

EFFECT OF METHANOL LEAF EXTRACT OF *ORTHOSIPHON STAMINEUS* BENTH. ON HEPATIC DRUG METABOLIZING ENZYMES IN SPRAGUE DAWLEY (SD) RATS

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Abstrak: *Orthosiphon stamineus* Benth. atau dikenali dengan nama tempatan sebagai Misai Kucing digunakan secara meluas di Malaysia untuk rawatan hipertensi, masalah sistem urinasi dan penyakit batu karang ginjal. Kajian ini bertujuan untuk mengkaji kesan *in vitro* ekstrak metanol *O. stamineus* ke atas aktiviti aminopirin N-demetilase, UDP-glukuronosiltransferase (UGT) dan glutathione-S-transferase (GST) di dalam hati tikus jantan dan betina muda Sprague Dawley (SD). Hepatosit, mikrosomal dan fraksi sitosolik (fraksi S-9) daripada hati tikus jantan dan betina muda telah digunakan sepanjang eksperimen *in vitro* ini untuk menguji aktiviti aminopirin N-demetilase, UGT dan GST. Keputusan yang didapati menunjukkan ekstrak metanol *O. stamineus* tidak mempengaruhi aktiviti aminopirin N-demetilase di dalam hati tikus jantan dan betina SD kecuali pada 0.001 mg/ml ($P < 0.05$) pada tikus betina. Walau bagaimanapun, kebanyakan aktiviti UGT dan GST di dalam hati tikus jantan dan betina dikurangkan secara signifikan oleh ekstrak metanol *O. stamineus* terutamanya pada kepekatan yang lebih tinggi seperti 0.1 mg/ml dan 1 mg/ml jika dibandingkan dengan kumpulan kawalan. Secara kesimpulan, kepekatan ekstrak metanol *O. stamineus* daripada 0.00001 mg/ml sehingga 0.0001 mg/ml tidak mempengaruhi aktiviti aminopirin N-demetilase, UGT dan GST di dalam hati tikus jantan dan betina muda SD. Kepekatan ekstrak metanol *O. stamineus* yang lebih tinggi daripada 0.0001 mg/ml mempunyai kesan yang lebih besar ke atas aktiviti UGT dan GST jika dibandingkan dengan aktiviti aminopirin N-demetilase di dalam hati tikus SD dan kesannya adalah berkadar dengan dos.

Kata kunci: *Orthosiphon stamineus*, Hati Tikus, Aminopirin, UGT, GST

Abstract: *Orthosiphon stamineus* Benth. or locally known as *Misai Kucing* is widely used in Malaysia for treating hypertension, urinary system ailments and kidney stone disease. This study aims to study the *in vitro* effect of methanol extract of *O. stamineus* on aminopyrine N-demethylase, UDP-glucuronosyltransferase (UGT) and glutathione-S-transferase (GST) activities in young male and female Sprague Dawley (SD) rat liver. Hepatocytes, liver microsomes and cytosolic liver fraction (S-9 fraction) from young male and female SD rats were used throughout the *in vitro* experiment to examine aminopyrine N-demethylase, UGT and GST activities. Results obtained showed that aminopyrine N-demethylase activities in young male female and rat liver were not affected by methanol extract of *O. stamineus* except at 0.001 mg/ml ($P < 0.05$) in female rats. However, most of the UGT and GST activities in young male and female SD rat liver were significantly decreased by methanol extract of *O. stamineus* treatment especially at the higher concentrations such as 0.1 mg/ml and 1 mg/ml as compared to their control group. In conclusion, concentrations of methanol extract of *O. stamineus* from 0.00001 mg/ml to

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0.0001 mg/ml did not affect the activities of aminopyrine N-demethylase, UGT and GST in young male and female SD rat liver. Methanol extract of *O. stamineus* higher than 0.0001 mg/ml had a greater influence on UGT and GST activities than the aminopyrine N-demethylase activities in SD rat liver and the effects were dose-dependent.

Keywords: *Orthosiphon stamineus*, Rat Liver, Aminopyrine, UGT, GST

INTRODUCTION

Herbal medicines have received great attention as alternative medicines in recent years and are sold as dietary supplements. *Orthosiphon stamineus* Benth. or locally known as *Misai Kucing* becomes one of the most popular herbal medicine in Malaysia (Indubala & Ng 2000). *O. stamineus* is traditionally used to treat urinary lithiasis, rheumatism, hepatitis and jaundice (Wiert 2002). The leaves are arranged in opposite pairs. The petiole is relatively short, about 0.3 cm in length and reddish purple in color. The flowers are borne on verticals about 16 cm length, white to bluish in color with long far-exserted filaments, making it look like cat's whispers (Wiert 2002). Many research institutes such as Institute of Medical Research and Hospital Kuala Lumpur are conducting clinical study to prove the efficacy of *O. stamineus* in treating kidney stones disease (Sim et al. 2003).

There are many documented reports about herb-drug interactions in human (Rotblatt & Ziment 2002). Induction of hepatic enzyme activities in animals following the treatment with many natural compounds or traditional medicines which include *Gingko biloba* (Fessenden et al. 2001), grapefruit juice (Lilja et al. 2000; Kane & Lipsky 2000), St. John's Wort (*Hypericum perforatum*) (Durr et al. 2000) and Kava (*Piper methysticum*) (Almeida & Grimsley 1996) had previously been reported. However, there is no previous scientific study reported on the effect of methanol extract of *O. stamineus* on herb-drug interaction and toxicity in laboratory animals and in man. It is possible that one substance may alter the bioavailability of another substance by inducing the phase I and phase II hepatic enzymes system, and subsequently affect drug therapeutic effect or even generate toxicity when two or more substances are given concurrently. There are two types of drug metabolizing enzymes; phase I and phase II enzymes (Timbrell 2000). Cytochrome P450 (CYP)-dependent monooxygenase is one of the major phase I enzymes. Aminopyrine N-demethylase enzyme is categorized as N-dealkylation reaction which belongs to one of the oxidation processes in the phase I hepatic drug metabolism reaction (Hodgson & Goldstein 2001). Aminopyrine is mainly N-demethylated by CYP P450 3A and 2B in rats (Kamataki 1993) and CYP 2C, such as CYP 2C19 in humans (Timbrell 2000) although many other isoforms of cytochrome P450 are also involved. Many drugs are metabolized through dealkylation reactions where alkyl groups attach to oxygen, nitrogen or sulphur via oxidation process, and rearrangement occurs resulting in loss of the alkyl group as aldehyde. Many cytochrome P450 system reactions involve an insertion of an oxygen atom into the bond between carbon and hydrogen, which is called hydroxylation; addition of an oxygen atom to a π -bond (epoxidation) or addition of an oxygen to the electron pair on a heteroatom (heteroatom oxidation) (Gibson & Skett 1994). UGT and GST are the major

phase II enzymes. GST activity is present in most animal tissues but is especially prevalent in the liver, which plays a major role in detoxification. GST utilizes glutathione (GSH) to scavenge electrophilic xenobiotics as part of an organism's defense mechanism against the mutagenic, carcinogenic and toxic effects of such compounds. Induction of GST activities towards 1-chloro-2,4-dinitrobenzene (CDNB) appears to be induced by a number of GST forms in rat liver. The liver is the major site for UGT enzymes and glucuronidation reactions in all living organisms, and catalyzing enzymes known as UGT are expressed in the centrilobular and periportal zones of liver (Ullrich *et al.* 1984). UGT enzymes and glucuronidation reactions have been reported to have important role in drugs and xenobiotic metabolism. The primary role of drug glucuronidation is to initiate clearance of drugs from the body in bile and urine. However, other endogenous compounds like steroid hormone, bile acids, and thyroid hormones are also metabolized through glucuronidation reaction (Dutton 1980). This study aims to examine the possible herb-drug interactions of *O. stamineus* by examining the *in vitro* effect of methanol leaf extract of *O. stamineus* on several hepatic drug metabolizing enzymes known as aminopyrine N-demethylase, UGT and GST activities in rat liver.

MATERIALS AND METHODS

Chemicals

Aminopyrine, glutathione (reduced form), *p*-nitrophenol (*p*NP), uridine diphosphate glucuronic acid (UDPGA), magnesium chloride (MgCl₂), and CDNB were purchased from Sigma Chemicals Co, St Louis, MO, USA.

Experimental Animals

The albino young male and female rats (7 weeks old \pm 1 week; 100 \pm 10 g) from SD strain bred in the animal house, School of Pharmaceutical Sciences, Universiti Sains Malaysia were used throughout this experiment. The rats had free access to water and food.

Preparation of Extract

The spray-dried powder-form *O. stamineus* extract was supplied by Professor Zhari Ismail from School of Pharmaceutical Sciences, Universiti Sains Malaysia.

The leaves of the plant were collected in the late afternoon, from 30–45 days old white flowered plants. The leaves were chopped and dried at approximately 40°C for three days. Methanol extract of *O. stamineus* was prepared using a proportion of 10 g dried leaves in 100 ml of methanol by warming for four hours at 40°C. The solution was filtered through filter paper (Whatman No. 1), concentrated and spray-dried to obtain the crude methanol extract (Akowuah *et al.* 2004).

Liver Samples Preparation

Hepatic cells contain a multitude of metabolizing enzymes that are usually localized in the specific organelles within the cells. This study used freshly

isolated perfused hepatocytes to examine the aminopyrine N-demethylase activity whereas S-9 liver cytosolic fraction and liver microsomes were used to examine the GST and UGT activities respectively in rats.

Preparation of Hepatocytes

Isolated hepatocytes from normal young male and female SD rats were prepared by the collagenase perfusion technique (Hussin & Skett 1988). The animals were anesthetized with ether and the liver was perfused *in situ* through the hepatic portal vein with calcium-free Hanks balanced salt solution (Hank's BSS) for about 15 min followed by the collagenase buffer (Hank's BSS supplemented with 4 mM calcium chloride and 0.5 mg collagenase/ml) until the liver appeared to have broken up. The liver was removed and homogenized. The cell suspension was filtered through gauze and centrifuged at $200 \times g$ for 5 min. The supernatant was removed and cells suspended in incubation medium (Hank's BSS supplemented with 1 g/l glucose, 100 mg/l $MgSO_4$, 100 mg/l $MgCl_2$ and 185 mg/l $CaCl_2$; pH 7.4). The cells were then counted using a haemocytometer and assayed for viability using trypan blue.

Preparation of S-9 Fraction and Liver Microsomes

S-9 fraction was prepared as described by Gibson and Skett (1994). The liver was homogenized and the homogenate was centrifuged at 9 000 rpm for 20 min at 4°C. The supernatant or known as S-9 contains microsomal and soluble cytosolic fraction. The S-9 were kept at -70°C until use. Protein concentration of the S-9 fractions was determined by Lowry's method using bovine serum albumin as the standard (Gibson & Skett 1994).

Liver microsomes were prepared using calcium precipitation technique (Gibson & Skett 1994). Part of the cytosolic fraction above was then used to prepare rat liver microsomes. Microsomal protein concentration was determined by the method of Lowry *et al.* (1951). The microsome was kept frozen at -80°C with 20% (v/v) glycerol added.

Enzyme Assay

Aminopyrine N-demethylase Assay

For aminopyrine N-demethylase assay, each petri dish contains aminopyrine (final concentration, 25 mM), 6.0×10^3 hepatocytes, incubation medium and an equal volume (1 ml) of *O. stamineus* (ranging 10 ng/ml to 1 mg/ml). The final volume of reaction mixture was 10 ml. The petri dishes were then incubated in an incubator for 18 minutes at 37°C. Control petri dishes essentially contained all of the above with the exception of the herbal preparation, being replaced with distilled water. The reaction was terminated by adding 25% (w/v) $ZnSO_4$ and followed by saturated $Ba(OH)_2$ after five minutes. The sample was then centrifuged by table-top-centrifuge machine (Eppendorf®) at 1000 rpm for five minutes. Aminopyrine N-demethylase activity was measured at 415 nm using Powerwave X340® microplate reader (Bio-Tek Instruments, inc. USA) to

determine the quantity of formaldehyde formed according to the colorimetric method of Nash (1953).

Glutathione-S-transferase (GST) Assay

Total GST activity was measured with CDNB as the substrate (Gibson & Skett 1994). The reaction mixture contained potassium phosphate buffer (100 mM; pH 6.5), 0.01 ml of GSH (30 mM), 0.01 ml of CDNB (30 mM) and equal volume of serial concentrations of methanol extract of *O. stamineus* (10 ng/ml to 1 mg/ml). The reaction was started by adding 10 mg/ml of cytosolic fraction. The final volume was 0.3 ml. Control petri dishes essentially contained all of the above with the exception of the herbal preparation, being replaced with distilled water. The reaction was monitored kinetically at 340 nm for every minute until five minutes by a continuous Powerwave X340[®] microplate spectrophotometer (Bio-Tek Instruments, inc. USA).

UDP-glucuronosyltransferase (UGT) Assay

UGT activity towards pNP was assayed following slight modifications of the method by Bock and Kohle (2004). 0.04 ml rat liver microsomes (2.5 mg/ml) were preincubated with 0.008 ml of Triton X-100 (0.75% w/v) for three minutes at 37°C in a water bath shaker. The reaction mixture contained 0.02 ml tris-HCl (1.0 M), 0.02 ml MgCl₂ (50 mM), 0.02 ml pNP (5 mM) as the substrate and 0.02 ml of serial concentrations of methanol extract of *O. stamineus* (10 ng/ml to 1 mg/ml). Distilled water was added to a final volume of 0.2 ml. Control petri dishes essentially contained all of the above with the exception of the herbal preparation, being replaced with distilled water. The reaction was started by adding 0.02 ml of 30 mM UDPGA. Reaction mixture was then incubated for another 15 min at 37°C. The reaction was stopped by adding 20% (w/v) trichloroacetic acid to precipitate the protein and centrifuged at 2000 rpm for 10 min using a centrifuge (Eppendorf[®]). Supernatant was mixed with 0.5 M NaOH to develop the color of the mixture. The absorbance was read at 405 nm on a Powerwave X340[®] microplate reader (Bio-Tek Instruments, inc. USA).

Statistical Analysis

Aminopyrine N-demethylase, UGT and GST enzyme activities were compared to the respective control group; and means and standard deviations were calculated. Analysis was carried out using Dunnett Test. The levels of significant were set at $P < 0.05$ and $P < 0.01$.

RESULTS

A total of six different concentrations of methanol extract of *O. stamineus* (0.00001 mg/ml to 1 mg/ml) were examined in normal young male and female SD rat liver. Lower concentrations of *O. stamineus* (0.00001 mg/ml to 0.0001 mg/ml) did not affect both aminopyrine N-demethylase, UGT and GST activities (Figs. 1, 2 & 3). A significant increase in aminopyrine N-demethylase (Fig. 1) and UGT activities (Fig. 2) were observed in normal young female SD rat liver in the

presence of 0.001 mg/ml ($P < 0.05$) of methanol extract of *O. stamineus*. However, both UGT and GST activities in young female SD rat liver were significantly decreased after being treated with 0.1 mg/ml ($P < 0.01$) and 1 mg/ml ($P < 0.01$) of methanol extract of *O. stamineus* as compared to their control group (Figs. 2 & 3). The *in vitro* effect of methanol extract of *O. stamineus* on phase II hepatic metabolizing enzymes that are UGT and GST activities in young female SD rats were dose-dependent from the concentration of 0.001 mg/ml to 1 mg/ml.

No significant change on aminopyrine N-demethylase activities were observed in normal young male SD rats (Fig. 1). A significant increase in UGT and GST activities were observed after being treated with 0.001 mg/ml ($P < 0.05$) and 0.01 mg/ml ($P < 0.05$) of methanol extract of *O. stamineus* respectively as compared to control group (Figs. 2 & 3). However, both UGT and GST activities in young male SD rat liver were significantly decreased after being treated 1 mg/ml ($P < 0.01$) of methanol extract of *O. stamineus* as compared to their control group (Figs. 2 & 3).

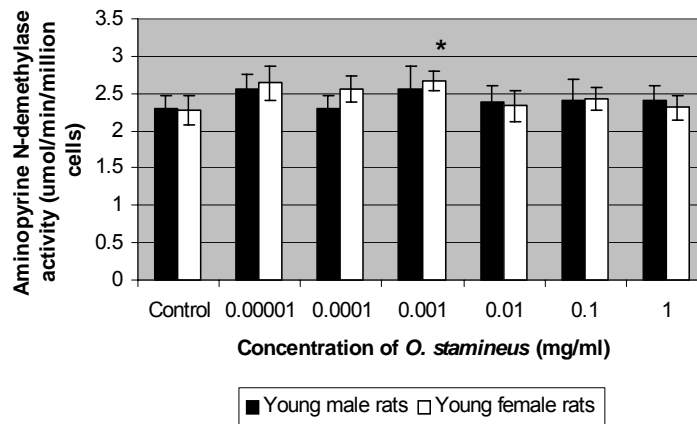


Figure 1: *In vitro* effect of methanol leaf extract of *O. stamineus* on aminopyrine N-demethylase activity in young male and female SD rat hepatocytes.

n = 6; values = mean \pm S.D

Results were analyzed by Dunnett test

* $P < 0.05$ as compared to control group

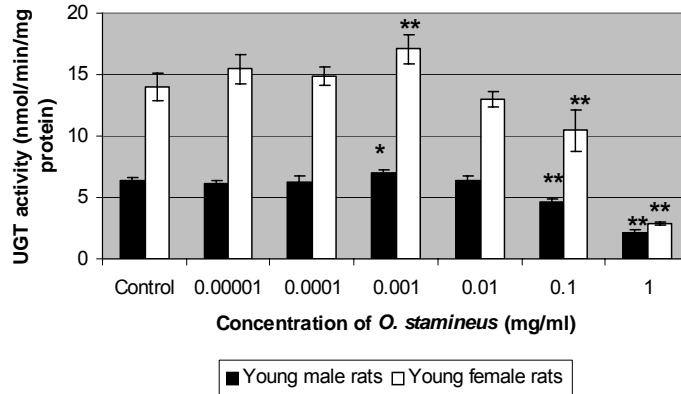


Figure 2: *In vitro* effect of methanol leaf extract of *O. stamineus* on UGT activity in young male and female SD rat liver microsomes.
 n = 6; values = mean ± S.D
 Results were analysed by Dunnett test
 * P < 0.05; ** P < 0.01 as compared to control group

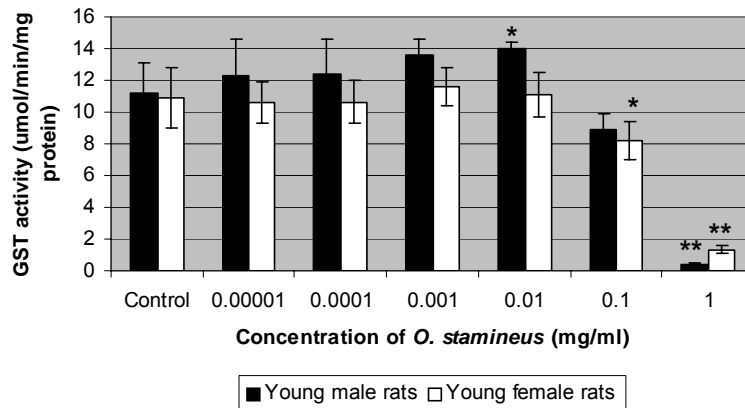


Figure 3: *In vitro* effect of methanol leaf extract of *O. stamineus* on GST activity in young male and female SD rat liver cytosolic fraction.
 n = 6; values = mean ± S.D
 Results were analysed by Dunnett test
 * P < 0.05; ** P < 0.01 as compared to control group

DISCUSSION

Methanol extract of *O. stamineus* was shown to be more effective in affecting phase II hepatic drug metabolizing enzymes, UGT and GST activities as compared to phase I hepatic drug metabolizing enzyme, aminopyrine N-demethylase activity in SD rats. At the higher concentration of *O. stamineus* such as 0.1 mg/ml and 1 mg/ml, it was significantly affected hepatic phase II enzymes, UGT and GST activities but not on phase I, aminopyrine N-demethylase activity in normal young female SD rat liver. Prochaska and Talalay (1988) termed the incident of induction in phase II enzymes but not in phase I enzymes (CYP) as 'monofunctional induction' in contrast to 'Ah receptor agonists' which induce both phase I and II enzymes (bifunctional induction). In addition, methanol extract of *O. stamineus* also exerted biphasic effect on UGT activity in which in the presence of 0.001 mg/ml, a significant increase in UGT activity was observed; on the other hand, at the higher concentrations, a significant decrease in UGT activity was observed in young male and female SD rat liver.

The major differences of the effect of *O. stamineus* extract on male and female SD rats were seen at GST and aminopyrine N-demethylase activities. The biphasic effect of methanol extract of *O. stamineus* on GST activity was only observed in normal young male SD rat liver but not in female rats, in which a significant increase in GST activity was observed in the presence of 0.01 mg/ml of methanol extract of *O. stamineus* and decreased its GST activity in the presence of 1 mg/ml of methanol extract of *O. stamineus*. The effect of *O. stamineus* extract on drug metabolizing enzymes in young male rat liver was exhibited to be 'monofunctional induction' pattern because the aminopyrine N-demethylase activities were not affected by any concentration of methanol extract of *O. stamineus*. Thus, different concentrations of *O. stamineus* extract and gender factor may be responsible for the different results obtained in our *in vitro* studies. If an *O. stamineus* extract exceeded certain concentration, a significant induction of hepatic drug metabolizing enzymes may occur, as shown in this study.

Although clinically relevant interaction between *O. stamineus* with other drugs have not been reported, it should be generalized that any induction to increase the rate of liver metabolized enzyme activity would result in herbs or drugs being metabolizing at a faster rate, and their overall clinical effectiveness would be reduced. For example, based on the results obtained, 0.001 mg/ml of methanol extract of *O. stamineus* should not be taken concurrently with erythromycin and diazepam because *O. stamineus* extract induces a major hepatic drug-metabolizing enzymes, aminopyrine N-demethylase and UGT activities in female rat and it will reduce the efficacy of drugs that are metabolized by CYP3A4 and UGT enzymes (Hussin & Skett 1988). In contrast, any inhibition of these liver enzymes activity would prolong effectiveness of the drug or herb in the body and remains in the body for a longer time. However, it would cause some unwanted side effects (Rotblatt & Ziment 2002). However, this preliminary study remains to be elucidated whether these *in vitro* data may be assigned to the *in vivo* situation.

O. stamineus contains many chemically active constituents including polyphenols (lipophilic flavonoids and phenolic acids), terpenoids (diterpenes and triterpenes) and sterols (Tezuka *et al.* 2000). Recent researchers from Universiti Sains Malaysia had reported that rosmarinic acid is the main component in the standardized methanol extract of *O. stamineus* with concentration ranging from 5.1% to 29.9% of the total dry leaf weight. Concentrations of 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF), eupatorin (EUP) and sinensetin (SEN) ranged from 0.05% to 0.69%, 0.34% to 3.37% and 0.22% to 1.76% respectively (Akowuah *et al.* 2004). The therapeutic effects of *O. stamineus* have been ascribed mainly to its polyphenols, which have enzyme inhibition and antioxidant activities (Akowuah *et al.* 2006). The mechanisms of modulation effect of these flavonoids on UGT and GST activities remain unknown and have not yet been extensively reported although it is generally accepted that flavonoids are inducer of UGT and GST activities in rat liver (Hodek *et al.* 2000). The structural-relationship of each compound such as EUP, SEN and rosmarinic acid on these modulation effects of hepatic drug metabolizing enzymes need to be clarified in future.

CONCLUSION

Methanol extract of *O. stamineus* had a greater influence on UGT and GST activities in young male and female rat liver as compared to aminopyrine N-demethylase activity. A biphasic effect of methanol extract of *O. stamineus* on UGT activity was generally observed in both young male and female SD rats. Higher concentrations of methanol extract of *O. stamineus* exhibited "monofunctional induction" pattern on phase II that are UGT and GST activities but not on hepatic phase I, aminopyrine N-demethylase activity in young male and female SD rats.

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