COMPARATIVE PHARMACOKINETICS OF PUERARIN IN PURE COMPOUND FORM AND PUERARIN IN WHITE KWAO KRUA EXTRACT IN FEMALE CYNOMOLGUS MONKEYS

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สุรีรัตน์ นามเคน : การเปรียบเทียบเภสัชจลนศาสตร์ของพูรารินในรูปสารบริสุทธิ์และพูรารินในสารสกัดกวาวเครือขาวในลิงแสมเพศเมีย. (COMPARATIVE PHARMACOKINETICS OF PUERARIN IN PURE COMPOUND FORM AND PUERARIN IN WHITE KWAO KRUA EXTRACT IN FEMALE CYNOMOLGUS MONKEYS) อ.ที่ปรึกษาหลัก : ศ. ดร.สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาร่วม : รศ. ภก.ดร.พิสิฐ เขมาวุฒฒ์

กวาวเครือขาว เป็นพืชเฉพาะถิ่นของไทย ที่มีประวัติการใช้ทางยามาอย่างยาวนาน โดยมีพูรารินเป็นสารหลักที่สามารถพบได้ในพืชชนิดนี้ ที่มีฤทธิ์ในการรักษาและป้องกันโรคชรา จากการศึกษาทางเภสัชจลนศาสตร์ของพูรารินก่อนหน้านี้ในสัตว์ฟันแทะ ซึ่งมีลักษณะทางกายภาพและสรีรวิทยาหลายอย่างที่แตกต่างกับมนุษย์ พบว่ายังไม่เพียงพอในการนำไปออกแบบและพัฒนาพูรารินให้เป็นผลิตภัณฑ์ที่อยู่ในรูปยาแผนปัจจุบันเพื่อใช้ในมนุษย์ ดังนั้น ในการทดลองนี้จึงทำการเปรียบเทียบเภสัชจลนศาสตร์ของพูรารินในรูปของพูรารินบริสุทธ์ (PUE) และสารสกัดกวาวเครือขาว (PME) ในลิงแสมเพศเมีย โดยให้ PME ในขนาด 826 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว (ที่มีสารพูรารินในปริมาณ 10 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว) และ PUE ในขนาด 10 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว โดยการให้ทางปากต่อเนื่อง 7 วัน และให้ PUE ในขนาด 1 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว 1 ครั้ง ทางหลอดเลือดดำ เพื่อใช้ในการวิเคราะห์ความสามารถในการดูดซึมพูรารินเข้าสู่ร่างกายลิงเมื่อให้ทางปาก จากนั้นเก็บตัวอย่างเลือด ปัสาวะ และอุจจาระ ตามเวลาที่กำหนด แล้วนำไปตรวจวิเคราะห์ด้วยเครื่อง liquid chromatography tandem mass spectrometry จากผลการศึกษาไม่พบอาการไม่พึงประสงค์ในสัตว์ทดลอง รวมถึงระดับ aspartate aminotransferase และ alanine aminotransferase ในพลาสมาที่ใช้บ่งบอกภาวะการทำงานของตับ และระดับ creatinine ในพลาสมา ที่ใช้ในการบ่งบอกภาวะการทำงานของไต จากการวิเคราะห์ค่าชีวประสิทธิผลของสารพูรารินเมื่อให้ทางปากพบว่ามีค่าเท่ากับร้อยละ 1.44 เมื่อให้ในรูปของ PME และเท่ากับร้อยละ 0.88 เมื่อให้ในรูปของ PUE แต่ครึ่งชีวิตของสารพูราริน ($T_{1/2}$) เมื่อให้ในรูปของ PUE มีค่ายาวกว่า (4.78 ชั่วโมง) เมื่อเทียบกับเมื่อให้ในรูปของ PME (2.61 ชั่วโมง) ภายหลังจากการป้อนสารพูรารินทั้งสองรูปแบบนานต่อเนื่อง 7 วัน ทำให้เกิดการสะสมของสารพูรารินในร่างกายลิงแสม ในขณะที่อวัยวะที่มีบทบาทในการเปลี่ยนสภาพของสารพูรารินให้เป็นสารเมตาบอไลต์ได้ทำให้เกิดการเปลี่ยนแปลงสารผ่านทาง 2 วิถี คือ hydroxylation และ deglycosylation ก่อนมีการขจัดสารออกนอกร่างกายทางปัสสาวะและอุจจาระ โดยสัดส่วนในการขับออกของสารพูรารินในรูปของสารต้นแบบมีค่าต่ำกว่าร้อยละ 1 โดยสรุป การให้สารพูรารินทางปากในรูปของ PME สามารถดูดซึมได้ดีกว่าการให้ในรูปของ PUE แต่มีครึ่งชีวิตที่สั้นกว่า และสารพูรารินสามารถสะสมในร่างกายลิงได้เมื่อให้ต่อเนื่องกันเป็นระยะเวลานาน ซึ่งข้อมูลการศึกษาเภสัชจลนศาสตร์ในลิงแสมเพศเมียนี้ สามารถนำไปประกอบการออกแบบขนาดและความถี่ในการให้สารพูรารินทางปากที่เหมาะสมต่อการนำไปใช้ในการรักษาโรคในมนุษย์ต่อไป

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Pueraria mirifica is an endemic Thai plant that puerarin is a major chemical found in this plant and shows several pharmacological activities in aging diseases. Although the pharmacokinetic data on puerarin have been reported in rodents, it is still inconclusive for the development of puerarin as phytopharmaceutical products for human use. This is because of the differences in anatomical and physiological characteristics between rodents and humans. Therefore, the comparative pharmacokinetics of puerarin in pure compound form (PUE) and puerarin in *P. mirifica* extract (PME) was conducted in female cynomolgus monkeys. PME at a dose of 826 mg/kg.BW (equivalent to 10 mg/kg.BW of puerarin) and PUE at a dose of 10 mg/kg.BW were daily orally administered to monkeys for 7 consecutive days. A single intravenous injection of 1 mg/kg.BW of PUE was also performed for the bioavailability analysis of puerarin orally administered to monkeys. Serial blood samples and excreta (urine and feces) were collected after dosing at designated times. The levels of puerarin in biological samples were determined by liquid chromatography tandem mass spectrometry. After PME and PUE orally dosing to monkeys, plasma levels of aspartate aminotransferase and alanine aminotransferase which were indicated the liver function, and plasma creatinine levels which were indicated the kidney function were fluctuated in the normal range, with no abnormal physical signs in animals. The absolute oral bioavailability of puerarin was 1.44% after the PME oral dosing and 0.88% after the PUE oral dosing, but the $T_{1/2}$ was prolonged for nearly two times in the PUE group (4.78 h) comparing to the PME group (2.61 h). After 7-day multiple oral dosing of puerarin in both preparations, the accumulations were occurred in the body of the animals. Major metabolite pathways of puerarin found in monkeys were hydroxylation and deglycosylation before excreted via urine and feces. A negligible amount of unchanged puerarin was detected for less than 1% in urine and feces. In conclusion, an oral dosing of a puerarin shows the better absorption in the extract form than in the pure compound form, but it has a shorter half-life. Puerarin can be accumulated in the body of the animals when it is continuously orally dosing. Thus, the pharmacokinetic profiles obtained from female cynomolgus monkeys in this study could help to design the prescribed remedy of the oral administration of puerarin as phytopharmaceutical products for human use.

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Co-advisor's Signature .....

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Sureerat Namken

TABLE OF CONTENTS

Page

List of Table

Page

List of Figures

Page

CHAPTER 1

GENERAL INTRODUCTION

Pueraria candollei var. *mirifica* (Airy Shaw and Suvatabandhu) Niyomdham or *P. mirifica*, which has been known in Thai as "Kwao Krua Khao", is an endemic Thai plant belonging to the family Leguminosae, subfamily Papilionoideae (Bodner and Hymowitz, 2002; Ingham et al., 2002). Its tuberous roots have long been used as a Thai traditional medicine for rejuvenation and health improvement including improving cognitive function, alleviating sleep disorder, and increasing general vigor (Kerr, 1932; Wanandorn, 1933). Later, at least 17 phytoestrogenic substances in *P. mirifica*'s roots are isolated and reported. They can be categorized into three main classes: (I) isoflavonoids, comprised of daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin (PUE), mirificin and puermiricarpene; (II) coumestans comprised of coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol; and (III) chromenes comprised of miroestrol, deoxymiroestrol and isomiroestrol (Pope et al., 1958; Ingham et al., 1986a; Ingham et al., 1988; Ingham et al., 1989; Chansakaow et al., 2000a; Cherdshewasart and Sriwatcharakul, 2007; Urasopon et al., 2008). During the past two decades, the estrogenic activities of the powder or the extract of *P. mirifica* tuberous roots were widely tested in various speceis of laboratory animals and humans on reproductive organs (Malaivijitnond et al., 2004; 2006; Trisomboon et al., 2004; 2005; 2006a; 2006b; Cherdshewasart et al., 2007a; Jaroenporn et al. 2006; 2014; Kittivanichkul et al., 2016), bones (Urasopon et al., 2007; 2008; Tiyasatkulkovit et al., 2012; 2014; Kittivanichkul et al., 2016; Suthon et al., 2016), anti-cancer effect (Cherdshewasart et al., 2007b), cardiovascular diseases

(Wattanapitayakul et al., 2005), climacteric symptoms (Muangman and Cherdshewasart, 2001; Kongkaew et al., 2018) and cognitive improvement (Anukulthanakorn et al., 2016). *P. mirifica* has minimal toxicity effects on cardiovascular function, blood lipid levels, and liver enzyme activity in rabbits (Wattanapitayakul et al., 2005) and in menopausal women (Muangman and Cherdshewasart, 2001; Chandeying and Sangthawan, 2007). Based on the OECD test guideline of acute toxicity, feeding of *P. mirifica* extract (PME) up to 2000 mg/kg in male rats was indicated as safe (Mohamad et al., 2019).

Among 10 isoflavonoids isolated from *P. mirifica* roots, PUE is the main constituent (Cherdshewasart et al., 2007b) and has several pharmacological effects such as increasing osteoblast activities (Tiyasatkulkovit et al., 2012; 2014; Wang et al., 2013), inhibiting osteoclast activities (Li and Yu, 2003; Tiyasatkulkovit et al., 2012; 2014), blood clotting (Coull et al., 2002) and neuronal apoptosis (Zhang et al., 2011), ameliorating the learning and memory deficit in rodents (Li et al., 2010; Liu et al., 2015; Hong et al., 2016; Li et al., 2019), and exhibiting anti-inflammation and antioxidant activities (Jin et al., 2012). A toxicity evaluation for PUE is very limited to cardiovascular function, hematological and biochemical parameters in rats while no toxicity was found (Zhang et al., 2006; Chung et al., 2009). PUE showed no signs of irritation and damage to intestinal mucosa after oral administration (Wu et al., 2008). Based on these diverse pharmacological and safety data of PUE and PME, the development of these two substances as phytopharmaceutical products for clinical use is considered.

Along the way of drug or pharmaceutical product development process, the pharmacokinetic data of the products are essential for approval by the regualtory authorities for the human use. Previously, pharmacokinetics of PUE were conducted in rat (Yang et al., 2011; Anukunwithaya et al., 2018), rabbit (Cui et al., 2005; Deng et al., 2006), and dog (Ren et al., 2006; Yi et al., 2015). However, the results were not aligned across the species. PUE could be absorbed through intestinal at 2.10 – 7.50% of the given dose (Su et al., 2016; Anukunwithaya et al., 2018) and reached a maximum plasma concentration (Cmax) at 0.19 – 1.83h in rats (Yang et al., 2011; Cao et al., 2013; Anukunwithaya et al., 2018) while the Cmax was 0.83 – 1.08h in rabbit (Cui et al., 2005) and 1.50 – 4.00h (Ren et al., 2006). PUE could be distributed into various organs of rat including heart, lungs, liver, spleen, kidneys and brain after 2.5h of intragastric administration (Luo et al., 2011), and also distributed to femurs, tibias, mammary glands after 1h of intravenous administration (Anukulwithaya et al., 2018). The biotransformation of PUE in humans and rats was reported to occur via different reactions (Yasuda et al., 1995; Prasain et al., 2004, Jung et al., 2014; Anukulwithaya et al., 2018). Phase I hydrolysis and phase II glucuronidation in humans changed PUE into daidzein and PUE glucuronides (Jung et al., 2014), respectively, while the daidzein was further biotransformed by phase I reduction to dihydrodaidzein and finally to equol in rats (Prasain et al., 2004). Daidzein was also metabolized by phase II (sulfation) to obtain daidzein-sulfate as a final product in rats (Yasuda et al., 1995). For excretion, PUE glucuronides were major metabolites of PUE in rat which were mainly excreted via urine and to a lesser extent via feces (Prasain et al., 2004; Anukulwithaya et al., 2018).

Based on these contradict resutls, the pharmacokinetic data of PUE in laboratory animal which has similar anatomical and physiological characteristics to those of humans are needed. Cynomolgus monkey (*Macaca fascicularis*) is one of the most commonly used animal models for pharmacokinetic studies of pharmaceutical products (Cauvin et al., 2015). This is because their anatomical and physiological characteristics are similar to those of humans. For example, the gastric pH and gastric emptying time after fasting in cynomolgus monkeys are 1.9 – 2.2 and 153 ± 87 mins (Chen et al., 2008), respectively, compared to pH 1.5 – 3.5 and 248 ± 39 mins in humans (Bolondi et al., 1985; Schwarz et al., 2002). Besides, CYP3A, a drug metabolizing enzyme found in the liver, showed 93% similarity of animo acid sequences between monkeys and humans (Komori et al., 1992). Thus, this study will assess the pharmacokinetics of PUE in female cynomolgus monkeys. Besides PUE, there are many other flavonoids in the PME such as daidzin, daidzein, genistin and genistein, the coexistence of these substances may affect the pharmacokinetic parameters of PUE in PME. Searching from the databases of Pubmed and Scopus, no publication of the pharmacokinetics of PUE in PME was found and also the comparison with the PUE alone. Here, the pharmacokinetic studies of PUE alone and PUE in PME in cynomolgus monkeys are conducted and compared. The results from this study should provide useful information for the prescribed remedy of PUE and PME as phytopharmaceutical products for the human use.

Objectives

1. To study the pharmacokinetics of PUE in pure compound form and in PME after a single intravenous or oral administration in female cynomolgus monkeys.

2. To study the pharmacokinetics of PUE as in pure compound form and in PME after oral administrations for 7 consecutive days in female cynomolgus monkeys.

3. To compare the pharmacokinetics of PUE as in pure compound form and in PME in female cynomolgus monkeys.

CHAPTER 2

LITERATURE REVIEW

1. Pueraria mirifica

1.1. Origin and description

Pueraria candollei var. *mirifica* (Airy Shaw and Suvatabandhu) Niyomdham or *P. mirifica* belongs to the family Leguminosae and subfamily Papilionoideae (Bodner and Hymowitz, 2002; Ingham et al., 2002). The plant distributes in deciduous forests or hill slopes at elevations between 300 and 800 m in the north, west and northeast of Thailand (Lakshnakara et al., 1952; Niyomdham, 1992; Van der Maesen, 2002). *P. mirifica* is a woody perennial climber (Figure 2.1) with slightly hairy branches and white starchy tuberous roots. The roots are approximately 10-70 cm diameter in a chain of round-shaped bulbs of various sizes (Van der Maesen, 2002; Niyomdham, 2004). Stems are elongated up to 5 m in length (Niyomdham, 2004). Leaves are 15-18 cm long and 10-15 cm wide with three entire lobed leaflets arranged pinnately (Van der Maesen, 2002; Niyomdham, 2004). The bluish-purple flowers are about 8-10 mm long, with an inflorescence up to 30 cm, somewhat similar to a bean flower, and calyx densely pubescent, appearing during February to March (Van der Maesen, 2002; Niyomdham, 2004). Following the plant flowers in February-March, the seedpods appear in April (Van der Maesen, 2002; Niyomdham, 2004). The pods are flat-shape, with 5-7 mm wide and 3 cm long, glabrous and hairy, and each pod contains up to 3-5 seeds (Van der Maesen, 2002; Niyomdham, 2004). *P. mirifica* is locally called Kwao Krua, Kwao Krua Kwao, white Kwao Krua or Guao Krua (Ingham et al., 2002).

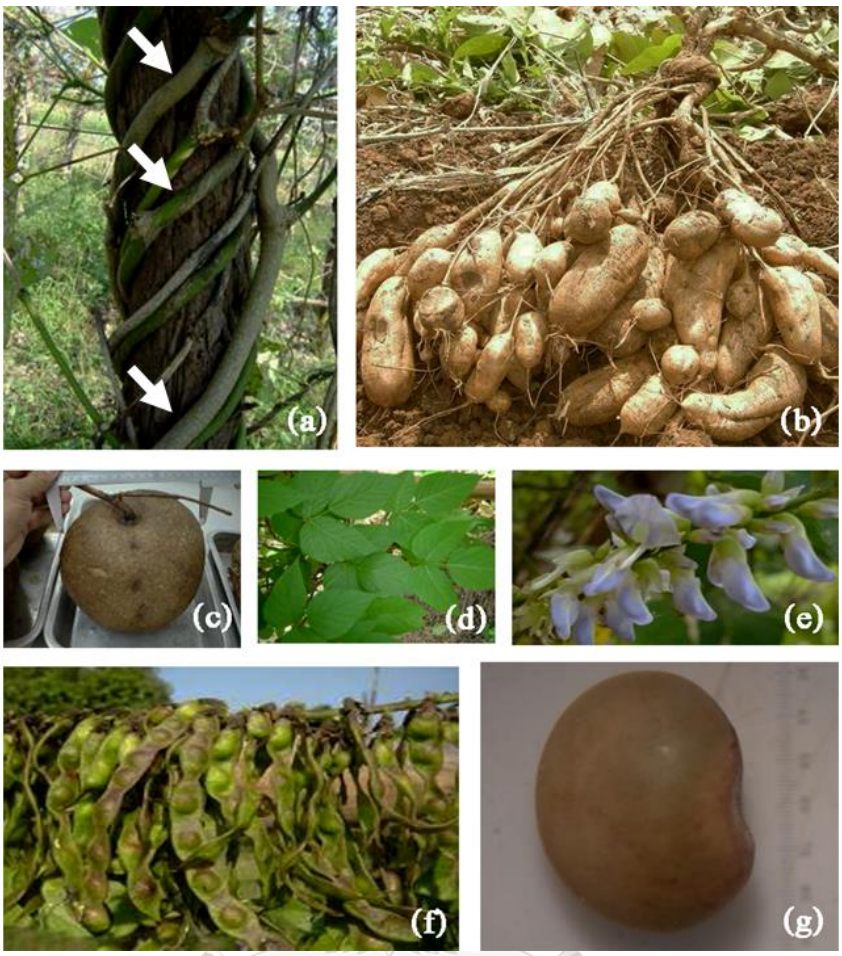

Figure 2.1 *Pueraria mirifica* in northeastern Thailand; (a) stem (white arrow), (b-c) tuberous roots, (d) leaves, (e) flowers, (f) pods, and (g) seeds (with permission from Chaowiset, 2007).

Historically, the usage of the tuberous roots of *P. mirifica* as traditional medicine was noted in palm-leaf manuscript by two Burmese authors which was translated into pamphlet by Nai Plian Kitisri and subsequently edited by Suntara (Suntara, 1931). The traditional use of *P. mirifica* was for a good health and rejuvenation, such as increasing energy and vigor, alleviating sleep disorder and memory loss, skin care, anti-wrinkling and improving vision (Suntara, 1931; Kerr, 1932). Without the knowledge on hormones at that moment of time, it was believed

that the roots of *P. mirifica* might contain estrogenic materials, and the rejuvenating qualities in menopausal women and andropausal men were noted over a past hundred years (Kerr, 1932; Wanandorn, 1933; Ingham et al., 2002).

1.2. Chemical constituents in tuberous root of *P. mirifica*

The constituents in the dried and powdered tuberous root of *P. mirifica* are characterized extensively and can be classified into three groups of chromenes, coumestans and isoflavones (Table 2.1). Chromenes comprise miroestrol, deoxymiroestrol and isomiroestrol (Pope et al., 1958; Jone and Pope, 1961). Noted, miroestrol is the first phytoestrogen isolated from *P. mirifica* (Pope et al., 1958; Jone and Pope, 1961; Chansakaow et al., 2000a). Although both miroestrol and deoxymiroestrol elicit strong estrogenic activity, deoxymiroestrol is more active (approximately 10-fold stronger) than the miroestrol (Chansakaow et al., 2000a). Coumestans, a minor component in *P. mirifica*, include coumestrol, mirificoumestan, mirificoumestan glycol and mirificoumestan hydrate (Ingham et al., 1986b; 1988). Coumestrol is the main component of the coumestans (Cvejic et al., 2012). Isoflavones include daidzin, daidzein, genistin, genistein, PUE, mirificin, tuberosin, puemiricarpene, kwakhurin and kwakhurin hydrate (Ingham et al., 1986a; 1986b; 1989; Tahara et al., 1987; Chansakaow et al., 2000b). The chemical structures of phytoestrogens in these three groups are shown in Figure 2.2. The isoflavones characterized in *P. mirifica* may occur as aglycone forms such as daidzein, genistein, kwakhurin and tuberosin (Ingham et al., 1986b; Tahara et al., 1987; Ingham et al., 2002) or glycosides, either *O*- or *C*-glycoside form. The isoflavones-*O*-glycosides are, for example, daidzin and genistin (Ingham et al., 1986a; 1989) and the isoflavones-*C*-glycosides are PUE and mirificin (Ingham et al., 1986a; 1986b). The isoflavonoids are

the major content in *P. mirifica*, mainly PUE, daidzin, daidzein, genistin and genistein, ranging by 18.61 – 198.29 mg/100 g of dry weight tuberous root (Malaivijitnond et al., 2004; Cherdshewasart et al., 2007a; Urasopon et al., 2008). The individual and total isoflavonoid contents are highly varied between locations; the high contents found in any locations are PUE and genistin (Cherdshewasart et al., 2007a), and also depend on genetic, growing condition, growth stage and preservation method (Chansakaow et al., 2000b; Cherdshewasart and Sriwatcharakul, 2007; Cherdshewasart et al., 2007a).

Table 2.1 Three groups of chemical constituents in the tuberous root of *P. mirifica*

Chromenes	**Coumestans**	**Isoflavones**
miroestrol	coumestrol	daidzin
deoxymiroestrol	mirificoumestan	daidzein
isomiroestrol	mirificoumestan glycol	genistin
	mirificoumestan hydrate	genistein
		puerarin
		mirificin
		kwakhurin
		kwakhurin hydrate
		tuberosin
		puemiricarpene

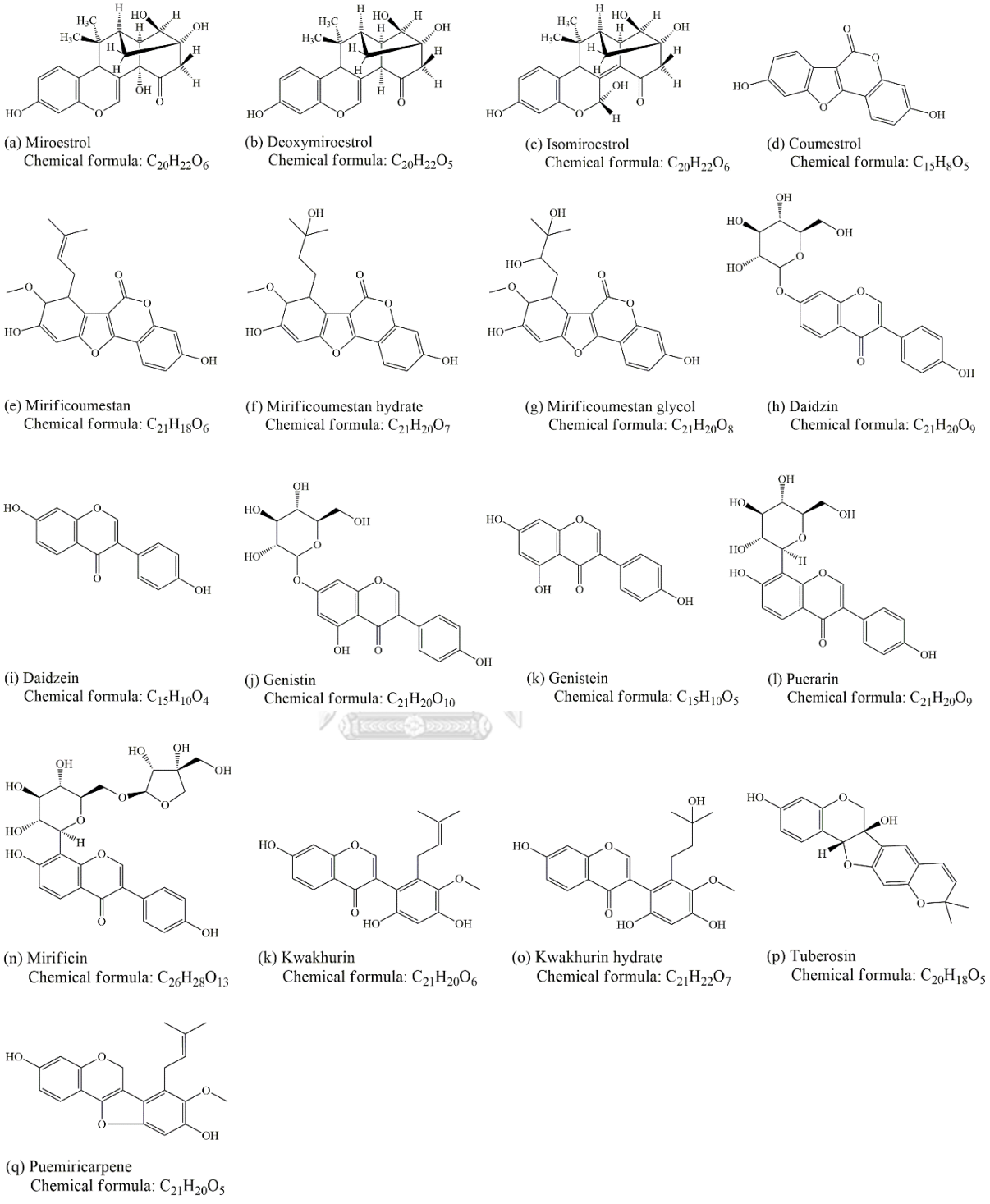

Figure 2.2 Structures of chromenes: (a) miroestrol, (b) deoxymiroestrol and (c) isomiroestrol, coumestans: (d) coumestrol, (e) mirificoumestan, (f) mirificoumestan hydrate and (g) mirificoumestan glycol, isoflavones: (h) daidzin, (i) daidzein, (j) genistin, (k) genistein, (l) puerarin, (m) mirificin, (n) kwakhurin, (o) kwakhurin hydrate, (p) tuberosin and (q) puemiricarpene.

1.3. Physicochemical properties of puerarin

As mentioned above that the major isoflavone isolated from the tuberous root of *P. mirifica* is PUE, the studies on this phytoestrogen has been extensively and intensively. The chemical structure of PUE (7, 4'-dihydroxy-8-C-glucosylisoflavone) is shown in Figure 2.3. The structural characteristics has presented a steric hindrance of carbonyl group of the pyran ring formed to ring B and two phenolic hydroxyl groups at the 7, 4′-site linked with hydrogen bond between intermolecular, leading to the intermolecular forces greater and higher melting and boiling points (Lv and Tan, 2009). Since a weak intramolecular hydrogen bond between 7-OH and the glycosyl group, which in chair conformation and introducing a glucose moiety, the bond length of 7-OH is a little longer than that of 4′-OH. (Zhou et al., 2019). A glucose moiety makes PUE strongly hydrophilic, but the n-octanol/water partition coefficient (Log P_{ow}) and solubility in water is only -0.35 (Han et al., 2009) and 0.46 (Wang and Cheng, 2005), respectively. Following the Biopharmaceutics classification system of the Food and Drug Administration (FDA, 2020) and European Medicines Agency (EMA, 2018), PUE is classified into class IV based upon permeability and solubility for the purpose of predicting oral drug absorption (Li et al., 2014). The highly intestinal permeability correlates to fractional absorption Fa>0.9, and the high solubility correlates to the highly completely soluble in 250 ml or less of aqueous media at the pH range of 1.2 – 6.8 and 37 ± 1°C (EMA, 2018). The physicochemical properties of PUE are described in Table 2.2.

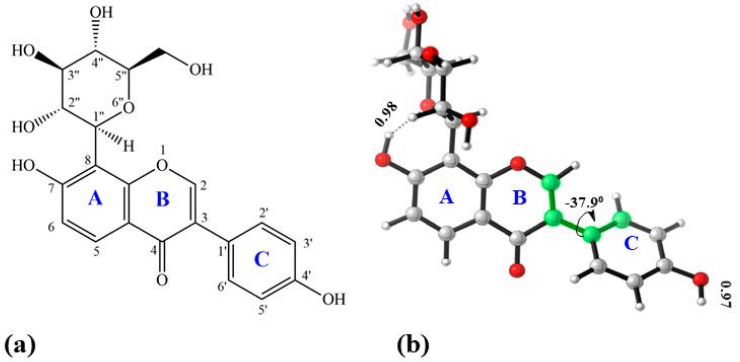

Figure 2.3 Puerarin and (a) its molecular structure and (b) stable conformation

Table 2.2 Physicochemical properties of puerarin

Property names	**Values**	**References**
Formula	$C_{21}H_{20}O_9$	Swiss Institute of Bioinformatics, 2019
Molecular weight (g/mol)	416.38	
Number of heavy atoms	30	
Number of H-bond donors	6	
Number of H-bond acceptors	9	
Number of rotatable bonds	3	
Molar refractivity	104.59	
Topological polar surface area	160.82 $Å^2$	
Solubility in water (mol/L)	0.45 - 0.46: poorly soluble	Wang and Cheng, 2005; Quan et al., 2007
Log P_{ow}	-0.35	Han et al., 2009
Intrinsic dissolution rate at 37 °C ($mg/min/cm^3$)	pH 1.0: 0.39 4.0: 0.36 6.8: 0.63 7.4: 1.09	Li et al., 2014

2. Pharmacological and toxicological studies of *P. mirifica* extract and puerarin

Pharmacological effects of PME and PUE have been investigated on the reproductive organs**,** cardiovascular systems, brain and bone in both males and females. As PME and PUE are considered to be developed as herbal medicine, their toxicological data are also essential. Thus, the pharmacological and toxicological studies of PME and PUE are described below

2.1. Pharmacological properties of *P. mirifica* extract

Reducing cancer risk

Cancer is one of major public health problems that causes a morbidity globally. There are multisteps in cancer development including cell adhesion, cell invasion, cell proliferation, cell transportation through circulatory system and growth in a secondary organ (Shen et al., 2009). PME reduces risk of cancers, mainly on hormone-dependent-cancer type such as cervical cancer (Jeon et al., 2005), breast cancer (Cherdshewasart et al., 2007b), and prostate cancer (Mohamad et al., 2019), through its mechanism of action is either on estrogen receptor-α (ER-α) or -β (ER-β) binding (Jeon et al., 2005; Messina and Wood, 2008). PME exhibits *in vitro* anti-proliferative effect on both breast cancer cells and ovarian cancer cells (Cherdshewasart et al., 2008). Daily feeding of 100 and 1,000 mg/kg of PME for 4 weeks to 7,12-DMBA-induced breast cancer female rats could reduce the expression of ER-α and ER-α/ER-β ratio together with a decrease in tumor development and multiplicity (Cherdshewasart et al., 2007b). PME exhibits antiandrogenic activity in Benign Prostatic Hyperplasia rats by inhibiting the activity of 5α-reductase enzyme, thus it indirectly reduces the conversion of testosterone to dihydrotestosterone (Mohamad et al.,2019). Another pathway for the reduction of prostate cancer by PME treatment in males is that PME suppresses the hypothalamic-

pituitary-gonad axis and reduces the development of prostate cancer (Huggins and Hodges, 1941; Mohamad et al.,2019).

Anti-osteoporotic activity

Since phytoestrogens in PME highly bind with ER-β which is mainly expressed on the osteoblast cells (Urasopon et al., 2007; 2008; Tiyasatkulkovit et al. 2014), the use of PME to mitigate the osteoporosis in the elderly is attracted attention. Feeding of PME at doses of 10-1,000 mg/kg/day for 90 days in both sexes of rats could prevent bone loss (Urasopon et al., 2007; 2008). Feeding of PME at a dose of 50 mg/kg/day to ovariectomy-induced osteoporotic female rats could maintain bone mineral density and bone histomorphometry (Suthon et al., 2016). Given *P. mirifica* powder at a dose of 1,000 mg/kg/day, by mixing with monkey chow, to postmenopausal osteoporotic monkeys for 16 months could ameliorate the reduction in bone loss at cortical diaphysis (Kittivanichhkul et al., 2016). This implies that PME could be used as an anti-osteoporotic agent to reduce bone fractures in humans (Kittivanichkul et al., 2016). Later, PME treatment at doses of 20-50 mg/kg/day for 6 months in postmenopausal women also showed a positive result on bone (Manonai et al., 2008). PME stimulates bone formation and suppresses bone resorption by increasing the mRNA expression of alkaline phosphatase (ALP) and decreasing receptor activator of nuclear factor kappa B ligand (RANKL)/ osteoprotegerin ratio in rat osteoblast-like UMR106 cells (Tiyasatkulkovit et al. 2012) and primary monkey osteoblast cells (Tiyasatkulkovit et al., 2014).

Anti-cardiovascular diseases

Cardiovascular disease remains a leading cause of disease burden in the world (Roth et al., 2019). Several evidences confirmed that an increase in low-density

lipoprotein (LDL) cholesterol and its major protein component apolipoprotein B (apo B) levels, and a decrease in high density-lipoprotein (HDL) cholesterol and its major protein component apolipoprotein A-1 (apo A-1) levels in plasma linked to incidence of cardiovascular disease (Roth et al., 2019). PME significantly increases HDL and apo A-1 levels and decreases LDL and apo B levels in postmenopausal women after binding to both ERs (Okamura et al., 2008). PME also increased endothelial vasorelaxation by inducing translocation of endothelial NO synthase via a NO-dependent pathway which decreased endothelial dysfunction (Wattanapitayakul et al., 2005).

2.2. Pharmacological properties of puerarin

Antidiabetic activity

It has been widely reported the protective effects of PUE on diabetes mellitus through its hypoglycemic effect in laboratory animals (Chen et al., 2004; Li et al., 2009; Shen et al., 2009; Zhang et al., 2010; Wu et al., 2013; Tanaka et al., 2016; Yang et al., 2016). The intravenous injection of PUE could reduce plasma glucose concentrations in streptozotocin (STZ)-induced diabetic rats (Chen et al., 2004; Li et al., 2009; Shen et al., 2009) and mice (Wu et al., 2013), ovariectomized mice (Tanaka et al., 2016), high-fat diet-induced insulin resistant rats (Zhang et al., 2010) and high-fat diet-induced diabetic mice (Yang et al., 2016) in a dose-dependent manner. The underlying mechanisms for its hypoglycemic effect are as follows:

Increase glucose absorption in muscle: PUE enhanced glucose absorption in myoblasts through the phospholipase C (PLC)/ protein kinase C (PKC) pathway (Hsu et al., 2002). In the soleus muscle of STZ-induced diabetic rats, PUE also increased the absorption of radioactive glucose and increased glucose transporter type 4 (GLUT4) expression (Hsu et al., 2003). In STZ-induced diabetic mice, PUE treatment increases

endogenous mRNA levels of the skeletal muscle insulin receptor and the peroxisome proliferators activated receptor (Wu et al., 2013).

Promote adipocyte differentiation and glucose uptake of adipocytes: In preadipocytes, PUE increases mRNA expression of peroxisome proliferators activated receptor-γ and its target genes, adipocyte-specific fatty acid binding protein 2 and GLUT4 (Lee et al., 2010). Insulin-induced preadipocyte differentiation is also aided by PUE (Xu et al., 2005).

Increase insulin resistance: Injected the coronary heart disease patients with 500 mg of PUE, in addition to usual therapy, for 3 weeks could lower fasting plasma insulin, plasma total cholesterol, triglyceride, LDL cholesterol, plasminogen activator inhibitor-1 activity, and increase insulin sensitivity index (Shi et al., 2002). PUE administration effectively reverses the increased body weight gain and impaired glucose/insulin tolerance in high-fat diet-induced insulin resistant rat (Zhang et al., 2010).

Protection of the islet cells: PUE effectively diminishes apoptosis and cell death caused by H_2O_2 (Xiong et al., 2006) and cobalt chloride (Li et al., 2014), and elevated glucose levels in isolated islet cells (Yang et al., 2016). PUE treatment reduces insulin levels, and also increases insulin receptor substrate-1 and insulin-like growth factor-1 protein levels in pancreas tissue of STZ-induced diabetic rats (Wu et al., 2013). PUE improves β-cell survival in high-fat diet and diabetic mice (Yang et al., 2016) by increasing the expression of anti-oxidative stress-related enzymes, including catalase and superoxide dismutase, and scavenging reactive oxygen species (Xiong et al., 2006).

Anti-Alzheimer's activity

Alzheimer's disease is a progressive deterioration of cognitive functions which usually occurs in elderly over 65 years of age, especially in people with lowered sex steroid hormone levels, for example, postmenopausal women (Solomon et al., 2014; Kumar et al., 2015). The neuropathological hallmarks of Alzheimer's disease are (i) overproduction and accumulation of amyloid-beta (Aβ) peptide, (ii) formation of neurofibrillary tangles, and (iii) loss of synaptic plasticity (Katzman and Saitoh, 1991; Selkoe and Hardy, 2016). Other effects including increased oxidative stress, mitochondrial apoptosis, and inflammation may also lead to neuronal death (Selkoe, 2001; Awasthi et al., 2016). PUE has the protective effects on Alzheimer's disease via several mechanisms, such as PUE blocks Aβ-stimulated oxidative stress and lipid peroxidation via the glycogen synthase kinase-3β/ nuclear factor erythroid 2-related factor 2 signaling pathway (Zhou et al., 2014). PUE also triggers Nrf2 accumulation by regulating Akt/ glycogen synthase kinase-3β signaling pathway, which leads to the induction of phase II expression of detoxifying enzymes and antioxidant enzymes and in turn decreases oxidative stress and inflammation (Zhang et al., 2011). PUE decreases Aβ levels and suppresses the hyperphosphorylation of tau protein in amyloid precursor protein/ presenilin 1 double transgenic mice (Mei et al., 2016).

Anti-osteoporotic activity

PUE has shown an immense therapeutic potential for increasing bone mass (Huang et al., 2009), and preventing and/or slowing down the process of bone loss (Huang et al., 2009; Liu and Li, 2012; Wang et al., 2012; Li et al., 2014). In a rodent model, PUE treatment could stimulate bone matrix collagen production that stimulates osteoblast proliferation and differentiation and subsequently bone formation (Wong

and Rabie, 2007; Zhang et al., 2012). PUE also acts as a regulator of autophagy by inhibiting the autophagic activity of osteoclast precursors, and suppresses differentiation of osteoclasts (Liu et al., 2015; Wang et al., 2019; Zhou et al., 2019). PUE stimulates the proliferation, increases the mRNA expression of ALP and osteoprotegerin, and decreases the mRNA expression of RANKL of rat osteoblast-like UMR106 cells (Tiyasatkulkovit et al., 2012). PUE enhances mRNA expression of ALP and type I collagen in primary monkey osteoblasts (Tiyasatkulkovit et al., 2014). The increasing of oxidative stress induced by various pathological and physiological factors breeds a series of bone disorders (Kalyanaraman et al., 2018; Li et al., 2019), while PUE displays an anti-oxidative stress capability and mediates osteoclastogenesis (Schroder, 2019).

Anti-cardiovascular diseases

Previous studies have shown that PUE has some therapeutic effects on cerebral ischemia (Gao et al., 2009), myocardial ischemia (Zhang et al., 2006), hypertension (Xu et al., 2005), and arteriosclerosis (Yan et al., 2006). In the case of myocardial ischemia/reperfusion injury, PUE significantly improves cardiac structural damage and dysfunction by reducing myocardial infarct size, apoptotic cell death (Wang et al., 2020), cardiac fibrosis (Chen et al., 2014), cardiac hypertrophy (Chen et al., 2014; Liu et al., 2015), inhibiting autophagy (Xu et al., 2019) and inflammation (Li et al., 2018), and increasing blood flow (Zhang et al., 2013). PUE exerts an inhibitory effect of myocardial apoptosis through phosphatidylinositol 3-kinase/ Akt pathway (Liu et al., 2012; Deng et al., 2017). PUE could also ameliorate oxidative stress, energy metabolism, and metabolic disturbance in myocardial tissues **(**Zhou et al., 2020).

2.3. Toxicity of P. mirifica

P. mirifica has minimal toxicity effects to cardiovascular function, blood lipid levels, and liver enzyme activity in rabbits (Wattanapitayakul et al., 2005) and in menopausal women (Muangman and Cherdshewasart, 2001; Chandeying and Sangthawan, 2007). PME orally administered to rats at doses of 10 and 100 mg/kg.BW/day for 90 days (Chivapat et al., 2000) and at doses of 2 and 40 mg/kg.BW/day for 180 days (Manosroi et al., 2004) has no effects on organ weight and histopathology. Treatment of PME at doses of 10, 100, and 1000 mg/kg.BW/day for 30 days in male rats that were testosterone-induced prostate hyperplasia did not change AST and ALT levels and weights of kidney and liver (Mohamad et al., 2019), but it could increase the body weights. Similarly, postmenopausal cynomolgus monkeys fed with 1000 mg/kg.BW/day of *P. mirifica* powder mixed with monkey chow for 365 days showed normal physical appearance and body weight (Kittivanichkul et al., 2016). Based on the OECD test guideline of acute toxicity, feeding of PME up to 2000 mg/kg in male rats was indicated as safe to the animals (J. Mohamad et al., 2019)

2.4. Toxicity of puerarin

PUE tested in experimental animals shows low toxicity. Regarding the intensive evaluation, PUE injection has been approved by the State Food and Drug Administration of China for the treatment of angina and myocardial infarction. Injection of PUE at a dose of 516.7 mg/kg in rats and at a dose of 273.1 mg/kg in rabbits for 30 days exhibits no allergy and no dermal irritation (Chen et al., 2018). Rats shows no signs of irritation or damage to three parts of the intestinal mucosa; duodenum, jejunum, and ilium, after a single oral dose of 100 mg/kg of PUE (Wu et al., 2018). The repeated oral administration of 50 and 250 mg/kg/day of PUE for 30 days in rats showed

no changes in hematological and biochemical parameters (Chung et al., 2009). A single oral administration of 6, 12, or 24 mg/kg of PUE in healthy Korean subjects indicated no clinically significant changes and no serious adverse effects on of vital signs (Kim et al., 2017).

3. Pharmacokinetics

Pharmacokinetics, which is derived from the Greek words pharmakon (the compound as a drug) and kinetikos (movement), is described as the study of the dynamic movements of drugs during passage through the body. It includes all four processes of absorption, distribution, metabolism, and elimination, or ADME (Turfus et al., 2017). The drug can be administered via many routes which require absorption of the drug from the site of administration to get into the blood circulation, while it can be administered directly into the blood circulation (intravenously), bypassing the absorption process. Once the drug is into the blood circulation, it is distributed throughout the body, where a fraction of the drug enters the target sites, binds to receptors, and exerts a therapeutic effect. During its voyage into the body, the drug is metabolized by various tissues (mainly liver) into other (mainly inactive) forms before being eliminated mainly via urine and feces (Merchant, 2022).

A previously published survey on the causes of failure in drug development indicated that inappropriate pharmacokinetics was a major cause (Merchant, 2022). Inappropriate pharmacokinetic behaviors include such factors as low bioavailability due to poor absorption characteristics, short elimination half-life leading to short duration of action and excessive variability due to genetic or environmental factors (Walker, 2004). Pharmacokinetic knowledge is used in the drug discovery process

across the pharmaceutical industry (Walker, 2004). Understanding and interpreting pharmacokinetic behavior from information of pharmacokinetic parameters can use to optimize dosage regimens for human use and can provide the best therapeutic effects and avoid adverse side effects (Saghir and Ansari, 2018). The accurate pharmacokinetic data in animal studies can provide useful insight on ADME in humans, and the appropriate human dosage regimens are also estimated based on predicted human pharmacokinetic exposure (Yim et al., 2020). A typical pharmacokinetic profile illustrates a drug concentration in blood over a period of time, it usually measures post-administration of drug until most of the drug is eliminated from the body. The shape of the plasma drug concentration-time profile is depended on the route of administration of the drug, i.e., intravenous or oral as shown in Figure 2.4 (Merchant, 2022).

The oral route is the most common route of drug administration that has various advantages over other routes such as easy for administration, patient preference, cost-effectiveness, and ease of large-scale manufacturing (Alqahtani et al., 2021). According to current estimates, oral formulations in liquids, capsules, tablets, or chewable tablets account for over 90% of all pharmaceutical formulations designed for human uses (Alqahtani et al., 2021). Orally administered medicines can also be targeted to specific areas of the gastrointestinal tract for localized therapy of pathological diseases; stomach and colorectal cancers, infections, inflammations, bowel diseases, gastro-duodenal ulcers, and gastroesophageal reflux disorders (Alqahtani et al., 2021). Despite these advantages, the development of oral formulations presents several challenges due to many factors govern oral drug absorption including: *physicochemical properties of drug* such as drug stability in the gastrointestinal fluid, ionization constant, lipophilicity of the drug, drug solubility, dissolution rate, salt form, protein binding, complex

formation, surface area, or particle size; *the anatomy and physiology of the drug absorption site* such as pH of various gastrointestinal segments, esophageal transit time, esophageal motility, food, gastric emptying, small intestinal transit time, bile salt, efflux transporters, or metabolic enzyme; and *dosage form factors* such as solutions, capsules, tablets, coated tablets, or suspensions (Alqahtani et al., 2021).

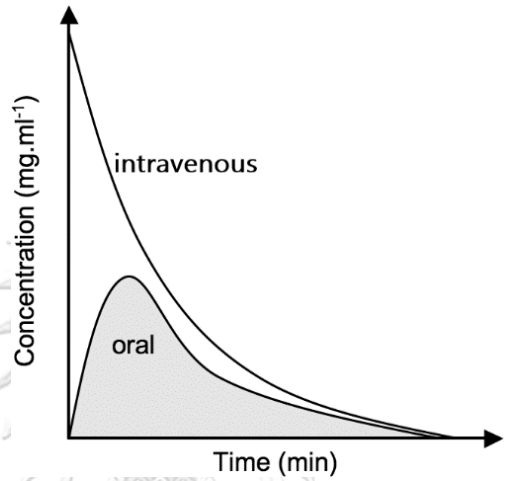

Figure 2.4 A typical pharmacokinetic profile (plasma drug concentration-time profile) of a drug following intravenous or oral administration.

3.1 Compartmental models (William et al., 2014)

Compartmental models are deterministic models that the observed drug concentrations determine the type of compartmental model which is required to describe the pharmacokinetics of the drug. To construct a compartmental model to predict a time-course of drug concentrations, simplifications of body structures are made. The models are categorized by the number of compartments needed to describe the drug's behavior in the body, that is, one-, two-, and multi-compartment models. The compartment that includes blood (plasma), heart, lungs, liver, and kidneys is usually referred to as the central compartment or the highly blood-perfused compartment, while

the other compartment that includes fat tissue, muscle tissue and cerebrospinal fluid is the peripheral compartment, which is less well perfused than the central compartment.

The one compartment model (Loftsson, 2015; Saghir and Ansari, 2018): It is the simplest mammillary model that describes drug distribution and elimination. In the model, the body is described as a single, uniform compartment into which the drug is administered and from which it is eliminated. This is a very simplistic view of the body, in which the drug enters the blood and is then rapidly equilibrated with other parts of the body. This model does not predict actual drug concentrations in the various tissues but assumes that drug tissue concentrations will be proportional to the drug plasma concentrations. The drug equilibrates rapidly in the body, and it is assumed that the concentration throughout the compartment is equal to the plasma concentration. For example, highly hydrophilic drugs which are confined to body water usually have single compartment pharmacokinetics.

As an example, following rapid intravenous administration the blood concentration decreases rapidly at the beginning and then falls more slowly thereafter. This typical first-order elimination can be described by the exponential term:

$$C_p = C_p^0 \, e^{-kt}$$

where Cp represents the blood (plasma) concentration of a drug at time t, C_p^0 is the blood initial concentration, and k is the first-order elimination rate constant. A more practical form of this equation is obtained by substituting the base 10 logarithm:

$$\log C_p = \log C_p^0 - \frac{kt}{2.3}$$

The two-compartment model (Saghir and Ansari, 2018): Though the drug is still distributed instantly and homogeneously into all of the central compartment, it now also

diffuses gradually into the peripheral compartment. A central compartment that roughly corresponds to the blood pool and a peripheral compartment that represents various fluids and tissues of the body for which drugs have a particular affinity. The distribution phase is the initial rapid decline in plasma drug concentration. The elimination phase is the slow decline in drug concentration, sustained by redistribution of drug from tissue stores. One can see how this modeling lends itself to ever-increasing complexity. For example, many lipophilic compounds (with high Vd), are better modeled with a two-compartment model.

This system can be described mathematically by a differential equation comprising two exponential terms, one for each segment of the curve. Taken individually, each one of these terms is essentially similar to the one used to describe the curve corresponding to the one-compartment model:

$$\log C_p = \log A - \frac{\propto t}{2.3} + \log B - \frac{\beta t}{2.3}$$

where A and B are proportionality constants for each compartment (A+B = Cp), and a and b are composite rate constants that can be regarded as the elimination rate constant of each segment of the curve. The first segment is known as the a-phase which is dominated by distribution among the various organs and tissues, whereas the second segment corresponds to the b-phase, which mainly characterizes the elimination of a drug. Accordingly, the $T_{½}$ of a drug displaying such kinetic behavior is calculated from the b-phase similar to the one-compartment model.

The three-compartment model (Schnider and Minto, 2011): Considering a highly fat-soluble drug, when given as a bolus, it distributes rapidly into all tissues including lean muscle and fat. However, lean muscle contains little fat and is therefore

a poor storage reservoir for the drug. The drug is eliminated from that compartment at approximately the same rate as it is from the blood. The fat compartment however soaks up a large amount of the drug. After a while, much of the drug has been cleared from the circulating blood and lean tissue and at this stage the fat compartment begins to act as a source of the drug, topping up the plasma levels as elimination takes place.

$$C_{(t)} = Ae^{-\propto t} + Be^{-\beta t} + Ce^{-\gamma t}$$

where Ct represents the drug concentration at time t, A, B and C are coefficients which describe the exponential functions of each phase. α, β and γ are exponents which describe the shape of the curve for each phase.

3.2 Basic pharmacokinetic parameters

Pharmacokinetic parameters are assessed by monitoring variations in concentration of the drug and/or its metabolites in physiological fluids (Tillement and Tremblay, 2007). The parameters give an overall indication of the behavior of the drug in the body. The pharmacokinetic parameters can be acquired after either a single dose or multiple doses of drug administration. For single dose (Newland, 2006), it is used within the safety assessment of a drug. The parameters of pharmacokinetic refer $AUC_{0\text{-}inf}$, $AUC_{0\text{-}tau}$, Cmax, Tmax, and $T_{½}$. While multiple doses (Newland, 2006) were conducted to find out that the drug is also safe and tolerable after comparing with a single dose, pharmacokinetic data of multiple doses were assessed on day 1 and the last day and the parameters as $AUC_{0\text{-}inf}$, $AUC_{0\text{-}tau}$, Cmax, Tmax, and $T_{½}$, and $AUC_{0\text{-}tau}$, Cmax, Tmax, and $T_{½}$ were analyzed, respectively. In addition, the accumulation of drug in the body after multiple doses were also assessed to confirm the rationale of safety and efficacy. The drug accumulation, which represents the relationship between the dosing interval and the rate of elimination for the drug will be observed (Schnider and

Minto, 2011). When the dosing interval is long, in association with the time needed to eliminate the drug, drug accumulation is low, while the dosing interval is short, drug accumulation is high. The steady state of plasma drug level is reached when concentrations rise and fall according to a repeating pattern and a continued administration of the drug at the same dose, with the same time period between doses. This repeated time period of dosing is often called the *dosing interval* and is abbreviated using the Greek letter tau (τ). Drug accumulation and attainment of steady state does not require IV bolus dosing. For most drugs, it takes roughly five half-lives to reach an approximate steady state.

Maximum plasma concentration (Cmax, Crotti et al., 2015): the highest drug concentration observed in plasma following administration. The value of the maximum plasma concentration is directly obtained from the experimental data without interpolation. When identical maximum concentrations occur at different time points in the same individual concentration *vs.* time profile, the first occurrence will be considered for Cmax.

Time until Cmax is reached (Tmax, Crotti et al., 2015): the time of the maximum plasma concentration is directly obtained from the experimental data without interpolation. Tmax may be useful where an immediate effect is desired.

Area under the concentration - time curve (AUC, Saha, 2018): the measure of the total systemic exposure to the drug. It represents the amount of unchanged drug that has reached the general circulation and it is useful to define the bioavailability of a drug. It is commonly estimated by the trapezoidal rule where the area between the curve and the axis is considered as series of smaller trapezoid and the areas of all the trapezoids are added to obtain the total AUC.

AUC_{0-t} represents the area under the plot of drug concentrations *vs.* time curve, from time of drug administration (time 0) to time "t". In most cases "t" is understood to be the last experimental point when a biological sample has been collected and evaluated.

$AUC_{0-\alpha}$ represents the area under the plot of drug concentration *vs.* time curve from time 0 till the time the concentration becomes zero. This involves calculation of the area of the triangle whose base is represented by the last measured concentration at time "t" and the apex of the triangle is the extrapolated point where the curve meets the time axis.

AUC_{0-tau} represents the area under the plot of the drug concentration *vs.* the time curve from time 0 to the end of the dosing interval.

Oral availability (F, Tillement and Tremblay, 2007): it is the fraction of the dose of drug given orally that reaches the systemic circulation. The F defines how much drug gets into the systemic circulation after oral ingestion. It is usually defined by comparison of the AUC in the systemic circulation after oral ingestion with the AUC after IV dosing.

Accumulation ratio (AR, Newland, 2006): is measured by AUC_{0-tau} day14/AUC_{0-tau} day 1.

Volume of distribution (Vd, Crotti et al., 2015): The Vd is often called the 'apparent' Vd, since the volume has no real anatomical meaning. The apparent volume into which the drug is dissolved. A drug distributes equally for its concentration in blood, plasma, or serum. It is expressed in units of volume. The Vd value depends on the binding of the drugs to plasma proteins and tissues and it is useful to correlate the

drug concentration in plasma with its amount in the body. Drugs with small Vd tend to be polar and water soluble, while drugs with large Vd tend to be highly lipid soluble.

Clearance (CL, Crotti et al., 2015): CL is defined as the volume of blood, plasma or serum from which drug is irreversibly removed per unit time. CL is an index to indicate how well a drug is removed irreversibly from the circulation. Drug clearance may occur via several different organs or pathways of elimination, including hepatic metabolism (liver), renal (kidneys removal of unchanged drug), and biliary (bile) excretion. CL is a systemic clearance following IV administration, while an apparent clearance (CL/F) is a parameter after PO dose or systemic clearance following an extravascular administration. The plasma concentration-time profile after extravascular administration depends on: 1) dose: lower doses usually produce a proportional decrease in plasma concentration at all times; 2) F: lower F results in a proportional decrease in plasma concentration; 3) rate of absorption: slower rate of absorption delays and reduces the magnitude of the peak of plasma concentration; 4) Vd: larger Vd is responsible for both a lower peak concentration and a longer elimination half-life, delaying the peak approach; and 5) CL: CL mainly affects the disposition phase; however, an increase in CL also results in a faster approach to a lower peak concentration.

Mean residence time (MRT, Saha, 2018): the average of the times that each drug molecule remains in the body.

Half-life in the terminal phase ($T_{½}$, Saha, 2018 and Sani, 2019): the $T_{½}$ provides an index of the time-course of drug elimination, the time-course of drug accumulation, and choice of dose interval.

Time-course of drug elimination: the amount of time required for the concentration of the drug to decrease by 50%. The $T_{½}$ is the net effect of all processes leading to removal of the drug. It is independent of the amount of drug in the body and it is useful for the determination of the frequency of drug administration. The dimension of half-life is in units of time. Half-life is directly proportional to drug volume of distribution but inversely proportional to its clearance.

Time-course of drug elimination and accumulation: if a drug is discontinued after an infusion, the drug concentration will decline exponentially to 10% of its starting value after four half-lives. Similarly, if a drug is started as a constant infusion, it will take four half-lives to accumulate to >90% of the final steady-state concentrations.

Choice of dose interval: the dose interval is usually chosen so that concentrations stay above the minimum effective concentrate but below the minimum toxic concentration. Other considerations in the choice of dose interval are the therapeutic index of the drug and compliance. A drug with a high therapeutic index may be dosed less frequently. Compliance is best with dosing once or twice daily. If drug CL decreases, it may be possible for a drug that is normally given three or four times a day to be given twice or once daily, with greater chance of compliance which is good therapeutics.

3.3 Analytical technique for pharmacokinetic analysis

In order to define the pharmacokinetic profile of a compound, the method and the analytical technique used are fundamental. The higher the method sensitivity, the better the description of the drug kinetics, in terms of a much longer monitoring of drug concentration in samples collected at specific time points, which also means a better measurement of the main pharmacokinetic parameters as AUC, T½, CL, or Vd. Then,

high-resolution analytical techniques play an important role in determining concentrations of the compound and its metabolites in biological samples. The analytical method such as gas chromatography-mass spectrometry-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography/tandem mass spectrometry (LC-MS/MS), and enzyme-linked immunosorbent assay (ELISA) have been used to analyze the compound and its metabolites in biological samples with very different concentration ranges. The general advantages and disadvantages of GC-MS, LC-MS, CE-MS, LC-MS/MS, and ELISA for measurements of drug concentration in biological samples are listed in Table 2.3.

Table 2.3 Advantages and disadvantages of GC-MS, LC-MS, CE-MS, LC-MS/MS, and ELISA for measurements of drug concentration in biological samples.

Analysis techniques	Advantages	Disadvantages
GC-MS (Dirks et al., 2018)	High resolution, ideal to resolve complex biological samples	Impossible analysis of thermolabile compounds (metabolites)
	Possible simultaneous analysis of different compounds classes	Non-volatile metabolites must be derivatized before analysis
		Difficulties in identification of unknown compounds after derivatization
LC-MS (Dirks et al., 2018)	High sensitivity	A few restrictions on LC eluents
	Average to high chromatographic resolution,	De-salting may be needed.
	Derivatization is unnecessary	Limited structural information
	Possible analysis of thermolabile compounds (metabolites)	Matrix effect
CE-MS (Dirks et al., 2018)	Useful for complex biological samples	Complex methodology and quantification
	Small volumes	Buffer incompatibility
	High resolution	Difficulties in interfacing

Table 2.3 (Continuous)

Analysis techniques	Advantages	Disadvantages
LC-MS/MS (Eisenhofer et al., 2016)	Minimal consumable costs High sample throughput Sample preparation relatively simple High analytical sensitivity High analytical specificity and relative freedom from interferences High versatility of LC-MS/MS instruments	High capital cost of instrumentation High level of operator expertise required Need to develop in-house methods
Immunoassay (ELISA, Eisenhofer et al., 2016)	Minimal expense of instrumentation Kit methods simple to set up Minimal operator expertise required	High costs of kit method consumables Lengthy sample preparation/analysis time Each metabolite must be measured separately Difficult to identify presence of interferences Poor accuracy-negative bias Poor analytical sensitivity

3.4 Animal model for pharmacokinetic study

In evaluating possible pharmacotherapeutics of drugs and medicines for any diseases, it is crucial to consider any potential animal species differences in pharmacokinetic characteristics. Rats are economical and practical screens for the initial *in vivo* studies of drug's metabolism and characterization of pharmacokinetic parameters. However, because of the dissimilarities between rats and humans in many physiological functions, a pharmaceutical industry requires the pharmacokinetic data of non-rodent species. For puerarin, it was reported that puerarin could affect the pharmacokinetics of some active compounds which were substrates of P-gp, MRP, and CYP 450 (Zhao et al., 2018; Wang et al., 2019; Zhang et al., 2020). Cytochrome P450 is a major enzyme involved in drug metabolism and bioactivation accounting for 70-80 % of the total number of different metabolic reactions (Evans and Relling 1999; Guengerich, 2008). The drug- drug interaction can induce or inhibit specific cytochromes P450 enzymes. Puerarin inhibited the metabolic activities of CYP in 2C and 1A families in *in vitro* (Kim et al., 2014), and decreased the activity of CYP3A (Wang et al., 2019), and induced the activity of CYP1A families *in vivo* (Zheng et al. 2010). The expression of CYP 1A, 2C, and 3A associated enzymatic activities were found in monkey, beagle dog, and rat hepatic microsomes as well as in human (Shimada et al., 1997; Bogaards et al., 2000). However, the expression levels of P450 forms in 1A family of cynomolgus and rhesus monkeys were higher than those of rats and dogs (Edwards et al., 1994). Besides, amino acid sequences of CYP3A in human and monkey were highly conserved and showed 93% homology (Komori et al., 1992), while only 80% homology was found in beagle dog (Ciacco et al., 1991; Ciacco and Halpert,

1989). Both of CYP 3A and 2C contents in rats were lower than in monkeys (Shimada et al., 1997).

Research study examined the molecular characteristics and species differences related to successful extrapolation to human pharmacokinetics by performing extensive meta-analyses of pharmacokinetic data from drugs evaluated in humans, monkeys, dogs, and rats (Ward and Smith, 2004; Jolivette and Ward, 2005; Ward et al., 2005). A literature survey compiling pharmacokinetic data from the monkey for 103 nonpeptide xenobiotics showed the most qualitatively and quantitatively accurate predictions of human pharmacokinetic parameters, in which extrapolated drug clearance and Vd, and the least biased predictions compared with other species (Ward and Smith, 2004). Based on their similar CYP profile, cynomolgus and rhesus macaques in general are considered as a species of choice in pharmacokinetic study of drugs and biologics (Farese et al., 2003; Ramakrishnan et al., 2003).

Generally, a number of non-human primates used in typical GLP safety studies were 4 animals per group in a single dosing or multiple dosings (<28 days, Hobson, 2000). To apply the preclinical pharmacokinetic data acquired from animals to design the doses for human use, the same route of administration should be done. As this thesis aimed to provide the used data to the prescribed remedy of PUE and PME as phytopharmaceutical products for the human use, the oral administration was planned. An oral administration can encounter with the efficiency of the drug absorption through the gastrointestinal tract, and the developmental changes in absorptive surfaces can influence the rate and extent of the bioavailability of the drugs (Fernandez et al., 2011). Since the PUE has been planned to be used in adult human, adult female cynomolgus monkeys, aged 5 to 7 years old, were selected as animal models for this study.

4. Pharmacokinetics of puerarin

The pharmacokinetics of PUE, as a single compound, in in vitro (Su et al., 2016; Zhao et al., 2018) and in vivo studies in healthy animals such as rats (Yang et al., 2011; Anukunwithaya et al., 2018), rabbits (Cui et al., 2005; Deng et al., 2006), and dogs (Ren et al., 2006; Yi et al., 2015) have been conducted. The pharmacokinetics of PUE in mice, rats, and dogs fitted a 2-compartment model, while it fitted a 3-compartment model in rabbits (Jin et al., 1992; Yang et al., 2011). Regarding the sites of absorption, PUE can be absorbed in all segments of the rat's intestine, where the jejunum and ileum are the main absorption sites (Chen et al., 2020), by P-glycoprotein-mediated and MRP-mediated transporters (Su et al., 2016; Zhao et al., 2018). In rats, PUE was absorbed at 2.10 – 7.50% of the given dose (Su et al., 2016; Anukunwithaya et al., 2018) and reached a Cmax at 0.19 – 1.00h (Yang et al., 2011; Cao et al., 2013; Anukunwithaya et al., 2018). In rabbits and dogs, PUE reached the Cmax at 0.83 – 1.08h (Cui et al., 2005) and 1.50 – 4.00h (Ren et al., 2006), respectively. After 1h of PUE IV administration in rats, PUE was distributed to liver, spleen, kidneys, femurs, tibias, mammary glands, lungs, heart, and brain (Anukunwithaya et al., 2018). Moreover, PUE administered to rats could cross placenta and blood-brain barrier (Cao et al., 2013; Kong et al., 2017) and become widely distributed in many areas of the brain such as hippocampus, cerebral cortex, and striatum (Kong et al., 2019).

There are many reports on PUE pharmacokinetics, however it is still inconclusive because of the contradictory results and the different anatomical and physiological characteristics between those animal models and humans. Among many existing species of animals, nonhuman primate such as cynomolgus macaque (*Macaca*

fascicularis) is the most commonly used animal model for pharmacokinetic studies of pharmaceutical products orally given to humans (Cauvin et al., 2015). The gastric conditions in cynomolgus macaques are similar to those of humans, but they are different from the rodents. The gastric pH and gastric emptying time after fasting in cynomolgus monkeys are 1.9 – 2.2 and 153 ± 87 mins (Chen et al., 2008), respectively, compared to pH 1.5 – 3.5 and 248 ± 39 mins in humans (Bolondi et al., 1985; Schwarz et al., 2002), while a gastric pH and a liquid orocecal transit after fasting in rats were 4 – 4.3 and 74 mins, respectively (Ward and Coates, 1987; Schwarz et al., 2002). Besides, CYP3A, a drug metabolizing enzyme found in the liver, showed 93% similarity of amino acid sequences between monkeys and humans (Komori et al., 1992). Therefore, the pharmacokinetics of PUE in cynomolgus monkey should provide a better valuable information than those in other animals when the PUE will be developed to be phytopharmaceutical products for clinical use.

CHAPTER 3

MATERIALS AND METHODS

1. Test compounds and synthetic compounds

The PUE powder (99.0% purity) was purchased from Pure Chemistry Scientific, Inc., USA, and the dried powder of *P. mirifica* (lot no. 141023) was kindly provided by the Smith Natural Co., Ltd, Thailand. The dimethyl sulfoxide (DMSO, purity > 99.5%), ethanol (purity > 95.0%) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich, Corp., USA. Glycyrrhetinic acid (purity > 90.0%) was acquired from Wako Pure Chemical Industries, Ltd., Japan. The HPLC grade methanol (100.0% purity) was acquired from Merck, Ltd., USA.

To prepare the PME, the dried powder of *P. mirifica* was extracted with 95% (v/v) ethanol using a Soxhlet extractor at 60 °C for 6h, filtered through Whatman no. 4 filter paper, and dried by a rotary evaporator at 60 °C, 500 rpm with a pressure of 100 - 300 mbar, until it became a viscous crude extract. The PME was stored in a dark bottle at 4 °C until used in pharmacokinetic study, and some portion was used for analysis of PUE content by LC-MS/MS technique.

2. Analysis of PUE content in the PME using LC-MS/MS technique

The PUE standard and PME were weighed for 1 mg, dissolved in 1 mL of DMSO, and diluted 100 folds in methanol. The diluted samples were mixed with a 10-fold volume of methanol containing 10 ng of glycyrrhetinic acid (an internal standard), and run-on Shimadzu 8060 LC-MS/MS system (Shimadzu Corp., Kyoto, Japan), which was equipped with a vacuum degasser, a binary pump, an autosampler, and a triple

quadrupole LC/MS with an electrospray ionization (ESI) source which was operated and controlled by LabSolution version 5.86 software (Shimadzu Corp., Kyoto, Japan). The Nexera Ultra-High-Performance LC system was equipped with C18 column, Phenomenex Synergi Fusion-RP, with an oven temperature of 40 °C. The mobile phase was 100% (v/v) methanol and 0.2% (v/v) formic acid in water (pH 2.5), at a flow rate of 0.5 mL/min, run as a gradient starting at 10% (v/v) methanol for 1.5 min, increased to 90% (v/v) methanol at 1.5 to 3.5 min, and then decreased to 10% (v/v) methanol at 4 to 4.5 min. The MS analysis was operated in a negative ionization mode by monitoring precursor ion to product ion transitions with mass to charge ratios of 415.05/267.00 (PUE) and 469.35/409.40 (glycyrrhetinic acid). Retention times of PUE and glycyrrhetinic acid were 1.46 and 2.09 min, respectively. Their chromatograms were essentially free from endogenous interference (Figure 3.1).

The PUE content in PME was then calculated, and the result showed that the PUE content was 1.21 mg/100 mg of PME.

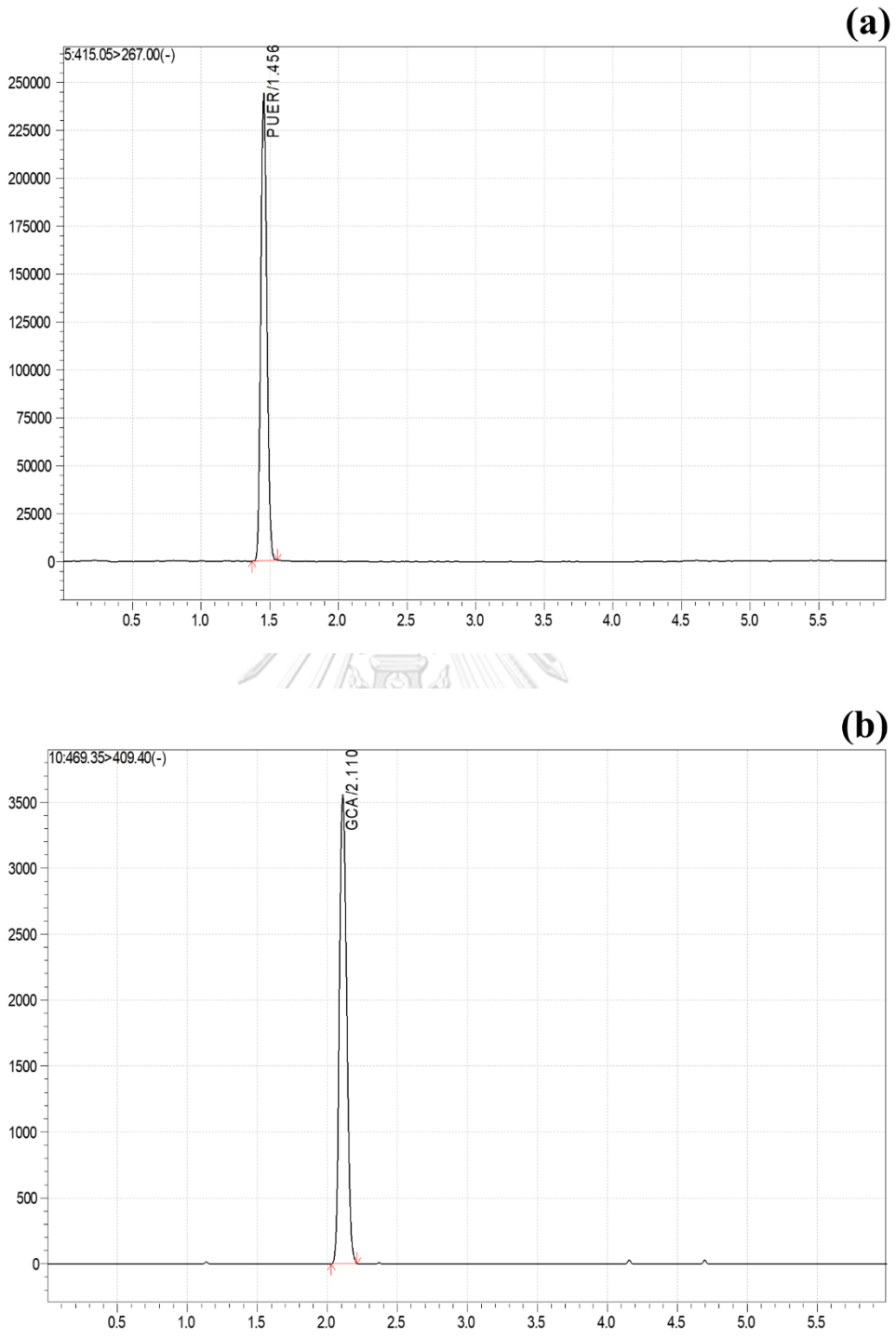

Figure 3.1 LC-MS/MS chromatograms of (a) puerarin and (b) glycyrrhetinic acid spiked in plasma

3. Preparation of PUE and PME for pharmacokinetic study

The PUE and PME were freshly prepared by dissolving in 100% DMSO until it became a clear solution. The solution was subsequently diluted with PBS (pH 7.4) to a concentration of 9: 91 (v/v) DMSO: PBS. Each test substance solution was filtered aseptically using a 0.22-μm pore size polytetrafluoroethylene syringe filter.

To compare the pharmacokinetics of PUE in pure form and in PME after dosing, the dosage of PME was adjusted to 826 mg/kg, which is equivalent to 10 mg/kg of PUE for oral administration. PUE was also administered intravenously at a dose of 1 mg/kg, and was used for comparison with and calculation of the bioavailability of PUE oral dosing. The 9: 91 (v/v) DMSO: PBS at 1 mL was used as a control for each dosing route.

4. Animals and experimental design

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Primate Research Center of Thailand-Chulalongkorn University (NPRCT-CU) (Protocol review number: 1975005; Approval date: April 17, 2019).

Sixteen adults female cynomolgus monkeys, aged 5 to 7 years old and 3.5 to 5.0 kg of body weight, were supplied by the Breeding Facility of the NPRCT-CU. The female monkeys were selected for the study due to the ease in handling for oral administration of the test items under non-anesthetized condition. Selected animals were kept in the acclimatized room for 3 days before transferring to the experimental room. At the experimental room, animals were individually housed in stainless-steel cages with controlled lighting (12h of dark-light cycle, light on from 06:00h to 18:00h)

at temperature of 25 ± 1 °C and a relative humidity of 50 ± 10% in the Animal Biosafety Level-1 facility of the NPRCT-CU. The facility has been AAALAC International Accredited (No. 1752). Animals were fed with standard monkey chow diet (Perfect Companion Group Co., Ltd., Thailand) in the morning (09:00-10:00h) and fresh fruits and vegetables in the afternoon (14:00-15:00h), with free access to hyperchlorinated water pH 7.3 – 7.7 which was provided *ad libitum*. Animal health were visually monitored daily by veterinary technicians or veterinarians.

Sixteen animals were randomly divided into 4 groups (n = 4 per group) and received a single IV administration of 1 mg/kg of PUE, a single oral administration of 1 mL of vehicle (9: 91 [v/v] DMSO: PBS), and a 7-day repeated oral administration of 10 mg/kg of PUE or 826 mg/kg of PME (equivalent to 10 mg/kg of PUE), respectively. To ensure the complete absorption of the PUE and PME after oral dosing, monkeys were fasted overnight before dosing. After oral dosing, animals were kept fasting further for at least 4 hours for complete absorption of the test items (Chen et al., 2008).

For single IV PUE group, blood samples were collected at 0 (pre-dose), 5 min, and 0.25, 0.5, 1, 2, 4, 8, and 24h after injection. For single oral vehicle group, serial blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 8, and 24h after oral dosing. For 7-day repeated oral PUE and PME groups, serial blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 8, and 24h after oral dosing on Day 1 and on Day 7. Serial blood collections on Day 1 were included in analysis of a single oral administration. In all 4 groups, 1 mL of blood samples were collected from saphenous vein at each time point, except at 0 and 24h that the 3 mL of blood samples were collected from the femoral vein. Blood was transferred into a heparinized tube, mixed well and centrifuged at 1,700

xg at 4 °C for 20 min. The blood plasma was harvested and kept at -20 °C until analysis of PUE and its metabolites.

Urine and fecal samples were collected only from PUE (IV and oral dosing) and PME (oral dosing) groups at 2 periods: 0-24 h and 24-48h after dosing (on Day 1 of single IV dosing and on Day 1 and Day 7 of 7-day repeated oral dosing). Urine and feces were collected from the tray placed under each individual cage. To prevent contamination between feces and urine, a tray was covered with an iron mesh before being placed under the monkey cage. Urine and feces were kept into tubes. The feces samples were submerged in 17 mL of methanol to prevent the plausible catalytic reaction which might occur by the microflora. The fecal sample was homogenized thoroughly in methanol by homogenizer and volume of the mixture was adjusted to 25 mL by methanol. Both excreta were centrifuged at 5,000 *xg*, 4 °C for 20 min, and the supernatant was harvested and kept at -20 °C until analyzed for PUE and its metabolites.

5. Blood chemistry analysis

At 0h (on Day 1 of single oral vehicle group) and 24h (on Day 1 of single IV PUE, and on Day 1 and Day 7 of 7-day repeated oral PUE and PME) after administration, blood samples (2 mL) were aliquot from the collection above, and centrifuged at 1,700 *xg* at 4 °C for 20 min. The blood plasma (500 µL) was harvested and analyzed for biochemical parameters in association with liver and kidney function. These are major organs involving drug metabolism and excretion. Liver function was determined by measuring the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), while the level of creatinine was a representative of kidney function. AST, ALT,

and creatinine levels were determined by using A Sysmex BX-3 0 1 0 automated biochemistry analyzer (Furuno Electric Co., Ltd, Japan).

6. Analysis of PUE and its metabolites in biological specimens using LC-MS/MS and QTOF LC/MS technique

Collected plasma, urine, and feces specimens were treated using the protein precipitation method (Prasain et al., 2004). In brief, the frozen samples were thawed at room temperature and 50 µL of each sample was mixed with 200 µL of methanol containing 100 ng glycyrrhetinic acid (as an internal standard) and vortex for 10 min. The mixture was then centrifuged at 12,000 *xg* at 4 °C for 10 min, and 150 µL of supernatant was collected to determine the concentration of PUE and its metabolites by LC-MS/MS and QTOF LC/MS, respectively.

The internal standard, and PUE at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.90625 ng/mL were spiked in the blank matrices (plasma, urine, and feces) and the different standard curves were constructed to measure the concentration of PUE in each biological specimen.

The determination of PUE concentrations in the biological specimens using LC-MS/MS technique was performed as did for the PME samples (as mentioned in **2. Analysis of PUE content in the PME using LC-MS/MS technique**). For metabolite identification, the experiment was analyzed using an Agilent mass spectrometer 6540 QTOF equipped with liquid chromatograph 1260 (Agilent technologies). The stationary phase was Phenomenex Luna C18 column, and the mobile phase was methanol and 0.2% (v/v) formic acid in water. It was run at a flow rate of 0.5 mL/min with gradient elution from 5% (v/v) methanol to 95% (v/v) methanol within 30 min, and then

maintained until 40 min. Column oven was kept constant at 35 °C, and the injection volume was 5 µL per sample. The MS analysis was conducted in negative mode with ESI. Mass spectra were screened from 100 – 1000 m/z, and the chromatogram analysis was conducted by Mass Hunter B.06.00 (Agilent Technologies). To identify the compounds, peak retention time, mass data, and their fragmented ions were compared to those of registered compounds on public databases: Human Metabolome Database, METLIN Metabolomics Database and Library (Agilent Technologies), and authentic compound. The mass error was calculated when comparing a theoretical m/z and an experimentally observed m/z of an assignment.

The analytical method was performed following to the US FDA guidelines for industry for bioanalytical method validation (U.S. food and drug administration, 2018). Thus, the validation parameters, including linearity, accuracy, precision, recovery, and stability, were evaluated. The linearity was defined as the range of analyst concentration that can be fitted with the calibration curve with $R^2 > 0.99$. The accuracy was determined by comparing the measured concentration to the actual low, middle and high concentrations of the quality control (QC) samples, in six replicates, on 3 consecutive days. The accuracy was reported as %bias which was calculated by (measured value-actual value)/ actual value × 100. The precision was determined concurrently with accuracy by analyzing QC samples for intra-assay (6 replicates within a day) and inter-assay variation (once a day for 3 consecutive days). The precision was reported as %RSD which was calculated by (SD/mean) × 100. The accepted precision should to be < 15%, and the accuracy should be within ±15%. The recovery was calculated by comparing the peak-ratio of the prepared sample to that of the standard solution containing the same concentration. The % recovery would be

indicated the quality of sample preparation. The stability test consisted of short-term stability at room temperature, long term stability at storage conditions, freeze thaw stability, and autosampler stability. Short term stability was evaluated the specimens kept at room temperature for 24 h. Long term stability was to assess the specimens kept at -20 °C for 3 months. The freeze-thaw stability of PUE was tested by analyzing QC at three different concentrations subjected to three freeze-thaw cycles (-20 °C and 25 °C). The autosampler stability was determined by measuring the peak area obtained from freshly prepared QC samples compared with samples that were stored in the autosampler compartment for 24 h. The PUE was considered stable, if the accuracy deviation was within ± 15%.

7. Pharmacokinetic analysis

PK analysis was performed using a noncompartmental method with the PK solutions software version 2.0 (Summit Research Services). The Cmax and Tmax are determined directly from the plasma concentration-time profile. The AUC_{0-t} is calculated using the trapezoidal rule, and AUC_{t-inf} is calculated as C_t/kel, where C_t is the last observed plasma concentration after administration and kel is the elimination rate constant calculated from the slope of the terminal phase of the plasma concentration time curve. The terminal elimination $T_{1/2}$ is calculated as 0.693/kel, where the kel is the apparent elimination rate constant of PUE from plasma. CL/F is the apparent total clearance and Vd/F is the apparent volume of distribution. The apparent clearance is calculated as dose/AUC_{0-inf}, and the apparent volume of distribution is equal to (CL/F)/kel. The MRT is calculated using trapezoid area calculations extrapolated to infinity, as equal to $AUMC_{0-inf}/AUC_{0-inf}$. The absolute oral bioavailability (F) is

calculated as (mean $AUC_{0\text{-}inf}$ of PO/mean $AUC_{0\text{-}inf}$ of IV) × (dose IV/dose PO) × 100. In the (7-day repeated) multiple dosing, the AR is calculated as the ratio of $AUC_{0\text{-}tau}$ on Day 7 to $AUC_{0\text{-}tau}$ on Day 1, where tau is the dosing interval (24h) and the AUC values are calculated by the mixed log-linear trapezoidal summations.

8. Statistical analysis

All statistical tests were conducted using the SPSS for Window Software (version 22.0). Data are presented as mean ± one standard deviation (SD) or the median with 25-75% interquartile range. Nonparametric tests were used to determine the significance among groups. The significance at the $p < 0.05$ level was accepted. Data were tested for a normal distribution using the Shapiro-Wilk test, as well as the histograms of distribution test. Significant difference in plasma biochemical levels between pre-dose (0h) and 24h post-dose, was compared utilizing a paired Student's t-test or Wilcoxon signed-rank test, where appropriate. Differences in pharmacokinetic parameters between the 2 related groups (multiple dosing on Day 1 and Day 7) were compared using a paired Student's t-test or Wilcoxon signed-rank test, while differences in pharmacokinetic parameters between PUE and PME (on Day 7) were evaluated using a student's t-test or a Mann-Whitney U test.

CHAPTER 4

RESULTS

1. Method validation

The retention time of PUE, and glycyrrhetinic acid are 1.46 and 2.09 min, respectively (Figure 4.1). There was no significant interference chromatographic peak which eluted at the same retention times as seen in PUE and glycyrrhetinic acid in any types of biological specimens. Since there was no other impurity interference around the retention times of the target compound and internal standard, it indicates a good specificity for the method used in this study.

The percent of accuracy, precision and recovery were assessed by determining the quality control samples at low, medium and high concentrations (15.63, 125 and 500 ng/mL) in six replicates for each day on 3 consecutive days. The results are summarized in Table 4.1. The intra- and inter-assay coefficients of variation (%CV) for accuracy and precision were between 1.04 – 9.09 and 0.88 – 6.81, respectively, which were lower than 10%. The %recovery of the quality control samples in this study was in the range of 83.74 – 97.37%.

The stabilities of PUE spiked in plasma at 3 concentrations (low, medium, and high) and stored in 4 procedural conditions are shown in Table 4.2. All parameters of 3 PUE's concentrations; low, medium and high, were within acceptable limits after being stored in 4 storage conditions; long term storage (20 °C for 3 months), freeze-thaw 3 cycles (-20 °C and 25 °C), room temperature for 10h, and autosampler (4 °C) for 24h. Statistical significant differences among 4 procedural conditions of each PUE concentration were analyzed, and no significant differences were detected (One-way

ANOVA; at high PUE concentration, p = 0.651; at medium PUE concentration, p = 0.344, and at low PUE concentration: p = 0.677).

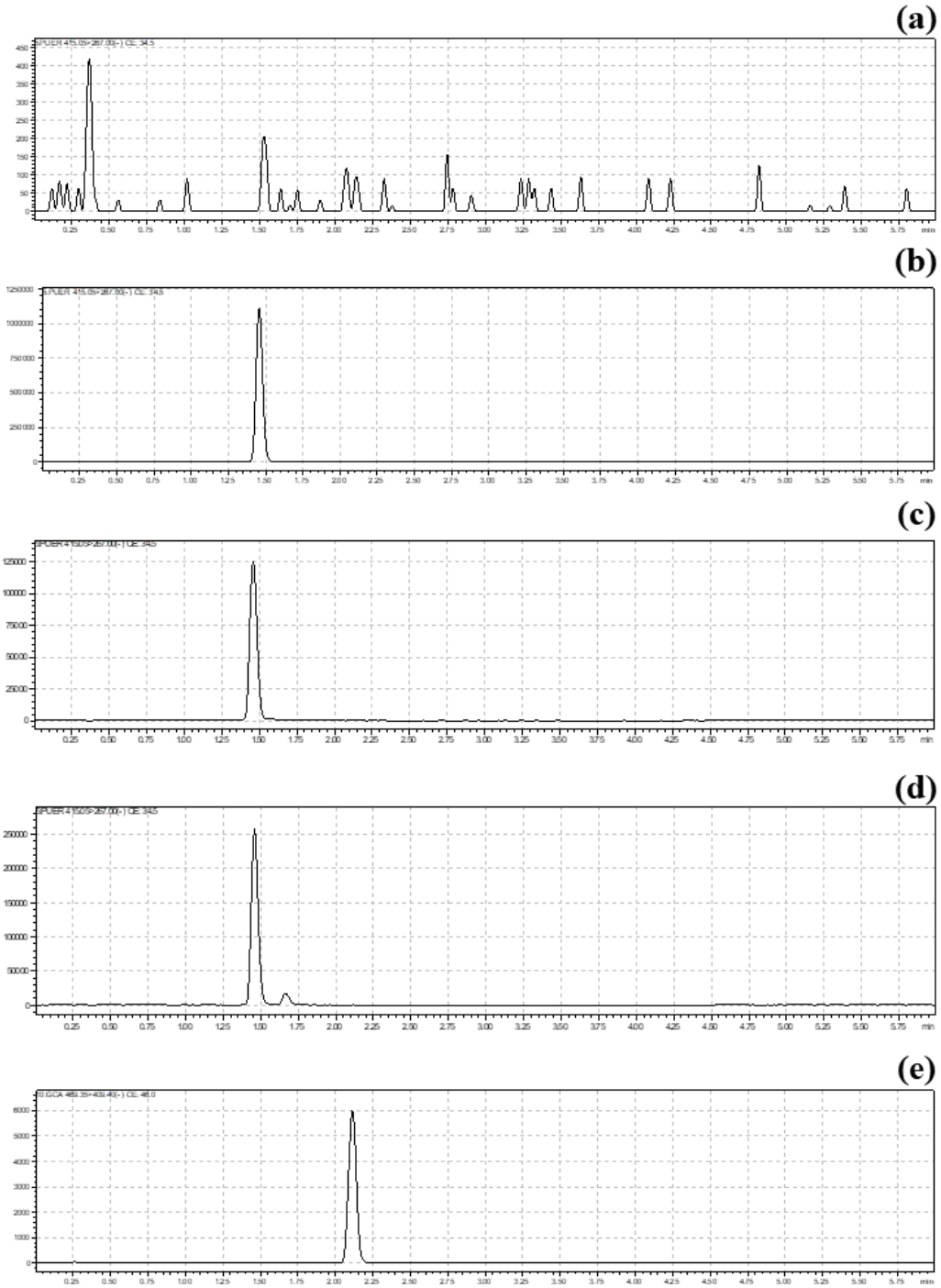

Figure 4.1 Representative chromatograms of pre-spiked (a) blank plasma, (b) puerarin in plasma, (c) puerarin in urine, (d) puerarin in feces and (e) glycyrrhetinic acid internal standard.

Table 4.1 The intra- and inter-assay coefficient of variation of the precision and accuracy, and the recovery of puerarin using LC-MS/MS

Spiked concentration (µg/L)		**Concentration measured (µg/L) (mean ± SD)**	**%Precision (RSD)**	**%Accuracy**	**%Recovery (mean ± SD)**
Intra-assay (n=6)					
High	500	505.20 ± 5.42	1.04	0.88	93.50 ± 9.85
Medium	125	114.12 ± 3.93	8.70	1.60	97.37 ± 6.42
Low	15.63	15.88 ± 0.41	1.63	2.85	91.67 ± 4.21
Inter-assay (n=3)					
High	500	479.78 ± 22.11	4.61	4.04	84.88 ± 12.16
Medium	125	117.73 ± 2.53	2.15	5.81	88.63 ± 9.66
Low	15.63	16.77 ± 1.52	9.09	6.81	83.74 ± 8.02

Table 4.2 The stability of puerarin in plasma at 4 storage conditions using LC-MS/MS

Plasma in storage conditions	Spiked concentration (µg/L)		Concentration measured (µg/L) (mean ± SD)	% Precision (RSD)	% Accuracy
10h at room temperature	High	500.00	478.16 ± 12.34	4.37	2.58
	Medium	125.00	117.03 ± 1.95	6.38	1.66
	Low	15.63	15.37 ± 0.64	1.65	4.15
3 freeze-thaw circles	High	500.00	478.71 ± 13.06	4.26	2.73
	Medium	125.00	120.46 ± 3.90	3.63	3.24
	Low	15.63	15.62 ± 1.06	0.03	6.78
3 months at -20 ℃	High	500.00	485.90 ± 9.84	2.82	2.03
	Medium	125.00	119.91 ± 3.74	4.08	3.12
	Low	15.63	15.26 ± 0.68	2.36	4.45
24h at autosampler	High	500.00	478.41 ± 11.98	4.37	2.58
	Medium	125.00	118.34 ± 4.07	5.33	3.44
	Low	15.63	15.79 ± 0.85	1.05	5.41

2. Animal health status

Plasma biochemical marker levels before (0h) and 24-h after a single oral administration (PO) of vehicle, 24-h after single intravenous administrations (IV) of 1 mg/kg PUE, and 24-h of Day 1 and Day 7 after 7-day repeated oral dosing of 10 mg/kg PUE or 826 mg/kg PME in female cynomolgus monkeys, which indicate liver and kidney functions, are shown in Table 4.3. After single oral administration of vehicle control group for 24h, only the AST level (36.54 ± 7.43 U/L *vs.* 165.13 ± 122.16 U/L) was significantly increased ($p < 0.05$), while no significant differences were detected for ALT and creatinine levels. No significant differences of AST levels between vehicle control group (24h) and PUE-IV (24h), PUE-PO (Day 1), and PME-PO (Day 1) after an administration of vehicle or PUE for 24h. Following 7-day repeated oral dosing of PUE and PME, the level of AST at Day 7 was significantly lowered than Day 1 ($p < 0.05$ between Day 7 and Day 1, PUE: 70.63 ± 25.04 U/L *vs.* 170.25 ± 19.87 U/L, PME: 67.25 ± 26.48 U/L *vs.* 244.63 ± 110.20 U/L), but no significant changes for ALT and creatinine levels. Throughout the study, all monkeys were in a good health, they were observed to consume food and water normally with no signs of illness.

Table 4.3 The plasma biochemical levels before and after single oral administration (PO) of vehicle, after single intravenous administrations (IV) of 1 mg/kg PUE, and multiple oral dosing of 10 mg/kg PUE or 826 mg/kg PME at Day 1 and Day 7 in female cynomolgus monkeys.

Treatment	**Biochemical parameters**		
	AST (U/L)	**ALT (U/L)**	**Creatinine (mg/dL)**
Vehicle			
0h	36.54 ± 7.43*	48.00 ± 43.98	0.94 ± 0.15
24h	165.13 ± 122.16	99.42 ± 69.83	0.90 ± 0.14
PUE-IV			
0h	35.13 ± 7.55*	35.00 ± 26.72	0.93 ± 0.14
24h	106.25 ± 49.00	46.38 ± 38.14	0.91 ± 0.09
PUE-PO			
Day 1 (24h)	170.25 ± 19.87[a]	103.00 ± 29.11	0.88 ± 0.10
Day 7	70.63 ± 25.04	89.63 ± 54.06	0.93 ± 0.10
PME-PO			
Day 1 (24h)	244.63 ± 110.20[a]	137.13 ± 66.81	0.90 ± 0.12
Day 7	67.25 ± 26.48	95.63 ± 55.76	0.81 ± 0.05

The data are expressed as mean ± SD (n = 4); *$p < 0.05$: 0h *vs.* 24h; [a]$p < 0.05$: Day 1 *vs.* Day 7.

3. Plasma concentration-time profiles and oral bioavailability

After a single intravenous administration of 1 mg/kg PUE, the plasma PUE concentration reached a maximum (Cmax) of approximately 3,162.48 µg/L, and gradually declined to 10.78 µg/L at 24h, as presented in Figure 4.2. Since the first blood collection after intravenous administration of PUE was 15 min, and the peak of plasma concentration has already been detected, thus it assumed that the peak concentration might occur earlier than 15 min. The total area under the curve ($AUC_{0\text{-}inf}$ and $AUC_{(0\text{-}24)}$) of PUE was 6,751.86 ± 9,781.80 µg × h/L, and the compound had an apparent volume of distribution (Vd/F) of 0.47 ± 0.34 L/kg, an apparent clearance (CL/F) of 0.44 ± 0.31 L/h/kg, and a half-life ($T_{1/2}$) of 0.71h, as shown in Table 4.4.

In comparison with a single intravenous administration of 1 mg/kg PUE, the Cmax, $AUC_{(0\text{-}24)}$ and $AUC_{(0\text{-}inf)}$ values after a single oral administration of 10 mg/kg PUE (data at Day 1) were lowered (Cmax = 125.97 ± 68.68 µg/L, $AUC_{(0\text{-}24)}$ = 541.79 ± 373.67 µg × h/L, and $AUC_{(0\text{-}inf)}$ = 595.67 ± 384.85 µg × h/L), while the values of an apparent volume of distribution, an apparent clearance, and a half-life were higher (Vd/F = 231.70 ± 333.87 L/kg, CL/F = 26.99 ± 23.75 L/h/kg and $T_{1/2}$ = 4.78h). Based on these data, the PUE fraction absorbed (bioavailability) amount was 0.88% after a single oral dosing with pure PUE compound.

Comparing between a single oral administration of 10 mg/kg PUE (data at Day 1) and a single oral administration of 826 mg/kg PME (at Day 1), the Cmax, $AUC_{(0\text{-}24)}$, and $AUC_{(0\text{-}inf)}$ of PUE in the PME group were rather higher than those of the PUE group, while the values of an apparent volume of distribution, an apparent clearance, and a half-life of the PUE group were higher than the PME group (see Table 4.4). Especially for the $T_{1/2}$, it was prolonged for nearly two times in the PUE group (4.78h)

comparing to the PME group (2.61h). Unexpectedly, the PUE fraction absorbed (bioavailability) amount after the PME oral dosing was higher than the pure PUE administration (1.44% for PME group and 0.88% for the PUE group).

After monkeys were daily oral dosing of PME and PUE for 7 days, all of the $AUC_{(0-24)}$, $AUC_{(0-inf)}$, mean residence time (MRT), and $T_{1/2}$ at Day 7 were higher than Day 1 for 2.5 – 9 times in both PME and PUE group, while the CL/F value was decreased. PUE in PME and PUE group had the accumulation ratio (AR) about 1.94 ± 1.57, and 3.19 ± 1.45, respectively. Noted, the CL/F at Day 7 of the PME group was two times higher than the PO group ($p < 0.05$: 6.25 ± 1.28 L/h/kg for PME group and 3.42 ± 0.96 L/h/kg for PO group).

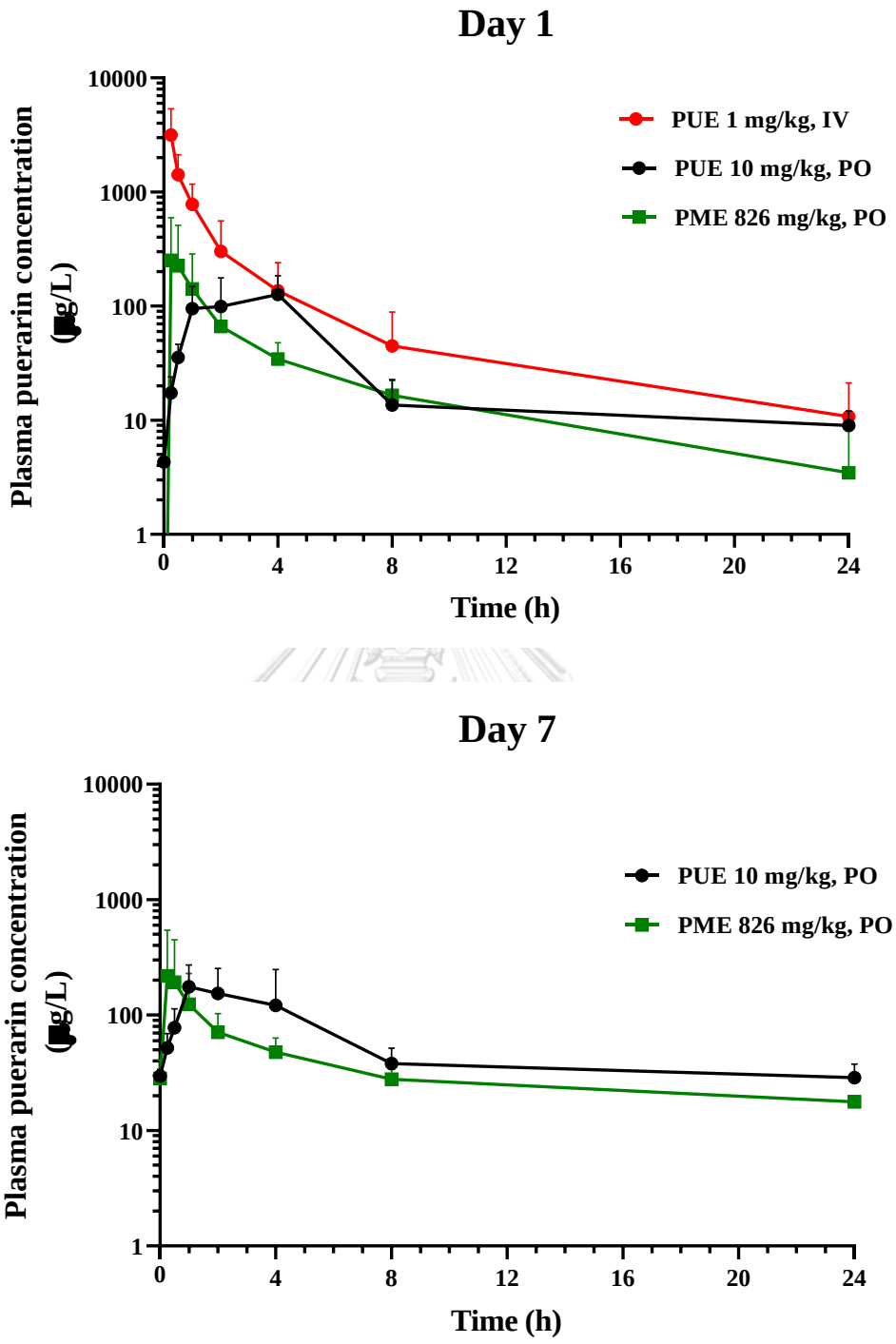

Figure 4.2 Plasma concentration-time curves (mean ± SD; n = 4) of PUE after a single intravenous administration (IV) of 1 mg/kg PUE, and 7-day repeated oral dosing of 10 mg/kg PUE and 826 mg/kg PME at Day 1 and 7.

Table 4.4 Pharmacokinetic parameters of PUE after single intravenous administration (IV) of 1 mg/kg PUE, and multiple oral administration of 10 mg/kg PUE and 826 mg/kg PME at Day 1 and 7.

Pharmacokinetic parameters	**PUE-IV**	**PUE-PO**		**PME-PO**	
		Day 1	**Day 7**	**Day 1**	**Day 7**
Cmax[a] (µg / L)	N/A	125.97 ± 68.68	219.94 ± 101.61	262.13 ± 335.70	237.03 ± 315.62
Tmax[b] (h)	N/A	1.50 (1.00; 3.50)	1.50 (1.00; 3.50)	0.25 (0.25; 0.81)	0.63 (0.25; 1.75)
AUC_{0-24}[a] (µg / L)	6,751.86 ± 9,781.80	541.79 ± 373.67	1,376.40 ± 704.45	648.31 ± 383.89	893.23 ± 330.38
AUC_{0-inf}[a] (µg × h / L)	6,751.86 ± 9,781.80	595.67 ± 384.85*	3,138.73 ± 1,035.35†	975.06 ± 656.42	1,658.74 ± 377.32
MRT[b] (h)	0.46 (0.24; 1.32)	6.03 (4.03; 17.22) *	45.69 (42.89; 79.01)	4.32 (2.94; 72.81)	39.40 (19.78; 50.31)
Vd/F[a] (L/kg)	0.47 ± 0.34	231.70 ± 333.87	222.55 ± 99.85	171.12 ± 275.37	258.49 ± 93.90
CL/F[a] (L / h / kg)	0.44 ± 0.31	26.99 ± 23.75	3.42 ± 0.96†	15.58 ± 11.62	6.25 ± 1.28
$T_{1/2}$[b] (h)	0.71 (0.66; 0.77)	4.78 (0.69; 18.33) *	37.96 (35.83; 60.98)	2.61 (0.66; 55.29)	34.33 (18.49; 36.05)
Bioavailability (%)	N/A	0.88	N/A	1.44	N/A
AR_{AUC}[a]	N/A	N/A	3.19 ± 1.45	N/A	1.94 ± 1.57

[a]Data are expressed as mean ± SD (n = 4); [b]Data are expressed as median (P25; P75); *p < 0.05: Day 1 *vs.* Day 7; †p < 0.05: PUE-PO *vs.* PME-PO at Day 7.

4. Metabolites and metabolic pathways of PUE after a single intravenous and multiple oral administration

After a single intravenous administration of 1 mg/kg PUE, and 7-day repeated oral dosing of 10 mg/kg PUE or 826 mg/kg PME in female cynomolgus monkeys, two metabolites were detected and identified in plasma, urine, and feces of monkeys (Figure 4.3). Two metabolic pathways were proposed. The hydroxylated PUE product possessed the ion at *m/z* 431.10 ($C_{21}H_{20}O_{10}$, error $\leq$ 10), and eluted at 28.85 min. While the deglycosylated PUE product possessed the ion at *m/z* 253 ($C_{15}H_{10}O_4$, error $\leq$ 7), and eluted at 15.45 min. By comparing the chromatographic retention times and MS/MS spectra, hydroxylation product of PUE was identified as daidzein.

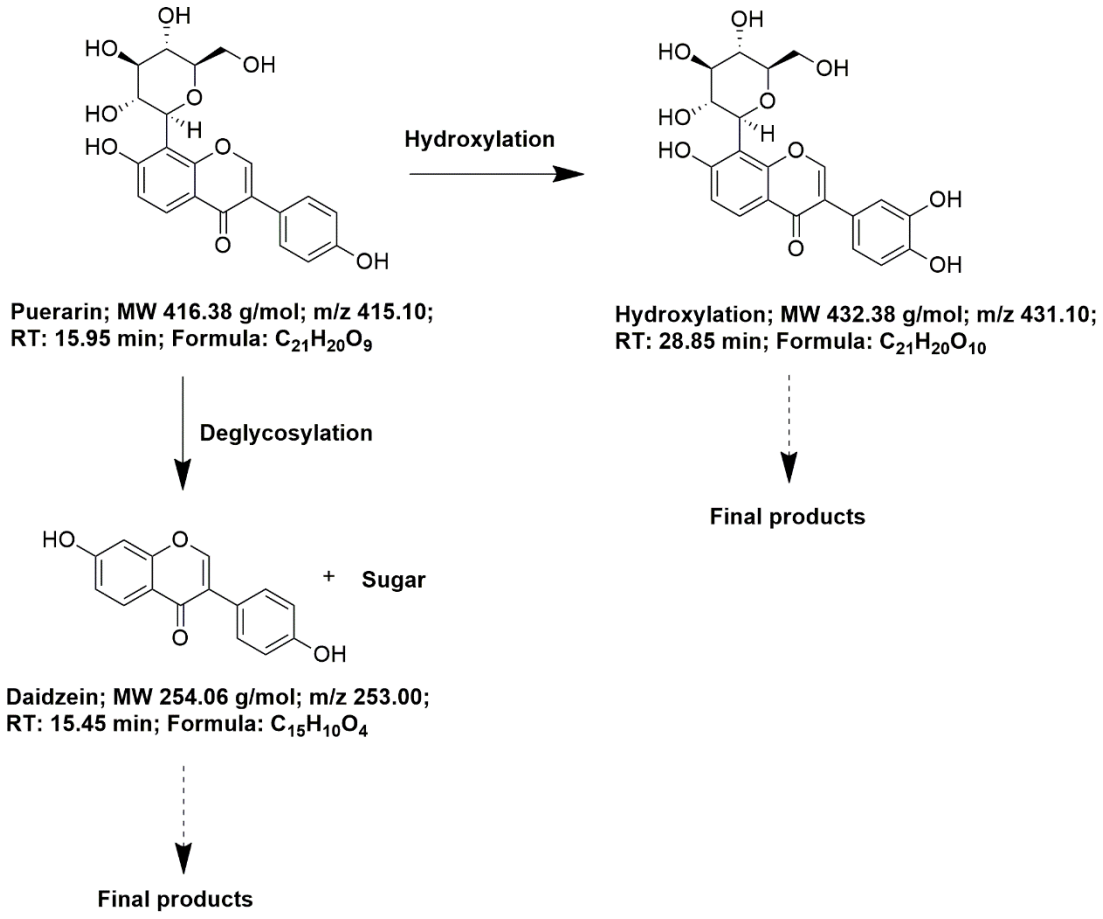

Figure 4.3 Proposed metabolic pathways of PUE after intravenous and oral administration.

5. Excretion study

Urine and feces collected from monkeys at 24 and 48h after a single intravenous administration of 1 mg/kg PUE, and after multiple oral dosing of 10 mg/kg PUE or 826 mg/kg PME (at Day 1 and Day 7) were quantified for the PUE level. Negligible amounts of unchanged PUE were removed from the monkey's body, as shown in Table 4.5. Within 48 hours of dosing, less than 2% of the unchanged PUE was excreted in urine and faces.

Table 4.5 The percent recovery of unchanged PUE during 0-24h and 24-48h after single intravenous administration (IV) of 1 mg/kg PUE, and multiple oral administration of 10 mg/kg PUE and 826 mg/kg PME at Day 1 and 7.

Percent recovery	**PUE-IV**	**PUE-PO**		**PME-PO**	
		Day 1	**Day 7**	**Day 1**	**Day 7**
Urine					
0-24 (h)	<1.00	<1.00	<1.00	<1.00	<1.00
24-48 (h)	<1.00	<1.00	<1.00	<1.00	<1.00
Feces					
0-24 (h)	<1.00	<1.00	<1.00	1.30 ± 1.60	1.91 ± 1.74
24-48 (h)	<1.00	<1.00	<1.00	<1.00	<1.00

Data are expressed as mean ± SD (n = 4).

CHAPTER 5

DISCUSSION AND CONCLUSION

Comparative pharmacokinetics of PUE alone and PUE in PME, at an equivalent PUE dose of 10 mg/kg, after oral dosing for 7 consecutive days was conducted to elucidate the pharmacokinetic profiles of PUE in 2 preparations. Oral dosing was selected as an administration route for this study because it is aimed to mimic the human use. Cynomolgus monkeys were selected as animal models for this study because the interspecies differences between other animal species, i.e. rats, and humans, were reported (Anukunwithaya et al., 2018), and it needs to be addressed before the PUE can be applied for human use. The pharmacokinetics were analyzed at day 1, which counted as a single oral dose, and at day 7, which was considered as a 7-day repeated oral dosing. The results at day 1 were also compared with that of a single IV injection of 1 mg/kg PUE which aimed to evaluate the oral bioavailability of PUE. The vehicle alone (single oral dosing) group was kept as an internal control group to validate the effects of oral dosing on stress and health of animals.

All female monkeys did not show any abnormal physical appearance or abnormal liver and kidney functions after 7-day repeated oral dosing. Constant levels of a plasma kidney marker (creatinine) were in line with those reported in cynomolgus monkeys (Satoh et al., 2016) and humans (David et al., 2016). A significant elevation of plasma AST level, a liver marker, at 24 h after oral dosing of the vehicle was assumed to indicate the stress effect on animal manipulations during the experiment. Stressful condition is one of the environmental factors that may be leading to alteration of AST (Wu, 1998; Kobayashi et al., 2010). Similar conditions were previously observed in

healthy rhesus monkeys (*M. mulatta*) after oral dosing of normal saline (Khemawoot et al., 2011). After multiple oral administrations of PUE and PME, there was a significant decrease in plasma AST levels on day 7, which returned to a basal level when compared to day 1 . The findings of this study indicate that the animals had adapted to the experimental conditions.

A single oral dosing of PME in cynomolgus monkeys showed a slightly higher oral bioavailability of PUE than the administration of PUE alone. This result is in line with the higher Cmax and shorter Tmax, indicating a better absorption, in the PME group compared to the PUE alone group. The phenomenon might be explained by the fact that PME is a mixture of several compounds from the tuberous root of *P. mirifica*, which contains bioenhancers and can inhibit both the efflux transporters and drug metabolizing enzymes at the brush border membrane of the small intestine.

The oral bioavailability of PUE in nonhuman primates in this study was lower than that previously reported in rodents (1.0 *vs.* 7.0%) (Anukulwithaya et al., 2018). The low level of oral bioavailability in non-human priamtes may be affected by first-pass metabolism, which occurs mainly in the enterocytes of the gastrointestinal epithelium and the hepatocytes of the liver by reducing the fraction of drug concentration between entering the portal vein directly from the small intestine and passing through the liver before it reaches the systemic circulation (Kuroda et al., 2000). This in line with the comparative study between rats and monkeys for the oral bioavailability of methotrexate where a percentage of the first-pass effect after oral administration of methotrexate in monkeys (about 62%) was higher than in the rats (only about 24%; Kuroda et al., 2000). Another possible explanation is that the small intestine of cynomolgus monkey had a lower membrane permeability than those in rats

(Takahashi et al., 2008). Similarly, a previous study reported that the bioavailability of a single oral dose of piroxicam, a nonsteroid anti-inflammatory drug, in rats was higher than that in cynomolgus macaques (Krause et al., 1983).

Note that multiple oral dosing of PUE and PME showed a similar tendency for systemic accumulation, with an AR of 1.94 – 3.19. The MRT, which is a summation of the ADME process, increased approximately 7- to 9-fold after multiple oral dosing in both groups. This phenomenon correlated well with the excretion parameters, with lower clearance and longer elimination half-life being observed on day 7. It was previously reported that the AUC0-inf for oral dosing of PME in humans after 3 consecutive days, 3 times a day, was higher than after a single dose and had a steady-state concentration of 40.98 μg/L (David et al., 2006). Since 2 preparations of PUE were given to monkeys in this study, PUE in PME and PUE alone, the drug-drug interaction and drug metabolizing reaction that affect the bioavailability could be different between these 2 preparations. The drug-drug interaction and drug metabolizing reaction might occur after multiple dosing of PME, while the drug metabolizing reaction should mainly be observed in the PUE-alone preparation. Drug metabolizing reaction was reported in the PUE treated to rats. PUE had the inhibitory effects on P-gp and CYP450 enzymes, such as CYP3A4, CYP2B6, CYP2C9, and P-gp (Zheng et al., 2010; Guo et al., 2014; Kim et al. 2014; Liu et al. 2015; Wang et al., 2019). P-gp is an ATP-dependent transmembrane efflux pump that is expressed in columnar epithelial cells of the lower gastrointestinal tract and canalicular surface of hepatocytes (Thiebut et al., 1987), while CYP3A4 is the most abundant cytochrome P450 presented in human hepatocytes and intestinal enterocytes (Paine et al., 2006; Thummel, 2007). It was also reported that PUE increased Cmax and AUC_{0-t}, prolonged

$T_{1/2}$, and decreased clearance rate of astragaloside IV by inhibiting P-gp or CYP3A4 (Zhang et al., 2022). Similarly, oral absorption of PUE after given in the form of *P. lobata* extract was higher than that given as a pure compound (Zhang et al., 2020). PME contains at least 17 phytoestrogenic substances (Ingham et al., 2002); drug-drug interaction can occur via bioenhancement or efflux transporter activity (Zhang et al., 2019) as mentioned earlier. Thus, multiple oral dosing of PUE and PME caused the 3- to 5-fold lower clearance rate and longer $T_{1/2}$. Accordingly, multiple dosing of PUE and PME could improve metabolic exposure and should result in better pharmacological outcomes in nonhuman primates as well as in humans.

Glucuronidation is reported to be the major reaction pathway in the biotransformation of PUE in rats and humans (Luo et al., 2012; Anukulthanakorn et al., 2016). However, this study revealed that hydroxylation and deglycosylation were the 2 major biotransformations of PUE in female cynomolgus monkeys. Indeed, hydroxylated PUE and daidzein were the 2 major metabolites found in the plasma after IV and oral dosing of PUE and PME. The hydoxylated PUE was also reported in rats and detected in the urine in a 0-4 h period (Prasain et al., 2004). Since the intestinal bioavailability (FI=F/[FH*Fa]) in female cynomolgus monkeys is very low (about 0.012 or 1.2%), this suggests that the intestine plays a major role in the metabolism of PUE. The plausible enzymes in intestinal enterocytes metabolizing the PUE are P-gp and CYP3A (Wang et al., 2019). PUE was reported as a plausible substrate of P-gp (Zhang et al., 2019). In monkeys, CYP3A4 might induce hydroxylation and produce hydroxylated PUE (Paine et al., 2006). CYP3A may also hydrolyze PUE to daidzein. Interspecies differences in the metabolic pathway of PUE and the enzyme(s) responsible for these biochemical reactions might need further exploration. Only

negligible amounts of unchanged PUE was found in the excreta of cynomolgus monkeys after dosing with PUE or PME for 48 h, and so most of the PUE was biotransformed into other products before excretion.

The pharmacokinetic study in female cynomolgus monkeys showed both similarity and difference from those of humans. Tmax, half-life, and clearance of PUE after a single oral administration in female cynomolgus monkeys were almost the same of those in humans (Qin et al., 2009; David et al., 2006). Tmax of cynomolgus monkeys were 0.25 – 1.5 h, while that of humans were 0.85 – 1.60h (Qin et al., 2009; David et al., 2006). The half-life in cynomolgus monkeys was 2.61 – 4.78h and was 3.86 – 4.7h in humans (David et al., 2006; Jung et al., 2014). After single oral dosing in this study, the CL/F was 15.58 – 26.99 (L / h / kg) in monkeys, while that of human was 22.12 (L / h / kg) . After multiple oral dosing of PUE, the accumulation values were occurred, and the parameter $AUC_{0\text{-}t}$ at last day were increased in both species (David et al., 2006). However, a species difference of PUE metabolites and CL/F formations was observed. In humans, glucuronides were the main metabolites of PUE and could form puerarin-7-O-glucuronide through conjugation reaction (Zhang et al., 2019), but the hydroxylation was the main metabolite for monkeys.

This is the first information for the pharmacokinetics study of PUE, comparing between the pure form and in the extract form, in female cynomolgus monkey. The dose of PME that was suggested to use in humans contains 10 mg of PUE which is safe for clinical trials in both single and multiple dosage regimen. Oral dosing of PUE showed a good absorption in the extract form and an accumulation (after 7-day repeated doses) in both forms. Thus, the pharmacokinetic profiles obtained from this study could

help to design the prescribed remedy of PUE and *P. mirifica* extract as phytopharmaceutical products for human use.

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