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Anti-inflammatory Activity of an Orange Peel Polymethoxylated Flavone, 3′,4′,3,5,6,7,8-Heptamethoxyflavone, in the Rat Carrageenan/Paw Edema and Mouse Lipopolysaccharide-Challenge Assays

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The anti-inflammatory properties of 3′,4′,3,5,6,7,8-heptamethoxyflavone (HMF), a citrus polymethoxylated flavone, were studied in the bacterial lipopolysaccharide (LPS)-challenge/tumor necrosis factor-α (TNFα) response in mice and in the carrageenan/paw edema assay in rats. In each of these trials, HMF administered by intraperitoneal (ip) injection exhibited anti-inflammatory activity, whereas HMF administered orally (po) produced no effects. The inhibition observed in the LPS-challenge/TNFα assay correlated with the HMF levels in the blood sera of mice dosed (ip) with either 33 or 100 mg/kg body weight. Low levels of HMF (0.035 ± 0.024 ppm) were detected in the blood sera of mice dosed orally [100 mg of HMF (suspended in vegetable oil)/kg], whereas ip injection led to higher levels (0.517 ± 0.051 ppm). This may account for the different levels of anti-inflammatory effects observed in mice following ip vs oral HMF administration. HMF metabolites, including a number of mono- and di-demethylated HMF metabolites and their glucuronic acid conjugates, were also detected, but results of these studies suggest that the glucuronidated metabolites of HMF are inactive in these inflammation models.

KEYWORDS: Flavonoids; polymethoxylated flavones; citrus; inflammation; cytokines; tumor necrosis factor-α; carrageenan; bacterial lipopolysaccharides

INTRODUCTION

Flavonoids in citrus have been widely studied for their potential anti-inflammatory actions and their effects on capillary permeability in mammals. Most thoroughly studied have been the flavonoid glycosides, diosmin and hesperidin, which block inflammation at several sites of action (*1, 2* and references therein). In addition to these compounds, the polymethoxylated flavone (PMF) aglycones represent another class of potentially active citrus flavonoids (*3*). The PMFs were previously observed to inhibit the production of certain pro-inflammatory protein cytokines by bacterial lipopolysaccharide (LPS)-stimulated human monocytes (*4*), and this action was shown to be linked to the inhibition of the phosphodiesterase-4 enzymatic activity in the activated human monocytes. This phosphodiesterase inhibition was similar to that reported earlier for other methoxylated flavones (*5*−*7*). Among the PMFs, the molecule 3′,4′,3,5,6,7,8-heptamethoxyflavone (HMF) was the most potent inhibitor of the monocyte phosphodiesterase-4 and the LPS-stimulated cytokine production. Yet, while these findings suggest that the citrus PMFs might be anti-inflammatory, there has been to date no thorough evaluation of their anti-inflammatory actions in vivo. The objective of this study was to ascertain the effects of both oral (po) and intraperitoneal (ip) administration of HMF in two preclinical inflammation trials.

MATERIALS AND METHODS

HMF Isolation. A HMF-containing PMF fraction was prepared from orange oil nonvolatile residues as previously described (*8*). PMF fractionation by silica gel column chromatography [Michel-Miller prepacked column, 170 g (Ace Glass, Vineland, NJ)] was achieved using linear gradients of hexane/ethyl acetate (4 L total), progressing from 1:3 to 2:1 (v/v). HMF in the column fractions was detected by analytical silica gel thin-layer chromatography (TLC) (*9*). Final HMF purification was accomplished using a RediSep reversed phase C18 (43 g) column (ISCO, Lincoln, NE) and an ISCO CombiFlash 100 chromatography system. Elution conditions included a two-solvent gradient composed initially of water/methanol (80/20, v/v) and increased in methanol content with linear gradients to 60/40 (v/v), then to 40/60 (v/v), and finally to 20/80 (v/v) over 2.5 h intervals at a flow rate of 10

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Table 1. Serum TNF-α Levels (pg/mL) Following LPS Challenge in Mice with HMF/VO Solution (ip) Administration[a]

treatment	serum TNF-α levels (pg/mL)	% inhibition
VO vehicle	6167 ± 182	0
50% PEG 400 vehicle	3774 ± 152	39[b]/0[c]
naringin (100 mg/kg)	4168 ± 239	−13[c]
dexmethasone (0.5 mg/kg)	2112 ± 50	66[b]/42[c,d,e]
HMF (33 mg/kg)	4313 ± 144	30[b,e]
HMF (100 mg/kg)	3411 ± 86	45[b,e]

[a] Values are averages ± standard deviations. The statistical difference ($p \leq 0.05$) was determined by Student's *t* test. [b] Relative to VO vehicle. [c] Relative to 50% PEG 400 vehicle. [d] Indicates different from 50% PEG 400 vehicle ($p \leq 0.05$). [e] Indicates different from VO vehicle ($p \leq 0.05$).

Table 2. Serum TNF-α Levels (pg/mL) following LPS Challenge in Mice with HMF/VO Suspension (po) and (ip) Administration[a]

group	treatment	serum TNF-α levels (pg/mL)	*p* vs vehicle
1	VO vehicle minus LPS	70.3 ± 35	
2	VO vehicle plus LPS	1920 ± 678	
3	prednisolone (10 mg/kg (op) + LPS	617 ± 143	0.001
4	HMF [50 mg/kg (op)] + LPS	2399 ± 843	0.232
5	HMF [100 mg/kg (op)] + LPS	2286 ± 653	0.290
6	HMF [200 mg/kg (op)] + LPS	2203 ± 793	0.456
7	VO vehicle (ip) + LPS	2079 ± 660	
8	prednisolone [10 mg/kg (ip)] + LPS	656 ± 177	0.0004
9	HMF [100 mg/kg (ip)] + LPS	1850 ± 762	0.53

[a] Values are averages ± standard deviations. The statistical difference (*p*) was determined by Student's *t* test.

mL min^{-1}. The purity of the HMF was established by analytical silica gel TLC (*9*) and high-performance liquid chromatography−mass spectrometry (HPLC-MS) analysis (*10*).

LPS Challenge in Mice with HMF ip Injection. HMF was dissolved in heated Wesson vegetable oil (VO) 80 °C to give doses of 33 and 100 mg HMF/kg body weight in dosing volumes of 10 mL/kg body weight. Naringin was dissolved in 50% polyethylene glycol 400 (PEG 400) (Fisher Scientific, Fair Lawn, NJ) in saline to give 100 mg naringin/kg body weight in 10 mL/kg. All dosing solutions were allowed to cool to room temperature prior to ip injection. Dexamethasone was combined with vehicles (50% PEG 400 in saline or VO) to create a 0.05 mg/kg body weight dosing solution in 0.1 mL. One hour before LPS injections, male Swiss Webster mice (5−6 weeks of age) (seven/group) were dosed ip with either HMF (33 mg/kg or 100 mg/kg), naringin (100 mg/kg), dexamethasone (0.5 mg/kg), or vehicle (100% VO or 50% PEG 400 in saline). Ninety minutes post LPS injection, mice were anaesthetized and bled by cardiac puncture. The collected blood was processed to serum and divided for tumor necrosis factor (TNF)-α measurements and metabolite analysis. The serum samples were assayed for TNF-α concentration by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

LPS Challenge in Mice with HMF Oral Administration. Prednisolone suspension in Wesson VO was prepared by homogenizing 10 mg of prednisolone in 10 mL of VO. HMF suspensions in VO were prepared at 5, 10, and 20 mg/mL. For 2 days prior to LPS challenge, animals [male Swiss Webster mice (5−6 weeks)] were orally dosed with their respective test materials to achieve 50, 100, and 200 mg HMF/kg body weight administrations. On the third day, mice in groups 1−6 (**Table 2**) were orally dosed with their respective test materials. Mice in groups 7−9 (**Table 2**) were injected with their respective test material solutions (0.1 mg/mL). At 1 h following dosing, mice were administered with LPS [10 mg/kg (ip)]. At 1.5 h post LPS challenge (2.5 h postdose), the mice were anesthetized and exsanguinated. The collected blood was processed to serum and divided for TNF-α measurements and metabolite analysis. The serum samples were assayed for TNF-α concentration by ELISA. All samples in groups 2−9 were initially diluted 1:2 into distilled water. All samples, standards, and internal controls were assayed in duplicate.

Carrageenan-Induced Paw Edema. The carrageenan-induced paw edema assay was a modification of that described by Winter et al. (*11*). Male Sprague−Dawley rats (284−312 g) were injected with caraggenan (right hind foot pad) and were evaluated for effects on inflammation edema by oral treatments of water, VO, and HMF at either 50 or 100 mg/kg (solubilized in VO). Injections (ip) of VO or HMF (100 mg/kg in VO) were also conducted, as were subcutaneous (sc) treatments of indomethacin [4 mg/kg (5% sodium bicarbonate)], 50% PEG 400 in saline, and hesperidin (100 mg/kg in 50% PEG 400 in saline). HMF was dissolved as a 10 or 20 mg/mL solution in VO for dosing at 5 mL/kg. All right paws were callipered at baseline, 2, and 4 h post-carrageenan injection. Efficacy evaluation was based on weight difference due to inflammation-induced swelling in injected (right) vs uninjected (left) paws. Four and a half hours after carrageenan injection, after CO_2 euthanasia, the hind paws were transected at the level of the malleoli and weighed, and differences between the injected and noninjected and the treated vs nontreated rats were determined. Experiments with live animals conformed to the standard operating procedures of the University of Colorado vivarium. The basic study design and animal usage were approved by Bolder BioPath's Institutional Animal Care and Use Committee (IACUC) for compliance with regulations prior to study initiation.

HPLC-MS Analysis of HMF Metabolites. The metabolites of HMF were analyzed by HPLC-MS as reported previously by Kurowska and Manthey (*12*), with minor modifications. To enhance the detection of the trace levels of metabolites in the blood sera, single ion response (SIR) was used as the detection method of the positive electrospray ionization-MS. Sera from treated mice were prepared and stored at −80 °C. Thawed serum (100 μL) was mixed with 600 μL of methanol containing 15 ppm hesperetin (3′,5,7-trihydroxy-4′-methoxyflavone) as an internal standard. HPLC separations of the HMF metabolites were achieved using a Discovery RP Amide C16 column (15 cm × 3 mm i.d.) (Supelco, St. Louis, MO). Elution conditions included a three solvent gradient composed initially of water/acetonitrile/2% formic acid (85/10/5, v/v/v) and increased with linear gradients to 81/14/5 (v/v/v) over 15 min, then to 77/18/5 (v/v/v) by 20 min, then to 70/25/5 (v/v/v) by 30 min, then to 55/40/5 (v/v/v) by 55 min, then to 0/95/5 (v/v/v) by 67 min with a flow rate of 0.75 mL/min. Instrumentation included a Waters 2695 Alliance HPLC coupled with a Waters 996 PDA and Waters ZQ single quadrupole MS detectors. Data handling was done with MassLynx software version 3.5 (Micromass, Division of Waters Corp., Beverly, MA).

Statistical Analysis. Statistical analyses of right paw weight and paw weight differences were done by comparing groups with the Student's *t* test with significance set at $p \leq 0.05$. Statistical analyses of TNF-α production differences were done by comparing groups with the Student's *t* test with significance set at $p \leq 0.05$.

RESULTS

LPS Challenge in Mice with HMF ip Injection. LPS challenge in mice induced rapid TNF-α production by circulating blood monocytes in a response similar to that of the cultured human monocytes (*4*). HMF, dissolved in VO, administered at 33 and 100 mg/kg (ip), significantly inhibited the LPS-induced TNF-α production in Swiss Webster mice by 30 and 45%, respectively, as compared to the VO vehicle group (**Table 1**). Treatment with dexamethasone (0.5 mg/kg) resulted in 66% inhibition as compared to the VO vehicle group and 42% as compared to the 50% PEG 400 in saline group. Treatment with naringin at 100 mg/kg (ip) provided no inhibition of TNF-α production as compared to the 50% PEG 400 in saline group.

LPS Challenge in Mice with HMF Oral Administration. In a second trial, HMF was administered both po and ip as a suspension in VO, in contrast to the initial trial where the HMF was dissolved in the VO. In this second trial, the TNF-α induction response was evaluated in LPS-challenged mice previously given three daily oral doses of the HMF/VO

Table 3. Right vs Left Paw Weight Differences (g) Following Carrageenan Injection in Rats with HMF/VO Solution (po) and (ip) Injection[a]

group	mean ± SE	% inhibition
1. water (po)	1.35 ± 0.07	0
2. VO (po)	0.94 ± 0.06	30[b]
3. HMF (in VO) [50 mg/kg (po)]	1.11 ± 0.07	18[b]
4. HMF (in VO) [100 mg/kg (po)]	1.07 ± 0.07	21[b]
5. VO (ip)	1.14 ± 0.10	16
6. HMF (in VO) [100 g/kg (ip)]	0.60 ± 0.06	56[b,c]
7. indomethacin [4 mg/kg (sc)]	0.92 ± 0.06	32[b,c]
8. hesperidin (in 50% PEG 400 + 50% saline) (100 mg/kg)	1.04 ± 0.5	23[b]
9. vehicle (50% PEG 400 + 50% saline)	0.95 ± 0.06	29[b]

[a] The statistical difference ($p \leq 0.05$) was determined by Student's t test. [b] Indicates different from water (po) vehicle ($p \leq 0.05$). [c] Indicates different from VO (ip) vehicle ($p \leq 0.05$).

suspension. The last dose was administered 1 h prior to the LPS challenge. Daily doses given were 50, 100, and 200 mg HMF/kg body weight. As shown in **Table 2**, no inhibition of the serum TNF-α production occurred following any of these oral doses (groups 4−6) relative to the VO vehicle control (group 2). In fact, the oral dose of HMF (200 mg/kg) increased the serum TNF-α levels by nearly 19% ($p = 0.456$). The positive control prednisolone (10 mg/kg; group 3) decreased TNF-α production by 68% (617 vs 1920 pg/kg). The ip administration of 100 mg HMF (suspended in VO)/kg to LPS-challenged mice (group 9) decreased the induced serum TNF-α levels by a nonsignificant ($p = 0.53$) 11% (1850 pg/mL) relative to the ip control (group 7) (2079 pg/mL). The positive control (group 8) of prednisolone [10 mg/kg (ip)] gave nearly identical inhibition as prednisolone administered orally (group 3).

Carrageenan-Induced Paw Edema with HMF Oral and ip Administration. The results of the carrageenan-induced paw edema assays with HMF administration (ip) and (po) are listed in **Table 3**. Treatment with HMF, 50 mg/kg (dissolved in VO) po (group 3), or HMF 100 mg/kg (group 4) produced less effect (18 and 21%, respectively) on weight differences and ankle swelling than the VO (po) control treatment (30%) (group 2), relative to the water treatment (0%) (group 1). Injection of VO vehicle (group 5) nonsignificantly decreased the difference in the weight between the right and the left paws (16%) as compared to the water treated animals. In contrast, ip treatment with HMF (group 6) at 100 mg/kg (in VO) significantly decreased the difference in weight between the right and the left paws (56%). Treatment with HMF 100 mg/kg (ip) also resulted in significant inhibition (12%) of ankle caliper measurements (AUC) as compared to the water treatment group and significant inhibition (7%) of ankle caliper measurements (AUC) as compared to VO ip-treated rats (data not shown).

Carrageenan-Induced Paw Edema with sc Administration of Hesperidin. Treatment with indomethacin (group 7) at 4 mg/kg, sc, significantly decreased the difference in weight between the right and the left paws (32%) and the weight of the right paw (14%) as compared to the water-treated control rats. Treatment with indomethacin also resulted in significant inhibition (9%) of ankle caliper measurements (AUC) (data not shown). However, treatment with hesperidin (100 mg/kg in 50% PEG 400) (sc) produced no significant difference in the inflammation parameters relative to the treatments with the 50% PEG 400 in saline vehicle alone (group 9).

Metabolite Study. The anti-inflammatory responses produced by HMF in the LPS-challenged mice were evaluated relative to the abundance of HMF and HMF metabolites in the blood sera of the treated mice. HMF was monitored 433 *m*/*z*, the

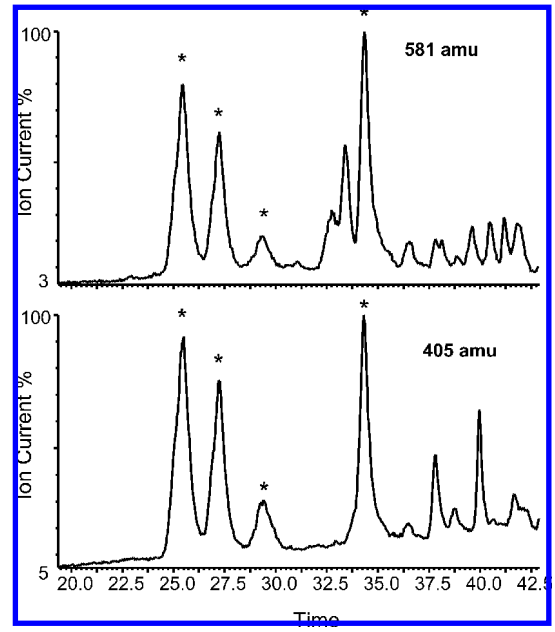

Figure 1. SIR total ion chromatograms (TICs) monitored at 581 and 405 amu, selected for the detection of the glucuronide conjugates (upper panel) of the di-demethylated HMF metabolites (lower panel), respectively. The peaks marked with an asterisk designate possible di-demethylated HMF metabolites that occurred as glucuronic acid conjugates (581 amu).

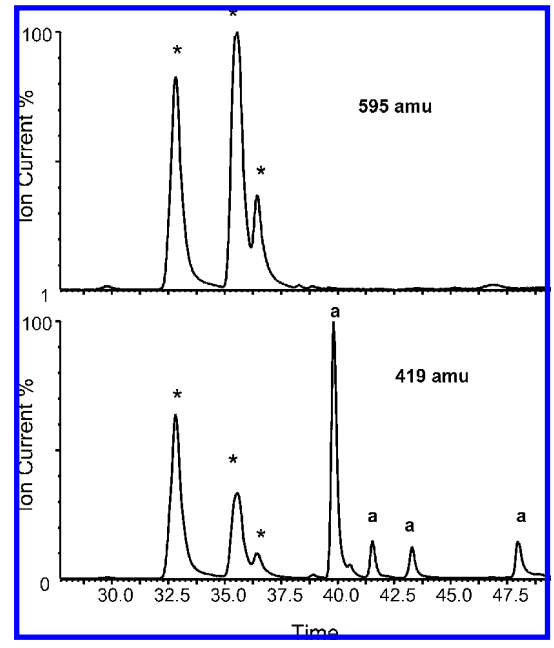

Figure 2. SIR TICs monitored at 595 and 419 *m*/*z*, selected for the detection of the glucuronide conjugates (upper panel) of the monodemethylated HMF metabolites (lower panel), respectively. The peaks marked with an asterisk designate possible monodemethylated HMF metabolites that occurred as glucuronic acid conjugates (595 amu). The four remaining peaks eluting after 38 min, designated with a, are likely monodemethylated aglycones.

protonated molecular ion $(M + H)^{+1}$ of HMF, while two other *m*/*z* values were also monitored corresponding to the protonated molecular ions for monodemethylated HMF (419 amu) and didemethylated HMF (405 amu) (**Figures 1** and **2**). Most of the major metabolites detected at 419 and 405 *m*/*z* were also detected at 595 and 581 *m*/*z*, respectively, corresponding to glucuronic acid conjugates. These compounds were absent in blood sera of the control animals (data not shown).

Table 4. Blood Serum Concentrations (μg/g) of HMF and the Main HMF Metabolites Detected at 433 and 419 and 405 *m/z*, Respectively, in Mice Administered 33 and 100 mg/kg HMF Dissolved in VO[a]

compound/elution time (min)	100 mg/kg (ip)	33 mg/kg (ip)
	HMF	
	14.20 ± 4.66	8.62 ± 1.56
	419 *m/z*	
32.8	0.866 ± 0.402	0.195 ± 0.017
35.5	0.344 ± 0.155	0.080 ± 0.040
36.4	0.350 ± 0.185	0.084 ± 0.045
39.8	0.529 ± 0.240	0.138 ± 0.057
41.5	0.109 ± 0.056	0.054 ± 0.053
43.2	0.067 ± 0.033	0.042 ± 0.007
	405 *m/z*	
25.5	0.104 ± 0.054	0.024 ± 0.012
27.2	0.031 ± 0.011	0.009 ± 0.004
29.4	0.039 ± 0.024	0.011 ± 0.005
34.3	0.047 ± 0.020	0.030 ± 0.023

[a] Blood samples were taken 2.5 h post HMF administration. Calculations of metabolite concentrations were made using the conversion factor (MS SIR peak area/ng) of a HMF standard.

For the LPS-challenged mice, administered with 33 and 100 mg/kg (ip) HMF (dissolved in VO), levels of the unmodified HMF in the blood serum were 8.62 ± 1.56 and 14.20 ± 4.66 ppm, respectively (**Table 4**). In the same way, most of the metabolites detected at 405 and 419 *m/z* were significantly higher in the animals treated with 100 mg of HMF (ip) as compared to the animals treated with 33 mg/kg (ip) (**Table 4**).

In the second LPS-challenge mouse study, HMF administered as a suspension in VO (po) produced only trace levels of unmodified HMF in the blood serum (0.035 ± 0.024 ppm for 100 mg HMF/kg and 0.048 ± 0.018 ppm for 200 mg HMF/kg). HMF at 100 mg/kg (ip), as a suspension in VO, produced nearly 10 times higher levels of HMF (0.517 ± 0.051 ppm) in the sera of the treated mice. This latter value is still only 4% of the level in the mice administered HMF (ip) dissolved in VO (see above).

In the study where HMF was administered (ip and po) as a suspension in VO, HMF metabolites were detected that were similar to those in the prior study shown in **Figures 1** and **2** (data not shown). Consistent with the low concentration of the unmodified HMF in the sera of these treated animals, levels of the main HMF metabolites were 10−15 times lower than in the mice administered HMF dissolved in VO (data not shown).

DISCUSSION

The results of this study demonstrate the in vivo anti-inflammatory properties of the citrus flavonoid, HMF, in two inflammation models. Administration of HMF (dissolved in VO) by injection (33 and 100 mg HMF/kg) (ip) produced significant dose-dependent inhibition of the TNF-α production in LPS-challenged mice. A single dose (100 mg/kg) (ip) of HMF dissolved in VO also produced 56% inhibition of the carrageenan-induced paw edema in rats. The HMF (ip) administration at 33 and 100 mg/kg produced 8.6 ± 1.56 and 14.2 ± 4.66 ppm HMF in the mice blood sera, respectively. No measurements were taken of the HMF levels following the treatments in the carrageenan-induced paw edema study. In the follow-up study comparing oral and ip administrations of HMF to LPS-challenged mice, the HMF was administered as a suspension in VO, rather than as the homogeneous solution in the first trials. Although the two HMF sample preparations (suspended in VO vs dissolved) were conducted separately with different sets of animals, the results indicate that the injection of the HMF/VO suspension (ip) produced far lower HMF levels (0.57 ppm) in the blood sera of these mice as compared to the mice administered (ip) HMF dissolved in VO. This sharply lower blood serum HMF concentration is a possible explanation for the nonsignificant (11%) (**Table 2**) inhibition of the TNF-α production in the mice administered the HMF/VO suspension.

Oral administration of HMF in either of the inflammation studies produced no anti-inflammatory effects. The administration of HMF suspended in VO (100 mg HMF/Kg) (po) produced only trace levels of HMF (0.02 ppm) in the blood sera of the treated mice, and these resulting levels were too low for efficacy. The absence of anti-inflammatory actions occurred in spite of the abundance of glucuronidated HMF metabolites. These findings are supported by the carrageenan-induced paw edema trial, where HMF administered by ip injection produced large anti-inflammatory effects, while the orally administered HMF produced no effect. Thus, it is likely that the active compound is the free, unmodified HMF and not the glucuronidated metabolites.

In each of the studies, the flavonoid glycosides, hesperidin or naringin, were included as positive controls based on previous reports. Kawaguchi et al. (*13*) showed that (ip) treatment of mice with naringin 3 h prior to LPS challenge significantly reduced, in a dose-dependent manner, the rise in serum TNF-α levels and the accompanying liver damage due to the LPS challenge. More recently, it was demonstrated that naringin (dissolved in 0.5% Tween 80 in saline) administered at 10, 30, and 60 mg/kg (ip) 1 h prior to LPS challenge in mice dose dependently suppressed TNF-α production and, similarly, increased mouse survival rates (*14*). Naringin, administered 1 h prior to the LPS challenge, also significantly inhibited TNF-α production. Yet, in our studies, naringin injection at 100 mg/kg (ip) 1 h prior to LPS challenge produced no inhibition of the LPS-induced TNF-α production (**Table 1**). One relevant observation is that the 50% PEG 400 vehicle used in our study suppressed the LPS-induced TNF-α production by 39% relative to the VO control. No further inhibition was observed with 50% PEG 400 with naringin, but the suppression by the vehicle may have masked any possible effects expressed by naringin. A similar lack of anti-inflammatory activity was observed for hesperidin at 100 mg/kg (sc) 1 h prior to carrageenan injection into rat paws. This is in contrast to the inhibition of carrageenan-induced edema formation in rats administered hesperidin at 50 and 100 mg/kg (sc) by 47 and 63%, respectively, in an early study (*15*). Two more recent studies have confirmed anti-inflammatory properties of hesperidin in LPS-challenged mice. Kawaguchi et al. (*16*) reported that hesperidin (suspended in saline) administered (ip) at doses of 0.3−3 mg/mouse 3 h prior LPS challenge significantly inhibited TNF-α production and increased infection-induced lethal shock survival rates of the treated mice. Oral administration of hesperidin at 200 mg/kg (suspended in saline) also downregulated TNF-α production in mice challenged with intratracheal LPS (*17*). Although the reasons for the discrepancies between these earlier studies and our present investigation are unclear, it is again possible that these contrasting findings may be the results of the use of different vehicles, in our cases, 50% PEG 400 in saline. Further differences may also occur due to differences in the dissolution, uptake, metabolism, or tissue accumulation of the flavonoids and their corresponding metabolites. Several of these parameters may be influenced by the choice of vehicle used to administer the flavonoids. In our current study, the presence of the metabolites of HMF is sharply influenced by differences in the routes of administration. Levels of serum HMF and HMF metabolites were dramatically higher in mice administered

HMF dissolved in VO than in mice administered HMF as a suspension in VO (**Table 4**). Factors influencing bioavailability obviously affect efficacy of the administered compounds and obviously, further in vivo studies are needed to better understand the parameters under which anti-inflammatory activities are exhibited by HMF and the other citrus flavonoids.

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