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# Biological Activity, Metabolism and Separation of Polymethoxyflavonoids from Citrus Peels

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## ABSTRACT

Polymethoxyflavonoids (PMFs) almost exclusively exist in citrus plants. In recent years, there has been increasing and particular interests in the exploration of the health benefits associated with PMFs. PMFs are reported to exhibit a broad spectrum of biological activities, such as anti-inflammatory, anti-carcinogenic, anti-tumor, anti-viral, anti-thrombogenic and anti-atherogenic properties. Metabolic studies, especially the identification of *in vivo* biotransformation products of PMFs, have revealed that the major metabolites are desmethylated or hydroxylated PMFs. Biological screening of some of these metabolites has revealed that they may possess more potent bioactivities such as anti-inflammatory and anti-cancer properties. Elucidation and biological activity study of PMF metabolites may lead to further exploration and understanding of PMFs' health benefits and the mechanism along with the beneficial properties in human beings. The increased demand of PMFs for efficacy and clinical trial studies has resulted in a demanding supply for pure PMFs in large quantities. Consequently, separation and characterization of PMFs have attracted increasing attention and new techniques have evolved dramatically in recent studies. However, the development of an efficient large-scale separation method(s) as well as robust and reliable analysis method(s) for PMFs and their metabolites are needed and eventually will be developed in the near future because of the significance and urgency. This review describes the occurrence, bioactivity, bioavailability, metabolism and chemistry of PMFs from citrus genus, especially the peels of citrus fruits.

**Keywords:** anti-inflammatory, anti-cancer, bioavailability, isolation, hydroxylated polymethoxyflavones
**Abbreviations:** **COX-2**, Cyclooxygenase-2; **LC**, liquid chromatography; **HPLC**, high performance liquid chromatography; **IL**, Interleukin; **iNOS**, Inducible nitric oxide synthase; **LPS**, Lipopolysaccharide; MEK, mitogen-activated protein/extracellular signal-regulated kinase; **MMP**, Matrix metalloproteinase; **NFκB**, Nuclear factor κB; **NO**, Nitric oxide; **OPE**, orange peel extract; **PMFs**, polymethoxyflavonoids; **ROS**, Reactive oxygen species; **SFC**, supercritical fluid chromatography; **TNF-α**, Tumor necrosis factor-α; **TPA**, 12-O-tetradecanoylphorbol-13-acetate

## CONTENTS



## INTRODUCTION

Citrus production in selected major producing countries in 2003/2004 was 73.1 million metric tons (currently 105 million metric tons). Total citrus production of the United States was 14.85 million metric tons (National Agricultural Statistics Service). Around 34% of these products were used for juice production, yielding approximately 44% of peels as by-products. Citrus peels have a natural resistance against UV, fungi, insects, *etc*. Also, some citrus peels, like orange and tangerine peels have been used as traditional medicines in some Asia countries for treatment of stomach

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Invited Review

upset, skin inflammation, muscle pain, and ringworm infections. The major constituents in citrus peels include flavonoids, mainly polymethoxylated flavonoids (PMFs); terpenoids, such as limonene and linalool; and other volatile components. Traditionally, citrus peels, particularly orange peels, have been processed for their volatile and nonvolatile fractions, which are used in various applications in food, drug and cosmetic products. However, the overall demand of citrus peels is of insignificance because the important nutraceutical and pharmaceutical application of PMFs have not been widely explored and recognized.

## CHEMISTRY OF FLAVONOIDS AND POLYMETHOXYFLAVONOIDS

Flavonoids, a class of chemically related polyphenols of plant origin, exist ubiquitously in nature and also exhibit a broad spectrum of pharmacological properties (Middleton *et al.* 2000; Lopez-Lazaro 2002; Murakami and Ohigashi 2004). These polyphenolic compounds have a basic 15-carbon basic chemical skeleton and can be represented as C6-C3-C6, consisting of two benzene rings (C6) joined by a linear three-carbon chain (C3). Flavonoids from citrus fruits have a benzo-γ-pyrone skeleton with a carbonyl group at the $C_4$ position (**Fig. 1**).

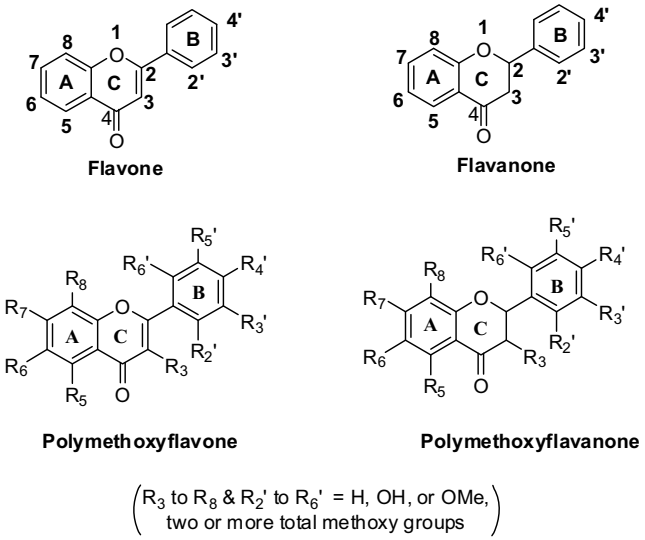


**Fig. 1 Chemical structure illustration of flavonoids from citrus.**

These particular flavonoids consist of two classes of compounds, named flavones and flavanones (**Fig. 1**). The major flavones in fruits and vegetables are glycosides of quercetin, luteolin and apigenin. It was reported that the edible source of flavones are limited to parsley and celery (Manach *et al.* 2004). However, flavones are very rich in citrus fruits, especially PMFs in which there are usually at least four methoxy groups on the A-, B- and/or C-ring (**Fig. 1**). PMFs exist almost exclusively in the *Citrus* genus, particularly in the peels of two citrus species, sweet orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*) (Dugo *et al*. 1996). So far, more than 20 PMFs have been isolated and identified from different tissues of citrus plant (Chen and Montanari 1998; Kawaii *et al.* 2001; Li S *et al.* 2006b). The types and content of PMFs vary among different varieties of citrus species (Wang *et al.* 2008). Thus the types and concentrations of PMFs may serve for taxonomic purposes in botanical and agricultural sciences. As an example, PMF concentration in 'Dancy' tangerine peels are almost 10-fold higher than that of other varieties of tangerine and oranges (Manthey and Grohmann 2001). In terms of the types of PMFs, the only PMF detected in the 'Ambersweet peel' was tangeretin, 'Temple' orange and Valencia peels were similar except that no 3,5,6,7,8,3',4'-heptamethoxyflavone was found in Temple orange peels. In sweet orange peels, the major PMFs are nobiletin, tangeretin, 3,5,6,7,8,3',4'-heptamethoxyflavone and 3,5,6,7,3',4'-hexamethoxyflavone (Li S *et al.* 2006b). Overall, the relatively common PMFs in high-yielding citrus are two polymethoxylated flavones, nobiletin and tangeretin, both present in sweet and bitter orange (*Citrus aurantium*) peels (Horowitz and Gentilli 1977). Sinensetin is the third common polymethoxyflavone in the skin of citrus fruits (Manach *et al.* 2004), whereas 3,5,6,7,8,3',4'-heptamethoxyflavone and 3,5,6,7,3',4'-hexamethoxyflavone are the two most abundant PMFs after nobiletin and tangeretin in sweet orange peels (Li S *et al.* 2006b; Wang *et al.* 2008). Flavanones are present in high concentrations only in citrus fruits, although they are also found in tomatoes and some aromatic plants, like mint. The most prevalent flavanones are hesperetin from oranges, naringenin from grapefruit, and eriodictyol from lemons (Manach *et al.* 2004). These flavanones are generally glycosylated by a di-

**Table 1** PMFs and their related compounds found in orange peel.

| Name | Reference | Name | Reference |
|---|---|---|---|
| Tangeretin | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 5-Hydroxy-6,7,8,4'-tetramethoxyflavone | Ghai *et al.* 2001; Li S *et al.* 2006b |
| Sinensetin | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 7-Hydroxy-3,5,6,8,3',4'-hexamethoxy-lavone | Ghai *et al.* 2001 |
| Nobiletin | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 7-Hydroxy-3,5,6,3',4'- pentamethoxyflavone | Ghai *et al.* 2001 |
| 5,7,8,3',4'-Pentamethoxyflavone | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 5,7-Dihydroxy-3,6,8,3',4'-pentamethoxyflavone | Ghai *et al.* 2001 |
| 5,7,8,4'-Tetramethoxy-flavone | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006a | 5-Hydroxy-6,7,4'-trimethoxyflavone | Li S *et al.* 2006b |
| 5,6,7,4'-Tetramethoxy-flavone | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 3-Hydroxy-5,6,7,4'-tetramethoxyflavone | Li S *et al.* 2006b |
| 3,5,6,7,4'-Pentamethoxyflavone | Boem and Voelcker 1959; Li S *et al.* 2006b | 3-Hydroxy-5,6,7,8,4'-pentamethoxyflavone | Li S *et al.* 2006b |
| 3,5,6,7,8,3',4'-Heptamethoxyflavone | Manthey *et al.* 2001a; Ghai *et al.* 2001; Li S *et al.* 2006b | 5-Hydroxy-3,7,3',4'-tetramethoxy-flavone | Manthey *et al.* 2001a; Li S *et al.* 2006b |
| 3,5,7,8,3',4'-Hexamethoxyflavone | Tatum and Berry 1978 | 5-Hydroxy-3,7,8,3',4'-pentamethoxyflavone | Li S *et al.* 2006b |
| 5,7,3',4'-Tetramethoxy-flavone | Mizuno *et al.* 1991; Manthey *et al.* 2001a; Li S *et al.* 2006b | 5-Hydroxy-6,7,3',4'-tetramethoxyflavone | Manthey *et al.* 2001a; Li S *et al.* 2006b |
| 3,5,6,7,3',4'-Hexamethoxyflavone | Tatum and Berry 1978; Li S *et al.* 2006b | 5,6,7,4'-Tetramethoxy-flavanone | Li S *et al.* 2006b |
| 5,7,4'-Trimethoxy-flavone | Mizuno *et al.* 1991 | 5-Hydroxy-6,7,8,3',4'-pentamethoxyflavanone | Li S *et al.* 2006b |
| 5-Hydroxy-7,8,3',4'-tetramethoxyflavone | Ghai *et al.* 2001 | 2'-Hydroxy-3,4,4',5',6'-pentamethoxy-chalcone | Li S *et al.* 2006b |
| 5-Hydroxy-3,6,7,8,3',4'-hexamethoxyflavone | Ghai *et al.* 2001; Li S *et al.* 2006b | 2'-Hydroxy-3,4,3′,4',5',6'-hexamethoxychalcone | Li S *et al.* 2006b |

saccharide at position 7 (a hydroxyl group). Hesperetin and naringenin are found in the fruit tissue and peel, largely as their glycosides, hesperidin and naringin.

The structures of PMFs usually differ in the numbers, types or positions of substitution on the 2-phenyl-γ-pyrone skeleton (**Fig. 1**). The general structures of PMFs and polymethoxyflavanones are also illustrated in **Fig. 1**. Interestingly, the majority of the identified compounds are derivatives from the base structure of flavones, although two flavanones were identified from 'Dancy' tangerine leaves (Chen and Montanari 1998). **Table 1** illustrates the profile of PMFs and their related compounds in terms of plant physiology in the orange peel, including 12 polymethoxyflavones, nine hydroxylated polymethoxyflavones, one hydroxylated polymethoxyflavanone, one polymethoxyflavanone and two polymethoxychalcones being isolated and characterized, and four hydroxylated PMFs having been identified. Among those identified PMFs, tangeretin and nobiletin have been intensively studied over the past 10 years because of their pharmacological properties and relatively readily availability. Recently, 3,5,6,7,8,3',4'-heptamethoxyflavone, 5-desmethylnobiletin, 5-desmethyltangeretin, 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone and some other hydroxylated polymethoxyflavones have led to a new research field because of their greater potency and better efficacy than their PMF counterparts (Li S *et al.* 2007b, 2007c; Pan *et al.* 2007).

## BIOLOGICAL ACTIVITIES OF POLYMETHOXYFLAVONOIDS

Citrus flavonoids, especially citrus fruit derived polymethoxyflavonoids and their metabolites exhibit a wide variety of biological activities including anticarcinogenic (Iwase *et al*. 2001; Manthey *et al.* 2001b; Sergeev *et al*. 2006; Lai *et al*. 2007), anti-inflammatory (Manthey *et al.* 1999; Murakami *et al.* 2000a, 2000b; Lin *et al.* 2003; Murakami *et al.* 2005), antitumor (Yoshimizu *et al*. 2004; Fan *et al*. 2007; Miyata *et al*. 2008), anti-viral (Kaul *et al*. 1985), anti-atherogenic (Kurowska *et al.* 2004; Whitman *et al*. 2005) and anti-thrombogenic (Tzeng *et al*. 1991) properties, etc. The following research results serve as examples and were obtained from molecular biological activity screening of PMFs in various cell lines (Murakami *et al.* 2000a, 2000b; Manthey *et al.* 2001b; Lin *et al.* 2003; Kurowska *et al*. 2004; Murakami *et al.* 2005; Lai *et al*. 2007; Li S *et al.* 2007b; Saito *et al*. 2007; Tang *et al*. 2007). PMFs have been shown to inhibit LPS-induced $NO^{\bullet}$ production and suppress the gene expression of iNOS and COX-2 in RAW264.7 macrophage (Murakami *et al.* 2000a; Choi *et al.* 2007; Li S *et al.* 2007c); hydroxyflavones to block adhesion molecule biosynthesis in cytokine-induced endothelial cell line (Gerritsen *et al*. 1995; Read 1995); to block activation-induced degranulation of neutrophils and mast cells (Middleton and Kandaswami 1992); to inhibit expression of tumor necrosis factor–α (TNFα) (Manthey *et al*. 1999); to induce the differentiation of myeloid leukemic cells (Sugiyama *et al*. 1993); to suppress proliferation while promoting apoptosis (Hirano *et al.* 1995); to reduce lymphocyte proliferation and platelet aggregation (Mookerjee *et al.* 1986; Beret *et al.* 1988); and to suppress ethanol-induced gastric hemorrhagic lesions while promoting chloride secretion by human colonic epithelial cells (Takase *et al.* 1994). Significant scientific data demonstrated that citrus flavonoids promote metal chelation, scavenging of free radicals, alteration of phase I cytochrome P-450 (CYP) CYP1A1 enzyme activation and stimulation of phase II conjugation enzymes (Murakami *et al.* 2004). The *in vivo* studies have shown PMFs to exhibit significant inhibitory effects on mouse skin tumor promoted by a two-stage carcinogenesis test (Iwase *et al*. 2000, 2001); to remarkably inhibit two distinct stages of skin inflammation induced by double TPA application and suppressed UVB-induced expression of COX-2 protein (Murakami *et al*. 2000a; Tanaka *et al.* 2004); to inhibit TPA-induced expression of iNOS and COX-2 through suppression of TPA-induced activation of ERK1/2, p38MAPK and P13K/Akt in mice (Lai *et al*. 2007); to significantly inhibit skin tumor formation induced by TPA-DMBA (7,12-dimethylbene[α] anthracene (Lai *et al*. 2007); to reduce azoxymethane-induced large bowel carcinogenesis in rats (Suzuki *et al*. 2004; Tang *et al*. 2007); and to inhibit intestinal tumor growth in $Apc^{Min/+}$ mice (Fan *et al*. 2007). Epidemiological studies indicate that flavonoid ingestion is associated with a reduced risk of certain forms of cancer (lung, breast and prostate) and lower incidence of chronic disease, such as ischemic heart disease mortality, typy-2 diabetes, and asthma etc. (Knekt *et al.* 2002). However, the results obtained from other epidemiological studies of flavonoids yielded either no association or inverse association between the effects of flavonoids and the human diseases in cardiovascular disease and in cancer (Knelt *et al.* 2002).

### Anti-inflammatory activities

Inflammation, an immune response to bacterial, viral, mechanical or chemical injuries on tissues, involves the activation and directed migration of leukocytes (neutrophils, monocytes and eosinophils) from the venous system to damage sites. The indication of chronic inflammation is the persistent accumulation and activation of leucocytes (Coussens and Werb 2002). A variety of proteins act as mediators in the process of leucocytes migration and activation. The first group of leucocytes to migrate to the injury site for tissue repair, including adhesion molecules (L- P-, and E-selectin), cytokines (TNF, IL-1, IL-6, etc.) and leukocyte-activating molecules, endothelial vascular cell-adhesion molecule-1 (VCAM-1), MadCAM-1, matrix metalloproteinases (MMPs), are involved in the process of recruitment and activation of neutrophils. Monocytes, the second group of leucocytes to be recruited, are mediated by chemokines; and after reaching damaged tissue, they are differentiated into macrophages, which become the major source of growth factor and cytokines. During the inflammation process, chemical agents that have the ability to modulate the above-mentioned proteins will lead to either pro- or anti-inflammatory effects. There are a few key mediators in the modulation of inflammation. It has been well established that the production of cytokines (interleukin-1β or IL-1β, TNF-α, IL-6, IL-12, IFN-γ), nitric oxide (NO), prostaglandins and leukotrienes are correlated with the inflammation process. The pro-inflammatory mediators, prostaglandins and NO, are produced by actions of COX-2 (cyclooxygenases) and iNOS (inducible nitric oxide synthase), respectively. These inflammatory mediators are soluble and diffusible molecules so that they act both locally at the site of tissue damage and infection, and remotely at more distant sites. COX-2 and iNOS are important enzymes that mediate most of the inflammatory processes. Improper up-regulation of COX-2 and/or iNOS has been associated with pathophysiology of certain types of human cancers as well as inflammatory disorders (Surh *et al.* 2001). The eukaryotic transcription factor, nuclear factor-kappa B (NF-κB) is involved in regulation of COX-2 and iNOS expression. NF-κB is activated by a variety of pro-inflammatory stimuli, such as cytokines, phorbol esters, bacterial or viral products, oxidants and UV radiations, etc. (Bremner and Heinrich 2002). Activator Protein-1 (AP-1) is another important transcription factor. AP-1 involves in the transcriptional regulation of cytokines and mediators. Many chemical agents affect the formation and activation of AP-1 proteins. COX-2 gene transcription is regulated by NFκB and by AP-1 (Adderley and Fitzgerald 1999). The activation of both AP-1 and NFκB may result in greater inflammation than that of any one transcription factor alone (Adderley and Fitzgerald 1999). One of the most significant biological properties of PMFs is their anti-inflammatory activity. As early as 1936, the capillary protective effects of certain citrus flavonoids in conjunction with ascorbic acid were reported (Manthey *et al.* 2001b). Research data obtained thereafter not only widely confirmed the early findings, but also provided additional

numerous evidence to have shown that citrus flavonoids, especially PMFs in recent reports, are directly associated with the inhibition of enzymes involved in the inflammation (Lai *et al*. 2007; Li S *et al*. 2007c).

One example of a potential anti-inflammatory agent within the *Citrus* PMF family, nobiletin, has been shown to inhibit matrix degradation of the articular cartilage and pannus formation in osteoarthritis and rheumatoid arthritis by effectively inhibiting the production of pro-matrix metalloproteinase-9 (MMP-9) and prostaglandin $E_2$ ($PGE_2$) in human synovial fibroblasts by selectively down-regulating cyclooxygenase-2 (COX-2) activity (Lin *et al.* 2003; Murakami *et al.* 2005). Molecular biological evidence has shown that nobiletin suppresses gene expression and the production of some matrix metallproteinases (MMP-1, MMP-3 and MMP-9) in rabbit articular chondrocytes and synovial fibroblasts (Ishiwa *et al.* 2000). Additional recent work from Ito's group has demonstrated that proMMP-1 and proMMP-3 were also inhibited by nobiletin, while the endogenous MMP inhibitor TIMP-1 was up-regulated (Lin *et al.* 2003). Also, the higher uptake rate of PMFs has been shown in the case of HL-60 cells (Murakami *et al*. 2000a). Gene expression of other pro-inflammatory cytokines, such as interleukin IL-1α, IL-1β, TNF-α, and IL-6 were found to be down-regulated by nobiletin, tangeretin, and 3,5,6,7,8,3′, 4′-heptamethoxyflavone (Manthey *et al.* 1999; Lin *et al.* 2003). It was shown in an LPS-induced, inflammatory response mouse macrophage model that nobiletin, tangeretin, 3′, -desmethylnobiletin, 4′-desmethylnobiletin, and 3′, 4′-didesmethylnobiletin moderately attenuates iNOS and COX-2 gene expression (Manthey *et al.* 1999; Li S *et al.* 2007c) and significantly suppressed the activation of AP-1, NF-κB, and CREB (Murakami *et al.* 2005). Ohigashi's research group reported that nobiletin and 3,5,6,7,8,3′,4′-heptamethoxyflavone significantly inhibited two distinct stages of skin inflammation induced by double TPA application and suppressed UVB-induced the expression of COX-2 protein (Murakami *et al.* 2000a; Tanaka *et al.* 2004). Recently, we demonstrated that 5-hydroxy-3,6,7,8, 3′,4′-hexamethoxyflavone inhibited TPA-induced expression of iNOS and COX-2 through the suppressing TPA-induced activation of ERK1/2, p38MAPK, and PI3K/Akt in mouse. Furthermore, 5-hydroxy-3,6,7,8, 3′,4′-hexamethoxyflavone significantly inhibited 7,12-dimethylbene[a]anthracene (DMBA)/TPA-induced skin tumor formation (Pan *et al.* 2007). Release of the important inflammatory mediator, $PGE_2$, was found to be inhibited by nobiletin. Of great interest was the finding that nobiletin selectively down regulates COX-2 mRNA expression, but not COX-1 (Lin *et al.* 2003). This feature resembles commercial anti-inflammatory nonsteroidal drugs, but with fewer side effects. These findings indicate that nobiletin could be a novel anti-inflammatory and/or immunomodulatory potential drug. All of the above work is based mostly on *in vitro* evaluation, while these anti-inflammatory functions have yet to be confirmed *in vivo*.

The anti-inflammatory activity of flavonoids has been reported to be associated with their free radical scavenging property. The free radicals like superoxide radical, are ubiquitous. These radicals may be transformed to the more reactive and damaging radical species. For instance, the superoxide radical, can be readily converted to hydroxyl radical by reacting with surrounding water or ferric ions in the Haber-Weiss and Fenton reactions (Deby and Goutier 1990). In view of the pharmacological interest in flavonoids, Fazilatun *et al*. determined the superoxide radical scavenging activity of some flavonoids and described the superoxide radical scavenging capacity of flavonoids at 100 μM in a decreasing sequence of the following flavonoids: quercetin > luteolin > 5,7,3′,5′-tetrahydroxyflavone > blumeatin > rhamnetin > tamarixetin > luteolin-7-methyl ether > dihydroquercetin-4'methyl ether > dihydroquercetin-4′,7-dimethyl ether (Fazilatun *et al.* 2005). Therefore, it is deduced that flavonoids with one or more free hydroxyl groups were more active than permethylated compounds in free radical scavenging activity. Hence, hydroxylated PMFs have the property of scavenging free radical species, whereas fully methoxylated flavonoids can effectively inhibit the enzymes such as inducible nitric oxide synthase (iNOS) and NADPH oxidase that generate free radicals like NO and super oxide anion (Murakami *et al.* 2000b; Choi *et al.* 2007).

Therefore, the polyhydroxylated flavones, or PHFs, like quercetin have strong free radical scavenging activity whereas the fully methoxylated flavones (PMFs) possess efficient inhibitory activity of inflammatory enzymes like iNOS. The combination of the two molecules that have both multimethoxy groups and hydroxyl group(s) have been searched and predicted to have much more potent anti-inflammatory property than that of PHFs (polyhydroxylated flavones) and PMFs (permethoxylated flavones). Hydroxylated polymerthoxyflavones (OH-PMFs), a subcategory of PMFs and isolated from citrus peels like sweet orange peels (Li S *et al.* 2006b), have both hydroxyl group(s) and polymethoxy groups. Biological activity studies of hydroxylated PMFs have confirmed their stronger and more potent anti-inflammatory property than that of flavonoids and permethoxylated PMFs (see references in **Table 2**). For example, A side-by-side study of nobiletin and its three metabolites (**Fig. 2**) against the LPS-induced inflammation found that the nobiletin metabolites, 3'-desnobiletin, inhibited the iNOS enzyme more effectively than nobiletin, whereas other two metabolites 4'-desnobiletin and 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (or 3',4'-didesmethylnobiletin) efficiently suppressed the gene expression of iNOS and COX-2, in

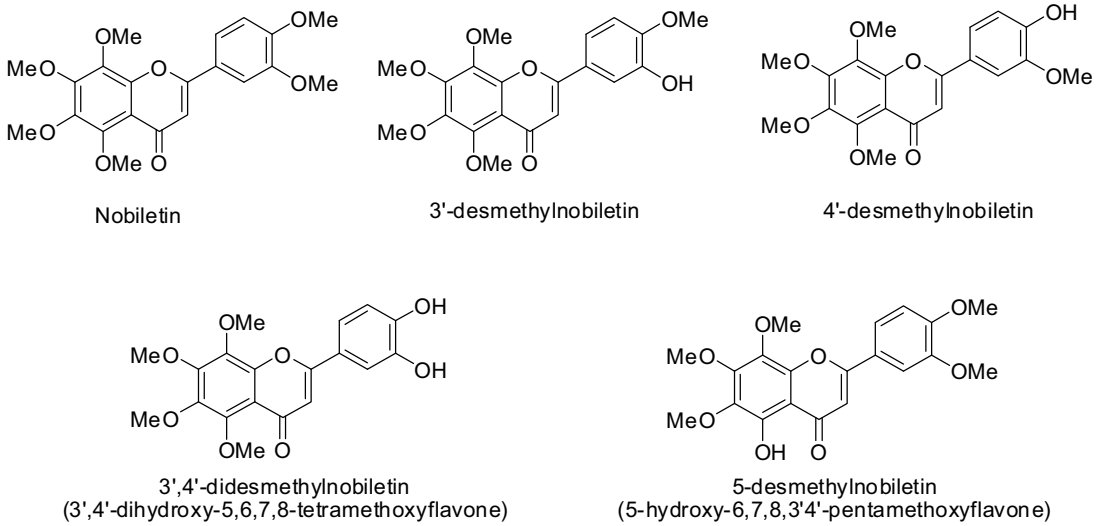


**Fig. 2 Structures of nobiletin and its metabolites and 5-desmethylnobiletin.**

**Table 2** Anti-inflammatory activity of citrus PMFs.

| Polymethoxyflavones | Proposed mechanisms | Reference |
|---|---|---|
| Nobiletin | 1. Inhibited LPS-induced both NO and $O_2^-$ generation, phorbol ester-induced oxidative stress, skin inflammation and tumor promotion. | Murakami *et al.* 2000a; Choi *et al.* 2007 |
| | 2. Interfered with the LPS-induced production of PGE2 and the gene expression of pro-inflammatory cytokines including IL-1β, IL-1β, TNF-α and IL-6. | Murakami *et al.* 2005; Lin *et al.* 2003 |
| | 3. Suppressed the activation of AP-1, NF-κB, and CREB as well as attenuated COX-2 mRNA expression. | Murakami *et al.* 2005 |
| | 4. Unregulated TIMP production and down-regulated IL-1-induced gene expression and production of proMMP-1/procollagenase-1 and proMMP-3. | Lin *et al.* 2003 |
| | 5. Suppressed the production and gene expression of MMP-9/gelatinase B in rabbit synovial fibroblasts | Ishiwa *et al.* 2000 |
| | 6. Suppressed the UVB-induced expression of COX-2, decreased the activity of cPLA2 and production of PGE2 | Tanaka *et al.* 2004 |
| Tangeretin | 1. Inhibited LPS-induced NO production in RAW264.7 cells. | Choi *et al.* 2007 |
| | 2. Suppressed IL-1β-induced COX-2 expressions through inhibition of p38 MAPK, JNK, and AKT activation. | Chen *et al.* 2007 |
| | 3. Suppressed the interleukin 1 (IL-1) induced production of proMMP-9/progelatinase B in rabbit synovial cells. | Lin *et al.* 2003 |
| 3,5,6,7,8,3',4'-heptamethoxyflavone | 1. Inhibited LPS-induced monocyte expression of tumor necrosis factor (TNFα). | Manthey *et al.* 1999 |
| | 2. Inhibited macrophage inflammatory protein-1α (MIP-1α) and interleukin-10 (IL-10) production. | Manthey *et al.* 1999 |
| | 3. Against (+/-)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide/TPA-induced two stage carcinogenesis | Iwase *et al.* 2001 |
| 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone | 1. Inhibited TPA-induced expression of iNOS and COX-2, suppress TPA-induced activation of ERK1/2, p38 MAPK, and PI3K/Akt in mouse skin. | Lai *et al.* 2007 |
| | 2. Inhibited DMBA/TPA-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity. | Lai *et al.* 2007 |
| 3'-demethylnobiletin, 4'-desmethylnobiletin and 3',4'-didesmethylnobiletin | 1. Inhibited LPS-induced NO production and iNOS, COX-2 protein expression in RAW264.7 macrophage | Li S *et al.* 2007c |

comparison to the parent compound, nobiletin (Li S *et al.* 2007c). The most recent result is the strong anti-inflammatory activity of a citrus OH-PMF: 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (**Fig. 2**). By a topical application of 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-HO-HexaMF) on 12-Otetradecanoylphorbol-13-acetate (TPA)-induced expression of iNOS and COX-2 in mouse skin, we found that 5-HO-HexaMF (5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone) can effectively inhibit the transcriptional activation of iNOS and COX-2 mRNA and protein. Pre-treatment with 5-HO-HexaMF resulted in the reduction of TPA-induced nuclear translocation of nuclear factor-kB (NF-kB) subunit and DNA binding by blocking phosphorylation of the inhibitor kB (IkB)-α and p65 and subsequent degradation of IkBα. In addition, 5-HO-HexaMF can inhibit TPA-induced phosphorylation and nuclear translocation of the signal transducer and activator of transcription-3. Moreover, 5-HO-HexaMF can suppress TPA-induced activation of extracellular signal-regulated kinase 1/2, p38 mitogen activated protein kinase and phosphatidylinositol 3-kinase/Akt, which are upstream of NF-kB. We also found that 5-HO-HexaMF significantly inhibited TPA-induced mouse skin inflammation by decreasing inflammatory parameters. Furthermore, 5-HO-HexaMF also significantly inhibited 7,12-dimethylbenz[α]anthracene/TPA-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity of papillomas at 20 weeks. Therefore, all these results revealed for the first time that 5-HO-HexaMF is an effective antitumor agent and its inhibitory effect is through the down-regulation of inflammatory iNOS and COX-2 gene expression in mouse skin, suggesting that 5-HO-HexaMF is a novel functional agent capable of preventing inflammation-associated tumorigenesis (Lai *et al.* 2007). The results of anti-inflammatory activity and its mechanism study of selected PMFs are listed in **Table 2**. By comparing the anti-inflammatory activity of polymethoxyflavones and their hydroxylated counterparts, it is evident that the hydroxylated polymethoxyflavones are more potent agents against inflammation.

## Anti-carcinogenic activity

Numerous evidence of the association of inflammation and infection with cancer initiation and proliferation exists and is compelling. The proposed mechanisms includes: direct integration of viral DNA into host genome; immunosuppression caused by viral infection leading to a failure to inhibit malignancy; production of ROS (reactive oxygen species) and RNS (reactive nitrogen species), which in turn cause damage to host DNA (Coussens and Werb 2002; Ohshima *et al.* 2003). Detailed studies have unveiled the relationship between inflammation and cancer. Chronic inflammation is induced by biological, chemical, and physical factors that have been found associated with the increased risk of human cancer at various sites. Inflammation causes the activation of a variety of inflammatory cells, which in turn induce and activate several oxidant-generating enzymes, such as NADPH oxidase, iNOS, myeloperoxidase, and eosinophil peroxidase. These enzymes generate high concentrations of diverse free radicals and oxidants, including super oxide anion ($O_2^-$), nitric oxide (NO), nitroxyl, nitrogen dioxide ($NO_2$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HClO) and hypobromous acid (HBrO), etc. These free radicals and oxidants react with each other and produce more potent reactive oxygen and nitrogen species, such as peroxynitrite. The adverse effects of these reactive species cause severe damages to DNA, RNA, lipids, and proteins by nitration, oxidation, chloronation, and bromination reactions, which consequently lead to increased mutations and altered functions of enzymes and proteins. These effects contribute to the multi-stage carcinogenesis process. Because of the intimate relationship between inflammation and cancer, a thorough exploration of inflammation and appropriate treatment of inflammation should be pursued, especially as it relates to early-stage cancer induction in human population (Balkwill and Mantovani 2001; Coussens and Werb 2002; Ohshima *et al.* 2003).

Many *in vitro* and *in vivo* studies indicate protective effects of polymethoxyflavonoids against the occurrence of cancer. For example, PMFs, like nobiletin, tangeretin, sinesetin, and 3,5,6,7,8,3',4'-heptamethoxyflavone, have been shown to selectively inhibit the growth of human leukemic

**Table 3** Anti-carcinogenic activity of citrus polymethoxyflavonoids and their derivatives

| Polymethoxyflavones | Proposed mechanisms | Reference |
|---|---|---|
| Nobiletin | 1. Inhibited the proliferation of different human cancer cells and migration of human umbilical endothelial cells. | Kawabata *et al.* 2005 |
| | 2. Induced HL-60 cells differentiated into mature monocyte/macrophage. | Kawaii *et al.* 1999b |
| | 3. Induced G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. | Kawaii *et al.* 1999c |
| | 4. Inhibited xanthine oxidase induced $O_2^-$ generation and TPA-stimulated $O_2^-$ generation in differentiated human promyelocytic HL-60 cells. | Morley *et al.* 2007 |
| | 5. Protected the development of adenocarcinoma of the prostate (TRAP) in transgenic mice and inhibited the growth of human prostate cancer cells. | Murakami *et al.* 2000a |
| | 6. Reduced AOM-induced cell proliferation and the prostaglandin $E_2$ content in colonic adenocarcinoma and/or colonic mucos, and increased the apoptotic index. | Tang *et al.* 2007 |
| | 7. Suppressed proMMPs and augmented the expression of TIMP-1 which was associated with the inhibition of tumor invasion. | Suzuki *et al.* 2004 |
| | 8. Inhibited the APMA-stimulated gelatinolytic activity and also the collagenolytic activity in rat brain and lung tissue used the film in-situ zymography method. | Miyata *et al.* 2004 |
| | 9. Directly inhibited MEK activity and the sequential ERK activation, resulted in suppressing the proMMP-9 production in HT-1080 cells. | Sasaki *et al.* 2005; Miyata *et al*. 2008 |
| | 10. Acted as a signal modulator to attenuate the activation of intrinsic pathway in $H_2O_2$-induced apoptosis in human neuroblastoma SH-SY5Y cell line. | Akao *et al*. 2008 |
| Tangeretin | 1. Suppressed mutagenic activity in the *S. typhimurium* test and induce HL-60 differentiation. | Kawaii *et al.* 1999a |
| | 2. Inhibited PhIP-DNA adduct formation in colon and induce glutathione transferase (GST) activity. | Miyazawa *et al.* 1999 |
| | 3. Inhibited P450 1A, P450 1A2 and P450 3A4 in human liver microsome. | Breinholt *et al.* 1999 |
| | 4. Inhibited the activity of 15-lipoxygenase *in vitro*. | Obermeier *et al.* 1995 |
| | 5. Modulated gap junctional intercellular communication (GJIC) in the rat liver epithelial cell line. | Malterud *et al.* 2000; Chaumontet *et al.* 1994 |
| | 6. Inhibited extracellular-signal-regulated kinases 1/2 (ERK1/2) phosphorylation and growth of human mammary cancer cells and cytolysis by natural killer cells. | Van *et al*. 2005 |
| | 7. Inhibited TCDD-induced transcriptional activity of the CYP1A1 promoter and the expression of mRNA and protein. | Hamada *et al.* 2006 |
| | 8. Reduced the number of metastatic nodules in Lentini's model. | Martinez *et al.* 2005 |
| | 9. Upregulated the function of the E-cadherin/catenin complex in human MCF-7/6 breast carcinoma cells and led to firm cell-cell adhesion and inhibition of invasion *in vitro*. | Brack *et al.* 2002 |
| 3,5,6,7,8,3',4'-heptamethoxyflavone | 1. Inhibited the activity of 15-lipoxygenase *in vitro*. | Malterud *et al.* 2000 |
| | 2. Inhibited the proliferation of different human cancer cells and induced HL-60 differentiation. | Kawaii *et al.* 1999a, 1999b |
| 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone | 1. Induced apoptosis by increase the concentration of intracellular $Ca^{2+}$resulting from both depletion of the endoplasmic reticulum $Ca^{2+}$ stores and $Ca^{2+}$ influx from the extracellular space in human breast cancer cell. | Sergeev *et al.* 2006 |
| | 2. Induced growth inhibition of human cancer cells and induction of apoptosis in HL-60 cells through modulation of mitochondrial functions regulated by ROS. | Pan *et al.* 2007 |
| 5-hydroxy-6,7,8,3',4'-pentamethoxy-flavone | 1. Induced apoptosis by increase the concentration of intracellular $Ca^{2+}$ resulting from both depletion of the endoplasmic reticulum $Ca^{2+}$ stores and $Ca^{2+}$ influx from the extracellular space in human breast cancer cell. | Sergeev *et al.* 2006 |
| | 2. Elevated the apoptotic cell population and caspase-3 activity synergistically by the combined treatment with tangeretin. | Akao *et al*. 2008 |

cell (HL-60) lines *in vitro* (Sugiyama *et al.* 1993), to induce the differentiation of myeloid leukemic cells, and to suppress proliferation while promoting apoptosis in HL-60 cell line (Sugiyama *et al.* 1993; Hirano *et al.* 1995), and to reduce the invasion of tumors in animal models (Vermeulen *et al.* 1996). Citrus PMFs inhibit carcinogenesis by mechanisms that are involved in blocking the metastasis cascade, inhibition of cancer cell mobility in circulatory systems, apoptosis, selective cytotoxicity, and antiproliferation (**Table 3**). These PMFs have been shown to reduce the invasion of tumors in animal models, to induce the differentiation of myeloid leukemic cells, and to suppress proliferation of cancer cells. PMFs have also been shown to reduce lymphocyte proliferation and platelet aggregation and can suppress ethanol-induced gastric hemorrhagic lesions, while promoting chloride secretion by human colonic epithelial cells (Ishiwa *et al.* 2000). Comparative studies of the antiproliferative effects between polymethoxyflavonoids (PMFs), nobiletin and tangeretin, and polyhydroxyflavonoids (PHFs), quercetin and taxifolin on the *in vitro* growth of a human squamous-cell carcinoma cell line revealed that the two polymethoxyflavones inhibited cell growth significantly, whereas the two polyhydroxyflavonoids showed no significant inhibition at any of the tested concentrations. A rational explanation for this observation was the higher lipophilicity and greater permeability of PMFs over the PHFs (polyhydroxyflavonoids), which suggests that the potent bioactivity of PMFs is due to their enhanced cellular absorption (Kandaswami *et al.* 1991).

Previously, studies of the biological activity of PMFs have been focused on the two most abundant PMFs in citrus peels: tangeretin and nobiletin. The anticancer activity of tangeretin is well accepted (Kawaii *et al.* 1999a; Miyazawa *et al.* 1999). The anti-cancer function of tangeretin has multiple mechanisms. It can inhibit the cancer initiation stage by modulating hepatic enzymes, thus affecting xenobiotic activation and detoxification in the liver. For example, glutathione transferase activity is enhanced by tangeretin (Breinholt *et al.* 1999), while P450 1A2, 3A4, and 2B, 3A were reported to be inhibited respectively to different extents (Obermeier *et al.* 1995; Siess *et al.* 1995). Tangeretin also plays multiple roles in the cancer-cell proliferation and the metastasis stage by inhibiting cell adhesion and invasion (Brack *et al.* 2002; Martinez *et al.* 2005). Although it is not a good free radical scavenger, tangeretin does inhibit 15-lipoxygenase activity (Malterud and Rydland 2000), suggesting that it may exert a modulating effect on enzymatic lipid oxidation. Tangeretin is able to enhance gap-junction intercellular communication between normal cells and mutated cells and thus inhibit cancer cell proliferation (Chaumontet *et al.* 1994). Tangeretin can arrest the cell cycle in the G1 phase by inhibiting cyclin-dependent kinases (Cdk)

and enhancing Cdk inhibitor proteins (Pan *et al.* 2002; Morley *et al.* 2007). Tangeretin has shown an inhibition mechanism of human mammary cancer cells and cytolysis in which it inhibits extracellular-signaling-regulated kinase (ERK) phosphorylation (Van *et al.* 2005). A recent study also showed that tangeretin exerts its antitumor activity by repressing induced and constitutively expressed cyclooxygenase-2 (COX-2) in human lung cancer cells (Chen *et al.* 2007).

Research on the anticancer activity of nobiletin originated from the anti-carcinogenic and anti-tumor activity of citrus flavonoids. Nobiletin can inhibit the proliferation of human prostate, skin, breast and colon carcinoma cell lines, inhibit the production of some MMPs, and inhibit the proliferation and migration of human umbilical endothelial cells (Murakami *et al.* 2000a, 2002). Nobiletin was also shown to have anti-proliferative and apoptotic effects on a gastric cancer cell line and also exhibited a disruptive effect on the progression of the cell-cycle. Nobiletin inhibited the growth of prostate cancer cells and reduced Azoxymethane (AOM)-induced large bowel carcinogenesis in rats (Suzuki *et al.* 2004; Tang *et al.* 2007). Furthermore, nobiletin has anti-invasion activity by inhibiting matrix metalloproteinase (MMP) activity in the rat (Miyata *et al.* 2004; Sasaki *et al.* 2005). In an evaluation of 42 flavonoids including PMFs and PHFs, nobiletin showed the strongest antiproliferative activity against six human cancer cell lines (Yoshimizu *et al.* 2004). Nobiletin has been shown to suppress prostaglandin $E_2$ ($PGE_2$) production and COX-2 protein expression *in vitro*. COX-2, induced by several stimuli associated with inflammation, is involved in carcinogenesis including colon tumorigenesis of humans and rodents (Kohno *et al.* 2001). Recently nobiletin was shown to be an inhibitor of both NO and $O_2^-$ generation in human cells, and a powerful inhibitor of two distinct stages of skin inflammation induced by double TPA application (Iwase *et al.* 2000). Nobiletin is also demonstrated to inhibit MEK (mitogen-activated protein/extracellular signal-regulated kinase) activity directly and to inhibit the sequential ERK activation, resulting in the suppression of proMMP-9 production in HT-1080 cells (Miyata *et al*. 2008).

Recent studies showed that 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-HO-HexaMF), 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, 3,5,6,7,8,3',4'-heptamethoxyflavone, and sinensetin induced apoptosis by increasing the concentration of intracellular $Ca^{2+}$ and by modulating mitochondrial function (Sergeev *et al.* 2006; Pan *et al.* 2007). Existing at about 10 ppm (10 mg/kg) in dried orange peels, 5-HO-HexaMF induced growth inhibition of human cancer cells and apoptosis in human leukemic (HL-60) cells by modulating mitochondrial functions regulated by reactive oxygen species (ROS). ROS generation occurs in the early stages of 5-HO-HexaMF induced apoptosis, preceding cytochrome c release, caspase activation, and DNA fragmentation. The changes occurred after single breaks in DNA were detected, suggesting that 5-HO-HexaMF induced irreparable DNA damage, which consequently triggered a caspase-independent pathway, as indicated by endonuclease G, and also contributed to apoptosis caused by 5-HO-HexaMF. Antioxidants suppressed 5-HO-HexaMF -induced apoptosis. 5-HO-HexaMF dramatically enhanced the growth arrest DNA damage-inducible gene 153 (GADD153) protein in a time-dependent manner. N-acetylcysteine (NAC) and catalase prevented the up-regulation of GADD153 expression caused by 5-HO-HexaMF. These findings suggest that 5-HO-HexaMF creates an oxidative cellular environment that induces DNA damage and GADD153 gene activation, which in turn assisted to trigger apoptosis in HL-60 cells. Meanwhile, ROS were proven an important inducer in this apoptotic process. The 5-hydroxyl group on the A-ring of 5-HO-HexaMF was found to be essential for its anti-proliferative and apoptosis inducing activity. This study identified the novel mechanisms of 5-HO-HexaMF-induced apoptosis and indicated that 5-HO-HexaMF could be a potential chemopreventive and chemotherapeutic agent (Pan *et al.* 2007).

Our recent results from a systematic anticancer screening of PMFs and hydroxylated PMFs have enabled us to establish a structure activity relationship (SAR) within this group of PMF compounds (Li S *et al*. 2007b). Among 15 PMF substances, five abundant PMFs and two hydroxylated PMFs were isolated from sweet orange peel. Also, seven hydroxylated PMF analogues are commonly found in sweet orange peel. Screening against cell proliferation and induction of apoptosis in human (HL-60) leukemia cells revealed that 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5-demethylnobiletin) and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone have the strongest activity, followed by 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (5-desmethylsinensetin). Overall anti-cancer activity of PMFs was significantly lower compared to hydroxylated PMFs (Li S *et al.* 2007b). **Table 3** is a summary of the available anticancer activities and mechanisms of citrus PMFs.

## Anti-atherosclerosis activity

Strong evidence indicates that citrus flavonoids may reduce the hepatic production of cholesterol containing lipoproteins, thus reducing total cholesterol concentration in the plasma, and consequently reducing the occurrence of car-

**Table 4** Anti-atherosclerosis activity of citrus polymethoxyflavonoids.

| Polymethoxyflavones | Proposed mechanisms | Reference |
|---|---|---|
| Nobiletin | 1. Inhibited class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages, which was associated with the prevention of atherosclerosis at the level of the vascular wall by inhibiting macrophage foam-cell formation. | Whitman *et al.* 2005 |
| | 2. Suppressed phorbol ester-induced phosphorylation of ERK 1/2, JNK 1/2, and c-Jun (Ser-63), attenuated expression of SR-A, SR-PSOX, CD36, and CD68 mRNA, leading to the blockade of DiI-acLDL uptake. | Eguchi *et al.* 2006 |
| | 3. Enhanced both differentiation and lipolysis of adipocyte through activation of signaling cascades mediated by cAMP/CREB. | Saito *et al.* 2007 |
| Tangeretin | 1. Reduced serum total and very low-density lipoprotein (VLDL) + LDL cholesterol and serum triacylglycerols. | Kurowska and Manthey 2004 |
| | 2. Reduction in apoB secretion observed in Hep G2 cells, suppression of TAG synthesis and activities of DAG acyltransferase, activate the peroxisome proliferator-activated receptor (PPAR). | Kurowska *et al.* 2004 |
| Other citrus flavonoids | 1. Decreased in serum triglyceride (TG) and cholesterol levels, reduced TG contents in the liver and heart, regulated adipocytokines by significantly suppressing TNF-α, INF-γ, IL-1β and IL-6 expression and increasing adiponectin, increased PPARα and PPARγ protein expression in the liver | Li RW *et al.* 2006 |
| | 2. Decreased apoB secretion and cholesterol esterification, decreased both the activity and expression of microsomal triglyceride transfer protein (MTP), increase in low density lipoprotein (LDL) receptor mRNA. | Wilcox *et al.* 2001 |
| | 3. Reduced fatty streak formation and neointimal macrophage infiltration and inhibited hypercholesterolemia-induced intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells | Choe *et al.* 2001 |

diovascular disease (Wicox *et al.* 2001; Kurowska and Manthey 2004; Guthrie *et al.* 2006). A recent study suggests that nobiletin can reduce the circulating concentrations of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in the blood and can directly inhibit foam-cell formation at the site of lesion development within a vessel wall (Whitman *et al.* 2005) (**Table 4**). It was recently reported that PMF supplementation may ameliorate hyper-triglyceridemia and its anti-diabetic effects in hamsters through adipocytokine regulation and peroxisome proliferators activated receptor-α (PPARα) and PPARγ activation (Li RW *et al.* 2006). Tangeretin was able to lower cholesterol and triacylglycerols, and modulate apoB-containing lipoprotein metabolism by activating the peroxisome proliferator-activated receptor (Kurowska *et al*. 2004; Kurowska and Manthey 2004). The anti-inflammatory activities of nobiletin are likely linked to the prevention of plaque formation during atherosclerosis. Nobiletin markedly reduced TPA-induced, lectin-like, ox-LDL receptor-1 (LOX-1) mRNA expression in THP-1 human monocyte-like cells in dose and time-dependent manners. It also suppressed the phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK) 1/2, and c-Jun (Ser-63), thereby inhibiting the transcriptional activity of activator protein-1. Furthermore, nobiletin attenuated the expression of SR-A, SR-PSOX, CD36, and CD68, but not CLA-1, mRNA, leading to the blockade of DiI-acLDL uptake (Eguchi *et al.* 2006). It was recently reported that nobiletin enhanced both differentiation and lipolysis of adipocyte through the activation of signaling cascades mediated by cAMP/CREB (Saito *et al.* 2007). Naringin, a bioflavonoid found in citrus fruit peel, significantly reduced fatty streak formation and neointimal macrophage infiltration and inhibited the expression of ICAM-1 in endothelial cells, suggesting that naringin may have antiatherogenic and hepatoprotective action (Choe *et al.* 2001). PMFs may play a role in the prevention of atherosclerosis because of the potential role of inflammation in the genesis of atherosclerosis (Ross 1999; Whitman *et al.* 2005; Li RW *et al.* 2006).

## METABOLISM OF POLYMETHOXYFLAVONOIDS FROM CITRUS PEEL

Nutrients and drugs have to be in solution in order to be absorbed from the gastrointestinal tract. During the course of absorption, the exogenous substances are metabolized in the intestine and later in the liver. Metabolism is the sum of the processes by which a particular substance is handled (as by absorption, incorporation, detoxification and excretion) in the living body. It is a natural response from the host body to defend itself from foreign substances. Generally, it may include a detoxification process performed by liver enzyme (phase I and II enzymes) systems in which the xenobiotic substances are transformed into more hydrophilic molecules so that they can be easily excreted from the body. Phase I enzymes, mainly cytochrome P450s which attach hydrophilic functional groups to the foreign compounds, are involved in oxidation and reduction reactions as well as the hydrolysis of ester, amide and ether linkage, and as a result, generate stronger hydrophilic groups such as –OH, $–NH_2$, –SH, and $–CO_2H$, etc. Phase II enzymes, also known as transferases or conjugation enzymes such as glutathione transferase, link the hydrophilic groups (–OH, $–NH_2$, –SH, and $–CO_2H$, etc.) to more hydrophilic groups such as glucuronic acid or sulphates. For instance, glutathione transferase provide glutathione to compounds that are metabolized by phase I enzymes. Consequently, the polarity and hydrophilicity of so formed metabolites are significantly increased and the tendency of these molecules being easily excreted through kidney is also greatly increased (Iersel *et al.* 1999; Yan and Caldwell 2001). The natural process starts with ingestion. Lipophilic substances bind to blood cells, albumen, lipoproteins, lymph cells and lymph proteins, etc. Then the bounded complex undergoes phase I and phase II transformations and generate more polar metabolites, which may be circulated in the blood system (Yan and Caldwell 2001). Some fractions of these metabolites are eventually excreted either by kidney through urine, or by bile through guts then feces. Smaller and highly polar molecules tend to be excreted by urine whereas larger conjugated metabolites are eliminated in feces through bile and intestine. Other fractions of metabolites are circulated in blood system and distributed to tissues and organs where are the action sites of the biological agents. Therefore, the metabolites in the blood or plasma are of key importance in elucidating the metabolite profile and the mechanisms involved in the biological actions of nutraceuticals and pharmaceuticals.

### Metabolism of flavonoids

The antioxidant and biological activity of flavonoids are closely associated with the number and position of hydroxyl groups on the 3-ring skeleton structure of flavonoids. Biological activities of nutrients and pharmaceuticals mainly depend on their bioavailability and sometimes the bioactivity of metabolites. For instance, some diets are rich in bioactive flavonoids, but they do not necessarily function as their desired biological activity because of the insufficient absorption of flavonoids by small intestine, extensive metabolizetion before reaching the site of action, or rapid elimination by kidney or bile. The biological activities and health properties of flavonoids such as quercetin, apigenin, narigenin and genistein, have been extensively studied and there are numerous reports concerning their metabolism and their pharmacokinetics (Middleton *et al.* 2000; Gradolatto *et al.* 2005). Biotransformation and biological activity of metabolites of bioactive molecules provide significant information regarding metabolic pathways and the molecules being responsible for the biologically activity. The pharmacokinetics and metabolism investigation of apigenin (3′-hydroxy-5,7-dihydroxy-flavone), a weak estrogenic flavonoid phytochemical present in aromatic plants (camomilla, rosemary and parsley), suggested a slow metabolism of apigenin, with a slow absorption rate and slow clearance (Gradolatto *et al.* 2005). These findings provide evidence for the hypothesis of the possible accumulation of apigenin in the body.

### Metabolism study of citrus polymethoxyflavonoids

The chemical and physical properties of polymethoxy groups are different from those of polyhydroxy groups on the flavone structure. Hence, it is evident that the properties of PMFs are separate and distinct from those of common flavonoids in absorption and metabolism *in vitro* (Murakami *et al.* 2001). It has been found that PMFs undergo *in vivo* biotransformation and produce metabolites with different bioactivities and pharmacological properties (Li S *et al.* 2007b, 2007c). Hence, it is of significance to elucidate the metabolism and metabolic fate of PMFs.

As early as 1980, it was observed that the addition of tangeretin or nobiletin to human liver microsomes activated both the hydroxylation of benzo[α]pyrene and the metabolism of aflatoxin B1 to mutagens (Conney *et al.* 1980). Later, it was demonstrated that the mixture of nobiletin and tangeretin dose-dependently induced liver mixed function oxidase (MFO) systems in rainbow trout (Nixon *et al.* 1984). Yang *et al.* first reported the effects of tangeretin on cytochrome P450 activity in 1995. They found that 7-ethoxyresorufin-*O*-deethylase (classified as CYP 1A) and nifedipine oxidase (CYP 3A4) in human liver microsomes were inhibited by tangeretin in a noncompetitive manner (Obermeier *et al.* 1995). But contradictory results from immune-blot analysis of rat liver samples showed that flavones like tangeretin increased the activity of some cytochrome P450 enzymes, such as ethoxyresorufin-*O*-deethylase, methoxyresorufin-*O*-demethylase and pentoxyresorufin-*O*-dealkylase (Canivenc-Lavier *et al.* 1996). Acetaminophen oxidation, catalyzed by rat liver P450 3A4, was stimulated by tangeretin, nobiletin and other PMFs, but inhibited by 40-60% by myricetin and quercetin (Li Y *et al.* 1994).

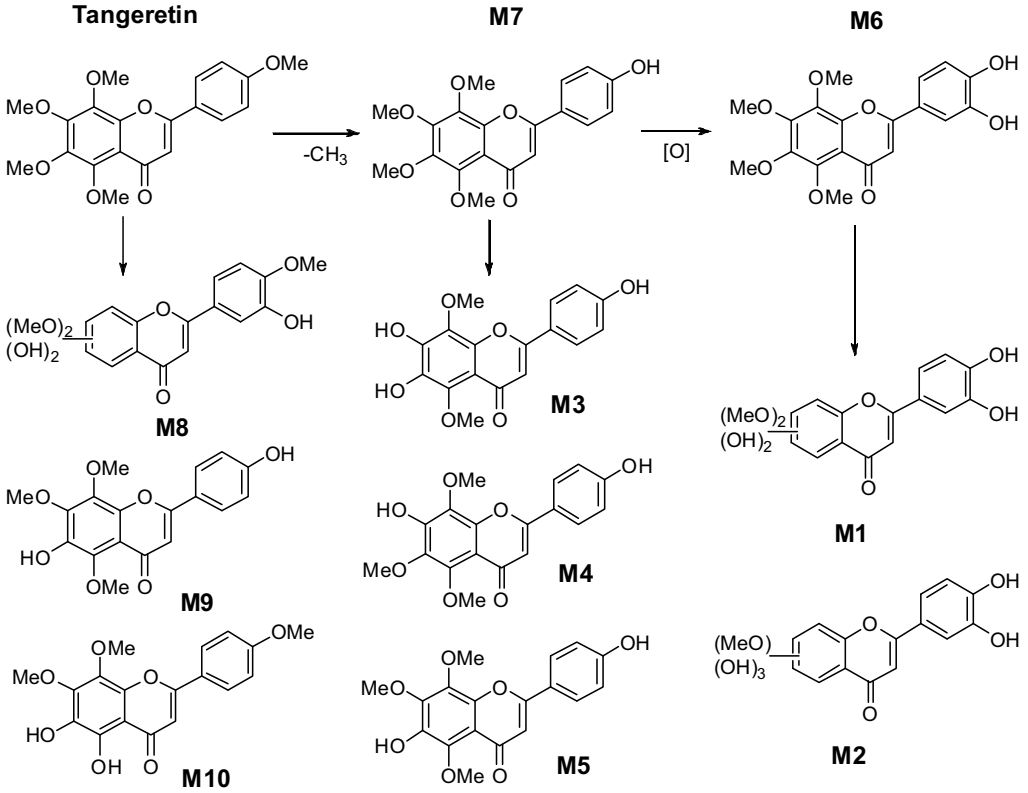


**Fig. 3 Biotransformation pathway of tangeretin in rat.**

These observations suggested an interaction between PMFs and liver cytochrome P-450 isozymes.

Cytochrome P450 (CYP) is the key enzyme system involved in metabolism of flavonoids and the P450 enzymes catalyze the hydroxylation and demethylation reactions on the C6-C3-C6 flavone skeleton. The biotransformation pathway of flavonoids is considered to be identical across the species. The 3' and 4' positions on the B-ring of flavonoid structure are the primary site of biotransformation. The number and position of the hydroxyl and methoxy groups on B-ring influence the metabolism to a great extent. The observation that CYP 1A2 plays a major role in the hepatic metabolism of genestein and tangeretin among different forms of CYP P450 enzymes suggests that inter-individual differences in the metabolism of PMFs may result in the discrepancy of biological activities (Nielsen *et al.* 1998, Breinholt *et al.* 2003; McKendall *et al*. 2008).

## Metabolism of tangeretin

Tangeretin was de-methylated by interacting with human and rat liver microsomes of P450 system (Vyas *et al.* 1983; Canivenc-Lavier *et al.* 1996). Breinholt and his coworkers carried out both *in vitro* and *in vivo* experiments to study the metabolic fate of tangeretin by analyzing various samples from following stated corresponding experiments In the *in vitro* experiment, tangeretin was incubated with Aroclor induced rat liver microsomes and three major metabolites of tangeretin were identified as 4'-desmethyltangeretin (M7 in **Fig. 2**), 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (M6 in **Fig. 2**) and 6,4'-dihydroxy-5,7,8-trimethoxyflavone by HPLC, LC/MS and proton NMR (M5, Nielsen *et al.* 1998). In their *in vivo* biotransformation study of tangeretin (**Fig. 3**), the rats were gavage fed repeatedly. Samples of rat urine and feces were collected and analyzed by HPLC-MS and $^1$H NMR techniques (Nielsen *et al.* 2000). Both major and minor metabolites were isolated and characterized. The HPLC profile of tangeretin metabolites with UV at 260 nm of rat urine and rat fecal sample was illustrated. They found a minimum of ten tangeretin metabolites. The dominant metabolite in rat urine was 4'-desmethyltangeretin and the other major metabolite 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone, though other metabolites with intact 4'-methoxy group and with desmethylation at various positions on A ring were also identified. Therefore, 4'-methoxy group of tangeretin might be the primary site for desmethylation and 3'-position might be the most vulnerable site for hydroxylation or oxidation by phase I enzymes.

## Metabolism of nobiletin

The metabolism study of nobiletin was conducted over the last few years. Ohigashi's research group examined the *in vitro* biotransformation of nobiletin by treating nobiletin with rat liver S-9 mixture for 24 hours. Based on high performance liquid chromatography (HPLC) analysis and proton nuclear magnetic resonance ($^1$H NMR) study, they identified the major metabolite as 3'-desmethylnobiletin (MN2 in **Fig. 4**). They also found that the demethylation rate of nobiletin was slow with a half-life greater than 24 hours, much lower than that of ethoxycoumarin ($t_{1/2}$ = 2 h) (Murakami *et al.* 2001), suggesting that the half-life of nobiletin in an *in vivo* system is considerably long. In contrast to the demethylation of PMFs, the 3' hydroxy group on the B-ring of polyhydroxylated flavonoids (PHFs), such as quercetin, was methylated in rat plasma, indicating that the active site of methylation and demethylation at 3'-position of certain PHFs and PMFs, respectively, is one of the key steps in the metabolic pathway of PMFs and PHFs bearing methoxy or hydroxy groups in the B-ring (Murakami *et al.* 2001). Ohigashi and his associates continued their experiments in male SD rat and they identified the dominant metabolite as 3'-desmethylnobiletin (MN2 in **Fig. 4**) with two other monodemethylated nobiletin and two di-desmethylnobiletin products. The result was consistent with the findings from their previous *in vitro* experiments (**Fig. 4**). Also in their *in vivo* experiment, they detected the only metabolite, 3'-desmethylnobiletin (MN2 in **Fig. 4**), in serum (Murakami *et al.* 2002). Another study of nobiletin biotransformation in the same species – male SD rat, and from urine sample after oral administration of nobiletin, Ohsawa's research group isolated and identified one major and two minor metabolites

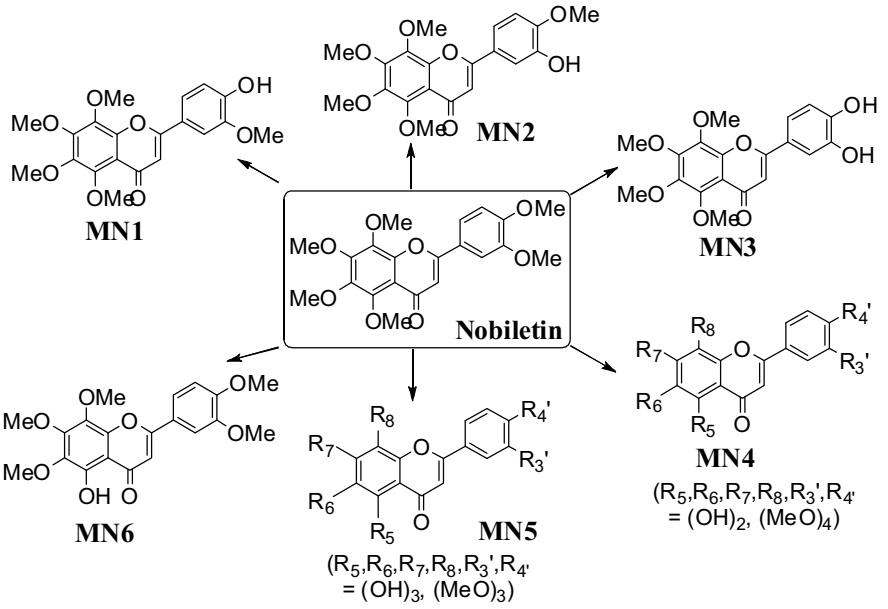


**Fig. 4 Identified metabolites of nobiletin.**

by three-dimensional HPLC equipped with a photodiode array detector (PDA or DAD). The structure of the major metabolite was characterized as 4'-desmethylnobiletin (MN1 in **Fig. 4**) by spectroscopic methods (Yasuda *et al.* 2003). One of the minor metabolites was also mono-demethylnobiletin, but not 5-demethylnobiletin by $^1$H NMR. The other minor metabolite was di-demethylated nobiletin by electrospray ionization mass spectroscopy (ESI-MS).

Furthermore, the biotransformation of nobiletin by *Aspergillus niger* and the anti-mutagenic activity of a metabolite were investigated (Okuno *et al.* 2004). After three days' cultivation of nobiletin in the *A. niger* culture medium, only one metabolite was isolated and identified as 4'-desmethylated nobiletin (MN1 in **Fig. 4**). They also investigated the anti-mutagenic activity of the metabolite, 4'-desmethylnobiletin and found that it had suppressive effects on gene expression of the SOS response to DNA damage in *Salmonella typhimurium* TA1535/pSK1002, induced by chemical mutagens furylfuramide, MeIQ and Trp-P-1. This was the first report on the biological and pharmacological activity study of 4'-desmethylnobiletin (MN1 in **Fig. 4**). Previously, it was reported by the same research group that nobiletin exhibits inhibition activity against the SOS response and anti-mutagenic activity against chemical mutagens (Miyazawa *et al.* 1999). In their comparitive study, nobiletin and 4'-desmethylnobiletin showed similar suppressive effects against furylfuramide, UV, Trp-P-1 and MeIQ, but 4'-desmethylnobiletin exhibited greater suppressive effects than nobiletin against activated Trp-P-1 and MeIQ (Okuno *et al.* 2004). The metabolism study of nobiletin on CD-1 mice found three metabolites: 3'-desmethylnobiletin (MN2), 4'-desmethylnobiletin (MN1), and 3',4'-di-desmethylnobiletin (Li S *et al.* 2006c, 2007c).

**Table 5** is a summary of nobiletin metabolites in different biofluids of different species. The metabolism study of PMFs indicates that the major metabolites are demethylated PMFs, such as mono- and di-demethylation products, and hydroxylation (oxidation) products detected in tangeretin biotransformation. The demethylation occurs mainly at the B-ring of C6-C3-C6 flavone skeleton with the formation of 4′-, 3′- and 3′,4′-hydroxylated PMFs. Hence, one conclusion can be drawn from the preliminary results, that is, the metabolic pathway of PMFs mainly undergoes B-ring demethylation. Further timely mechanism investigations of PMF biotransformation, especially in various animal species and different organs are necessary to advance the understanding of PMF metabolic pathway.

## BIOAVAILABILITY OF POLYMETHOXYFLAVONOIDS

Bioavailability of a drug or a nutrient refers to the amount of the drug or the nutrient that reaches the blood circulation system and to the tissue (van de Waterbeemd *et al.* 2003). Bioavailability is an overall effect of absorption, distribution, metabolism and excretion (ADME). Hence, the determination of bioavailability is a combined rate of absorption, metabolism, distribution and excretion. Absorption describes how well a drug or a nutrient passes to the systemic circulation after oral administration. Metabolism is how fast a drug or a nutrient is eliminated from the systemic circulation. Distribution refers to how well a drug or a nutrient reaches the tissues. Excretion is the rate of a drug or a nutrient being secreted from the systemic circulation. One of the most influential factors of bioavailability of a nutrient or a drug is absorption, which can be defined as the product of solubility and permeability. Absorption is usually the first pharmacokinetic component studied. Solubility refers to aqueous solubility, usually across a pH range. A nutrient or a drug substance can only be absorbed from the gastrointestinal tract when it is in solution. Therefore, the fraction of drug absorbed into the portal vein is a function of the aqueous solubility of the substance. Permeability is a compound's ability to across into the blood circulation system through intestinal membranes (Kansy *et al.* 1998). Both solubility and permeability affect absorption and they are considered together, not regarded as independent parameters. Normally, compounds with high solubility and high permeability are usually well absorbed, whereas compounds with low solubility and low permeability are poorly absorbed. Compounds

**Table 5** Identified nobiletin metabolites.

| Biofluids | Metabolites (Fig. 4) | Reference |
|---|---|---|
| Urine, male SD rat | MN1 (major), one other mono-desnobiletin, one MN4, no MN6 | Yasuda *et al.* 2003 |
| Urine, male SD rat | MN2 (major), two other mono-desnobiletin, two MN4 | Murakami *et al.* 2002 |
| Serum, male SD rat | MN2 only | |
| Liver S-9 mixture, rat | MN2 (major) | Murakami *et al.* 2001 |
| Urine, female CD-1 mice | MN2 and MN3 (major), MN1 | Li S *et al.* 2006c, 2007c |
| Plasma, female CD-1 mice | MN2, MN3, MN4, MN5, no MN6 | Li S *et al.* 2008 |
| *Aspergillus niger* medium | MN1 only | Okuno *et al.* 2004 |

with mixed properties have to be carefully characterized to ensure that they show sufficient absorption for therapeutic or nutritional use.

Permeability represents the overall effects of influx and efflux in the body (Ritschel 1987). Influx refers to a drug or a nutrient being transported from small intestine to blood system by absorptive transporters such as multidrug resistance proteins (MRP1 and MRP3), etc., whereas efflux is that the drug or nutrient is pumped out from blood system to intestine by secretory transporter like BCRP, P-glycoprotein and MRP2, etc. Cultured "intestine-like" cells such as monolayer cultured cells (Caco-2) have been used for many years to test permeability. The Caco-2 cell monolayers are human carcinoma cell lines that have many enterocytes-like properties. They are used by pharmaceutical companies to evaluate the oral absorption potential of drugs and/or to study their absorption mechanism. Transport studies through Caco-2 cells provide information about: (i) intestinal permeability, (ii) transport mechanism (paracellular or transcellular or active carrier), (iii) role of intestinal metabolism, and (iv) influence of P-glycoprotein efflux system (Walle *et al.* 2003). In other words, Caco-2 permeability can provide valuable information in the early lead discovery phase of biologically active compounds and development phase for intestinal permeability and absorption prediction through membranes. Hence, solubility and permeability are essential parameters in the prediction of absorption, which affects bioavailability of substances interested. Another type of permeability test known as PAMPA (Parallel Artificial Membrane Permeation Assay) was developed by Roche scientists in 1998 (Kansy *et al.* 1998). PAMPA carried out in microplates, measures the permeation of a compound through a phospholipid-coated filter medium that mimics intestinal cell structures. The idea behind the PAMPA assay is to predict the human intestinal permeability. Among three possible pathways through a membrane (paracellular, transcellular and active transport), 80-90% of all small molecular drugs choose the transcellular route. Transcellular permeation is based on passive diffusion, driven by a concentration gradient between donor and acceptor. The small intestine (duodenum, jejunum and ileum) is the major absorption site with the largest absorption area. PAMPA assay mimics these absorption conditions using an artificial phospholipid membrane. Therefore, PAMPA is a passive assay which can be measured in a highthroughput way.

The bioavailability of PMFs has been studied sporadically and the knowledge of their absorption and bioavailability were based on the assumption of good permeability due to their high lipophilicity because of the multiple methoxy groups. Ohigashi and his colleagues investigated the *in vitro* absorption of nobiletin and luteolin and found that nobiletin preferably accumulated in a differentiated Caco-2 cell monolayer while luteolin did not (Murakami *et al.* 2001). Based on their findings, it was concluded that nobiletin had a much higher permeability and tendency to accumulate in the intracellular compartment than that of luteolin. The same research group conducted an *in vivo* study of nobiletin in SD male rats and concluded that nobiletin has a distinct property to accumulate in a wide range of organs including the stomach, small and large intestines, liver and kidney during the 1 to 4 h periods after a single dose.

We performed bioavailability studies of some PMFs by measuring their solubility and permeability to further understand the cause of their absorption and bioavailability. The high throughput screening method, lyophilisation solubility assay (LYSA), was used to measure the solubility of PMFs. Some permeability data were obtained from both PAMPA (Parallel Artificial Membrane Permeation Assay) and Caco-2 experiments. Based on the data we can see that the solubility of PMFs was generally poor, but their permeability was good (**Table 6**). Therefore, the overall absorption of PMFs was good, which might contribute to the good bioavailability of PMFs. The multi methoxy groups are hydrophobic, which contributes to the poor solubility of PMFs. However, the lipophilic nature of methoxy groups is closely related to the good permeability of PMFs. Therefore, the PMFs may have higher permeability through small intestine wall and are readily absorbed into the blood circulation system of the human body (Murakami *et al.* 2004).

In the lyophilisation solubility assay (LYSA), the maximum reading is 500 μg/mL. In general, any number that is greater than 200 μg/mL is considered soluble. The number between 100 and 200 μg/mL is considered medium solubility. However, there is a very close relationship among solubility, permeability and potency. Solubility alone is insufficient to describe the bioavailability of an active compound in oral administration. Lipinski depicted the minimum acceptable solubility that is required for an orally active drug. He grouped the compounds in sets of three and showed that at pH 6.5 or 7.0, the minimum thermodynamic aqueous solubility required for low, medium and high permeability values at a particular clinical dose. For instance, to achieve oral absorption, a compound with medium intestinal permeability and a projected human dose of 1 mg/kg requires a minimum aqueous solubility of 52 μg/mL. For a high permeable compound with 0.1 mg/kg of projected human dose, the required minimum solubility is only 1 μg/mL (Lipinski 2000).

## Solubility of PMFs

The LYSA data are shown in **Table 6**. The overall solubility of PMFs is low. The solubility of hydroxylated PMFs is better than their fully methoxylated counterparts, such as the solubility of 5-desmethylnobiletin (32 μg/mL), 3'-desmethylnobiletin (29 μg/mL) and 4'-desmethylnobiletin (22 μg/mL) is higher than that of nobiletin (12 μg/mL). Another example, the solubility of 3-hydroxynobiletin (37 μg/mL) is significantly higher than 3-methoxynobiletin (8 μg/mL). The more hydroxyl groups the PMFs have, the better their solubility. Thus, 3',4'-dihydroxy-5,6,7,8-tetramethylflavone has the highest solubility (53 μg/mL) among the 10 PMFs screened by LYSA. Therefore, the sequence of the PMF solubility from high to low is: 3',4'-dihydroxy-5,6,7,8-tetramethylflavone > 5-desmethylnobiletin > 3'-desmethylnobiletin > 4'-desmethylnobiletin > nobiletin. Among the polymethoxyflavones, there is a trend that the more methoxy groups the PMF has, the less soluble, but the solubility difference is insignificant: tangeretin (19 μg/mL), nobiletin (12 μg/mL), 5'-methoxynobiletin (14 μg/mL). As noted in **Table 6**, the solubility of nobiletin was obtained using HPLC measurement which is more accurate than LYSA. Another observation was that hydroxyl group at 3-position of a PMF C-ring tends to increase the PMF solubility dramatically: the solubility of 3-hydroxynobiletin (37 μg/mL) is greatly higher than 3'-methoxynobiletin (8 μg/mL). Interestingly, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, with a LYSA solubility of 6 μg/mL, is nearly insoluble. Presumably, the absence of 8-methoxy group on the A-ring affected the solubility, in comparison with 3'-desmethylnobiletin. The solubility of 5,6,7,3',4'-pentamethoxyflavone was not obtained and one major reason was that its solubility is too low to detect in the LYSA assay.

## Permeability of PMFs

**Table 6** also shows the passive permeability data obtained from PAMPA. Overall, the permeability of PMFs is high, which means PMFs can easily across the phospholipids membrane. This may be due to their hydrophobic property since they have multiple methoxy groups. In contrast to the general flavonoids which possess multiple hydrophilic hydroxyl groups, the PMFs have strong tendency of lipophilicity because the hydrophobic feature of methoxy groups. Tangeretin has the highest permeability ($1.62 \times 10^{-6}$ cm/s), followed by nobiletin ($1.38 \times 10^{-6}$ cm/s), 4'-desmethylnobiletin ($1.14 \times 10^{-6}$ cm/s), and 3'-desmethylnobiletin ($1.05 \times 10^{-6}$ cm/s). All the PMFs tested have or close to high permeability except 3-hydroxynobiletin whose permeability is in the medium range ($0.55 \times 10^{-6}$ cm/s). Surprisingly, 3',4'-di-

**Table 6** Solubility and permeability data of citrus PMFs.

| Name | LASA (μg/mL) | PAMPA | | Caco-2 ($P_{app}$) | | |
|---|---|---|---|---|---|---|
| | | (cm/s × $10^{-6}$) | Permeability | A to B (cm/s × $10^{-6}$) | B to A (cm/s × $10^{-6}$) | Ratio (B to A)/(A to B) |
| Sinensetin | - | 1.02 | H | - | - | - |
| Tangeretin | 19 | 1.62 | H | - | - | - |
| Nobiletin | 12* | 1.38 | H | - | - | - |
| 5,6,7,8,3',4',5'-Heptamethoxy-flavone | 14 | 0.75 | M | - | - | - |
| 3,5,6,7,8,3',4'-Heptamethoxy-flavone | 8 | 0.93 | M | 802 | 535 | 0.7 |
| Gardenin A | - | 0.9 | M | - | - | - |
| 3'-Hydroxy-5,6,7,4'-tetra-methoxy-flavone | 6 | 0.98 | M | 558 | 507 | 0.9 |
| 3'-Hydroxy-5,6,7,8,4'-penta-methoxy-flavone | 29 | 1.05 | H | - | - | - |
| 4'-Hydroxy-5,6,7,8,3'-penta-methoxy-flavone | 22 | 1.14 | H | - | - | - |
| 3',4'-Dihydroxy-5,6,7,8-penta-methoxy-flavone | 53 | 0.98 | H | - | - | - |
| 3-Hydroxy-5,6,7,8,3',4'-hexa-methoxy-flavone | 37 | 0.55 | M | 1700 | 1000 | 0.6 |
| 5-Hydroxy-6,7,8,3',4'-penta-methoxy-flavone | 32 | - | - | - | - | - |

*Data measured from HPLC method.

hydroxy-5,6,7,8-tetramethoxyflavone is close to high permeability (0.98 × $10^{-6}$ cm/s), indicating that the hydroxyl groups on the B-ring of PMFs have none or minimal influence on PMF permeability.

Usually, compounds that are highly soluble have poor permeability and lipophilic compounds have high permeability but poor solubility. Based on **Table 1**, PMFs generally have poor solubility. However, PMFs that have better solubility are also highly permeable to the membrane, which in turn contribute to their high absorption and bioavailability. The examples are tangeretin, 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone, 4'-demethylnobiletin, 3'-demethylnobiletin, and nobiletin. In analysis of Caco-2 data, the apparent permeability ($P_{app}$) from A (apical side) to B (basolateral side) represents the overall effects of transportation carried out by both absorptive transporters and secretory transporters. The compound has good permeability when $P_{app}$ >100 × $10^{-7}$ cm/s. The apparent permeability ($P_{app}$) from B (basolateral side) to A (apical side) only measures the effects of secretory transport. If the ratio of $P_{app}$ from B to A over A to B is greater than 3, there will be efflux liability, which means that the compound is pumped out too fast from blood circulation system, which will affect the concentration of the substance in the systemic circulation, and consequently affect the absorption and bioavailability of the compound. There are only three PMFs whose Caco-2 data (**Table 6**) are currently available. From the available Caco-2 data of PMFs assayed, we can see that the $P_{app}$ for the three PMFs from A to B are 508 × $10^{-7}$ cm/s for 3'-demethylsinenstin, 802 × $10^{-7}$ cm/s for 3-methoxynobiletin, 1700 × $10^{-7}$ cm/s for 3-hydroxynobiletin. They are significantly greater than 100 × $10^{-7}$ cm/s, indicating that the PMFs have superb permeability. For all the compounds tested, the $P_{app}$ ratios of B to A over of A to B were less than 3. They are 0.9 for 3'-desmethylsinenstin, 0.7 for 3-methoxynobiletin, and 0.6 for 3-hydroxynobiletin. Therefore, the PMFs have no efflux problem.

In consideration of absorption and bioavailability, the data set (LYSA, PAMPA and Caco-2) of each compound should be considered as a whole. To this extent, conclusion can be drawn that PMFs are marginally soluble, with 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone, 4'-demethylnobiletin and 3'-demethylnobiletin having better solubility among the PMFs assayed. Tangeretin and nobiletin are highly permeable but have poor solubility. However, all the PMFs assayed are highly permeable across the small intestine wall. Considering the solubility and permeability together, the overall good absorption of PMFs can be predicted. Further *in vivo* bioavailability exploration of PMFs is needed.

## ANALYSIS AND PREPARATION OF CITRUS PMFs

### Analysis of PMFs

The analysis of the PMF content in various citrus tissues has been conducted using separation methods including GC (gas chromatography, Stremple 1998), supercritical fluid chromatography (SFC, Morin *et al.* 1991; Dugo *et al.* 1996), and high performance liquid chromatography (HPLC, Bianchini and Gaydou 1980; Heimhuber *et al.* 1980; Bianchini *et al.* 1987; Manthey and Buslig 2003; Weber *et al.* 2006). Six major PMFs in citrus oil were successfully separated using GC at high temperature (310.8°C) and were identified by GC-MS gas chromatography – mass spectroscopy), but no quantitative result was reported (Stremple and High 1998). SFC separation using methanol modified $CO_2$ and a packed silica column gave good resolution and quantitative analysis for some PMFs in citrus oils (Morin *et al.* 1991; Dugo *et al.* 1996). Many reports have been published on the determination of PMFs by HPLC on the normal phase mode or the reverse phased (RP) mode. Hyphenated HPLC-MS and HPLC-NMR (nuclear magnetic resonance) have also been used in the PMF structure elucidation.

However, a validated analytical method for the quantitative analysis of PMFs did not come out until this year (Wang *et al.* 2008). The research findings provide a very detailed, validated quantitative method involving the use of a reversed phase HPLC system. It is the first report on the development of a useful, simple and reliable analytical method focused on the analysis of the six major PMFs, namely sinensetin, 3,5,6,7,3',4'-hexamethoxyflavone, nobiletin, 5,6,7,4'-tetramethoxyflavone, 3,5,6,7,8,3',4'-heptamethoxyflavone, and tangeretin. It is also necessary and significant to have a validated methodology in PMF analysis, since presently, little is known about the correlation between the bioactivity and the individual PMF contents in the citrus genus extract, due to the disconnection between the biological testing and a quantitative PMF analysis. In this study, a validated LC method with UV detection was developed for the quantitative analysis of the six major PMFs. The method was successfully utilized for the direct quantitation of each PMFs in four commercial orange peel extract (OPE) products (**Table 7**). This is the first validated study on the simultaneous quantitative analysis of six PMFs in OPEs using liquid chromatography (LC). Using a polar embedded reverse phase column, this LC method has been successfully developed to separate six major PMFs in citrus genus. The analytical method was validated in linear range, LOD (limit of detection), LOQ (limit of quantitation), repeatability,

**Table 7** Analysis of six PMFs in OPEs from different OPE manufactures.

| PMFs | Weight percentage (%) | | | |
|---|---|---|---|---|
| | Extract A | Extract B | Extract C | Extract D |
| Sinensetin | 3.9 | 3.2 | 1.4 | 1.0 |
| Hexamethoxyflavone | 4.1 | 3.7 | 0.4 | - |
| Nobiletin | 26.1 | 24.4 | 33.2 | 10.5 |
| Tetramethoxyflavone | 11.4 | 12.5 | 0.6 | 0.4 |
| Heptamethoxyflavone | 31.2 | 20.7 | 62.3 | - |
| Tangeretin | 15.4 | 18.1 | 2.8 | 8.2 |
| Total% of PMFs | 92.1 | 82.6 | 100.7 | 20.1 |

**Table 8** Comparison of selectivity and resolution on LC and SFC with non-chiral columns.

| Stationary phase | Selectivity (α) | | Resolution (Rs) | |
|---|---|---|---|---|
| | LC | SFC | LC | SFC |
| Cyano | 1.0 | 1.0 | 0 | 0 |
| Diol | 1.06 | 1.07 | 1.11 | 1.99 |
| Pyridine | 1.05 | 1.07 | 1.10 | 2.16 |
| Silica | 1.05 | 1.18 | 1.06 | 2.20 |

**Table 9** Comparison of selectivity and resolution on LC and SFC with chiral columns.

| Stationary phase | Selectivity (α) | | Resolution (Rs) | |
|---|---|---|---|---|
| | LC | SFC | LC | SFC |
| AD | 1.40 | 3.05 | 1.70 | 22.5 |
| OD | 1.0 | 1.51 | 0 | 7.56 |
| AS | 1.0 | 1.15 | 0 | 1.09 |
| OJ | 1.31 | 1.45 | 6.20 | 6.29 |

intermediate precision, and system suitability. For instance, in this study, with a 5 μL injection volume, LOD was 0.15 μg/mL and LOQ was 0.5 μg/mL for all standard PMFs. This method allows for a simple, accurate, and precise determination of PMFs in OPEs. In addition, this LC method can easily be utilized as an analytical procedure for quality control (QC) of PMFs and OPE production (Wang *et al.* 2008). Big discrepancies have been found between the manufacture claimed PMF content and the tested contents from our testing, in terms of both individual PMFs percentage and total amount of PMFs in the OPEs. Therefore, the development of this analytical method is of crucial significance.

With a chiral packed column, we have successfully developed an SFC (supercritical fluid chromatography) method to separate nobiletin metabolites - two chemical regioisomers of hydroxylated PMFs (Wang *et al.* 2006). The result was also compared with high performance liquid chromatography (HPLC) under normal phase mode by using chiral and non-chiral supporting. The separation of 3'-desmethylnobiletin and 4'-desmethylnobiletin mixture (by mixing synthesized standards) was trialed on reversed phase liquid chromatography-mass spectrometry initially and two compounds were co-eluted. Additionally, both display same mass spectrometric fragmentation resulting in difficulties for metabolite identification in metabolism study. Four non-chiral columns, cyano, diol, pyridine and silica, were tested on both normal phase liquid chromatography and supercritical fluid chromatography. For LC, among the four columns, diol, pyridine and silica columns gave partial separations to the compounds. No separation was obtained on a cyano column (**Table 8**). At the same mobile phase condition (40% hexane and 60% ethanol), the retention times of the analytes of these four columns are all around 5-6 minutes. For silica, diol and cyano columns, the dominant interaction between stationary phase and analytes is hydrogen bonding. For pyridine column, both hydrogen bonding and π-π interaction have effects. However, the structures of these two compounds are too similar to be fully resolved based on the difference in molecular interaction. It is also widely known that SFC is a normal phase separation technique. Polar organic solvent, such as methanol, is usually used as modifier of $CO_2$ to enhance the polarity of the mobile phase. A condensed modifier layer is adsorbed on the stationary phase and as a result, the mobile phase is almost always less polar than the modified stationary phase (Berger 1995). As most used modifier, 20% methanol was used with supercritical $CO_2$ and same columns were tested on SFC. Similar results were observed. Still no separation was obtained on the cyano column, but fully baseline separations were achieved on diol, pyridine and silica columns. The selectivity and resolution of the separations on LC and SFC were compared and in terms of selectivity, there was no significant difference between the results from LC and SFC (**Table 8**). For the mobile phases, the different physical properties of organic solvent (in LC) and modified supercritical $CO_2$ (in SFC) have little influence on selectivity. The improved resolution in SFC separation is due to the higher separation efficiency (higher theoretical plates) of this separation technique. Supercritical $CO_2$ has higher diffusivity and lower viscosity, allows for fast separations at higher flow rate and more efficient mass transfer. On LC, only AD and OJ columns gave separations with the selectivity of 1.40 and 1.31, respectively. No separation was obtained on OD and AS columns (**Table 9**). As the comparison, SFC achieved much better results. AD column gave the best separation with the resolution of 22.5 (selectivity of 3.05), and the elution time difference between the two pair up to 10 minutes. Except for the partial separation on AS column, OD and OJ also gave good separations on SFC with the selectivity of 1.51 and 1.45, respectively (**Table 9**).

A more symmetric peak shape was also noticed during the SFC separation. The two metabolites have a hydroxyl group on position 3'- and 4'-position on the B-ring of PMF skeleton, respectively, which are able to donate a proton, and as the result, the compounds behave as weak acids. On the normal phase LC with neutral mobile phase (hexane/ethanol mixture), the protonated analytes may have secondary interaction with the uncovered silanol groups on the silica gel (the supporting material of the stationary phase), and tailing peaks were observed on all of the four columns. As the SFC modifier, methanol may contain low percentage of water, and the reaction of $CO_2$ with water yield carbonic acid. Therefore, in supercritical $CO_2$ chromatography, the analytes are separated under acidic environments. Low pH suppressed the ionization of the analytes, avoided the secondary interaction occurred. Therefore, the peaks resolved on SFC were all symmetric. The nobiletin metabolites in mouse urine were then profiled under optimized SFC condition. Based on the optimized SFC method, 3′-desmethylnobiletin was identified as the major hydroxylated metabolite of nobiletin in the mice urine.

## Isolation of polymethoxyflavones from citrus peels

The biological activity screening of PMFs has been mainly performed *in vitro*, which consumes only small amount (milligram scale) of materials. The efficacious study, pharmacokinetics and thermodynamics, and clinical trials of PMFs, especially with interested individual PMF molecules, have been rarely performed and also limited to use a mixture of extracts from citrus plants to the most extent. The bottle neck to advance to more complicated biological study with pure PMF substances is the dearth of PMF supply in large quantity and single pure compounds. Some PMFs are commercially available, but the cost is too high to perform an efficacy study. For instance, 3,5,6,7,8,3′,4′-heptamethoxyflavone from citrus peels has been reported to exhibit potent anti-tumor activity and to be a chemopreventive agent against nitric oxide carcinogenesis (Iwase *et al.* 2000, 2001). However, the high cost ($300/mg) of 3,5,6,7,8,3′,4′-heptamethoxyflavone limited more thorough *in vitro* investigation and efficacy study, because in animals of lower species (mice, rats, etc.) may easily consume grams of 3,5,6,7,8,3′,4′-heptamethoxyflavone at a price of $300,000/g. Additionally, billions of dollars are required for clinical trial studies with the same compound, which is not feasible in the nutraceutical and pharmaceutical industries. Therefore, it is necessary and urgent to develop an efficient method for large scale separation of PMFs, to dramatically reduce the cost of pure PMF and to remove the bottle-neck in the discovery of PMF as novel nutraceuticals and potential pharmaceuticals.

The isolation and purification of PMFs has been rarely investigated until recently. A separation method of PMFs using high speed counter-current chromatography was reported (Wang *et al.* 2005). Although this method was able to isolate some PMFs in multi-milligram quantities, it is laborious and time-consuming, which consequently limited its scalability and application in larger scale separations.

**Table 10** Comparison of different methods for the purification of citrus PMFs.

| Chromatography method | Throughput (g/h) | Purity (%) | Reference |
|---|---|---|---|
| HSCCC | 0.03 | 98-95 | Wang *et al.* 2005 |
| SFC | 0.8 | 99-100 | Li S *et al.* 2007a |
| HPCCC | 4 | 97-99 | Li S *et al.* 2008 |
| Chiral HPLC | 0.2-1 | 99-100 | Li S *et al.* 2006a |

The first reported large scale separation of PMFs is the nobiletin isolation (Li S *et al.* 2006a). The cold pressed orange peel was first passed a silica gel column to remove orange peel oils and to divide the PMFs into six groups in which the group V, mainly containing nobiletin and 5,6,7,4′-tetramethoxyflavone, was loaded onto a Welk-O chiral column (Regis Welk-O 1 R,R 450 gram column). Using ethanol and hexanes as eluting solvents, from one preparation, more than 2 grams of nobiletin was obtained in 45 minutes. The application of this chiral preparative HPLC method not only opened a new era of nobiletin research in both *in vitro* and *in vivo* studies, because of the availability of PMFs in large amount, but also widened the application of chiral column in PMF isolation. This was the first application of chiral chromatography to the isolation of PMFs, which solved the difficult separation problem of PMFs due to their similarities in chemical structures and physical properties. More general separation method of large quantities of PMFs using SFC technology has been recently developed (Li S *et al.* 2007a). During the course of isolation and biological activity studies of PMFs from sweet orange peel, by screening various separation methods, such as normal phase chromatography (silica gel, diol, cyano and amine columns), C18 reverse phase HPLC, chiral HPLC and SFC separation techniques, we developed an efficient and scalable SFC method for the large scale separation of four common PMFs: nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone. With potential application to becoming a common technology in large scale isolation, this SFC technology has many advantages over the other separation methods in cost effectiveness, time efficiency and full automation. This is the first reported SFC application in preparative separation of PMFs. It is of significance because it has not only provided an efficient and large scale preparation of PMFs, but also explored a new application of the SFC technology in the field of PMF research. Most recently, we have also developed a more efficient method for isolating individual PMFs from crude sweet orange peel extract (Li S *et al.* 2008). By using the high performance counter current chromatography (HPCCC) technology, we were able to obtain six pure PMFs and one of 5-hydroxylated polymethoxyflavone in a single separation in our analytical HPCCC system. This method can be easily scaled up in our preparative HPCCC systems for the quick isolation of PMFs in gram quantity. **Table 10** presents a comparison of different methodologies employed in the isolation of PMFs from OPE (Li S *et al.* 2008).

## CONCLUDING REMARKS

In summary, polymethoxyflavonoids from Citrus genus have potent biological activities in anti-cancer, anti-inflammation and anti- atherosclerosis. They have good permeability and bioavailability. The metabolites of PMFs are mainly mono- and di-hydroxylated polymethoxyflavones. A validated analytical liquid chromatography method has been developed and applied in the quality control of citrus peel extract from various sources. The technology for isolation and purification of the PMFs has advanced at a rather rapid pace, which consequently will facilitate the discovery of PMFs as neutraceuticals and/or pharmaceuticals from citrus.

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