THE EFFECT OF STANDARDIZED EXTRACT OF EURYCOMA LONGIFOLIA ON PHASE I AND PHASE II METABOLIC PATHWAYS AND ROSIGLITAZONE PHARMACOKINETICS

by

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UNIVERSITI SAINS MALAYSIA

DECEMBER, 2013

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Thesis submitted in fulfillment of
the requirements for the degree of
Doctor of Philosophy

DECEMBER, 2013

ACKNOWLEDGEMENTS

Praise to Allah SWT who has given His mercy and grace, so the author is able to complete this thesis. Even though numerous obstacles must be passed, thanks and sincere appreciation from the author to those of who have assisted and supported her, so that this thesis can be completed. First, I gratefully acknowledge to Professor Chan Kit Lam, Professor Abas Hj Hussin and Associate Professor Dr. Sabariah Ismail for their supervision, advice and guidance during the study period. Second, I thank my laboratory mates, Moath Kahtan Bashir, Mahfoudh al Musli, Rukhsana Anwar, Juzaili Azizi, Nur Aziah, Syima Muslim, Low Bin Seng and Ma Hi Qiu who supported me in my work. Third, I would like to acknowledge the financial, academic and technical support of the Universiti Sains Malaysia and its staff, particularly in the award of Postgraduate Research Grant. Fourth, my deepest gratitude to my parents, brother, sister and my sons, who gave me the moral support and thank to my husband for his love, personal support and great patience at all time. Finally, I thank all who supported me during the completion of the project, my apologies for I could not mention their names personally one by one.

Purwantiningsih, 2013

Universiti Sains Malaysia

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ADP	adenosine-5’-diphosphate
Ad libitum	free feeding
ACN	acetonitrile
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
AUC	area under plasma concentration-time curve
AUC_{0-t}	under the curve from zero time to the last of sampling time
BCC	Business Communications Company, Inc
BSA	bovine serum albumin
Bw	body weight
Ca/CaM	calcium calmodulin complex
Ca^{2+}	calcium ion
CAGR	compounded annual growth rate
cAMP	cyclic adenosine-3’,5’-monophosphate
cGMP	cyclic guanosine-3’, 5’-monophosphate
CDNB	1-chloro-2,4-dinitrobenzene
CL	clearance
C_{max}	maximum drug concentration in plasma
COX-2	cyclooxygenase-2
CYP	cytochrome
CV	coefficient of variation
DAG	diacylglycerol
DMSO	dimethylsulphoxide
ESI	electrospray ionization
et al	elsewhere or and others
EC_{50}	effectiveness concentration of 50% effect
ED	erectile-dysfunction
GC	guanylate cyclase
Gpp	guanylyl-5’-imidodiphosphate
GSH	glutathione

GST	glutathione-S-transferase
GTP	guanosine tri-phosphate
HBSS	Hank's balanced salt solution
hcG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography-ultraviolet
IBMX	3-isobutyl-1-methylxanthine
iNOS	inducible nitric oxide-synthase
IC_{50}	inhibition concentration of 50% activity
IL	interleukin
i.v.	intravenous
IS	internal standard
k_e	elimination rate constant
K_i	inhibition constant
KT5720	9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester
KT5823	9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epxoy-1H,8H,11H-2,7b-11a-triazadibenzo(a,g)cycloocta(cde)-trinden-1-one
LC-MS	liquid chromatography–mass spectrometry
LOQ	limit of quantification
MMFO	microsomal mixed-function oxidase
MSD	mass selective detector
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	necrosis factor- κB
NO	nitric oxide
OECD	Organization for Economic Cooperation and Development
OKA	okadaic acid
OTC	over-the-counter
*p*NP	*p*-nitrophenol
PDE	phosphodiesterases
PEG 400	polyethylene glycol-400

PFF	percentage of formaldehyde formed
PgP	P-glycoprotein
PK_A	protein kinase A
PK_C	protein kinase C
PK_G	protein kinase G
PMA	phorbol-12-myristate-13-acetate
PPAR-γ	peroxisome proliferator-activated receptor-γ
SD	standard deviation
STZ	streptozotocin
T_{max}	time to reach of maximum drug concentration in plasma
$t_{1/2}$	elimination half life
TNF-α	tumor necrosis factor-α
TPA	trifluoperazine
UDP	uridine diphosphate
UDPGA	uridine 5'-diphosphoglucoronic acid
UGT	UDP-glucuronosyltransferase
V_d	volume of distribution

KESAN EKSTRAK TERPIAWAI EURYCOMA LONGIFOLIA KE ATAS LINTASAN METABOLIK FASA I DAN FASA II DAN FARMAKOKINETIK ROSIGLITAZON

ABSTRAK

Kepercayaan bahawa produk semula jadi adalah lebih selamat daripada ubat-ubat sintetik telah membawa kepada pertumbuhan dramatik penggunaan persediaan herba. Walau bagaimanapun, persediaan herba juga mempunyai potensi untuk menyebabkan ketoksikan, interaksi ubat-herba dan boleh menjejaskan kesan terapeutik ubat. *Eurycoma longifolia* Jack (Tongkat Ali) telah digunakan secara meluas oleh penduduk tempatan sebagai aprodisiak dan mengandungi banyak sebatian kimia yang perlu dipantau samada wujudnya interaksi ubat-herba. Oleh itu, kajian mengenai kesan-kesan ekstrak *E. longifolia* terpiawai (TAF-273) ke atas profil farmakologi membabitkan metabolisme fasa I dan II, dan farmakokinetik rosiglitazon perlu dijalankan untuk memastikan keberkesanan dan keselamatan penggunaannya.

Kesan *in vitro* ekstrak TAF-273 *E. longifolia* kaya quassinoid terpiawai ke atas metabolisme aminopirina dan rosiglitazon telah dikaji. TAF-273 meningkatkan metabolisme aminopirina secara signifikan ($P < 0.05$) khasnya dalam tikus normal dewasa jantan dan betina. Pada sisi lain, TAF-273 meningkatkan secara signifikan ($P < 0.05$) metabolisme rosiglitazon khasnya dalam tikus jantan tua normal dan diabetik. Kesan *in vivo* TAF-273 dalam tikus ke atas fasa I metabolisme rosiglitazon adalah meningkat secara signifikan ($P < 0.001$) untuk semua haiwan bagi semua dos (5 to 1000 mg/kg) kecuali pada 1 mg/kg ($P > 0.05$). Mekanisme TAF-273 meningkatkan metabolisme aminopirina dalam tikus dewasa jantan mungkin diperantarakan melalui

perencatan tirosina kinase dan pengaktifan protein G, PK_G, PK_A dalam laluan cAMP, kalmodulin dan PK_C, manakala dalam tikus dewasa betina, mungkin diperantarakan melalui pengaktifan protein G, PK_G, PK_A dalam laluan cAMP dan PK_C. Kesan TAF-273 keatas metabolisme fasa I rosiglitazon dalam tikus tua jantan diabetik mungkin diperantarakan melalui pengaktifan protein G, PK_A dalam laluan cAMP, calmodulin dan PK_C manakala dalam tikus tua jantan normal mungkin diperantarakan melalui perencatan protein fosfatase dan tirosina kinase.

Dalam kajian ke atas metabolism fasa II, TAF-273 menurunkan secara signifikan aktiviti UGT *in vitro*, dengan IC_{50} 0.74 µg/mL, manakala kesan TAF-273 ke atas aktiviti GST hanya ditemui pada kepekatan tertinggi yang dikaji (1000 µg/mL; $P < 0.01$). Dalam kajian *in vivo*, penurunan signifikan ($P < 0.01$ dan $P < 0.001$) aktiviti UGT telah ditemui dalam kajian akut dan sub-akut. Kesan TAF-273 ke atas aktiviti GST *in vivo* didapati berbeza, TAF-273 menurunkan secara signifikan ($P < 0.001$) aktiviti GST dalam kajian akut manakala dalam kajian sub-akut, TAF-273 meningkatkan secara signifikan ($P < 0.001$) aktiviti GST 10% sampai 13%. IC_{50} TAF-273 pada perencatan CYP2D6, CYP3A4 dan CYP1A2 aktiviti tidak dapat ditentukan, sebaliknya kajian perencatan TAF-273 dan eurikomanon ke atas aktiviti CYP2C9 dan CYP2C19 menunjukkan bahawa TAF-273 dan eurikomanon bukan perencat poten untuk CYP2C9 dan CYP2C19 dengan IC_{50} eurikomanon 47.33 µM dan 167.88 µM. Kesan TAF-273 ke atas farmakokinetik rosiglitazon telah diperhatikan untuk kajian kedua-dua oral dan intravena. TAF-273 meningkatkan secara signifikan ($P < 0.05$) isipadu (Vd) dan klearans (CL) rosiglitazon sebesar 19.69 % dan 12.41 % dalam kajian oral, tetapi tidak menunjukkan perubahan

signifikan keatas profil farmakokinetik dan bioavailabiliti rosiglitazon dalam kajian intravena.

THE EFFECT OF STANDARDIZED EXTRACT OF EURYCOMA LONGIFOLIA ON PHASE I AND PHASE II METABOLIC PATHWAYS AND ROSIGLITAZONE PHARMACOKINETICS

ABSTRACT

The belief that natural products are safer than synthetic drugs has led to the dramatic growth in herbal medicine usage. However, herbal preparations have the potential to cause toxicity, herb-drug interactions and may affect the drug therapeutic effects. *Eurycoma longifolia* Jack (Tongkat Ali) has been widely used by the local population as an aphrodisiac, and contains many chemical constituents which should be monitored for herb-drug interactions. Therefore, study about the effects of the standardized extract of *E. longifolia* on the pharmacological profile involving metabolism phase I and II, and the pharmacokinetics of rosiglitazone needs to be conducted to ensure the efficacy and safety of use.

The *in vitro* effect of a standardized quassinoid-rich *E. longifolia* extract (TAF-273) on aminopyrine and rosiglitazone metabolism was studied. TAF-273 increased significantly aminopyrine metabolism ($P < 0.05$) especially in the adult normal male and female rats. On the other hand, TAF-273 increased rosiglitazone metabolism *in vitro* significantly ($P < 0.05$) especially in the old normal and diabetic male rats. The *in vivo* effect of TAF-273 in rats on phase I rosiglitazone metabolism was significant increase ($P < 0.001$) in all animals for all the doses (5 to 1000 mg/kg) except at 1 mg/kg ($P > 0.05$). The mechanism of TAF-273 in increasing the aminopyrine metabolism in adult male rat was probably mediated through the inhibition of tyrosine kinases and the activation of G-protein, PK_G, PK_A in the cAMP pathway, calmodulin and $PK_{C,}$ while in the adult female rat was probably mediated

through the activation of G-protein, PK_G, PK_A in cAMP pathway and PK_C. The effect of TAF-273 on phase I rosiglitazone metabolism in the old diabetic male rat was probably mediated through the activation of G-protein, PK_A in the cAMP pathway, calmodulin and PK_C, while in the old normal male rat was probably mediated through the inhibition of protein phosphatase and tyrosine kinase.

In phase II metabolism studies, TAF-273 decreased significantly the *in vitro* UGT activity, with IC_{50} of 0.74 µg/mL, while TAF-273 effect on GST activity *in vitro* was only found at the highest concentration tested (1000 µg/mL; $P < 0.01$). In the *in vivo* assay, a significant decrease ($P < 0.01$ and $P < 0.001$) in the UGT activity was found in the acute and sub-acute studies. Effect of TAF-273 on the GST activity *in vivo* was found different: TAF-273 decreased significantly ($P < 0.001$) the GST activity in the acute study, whereas in the sub-acute study, TAF-273 increased significantly ($P < 0.001$) the GST activity from 10 to 13%. The IC_{50} of TAF-273 on the inhibition of CYP2D6, CYP3A4 and CYP1A2 activity could not be determined, while the inhibition studies of TAF-273 and eurycomanone on the CYP2C9 and CYP2C19 activities indicated that TAF-273 and eurycomanone were not potent inhibitors for both CYP2C9 and CYP2C19 with the eurycomanone IC_{50} of 47.33 µM and 167.88 µM. The effect of TAF-273 on the rosiglitazone pharmacokinetics has been observed for both oral and intravenous studies. TAF-273 increased significantly ($P < 0.05$) the volume of distribution (Vd) and clearance (CL) rosiglitazone by 19.69 % and 12.41 % in the oral study, but did not show significant changes on the pharmacokinetic profile and bioavailability of rosiglitazone in the intravenous study.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Herbal medicines have been used for a long time (Zhang *et al*., 2005) and the market has been rapidly growth. The usage of herbal preparations is widespread especially in China, South and Central America, Africa, Indonesia, India and Pacific Islands, United States, Suriname, Ghana and Peru (Bhattaram *et al*., 2002, Barnes *et al*., 2008; Bussmann & Sharon, 2009; Van-Andel *et al*., 2012; Van-Andel & Carvalheiro, 2013). Demand for natural supplements including traditional medicines increase over the years. According to analysis of BCC Research, herbal supplements have grown at a Compounded Annual Growth Rate (CAGR) of 3 % and contribute more than $83 billion to the overall healthcare market (Valvis, 2011). In Europe, the total herbal products over-the-counter (OTC) in 1991 and 1992 were £1.45 billion and £3.31 billion, respectively (Barnes *et al*., 1998). The increase of herbal sales in the United States was approximately 25 % per year. The data showed a rise from $1 billion in 1994 to $4 billion in 1998 (Bent & Ko, 2004). The estimation of traditional medicines market and other health foods in Malaysia are between US$526 to US$790 million (Noordin *et al*, 2008). Abu Kasim (2007) reported that the total value of herbal industry in the Malaysian market reached RM4.55 billion in 1999 and the market value was estimated at RM7.97 billion in 2005.

A herb-drug interaction can be defined as pharmacologic or clinical response to the coadministration of a traditional drug or pharmaceutical preparation and a herbal product (Brazier & Levine, 2003). The use of herbal remedies that are often coadministered with prescribed drugs could raise the potential of herb-drug

interactions (Zhou *et al*., 2004). The belief among many users and suppliers of herbal drugs that these preparations are natural and safe for consumption have resulted in many herbal products on sale in the market (Zhang *et al*., 2005; Newall *et al*., 1996). However, it is possible that one substance may alter the bioavailability of another substance by inducing phase I or phase II hepatic metabolism that can affect the drug therapeutic response. There are many reports on herb-drug interactions, adverse effects of herbs and their abilities to influence the therapeutic effect of clinical drugs (Ernst, 2002; Mills *et al*., 2005; Low & Tan, 2007). The concurrent use of herbal medicines with synthetic drugs, can mimic, synergistic or contradict the effects of the drugs (Fugh-Berman, 2000), where the interactions may cause some serious clinical consequences (Izzo & Ernst, 2001; Zhou *et al*., 2004; Saxena *et al*., 2008). Effects of herbal preparations on metabolism and pharmacokinetic processes are shown in Tables 1.1 and 1.2, while, Table 1.3 shows the effects of some herbal preparations or compounds derived from medicinal plants on UDP-glucuronosyltransferase (UGT) and glutathione-S-transferase (GST) activities. Thus, studies about the effect of herbal medicines on pharmacological profile involving drug metabolism phase I or II, and the pharmacokinetics study of drugs will become the important findings for rational usage of herbal remedies (Zhang *et al*., 2005).

Table 1.1 Assessment of medicinal plants on drug interactions and effects on cytochrome (CYP) activities (Saxena *et al*., 2008)

No	Medicinal plants and herbal constituent	Drug	Experimental model	Symptom of interaction	Mechanism
1	St. John's Wort (*Hypericum perforatum*): Hyperforin	Cyclosporin	Human hepatocytes	Reduction of serum cyclosporin concentration	Induction of CYP3A4 by hyperforin
2	St. John's Wort (*Hypericum perforatum*): Hyperforin	Omeprazole	Human clinical trial	Decrease of omeprazole plasma concentration	Induction of CYP2C19, 3A4 by hyperforin
3	Garlic (*Allium sativum*): Allicin	Saquinavir	Human clinical trial	Decrease of saquinavir bioavailability	Induction of CYP3A4 by allicin
4	*Piper nigrum*: Piperin	Phenytoin, propranolol, theophylline	Human clinical trial	Increase the bioavailability of phenytoin, propranolol and theophylline	Piperine suppressed CYP2E1 expression
5	Gingko (*Gingko biloba*): Ginkgolic acid	Diltiazem	*In vitro* and *in vivo* analysis on rat hepatic and intestinal CYP enzymes	Inhibition of diltiazem metabolism	The leaf extract inhibited CYP3A activity and ginkgolic acids showed inhibition on CYP1A2, CYP2C9, CYP2C19 activity

Table 1.2 Pharmacokinetic effect of herbal medicines on some drugs (Izzo & Ernst, 2001; Zhang *et al*., 2005; Zhou *et al*., 2004)

No	Herbal Medicines	Pharmacokinetic effect
1	St John's Wort/ SJW (*Hypericum perforatum*)	a. Decreases the plasma concentration of simvastatin and the active metabolite (simvastatin acid) b. Decreases cyclosporine blood concentration leading to organ rejection c. Increases the clearance of alprazolam d. Does not alter the pharmacokinetics of tolbutamide, but increases the incidence of hypoglycemia
2	Garlic (*Allium sativum* L.)	a. Decreases the AUC of saquinavir and ritonavir, and C_{max} of saquinavir b. Changes pharmacokinetic variables of paracetamol c. Decreases blood concentrations of warfarin and produces hypoglycaemia when taken with chlorpropamide
3	Echinacea (*Echinacea purpurea* root)	a. Reduces the oral clearance of caffeine and tolbutamide b. Increases the systemic clearance of midazolam
4	Ephedra (*Ephedra sinica*)	The pH dependence of renal elimination of the ephedrine alkaloids which could lead to undesirable side effects in persons with high urine pH values
5	Ginseng (*Panax ginseng*)	Lowers the blood concentrations of alcohol and warfarin

Table 1.3 Effect of herbal medicines or compounds derived from medicinal plants on UDP-glucuronosyltransferase (UGT) and glutathione-S-transferase (GST) activities

No	Herbal medicines or compounds derived from medicinal plants	Effect on UGT and GST activities	References
1	Carnosol (phenolic diterpene)	Increased GST activity in human cells in rat liver	Singletary, 1996
2	Tannic acid and butein	Decreased GST activity	Zhang *et al.*, 1997
3	Flavones and flavanones	Increased UGT and GST activities in rat liver	Hodex *et al.*, 2002
4	Protocatechuic acid	Increased UGT and GST activities in rat liver	Krajka-Ku´zniak *et al.*, 2004
5	*Orthosiphon stamineus* leaf extracts	Increased both UGT and GST activity in diabetic rat liver	Han *et al.* 2009
6	Flavonoids of Ginkgo biloba extract	Induced the expression of UGT1A1 and CYP1A2 in HepG2 cells	Li *et al*., 2009
7	*Morinda citrifolia* (Noni) juice	Decreased the UGT activity and increased the GST activity in rat liver	Mahfoudh *et al*., 2009
8	*Mitragyna speciosa* Korth extract	Increased GST activity in rat liver	Azizi *et al.*, 2010
9	*Andrographis paniculata* and *Orthosiphon stamineus* extracts	Decreased of some human UGT isoforms activities	Ismail *et al*., 2010

1.2 LITERATUR REVIEW

1.2.1 Testosterone level and erectile dysfunction (ED)

Based on one analysis that used a large member of male subjects, there was a relationship between testosterone level and health. The increase of testosterone levels in men may reduce some symptoms such as depression, obesity, high blood pressure, frequent colds and heart attacks (Booth e*t al*., 1999). The relationship between

testosterone and obesity was on the relationship of testosterone and muscle mass. Testosterone was related to the development of skeletal muscle mass especially in protein synthesis (Freedman *et al*., 1991; Ozata *et al*., 1996; Booth *et al*., 1999). Most of medical research literatures have focused on the treatment of hypogonadism and at normal levels, testosterone could improve sexual function and mood (Davidson *et al*., 1979; Billington *et al*., 1983; Arver *et al*., 1996; Wang *et al*., 1996) and increase skeletal muscle mass (Brodsky *et al*., 1996).

Many chronic diseases, such as cardiovascular diseases, hypogonadism and diabetes mellitus, may affect the sexual function and have been significantly related to sexual dysfunction (Wespes, 2002). According to previous studies, testosterone deficiency, previously recognized as common in men with type 2 diabetes, was also common in men with type 1 diabetes (Rhoden *et al*., 2005; Tomar *et al*., 2006; Chandel *et al*., 2008). Low testosterone levels may cause health problems that lead to erectile dysfunction (Marbeger *et al*., 2006).

1.2.2 Erectile dysfunction therapies

As many as 70 million men were affected by erectile dysfunction (ED) in Europe and North America (Wood, 2000) and around 30 million men were affected in the United States. With aging, the frequency of ED can increase although not always due to aging process. About 25% of men over 65 years of age have some degree of ED. According to one study on ED prevalence (The Massachusetts Male Aging Study), about 52 % of men suffered from ED, at 40 to 70 years (Feldman, 1994).

Not only in America and Europe, ED has been known to be a common sexual problem amongst Asian men. In a study of men aged from 40 to 70 years in four Asian countries, including Malaysia and Japan, it was found that the prevalence of ED (from moderate to complete ED) was 22 % and 34 % (Nicolosi *et al*., 2003). In China, the prevalence of ED was 28.3 % (Bai *et al*., 2004), in Korea, it was 32.2 % (Cho *et al*., 2003) and in Taiwan, 17.7 % (Chen *et al*., 2004). Many Asian men have used traditional medicine for the treatment of ED problems (Fisher *et al*., 2005). Some of traditional medicines used in Asia to increases libido, vitality and sexual energy are shown in Table 1.4 (Low & Tan 2007).

In "The Asian Men's Attitudes towards Life Events and Sexuality (Asian MALES) Study", 65 % of the Malaysia's respondents preferred using traditional remedies because they believed that they can improve overall well being including blood circulation, cure ED with fewer adverse events (Low & Tan., 2007). Therefore, many Asians choose traditional medicine for treatment rather than Westerns medicine (MacKay, 2004).

Table 1.4 Various traditional medicines used in Asia-Pacific (Low & Tan, 2007)

Traditional medicines	**Country**	**Traditional medicines**	**Country**
Tongkat Ali	Malaysia	Three penis wine	Asia
Badam	India	*Panax ginseng* (Rhen Shen)	China
Horny goat weed	Australia	Gingko + *Panax ginseng*	China
Tiger penis	Asia	Yin Guo + Sarsaparilla	China
Powered Rhino horn	Asia	*Panax ginseng* + *Epimedium*	China
Stag penis	Asia	*Curculi orchlodes* (Xian Mao)	China

Some natural products that were used to cure erectile-dysfunction included dehydroepiandrosterone, yohimbine, l-citrulline, pyrano-isoflavones, berberine, papaverine, prostaglandin E_1 and forskolin. Some preparations contained the crude extracts or purified extracts and others contained a mixture of identified compounds derived from the natural sources, such as preparations of *Tribulus terrestris*, lipidic extract from *Lepidium meyenii* (maca), ginseng from *Panax ginseng* or *P. quinquefolius*, *Eurycoma longifolia* extracts, products from *Cordyceps sinensis* and catuama herbal medicine that consists of *Trichilia catigua, Paullina cupana, Ptychopetalum olacides* and *Zingiber officinalis* (Drewes *et al*., 2003). In Malaysia, some of the traditional remedies include the animal parts such as deer antlers and tails, rhinoceros horn and tiger penis, or medicinal herbs including ginseng (*Panax ginseng*), Linzhi Jamu (*Ganoderma*) and Tongkat Ali (*Eurycoma longifolia*) (Low *et al*., 2002; 2004).

1.2.3 *Eurycoma longifolia* Jack (*E. longifolia*)

E. longifolia is a tall tree belonging to the Simaroubaceae family, has been popular in Asia and is distributed in the forests of Burma, Southern Myanmar, Thailand, Indochina (Cambodia, Laos and Vietnam), Sumatra, Borneo, Philippines and Malaysia. *E. longfolia* has many local names: in Brunei is known as tungat ali, langsia siam or pasak bumi; in Indonesia is known as beseng, bidara laut or pasak bumi; in Cambodia is known as antoung sar or antong sar; in Laos is known as tho nan; in Thailand is known as plaalai phuenk, hae phan chan or phiak; in Vietnam is known as Cay ba binh and in Malaysia is known as bedara putih, bedara merah or tongkat ali (Chan *et al*, 1998; Uji, 1999). The plant has a habit as spindly unbranched

tree or grows to a height of 10 m with a few upright branches with stems of grayish brown colour and each forming an umbrella-like rosette of leaves (Figure 1.1).

Figure 1.1 *Eurycoma longifolia* Jack (Tongkat Ali)

The *E. longifolia* has a classification as follows:

Kingdom **: Plantae**

Super Division **: Spermatophyta**

Division **: Magnoliophyta**

Class **: Magnoliopsida**

Subclass **: Rosidae**

Order **: Sapindales**

Family **: Simaroubaceae**

Genus **: *Eurycoma***

Species **: *Eurycoma longifolia* Jack** (Cronquist, 1981**)**

In Malaysia, Tongkat Ali has been claimed to improve the stamina of men during sexual activities, increase the vitality and restore erection. Therefore, it has been reputed as an aphrodisiac. The herb is commonly taken as a decoction of roots in water. Nearly 200 Tongkat Ali products are available on the domestic Malaysian market, in either in combination of herbs or single preparation (Cyranoski, 2005).

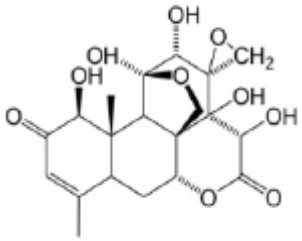
Many publications have revealed the aphrodisiac activity of *E. longifolia* which has been used to enhance male virility during sexual activities. The effects of some *E. longifolia* root extracts (methanol, butanol, chloroform and water) to enhance the libido of male rats had been evaluated by Ang & Sim (1997). The rats were treated with *E. longifolia* at various doses (200, 400 and 800 mg/kg) for ten days, twice daily. The results showed a rise in the mounting frequency. The results suggesting that *E. longifolia* have an ability to stimulate the sexual arousal in male rats. Ang & Ngai (2001) examined the aphrodisiac activity of *E. longifolia* in noncopulator male rats. They used the electrical cage method. Fractions of *E. longifolia* (0.5 g/kg) decreased the hesitation time of male rats during the investigation period. The results support the usage of the plant as a traditional medicine for the aphrodisiac ability. Another experiment by Ang & Lee (2002) gave the evidence that the middle-aged male rats which were treated with different fractions of *E. longifolia* extract (800 mg/kg) showed the changes in sexual behavior. The rats exhibited an increase in the orientation activities including licking, sniffing and mounting towards the females. The sexual motivation activity of male mice with different ages after being treated with extract of *E. longifolia* daily for ten days (500 mg/kg) reported by Ang *et al.* (2003a). A momentary increase occurred in the percent of mice responding after administering the extract by 50 % of the adult

middle-aged male mice. Later, the effect of the *E. longifolia* crude extract in ethanol on the release of testosterone had been identified. It was reported that the ethanolic crude extract decreased the basal release of testosterone. On the other hand, the crude extract increased the production of testosterone induced by the human chorionic gonadotropin (hcG) in rat Leydig cells (Lin *et al.*, 2001).

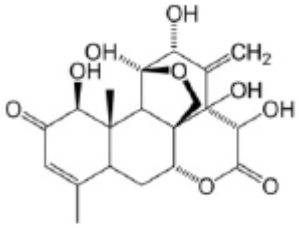
The plant roots of *E. longifolia* are widely used in traditional medicine for healing of boils, after birth, for fever, syphilis, wound ulcer and bleeding gums (Burkill, 1966). *E. longifolia* roots have many chemical constituents. Several of the chemical constituents have been isolated from the roots and characterized, consisting of alkaloids (Mitsunaga *et al.*, 1994; Kanchanapoom, 2001), squalene derivatives (Morita *et al.*, 1993; Itokawa *et al*., 1991a; 1991b), quassinoids (Chan *et al*., 1989; 1991; 1992; Ang *et al*., 2002), tirucallane-type triterpenes (Itokawa *et al*., 1992) and biphenylneolignan (Morita *et al*., 1992). Low *et al.* (2011) determined the major quassinoids in the standardized extract of *E. longifolia*. The extract contained 4.0%, 18.5%, 0.7% and 9.5% of 13α(21)-epoxyeurycomanone, eurycomanone, 13α,21-dihydroeurycomanone and eurycomanol**,** respectively (Figure 1.2).

Many studies on *E. longifolia* showed that the constituents in *E. longifolia* possess antiplasmodial activity (Chan *et al*., 2004; 2005), cytotoxic effect (Kardono *et al*., 1991), plant growth inhibitors, anti-schistosomal, anti-tumor promoting agent and anti-parasitic activity (Jiwajinda *et al*., 2001; 2002), apoptosis effects in HepG2 cells (Zakaria *et al*., 2009), antipyretic activity (Chan *et al*.,1995) and aphrodisiac property (Ang & Sim 1997; Ang & Ngai, 2001; Ang & Lee, 2002; Ang *et al*., 2003b). Hitherto, only a few studies have been published on herb-drug interaction. One study

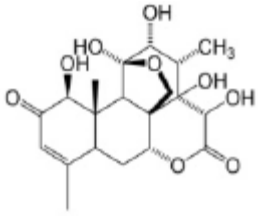
by Salman *et al* (2010) on the effect of *E. longifolia* water-based extract on the bioavailability of propranolol showed that the extract decreased AUC (29%), reduced C_{max} (42%) and prolonged T_{max} (86%) of propranolol.



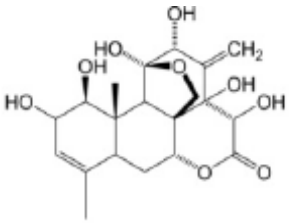
13α(21)-epoxyeurycomanone (**1**)



Eurycomanone (**2**)



13α,21-dihydroeurycomanone **(3)**



Eurycomanol (**4**)

Figure 1.2 Chemical structures of 13α(21)-epoxyeurycomanone (1), eurycomanone (2), 13α,21-dihydroeurycomanone (3) and eurycomanol (4) in the standardized extract of *E. longifolia* (Chan *et al*., 2009; Low *et al*., 2011)

Tongkat Ali is claimed as a testosterone booster (Taufiqurrachman & Wibowo, 2000; Low *et al.,* 2013)**,** has been widely used in society and contains many constituents. It does not affect in the same way as other aphrodisiacs, which show their effects immediately. It should be used regularly over time. Patient may use concurrently with another medicine, so a herb-drug interaction may occur and therefore affect the drug therapeutic effect or even generate toxicity. Therefore, study about the effects of the standardized extract of *E. longifolia* on the pharmacological profile involving drug metabolism phase I and II, and pharmacokinetics effect when

used concurrently with a modern drug, for example rosiglitazone, needs to be conducted to further examine efficacy and safety of the herbal remedies.

1.2.4 Rosiglitazone

Rosiglitazone is used as a model drug in this study because of the metabolic pathways through the N-demethylation. Rosiglitazone is a potent antidiabetic agent (Young *et al*., 1998; Patel *et al*., 1998) and is usually combined with metformin to treat the type 2 diabetes patients (Matthews *et al*., 1999). Rosiglitazone decreases the insulin resistance by interacting with the peroxisome proliferator activated receptor-γ (PPAR-γ). The activation of PPAR-γ will increase insulin sensitivity in the liver, adipose tissue and skeletal muscles, which lead to changes of gene expression that involves metabolism of glucose (Lehmann *et al*., 1995; Matthews *et al*., 1999). PPAR-γ is an important factor that influences various genes involved in the induction of adipocyte-specific gene expression, lipid metabolism and glucose homeostasis. One study has shown that changes in lipid metabolism may mediate the therapeutic effects of rosiglitazone (PPAR-γ agonist) (Shockley *et al*., 2009; Muurling *et al*., 2003; Jucker *et al*., 2003). PPAR-γ activation will inhibit the nitric oxide (NO) overproduction, tumor necrosis factor (TNF)-α and interleukin (IL)-6 expression, suppresses COX-2 and induction of inducible NO-synthase (iNOS) by repression of NF-κB and activation of the activator protein-1 (Inoue *et al*., 2000; Maggi *et al*., 2000). NO gas release is one factor that can alter erection process through the activation of guanylate cyclase (GC) enzyme that converts the nucleotide guanosine tri-phosphate (GTP) into cyclic guanosine monophosphate (cGMP) (Palmer, 1999).

Rosiglitazone is used in the treatment of diabetes and is usually administered at doses 4 to 8 mg/day (Sahi *et al.*, 2003). The absorption of rosiglitazone is rapid and essentially complete, with absolute bioavailability estimated to be 99 % after oral tablet dosing and 95 % after oral solution dosing, and clearance was primarily in metabolic form. In human, the major routes of metabolism are N-demethylation and hydroxylation with subsequent conjugation. Figure 1.3 shows the metabolic pathways of rosiglitazone *in vitro*. Glucuronide conjugation is found lower (7.5 % of administered) than sulphate conjugation (37.8 %) (Baldwin *et al.*, 1999; Cox *et al.*, 2000). It was reported that rosiglitazone was a strong inhibitors of CYP2C8 and CYP3A4, *in vitro*, and CYP2D6 was slightly inhibited by rosiglitazone (Sahi *et al.*, 2003). CYP2C8 was primarily responsible for the hydroxylation and N-demethylation of rosiglitazone in human liver microsomes; with minor contributions from CYP2C9 (Baldwin *et al.*, 1999).

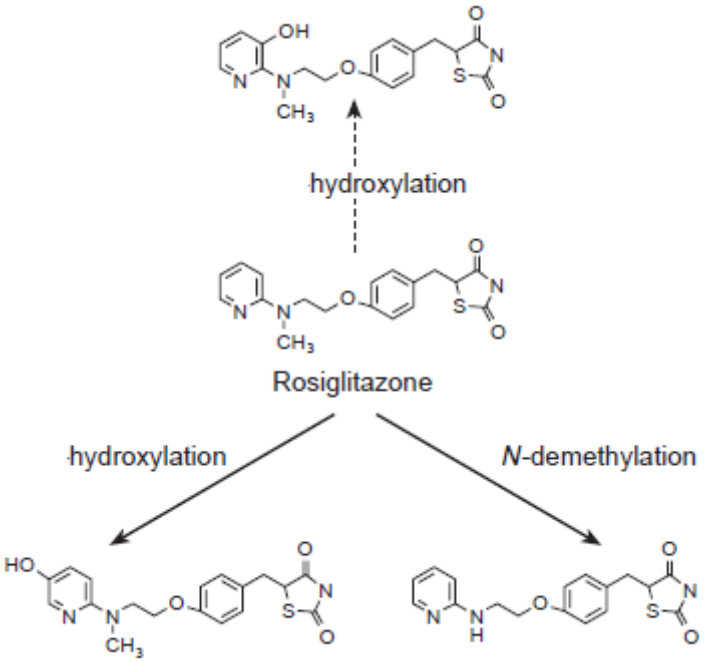


Figure 1.3 Metabolic pathways of rosiglitazone *in vitro* with the major routes of metabolism are N-demethylation and hydroxylation (Baldwin *et al*., 1999)

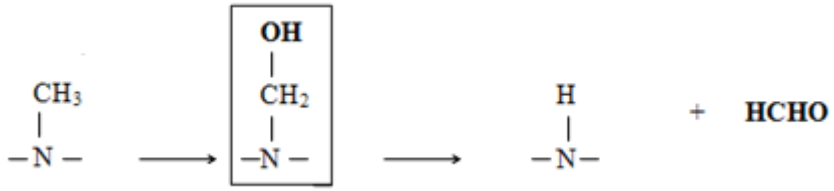
1.2.5 Aminopyrine

Aminopyrine is an analgesic and antipyretic drug, is rapidly and almost completely absorbed after oral administration. The time to reach the maximum plasma concentration is about 1.5 h and the time of half-life is 2 to 3 h. The major route of aminopyrine metabolism is through demethylation and acylation pathways, with unchanged aminopyrine excreted in small quantities (10%). Aminopyrine is mainly N-demethylated by CYP3A and CYP2B in rats (Kamataki, 1993; Timbrell, 2001) and CYP2C in humans (Woolf, 1999). Some studies of drug-drug interaction by using aminopyrine as substrate have been performed (Gemayel *et al*, 2001; Di Nucci *et al*, 1979; Vessel *et al*, 1976). Herb-drug interaction study of aminopyrine with some herbal medicines has been reported. Abas *et al.* (1998) reported about the interaction of aminopyrine with seven traditional medicines in rat hepatocytes. The results showed that some of these preparations affected significantly aminopyrine metabolism. Han & Hussin (2007) had studied about the effect of *O. stamineus* Benth on aminopyrine metabolism. *O. stamineus* showed significant increase in the aminopyrine N-demethylase activity. Interaction study of *O. stamineus* and *M. citrifolia* with aminopyrine metabolism in rats have been reported by Chin *et al.* (2009). Recently, it was reported that *in vivo* administration of Faizol Ubat Batuk (FUB) in rat affected phase I aminopyrine metabolism. The effects of FUB were related with disease and gender (Taher & Hussin, 2011). On the other hand, Ravindranath & Varada (1989) studied about aminopyrine N-demethylase activity in mouse liver and brain that was associated with cytochrome P-450 acitity and sex-difference. Activity of aminopyrine N-demethylase in human liver microsomes from 31 different patients has been reported. The results suggested that in the population studied, inter-individual differences may be due to the occurrence of different

amounts of the same enzyme, rather than the presence of different enzymes (Garcia-Agundez *et al*., 1990).

In phase I metabolism, many reactions such as hydroxylation, reduction or dealkylation can occur. Dealkylation reaction occurs with drugs or xenobiotics that have the functional groups such as an alkoxy group or an alkyl substituted thiol, a secondary and tertiary amine and will produce the formaldehyde (HCHO) (Gibson & Skett, 2001). Depending on the type of atom that binds the alkyl group, these reactions are divided to the N-, O- or S-dealkylations (Timbrell, 2001, Gibson & Skett, 2001). Aminopyrine and rosiglitazone are examples of drugs that are metabolized through N-demethylation. The reaction occurs in two stages, the first is attachment of the hydroxyl group to the methyl group next to the nitrogen and then the decomposition process of this intermediate with the loss of formaldehyde. The formaldehyde formation can be determined by Hantzsch reaction (Figure 1.4)

A



Unstable intermediate **Formaldehyde**

B

Acetylacetone **Formaldehyde** **3,5-Diacetyl-1-4 Dihydrolutidine (λ_{max} 415 nm)**

Figure 1.4 The mechanism of N-demethylation reaction (A) and colorimetric determination of formaldehyde by Hantzsch reaction (B) (Gibson & Skett, 1994)

1.2.6 Theoretical framework

The belief in society that natural products are safer than synthetic drugs has led to the dramatic growth of herbal medicine usage. Like synthetic drugs, herbal remedies may also cause herb-drug interactions or adverse effects. Knowledge of the effect of herbal medicines on the pharmacological profile and pharmacokinetics of drugs are needed to explain and predict the various events related to the efficacy and toxicity of herbal preparations. However, study about the herb-drug interactions of herbal preparations have not been systematically studied, especially for *Eurycoma longifolia* that is widely used in Indonesia, Malaysia and Vietnam.

Tongkat Ali (*E. longifolia*) is claimed as an aphrodisiac. The effect will be felt gradually after a certain period of usage in about one week or more. It is possible that type 2 or type 1 diabetic patients that had experience on erectile dysfunction uses concurrently Tongkat Ali with an antidiabetic agent (such as rosiglitazone). An antidiabetic agent is usually used for a long period of time. While rosiglitazone is a ligand for PPAR-γ, PPAR-γ is a critical transcription factor that influences numerous genes related to lipid metabolism and glucose homeostasis. This suggests that changes in lipid metabolism may mediate the therapeutic effects of PPAR-γ agonist. This is due to the fact that PPAR-γ activation will inhibit NO overproduction, and NO gas release is one factor that can alter erection process through the activation of GC enzyme that converts GTP into cGMP. Herb-drug interaction may occur if Tongkat Ali and rosiglitazone are used concurrently for a long time.

1.2.7 Objectives of study

There are five objectives in this study:

a. To examine the *in vitro* and *in vivo* effects of the standardized extract of *E. longifolia* on phase I hepatic drug metabolizing enzymes on Sprague-Dawley rats.

b. To evaluate the effect of the standardized extract of *E. longifolia* on the mechanism of drug metabolism in rat liver.

c. To examine the *in vitro* and *in vivo* effects of the standardized extract of *E. longifolia* on phase II hepatic drug metabolizing enzymes on Sprague-Dawley rats.

d. To examine the effect of the standardized extract of *E. longifolia* on the human cytochrome P450 (CYP450) activity.

e. To examine the effect of the standardized extract of *E. longifolia* on the pharmacokinetic profile and bioavailability of rosiglitazone when co-administered orally.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Sources of chemicals used

Table 2.1 Chemicals for standardization of *Eurycoma longifolia* extract (TAF-273) by using LC-MS

No	Name of chemicals	Company, Country
1	ACN (acetonitrile) (HPLC grade)	Merck, Germany
2	Formic acid of HPLC grade	Sigma Chemicals Co, USA
3	Methanol of HPLC grade	Merck, Germany
4	Eurycomanone standard (purity ≥95%)	Kind gift from Prof. Chan Kit Lam

Table 2.2 Chemicals for phase I metabolism studies and mechanism study

No	Name of chemicals	Company, Country
1	Aminopyrine (4-Dimethyl-amino-antipyrine)	Sigma Chemicals Co, USA
2	Barium hydroxide	R & M Chemicals, UK
3	Calcium chloride	Riedel-deHaen, France
4	Dimethyl sulfoxid (Methyl sulfoxide)	Fluka, Switzerland
5	Diethyl ether	BDH Laboratory Supplies, UK
6	Disodium hydrogen phosphate	R & M Chemicals, UK
7	Formaldehyde solution 37%	Merck, Germany
8	Furafylline	Sigma Chemicals Co, USA
9	Genistein	Calbiochem, Germany
10	Glucose monohydrate	Riedel-deHaen, France
11	Gpp [guanylyl-5'-imidodiphosphate tetralithium salt]	Calbiochem, Germany
12	IBMX [3-isobutyl-1-methylxanthine]	Calbiochem, Germany
13	KT5823 [9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epxoy-1H,8H,11H-2,7b-11a-triazadibenzo(a,g)cycloocta(cde)-trinden-1-one]	Calbiochem, Germany
14	KT5720 [9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester]	Calbiochem, Germany
15	L-ornithine [L-N5-(1-iminoethyl)-ornithine]	Calbiochem, Germany
16	Magnesium chloride	BDH Laboratory Supplies, UK
17	Magnesium sulphate	BDH Laboratory Supplies, UK
18	OKA [okadaic acid potassium salt]	Calbiochem, Germany
19	PMA [phorbol-12-myristate-13-acetate]	Calbiochem, Germany
20	Potassium chloride	R & M Chemicals, UK
21	Potassium dihydrogen orthophosphate	Ajax Chemicals, Australia
22	Rosiglitazone maleate	Wuhan Sunrise Technology, China
23	Sodium hydrogen bicarbonate	R & M Chemicals, UK
24	Sodium chloride	BDH Laboratory Supplies, UK
25	Streptozotocin	Sigma Chemicals Co, USA
26	TPA [trifluoperazine dihydrochloride]	Sigma Chemicals Co, USA
27	Trypan blue	Sigma Chemicals Co, USA
28	Zinc sulphate heptahydrate	R & M Chemicals, UK

Table 2.3 Chemicals for phase II metabolism studies (UGT and GST assay)

No	Name of chemicals	Company, Country
1	BioRad protein assay kit	Hercules, CA, USA
2	CDNB [1-chloro-2,4-dinitrobenzene]	Sigma Chemicals Co, USA
3	Diethyl ether	BDH Laboratory Supplies, UK
4	Disodium hydrogen phosphate	R & M Chemicals, UK
5	Glutathione, reduced form	Sigma Chemicals Co, USA
6	Glycerol	Sigma Chemicals Co, USA
7	Magnessium chloride	ChemAR, Systerm, Malaysia
8	p-Nitrophenol	Sigma Chemicals Co, USA
9	Potassium chloride	R & M Chemicals, UK
10	Potassium dihydrogen orthophosphate	Ajax Chemicals, Australia
11	Sodium diclofenac	Sigma Chemicals Co, USA
12	Sodium hydroxide	J&J Laboratory Chemicals, Austria
13	Tannic acid	HmbG Chemicals, Malaysia
14	Trichloroacetic acid	Fisher Scientific, USA
15	Tris-HCl	Sigma Chemicals Co, USA
16	UDPGA [Uridine 5'-diphosphoglucoronic acid	Sigma Chemicals Co, USA
17	Triton X-100	ICN Biomedicals, Inc, USA

Table 2.4 Chemicals for human cytochrome P-450 study

No	Name of chemicals	Company, Country
1	a. P450-GLO™ CYP1A2 Assay kit, includes: - Luciferin-ME (luciferin 6'methyl ether), 5 mM - Luciferin detection reagent (lyophilized) - Reconstitution buffer b. Human CYP1A2 Enzyme System kit, includes: - CYP1A2 (1pmol/µL) + reductase - Control membranes - 1M Potassium phosphate buffer (pH 7.4) - Luciferin-free water - Solution A, NADPH regeneration system - Solution B, NADPH regeneration system	Promega, USA
2	a. P450-GLO™ CYP2C9 Assay kit, includes: - Luciferin-H (6' deoxyluciferin), 5 mM - Luciferin detection reagent (lyophilized) - Reconstitution buffer b. Human CYP2C9 Enzyme System kit, includes: - CYP2C9 (1pmol/µL) + reductase + b5 - Control membranes - 1M Potassium phosphate buffer (pH 7.4) - Luciferin-free water - Solution A, NADPH regeneration system - Solution B, NADPH regeneration system	Promega, USA
3	a. P450-GLO™ CYP2C19 Assay kit, includes: - Luciferin-H EGE (ethylene glycol ester of 6' deoxyluciferin), 123 µg/bottle - Luciferin detection reagent (lyophilized) - Reconstitution buffer with esterase b. Human CYP2C19 Enzyme System kit, includes: - CYP2C19 (1pmol/µL) + reductase + b5 - Control membranes - 1M Potassium phosphate buffer (pH 7.4) - Luciferin-free water - Solution A, NADPH regeneration system - Solution B, NADPH regeneration system	Promega, USA

Table 2.4 Continued

No	Name of chemicals	Company, Country
4	a. P450-GLO™ CYP2D6 Assay kit, includes: - Luciferin-ME EGE(ethylene glycol ester of luciferin 6' ethyl ether), 900 µg/bottle - Luciferin detection reagent (lyophilized) - Reconstitution buffer with esterase b. Human CYP2D6 Enzyme System kit, includes: - CYP2D6 (1pmol/µL) + reductase - Control membranes - 1M Potassium phosphate buffer (pH 7.4) - Luciferin-free water - Solution A, NADPH regeneration system - Solution B, NADPH regeneration system	Promega, USA
5	a. P450-GLO™ CYP3A4 Assay kit, includes: - Luciferin-BE (luciferin 6' benzyl ether), 5 mM - Luciferin detection reagent (lyophilized) - Reconstitution buffer b. Human CYP3A4 Enzyme System kit, includes: - CYP3A4 (1pmol/µL) + reductase + b5 - Control membranes - 1M Potassium phosphate buffer (pH 7.4) - Luciferin-free water - Solution A, NADPH regeneration system - Solution B, NADPH regeneration system	Promega, USA
6	Beetle luciferin [(4S)-4-5-dihydro-2-(6-hydroxybenzothiazolyl)-4-thiazolecarboxylic acid or D-luciferin] potassium salt	Promega, USA

Table 2.5 Chemicals for pharmacokinetic study

No	Name of chemicals	Company, Country
1	Acetonitrile (ACN) (HPLC grade)	Merck, Germany
2	Ethyl acetate (HPLC grade)	Merck, Germany
3	Methanol (HPLC grade)	Merck, Germany
4	Disodium hydrogen phosphate	R & M Chemicals, UK
5	Hydrochloric acid (HCL, analytical grade)	Fisher Scientific, UK
6	Potassium dihydrogen orthophosphate	Ajax Chemicals, Australia
7	Rosiglitazone maleate	Wuhan Sunrise Technology, China
8	Triethylamine (analytical grade)	Acros Organics, USA
9	Polyethylene glycol-400 (PEG 400)	Sigma Chemicals Co, USA