CHAPTER III
RESEARCH METHODOLOGY

1. Chemicals

Acetonitrile (analytical and HPLC grade, LabScan® Asia Co., Ltd. Thailand, EMD Chemicals, USA), acetyl acetone (SD Fine-Chem Limited, India), ammonium acetate (Ajax Finechem Pty, Ltd, Australia), antipyrine (Fluka®, China), ascorbic acid (Carlo Erba Reagenti Spa, Italy), barium hydroxide (Sigma-Aldrich Co., USA), bovine serum albumin (BSA, Fluka®, Switzerland), capric triglyceride (Miglyol 810N, Sasol, Germany), carboxymethyl cellulose (CMC), castor oil (Sigma-Aldrich, India), copper sulfate (Carlo Erba Reagenti Spa, Italy), β-CD (Sigma-Aldrich, USA), dexamethasone (Fluka®, China), dichloromethane (commercial grade), diethylene glycol monoethyl ether (DGME, Sigma-Aldrich, USA), dipotassium hydrogen phosphate (Acros® Organics, USA, Ajax Finechem Pty, Ltd, Australia), dimethyl sulfoxide (DMSO, Labscan Asia Co., Ltd, Thailand), disodium hydrogen phosphate (Ajax Finechem Pty, Ltd, Australia), diethyl ether (RCI Labscan Limited, Thailand), ethanol (commercial grade: Liquor Distillery Organization Excise Department, Thailand; analytical grade: AnalaR® VWR International Ltd., England; and 95.0% spectrophotometric grade: Acros® A.C.S., USA), ethylene diaminetetra-acetic acid disodium salt (Asia Pacific Specialty Chemical Limited, Australia), erythromycin (Fluka®, USA), Folin reagent (Merck, Germany), formic acid (Fisher® Scientific, England and Fluka®, Germany), furosemide (Sigma-Aldrich, Italy), d-(+)-glucose solution (45%, Sigma-Aldrich, USA), HBSS with calcium and magnesium (Cellgro®, USA), HBSS without calcium and magnesium (Gibco® Invitrogen™, USA), heparin (Leo Pharmaceutical Products, Denmark), HEPES solution (HyClone®, USA), hydrochloric acid (Labscan Asia Co., Ltd, Thailand), hydroxypropyl-β-CD (HP-β-CD, Aldrich, Japan), 3-methylchrolanthrene (Sigma-Aldrich, USA), β-naphthofla vone, nicotinamide adenine dinucleotide phosphate (NADPH), 4-nitrocatechol (Sigma -Aldrich, USA), normal saline solution (NSS, A.N.B Laboratories Co., Ltd., Thailand), olive oil (Sigma-Aldrich, USA), ouabain octahydrate (Sigma- Aldrich, Italy), perchloric acid (Sigma-Aldrich, USA), phenobarbital (Sanofi Aventis, France),

p-nitrophenol (Carlo Erba Reagenti Spa, Italy), poly ethylene glycol 400 (PEG 400, S. Tong Chemical Co., Ltd., Thailand and Spectrum, USA), polyoxyethylene castor oil (Cremophor® EL, Sigma-Aldrich, Germany), potassium chloride (BDH AnalaR, VWR International Ltd., England), potassium dihydrogen phosphate (Asia Pacific Specialty Chemical Limited, Australia), potassium phosphate monobasic sodium hydroxide buffer solution (0.05 M, Fisher® Scientific, USA), propylene glycol (Namsiang Co., Ltd., Thailand and Spectrum, USA), resorufin ethyl ether, resorufin methyl ether, resorufin pentyl ether, resorufin standard (Sigma-Aldrich, USA), sodium carbonate (Ajax Finechem Pty, Ltd, Australia), sodium dihydrogen phosphate (Acros Organics, USA), sodium dithionite, sodium chloride (Ajax Finechem Pty, Ltd, Australia), sodium hydroxide (Labscan Asia Co., Ltd, Thailand, Acros Organics, USA), sodium potassium tartate, tris (hydroxymethyl)-aminomethane, soybean oil (Spectrum, USA), triglyceride of coconut oil (Neobee® M-5, Spectrum, USA), Tween20, Tween40, Tween60, Tween80 (Sigma-Aldrich, USA), verapamil HCl (Sigma-Aldrich, China), and zinc sulfate (Asia Pacific Specialty Chemical Limited, Australia).

2. Materials

Beakers, C8 column 5 µm 4 x 250 mm (Hypersil, Agilent®, USA), C18 column 5 µm 2 x 50 mm (Chromolith, EMD chemicals, USA), conical tube 15 and 50 ml, cottons, cryo 1°C freezing container (Nalgene™, USA), feed needle, filter papers No.1, forceps, funnel, glass column, guard column (C18, All-Guard™ Grace Davison Discovery Science, IL, USA), IV catheter, metabolic cages, micropipette, microtube 1.5 and 2.0 ml, needles, nylon syringe filters 0.45 µm (Fisherbrand®, Ireland), operating set, pipette, round bottom flasks, stirring rods, syringes, thin layer chromatography (TLC) silica gel 60 F_{254} aluminium sheets, tips

3. Instruments

Centrifuge (Kubota® Series 5922, Japan), incubator (Memmert®, Germany), high performance liquid chromatography (HPLC, Agilent® 1200 series VWD detector with quaternary pump, Germany and Shimadzu®-UFLC DAD detector with quaternary pump, Japan), homogenizer (Janke & Kunnel IKA® Labortechnik RE16,

Germany), Liquid chromatography-Mass spectrometer (LC-MS, TSQ Quantum Access, Thermal Scientific), lyophilyzer (Christ® Amma 1-16 LS,Germany and VisTis adVantage wizard 2.0, USA), mixer (Vortex mixer® VM-300, Taiwan), precision balances (Mettler Toledo® Dragon204, Switzerland), water bath (Julabo® SW22, Germany), liquid scintillator analyzer (Tri-Carb 3110 TR, PerkinElmer®, USA), rotary evaporator (Eyela® N-1000 and Digital water bath SB-1000, Japan), spectrofluorometer (PerkinElmer® LS-50B, UK), ultracentrifuge (Soevall® UltraPro80, Dupont, USA), ultrasonic bath (Elma® S60, Germany), UV cabinet (Spectroline® CM-10, USA), UV-VIS spectrophotometer (Shimazu® UV-1700, Japan), water-bath shaker (New Brunswick Scientific Classic series C76, NJ),

4. Preparation of KP crude extract

KP rhizomes (Romkaou strains) were collected from cultivable source at Phurue, province of Loei, Thailand. The extraction process was followed the Pretty Patent, Thailand No. 4048. The rhizomes were sliced to small pieces, dried at 45^{o}C, and grinded by using grinding machine to get the dried powder. Dried powder of KP rhizomes was extracted by using maceration techniques with 95% ethanol (base on non-polarity of methoxyflavones) for three days with occasionally stirring. After filtration, filtrate was evaporated to discard the solvent. The crude extract was obtained by freeze-dried method using lyophilizer.

5. Isolation of major compounds in KP crude extract

Methoxyflavones from KP, including DMF, TMF, and PMF, were isolated by using column chromatography and TLC as previously described by Sutthanut et al. (2007). Briefly, column chromatography was carried out on silica gel 60. Ethyl acetate in hexane and methanol in ethyl acetate were sequentially applied into the column by gradually increasing of the polarities of the solvents. Then, each fraction from the column was collected, evaporated, and detected using TLC technique under UV cabinet. The fractions having the same TLC pattern were pooled and dissolved with optimal solvent, which can volatile and dissolve the compound. The mixture was placed at room temperature until forming crystals. After that, the crystals were

separated from the mixture and checked for the purity by using TLC and HPLC methods.

6. Pharmacokinetics and bioavailability study

6.1 Animals

Male Wistar rats were used at 6-8 weeks of age and 270-310 g of BW. Animals were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. They were housed at ambient temperature of 22 ± 2^{o}C with 12 h light/dark cycles. The animals were looked after for seven days before the experiment and received the standard rat food (C.P. rat feed 082, S. WT Co Lid, Thailand) and water. All the procedures were carried out with the approval of the Khon Kaen University ethics committee for animal research at No. AE.KKU.13/2552 following internationally accepted principles for laboratory use and care of European community (EEC directive of 1986; 86/609/EEC).

KP crude extract formulation was prepared at the dose of 250 mg/kg BW which that dose showed pharmacological activity in rat namely antidepression and cognitive enhancement (Hawiset et al., 2011). Moreover, this dose was able to detect methoxyflavones level in blood, and can be intravenously administrated. Co-solvent, including propylene glycol, PEG 400, and ethanol were selected to increase solubility and can be injected through intravenous route.

Rx. KP crude extract formulation (KP solution)

KP crude extract		2.5 g
Propylene glycol		2.8 ml
PEG 400		3.5 ml
Ethanol		0.2 ml
DI water	qs to	10 ml

Rats were divided into two groups of 10 animals each. The rats were intravenously through tail vein and orally administered 250 mg/kg BW of KP solution for IV-group and oral-group, respectively. Blood samples (200 µl) were taken from tail vein before dosing and at 10, 20, 30 min, 1, 2, 6, 12, and 24 h after administration to the heparinized microtube and kept at -20^{o}C before analysis.

6.2 Blood extraction

6.2.1 Optimization of extraction method

The blood extraction was optimized by liquid-liquid extraction in various solvents namely ethanol, methanol, dichloromethane, acetonitrile, and hexane. Blank blood samples were added with 0.10 g of KP crude extract containing 10.72 µg of PMF/g of KP crude extract, 10.72 µg of TMF/g of KP crude extract, and 3.67 µg of DMF/g of KP crude extract. After that, the various solvents were added to the mixture of blood and KP crude extract. Then, the mixture was mixed, sonicated, and centrifuged at 2,000xg for 2 min. The pellet was further extracted for two times. The supernatant was pooled and determined for the methoxyflavones concentration, and then the % recovery was calculated. The solvent that showed the highest of % recovery was selected for blood extraction.

6.2.2 Extraction

The 200 µl aliquot of rat blood sample was added with 1 ml of selected solvent. The mixture was vortex-mixed for 2 min and sonicated for 5 min. After centrifugation at 2,000xg for 2 min, the supernatant was removed to clean microtube and evaporated to dryness. The residue was reconstituted with 200 µl of acetonitrile which was mobile phase in HPLC system. Then, the solution was filtered through 0.45 µm-syringe filters and analyzed by using HPLC method.

6.3 Optimization of chromatographic conditions

The chromatographic system consisted of C8 column which was controlled at 20^{o}C, VWD detector (335 nm) by Agilent® 1200 Series with quaternary pump and automate injector for quantitative analysis. Mobile phases were acetonitrile (A) and 0.5% formic acid (B) with gradient system. Linear gradient elution was employed within 55 min running time. The gradient elution was as follows: 0-10 min: 29% A at flow rate from 1.0 to 0.8 ml/min, 10-20 min: 29% A to 46% A at flow rate from 0.8 to 0.6 ml/min, 20-40 min: 46% A at flow rate from 0.6 to 0.8 ml/min, and 40-55 min: 46% A to 29% A at flow rate 0.8 to 1.0 ml/min.

The chromatography systems were developed to obtain an analytical method with desirable characteristics including adequate resolution of separation, high sensitivity, selectivity, accuracy, and precision. Calibration curves were performed by analyzing mixture of methoxyflavones at five concentrations. The limit

of detection (LOD) and limit of quantitation (LOQ) which were concentrations at S/N ratio of about 3 and 10, respectively were assayed by using the diluted mixture of methoxyflavones. The precision and accuracy were determined in three validation batches on three different days, each batch containing three replicates of mixture of methoxyflavone samples at several concentration levels.

6.4 Pharmacokinetic parameters calculation

The parameters were calculated by Phoenix® WinNonlin® program (Pharsigth Corporation, USA) based on one-compartment model for IV-bolus and per oral. Bioavailability (F) which is defined as the fraction of unchanged drug that reaches the systemic circulation after the administration of a drug dose is calculated from AUC of oral route and AUC of intravenous route as shown in this equation.

$$F = \frac{AUC_{oral} / Dose_{oral}}{AUC_{iv} / Dose_{iv}}$$

7. Organ distribution study

7.1 Animals

Wistar rats (male, 250-300 g) from the National Laboratory Animal Center, Mahidol University, Thailand were used. After 7-day acclimatization period, animals were housed 3-4 per cage in room designed to maintain adequate conditions at 22 ± 2°C with 12 h light/dark cycles. The animals were freely fed with the standard rat food (C.P. rat feed 082, S. WT Co Lid, Thailand) and water. All animal procedures were in accordance with the regulations of Khon Kaen University ethics committee for animal research at No. AE.KKU.13/2552 following internationally accepted principles for laboratory use and care of European community (EEC directive of 1986; 86/609/EEC).

The rats were divided to four groups of seven animals each. Since the methoxyflavones concentrations in several organs were lower than the LOQ when administration of KP at the dose lower than 750 mg/kg BW and single administration of KP at dose of 2 g/kg BW was safe in rats (Chivapat et al., 2004). Therefore, 750 mg/kg BW of KP solution (prepared as described below) was orally administered.

Then, the rats in each group were killed by cervical dislocation at several time points after administration. In order to clean the blood out from body, NSS was perfused into left ventricle of heart and the blood vessel at right atrium of heart was cut. Finally, the major organs including liver, lung, kidney, testes, and brain were removed out and weighed. The organs were kept at -20^{o}C before further analysis.

Rx. KP solution

KP crude extract		7.5 g
Propylene glycol		2.8 ml
PEG 400		3.5 ml
Ethanol		0.2 ml
DI water	qs to	10 ml

7.2 Organ extraction

Optimization of the method was performed as described in Section 6.2.1. Blank organs were added with 0.05 g of KP crude extract containing PMF, TMF, and DMF at 5.56, 5.56, and 1.88 µg/g of KP crude extract.

The whole organs were chopped to small pieces, and then homogenized and mixed in phosphate buffer solution (PBS) pH 7.4 by using homogenizer. Then, the mixture was added with selected solvent. After centrifugation at 2,000xg for 5 min, the supernatant was collected and evaporated to dryness. The samples were extracted for three times. The residue samples were reconstituted with 200 µl of acetonitrile which was mobile phase and filtered via 0.45 µm-syringe filter. PMF, TMF, and DMF concentrations in several organs were determined by HPLC systems as described in Section 6.3.

8. Effect of KP crude extract on cytochrome P450 enzymes

8.1 Animals

Male ICR mice at 6-8 weeks of age and 25-30 g of BW were purchased from the National Laboratory Animal Center, Mahidol University, Saraya, NP, Thailand. Animals were housed at ambient temperature of 22 ± 2^{o} C with 12 h light/dark cycles and free access of food (C.P. Mice feed 082, S. WT Co Lid, Thailand) and *ad libitum* water. All animals were acclimated for seven days prior to the experiment. The protocols of animal housing and treatments used were approved

by the Khon Kaen University Ethics Committee for animal research (Approval no. AE.KKU.31/2550) following internationally accepted principles for laboratory use and care of European community (EEC directive of 1986; 86/609/EEC).

Mice were divided into 12 groups of 10 animals each. Group 1-5 were positive control groups. Group 1 orally received 100 mg/kg of 3-methylchrolanthre in olive oil in order to induce CYP1A1 activity for 3 days (Jarukamjorn et al., 2006), group 2-5 were intraperitoneally administered with 80 mg/kg of β-naphthoflavone in DI water (CYP1A2, Bray et al., 2002), 100 mg/kg of phenobarbital in DI water (CYP2B, Jarukamjorn et al., 2006), 2.5 g/kg of 35% ethanol in DI water (CYP2E1, Wang et al., 2007), and 75 mg/kg of dexamethasone in DI water (CYP3A, Bray et al., 2002) for 7 days, respectively. Group 6-8 were orally administered of 250 mg/kg of KP extract dissolved in 2% CMC for 7, 14, and 21 days, respectively. Group 9-12 were negative control groups that were orally administered 2% CMC for 7, 14, 21 days, and untreated, respectively. In this study, propylene glycol, PEG 400, and ethanol which were the solvent in KP solution may influence on CYP450 enzyme activity. Thus, CMC was selected as vehicle for KP crude extract. The mice were then cervically decapitated 24 h after the last treatment. The liver was collected for microsome preparation.

8.2 Hepatic microsome preparation

Hepatic microsome preparation was adapted from Jarukamjorn et al. (2006) and Dudda and Kurzel (2006). Before removal of liver, it was cleaned by perfusion with 1.15% KCl via inferior venacava. The samples were cut to small pieces by scissor and put in the cool solution of 1.15% KCl, then homogenized at 2,000 cycle/min and centrifuged at 10,000xg at 4^{o}C for 10 min. The supernatant was further centrifuged at 104,000xg at 4^{o}C for 60 min. The microsomal fraction was obtained by suspending the pellet in Tris-alkylresorufin buffer (pH 7.8) for the alkylresorufin *O*-dealkylation assay, PBS pH 6.8 for the *p*-nitrophenol hydroxylation assay, or PBS pH 7.4 for the erythromycin *N*-demethylation assay. In order to obtain the appropriate concentration of microsomal protein, the microsome was further diluted by using cool distilled water. Finally, the protein concentrations of the microsome were determined and stored at -80^{o}C until used.

8.3 Determination of protein concentration

Protein concentrations in microsomes were determined as previously described (Lowry et al., 1951) by using BSA as a standard. Microsome samples were added with color forming reagent which composed of 2% w/v Na_2CO_3 in 0.1 N NaOH, 1% w/v $CuSO_4.5H_2O$, and 2% sodium potassium tartate and incubated at room temperature for 10 min. Then, 0.3 ml of 1 N folin reagent was added and incubated at room temperature for 30 min. The absorbance at 650 nm was measured by using UV-VIS spectrophotometer. The protein concentration in microsome samples were calculated from BSA standard curve.

8.4 CYP450 enzymes activities study

8.4.1 Alkylresorufin *O*-dealkylation (AROD) assay was used for determining CYP1A1, 1A2, and 2B enzymes activity following Sakuma et al. (1999). The substrates were ethoxyresorufin, methoxyresorufin, and pentoxyresorufin for CYP1A1, 1A2, and 2B enzymes, respectively. Mixtures of 0.1 M Tris buffer pH 7.8, distilled water, substrates (10 mM), microsomal protein (1 mg), and 10 mM NADPH (total volume = 3.52 ml) were incubated in a shaking water bath at 37^{o}C and the fluorescent intensity was measured at excitation wavelength 530 nm, and emission wavelength 585 nm at every 30 s for 30 min by using spectrofluorometer. Distilled water was used as a blank. The enzyme specific activities were expressed as pmole of resorufin/min/mg protein by comparing with resorufin standard curve following the equation.

$$\text{AROD activity} = [(\Delta Fs - \Delta Fb) \times (500/Fstd)] \div \text{microsome concentration (mg/ml)}$$

When; ΔFs is the slope of curve between time and fluorescent intensity of sample,

ΔFb is the slope of curve between time and fluorescent intensity of blank

Fstd is fluorescent intensity of alkoxyresorufin

8.4.2 *p*-Nitrophenol hydroxylation (PNPH) assay was used to determine CYP2E1 activities (Kim et al., 1988). Reactions were initiated by adding NADPH to the mixture of substrate (10 mM), microsomal protein (1 mg), ascorbic

acid (1 mM), *p*-nitrophenol (490 µM), and 0.1 M PBS pH 6.8 (total volume = 1 ml) at 37°C for 10 min. After that, 0.6 N perchloric acid was added to stop reaction. Then the mixtures were centrifuged at 3,000xg for 5 min. Sodium hydroxide (10 N) was added into the supernatants. Finally, the absorbance at 546 nm was determined by using UV-VIS spectrophotometer. The enzyme specific activities (mmole/min/mg protein) were determined from *p*-nitrocatechol standard curve.

8.4.3 CYP3A activity was determined by using erythromycin *N*-demethylation (ERDM) reaction (Bray et al., 2002). Mixtures of microsomes (1 mg), 0.1 mM EDTA, 0.4 mM erythromycin, and 0.1 M PBS pH 7.4 (total volume = 1 ml) were incubated in shaking water bath at 37°C for 2 min. Then, 10 mM NADPH was added and incubated at 37°C for 30 min. Zinc sulfate (15%, 0.33 ml) solution was added into the mixtures. Barium hydroxide (0.33 ml) was added in five min later. After that, the mixtures were centrifuged at 10,000xg for 3 min. Nash reagent containing 30% of ammonium acetate and 0.4% acetyl acetone (0.33 ml) was further added into the mixtures. Then, the absorbance of sample was determined at 415 nm by using UV-VIS spectrophotometer. The enzyme specific activities (µmole/min/mg protein) were determined from formaldehyde standard curve.

8.4.4 Determination of CYP450 content

CYP450 contents were determined from the CO-difference spectra of the liver microsomes by using UV-VIS spectrophotometer, as described by Omura and Sato (1964). When a CYP450 attaches with CO, Fe^{3+} (ferric) in CYP450 was changed from oxidized to reduced form (Fe^{2+}) by using sodium dithionite as a catalyst. CYP450-CO complex can absorb the light at 450 nm.

Microsomal protein was diluted by 0.22 M Tris buffer pH 7.4 containing 22.22% glycerol. The solution was scanned at 400 – 500 nm by UV-VIS spectrophotometer for adjust baseline. Then, the mixture in cuvette was bubbled with CO for 1 min at rate of 1 bubble/sec and a few milligrams of sodium dithionite were added. A 30 sec later, the mixture was rescanned at 400 – 500 nm. CYP450 contents were calculated from following formula as nmol/mg protein.

$$\text{P450 content (nmol/mg protein)} = \frac{[(A450 - A490)/91] \times 1{,}000 \times \text{dilution factor}}{\text{Microsomal concentration (mg/ml)}}$$

When: A450 = absorbance at 450 nm, A490 = absorbance at 490 nm

8.4.5 *In vitro* effect of KP crude extract

8.4.5.1 IC_{50} and inhibition constant (Ki) determination

A non-induced hepatic microsome of untreated mice was used in this study. IC_{50} and Ki values were evaluated using fixed concentrations of substrates including 10 mM of resorufin ethyl ether, 10 mM of resorufin methyl ether, 10 mM of resorufin pentoxyl ether, 490 µM of *p*-nitrophenol, and 0.4 mM of erythromycin for CYP1A1, CYP1A2, CYP2B, CYP2E1, and CYP3A, respectively. Varying amounts of KP crude extract with concentrations ranging from 0.071-5.682 µg/ml for CYP1A1, CYP1A2, and CYP2B, 0.25-20.00 µg/ml for CYP2E1, and 1.00-20.00 µg/ml for CYP3A were added to a series of incubation tubes in a shaking water bath at 37^{o}C. The reaction mixtures were incubated with NADPH (10 mM) for 10 min for CYP1A1, CYP1A2, CYP2B, and CYP2E1, and 30 min for CYP3A. Then, the CYP450 activities were measured as previous described. A negative control, containing all components of the incubation mixture except the test compound was processed in parallel. The IC_{50} value, which is at the concentration of KP crude extract yielding 50% reactivity, was determined using linear regression analysis of the plot of the percentage of the activity remaining after inhibition versus log concentration of KP crude extract. The Ki was determined from x-intercept of Dixon plot (Sawhney and Singh, 2005).

8.4.5.2 Investigation of Michaelis-Menten kinetic parameters, and type of reaction

Michaelis-Menten kinetic parameters including Vmax and Km were determined for CYP450-catalyzed KP crude extract in non-induced mouse liver microsomes. A fixed amount of KP crude extract (5 µg for CYP1A1, CYP1A2, and CYP2B and 10 µg for CYP2E1 and CYP3A) was added to the reaction mixture. The CYP450 activities were measured as previously described. Serial concentrations of substrates were varied from 20-100 pmole for both resorufin ethyl ether and resorufin methyl ether for CYP1A1 and CYP1A2. The following substrates were used: resorufin pentoxyl ether (10-70 pmole) for CYP2B, *p*-nitrophenol (5-80 µmole) for CYP2E1, and erythromycin (20-120 nmole) for CYP3A. Control incubations did not contain any test compound. Vmax and Km values were obtained

from the 1/y- and 1/x-intercepts of a Lineweaver-Burk plot after incubating at 37^{o}C for 10 min for CYP1A1, CYP1A2, CYP2B, and CYP2E1, and 30 min for CYP3A.

9. Excretion study

9.1 Animals

Male Wistar rats (6-8 weeks old) weiging 250-300 g were purchased from the National Laboratory Animal Center, Mahidol University, Saraya, NP, Thailand. All animal procedures were approved and under the regulations of the Khon Kaen University Ethics Committee for animal research (Approved no. AE.KKU.43/2552) following internationally accepted principles for laboratory use and care of European community (EEC directive of 1986; 86/609/EEC). The animals had free access to food and water *ad libitum* and maintained at 22 ± 2^{o}C with 12 h light/dark cycles. They were allowed to acclimatize for a week prior to their use in the study.

The oral dosage was 750 mg/kg BW of KP solution (n=10). The KP solution was prepared as described below. After administration, the rat was put in the metabolic cage. Then, the urine and feces samples were collected at every six hours within 72 h and kept at 4^{o}C before analysis.

Rx. KP solution

KP crude extract		7.5 g
Propylene glycol		2.8 ml
PEG 400		3.5 ml
Ethanol		0.2 ml
DI water	qs to	10 ml

9.2 Feces and urine extraction

Optimization method was performed as described in Section 6.2.1. Blank urine and feces samples were added with 0.06 g of KP crude extract (containing PMF, TMF, and DMF at 5.32, 5.32, and 1.85 µg/g of KP crude extract, respectively).

Feces samples were pre-mixed with PBS pH 7.4 Urine samples and the mixture of feces samples were extracted by liquid-liquid extraction using selected solvent (3 volumes of weight). The samples were mixed, ultra-sonicated, and

centrifuged at 2,000xg for 2 min. The supernatant was collected and evaporated in water bath at temperature of 50°C to dryness. Finally, the precipitate was reconstituted with 1 ml of acetonitrile which was the mobile phase and filtrated through 0.45 µM of syringe filter before injecting to HPLC systems as described in Section 6.3 for quantitative and LC-MS for qualitative. The concentrations of methoxyflavones in urine and feces were displayed as micrograms of methoxyflavone/gram of urine or feces sample.

9.3 Optimization of LC-MS systems

Metabolites in urine and feces samples were identified by using LC-ESI-MS (Thermo Scientific, TSQ quantum). Full scan MS and MS/MS modes were simultaneously used to confirm the metabolites. The samples were analyzed in Full scan as positive mode for mass screening. The mass scan began at 30 to 1,000 m/z. The unknown masses were further determined in MS/MS mode. Collision energy was optimized ranged 10 to 70 eV to define fragment ions of the metabolites. LC system composed of acetonitrile (A) and 0.5% formic acid in water (B) as the mobile phase running in 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 ul. The analytical column was a Chromolith®, EMD C18 column (2.0x50 mm, 5 µm). Xcaliber® 2.0.6 program (Thermo Fisher Scientific. Inc. 1998-2007) was used to integrate the chromatogram.

10. Products development for oral bioavailability enhancement of KP crude extract

10.1 Optimization of HPLC system

HPLC analysis in all studies of product development was performed by using Shimadzu®-UFLC DAD detector. C18 column (5 µm, 2 x 50 mm) was eluted at 25°C with a linear gradient from 5% of acetonitrile (A) and 95% of 0.5% formic acid in water (B) to 50% A and 50% B in 60 min at flow rate of 0.2 ml/min. Injection volume was 20 µl. Methoxyflavones were detected by using DAD detector at 335 nm. HPLC system was validated as previously described in section 6.3.

10.2 Pre-formulation study

10.2.1 Solubility test

KP crude extract was determined for its solubility properties in various solvents and co-solvents including water, ethanol, 0.01 M HCl, 0.1 M NaOH, 0.9% normal saline, 0.2 M PBS pH 7.4, PEG 400, propylene glycol, and glycerin at 25^{o}C. The solubility test was carried out following Tietgen (2006). The crude extract was weighed, and then the solvent or co-solvent was added until the saturation visibly occurs. The mixture was centrifuged and the supernatant was later collected. Methoxyflavones in supernatant were extracted by liquid-liquid extraction using acetonitrile and then were assayed by HPLC technique. Methoxyflavones (PMF, TMF, and DMF) were used as the markers for determination of the solubility value. All experiments were performed in triplicate.

10.2.2 Determination of partition coefficient (logP)

LogP was determined by using shake flask method that was adapted from Tietgen (2006) and the European Commission Joint Research Centre Institute for Health and Consumer Protection. In order to determine a logP, equilibrium between all interacting components of the system must be achieved, and the concentrations of the substances dissolved in the two phases must be determined. Octanol and water are standard solvents for the equilibrium experiments. KP crude extract was added into the equilibrium mixture solvents between octanol and water. Then, the mixture was quickly rotated through 180^{o} for 100 times in five min, and placed for 24 hours at 25^{o}C to separate two phases. Finally, each phase was extracted by liquid-liquid extraction using acetonitrile and analyzed by HPLC technique. All experiments were performed in triplicate.

10.3 Preparation of KP-SMEDDS

10.3.1 Determination of KP solubility in oils, surfactants, and co-surfactants

In order to identify the suitable compositions of SMEDDS, the solubility of KP crude extract in various oils, surfactants, and co-surfactants were performed. Each experiment was performed in triplicate. An excess amount of KP crude extract was added to 5 ml of the vehicles in 10-ml bottle. The mixtures were shaken at 25^{o}C for 48 h by using water-bath shaker (New Brunswick Scientific

Classic series C76, NJ). After reaching equilibrium, each bottle was centrifuged at 112xg for 5 min and the excess insoluble was discarded by filtration using 0.45 μm syringe filter. The concentrations of PMF, TMF, and DMF in soluble part were further quantified by using HPLC as previously described.

10.3.2 Compatibility tests

In order to determine the compatibility between surfactant and oil, oil was dropped into 1 ml of surfactant. The mixture was gently mixed for 5 min by using vortex mixer. Added amount of oil and appearance of resulting solution were recorded. Under the condition of clearance and transparency of resulting solution, the more amount of added oil showed the better compatibility with surfactant (Cui et al., 2009). Three replicate assessments were carried out.

In addition, co-surfactants were screened on the basis of their compatibility with the mixed system of selected oil and surfactant solution. The selected oils were dropped to the mixture of selected surfactant and co-surfactants. The added amounts of oils were recorded at the mixture becoming turbid. Under the condition of clearance and transparency of resulting solution, the more amount of added oil showed the better compatibility with surfactant and co-surfactant (Cui et al., 2009).

10.3.3 Pseudo-ternary phase diagram study

Pseudo-ternary phase diagrams were constructed by using water titration method in order to identify the self-emulsifying regions and optimize the concentration of surfactants, co-surfactants, and oils. The ratios of surfactant and co-surfactant were varied. Surfactant was mixed with co-surfactant in fixed weight ratios (1:1 and 2:1 weight ratios). The formulations (S1-S7) as shown in Table 6 were prepared for constructing the pseudo-ternary phase diagrams. For each phase diagrams at a specific ratio of surfactants and co-surfactants, aliquots of each surfactant-co-surfactant were mixed with oil. Then, each mixture was titrated with water under magnetic stirring drop by drop until the mixture become clear at a certain point. The concentrations of the components were recorded to complete the pseudo-ternary phase diagram. The contents of oil, surfactant, and co-surfactant which showed most stable after formation and broadest self-emulsifying regions with using

small amount of surfactants were selected. Three replicate assessments were performed for each mixture.

Table 6 SMEDDS developed formulations

Formulation codes	Surfactants	Co-surfactants	Oils
S-1	Cremophor® EL	propylene glycol	Miglyol 810N
S-2	Cremophor® EL	propylene glycol and ethanol	Miglyol 810N
S-3	Cremophor® EL	propylene glycol	triglyceride of coconut oil
S-4	diethylene glycol monoethyl ether	propylene glycol	Miglyol 810N
S-5	Tween 80	Ethanol	Miglyol 810N
S-6	Tween 80	Ethanol	triglyceride of coconut oil
S-7	Tween 80	propylene glycol and ethanol	triglyceride of coconut oil

10.3.4 Preparation of KP-SMEDDS formulations

A series of KP-SMEDDS formulations were prepared with varying ratio of selected oil (15-30%), and the mixture of surfactant and co-surfactant (2:1, 70-85%). KP crude extract with accurate weighed (0.1 g) was added to the vial and mixed by gentle stirring and vortex mixing until a homogeneous mixture formed. The formulations were further analyzed for droplet size, self-emulsification, and precipitation assessment. All experiments were performed in triplicate.

10.3.5 Droplet size analysis

KP-SMEDDS containing 0.25 mg of KP crude extract (10 µl) was gentle dispersed with 1 ml of DI water (filtered via 0.2 µm syringe-filter before used) resulting in the formation of the microemulsion.. The microemulsion (1 ml) was loaded in cuvette and measured its particle size by using Malvern Instruments

Zetasizer Nano series, USA. Blank SMEDDS was used to evaluate for the drug-loaded on SMEDDS.

10.3.6 Self-emulsification and precipitation assessment

KP-SMEDDS (4 ml) containing 1.0 g of KP crude extract was added in 40 ml of DI water at room temperature. After that, the microemulsion was visually assessed on the speed of emulsification, clarity, and apparent stability.

10.4 Preparation of KP-CD complex

10.4.1 Phase solubility study

Phase solubility diagram was conducted in water followed the previous reported by Higuchi and Connors (1965). This diagram is useful for studying inclusion complex of poorly water-soluble drugs with CD in water, assessing the effect of the CD on the apparent solubility of the drug, and giving the stoichiometry of the equilibrium. Furthermore, the diagram indicates which derivatives of CD can dissolve the drug. In this study, β-CD and HP-β-CD were used. The excess amount of KP crude extract was added to 20-ml glass vials containing various concentrations of β-CD (0-30 mM) or HP-β-CD (0-60 mM) in water. Each experiment was carried out in triplicate. The mixtures were shaken at 30^{o}C until equilibrium (5-7 days). Then, the mixtures were centrifuged, and then the supernatant was filtered through 0.45 μm membrane filter in order to discard insoluble drugs. The filtrate was analyzed for drug concentration by using HPLC technique. Finally, the dissolved methoxyflavones concentrations were plotted with CD concentrations. The apparent binding constants (Kc) of KP-CD complexes were calculated from the slope and intercept of the straight lines of the phase solubility diagrams according to the following equation:

$$Kc = \frac{(slope)}{So\ (1\text{-}slope)}$$

where So is the drug solubility in the absence of CD

10.4.2 Preparation of KP-CD complex

KP-CD complex was prepared by using freeze-drying technique (Wen et al., 2010). Stoichiometric quantities of KP and CD were 1:1 mole ratio which was obtained from phase solubility diagrams. Three replicate analyzes were carried out for each formulation. KP crude extract with known flavonoids contents, was dissolved in ethanol to facilitate its solubility. Then, the CD solution

(equal mole to mole of PMF, TMF, and DMF) in water was added. The mixture was gentle stirred for 24 h. The ethanol was evaporated out. The residue was reconstituted with water and the insoluble KP was separated out by filtration. The soluble KP was keep at -80^{o}C for 12 h. Finally, KP-CD complex was obtained by using freeze-dryer (VisTis adVantage wizard 2.0, USA). The complexes were further characterized whether they were inclusion complex or physical mixture by using DSC, and then evaluated for the physical properties and drug absorption.

10.4.3 Preparation of physical mixture

The equal molar of KP crude extract and CD were gentle mixed in mortar and pestle.

10.4.4 Differential scanning calorimetry (DSC)

KP-CD complex, physical mixture, KP crude extract, and CD were weighed (40 mg of each) and sealed in the aluminum crimped pan. DSC thermal curves were recorded by using thermal analyzer equipped with a differential scanning calorimeter (DSC-6 Perkin Elmer, USA). Measurements were made in triplicate. The system of DSC composed of holding at 25^{o}C for 1.0 min, heating from 25^{o}C to 300^{o}C at 10^{o}C/min, holding at 300^{o}C for 1 min, finally cooling from 300^{o}C to 25^{o}C at 40^{o}C/min. The total run of each sample was 60 min.

10.5 Evaluation of physicochemical properties and drug absorption

10.5.1 *In vitro* dissolution study

In order to compare the dissolution behaviors with KP crude extract, KP-SMEDDS, and KP-CD complex were tested *in vitro* dissolution by using USP30 Dissolution apparatus II with paddle. The formulations were filled in hard gelatin capsules No.0. 0.2 M PBS pH 6.8 and 0.1 N HCl solutions containing 0.5% of Tween 20 were used as medium and were controlled at 37 ± 0.5^{o}C. Six replicate assessments were performed for each formulation in each of the media. The paddles were rotated at 100 rpm. During the release studies, the aliquot of 5 ml was withdrawn at 0, 10, 20, 30, 40, 50, and 60 min. The removed volume was immediately replaced each time with 5 ml of fresh medium at 37 ± 0.5^{o}C. Then, the samples were injected to HPLC. The pH of each media was measured both at before and after the experiments.

10.5.2 Stability study

To confirm the stability of the developed products, KP-SMEDDS and KP-CD complex, the formulations were stored at 4°C (RH = 70±5%), 25°C (RH = 75±5%), and 40°C (RH = 75±5%) for three months in sealed glass vial and protected from light. Droplet size, phase separation, drug precipitation, and methoxyflavones contents were determined at 0 day, 2 weeks, 1, 2 , and 3 months for KP-SMEDDS. DSC analysis to confirm the inclusion complex and methoxyflavones contents were determined at several time points for 3 months for KP-CD complex. All experiments were performed in triplicate.

10.5.3 *In vitro* KP absorption study by using Caco-2 cells

Caco-2 cells were cultured in minimum essential medium with Eargle's salt and L-glutamine supplemented with 10% of fetal bovine serum, 1% of non-essential amino acid, and 1% of Antibiotic-Antimycotic at 37°C in 5% CO_2-incubator. The cells were passaged by using trypsin-EDTA solution after reaching 90% confluency, and then plated at a 1:5 ratio in 75cm^2-tissue culture flask (Costar®).

To prepare the Caco-2 cells for transport experiment, the cells (passage number 27-35) were seeded onto the apical site of Transwell tissue culture plate (12 mm diameter, 0.4 µm polycarbonate membrane, Costar®) at a concentration of 120,000 cells/ml for 0.4 ml. Medium was changed in the next day to remove any dead or non-adherent cells. The old cell culture medium was suctioned off and replaced with fresh medium both of apical (0.5 ml) and basolateral (1.5 ml) sites every two days. The cell monolayers after seeding for 21-28 days with Trans Epithelial Electric Resistance (TEER) values greater than 400 Ω.cm^2, which measured by using Epithelial Tissue Voltohmmeter and Endohm-12 chopstick electrode (World Precision Instruments), were further used for transport study (Lee et al., 2002).

In transport experiments, the cell monolayer was pre-incubated both of apical (0.5 ml) and basolateral (1.5 ml) sites with transport buffer (Hank's balanced salt solution with calcium and magnesium containing 25 mM D-glucose solution and 10 mM HEPES buffer) at 37°C at least 30 min. After that, the transport buffer was replaced by 0.4 ml of KP crude extract (80 µg/ml), KP-SMEDDS (80 µg/ml), KP-CD (500 µg/ml), ^{14}C-mannitol (0.20 µCi/ml), or antipyrine (100 µM) at time zero. KP crude extract was dissolve in DMSO, ensuring that total DMSO

concentration was less than 1% DMSO final concentration. All formulations were diluted in transport buffer. Three replicate analyzes were used for each test substances. The inserts were transferred to new 12-well plates (Costar®), containing 1.5 ml of transport buffer, at 0, 10, 30, 45, and 60 min in order to maintain the sink condition in basolateral site. The plates were kept in a 37°C incubator or on a hot plate set to 37°C all duration of the experiment. Finally, the methoxyflavones concentrations in each formulation in basolateral site at various time points were analyzed by HPLC techniques as previously described in Section 10.1. Antipyrine concentrations were determined by HPLC (Kimoto et al., 2009).For ^{14}C-mannitol activity, the sample (1 ml) from basolateral site was added with Ultima Gold™ Perkin Elmer (10 ml) and then the radioactive activity was analyzed by using a Liquid Scintillator (Tri-Carb 3110 TR Model no. A311000, Perkin Elmer, USA).

10.5.4 *In vivo* oral bioavailability study in rats

Male Sprague-Dawley rats (6-8 weeks of age, weighing 250-300 g) with surgically implanted jugular vein catheters were obtained from Charles River (a Division of Laboratory Animal Medicine, the University of North Carolina at Chapel Hill approved supplier). These came secured with wound clips, and needed re-securing after each blood draw to prevent the rat from damaging the catheter. All animal protocols were approved by Institutional Animal Care and Use Committee, the University of North Carolina at Chapel Hill (No. 11-254.0). The animals were divided into six groups of three animals each. They were housed singly as the ones having implanted catheters at 22±2°C with a controlled 12 h light-dark cycle. Test article was administered either by oral gavage or intravenously via tail vein injection under isoflurane anesthesia (2-5% vaporizer) according to schedules below.

- KP solution, IV, 165 mg/kg single dose
- KP solution, po, 250 mg/kg single dose
- KP-SMEDDS, IV, 250 mg/kg single dose
- KP-SMEDDS, po, 250 mg/kg single dose
- KP-CD complex, IV, 250 mg/kg single dose
- KP-CD complex, po, 250 mg/kg single dose

KP solution was prepared by dissolving the KP crude extract in 35% of PEG 400, 28% of propylene glycol, 2% of ethanol and water to complete 100% as previously describe in section 6.1.

Blood samples (200 µl) were drawn from jugular vein catheter at pre-dose and 5, 15, 30 min, 1, 3, 6, and 12 h in 1.5-ml heparinized microcentrifuge tubes to determine the blood concentration of the methoxyflavones by HPLC technique. After each blood drawn, catheter was flushed with 0.1 ml NSS and 0.1 ml Heparin Lock flush (10 units/ml) to maintain patency. All animals were euthanized at the end of the study by CO_2 with subsequent thoracotomy (open the chest cavity using sharp scissors or scalpel) to ensure that animals were dead.

Blood samples were extracted by liquid-liquid extraction using acetonitrile. The samples were injected (20 µl) to HPLC system. The methoxyflavones (PMF, TMF, and DMF) concentrations in blood were plotted against times. Pharmacokinetic parameters were calculated by using Phoenix® WinNonlin® program (Pharsight Corporation, USA). One-compartment model for IV-bolus and per oral modes were selected to calculate the pharmacokinetic parameters for IV and oral routes, respectively.

11. Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA and Independent T-test were utilized to compare the difference groups. The statistical significance was taken at P-value less than 0.05.