MODULATION OF CYTOCHROME P450s BY *STROBILANTHES CRISPUS* ANTICANCER SUB-FRACTION AND ITS POTENTIAL INTERACTION WITH TAMOXIFEN METABOLISM

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LIST OF SYMBOLS

%	Percentage
±	More or less
>	Greater than
<	Smaller than
°C	Degree Celsius
µ	Micro
Σ	Summation
α	Alpha
β	Beta
×g	Gravity

LIST OF ABBREVIATIONS

AUC	Area under the concentration-time curve
CL	Clearance
C_{max}	Maximum plasma concentration
C_p	Plasma concentration
CV	Coefficient of variation
CYP	Cytochrome P450
DPBS	Dulbecco's Phosphate Buffered Saline
E	Free enzyme
EDF	Endoxifen
EI	Enzyme-inhibitor complex
ES	Enzyme-substrate complex
ESI	Enzyme-substrate-inhibitor complex
ESI	Electrospray ionization
eV	Electron volt
F3	The standardized sub-fraction of *Strobilanthes crispus*
g	Gram
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
h	Hour
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
HQC	Quality control sample at high concentration level
HTF	4-hydroxytamoxifen
I	Inhibitor
IC_{50}	The half maximal inhibitory concentration
IS	Internal standard
IVCI	*In vitro* CYP inhibition
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Potassium dihydrogen phosphate
K_i	Inhibitory constant

K_m	The Michaelis constant
kV	Kilovolt
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
l/min	Liter per minute
LQC	Quality control sample at low concentration level
M	Molar
$MgCl_2 * 6H_2O$	Magnesium chloride hexahydrate
Mg/mL	Milligram per milliliter
min	Minutes
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mM	Millimolar
MP	Mobile phase
MQC	Quality control sample at medium concentration level
MRM	Multiple reaction monitoring
ms	Millisecond
n	Number
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NDT	*N*-desmethyltamoxifen
ng/mL	Nanogram per milliliter
nm	Nanometer
NRS	NADPH regeneration system
pmol/min/pmol CYP	Picomole per minute per picomole cytochrome P450
pmol/mL	Picomole per milliliter
PPB	Potassium phosphate buffer
P/S	Penicillin-streptomycin
QC	Quality control
QQQ	Triple quadrupole
r^2	Coefficient of determination

RE	Relative error
S	Substrate
TAM	Tamoxifen
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar
U/mL	Unit per milliliter
V	Volt
V_{max}	Maximum reaction velocity

MODULASI SITOKROM P450s OLEH ANTIKANSER SUB-FRAKSI *STROBILANTHES CRISPUS* DAN POTENSI INTERAKSI DENGAN METABOLISMA TAMOXIFEN

ABSTRAK

Sifat antikanser *Strobilanthes crispus* (*S. crispus*) mencadangkan potensi manfaat penggunaannya sebagai adjuvan dalam rawatan kanser payudara. Potenesi interaksi ubat herba (HDI) menimbulkan kebimbangan terhadap keselamatan dalam farmakoterapi kerana perubahan metabolisme sitokrom P450 (CYP) boleh mengakibatkan kegagalan rawatan dan ketoksikan. Dalam kajian ini, kesan modulasi *in vitro* sub-fraksi *S. crispus* (F3) terhadap lima CYP manusia (CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) telah dikaji. Kajian menunjukkan tiada kesan perencatan yang ketara oleh F3 ke atas kesemua lima CYP tersebut dengan nilai IC_{50} 100 kali melebihi perencat CYP yang terdapat di pasaran. Penggunaan F3 dengan terapi kanser payudara konvensional yang menggunakan tamoxifen (TAM) boleh menjadi satu strategi untuk memaksimumkan keberkesanan terapeutik. Oleh itu, kajian interaksi antara F3 dan TAM adalah penting. Potensi HDI disiasat terlebih dahulu dengan menilai pengaruh F3 ke atas metabolisma TAM yang dimangkin oleh CYP secara *in vitro*. F3 menunjukkan perencatan jenis campuran yang lemah terhadap 4-hidroksilasi TAM yang dimangkin oleh CYP2D6 dan *N*-desmetilasi TAM yang dimangkin oleh CYP3A4. Kesan gabungan TAM dan F3 ke atas viabiliti sel MCF-7 kemudiannya dikaji. Kesan antagonistik timbal dikesan daripada rawatan gabungan. Hasil kajian ini mencadangkan kemungkinan interaksi antara F3 dan TAM dan juga metabolisma ubat-ubatan yang dimangkin oleh lima CYPs adalah rendah. Namun

begitu, kajian lanjut diperlukan untuk menilai keberkesanan dan keselamatan rawatan kombinasi TAM dan F3 secara *in vivo*.

MODULATION OF CYTOCHROME P450s BY *STROBILANTHES CRISPUS* ANTICANCER SUB-FRACTION AND ITS POTENTIAL INTERACTION WITH TAMOXIFEN METABOLISM

ABSTRACT

The anticancer property of *Strobilanthes crispus* (*S. crispus*) suggests its potential benefit as an adjuvant in breast cancer treatment. Potential herb-drug interaction (HDI) has always been a safety concern in pharmacotherapy as alteration in cytochrome P450 (CYP) mediated metabolism may lead to treatment failure and toxicity. In this study, the *in vitro* modulatory effect of a standardized sub-fraction of *S. crispus* (F3) on five human CYPs (CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) was first investigated. Negligible inhibitory effects of F3 on all the five CYPs were observed, with IC_{50} values more than 100-fold higher in comparison to known CYP inhibitors. The use of F3 in conjunction with conventional breast cancer therapy using tamoxifen (TAM) could be a strategy to maximize the therapeutic efficacy. Thus, the investigation of the interaction between F3 and TAM is crucial. The potential HDI was first investigated through evaluating the influence of F3 on CYP mediated TAM metabolism *in vitro*. F3 demonstrated weak mixed-type inhibition towards CYP2D6 catalyzed TAM 4-hydroxylation and CYP3A4 catalyzed TAM *N*-desmethylation. The combined effect of TAM and F3 on the viability of MCF-7 cell line was then investigated. A buffering antagonistic effect was observed from the combined treatment. The outcome of this study suggests the low possible interactions between F3 and TAM as well as the five CYPs catalyzed drug metabolism. Nevertheless, further studies are warranted to evaluate the efficiency and safety of the drug combination treatment *in vivo*.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Herbs have been used since ancient times and they are still being taken for medicinal purposes. Some herbal medicines are used together with radiotherapy or chemotherapy to reduce the complications and side effects as well as to improve treatment effectiveness (Yin *et al.,* 2013). A standardized sub-fraction of *S. crispus* (F3) has been reported to induce cytotoxicity and apoptosis on breast cancer cell lines, which includes MCF-7 and MDA-MB-231 (Yaacob *et al.,* 2015). F3 also demonstrated visible inhibition towards mammary tumor growth in animal studies (Yaacob *et al.,* 2015). With the anticancer properties found, *S. crispus* extracts have the potential to be used as an adjuvant in breast cancer therapy.

Tamoxifen (TAM) is widely used for treating estrogen receptor (ER)-positive breast cancer. As with many anti-cancer agents, TAM use is related to various adverse effects such as elevated risk of endometrial cancer (Bergman *et al.*, 2000; Hu *et al.*, 2015) and gastric adenocarcinoma (Chandanos *et al.*, 2006). TAM is a prodrug and is metabolized by cytochrome P450 (CYP) into a few metabolites, which are *N*-desmethyltamoxifen (NDT), (*Z*)-4-hydroxytamoxifen (HTF) and (*Z*)-endoxifen (EDF) (Jaremko *et al.*, 2010), mainly through CYP2D6, CYP3A4 and CYP3A5. Co-administration of TAM with paroxetine, an anti-depressant during treatment for breast cancer has been reported to result in higher risk of mortality, associated with irreversible inhibition of paroxetine on CYP2D6 (Kelly *et al.*, 2010). To alleviate side effects or enhance the therapeutic effect of TAM, some patients also resort to alternative treatments such as the consumption of herbal products.

Hepatic elimination of drugs or their metabolites from the body occurs through metabolism by CYP enzymes or non-CYP enzymes. Aberrations in drug metabolism resulting from modulation of CYP enzyme activity is a contributing factor to drug-drug interactions (DDI) (Polasek *et al.*, 2011; Tornio *et al.*, 2019). Prediction of herb-drug interaction (HDI) or DDI is critical in clinical drug development to minimize adverse drug reactions or loss of efficacy resulting from co-administration of supplementary and therapeutic drugs.

1.2 Cytochrome P450 (CYP)

CYP enzymes mediate the oxidative metabolism and biosynthesis of many endogenous and exogenous substances such as steroids, prostaglandins, drugs, environmental pollutants, alcohol, and organic solvents (McFadyen *et al.*, 2004; Guengerich, 2017). CYPs are membrane-bound hemeproteins and individual CYP contains an iron-protoporphyrin IX molecule as the prosthetic group (Hasler *et al.*, 1999). The thiol-group of CYP serves as a ligand that changes the electron density of the heme porphyrin ring thereby activating atmospheric dioxygen (Hasler *et al.*, 1999). The membrane-bound form of CYP reductase in eukaryotes mediates electron transfer from NADPH to the CYP heme center (Figure 1.1) (Das & Sligar, 2009). Oxidative metabolism is catalyzed by CYP through inserting an oxygen atom in an organic substrate and reducing another to water.

The activity of a CYP enzyme may give a pleiotropic effect on the efficacy of a treatment by either activating prodrugs such as TAM to produce metabolites that have greater potency on treating a disease or inactivating the drugs so that their effects cannot be exerted (Desta *et al.*, 2004; Vaclavikova *et al.*, 2007). CYP19A1 enzyme which converts androgens to estrogens plays a role in estrogen-dependent diseases development such as breast and endometrial cancer (Lamb *et al.*, 2007). CYP3A4 in

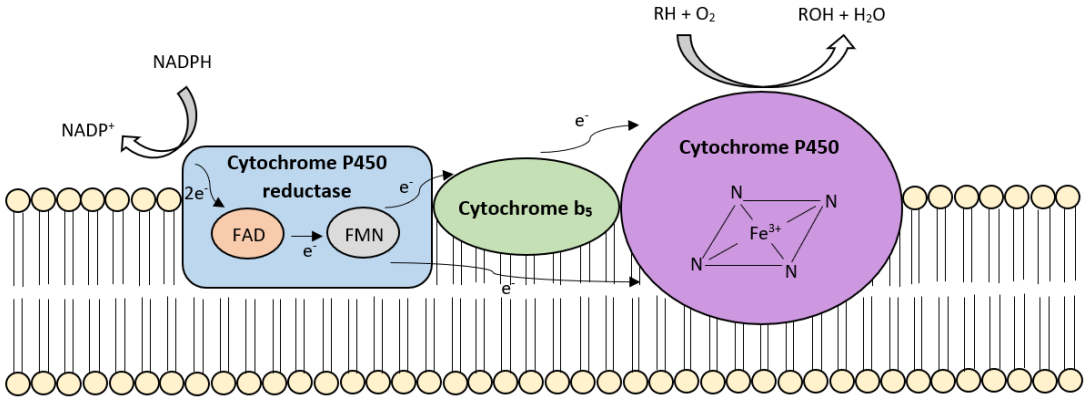

Figure 1.1: Schematic diagram of CYP catalyzed biotransformation of chemical compound. Two electrons from NADPH are transferred to the heme group of CYP via CYP reductase which has two flavin-containing domains, FAD and FMN or via cytochrome b_5. CYP catalyzes the oxidative breakdown of substrate by inserting one oxygen atom into the chemical compound while reducing another one to water.

cancer cells and CYP1B1 have been reported to inactivate tamoxifen (Vaclavikova *et al.*, 2007). CYP enzymes are also critical in tumor development as some CYP enzymes enable the activation of tumor-inducing compounds, for example, 4-hydroxyestradiol or inactivation of anti-tumor agents (McFadyen *et al.*, 2004). CYP1A1, CYP1B1 and CYP2E1 can promote tumor formation due to their capacity to activate pro-carcinogens (McFadyen *et al.*, 2004; Vaclavikova *et al.*, 2007).

CYPs from CYP1, CYP2 and CYP3 families play a major role in metabolizing over 90% of clinical drugs (Preissner *et al.*, 2013). Human CYP2B6 represents the minor hepatic CYP accounting for around 2-5% of the total minor hepatic CYP, but it demonstrates approximately 300-fold expression variability (Zanger & Schwab, 2013). CYP2B6 catalyzed conversion of cyclophosphamide to 4-hydroxycycphosphamide, which is the precursor of cytotoxic metabolites (phosphoramide mustard and acrolein) (Zanger *et al.*, 2007). CYP2C9 commonly metabolized drugs with a weakly acidic property such as the anticoagulant, anticonvulsants, oral antidiabetics and angiotensin receptor blockers (Zanger & Schwab, 2013). The prominent role of CYP2C19 in the metabolism of antidepressants such as imipramine and sertraline are known (Brøsen, 2004). CYP2D6 contributes to metabolizing nearly 25% of currently available drugs (Zhou, 2009). This isoform displays a genetic polymorphism which consequently, leads to large discrepancies in drug metabolism catalyzed by CYP2D6 (Maréchal *et al.*, 2008). With a large malleable active site and capability to catalyze different chemical reactions, CYP3A4 is able to convert various endogenous and exogenous compounds (Sevrioukova & Poulos, 2015). CYP3A4 is known as an efficient steroid hydroxylase in catabolizing endogenous steroids such as testosterone, progesterone and cortisol (Zanger & Schwab, 2013). In view of the critical role of P450s in phase I

drug metabolism, alteration in CYP mediated reaction can lead to drug interactions (Lynch & Price, 2007).

1.3 Enzyme inhibition

CYP inhibition by chemicals can be characterized by performing enzyme inhibition kinetic studies. The experiment is conducted through using varied concentrations of substrate (*S*) and inhibitor (*I*), and the attained data is analyzed using the transformed Michaelis-Menten equation, including the Eadie-Hofstee plot (v vs. v[*S*]), the Lineweaver-Burk plot ($1/v$ vs. $1/S$) and the Dixon plot ($1/v$ vs. S). Reversible enzyme inhibition is found as the common cause of DDI or HDI (Zhou *et al.*, 2003). Reversible inhibitor which forms non-covalent interaction with enzyme surface demonstrates a transient inhibitory effect in which normal enzymatic functions will resume after the removal of inhibitors from the body (Saboury, 2009; Chen, Qin, *et al.*, 2014). Reversible enzyme inhibition can be categorized into competitive, non-competitive, uncompetitive and mixed types.

A competitive inhibitor has a similar structure to *S*, thus competing with *S* for the enzyme active site (Figure 1.2A). The inhibitory potency of this type of inhibitor can be attenuated through an increasing concentration of *S* ([*S*]). In non-competitive type inhibition, the binding of substrate to an enzyme is not influenced by the inhibitor as they bind to different sites of enzyme independently. The non-competitive inhibitor does not inactivate enzyme upon binding to it but exerts inhibitory effect through reducing the amount of enzyme available for drug metabolism (Houston *et al.*, 2003; Ring *et al.*, 2014). The scheme of non-competitive inhibition is presented in Figure 1.2B. Since the inhibitor and substrate do not compete for the catalytic site, the inhibitory potency is not affected by alteration in [*S*].

Theoretical uncompetitive inhibition is uncommon in drug metabolism studies *in vitro* (Houston *et al.*, 2003). The uncompetitive inhibitor only binds to the substrate-bound enzyme (*ES*) (Figure 1.2C). Upon substrate binding to the enzyme, the structure of enzyme changes, therefore, exposes the inhibitor binding site (Saboury, 2009). Alteration in [*S*] can increase the degree of inhibition. Mixed-type inhibition affects substrate binding and reaction velocity (Ring *et al.*, 2014). It is sometimes referred to as non-competitive inhibitor. Mixed-type inhibitors demonstrate different binding affinities towards free enzyme (*E*) and *ES* complex. Two dissociation constants are obtained from the two equilibria of reaction, which include: (A) K_i denotes the dissociation of the enzyme-inhibitor (*EI*) complex; (B) αK_i denotes the dissociation of the inhibitor from the enzyme-substrate-inhibitor (*ESI*) complex (Figure 1.2D). When the constant value of α is equal to 1, the *I* demonstrates the same affinity for both the *E* and *ES* complex. When α value is greater than 1, the *I* has a greater affinity for the *E*, whereas the *I* binds more favorably to the *ES* complex when α value is less than 1 (Copeland, 2005; Ring *et al.*, 2014).

1.4 Drug combination

Drug combination is a strategy used for treating dreadful diseases including cancers, AIDS, hypertension and asthma with the purpose of attaining synergistic therapeutic effect (Chou, 2006; Foucquier & Guedj, 2015). For instance, the combination of rapamycin and chemotherapeutic agent (paclitaxel, carboplatin or vinorelbine) has synergistically enhanced the cytotoxicity in breast cancer cell lines due to aberrations in the phosphatidylinositol 3'-kinase/Akt pathway (Mondesire *et al.*, 2004). Besides the combination of synthetic drugs, a combination of drug and natural product can also achieve therapeutic synergism. Aloe-emodin, derived from *Aloe vera* and *Rheum palmatum* demonstrated synergistically enhancement in TAM induced cytotoxicity

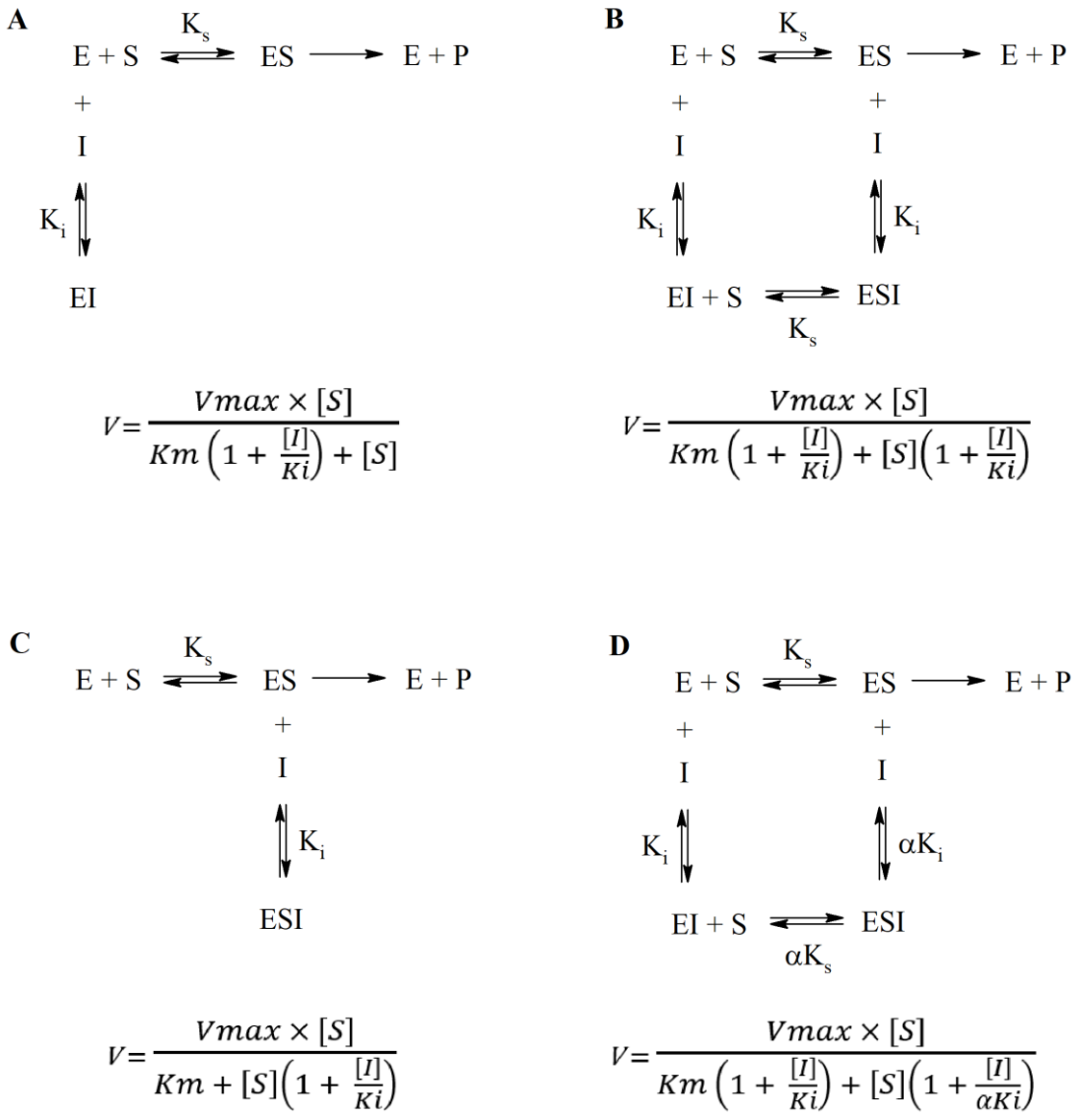

Figure 1.2: Scheme and equation for reversible enzyme inhibition: (A) competitive inhibition; (B) non-competitive inhibition; (C) uncompetitive inhibition; (D) mixed-type inhibition.

E: free enzyme, *S*: substrate, *P*: product, *I*: inhibitor, *ES*: enzyme-substrate complex, *EI*: enzyme-inhibitor complex, *ESI*: enzyme-substrate-inhibitor complex, K: dissociation constant.

towards MCF-7 cell (Tseng *et al.*, 2017). In addition to maximizing treatment efficacy, drug synergism minimizes toxicity through lowering doses of drugs and minimizing the occurrence of drug resistance (Roell *et al.*, 2017). Confront difficulties in finding and approval of new chemical or molecular entities, the combined use of currently available drugs is a potential strategy to treat incurable diseases for instances, Alzheimer's disease (Herrick & Million, 2007; Pangalos *et al.*, 2007).

The combination index (CI) method that can be used to quantitatively determined combined effects of drugs combination effects is developed based on the median effect principle of the mass-action law (Chou, 2010). The synergism of drug combination is depicted with CI value less than 1, additivity with CI value equal to 1 and antagonism with CI value greater than 1 (Chou & Talalay, 1983; Chou, 2010). This method has been widely applied in studying drug combination effects over the last few decades (Roell *et al.*, 2017).

1.4.1 Herb-drug interaction (HDI) mediated by CYP

Herbs are traditionally used in folk medicine and are continually used worldwide for promoting health and treating various diseases (Chen *et al.*, 2012). Co-administration of herbal medicines with therapeutic drugs raises concerns about potential HDI, which could lead to significant clinical outcomes. Drug interaction mediated by modulation of CYP450 activities can result in adverse effects such as alteration in drug clearance thus leading to toxicity (Zlokarnik *et al.*, 2005). St. John's wort (SJW) is known to induce CYP3A4 and triggers clinically significant HDI (Borrelli & Izzo, 2009; Oga *et al.*, 2016). Hyperforin in SJW exerts agonistic effect towards the pregnane X receptor (PXR) (Zhou *et al.*, 2004). PXR together with the retinoid X receptor (RXR) forms a heterodimer when activated by hyperforin and then binds to the pregnane response element in the DNA as a transcription factor (Zhou *et al.*, 2004; Kubin *et al.*, 2005),

thus inducing CYP3A4 activity. Some observed HDI cases mediated by CYP are summarized in Table 1.1.

1.4.2 *In vitro* CYP inhibition (IVCI) assay

In vitro assessments of metabolism contribute to the understandings of biotransformation across different species. Utilization of *in vitro* systems provides the advantage of isolating metabolism pathways from other complex processes, such as phase I metabolism from phase II metabolism which enables identification of enzyme(s) responsible for biotransformation of xenobiotics (Lipscomb & Poet, 2008). With the advantage, the use of *in vitro* system supports the clinical drug development in predicting DDI or HDI.

For evaluation of inhibitory effects of drug candidates, *in vitro* systems using human liver microsomes (HLM) or recombinant CYPs are employed to monitor the effects on the metabolism of CYP substrates (Zhang *et al.*, 2012). Screening for potential drug interaction has been performed using various IVCI assays, whereby the rate of drug metabolism by CYP enzymes have been analyzed using fluorescence (Ponnusankar *et al.*, 2011), radiometric (Moody *et al.*, 1999), liquid chromatography-UV (LC-UV) (He *et al.*, 2015) and liquid chromatography-tandem mass spectrometry (LC-MS) (Yadav *et al.*, 2018) based methods. High selectivity and sensitivity of LC-MS/MS for the monitoring target analytes has been widely utilized in IVCI (Pillai *et al.*, 2013; Chen *et al.*, 2017; Valicherla *et al.*, 2019). Data obtained from *in vitro* CYP inhibition assessment can be used as a guideline for the clinical study of the interaction between drugs and can provide information for designing new drug candidates or removing CYP inhibiting compounds (Walsky & Obach, 2004; Hughes *et al.*, 2011).

Table 1.1: CYP mediated herb-drug interactions.

Herbal medicine	Extract or compound	Modulatory effect on CYPs	Clinical outcome	References
Hypericium perforatum (St. John's wort)	Standardized extract containing 0.3% hypericin	Induction of CYP3A4	Elevated ratio of urinary 6β-hydroxycortisol/cortisol	(Roby *et al.*, 2000)
	Standardized extract containing 0.3% hypericin		Elevated clearance (*CL*) of midazolam	(Dresser *et al.*, 2003)
	300 mg tablet		Significant decrease in plasma concentration (C_p) of active SN-38 by 42% when SJW co-administered with irinotecan	(Mathijssen *et al.*, 2002)
	300 mg tablet		Reduction in mean of area under the concentration-time curve (AUC) of indinavir by 57%	(Piscitelli *et al.*, 2000)
	300 mg extract		Increase in imatinib *CL* by 43% and decline in imatinib AUC by 30%	(Frye *et al.*, 2004)
			Increase in *CL* of nevirapine by 35%	(de Maat *et al.*, 2001)

Table 1.1. Continued

Herbal medicine	Extract or compound	Modulatory effect on CYPs	Clinical outcome	References
Ginkgo biloba (Ginkgo)	Powder extract contained 12.4% quercetin and 9.4% terpenes	Induction of mRNA expression of CYP2B1/2, CYP3A1 and CYP3A2	Significant reduced the hypotensive effect of nircardipine in rat	(Shinozuka *et al.*, 2002)
	EGb761 extract (terpenoidic and flavonoidic fractions)	Strong inhibition on CYP1A2, CYP2C9, CYP2E1 and CYP3A4 *in vitro*		(Gaudineau *et al.*, 2004)
	120 mg extract	Induction of CYP3A4	Decrease in AUC and maximum plasma concentration (C_{max}) of midazolam by 34% and 31%, respectively	(Robertson *et al.*, 2008)
	Ginkgolide A	Induction of CYP3A4		(Liu *et al.*, 2011)
	Ginkgolic acids I and II	Inhibition on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 activities *in vitro*		(Zou *et al.*, 2002)

Table 1.1. Continued

Herbal medicine	Extract or compound	Modulatory effect on CYPs	Clinical outcome	References
Allium sativum (Garlic)	Garlic juice	Induction of CYP1A2 and CYP2E1 expressions in mouse		(Kishimoto *et al.*, 1999)
	Different products (freeze-fried, oil, odorless, aged)	*In vitro* inhibition on CYP2C9*1, CYP2C19, CYP3A4, CYP3A5 and CYP3A7 activities and increased CYP2C9*2 activity	Decrease in *CL* of docetaxel by 36% Significant elevation of AUC and C_{max} of atorvastatin	(Foster *et al.*, 2001; Fasinu & Rapp, 2019; Shaikh *et al.*, 2020)
	Diallyl disulphide	Induction of CYP2B1/2 activity in rats and inhibition on CYP2E1 activity in recombinant rat and human CYPs		(Jin & Baillie, 1997; Teyssier *et al.*, 1999)
	Allicin	*In vitro* inhibition on CYP1A2, CYP2C9 and CYP2C19 activities		(Teyssier *et al.*, 1999; Zou *et al.*, 2002)
	Garlic caplets (GarliPure, maximum allicin formula)		Significant decrease in mean saquinavir AUC by 51% and C_{max} by 54%	(Piscitelli *et al.*, 2002)

Table 1.1. Continued

Herbal medicine	Extract or compound	Modulatory effect on CYPs	Clinical outcome	References
Panax ginseng (Ginseng)	Ginsenoside Rd	Weak inhibition on CYP2C9, CYP2C19, CYP2D6 and CYP3A4 *in vitro* activities		(Henderson *et al.*, 1999)
	Ginsenosides Rc and Rf	Increase in CYP2C9 and CYP3A4 *in vitro* activities		(Henderson *et al.*, 1999)
			Elevated C_{max} of nifedipine by 29%	(Smith *et al.*, 2001)
	500 mg ginseng	Induction of CYP3A activity	Significant reduced the *CL* and C_{max} of midazolam	(Malati *et al.*, 2012)

1.5 *Strobilanthes crispus*

Strobilanthes crispus (L) Bremek (Figure 1.3) from the family of Acanthaceae is a bush-like plant that can be found on riverbanks and abandoned fields and is native to countries such as Madagascar and Indonesia (Nurraihana & Norfarizan-Hanoon, 2013). The common names of this plant include picah beling (Jakarta), kejibeling (Java) and pecah beling (Malaysia) (Abu Bakar *et al.*, 2006). *S. crispus* has been used in folk medicine as an antidiabetic, anticancer, diuretic, antilytic, and laxative agent in Malaysia and Indonesia (Perry & Metzger, 1980; Nurraihana & Norfarizan-Hanoon, 2013). The local people had boiled *S. crispus* leaves in water and drank the filtrates as traditional medicine. It has been reported that the indigenous peoples of Perak in Peninsular Malaysia eat the fresh leaves to strengthen the immune system (Samuel *et al.*, 2010).

A few products made from this herbal plant including herbal tea and capsules containing powdered crude leaves are now available in the health-food market. *S. crispus* has been increasingly consumed by the public due to its anti-carcinogenic, antioxidant properties and wound healing properties (Nurraihana & Norfarizan-Hanoon, 2013). Anticancer activity of *S. crispus* including anti-proliferative and apoptotic activity in cancer cells has been previously reported (Abu Bakar *et al.*, 2006; Yaacob *et al.*, 2010). In the previous study conducted by Yaacob *et al.* (2015), F3 is the sub-fraction that showed the strongest cytotoxic effect on both MDA-MB-231 and MCF-7 cells among the five fractions obtained from dichloromethane (DCM) extract of *S. crispus*. F3 significantly inhibited mammary tumor growth (Yaacob *et al.*, 2015) and activated the immune system (Yankuzo *et al.*, 2018). Additionally, previous studies revealed that *S. crispus* extracts displayed no significant effects on hepatic and renal functions of rats (Lim *et al.*, 2012; Yaacob *et al.*, 2015). The major chemical

Figure 1.3: The leaves of *S. crispus*.

constituents of F3 had been identified. These include lutein, 13^1-hydroxy-13^2-oxo-pheophytin a, phytosterols (campesterol, stigmasterol and β-sitosterol), pheophytin a, and 13^2-hydroxy-pheophytin α.

1.6 Breast cancer and use of tamoxifen (TAM)

Advancement in diagnostic and therapeutic strategies has greatly reduced breast cancer mortality by 25% over the last two decades (Yue *et al.*, 2013). However, the worldwide incidence of breast cancer remains the highest among all cancer types in women with approximately 2.1 million newly diagnosed cases and over 0.6 million deaths reported in 2018 (Bray *et al.*, 2018). A comprehensive understanding of the mechanism underlying carcinogenesis is required to develop effective prevention strategies. The occurrence of breast cancer is primarily contributed by genetic factors, in which mutations in tumor suppressor genes including *BRCA1*, *BRCA2*, *TP53*, *CHEK2* and *PTEN* are known (Thompson & Easton, 2004). In addition to genetic factors, hormones are also involved in the development of breast cancer (Clemons & Goss, 2001). Hormonal factors together with gene mutation were suggested promoting breast tumorigenesis through supporting cancer cell growth or involving in de-differentiation of oncogenic epithelial cells (Wang & Di, 2014).

1.6.1 Role of estradiol in breast cancer development

Estradiol (E2) is a key steroid hormone in development of breast cancer (Yue *et al.*, 2013). Progression of breast cancer was observed in different animal models administered with E2 (Hollingsworth *et al.*, 1998; Zumoff, 1998). Abrogation of E2 effect with anti-estrogenic agents and inhibition of its synthesis using aromatase inhibitors were found preventing contra-lateral breast cancer development (Howell *et al.*, 2005; Thürlimann *et al.*, 2005). Breast cancer associated with elevated estrogen

level is developed through either estrogen receptor (ER) dependent or independent mechanisms.

1.6.2 Estrogen receptor (ER)-dependent breast cancer

Estrogen can bind to one of the ER subtypes (ERα and ERβ) (Thomas & Gustafsson, 2011). ERα is the key ER subtype in mammary epithelium (Curtis Hewitt *et al.*, 2000) which comprises a N-terminal domain (AF1), a DNA-binding domain, and a C-terminal ligand-binding region that has an AF2 domain (Kumar *et al.*, 1987). Once estrogen binds to ERα, the activated ERα binds to the specific hormone-responsive element in or near the gene promoter, thus triggers transcription of the gene (Saha Roy & Vadlamudi, 2012). ER-mediated mechanism enhances cell proliferation thus enables cancer development (Preston-Martin *et al.*, 1990). Increase in cell division numbers raises possible errors in DNA replication and rapid cell division results in the ineffective repair of mutations (Preston-Martin *et al.*, 1990). DNA adducts or breaks in fast-dividing cells could become fixed mutations and passed on to daughter cells (Preston-Martin *et al.*, 1990).

1.6.3 Estrogen receptor (ER)-independent breast cancer

Carcinogenesis of estrogens via ER independent mechanism is facilitated by direct genotoxicity of estrogen metabolites (Russo *et al.*, 2002; Russo *et al.*, 2006). The direct genotoxic effect was evidenced by DNA points mutations resulting from 4-OH estradiol in mutation assay using BB Rat2 embryonic cells (Zhao *et al.*, 2006). Human CYP enzymes including CYP1B1, CYP1A1 and CYP3A4 at phase I metabolism play critical roles in the oxidative metabolism of estrone (E1) and E2 to form 2,3- and 3,4-catechols (Yue *et al.*, 2013). The catechols undergo secondary metabolism to produce 2,3- and 3,4-quinones which form DNA adducts (Cavalieri *et al.*, 2006). Previous studies revealed that women at high risk or with breast cancer had

their urine detected with a high level of depurinating adducts (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009).

1.6.4 Tamoxifen (TAM)

TAM is a selective estrogen receptor modulator (SERM) that binds competitively to ER and consequently inhibits the estrogen-regulated gene expressions (Criscitiello *et al.*, 2010). TAM displays tissue-selective pharmacology, in which TAM acts as an estrogen antagonist in breast tissue and an agonist in the endometrium and bone tissues (Lewis-Wambi & Jordan, 2004). Furthermore, TAM inhibits angiogenesis, downregulates insulin-like growth factor 1 (IGF-1) and transforming growth factor (TGF) activities as well as induces programmed cell death (Ellis *et al.*, 1997; Karn *et al.*, 2010). Imbalance of cell proliferation and cell loss due to slow cell proliferation may cause the regression of tumors (Criscitiello *et al.*, 2010). TAM is used as hormone therapy for breast cancer at all stages and has successfully decreased contralateral breast cancer and reoccurrence risk by up to 50% (Martinkovich *et al.*, 2014; Bens *et al.*, 2019).

TAM is a prodrug that must be metabolized by CYP enzymes to produce two active metabolites: HTF and EDF (Gorman *et al.*, 2013). Biotransformation of TAM mediated by CYP enzymes is depicted in Figure 1.4. EDF is equally as potent as HTF in inhibiting estrogen-responsive gene transcription (Desta *et al.*, 2004; Gorman *et al.*, 2013). *N*- demethylation of TAM is found predominantly catalyzed by CYP3A4 and CYP2D6 with smaller contributions by CYP1A1 and CYP1A2 (Crewe *et al.*, 2002; Desta *et al.*, 2004). 4-hydroxylation of TAM is reported to be mainly catalyzed by CYP2D6, 2B6, and 2C9 (Wiseman & Lewis, 1996; Crewe *et al.*, 2002; Desta *et al.*, 2004). The biotransformation of NDT to EDF is primarily mediated by CYP2D6 (Desta *et al.*, 2004).

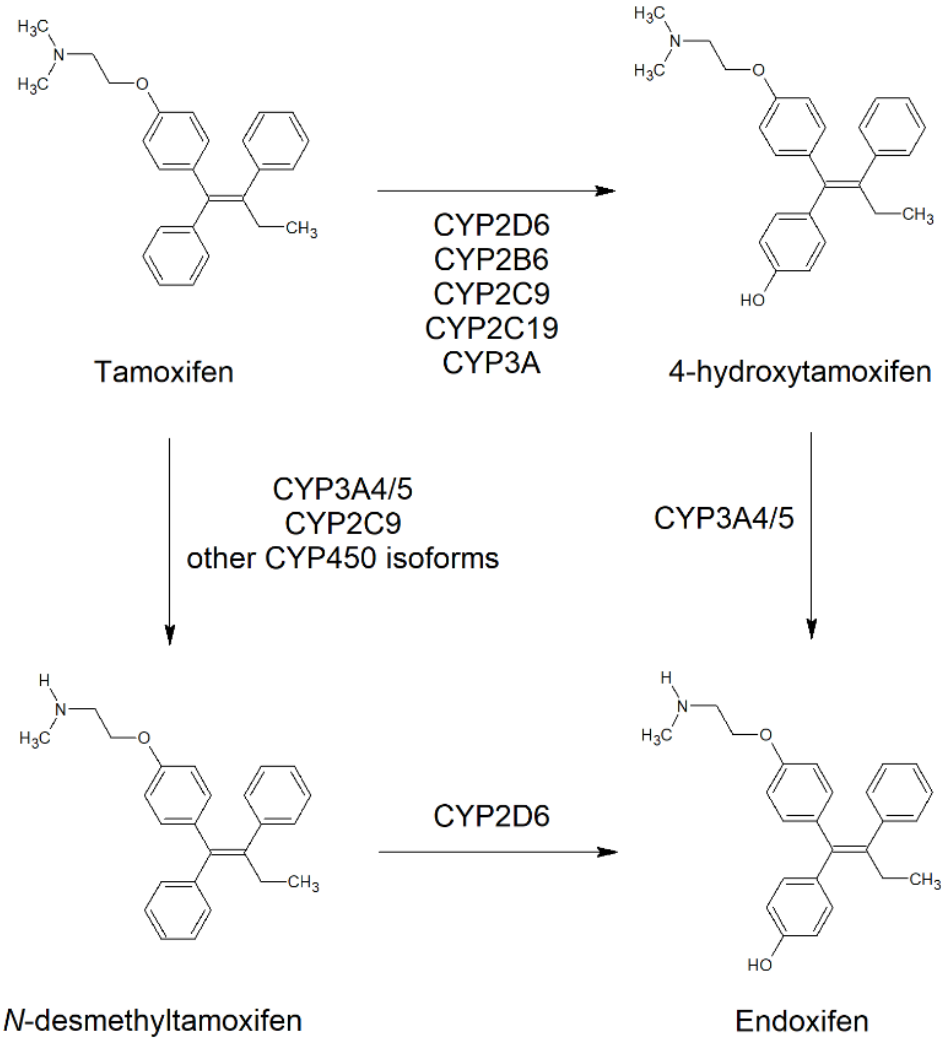

Figure 1.4: CYP450 mediated biotransformation of tamoxifen to active metabolites (HTF, NDT and EDF).

Widespread use of TAM has raised the concern about the side effects, especially carcinogenicity of this drug (White *et al.*, 1992; Phillips, 2001). High risk for developing endometrial cancer was found associated with long-term use of TAM in breast cancer patients (Chen, Kuo, *et al.*, 2014). Two primary metabolites, alpha-hydroxytamoxifen (α-HTF) and HTF were identified in TAM metabolic studies using rodent liver microsomes (Lim *et al.*, 1994). The two metabolites can be further metabolized to form DNA adducts which potentially trigger carcinogenesis.

1.7 Research objectives

The potential for herb-drug interaction (HDI) resulting from co-administration of TAM with *S. crispus* is unknown. The aim of the current study is therefore to evaluate the modulatory effect of F3 on human CYP enzymes and the potential interaction between F3 and TAM using *in vitro* models. The findings can be used as a guide for further *in vivo* study on predicting the risk of using F3 in combination with therapeutic drugs.

The specific objectives are:

1. To investigate the modulatory effects of F3 on the enzymatic activity of CYP2B6, 2C9, 2D6, 2C19, and 3A4 via fluorescence-based screening

2. To investigate the effect of F3 on the formation of substrate metabolites of major CYP enzymes using HPLC MS/MS

3. To evaluate the effects of F3 on tamoxifen metabolism by HPLC MS/MS by measuring formation of 4-hydroxytamoxifen and *N*-desmethyltamoxifen

4. To assess the cytotoxic effect of combination of F3 and tamoxifen on MCF-7 cell line using Alamar Blue assay

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Vivid® CYP450 (2B6 blue, 2C9 blue, 2C19 blue, 2D6 blue and 3A4 blue) screening kits, CYP2C9, CYP2D6 and CYP3A4 BACULOSOMES® Plus Reagents were purchased from Life Technologies Corporation (Carlsbad, USA). Advanced RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin (P/S) (10,000 U/mL) and L-glutamine (200 mM), Dulbecco's Phosphate Buffered Saline (DPBS) and trypsin-EDTA (0.25%) were purchased from Gibco Laboratories. TAM, (*E/Z*)-EDF, propranolol, formic acid (FA) (liquid chromatography grade), quinidine and resazurin sodium salt were purchased from Sigma (Saint Louis, USA). Diclofenac (sodium salt), HDF, meclofenamate (sodium salt), NDT, (*Z*)-HTF, ketoconazole, miconazole (nitrate) and sulfaphenazole were purchased from Cayman Chemical (Ann Arbor, USA). Chloroform, dichloromethane, ethyl acetate, hexane (analytical grade), acetonitrile (liquid chromatography grade), methanol (liquid chromatography grade), silica gel 60 (0.040 – 0.063 mm), nicotinamide adenine dinucleotide phosphate (NADP) disodium salt, α-D-glucose-6-phosphate (G6P) (monosodium salt), glucose-6-phosphate dehydrogenase (G6PD), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and magnesium chloride hexahydrate ($MgCl_2 * 6H_2O$) were purchased from Merck (Darmstadt, Germany).

2.2 Plant material and F3 extract preparation

The leaves of *S. crispus* were acquired from a cultivated source at Tasek Gelugor, Penang, Malaysia. The *S. crispus* extract (F3) was prepared as previously described (Yaacob *et al.*, 2015). In brief, the leaves of *S. crispus* were first cleaned,

air-dried and then pulverized into powder. The leaves powder was sequentially extracted with hexane followed by dichloromethane and was sonicated in Fisher Scientific ultrasonic bath FB15051 (Pittsburgh, USA) for 20 min followed an overnight soak. The extract obtained with dichloromethane was filtered and the solvent was evaporated *in vacuo* to yield dried dichloromethane extract. The extract was chromatographed on silica gel 60 through dry vacuum liquid chromatography. Step gradient elution was performed with hexane : chloroform : ethyl acetate : methanol (2:3:0:0 to 0:0:9:1, v/v/v/v, final volume of 300 mL each). The extract was dried using IKA RV 10 C rotary evaporator (Staufen, Germany) and kept in vials for storage at -20°C.

Qualitative analysis of F3 extract by HPLC-UV was performed to ascertain the presence of lutein, a chemical marker in the extract. An Agilent 1260 Infinity Diode Array Detector (California, USA) coupled to an Agilent 1260 Infinity II HPLC system (California, USA) was used for sample analysis. A Thermo Scientific Hypersil GOLD^{TM} C18 column (150 × 46 mm, 3 µm) (Waltham, USA) was used for separation with flow rate of 0.85 mL/min. Ultraviolet detection was set at 444 nm. The column thermostat was set at 40 °C. An aliquot (20 µL) of sample was injected for analysis. The mobile phase (MP) consisted of 0.1% formic acid in HPLC grade water (MP A) and acetonitrile (MP B). A stepwise gradient elution (Table 2.1) was employed for separation.

2.3 CYP inhibition assays

2.3.1 Vivid CYP inhibition assay

The inhibitory effect of F3 on CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 was determined using Vivid® CYP (2B6 blue, 2C9 blue, 2C19 blue, 2D6

Table 2.1: HPLC gradient elution program.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (µL/min)
0.00	70.0	30.0	0.850
1.00	70.0	30.0	0.850
8.00	0.0	100.0	0.850
27.00	0.0	100.0	0.850
28.10	70.0	30.0	0.850
30.00	70.0	30.0	0.850

blue and 3A4 blue) screening kits. The CYP BACULOSOMES reagent contains recombinant human CYP enzyme and NADPH-CYP reductase. A NADPH regeneration system (NRS) which contains 333 mM G6P and 30 U/mL G6PD converts $NADP^+$ into NADPH. The substrate (BOMCC or EOMCC) was metabolized by CYP enzymes into highly fluorescent products (Figure 2.1).

Procedures of the Vivid CYP450 enzyme assay were conducted according to the manufacturer's instructions. Briefly, the stock solutions of F3 and known CYP inhibitors were serially diluted with reaction buffer provided in the kits to several concentrations (Table 2.2). A white NUNC® 96-well plate (Roskilde, Denmark) was used in this assay. In each well, a master pre-mix solution (50 µL) was added to F3 or solvent controls (40 µL). Pre-incubation of the reaction mixture at room temperature was set for 20 minutes. The reaction was started by addition of a mixture of substrate and $NADP^+$ (10 µL). Known inhibitors of the five CYP enzymes as listed in Table 2.2 were used as positive controls. The fluorescence readings were measured every 30 seconds using an Agilent Cary Eclipse Fluorescence Spectrophotometer (California, USA) over different incubation time (listed in Table 2.2) with an excitation wavelength of 415 nm and an emission wavelength of 460 nm.

The rate of reaction was calculated based on the fluorescence change per unit time over the linear range of reaction progress curve. Percentage of enzyme inhibition of test compounds was calculated using the formula as stated below:

$$\text{Percentage of enzyme inhibition} = \left(1 - \frac{X-B}{A-B}\right) \times 100\%$$

Where,