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ORIGINAL ARTICLE

Inhibitory Effects of *Ficus deltoidea* Extracts on UDP-glucuronosyltransferase and Glutathione S-transferase Drug-Metabolizing Enzymes

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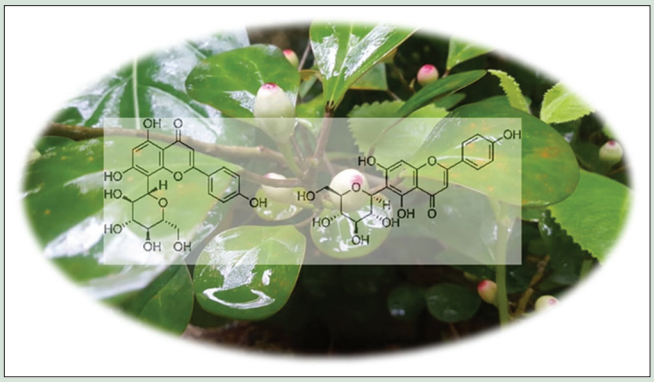
ABSTRACT

Background: Mas cotek or *Ficus deltoidea* is conventionally used to treat various diseases and often consumed with other medication and this may give rise to herb–drug interaction. The potential of *F. deltoidea* for interactions with drug-metabolizing enzymes of UDP-gucuronosytranserase (UGT) and glutathione S-transferase (GST) have not been investigated. **Objective:** We had evaluated the potential of methanol, ethanol, and aqueous extracts of *F. deltoidea* to cause UGT- and GST-mediated herb–drug interaction *in vitro*. **Materials and Methods:** The total phenolic content and total phenolic content were determined using modified colorimetric method. In the UGT study, *para*-nitrophenol (*p*-NP) was employed as a substrate to determine the UGT enzymes activity in rat liver microsomes (RLM) and human liver microsomes (HLM). For the GST study, 1-chloro-2,4-dinitrobenzene was employed as a substrate to determine GST activity in rat liver cytosolic fraction. **Results:** The total phenolic content in *F. deltoidea* extracts can be ranked as follows: Methanol extract > aqueous extract > ethanol extract, whereas the content of flavonoid compounds in *F. deltoidea* extracts can be ranked as: Methanol extract > ethanol extract > aqueous extract. Assessment using the UGT enzymes of RLM (IC_{50} [Half-maximal inhibitory concentration] = 881.40 ± 1.14 μg/mL) and HLM (IC_{50} = 63.44 ± 1.20 μg/mL) showed that the methanol extract of *F. deltoidea* significantly inhibited *p*-NP glucuronidation compared with ethanol and aqueous extracts. For GST inhibition study, methanol extract strongly inhibited GST activity (IC_{50} = 70.73 ± 1.07 μg/mL), whereas no IC_{50} values were determined for ethanol and aqueous extracts. **Conclusion:** The methanol extract of *F. deltoidea* containing the highest flavonoid content highlights the possibility of herb–drug interaction through the modulation of *p*-NP UGT and GST activity.

Key words: 1-chloro-2, 4-dinitrobenzene, *Ficus deltoideia*, glutathione S-transferases, herb–drug interactions, p-nitrophenol, uridine 5′-diphospho glucuronosyltransferases

SUMMARY

- The potential of methanol, ethanol, and aqueous extracts of *Ficus deltoidea* to cause UDP-gucuronosytranserase (UGT)- and glutathione S-transferase (GST)-mediated herb–drug interaction in rat liver microsomes and human liver microsomes by *in vitro* were evaluated. The UGT inhibition study showed that the methanol extract of *Ficus deltoidea* significantly inhibited *para*-nitrophenol (*p*-NP) glucuronidation compared with ethanol and aqueous extracts. For GST inhibition study, methanol extract strongly inhibited GST activity, whereas no IC_{50} values were determined for ethanol and aqueous extracts. The methanol extract of *Ficus deltoidea* containing the highest flavonoid content highlights the possibility of herb–drug interaction through the modulation of *p*-NP UGT and GST activity.



Abbreviations Used: UGT: UDP-gucuronosytranserase; GST: Glutathione S-transferase; *para*-nitrophenol; RLM: Rat liver microsomes; HLM: Human liver microsomes; *p*-NP: CDNB: 1-chloro-2,4-dinitrobenzene; IC_{50}: Half-maximal inhibitory concentration; CYP450: Cytochrome P450; SULTs: Sulfotransferases; GAE: Gallic acid equivalent; QE: Quercetin equivalent; DPPH: 2,2-diphenyl-1-picrylhydrazyl; V_{max}: Maximal velocity of reaction; K_m: Michaelis constant; SD: standard deviation; ND: not determined.

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INTRODUCTION

Over the past decades, herbal plants have been increasingly used in disease treatment and are assumed to be harmless. This is because herbal plants are natural-based product, and for this reason, herbal plants are also not subjected to the scrutiny of the approval process as applied to the new drug applications.[1,2] Herbal plants also contain numerous important chemical constituents such as alkaloids, flavonoids, anthraquinones, polyphenols, terpenoids, glycosides, coumarins, saponins, and tannins. These chemical constituents in herbal plants are likely to be substrates, inhibitors, or inducers of drug-metabolizing enzymes.[3]

"Mas cotek" scientifically known as *Ficus deltoidea* is a traditional medicinal plant of Malaysia. Conventionally, mas cotek is used for treating diabetes, high blood pressure, heart problems, gout, diarrhea, pneumonia, and skin diseases.[4] Mas cotek also exhibits antioxidant

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hypoglycemic and antinociceptive properties.[5,6] Other than that, the plant has also been used in the treatment of migraine and conventionally or has been consumed as herbal drink by post delivery women to strengthen the uterus.[5] However, some of the herbal medicines may result in herb–drug interactions on co-administration with prescribed drugs.[7] Interaction of several plants with phase I and phase II enzymes has been reported.[7] The interaction of herbal extracts or their constituents with drug-metabolizing enzymes has been associated with alterations in the pharmacokinetics of drugs such as midazolam.[7] The interaction may involve both induction and inhibition of enzymes activities, the latter being more common and sometimes cause harmful side effects.[8,9]

In general, drug-metabolizing enzymes play a vital role in eliminating foreign compounds (xenobiotics), herbal plant constituents, and endogenous substances by increasing the compound solubility through the molecular structure functionalization processes in Phase I and/or conjugation reactions in Phase II. The major is cytochrome P450 (CYP450) of phase I-metabolizing enzymes, chemically modify and prepare the compounds molecular structure for the subsequent Phase II metabolism.[10,11] Phase II is considered as the true "detoxification" reaction as it produces metabolites that are generally water-soluble and easily excreted.[12] Examples of Phase II drug-metabolizing enzymes are UDP-glucuronosyltransferases (UGTs), sulfotransferases, and glutathione S-transferases (GSTs). Inhibition of drug-metabolizing enzymes can cause harmful side effects such as increased parent drug's plasma level, prolonged pharmacological effects of the parent drug, and enhancement of drug-induced toxicity.[13]

Glucuronidation, catalyzed by UGT enzymes is the most important phase-II conjugation reaction.[12] During glucuronidation reaction, a glucose-derived moiety glucuronic acid is conjugated to a suitable functional site (hydroxyl, carboxyl, carbonyl, sulfhydryl, and amine) on a substrate modulated by UGT proteins. In general, this reaction leads to the formation of the respective β-D-glucuronides with easy elimination by bile or urine.[12]

The second-most important phase II detoxification enzyme is GST.[14] GSTs are involved in the metabolism of xenobiotics and play an important role in cellular protection against oxidative stress.[12] GSTs have shown antioxidant properties and account for multifunctional roles in cell defense systems against electrophilic compounds.[15] Generally, GST enzymes catalyze conjugation reactions between glutathione (GSH) and an electrophile compound by the formation of a thioether. This reaction generally used 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, which can react enzymatically with the nucleophile.

Many drugs are highly metabolized by the UGT and GST enzymes without the requirement of CYP450 metabolism, for example, zidovudine, morphine, and codeine, which are metabolized primarily through UGT enzymes. Meanwhile, cancer chemotherapeutic agents that are potent substrates for GSTs include adriamycin and thiotepa.[16-18] Many herbal plant extracts can inhibit UGT enzymes, for example, *Silybum marianum*, *Serenoa repens*, *Vaccinium macrocarpon*, *Camellia sinensis,* and *Rographis paniculate,* and *Orthosiphon stamineus*.[19-21] Similarly, several plant extracts were also known to inhibit GST enzymes, for example, *Cinnamomum iners*, *O. stamineus,* and *Croton argyratum*.[15] Therefore, there is a potential for herb–drug interaction through competition with numerous drugs for this conjugation pathway.[19] When herbal plants and drugs compete for the same drug-metabolizing enzymes and depending on their relative affinities and inhibitory potencies, this may result in inhibition of metabolism either of herbal plants or drugs.[10]

Till date, the inhibitory effect of *F. deltoidea* on UGT and GST drug-metabolizing enzymes has yet to be reported. Even though CYP450 in Phase I contribute toward the bioactivation of numerous drugs and endogenous compounds, the present study is focused on the phase-II pathways that would ultimately affect detoxification fate of the drugs, i.e., the UGT and GST enzymes. Therefore, we aim to determine the effect of methanol, ethanol, and aqueous *F. deltoidea* extracts on Phase-II UGT and GST drug-metabolizing enzymes activities. The three different extraction solvents with respective pf phenolic and flavonoid content along with antioxidant capacity may have different potential in modulating drug-metabolizing enzymes activities. Methanol was used in the extraction process of *F. deltoidea* because this solvent has been recorded to elute more phenolic and flavonoid compounds.[22] Ethanol was used in the extraction process of *F. deltoidea* because it is more likely to be used in industry, and it is less toxic compared to other organic solvent. In addition, the aqueous extraction process was also studied because it creates the same classical preparation of traditional beverage consumed by most people for example tea.

Besides, the determination of total phenolic, flavonoid content, and antioxidant activity of the herb extracts were also carried out to investigate the involvement of these phytochemicals in modulating drug-metabolizing enzymes.

MATERIALS AND METHODS

Materials

The whole plant of *F. deltoidea* (1 kg) was purchased from Herbagus Sdn. Bhd., Bertam, Kepala Batas, Penang, Malaysia, authenticated by Dr. Rahmad Zakaria and deposited at the Herbarium of School Biological Science, Universiti Sains Malaysia (USM) with assigned voucher specimen number 11517. Standard solution: Gallic acid, ascorbic acid, and diclofenac, were purchased from Sigma Aldrich, USA, while tannic acid was purchased from R and M Chemicals, Canada. Pooled human liver microsomes (HLM) Product No. M0567 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The pooled HLM were stored at −80°C fridge until used.

Methods

Preparation of the methanol, ethanol, and aqueous extracts of Ficus deltoidea

The dried leaves of *F. deltoidea* (100 g) were ground into powder followed by extraction with water at 50°C for 3 h in the water bath. Extraction of *F. deltoidea* with methanol and ethanol was done using respective solvents (1 L) through maceration for about 24 h at ambient temperature. The extraction processes were repeated three times on the Marc.[23] The filtered ethanol and methanol extracts were then concentrated under reduced pressure using a rotary evaporator. The extracts were then subjected to lypophilization by a freeze-dryer to produce powdered forms of the extract. The methanol, ethanol, and aqueous extracts were prepared in methanol, ethanol, and distilled water, respectively, to obtain a stock solution of 100 mg/mL and stored at −20°C until use.

Determination of the total phenolic content in Ficus deltoidea extracts

Total phenolic content of *F. deltoidea* extracts were determined using the Folin–Ciocalteu method described by Juan and Chou.[24] An aliquot of 0.1 mL gallic acid or 0.1 mL extract (from 100 μg/mL stock solution) was mixed with 1.0 mL Follin–Ciocalteu phenol reagent and allowed to react for 3 min. Then, 300 μL of 1N sodium carbonate was added and allowed to stand for 90 min at ambient temperature. After this, the absorbance at 725 nm was measured using microplate reader (PlateCHAMELEON™ multi-technology plate reader 425–106). The experiment was carried out in triplicate for each concentration in the standard curve of gallic acid (0.02–0.8 μg/mL) and sample (100 μg/mL). Total phenolic content in the *F. deltoidea* extracts were calculated using the following formula:

$$C = c \cdot \frac{V}{m},$$

Where C is the total phenolic content, mg per g plant extracts in gallic acid equivalent (GAE); c is the concentration of gallic acid established from the calibration curve, mg/L. Where, V is the volume of extract, L; m' is the weight of plant extract, g. Results were expressed as milligrams of GAE/g of dry weight (mg GAE per g extracts). All experiments were carried out in triplicates.

Determination of total flavonoid content in Ficus deltoidea extracts

The total flavonoid content of the sample was determined using a modified colorimetric method, which was previously described by Zhishen *et al*.[25] Quercetin was used as the standard. An aliquot of extract solution (250 µL from 100 µg/mL stock solution) was mixed with 1.25 mL distilled water, respectively. The mixture was mixed with 75 µL of 5% (v/v) sodium nitrite solution. After standing for 6 min, the mixture was combined with 1500 µL of 10% aluminum chloride. About 0.5 mL of 1M sodium hydroxide and 275 µL of distilled water were added after 5 min later. The absorbance of the solution at 510 nm was then measured using microplate reader (PlateCHAMELEON™ multitechnology plate reader 425–106). A calibration curve using quercetin in a concentration range of 0.02–0.4 mg/mL was prepared. The total flavonoid content of *F. deltoidea* extracts was expressed as quercetin equivalent (QE), which reflected the flavonoid content as the amount of quercetin in *F. deltoidea* extract. All experiment was performed in triplicates.

Determination of 2,2-diphenyl-1-picrylhydrazyl scavenging capacity of Ficus deltoidea extracts

The free radical scavenging activity of *F. deltoidea* extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH).[26] One mg/mL of all samples were dissolved in methanol. All samples were prepared from different concentrations (6.25–100 µg/mL) and 200 µL of samples from each concentration was loaded into 96 well plate. To this mixture, 50 µL of DPPH (1 mM) solution was added into each of the well plate. After 30 min incubation at room temperature (22–24°C) in a dark place, the absorbance was measured at 517 nm using microplate reader (PlateCHAMELEON™ multitechnology plate reader 425–106) against methanol as the blank. Free radical scavenging activity of the *F. deltoidea* extracts and ascorbic acid as positive control were determined according to the following formula:

$$\text{Free radical scavenging activity } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

Where A_s is the absorbance of DPPH and sample, A_c is the absorbance of control.

Human liver microsomes and preparation of rat liver microsomes

Pooled HLM were obtained from Sigma-Aldrich (St. Louis, MO, USA). The concentration of protein in the test sample was interpolated by comparing the absorbance values to the BSA standard curves. Sprague-Dawley male rats were obtained from the Animal House, USM. Since the sex was not accounting for a biological variable in this study, Sprague-Dawley male rats were preferably used as *in vitro* model. This is because male rats have no hormone fluctuations associated with the female reproductive cycle, and it may influence the result.[27] The rats were placed under 12 h light and 12 h dark conditions with controlled temperature (25°C ± 2°C) for 7 days before experiments to give them a stable habituation period before the actual experiment started.[28] Water and food *ad libitium* were given to the rats. Animals were maintained and handled according to the recommendations of the USM ethical committee, which approved the design of the animal experiments with the reference number USM/Animal Ethics Approval/2011/(72) (340).

Six rats were obtained and remain untreated. Euthanasia was performed with overdose of carbon dioxide in a chamber until the rats were sacrificed. The livers were then removed and weighed using analytical balance (MettlerToledo AL204 laboratory balance) before experiments. The livers were rinsed with distilled water (ice-cooled) followed by ice-cooled 67 mM potassium phosphate buffer (pH 7.4) to flush out the blood, blotted dry, and weighed using analytical balance (MettlerToledo AL204 laboratory balance). Isolated rat liver samples were homogenized in 67 mM potassium phosphate buffer (pH 7.4) with 1.15% (w/v) KCl. The volume of buffer used for homogenization using Potter-Elvehjem homogenizer is three times the weight of the liver samples. After centrifugation of the homogenate fraction at 12,500 ×*g* for 20 min at 4°C, the resultant supernatant was transferred to ultracentrifuge tubes (Optiseal™) and centrifuged at 100,000 ×*g* for 60 min in a refrigerated ultracentrifuge (Optima™ TLX, Beckman Coulter, Inc., USA). The microsomal pellets were resuspended in 300 µL 67 mM potassium phosphate buffer with 1.15% KCl and 20% (v/v) glycerol. The pooled microsomes were homogenated again to mix the solution properly and stored frozen at −80°C until used. Protein concentrations were determined by the method described previously[29] with modification.[30]

Maximal velocity of reaction and Michaelis constant determination

Incubation conditions were chosen such that product formation was linear with respect to both microsomal protein amount and incubation time for the determination of UGT activities toward *para*-nitrophenol *(p*-NP) in rat liver microsomes (RLM) (0.5 mg/L) and HLM (0.1 mg/L). The incubation time for the determination of UGT activities in RLM and HLM was 30 min, respectively. Substrate concentrations for the determination of UGT activities were 50–3000 mM. The Michaelis–Menten parameters, such as michaelis constant (K_m) and maximal velocity of reaction (V_{max}), were determined using GraphPad Prism® 5 (Version 5.01, GraphPad Software, Inc., USA) and expressed as means ± standard error of the mean of triplicates.

UDP-glucuronosyltransferase enzymes activity assay

The effect of *F. deltoidea* UGT enzymes activity in rat liver and HLM toward *p*-NP as substrates was determined using the spectrophotometric method. The assay was conducted following previously published procedure[31] with slightly modification on optimization in our own laboratory. The assays were performed at a substrate concentration that was closed to the apparent K_m value.

The p-nitrophenol UDP-glucuronosyltransferase activity assay in rat liver microsomes and human liver microsomes

The *p*-NP was used as probe substrate for UGT enzyme activity in RLM and HLM. In brief, in the incubation mixture (final volume, 200 µL) consisted of microsomal protein (0.5 mg/mL and 0.1 mg/mL for RLM and HLM, respectively), Triton X-100 (0.01% and 0.003% for RLM and HLM, respectively), 50 mM $MgCl_2$, 1M Tris-HCl (pH 7.4) and *p*-NP. The concentration of *p*-NP in incubation was 0.5 mM, which correspond to the K_m in RLM and HLM. The reaction was started by adding 30 mM of UDPGA. After the mixture was incubated for 30 min at 37°C, the reaction was stopped by adding 20% trichloroacetic acid vortex mixing and placing tubes on ice. After 5 min, the tubes were centrifuged at 2000 rpm for 10 min and the supernatant was transferred to the other tubes and mixed with 0.5M NaOH to develop the yellow color of *p*-NP. After 10 min incubation, 200 µL of the mixture was transferred into the 96-well plate and measured the absorbance at 405 nm. Screening experiments were performed by adding *F. deltoidea* (aqueous, ethanol,

and methanol extracts) at five different concentrations to the incubation mixture. Incubation with and without diclofenac (0.1–1000 μM) were performed to serve as positive and negative controls, respectively.

Calculation of para-nitrophenol UDP-gucuronosytranserase specific activity

The *p*-NP glucuronidation was quantified by measuring the decrease in absorbance at 405 nm. The results were expressed as nmol glucuronide formed/min/mg microsomal protein. UGT-specific activity was reported as percent specific activity over control. The equation can be illustrated as follows:

$$\text{UGT specific activity} = \frac{\left[p-NPG\right](\mu M)}{Incubation\ time\ (min)\times Amount\ of\ protein\ used\ (mg/mL)}$$

Glutathione S-transferase enzymes activity assay

The effect of *F. deltoidea* GST enzymes activity toward CDNB as substrates was also determined using the spectrophotometric method. The method of GST enzymes assay was performed based on Habig *et al*.[32] with slightly modification and optimization in our own laboratory. The assays were performed at a substrate concentration that was closed to the apparent K_m value.

The glutathione S-transferase enzymes activity assay in rat liver cytosolic fractions

Briefly, 300 μL incubation mixture in the 96-well plates contained 10 μL of distilled water, 150 μL of 200 mM potassium buffer (pH 6.5), and 10 μL of 30 mM of GSH. After that, 60 μL of 0.625 mg/mL of rat liver cytosolic fraction was added into the mixture so that the final concentration was 0.125 mg/mL, followed by the addition of 60 μL of five times concentration of test samples (*F. deltoidea* and tannic acid as positive control, respectively). The reaction was started on the addition of 10 μL of 30 mM CDNB. Conjugation activity was measured at 340 nm for 5 min with 30 s time intervals. Blank group for each test sample contained denatured rat liver cytosolic fraction. Blank group for control group contained no test sample. The reaction was conducted in five replicates.

Calculation of glutathione S-transferase specific activity

The conjugation activity was expressed as μmol of CDNB conjugated produces per min per mg of protein. The extinction coefficient for CDNB for GST assay is 9.6 mM^{-1} for 1 cm solution path length. Since this present experiment was conducted in 300 μL reaction mixture in the microtiter well plate, path length equivalent to 0.786 cm. Hence, the extinction coefficient of CDNB for this experiment was calculated to be 7.55 mM^{-1}. GST-specific activity was calculated using the equation below:

$$\text{GST specific activity} = \frac{\left(\Delta ABS_{340}\right)\frac{\text{test}}{\text{minute}}-\frac{\left(\Delta ABS_{340}\right)\text{blank}}{\text{minute}}}{\text{Extinction Coefficient}_{CDNB}\times \text{DNBnction Coefficient seasen}\left(;\frac{mg}{mL}\right)}$$

Statistical analysis

The remaining enzyme activities were calculated and expressed as a percentage of control. The remaining enzyme activities and herbal extract concentration data were fitted in IC_{50} equation using GraphPad Prism® 5 Version 5.01, GraphPad Software, Inc., USA) and expressed as means ± standard deviation of five replicates. The goodness of fit R^2 values was ≥0.9 for all reported IC_{50} value. The significant difference of the results obtained was evaluated using one-way analysis of variance used of Dunnet test. A value of $P < 0.5$ was considered statistically significant.

RESULTS AND DISCUSSION

Solvent extraction yield

In this study, methanol and distilled water were used as solvents with different polarities to extract *F. deltoidea* leaves. The solvent extraction method is commonly used to obtain antioxidants from plant materials.[24] By using different polarities of solvent extraction, it is expected that there would be variable amounts of phenolic and nonphenolic compounds such as protein sugars organic acid and pigments present in the prepared solvent extracts.[33]

Table 1 shows the extraction yields of *F. deltoidea* leaves from various solvents. All the extraction yields of *F. deltoidea* ranged from 5.28% to 5.89%. The extraction yields found closed each other. Therefore, this study showed that the extraction yields were not dependent on the extraction solvent. Contrary to Sun and Ho[33] and Juan and Chou,[24] variation in the extraction yield was found to be dependent on extraction solvent.

Determination of total phenolic content, total flavonoid and 2,2-diphenyl-1-pi crylhydrazyl scavenging capacity of *Ficus deltoidea* extracts

The total phenolic content of *F. deltoidea* extracts was expressed as mg of GAE/g of extract (mg GAE/g). It is calculated from a gallic acid standard curve and summarized in Table 1. The methanol extract showed the highest total phenolic content, followed by aqueous extract and ethanol extract. Therefore, this study showed that the total phenolic content depends on the type of solvent used for extraction. A study by Juan and Chou[24] had also found similar results. In their study, the acetone extract of black soybean showed the highest total phenolic content followed by ethanol, methanol, and water extract of the black soybean.

Flavonoids are plant secondary metabolites. The classes of flavonoids include chalcones flavones, flavonols, flavanones, flavonols, anthocyanins, and isoflavones.[34] Consumption of flavonoid-containing fruits and vegetables has been linked to protection against cancer and heart disease.[35] The total flavonoids of *F. deltoidea* extracts were expressed as mg QE per g of extract (mg QE/g). It is calculated from a quercetin standard curve and summarized in Table 1. Similar to total phenolic content, methanol extract also exhibits the highest total flavonoid content, followed by ethanol extract and aqueous extract.

Table 1: Percentage yield, total phenolic, total flavonoid, Half-maximal inhibitory concentration (IC_{50}) of 2,2-diphenyl-1-picrylhydrazyl radical scavenging and the amount of vitexin and isovitexin in *Ficus deltoidea* extracts

Ficus deltoidea extract	Percentage yield	Total phenolic (mg GAE/g)	Flavonoid content (mg QE/g)	DPPH scavenging IC_{50} (μg/mL)	Vitexin (mg/g)	Isovitexin (mg/g)
Methanol	5.28	159.80±2.95	457.79±1.26	37.07±0.67	21.18±0.87	18.39±0.67
Ethanol	5.89	74.29±1.29	208.48±0.75	275.85±0.15	3.95±0.20	3.88±0.17
Aqueous	5.48	90.80±0.65	110.17±0.58	129.00±1.09	4.06±0.03	6.22±0.03
Reference					Zulkiffli, 2015	Zulkiffli, 2015

Results are expressed as mean values ± SD for three replicates (n=3). SD: Standard deviation; IC_{50}: Half-maximal inhibitory concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic acid equivalent; QE: Quercetin equivalent

Antioxidant activities of *F. deltoidea* extracts were investigated by DPPH scavenging inhibition method. DPPH is a stable free radical. The DPPH percent radical scavenging activity (% RSA) of the *F. deltoidea* extracts compared with ascorbic acid is shown in Figure 1 and summarized in Table 1. Table 1 shows the calculated half-inhibition concentration (IC_{50}), which is the effective concentration of *F. deltoidea* extract required to decrease initial DPPH concentration to 50%. These IC_{50} values were obtained by interpolation from the linear regression analyses of data shown in Figure 1. The methanol extract showed the lowest IC_{50}, corresponding to the highest DPPH-radical scavenging activity followed by aqueous extract and ethanol extract.

Based on the data obtained, the result of antioxidant activities of this plant showed that the fraction that is rich with phenolic compounds showed the highest total antioxidant capacities.[36] This is in agreement with other studies that support the role of phenolic compounds in antioxidant activity.[37] This study showed that the higher content of vitexin and isovitexin compounds in *F. deltoidea* extracts showed a stronger antioxidant activity.

Vitexin and isovitexin content in *F. deltoidea* extracts were quantified previously in our laboratory.[38] The weight percentage of vitexin and isovitexin in each extract of *F. deltoidea* is presented in Table 1. Study from our laboratory have shown that methanol extract of *F. deltoidea* contained the highest amount of vitexin (21.18 ± 0.87 mg/g) and isovitexin (18.39 ± 0.67 mg/g) followed by aqueous extract (4.06 ± 6.22 mg/g and 6.22 ± 0.03 mg/g, respectively) and ethanol extract (3.95 ± 0.02 mg/g and 3.88 ± 0.17 mg/g, respectivey).[38] While, this study shown that *F. deltoidea* methanol extract showed good antioxidant activity compared to the other extracts. Therefore, it is suggested that the higher the content of vitexin and isovitexin in each extract, the stronger is the antioxidant activity.

Vitexin and isovitexin are main flavone *C*-glycosides and had been documented to correlate with good antioxidant activity.[39] Flavone *C*-glycosides is a kind of important constituents of the flavonoid family. It present in foodstuffs and nutraceuticals and received much attention recently because of their antioxidant and anticancer properties.[40] The vitexin and isovitexin structure are shown in Figure 2. Vitexin possesses glucose at C8 position of A ring, whereas in isovitexin glucose is present at C6 position of A ring. Based on a study by Swati *et al*.[41] the ability of vitexin and isovitexin to scavenge radicals such as DPPH and $ABTS^{\bullet+}$ gives preliminary information about electron or hydrogen donating capacity of the antioxidant molecule. Their data revealed that the activity of vitexin and isovitexin against DPPH associated with the 8th and 6th positioned C-glycosylation, respectively. However, vitexin scavenged DPPH more efficiently than isovitexin. The lower activity of isovitexin against DPPH radical could be due to stearic hindrance associated with the 6th positioned C-glycosylation.

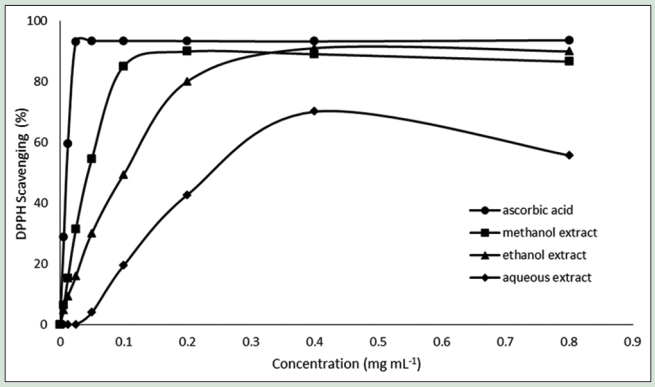

Figure 1: 2,2-diphenyl-1-picrylhydrazyl scavenging activities of *Ficus deltoidea* extracts compare with ascorbic acid. Results are expressed as mean of percent of 2,2-diphenyl-1-picrylhydrazyl free radical scavenging capacity ± standard deviation for three replicates (*n* = 3)

Inhibition of *para*-nitrophenol UDP-glucuronosyltransferase

All the UGT enzyme assay parameters were optimized in the laboratory to ensure glucuronidation inhibition experiments were carried out under initial rate conditions. Besides, the V_{max} and K_m values for UGT enzymes of both rat liver and HLM were also determined. From the kinetic analysis, the V_{max} for UGT enzymes from RLM employing *p*-NP was 43.04 ± 1.64 nmol/min/mg and the K_m value was 860 ± 86.81 μM. Meanwhile, the V_{max} value for UGT enzymes from HLM was 99.35 ± 8.44 nmol/min/mg and the K_m value was 468.40 ± 111.80 μM. The V_{max} and K_m value of UGT from RLM and HLM are summarized in Table 2.

For competitive inhibition, experiments that measure the half-maximal inhibitory concentration (IC_{50}) values, a substrate concentration at around or below the K_m must be used.[42] Using substrate concentration higher than the K_m will make the identification of competitive inhibitors more difficult as the velocity is insensitive to changes in substrate concentrations.[42] Further, the inhibition of *p*-NP UGT in RLM and HLM by *F. deltoidea* extracts were performed at the optimized condition and at *p*-NP concentration that were less than the apparent K_m value (500 μM).

Aqueous, ethanol, and methanol extracts of *F. deltoidea* at 10, 100, and 1000 μg/mL were screened for their effect on *p*-NP UGT activity by determining the percentage of inhibition [Figure 3]. The study was first carried out in pooled RLM. In this study, diclofenac was used as a positive control for UGT enzyme inhibition assay. Diclofenac was chosen as a positive control since it was reported to be a nonselective inhibitor of several major hepatic UGT enzymes.[43]

Result in Figure 3 showed that the methanol extract of *F. deltoidea* is the most effective inhibitor for *p*-NP glucuronidation in RLM with a percentage of inhibition of >70% at the highest concentration compared to the negative control 100%. Ethanol and aqueous extracts both inhibited *p*-NP glucuronidation in RLM with the percentage of inhibition of 34% and 47%, respectively. Further, the concentration of methanol extract to inhibit 50% of *p*-NP UGT activity (IC_{50}) were determined by plotting the remaining enzyme activity against methanol extract concentration

Table 2: The maximal velocity of reaction and Michaelis constant values for *para*-nitrophenol UDP-gucuronosytranserase of rat liver and human liver microsomes and glutathione S-transferase of rat liver cytosolic fraction

	V_{max} (nmole/min/mg)	K_m (μM)
RLM	43.04±1.64	860.50±86.81
HLM	99.35±8.44	468.40±111.80
Rat liver cytosolic fraction	240.48±0.02	1010.08±0.07

Results are expressed as mean values±SD for three replicates (*n*=3). SD: Standard deviation; HLM: Human liver microsome; RLM: Rat liver microsome; V_{max}: Maximal velocity of reaction; K_m: Michaelis constant

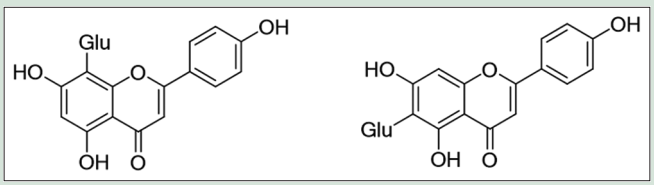

Figure 2: Chemical structures of A: Vitexin and B: Isovitexin. Glu: Glucose

using sigmoidal plot log (inhibitor) versus normalized response equation [Figure 4]. Based on the sigmoidal plot in Figure 4, methanol extract of *F. deltoidea* showed an IC_{50} value of 881.40 ± 1.14 µg/mL. However, the aqueous and ethanol extracts were not determine their IC_{50} values since the percentage of inhibition was <50%, respectively. Therefore, aqueous and ethanol extracts showed no notable effect of inhibition for *p*-NP glucuronidation (IC_{50} >250 µg/mL).

Further, the *F. deltoidea* extracts (methanol and aqueous) were evaluated for their potential to inhibit *p*-NP glucuronidation in HLM as HLM is the most relevant system in evaluating *in vitro* drug metabolism in human. The methanol extract was studied since it gave the highest inhibition among the extracts in RLM, whereas the aqueous extract was also studied as it represents the traditional preparation used by consumers. Result in Figure 5 showed that the methanol extract strongly inhibited glucuronidation of *p*-NP with an IC_{50} value of 63.44 ± 1.20 µg/mL, whereas aqueous extract gave an IC_{50} value of 138.00 ± 1.09 µg/mL, twice the value of the methanol extract [Figure 5]. The IC_{50} values of *F. deltoidea* extracts on *p*-NP glucuronidation in RLM and HLM are summarized in Table 3.

Inhibitory effect of methanol extracts of *F. deltoidea* on *p*-NP glucuronidation in pooled HLM gave an IC_{50} a much lower almost 14 times than in RLM (881.40 ± 1.14 µg/mL for RLM and 63.44 ± 1.20 µg/mL for HLM). The aqueous extract also showed inhibition of *p*-NP glucuronidation a much lower in HLM than in RLM (138.00 ± 1.09 µg/mL for HLM and no notable effect of inhibition in RLM). The difference in the degree of inhibition of *p*-NP glucuronidation observed between RLM and HLM may be due to the differences in K_m values for the models used, HLM and RLM. In our study, we found that the K_m for human was 468.4 µM; relatively lower compared to the K_m of rat which was 860.5 µM. V_{max} is the maximum rate of activity the enzyme can attain as further increases in substrate concentration did not increase the rate [Table 2]. A low value of V_{max} means that the enzyme does not convert much substrate to product per unit of time when it is too saturated with the product. Thus, the maximal velocity of the enzyme is relatively small. Meanwhile, the K_m value is the substrate concentration at half-maximal rate (V_{max}). This means that half of the enzyme molecules have a substrate molecule bound, while the other half of the enzyme molecules are free of the substrate. Enzymes with a low value of K_m have a high affinity to bind with the substrate. Therefore, human liver as a model with a lower K_m value compared with rat liver is most likely to have a lower IC_{50} value for inhibition. Furthermore, the fact that UGT enzymes may be present at different levels in RLM compared to HLM may be one of the reasons contributing to the difference in the degree of inhibition between the two species.[44]

The results for UGT inhibition in this study showed that the methanol extract gave the highest inhibition, followed by ethanol and aqueous extracts in both RLM and HLM. As described earlier, this study had shown that *F. deltoidea* extracts contained total phenolic content in the order of methanol > aqueous > ethanol, while for total flavonoid content the rank order would be methanol > ethanol > aqueous. Therefore, there seemed to be a correlation between the inhibitory potential of UGT with the amount of flavonoid in the extracts.

The ability of flavonoids as the main constituent that could modulate UGT activity has been reviewed by Moon *et al*.,[45] and this is also shown by the results of this study. Many flavonoids contain Michael reaction center(s) in their molecules. Thus, this characteristic may be related to their effects on phase II enzymes.[45] Flavonoids are present in many

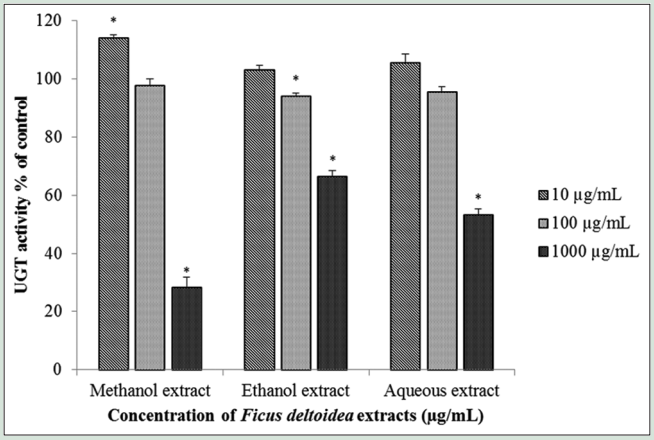

Figure 3: Inhibitory effect of *Ficus deltoidea* extracts on *para*-nitrophenol glucuronidation in rat liver microsomes. Values represent the mean of percentage activity over control ± standard deviation for five replicates (n = 5). Statistical analysis was performed using one-way analysis of variance followed by Dunnet's test. *Indicates significant difference from control (P < 0.05)

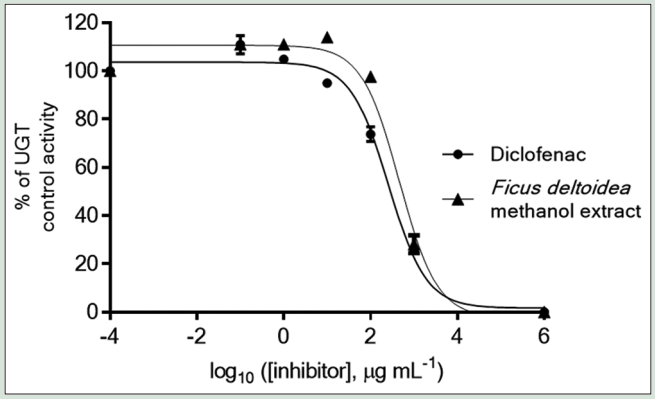

Figure 4: Inhibition of *para*-nitrophenol glucuronidation in rat liver microsomes by *Ficus deltoidea* methanol extract compared to positive inhibitor (diclofenac). Data are expressed as the mean percentage activity relative to negative control ± standard deviation for five replicates (n = 5). Error bars represent two-sided standard error of the mean. Goodness of fit R^2 values were >0.9

Table 3: Half-maximal inhibitory concentration values (µg/mL) for rat liver microsome and human liver microsome *para*-nitrophenol UDP-gucuronosytranserase and rat cytosolic fraction glutathione S-transferase with *Ficus deltoidea* extracts

Ficus deltoidea extract	*p*-NP UGT		GST
	RLM (µg/mL)	HLM (µg/mL)	Rat cytosolic fraction (µg/mL)
Methanol	881.40±1.14	63.44±1.20	70.73±1.07
Ethanol	ND	ND	ND
Aqueous	ND	138.00±1.09	ND

ND: Not determined. Results are expressed as the best-fit IC_{50} values±SD for five replicates (n=5). SD: Standard deviation; IC_{50}: Half-maximal inhibitory concentration; *p*-NP: *para*-nitrophenol; GST: Glutathione S-transferases; UGT: UDP-glucuronosyl transferase; HLM: Human liver microsome; RLM: Rat liver microsome

dietary supplements which are usually derived from natural plants including *Ginkgo biloba*, soy isoflavonones, tea, and red wine.[45] The effect of flavonoids on drug-metabolizing enzymes activity is generally dependent on the concentrations of flavonoids on enzymes activity is generally dependent on the concentrations of flavonoids present and also on the different flavonoids ingested.[45] The effect of natural plants such as ketum (*Mitragyna speciosa*), ginkgo (*G. biloba*) and milk thistle (*S. marianum*) on UGT activity in RLM had also indicated that flavonoids content is responsible for UGT inhibitory effect.[46-50]

Inhibition of glutathione S-transferases

Inhibition of GST-mediated conjugation of GSH to CDNB by *F. deltoidea* extracts was investigated with GSTs in rat liver cytosolic fraction. The K_m and V_{max} values were 1010.08 ± 0.07 μM and 240.48 ± 0.02 μmol/min/mg, respectively. In order for competitive inhibitors to be identified in a competitive inhibition experiment that measures IC_{50} values, a substrate concentration around or below the K_m must be used. Therefore, the inhibitory effect of positive inhibitor (tannic acid) and *F. deltoidea* on GST enzymes activity by rat liver cytosolic fraction were performed at optimized conditions and at CDNB concentration that were close to the apparent K_m value (1.0 mM).

Figure 6 shows the decreased of GST enzymes activity by *F. deltoidea* extracts. Methanol, ethanol, and aqueous extracts decreased the GST enzymes activity from 86%–30%, 95%–50%, and 97%–79%, respectively. Further, the concentration of methanol extract to inhibit 50% of GST enzymes activity (IC_{50}) were determined by plotting the remaining enzyme activity against methanol extract concentration using sigmoidal plot log (inhibitor) versus normalized response equation. The IC_{50} values were not determined for aqueous and ethanol extracts since the percentage of inhibition was <50%. Therefore, aqueous and ethanol extracts showed no notable effect of inhibition for GST enzymes activity (IC_{50} >250 μg/mL). Methanol extract showed the IC_{50} value of 70.73 ± 1.07 μg/mL [Figure 7], whereas IC_{50} value were not determined for ethanol and aqueous extract due to percentage of inhibition was <50% at the highest concentration. The IC_{50} value of *F. deltoidea* methanol extract on GST enzymes activity rat liver cytosolic fraction are summarized in Table 3.

GST is a phase II enzyme that is one of the major enzyme systems in protecting against chemicals that are carcinogenic.[51] All the extracts of *F. deltoidea* that were investigated showed inhibition on GST activity. The percentages of inhibition of GST enzyme were evaluated in the presence of varied extract concentrations (0.01 μg/mL–250 μg/mL). The IC_{50} values were obtained graphically by nonlinear regression analysis of the remaining percentage enzyme activity (μg) versus the logarithm of natural product concentration (μg/mL).[15] Tannic acid, which was used as a positive control in the GST assay, showed an inhibition with an IC_{50} value of 15.34 ± 1.08 μg/mL and compared to the inhibition of GST activity by *F. deltoidea*. Comparing all the IC_{50} values of the extracts for the GST assay methanol extract gave the highest inhibition with the value of 70.73 ± 1.07 μg/mL. IC_{50} values were not being determined for ethanol and aqueous extracts due to the percentage of inhibition being <50% at the highest concentration (500 μg/mL). In addition, phenolic compounds have shown significant inhibition on GST activity rat liver cytosolic fraction.[15] Previously, a research reported that plant polyphenols such as tannic acid, ellagic acid, ferulic acid, stilbene caffeic acid, quercetin and curcumin gave inhibitory effects against GST.[52]

The results for GST inhibition in this study showed that the methanol extract gave the highest inhibition, followed by ethanol and aqueous extracts. As described early, this study had shown that *F. deltoidea* extracts contained total phenolic content in the order of methanol > aqueous > ethanol, while for total flavonoid content, the rank order would be methanol > ethanol > aqueous. Therefore, there

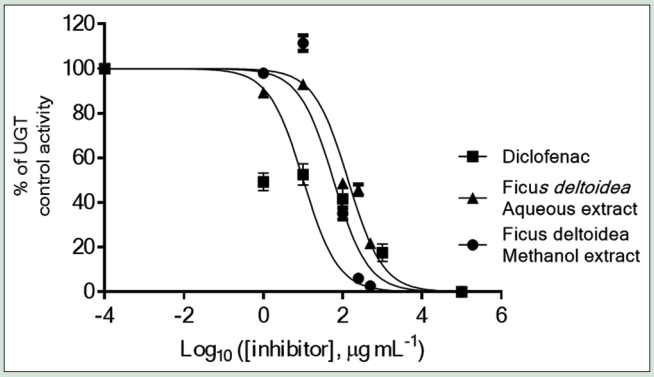

Figure 5: Inhibition of *para*-nitrophenol glucuronidation in human liver microsomes by *Ficus deltoidea* methanol and aqueous extracts compared to positive inhibitor (diclofenac). Data are expressed as the mean percentage activity relative to negative control ± standard deviation for five replicates (n = 5). Error bars represent two-sided standard error of the mean. The goodness of fit R^2 values were >0.9

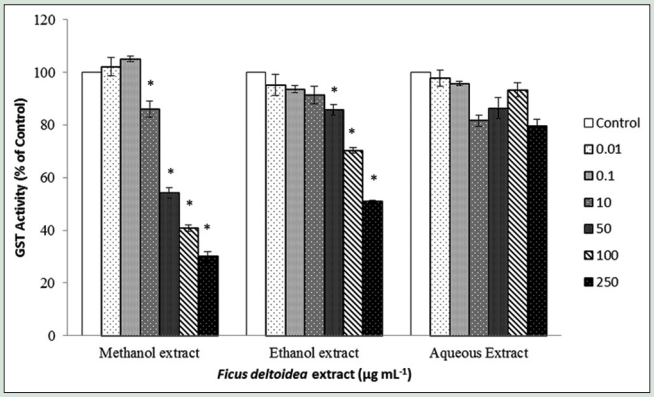

Figure 6: Inhibitory effect of *Ficus deltoidea* extracts on glutathione S-transferase enzymes activity in rat liver cytosolic fraction. Values represent the mean of percentage activity over control ± standard deviation for five replicates (n = 5). Statistical analysis was performed using one-way analysis of variance followed by Dunnet's test. *Indicates significant difference from control (P < 0.05)

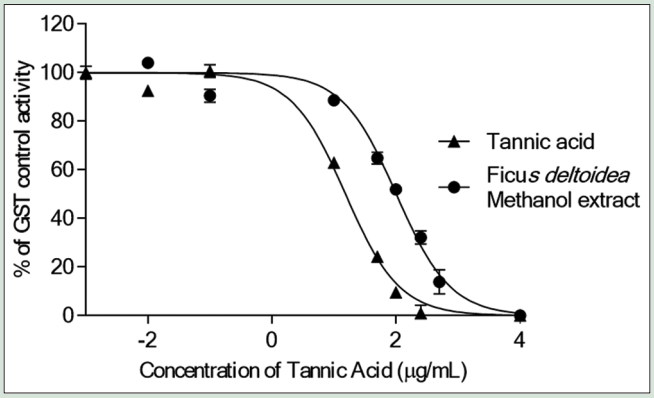

Figure 7: Inhibition of glutathione S-transferase enzymes activity in rat liver cytosolic fraction by *Ficus deltoidea* methanol extracts compared to positive inhibitor (tannic acid). Data are expressed as the mean percentage activity relative to negative control ± standard deviation for five replicates (n = 5). Error bars represent two-sided standard error of the mean. Goodness of fit R^2 values were >0.9

seemed to be a correlation between the inhibitory potential of GST with the amount of flavonoid in the extracts.[53]

Flavonoids from different classes of flavonol and flavonone such as morin, quercetin, isorhamnetin, myricetin, acacetin, and apigenin showed a potent inhibitory potency on GST enzyme activity.[54] In addition, flavonoids also have been shown to inhibit GST activity in human blood platelets rat liver and rat kidney.[55] Furthermore, other plant polyphenols, including tannic acid, ethacrynic acid, ellagic acid, and caffeic acid, have been shown to inhibit the GST enzymes activity.[54] Thus, this finding suggests that high GST inhibitory activity of *F. deltoidea* methanol extract could be attributed to the high flavonoids content as indicated in the literature for the GST inhibitory effects of *M. speciosa* korth leaf extracts.[53]

Overexpression of GST in tumor cells has received massive attention as they impede the chemotherapy treatment such as chlorambucil.[56] Many studies have focused on recognizing chemopreventive substances to modulate carcinogenesis, particularly natural occurring substances, and it has been proven that GST inhibitors could be useful therapeutic agents to modulate anticancer drug-resistance.[57,58] GST enzyme participates in the metabolism of various types of anticancer drug, including chlorambucil.[59] Hence, the overexpression of GST in tumor cells could enhance the metabolism of anticancer drugs and reduce its therapeutic effect. Ellagic acid and curcumin have also been reported to inhibit GST enzyme.[60] Both of them possess antitumor activity, and the authors had also suggested that these two compounds could inhibit the growth of tumor cell and prevent resistance in detoxification of the co-drug at the same time. Flavonoids are known for its anti-oxidative properties, anti-atherosclerotic effect, anti-inflammatory, antithrombogenic effect, and anti-tumor effect.[61] Besides, flavones and flavonols show inhibition in cancer cell growth through cell cycle arrest and apoptosis induction.[62] Therefore, the inhibition of GST by *F. deltoidea* could possibly result in reduced protection against toxic effects of electrophilic substances and influence other GST-related biological functions.[63,64]

CONCLUSION

The present study has comprehensively shown the inhibitory potential of *F. deltoidea* methanol extract toward phase-II drug metabolism enzymes (UGT and GST). The methanol extract of *F. deltoidea* gave the highest inhibition toward *p*-NP glucuronidation in RLM and HLM. Therefore, this may well result in *p*-NP UGT-dependent herb–drug interactions. *F. deltoidea* methanol extract also the strongest inhibitor of the GSTs tested. Inhibition of GSTs may be beneficial for cancer therapy, but in normal cells, it may also result in increased toxicity due to reduced protection against electrophilic chemicals or metabolites.

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Conflicts of interest

There are no conflicts of interest.

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