

THE *IN VITRO* AND *EX VIVO* EFFECT OF *PHYLLANTHUS NIRURI* METHANOL EXTRACT ON HEPATIC GLUTATHIONE S-TRANSFERASE ACTIVITY IN STZ-INDUCED DIABETIC SPRAGUE DAWLEY RATS

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ABSTRACT

Objective: The aim of this study was to investigate the *in vitro* and *ex vivo* (acute and sub-chronic doses) effect of *Phyllanthus niruri* methanol extract (PNME) on the activity of cytosolic glutathione S-transferase (GST) 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 intravenous). The *in vitro* study was performed on a cytosolic fraction of diabetic rat livers using PNME in concentrations of 0.01, 1, and 10 µg/ml. Meanwhile, *ex vivo* studies were performed on diabetic rats using PNME in doses of 500, 1000, 2000 and 5000 mg/kg p.o. for acute study (1 day) and 100, 500 and 2000 mg/kg/day p.o. for sub-chronic study (14 days). The GST activity in the cytosolic liver fraction was measured using spectrophotometric analysis.

Results: The *in vitro* study showed that, there is no significant effect of the three concentrations of PNME versus control on GST activity in cytosolic fraction of diabetic young female SD rat livers, while for *ex vivo* study, there is a significant dose-independent induction effect on GST enzyme activity at all doses used in acute study. Meanwhile, the sub-chronic study showed significant dose-independent induction effect at doses 500 and 2000 mg/kg.

Conclusion: This study suggests that *P. niruri* may increase the clearance of drugs that are metabolized by GST enzyme in Phase II metabolism when given concomitantly with this plant extract.

Keywords: *Phyllanthus niruri*, Glutathione S-transferase, Diabetic rats, Cytosolic liver fraction, Phase II metabolism.

INTRODUCTION

Phyllanthus niruri Linn. is a small annual herb that belongs to *Phyllanthus* genus from *Euphorbiaceae* family. This genus consists of more than 600 species which are widely distributed in tropical areas of the world [1,2]. *P. niruri* is commonly used as a folk medicine in Malaysia (traditionally named as Dukong anak), India, China and Brazil for the treatment of kidney stones, gall stones [3], and as a diuretic and tonic [4]. *P. niruri* is also used for dysentery, dyspepsia, and hypertension and has been proven to possess a lipid lowering activity [5]. Recent studies showed that this plant has an antiviral activity, especially on hepatitis B virus [6]. This plant has also been shown to have an inhibitory action against the human immune deficiency virus (HIV) [7].

The main active constituents of this plant are lignans, flavonoids, alkaloids, tannins, terpenes and phenols [2]. However, many components of this plant can be metabolized by Phase I and Phase II liver enzymes and these components may alter the activity of drug metabolizing enzymes of the liver [8]. Phase I detoxification system is mainly comprised of the cytochrome P450 family of enzymes, which are regarded as the first defense of the body against xenobiotics. The metabolites from Phase I metabolism which become more water soluble enter Phase II conjugation reactions to increase the water solubility of the metabolites so that they can be easily excreted in urine or bile. The main conjugation reactions are glucuronidation, methylation, sulfation, acetylation, and glutathione (GSH) conjugation.

GSH conjugation is an important Phase II metabolic reaction. It is a protective reaction to remove electrophilic compounds such as epoxides, nitroalkanes, haloalkanes, alkenes and aromatic halo and nitro compounds. The conjugation of GSH to these electrophilic

compounds is catalyzed by GSH S-transferase (GST) enzyme, which is mainly located in the cytosolic fraction of the liver, kidney, gut, and other tissues [9].

A previous study showed that there is a significant dose-independent increase in diabetic young female Sprague–Dawley (SD) rats on Phase I aminopyrine N-demethylase enzyme activity when treated with *P. niruri* methanol extract (PNME) [10]. Therefore, it is important to investigate if Phase II drug metabolizing enzyme will also being affected by PNME. In this study, we will examine the effect of PNME on GST activity in this group of rats.

METHODS

Chemicals and reagent

Bio-Rad reagent was supplied from Bio-Rad Laboratories, Inc., USA. The bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH reduced form were purchased from Sigma Chemicals Co, USA. Dipotassium hydrogen phosphate (K_2HPO_4) and tween 20 was supplied from System[®], Malaysia. Potassium chloride (KCl) was obtained from R&M Chemicals, UK. Potassium di-hydrogen orthophosphate (KH_2PO_4) was supplied from Ajax Chemicals, Australia and streptozotocin (STZ) was supplied from CalBioChem., USA.

Experimental animals

Young female SD 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), Pulau Pinang, 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 performed in accordance with Organization for Economic Cooperation Development (OECD) guidelines and approved by the Ethical Committee of USM with reference to the number USM/PPSF/50 (012) Jld.2.

Preparation and standardization of plant extract

P. niruri Linn. was generously provided by from Prof. Chan Kit Lam from the Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia. The aerial parts of the plant were dried 4-5 days in an oven at 40°C. The dried plant material was subsequently pulverized, and the active constituents were extracted by methanol (5 L for 5 days) using soxhlet apparatus (Quickfit, England). The extract was then dried using rotary evaporator (Buchi, Switzerland) and freeze dryer (Labconco corporation, USA) to yield the dried crude methanol extract of *P. niruri* (20.48% yield of powdered plant). The obtained extract has a poor solubility. Therefore, Tween 20 in 20% distilled water was used to solubilize it [11].

The methanol extract of *P. niruri* was standardized using phyllanthin as a marker compound through a simple analytical HPLC-UV method. After drying of the extract, a residue was reconstituted in methanol to produce 1 mg/ml concentration, and 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 × 250 mm; Agilent; USA). The mobile phase used was acetonitrile: de-ionized water (55:45 v/v) and the chromatographic peaks of the sample were identified by a UV detector (Tokyo, Japan) at a wavelength 230 nm [11].

Induction of diabetes

STZ was freshly prepared with 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 hrs of STZ injection, the diabetic state was confirmed by measuring fasting blood glucose. Blood was withdrawn from the tail and tested for glucose level using a glucometer (Accutrend, Germany). Rats with glucose level <15.5 mmol/l were excluded from the study.

In vitro study

6 young female SD rats were diabetically induced by STZ and sacrificed to obtain liver samples, from which cytosolic fractions were prepared. Three concentrations of PNME 0.01, 1 and 10 µg/ml were added *in vitro* to the cytosolic fraction from liver samples while for control the extract was replaced with de-ionized water.

Ex vivo study

For the *ex vivo* study, after induction of diabetes, 54 diabetic young female SD rats were divided into nine groups of six animals in each group. For 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 2 weeks in a single daily dose and acted as a control, whereas Groups VII, VIII and IX were given a 2 weeks 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 hrs of the dosing, while the groups of the sub-chronic study were sacrificed after 24 hrs of the last dose (i.e. after the 14th dose). In all groups, liver was removed immediately and weighed to prepare the cytosolic fraction.

Preparation of liver cytosolic fraction

Rat liver samples were homogenized in three volumes of 50 mM potassium phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer (Kontes, USA) at 4°C. This homogenate was then centrifuged for 20 minutes at 12,000 g (Vision, Korea). The supernatant was further centrifuged at 4°C using an ultra-centrifuge (Beckman coulter, USA) for 60 minutes at 100,000 g. The supernatant obtained after ultra-centrifuge represented the cytosolic fraction. The cytosolic fractions for all groups were kept at -80°C until use [12]. Protein

concentration was determined for each group using Bradford method [13], using BSA as a standard curve.

Determination of GST enzyme activity

The method for GST enzyme activity was obtained from the work published by Habig *et al.* [14] and was optimized for the current study. Incubation mixtures (300 µL) contained 0.1 M potassium phosphate buffer (pH 6.5), 30 mM CDNB, 30 mM GSH, and GST enzymes (0.08 mg/ml rat liver cytosolic fraction). The experiments were divided into the blank group, control group and test groups for both *in vitro* and *ex vivo* studies. For blank groups, the cytosolic fraction was first denatured by heating it to 60°C for 10 minutes in a water bath. For the control group, the cytosolic fraction used was not being denatured. The plant extracts at concentrations 0.01, 1 and 10 µg/ml were added *in vitro* to the reaction mixture. Meanwhile, for *ex vivo* study, the cytosolic fractions from treated rats were used without the addition of plant extracts to the reaction mixture. For the analysis of GST enzyme activity, a simple spectrophotometric method was used. This method is based on the principle that GST enzyme catalyzes the condensation of GSH with the model substrate, 1-chloro-2,4-dinitrobenzene (CDNB) yielding 2,4-dinitrophenyl- GSH, which absorbs light at 340 nm. GST enzyme activity was measured kinetically for every minute until 5 minutes using the microplate reader.

Statistical analysis

Results obtained were compared with the control. Mean and standard deviation were calculated. One-way ANOVA with *post-hoc* Dunnett's test were used to analyze data, and the level of significance was set at $p < 0.05$.

RESULT

In vitro effect of standardized PNME on GST enzyme activity

Fig. 1 shows the *in vitro* effect of PNME at three concentrations 0.01, 1 and 10 µg/ml on GST activity in diabetic young female SD rats. All *in vitro* concentrations showed no significant effect on GST activity compared with the respective control group.

Acute ex vivo effect of standardized PNME on GST enzyme activity

The protein concentration of cytosolic liver fraction of young female diabetic rats was only reduced significantly at a dose of 500 mg/kg of extract. All acute oral doses of PNME showed a significant dose-independent increase in GST activity compared with the respective control group (all $p < 0.05$). Fig. 2 shows the effect of acute oral dose of PNME on GST activity in diabetic young female SD rats.

Sub-chronic ex vivo effect of PNME on GST enzyme activity

Fig. 3 demonstrates the effect of sub-chronic oral doses of PNME on GST activity in young diabetic SD rats. Similarly, GST activity showed a significant dose-independent increase in groups treated with 500 and

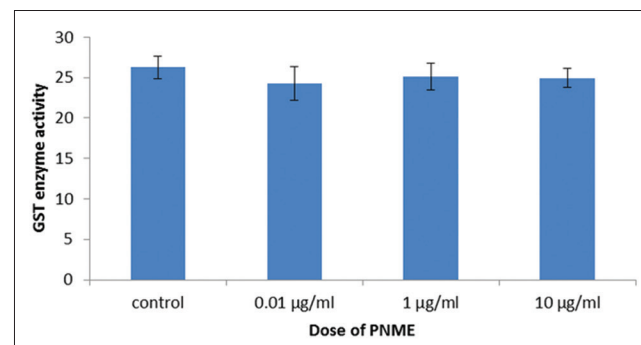


Fig. 1: In vitro effect of *Phyllanthus niruri* methanol extract (PNME) on cytosolic glutathione S-transferase (GST) activity in diabetic young female Sprague-Dawley rats. Each value is expressed as mean ± standard deviation: n=6. GST activity is expressed as µmol product formed/min/mg of cytosolic protein

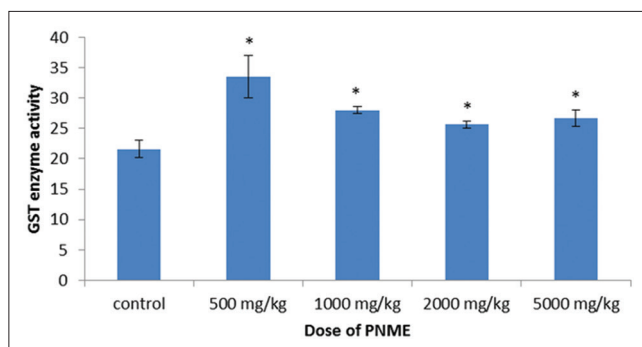


Fig. 2: Effect of acute oral dose of *Phyllanthus niruri* methanol extract on cytosolic glutathione S-transferase (GST) activity in diabetic young female Sprague-Dawley rats. Each value is expressed as mean \pm standard deviation: n=6; significant difference with respect to control: *p<0.05. GST activity is expressed as μ mol product formed/min/mg of cytosolic protein.

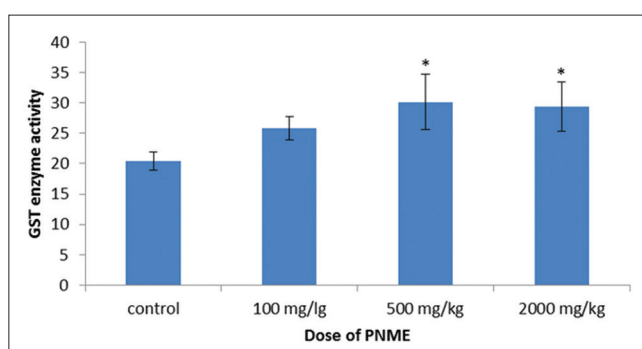


Fig. 3: Effect of sub-chronic oral dose of *Phyllanthus niruri* methanol extract on cytosolic glutathione S-transferase (GST) activity in diabetic young female Sprague-Dawley rats. Each value is expressed as mean \pm standard deviation: n=6; significant difference with respect to control: *p<0.05. GST activity is expressed as μ mol product formed/min/mg of cytosolic protein

2000 mg/kg (all p<0.05) of PNME as compared to the respective control. No significant effect on GST activity was observed with 100 mg/kg sub-chronic dose compared to the respective control.

DISCUSSION

Redox status is a concept that is used to describe the ratio of interconvertible of reduced and oxidized forms of a molecule. The disturbance of redox status causes an increase in reactive oxygen species (ROS), which oxidizes macromolecules of the cell and consequently affects proteins and transcription factors that are important for cell survival. GSH is regarded as one of the most important redox molecule that possesses a protective antioxidant activity inside the cell. This is because of its high concentration inside the cell compared to other redox molecules. It performs the antioxidant action due to the oxidation of the thiol group in its cysteine residue to the disulfide form [15]. GSH is the cofactor of cytosolic GST S-transferase enzyme (GST) which conjugates with other electrophilic molecules and free radical species. Seven sub-family classes have been recognized for cytosolic GST in mammals namely alpha, mu, pi, zeta, theta, omega and sigma [16]. Different gene families have the same function but differ in their substrate specificities [17]. Mu and pi GST subfamilies are the major members of GST family. They are involved in GSH conjugation with chemotherapeutic agents, ROS and a wide range of xenobiotic [18].

Diabetes mellitus is regarded as the main cause of oxidative stress in the body. This may be due to the reduction of redox molecules concentration

like GSH, which consequently leads to a reduction in GST activity as this enzyme conjugates GSH to electrophilic molecule [19,20]. GSH was proven to possess a protective role against diabetes mellitus inside the body [21].

The selection of diabetic young female SD rats in this study was due to a previous study which showed that this group of rats possessed the highest significant effect on Phase I aminopyrine N-demethylase enzyme activity after treatment with PNME [10]. Furthermore, diabetes mellitus is considered as a good model to study the cellular, molecular, and morphological changes in liver [22]. The diabetic state was induced by intravenous injection of STZ (60 mg/kg), which is used widely to induce Type I diabetes mellitus in experimental rats. This effect is primarily due to the destruction of beta-cells of the pancreas that synthesize and secrete insulin. Consequently, there will be a reduction in insulin secretion and elevation in blood glucose level [23]. Besides, it has been reported that STZ causes a reduction of GSH content and a simultaneous increase in the free radicals concentration. These entities can perhaps interfere with DNA, hence causing cellular damage [24].

Supplementation with dietary antioxidants like herbs helps in protecting the body from carcinogenic chemical compounds. The possible mechanism of such action may be due to antioxidant dependent induction of detoxifying enzymes [25].

P. niruri is a herb that is widely distributed in tropical areas such as India, China and Malaysia. Several bioactive molecules have been discovered in *P. niruri* such as lignans, flavonoids, glycosides, and tannin [26]. Phyllanthin and hypophyllanthin, are the polyphenolic lignan compounds in *P. niruri* which found to have a protective role from carbon tetrachloride cytotoxicity in rat hepatocytes [27]. *P. niruri* is one of the components of a multi-herbal preparation for the treatment of liver ailment [28]. Several studies have shown that the hepatoprotective role of the plant is associated with the antioxidant effect of its extract [29,30].

Alteration of hepatic drug metabolizing enzymes activity is an important concept in drug interactions, which leads to adverse drug reactions [31]. Although *P. niruri* received much attention from researchers, its modulatory effect on hepatic drug metabolizing enzymes has not yet been studied. Therefore, in this study, we have examined the effect of PNME on cytosolic GST enzyme activity.

In vitro results showed no significant effect of PNME on GST enzyme activity. This indicates that there is no direct effect of PNME on GST enzyme activity, while *ex vivo* results showed induction of cytosolic GST enzyme activity after acute and sub-chronic administration of PNME. The results indicate that induction of PNME toward GST enzyme activity may be partly due to intracellular induction of GSH caused by its flavonoids and lignans content [32,33] and partly to the scavenging antioxidant activity. This is agreed with other studies which showed that *P. niruri* has a high antioxidant activity and free radical scavenging activities [34,35]. This scavenging antioxidant activity is most likely attributable to lignans and flavonoids as potent antioxidants [36,37]. The increase of cytosolic GST enzyme activity after administration of *P. niruri* may accelerate the clearance of drugs that are metabolized by GST in Phase II metabolism and given concomitantly with the plant extract.

CONCLUSION

In summary, the Phase II *in vitro* study showed no significant effect of PNME on GST enzyme activity in diabetic young female rats. However, acute and sub-chronic *ex vivo* studies showed induction effect on GST activity at dose 500 mg/kg of the PNME. The increase in GST activity is likely to increase the scavenging activity toward free radicals and toxic compounds that are mainly produced due to diabetic state after STZ injection. The increase in GST activity may also increase the clearance of aminopyrine and drugs metabolized through the GST pathway if they are to be given concomitantly with *P. niruri* extract.

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