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Research Article

ANTIOXIDANT PROPERTIES OF *GYNURA PROCUMBENS* EXTRACTS AND THEIR INHIBITORY EFFECTS ON TWO MAJOR HUMAN RECOMBINANT CYTOCHROME P450S USING A HIGH THROUGHPUT LUMINESCENCE ASSAY

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ABSTRACT

Objective: Gynura procumbens is an evergreen herb traditionally used to treat various diseases such as fever, kidney disease, and diabetes. Despite various studies conducted on the pharmacological activities of G. procumbens, the interaction between this herb with drug-metabolizing enzymes is still unknown.

Methods: This study investigates the effects of ethanol, methanol, and aqueous extracts of G. procumbens on CYP3A4 and CYP1A2 enzyme activities along with its antioxidant capacity using high throughput luminescence assay.

Results: The content of phenolic compounds in G. procumbens extracts can be ranked in decreasing order as follows: Methanol extract > ethanol extract > aqueous extract, whereas the content of flavonoid compounds in G. procumbens extracts can be ranked in decreasing order as follows: Ethanol extract > methanol extract > aqueous extract. The methanol extract of G. procumbens exhibited the most active free radical scavenger compared to the other extracts. Ethanol extract of the G. procumbens exhibited strong inhibitory effect on CYP3A4 and CYP1A2 enzyme with IC_{50} values of 32.01±1.11 µg/mL and 7.87±1.22 µg/mL, respectively. The methanol extract exhibited weak inhibitory effect on CYP3A4 and CYP1A2 enzymes with IC_{50} values more than 100 µg/mL. The aqueous extract, on the other hand, demonstrated no inhibitory effect on both enzymes.

Conclusion: The inhibition of CYP3A4 and CYP1A2 in this study follows the rank order of total flavonoid content (ethanol extract > methanol extract > aqueous extract) in which the higher the total flavonoid content in the extract, the higher would be the inhibition of drug metabolizing enzymes studied.

Keywords: Gynura procumbens, Antioxidant properties, CYP3A4, CYP1A2.

INTRODUCTION

Herbal medicines have gained great attention throughout the world, however, they are not subjected to undergo evaluation of metabolic interaction which is a prerequisite for registered drugs [1]. Currently, herbal medicines are usually taken together with conventional drugs, and this increases the possibility of interaction between herbal medicines and conventional drugs [2]. Pharmacological studies reported that kava extract and/or kavalactones, potent inhibitors of CYP3A4, may decrease the elimination of alprazolam upon coadministeration of kava and alprazolam [3]. Other studies reported that the tanshinone an active constituent in Danshen (Salvia miltiorrhiza) inhibited various CYP probe substrates in both human liver microsomes and specific human isoforms in vitro [4]. In addition, drug interaction has also been observed for Schisandra fruit extract that showed a potent inhibitory effect on CYP3A4 activity. The components responsible for CYP3A4 activity inhibition were identified as gomisin B and gomisin C comparable with that of ketoconazole [5].

Among CYP450 enzymes, only five accounts for major drug metabolism, namely CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2 [6]. CYP3A4 is the most important enzyme as it metabolizes almost all CYP450-mediated reactions [7]. In general, xenobiotic compounds are removed from our body by oxidation reaction of CYP450 enzymes resulting in the formation of more water-soluble and less toxic metabolite. However, metabolic activation of carcinogens may also occur by some CYP enzymes [8]. For instance, CYP1 family is responsible for metabolic activation of carcinogens such as benzo(α)pyrene and 7,12-dimethylben(α)anthracene [9] and, therefore, an inhibitor of CYP1A2 might be correlated with chemopreventive effects [10].

Gynura procumbens is an annual evergreen shrub of the compositae family. It is widely distributed in South East Asian countries, including Indonesia, Thailand, and Malaysia. G. procumbens is locally known as "Akar Sebiak," "Kecam Akar," and "Sambung Nyawa" in Malaysia [11]. The plant is traditionally used to treat various types of illnesses such as fever, rash, inflammation, kidney disease, hemorrhoids, and diabetes mellitus [12,13]. The leaves of G. procumbens are not toxic [14] and exhibited anti-diabetic [15,16], wound healing potential [17], anti-inflammatory [18], and antihypertensive activities [13]. Numerous studies have exposed that G. procumbens leaves extract contains several active chemical constituents such as saponins, tannins, terpenoids, sterol glycoside, and flavonoids [17]. Previous studies had also reported that G. procumbens leaves extracts contained two potential antioxidant components which are kaempferol-3-O-rutinoside and astragalin [19]. Flavonoids are polyphenolic compounds with potential beneficial effects on human health; they reportedly have anti-allergic, anti-platelet, anti-viral, anti-inflammatory, anti-tumor, and anti-oxidant activities.

Despite numerous studies on the various pharmacological effects of G. procumbens, its inhibitory effects on CYP enzymes have not been investigated to date. As far as we know, this is the first study about the inhibitory effect of G. procumbens extracts on recombinant human CYP3A4 and CYP1A2 along with its total phenolic content, total flavonoid content, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity. Previous studies had reported that certain plant polyphenolic compounds are known to be CYP450 inhibitors, especially CYP3A4 and CYP1A2 [20,21]. The purpose of this study, therefore, was to investigate the inhibitory effects of G. procumbens extracts on CYP3A4 and CYP1A2 enzyme activities. The findings of this study would be

valuable in providing a guideline for future in vivo and clinical studies on the possible interaction of this herb with drugs highly metabolized by these two enzymes.

METHODS

Chemicals and reagents

Gallic acid, quercetin, DPPH, Follin-Ciocalteu's reagent, sodium carbonate, sodium nitrite, aluminum trichloride, and ascorbic acid were purchased from sigma aldrich, USA. P450-Glo™ CYP1A2 screening system, P450-Glo™ CYP3A4 screening system and beetle luciferin, potassium salt were acquired from Promega Corporation, USA.

Plant collection

Fresh leaves of G. procumbens were collected on Penang Island, Malaysia. The plant was deposited in the herbarium of the school of biological sciences; Universiti Sains Malaysia. The number of voucher specimen is 10833. The leaves of G. procumbens were dried in the oven at 40°C for 3 days and ground into a fine powder using a milling machine.

Perparation of methanol extract of *G. procumbens*

The dried leaves of G. procumbens (300 g) were extracted in a soxhlet for 2 days. The leaves of G. procumbens was firstly extracted with petroleum ether (60-80°C) and followed by dichloromethane so that the nonpolar and semi-polar compound can be extracted out. Then the extraction process was continued with methanol in soxhlet apparatus. Methanol was removed using a rotary evaporator (Buchi R-215, Flawil, Switzerland) under reduced pressure (Buchi V-700). Finally, the extract was freeze-dried (Freeze dryer-Labconco, Kansas City, Missouri) to yield 12.6 g crude methanol extract and stored at −20°C.

Preparation of ethanol and aqueous extract of *G. procumbens*

Briefly, the dried leaves of G. procumbens (100 g) was macerated with ethanol and water (1L) for 3 days in the water bath at 60° to produce an ethanol extract of G. procumbens and aqueous extract of G. procumbens, respectively. Ethanol and water were drained and macerated for the second time, the following day under the same conditions. On the last day of the maceration process, the suspension was filtered, and the filtrate was centrifuged at 3000 rpm for 20 minutes. Finally, the ethanol was removed using a rotary evaporator, and the remaining extract was freeze-dried to yield 7.8 g of ethanol extract. In contrast with aqueous extract, water was removed by freeze drying directly after the centrifugation process to yield 27.5 g of aqueous extract.

Total phenolic content

Determination of total phenolic content in G. procumbens extracts was completed by applying Folin-Ciocalteu method described by previous studies [22]. Briefly, extracts (100 µL) and Folin–Ciocalteu phenol reagent (1 mL) were mixed together and allowed to react for 3 minutes. Then, 300 µL of Na_2CO_3 (1 N) was added into the mixture and allowed to react at room temperature for 90 minutes before proceeding with measurement of absorbance at 725 nm. To calculate the total phenolic content in G. procumbens extracts, the standard curve of gallic acid was constructed. Total phenolic content was calculated according to the formula below:

$$C=(c \times V)/m$$

Where C is denotes as the total phenolic content in G. procumbens extracts, µg/mg plant extract in gallic acid equivalent (GAE); c is the concentration of gallic acid from the standard curve (mg/mL); V is the volume of the plant extract; and m is the weight of pure G. procumbens extract, g. The results were expressed as µg gallic acid/mg of extract.

Total flavonoid content

Total flavonoid content in the G. procumbens extract was determined [22] with slight modification and employing quercetin as the standard. Extracts and quercetin (0.25 mL), each was combined with distilled water (1.25 mL), and $NaNO_2$ solution (5%) and allowed to react for 6 minutes. Then, 10% of $AlCl_3$ solution (0.15 mL) was added to the mixture and after standing for 5 minutes, 0.5 mL of NaOH (1M) and 0.275 mL distilled water were added and absorbance was read at 510 nm. The results were stated as µg quercetin equivalent (QE) (µg quercetin/g extract) which was calculated from formula below:

$$C=(c \times V)/m$$

Where C is denotes as the total phenolic content in G. procumbens extracts, µg/mg plant extract in QE; c is the concentration of quercetin from the standard curve (mg/mL); V is the volume of the plant extract, and m is the weight of pure G. procumbens extract, g. Total phenolic and total flavonoid content were measured using Multiskan™ go microplate spectrophotometer, thermo scientific.

DPPH free radical scavenging capacity

The DPPH free radical scavenging activity of the ethanol, methanol, and aqueous extract of G. procumbens was measured using the method previously described by Shimada and co-workers [23]. All extracts were prepared at concentrations of 1 mg/mL in methanol. Each extract (200 µL) with a series of dilution ranging from 0.00625 to 0.8 mg/mL was loaded into 96 wells plate. DPPH, which was dissolved in methanol (50 µL, 1 mM), was added into the wells and incubated in the dark for 30 minutes at room temperature with DPPH in methanol to serve as a blank. Ascorbic acid (0.00625-0.8 mg/mL) was used as positive control. DPPH free radical scavenging capacity was measured at 517 nm (Multiskan™ go microplate spectrophotometer, thermo scientific). The radical scavenging activity was calculated using the equation as follows:

$$\text{Percentage of inhibition} = (A_0 - A_T)/A_0 \times 100$$

Where A_0 and A_T are absorbance values for blank and test sample, respectively.

Determination of CYP450 enzymatic activities using P450-Glo™ CYP3A4 and CYP1A2 assay

Briefly, ethanol, methanol, and aqueous extract of G. procumbens (0.1-500 µg/mL) were treated in a 96-well microplate with 12.5 µL of four times the concentration of CYP3A4 reaction mixture containing 1 picomol of CYP3A4 membrane and 50 µM luciferin-BE. As for CYP1A2 assay, ethanol, methanol, and aqueous extract were incubated with 12.5 µL of four times the concentration of CYP1A2 reaction mixture containing 0.5 pmol of CYP1A2 membrane, 100 µM luciferin-ME, and potassium phosphate buffer (100 mM). The incubation was started according to the manufacturer's instructions. Solvent content was kept at <2% per well. Net CYP-dependent luminescence was calculated by subtracting the mean luminescence of the blank (no CYP3A4 membrane) from test compound and untreated (control) luminescence value. Net luminescence values from the untreated represent the total CYP activity. The decrease in activity of the test compound reflects the modulation of this test compound toward CYP activity.

Data analysis

IC_{50} values were determined graphically by nonlinear regression analysis of the plots of the logarithm of G. procumbens extracts concentration versus percentage of remaining activity using the GraphPad Prism® version 5.0 software (Graph Pad Software, Inc., USA). All experiments were carried out in three replicates and expressed as the mean±standard error. The significance of the differences between the groups was assessed by one-way analysis of variance and Dunnett test with the level of significance set at *$p<0.05$.

RESULTS

Plant extraction

Two types of methods were used to produce methanol, ethanol, and aqueous extracts of G. procumbens. Soxhlet extraction of the methanol extract of G. procumbens gave a yield 4.2% dry weight. On the other hand, maceration of G. procumbens with ethanol and water gave a yield of dry weight of 7.8% and 27.5% respectively.

Total phenolic content and total flavonoid content

Table 1 demonstrates the total phenolic, and total flavonoid content in the G. procumbens extracts stated as mg GAE/g extract and mg QE/g extract, respectively. The methanol extract of G. procumbens exhibits the highest total phenolic content (75.70±2.69 mg GAE/g extract) followed by ethanol extract (70.70±2.58 mg GAE/g extract). On the other hand, the total phenolic content in an aqueous extract was observed to be lower than the latter (40.00±0.59 mg GAE/g extract). The total phenolic content of G. procumbens extracts was calculated from a gallic acid standard curve with linear regression equation of y=2.2716x−0.0164 (r^2=0.9996).

Similar to total phenolic content, the total flavonoid content also varied with the type of solvent used in extraction. In this case, ethanol extract reveals the highest total flavonoid (219.35±1.17 mg of QE/g extract). It was found that the aqueous extract shows the lowest amount of total flavonoid content than the methanol extract with 64.59±0.68 mg QE/g extract and 164.31±0.67 mg QE/g extract, respectively. The total flavonoid content of G. procumbens extracts was calculated from a quercetin standard curve with linear regression equation of y=0.2898x+0.0019 (r^2=0.9971).

DPPH free radical scavenging capacity

Fig. 1 clearly shows that the DPPH radical scavenging effects of the G. procumbens extracts increased in the order of methanol > aqueous > ethanol. As shown in Fig. 1, the percentage of DPPH scavenging effect increased as the concentration of the extracts was increased until it reached a plateau. From the results obtained (Fig. 1), the methanol extract showed the lowest IC_{50} value (0.15±0.02 µg/mL) compared to the ethanol extract and aqueous extract (0.19±0.05 µg/mL and 0.21±0.02 µg/mL, respectively) (Table 2). In addition, among these three extracts of G. procumbens, the methanol extract showed the highest percentage of the DPPH scavenging effect of the lower concentration (0.0625 µg/mL). Fig. 1 also demonstrates that at concentration of 0.05 µg/mL of ascorbic acid, the graph started to plateau, which indicated that almost all free radicals in ascorbic acid were being scavenged by DPPH.

Effect of the treatment of *G. procumbens* ethanol, methanol and aqueous extract on P450-Glo™ CYP3A4 and CYP1A2 assay

In the present study, the inhibitory effects of G. procumbens extracts were investigated in recombinant human CYP3A4 and CYP1A2. The inhibitory effects of G. procumbens extracts toward CYP3A4 and CYP1A2 are shown in Fig. 2. As shown in Fig. 2a, when 250 µg/mL of ethanol extract of G. procumbens was incubated with CYP3A4 enzyme, the CYP3A4-mediated D-luciferin formation was decreased by more than 90% compared with the methanol extract. However, the result in Fig. 2a clearly demonstrates that the aqueous extract did not show significant inhibition toward CYP3A4-mediated D-luciferin formation.

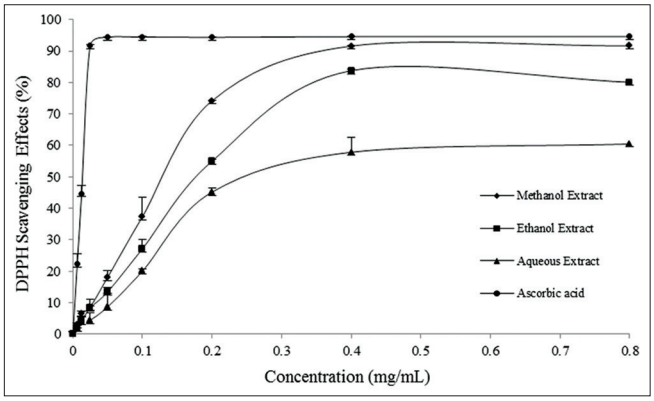

Fig. 1: 2,2-diphenyl-1-picryl-hydrazyl scavenging effect of the *Gynura procumbens* extracts with concentration range from 0.00625 and 0.8 mg/mL for all extracts. Values are expressed as mean in mg per mL ± standard error of the mean for four replicates (n=4)

The inhibitory effect of G. procumbens extracts toward CYP1A2 shows a similar pattern to CYP3A4 (Fig. 2b). At 250 µg/mL, G. procumbens extracts showed different degrees of inhibition. The ethanol extract almost depleted CYP1A2 activity with percentage of the remaining activity of 0.51%. At 500 µg/mL, both the methanol and aqueous extract of G. procumbens inhibited CYP1A2 activity with the remaining activities of 22.8% and 64.2%, respectively.

To measure the effectiveness of a compound to inhibit CYP3A4 and CYP1A2, half-maximal inhibitory concentration (IC_{50}) were determined. Based on nonlinear regression analysis shown in Fig. 3, the IC_{50} values for ethanol, methanol, and aqueous extract were 32.01±1.11 µg/mL, 229.70±1.10 µg/mL and 1611.00±1.20 µg/mL, respectively for CYP3A4 and 7.87±1.22 µg/mL, 179.90±1.17 µg/mL and 1245.00±1.24 µg/mL, respectively, for CYP1A2. Based on the IC_{50} values (Table 3), the inhibitory effects of G. procumbens toward recombinant human CYP3A4 and CYP1A2 isoforms were found to be in the following order: Ethanol extract > methanol extract > aqueous extract.

DISCUSSION

In an effort to ascertain the potential herb-drug interaction between G. procumbens and co-administered drugs, the present study had evaluated the effect of G. procumbens extracts on the enzyme activities of CYP3A4 and CYP1A2, which are known to be involved in the metabolism of most commonly prescribed drugs. The potential inhibitory effects of

Table 1: Total phenolic content and total flavonoid *Gynura procumbens* extracts. Values are expressed as mean of mg of GAE or QE per gram of extract±SEM for five replicates (n=4)

Gynura procumbens extract	Total phenolic content	Total flavonoid content
	mg GAE/g extract	mg QE/g extract
Methanol	75.70±2.69	164.31±0.67
Ethanol	70.70±2.58	219.35±1.17
Aqueous	40.00±0.59	64.59±0.68

GAE: Gallic acid equivalent, QE: Quercetin equivalent, G. procumbens: Gynura procumbens, SEM: Standard error of the mean

Table 2: IC_{50} values for DPPH scavenging activity of *G. procumbens* extracts. Values are expressed as mean in milligrams per mL±SEM for four determination (n=4). IC_{50} value for ascorbic acid was not calculated due to the high DPPH scavenging effect at the lowest concentration

Gynura procumbens extract	IC_{50} value (mg/mL)
Methanol	0.15±0.02
Ethanol	0.19±0.05
Aqueous	0.21±0.02

G. procumbens: Gynura procumbens, DPPH: 2,2-diphenyl-1-picryl-hydrazyl, SEM: Standard error of the mean

Table 3: Modulatory effects of *G. procumbens* extracts on CYP450 isoforms. Values represent the mean in microgram per mL of *G. procumbens* extracts±SEM for three replicates (n=3)

Type of extract/ positive inhibitor	IC_{50} values (µg/mL) (goodness of fit value, r^2)	
	CYP3A4	CYP1A2
Ethanol	32.01±1.11 (0.988)	7.87±1.22 (0.953)
Methanol	229.70±1.10 (0.971)	179.90±1.17 (0.939)
Aqueous	>500	>500

G. procumbens: Gynura procumbens, SEM: Standard error of the mean

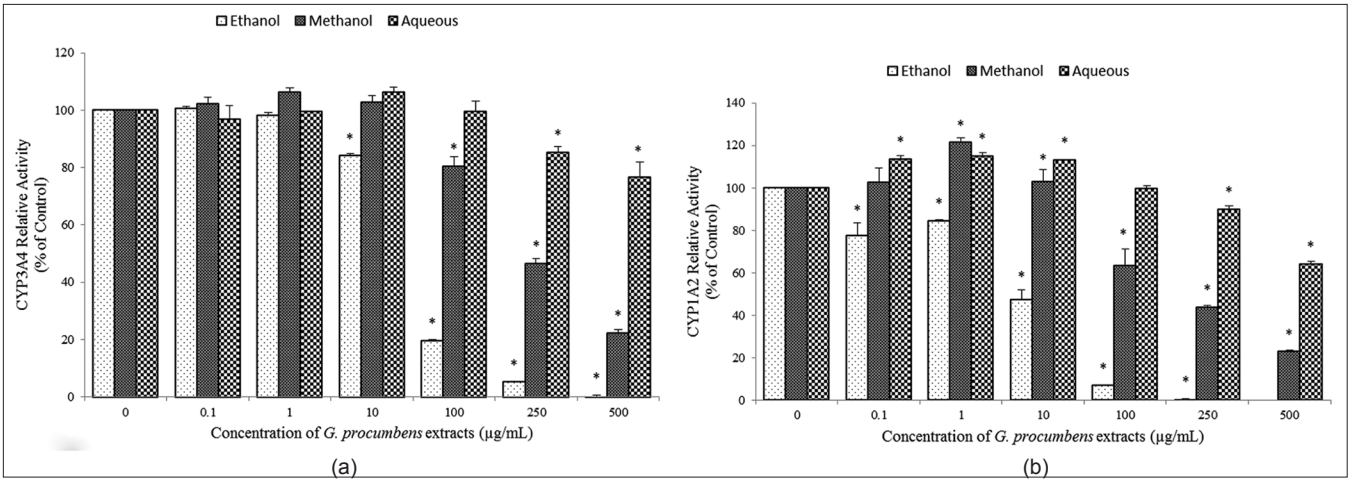

Fig. 2: Inhibitory effects of ethanol, methanol and aqueous extract of *Gynura procumbens* (0.1-500 µg/mL) towards recombinant human (a) CYP3A4 and (b) CYP1A2 isoforms activity. The value corresponds to mean of CYP450 relative activity ± standard error of the mean of three replicates (n=3). Statistical analysis was performed using one-way analysis of variance and Dunnet test. *p<0.05 versus control (without *G. procumbens* extracts)

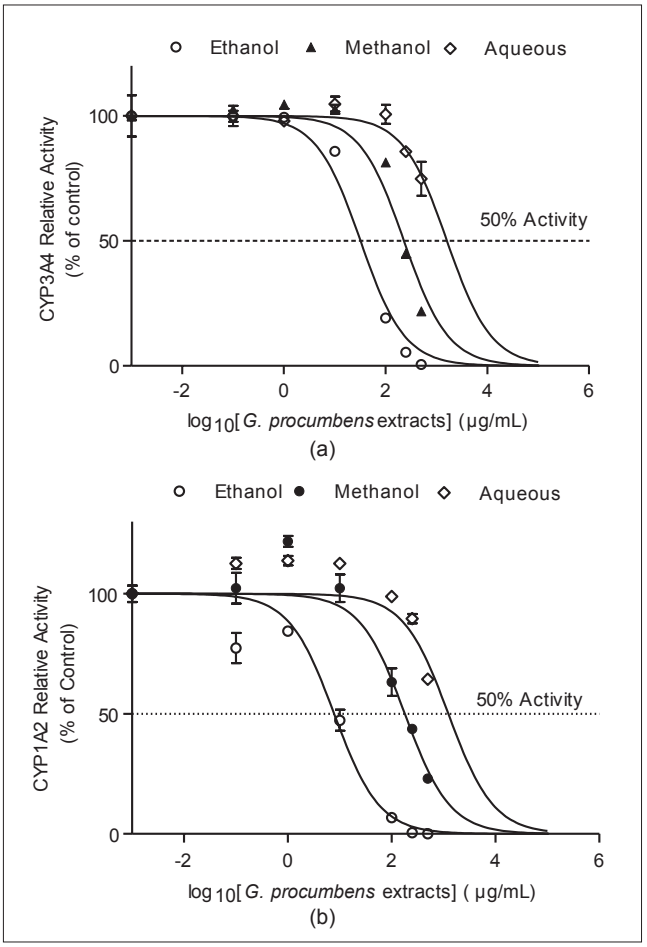

Fig. 3: Inhibitory effect *Gynura procumbens* extracts towards (a) CYP3A4-mediated; (b) CYP1A2-mediated D-luciferin formation. Each point represents the means of CYP450 relative activity ± standard error of the mean for three replicates (n=3). Goodness of fit r^2 values for both enzymes was ≥0.9

G. procumbens standardized extracts on CYP3A4 and CYP1A2 enzyme activities along with its antioxidant capacities were determined, since herbal medicines is a complex mixture of constituents that are known to cause herb-CYP interactions [3].

In accordance with the observation of this study, total phenolic content in the G. procumbens extracts can be listed in the decreasing order as follows; methanol > ethanol > aqueous. Dependence on the extraction solvent used, there could be various kinds and amount of phenolic and nonphenolic compounds extracted [24]. The mean values for total flavonoid content in this study ranged from 64.59 to 219.35 mg QE/g extract. The aqueous extract in this study showed the lowest percentage of flavonoid compounds and this result may be explained by the fact that flavonoids have low solubility in water [25]. The antioxidant activity of a compound is expressed as a relative decrease in DPPH concentration or as the concentration of a compound decrease the absorbance of DPPH by 50%. From the DPPH scavenging activity analysis, the methanol extract shows the highest radical scavenging activity followed by the ethanol and aqueous extract in decreasing order, and this pattern was similar to the rank order of the total phenolic content analysis. There was a positive connection between the two data obtained. Phenolic compounds which are present in the highest percentage in the methanol extract might be responsible for the highest radical scavenging activity observed. Mellilotis officinalis exhibited the highest content of flavonoid and phenolic compounds and showed the highest antioxidant activity among the other plants [26]. Hydroxyl groups present abundantly in the phenolic compounds may contribute to the high free radical scavenging activity [26].

There are immense numbers of publications regarding the effect of herbal medicines on drug metabolizing enzymes. However, the effects of G. procumbens on drug metabolizing enzymes have not been explored. CYP450 are the major enzymes that metabolize most pharmaceutical compounds and can be found abundantly in the liver and small intestine [6]. Most drugs are metabolized by CYP3A4, CYP2D6, CYP1A2, CYP2C9, and CYP2C19 with some contribution from the other CYPs. There is a possibility that herbal constituents may be substrates, inducers, and inhibitor of CYPs [3] and thus, may affect the biotransformation of any co-administered drugs that are metabolized using the same enzymes as the herbal constituents.

This study had investigated the effect of the whole herb on the effect of CYP450 enzyme activities for two reasons. First, herbs are believed to be a traditional medicine and people tend to eat raw the whole herb and secondly, there are possibilities of the existence of other active compound in the herb that are able to affect CYP450 expression [27]. In this study, ethanol, methanol, and aqueous extracts of G. procumbens were screened for their inhibitory effect toward P450-Glo™ CYP3A4 and P450-Glo™ CYP1A2 assay. All parameters (protein concentration and time incubation) have been optimized by the manufacturer, and

each substrate has been incubated at its K_m concentration. The ethanol extract was found to exhibit the highest inhibitory effect toward CYP3A4 compared to the methanol and aqueous extracts. Based on the result as judged by the experimentally derived IC_{50} values, the ethanol extract inhibited CYP3A4 about 10-fold more potent than the methanol extract (ethanol extract: IC_{50} value of 32.01±1.11 µg/mL; methanol extract: IC_{50} value of 229.70±1.10 µg/mL). The ethanol extract of G. procumbens was also found to exhibit the highest amount of flavonoid compounds compared to the other two extracts, and it is probable that compounds, particularly flavonoids, which exist in the ethanol extract, may be responsible for the inhibition of CYP3A4 enzyme activity. This study shows that flavonoids may be responsible for CYP3A4 inhibition as the order of CYP3A4 inhibition follows the rank order of flavonoid content (CYP3A4 inhibition order: Ethanol extract > methanol extract > aqueous extract; total flavonoid content: Ethanol extract > methanol extract > aqueous extract). Ho and co-workers found that flavones with more hydroxyl groups attach to both of the A and B rings of flavonoid showed strong inhibition toward CYP3A4 activity [21].

However, insignificant inhibitory activities of CYP3A4 by the aqueous extract were observed. It is possible to hypothesize that the aqueous extract of G. procumbens is less likely to cause herb-drug interaction. Further evidence for this hypothesis can be seen from the aqueous extract of this plant which shows no inhibition toward CYP3A4 activity, and it also contains low amount of phenolic and flavonoid compounds. The IC_{50} values obtained in this study can be categorized into classes with high (IC_{50} below 1 µM), medium (IC_{50} between 1 and 10 µM), and low (IC_{50} above 10 µM) [28]. In short, the inhibitory potential of G. procumbens (ethanol extract) toward CYP3A4 (IC_{50}=32.01±1.11 µg/mL) could have potential herb-drug interaction in the liver or intestine and hence, may significantly increase the bioavailability of co-administrated drugs and consequently increased plasma concentrations of drugs and lead to toxicity [29].

CYP1A2 is the third highest in the pool of CYP450 enzymes after CYP3A4 (~30%) and CYP2C9 (~20%) [6]. CYP1A2 metabolizes the activation of the procarcinogen to reactive intermediate which then triggers the production of cancer [30]. Numerous studies on the inhibition of CYP1A1/1A2 by flavonoids have been conducted, and most of them showed potent inhibition [20,31]. In the present study, ethanol, methanol, and aqueous extracts of G. procumbens were screened for their inhibitory effect toward human recombinant CYP1A2 the isoform. This study shows that the ethanol extract potently inhibited CYP1A2 activity to a greater extent with an IC_{50} value of 7.87±1.22 µg/mL, whereas the methanol extract inhibited CYP1A2 with an IC_{50} value of 179.90±1.17 µg/mL. The aqueous extract, however, exhibits a negligible inhibition toward CYP1A2 similar to the CYP3A4 enzyme activity. The ethanol extract of G. procumbens is the most potent CYP1A2 inhibitor of all tested extracts, similar to CYP3A4, which followed the total flavonoid content rank order (ethanol extract > methanol extracts > aqueous extract). In short, the ethanol extract shows good inhibition of CYP1A2 and CYP3A4 isoform and suggested that flavonoids, which exist abundantly in the ethanol extract of G. procumbens may be responsible for this inhibition.

In vitro screening for herbs that inhibit cytochrome P450 is well-established, however, further in vivo studies are warranted for confirmation. In deciding whether the in vitro assessment requires further investigation in vivo, Strandell et al. (2004) proposed determination of volume per dose index (VDI)[32]. To aid in measuring the potential of IC_{50} concentration of G. procumbens toward CYP450 in vivo, VDI was calculated. The VDI is known as the volume in which one dose should be dissolved to reach IC_{50} concentration. The calculation was carried out based on G. procumbens product which was available in the market. Table 4 shows the estimation of IC_{50} in volume per dose unit based on 400 mg per capsule of G. procumbens sold in the market. Only the ethanol extract exceeded the 0.8 L/dose which is the cutoff point proposed by Strandell et al. (2004) for CYP3A4 and CYP1A2 [32]. The aqueous extract shows negligible effect on CYP3A4 and CYP1A2 while the methanol extract exhibits a moderate degree of inhibition

Table 4: Inhibition of CYP enzymes by *G. procumbens* extracts. The data represent as liter per dose unit to a cutoff value of 0.8 L/dose

G. procumbens extract	VDI (L/dose)	
	CYP3A4	CYP1A2
Ethanol	12.05	50.83
Methanol	1.74	2.22
Aqueous	n.d	n.d

*n.d: No observed inhibition, i.e., <0.8 L/dose unit, VDL: Volume per dose index, G. procumbens: Gynura procumbens

(IC_{50} values of 1.74 and 2.22 L/dose). Nevertheless, the ethanol extract of G. procumbens shows the most potent inhibition among the other two extracts for CYP3A4 and CYP1A2 enzymes with a VDI value of 12.05 L/dose and 50.83 L/dose, respectively. VDIs of the ethanol extract for both CYP3A4 and CYP1A2 implicate strong degree of inhibition as only volumes approaching and exceeding 4 L/dose (the body blood volume) are considered large enough to cause in vivo interaction [32].

CONCLUSION

The results of the total phenolic content analysis and DPPH scavenging effect were as follows: Methanol extract > ethanol extract > aqueous extract. In contrast, the result of total flavonoid content analysis shows a different ranking as follows: Ethanol extract > methanol extract > aqueous extract. This study revealed that the extract with the highest content of flavonoids shows the highest inhibition of CYP3A4 and CYP1A2 enzyme activities. Taken together, we conclude that the ethanol extract of G. procumbens shows the most potent inhibitory toward CYP3A4 and CYP1A2 enzyme while the methanol extract exhibits moderate inhibitory effect. On the other hand, the aqueous extract shows negligible effect on CYP activities. Since most individuals consume the leaves of G. procumbens either raw or boiled with water, the findings of this study suggest that the aqueous extract may be safe to be taken with most other drugs, and herb-drug interaction is unlikely to occur. Nevertheless, the inhibitory effect of ethanol extract of G. procumbens on CYP1A2 suggests that this extract may have the possibility to act as anti-carcinogen in human. Nonetheless, in vivo studies in animals and ultimately in humans should be done in order to verify this in vitro study.

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REFERENCES

1. Hansen TS, Nilsen OG. In vitro CYP3A4 metabolism: Inhibition by Echinacea purpurea and choice of substrate for the evaluation of herbal inhibition. Basic Clin Pharmacol Toxicol 2008;103(5):445-9.
2. Ernst E. Herb-drug interactions: Potentially important but woefully under-researched. Eur J Clin Pharmacol 2000;56(8):523-4.
3. Zhou S, Koh HL, Gao Y, Gong ZY, Lee EJ. Herbal bioactivation: The good, the bad and the ugly. Life Sci 2004;74(8):935-68.
4. Wang X, Cheung CM, Lee WY, Or PM, Yeung JH. Major tanshinones of Danshen (Salvia miltiorrhiza) exhibit different modes of inhibition on human CYP1A2, CYP2C9, CYP2E1 and CYP3A4 activities in vitro. Phytomedicine 2010;17(11):868-75.
5. Iwata H, Tezuka Y, Kadota S, Hiratsuka A, Watabe T. Identification and characterization of potent CYP3A4 inhibitors in Schisandra fruit extract. Drug Metab Dispos 2004;32(12):1351-8.
6. Guengerich FP. Cytochromes P450, drugs, and diseases. Mol Interv 2003;3(4):194-204.
7. Guengerich FP. In vitro techniques for studying drug metabolism. J Pharmacokinet Pharmacodyn 1996;24(5):521-33.
8. Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994;26(1-2):165-83.
9. Slaga TJ, Gleason GL, DiGiovanni J, Sukumaran KB, Harvey RG. Potent tumor-initiating activity of the 3,4-dihydrodiol of 7,12-dimethylbenz(a) anthracene in mouse skin. Cancer Res 1979;39:1934-6.

10. Kim H, Choi HK, Jeong TC, Jahng Y, Kim DH, Lee SH, et al. Selective inhibitory effects of mollugin on CYP1A2 in human liver microsomes. Food Chem Toxicol 2013;51:33-7.
11. Bhore S, Vaishana K. Comparison of three plant tissue culture media for efficient micropropagation of an important tropical medicinal plant, Gynura procumbens (Lour) Merr. Am Eurasia J Agric Environ Sci 2010;8(4):474-81.
12. Saiman MZ, Mustafa NR, Schulte AE, Verpoorte R, Choi YH. Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of Gynura procumbens. Plant Cell Tissue Organ Cult 2012;109(3):465-75.
13. Kim MJ, Lee HJ, Wiryowidagdo S, Kim HK. Antihypertensive effects of Gynura procumbens extract in spontaneously hypertensive rats. J Med Food 2006;9(4):587-90.
14. Rosidah, Yam MF, Sadikun A, Ahmad M, Akowuah GA, Asmawi MZ. Toxicology evaluation of standardized methanol extract of Gynura procumbens. J Ethnopharmacol 2009;123(2):244-9.
15. Hassan Z, Yam MF, Ahmad M, Yusof AP. Antidiabetic properties and mechanism of action of Gynura procumbens water extract in streptozotocin-induced diabetic rats. Molecules 2010;15(12):9008-23.
16. Lee HW, Hakim P, Rabu A, Sani HA. Antidiabetic effect of Gynura procumbens leaves extracts involve modulation of hepatic carbohydrate metabolism in streptozotocin-induced diabetic rats. J Med Plants Res 2012;6(5):796-812.
17. Zahra A, Kadir F, Mahmood A, Al Hadi A, Suzy S, Sabri S, et al. Acute toxicity study and wound healing potential of Gynura procumbens leaf extract in rats. J Med Plants Res 2011;5(12):2551-8.
18. Iskander MN, Song Y, Coupar IM, Jiratchariyakul W. Antiinflammatory screening of the medicinal plant Gynura procumbens. Plant Foods Hum Nutr 2002;57(3-4):233-44.
19. Yam M, Sadikun A, Asmawi M. Antioxidant potential of Gynura procumbens. Pharm Biol 2008;46(9):616-25.
20. Zhai S, Dai R, Friedman FK, Vestal RE. Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. Drug Metab Dispos 1998;26(10):989-92.
21. Ho PC, Saville DJ, Wanwimolruk S. Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. J Pharm Pharm Sci 2001;4(3):217-27.
22. Juan MY, Chou CC. Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with Bacillus subtilis BCRC 14715. Food Microbiol 2010;27(5):586-91.
23. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992;40(6):945-8.
24. Sun T, Ho CT. Antioxidant activities of buckwheat extracts. Food Chem 2005;90(4):743-9.
25. Algariri K, Meng KY, Atangwho IJ, Asmawi MZ, Sadikun A, Murugaiyah V, et al. Hypoglycemic and anti-hyperglycemic study of Gynura procumbens leaf extracts. Asian Pac J Trop Biomed 2013;3(5):358-66.
26. Pourmorad F, Hosseinimehr S, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr J Biotechnol 2006;5(11):1142-5.
27. Pekthong D, Martin H, Abadie C, Bonet A, Heyd B, Mantion G, et al. Differential inhibition of rat and human hepatic cytochrome P450 by Andrographis paniculata extract and andrographolide. J Ethnopharmacol 2008;115(3):432-40.
28. Krippendorff BF, Lienau P, Reichel A, Huisinga W. Optimizing classification of drug-drug interaction potential for CYP450 isoenzyme inhibition assays in early drug discovery. J Biomol Screen 2007;12(1):92-9.
29. Appiah-Opong R, Commandeur JN, van Vugt-Lussenburg B, Vermeulen NP. Inhibition of human recombinant cytochrome P450s by curcumin and curcumin decomposition products. Toxicology 2007;235(1-2):83-91.
30. Guengerich FP. Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. Cancer Res 1988;48(11):2946-54.
31. Tsyrlov IB, Mikhailenko VM, Gelboin HV. Isozyme- and species-specific susceptibility of cDNA-expressed CYP1A P-450s to different flavonoids. Biochim Biophys Acta 1994;1205(2):325-35.
32. Strandell J, Neil A, Carlin G. An approach to the in vitro evaluation of potential for cytochrome P450 enzyme inhibition from herbals and other natural remedies. Phytomedicine 2004;11(2-3):98-104.