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Evaluation of Selected Malaysian Medicinal Plants on Phase I Drug Metabolizing Enzymes, CYP2C9, CYP2D6 and CYP3A4 Activities *in vitro*

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Abstract: This study investigated the effects of selected Malaysian medicinal plant extracts towards human recombinant cytochrome P450 (CYP450) enzyme activities *in vitro*. Five Malaysian medicinal plants were tested on the three main CYP450 enzyme activities of CYP2C9, CYP2D6 and CYP3A4. The abilities of these extracts to inhibit human cytochrome P450 enzyme activities were analyzed using a luminescent assay. *Orthosiphon stamineus* showed the most potent inhibitory activity against CYP2C9 with an apparent IC_{50} value of 77.5±1.1 μg mL^{-1}, while *Andrographis paniculata*, *Curcuma xanthorrhiza*, *Eurycoma longifolia* and *Mitragyna speciosa* extracts showed negligible inhibition. On the metabolism mediated by CYP2D6, *Mitragyna speciosa* showed the most potent inhibitory activities with IC_{50} values of 3.6±0.1 μg mL^{-1}, followed by *Orthosiphon stamineus*, *Andrographis paniculata* and *Curcuma xanthorrhiza* with IC_{50} value of 11.7±1.1, 44.2±4.5 and 215.3±71.6 μg mL^{-1}, respectively. *Andrographis paniculata* ethanolic extract gave the lowest IC_{50} value towards CYP3A4 with an apparent IC_{50} value of 27.6±3.7 μg mL^{-1}, followed by *Orthosiphon stamineus* (78.4±20.3 μg mL^{-1}), *Mitragyna speciosa* (142.8±13.8 μg mL^{-1}) and *Curcuma xanthorrhiza* (285.3±61.7 μg mL^{-1}). Sulfaphenazole, quinidine and ketoconazole were used as positive controls for CYP2C9, CYP2D6 and CYP3A4 respectively. The findings suggest that *Orthosiphon stamineus*, *Mitragyna speciosa* and *Andrographis paniculata* may contribute to herb-drug interactions if they are administered concomitantly with drugs metabolized by CYP2C9, CYP2D6 and CYP3A4 respectively.

Key words: Cytochrome P450 inhibition, *Mitragyna speciosa*, *Orthosiphon stamineus*, *Andrographis paniculata*, herb-drug Interactions

INTRODUCTION

Drug metabolism plays an important role in our body to increase the drug's water solubility in order to make them more readily excreted in bile or urine (Subehan *et al*., 2006). Drug metabolism can be divided into two phases that are Phase I and II. Phase I drug metabolism involves redox (oxidation and reduction) and hydrolysis reactions. Cytochrome P450 (CYP450) and flavin-containing monooxygenases (FMOs) are enzymes that catalyzed most of these reactions (Hasler *et al*., 1999; Parkinson, 2001; Nor Afifah *et al*., 2010; Rendic, 2002). This study focuses on cytochrome P450, which seems to be the most important enzyme family. CYP450 is a drug-metabolizing enzyme that is made up of hemoproteins, present abundantly in the endoplasmic reticulum in hepatocytes. Oxidations of most endogenous and exogenous compounds are catalyzed by CYP450 (Gonzalez, 1988). Structurally different types of drugs have the capability to be metabolized by the same enzymes, thus can lead to CYP450 inhibition due to their wide substrate specificity. More than half of the drugs in the market currently are metabolized by CYP2C9, CYP2D6 and CYP3A4 (Wienkers and Heath, 2005).

CYP450 enzyme inhibition may probably contribute to the undesirable consequences such as the decrease in one of the drug clearances when two or more drugs are administered at the same time because of drug-drug interactions and decrease in the formation of pro-drugs reactive metabolites, which can lead to a decrease in the pharmacological effects of drug metabolism. Thus, the decrease in drug metabolism rate will increase drug toxicity (Wienkers and Heath, 2005; Jamal *et al*., 2010).

There are a lot of herbal plants that have been used both in primary forms or combined into mixtures as traditional medicines. Traditional medicine has been used as folk medicines as they are assumed to be non-toxic due to their origin from natural sources (Zhou *et al*., 2004). Pharmacologically active constituents such as alkaloids, flavonoids, anthraquinones, polyphenols, terpenoids, glycosides, coumarins, saponins, tannins and essential oils can be found in these plants and have the ability to take part in herb-drug interactions (Zhou *et al*., 2004; Markowitz *et al*., 2000). Garlic (*Allium sativum*), ginseng (*Panax ginseng*), St John's wort (*Hypericum perforatum*) and liquorice (*Glycyrrhiza glabra*) are medicinal herbs

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Table 1: Reported therapeutic applications of Malaysian medicinal plants selected for this study

Plant name	Family	Part used	Local name	Therapeutic application
Andrographis paniculata Nees.	Acanthaceae	Aerial	Hempedu bumi	Infectious-fever causing disease, cold, loss of appetite, irregular stools and diarrhea
Curcuma xanthorrhiza Roxb.	Zingiberaceae	Rhizome	Temulawak	Stomach disease, liver disorder, constipation, bloody diarrhea, dysentery, children's fevers, hemorrhoids
Eurycoma longifolia Jack.	Simaroubaceae	Leaves	Tongkat ali	Antipyretic, antimalaria, restorative activities, antiulcer
Mitragyna speciosa Korth.	Rubiaceae	Leaves	Ketum	Alleviate pain, coughing, diarrhea and intestinal infections by amoeba and protozoa.
Orthosiphon stamineus Benth.	Lamiaceae	Leaves	Misai kucing	Eruptive fever, epilepsy, gallstones, hepatitis, rheumatism, hypertension, kidney or bladder inflammation and diabetes

that have been reported to be involved in herb-drug interactions (Zhou *et al*., 2004; Ioannides, 2002).

In vitro assay is one of the simple and effective methods to evaluate the potential of drugs or medicinal plants inhibition towards CYP-mediated metabolism as well as to minimize potential herb-drug interactions (Zientek *et al*., 2008). Thus, in this study, we have used the recombinant expressed enzymes, *in vitro* in a simple luminescent assay. Through this model, the activities of CYP450 enzymes could be evaluated individually. This recombinant expressed enzyme system is produced in the endoplasmic reticulum of eukaryotic host cells. In this study, we report the CYP450 enzyme inhibition of selected Malaysian medicinal plants and herb-drug interaction potential. In this study, five selected Malaysian medicinal plants, which were *Andrographis paniculata*, *Curcuma xanthorrhiza*, *Eurycoma longifolia*, *Mitragyna speciosa* and *Orthosiphon stamineus* (Table 1) were tested on human recombinant CYP2C9, CYP2D6 and CYP3A4 enzymes.

MATERIALS AND METHODS

Chemicals: The enzyme assays were carried out using the following chemicals and reagents: The P450-Glo™ Screening Systems was obtained from Promega, USA. This system contained recombinant human cytochrome P450 2C9, 2D6 and 3A4 enzymes in the microsome produced by baculovirus-infected insect cells, membrane fraction devoid of cytochrome P450 enzyme activity, the luminogenic cytochrome P450 substrates [6'deoxyluciferin (Luciferin-H), ethylene glycol ester of luciferin 6' methyl ether (Luciferin ME-EGE) and luciferin 6' benzyl ether (Luciferin-BE)], an NADPH regeneration system, 1M potassium phosphate buffer, pH 7.4, Luciferin Detection Reagent and Luciferin-Free Water. Positive controls, sulfaphenazole, quinidine and ketoconazole were purchased from Sigma Chemicals (St. Louis, USA). This study was conducted from May 2009 until February 2010 at Center for Drug Research, Universiti Sains Malaysia and Malaysia.

Plant materials: The *Andrographis paniculata* and *Eurycoma longifolia* ethanolic extracts were obtained from Prof. Chan Kit Lam, Pharmaceutical Chemistry Discipline, School of Pharmaceutical Sciences, Universiti Sains Malaysia (USM). The *Orthosiphon stamineus* methanolic extract was obtained from Professor Zhari Ismail, Pharmaceutical Chemistry Discipline, School of Pharmaceutical Sciences, Universiti Sains Malaysia (USM). The *Curcuma xanthorrhiza* ethanolic and *Mitragyna speciosa* methanolic extract were prepared in our laboratory at Centre for Drug Research, Universiti Sains Malaysia (USM). The parts of plant used in this study were aerial (*Andrographis paniculata*), rhizome (*Curcuma xanthorrhiza*) and leaves (*Eurycoma longifolia*, *Mitragyna speciosa* and *Orthosiphon stamineus*). All extracts were standardized extracts.

Enzyme assay: The luminescent assay was carried out as described in P450-Glo™ Screening Systems by Promega, USA. Essentially, this assay was performed in the 96 white flat bottom plates (Greiner Bio-one, USA). The concentration of extracts and positive control ranging from 0.01-1000 μg mL^{-1} and 0.02-200 μM were used respectively. The concentrations of the plant extracts and positive controls were prepared four times of the original concentrations. Firstly, 12.5 μL of the plant extracts were added to the treated wells. For the untreated wells, 12.5 μL of the luciferin-free water was added. This well presents the total activity of the cytochrome P450 enzyme activity. Positive control (12.5 μL) was added into wells appropriate to their enzymes. The minus-P450 wells, also known as negative control were added with 12.5 μL of the luciferin-free water. After that, 12.5 μL of the 4X reaction mixtures (contains the human CYP membrane preparations, appropriate luminogenic substrate, 1M potassium phosphate buffer) were added to all wells except minus-P450 control wells. For the minus-P450 wells, 12.5 μL of the 4X control reaction mixtures (contains the membrane fractions devoid of cytochrome P450 enzyme activity, appropriate luminogenic substrate, 1M potassium phosphate buffer) was added. These wells represent the cytochrome P450-independent background luminescence of the assay. The 6'deoxyluciferin (Luciferin H), ethylene glycol ester of luciferin 6'methyl ether (Luciferin ME-EGE) and luciferin 6'benzyl ether (Luciferin-BE) were used as luminogenic substrates for CYP2C9, CYP2D6 and CYP3A4, respectively. The mixture was mixed properly by tapping the wells gently. Then, the mixture was

pre-incubated for 10 min at room temperatures (25°C). In order to initiate the reaction, 25 μL of the 2 X NADPH regeneration systems was added to all wells. This system contains $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $MgCl_2$, functioning to initiate and sustain the cytochrome P450 reaction by maintaining a nonlimiting NADPH system. The mixture was incubated 30 min (45 min for CYP2D6) at room temperature. In addition, 50 μL of Luciferin Detection Reagent was added. This will stop the reaction and counts the luminescent signal. The luminescent values, Relative Light Unit (RLU) were read using microplate reader after 20 min incubation. The purpose of the incubation is to stabilize the luminescent signal. This luminescent signal is stable for about 4 h.

Statistical analysis: Results were presented as the Mean±SEM of three replicates for two independent experiments. The significant differences were evaluated using ANOVA, followed by Dunnett's test.

RESULTS AND DISCUSSION

In this study, five selected Malaysian medicinal plants were investigated for their effects on human cytochrome P450 enzyme activity. This is the first study about the effect of selected Malaysian medicinal plants on human CYP450 using a luminescent assay. This luminescent assay employed luminogenic P450 probe substrates that are derivatives of beetle luciferin, as substrates for luciferase enzymes. These luminogenic P450 probe substrates were converted by different P450 enzymes to luciferin. Luminogenic substrate assays are based on the enzymatic release of free luciferin by a CYP450 from an inactive luciferin precursor. The luciferase enzyme uses the CYP450 generated luciferin as a substrate with ATP and oxygen to create light. The light produced was then measured using a microplate reader and is directly proportional to the activity of CYP450 enzymes. The plant extracts that inhibit CYP450 enzyme activity result in less light production (Bosetti *et al*., 2005). Unlike fluorescent assay, luminescent assay has no fluorescent interferences. This is because no fluorescent excitation and emission overlaps between analytes (Bosetti *et al*., 2005).

The IC_{50} values were determined for the extracts that showed more than 50% inhibition (Appiah-Opong *et al*., 2007). The IC_{50} values were calculated using the GraphPad Prism® 5 (Version 5.01, GraphPad Software, Inc., USA) by plotting the percentage inhibition of CYPs enzyme activities versus log concentration of the plant extracts. For CYP2C9, *Orthosiphon stamineus* methanolic extract (OSME) showed the strongest inhibition with an apparent IC_{50} value of 77.5±1.1 μg mL^{-1}. All other extracts did not show any significant inhibition of CYP2C9. For CYP2D6, *Mitragyna speciosa* methanolic extract (MSME) showed the strongest inhibition with an apparent IC_{50} value of 3.6±0.1 μg mL^{-1}. The rank order of inhibition is MSME > OSME > APEE > CXEE. For CYP3A4, *Andrographis paniculata* ethanolic extract (APEE) showed the strongest inhibition with an apparent IC_{50} value of 27.6±3.7 μg mL^{-1}. The rank order of inhibition is APEE > OSME > MSME > CXEE. *Eurycoma longifolia* ethanolic extract did not show any significant inhibition of all the CYPs tested. Sulfaphenazole, quinidine and ketoconazole were used as positive controls towards CYP2C9, CYP2D6 and CYP3A4, respectively. These positive controls showed IC_{50} values of 0.09±0.05, 1.09±36 and 0.056±0.002 μg mL^{-1} correspondingly. However, the IC_{50} values obtained with the extracts are higher than the IC_{50} values obtained with the positive controls suggesting that these plants showed weak inhibition.

At present, the CYP450 metabolizes more than 50% of the drugs in the market. Cyclosporine A, dihydropyridines, ethinylestradiol, midazolam, terfenadine and triazolam are among 50% of clinical drugs metabolized by CYP3A4, which is the most important CYP450 enzyme (Rendic and di Carlo, 1997). In addition, about 30% of all drugs are catalyzed by CYP2D6, including amitriptyline, imipramine, haloperidol, propanolol and dextromethorphan (Clarke and Jones, 2002). The clearance of the drugs such as S-warfarin, phenytoin, tolbutamide, torsemide and many non-steroidal anti-inflammatory agents in human bodies are metabolized by CYP2C9 (Miners and Birkett, 1998). Herbal products and western drugs are reported to have potential in herb-drug interaction if they are administered simultaneously (Foster *et al*., 1999; Nebel *et al*., 1999; Taylor and Wilt, 1999). If a first drug inhibits the metabolism of a second co-administered drug, the second drug may accumulate in human body. Thus, the study of herbal medicines as potential drug inhibitors is particularly important to minimize or prevent the consequences of herb-drug interactions. In addition, most of the plant used in this study contains many of the active constituents such as alkaloids, flavonoids, polyphenol and terpenoids. The presence of these active constituents may contribute to the inhibitory activity observed.

Eurycoma longifolia ethanolic extract showed negligible inhibitory activities towards CYP2C9, CYP2D6 and CYP3A4. This negligible inhibitory activity was observed because the percentage inhibitions of all the CYPs tested were less than 50%. The effect of *Curcuma xanthorrhiza* ethanolic extract on CYP2C9 can be ignored because the percentage of inhibition is less than 20%. *Curcuma xanthorrhiza* showed the highest IC_{50} values towards CYP2D6 and CYP3A4 enzyme activities but the values are still less than the highest concentration, 1000 μg mL^{-1}. This outcome is parallel to the result

showed by Usia *et al.* (2006) where the inhibition is less than 30% against CYP2D6 and CYP3A4.

In China, Thailand and Ayurvedic medicine, *Andrographis paniculata* is one of the most essential medicinal plants that have been used to treat colds, influenza, gastric disorder and other infectious disease. Figure 1a-e showed that *Andrographis paniculata* inhibited more than 90% of CYP2D6 and 60% of CYP3A4

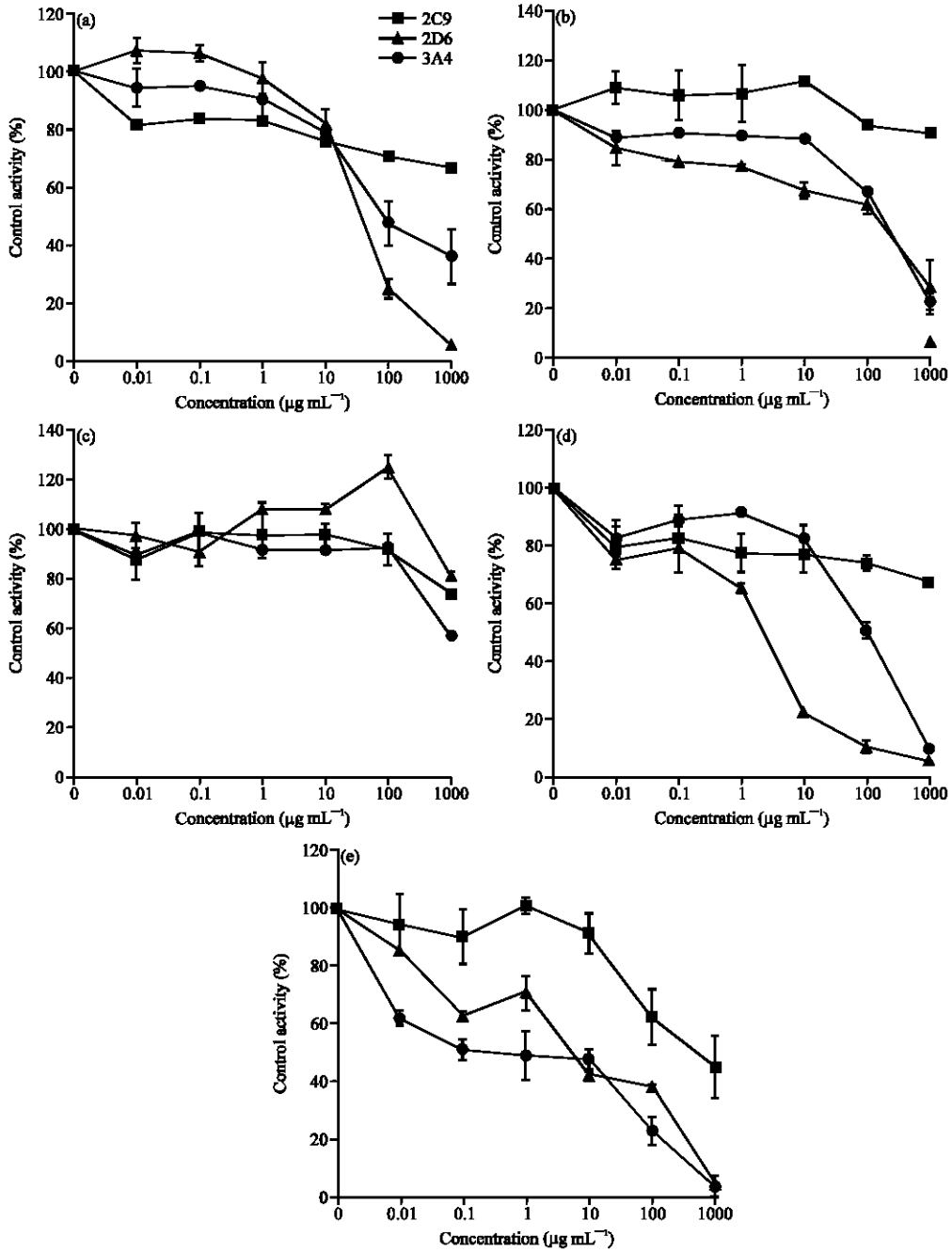

Fig. 1: The percentages of CYP2C9, CYP2D6 and CYP3A4 control activities versus Malaysian medicinal plant extract concentrations. Values are Mean±SEM for three determinations (n = 3) from two independent experiments. Statistical analysis was conducted using one-way ANOVA and Dunnett's test. (a) *Andrographis paniculata*, (b) *Curcuma xanthorrhiza*, (c) *Eurycoma longifolia*, (d) *Mitragyna speciosa* and (e) *Orthosiphon stamineus*

enzyme activities. Our findings are in line with Usia *et al.* (2006), whereby *Andrographis paniculata* was found to inhibit 92.6% (CYP2D6) and 30-70% (CYP3A4) of their enzyme activities (Usia *et al.*, 2006). In addition, andrographolide is the major active constituent extracted from the leaves of the *Andrographis paniculata*, which is a bicyclic diterpenoid lactone and it is believed that andrographolide is responsible for many of its biological activities. A study done by Pekthong *et al.* (2008) reported significant inhibition of CYP 2C and CYP3A by *Andrographis paniculata* ethanolic extract with minimal inhibition of the enzymes by andrographolide.

Terpenoids and polyphenols are among the active constituents present in the *Orthosiphon stamineus* methanolic extract (Tezuka *et al.*, 2000). Rosmarinic acid is a caffeic acid derivative, present abundantly in the *Orthosiphon stamineus* methanolic extract, followed by eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and sinensetin in the percentages of 7.58, 0.3, 0.24 and 0.2, respectively (Akowuah *et al.*, 2005; Yam *et al.*, 2009). One of these active constituents may contribute to the inhibition of CYPs, but further study is required for the confirmation of this assumption.

Our findings showed that *Mitragyna speciosa* ethanolic extract gave inhibition of more than 90% for both CYP2D6 and CYP3A4. *Mitragyna speciosa* or ketum had long been used traditionally in Thailand and some states in Malaysia as a medicinal herb to boost physical stamina, as well as a folk medicine for a variety of disease such as pain and fever (Vicknasingam *et al.*, 2009). However, based on an increase in the number of internet postings on ketum purchase, preparation, use and benefits and management of withdrawal pains there is an assumption that it is increasingly being used to treat opiate withdrawal pains (Vicknasingam *et al.*, 2009). *Mitragyna speciosa* is ingested as a juice in Malaysia, while in Thailand, it is consumed by chewing (Vicknasingam *et al.*, 2009). Mitragynine is one of the 25 alkaloids that have been found in *Mitragyna speciosa*. Mitragynine, which is its major alkaloid, has been established to produce antinociceptive effect (Ponglux *et al.*, 1994). According to Vicknasingam *et al.* (2009), a daily consumption of *Mitragyna speciosa* solution (3×250 mL) by *Mitragyna speciosa* users to ease opiate withdrawal symptoms contains 75 mg of mitragynine (Vicknasingam *et al.*, 2009). As we can see, the content of the mitragynine in *Mitragyna speciosa* consumed by the users is quite high, thus the potential of herb-drug interaction should be taken into account. In addition, our findings showed that *Mitragyna speciosa* methanolic extract gave inhibition of more than 90 % for CYP2D6 and more than 80% for CYP3A4 (Fig. 1). The IC_{50} value of *Mitragyna speciosa* methanolic extract is 3.6±0.1 μg mL^{-1}, which is in the same concentration range compared to the quinidine (1.09±0.36 μg mL^{-1}) towards CYP2D6 (Table 2).

Table 2: The IC_{50} values of the selected Malaysian medicinal plants

	IC_{50} value (μg mL^{-1})		
Medicinal plants	CYP2C9	CYP2D6	CYP3A4
Andrographis paniculata ethanolic extract	ND	44.2±4.5*	27.6±3.7*
Curcuma xanthorrhiza ethanolic extract	ND	215.3±71.6*	285.3±61.7*
Eurycoma longifolia ethanolic extract	ND	ND	ND
Mitragyna speciosa methanolic extract	ND	3.6±0.1*	142.8±13.8*
Orthosiphon stamineus methanolic extract	77.5±1.1	11.7±1.1*	78.4±20.3*
Control			
Sulfaphenazole	0.09±0.05		
Quinidine		1.09±0.36	
Ketoconazole			0.056±0.002

All values are the Mean±SEM of three determinations (n = 3) with two independent experiments. ND (not determined) due to the less than 50% of inhibition (<50%). *p<0.05 vs. control. Statistical analysis was conducted using one-way ANOVA and Dunnett's test

CONCLUSION

In conclusion, our findings showed that *Orthosiphon stamineus*, *Mitragyna speciosa* and *Andrographis paniculata* have the potential to inhibit enzyme activities of CYP2C9, CYP2D6 and CYP3A4, respectively. *Eurycoma longifolia* ethanolic extract however, showed no significant inhibition towards the three CYPs. The lowest IC_{50} value is observed with *Mitragyna speciosa* methanolic extract on CYP2D6. The findings also provide important information regarding the safety of these selected Malaysian medicinal plants as important nutraceuticals. At present, the active constituents responsible for the CYPs inhibition for each medicinal plant investigated are unknown. Therefore, further work is warranted to identify the active constituents responsible for CYP2C9, CYP2D6 and CYP3A4 inhibition.

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