CHAPTER V
DISCUSSION

1. Pharmacokinetics of KP crude extract in rats

In this study, KP crude extract in 95% ethanol showed the high amount of three methoxyflavones including PMF (2.33%), TMF (3.11%), and DMF (2.11%). These methoxyflavones also possessed many pharmacological activities such as antimicrobial, anti-hyperuricemia, anti-cholinesterase, antimutagenicity, inhibition of P-glycoprotein, and chemoprotective agents. Therefore, PMF, TMF, and DMF were used as markers in pharmacokinetic study.

Pharmacokinetic parameters are beneficial to describe and predict information related to the efficacy and toxicity of drugs, therefore it is essential to study the pharmacokinetic. The understanding of pharmacokinetic is very important to provide the information on blood and tissue levels, optimum dosage regimen, correlation of drug concentration with pharmacological or toxicological activity, and drug interaction (Shargel et al., 2005).

Analysis method for biological samples namely blood, tissues, urine, and feces sample is one of the key steps of pharmacokinetic study. This HPLC method on condition as mentioned in the methodology was successful to determine the methoxyflavones in biological samples with adequate resolution of separation, high sensitivity, selectivity, accuracy, and precision. The extraction method of methoxyflavones using acetonitrile provided good recovery for biological samples in pharmacokinetic, organ distribution, and excretion studies. Acetonitrile used as extraction solvent in this study, could be not only the good solvent for methoxyflavones, water insoluble compounds, but it also acts as protein precipitating agent.

Methoxyflavones in KP crude extract were quickly absorbed into blood circulation reaching the maximum concentration within 1-2 h after oral administration following by broadly distributed in whole body with taken the pharmacological activity, finally gradually excreted out. These data support the previous report that DMF was found in plasma of rat that treated with DMF 5 mg/kg at Tmax of 1 h

(Walle at al. 2007). From blood concentration-time profile of methoxyflavones after IV administration of KP crude extract in this study, the methoxyflavones were rapidly cleared out from blood circulation. After the first 6 h of administration, PMF, TMF, and DMF were excreted. The compounds were found in urine and feces. In organ distribution study, methoxyflavones were found at highest levels in liver and kidney, suggesting their main elimination by liver and kidney.

According to pharmacokinetic parameters, 4-5 times of $t_{1/2}$ which were about 15, 25 and 30 h for PMF, TMF, and DMF, respectively, it is suggested that they might spend about 1-2 days to excrete out of the body. PMF was quickly excreted out of the body with the lowest of $t_{1/2}$ and highest clearance resulting in the lowest of Cmax and AUC which is used as a measure of the total amount of unchanged drugs reaching the systemic circulation after administration. The high Vd value of methoxyflavones suggested that the methoxyflavones might be broadly distributed into extravascular systems.

The results showed that oral bioavailability of methoxyflavones were low about 1-4%, suggesting that these methoxyflavones may be destroyed by the first-pass metabolism in gastrointestinal (GI) tract or liver, potential hydrolysis in GI tract, poor permeability via intestine, and binding with plasma protein. Moreover, the low water solubility and high lipophilicity of methoxyflavones (logP values were less than 2.0) may also impact on the low bioavailability.

Regarding the organ distribution study, the amount of methoxyflavones in KP crude extract can be determined in the tested organs including liver, lung, brain, kidney, and testes. Walle et al. (2007) reported that after oral administration, DMF was found mainly in liver much more than in plasma, kidney, and lung,. Tsuji et al. (2006) also reported that after administration of DMF to Atlantic killifish, DMF had highest concentration in liver and following in brain, intestine, gill, and skin, respectively. In current study, the methoxyflavones were detected in brain and testes indicating the ability of their penetration into the blood-brain and blood-testicle barriers. The highest amounts in brain and testes were possessed by TMF and DMF. These substances had probably lower water solubility and smaller molecules than those of PMF to give better permeability across blood-brain and blood testicle barriers.

After absorption and distribution, the methoxyflavones were further metabolized and elimination. In the study, the metabolite forms after administration of KP crude extract were firstly presented. The chemical structures of the metabolites were clarified by LC-MS both of full scan MS and MS/MS modes providing $[M+H]^+$ of parent compounds and daughter ions to obtain more structural information. Since the metabolites commonly maintain most of core structure of the drug, thus it is the key of structure elucidation of unknown metabolites. The identification of metabolites indicated that methoxyflavones in KP crude extract were mainly metabolized to be demethylated forms and were minor conjugated to be sulfate and glucuronides forms. The major parent compounds (PMF, TMF, and DMF) were also detected in urine and feces less than 2% of dose suggesting that the major forms of excretion were probably the metabolite forms. Tsuji et al. (2006) also found demethylation and glucuronidation of DMF after administration of DMF. The metabolites of KP crude extract can be found in rat feces and urine samples after administration of KP crude extract but it was not appear in blood samples and basolateral side in Caco-2 cell. These finding indicated that the metabolites of KP may not be metabolized from gastrointestinal tract but it might be metabolized via the metabolizing enzyme in liver. In addition, the undetectable level of metabolites in blood samples may indicate that the active substances possessing pharmacological activity of KP crude extract are probably the methoxyflavones especially PMF, TMF, and DMF.

In term of the effect of KP crude extract on CYP450 enzyme activities, mouse hepatic microsomes both *in vitro* and *in vivo* studies were performed in this study. CYP1A1, CYP1A2, CYP2B, CYP2E1, and CYP3A were selected for this study because of they are the main enzymes that play the important roles in metabolism of many drug. Moreover, there are many reports that these enzymes involve in drug interaction. The *in vitro* studies measured a direct effect of KP crude extract on CYP through binding the enzymes, while the *in vivo* study demonstrated its influence on CYP regulation, both of inhibition and induction. The results showed for the first time that KP crude extract had a significant impact on CYP-metabolizing enzymes.

The positive controls used in this study including 3-methylchrolanthrene, β-naphthoflavone, phenobarbital, ethanol, and dexamethasone showed significant induction of CYP1A1, CYP1A2, CYP2B, CYP2E1, and CYP3A activities,

respectively. The vehicle (2% CMC) that was used to suspend the KP crude extract did not affect the CYP450 contents, CYP1A1 and CYP3A activities in all durations of treatment, and CYP1A2, CYP2B, and CYP3A activities after 7- and 14-day of treatments. However, the vehicle slightly affected on CYP1A2, CYP2B, and CYP2E1 activities at 21-day of treatment. This is in consistent with the findings of Cantoni et al. (2003) who reported that using 1.6% CMC as a vehicle for St. John's wort (*Hypericum perforatum*) extract for 12-day treatment, did not affect CYP450 enzymatic activity. These findings implied the possible effect of long-term utilization of CMC as the vehicle. Moreover, the hepatic microsomal CYP450 contents of the mice after 7 and 21 days KP crude extract treatment were not significantly different from the vehicle control groups. However, the elevated levels of CYP 450 after 14 days of treatment suggested a time-dependent induction.

The KP crude extract met the criteria for a non-competitive interaction with CYP1A1 based on the decrease of Vmax and unchanged Km. The high Ki value and low IC_{50}, as well as the high Km value was indicative of the weakness of the interaction between the KP crude extract and CYP1A1. In the *in vivo* study, CYP1A1 activity was markedly induced by the short-term KP crude extract treatments. This finding was similar to previous reports on *Andrographis paniculata* extract (Jarukamjorn et al., 2006), and *Curcuma comosa* extract (Kittichanun et al., 2010), both of which there was increase of CYP1A1 activity. However, the enzyme activity was slightly induced by KP after long-term treatment and may indicate a recovery of the induction of this enzyme. KP crude extract may have hepatoprotective activity following long-term treatment. In addition, the induction of KP crude extract on CYP1A1 may have an impact on the activation of carcinogenesis due to its ability to activate certain procarcinogens including benzo[a]pyrene and other polycyclic hydrocarbons (Guengerich and Shimada, 1991).

With respect to CYP1A2, the KP crude extract presented a mixed-type interaction pattern with an increase in both Vmax and Km suggesting that it might bind to both the active site and the allosteric site of the enzyme. Interference with the allosteric site of CYP1A2 could modify the conformation of the enzyme and prevent the binding and subsequent metabolism of concomitantly administered drugs (Volak et al., 2008). The high Vmax and low Ki values were indicative of a potent interaction

between the KP crude extract and CYP1A2. In addition, short-term treatments with the KP crude extract resulted in the induction of CYP1A2 activity; this returned to baseline levels after prolonged administration. However, the induction was lower than that of the positive control, β-naphthoflavone. Since, CYP1A2 is the main metabolic enzyme for many drugs, e.g., paracetamol, caffeine, ondansetron, phenacetin, tacrine, tamoxifen, and theophylline (Lin and Lu, 1998), the co-administration of KP crude extract with these drugs raises concerns about possible drug interactions. Moreover, CYP1A2 enzyme plays an important role in the activation of procarcinogens to carcinogens such as 2-aminoanthracene, 2-aminofluorene, quinoline derivatives, quinoxaline derivatives, 2-amino-1-methyl-6-phenylimidazole[4,5-b]pyridine (PhIP), and imidazole derivatives (Guengerich and Shimada, 1991). Hence, the KP crude extract may have an influence on carcinogenesis. The observations that the KP crude extract affects CYP1A1 and CYP1A2 activities is in accordance with the report of Walle and Walle (2007) on the effect of the methoxyflavones including DMF and 5-methoxyflavone on CYP1A1 and CYP1A2 activities.

Regarding the effect of KP crude extract on CYP2B, a competitive interaction in which the substrates may bind with the active site of the enzyme with low Km values are reflective of high potency. The *in vivo* study demonstrated that the KP extract markedly induced CYP2B activity, similar to that observed for *A. paniculata* (Jarukamjorn et al., 2006) and *C. comosa* (Kittichanun et al., 2010). CYP2B is an isoform that plays an important role in the metabolism of various drugs including bupropion, coumarins, cyclosphosphamide, mephenytoin, methadone, ketamine, and efavirenz, as well as the pesticide methoxychor (Coleman, 2010). It has also been reported that diabetic rats have markedly increased CYP2B activity (Ioannides et al., 1996). Thus, the consumption of KP extract by diabetic patients raises concerns for the possibility of herbal-drug interactions.

The interaction of the KP crude extract on CYP2E1 was uncompetitive with low Vmax and Km values. When compared to vehicle group, KP crude extract significantly induced CYP2E1 activity only long-term treatment. CYP2E1 is an enzyme that metabolizes many commonly used drugs and xenobiotics including ethanol, paracetamol, caffeine, chlorzoxazone, enflurane, and theophylline (Lin and Lu, 1998). This enzyme also plays a key role in the activation of procarcinogens such

as carbon tetrachloride, ethylene dichloride, ethylene dibromide, vinyl chlorine, vinyl bromide, and ethyl carbamate (Guengerich and Shimada, 1991). Furthermore, CYP2E1 generates reactive oxygen species that may interact with DNA to induce mutations that involve in the etiology of many diseases including diabetes and rheumatoid arthritis, as well as in the process of aging. As a result of CYP2E1 induction, diabetic animals are sensitive to carcinogens that are activated by this enzyme (Ioannides et al., 1996). The induction of CYP2E1 at an advanced stage of alcoholic liver disease also increases basal lipid peroxidation and carbon tetrachloride-induced peroxidation (Castillo et al., 1992). Therefore, the induction of CYP2E1 by the KP crude extract might cause herbal-drug interactions and promote carcinogenesis.

On the other hand, the interaction of the KP crude extract with CYP3A was uncompetitive with a low Vmax value. In the *in vivo* study, the KP crude extract did not interfere with CYP3A activity throughout the entire duration of treatment. This is fortunate as CYP3A isoforms are important enzymes involved in the metabolism of many drugs and xenobiotics including clarithromycin, codeine, cyclosporin, dapsone, diazepam, erythromycin, indinavir, lovastatin, nifedipine, carbamazepine, losartan, quinidine, taxol, terfenadine, and verapamil (Lin and Lu, 1998).

There were several reports of commercial methoxyflavones affecting CYP450 activity. Wen et al. (2005) reported that DMF and 5-hydroxy-7-methoxyflavone inhibited CYP1A1 activity. Walle and Walle (2007) also demonstrated that DMF and TMF affected on CYP1A1, CYP1A2, and CYP3A4 activity. In addition, 2'-methoxyflavone, 3'-methoxyflavone, 4'-methoxyflavone, and 3',4'-dimethoxyflavone inhibited CYP1A1, CYP1A2, and CYP2A6 activities (McKendall et al., 2008). Our results revealed that KP crude extract, which is a traditional used preparation that contains more than 11 methoxyflavones (Sutthanut et al., 2007), modulated CYP450 metabolizing enzymes however, with weak/medium modulations. To better understand the modulatory effect of methoxyflavones of the KP, each ingredient should be further studied.

2. Product developments

The utilization of KP is limited by the reason of low water solubility and low oral bioavailability. In order to improve its solubility, dissolution and oral absorption, SMEDDS and CD complex of KP crude extract were developed and characterized resulting in the efficient utilization of KP crude extract.

Methoxyflavones in KP crude extract showed a low solubility in water, acidic, and basic solution. The methoxyflavones showed low solubility among the oils tested such as castor, corn, and soy bean oils. These suggested that the methoxyflavones have no lipophilic properties although their solubility in water is very low. Nevertheless, the methoxyflavones were soluble in co-solvent namely ethanol, propylene glycol, Tween 20, and PEG.

SMEDDS spontaneously forms fine oil-in-water microemulsion in mild agitation with the presence of water or gastrointestinal fluids. The system composes of oil, surfactant, and/or co-surfactant. A pseudo-ternary phase diagram which is used to determine the efficient self-microemulsifying regions was constructed to optimize the amounts of oil, surfactant, and co-surfactant (Sachan et al., 2010). SMEDDS in this study composed of oil (triglyceride of coconut oil), a surfactant (Cremophor® EL), and also a co-surfactant (propylene glycol) with the 2:1 ratio between surfactant and co-surfactant. These components possessed the broadest self-microemulsifying region and good solubility of methoxyflavones. The appearance, precipitation within 24 h, speed of self-microemulsifying forming when adding water, *in vitro* dissolution test, droplet size analysis, formulation stability study, *in vitro* permeability study in Caco-2 cells, and oral bioavailability study in rats were evaluated compared with KP crude extract.

When the concentration of triglyceride of coconut oil was less than 25% of the system and the mixture of Cremophor® EL and propylene glycol (2:1) was higher than 75%, the particle size of the system was lower than 50 nm. There was no effect of drug-loading on particle size. The increasing of surfactant and co-surfactant in SMEDDS decreased the droplet size whereas the increasing of oil in the system increased the droplet size. This was probably described by the stabilization of the oil droplets as resulted of the localization of molecules of surfactant at the interface between oil and surfactant (Gursoy and Benita, 2004). However, the concentration of

oil was higher than 20%, the visual observations of the formulation presented the turbidity. When the concentration of mixture of Cremophor® EL and propylene glycol (2:1) increased higher than 80%, the spontaneity of the self-microemulsification process was increased and showed no precipitation at 0 and 24 h. Therefore, the 80 and 85% of mixture of Cremophor® EL and co-surfactant (2:1 ratio) with triglyceride of coconut oil (S-3-80 and S-3-85) showed good efficacy.

Moreover, the CD complex was also developed to enhance the oral bioavailability of methoxyflavones in KP crude extract. Phase solubility diagram is assessment the effect of CD on the apparent solubility of drug, and giving the stoichiometry of binding between the CD and the drug and the equilibrium binding constant (Higuchi and Connors, 1965). The methoxyflavones were soluble in HP-β-CD at 10-fold greater than that of β-CD. Stella and Rajewski (1997) reported the advantages of HP-β-CD that HP-β-CD has higher water solubility than β-CD. It also can be applied in parenteral route while β-CD is unsafe due to severe nephrotoxicity. As a result, HP-β-CD was further applied to form inclusion complex with the methoxyflavones.

The phase solubility diagram indicated that the methoxyflavones included in HP-β-CD cavity at 1:1 molar ratio of methoxyflavones and HP-β-CD molecules. The higher apparent biding constant (Kc) suggests that HP-β-CD can form more stable inclusion complex with the guest molecule. From our results, TMF having the highest Kc formed more stable inclusion complex than that of DMF and PMF, respectively. The structure of TMF may best fit in the cavity of HP-β-CD.

The inclusion complex of KP and HP-β-CD was confirmed by the absence of the melting endotherm of KP crude extract at 120^{o}C. DSC curves confirm not only the interaction between the methoxyflavones and CD but also a real inclusion. The absence of endotherm curve of KP-HP-β-CD complex indicated the complete inclusion of guest molecule in the cavity of HP-β-CD.

The release of methoxyflavones in KP from KP-SMEDDS and KP-HP-β-CD formulations were assessed in 0.1 N HCl and 0.2 M PBS pH 6.8 representing gastric and intestinal fluids, respectively comparing to KP crude extract. For very poorly soluble compound, medium may contain a percentage of surfactants (SLS, polysorbate, or lauryldimthylamine oxide) that use to enhance drug solubility as USP

recommended. Therefore, 0.5% of Tween 20 was added in both of mediums. S-3-80 reached 100% drug release within 20 and 30 min in 0.01 N HCl and 0.2 M PBS pH 6.8, respectively. While, S-3-85 got to 100% drug release within 20 min in 0.01 N HCl and 0.2 M PBS pH 6.8. The increase in concentration of Cremophor® EL and propylene glycol in KP-SMEDDS, improved dissolution profiles of the methoxyflavones. Moreover, KP-HP-β-CD complex was evaluated its dissolution profiles. The results showed that the complex rapidly achieved the 100% drug release within 10 min. From these findings, KP-SMEDDS took longer time to reach 100% drug release than that of KP-HP-β-CD complex. KP-SMEDDS may spend the short time in order to form the microemulsion before dissolving in the GI fluids. While, KP-HP-β-CD complex immediately dissolved in GI fluids and can be further absorbed into systemic circulation. In addition, the results suggested that the dissolution rate of methoxyflavones were not affected by the different pH. When comparing the dissolution profiles of S-3-80, S-3-85, and KP-HP-β-CD with KP crude extract, the developed formulaiton can improve the dissolution of methoxyflavones in KP. These results indicated the advantage of both of SMEDDS and CD formulation in improving the rate of drug release over KP crude extract. The increasing of solubility and dissolution rate by S-3-80, S-3-85, and KP-HP-β-CD complex may cause the enhancement of intestinal permeability and oral absorption. Since, the dissolution rate and drug solubility play an important role in drug absorption.

In order to screen the ability of the developed formulations to increase the delivery of methoxyflavones across the intestinal mucosa, the Caco-2 cell permeability study was performed. Yee (1997) confirmed that Caco-2 cells can be *in vitro* approach to predict *in vivo* human absorption. In this study, mannitol and antipyrine following the FDA guideline were used as low and high permeability substances, respectively. Moreover, mannitol which possesses only passive diffusion via paracellular route is used to assess the integrity of the monolayes of Caco-2 cells as well as the paracellular maker (Artursson et al., 2001). Antipyrine which crosses the intestinal cells by passive diffusion via transcellular route is used as transcellular marker (Kimoto et al., 2009).

KP-HP-β-CD complex showed the Papp greater than that of KP crude extract at 3.8, 5.0, and 5.1-fold for PMF, TMF, and DMF, respectively since CD complex improves drug solubility and dissolution rate (Brewster and Loftsson, 2007). The increase Papp values of methoxyflavones in Caco-2 cells by S-3-80 and S-3-85 (about 10-fold comparing with KP crude extract) might be resulted from not only the increase of membrane fluidity to facilitate the permeation by surfactant and co-surfactant but also the enhanced drug solubility of S-3-80 and S-3-85 (Sachan et al., 2010). These reasons explained why S-3-80 and S-3-85 showed higher Papp values of methoxyflavones than that of CD and KP crude extract. In addition, S-3-80 and S-3-85 showed no significant difference of Papp values. Therefore, S-3-80 that composed of the lower of surfactant and co-surfactant was selected for further *in vivo* oral absorption study.

Noticeable, Papp values among PMF, TMF, and DMF in three tested formulations were higher than that of mannitol (paracellular marker) and antipyrine (transcellular marker). These findings may imply that the methoxyflavones in KP crude extract could be transported across the Caco-2 cells by many pathways not only the passive diffusion via paracellular and transcellular route but also the carrier-mediate, transcytosis, and active transport.

There was previous study of transepithelial permeability experiment in Caco-2 cell of DMF and TMF compounds (Wen and Walle, 2006). The Papp values of DMF and TMF (23.3 and 27.6 x 10^{-6} cm/sec, respectively) were lower than that of DMF and TMF treating with KP crude extract about 10-fold and lower than that of DMF and TMF treated with S-3-80 and KP-HP-β-CD complex about 100-fold. These findings may imply that the other components in KP crude extract may influence on permeability of the extract through Caco-2 cells.

The stability study of S-3-80, S-3-58, and KP-HP-β-CD complex for 3 months indicated that both of SMEDDS formulations were stable in conditions of 4, 25, and 40^{o}C by no significant changing of methoxyflavones contents. However, the methoxyflavones contents in KP-HP-β-CD complex when kept at 40^{o}C were declined after storage for 2 and 3 months. These results suggested that KP-HP-β-CD complex should not be kept at 40^{o}C. Nevertheless, the stability of developed products should be continually study until 6-12 months.

Based on the dissolution study and *in vitro* absorption study by using Caco-2 cells, S-3-80 and KP-HP-β-CD complex were selected for *in vivo* oral bioavailability study.

The *in vivo* absorption study of developed formulations was investigated in rats after oral or intravenous administrative of S-3-80 or KP-HP-β-CD complex comparing to KP crude extract in propylene glycol, PEG400, ethanol and water. The AUC and Cmax values of methoxyflavones were enhanced by two developed formulations. The enhancement of Cmax is reasonable due to CD complex or SMEDDS speed up drug dissolution, and higher levels of drug in solution in the gastrointestinal fluid resulting in the greater drug absorption. The oral bioavailability of methoxyflavones in both of developed formulation also considerably increased. The bioavailability for PMF, TMF, and DMF of KP-HP-β-CD complex were 21.63, 34.20, and 22.90-folds greater than those of KP crude extract, respectively. For S-3-80, the oral bioavailability values (25.38, 42.00, and 26.01–fold for PMF, TMF, and DMF, respectively) were higher than those of KP crude extract. These results suggested that the oral absorption of methoxyflavones could be improved by SMEDDS and CD complex.

There are several probably reasons of the improvement of oral bioavailability by SMEDDS. The improved oral absorption is probably due to the increased drug solubilization and dissolution rate. SMEDDS possessing the nano-size (less than 50 nm) providing a large surface area may be one approach to enhance oral absorption. Oil in SMEDDS also protects the drug form enzyme degradation in GI tract (Gursoy and Benita, 2004) and increases the amount lipophilic drug transport through the intestinal lymphatic system which plays an important absorption pathway of poorly water-soluble drugs (Patel et al., 2010). In addition, the surfactants may penetrate through intestinal cell membranes following with disturbance of lipid bilayers resulting in the enhancement of drug permeability (Gursoy and Benita, 2004). Co-surfactants increase the fluidity of the interface as well as damage liquid crystalline or gel structure which is the barrier of microemulsion formation. These reasons may result in the slightly higher bioavailability of SMEDDS than that of CD complex. While, CD improves only drug solubility and dissolution rate to enhance of drug absorption (Brewster and Loftsson, 2007).