

Original Article

THE *IN VITRO* AND *EX VIVO* EFFECT OF *PHYLLANTHUS NIRURI* METHANOL EXTRACT ON HEPATIC UDP-GLUCURONYLTRANSFERASE ENZYME ACTIVITY IN STZ-INDUCED DIABETIC SPRAGUE DAWLEY RATS

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ABSTRACT

Objective: The aim of the study was to investigate the *in vitro* and *ex vivo* (acute and sub-chronic doses) effect of *Phyllanthus niruri* methanol extract (PNME) on the microsomal UDP-glucuronyltransferase (UGT) enzyme activity in streptozotocin (STZ)-induced diabetic young female Sprague Dawley (SD) rats.

Methods: Young female SD rats were induced type I diabetes mellitus using STZ (60 mg/kg i. v.). The *in vitro* study was performed on a microsomal fraction of diabetic rat livers using PNME in doses of (0.01 µg, 1 µg and 10 µg)/ml. While *ex vivo* studies were performed on the microsomal fraction of diabetic rats using PNME in doses of 500, 1000, 2000 and 5000 mg/kg p. o. for acute *ex vivo* study (one-day treatment) and 100, 500 and 2000 mg/kg/day p. o. for sub-chronic one (daily dose for two weeks). *p*-nitrophenol (*p*-NP), was used as a marker substrate for UGT enzyme activity and analyzed using the spectrophotometer to determine UGT enzyme activity.

Results: The *in vitro* result showed that, there is no significant effect of the three concentrations of PNME *versus* control on UGT activity in the microsomal fraction of diabetic young female SD rat livers, while for *ex vivo* study, the result showed that UGT activity in the microsomal fraction of diabetic young female SD rats significantly and dose-independently increased at doses 1000, 2000 and 5000 mg/kg p. o in acute study (all $p < 0.05$ vs control). However, no significant effect of PNME has been seen in the three doses used in the sub-chronic study.

Conclusion: The three different concentrations of PNME have no significant effect as compared to control on UGT activity in the *in vitro* study. However, *ex vivo* study showed significant and dose-independent increased in UGT activity at doses 1000, 2000, and 5000 mg/kg p. o in acute study (all $P < 0.01$ vs control), but no significant effect on sub-chronic study.

Keywords: *Phyllanthus niruri*, UGT, Diabetic rats, Microsomal liver fraction, *p*-NP.

INTRODUCTION

Phyllanthus niruri is commonly used as a folk medicine in Malaysia (traditionally named as Dukong anak), India, China and some South American countries like Brazil and Paraguay for the treatment of various illnesses especially for viral hepatitis and HIV infection due to its potent anti-viral activity [1, 2]. It has also been found to be effective to remove kidney and gall stones [3], and as an antinociceptive and anti-inflammatory agent [4], antihyperlipemic and anti-hypertensive agent [5, 6], antiparasitic, antimicrobial and antihyperuricemic agent [7-9]. Many chemical compounds have been isolated from this plant like, lignans, alkaloids, coumarins, flavonoids, sterols, tannins and triterpenoids [10].

Hepatic drug metabolizing enzymes are divided into phase I enzymes (functionalization reactions) and phase II (conjugation reactions). The phase I system is mainly comprised of the cytochrome P450 family of enzymes, which are regarded as the first defense of the body against xenobiotic. The metabolites from phase I metabolism which becomes more water soluble enter phase II conjugation reactions to increase the water solubility of the metabolites to be easily excreted into urine or bile [11].

Glucuronidation is the major phase II biotransformation reaction. It involves the transfer of glucuronic acid from uridine 5'-diphosphoglucuronic acid (UDPGA) to countless structurally unrelated endobiotics and xenobiotic substances possessing hydroxyl, carboxyl, amino or sulfhydryl groups, converting them to water-soluble glucuronides. This reaction leads to the synthesis of ether, ester, carbamoyl, carbonyl, and carboxyl which characterized by high polarity and water solubility, hence, suitability for excretion. A family of enzymes known as UDP-glucuronyl transferases (UGT) is responsible for this reaction. UGT enzymes are presents in liver,

lung, brain, skin and olfactory epithelium, but the major site of glucuronidation is the liver [12].

UGT isoforms have been classified into two main families UGT1 and UGT2, depending on gene structure and amino acids sequence similarities [13]. The gene product of the UGT1 family is 38-40 % identical with UGT2 family gene product. About 50 % is the similarity in gene sequence within a single family while the similarity increased up to 60 % within the same sub-family [14].

For UGT1 family, nine isoforms have been discovered in human and seven isoforms in rat, while for UGT2 family, six isoforms have been discovered in human and seven isoforms in rat [15, 16]. Some UGT isoforms have evolved to prevent the accumulation of potentially toxic endogenous compounds, for example, UGT1A1 isoform is responsible for bilirubin glucuronidation, which is the end product of heme catabolism [17].

Recently, due to the wide spread use of herbs for treatment of various illnesses, many attention has been given to the effect of herbs on drug metabolizing enzymes to predict any herb-drug interaction which may lead to serious side effects [18-20]. The measurement of UGT enzyme activity is an example of such studies to examine the herbal effect on its activity and the possibility of interactions. No previous study has been done to see the effect of *Phyllanthus niruri* on UGT enzyme activity, therefore in this study; we will examine the effect of *Phyllanthus niruri* methanol extract (PNME) on UGT enzyme activity in young female diabetic rats group. This group of rats treated with the PNME has previously showed a significant dose independent increase on phase I aminopyrine N-demethylase enzyme activity [21]. Therefore, it is crucial to investigate if the phase II drug metabolizing enzyme (UGT) will also be affected.

MATERIALS AND METHODS

Chemicals used

Bio-Rad reagent was supplied from Bio-Rad laboratories, Inc., USA. The bovine serum albumin (BSA), p-nitrophenol (p-NP), and UDP-glucuronic acid, were purchased from Sigma Chemicals Co, USA. Dipotassium hydrogen phosphate (K_2HPO_4) and Tween 20 were supplied from System[®], Malaysia. Potassium chloride (KCL) was obtained from BDH laboratory supplies, UK. Potassium di-hydrogen orthophosphate (KH_2PO_4) was supplied from Univar analytical reagent, Australia. Triton X-100 and Tris ultra purine were supplied from ICN Biomedicals, Inc., USA. Sodium hydroxide and Trichloroacetic acid were supplied from Ajax chemicals, Austria, and streptozotocin (STZ) was supplied from CalBioChem., USA.

Experimental animals

Young female Sprague Dawley rats (120-130 g) were used in this study. The animals were obtained from the Animal House Facility, School of Pharmaceutical Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia. The rats were housed in standard environmental conditions (25 °C, 60-70 % humidity) under natural lighting and fed with normal commercial rat chow (Gold Coin Sdn Bhd, Malaysia) and water *ad libitum*. All experimental protocols and procedures on animals were approved by the ethical committee of USM.

Preparation and standardization of plant extract

A voucher specimen of *Phyllanthus niruri* Linn. with No. 10843 has been deposited at the herbarium of School of Biological Sciences, Universiti Sains Malaysia.

The aerial parts of the plant were dried 4-5 d in an oven at 40 °C. The dried plant material was subsequently pulverized and the active constituents were extracted by methanol (5 liters for 5 d) using soxhlet apparatus (Quickfit, England). The extract was then dried using rotary evaporator (Buchi, Switzerland) and freeze dryer (Labconco Corporation, U. S. A.) to yield the dried crude methanol extract of *Phyllanthus niruri* (20.48 % yield of powdered plant). The obtained extract has a poor solubility, so Tween 20 in 20 % distilled water was used to solubilize it. The methanol extract of *Phyllanthus niruri* was standardized according to Murugaiyah and Chan method [22], using phyllanthin as a standard substance through a simple analytical HPLC-UV method. After drying of the extract, a residue was reconstituted in methanol to produce 1 mg/ml concentration, then this sample was filtered through a 0.45 µm PTFE syringe filter (Whatman, Maidstone, England) and a sample of 25 µl was injected into the HPLC (Jasco PU-980; Japan) column (SB-C18; 4.6 i.d. x 250 mm; Agilent; U. S. A.). The mobile phase used was acetonitrile: de ionized water (55:45 v/v) and the chromatographic peaks of the sample were identified through a UV detector (Tokyo, Japan) at a wavelength 230 nm. Fig. 1 shows the plant extracts chromatogram and the standard phyllanthin chromatograms at three different concentrations (0.1 ppm, 0.5 ppm and 5 ppm) depending on retention time and quantified using the external standard method.

Induction of diabetes

Streptozotocin (STZ) was freshly prepared in ice-cold distilled water and injected intravenously via the tail vein of the animal at a dose of 60 mg/kg body weight. After 72 h of STZ injection, the diabetic state was confirmed by measuring fasting blood glucose (FBG). Blood was withdrawn from the tail and tested for glucose level using a glucometer (Accutrend, Germany). Rats with less than 15.5 mmol/l of fasting blood sugar were excluded from the study.

Animals

For *in vitro* study, a six young female SD rats were induced diabetic by STZ and sacrificed to obtain liver samples, from which microsomal fractions were prepared. During experiment, 3 doses of PNME (0.01 µg, 1 µg and 10 µg)/ml were added *in vitro* to the microsomal fraction from liver samples while for control; the extract was replaced with the solvent.

For the *ex vivo* study, after induction of diabetes, 63 diabetic young female SD rats were divided into 9 groups of 6 animals in each

group. The acute study, group I was fed with distilled water+Tween 20 (20 %) and therefore served as a control, while groups II, III, IV and V were fed with a single oral dose of 500, 1000, 2000 and 5000 mg/kg of PNME, respectively. For the sub-chronic study, group VI was administered distilled water+Tween 20 (20 %) for two weeks in a single daily dose and acted as a control, whereas groups VII, VIII and IX were given a two-week treatment of a single daily dose of 100, 500 and 2000 mg/kg of PNME, respectively. The groups of the acute study were sacrificed after 24 h of the dosing while the groups of the sub-chronic study were sacrificed after 24 h of the last dose (i.e. after the 14th day dose). In all groups, livers were removed immediately and weighed to prepare the microsomal fraction.

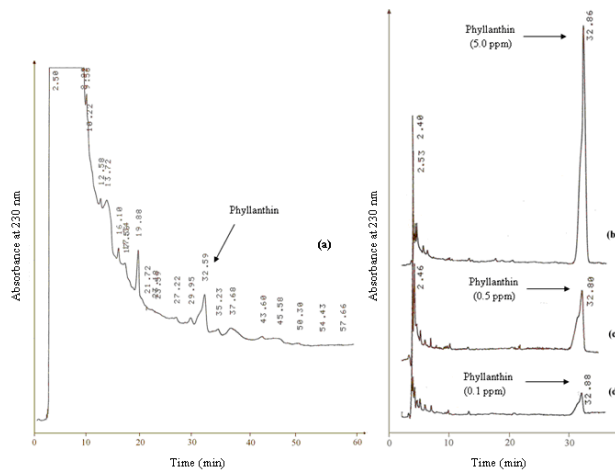


Fig. 1: Chromatograms from the analysis of phyllanthin, where (a) is PNME, (b) standard phyllanthin (5 ppm), (c) standard phyllanthin (0.5 ppm) and (d) standard phyllanthin (0.1 ppm)

Preparation of microsomal liver fraction

Rat livers were homogenized in 3 volumes of 50 mM potassium phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer (Kontes, U. S. A.) at 4 °C. This homogenate was then centrifuged for 20 min at 12 000 g (Vision, Korea). The supernatant was further centrifuged for 60 min at 100 000 g using an ultra-centrifuge (Beckman Coulter, U. S. A.). Following the final centrifugation, the supernatant obtained represented the cytosolic fraction while the sediment was the microsomal fraction. After separation of the cytosolic fraction, the microsomal fraction was re-suspended in 3 volumes in potassium phosphate buffer 50 mM (pH 7.4) [23]. Protein concentration was determined for each group according to Bradford method [24], using bovine serum albumin (BSA) as a standard curve.

Determination of UGT enzyme activity

UGT enzyme activity toward p-nitrophenol (p-NP) assayed with a slight modification according to the method described by [25, 26]. p-NP is commonly used because the unreacted amount of p-NP can be measured by the spectrophotometric procedure. The microsomal protein concentration used in the experiments was 2.5 mg/ml [27]. For blank groups, the microsomal fractions of both *in vitro* and *ex vivo* studies were denatured by heating up to 60 °C for 10 min before experiments. 40 µl of microsomal protein were incubated with 8 µl of Triton X-100 (0.75 w/v) for 3 min, then it is added to the mixture that contains 20 µl tris-HCL (1 M), 20 µl of $MgCl_2$ (50 mM) and 20 µl of p-NP (5 mM). For *in vitro* study 20 µl of serial dilutions of PNME were added and complete the volume of mixture to 200 µl by deionized water, then the reaction was started by the addition of 20 µl of UDP-GA and after 15 min of incubation at 37 °C, the reaction stopped by the addition of 80 µl of 20 % trichloroacetic acid. The solution was centrifuged at 2000 rpm for 10 min, then 200 µl of supernatant was added to 800 µl of 0.5 M NaOH and reading was taken using the microplate reader at absorbance length 405 nm. In control groups, the same experiment was done for other groups, but with replacement of PNME with the solvent solution (distilled water+20 % of Tween 20) for both *in vitro* and *ex vivo* studies.

Statistical analysis

Results were compared with the control and mean and standard deviation was calculated. Dunnett test was used to analyze data and the level of significance was set at $p < 0.05$.

RESULTS

Fig. 2 shows the *in vitro* effect of PNME in 3 doses (0.01 μg , 1 μg and 10 μg)/ml on UGT activity in diabetic young female SD rats. All *in vitro* doses showed no significant effect on UGT activity compared to the respective control group.

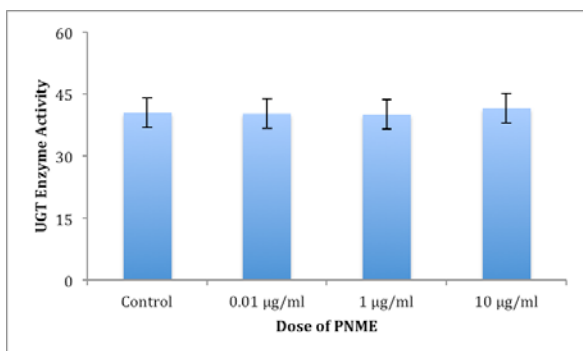


Fig. 2: *In vitro* effect of PNME on microsomal UGT activity in diabetic young female Sprague Dawley rats. Each value is expressed as mean \pm SD: n=6; $p > 0.05$, not significantly different than control

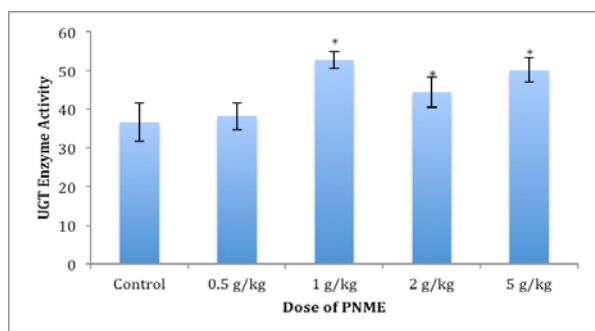


Fig. 3: Effect of acute oral dose of PNME on microsomal UGT activity in diabetic young female Sprague Dawley rats. Each value is expressed as mean \pm SD: n=6; * $p < 0.05$, significantly different than control. UGT activity is expressed as nmol product formed/min/mg of microsomal protein

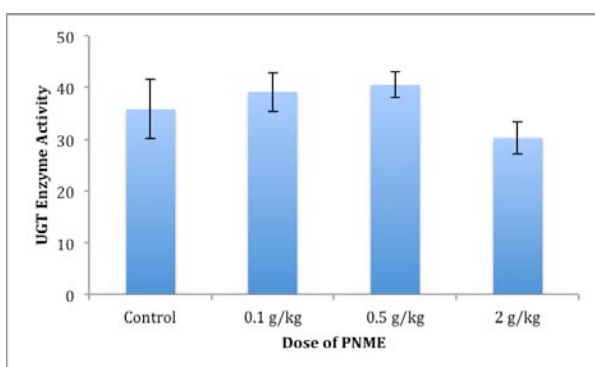


Fig. 4: Effect of sub-chronic oral dose of PNME on microsomal UGT activity in diabetic young female Sprague Dawley rats. Each value is expressed as mean \pm SD: n=6; $p > 0.05$, not significantly different than control

Fig. 3 shows the effect of an acute oral dose of PNME on UGT activity in diabetic young female SD rats.

The doses (1000, 2000 and 5000) mg/kg of PNME showed a significant dose independent increase in UGT activity compared to the respective control group (all $p < 0.05$). With control showing 36.63 nmol/min/mg, and significantly increase at 52.64 nmol/min/mg of dosage 1 g/kg, 44.42 nmol/min/mg of dosage 2 g/kg, and 50.03 nmol/min/mg of dosage 5 g/kg.

Fig. 4 shows that no significant effect of PNME was obtained in the sub-chronic *ex vivo* study for all doses used.

DISCUSSION

Glucuronide conjugation is an important pathway in the inactivation and excretion of the variety of drugs and endogenous compounds. This reaction is catalyzed by microsomal UDP-glucuronyl transferase, a family of isoenzymes with different substrate specificity [12]. UGT detoxification role is performed, either by preventing the potentially toxic xenobiotics accumulation or by avoiding their subsequent bio-activation to a more reactive toxic intermediate [28]. UGT isoenzymes may also have a crucial role in controlling endogenous signal compounds, like hormones, where direct inhibition of UGTs by competitive xenobiotics may significantly affect steroid glucuronides production that possess an important pharmacological activity leading to affect the physiological function of the responsive tissue [29].

Glucuronidation is under complex regulation factors. The first factor occurs either at the gene transcription level, which results in mRNA and proteins level changes, and/or on the level of post-translational processing. The second factor is the modulation of UGT functional state because of its association with the lipid environment; however, many studies have been done to determine the factors that may affect UGT isoformal enzymes activities like effect of age, gender, diseases, hormones and administration of drugs, dietary or natural product like herbs. Also, restrictions of UDP-glucuronic acid to access the active site of enzyme [30, 31], and/or the protein-protein interactions from oligomer formation may be another factors that may affect UGT activity [32]. Diabetes mellitus, which is the main characteristic of our rat study group, has been shown to cause a significant reduction of UGT enzyme activity especially in *p*-nitrophenol glucuronidation [33].

Many studies have been done on herbal extracts and herbal chemical compounds to show its effects on phase II enzymes activity and especially UGT enzyme due to its importance, for example, herbal tea and coffee was found to cause a significant increase in UGT enzyme activity [34, 35]. Cruciferous vegetables like garden cress (*Lepidium sativum*), water cress (*Nasturtium officinale* R. Br) and mustard (*Sinapis alba* L.), were found to induce UGT enzyme activity in human derived hepatoma cell line (HepG2) [36]. A Thai vegetable known as *Siamese cassia*, was found to significantly increase the activity of UGT enzyme (220 % of control) in male Wistar rats [37]. Dietary flavonoids were found to cause significant induction effect on UGT1A1 enzyme activity [38], and this agreed with a study done by Li and his colleagues, which found that, flavonoids and terpenoids isolated from *Ginkgo biloba* significantly increase the activity of UGT1A1 enzyme in human primary hepatocytes and HepG2 cells [39].

However, our *in vitro* study for the effect of PNME (0.01 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$) on microsomal UGT enzyme activity showed no significant effect comparing with the control group (fig. 2), so this may indicate that there is no direct effect of our extract on microsomal UGT enzyme activity.

However, in the *ex vivo* study and although rats suffered from experimental diabetes, which suppresses UGT enzyme activity, the rats that were dosed with a single acute dose of PNME at (1000, 2000, and 5000 mg/ml) showed a significant induction ($P < 0.01$) of microsomal UGT enzyme activity (fig. 3), while no significant effect can be seen in sub-chronic doses (fig. 4).

A possible explanation of the discrepancy in results between *in vitro* and *ex vivo* studies is that UGT enzyme location in the endoplasmic reticulum prevents substrates and glucuronidated products

movement in and from the active sites. Furthermore, the presence of activated surfactants in the *in vitro* enzymatic incubation system may have altered the inhibitory kinetics of UGT enzymes. In addition, the generated glucuronidated products are eliminated *in vivo*, but not in *in vitro* incubation system, hence, the accumulation of conjugated products in the reaction medium may inhibit the enzyme activity [40]. Talalay and his colleagues suggested that, the significant increase in the activities of phase II enzymes especially UGT and GST enzymes may be a critical and sufficient condition to achieve chemo protection [41].

Recent studies found that, UGT enzyme expression is controlled by a promoter elements or transcription factors, and such factors can be activated through xenobiotic or hormones or carcinogens attachment to specific receptors in these factors leading to increase of the protein enzyme expression and induction of activity. Some receptors have been identified like aryl hydrocarbon receptor, constitutive androstane receptor, and pregnane X receptor [42]. An example of such induction effect of xenobiotics, like plant extracts, on UGT enzyme activity, is the induction effect of *Cajanus cajan* Linn. on UGT2B activity through the induction of transcription factors that regulate the expression of UGT2B cytoprotective gene [43], and it may be the possible pathway of our extract's induction effect in the acute *ex vivo* study. However, the absence of significant effect of our extract in the sub-chronic *ex vivo* study may be attributed to the diabetic condition of our group under study, which tends to decrease UGT enzyme activity [33].

CONCLUSION

P. niruri is an important medicinal plant in tropical and subtropical regions of the world. Due to the wide use of this plant, possible herb-drug interactions may occur. This study investigated the *in vitro* and *ex vivo* effect of standardized methanol extract of *P. niruri* on phase II (UGT) enzymes activities and the elucidation of the mechanism of such effect. In phase II *in vitro* study, no significant effects were observed on UGT enzyme activities in diabetic young female rats. However, acute *ex vivo* study showed significant induction effect on UGT enzyme activity at doses (1000 mg/kg, 2000 mg/kg, and 5000 mg/kg) of the PNME. While for sub-chronic study, no significant effect of PNME was observed on UGT enzyme activity.

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CONFLICT OF INTERESTS

Declared None

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