Pharmacokinetics, Bioavailability, Tissue Distribution, Excretion, and Metabolite Identification of Methoxyflavones in *Kaempferia parviflora* Extract in Rats

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ABSTRACT:

Kaempferia parviflora (KP) is an herbal plant in the family of Zingiberaceae. KP mainly contains methoxyflavones, especially 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF), and 3,5,7,3′,4′-pentamethoxyflavone (PMF). The present study was designed to characterize the pharmacokinetics, including bioavailability, distribution, excretion, and identification of metabolites after administration of a KP ethanolic extract. Male rats were orally or intravenously administered a 250 mg/kg concentration of a KP extract, and blood samples were obtained at selected times to determine pharmacokinetic parameters of PMF, TMF, and DMF. For distribution and excretion studies, the organs, urine, and feces samples were collected at various times after oral administration of a larger (750 mg/kg) dose of KP extract. Methoxyflavones in the biological samples were quantified by high-performance liquid chromatography-UV, and the metabolites in urine and feces were further identified by using liquid chromatography-tandem mass spectrometry. After oral administration, concentrations of the three methoxyflavones quickly approached their maximal concentration, ranging from 0.55 to 0.88 μg/ml within 1 to 2 h after administration, and then were gradually excreted with half-lives of 3 to 6 h. The methoxyflavones showed low oral bioavailability of 1 to 4%. Three methoxyflavones were detected at their highest levels in liver followed by kidney. They were also found in lung, testes, and brain. After absorption, organ distribution, and metabolism, the components of KP were mainly eliminated through urine in the forms of demethylated, sulfated, and glucuronidated products and as demethylated metabolites in the feces. The parent compounds were found to have 0.79, 1.76, and 3.10% dose recovery in urine and 1.06, 1.77, and 0.96% dose recovery in feces for PMF, TMF, and DMF, respectively. These studies are the first to describe the pharmacokinetics of KP extract to provide the information on blood and tissue levels.

Introduction

Kaempferia parviflora (KP), a medicinal herb in the Zingiberaceae family, is found in the upper Northeastern regions of Thailand. Its rhizome has been used in folk medicine reportedly for health promotion and stimulation. Several pharmacological responses to KP and its components have been claimed, including aphrodisiac (Sudwan et al., 2006; Wattanapitayakul et al., 2007; Chaturapanich et al., 2008; Wattanathorn et al., 2012), antipeptic ulcer (Rujjanawate et al., 2005),

anti-inflammatory (Tewtrakul and Subhadhirasakul, 2008; Sae-wong et al., 2009; Nakao et al., 2011), antiallergenic (Tewtrakul et al., 2008), antimutagenic (Azuma et al., 2011), antidepressive (Wattanathorn et al., 2007), antimicrobial (Yenjai et al., 2004, 2007; Kummee et al., 2008), anticholinesterase (Sawasdee et al., 2009), anticancer (Wen et al., 2005; Banjerdpongchai et al., 2008; Leardkamolkarn et al., 2009; Wudtiwai et al., 2011), cardioprotective (Tep-areenan et al., 2010; Malakul et al., 2011a,b), and antiobesity activity (Akase et al., 2011). In addition, inhibition of P-glycoprotein function and multidrug resistance-associated protein by KP have been demonstrated (Patanasethanont et al., 2007a,b). Components of KP can be found in traditional medicine products, such as pill and alcohol extract solution dosage forms. They are also available as tea and wine products.

Phytochemical investigations have shown that KP contains the following methoxyflavonoids (in decreasing order): 3,5,7,3′,4′-pentamethoxyflavone (PMF) > 5,7-dimethoxyflavone (DMF) > 5,7,4′-trimethoxyflavone (TMF) > 3,5,7,4′-tetramethoxyflavone > 5,7,3′,4′-tetramethoxyflavone > 5-hydroxy-7-methoxyflavone

ABBREVIATIONS: KP, *Kaempferia parviflora*; PMF, 3,5,7,3′,4′-pentamethoxyflavone; TMF, 5,7,4′-trimethoxyflavone; AUC, area under the blood concentration-time curve; DMF, 5,7-dimethoxyflavone; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; AUC, area under the curve; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation.

TABLE 1

Method validation of optimized HPLC systems

Flavonoids	Precision (%RSD)			LOD	LOQ	Linearity	Accuracy(% Error)	
	Conc.	Intraday	Interday				1	50
	$\mu g/ml$			$\mu g/ml$		R^2	$\mu g/ml$	
DMF	2	3.56	5.99	0.93	1.86	$y = 9.027x + 3.646$ ($R^2 = 0.996$)	6.12	0.43
	5	1.31	5.51					
	10	2.05	2.60					
	20	1.23	4.18					
	40	0.53	2.29					
PMF	2	3.85	5.00	0.19	0.37	$y = 20.771x + 11.987$ ($R^2 = 0.993$)	3.23	2.16
	5	0.86	3.66					
	10	1.39	6.38					
	20	1.40	7.47					
	40	0.32	3.55					
TMF	2	4.89	5.00	0.09	0.19	$y = 74.471x + 56.212$ ($R^2 = 0.993$)	2.80	2.49
	5	1.04	6.38					
	10	0.71	7.31					
	20	0.32	7.73					
	40	0.39	4.07					

Conc., concentration: RSD, relative S.D. (calculated from S.D. divided by mean and multiplied by 100).

>5-hydroxy-3,7-dimethoxyflavone > 5-hydroxy-7,4′-dimethoxyflavone >5-hydroxy-3,7,4′-trimethoxyflavone > 3,5,7-trimethoxyflavone >5-hydroxy-3,7,3′,4′-tetramethoxyflavone (Sutthanut et al., 2007). Acute toxicity studies in mice have demonstrated an LD_{50} value of KP of >13.3 g/kg, whereas chronic toxicity studies in rats treated with 1 g/kg KP for 6 months did not show any abnormalities upon histopathological examination of organs, behavioral assessments, physical examination, or body weight (Chivapat et al., 2004). Thus, on the basis of past efficacy and safety evaluations, KP has the potential to be developed as an alternative medicine from a natural source. However, basic pharmacokinetic data on the absorption, distribution, metabolism, and excretion of KP are still lacking. These data are important to establish recommended dosage regimens; to support drug labeling; to predict the possible accumulation of drug and/or metabolites in plasma, tissue, feces, and urine; to estimate the rate and the extent of drug absorption; and to determine how changes in physiology or disease affects pharmacokinetics of KP components. Thus, the purpose of this study was to investigate the pharmacokinetics of the major methoxyflavones in KP ethanolic extracts in rats and identify their metabolites.

Materials and Methods

Chemicals. Acetonitrile (LabScan, Bangkok, Thailand), formic acid (Thermo Fisher Scientific, Leicester, UK), heparin (Leo Pharmaceutical Products, Ballerup, Denmark), polyethylene glycol 400 (PEG400; S. Tong Chemical Co., Ltd., Bangkok, Thailand), and propylene glycol (Namsiang Co., Ltd., Bangkok, Thailand) were used in this study. All other chemicals were of analytical grade.

Preparation of KP Ethanolic Extract and Isolation of Methoxyflavones in KP. The ethanolic extract of KP rhizomes obtained from the 2007 harvest of the Romkaou strains in the Loei province of Thailand was prepared following our previously described procedure (Mekjaruskul et al., 2012), which provided a 5.71% yield. The methoxyflavones (PMF, TMF, and DMF) in KP were isolated using column chromatography as described previously (Sutthanut et al., 2007). The KP ethanolic extract contained 23.318 mg/g PMF, 31.056 mg/g TMF, and 21.103 mg/g DMF. The KP formulation was prepared by dissolving the KP extract in a mixture of propylene glycol (28%), polyethylene glycol 400 (35%), ethanol (2%), and deionized water (for adjusting to 100%).

Animals. Male Wistar rats were used at 6 to 8 weeks of age and 270 to 310 g body weight. Animals were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. They were housed at ambient temperature of 22 ± 2°C with 12-h light/dark cycles and were observed for 7 days before the experiment. The animals received standard rat food (C.P. rat feed 082; S.W.T. Co. Ltd, Samutprakan, Thailand) and water. The experimental protocols involving animals were reviewed and approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (approval numbers. AE.KKU.13/2552 and AE.KKU.43/2552) following internationally accepted principles for laboratory use and care of European community (EEC directive of 1986; 86/609/EEC).

Pharmacokinetics and Bioavailability. Rats were divided into two groups of 10 animals each and administered KP formulations (250 mg/kg) via tail vein
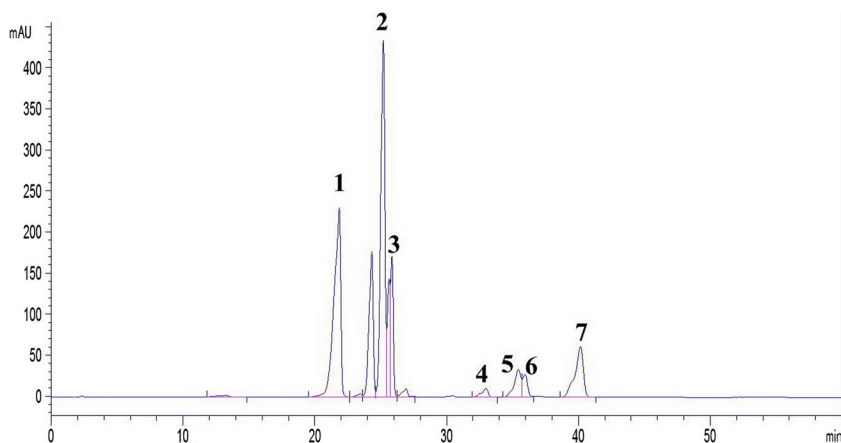

FIG. 1. HPLC-UV chromatogram of KP formulation (0.75 mg/ml; 1, PMF; 2, TMF; 3, DMF; 4, 5-OH-3,7,3′,4′-tetramethoxyflavone; 5, 5-OH-7-methoxyflavone; 6, 5-OH-3,7,4′-trimethoxyflavone; 7, 5-OH-3,7-dimethoxyflavone).
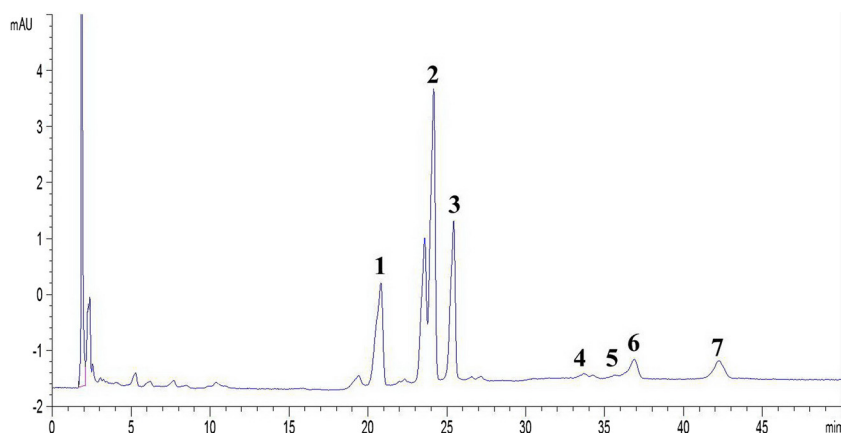
FIG. 2. HPLC-UV profile of the representative of rat blood after oral administration of 250 mg/kg KP (1, PMF; 2, TMF; 3, DMF; 4, 5-OH-3,7,3′,4′-tetramethoxyflavone; 5, 5-OH-7-methoxyflavone; 6, 5-OH-3,7,4′-trimethoxyflavone; 7, 5-OH-3,7-dimethoxyflavone).

injection or by oral gavage. The actual dose in both intravenous and oral routes calculated from the concentrations of three methoxyflavones in KP extract formulation was 9.44, 9.75, and 10.58 mg/kg for PMF, TMF, and DMF, respectively. For each group, blood samples (200 μl) were taken from the tail vein before dosing and at 10, 20, and 30 min and 1, 2, 6, 12, and 24 h after administration. Samples were collected in heparinized tubes and stored at $-20°C$.

Tissue Distribution and Excretion. Twenty-eight rats were orally administered the KP formulation (750 mg/kg) and subsequently sacrificed by cervical dislocation at 5 and 30 min and 2 or 4 h after administration (seven rats per group). Major organs, including liver, lung, kidney, testes, and brain, were removed and weighed. The organs were kept at $-20°C$ for further analysis. Another group of rats ($n = 10$) that was also administered a 750 mg/kg concentration of KP formulation was placed in the metabolic cages. Urine and feces samples were collected every 6 h for 72 h and subsequently stored at $-20°C$.

Biological Sample Extraction. Whole organs were diced into small pieces and homogenized in 0.01 M phosphate buffer solutions, pH 7.4 (three volumes of organ weight). The homogenates were subsequently added to 200 μl of acetonitrile. Feces samples were premixed with three volumes of 0.01 M phosphate buffer solutions, pH 7.4; urine and feces samples were then added to three volumes of acetonitrile. Blood samples (200 μl) were added to 1 ml of acetonitrile. All samples were extracted by liquid-liquid extraction three times. The mixtures were vortexed for 2 min and sonicated for 5 min. After centrifugation at 2000g for 2 min, the supernatants were transferred to clean microtubes and evaporated to dryness. The residues were reconstituted with 200 μl of acetonitrile, filtered through 0.45-μm syringe filters, and analyzed using HPLC-UV for quantitative evaluation as described previously (Mekjaruskul et al., 2012) and by LC-MS and LC-MS/MS for qualitative analysis.

Metabolite Identification by Using LC-MS. Metabolites in urine and feces samples were identified using LC-electrospray ionization-MS (Thermo

Fisher Scientific TSQ quantum). Full-scan MS and MS/MS modes were simultaneously used to confirm the structures of the metabolites. The samples were analyzed in full scan in positive mode for mass screening. The mass scan began at m/z 30 to 1000. The unknown masses were further analyzed in MS/MS mode. Collision energy was optimized ranging from 10 to 70 eV to define fragment ions of the metabolites. An LC system composed of acetonitrile (A) and 0.5% formic acid in water (B) as the mobile phase was running the following gradient: 0 to 60 min: gradient from 5% A/95% B to 50% A/50% B at a flow rate of 200 μl/min. The injection volume was 20 μl. The analytical column used was a Chromolith C18 column (2.0 × 50 mm, 5 μm; EMD/Millipore, Billerica, MA). The Xcaliber 2.0.6 program (1998–2007; Thermo Fisher Scientific) was used to integrate the chromatograms.

Data Analysis. Pharmacokinetic parameters were calculated by Phoenix WinNonlin program (Pharsight, Mountain View, CA) based on a one-compartment model for intravenous bolus and oral doses. Bioavailability (F), defined as the fraction of unchanged drug that reached the systemic circulation after the administration of a drug dose, was calculated from the AUC of the oral route and AUC of the intravenous route. The results were expressed as mean ± S.D. One-way analysis of variance and the independent t test were used to compare the different groups. Statistical significance was determined when P values were <0.05.

Results

Pharmacokinetic Parameters and Bioavailability. The HPLC assays were demonstrated to be of good linearity, precision, and high accuracy as shown in Table 1. Intraday and interday precision of the assays for PMF, TMF, and DMF at concentrations of 2 to 40 μg/ml ranged from 0.32 to 4.89% relative S.D. and from 2.29 to 7.73% relative S.D., respectively. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.09 to 0.93 and 0.19 to 1.86 μg/ml, respectively. The
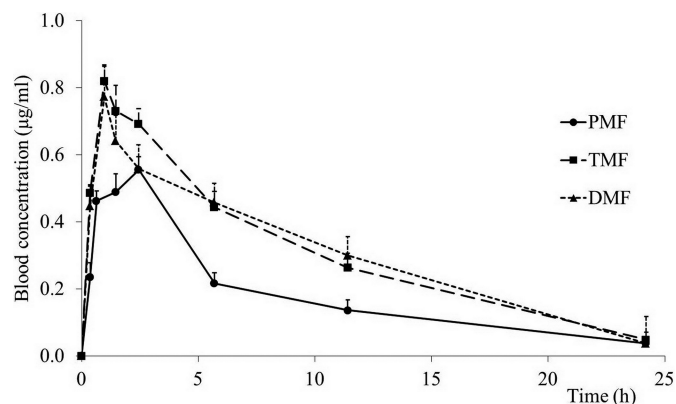

FIG. 3. Blood concentration-time profile of methoxyflavones after a single oral administration of 250 mg/kg KP to rats by HPLC-UV determination. Each data point represents the mean ± S.D. ($n = 10$).
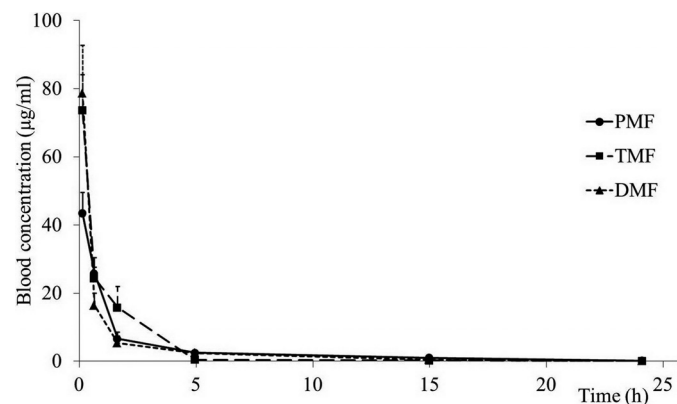

FIG. 4. Blood concentration-time profile of methoxyflavones after a single intravenous administration of 250 mg/kg KP to rats by HPLC-UV determination. Each data point represents the mean ± S.D. ($n = 10$).

TABLE 2

Pharmacokinetic parameters and oral bioavailability of methoxyflavones after single oral or intravenous administration of 250 mg/kg KP in rats

PK Parameters	Route	PMF	TMF	DMF
AUC (h · μg/ml)	Oral	3.65 ± 0.63**·***	6.96 ± 1.11***	7.01 ± 1.37***
	IV	76.77 ± 19.50**	275.66 ± 86.06	233.48 ± 71.57
$t_{1/2}$ (h)	Oral	3.12 ± 1.34	5.04 ± 1.10	5.85 ± 1.72
	IV	2.36 ± 1.89	4.19 ± 1.45	3.75 ± 1.01
K_e (h^{-1})	Oral	0.28 ± 0.17***	0.15 ± 0.04***	0.13 ± 0.03***
	IV	0.61 ± 0.22*	0.32 ± 0.12	0.32 ± 0.11
Cl (mL/h)	Oral	622.85 ± 114.86*	337.00 ± 62.17	367.28 ± 82.35
	IV	21.56 ± 7.18*·***	6.30 ± 2.17***	7.98 ± 2.34***
T_{max} (h)	Oral	1.71 ± 0.36*	0.85 ± 0.40	0.76 ± 0.40
C_{max} (μg/ml)	Oral	0.55 ± 0.05**	0.88 ± 0.11	0.78 ± 0.11
V_d (ml)	Oral	2637.13 ± 846.59	2385.10 ± 364.37	2957.53 ± 458.19
K_a (h^{-1})	Oral	1.23 ± 0.54	8.53 ± 3.64	8.69 ± 2.33
Bioavailability (%)		3.32	1.75	2.10

Data are expressed as mean $\pm$ S.D. ($n = 10$). IV, intravenous.
* Polyethylene glycol 400: significant higher than the others in the same route at $P < 0.05$.
** Significance lower than the others in the same route, $P < 0.05$.
*** Significance lower than the other route.

linear regression equations of the three major compounds, which were constructed by plotting the peak area versus concentrations, showed good linear relationships with R^2 values >0.990. The accuracy of the method for two concentrations (1 and 50 μg/ml) was $<7.0\%$ error. The extraction method for blood samples was successful with the highest yield at $91.63 \pm 0.23\%$.

The major compounds in KP, PMF, TMF, and DMF were found at the high concentrations in rat blood after administration of 250 mg/kg KP. HPLC-UV chromatograms of the KP formulation and blood samples after single oral administration of the formulation are shown in Figs. 1 and 2, respectively. The blood concentrations of PMF, TMF, and DMF at several time points for the oral and intravenous routes are illustrated in Figs. 3 and 4. Pharmacokinetic parameters for the oral and intravenous routes, which were calculated using the Pharsight WinNonLin program, are presented in Table 2. The one-compartment oral input model was the best fit.

In the oral route, maximum concentrations of PMF, TMF, and DMF were rapidly achieved with C_{max} (0.55–0.88 μg/ml) within 0.76 to 1.71 h (Figs. 3 and 4). K_a (absorption rate constant) of PMF, TMF, and DMF were 1.23 ± 0.54, 8.53 ± 3.64, and 8.69 ± 2.33 h^{-1}, respectively. After that, the concentrations slowly dropped to where they were below the LOD at 24 h after dosing with K_e (elimination rate constant) of 0.28, 0.15, and 0.13 h^{-1} for PMF, TMF, and DMF, respectively. Their half-lives ($t_{1/2}$) were 3.12, 5.04, and 5.85 h for PMF, TMF, and DMF, respectively. Clearance of PMF had the highest value at 622.85 ± 114.86 ml/h, followed by DMF (367.28 ± 82.35 ml/h) and TMF (337.00 ± 62.17 ml/h). The volumes of distribution (V_d) of PMF, TMF, and DMF ranged from 2385 to 2951 ml.

In the intravenous route, methoxyflavones concentrations rapidly decreased and cleared out from the systemic within 24 h. AUC value of PMF was found lowest at 76.77 ± 19.50 h · μg/ml. The clearance of PMF was 21.56 ± 7.18 ml/h, which was higher than that of TMF and DMF. In terms of different routes, the clearance of three methoxyflavones in the intravenous route were significantly lower than that of the oral route ($P = 0.05$). PMF possessed the highest value of K_e (0.61 ± 0.22 h^{-1}) followed by TMF and DMF. Comparing the K_e between the oral and intravenous routes, K_e of three methoxyflavones in the intravenous route were significantly higher than those of the oral route. The $t_{1/2}$ of methoxyflavones ranged from 2.36 to 4.19 h of which there was no significant difference compared between the oral and intravenous routes.

Oral bioavailability (F) was calculated based on the AUC from the oral and intravenous routes. The results showed that PMF had the

greatest bioavailability ($F = 3.32\%$) followed by DMF (2.10%) and TMF (1.75%), respectively.

Tissue Distribution of Methoxyflavones in Rats. The tissue extraction method demonstrated high recovery at 90 to 92%. The quantitative data of methoxyflavones, PMF, TMF, and DMF, in various organs after administration of 750 mg/kg KP are presented in Table 3. The highest levels of PMF, TMF, and DMF both in AUC$_{0.083–4 h}$ and C_{max} were found in liver followed by kidney. PMF concentrations detected in lung were higher than those of testes followed by brain. For TMF, the highest concentration was found in liver followed by kidney, brain, testes, and lung. The concentration of DMF in liver was significantly higher than that of kidney followed by lung, brain, and testis. In terms of the amounts among three methoxyflavones, the concentrations of PMF, TMF, and DMF in liver were not significantly different at $P > 0.05$; likewise, TMF and DMF concentrations in kidney, lung, brain, and testes samples were not different at $P > 0.05$. For PMF, the concentrations in kidney and lung were lower than those of TMF and DMF, respectively ($P < 0.05$). In brain and testes, TMF and DMF concentrations were significantly higher than those of PMF ($P < 0.05$). Three methoxyflavones were first found in rat lung—the

TABLE 3

Accumulative tissue contents, C_{max}, and T_{max} of PMF, TMF, and DMF in various rat organs after oral administration of 750 mg/kg KP

Organ	Methoxyflavone	Parameter		
		AUC$_{0.083–4 h}$	C_{max}	T_{max}
		h · μg/g organ	μg/g organ	h
Liver	PMF	9.07 ± 1.37	3.10 ± 1.78	3.43 ± 0.98****
	TMF	7.83 ± 0.33	3.01 ± 1.49	2.64 ± 1.38
	DMF	8.60 ± 1.97	3.85 ± 1.45	2.57 ± 1.10
Kidney	PMF	2.03 ± 0.67*	1.00 ± 0.39	3.43 ± 0.98
	TMF	3.01 ± 0.85	1.64 ± 0.59	3.43 ± 0.98
	DMF	2.51 ± 0.68	1.33 ± 0.49	3.43 ± 0.98
Lung	PMF	1.60 ± 0.37**	2.23 ± 1.13	0.08 ± 0.17***
	TMF	1.88 ± 0.35	1.41 ± 0.58	1.76 ± 0.19
	DMF	2.17 ± 0.31	2.00 ± 1.20	1.20 ± 0.19
Brain	PMF	0.76 ± 0.13***	0.26 ± 0.10***	3.14 ± 1.07
	TMF	2.50 ± 0.37	1.43 ± 0.58	3.43 ± 0.98
	DMF	1.91 ± 0.56	0.96 ± 0.37	3.14 ± 1.07
Testes	PMF	1.01 ± 0.24***	0.50 ± 0.31***	2.36 ± 1.25
	TMF	1.96 ± 0.67	1.10 ± 0.43	3.14 ± 1.07
	DMF	1.90 ± 0.62	0.91 ± 0.44	2.86 ± 1.07

Data are mean $\pm$ S.D. ($n = 7$).
* Significance lower than TMF at $P < 0.05$.
** Significance lower than DMF at $P < 0.05$.
*** Significance lower than that of TMF and DMF at $P < 0.05$.
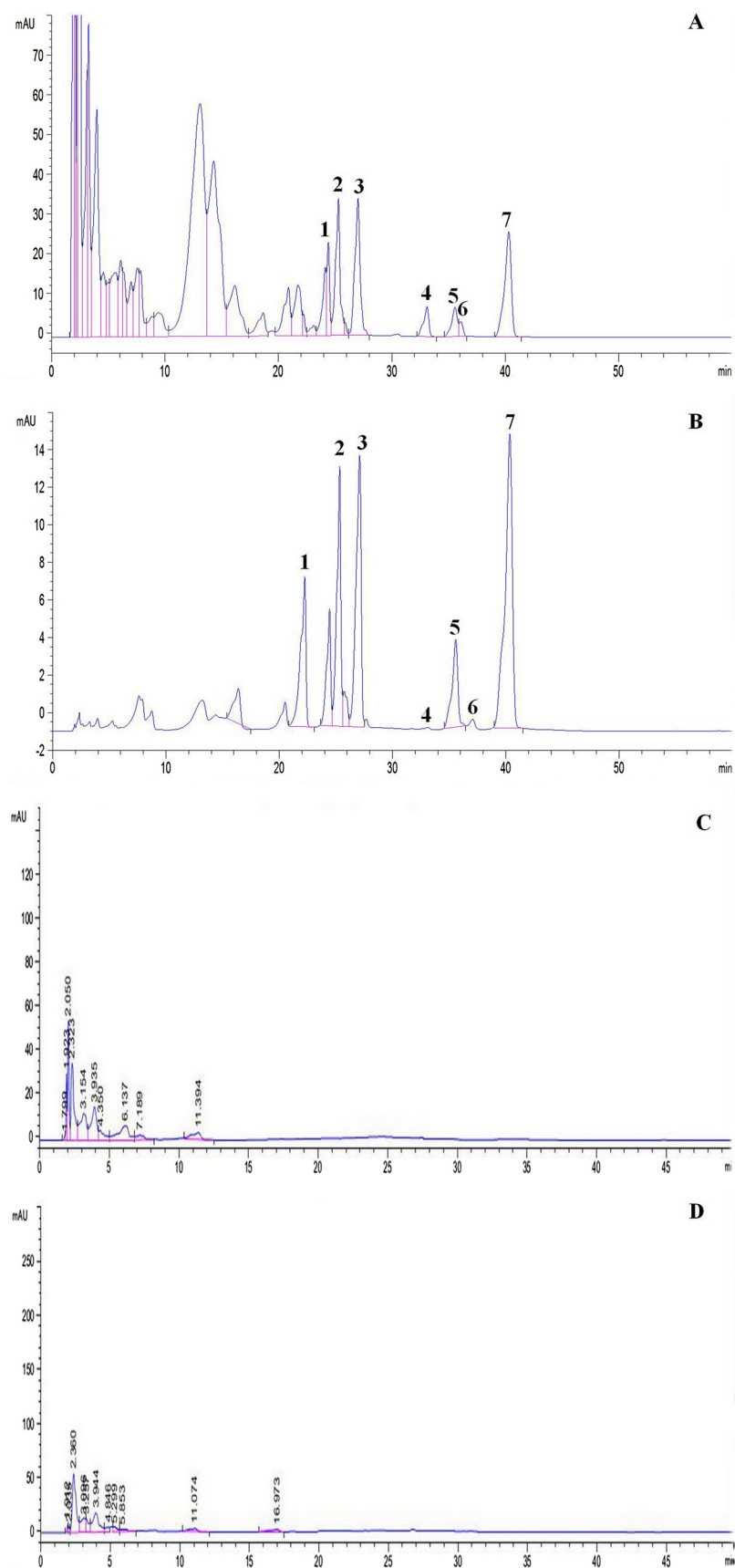**** Significance higher than that of TMF and DMF at $P < 0.05$.

FIG. 5. HPLC-UV profiles of rat urine (A) and feces (B) 12 h after oral treatment with 750 mg/kg KP and untreated rat urine (C) and feces (D) (1, PMF; 2, TMF; 3, DMF; 4, 5-OH-3,7,3′,4′-tetramethoxyflavone; 5, 5-OH-7-methoxyflavone; 6, 5-OH-3,7,4′-trimethoxyflavone; 7, 5-OH-3,7-dimethoxyflavone).
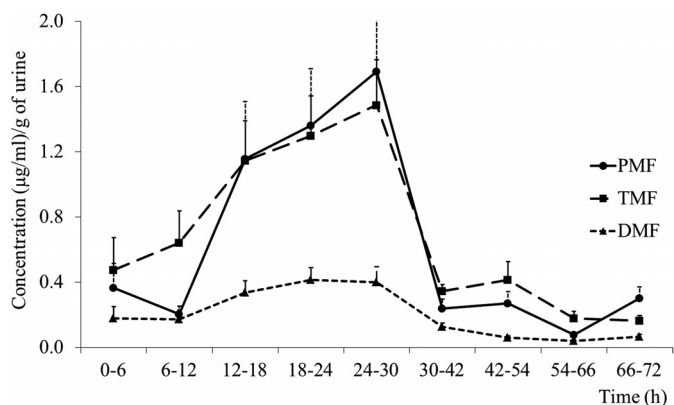
FIG. 6. Profile of methoxyflavones concentrations with time for urine samples of rats receiving 750 mg/kg KP for 72 h.

minimum of time to maximal concentration (T_{max}) in lung followed by liver, testes, brain, and kidney after administration of 750 mg/kg KP formulation.

Urinary and Fecal Excretion of Methoxyflavones in Rats. The extraction of methoxyflavones from urine and feces samples showed high recovery (92–98%). HPLC-UV chromatograms of rat urine and feces after administration of 750 mg/kg KP are shown in Fig. 5, A and B, respectively. All compounds presented in rat urine and feces after administration of 750 mg/kg KP were not found in control rats as shown in Fig. 5, C and D, for untreated rat urine and feces, respectively. The profiles of PMF, TMF, and DMF in urine and feces are illustrated in Figs. 6 and 7, respectively. In urine, the amounts of PMF and TMF were highest at 24 to 30 h. After that, the amounts rapidly declined until 72 h. The concentration of DMF in urine was highest at 18 to 24 h, and then the levels slowly diminished below the limit of detection at 72 h. It was observed that DMF concentrations were markedly lower than those of PMF and TMF as measured at 0 to 6, 12 to 28, 18 to 24, 24 to 30, and 42 to 54 h. At 6 to 12 and 54 to 66 h of collecting time, TMF levels were significantly higher than those of PMF and DMF. PMF concentration at 66 to 72 h was greater than that of TMF and DMF.

In feces samples of rats receiving 750 mg/kg KP, concentrations of PMF, TMF, and DMF quickly reached their highest levels within 24 h and then gradually decreased. The highest amounts of PMF and TMF were observed at the 18- to 24-h collecting time; whereas DMF concentration showed the highest levels at 12 to 18 h after administration. In general, the patterns of concentrations of these three methoxyflavones in feces were not different, but the level of DMF at 0 to 6 h was significantly lower than that of PMF and TMF.

Metabolite Identification in Urine and Feces. The identification of the KP metabolites was based on different retention times from the known compounds in the KP formulation, the m/z ratios, and fragment ions by LC-MS and LC-MS/MS. The m/z ratios of the metabolites in urine and feces samples were determined from full scans in positive mode from m/z 30 to 1000. Structural elucidation of the metabolites in urine and feces samples were based on the fragmentation patterns of parent ion from MS/MS mode at collision energy of 35 to 40 eV generating daughter ions. MS/MS spectra of the metabolites were shown in Fig. 8. Metabolic reactions, proposed structures of the metabolites in urine and feces, and their fragment ions from MS/MS are presented in Table 4. A proposed metabolic scheme of the methoxyflavones in KP is illustrated in Fig. 9. M1 (m/z 255) was 28 Da lower than the $[M+H]^+$ signal of DMF (m/z 283), indicating the loss of two methyl groups (-CH$_3$) by demethylation reactions at the C-5 and C-7 positions of DMF. From the ion at the $[M+H]^+$ signal of M2

at 285, there appeared to be a loss of methyl groups at C-5 and C-4′ of TMF (m/z 313) by demethylation reactions. M3 had a $[M+H]^+$ signal at 315, which was the demethylated metabolite of 3,5,7,4′-tetramethoxyflavone (m/z 343) at the C-3 and C-5 positions. M4 gave a $[M+H]^+$ signal at 345, 28 Da less than PMF (m/z 373), indicating the loss of two methyl groups, most likely at C-5 and C-3′ of PMF. In the case of M5, DMF was demethylated at the C-5 position, yielding an m/z ratio at 269, and then further sulfated (80 Da), resulting in a $[M+H]^+$ signal at 349. The molecular mass of M6 (m/z 359) was 14 Da lower than that of PMF, indicating demethylation of PMF at C-3′ position. M7 was assumed to be metabolized from TMF by demethylation reactions at C-5 and then further by demethylation and sulfation at C-7. M8 had a $[M+H]^+$ signal at 379, indicating that it was metabolized by demethylation and further by sulfation at the C-5 position of TMF. For M9, the oxidation at C-7 of M1 occurred, resulting in a $[M+H]^+$ signal at 239, this was further conjugated to the glucuronide, providing an m/z ratio at 415. M3 was metabolized at the C-4′ position by demethylation, yielding M10 at an m/z ratio of 301. M11, which showed a protonated molecular ion at m/z 331, was assumed to be demethylated at the C-3′, C-5, and C-3 positions of PMF.

Discussion

The pharmacokinetic parameters of the KP crude extract after oral or intravenous administration to rats, including bioavailability, tissue distribution, excretion, and metabolite identification, were determined in the present study. These parameters are useful in describing and predicting information related to its efficacy and toxicity, including blood and tissue levels, determination of optimum dosage regimens, and correlation of drug concentration with pharmacological or toxicological activity (Shargel et al., 2005).

Investigations on the pharmacokinetics and bioavailability of KP formulation at doses of 250 mg/kg showed pharmacological activity in rat, namely antidepressive effects and cognitive enhancement (Hawiset et al., 2011). Moreover, methoxyflavones were quantified in blood at these doses. Therefore, the 250 mg/kg dose of the KP extract was selected for the current studies. KP extracts contain high amounts of three methoxyflavones: PMF, TMF, and DMF (Sutthanut et al., 2007). These methoxyflavones also possess many pharmacological activities such as those listed above as well as anticholinesterase (Sawasdee et al., 2009) and antimutagenic (Azuma et al., 2011) activity. Therefore, PMF, TMF, and DMF were selected to be the markers for this pharmacokinetic study. Development of an analytical method for blood, tissue, urine, and feces samples is a key step of pharmacokinetic study. The HPLC method described was successful
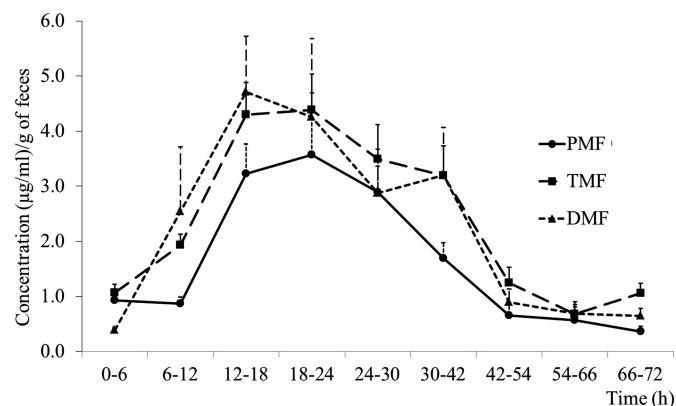

FIG. 7. Profile of methoxyflavones concentrations with time for feces samples of rats receiving 750 mg/kg KP for 72 h.
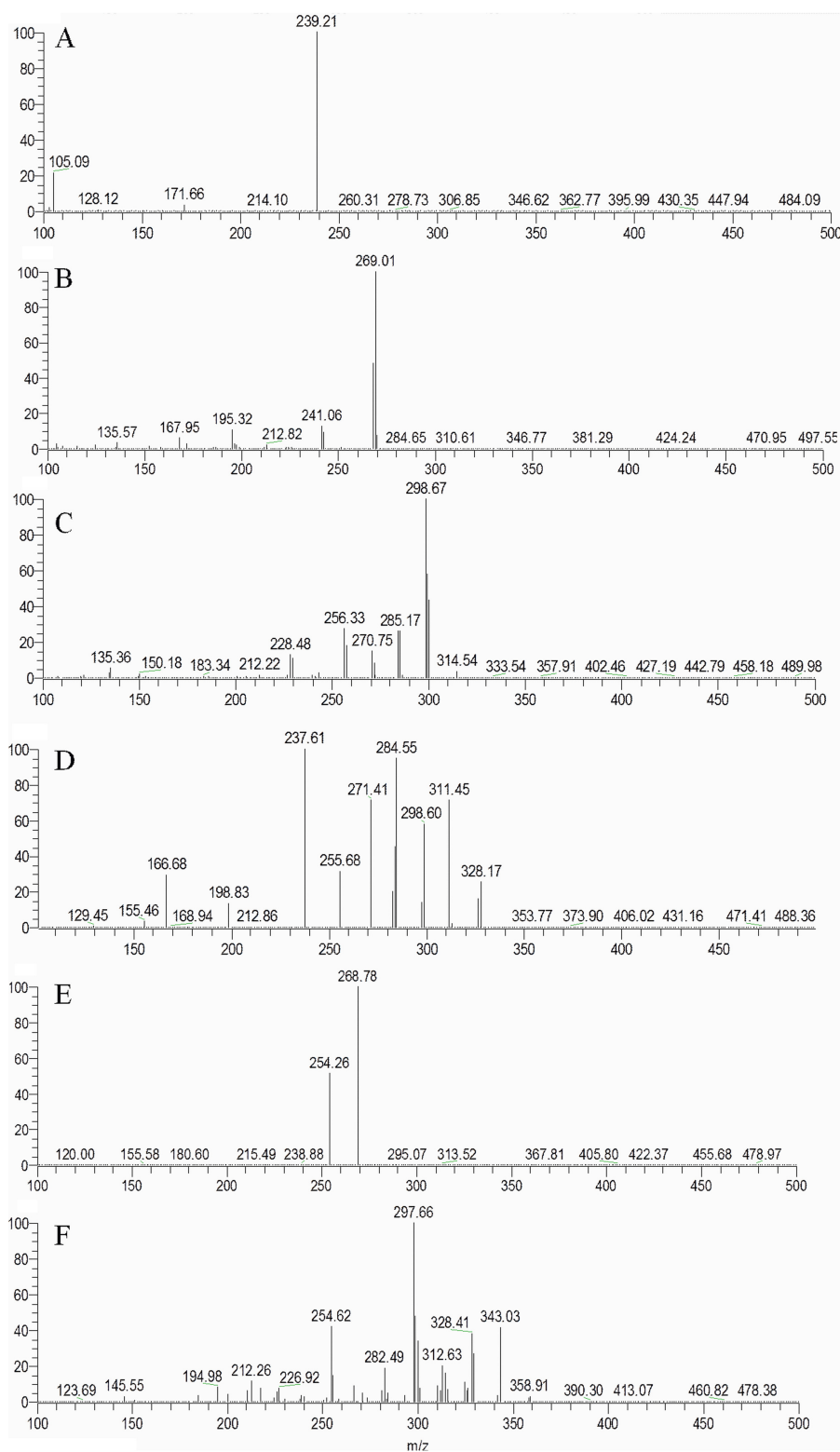
FIG. 8. MS/MS spectra of metabolites (A, M1; B, M2; C, M3; D, M4; E, M5; F, M6; G, M7; H, M8; I, M9; J, M10; K, M11) in rat urine and feces after oral administration of 750 mg/kg KP.

in determining the methoxyflavones in biological samples with adequate resolution of separation, high sensitivity, selectivity, accuracy, and precision. The extraction method of methoxyflavones using acetonitrile also yielded good recoveries for biological samples in the pharmacokinetic, organ distribution, and excretion studies. Acetonitrile used as the extraction solvent in this study was not only a good solvent for water-insoluble methoxyflavones but it also acted as a protein-precipitating agent.

After a single oral administration of KP, the methoxyflavones in KP were rapidly absorbed, reaching maximal concentrations in blood within 1 to 2 h followed by broad distribution in organs and gradual excretion. These data support the previous report that DMF was detected in plasma of rats treated with 5 mg/kg DMF with a T_{max} of 1 h (Walle at al., 2007). From the blood concentration-time profile of methoxyflavones after intravenous administration of KP in this study, the methoxyflavones were rapidly cleared from the circulation. After
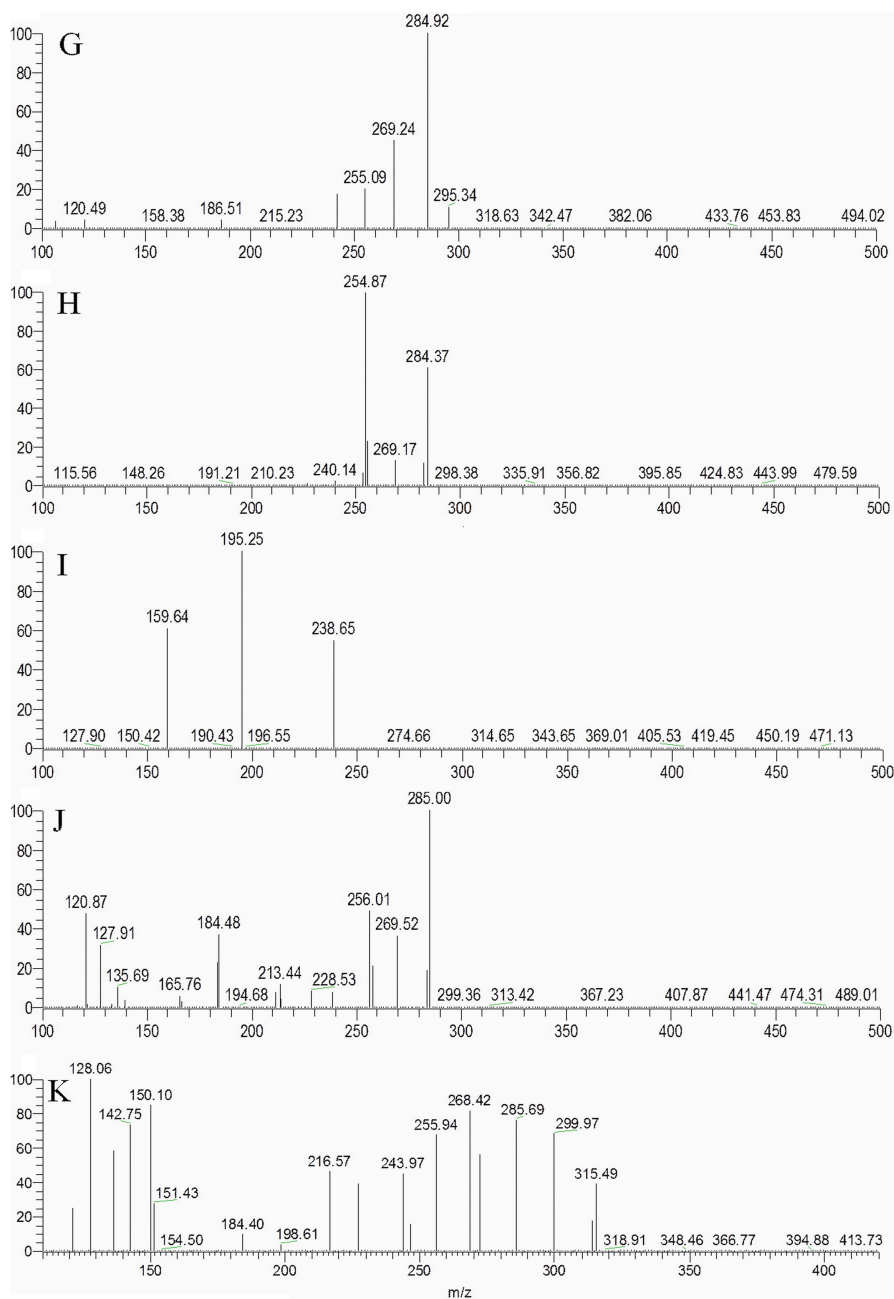
FIG. 8. *Continued.*

the first 6 h of administration, PMF, TMF, and DMF were mainly excreted. These compounds were subsequently found in urine and feces.

In a preliminary study, the methoxyflavone concentrations in several organs were lower than the LOQ when administration of KP was at doses of <750 mg/kg. Moreover, single administration of KP at dose of 2 g/kg was safe in rats (Chivapat et al., 2004). Thus, 750 mg/kg KP was selected for the organ distribution and excretion studies. Walle et al. (2007) reported that after oral administration of DMF, it was found mainly in the liver with much higher levels than in plasma, kidney, and lung. Tsuji et al. (2006) also reported that after administration of DMF to Atlantic killifish, the highest concentration of DMF was found in liver followed by brain, intestines, gill, and skin. In the current study, the methoxyflavones were detected in brain and testes, indicating their ability to penetrate the blood-brain and blood-testicular barriers. In the organ distribution study, methoxyflavones were found at highest levels in liver and kidney, suggesting their main elimination by these two organs. The given doses of 250, 500, and 1000 mg/kg body weight to rats from the study of our research teams showed the linear relationship of the doses and the blood concentrations (B. Sripanidkulchai and C. Mekjaruskul, unpublished data). Therefore, the estimated AUC in blood of the dose of 750 mg/kg was 10.95, 20.88, and 21.03 h · μg/ml for PMF, TMF, and DMF, respectively, in which these values were similar to the AUC of all organs. It suggested that the methoxyflavones may distribute in the organs rather than in blood. The calculated V_d values of methoxyflavones were 8.79, 7.95, and 9.85 l/kg for PMF, TMF, and DMF, respectively. The body water of rat is 57 to 72% body weight (Foy and Schnieden, 1960). Therefore, the methoxyflavones may mostly distribute in body water.

These results suggest that approximately 15, 25, and 30 h are required for complete elimination (4–5 times elimination $t_{1/2}$) of

TABLE 4

Proposed structures and metabolic pathways of metabolites in rat urine and feces after administration of 750 mg/kg KP
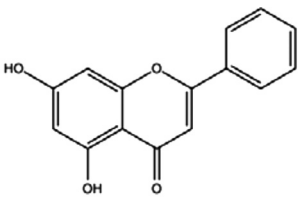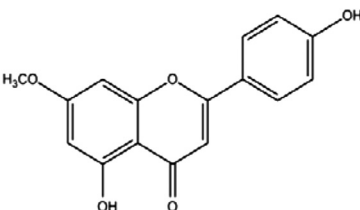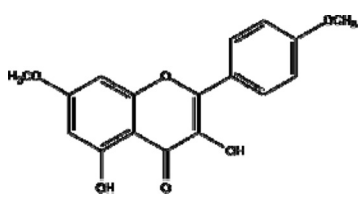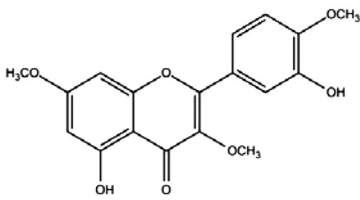
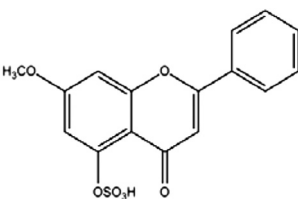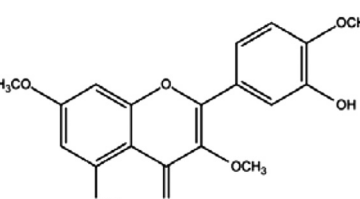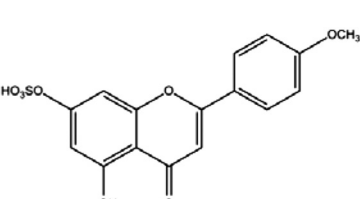| No.	$[M + H]^{+1}$	Fragment Ions in MS/MS	Structure of Proposed Metabolites	Reactions	Source	Possible Substrate of the Metabolite
M1	255	239.11		Demethylation	Urine/feces	DMF
M2	285	269.01		Demethylation	Urine/feces	TMF
M3	315	298.67 285.17 270.75 256.33		Demethylation	Urine/feces	3,5,7,4′-Tetramethoxyflavone
M4	345	328.17 311.45 298.60 284.55 277.41 255.68 237.61		Demethylation	Urine/feces	PMF
M5	349	268.78 254.26		Demethylation, sulfation	Urine	DMF
M6	359	343.03 328.41 297.66 282.49 254.62		Demethylation	Urine	PMF
M7	365	284.92 269.24 255.09		Demethylation, sulfation	Urine	TMF

TABLE 4—*Continued*

| No.	$[M + H]^{+1}$	Fragment Ions in MS/MS	Structure of Proposed Metabolites	Reactions	Source	Possible Substrate of the Metabolite
M8	379	298.38 284.37 269.17 254.87		Demethylation, sulfation	Urine	TMF
M9	415	238.65		Glucuronidation	Urine	DMF
M10	301	285.00 269.52 256.01		Demethylation	Feces	3,5,7,4′-Tetramethoxyflavone
M11	331	315.49 299.97 285.69 268.42 255.94		Demethylation	Feces	PMF
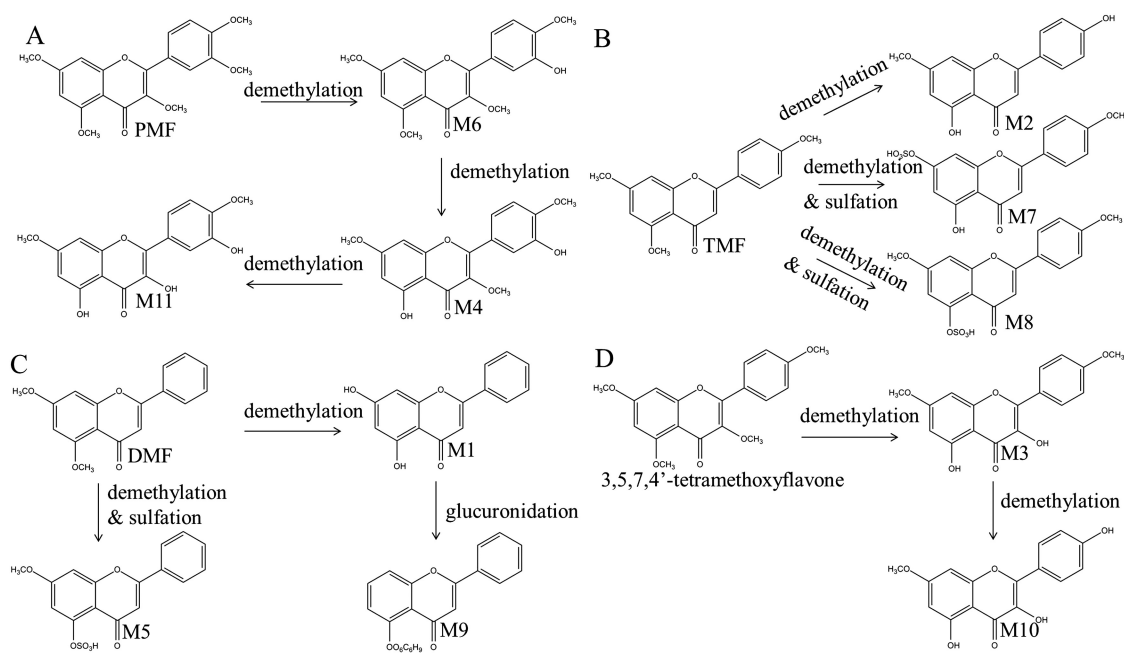
FIG. 9. Proposed metabolic pathways of PMF (A), TMF (B), DMF (C), and 3,5,7,4′-tetramethoxyflavones (D) in rats.

PMF, TMF, and DMF, respectively. PMF was quickly excreted, with the lowest of $t_{1/2}$ and highest clearance resulting in the lowest C_{max} and AUC. This can be used as a measure of the total amount of unchanged drug reaching the systemic circulation after administration.

Furthermore, the results showed that the oral bioavailability of methoxyflavones was low, approximately 1 to 4%. K_e of three methoxyflavones in the oral route were significant lower than those of the intravenous routes. It implied that the clearance process was not limited by the absorption process, suggesting that these methoxyflavones may be hydrolyzed in the gastrointestinal tract, destroyed by first-pass metabolism in the gastrointestinal tract or liver, or poorly absorbed in the intestines. The extraction ratios of PMF, TMF, and DMF (extraction ratio $= 1 - F$; Shargel et al., 2005) were 0.97, 0.98, and 0.98, respectively. These results showed that the methoxyflavones characterize as high-extraction molecules, resulting in the low bioavailability of these molecules. In addition, the low water solubility and high lipophilicity of the methoxyflavones, with logP values ranging from 2.0 to 3.5 (Sutthanut et al., 2009), may explain the low bioavailability.

After absorption and distribution, the methoxyflavones were further metabolized and eliminated. The percentage doses of PMF, TMF, and DMF left in urine at 0 to 72 h were 1.06, 1.77, and 0.96%, respectively. The percentage doses of PMF, TMF, and DMF left in feces at 0 to 72 h were 0.79, 1.76, and 3.10%, respectively. These results indicated that the excretion of KP was mainly in the form of the metabolites of the methoxyflavones. In terms of renal clearance, it can be calculated from the total amount of the unchanged drug in urine and the AUC (Hedayan, 2007). Therefore, renal clearance of PMF, TMF, and DMF was 115.21, 88.26, and 25.60 ml/h, respectively. Because the total clearance (calculated from WinNonlin program) is the sum of renal clearance and hepatic clearance, the calculated hepatic clearance of PMF, TMF, and DMF was 507.64, 248.74, and 341.68 ml/h, respectively. These results implied that the unchanged methoxyflavones preferred to eliminate in liver. The chemical structures of the metabolites after oral administration of KP were elucidated by LC-MS, both full-scan MS and MS/MS modes providing the $[M+H]^+$ of parent compounds and daughter ions to obtain more structural information. The components that presented at high concentrations in KP, including PMF, TMF, DMF, and 3,5,7,4'-tetramethoxyflavone, were assumed to be the parent compounds (Sutthanut et al., 2007). The identification of metabolites indicated that methoxyflavones in KP were mainly metabolized to their demethylated forms and were conjugated to the sulfate and glucuronide forms to a minor extent. Tsuji et al. (2006) also found demethylation and glucuronidation of DMF after administration of DMF. The metabolites of KP can be found in rat feces and urine samples after administration of KP, but they were not detected in blood samples. This finding may indicate that the active substances possessing pharmacological activity of KP are probably the intact methoxyflavones.

In conclusion, this study resulted in the first report on defining the pharmacokinetic parameters of KP as well as the identification of specific metabolites. Methoxyflavones levels rapidly reached a maximal concentration within 1 to 2 h after oral administration of KP extract and then widely distributed to the organs following by slow elimination. The oral bioavailability values of the methoxyflavones were found to be very low. The metabolites of methoxyflavones in urine were the demethylated, glucuronide, and sulfide forms, whereas only demethylated forms of methoxyflavones were found in feces.

Authorship Contributions

Participated in research design: Mekjaruskul and Sripanidkulchai.
Conducted experiments: Mekjaruskul.
Contributed new reagents or analytic tools: Jay and Sripanidkulchai.
Performed data analysis: Mekjaruskul.
Wrote or contributed to the writing of the manuscript: Mekjaruskul, Jay, and Sripanidkulchai.

References

Akase T, Shimada T, Terabayashi S, Ikeya Y, Sanada H, and Aburada M (2011) Antiobesity effects of *Kaempferia parviflora* in spontaneously obese type II diabetic mice. *J Nat Med* **65:**73–80.

Azuma T, Kayano SI, Matsumura Y, Konishi Y, Tanaka Y, and Kikuzaki H (2011) Antimutagenic and α-glucosidase inhibitory effects of constituents from *Kaempferia parviflora*. *Food Chem* **125:**471–475.

Banjerdpongchai R, Suwannachot K, Rattanapanone V, and Sripanidkulchai B (2008) Ethanolic rhizome extract from *Kaempferia parviflora* Wall. ex. Baker induces apoptosis in HL-60 cells. *Asian Pac J Cancer Prev* **9:**595–600.

Chaturapanich G, Chaiyakul S, Verawatnapakul V, and Pholpramool C (2008) Effects of *Kaempferia parviflora* extracts on reproductive parameters and spermatic blood flow in male rats. *Reproduction* **136:**515–522.

Chivapat S, Chavalittumrog P, Phadungpat S, Pama KK, Chansuvanich N, Attawish A, and Punyamong S (2004) Acute and chronic toxicity study of *Kaempferia parviflora* Wall ex. Baker powder. *J Thai Tradit Alternat Med* **2:**3–16.

Foy JM and Schnieden H (1960) Estimation of total body water (virtual tritium space) in the rat, cat, rabbit, guinea-pig and man, and of the biological half-life of tritium in man. *J Physiol* **154:**169–176.

Hawiset T, Muchimapura S, Wattanathorn J, and Sripanidkulchai B (2011) Screening neuropharmacological activities of *Kaempferia parviflora* (Krachai Dam) in healthy adult male rats. *Am J Appl Sci* **8:**695–702.

Hedaya MA (2007) *Basic Pharmacokinetics.* CRC Press, Boca Raton, FL.

Kummee S, Tewtrakul S, and Subhadhirasakul S (2008) Anitimicrobial activity of the ethanol extract and compounds from the rhizomes of *Kaempferia parviflora*. *Songklanakarin J Sci Technol* **30:**463–466.

Leardkamolkarn V, Tiamyuyen S, and Sripanidkulchai BO (2009) Pharmacological activity of *Kaempferia parviflora* extract against human bile duct cancer cell lines. *Asian Pac J Cancer Prev* **10:**695–698.

Malakul W, Ingkaninan K, Sawasdee P, and Woodman OL (2011a) The ethanolic extract of Kaempferia parviflora reduces ischaemic injury in rat isolated hearts. *J Ethnopharmacol* **137:**184–191.

Malakul W, Thirawarapan S, Ingkaninan K, and Sawasdee P (2011b) Effects of Kaempferia parviflora Wall. Ex Baker on endothelial dysfunction in streptozotocin-induced diabetic rats. *J Ethnopharmacol* **133:**371–377.

Mekjaruskul C, Jay M, and Sripanidkulchai B (2012) Modulatory effects of *Kaempferia parviflora* extract on mouse hepatic cytochrome P450 enzymes. *J Ethnopharmacol* **141:**831–839.

Nakao K, Murata K, Deguchi T, Itoh K, Fujita T, Higashino M, Yoshioka Y, Matsumura S, Tanaka R, Shinada T, et al. (2011) Xanthine oxidase inhibitory activities and crystal structures of methoxyflavones from *Kaempferia parviflora* rhizome. *Biol Pharm Bull* **34:**1143–1146.

Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai BO, Yumoto R, and Takano M (2007a) Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. *Eur J Pharmacol* **566:**67–74.

Patanasethanont D, Nagai J, Yumoto R, Murakami T, Sutthanut K, Sripanidkulchai BO, Yenjai C, and Takano M (2007b) Effects of Kaempferia parviflora extracts and their flavone constituents on P-glycoprotein function. *J Pharm Sci* **96:**223–233.

Rujjanawate C, Kanjanapothi D, Amornlerdpison D, and Pojanagaroon S (2005) Anti-gastric ulcer effect of *Kaempferia parviflora*. *J Ethnopharmacol* **102:**120–122.

Sae-wong C, Tansakul P, and Tewtrakul S (2009) nti-inflammatory mechanism of Kaempferia parviflora in murine macrophage cells (RAW 264.7) and in experimental animals. *J Ethnopharmacol* **124:**576–580.

Sawasdee P, Sabphon C, Sitthiwongwanit D, and Kokpol U (2009) Anticholinesterase activity of 7-methoxyflavones isolated from *Kaempferia parviflora*. *Phytother Res* **23:**1792–1794.

Shargel L, Wu-Pong S, and Yu A (2005) *Applied Biopharmaceutics and Pharmacokinetics*, 5th ed, McGraw-Hill, New York.

Sudwan P, Saenphet K, Saenphet S, and Suwansirikul S (2006) Effect of Kaempferia parviflora Wall. ex. Baker on sexual activity of male rats and its toxicity. *Southeast Asian J Trop Med Public Health* **37:**210–215.

Sutthanut K, Lu X, Jay M, and Sripanidkulchai B (2009) Solid lipid nanoparticles for topical administration of Kaempferia parviflora extracts. *J Biomed Nanotechnol* **5:**224–232.

Sutthanut K, Sripanidkulchai B, Yenjai C, and Jay M (2007) Simultaneous identification and quantitation of 11 flavonoid constituents in Kaempferia parviflora by gas chromatography. *J Chromatogr A* **1143:**227–233.

Tep-areenan P, Sawasdee P, and Randall M (2010) Possible mechanisms of vasorelaxation for 5,7-dimethoxyflavone from *Kaempferia parviflora* in the rat aorta. *Phytother Res* **24:**1520–1525.

Tewtrakul S and Subhadhirasakul S (2008) Effects of compounds from *Kaempferia parviflora* on nitric oxide, prostaglandin E_2 and tumor necrosis factor-alpha productions in RAW264.7 macrophage cells. *J Ethnopharmacol* **120:**81–84.

Tewtrakul S, Subhadhirasakul S, and Kummee S (2008) Anti-allergic activity of compounds from *Kaempferia parviflora*. *J Ethnopharmacol* **116:**191–193.

Tsuji PA, Winn RN, and Walle T (2006) Accumulation and metabolism of the anticancer

flavonoid 5,7-dimethoxyflavone compared to its unmethylated analog chrysin in the Atlantic killifish. *Chem Biol Interact* **164:**85–92.

Walle T, Ta N, Kawamori T, Wen X, Tsuji PA, and Walle UK (2007) Cancer chemopreventive properties of orally bioavailable flavonoids–methylated versus unmethylated flavones. *Biochem Pharmacol* **73:**1288–1296.

Wattanapitayakul SK, Suwatronnakorn M, Chularojmontri L, Herunsalee A, Niumsakul S, Charuchongkolwongse S, and Chansuvanich N (2007) Kaempferia parviflora ethanolic extract promoted nitric oxide production in human umbilical vein endothelial cells. *J Ethnopharmacol* **110:**559–562.

Wattanathorn J, Pangpookiew P, Sripanidkulchai K, Muchimapura S, and Sripanidkuchai B (2007) Evaluation of the anxiolytic and antidepressant effects of alcoholic extract of *Kaempferia parviflora* in aged rats. *Am J Agric Biol Sci* **2:**94–98.

Wattanathorn J, Pangpookiew P, Sripanidkulchai K, Muchimapura S, Sripanidkuchai K, and Sripanidkulchai B (2012) Aprodisiac activity of *Kaempferia parviflora. Am J Agric Biol Sci* **7:**114–120.

Wen X, Walle UK, and Walle T (2005) 5,7-Dimethoxyflavone downregulates CYP1A1 expression and benzo[a]pyrene-induced DNA binding in HepG2 cells. *Carcinogenesis* **26:**803–809.

Wudtiwai B, Sripanidkulchai B, Kongtawelert P, and Banjerdpongchai R (2011) Methoxyflavone derivatives modulate the effect of TRAIL-induced apoptosis in human leukemic cell lines. *J Hematol Oncol* **4:**52.

Yenjai C, Prasanphen K, Daodee S, Wongpanich V, and Kittakoop P (2004) Bioactive flavonoids from *Kaempferia parviflora. Fitoterapia* **75:**89–92.

Yenjai C, Sutthanut K, Sripanidkulchai B, Mungkhun N, Wongma C, Mukdasai S, and Kongkaew T (2007) Further studies of bioactive flavonoids from *Kaempferia parviflora. KKU Sci J* **35:**37–41.

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