suitable for screening oral bacterial species which associated with high and low degrader of phenolics.

In Caco-2 transport study, because glucuronide conjugates are expected to be major and less bioactive metabolites from herbal phenolics, and based on the current preliminary result, the apparent permeability were quite low among the most phenolic compounds, the conjugated compounds may be dominated during the Caco-2 cell treated with these active phenolics. The next step, we need to further conduct the method to investigate the inducibility of cytochrome P-450, glucuronidation, sulfation, and amino acid conjugation transformation by herbal components.

With regard to the protection effect of caffeic acid on DSS- induced colitis in animal, the further human study including the epidemiological investigation or human IBD disease-treated with different dose are the priority for consideration. In first caffeic acid human study, the subjects who we will focus on will be separated for higher dose of caffeic acid intake and lower or non caffeic acid intake to compare the colitis morbidity or with morbidity risk survey. The second human study, with consuming different doses of caffeic acid, we will observe the effect of caffeic acid on human colitis-treating recovery results. These may lead to well understanding of the interaction between animal study and human observation, which will help develop new drugs in clinic use.

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