INHIBITORY AND INDUCTIVE EFFECTS OF *BACOPA MONNIERI* STANDARDIZED EXTRACT AND ITS CONSTITUENTS ON HUMAN LIVER CYTOCHROME P450

MARISA KHUMYAT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Pharmaceutical Chemistry and Natural Products)
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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Pharmaceutical Chemistry and Natural Products of Naresuan University

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ABSTRACT

Bacopa monnieri (L) Wettst. (*B. monnieri*) has been beneficial for neuropharmacological disorders treatment. Herb-drugs interaction (HDIs) could occur due to inhibition or induction potential of drug metabolism enzymes. It could alter the pharmacokinetics and lead to toxicity or therapeutic failure. Currently, there are a few studies that reported *B. monnieri* extract could inhibit drug-metabolizing enzymes using rat liver microsome and human recombinant Cytochrome P450 (rCYPs). Those results align with an *in vivo* study that reported *B. monnieri* extract relatively inhibits CYP2C and 3A in rats after co-administration of *B. monnieri* extract and amitriptyline. However, no study regarding the inhibition potential on CYPs system using human liver microsome (HLMs) has been conducted. In addition, the induction potential of *B. monnieri* extract is still unclear. To achieve this purpose, the extract's inhibition, and the induction effect of the extract on CYP isoforms were evaluated using the *in vitro* gold standard models i.e., the pooled HLMs and primary human hepatocytes, respectively.

As the results, *B. monnieri* extract demonstrates strongly inhibit CYP2C19 activity with IC_{50} values of 17.68 µg/mL, and moderate inhibit CYP1A2, 2B6, and 2C9 with IC_{50} values of 75.98, 59.69, and 47.72 µg/mL, respectively. Whereas this extract showed weak inhibition potential on CYP3A4 with IC_{50} values of 101.40 µg/mL. To

solve the puzzle of inhibition mode of *B. monnieri* extract and bacopaside I which is assumed as a major bioactive compound derived from the pharmacological activity, the Lineweaver–Burk plots were conducted, and the secondary plots of Lineweaver-Burk plots were conducted to determine the inhibition constant (Ki) values. The results found that *B. monnieri* extract could inhibit CYP1A2 with competitive mode (Ki = 48.06 µg/mL) and inhibit CYP2B6, 2C9, and 2C19 (Ki = 5.16, 19.93, and 3.04 µg/mL, respectively) with mixed-type inhibition. In addition, the CYP2B6 was competitively inhibited by bacopaside I with Ki values of 7.02 µg/mL. Whereas this saponin glycoside could inhibit the CYP2C9 and CYP2C19 enzyme activity with mixed type inhibition (Ki = 25.92 and 13.25 µg/mL, respectively). Consequently, *B. monnieri* extracts also significantly enhanced the enzyme activity and mRNA expression of CYP1A2, and 2B6. In addition, the bacopaside I showed moderate inhibit CYP2B6 and CYP2C9 activity while others phytochemical compounds did not inhibit any CYPs isoform. Although in *in vitro* results demonstrated the inhibition and induction potential on the CYPs system, at oral administration of 300 mg/day *B. monnieri* extract, which is a recommended dose, may not produce HDIs.

Since the predicted fraction absorbed of five major bioactive compounds did not exceed 20% and oral bioavailability was equal to 6.2% in humans after orally receiving 10 mg of isolated compounds. Besides, consuming the *B. monnieri* extract might not produce hepatotoxicity. Because of the cell viability are still over 75% when treated with *B. monnieri* extract at high concentration in primary human hepatocyte. However, oral intake of simultaneous medications and *B. monnieri* extract should be done with caution. It cannot conclude from the present study whether *B. monnieri* extract has been a cause of herb-drug or herb-herb interactions in humans.

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CHAPTER I

INTRODUCTION

1. Statement of purpose

Currently, millions of people use herbs in combination with prescribed and non-approved therapy to achieve a synergistic effect. According to the World Health Organization (WHO), the trend of herbal uses in the global market is increasing from three trillion US dollars in 2006 to five trillion US dollars in 2050 (1). According to Eisenberg DM et al., there has been a 380 percent increase in the use of herbal remedies of the total US population with an estimated 15 million adults (18.4% of all prescription users) taking herbs. Consequently are a risk for potential adverse effects occurred from Herb-drug interactions (HDIs) which are involving with alterations of drug bioavailability or efficacy and chronic medical illness individuality, especially those with liver or kidney abnormalities (2).

Cytochrome P450 (CYPs) are the category of xenobiotic enzymes that play an important role in the initial step (Phase I metabolism) or the rate-limiting step for the metabolism of most medicines, endogenous factors and foreign compounds, including herbal components as effective substrates modulate the metabolism of the numerous chemical compounds found in herbs lead to induction or inhibition of specific CYP enzymes (3, 4) that this mechanism main causes of occurring HDIs. There is currently no suitable mechanism to collect meaningful data on herb-drug interactions that have been published.

Bacopa monnieri (L.) Wettst. commonly known as 'Brahmi' is an herb belonging to the family Plantaginaceae. Brahmi is an aquatic-perennial plant that grows in marshy environments. It is a little creeping herb with oblong green leaves and white-purple flowers with four or five petals. Summer was the time for flowering and fruiting. It has been used in Ayurvedic medicine with properties in the treatment of neuropharmacological disorders including cognitive enhancement and memory improvement (5, 6). The major compounds responsible for memory and cognition enhancement are steroidal saponin glycosides that were classified to the section of Jujuberginin and Pseudojujuberginin. Bacoside A_3, Bacopaside I, Bacopaside II,

Bacopaside X, and Bacopasaponin C are among the compounds discovered in these subclasses, according to a prior study (7). Moreover, *Bacopa monnieri* extracts (*B.monnieri* extracts) have protective effects against β-amyloid toxicity (8), anti-oxidant (9), and anti-inflammatory effects (10) supported by pharmacological data and scientific studies for assessment efficacy (5, 11) and safety (12) in clinical trials of *B. monnieri* extracts., Several Brahmi products, including nutritional supplements and herbal medicines, have been developed and are widely available on market.

According to a recent systematic review, *B. monnieri* extracts improved memory free recall, suggesting that it might be utilized as a memory booster that exhibited similar efficacies obtained from Ginseng (*Panax ginseng*) (13), Ginkgo (*Ginkgo biloba*) (14), and Garlic (*Allium sativum*). However, often the simultaneous ingestion of the herb with the drug results in HDIs. For example, co-administration of the herb extract (i.e., Garlic, Chamomile, and Mango) and warfarin which is an anti-platelet drug have been increased the risk of bleeding due to its inhibition potential on drug-metabolism enzymes (15). Furthermore, HDIs generated by consuming *B. monnieri* extracts and another medicine combination were caused by a response involving drug-metabolizing enzymes reaction. For instance, using the recombinant CYPs model, *B. monnieri* extracts may produce a possible inhibition of drug-metabolizing enzymes including CYP1A2, CYP2C9, CYP2C19, and CYP3A4 (16, 17). In addition, this extract has been shown a relatively inhibition potential on CYP2C and 3A activity in rats after co-administration of *B. monnieri* extract and amitriptyline (18). Few studies have been conducted using pool human liver microsome and primary human hepatocytes to investigate the inhibitory and induction effects of *B. monnieri* extracts and their phytochemicals on drug-metabolizing enzymes. Therefore, our study will evaluate the inhibition and induction effect of *B. monnieri* extracts and five bioactive compounds on drug-metabolizing enzymes, especially CYP450 to investigate drug safety follow the *in vitro* experiment guidelines from the US FDA Guidance for industry 2020 (19). This finding will be useful to evaluate the HDIs the extracts and its bioactive compounds when co-administration with other drugs as well as to predict further clinical interaction to ensure the patient's medication use for effective with maximum safety.

2. Purposes of the Study

2.1 To investigate the inhibitory effect of the *B. monnieri* standardized extract and its constituents on pool human liver microsome (Pooled HLM) as major CYPs using enzyme kinetic parameter

2.2 To evaluate the induction effect of *B. monnieri* standardized extract and constituents on primary human hepatocytes

3. Hypothesis

The *B. monnieri* standardized extract and its constituents may have inhibitory and inducing effects on the human liver cytochrome P450 enzyme.

4. Expected benefits from research

4.1 The effect of *B. monnieri* standardized extract constituents on cytochrome P450 enzymes in the human liver, as well as the inhibition and induction mechanisms of *B. monnieri* standardized extract constituents.

4.2 The outcome can estimate the likelihood of clinical herb-drug interaction.

4.3 To make research available to the general public and to use as data for the creation of *B. monnieri* products.

5. Scope of the Study

This research will evaluate the inhibitory and induction effects of *Bacopa monnieri* standardized extract and its constituents on human liver enzymes using pool human liver microsome and the induction effect on human liver enzymes using primary human hepatocytes.

6. Key Words

Inhibition, Induction, *Bacopa monnieri*, Herb-drugs interaction, Cytochrome P450, Enzyme kinetics

CHAPTER II

LITERATURE REVIEW

1. Description of plant

Bacopa monnieri, also referred to as *Bacopa monniera*, *Herpestis monniera*, water hyssop, commonly known as "Brahmi" is an herb from a family Plantaginaceae. BM is an aquatic plant often found in tropical regions and includes a genus of over 100 species distributed throughout the world, commonly found in India, Nepal, Sri Lanka, China, Taiwan, Vietnam, Thailand, and other southern states of the USA (20). The leaves are arranged oppositely, bright green, oblong shape with relatively thick, margin entire range 0.4-1.0 cm wide and 0.5-2.5 cm long with no petiole. The stems are a succulent lighter green, with numerous branches, soft, 10-40 cm long, 1-3 mm thick, and often showing sprouting rootlets. The flowers are light purple, blue or white color, axillary and solitary on pedicels 1.0-1.5 cm long. The corolla consists of 5 petals and 3 sepals that are 8-10 mm long and bright green capitate stigma. and the fruits are capsules up to 5 mm long, ovoid and glabrous, fruiting found in summer. Seeds yellow-brown, ellipsoid, truncate at one end, longitudinally striate (6, 21). BM has a long history of use in the Ayurvedic system of medicine tradition in the treatment of neuropharmacological and mental disorders (21). Its major constituents include the saponins bacoside A_3, bacopaside I, bacopaside II, bacopaside X, and bacopasaponin C used to treat for mind-related health and brain concerns such as Alzheimer's disease, memory loss, anxiety, attention deficit-hyperactivity disorder, epilepsy, and tonic for fight stress (22).

Figure 1 *Bacopa monnieri* or Brahmi, (a) whole plant, (b) leaf, (c, d) flower, (e) fruit (6)

2. Chemical constituents of *Bacopa monnieri*

The pharmacological activity of *B. monnieri* has several compound groups; the active constituents as an important role that is responsible for memory and cognition enhancement are bacosides A and B. The constituent most studied has been bacoside A, which was found to be a blend of other major structural that chemicals structure in a group of triterpenoid saponin glycosides have based on aglycone unit, they classified a group of Dammarane-type comprises a family of 12 analogs.

Currently, the saponin glycoside was divided into 2 subgroups are Jujubogenin and Pseudojujubogenin especially bacoside A_3, bacopaside II, bacopasaponin C, bacopaside X, and other saponins called bacopasides I-XII (22, 23). The chemical structures and chemical names were shown in Figure 2 and Table 1 respectively.

In this study, *Bacopa monnieri* standardized extract and its constituents are beneficially and commercially available that an important role includes bacoside A_3, bacopaside I, bacopaside II, bacopaside X, and bacopasaponin C and were chosen representative for inhibition and induction effects assessment on major CYPs such as CYP1A2, CYP3A4, CYP2B6, CYP2C9, CYP2C19, CYP2E1, and CYP2D6.

Table 1 Chemical structures of Jujubogenin and Pseudojujubogenin glycosides from *B. monnieri* (24, 25)

Compound	Structure
Jujubogenin glycosides	
Bacoside A_1	3-0-[α-L-arabinofuranoyl(1—>3)-β-L-arabinopyranosyl]
Bacoside A_3	3- β - [0- β -D-glucopyranosyl(l—>3)-0- [α -L-arabinofuranosy (1—>2)]- 0- β -D-glucopyranosyl]
Bacopasaponin A	3-O- α -L-arabinopyranosyl-20-O- α -L-arabinopyranosyl
Bacopaside III	3-0- α -L-arabinofuranosyl-(1 —>2)- β -D-glucopyranosyl
Bacopaside IV	3-0- β -D-glucopyranosyl-(1—>3)- α -L-arabinopyranosyl
Bacopasideaponin G	3-0- [α -l-arabinofuranosyl-(1—>2)]- α-L-arabinopyranosyl
Becopaside X	[α-L-arabinofuranosyl(1—>2)-[β-D-glucopyranosyl-(1—>3)]-α-L-arabinofuranosyl
Pseudojujubogenin glycosides	
Bacogenin A_4	Ebelin lactone
Bacopasaponin B	3-0- [α -L-arabinofuranosyl (1—>*2)*- α -L- arabinopyranosyl]
Bacopasaponin C	3-0- [β -D-glucopyranosyl (1—>3) [α -L-arabinofuranosyl(l—>2)]- α -L-arabinopyranosyl]
Bacopasaponin D	3-0- [α -L-arabinofuranosyl(1—»2)- β -D-glucopiranosyl]
Bacopaside I	3-0- α -L-arabinofuranosyl-(1—>-2)- [6-O-sulphonyl- β -D-glucopyranosil-(1—>3)]- α –L-arabinopyranosyl
Bacopaside II	3-0- α -L-arabinofuranosyl-(1—>-2)- [β -D- glucopyranosil-(1—>3)]- β –D-glucopyranosyl
Bacopaside V	3-0-β-D-glucopyranosyl-(l—>3)-α-L-arabinofuranosyl

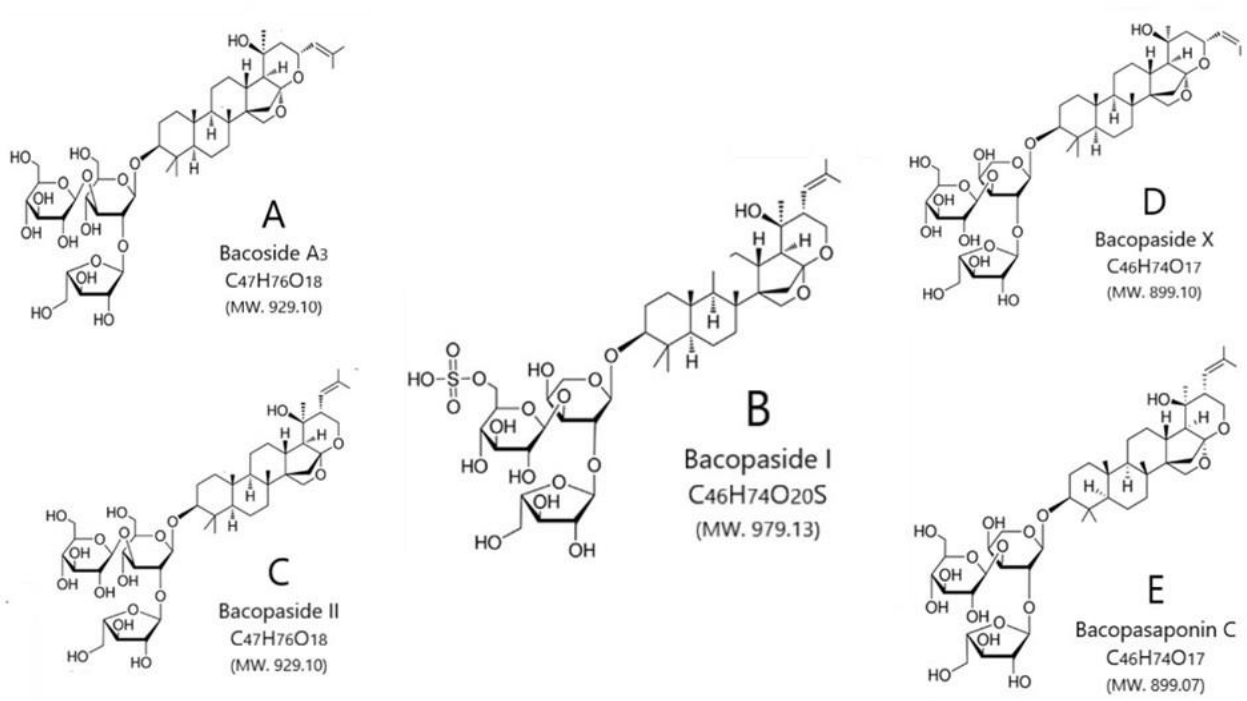

Figure 2 Structures of *B. monnieri* constituents, (A) bacoside A_3; (B) bacopaside I; (C) bacopaside II; (D) bacopaside X and (E) bacopasaponin C

3. Pharmacological activity

The *B. monnieri* is a traditional Ayurvedic medicinal plant and is extensively used in India as a nerve tonic for promoting mental health and improving memory brain function (26). In light of pharmacological effects, *B. monnieri* and five bioactive have been rich in the effect such as anti-oxidant (27-29) , anti-inflammatory (10, 30, 31), anti-epileptic (32), anti-depressant (20, 33) and etc. The detail has been discussed briefly below.

3.1 Neuropharmacological effects

Learning and memory actions: The *B. monnieri* extracts and bioactive compounds like bacosides have been widely investigated in their brain-memory enhancement effects in several studies. These natural compounds can repair damaged neurons by increase enzyme activity, the protein involved in the neuronal synthesis, restoring synaptic activity, and accumulating nerve impulse transmission (26), which leads to increased memory functions *B. monnieri* has been shown to affect a number of neurotransmitter neuron. The central cholinergic framework is considered the foremost important neurotransmitter included within the direction of cognitive capacities.

The effect of *Bacopa monnieri* leaf ethanolic extract (BMEE) on the serotonergic system of postnatal rats by oral administration the result showed that BMEE improved the learning and retention of memory significantly in all behavioral tasks. It's can increase the level of serotonin (5-HT) while dopamine (DA) decreased. This process is possible by regulating the expression of TPH2, 5-HT metabolism, and transport (34). Kamkaew et al. study chronic and acute effects of the *B. monnieri* (40 mg/kg) on cerebral blood flow (CBF) compared with *Ginkgo biloba* (60 mg/kg) and donepezil (1 mg/kg) in rats, after 8 weeks of daily oral dosing and systolic blood pressure was also measured using the tail-cuff method or via arterial cannulation. The result suggests that the chronic administration of *B. monnieri* had no effect on blood pressure. In contrast, intravenous acute infusion of these herbals (20–60 mg/kg) had marked dose-dependent hypotensive actions which increased CBF up to 25% when treated *B. monnieri* 40 mg/kg/day. These results may account for its reported pro-cognitive effect, and its further exploration as an alternative nootropics drug is worthwhile (35).

In 2015, the studies found that *B. monnieri* was beneficial for the prevention of cognitive deficits related to cerebral ischemia. In addition, bacopaside I played a role in the neuroprotective effects *via* PKC and PI3K/Akt mechanisms in the mouse model (36). The systematically assess and critically summarize the clinical trial of *B. monnieri* can effectiveness outcomes and safe in children and adolescents' populations for improving elements cognition as well as behavior and attention-deficit domains (37).

In 2018, The study effect on *Brahmi vati* and bacoside A of *B. monnieri* on acute pentylenetetrazole-induced seizures, an amphetamine-induced model of schizophrenia, and scopolamine-induced memory loss in animals model (38). The *B. monnieri* prevents mitochondrial and oxidative stress in the cultured cells. Furthermore, it can prolong the healthy lifespan of *C. elegans*, indicating that *B. monnieri* has the potential for therapeutic and preventative use in neurodegenerative disease. Protective against glutamate toxicity and increases the longevity of life (39).

Clinical studies: clinical studies have examined the effects of *B. monnieri* and constituents that focused on its cognitive enhancing activity. Roodenrys et al. reported that a double-blind randomized, placebo management study performed on 76 adults aged between 40-65 years old in which various memory functions were tested and levels of tension measured. The results show a significant effect of the *B. monnieri* on retention of new information; the rate of learning was unaffected and decreased the rate of forgetting of freshly information data. The verbal tasks, visual short-term memory tasks, and retrieval knowledge were unaffected (11). Besides, oral administration of 300 mg/day *B. monnieri* extracts for 12 weeks in the elderly (55 years old) has been improved memory acquisition and retention (22).

The commercially, *B. monnieri* extracts supplement in trade name as KeenMind™. This product is made from the stems, leaves and roots of *B. monnieri* and is extracted with fermentation alcohol. It's standardized to contain active bacosides at levels of 55th ± five-hitter. KeenMind™ facilitates develop novel preventative health practices and nutritional/pharmacological targets within the old for psychological features and brain health (22).

Pravina et al. evaluate the efficacy and tolerability of *B. monnieri* extracts (BacoMind ®) in 65 adults aged between 50-75 years with a single oral dose of 450 mg daily for the duration of 12 weeks. The neuropsychological tests revealed that BacoMind improved performance in tests associated with attention and verbal memory such as digit span backward test, list learning delayed recall test, paired associates dissimilar delayed recall test, and in visual retention -I test (12)

Overall, *B. monnieri* gave the functional to have multiple modes of action within the brain and the memory-boosting alternative source for the development of new neurological agents. Currently, researchers were found that *B. monnieri* extracts as a viable medicine for improving mental health and chemical constituents that are used for the treatment of many fatal or life-threatening diseases, which is quite helping the body in numerous ways are classified are Direct pro-cholinergic action, Antioxidant (flavonoid) activity, Metal chelation, Medicine effects, and Improved blood circulation. The neuropharmacological effect of *B. monnieri* extracts on clinical studies have been shown in Table 2.

Table 2 Summary of the neuropharmacological effects of *B. monnieri* on clinical studies

Substances/treatments	Subjects/patients	Results/comments	References
B. monnieri extract; chronically for 4 weeks	Patients with anxiety neurosis	Enhancing of memory	(26)
B. monnieri extract; chronically for 12 weeks	Children	Enhancing memory and learning	(40)
B. monnieri standardized extract (300 mg); acute treatment for 2 h	Healthy adult subjects	No significant changes were found	(27)
B. monnieri standardized extract	Mentally retarded children	Effective in enhancing learning and in controlling abnormal behavior	(41)
B. monnieri standardized extract (300 mg); chronically for 12 weeks	Healthy adult subjects	Improving early information processing, verbal learning and memory consolidation	(5)

Substances/treatments	Subjects/patients	Results/comments	References
B. monnieri standardized extract (300 mg); one trial after chronic treatment for three months and another trial and for 6 weeks after the completion of the trial	Healthy adult subjects	Significant effect on a test for retention of new information. Tasks assessing attention, verbal and visual short term memory and the retrieval of pre experimental knowledge were unaffected	(11)

3.2 Alzheimer's disease (AD)

Evaluation of the effect of 300 mg *B. monnieri* (Bacognize®) orally twice a day for 6 months in the newly diagnosed patients of AD in the patients mean age of 39 who completed the study was 65.23 years. Study patients showed statistically significant improvements in some aspects of cognitive functions in geriatric patients suffering from AD (42).

The neuroprotective properties studies of *B. monni*eri 100 mg /kg bodyweight for 180 days on memory deficits and biochemical changes in the ATPase system of AD-induced mice. Results revealed that BME showed positive effects on body weight, learning skills, memory, and concentration, whereas D-Gal and NaNO2 caused learning and memory deficits in mice which could be ameliorated by simultaneous administration of *B. monnieri* extracts. Similar, protective effects of *B. monnieri* extracts were noticed on the ATPase system could revert all the constituents of the ATPase system to normal levels in AD induced mice and to maintain ion gradients across biological membranes, thus confer significant neuroprotection against AD by stabilizing the structural and functional integrity of the membrane (43). Moreover, several studies were examined *B. monnieri* extracts effect on AD both in an animal model and clinical tries, thus collected data as shown in Table 3.

Table 3 Effects of *B. monnieri* extract on various study designs of AD (43)

The model used and study design	Dosage	Effect of *B. monnieri* extract treatment
In vivo model		
Ethylcholine aziridinium ion (2 nmol/2 μL)—ICV-induced male Wistar rats (44)	20, 40, and 80 mg/kg *B. monnieri*	*B. monnieri* enhanced the escape latency time (P <.01) in the Morris water maze test. Both cholinergic neuron and neuron densities reduction
Oral administration of aluminum chloride ($AlCl_3$; 50 mg/kg, p. o.)—IP-induced male Wistar rats	40 and 50 mg/kg	*B. monnieri* treated significantly prevented the reduction in SOD activity and decreased the lipid peroxides and protein oxidation.
Streptozotocin (STZ) (3 mg/kg, p. o.)—ICV-induced male Wistar rats	30 mg/kg	*B. monnieri* treated improved the memory and learning capability in ICV-STZ rats. BME treated significantly reduced in LPO levels.
d-Galactose (120 mg/kg, p. o.) and sodium nitrite (90 mg/kg, p. o.)—IP-induced male Albino mice	100 mg/kg	*B. monnieri* lessened both the $NaNO_2$ and d-Gal levels, which improved the body weight, memory, and learning skills. *B. monnieri* also normalized the ATPase system in AD-induced

The model used and study design	Dosage	Effect of *B. monnieri* extract treatment
In vivo model		
Okadaic Acid (300 mg/kg, p. o.)—ICV-induced male Sprague Dawley rats	40 and 80 mg/kg	*B. monnieri* treated significantly enhanced the memory-enhanced memory dysfunction in AD rats as appeared by a reduction in path length *B. monnieri* (bacosides) significantly (P <.05) prevents the lipofuscin aggregation in the middle-aged and aged rat brain cortex. Bacosides also enhances the synthesis of cholinergic neurotransmitter acetylcholine, modulates the metabolism of monoaminergic neurotransmitters and inhibits lipid
Female Wistar rats: young (2-3 months), middle-aged (17-18 months), aged (>24 months old)	200 mg/kg	

The model used and study design	Dosage	Effect of *B. monnieri* extract treatment
In vivo model		
Adult albino Wistar rats	20 mg/kg	PLGA nano *B. monnieri* formulation with a size range of 70-200 nm and a relatively low polydispersity index of 0.391 ± 1.2 showed that encapsulation efficiency was 57.11% ± 7.11%, with a drug loading capacity of 20.5% ± 1.98%. SEM revealed the PLGA nanoparticles' spherical shape, as well as it appeared to have low crystallinity by X-ray studies. This verified there were no chemical interactions between both polymer and drug molecules. The *in vitro* study showed a constant pattern with a maximum release of

The model used and study design	Dosage	Effect of *B. monnieri* extract treatment
In vivo model		
Male Swiss albino mice and Wistar rats were used to evaluate inotropic activity and bioavailability studies, respectively.	40 mg/kg	Bacopaephospholipid complex (BPC) portrayed 2 endothermal peaks (80.90°C and 171°C) in DSC studies. BPC treated significantly improved cognitive ability and anti-amnesic activity in aged mice in most memory-related models studied. BPC also retained effective bacopasides concentration for a longer period in rat serum.
Scopolamine—IP-induced male Swiss albino mice	120 mg/kg	*B. monnieri* treated reversed both retrograde and anterograde amnesia.
PSAPP mice	40 and 160 mg/kg	*B. monnieri* reversed both Y-maze and open-field hyperlocomotion behavioral changes in PSAPP mice. Thus, suggested *B. monnieri* reduced Aβ 1-40 and 1-42 levels in the cortex by 60%.

The model used and study design	Dosage	Effect of *B. monnieri* extract treatment
In vitro model		
PC12 cell line	100 μg/mL	*B. monnieri* ameliorated the mitochondrial and plasma membrane damage induced by 3 μg/ml scopolamine to 54.83% and 30.30%.
SK-N-SH cell line	12.5, 25, 50, 75, and 100 μg/mL	*B. monnieri* pretreatment significantly protects against H_2O_2 and acrolein-induced cytotoxicity and inhibited the generation of intracellular reactive oxygen species in addition to preserving the mitochondrial
Primary cortical cultured neurons cell line	100 g/mL	*B. monnieri* protected neurons from beta-amyloid-induced cell death. *B. monnieri* inhibited the lipid peroxidation reaction of brain homogenate in a dose-dependent

The model used and study design	**Dosage**	**Effect of *B. monnieri* extract treatment**
In vitro model		
SH-SY5Y cell line	0.1 to 25 μM	*B. monnieri* diminished the neurotoxicity of oxidized LDL in a dose-dependent manner potentially by suppression of cellular oxidative stress
SH-SY5Y cell line	Bacoside A (50 μM)	Bacoside A exerted significant inhibitory effects upon cytotoxicity, fibrillation, and particularly membrane interactions of amyloid-beta (Aβ42).

3.3 Anxiety and depression

Both animal and clinical research support *B. monnieri* extract standardization uses as an anti-anxiety remedy in traditional medicine. Research using *B. monnieri* extracts consist of 25%bacoside A exerted anxiolytic activity comparable to Lorazepam and benzodiazepine anxiolytic drug and it was attentively noted that the *B. monnieri* extract did not induce amnesia, side effects associated with lorazepam, but instead had a memory-enhancing effect (45, 46). The *B. monnieri* extract did not occur side effects but inversely it had a memory-enhancing effect. Besides, the *B. monnieri* syrup significantly decreases anxiety symptoms and levels of anxiety. Newly, in 2019 found that discovered mechanism of *B. monnieri* extract abrogates anxiety-like behavior by modulating alcohol markers and Gabra1, Gabra4, Gabra5 gene expression of GABA A receptor signaling pathway in rats (47).

3.4 Epilepsy

As well known, *B. monnieri* has been indicated as Ayurvedic medicine for epilepsy, research in animals demonstrated that Hersaponin as a constituent of *B. monnieri* extract exhibited protection against seizures in mice and showed the possibility for as an adjuvant in the treatment of epilepsy (47). The study examined the anticonvulsant properties of *B. monnieri* extracts in mice and rats were determined by intraperitoneal injections of *B. monnieri* extract (overdose to 50% of LD_{50}) and when administered acutely at lower doses (approaching 25% of LD_{50}), anticonvulsant activity was not observed. It was hypothesized that the anticonvulsive effects could be mediated through the GABA system which is involved in neural impulse transmission, likewise anticonvulsant, pain-relieving, and sedative activity (48).

3.5 Parkinson's disease (PD)

PD is associated with aggregation of protein alpha-synuclein and selective death of dopaminergic neurons, thereby leading to cognitive and motor impairment in patients. This information led to the current therapeutic strategies involves the prescription of dopamine agonist drugs which turn ineffective after prolonged use.

The study of model system *Caenorhabditis elegans* towards exploring the anti-Parkinsonian effects of a neuroprotective botanical *B. monnieri* by using two different strains of *C. elegans* from a transgenic model expressing, these studies show that *B. monnieri* reduces alpha-synuclein aggregation, prevents dopaminergic

neurodegeneration, and restores the lipid content in nematodes, thereby proving its potential as a possible anti-Parkinsonian agent (49).

The present study was examined the neuroprotective effect of Rotenone (RT) induced PD with reference to glutamate metabolism in different regions of the rat brain. The results indicated that the treatment with BM and Levodopa caused a significant elevation in glutamine content and the activity levels of Glutamate dehydrogenase (GDH), Glutamate synthase (GS) depletion in glutaminase activity when compared to induced PD rats. So the results of Swathi et al. (2012) suggest the ability of *B. monnieri* extract to modulate glutamate metabolism in different brain regions of induced rodents (50). In 2017, it found the research about the *B. monnieri* extract effect on PD indicates that *B. monnieri* extracts treatment provides nigrostriatal dopaminergic neuroprotection against MPTP induced Parkinsonism by the modulation of oxidative stress and apoptotic machinery possibly accounting for the behavioral effects (51).

3.6 Bronchitis and asthma effect

B. monnieri extract has shown the relaxant effect on chemically induced bronchoconstriction in *in vivo* studies. The broncho dilation effect of *B. monnieri* extract similar to disodium cromoglycate suggested this plant can be used as an alternative treatment of broncho constrictive conditions (32).

3.7 Gastrointestinal disorders effect

B. monnieri extract could direct spasmolytic effect on the gastrointestinal tract and intestinal smooth muscle. In addition, *B. monnieri* extract demonstrated the anti-microbial activity against *Helicobacter pylori*, which is bacteria responsible for chronic gastric ulcers, an *in vitro* study (33).

3.8 Cardiovascular effects

According to Ayurvedic medicine a text, *Bacopa monnieri* has been used as a cardiotonic agent. In addition, *B. monnieri* extracts demonstrated the vasodilation effect interfering with calcium channel flux in tissue cells (32).

3.9 Immunological benefits and others

B. monnieri extract had shown cytotoxic to sarcoma-180 cells due to DNA replication in the cancerous cell line (32). In addition, *B. monnieri* extract and bacoside also demonstrated anti-hyperglycemic through modulation of oxidative-nitrosative

stress and reduction in AGEs formation in diabetic rats. Thus, *B. monnieri* extract may be used as an alternative medicine for diabetic and neuropathic treatment (52).

3.10 Antioxidant activity

According to available published, *B. monnieri* extract showed modulate antioxidant activity. The possible mechanisms were metal chelation, free radical scavenging, and lipid peroxidation inhibition, resulting in the enhancement of antioxidant enzymes (27, 53). The antioxidant effect of *B. monnieri* extract also is observed in human fibroblast cells (29), rat astrocytes (28), rat liver homogenate, (54) and rat brain (55).

3.11 Anti-inflammatory activity

The *B. monnieri* extract is reported as a strong anti-inflammatory agent *via* inhibition of prostaglandin. The claim is supported by *in vivo* study. The extract can against carrageenan-induced paw edema in mice and rats, an acute inflammatory model (30). In addition, the extract also demonstrated the inhibition of COX-2 and 5-LOX, enzymes that catalyze the formation of mediators involved in the inflammatory process, in rat monocytes. The researcher found that another mechanism of action in the anti-inflammation effect of the extract was down-regulation of a pro-inflammatory agent, which is inhibition of TNF production human whole blood after LPS stimulation (56). The extract can be an alternative medicine for many anti-inflammation disorders (10).

4. Side effects and toxicity

To our knowledge, *B. monnieri* extract has a record of several hundred years of safe therapeutic use in Ayurvedic medicine. A double-blind, placebo-controlled clinical trial of healthy male volunteers investigated the safety of pharmacological doses of isolated bacosides over a four-week period. Likewise, the adverse effects of *B. monnieri* extract could not be found in humans following daily single (20-30 mg) and multiple (100-200 mg) oral doses (26).

The *B. monnieri* extract did not show any acute, sub-acute, and chronic toxicity in male-female Spargue-Dawlry rats following oral administration of the extracted. From the study, it was found that *B. monnieri* extract had no acute toxicity. The rats were fed extract in various dosage amounts once observed (1400, 2000, 2800 mg/kg/ day, respectively), found that no rodents died within 48 hours (57). Sub-acute toxicity

studies showed no change of rats in the experiment when feeding extract in varied dosages (250, 500, 1000 mg /1 kg /day respectively) for 14 days (57). In chronic toxicity studies, it was found that *B. monnieri* extract did not cause chronic toxicity when rats were fed various dosages of *B. monnieri* extract (0, 85, 210, 500 mg/kg/day respectively) for 90 days (57). As well as studying the pathological anatomy and histopathology found that rat organs did not differ between the test group and the control group (57).

The *B. monnieri* extract did not produce the hepatotoxic but protect the liver from the hepatotoxic agent. *In vivo* study, co-administration of 500 mg/kg/day *B. monnieri* extract with rifampicin, which is toxic to the liver, did not produce any hepatotoxic. These results indicated the protective effect against both liver toxicities in both rats (58). In addition, oral dosing of 180 mg / kg / day *B. monnieri* extract that has a protective effect against liver toxicity from CCl_4 by the antioxidant properties of Brahmi (59). Shalid et al. reported that the hepatotoxic property of *B. monnieri* extracts is due to its antioxidant effect (60).

5. Pharmacokinetics

The pharmacokinetics studies of *B. monnieri* were found from a database named PubMed. This electronic search was begun in September 2020. The following search terms were used "*B. monnieri*" AND "pharmacokinetics OR metabolism". The inclusion criteria consisted of studies investigating the pharmacokinetics information related to solubility, dissolution, absorption, distribution, and metabolism, which were performed both in *in vitro* and *in vivo* study, of *B. monnieri* and its five bioactive compounds. The studies not written in the English language were excluded. Of the 123 articles identified from PubMed, only 5 articles fitted the eligibility criteria.

The *B.monnieri* and its five bioactive compounds have been lipophilic compounds with log P of more than 1, low solubility(23), low dissolution property (61), and low absorbed (21%w/w) through rat intestine with three hours(23). Thus, the low oral bioavailability of the bacopa extract was expected. Besides, *B. monnieri* could distribute into the brain in rats following oral administration (61). Presently, the metabolism and excretion of bacopa are still unclear.

Based on *in vitro* studies using recombinant CYPs, *B. monnieri* extracts non-competitively inhibited CYP2C19, 2C9, and 1A2 with IC_{50}/Ki values of 23.67/9.5 µg/mL, 36.49/12.5 µg/mL, and 52.20/25.1 µg/mL respectively. Whilst CYP3A4, and 2D6 enzyme activity were competitively inhibited by *B. monnieri* extracts. The IC_{50} values for CYP3A4 and 2D6 were 83.95 and 2061.5 µg/mL respectively (17). Besides, the inhibition effect of *B. monnieri* extracts on CYP1A2 and 2C9 was confirmed using rat liver microsome (62). The IC_{50} values of CYP1A2 and 2C9 were 2.6 and 1.9-fold higher than the results obtained from Ramasamy et al.(17). Based on animal study, *B.monnieri* extracts demonstrated to increase the CYP1A2 enzyme activity in Sprague Dawley rats following oral administration of 20 and 40 mg/kg in a dose-dependent manner (63).

The *B. monnieri* gut concentration was expected higher than liver concentration when orally administered, consequently, this herb could exhibit stronger inhibition of intestinal CYPs than hepatic CYPs. When the humans consumed 300 mg/day of *B.monnieri* extract, the estimated gut concentration was 600 µg/mL (gut concentration = dose/500 mL) suggested that *B.monnieri* extract could contribute to HDIs (17). However, there are very few pharmacokinetics as well as the drug safety of this plant. In order to assess the safety of *B. monnieri* extract and its five bioactive compounds followed by US FDA Guidance for the industrial 2012 to 2020, the mechanism of inhibition and induction of it on CYPs in the primary human hepatocytes should be performed.

6. Drug-metabolizing enzyme

The drug-metabolizing enzyme (DMEs) play central parts within the digestion system, disposal, and/or detoxification of xenobiotic or exogenous compounds presented into the body. In common, DMEs ensure the body against the possibly hurtful presentation to xenobiotic from the environment. In arrange to play down the potential harm caused by these compounds, most of the tissues and organs are well prepared with different and different DMEs counting stage I, stage II metabolizing proteins (Figure 3) as well as stage III transporters, which are shown in plenitude either at a basal expression level and/or are actuated and display at an expanded level after xenobiotic introduction (64, 65).

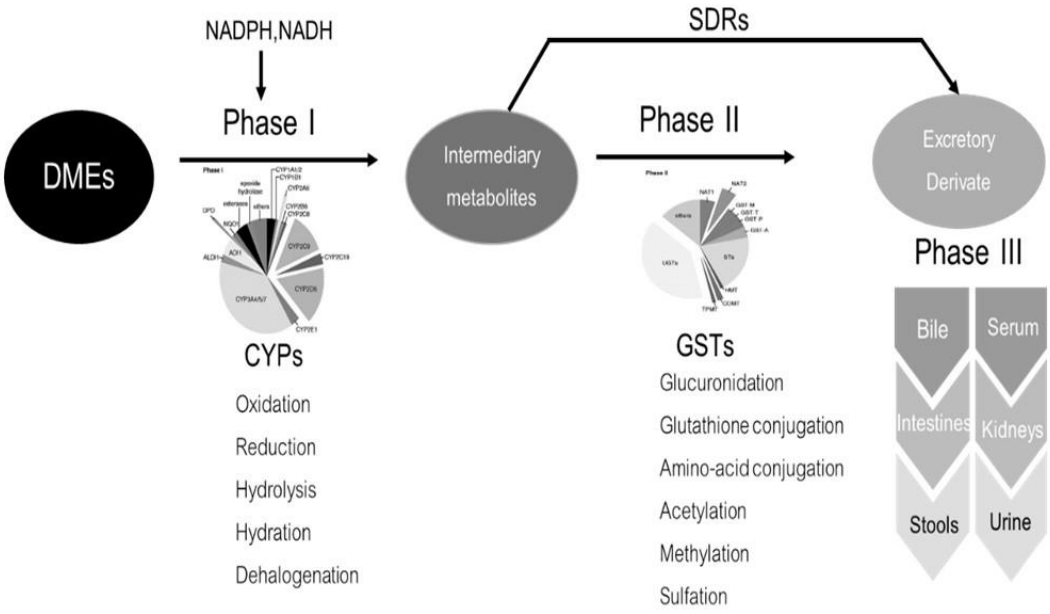

Figure 3 Cellular detoxification (drug metabolism); This process entails two phases including phase1 (functionalization) and phase 2 (conjugation); CYPs = cytochromeP450; SDRs=short-chain dehydrogenase reductases; GSTs = Glucosyltransferase (3)

Stage I DMEs comprise essentially of the CYPs superfamily of microsomal chemicals, which are found inexhaustibly within the liver, gastrointestinal tract, lung, and kidneys, comprising of families and subfamilies of proteins that are classified based on their amino corrosive grouping characters or likenesses. In humans, five CYP quality families, i.e. CYP1, CYP2, CYP3, CYP4, and CYP7 are accepted to play significant parts in hepatic and additional hepatic digestion systems and disposal of drugs (3, 4).

The phase II metabolizing or conjugating enzymes consists of a superfamily of enzymes including sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), epoxide hydrolases (EPH), glutathione *S*-transferases (GST), and *N*-acetyltransferases (NAT). Each superfamily of stage II DMEs comprises families and subfamilies of qualities encoding the different isoforms with diverse substrate specificity, tissue, and

formative expression, as well as inducibility and inhibitory by xenobiotics (66). Conjugation with phase II DMEs generally increases hydrophilicity, and thereby enhances excretion in the bile and/or the urine.

Drug metabolism can be divided into 2 main phases: phase I are oxidation, reduction and hydrolysis which alter morphology, and metabolite of drugs into more polarization while phase II is Synthesized or combination substances containing glucuronic acid, sulfate, acetyl, or others (conjugation phase). The major position of drugs metabolism as liver which found are more the endoplasmic reticulum, it is the area where drug metabolism activating as well as cytosol, mitochondria, nuclear envelopes, and plasma respectively. The enzymes in phase I are in the endoplasmic reticulum while in the phase II conjugation phase most enzymes are in the cytosol.

Phase I metabolism includes oxidation, reduction, hydrolysis, and hydration reactions. Oxidations performed by the microsomal mixed-function oxidase system (CYP450-dependent) are considered separately because of its importance and the diversity of reactions performed by this enzyme system. Oxidations involving cytochrome P450 system found in microsome (endoplasmic reticulum) of many cells (notably of the liver, kidney, lung, and intestine) perform many different functionalization reactions e.g., aromatic hydroxylation, aliphatic hydroxylation, epoxidation, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, oxidative deamination, *N*-oxidation, *S*-oxidation, dehalogenation, and alcohol oxidation. All these reactions require the presence of molecular oxygen and NADPH as well as the complete mixed-function oxidase system (CYP450, NADPH-cytochrome P450 reductases and lipid) All reactions involve the initial insertion of a single oxygen atom into the drug molecule. A subsequent rearrangement and/or decomposition of this product may occur, leading to the final product seen (Figure 4). Reductive reactions usually require NADPH but are generally inhibited by oxygen, unlike the mixed-function oxidase reactions that require oxygen. Examples of compounds undergoing reduction are Azo-compound, nitro-compound, epoxide, heterocyclic ring compound, and halogenated hydrocarbon. In most cases the final product after phase I contains a functional group, such as –OH, $–NH_2$, –SH, –COOH, etc. in the adequate chemical state to be by substrates of phase II or conjugative enzyme.

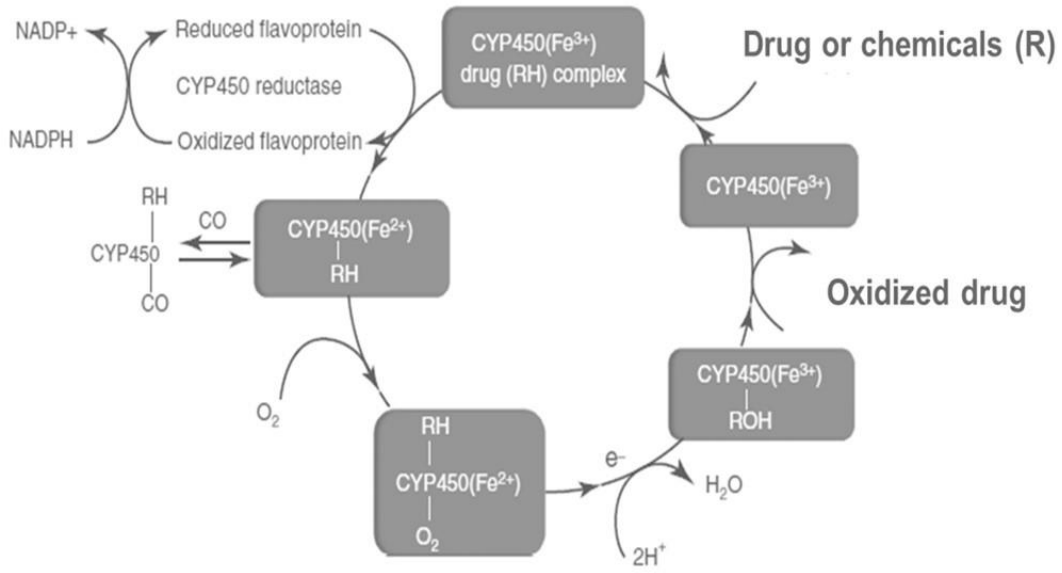

Figure 4 The catalytic cycle of cytochrome P450 (3)

The CYP450 system is an enzyme important for the metabolism of phase I drug metabolism and is an important system. Because the drug is metabolized through this system, the cytochrome P450 system is a group of the CYP450 enzyme or isozyme on the cell wall of the endoplasmic reticulum. Each CYP450 enzyme contains protein and heme (isozyme), which causes interest in the study of each type of drug that has metabolized through this system.

Isozyme is categorized and called family and subfamily based on the similarities of amino acid sequences. The participation of amino acid> 40% is classified as the same family and the participation of amino acid sequence> 55% is classified as a subfamily. Naming system of the CYP450, for example, CYP1A2 as follows:

CYP = cytochrome P450, 1 = Family, A= subfamily, 2 = type of enzyme

Currently, CYP450 is found in 12 family mammals and CYP1-4 about drug metabolism enzyme. According to studies on the amount of enzyme in the liver, hepatic isozyme contains approximately 30% of CYP3A, 20% of CYP2C, 13% of CYP1A2, 7% of CYP2E1, 4% of CYP2A6, 2% of CYP2D6, which includes more than 70% (67).

In humans, more than 50 isoforms have been isolated and 35 CYP isozymes are clinically relevant, although CYPs 1, 2, and 3 families appear to be mainly responsible for the metabolism of drugs and other xenobiotics (Table 4) and are also involved in the metabolic conversion of a variety of endogenous compounds such as vitamins, bile acids, and hormones. The CYP isozymes from the other families are generally involved in endogenous processes, particularly hormone biosynthesis.

Table 4 Summary of xenobiotic-metabolizing human hepatic CYPs

CYPs	Relative amount in liver (%)	Substrates (reaction in parenthesis)	Selective inhibitors	Other characteristics
1A2	~10	Ethoxyresorufin (*O*-deethylation)	Furafylline	Inducible
		Phenacetin (*O*-deethylation)		
2A6	~10	Coumarin (7-hydroxylation)		Polymorphic
2B6	~1	*S*-Mephenytoin (*N*-demethylation)	Orphenadrine	
		Bupropion (hydroxylation)		
2C8	<1	Paclitaxel (6α-hydroxylation)	Quercetin	
2C9	~20	Tolbutamide (hydroxylation)	Sulfaphenazole	Polymorphic
		Diclofenac (hydroxylation)		
		S-Warfarin (7-hydroxylation)		
2C19	~5	S-mephenytoin (4'-hydroxylation)	Ticlopedine	Polymorphic
		Omeprazole (oxidation)		
3A4	~30	Midazolam (1'- and 4-hydroxylation)	Diazepam	Inducible
		Testosterone (6β-hydroxylation)		
		Nifedipine (dehydrogenation)		

The major CYPs involved in the hepatic metabolism of most drugs including CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (66). The function of each enzyme is as follow:

CYP1A2: CYP1A2 is expressed mainly in the liver and is not or less expressed in extrahepatic tissues in humans, rats, and mice. In the human liver, CYP1A2 accounts for 13% of the total CYP content and is involved in the metabolism of ~4% of total drugs. In contrast, in monkeys and dogs, CYP1A2 is expressed at low levels in the liver of untreated animals, even though a quite strong similarity in amino acid sequence to humans (95% in monkeys). In humans, CYP1A2 metabolizes several drugs, including phenacetin, tacrine, ropinirole, acetaminophen, riluzole, theophylline, and caffeine (68).

CYP2B6: In humans, the CYP2B family is CYP2B6 and CYP2B7. CYP2B6 is expressed in the liver and in some extrahepatic tissues; CYP2B6 is involved in the metabolism of nearly 25% of drugs, such as the anticancer drugs cyclophosphamide and tamoxifen, the anesthetics ketamine and propofol and procarcinogens such as the environmental contaminants aflatoxin B1 and dibenzanthracene. The average relative abundance of CYP2B6 in the human liver ranges from 2 to 10% of the total CYP content. In addition, significant interindividual differences in hepatic CYP2B6 expression, which varies in some studies from 25- to 250- fold, have been reported. These large differences may be due to both polymorphism and induction. This finding of CYP2B6 variability suggests that there are significant interindividual differences in the systemic exposure to a variety of drugs that are metabolized by CYP2B6, with the consequent variation in therapeutic and toxic responses (68).

CYP2C9: It is involved in the metabolism of about 16% of drugs currently on the market. CYP2C9 are the major forms, accounting for 60%, respectively, of total human CYP2C, CYP2C9 proteins are primarily located in the liver where they account for approximately 20% of total cytochrome P450. CYP2C9 metabolizes many clinically important drugs including tolbutamide, phenytoin, *S*-warfarin, losartan, glipizide, torasemide, and numerous anti-inflammatory drugs such as ibuprofen, diclofenac, piroxicam, tenoxicam, mefenamic acid.

CYP2D6: CYP2D6 is expressed at a low level in the human liver accounting for about 4% of total P450 (12.8 pmol/mg microsomal protein), this enzyme is involved in the biotransformation of 30% of drugs. In humans, only one isoform, CYP2D6, is expressed in various tissues including the liver, kidney, placenta, brain, breast, lung, and intestine. Approximately 7 to 10% of the Caucasian population inherits mutant CYP2D6 alleles as an autosomal recessive trait, leading to individual variation in response to many drugs that are cleared by CYP2D. Another polymorphism stratifies the population depending on the copy number of wild-type alleles between poor metabolizers (PM, zero copies), intermediate metabolizers (one copy), extensive metabolizers (EM, two copies), and ultra-rapid metabolizers (multiple copies). In addition, this genetic variation in CYP2D6 is associated with risk for diseases and cancer, for example, Parkinson's disease, lung cancer, liver cancer, and melanoma. The rat and human CYP2D isoforms share a high sequence identity (>70%). Like human CYP2D6, the six isoforms are expressed in various tissues such as liver, kidney, and brain. Among the six isoforms, CYP2D1 is the rat orthologue of human CYP2D6. CYP2D15 is the major CYP2D in dogs with enzymatic activities like human CYP2D6. CYP2D15 is expressed in the liver, with lower but detectable levels in several other tissues (69). Bogaards et al. reported that dog and human liver microsomes showed similar enzyme kinetics with respect to the 1'- hydroxylation of bufuralol. In cynomolgus monkey, a full-length cDNA (called CYP2D17) encodes a 497-amino acid protein that is 93% identical to human CYP2D6. The recombinant CYP2D17 catalyzed the oxidation of bufuralol to 1'-hydroxybufuralol and dextromethorphan to dextrorphan, reactions shown to be mediated by CYP2D6 in humans, and strongly inhibited by quinidine (69).

CYP2E1: In humans, CYP2E1 accounts for approximately 6% of total P450 in the liver and is involved in the metabolism of 2% of the drugs. The CYP2E1 appears to have a dual physiological role, namely a role in detoxification, and in nutritional support. CYP2E1 is expressed in many tissues, such as the nose, the oropharynx (exposed to airborne xenobiotics), the lung, and the liver. Also, the inducible and adaptive responsiveness to xenobiotics is suggestive of a protective role. Regarding xenobiotics such as ethanol, CYP2E1 plays a detoxification role in preventing ethanol to reach excessive levels. In terms of its nutritional role, the up-regulation of CYP2E1

plays a useful physiologic role when starvation and/or low carbohydrate diet prevail because of its contribution to the metabolism of fatty acids and its capacity to convert ketones to glucose. However, like many other useful adaptive systems, when the adaptation ceases to be homeostatic and becomes excessive, adverse consequences prevail. CYP2E1-mediated metabolism generates oxygen radicals and, when this exceeds the cellular detoxification systems, it results in oxidative stress with its various pathologic consequences. This is true not only when excess alcohol must be metabolized, but also when CYP2E1 is confronted with an excess of ketones and fatty acids associated with diabetes and/or obesity. A few drugs are metabolized by CYP2E1, such as acetaminophen, caffeine and chlorzoxazone, the latter being considered a marker of CYP2E1 activity. Although relatively few drugs are oxidized by CYP2E1, the list of carcinogens that can be activated by CYP2E1 is quite extensive and includes benzene, styrene, acrylonitrile, and nitrosamines. CYP2E1 may generate reactive oxygen intermediates, such as superoxide radicals, which play a key role in liver injury because of the interaction with cellular proteins or DNA. CYP2E1 activity is inducible by ethanol and by acetone in both rodents and non-rodents. Like humans, many substrates such as organic solvents, nitrosamines, and drugs such as paracetamol are metabolized by rodent CYP2E1. Therefore, rodents such as rats may be an appropriate model to study CYP2E1 dependent metabolism in man. However, in dogs and monkeys some discrepancies with human have been found. In the dog microsome, the antibody against human CYP1A was shown to influence the 6-hydroxylation of chlorzoxazone, a typical activity of CYP2E1 in man. In monkeys, CYP2E1 activities in liver microsome seem to be like human CYP2E1. Disulfiram and diethyldithiocarbamate are mechanism-based inhibitors of CYP2E1 in man. In addition, diethyldithiocarbamate is a potent mechanism-based inhibitor of 6 OH-chlorzoxazone formation in microsomes of rodents and non-rodents' species, indicating a species-conserved mechanism for the oxidative biotransformation of chlorzoxazone among species (69).

CYP3A4: The CYP450 3A subfamily plays very important roles in the metabolism of xenobiotic and has very broad substrate specificity. It is highly inducible and can be inhibited by numerous drugs. Therefore, large interindividual differences in CYP3A-mediated metabolism have been reported (69).

The CYP3A subfamily is the most important of all human drug-metabolizing enzymes because this subfamily is involved in the biotransformation of approximately 50% of therapeutic drugs currently on the market, although its content in the liver is only 30% of total P450. Some examples of drugs metabolized by CYP3A are terfenadine, benzodiazepines midazolam, and triazolam, quinidine, lidocaine, carbamazepine, nifedipine, tacrolimus, dapsone, erythromycin, dextromethorphan, etc. In addition to drugs, CYP3A is involved in the oxidation of a variety of endogenous substrates, such as steroids, bile acids, and retinoic acid. CYP3A4 and its related CYP3A5 are the most abundant CYP isoforms in the human liver. It is also located at the apex of the enterocytes and plays a major role in the first-pass metabolism of xenobiotics. There are several factors in drug biotransformation and the detail is described as below (69).

Genetic polymorphism: Genetic differences are important contributors to the inter-individual differences in drug biotransformation seen within a population of patients. These differences are called genetic polymorphisms and are linked to inherited autosomal recessive traits. The definition of polymorphism is the presence within a population of at least 2 groups with distinctly different abilities to metabolize drugs (70). Individuals can be characterized as extensive (rapid) or poor (slow) metabolizers. Poor metabolizers often have an increased incidence of adverse effects. Among P450 polymorphisms, those affecting CYP2C9, CYP2C19, and CYP2D6 have the highest impact on drug metabolism (Table 5). CYP2D6 polymorphism requires special mention as it has been estimated that this isoform accounts for the metabolism of 25–30% of drugs used in clinical practice. In contrast, CYP1A1, CYP2E1, and CYP3A4 genes are relatively well conserved and only a few, if any, rare variants yielding changes in catalytic enzyme activity have been found (71).

Table 5 Polymorphic CYPs drug-metabolizing enzymes (71)

Enzyme	Functional allelic variants	Major variant	Allele frequency	Phenotype
CYP1A2	13	CYP1A2*1B	12 % Japanese	Decreased activity
CYP2A6	16	CYP2A6* 2	1–3% Caucasians	Inactive enzyme
CYP2C9	12	CYP2C9* 3	7–9% Caucasians	Altered substrate specificity
CYP2C19	16	CYP2C19* 2	13% Caucasians	Inactive enzyme
CYP2D6	46	CYP2D6* 4	12–21% Caucasians	Inactive enzyme
CYP2E1	2	CYP2E1* 3	<1% Caucasians	No effects

Disease: Impaired liver functions can lead to decreased drug biotransformation and is a function of the severity of the disease. Disease states that can impair liver function include hepatitis, alcoholic liver disease, biliary cirrhosis, and hepatocarcinoma. Infection can also alter drug biotransformation. There have been reports of impaired drug elimination during viral infections such as influenza, rhinovirus, adenovirus, herpes simplex, virus and infectious mononucleosis (3, 72).

Age: Infants do not develop a mature enzyme system until more than two weeks after birth. The elderly has age-related decreases in liver mass, hepatic enzyme activity, and hepatic blood flow. In addition, the overall metabolic capacity of the liver is decreased, although the considerable inter-individual variability in age and disease-related changes in organ function makes it difficult to form generalizations (3, 72).

7. Induction of CYPs

Upon repeated administration, certain drugs can alter their own metabolism, or that of other simultaneously or subsequently administered therapeutic agents, by changing the expression of drug-metabolizing enzymes. Exposure to certain chemicals (pollutants, cigarette smoke, alcohol, and dietary constituents) can also induce drug-

metabolizing enzymes. Induction may result in rapid metabolism of the drug and lower plasma concentrations to levels that are no longer efficacious (73). Enzyme induction also accounts for the onset of tolerance to some therapeutic agents. On the other hand, a possible consequence of enzyme induction is the increased formation of pharmacologically or toxicologically active metabolites. Thus, enzyme induction significantly contributes to interindividual differences in drug metabolism and toxicity (74). The phenomenon of CYP induction was first discovered and studied in experimental animals, but it was soon recognized to occur in humans. All human CYPs can be influenced to a certain extent, some of them being clearly induced by xenobiotics (74). Notably, there appears to be more variation in response to enzyme inducers among humans than in animals, probably due to varying genetics, lifestyles, and dietary habits (75). Enzyme inducers are usually classified based on their action on individual CYP isozymes. Table 6 shows representative inducers for human CYPs. Some compounds show CYP inductive potential across species (particularly for the CYP1A family). For other substances, significant differences exist in their inducing abilities in animals. Rifampicin (rifampin) for example is a potent inducer in humans and rabbits but it is a poor inducer in rats (76). In contrast, pregnenolone 16α-carbonitrile, a potent inducer of CYP3A in the rat, is not an inducer in either rabbits or humans (77). Different mechanisms are known to operate in CYP enzyme induction, but in general, it involves transcriptional activation of CYP genes by a receptor-dependent mechanism (figure 5), resulting in increased levels of specific CYP mRNAs. The first discovered inducing receptor, the aryl hydrocarbon (Ah) receptor, belongs to the PAS family of transcription factors. It stimulates transcription of CYP1A genes via direct interaction with the promoter region of the gene. Upon binding of the inducer to cytosolic Ah receptor, the complex is translocated to the nucleus, where it heterodimerized with the nuclear factor Arnt (Ah receptor nuclear translocator protein) and binds to an enhancer/promoter DNA region of CYP1A genes. This is a well conserved mechanism across species and accounts for the consistent induction of CYP1A by polycyclic aromatic hydrocarbons in many cell types (3).

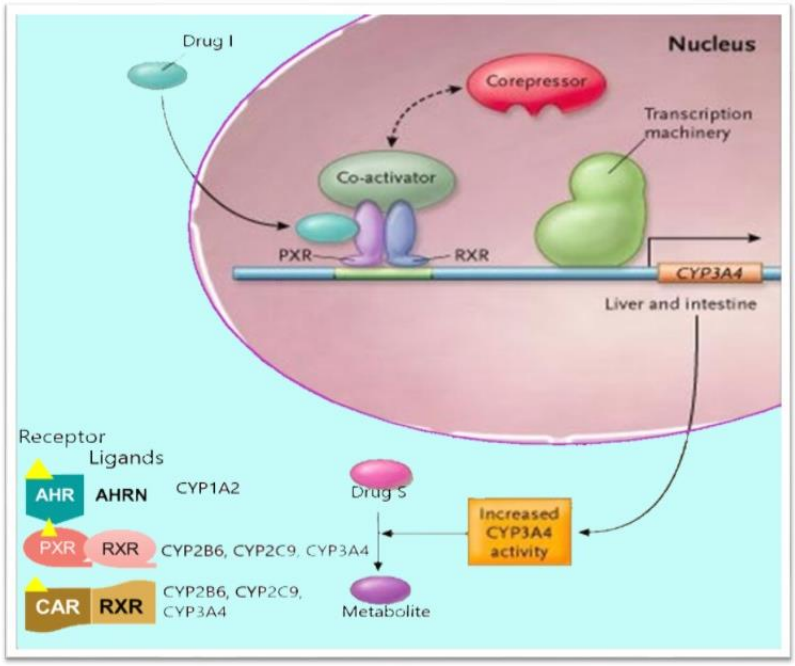

Figure 5 The mechanism of induction involves transcriptional activation of CYP genes by a receptor-dependent mechanism

Table 6 Inducers of human CYP450 isozymes

CYPs	Inducer
1A2	Polycyclic aromatic hydrocarbons, 3-methyl-indole (cruciferous vegetables), omeprazole, hyperforin, Chargilled meat
2B6	Nevirapine, Phenobarbital, Pesticides
2C9	Hyperforin, Rifampicin, Isoniazid
3A4	Corticosteroids, Macrolide Antibacterials, Phenobarbital, Lovastatin, Simvastatin, Omeprazole, and Rifampicin
4A	Peroxisome proliferators (fibrates), Clofibrate

8. Inhibition of CYPs

Drugs can also bind irreversibly (mechanism-based inhibition) or reversibly with the haem-binding site of the enzyme and inhibit other drugs from binding. Mechanism-based inhibition occurs when certain drugs are metabolized by the CYP450 system to active metabolites that bind to the enzyme and cause irreversible loss of function. Activity can only be restored by the synthesis of new enzymes, which may take several days (72).

More complex mechanisms of inhibition can also occur. Some drugs undergo metabolic activation by the cytochrome P450 system to inhibitory products. The metabolites may generate relatively stable complexes with CYP450 so that the cytochrome is held in an inactive state. There can be great clinical significance to this interaction since it is relatively long in duration. Additionally, when the interaction involves drugs with narrow therapeutic ranges, there is the potential for toxicity (72).

Unlike induction, enzyme inhibition usually begins with the first dose of the inhibitor. Inhibition is maximal when the inhibitor reaches a steady-state (four to seven half-life), and the maximum concentration of the inhibitory drug occurs when it reaches a steady-state at its new, longer half-life. The time required for the interaction to resolve also depends on the half-life of the drugs involved (3, 78).

Inhibition analyses through double reciprocal plots (Figure 5) are most useful as their slope and/or intercept effects talk directly about the effect of that inhibitor on the rate constants. Like before, we monitor initial velocity "v" but by varying the concentration of the inhibitor at different fixed concentrations of the substrate in the equation. The enzyme reaction following the Michaelis-Menten equation 1

$$v_o = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \text{equation 1}$$

The plotting between the changed velocity (v_0) with substrate concentration [S] Where V_{max} is the maximum velocity and K_m is Michaelis-Menten constant which is Molar or µg/mL unit (79).

To find the parameters more convenient and the value of parameters were extremely low, one of the limitations Hans Lineweaver and Dean Burk converts the equation to the reciprocal as namely: Lineweaver- Burk (equation 2).

$$\frac{1}{V} = \frac{K_m}{V_{max}+[S]} + \frac{1}{V_{max}} \qquad \text{equation 2}$$

When graph plotting between $1/v_o$ is the Y-axis and 1/ [S] is the X-axis, the linear regression graph is shown in the figure namely the Lineweaver-Burk plot which makes it possible to find the K_m and V_{max} values more accurately (79).

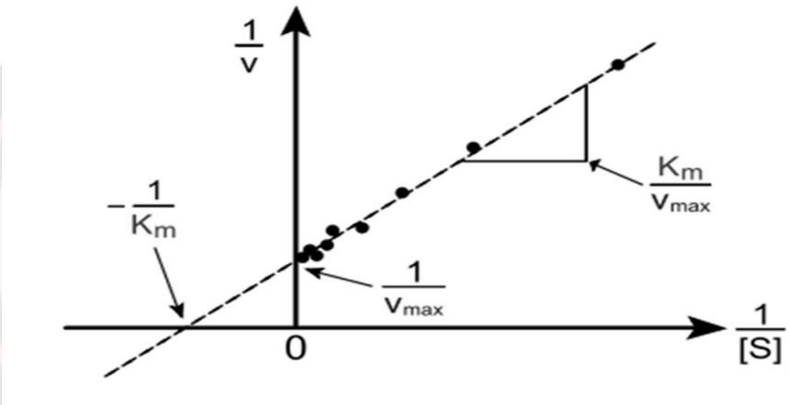

Figure 6 Double reciprocal plot between $1/v_o$ (Y-axis) and 1 / [S] (X-axis) according to Lineweaver-Burk equation so that the Y-axis is $1/V_{max}$, the X-axis is $-1/K_m$.

Enzyme inhibitors

Enzyme inhibitors are molecules that can bind to enzymes and reduce their catalytic activity. This principle is applied to antibiotics and enzyme-related therapies in the metabolic system, especially the CYP450 system. The activity of enzyme inhibitors can be classified into two types: permanent or irreversible inhibitors and reversible inhibition (79).

Irreversible inhibition

Irreversible inhibition or permanent inhibition (Figure 6), is produced by the addition of binding agents that bind to covalent enzymes, which are very stable and destroy functional groups necessary for catalysis (79).

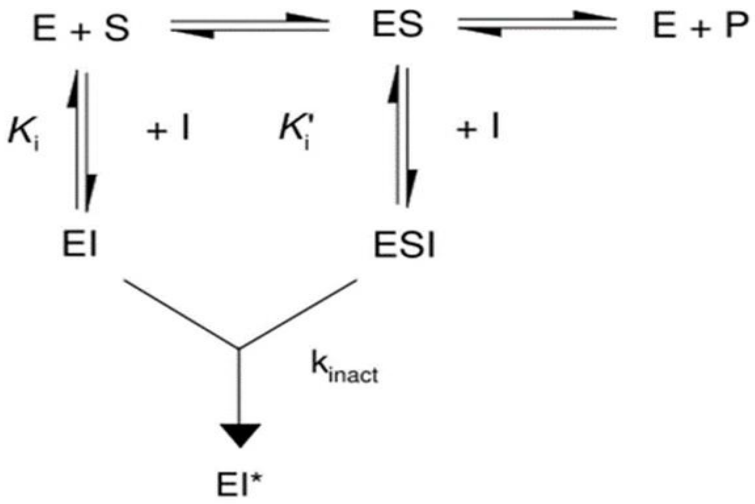

Figure 7 inhibition model equation (79)

Reversible inhibition

Reversible inhibition can either isolate the inhibitor from the enzyme or dilute the inhibitor to reverse the enzyme activity. with the same efficiency, reversible inhibition can be divided into 3 types: Competitive, Uncompetitive, and Noncompetitive inhibition. However, the Drug metabolism field found that involving 3 types were classified as competitive, non-competitive, and mixed type inhibition (79).

1. Competitive inhibition

The chemical structure of the competitive inhibitor has been similar to the substrates. Thus, it can bind to the free form of the enzyme (E) at the active site. Nonetheless, the competitive inhibitor cannot bind to the enzyme complex (ES) because the active site is bound by the substrates. The competitive inhibition can reverse (Figure 7). When either the concentration of substrate or inhibitor is more intense, it has a better chance of binding to the active site. Therefore, Factors affecting enzyme binding depend on the concentration. The result in the pharmacokinetics of competitive inhibition is K_m increase whereas V_{max} constant (79).

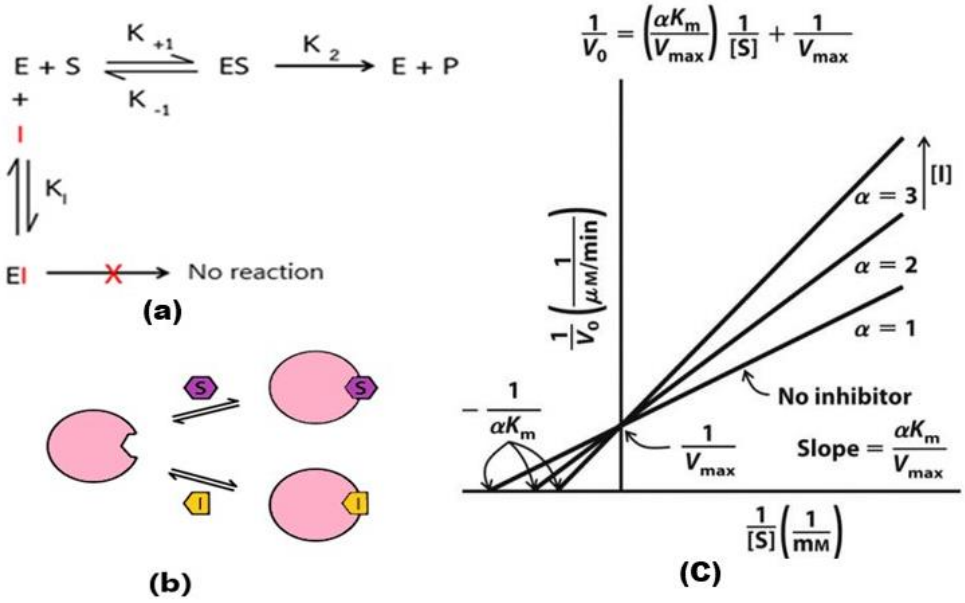

Figure 8 (a) Competitive inhibition equation, (b) Competitive inhibition model, (c) The Lineweaver-Burk plot of competitive inhibition when Inhibitor concentration at (α=1) is no inhibition that increasing on α=2,3, respectively (79).

2. Uncompetitive Inhibition

The inhibitor can specifically bind with ES complex only whereas not occurred with free enzyme (E), one of reversible inhibition type which resulting occur ESI complex, the final product not production and cause change the shape of enzyme until best fit for inhibitor binding site (Figure 8). This phenomenon not competing with the substrate. The result in the pharmacokinetics of Uncompetitive inhibition is K_m and V_{max} values are decreasing (79).

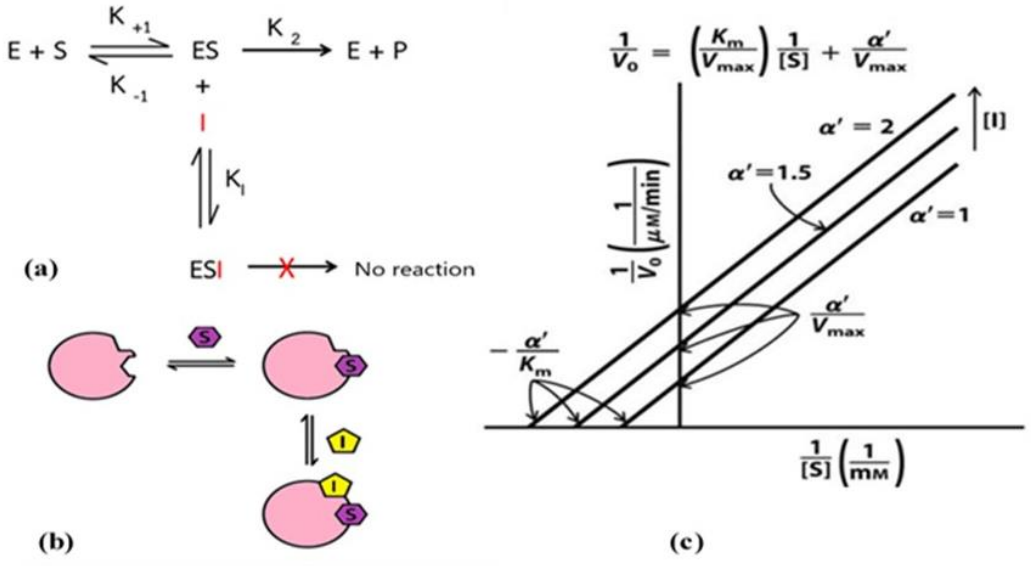

Figure 9 (a) Uncompetitive inhibition equation, (b) Uncompetitive inhibition model, (c) The Lineweaver-Burk plot of uncompetitive inhibitor when Inhibitor concentration at (α=1) is no inhibition that increasing on α=2,3, respectively (79)

3. Noncompetitive Inhibition

Noncompetitive self-inhibited prevents the capture of the non-binding at the active site, which can bind with or ES complex (Figure 9). The inhibitor changes the structure of the enzyme, causing the catalytic group to be unable to function normally or not in a suitable location to catalyze the reaction. Therefore, the substrate can be converted to a product less or not at all. If the binding of the substrate or inhibitor is independent of each other (KI = K´I), its namely pure noncompetitive inhibition (79).

If the substrate-binding influences inhibitor binding or inhibitor binding influences substrate binding (KI ≠ K´ I), it is to resemble. Therefore, this type of inhibition is called mixed inhibition (79).

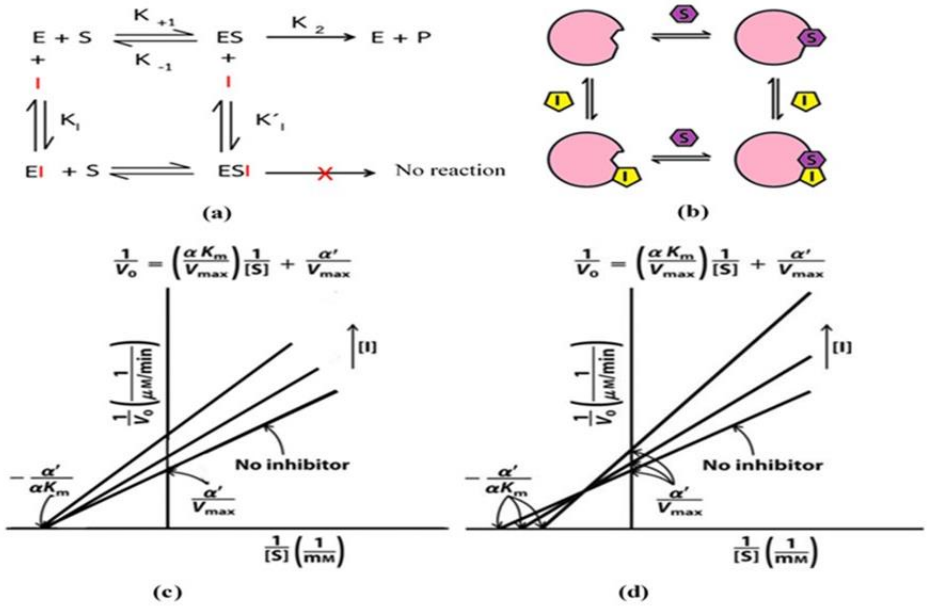

Figure 10 (a) Noncompetitive inhibition equation, (b) Noncompetitive inhibition model, (c) The Lineweaver-Burk plot of Pure noncompetitive inhibition when varying the inhibitor concentration. The kinetic results of the pure noncompetitive inhibitors were constant K_m and decreased V_{max}, (d) The Lineweaver-Burk plot of Mixed type inhibition when varying the inhibitor concentration. The kinetic results of the mixed type inhibitors were increased K_m and decreased V_{max} (79)

The nature of kinetic experiments results, and the information sought from reversible enzyme inhibition, which these main types of inhibition, must be established before embarking on its use to study enzyme mechanisms. data are as follows (79).

Competitive inhibition is the most common mechanism of inhibition and occurs when two or more drugs compete for the same enzyme. The clinical significance of an inhibition interaction depends primarily on the relative concentrations of the drugs, as well as a variety of other patient-specific factors. Some drugs are capable of binding to and acting as competitive inhibitors of different CYP450 enzymes from the ones that are responsible for the biotransformation of that particular drug (72).

9. The herb-drugs interaction (HDIs)

The multiple phytochemical components which may involve the pharmacological activities can be a cause of herb-drug interaction. Interactions can be in both pharmacokinetics and pharmacodynamics. The pharmacokinetic interactions of herbs and drugs were a target for several published. Herbs may affect the pharmacokinetics behavior including absorption, distribution, metabolism, and excretion process of drugs (15).

HDIs may change the drug levels in plasma and drug activities and lead to either therapeutic failure or toxicities. Sometimes it can be fatal. The combination uses of herbs and drugs together without the knowledge in the potential adverse herb–drug interactions, will risk to patient’s safety in medical services. In constant, some interactions can be beneficial to the therapeutic potential. It may be used for the development of new therapeutic strategies in the future.

Phytochemical containing in herbal products is eliminated by various metabolic enzymes and may be substrates for various transporters. The potential for involvement of drug metabolizing enzymes and transporters in the handling of herbal components leads to a predisposition of herb–drug interactions in several ways.

First, an herbal component can be a substrate for one or many isoforms of CYP enzymes and/or efflux systems (P-gp, MRP, and BCRP). Therefore, one substrate can compete for another substrate for either metabolism by the same CYP isozyme and/or efflux system resulting in higher plasma concentrations due to competitive inhibition,

Second, an herbal can also be either inhibitor or inducer of one or many CYP isoforms and/or efflux systems. Such interaction may produce the changing of drugs level in plasma, resulting in increasing or reducing of therapeutics effect.

In order to prevent adverse outcomes in patients taking combinations of herbal supplements and drugs, the potential herb-drug interactions should be identified. In addition, the identified mechanisms of interaction can also be offer insight into the approaches to be taken to minimize their impact and to design appropriate studies in humans. The described pharmacokinetic interactions of the popular herbal products with other medicinal agents are summarized in Table 7 (15).

The herbals or phytomedicines Trend: In recent times, herbs or phytomedicines have become popular due to the global trend of herbal uses is increasing. The estimated and predicted sizes of the global market of herbal uses are approximately US $80 - $100 billion and the US $2500 billion by the year 2010. Likewise, the trend in the West, Europe, Japan, Asia, North America, and Thailand are increasing continuously. However, the problem of the marketed herbal products including scientific evidence of efficacy and safety has lacked. This results in herbs- or herbs-drug interaction induced unfavorable clinical outcomes without crucial documentation on their temporal relations and concomitant use (15).

Table 7 Examples of Herb-drug interactions

Drug	Interaction	Comment
Garlic (Allium sativum)		
Warfarin (anticoagulant)	Additive blood thinning effects	Possible of spontaneous bleeding
Saquinavir (protease inhibitor)	Herb reduces blood level drug, lowers drug effect	May cause failure of therapy in AIDS
Ginko (Ginkgo biloba)		
Aspirin	Increased anticoagulation	Spontaneous bleeding
Warfarin	Increased anticoagulation	Brain hemorrhage (intracerebral)
Acetaminophen, caffeine and ergotamine	Unknown	Brain hemorrhage (subdural)

Drug	Interaction	Comment
Ginseng (Panax ginseng)		
Warfarin	Potentiation	Possible of spontaneous bleeding
Alcohol	Increased alcohol clearance	Stimulate alcohol metabolism
Influenza vaccine	Synergy, enhances resistance	Reduces flu symptoms frequency and severity
Insulin	Synergy, antidiabetic actions of herb	Improves blood sugar and diabetic
St. John's wort (Hypericum perforatum)		
SSRIs	Synergy-additive serotonin like effects	Risk of serotonin syndrome. avoid concurrent uses
Amitriptyline	↓ AUC of amitriptyline and its metabolite nortriptyline	Notify prescribing physician
Warfarin	↓ INR	
Midazolan	↓ Oral bioavailability by 39.3%	
Indinavir, ritonavir, saquinavir, lopinavi, amprenavir	Herb reduces blood level drug and lowers drug effect	May cause treatment failure due to decrease of drug concentration
Theophylline	Herb reduces blood drug level	probably by enhancing CYP3A4
Cyclosporine	Herb reduces blood level drug and lowers drug effect	May cause failure of therapy in AIDS
Digoxin (cardiac glycoside)	Herb reduces blood level drug and lowers drug effect	
Oral contraceptives	Herb possibly reduces blood level drug and lowers drug effect	enhances the metabolism of theophylline Risk of transplant rejection. notify prescribing physician May cause arrhythmias; notify prescribing physician Intermenstrual bleeding and unplanned pregnancies

Herb-Drug Interactions and Hepatotoxicity: Globally, patients have been attracted to herbal remedies without the correct conception, resulting to the therapeutics failure. The truth should use taking a combination of the herbs with drugs under prescription to avoid the adverse side effects, including hepatotoxicity. The toxicological effect of a drug or herb is perhaps caused by the inhibition of drug metabolism enzymes especially CYP450, leading to interactions with certain prescription drugs through various mechanisms (15).

The most appropriate systems allow us to extract clinically relevant information on the metabolic pathways and the potential drug interaction of herb-derived compounds. In recent times, there are several study models to investigate the metabolic pathways or metabolic stability. Human primary hepatocytes are the best model that can be applied in any metabolic study while human liver microsome are a tool for preliminary drug metabolism studies. Recombinant microsome which express specific enzymes and CYP-isoform-specific monoclonal antibodies has been used as a tool for enzyme inhibition and induction studies (80).

Metabolic-based drug interactions: The drugs metabolic of the same enzyme may competitively bind to the enzyme at the binding site, which is resulting to reduce in the metabolism of drugs with low affinity or an increase in parent drugs and pharmacological effects when oral administration. Metabolic drug interaction reported about the cytochrome P450 system can be classified as below.

1. **First-pass metabolism:**

This process occurs after the oral administration of the drug. The drug might be degraded in the gastrointestinal tract or eliminated via the liver before entered the blood circulation system and the action. For example, when the combination of oral dosing of cyclosporine which is metabolized by a CYP3A4 (was found in the digestive system) with erythromycin, which is CYP3A4 inducer, the first pass-metabolism will be increasing. In contrast, this phenomenon is absented when intravenous injection of erythromycin.

2. **Foreign compounds and environments:**

Increasing the metabolism rate by induction of the enzyme must be given for a period to synthesis the enzyme. For example, the smoking or exposure of

chlorinated hydrocarbon pesticides can induce the CYP1A2 metabolic pathways, leading to reduce the therapeutic effect of CYP1A2 substrates.

3. **Food and alcohol:**

The foods containing CYP450 inhibitors can change the pharmacokinetics of drugs, leading in reducing of therapeutic effect. For example, co-administration of bioflavonoids in grapefruit juice and nifedipine or felodipine could increase the area under the curve of nifedipine or felodipine up to approximately 3-fold. In addition, drinking small amounts of alcohol in short and not continuous periods is resulting in non-specific inhibition CYP450 effect while drinking alcohol for weeks or months could induce the CYP450 activity.

4. **Drugs:**

Drug metabolizing enzymes may change both quantitatively and qualitatively by internal factors such as genetics, age, gender, ethnicity, pathological, stress, or pregnancy, etc. (3).

The evaluation of drug-drug and herb-drug interaction: DDIs and HDIs were evaluated both in *in vitro* and *in vivo* studies. *In vivo* studies in animals, involved treating laboratory animals with the test compound, followed by the analysis of liver CYP enzymes *ex vivo*. However, this method is still limited by many issues. First, there is the requirement of large numbers of animals. Second, *in vivo* studies are not high throughput. Third, the extrapolated results may be unreliable due to the difference of CYP containing between species. In addition, *in vivo* study is interfered with by some pharmacokinetics processes including the absorption process and distribution process. At the same time, *in vivo* study in humans is costly, time-consuming, may be unethical in certain cases, difficult and may not provide mechanistic information.

In vitro studies are used for evaluating multiple products and multiple components, providing mechanistic information about any potential interaction. Another advantage is easy to uses. Recently, *in silico* approaches for evaluation of drug metabolism, drug transport, and drug interaction studies are increasing. Such an approach is expected to be of use to predict herb-drug interactions as well in the future. Most research on HDIs has been focus on the *in vitro* evaluation of herbal constituents in microsomal systems, supersomes, cytosols, expressed enzymes or cell culture systems such as transfected cell lines, primary cultures of human hepatocytes and tumor

derived cells. In addition, studies have also been carried out *in vivo* in animals (normal, transgenic, humanized) and in humans (primarily healthy individuals). Only a small number of studies have examined the effects of herbal products on phase II metabolism or drug transport (62). There are advantages and limitations to each of the method used (Table 8).

Table 8 Limitations and advantages of *in vivo* and *in vitro* systems for the evaluation of drug-drug and herb-drug interaction studies

Drug interaction studies	Limitations	Advantages
In vitro studies	- Single components used in test. - Typically use higher concentrations than clinically relevant - Does not account for poor bioavailability of the active component *in vivo* - Does not account for binding of active component *in vivo* to plasma proteins	- Easy to carry out - Simple systems - Provide mechanistic information
In vivo studies in humans	- Selection of products: There is large variability in actual content of different ingredients-batch to batch, product to product variations in disintegration and dissolution complicates interpretation. Difficult to extrapolate from one formulation to another. There are differences in the bioavailability of selected components from different formulations. - Study design: Often carried out in healthy humans and not in patients who may have additional compromising conditions - Positive and reference controls not normally used (use of rifampin to show induction and use of ketoconazole or other inhibitors to show inhibition of pathways tested and to serve as a reference for magnitude of any changes observed). - Need an evaluation of the bioavailability of the known ingredients.	- Will provide the final answer

Drug interaction studies	Limitations	Advantages
	- Does not distinguish gut versus liver effect of herbal products. - Generally costly. Difficult to obtain mechanistic information	

***In vitro* studies using microsome:** Microsome are contained in vesicles of the hepatocyte endoplasmic reticulum. Microsome are prepared from fresh human liver, liver slices, liver cell lines, and primary hepatocytes. This approach is a convenient tool to provide the CYP and UGT metabolic profiles. In addition, this study model can be used to investigate both the inhibition and induction effects of the interesting herbs and drugs. However, the microsomal study did not provide complete mechanistic information of any interactions (effects on mRNA or protein and the potential role of any metabolite formed).

***In vitro* studies using primary cultures:** In selecting *in vitro* systems one must pay particular attention to the test system used. The uses of more physiologically relevant *in vitro* models (such as primary human hepatocytes) are necessary if better predictions of DDIs and HDIs in humans. These models are suitable for the determination of whether there is a need to conduct more demanding clinical studies. The primary human hepatocytes can keep for 2 weeks or one month if placed in a three-dimensional culture. It can maintain all co-factors and the important co-substrates for phase I&II metabolic pathways and transporter function. Hence, the primary human hepatocyte is a fixable model for the study induction and inhibition of drug-metabolizing enzymes and certain transporters (19).

10. Interaction of *B. monnieri* on drug-metabolism enzymes

Using rat liver microsome, *B. monnieri* extract demonstrated very weak inhibit CYP1A2, 2C9, 2D6, and 3A with IC_{50} values of 135.59 µg/mL, 72.97 µg/mL, 116.47 µg/mL, and 143.23 µg/mL, respectively. Also, the saponin glycoside namely bacoside A_3 demonstrated very weak inhibition potential on CYP1A2, 2C9, 2D6, and 3A with IC_{50} values greater than 150 µg/mL (16).

Using recombinant CYPs experiment, *B. monnieri* has been a CYP2C9, 2C9, 1A2, and 3A4 inhibitors. The inhibition mode of CYP1A2 was identified as non-competitive inhibition with IC_{50} value equal to 52.20 µg/mL while Ki value equal to 25.10 µg/mL. Likewise, the IC_{50} and Ki values of CYP2C9 and 2C19 inhibition were 36.49/12.50 µg/mL, and 23.67/9.50 µg/mL with non-competitive mode respectively. Whilst the inhibition effect of *B. monnieri* on CYP3A4 was competitive mode with IC_{50} value and Ki value was 83.95 and 14.50 µg/mL respectively. In addition, the research also reported the weak inhibition against CYP2D6 (IC_{50} = 2,061.50 µg / mL) of *B. monnieri* (17). These results are in line with a published by Kar et al. (16). The CYP1A2 and 2C9 activities were inhibited by BM with IC_{50} values of 135.59 µg/mL and 72.97 µg/mL respectively. According to research, no experiments have been conducted to study the mechanism of inhibition and induction of *B. monnieri* extract on CYPs in the liver using primary human hepatocytes, which are studies as recommended by the US FDA. This will be important information to assess the safety of supplements or herbal when used co-administrate with drugs.

Based on *in vivo* study, the *B. monnieri* extract might relatively inhibit the CYP2C and 3A enzymes activity and enhance the oral bioavailability of amitriptyline. Since the pharmacokinetic parameters including maximum plasma concentration (C_{max}), the area under the curve from zero to time (AUC_{0-t}), and the haft life ($T_{1/2}$) of amitriptyline increased by 16.8%, 26.5%, and 15.5%, respectively in rats following simultaneously oral administration of *B. monnieri* extract and amitriptyline. Also, the oral clearance (CL/F) and volume of distribution (V_d) of amitriptyline were decreased by 26.2% and 15.5%, respectively (18).

CHAPTER III

RESEARCH METHODOLOGY

1. Materials and methods

1.1 Chemicals and authentic metabolite standards

The standard solution was used as substrates or products or metabolites, including reference inhibitors related to the conversion of reactants into products using enzyme functions. CYP450 (CYP activities) are CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. All CYP activity using catalytic enzyme was nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma–Aldrich (France). All other laboratory chemicals were used for the highest purity and from commercial suppliers. The standardized products, inhibitors, inducers as well as analytical methods were shown in Table 10.

B. monnieri standardized extract (batch No.: R63001) was purchased from GPO Ltd. (BKK, Thailand). Bacoside A_3, Bacopaside I, II, X, and Bacopasaponin C were obtained from GPO Ltd. (BKK, Thailand) and Pharmaceutical Sciences, Naresuan University (PHT, Thailand). Phenacetin, Acetaminophen, Bupropion, Hydroxybupropion, Tolbutamide, 4-Hydroxy tolbutamide, S-Mephenytoin, 4-Hydroxy mephenytoin, Bufuralol, 1'-Hydroxy bufuralol, Midazolam, and 1'-Hydroxy-midazolam were purchased from Sigma–Aldrich (Thailand). All other laboratory chemicals were used as the highest purity and from commercial suppliers. HepG2 cells were purchased from Biomedia Co., Ltd. (BKK, Thailand) and used with the permission of The Research Ethics Committee.

2. Preparation of human liver microsome

Pooled Human liver microsome (PHLM) from Sixth French autopsy samples and the pooled human liver microsomal (HMMCPL) were purchased form GIBCO Ltd. (BKK, Thailand) (Table 9) were used with permission of the Local Research Ethics Committee. Human liver microsome and pool human microsome were prepared as below. Protein content was determined using the bicinchoninic acid method (BCA protein determination) which were shown in the details in this table.

Table 9 Information of human liver microsome and the protein concentration of pool human liver microsome in all experimental

Human liver microsome code	The protein concentration of individual microsome (mg/ml)	The protein concentration of pooled human liver microsome (mg/ml)
L12	42.7	
S79	35.5	
S12	39.1	39.78
AL8	46.4	(Lot No. K001)
L22	38.2	
S68	36.8	
The pooled human liver microsomal (HMMCPL)	20.00	20 mg/ml (Lot No. PL050E-C) GIBCO Ltd. (BKK, Thailand)

Table 10 Standardized substrate, products, and selective inhibitors

CYP isoform	Selective substrate oxidation reaction, substrate concentration	Substrate	Authentic metabolite standards	Selective Inhibitor/Inducer	Analytical method	Reference
1A2	7-Ethoxyresorufin deethylation,1 µM	7-Ethoxyresorufin (ER)	Resorufin	α- naphthoflavone/ Rifampin	Microplate reader λ_{ext}=530 nm, λ_{emi}=580 nm	Burke et al.(81)
2B6	Bupropion hydroxylation, 50 µM	Bupropion	Hydroxyupropion	Proadifen/ Phenytoin	HPLC-UV with 210 nm	Faucette et al.(82)
2C9	Tolbutamide hydroxylation, 50 µM	Tolbutamide	4-Hydroxy tolbutamide	Sulfaphenazole/ Rifampin	HPLC-UV with 230 nm	Relling et al.(83)
2C19	S-Mephenytoin 4'hydroxylation, 10 µM	S-Mephenytoin	4-Hydroxy mephenytoin	Ticlopidine/-	HPLC-UV with 204 nm	Lasker et al.(84, 85)
2D6	1'-Bufuralol hydroxylation, 1 µM	Bufuralol	1'-Hydroxy bufuralol	Quinidine/-	HPLC-FLD Fluorescence λ_{ext}=252 nm, λ_{emi}=302 nm	Kernset et al.(86)
2E1	p-nitrophenol hydroxylation, 100 µM	p-nitrophenol	4-nitrocatechol	Disulfiram/ Ethanol	Microplate reader Absorbance=530 nm	Thomas et al.(87)
3A4	testosterone 6β-hydroxylation, 100 µM	Testosterone	6β-hydroxy testosterone	Ketoconazole/ Dexamethasone	HPLC-UV with 254 nm	Mc Killop et al. (88)

3. Preparation of *B. monnieri* extract

B. monnieri extracts prepared by the spray-dried method that received courtesy of Natural Products Research Group Research and Development Institute Government Pharmaceutical Organization (GPOs). The extract powder, containing the amount of total saponin approximately is 18% (by weight), which is a mixture of bacoside A_3, bacopaside II, bacopasoponin X, bacopasaponin C and bacopaside I. Five saponin standards are bacoside A_3, bacopasaponin C, bacopasides I, II and X were purchased from Natural Remedies, Bangalore, India (purity > 96% by HPLC).

Determination of activities in the *B. monnieri* extracts and compound according to the method of Nuengchamnong et al. (89) to selective the concentration of the standard substance for the ability test effect of inhibiting or inducing comprehensively. Whilst it corresponded with the amount in the extract and its constituent standardization should be collected at a temperature of -20ºC in the light-resistant bottle, until used for experimentation.

Table 11 Chemical Properties of five active compound of *B. monnieri*

Compounds	Molecular Weight (< 450)	Log P (< 5)	H-bond donor (< 3)	H-bond acceptor (< 7)	No. of rotatable bonds (< 8)
Bacopasaponin C	899.07	3.72	9	17	9
Bacopaside X	899.07	3.54	9	17	9
Bacopaside II	929.10	3.48	10	18	10
Bacoside A_3	929.10	3.30	10	18	10
Bacopaside I	979.10	1.10	9	20	11

4. Cytochrome P-450 monooxygenase activities

Determination of CYP1A2, 3A4, 2B6, 2C9, 2C19, 2D6, and 2E1 activity of the human liver microsome was described below. The metabolite, substrate, and internal standard were detected using HPLC-UV and fluorescence as shown in Table 10.

4.1 CYP1A2-dependent ethoxyresorufin-O-deethylase assay

Microsomal ethoxyresorufin-O-deethylation (EROD) was determined based on a previous study conducted by Burke et al. Briefly, human liver microsomes (0.04 mg protein) were incubated for 2 min at 37^{o}C with the substrate which is 7-ethoxyresorufin (6.5 μM) in a final volume of 100 μL. Initially, adding NADPH (10 mM) was used to activate the reaction and then $ZnSO_4$ (87 mM) and $Ba(OH)_2$ (79 mM) were used to terminate activate the reaction. The precipitated protein was removed by centrifugation (800 g: 10 min). The fluorescent metabolite resorufin was measured at excitation 530 nm and emission 580 nm. Results were expressed as pmol resorufin formed/min/mg microsomal proteins (81).

4.2 CYP2B6-depentdent bupropion hydroxylase assay

Bupropion hydroxylation (BuOH) was measured based on a previous study conducted by Faucette et al. Briefly, human liver microsome (0.2 mg protein) were incubated with bupropion (0.5 mM) and NADPH (1 mM) for 30 min at 37^{o}C. The reaction was stopped by adding ice-cold acetonitrile and placing samples on ice. After centrifugation, The HPLC-UV spectrophotometric at 210 nm was used to analyze the supernatants. The internal standard curve of hydroxybupropion was used as quantification. Results were expressed as pmol hydroxybupropion formed/min/mg microsomal proteins (82).

4.3 CYP2C9-dependent tolbutamide hydroxylase assay

Tolbutamide hydroxylation (TolOH) was measured based on a method conducted by Relling et al. Briefly, human liver microsome (0.1 mg protein) were incubated with tolbutamide (0.25 mM) and NADPH (1 mM) for 45 min at 37^{o}C. The reaction was stopped by adding ice-cold acetonitrile and storing samples on ice. The HPLC-UV spectrophotometric at 230 nm was used to analyze the supernatants. The internal standard curve of 4-hydroxytolbutamide was used as quantification. Results were expressed as pmol 4-hydroxytolbutamide formed/min/mg microsomal proteins (83).

4.4 CYP2C19-dependent S-Mephenytoin hydroxylase assay

Hydroxylation of S-Mephenytoin was measured based on a method conducted by Lasker et al. Briefly, human liver microsome (0.2 mg protein) were incubated with S-Mephenytoin (0.2 mM) and NADPH (1 mM) for 30 min at 37^{o}C. The

reaction was stopped by adding ice-cold acetonitrile and storing samples on ice until use. After centrifugation, the metabolite was analyzed by HPLC-UV spectrophotometric at 211 nm. The internal standard which is hydroxy-mephenytoin was used to conduct a standard curve for quantification. Results were expressed as pmol hydroxy-mephenytoin formed/min/mg microsomal proteins (84, 85).

4.5 CYP2D6-dependent bulfuralol hydroxylase assay

Hydroxylation of bufuralol was measured based on a method described following. Briefly, human liver microsome (0.1 mg protein) were incubated with bulfuralol (0.01 mM) and NADPH (0.5 mM) for 30 min at 37ºC. The reaction was stopped by adding 25 μL of 70% perchloric acid and places the samples on ice. The precipitated protein was removed by centrifugation. The HPLC-fluorescent metabolite 1-hydroxybufuralol was measured at excitation 252 nm and emission 302 nm. Results were expressed as pmol 1-hydroxybufuralol formed/min/mg microsomal proteins. (86).

4.6 CYP2E1-dependent *p*-nitrophenol hydroxylation assay

Paranitrophenol hydroxylation (PNP-OH) was measured based on a method described by Thomas et al. Briefly, human liver microsome (0.25 mg protein) were incubated with *p*-nitrophenol (0.2 mM) and NADPH (0.8 mM) at 37 ºC. The reaction was stopped with 50% trichloroacetic acid and places the samples on ice. After centrifugation and adding of NaOH (10M, 20μL) to the supernatant, the HPLC-UV with 535 nm was used as detection. Results were expressed as pmol 4-nitrocatheol formed/min/mg microsomal proteins (87).

4.7 CYP3A4-dependent testosterone hydroxylation assay

Testosterone 6β-hydroxylation (6β-Testo-OH) was measured based on a previous study conducted by McKillop et al. Briefly, human liver microsome (0.20 mg protein) with testosterone (0.25 mM) and NADPH (1 mM) for 30 min at 37ºC in a final volume of 250 μL. The ice-cold acetonitrile was added to stop the reaction and the samples were stored on ice. After centrifugation, The HPLC-UV spectrophotometric at 230 nm was used to analyze the supernatants. Results were expressed as pmol 6β-hydroxytestosterone formed/min/mg microsomal proteins (88).

5. IC_{50} determinations

Initially, the appropriate concentrations of ethoxyresorufin, testosterone, bupropion, tolbutamide, mephenytoin, bulfuralol, and nitrophenol were selected to determine the effect of *B. monnieri* extract and its bioactive compounds on the high capacity/low-affinity components (final concentrations of *B. monnieri* extract in the incubation mixture were 10, 20, 50, 100, 200, 500, 1000 μg/L and 5, 10, 12.5, 25 μM for its bioactivity compounds) of metabolism for each substrate. The compounds α-naphthoflavone, proadifen, sulfaphenazole, ticlopidine, quinidine, disulfiram, and ketoconazole were used as selective inhibitors of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 activity, respectively. The IC_{50} values for inhibitors, *B. monnieri* extract and its bioactive compounds were determined graphically by nonlinear regression analysis of the plot the logarithm of inhibitor concentration versus the percentage of remaining activity using GrapPadPrism8 (GraphPad Co.Ltd., USA).

6. Determination of enzyme kinetics

In order to determine the apparent K_i values and mode of inhibition of *B. monnieri* extract and its bioactive compounds, 4 to 5 concentrations of the specific substrate corresponding to $K_m/4$, $K_m/2$, K_m, 2 x K_m and 4 x K_m were incubated with a range of inhibitor concentration in a presence of human liver microsome as described from IC_{50} determination section. In addition, one of five bioactive compounds, which exhibited the strongest inhibition on CYPs enzyme activities, was determined the apparent Ki values and mode of inhibition.

All incubations were carried out in triplicate and the average was presented as the result; the variation was usually less than 10%. The kinetic parameters such as V_{max} and K_m were estimated from the best-fit least-squares linear regression of a double reciprocal plot of velocity versus substrate concentration as Lineweaver–Burk plots. The enzyme inhibition models and Lineweaver–Burk plots were used to estimate the mechanism of inhibition. The K_i values were calculated *via* the second plot of the slopes from Lineweaver–Burk plots versus inhibitor concentrations.

7. Human hepatocyte isolation, culture, and treatment

HepG2 cells, which were purchased from JCRB Cell Bank (Japan)., were cultured based on a previous study by Phokrai et al. (90). Initially, the 75% of cell viability or more with a density equal to 0.4 x 10^6 and 0.2 x 10^6 cells/well of HepG2 cells were added to 12 and 24-well plates for determination activities and CYP mRNA expression, respectively. In order to determine CYP activity, the 12 and 24-well bio-coat and multi-well culture plates with the density were determined as 0.4 x 10^6 and 0.2 x 10^6 cells/well was incubated in DMEM low glucose (GIBCO, cat. no 11885084) which contained 10%FCS, 1% Penicillin/Streptomycin at 37 °C and 5% CO_2 for 24 h. After that the medium was changed, the systems will be treated with the reference inducers, which included 25 µM β-naphthoflavone (βNF), and 500 µM Phenobarbital (PB) or 10 µM Rifampicin (RIF), *Bacopa monnieri* extract (BME), Bacoside A_3, Bacopaside I, Bacopaside II, Bacopaside X and Bacopasaponin C for 72 h. The medium includes the treatment compound will be changed every 24 h. The DMSO 0.1% v/v will be used as a control group.

Cryopreserved Human hepatocytes, which were purchased from KaLy Cell Company. Initially, cryopreserved Human hepatocytes were defrosted from cryotube in suspensions. The 75% of cell viability or more with a density equal to 1.35 x 10^6 of Human hepatocytes, was added to 6 well plates bio-coat collagen type I (GIBCO, Thailand) for determination of CYP mRNA expression and CYP enzyme activity. The human hepatocyte was thawed and seeded in CHRM (Invitrogen, Cat. No CM7000) and CHPM (Invitrogen, Cat. No CM9000), respectively then incubated at 37 °C and 5% CO_2 for 4-6 h. After that the medium was changed to Willium's E Medium (GIBCO, cat. No 32551), which contained ITS are containing insulin 6.25 µg/ml, Transferrin 6.25 µg/ml, and selenium 6.25 ng/ml (GIBCO, cat. No 41400), Dexamethasone 0.1 µM (Sigma D2915) and 1%Pen/Strep (GIBCO, cat. No 15140). Then, the systems will be treated with the reference inducers, which included 25 µM β-Naptholflavone (BNF), and 500 µM Phenobarbital (PB) or 10 µM Rifampicin (RIF), *Bacopa monnieri* extract (BME), bacoside A_3, bacopaside II, bacopaside X, bacopasaponin C and bacopaside I for 48 h. The medium will be changed every 24 h. The DMSO 0.1% v/v will be used as a control group.

8. Cytotoxicity

Initially, the HepG2 cell and primary human hepatocytes cultures equal to 60,000 cells/well and 500,000 cells/well, respectively were cultured in 96-multicoat well-plates. The bacopa extract concentrations were ranged as 0, 0.1, 1, 30, 60, 90, 120, 300 and 600 µg/ml for HepG2 cell. The bioactive compounds concentrations were ranged as 0, 0.01, 0.1, 1, 2.5, and 5 µM to measure cellular viability using MTT assay (91). In order to confirm the cellular viability in primary human hepatocytes, the bacopa extract was ranged as 0, 0.5, 5.0, 50.0, 500, and 5000 µg/ml (equivalent to 0-100 µM of bacopaside I). The percentage of cell viability was observed at 24, 48, and 72 h.

9. Induction effect of *B. monnieri* extract and its constituents on CYPs

HepG2-cells (24-well plates) and Human hepatocyte cells (6-well plates) were treated with 3 concentrations (non-cytotoxic concentration) reference compound or *B. monnieri* extract for 72 h (HepG2-cells) and for 48 h (human hepatocyte cells), was incubated with the substrates of each CYPs at 37°C 5% CO_2 and 95% air. The concentrations of substrates are phenacetin (30µM, CYP1A2) bupropion (50 µM, CYP2B6), tolbutamide on HepG2-cells (10 µM, CYP2C9) and diclofenac on human hepatocyte (25µM, CYP2C9), S-Mephenytoin (10 µM, CYP2C19), Bufuralol (1 µM, CYP2D6) and midazolam (2µM, CYP3A4). The biotransformation was terminated by adding 1:1 of 1% formic acid with acetonitrile. The samples were centrifuged at 12,000 rpm for 10 min, and then the resulting supernatant was aliquot as 20-100 µL to further analysed by LC-ESI-QTOF-MS/MS.

10. LC-ESI-QTOF-MS/MS condition

In brief, the P450 isoform-specific metabolite was analyzed using LC-ESI-QTOF-MS/MS analysis followed by a previous study by Nitra et al. (89). An Agilent 6540B Q-TOF-MS spectrometer (Agilent Technologies, Singapore) coupled with an Agilent 1260 Infinity Series HPLC system (Agilent, Waldbronn, Germany) was used to perform in this study. The HPLC was coupled to an electrospray ionization (ESI) source and proprietary Agilent dual nebulizer. The injection volume of the sampler and standards were adjusted to 20 µL. The 0.1% formic acid in water v/v (A) and 50% Methanol in acetonitrile v/v (B) was used as the mobile phase. It was employed in the

gradient mode with 95% solvent A for 0-10 minutes, after that change solvent composition to 95% B at 10-13 minutes, then stop time and post-run for 5 min. The ZORBAX Eclipse Plus C-18 column (4.6 mm x 100 mm, 3.5 μm) was purchased from Agilent Technologies, USA to separate compounds. The operating parameters in MS detection were described as follows: drying gas (N2) flow rate, 10.0 L/min; drying gas temperature, 350 °C; nebulizer pressure, 30 psig; capillary, 3500 V; skimmer, 65 V; octapole RFV, 750 V; and fragmentor voltage, 250 V in negative mode and 100 V in positive mode. The mass was set at m/z 100-1200 with a 250 ms/spectrum. Agilent LCMS-QTOF Mass Hunter Data Acquisition Software Version B.05.01 and Agilent Mass Hunter Qualitative Analysis Software B 06.0 (Agilent Technologies, USA) were used to collecting all of the acquisition and analysis of the data respectively. Each sample has identified the structure using in both positive and negative mode including targeted MS/MS analysis. In this study, a dual-nebulizer ESI source (calibrant solution A, Agilent Technologies, USA) was used to provide accurate mass measurements (error less than 5 ppm for analysis).

11. CYPs isoform mRNA expression

HepG2 cells (12-well plates) and Primary human hepatocyte cells (6-multiwell plate), treated with non-cytotoxic concentration from recommendation dose 300 or 600 mg/day, calculated by full adsorption 100% following BME 60-120 μg/mL and five bioactive compounds do not exceed than 2.5μM) reference compound is 10 μM rifampicin, 25 μM β-naptholflavone, 500 μM phenobarbital incubated for 48 h, then remove out supernatant for CYP activities analysis. It was kept by washing out the cells with PBS 1.5 mL two times before the mRNA extraction process. The total mRNA obtained from HepG2-cells and hepatocytes cells was extracted using TriZol® reagent (Invitrogen, France). The RNA was measured mRNA concentration using NANODROP 2000/2000C spectrometer. The cDNA was synthesized by T100 Thermal Cycler (Biorad, France) The volume begins from 0.5-1 μg of total RNA using iScript kit from Biorad company (France) at 45 °C for 40 min. Then the solutions were diluted RT reaction (1:10) as 10 μL will be used for real-time PCR amplification using SYBR Green kit and denaturation step software at 95°C for 3 min with 40 cycles of PCR (denaturation, 95°C, 30 secs; gradient for annealing temperature, 70-55°C, 1 min) and

one cycle at 55 °C for 1 min. The measuring of PCR product infusion step using human sense and reverse primers are described as follows Table 12. These results will be shown as fold change and compared to the control group (i.e., DMSO treated cultures). The over 2-fold change will be the ability as CYPs inducing or repressing (> 2-fold mRNA expression).

Table 12 RNA sequencing of CYPS and Actin for Identification

CYPs	**RNA sequencing**
CYP1A2	5’-GGGCACTTCGACCCTTACAA-3’and
	5’-GCACATGGCACCAATGACG- 3’
CYP2B6	5’-AGGGCCCCTTGGATTTCCG-3’ and
	5’-GGCCATACGGGAGGCCCTTG - 3’
CYP2C9	5’-CCTATCATTGATTACTTCCCG- 3’ and
	5’-AACTGCAGTGTTTTCCAAGC-3’
CYP3A4	5’-CACAAACCGGAGGCCTTTTG-3’ and
	5’-ATCCATGCTGTAGGCCCCAA- 3’
Actin	5’-GGGCACGAAGGCTCATCATT- 3’ and
	5’-AGTCGGTTGGAGCGAGCATC-3’

12. Statistics

The one-way analysis of variance and the Dunnett multiple range tests were used to assess the significance of the difference between the groups. The level of significance was set at $P < 0.05$.

CHAPTER IV

RESULTS

This section was divided into three main parts including 1) the cytotoxicity of the test compounds on the human liver cell line, 2) the inhibition effect of the test compounds on human liver microsome, and 3) the induction effect of the test compounds on both human liver cell line and normal cell. The insight information is described below. The percentage of chemical constituents in the standardized extracts, purchased from GPO (BKK. Thailand), was analyzed using the HPLC-UV followed by Limpeanchob et al. (8) and Saesong et al. (6) methods. Briefly, the bacopa extract peak was separated by comparison with each purified compound using a mobile phase consisted of 0.2% phosphoric acid and acetonitrile (65:35 v/v) adjusted pH equal 3.0 with 6 M NaOH. The flow rate was 1.0 ml/min by the total run time was 30 min. All peaks were integrated at the wavelength of 205 nm. Quantitative analysis was compared with the standard purified compound were known certainly concentration. All data were processed using LC solution software (Shimadzu, Japan). The quantities confirmation results were summarized in table 13.

Table 13 The amount of saponin glycosides in 100 mg Brahmi extract

Saponin glycosides	**Percent yields (%W/W)**
Bacoside A_3	2.117
Bacopaside I	6.775
Bacopaside II	4.421
Bacopaside X	2.581
Bacopasaponin C	2.268
Total	18.16

1. Cytotoxicity

The cytotoxicity of *B. monnieri* extract, as well as its five bioactive compounds, were evaluated on cell lines using MTT assay. The solvent effect from the 0.1-0.5% DMSO did not occur because the cell viability was equal to or higher than 75%. Using the HepG2 cells, bacopasaponin C showed the most cytotoxic to the cells with CC_{50} value of 2.327 µM following bacopaside II, bacopaside I, bacopaside X, bacoside A_3, and *B. monnieri* extract, respectively. Whilst the CC_{50} value of a control group (DMSO) was 2.817 µM. Using the primary human hepatocyte at 24, 48, and 72 h., the bacopa extract demonstrated the highest CC_{50} value. The CC_{50} values of *B. monnieri* extract and bioactive compounds on HepG2 cells and human hepatocytes were summarized in Table 13. The sigmoidal curves which represent the cytotoxicity of *B. monnieri* extract in cell line and normal cell were demonstrated in Figure 6-7. Although the CC_{50} value of each test compound showed extremely low, it did not produce any cytotoxicity on both human liver cell lines and human hepatocytes. Since the percentage of cell viability is greater than 75% at high concentration. The bar graphs showing the concentration of *B. monnieri* extract and bioactive compounds versus the percentage of cell viability were showed in Figure 5 and 7. This result suggested that consumption of 600 mg/day *B. monnieri* extract (dose/human plasma volume) might not produce hepatotoxic.

Table 14 The haft of cytotoxicity concentration test in HepG2-cells and primary human hepatocyte cells

CC_{50} (µM)	**BME**	**Ba A_3**	**Ba I**	**Ba II**	**Ba C**	**Ba X**
HepG2 Cells	788.80	45.02	254.80	369.7	50.89	63.12
Human hepatocyte	>1000	-	-	-	-	-

BME is *B. monnieri* extract, Ba A_3 is Bacoside A_3, Ba I is Bacopaside I, Ba II is Bacopaside II, Ba C is Bacopasaponin C, Ba X is Bacopaside X

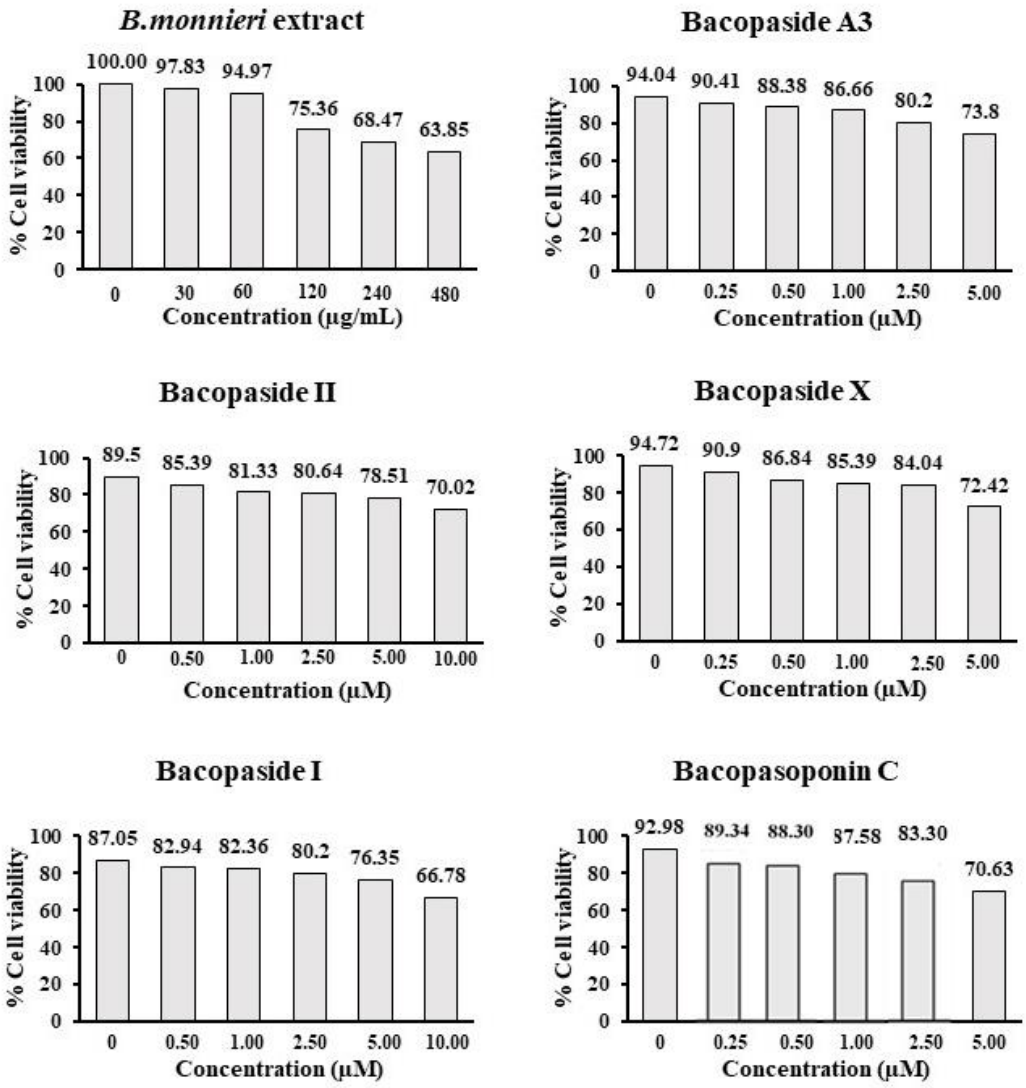

Figure 11 Illustrate the percentage of cell viability versus concentration of the *B. monnieri* extract and its five bioactive compounds in HepG2-cells

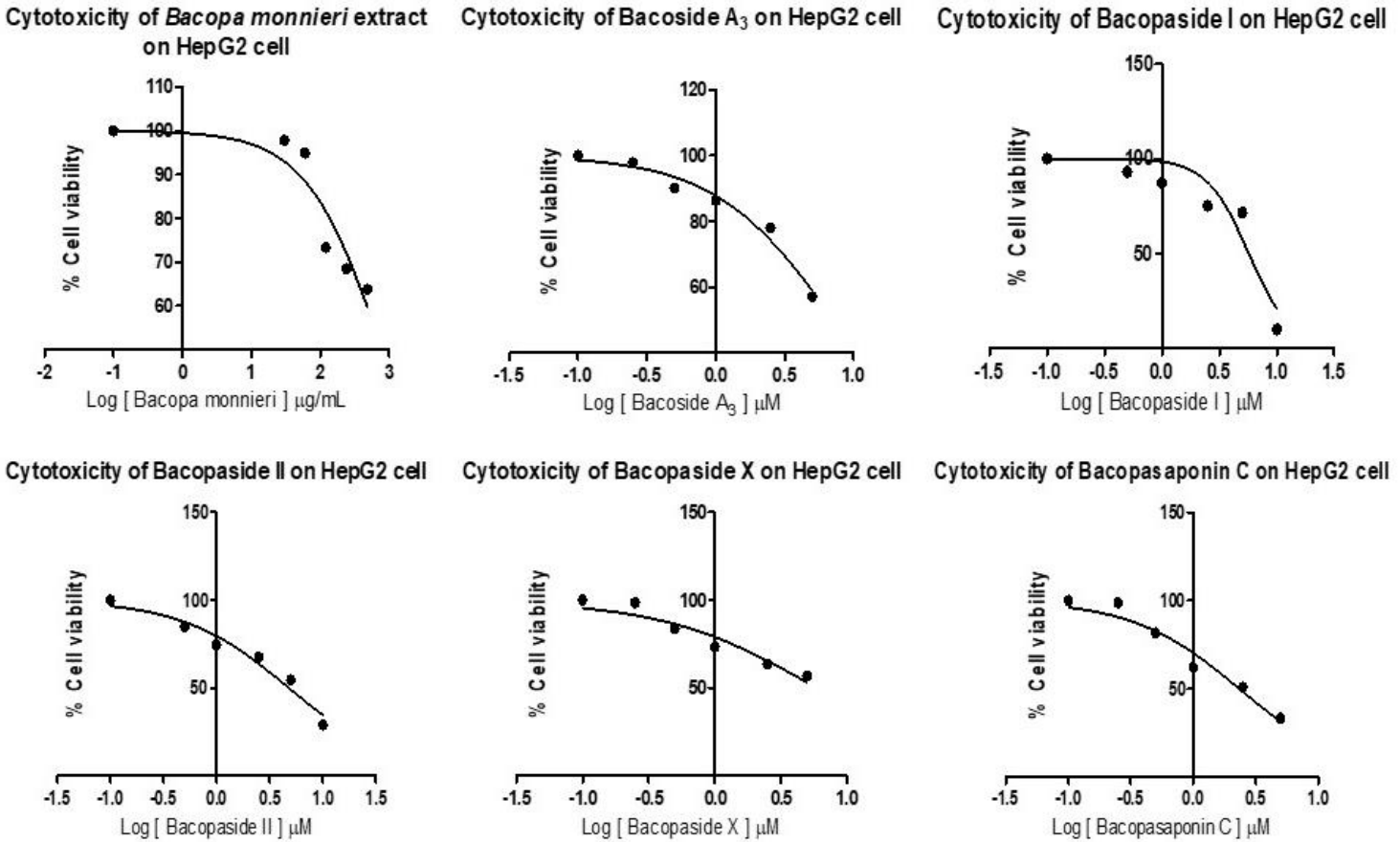

Figure 12 The sigmoidal curves which represent the cytotoxicity of the bacopa extract and five bioactive compounds on HepG2 cell

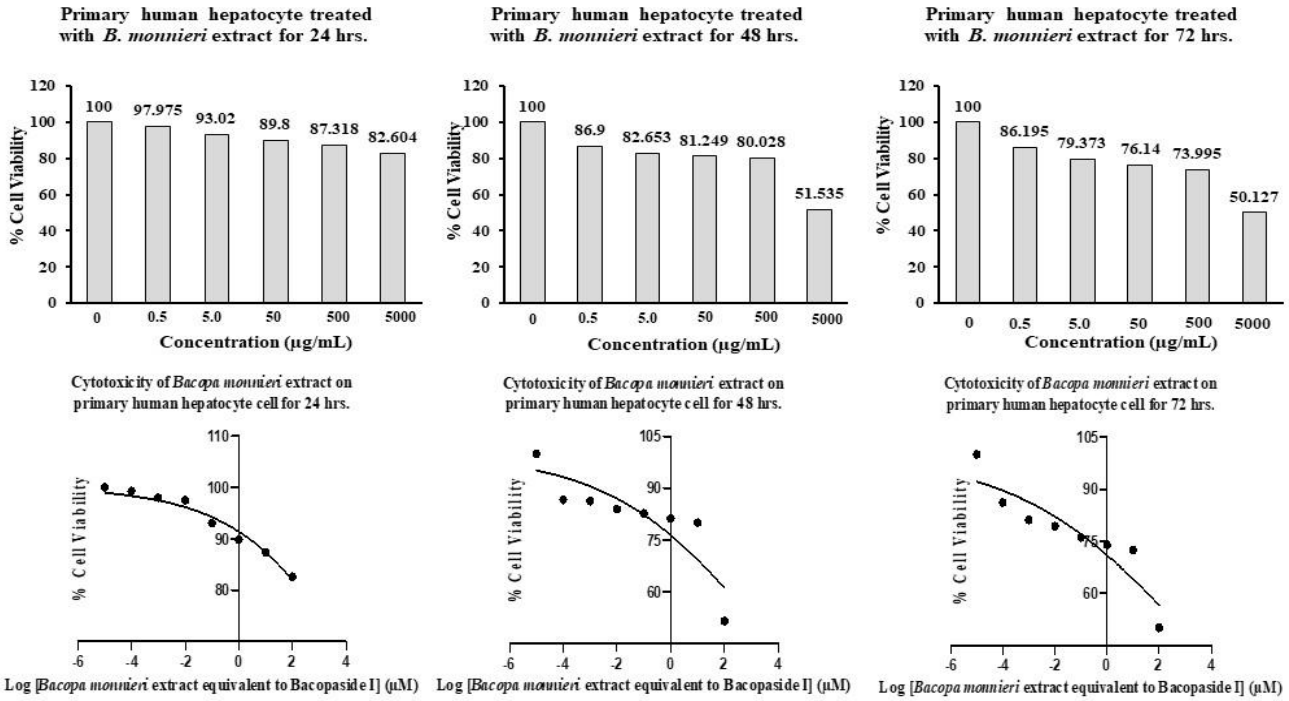

Figure 13 Illustrate the percentage of cell viability versus concentration of *B. monnieri* extract in human hepatocytes and the half of cytotoxicity concentration graph (CC_{50}) of *Bacopa monnieri* extract on primary human hepatocyte cells for 24,48, and 72 h respectively

2. The inhibition effect of the test compounds on human liver microsome

This sub-section provided information regarding the inhibition potential of the selective inhibitors on the human liver microsome, and the determination of the Apparent Half-Maximal Inhibitory Concentration (IC_{50}) for the test samples and standard inhibitors. Also, the inhibition constant (Ki) values and the modes of inhibition of *B. monnieri* extract were further determined with this sub-section.

2.1 Inhibition of human CYPs by selective inhibitors

Compounds known as selective inhibitors of CYP1A2, 2B6,2C9, 2C19, 2D6, 2E1, and 3A activities were used to confirm the selectivity of our assays. In this experiment, the selective inhibitors caused the greatest reduction in CYP-dependent activity with an IC_{50} as 0.3407, 4.445, 1.439, 4.143,1.705, 0.2538, and 4.878 μM of α-Naphthoflavone, Sertraline, Sulfaphenazole, Ticlopidine, Quinidine, Disulfiram, and Ketoconazole, respectively. In accordance with the report of Ramasamy et al. (2014) reported that the IC_{50} are 0.78, 0.03, 7.81, 0.02, and 0.32 μg/mL of α-Naphthoflavone, Sulfaphenazole, Ticlopidine, Quinidine, and Ketoconazole using recombinant CYPs respectively (17). These results confirmed the differences between the generated kinetic parameters acquired from the human liver microsome and recombinant CYPs. The summary of IC_{50} values of the selective inhibitors acquired from this experiment was shown in Table 14.

2.2 The determination of the IC_{50} values

The potential inhibitory action of bacopa extract and its constituents on various CYP isoforms was investigated using pooled liver microsome. According to Ramasamy et al. (17), the inhibitory effect towards CYPs in each isoform was shown in Figure 8. The Inhibition potency of a test compound can be classified according to its IC_{50} values, as potent if $IC_{50} \leq 20$ μg/mL or ≤10 μM, moderate if IC_{50} 20–100 μg/mL or 10–50 μM, or weak if $IC_{50} \geq 100$ μg/mL or ≥50 μM. The results showed that *B. monnieri* extract was a strong inhibitor of CYP2C19 with an IC_{50} of 17.68 μg/mL. Whilst *B. monnieri* extract moderately inhibited CYP1A2, CYP2B6, and CYP2C9 with IC_{50} of 75.98, 59.70, and 47.72 μg/mL, respectively. Bacopaside I showed moderate inhibit CYP2B6 and CYPC9 activity while it weak inhibit other CYP isoform activity. Another four bioactive compounds showed weak inhibit CYP450 activities with IC_{50} values larger than 100 μM.

Table 15 IC_{50} value of *B. monnieri* extract, its constituents, and selective inhibitors on P450 specific model activities

Test compounds	IC_{50} for CYP450 enzyme inhibition assay (µg/mL)						
	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
B. monnieri extract	75.9±5.8	59.7±4.7	47.7±8.3	17.7±1.5	126.0 ±25.6	113.6±12.2	101.4±23.1
bacoside A_3	>100	>100	>100	>100	>100	>100	>100
bacopaside I	>100	>50	>50	>100	>100	>100	>100
bacopasside II	>100	>100	>100	>100	>100	>100	>100
bacopaside X	>100	>100	>100	>100	>100	>100	>100
bacopasaponin C	>100	>100	>100	>100	>100	>100	>100
Positive control	0.1	0.9	0.5	1.1	0.6	0.1	2.6
(Compound)	α-Naptholflavone	Sertraline	Sulfaphenazole	Ticlopedine	Quinidine	Disulfiram	Ketoconazole

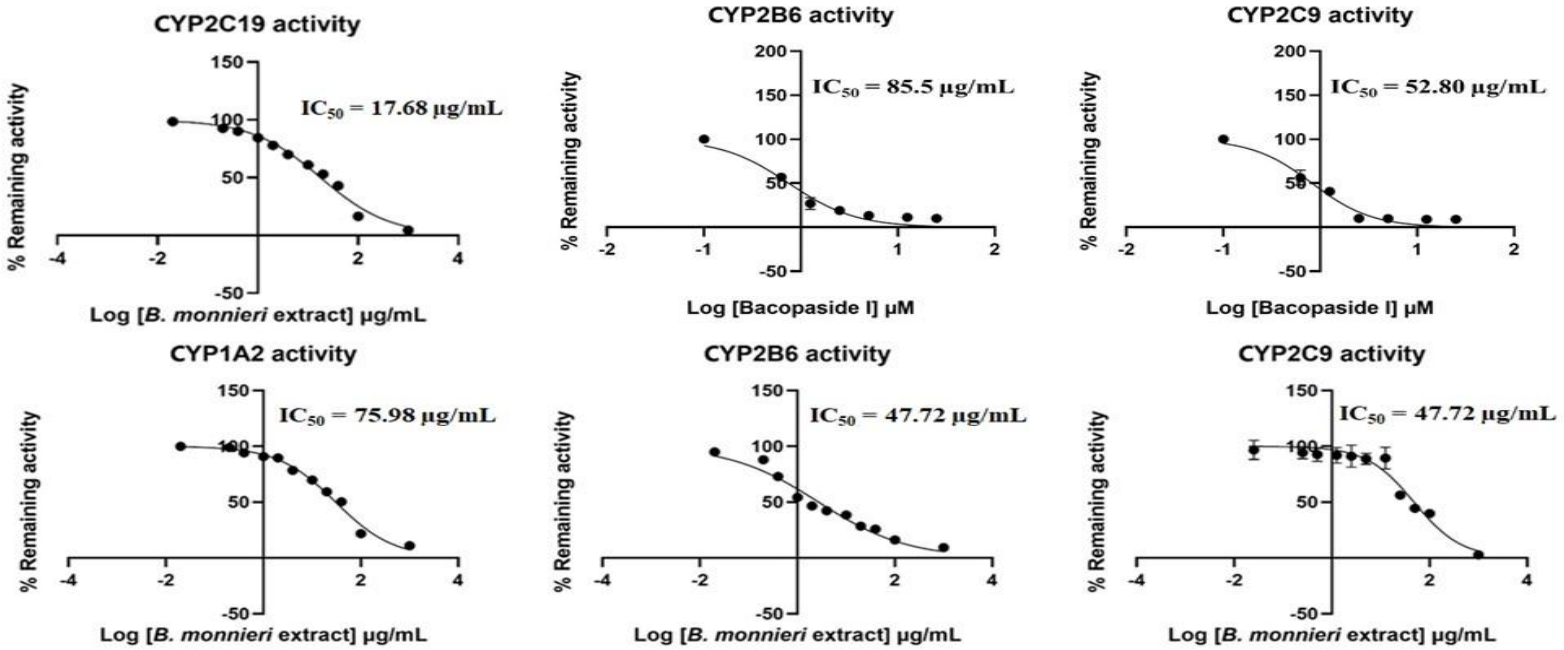

Figure 14 *B. monnieri* extract showed strongest inhibitory effects towards CYP2C19 (IC_{50} = 17.68 µg/mL) followed by CYP1A2 (IC_{50} = 75.98 µg/mL), CYP2B6 (IC_{50} = 59.70 µg/mL), CYP2C9 (IC_{50} = 47.72 µg/mL).Whereas, Bacopaside I showed moderate inhibitory effects towards CYP2B6 (IC_{50} = 85.5 µg/mL) and CYP2C9 (IC_{50} = 52.80 µg/mL), respectively. Each point represent the average of triplicate determination

2.3 Determination of the Ki Values and the modes of inhibition

We further characterized the CYP450 inhibitory properties of *B. monnieri* and its constituent were conducted with multiple of the extract and multiple concentrations of the substrates. Lineweaver–Burk plots for the inhibition of CYPs isoform was conducted to determine the Ki values (Figure 9-11). The binding affinity of the inhibition for CYP1A2, CYP2B6, CYP2C9, and CYP2C19 enzymes were 48.06, 5.16, 19.93, and 3.04 µM, respectively. The possible mode of inhibition for *B. monnieri extract* toward CYP2B6, CYP2C9, and CYP2C19 was mixed-type inhibition while CYP1A2 was competitive inhibition type. These results are expressed as the average of triplicate measurements and showed in Table 15. In addition, bacopaside I was determined the Ki value and the mode of inhibition. The Lineweaver–Burk plots for the inhibition of CYP2B6 was conducted Ki value as shown in Figure 11. There results showed that bacopaside I could competitively inhibit CYP2B6 activity with Ki value of 7.02 µg/mL. Whilst the compound could inhibit CYP2C9 and CYP2C19 activity with Ki value of 13.25 and 25.92 µg/mL, respectively as mixed-type inhibition mode.

Table 16 Inhibition constant (Ki), mode of inhibition, Plasma concentration, and *in vivo* interaction for *B. monnieri* extract and bacopaside I of human CYP enzymes

CYP enzyme	Ki (µg/mL)	Mode of inhibition	Plasma concentration [I] (µg/mL)	Prediction for an *in vivo* interaction [I]/Ki (92)
***B. monnieri* extract**				
CYP1A2	48.06	Competitive	3.6 – 7.2	0.075 – 0.15(Remote)
CYP2B6	5.16	Mixed type		0.69 – 1.39 (Possible)
CYP2C9	19.93	Mixed type		0.18 – 0.36 (Possible)
CYP2C19	3.04	Mixed type		1.18 – 2.36 (Likely)
Bacopaside I				
CYP2B6	7.02	Competitive	0.06-0.13	0.00084 (Remote)
CYP2C9	13.25	Mixed type		0.00044 (Remote)
CYP2C19	25.92	Mixed type		0.00022 (Remote)

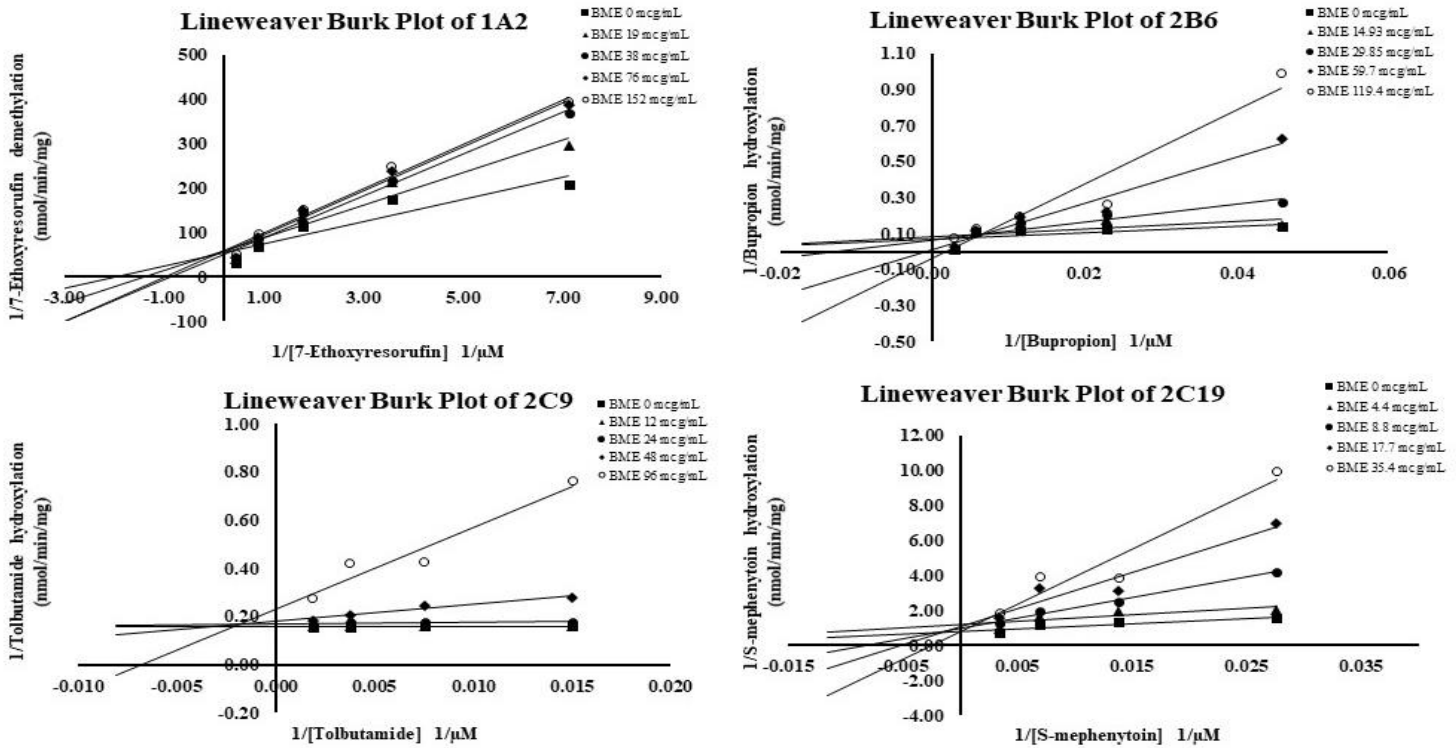

Figure 15 *B. monnieri* extract as moderate inhibited CYP2C19, CYP2C9, CYP2B6, and CYP1A2 as demonstrated by Lineweaver-Burk plots. The mixed type of inhibition of CYP2C19, CYP2C9 and CYP2B6 activity and competitively inhibition of CYP1A2 activity were determined by incubating different concentrations of *B. monnieri* extract with increasing concentrations of substrates. Each point represents the average of triplicate measurements

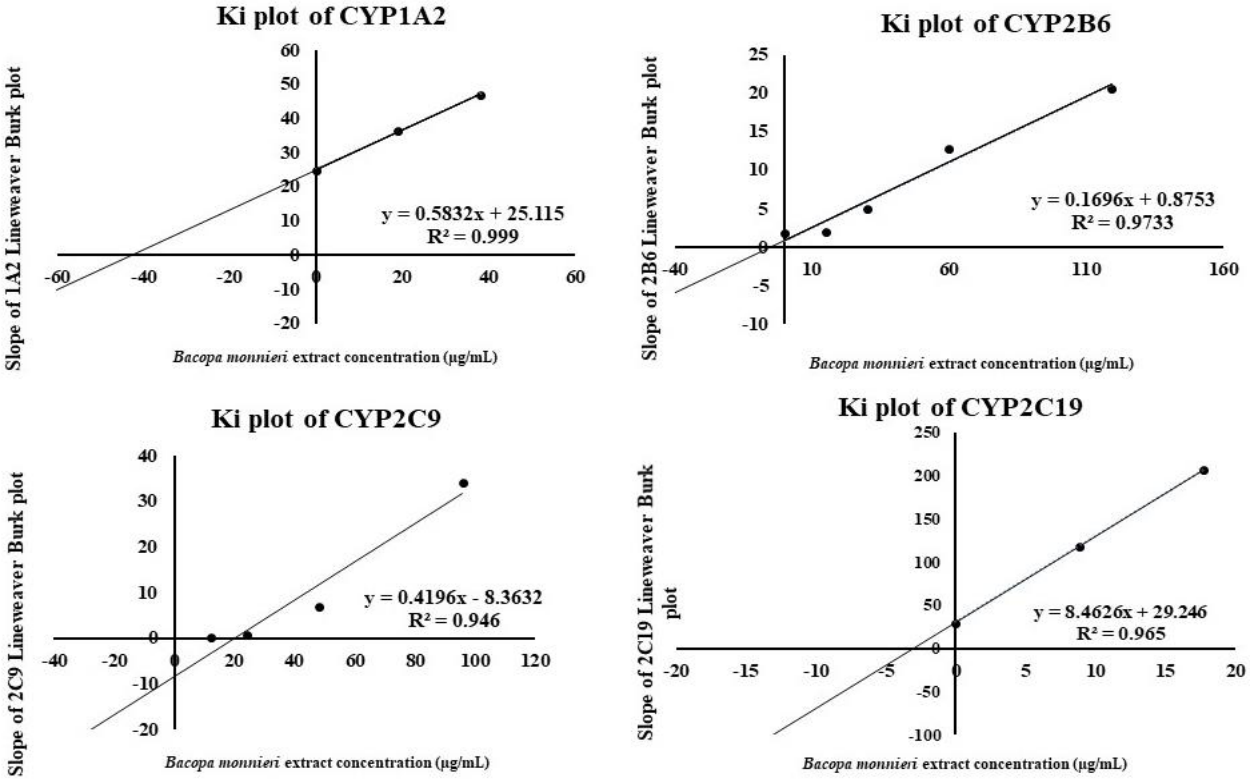

Figure 16 Ki values of *B. monnieri* extract for CYP1A2; CYP2B6; CYP2C9 and CYP2C19. Ki values were determined from secondary plots of CYP activity using the slopes of primary Lineweaver–Burk plot *versus* concentrations of bacopa extract. *B. monnieri* Ki values for CYP1A2, CYP2B6, CYP2C9 and CYP2C19 are of 48.0641, 5.1610, 19.93, and 3.04 µg/mL, respectively. Each point represents the average of triplicate measurements

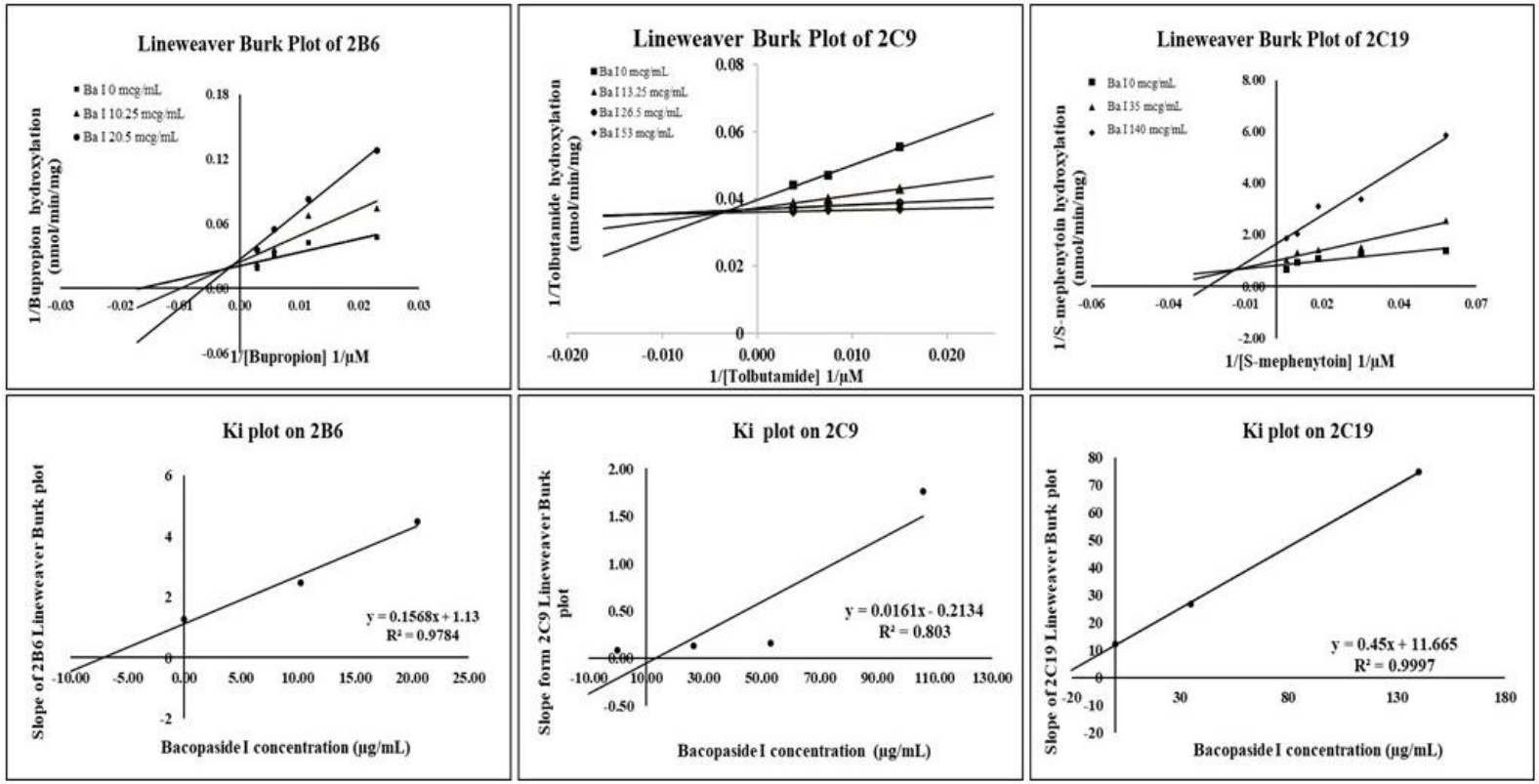

Figure 17 The Lineweaver Burk plot of bacopaside I on CYP2B6, 2C9, and 2C19. Ki values were determined from secondary plots of CYP2B6, 2C9, and 2C19 activity using the slopes of primary Lineweaver–Burk plot versus concentrations of bacopaside I.

3. The induction effect of the test compounds

This sub-section revealed the induction effect of the *B. monnieri* extract and its bioactive compounds on both the human liver cell line (HepG2 cell) and primary human hepatocytes. The enzyme activity of CYP450 was evaluated using LC-ESI-QTOF-MS/MS analysis. Whilst the mRNA expression of isolated CYP450 was investigated using real-time polymerase chain reaction (RT-PCR).

3.1 LC-ESI-QTOF-MS/MS analysis

In vitro induction effect of *B. monnieri* extract and bacopaside I, which assumed as a major bioactive constituent drive several pharmacological effects, were analyzed in HepG2 and primary human hepatocytes cells using LC-ESI-QTOF-MS/MS (positive mode). The metabolites peaks including acetaminophen, hydroxybupropion, 4'-hydroxy tolbutamide on HepG2 cells or 1'-hydroxydiclofenac on human hepatocytes cells, 4'-hydroxy mephenytoin, 1'-hydroxy bufuralol, and 1'-hydroxy midazolam were identified at 157.033, 256.111, 235.110 ,287.175 or 312.0189, 278.1751 and 342.079 *m/z* as demonstrated in Figure 12-13.

As the result, the bacopa extract significantly increased CYP1A2 and 2B6 activity in both human liver cell line and primary cell, whereas the drug-metabolizing enzyme including CYP2C9 and 3A4 in the human liver cell line was increased by the extract. The major bioactive compound derived from the CYP3A4 enzyme activity enhancement in the human liver cell line might be the bacopaside I (Figure 14). Using the primary human hepatocytes, bacoside A_3 significantly enhanced the enzyme activity of CYP1A2 while bacopasaponin C significantly enhanced CYP2B6 activity (Figure 15).

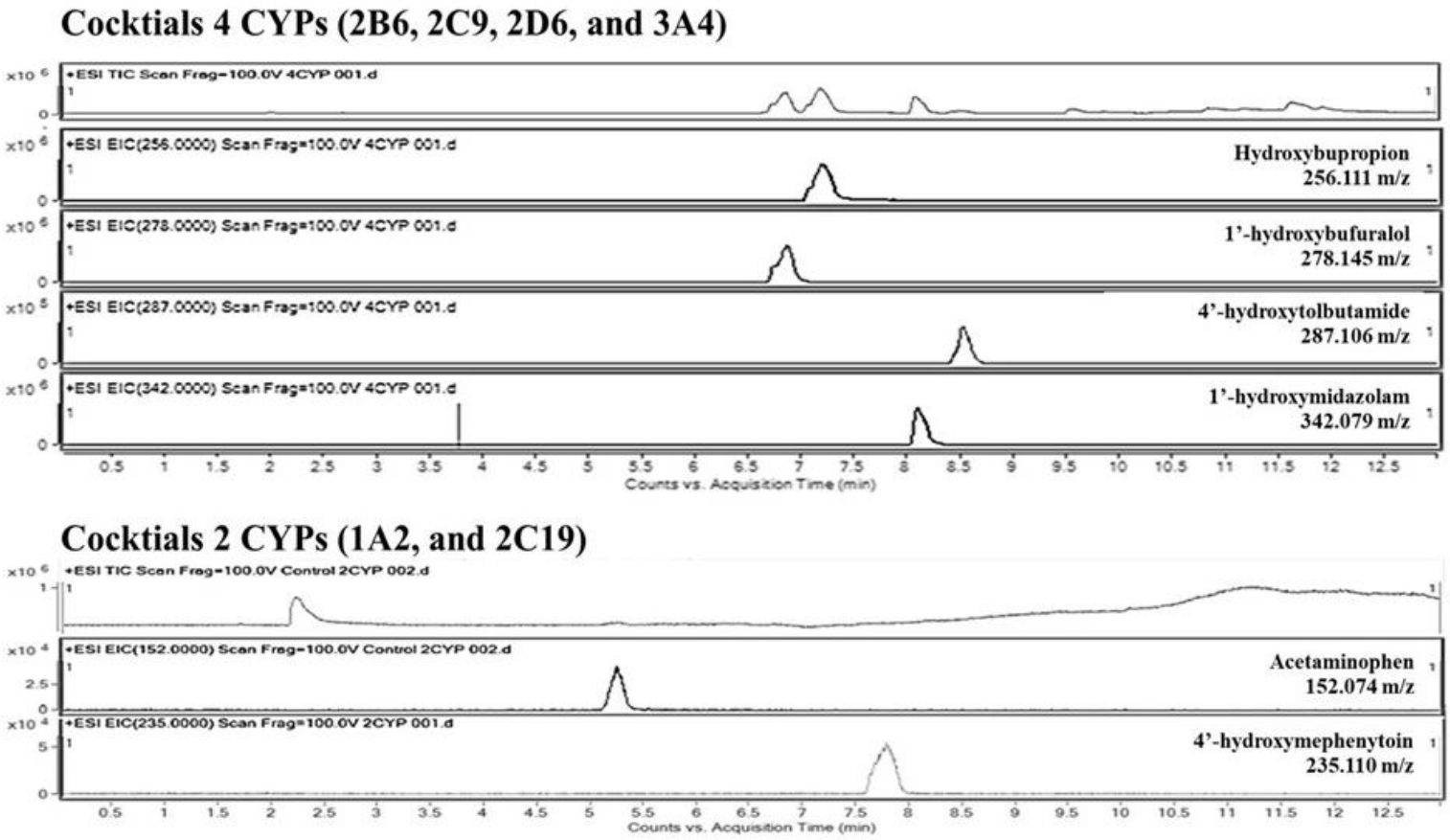

Figure 18 The cocktails substrate were shown the production of the metabolites on HepG2 Cells for incubation adapted following protocol by Kajsa Kanebratt et al. (93)

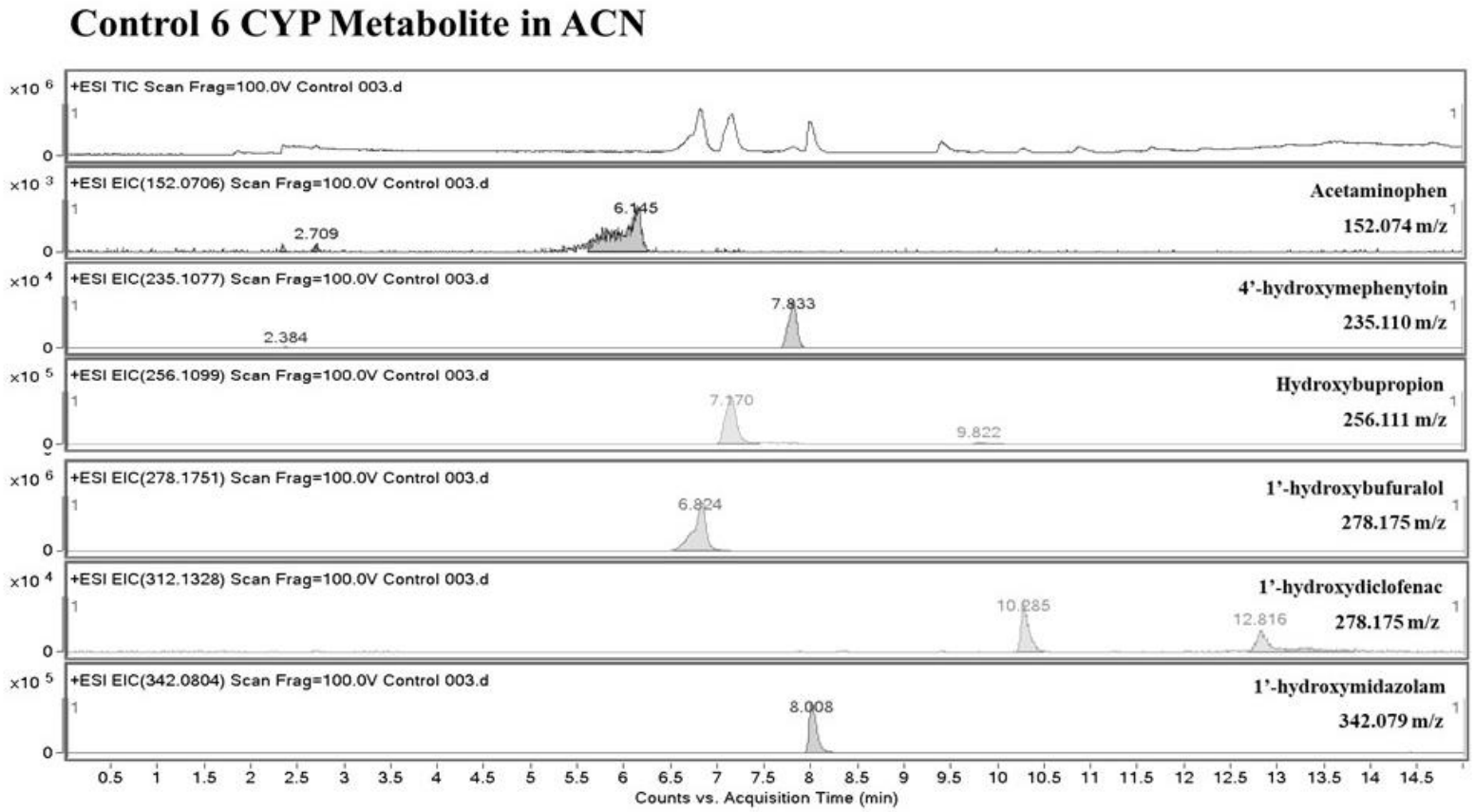

Figure 19 The cocktails substrate were shown the production of the metabolites on Human Hepatocyte Cells for incubation adapted following protocol by Kajsa Kanebratt et al. (2020) (93) and Chen, ZH et al. (2016) (94)

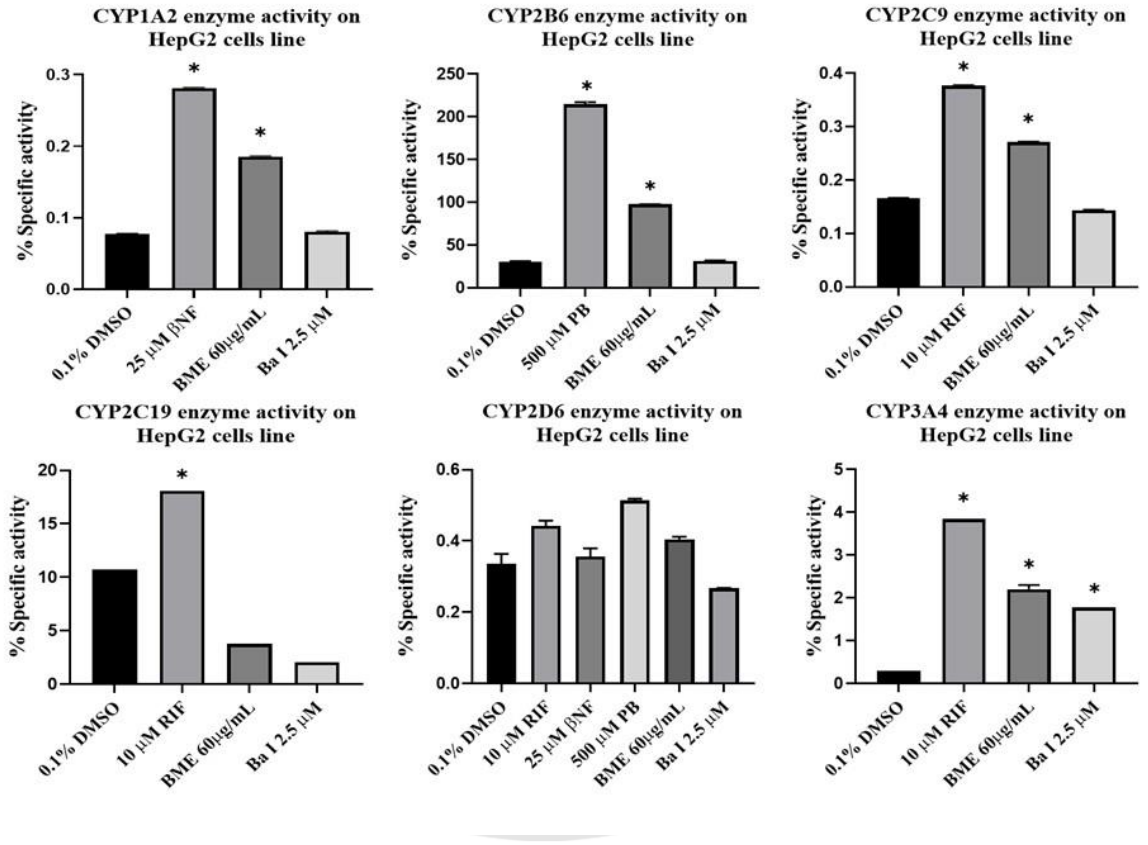

Figure 20 Illustration of the induction effect of HepG2 cells effect on *B. monnieri* extract, bacopaside I, and the positive control on CYPs activities. (*) $p > 0.05$ with respect to control

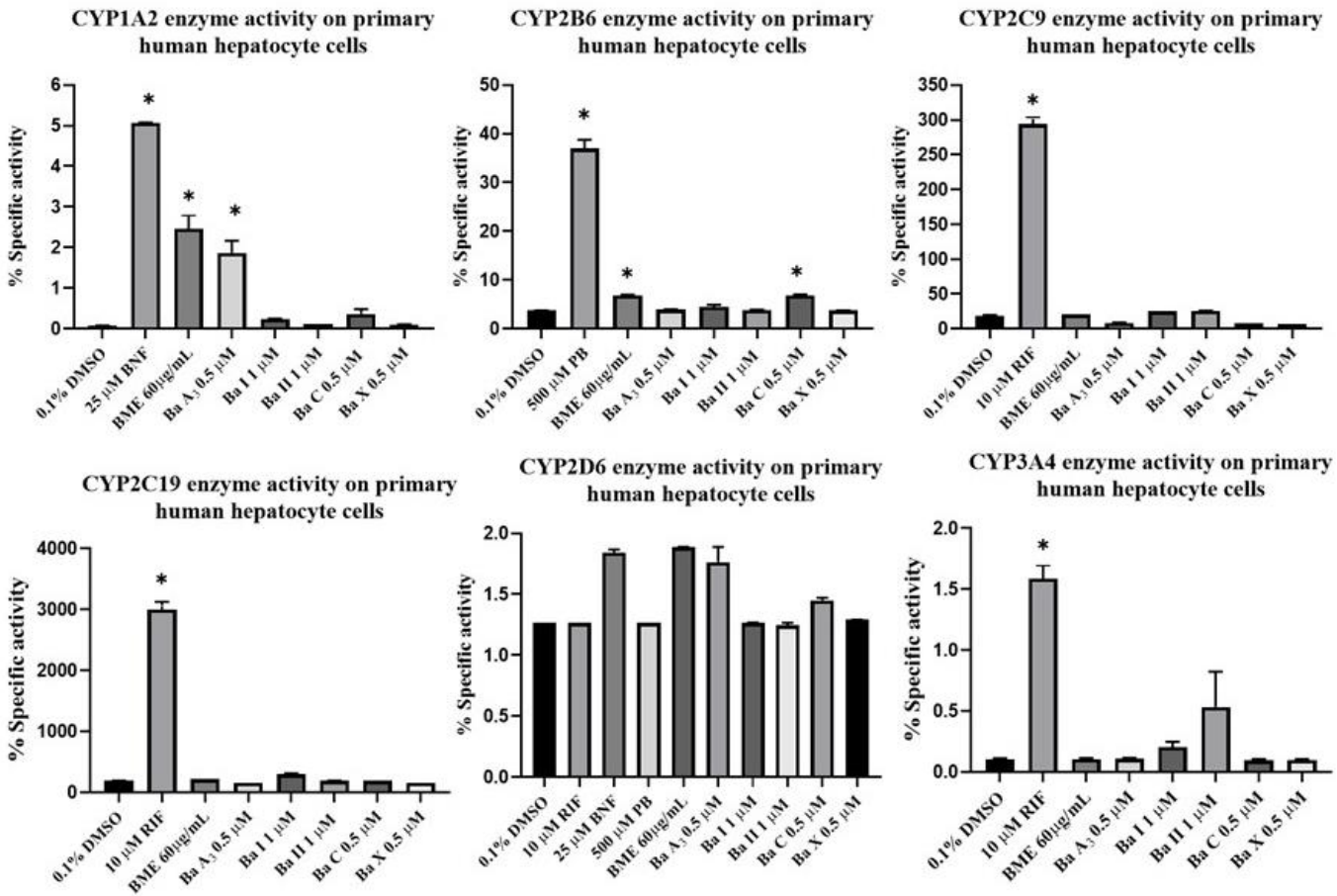

Figure 21 Illustration of the induction effect of primary human hepatocyte effect on *B. monnieri* extract, five bioactive compounds, and the positive control on CYPs activities. (*) $p > 0.05$ with respect to control

3.2 CYPs isoform mRNA expression

To investigate the induction effect of the extract and each bioactive compound in HepG2-cells and primary human hepatocyte, the mRNA expression was determined using RT-PCR. The expression levels of CYP1A2, 2B6, and 3A4 were shown in Figures 16-17.

Using HepG2 cell model, at 60 and 120 µg/mL *B. monnieri* extract showed 8-fold and 10-fold increasing of CYP1A2 mRNA expression respectively while a positive control, is BNF, could increase 24-fold mRNA expression. Also, at 60 and 120 µg/mL *B. monnieri* extract showed 2-fold and 7-fold increase of CYP2B6 mRNA expression respectively while a positive control, is PB, showed the highest mRNA expression induction. These results were in line with the mRNA expression using primary human hepatocytes. Despite at 60 µg/mL did not show the induction effect on CYP3A4 mRNA expression in the cell line, at 120 µg/mL, showed an approximately 4-fold increase of CYP3A4 mRNA expression. These results suggested the induction effect of *B. monnieri* extract might be in a concentration-dependent manner. However, *B. monnieri* extract and five bioactive compounds had no induction effect on CYP2C9 mRNA expression in both HepG2 cell and human liver cell as shown in Figure 15-16.

Of five bioactive compounds, bacoside A_3 and bacopasaponin C showed the highest induction of CYP1A2 in both HepG2 cells and normal cells. Besides, bacopaside II showed the highest induction of 3A4 expression followed by bacoside A_3 in cell line but not in normal cell. These results might suggest that bacoside A_3 might be the major bioactive compounds that drive CYP1A2 mRNA expression induction. Based on our results, concomitantly consuming *B. monnieri* extract and CYP1A2, and 2B6 substrates might occur HDIs and alter the pharmacokinetics, resulting in therapeutic failure. However, the bacopa extract and bioactive compounds did not alter the CYP2C9 mRNA levels. This result suggested co-administration of the bacopa extract and CYP2C9 substrates might not produce any effect on the body.

Based on qRT-PCR results, the western blotting assay was performed to further confirm the induction effect of the extract, bacoside A_3, and its isomer compound namely bacopaside II in HepG2-cells. Our experiment results showed that the extract at low concentration did not increase the CYP2B6 and 3A4 mRNA levels while at high concentration could increase the CYP2B6 and 3A4 mRNA levels. This

phenomenon suggested a concentration-dependent manner. However, the concentration of bacoside A_3 and bacopaside II did not enough to produce the induction effect on HepG2-cells (the figure did not show).

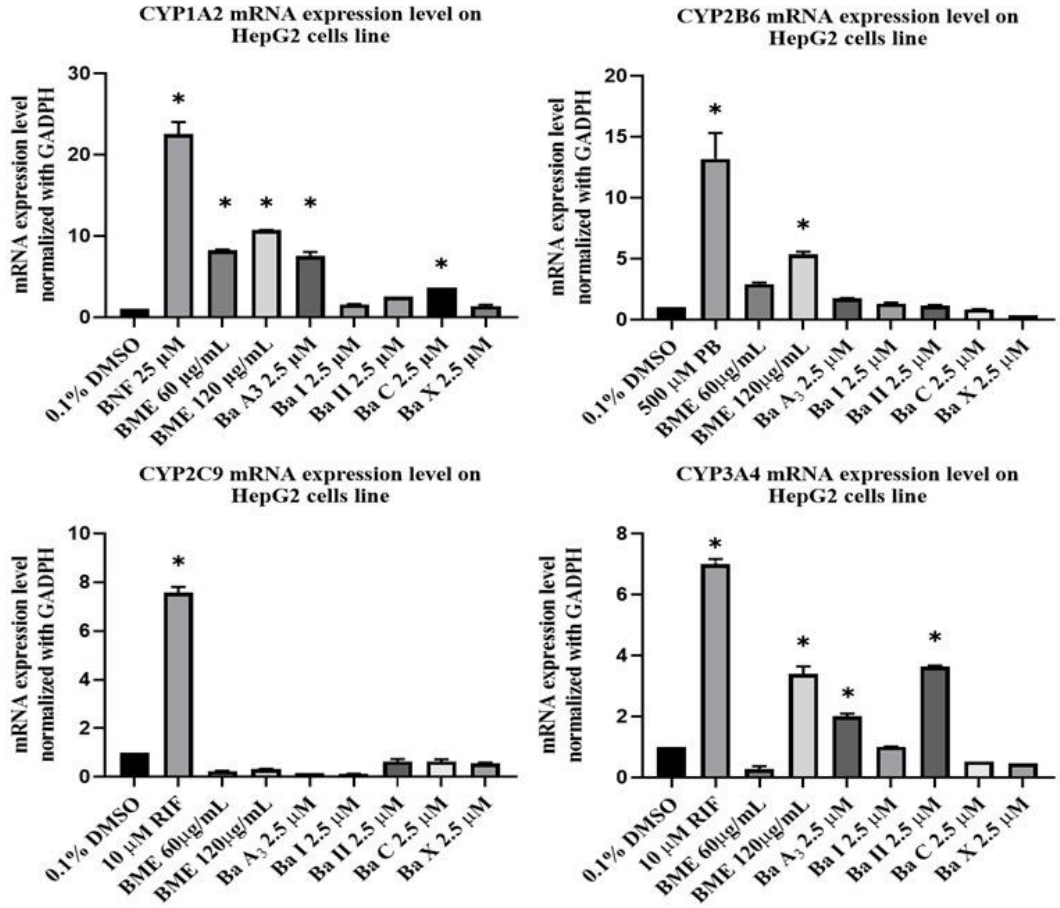

Figure 22 Illustration of CYPs isoform mRNA expression level of *B. monnieri* extract as well as five bioactive compounds on HepG2 cells. (*) $p > 0.05$ with respect to control

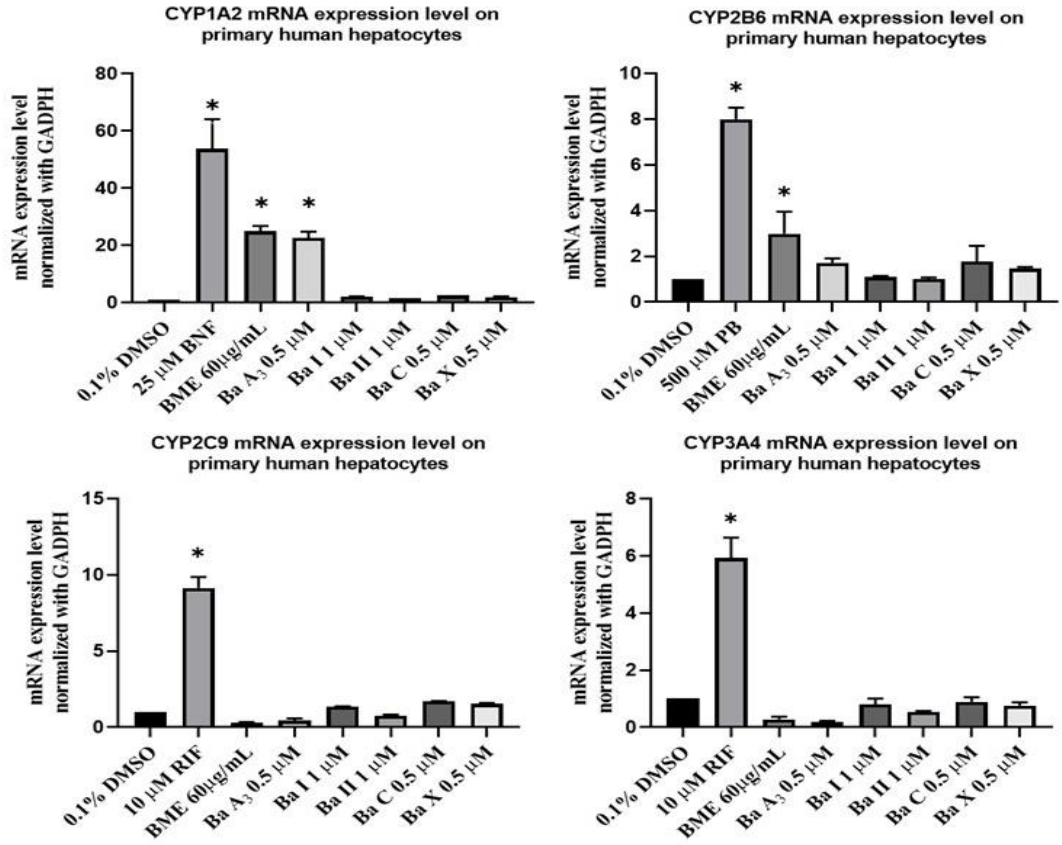

Figure 23 Illustration of CYPs isoform mRNA expression level of *B. monnieri* extract as well as five bioactive compounds on primary human hepatocyte. (*) $p > 0.05$ with respect to control

Table 17 The overall results of induction effects both activities and mRNA expression for *B. monnieri* extract and purified compounds on CYP450

Induction analysis	HepG2 cells	Human Hepatocytes
Enzyme activity	CYP1A2= BME	CYP 1A2= BME, A_3
	CYP 2B6= BME	CYP 2B6= BME, Ba C
	CYP 2C9= BME	
	CYP 3A4= BME, Ba I	
mRNA expression	CYP 1A2= BME, Ba A_3, Ba C	CYP 1A2= BME, Ba A_3
	CYP 2B6= BME	CYP 2B6= BME
	CYP 3A4=BME, Ba A_3, Ba II	

BME is *B. monnieri* extract, Ba A_3 is Bacoside A_3, Ba I is Bacopaside I, Ba II is Bacopaside II, Ba C is Bacopasaponin C, Ba X is Bacopaside X

CHAPTER V

DISCUSSION AND CONCLUSION

1. Discussion

CYP isoforms including CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were the major metabolic enzymes that contribute to 80% of the total hepatic metabolism (3). Drug-drug interaction *via* the CYP450 system has been widely studied because this interaction could often be clinically significant. However, other foreign compounds such as food or herbs might produce chemical interaction too (3). In this study, the inhibition potential of bacopa extract and purified compounds on CYP450 was assessed using HLMs, whereas the induction potential of that such compounds was assessed using HepG2 cell and primary human hepatocytes. Our findings are critical for developing guidelines for sensible administration precautions while utilizing this herbal medication. In this study, the *B. monnieri* extract was purchased for GPO Ltd. (Bangkok, Thailand) claiming to contain at least 18% (w/w) of total saponin glycosides. In order to confirm this claiming, the amount of total saponin glycoside (i.e., jujubogenin and psuedojujubogenin glycosides) containing in the *B. monnieri* extract was evaluated using the HPLC-UV method followed by Limpeanchob et al. (8). As the results found that 100 mg of the *B. monnieri* extract detected total saponins about 18.16%, which divided into bacoside A_3 2.12 %, bacopaside X 2.57%, bacopaside I 6.78%, bacopaside II 4.42%, and bacopasaponin C 2.27%. These results imply that about 74% of total saponins were pseudojujubogenin glycosides of which bacopaside I was the majority (~50%). Our result was in line with the quantitative analysis of saponins from Limpeanchob et al. (8), and Saesong et al. (6). Therefore, we could be sure that the *B. monnieri* extract was used in this study is rich and full of jujubogenin glycosides (bacoside A_3, and bacopaside X) and pseudojujubogenin glycosides (bacopaside I, bacopaside II, and bacopasaponin C).

The *in vitro* enzyme kinetic parameters i.e., IC_{50} and Ki values have been reflected on the inhibition ability of the test compounds. Based on the experimental results, the bacopa extract exhibited strongly inhibit CYP2C19 with IC_{50} and Ki values of 17.68 and 3.04 μg/mL, respectively. Therefore, this implies that concomitantly

intake the bacopa extract and CYP2C19 substrates (i.e., clopidogrel, diazepam, omeprazole, and methadone) might produce the HDIs. In addition, the bacopa extract expressed the moderate inhibition of CYP2C9, 2B6, and 1A2 with IC_{50} values of 47.72, 59.69, and 75.98 µg/mL, respectively. Thus, the bacopa extract and CYP2C9 (i.e., warfarin, diclofenac, and ibuprofen), 2B6 (i.e., artemisinin, bupropion, and cyclophosphamide), and 1A2 (i.e., caffeine, melatonin, and theophylline) should be taken with caution. However, five major bacosides group exhibited negligible inhibition towards CYP isoforms except for CYP2B6 and CYP2C9 (IC_{50} for bacopaside I equal to 84.02 and 52.80 µg/mL). There might be other compounds present along with bacopaside I in the herb able to modulate CYP activity. Additionally, the IC_{50} and Ki parameters have useful in predicting the likelihood of developing HDIs using *in silico* study i.e., development of the pharmacokinetic model or physiologically based pharmacokinetic model.

Based on animal study, the CYP3A1, and 3A2 mediated testosterone 6β-hydroxylation activity of the liver and intestine were 2 and 1.5-fold decreased in rats after oral administration of the bacopa extract at 31 mg/kg/day for one week (95). However, the inhibition potential of the bacopa extract on CYP3A using human recombinant CYPs (rCYPs) and HLMs gave the opposite results. The rCYPs have been uses as a tool to investigate metabolic stability and CYP reaction phenotyping. The strength point of rCYP was amenable to high-throughput screening. However, rCYPs have expressed only CYPs and are absent from competing enzymes. Whilst, HLMs have expressed both oxidative phase I enzymes and UGTs. Also, HLMs have been a gold standard model for *in vitro* assessment of inhibition potential on CYP system (96). These could become an explanation for the slight differences in experimental results obtained from this study and a previous study by Ramasamy et al. (17). Following our results, *B. monnieri* extracts demonstrated strong inhibit the enzyme activity of CYP2C19 with IC_{50} of 17.68 µg/mL that is close to the value from a previous study using rCYP (IC_{50} equal to 23.67 µg/mL). In addition, the bacopa extract demonstrated moderately inhibit CYP1A2, and 2C9, which were in line with a study by Ramasamy et al. (17). Despite our results suggested the bacopa extract did not inhibit CYP3A4 when the IC_{50} value was equal to or higher than 100 µg/mL, the IC_{50} value (101.4 µg/mL) acquired from our study differs from the value (83.95 µg/mL) using rCYP only

18.83%. However, those values suggested that the bacopa extract was weakly a CYP3A4 inhibitor.

To our knowledge, the bacosides group was found to be a blend of triterpenoid saponins glycosides that have one or more sugar chains linked to a nonpolar triterpene aglycone skeleton. Also, it was classified as dammarane-type triterpenoid saponins. Besides, the bacoside structure was the presence of three glycosides. These molecules have a high number of free hydrogen bonds suggested high polarity. Additionally, the binding affinity has been used to measure the strength of attraction between the CYP enzymes and the tested compounds. Also, the binding affinity for an active site depending on its chemical structure and physical properties. Hence, bacosides resulted in low affinities to CYP active sites and negligible inhibition of the CYP isoforms (17).

The HepG2 cell and human hepatocytes respond to CYP inducers successfully and have been employed as an *in vitro* model for testing compounds with unknown CYP induction potential (97). Despite human liver cell lines having several times less mRNA expression and high variability than normal liver cells, they have been functional in a preliminary screening of the induction potential on the CYP system (97, 98). Based on our induction assay, the bacopa extract significantly enhanced the enzyme activity and mRNA expression of CYP3A4. Whereas the results using primary human hepatocytes found that the bacopa extract and other purified compounds did not alter the enzyme activity and mRNA expression of CYP3A4 (Figure 15-16). Although the primary human hepatocytes usage *in vitro* experiment has been found phenotypic instability, which leading to low mRNA levels and decreased *de novo* synthesis, they still retain significant activity levels of both phase I and II enzymes for several days in culture. Besides, these cells respond to enzyme inducer, and enzyme activity and/or mRNA changes can be properly monitored. Also, the primary human hepatocytes have been recognized as the gold standard model for the evaluation of the impact of drugs and other xenobiotics (95). Therefore, we decided to use the results using primary human hepatocytes to indicate that the bacopa extract and five bioactive compounds did not affect the enzyme activity and mRNA expression of CYP3A4.

Based on our results, the *B. monnieri* acts as the CYP1A2 and 2B6 inhibitor and inducer that this phenomenon can occur. However, the inhibitory effect remains the most important metric considering two main points. First, during the enzyme

reaction, the inhibition process can occur immediately after ingestion of Brahmi with CYP1A2 and CYP2B6 substrates. Whilst induction process It takes up to 24-72 h to induce increased activity and mRNA in CY1A2 and CYP2B6. In addition, the inhibition effect of *B. monnieri* on CYP450 is depending on drug plasma concentration or dosage regimen. As our results found that the Ki values for CYP1A2 and 2B6 are 48.06 and 5.16 µg/mL, respectively. This suggests that consumers need to take up to 22.25 g/day of *B. monnieri* for CYP1A2 inhibition while ingestion 27.78 g/day of *B. monnieri* may inhibit CYP2B6 activity. Noted that oral administration of 27.78 g/day of *B. monnieri* can produce the drug plasma concentration as 60 µg/mL which is enough to generate the CYP1A2 and 2B6 induction potential. Therefore, the inhibition and induction effect of *B. monnieri* on CYP1A2 and 2B6 in humans after concomitantly oral intake of the *B. monnieri* with CYP1A2 and CYP2B6 substrates.

In this study, we further predicted the pharmacokinetics (first-order assumption) of bacoside A_3, bacopaside I, bacopaside II, bacopaside X, and bacopasaponin C using GastroPlus™ software (version 9.8.0002). Based on their chemical structure, the predicted log P value was 1.76 approximately which suggested a lipophilic compound when the value was greater than 1. The mean predicted permeability (P_{eff}) value equal to 4.67 x 10^{-6} cm/s which suggested highly intestinal absorption (>70%) when the value larger than 10 x 10^{-6} cm/s (99, 100). The fraction absorbed (F_a) of bacoside A_3, bacopaside I, bacopaside II, bacopaside X, and bacopasaponin C were found as 0.08, 0.09, 0.27, 0.18, and 0.31, respectively in human following oral administration of purified compounds at 10 mg. Those predicted results were in line with previous *in situ* studies (23). Even though the extract showed the strong inhibition potential on CYPs system, the among of five purified compounds in human blood circulation ($AUC_{0\text{-}inf}$) after intakes 10 mg of the isolated compound was not excess 0.05 µg.h/mL while the IC_{50} value for CYPs activity inhibition was larger than 17.68 µg/mL on CYP450. Therefore, consumers need to consume larger than 100-fold the extract to be able to cause an inhibition effect on CYPs.

Based on the predicted output, the protein-bound drug of five purified compounds was 70% approximately after oral administration of the bacopa extract in humans due to the predicted fraction unbound in plasma (f_{up}) equal to 30%. Those compounds in free form could distribute into many organs in the body. The bacoside

A_3, bacopaside II, bacopaside X, and bacopasaponin C were extensively first-pass metabolism. The predicted hepatic extraction ratio (E_H) was 0.70 which suggested a high extraction ratio, thus, the fraction of the dose that escaped hepatic first-pass metabolism (F_H) should be not excess 0.30. The mean predicted liver clearance for those compounds was 45.38 L/h. In contrast, bacopaside I was a low degree of the first-pass metabolism. Since, the E_H and F_H values were 0.25 and 0.75, respectively. Thus, the alteration in plasma protein binding sensitively affects oral clearance. The predicted liver clearance of bacopaside I was found as 14.90 L/h which is 3-fold lower than other compounds. However, the mean oral bioavailability (F_{oral}) of those compounds was predicted as 6.20%. These results reinforced the safety of ingesting the bacopa extract in light of HDIs.

Based on chemical structure, GastroPlus™ software could classify which compound was the transporter substances and inhibitors. As the predicted results, five major bioactive compounds were P-gp substrates (except bacopaside I) in human while the Western blot analysis results by Singh et al. (95) found that the bacopa extract altered the expression of intestine P-gp in rats but not in hepatic P-gp. The predicted output also demonstrated that five major bioactive compounds could be the OATP1B1 inhibitor. However, further pharmacokinetic studies in *in vivo* should be conducted to confirm this prediction.

Prospective study, this model has been developed further so that the extent of HDI evaluation performs precisely. According to the prediction for an *in vivo* interaction follow the criteria of Austin et al. (92), The BME possibly occurs *in vivo* interaction on CYP2C19, 2B6, and 2C9 respectively. Whereas Bacopaside I show remote an *in vivo* interaction, its good safety profile. However, based on predicted software and our results reinforce the safety of ingesting the bacopa extract in light of HDIs. Subsequently, we should be follow up *in vivo* and clinical trials with bacopa extract on CYP 2C19, 2B6, 2C9 combine with its substrate respectively. Another point of view, a previous study show efficiency of bacopa extract can action on the brain target excellently. In contrast, the fraction absorbed ($\%F_a$) and oral bioavailability ($\%F_{oral}$) values were predicted at just 20% and 6.20%, respectively. This suggested there is a very low amount of drugs that arrive in the blood circulation and can distribute

into the brain target. Therefore, the pharmacological activity on the brain target of bacopa extract should be shown extremely potential.

2. Conclusion

Our finding found that oral administration of the bacopa extract at 300-600 mg/day did not produce hepatoxic. In addition, following the finding from the present *in vitro* study confirmed the inhibitory effect of *B. monnieri* extract on CYP2D6, 2E1, and 3A4 activities cannot be excluded to cause HDIs. The bacopa extract demonstrated the inhibition and induction potential on CYP1A2, and 2B6. It was important to note that this extract strongly inhibited CYP2C19 activity and moderately inhibited CYP2C9. However, the likelihood of HDIs following intake of the *B. monnieri* extract remains low due to low values of F_a and F_{oral}. Therefore, simultaneously consuming the bacopa extract and CYP1A2, 2B6, 2C19, and 2C9 (such as caffeine, bupropion, omeprazole, and ibuprofen, respectively) did not seem to cause of HDIs. Last, we still need more clinical studies to confirm these results.

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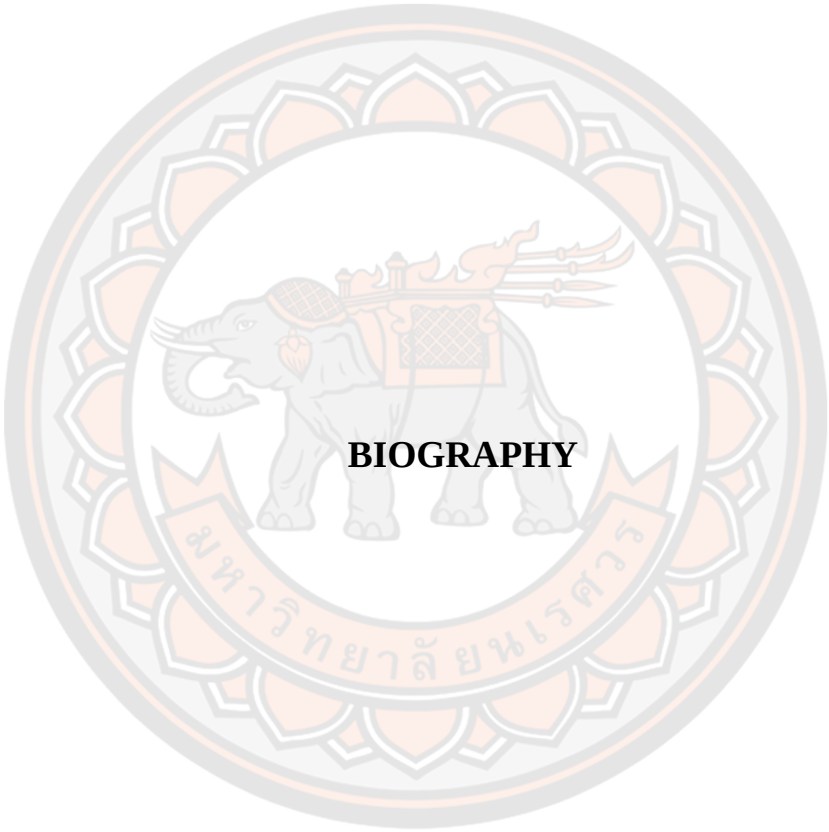

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