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Citrus Flavonoids as Functional Ingredients and Their Role in Traditional Chinese Medicine

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ABSTRACT

Citrus flavonoid concentration in different tissues of citrus from different species around the world were compared together with citrus related traditional Chinese Medicine (TCM) ingredients. The citrus species with high flavonoid content like *Citrus aurantium* and the flavonoid-enriched tissues such as peel and young fruit are frequently utilized in TCM recipes for their gastric protective, antiulcer, cholesterol lowering and anti-cancer effects. Meanwhile they are commonly used as food ingredients in China. Along with the metabolism and absorption, citrus flavonoids are reviewed by their structure-activity relationships and insight is provided about how this compares with the functionalities in TCM treatments. Similar to the interaction with drug absorption, citrus flavonoid-enriched ingredients in TCM recipes usually contribute as "helper" through protecting main active components, enhancing the absorption of the main drugs, and exerting synergistic effects in the overall prescriptions.

Keywords: *Citrus aurantium*, citrus flavonoid, *Fructus Aurantii*, *Fructus Aurantii Immaturus*, hesperidin, naringin, nobiletin, polymethoxylated flavone, structure-activity relationship, tangeretin

Abbreviations: CHI, chalcone isomerase; **CHS**, chalcone synthase; **EBV-EA**, Epstein-Barr virus early antigen; **F3H**, flavanone 3β-hydroxylase; **TCM**, traditional Chinese medicine

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INTRODUCTION

The wide availability and frequent consumption of citrus fruit and citrus flavonoids have raised continuous interest in the past decades regarding their distribution, food application and bioactivities. Naturally occurring citrus flavonoids can be categorized into two groups: (1) flavanone glycosides such as naringin, hesperidin, neohesperidin and (2) polymethoxylated flavones such as nobiletin, sinensetin, tangeretin (**Fig. 1**). The flavanones predominate among citrus flavonoids, occurring as mono and diglycosides while the methoxylated flavones occur as free aglycons (Ooghe *et al*. 1994). Following the Chinese perception that food and medicine come from the same source, citrus is one of the most commonly used ingredients in traditional Chinese medicine (TCM) with preparations obtained from peel, young fruit, mature fruit, flower and other tissues. These TCM ingredients are all edible food ingredients in China.

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Invited Review

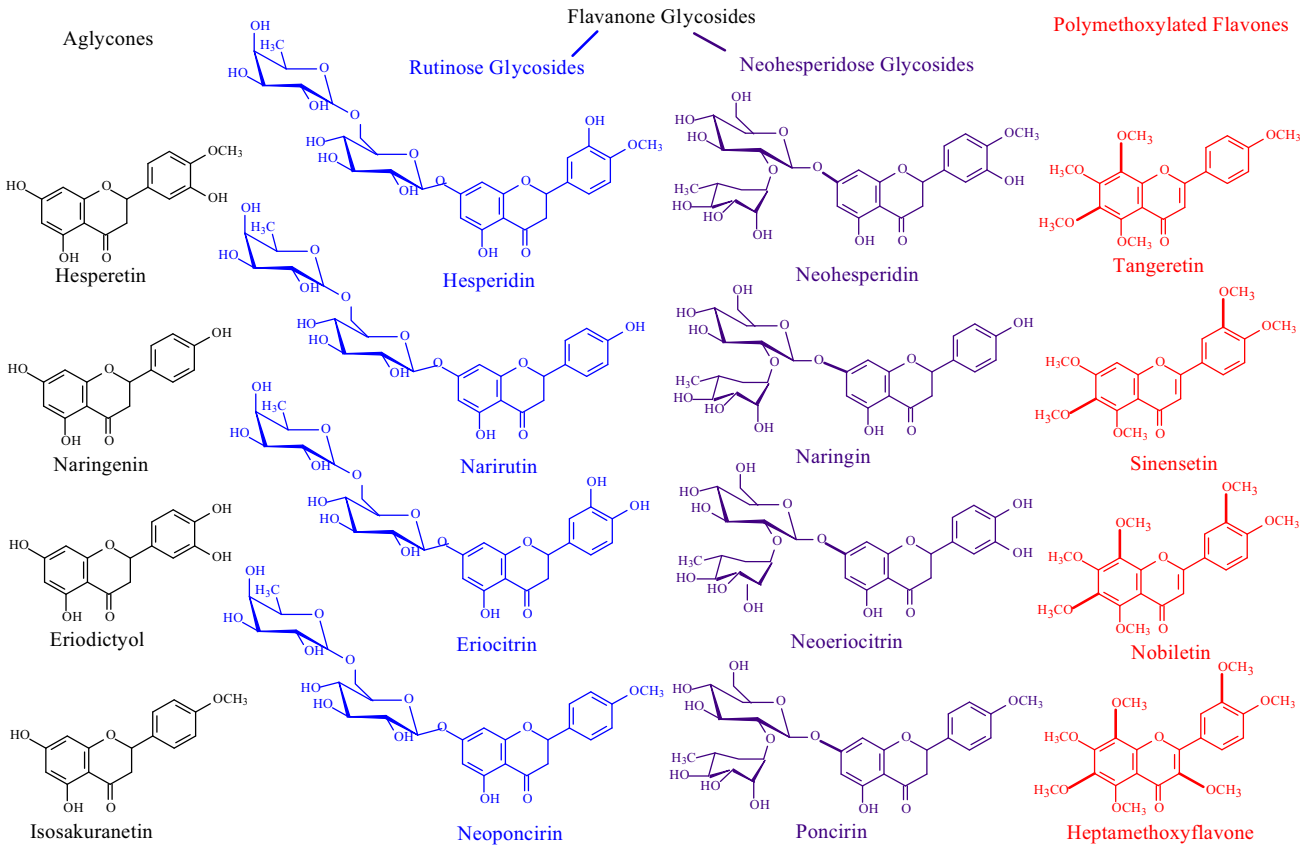

Fig. 1 Chemical structures of citrus flavonoids.

DISTRIBUTION

Distribution in tissues

Citrus flavonoids are widespread in plants, especially in the genus *Citrus* of the Rutaceae family. Their composition varies in different tissues during different stages of development. Jourdan *et al.* (1985) studied the distribution of naringin in *Citrus paradisi* Macfad (cv. 'Duncan') by radioimmunoassay. They found that seed coats of ungerminated seeds and young shoots had high naringin concentrations whereas cotyledons and roots had very low concentrations. Light-grown seedlings contained nearly twice as much naringin as etiolated seedlings, and in young plants and branches, the naringin content was highest in developing leaves and stem tissue. In flowers, the ovary had the highest levels of naringin accounting for nearly 11% of the fresh weight (Jourdan *et al.* 1985). In *Citrus aurantium*, the concentration of flavanones (naringin and neohesperidin) varied in different organs according to the following ranking: 10 mm diameter fruit (younger fruit) > 25 mm diameter fruit > fluids in flower receptacle (30 days old buds) > leaf (130 mm in length) > stems attaching the 30 days old buds to the tree (Castillo *et al.* 1992). Furthermore, citrus flavonoids mainly exist in fruit pericarp (albedo, membranes and the pith) (Kawaguchi *et al.* 1997; Barthe *et al.* 1998) while in citrus juices, only three types of flavonoids occur: the flavanones, the flavones, and the flavonols (Park *et al.* 1983). These flavanones are mainly accumulated or synthesized during an early stage of growth like in seeds, ovary and young/immature fruit (Jourdan *et al.* 1985; Castillo *et al.* 1992; del Rio *et al.* 1997). After they reach the highest level, the flavanones decrease due to the dilution effect of tissue growth (Jourdan *et al.* 1985; Castillo *et al.* 1992). The biosynthesis of flavanone glycosides occurs constitutively during cell division and differentiation and not during periods of cell elongation and subsequent maturation. A high level of polymethoxylated flavones were also found during early development of *C. aurantium* (cv. 'Sevillano') fruits and they were located in the peel (del Rio *et al.* 1998).

Citrus flavonoid biosynthesis

The flavonoid biosynthesis pathway is summarized in **Fig. 2** (modified from Moriguchi *et al.* 2003). Chalcone synthase (CHS) is the first enzyme in the biosynthesis of all classes of flavonoids in plants. It catalyzes the stepwise condensation of three acetate residues from malonyl CoA with *p*-coumaroyl CoA. The latter *p*-coumaroyl CoA is supplied from the phenylpropanoid pathway, which converts phenylalanine into phenolic secondary metabolites in plants. Naringenin chalcone, the product of the CHS reaction, is

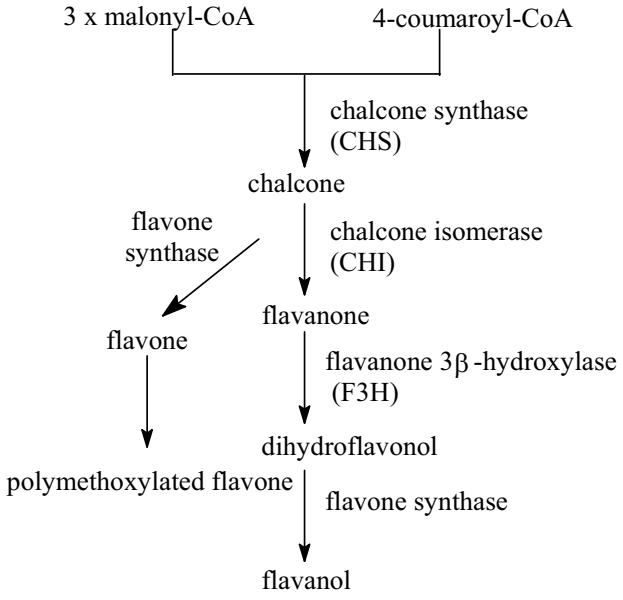

Fig. 2 Flavonoid biosynthesis in citrus.

Table 1 List of the three citrus cultivars from California with the highest flavonoid content (naringin, hesperidin and neohesperidin). (Based on Berhow *et al*. 1998).

Scientific name	Local name	Content %, w/w in fresh weight [1]	
C. paradisi	Star Ruby, CRC #3770	1.13	Naringin
C. reticulata × *Poncirus trifoliata*	Unknown, CRC #2619	0.78	
C. paradisi	Camulos, CRC #3139	0.58	
C. limon	Santa Teresa #1, CRC #3894	0.66	Hesperidin
C. reticulata × *Poncirus trifoliata*	Unknown, CRC #2619	0.51	
C. sinensis Navel cultivars	Fisher, CRC #3645	0.32	
C. paradisi	CRC #343	0.42	Neohesperidin
C. reticulata × *C. paradisi*	Sacaton, CRC #3331	0.27	
C. aurantium	Keen #1–10, CRC #2624	0.18	

[1] Sample preparation: citrus peel was separated into flavedo and albedo, frozen at -20°C and grounded into small pieces; samples in highest content are all from albedo.

Table 2 List of the three citrus cultivars from Central and South China with the highest flavonoid content (naringin, hesperidin, neohesperidin, sinensetin and nobiletin). (Based on Lu *et al*. 2006).

Scientific name	Local name	Content % per dry weight [1]	
C. paradisi cv. 'Changshanhuyou'	Huyou	3.25	Naringin
C. aurantium L.	Goutoucheng	2.11	
C. unshiu Marc. *Ueda unshiu*	Wenzhoumiju mid-late season	6.25	Hesperidin
C. unshiu var. *praecox* Tanaka	Gongchuan early season	6.18	
C. unshiu Marc.	Wenzhoumiju Jinghua	5.86	
C. paradisi cv. 'Changshanhuyou'	Huyou	2.76	Neohesperdin
C. aurantium L.	Goutoucheng	1.04	
C. sinensis Osbeck	Hamlin orange	0.053	Sinensetin
C. kinokuni Hort. ex Tanaka	Gongju	0.049	
C. reticulata Blanco (*C. poonensis* Hort. ex Tanaka)	YongChunLugan	0.047	
C. erythrosa Hort. ex Tanaka	Zhuhong	0.035	Nobiletin
C. succosa Hort. ex Tanaka	Bendizao	0.011	
C. reticulata Blanco (*C. poonensis* Hort. ex Tanaka)	YongChunLugan	0.010	

[1] Sample preparation: citrus peel (flavedo and albedo) and fruit pulp (juice sac and segment membrane) after juice squeezing were oven-dried at 55°C until constant weight was reached and grounded into powder; samples in highest content are all from peel.

then converted into a flavanone form by an intra-molecular reaction in which the C-ring is closed by the enzyme chalcone isomerase (CHI). These two forms of naringenin, the chalcone form and the flavanone form, appear to be the precursors for the compounds produced by plants with this related structure. By 3β-hydroxylation, flavanone 3β-hydroxylase (F3H) catalyzes the conversion of (2S)-flavanones to (2R, 3R)-dihydroflavonols, which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins. Flavanone is probably modified in a stepwise fashion to the various derivatives by hydroxylation, methylation, glucosylation and then rhamnosylation (Moriguchi *et al*. 2003).

On a genetic level, cDNA clones of the genes for CHS, CHI and F3H were isolated from citrus and the analysis of the related messenger RNA indicated that transcript levels for CHS, CHI, and F3H were generally high in young tissues such as leaves and fruitlets but decreased and disappeared in senescent tissues and/or towards fruit development (Moriguchi *et al*. 2001, 2003).

Distribution among species and TCM

As characteristic secondary metabolites, citrus flavonoids are taxonomically distributed among the *Citrus* genus. For examples, hesperidin and narirutin, the flavanones with rutinose (6-*O*-α-L-rhamnosyl-β-D-glucose), are relatively high in sweet oranges (*C. sinensis*), tangerines (*C. clementina*, *C. deliciosa*, *C. nobilis*, and *C. unshiu*) and tangors (such as *C. reticulate* x *C. sinensis*) whereas neohesperidin and naringin, the ones with neohesperidose (2-*O*-α-L-rhamnosyl-β-D-glucose), are high in sour oranges (*C. aurantium*) and tangelos (like *C. reticulata* x *C. paradisi* or *C. reticulata* x *C. grandis*) (Peterson *et al*. 2006). This finding is consistent with the collected compositional data of five citrus flavonoids (naringin, hesperidin, neohesperidin, sinensetin and nobiletin) in 114 cultivars from California (**Table 1**), 17 cultivars from China (**Table 2**), 66 cultivars from Japan (**Table 3**) and 8 from Cuba (**Table 4**). *C. paradisi* is the best known resource of naringin for cultivars grown in the USA (Star Ruby, 1.13% of fresh weight in albedo), China (Changshanhuyou, 3.25% of dry weight in the peel), Japan (Marsh grapefruit, 1.46% of fresh weight in edible part) and Cuba (Macf Isaac' grapefruit, 88.3% of dry weight in 5~8 mm immature fruit). On the other hand, only a few reports from Japan and China provided data about polymethoxylated flavones (e.g. sinensetin, nobiletin, tangeretin). Mostly they were found in the flavedo. Highest contents were found in Chinese cultivars of *C. sinensis*, *C. kinokuni*, *C. reticulate* (mandarin, Yongchun lugan), *C. erythrosa* and *C. succos*, while *C. tachibana*, *C. rippokorean*, *C. depressa*, *C. kinokuni* and *C. reticulata* (Ponkan) were the richest for Japanese cultivars. The distribution of tangeretin is often similar to that of nobiletin, which is the richest in tangerine and Ponkan (Nogata *et al*. 2006).

Table 3 List of the three citrus cultivars from Japan with the highest flavonoid content (naringin, hesperidin, neohesperidin, sinensetin and nobiletin). (Based on Kawaii *et al*. 1999c, 2001).

Scientific name	Local name	μg/100 mg in fresh weight [1]	
C. paradisi	Marsh grapefruit	1.459	Naringin
C. paradisi	Red blush	1.143	
C. natsudaidai	Natsudaidai	0.636	
C. hanayu	Hanayu	2.001	Hesperidin
C. reshni	Cleopatra	1.905	
C. unshiu	Unshu	1.596	
C. bergamia	Bergamot	0.508	Neohesperdin
C. aurantium	sour orange	0.324	
C. sudachi	Sudachi	0.220	
C. tachibana	Tachibana	0.067	Sinensetin
C. nippokorean	Kourai tachibana	0.011	
C. depressa	Shiikuwasha	0.006	
C. depressa	Shiikuwasha	0.021	Nobiletin
C. reticulata	Ponkan	0.013	
C. kinokuni	Mukaku kishu	0.012	

[1] Sample preparation: samples were separated into peel and edible part (consisting of juice sac and segment epidermis), freeze-dried and grounded into powder; samples in highest content are all from edible part.

Table 4 List of the three citrus cultivars from Cuba with the highest flavonoid content (naringin, hesperidin and neohesperidin). (Based on Ortuno *et al*. 1997).

Scientific name	Local name	Content %, w/w in dry weight [1]	
C. paradisi Macf.	'Isaac' grapefruit immature fruit	88.3	Naringin
C. paradisi Macf.	'Isaac' grapefruit mature fruit	22.4	
C. sinensis × *C. paradisi*	'Agrionja' mature fruit	9.3	
C. reticulata Blanco	'Galleta' mandarin immature fruit	49.2	Hesperidin
C. reticulata × (*C. reticulata* × *C. paradisi* Macf.)	'Nova' hybrid immature fruit	47.9	
C. reticulata Blanco	'Galleta' mandarin mature fruit	18.7	
C. aurantium L.	'Bouquet de Fleur' sour orange immature fruit	29.3	Neohesperidin
C. aurantium L.	'Afin' sour orange immature fruit	27.2	
C. macroptera	'Kerrii' immature fruit	13.1	

[1] Sample preparation: whole citrus fruits were oven-dried at 55°C and grounded into fine powder; immature fruit is only ~5 mm in diameter.

Table 5 List of the three TCM ingredients from China with highest flavonoid content (naringin, hesperidin, neohesperidin, sinensetin and nobiletin). (Based on Lu *et al*. 2006).

Scientific name	Local name	Content % w/w in dry weight [1]	
C. aurantium L.	*Fructus Aurantii Immaturus* Hua	10.78	Naringin
C. aurantium L.	*Fructus Aurantii Immaturus* Zhejiang	6.81	
C. aurantium L.	*Fructus Aurantii Immaturus* Xingan	4.68	
C. aurantium L.	*Fructus Aurantii* Hua	2.76	Hesperidin
C. aurantium L.	*Fructus Aurantii Immaturus* Xingan	2.12	
C. aurantium L.	*Fructus Aurantii Immaturus* Hua	0.72	
C. aurantium L.	*Fructus Aurantii Immaturus* Hua	6.89	Neohesperdin
C. aurantium L.	Daidai Flower	6.89	
C. aurantium L.	*Fructus Aurantii Immaturus* Zhejiang	6.49	
C. aurantium L.	Xingan *Fructus Aurantii*	6.10	
C. aurantium L.	*Fructus Aurantii Immaturus* Xingan	6.10	
Poncirus trifoliata (L.) Rafin.	Green *Fructus Aurantii*	0.179	Sinensetin
C. aurantium L.	*Fructus Aurantii Immaturus* Zhejiang	0.013	
C. aurantium L.	*Fructus Aurantii Immaturus* Hua	0.012	
C. aurantium L.	*Fructus Aurantii Immaturus* Hua	0.112	Nobiletin
C. aurantium L.	*Fructus Aurantii Immaturus* Zhejiang	0.070	
C. aurantium L.	*Fructus Aurantii Immaturus* Xingan	0.057	

[1] Sample preparation: TCM ingredients were milled into powder and oven-dried at 55°C until constant weight.

Interestingly, citrus is one of the most commonly used ingredients in TCM including parts of the peel (flavedo, albedo, Chenpi), young fruit (*Fructus Aurantii Immaturus*, Zhishi), mature fruit (*Fructus Aurantii*, Zhiqiao), flower (Daidai flower), seed (Ju He) and stem (Ju Gen). The most commonly used species in TCM are *C. aurantium*, *C. reticulata* and *Poncirus trifoliata* (Green Zhishi). **Table 5** gives an overall view of the TCM citrus ingredients with high flavonoid content. Most of these ingredients are from young fruit of *C. aurantium* which are characterized by high contents of flavanones and polymethoxylated flavones.

The collection of data on citrus flavonoids can help to characterize citrus species and find suitable flavonoid sources for food supplements. In addition, it can also support the development of fingerprinting technology of TCM.

ABSORPTION AND METABOLISM

Of the citrus flavonoids, hesperidin has been the most widely studied regarding its absorption and metabolism (reviewed by Garg *et al*. 2001). In one of the first clinical studies with pure compounds, Ameer *et al*. (1996) evaluated the disposition of citrus flavonoids after single oral doses of pure compounds (500 mg naringin and 500 mg hesperidin) and after multiple doses of combined grapefruit juice and orange juice, and of once-daily grapefruit. They were the first to demonstrate the oral absorption in humans of the citrus flavonoids naringenin and hesperetin. Volunteers, given single oral doses of hesperidin (500 mg) excreted hesperetin glucuronides within 3 h of administration and for 38 h thereafter. Cumulative urinary recovery of hesperetin, the aglycone, largely from orange juice administration, did not exceed 24%. Kim *et al*. (1998) observed that hesperidin was transformed in the intestine by bacterial glycosidases to its aglycone, hesperetin. Two other human studies tested the bioavailability of hesperetin and hesperidin after ingestion of orange juice. The peak plasma concentration for hesperetin from orange juice was 2.2 ± 1.6 μmol/L, with an elimination half-life of 1.3 to 2.2 h (Erlund *et al*. 2001). Upon ingestion of hesperidin, metabolites appeared in plasma 3 h after juice ingestion, reaching a peak at between 5 and 7 h, before returning to baseline at 24 h (Manach *et al*. 2003). The circulating forms of hesperetin were glucuronides (87%) and sulpho-glucuronides (13%). More recently, a randomized, double-blind, cross-over study on 16 healthy volunteers (Nielsen *et al*. 2006) indicated that enzymatic modification of hesperidin can improve bioavailability significantly, via the enzymatic conversion to hesperetin-7-glucoside. This was as judged by the total hesperetin AUC, C_{max}, and urinary excretion data that were significantly higher for hesperetin-7-glucoside compared to the low (2 mg/kg body weight) and high doses (6 mg/kg body weight) of hesperidin in orange juice. The authors proposed a model for the absorption of hesperidin (**Fig. 3**).

Another intriguing flavonoid is diosmin, which is synthesized from hesperidin, and used in the medical field as micronized purified flavonoid fraction (MPFF). It is an oral drug indicated in the treatment of venous disease, such as chronic venous insufficiency and all grades of hemorrhoidal disease. Katsenis (2005) has reviewed its pharmacological effects, therapeutic efficacy and benefits. Diosmin is absorbed as its aglycone, diosmetin, which is formed in the gut by bacteria. Interestingly, the reduction in particle size of diosmin led to a significant increase in absorption, based on urinary elimination of radioactivity from the initial ^{14}C-labelled diosmin in a double-blind, cross-over study in 12 healthy male volunteers (Garner *et al*. 2002). About half of the dose was eliminated in the faeces as unchanged diosmin and diosmetin. The predominant metabolite of diosmetin in man is 3-hydroxyphenyl propionic acid which is mainly eliminated in its conjugated form (Cova *et al*. 1992).

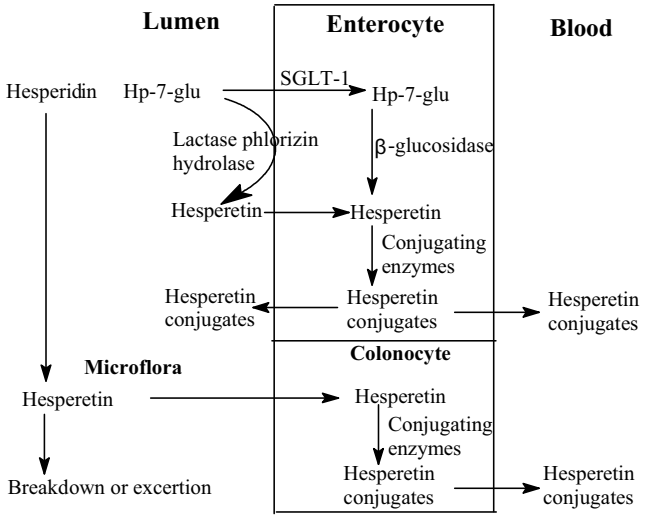

Fig. 3 Possible pathway for hesperidin and hesperetin-7-glucoside absorption showing the proposed shift in the site of absorption of hesperetin from the colon to the small intestine.

Very recently, the bioavailability of glucosyl hesperidin, which is a water-soluble derivative of hesperidin, has been compared in rats with hesperidin (Yamada *et al*. 2006). After oral administration of glucosyl hesperidin or hesperidin, the total AUC of rats administered glucosyl hesperidin was ca. 3.7 times greater than that obtained with hesperidin. The authors concluded that glucosyl hesperidin is absorbed more efficiently than hesperidin, giving the same metabolic profile as hesperidin. These results will have to be verified in human trials. Despite important hurdles regarding costs and regulations, the use of glucosyl hesperidin may become in the future an alternative to the poor solubility and low bioavailability of hesperidin.

The metabolic fate of nobiletin, a major component of polymethoxyflavones in citrus fruits, has been little studied. By using supercritical fluid chromatography, Li *et al*. (2006) recently identified 4'-demethylnobiletin as the major metabolite of nobiletin in mouse urine. They speculated that this was the result of methyl transferase enzymes in mice, giving free hydroxyl groups that can be easily conjugated with glucuronic acid or sulfates. This confirmed earlier results (Yasuda *et al*. 2003).

Naringin, the major flavonoid of grapefruit, was subject of a number of studies looking at the fate upon digestion. In an animal study, naringenin, the predominant flavanone found in grapefruit, and mainly found in a glycoside form (naringenin-7-rhamnoglucoside and naringenin-7-glucoside) was efficiently absorbed after feeding to rats (Felgines *et al*. 2000).

The possible interactions of citrus flavonoids with other food ingredients have been rarely studied. In that respect, the observations of Hou *et al*. (2000) are of interest. Their randomized, cross-over study with 6 healthy men indicated that honey reduced naringin/naringenin absorption by 33% when absorbed as untreated and honey-treated decoctions of pummelo pericarp.

BIOACTIVITIES AND STRUCTURE ACTIVITY RELATIONSHIP

Citrus flavonoids have attracted great interest on their bioactivities varying from antioxidant and anti-allergic activity (Matsuda *et al*. 1991; Lee *et al*. 2004; Kobayashi and Tanabe 2006) to anti-inflammatory and antiviral activity like anti-influenza A virus (Kim *et al*. 2001) and anti-hepatitis C virus from nobiletin (Suzuki *et al*. 2005). In addition to conventional use to treat capillary fragility (Martin 1955), naringin was reported to reduce the plasma cholesterol concentration in mice and rabbits (Jeon *et al*. 2004; Kim *et al*. 2004a, 2006) while hesperidin inhibited bone loss with lowering serum and hepatic cholesterol in mice (Chiba *et al*. 2003; Uehara 2006). Recent studies focused on polymethoxylated flavones and in particular on their potent antitumor activities (Manthey *et al*. 1999; Pan *et al*. 2002; Kawabata *et al*. 2005). In other studies, the potential hypopigmenting effects of nobiletin and hesperidin through tyrosinase inhibition were also reported (Sasaki and Yoshizaki 2002; Zhang *et al*. 2007).

Some of the bioactivities of citrus flavonoids were associated with their characteristic functional groups on the flavonoid nucleus such as hydroxyl, methoxyl, the 4-oxo/4-keto function and the C2-C3 double bond.

Hydroxyl group

Both the configuration and total number of hydroxyl groups substantially influence the mechanisms of antioxidant action including (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production, (2) scavenging reactive oxygen species and (3) upregulating or protecting antioxidant defenses (Pietta 2000). Due to the scarce presence of a free 3-hydroxyl group in citrus flavonoids, the 3',4'-catechol structure in the B-ring (Pietta 2000; Heim *et al*. 2002) gives the major contribution to the antioxidant activity of molecules such as eriodictyol (3',4',5,7-tetrahydroxyflavanone), neoeriocitrin (eriodictyol-7-*O*-neohesperidoside) and eriocitrin (eriodictyol-7-*O*-rutinoside) (Miyake *et al*. 1997; Yu *et al*. 2005). The presence of 5-OH is also important among citrus flavonoids. As for the total hydroxyl numbers, besides the tetrahydroxyl and two trihydroxyl flavanones above, another two trihydroxyl group flavanones, naringenin and hesperetin, also show significant antioxidant activity in lipid peroxidation assays (Saija *et al*. 1995; Heo *et al*. 2004; Kim *et al*. 2004c). Moreover, naringenin shows strong iron chelating activity (van Acker *et al*. 1996). In addition, eriodictyol, the 3',4'-dihydroxyl flavanone, was shown to be important for inhibiting granulomatic inflammation through down-regulation of COX-2 expression in various cells (Kim *et al*. 2004b).

Hydroxyl substituents in 4' and 7 positions were concluded to possess estrogenic activity (Miksicek 1995) and the main feature was the presence of a single hydroxyl group in the 4'-position of the B-ring of the flavan nucleus (Breinholt and Larsen 1998). As an example among citrus flavonoids, naringenin can bind both estrogen receptors, ERα and ERβ. Furthermore, it competes stronger with 17β-estradiol binding to ERβ than to ERα (Kuiper *et al*. 1998). On the other hand, estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. Inhibition of aromatase is an important approach for reducing growth stimulatory effects of estrogens in hormone-dependent breast cancer. Possessing the functional feature of multiple hydroxyl groups in 5 and 7 positions (Hodek *et al*. 2002), naringenin and hesperetin show significant inhibitory activities in human cytochrome P450 subenzymes, CYP1A2 (Fuhr *et al*. 1993) and CYP3A4 (Wilcox *et al*. 1999; Ho *et al*. 2001).

O-methylation/methoxyl

In general, the replacement of hydroxyl groups by methoxyl substituents anywhere on the entire structure significantly reduced or completely abolished antioxidant activity because O-methylation may reflect steric effects that perturb planarity. Although the ratio of methoxy to hydroxyl substituents does not necessarily predict the scavenging ability of a flavonoid, the B-ring is particularly sensitive to the position of the methoxy group (Heim *et al*. 2002), e.g. neohesperidin with 4'-methoxyl comparing to neoeriocitrin, hesperetin versus eriodictyol (heridictyol), and naringenin versus isosakuratenin (di Majo *et al*. 2005). In diosmetin, comparing to luteolin, the 4'-methoxyl group also reduces antiprotozoal and leishmanicidal activity (Tasdemir *et al*.

2006).

However, methoxylation enhances bioactivities through its different mechanism from hydroxylated flavonoids. Methoxylation in positions 3' and 4' is important to anti-inflammatory activity, like 5,6,7,3',4'-pentahydroxyflavone (sinensetin) which shows high inhibition of granulomatic inflammation. It also suggested the importance of 5,7,4'-methoxyl groups as nobiletin showed overall anti-inflammatory activity by down-regulation of both iNOS expression and COX-2 expression (Lin *et al*. 2003; Kim *et al*. 2004b; Kwak *et al*. 2005). In addition, COX-2 selective inhibition is associated with analgesic activity. The structure activity relationship for flavonoids supports that methoxylation at the 6- or 4'-position produced a two-fold increase in activity but substitution at 7- or 2' slightly decreased the potency (Thirugnanasambantham *et al*. 1993). Hesperidin, tangeretin and nobiletin may show the influence of methoxylation on their activities (Galati *et al*. 1994; Li *et al*. 1994; Park and Pezzuto 2002).

Citrus polymethoxylated flavones were reported for their potent antiproliferation activity and the total number of methoxyl groups was considered as the main contributor. For example, tangeretin inhibits the growth of B16F10 and SK-MEL-1 melanoma cell lines suggesting that at least three adjacent methoxyl groups confer a more potent antiproliferative effect (Rodriguez *et al*. 2002; Yanez *et al*. 2004). 3-OH and 8-methoxyl groups in polymethoxylated flavones were summarized to be important to antiproliferative activity in various human cancer cell lines, and tangeretin, nobiletin and heptamethoxyflavone were shown as best examples (Kawaii *et al*. 1999a, 1999b; Manthey and Guthrie 2002). An orange peel extract of 30% polymethoxyflavones containing tangeretin (19.0%), heptamethoxyflavone (15.24%), tetramethoxyflavone (13.6%), nobiletin (12.49%), hexamethoxyflavone (11.06%), and sinensitin (9.16%) was recently reported to potently suppress intestinal tumor growth in $Apc^{Min/+}$ mice, a mouse model for human familial adenomatous polyposis (Fan *et al*. 2007).

Antiviral flavonoids, mainly possessing methoxyflavones, poly-substituted in the A-ring, show a higher antiviral activity than do mono-substituted compounds (de Meyer *et al*. 1991). It was further supported that the presence of a methoxyl group at C-3 seems to enhance the inhibitory activity. This conclusion was drawn from the result that 3,5,6,7,3',4'-hexamethoxyflavone, 3,5,7,8,3',4'-hexamethoxyflavone, 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone and heptamethoxyflavone, all possessing a 3-methoxy group, showed strong inhibitory activity on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate (Iwase *et al*. 2000).

The 4-oxo/4-keto function and C2-C3 double bond

4-oxo group is very common to citrus flavonoids in both flavanones and polymethoxylated flavones. Aside from the 3',4'-catechol, 3-OH and overall hydroxylation pattern, the 4-oxo group helps to enhance antioxidant activity as well (van Acker *et al*. 1996; Pietta 2000; Heim *et al*. 2002). As to the C2-C3 double bond, it is specific to citrus flavones. In conjugation with the 4-oxo group, it significantly enhances antioxidant and antiproliferative activity (Kawaii *et al*. 1999a, 1999b).

Carbohydrate moieties

In general, carbohydrate moieties, in citrus flavonoids as flavanone glycosides are known to be hydrolyzed into their aglycone forms during digestion and metabolism, and appear to have negative influence on various bioactivities (Hollman and Katan 1997; Heim *et al*. 2002).

FUNCTIONALITY IN TRADITIONAL CHINESE MEDICINE

The therapy using TCM is a collection of thousands of years' clinical experience in China. The TCM ingredients related to citrus are: Chenpi, the peel of mature fruit (*Pericarpium Citri Reticulatae*); Qingpi, the peel of immature fruit (*Pericarpium Citri Reticulatae Viride*); Zhishi, the young fruit (*Fructus Aurantii Immaturus*); Zhiqiao, the mature fruit (*Fructus Aurantii*); Daidai flower, the flower; Ju He, the seed and Ju Gen, the stem. *C. aurantium*, *C. reticulata* and *Poncirus trifoliata* (Green Zhishi) are the main citrus species used in TCM. The full exploitation of the flavonoid-enriched tissues and of the young fruits can be associated to the key role citrus flavonoids play despite that sympathomimetic amine and synephrine in citrus were known to play also active roles. Furthermore, the application of TCM recipes is the most sophisticated part taking advantage of bioactivities of individual ingredients and their synergistic effects. Regarding citrus ingredients, they sometimes play a role as the "king ingredient" to exert their main bioactivities and other times as a "helper ingredient" to enhance absorption and/or interaction of other TCM ingredients. Based on modern bioactivity research methods and approaches reviewed above, a collection of TCM applications specifically associated with bioactivities of citrus flavonoids will be discussed next.

Gastric protective effect

Chenpi, Zhiqiao and Zhishi are common ingredients for treating gastric disorders. For example, Zhishi was shown to enhance gastrointestinal transit when compared to a cisapride treated group in adult Wistar rats (Zhu *et al*. 2000). A classic TCM soup recipe "Liu Jun Zi Tang" (also called Rikkunshi-to in Japan) was widely prescribed for gastric flatulence, anorexia, nausea, and vomiting. It was recently reported by a Japanese team that this recipe ameliorated abnormalities of NO-mediated gastric functions such as delayed gastric emptying. This recipe contains *Glycyrrhizae radix* (4.7%), *Zingiberis rhizoma* (2.3%), *Atractylodis lanceae rhizoma* (18.6%), *Zizyphi fructus* (9.3%), *Aurantii nobilis pericarpium* (Chenpi, 9.3%), *Ginseng radix* (18.6%), *Pinelliae tuber* (18.6%), and *Hoelen* (18.6%). Hesperidin and L-arginine were identified as two of the active ingredients contributing to the ability of Rikkunshi-to to facilitate gastric emptying (Kido *et al*. 2005). A similar gastric protective effect was observed in grapefruit seed extract by enhancing the expression of constitutive nitric oxide synthase. In consequence, nitric oxide and neuropeptides such as calcitonin gene related peptide (CGRP) released from sensory afferent nerves increased gastric microcirculation probably due to the antioxidant activity of naringenin (Brzozowski *et al*. 2005; Zayachkivska *et al*. 2005).

Antiulcer

As many Western researchers reported, citrus flavonoids such as hesperidin, naringin, diosmetin and their aglycons, hesperetin and naringenin showed significant protective effect from gastrointestinal ulcers. For example, ascorbic acid and hesperidin upon histamine-induced gastric ulcers in guinea pigs (Rossi *et al*. 1957), naringin on ethanol-induced gastric lesions in rats (Martin *et al*. 1994), Daflon 500 mg (the purified micronized flavonoid fraction containing 90% diosmin and 10% hesperidin), on improvement of clinical healing ulcer (Bergan *et al*. 2001). Similar clinical studies were reported in using the TCM, Zhishi. Zhi Tong Gao, the extract of ethyl acetate fraction from Zhishi, enriched in flavonoids, showed strong antiulcer activity (total effect 96%) in 156 patients of clinical studies (Zhou and Peng 1993). The related recipe Baiji Zhishi San was successfully used to treat gastric and duodenal ulcer for 288 patients in clinical studies (Su 1997).

Anti-*Helicobacter pylori*

Helicobacter pylori (HP) is a major causative bacterium of chronic gastritis, peptic ulcer and is associated with an increased risk of gastric carcinoma (Parsonnet *et al*. 1991). Zhishi was reported for its dose-dependent anti-HP effect (Liu *et al*. 2002), and Chenpi and Zhiqiao also showed positive activity against HP (Xu *et al*. 2000). Biochemical research provided evidence that hesperetin, naringenin and diosmetin inhibit the growth of HP though not by inhibiting the urease activity of HP (Bae *et al*. 1999).

Lowering cholesterol and anti-atherosclerosis

Like the lowering cholesterol effects of naringin and hesperidin, Jiu Xiang Xin Tong Shu containing Chenpi and Cheng Qi Tang containing Zhishi, the two TCM recipes, were demonstrated to reduce the serum cholesterol level in a hyperlipidemia rat model (Teng *et al*. 2004) and in a clinical evaluation on apoplectic hyperlipidemia patients (Yu and Yu 1999), respectively. Moreover, nobiletin may prevent atherosclerosis at the level of the vascular wall by inhibiting macrophage foam-cell formation (Whitman *et al*. 2005). Naringin and naringenin showed anti-atherogenic effect in high cholesterol-fed rabbits (Lee *et al*. 2001). Similarly, TCM recipes Hua Tan Qu Yu Tablet and Xue Fu Zhuo Fu pill, a famous formula of the Qing Dynasty, containing Chenpi and Zhiqiao, respectively, were demonstrated to ameliorate atherosclerosis in clinical studies (Li and Zhang 1997; Wang 2003a). In contrast to the conventional use of hesperidin and narigin to improve capillary fragility and vascular integrity, Zhishi is used as single ingredient to improve endothelial function and the platelet activation in patients with acute coronary syndrome (Wu *et al*. 2007).

Anti-cancer

Citrus associated TCM ingredients mostly show an antitumor effect on gastric cancer. A typical example is the clinical study on the recipe Liu Jun Zi Tang, containing Chenpi, that indicated significant improvements among 60 patients with advanced gastric cancer (Huang 2004). Another example is Zhishi Xiaopi pill treating gastric precancerous lesions among 30 patients with a total effect of 87% when compared to the control group with 67% (Mo and Luo 2002). The main contributors of these improvements may be the potent anti-cancer agents such as polymethoxylated flavones like nobiletin (Minagawa *et al*. 2001; Yoshimizu *et al*. 2004) and tangeretin. In addition, naringenin was reported to inhibit gastric cancer with unknown mechanisms (Lee *et al*. 2005).

Interaction with food and/or drug during absorption

Due to the Chinese perception that food and drug come from the same source, and the fact that an individual TCM recipe contains several active compounds which may affect each other, it seems very difficult to elucidate TCM mechanisms. Very limited research has been reported on how citrus related TCM ingredients play the role as "helper ingredients" from a biochemical point of view. During the preparation of recipe Zhi Shu Wan (Zhishi and Baishu, *Atractylodes Maeroeephata* Koid), the content of the active compound atractylon from Baishu was analyzed to be significantly higher than that in individual preparation of Baishu. It was speculated that the compounds in Zhishi (probably flavonoids) prevent the oxidation of atractylon to atractylolide and hydroxyatroctylolide (Luo *et al*. 1994). A synergistic effect of citrus flavonoids and paeoniflorin in the recipe Pai Nong San/Haino-san (Zhishi and Shaoyao, *Radix paeoniae* Rubra) was reported to inhibit carrageenin edema formation (Kano *et al*. 1983). Chenpi can also enhance the absorption of vitamin B, digoxin and griseofulvin in the upper small intestine through its ability to delay gastric emptying (Wang 2003b).

Similarly, food and drug interactions are used in Western therapies. The typical case is flavonoid-enriched grapefruit juice, which increases the bioavailability of certain drugs, by reducing presystemic intestinal metabolism. Naringenin and furanocoumarins were assumed to affect activity of intestinal cytochrome P450 sub-enzyme CYP3A4 but not hepatic CYP3A4 or colon CYP3A5 (Singh 1999; Evans 2000).

CONCLUSION

As secondary metabolite, citrus flavonoids are widely distributed in the dietary citrus fruit, and sometimes at high concentration. Although citrus flavonoids show mild bioactivities in human metabolism, the common consumption of citrus and its low toxicity have promoted a continous research interest in these molecules. Interestingly, on the other hand, by frequently using citrus flavonoids-enriched TCM and foods, Chinese have successfully developed and exploited the benefits of bioactivities from citrus flavonoids for thousands of years. Up to date, it is still very complicated to explain the interaction mechanism of citrus related and other TCM prescriptions. Nevertheless, the fusion of TCM application with Western biochemistry theory is improving the understanding of each other to unveil more on citrus flavonoids for the well-being of humans.

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APPENDIX I. CHINESE NAME LIST OF THE TCM INGREDIENTS

In case of any translation problems, all Chinese characters are shown as follows for references:

TCM ingredients:

1. peel of mature citrus fruit (*Pericarpium Citri Reticulatae*), Chenpi, 陈皮
2. peel of immature citrus fruit (*Pericarpium Citri Reticulatae Viride*), Qingpi, 青皮
3. (*Fructus Aurantii Immaturus*), Zhishi, 枳实
4. mature fruit (*Fructus Aurantii*), Zhiqiao, 枳壳
5. sour orange flower, Daidai flower, 代代花, 玳玳花, 酸橙花
6. citrus seed, Ju He, 橘核
7. citrus stem, Ju Gen, 橘梗
8. young citrus fruit of *Poncirus trifoliate*, Green Zhishi, 绿衣枳实

TCM recipes:

1. Liu Jun Zi Tang" (also called Rikkunshi-to in Japan) 六君子汤
2. Baiji Zhishi San, 白芨枳实散
3. Jiu Xiang Xin Tong Shu, 九香心痛舒
4. Cheng Qi Tang, 承气汤
5. Xiao Cheng Qi Tang, 小承气汤
6. Hua Tan Qu Yu Tablet, 化痰祛瘀片
7. Xue Fu Zhuo Fu pill, 血府逐瘀丸
8. Zhishi Xiaopi pill, 枳实消痞丸
9. Zhi Shu Wan, 枳术丸
10. Pai Nong San/Haino-san, 排脓散