Pharmacognosy Research [Phcog Res.]
Vol 1, Issue 2, Mar-Apr, 2009 Page 53-58
[Rapid Publication]
A Supplement to Pharmacognosy Magazine – [Phcog Mag.]
Rapid publication process initiated: 2nd February, 2009
Accepted: 26th February, 2009

PHCOG RES.: Research Article

Anti-hepatotoxicity Effect of *Orthosiphon stamineus* Benth against Acetaminophen-induced Liver Injury in Rats by Enhancing Hepatic GST Activity

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ABSTRACT

The present study was undertaken to assess the sub-chronic (14-day treatment) effect of oral administration of *O. stamineus* on hepatic drug metabolising enzymes and the protective effect against acetaminophen-induced liver injury in Sprague Dawley (SD) rats. Healthy male SD rats weighing 200 g ± 10 g were used throughout the experiments. Isolated hepatocytes and liver cytosolic fraction were used to examine the effect of *O. stamineus* on the activity of aminopyrine N-demethylase and glutathione-S-transferase respectively. Hepatoprotective effect of *O. stamineus* was judged by comparing the serum levels of hepatic markers, i.e. AST, ALT and ALP and the percentage of hepatocytes viability between *O. stamineus* treatment groups and acetaminophen (APAP) control group. *O. stamineus* extract at 125 mg/kg ($P<0.01$) and 500 mg/kg ($P<0.01$) exhibited significant hepatoprotective effect by restoring the elevation of serum levels of AST and ALT and increased the percentage of hepatocytes viability in rats. The hepatoprotective effect exhibited by *O. stamineus* at dose 500 mg/kg was comparable to silymarin at dose 20 mg/kg in acetaminophen-induced liver injury rats. The activity of GST was 17% higher in the rats treated with 500 mg/kg ($P<0.01$) of *O. stamineus* compared with the control group. There was no significant difference in body weigh gained, food consumption, water intake and relative organ weight between the treatment group and the control group. Methanol extract of *O. stamineus* protects against acetaminophen-induced liver injury in rats by enhancing the activity of GST in liver.

Key words: Acetaminophen, Glutathione-S-transferase, Hepatoprotective, *Orthosiphon stamineus*, serum enzymes

INTRODUCTION

Orthosiphon stamineus Benth. (Family: Lamiaceae) or locally known as "Misai Kucing" is easily cultivated in Southeast Asia countries likes Malaysia, Indonesia and Thailand (1). A decoction of the leaves is commonly consumed by Malaysian for the treatment of several pathological conditions, including gout, rheumatism, kidney problem and arteriosclerosis (2). Three major flavonoids, namely, sinensitin, eupatorin and 3'-hydroxyl-5,6,7,4'-tetramethoxyflavone were identified from the methanol leaf extract of *O. stamineus* (3). The beneficial therapeutic effects of *O. stamineus* to treat diabetes mellitus, kidney problems and inflammation have been scientifically proven using animal models (4-6). Oral administration of methanol leaf extract of *O. stamineus* at doses of 0.5 g/kg to 5 g/kg to young female SD rats for fourteen days did not cause any adverse effects or organ damages. There was no significant change in serum hepatic and kidney

markers between treatment groups and control group (7).

Acetaminophen is an effective analgesic when used at therapeutic doses. An overdose of acetaminophen induces severe hepatotoxicity in humans and experimental animals. An overdose of acetaminophen leads to increased formation of the active metabolites, namely *N*-acetyl-*p*-benzoquinoneimine (NAPQI) *via* cytochrome P450 system (8). Several mechanisms of hepatotoxicity induced by an overuse of acetaminophen have been extensively reported. These mechanisms are including the covalent binding of the NAPQI to cellular macromolecules, lipid proxidation, the oxidation of critical sufhydryl groups of cytochrome and the alteration of calcium homeostasis have been extensively reported (9). Increased the rate of biotransformation of acetaminophen by cytochrome P450 system increases the susceptibility to hepatotoxicity induced by acetaminophen while decreased the intracellular pool of glutathione (GSH) and phase II durg metabolising enzymes such as GST causes in inadequate detoxification mechanism (10). Some of the phytochemicals in herbal remedies have been demonstrated capable of causing profound effects on the metabolism of drugs by modulating hepatic drug metabolising enzymes. Thus, in this study, we examined the relationship between induction of hepatic drug metabolising enzymes and liver protective effects of methanol leaf extract of *O. stamineus* against acetaminophen-induced liver injury in rats.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the analytical grade. Acetaminophen, aminopyrine, 1-chloro-2,4-dinitrobenzene (CDNB), magnesium chloride, reduced glutathione were purchased from Sigma (St. Louis, M.O, USA).

Preparation of plant extract

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 (3).

Selection of experimental animals

All animals handling procedures were accordance with the guideline of the Animal Ethic Committee, Universiti Sains Malaysia. Healthy male Sprague Dawley (SD) rats weighing 200 g ± 10 g bred in Animal House Unit, Universiti Sains Malaysia were used throughout the experiments. The animals were kept in a room with controlled temperature (25°C ± 2°C) and in a 12-hr light/dark cycle for 1 week before experiments. All animals were free access to water and food *ad libitium*.

Determination of hepatic aminopyrine N-demethylase activity

The animals were divided into four groups, each group had six animals (n=6). First group served as control group which was received a single dose daily of distilled water while second, third and fourth group received orally 5 mg/kg, 125 mg/kg and 500 mg/kg body weight of methanol leaf extract of *O. stamineus* respectively for fourteen days. All rats were sacrificed twenty four hours after the last dose treatment. Isolated hepatocytes were prepared by collagenase perfusion technique (11). Isolated hepatocytes were used to examine the *in vivo* effect of *O. stamineus* on the activity of aminopyrine N-demethylase using spectrophotometer (12). The activity of aminopyrine N-demethylase was measured at 415 nm using Powerwave-X340® microplate reader (Bio-Tek Instruments, USA) to determine the quality of formaldehyde formed according to the colourimetric method by Nash (13).

Determination of hepatic GST activity

All animals were randomly grouped as described above and received the same treatment through the same route under similar conditions. All rats were sacrificed twenty four hours after the last dose treatment. The liver tissue was removed and homogenised to prepare liver cytosolic fraction (12). Liver cytosolic fraction was used to determine the activity of GST (14). The reaction was monitored kinetically at 340 nm for every minute until five minutes by a continuous Powerwave-X340® microplate reader (Bio-Tek Instruments, USA).

Cage-side observation

All rats were observed closely at the first four hours after each *O. stamineus* treatment. Any toxic symptoms and behavioural changes were recorded. The body weight gained, water intake and food consumption were recorded at day-3, day-7 and day-14 during the experimental period. Relative weight of organs such as liver, kidney, heart, spleen and lung were calculated. Gross examination was conducted to examine of any abnormalities developed in the organ.

Hepatoprotective activity

The rats were randomly assigned into six experimental groups, each contained six animals, including (i) control group was not received any treatment, (ii)

APAP group received a single dose daily of vehicle, distilled water, (iii) Silymarin+APAP group received a single dose daily of 20 mg/kg of silymarin and (iv-vi) *O. stamineus*+APAP groups received a single dose daily of 5 mg/kg, 125 mg/kg and 500 mg/kg of *O. stamineus* respectively. All rats were orally treated for fourteen days. 2 g/kg of acetaminophen was orally fed to all animals except control group, 2-hr after the last dose treatment. All rats were fasted at least 16 hours prior to the blood collection *via* cardiac puncture. The collected blood sample was allowed to clot and serum was separated at 2,500 rpm for 15 mins. The serum hepatic markers such as AST, ALT and ALP were assayed. Later, the rat livers were perfused using Collagenase Perfusion Technique. The cells were counted using haemocytometer and the viability of hepatocytes was assayed using trypan blue.

Statistical analysis

All the data were presented as mean ± S.D and analysed using Dunnett's Multiple Comparison Test. A values of $P<0.05$ and $P<0.01$ was considered statistical significant as compared to the respective control group.

RESULTS

Assessment of hepatic drug metabolising enzymes

The activity of aminopyrine N-demethylase and GST in the liver of control group was 2.42±0.17 µmol formaldehyde formed/min/million cells and 22.36±0.83 µmol/min/mg protein respectively. As shown in Fig. 1, the activity of hepatic GST was significantly increased by 17% in the rats treated with 500 mg/kg ($P<0.01$) of methanol leaf extract of *O. stamineus* compared to control group. There was no significant difference in the activity of aminopyrine N-demethylase between *O. stamineus* treatment groups and control group (Figure 1).

Cage-side Observation

No lethality incident was observed in *O. stamineus* treatment group and control group during the experimental period. Body weights changed, food consumption and water intake at day-3, -7 and -14 did not significantly change between control and *O. stamineus* treatment groups (Table 1). There was no significant change in relative weight of organs between *O. stamineus* treatment groups and control group (Table 2).

Hepatoprotective study

The serum biochemical analyses and percentage of hepatocytes viability revealed clearly the development of liver injury in the APAP group compared to control group (Figure 2 & 3).

Table 1: The effect of oral administration of methanol extract of Orthosiphon stamineus on body weight changed, water intake and food consumption in male SD rats.

Dose of *O. s* (mg/kg)	Body Weight Changed (g)			Food Consumption (g/rat/day)			Water Intake (ml/rat/day)		
	Day-3	Day-7	Day-14	Day-3	Day-7	Day-14	Day-3	Day-7	Day-14
Control	198.3±1.2	210.2±2.0	234.0±1.6	13.2±1.2	15.8±2.0	20.5±1.8	26.5±2.0	28.8±2.0	32.5±1.2
5	195.6±1.5	207.4±1.6	230.9±1.8	12.6±1.0	15.6±1.4	19.0±1.0	26.0±1.8	28.5±2.0	31.3±1.8
125	197.4±2.0	208.6±1.8	232.2±2.0	13.1±2.0	15.9±1.4	20.9±0.8	26.4±2.2	29.0±1.4	32.0±1.6
500	201.0±1.8	213.8±2.0	236.3±2.2	14.0±0.6	16.2±0.4	21.5±0.8	27.4±1.6	30.1±1.8	33.8±1.2

n=6; values = mean ± S.D; Results were analysed by Dunnett test.

Table 2: The effect of methanol extract of O. stamineus on the relative organ weight in male SD rats.

Dose of *O. s* (mg/kg)	Relative organ weight (g/100g body weight)				
	Liver	Heart	Kidney	Lung	Spleen
Control	2.71±0.10	0.32±0.02	0.57±0.02	0.54±0.03	0.20±0.01
5	2.68±0.08	0.31±0.02	0.56±0.03	0.53±0.0	0.20±0.01
125	2.68±0.06	0.31±0.02	0.58±0.04	0.53±0.02	0.20±0.01
500	2.74±0.10	0.32±0.02	0.59±0.04	0.54±0.02	0.20±0.01

n=6; Results are expressed as mean ± S.D; Results are analyses using Dunnett Test.

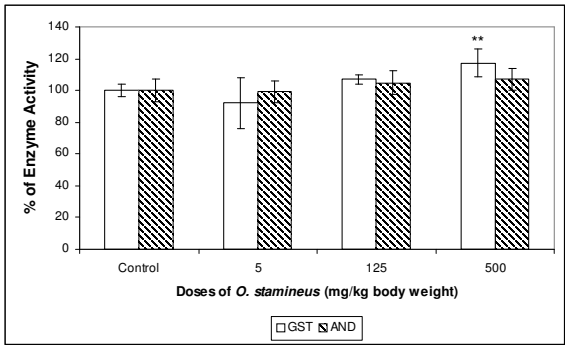

Figure 1: The effect of methanol leaf extract of Orthosiphon stamineus on hepatic glutathione-S-transferase (GST) and aminopyrine N-demethylases (AND) activity in male SD rats.

*n=6; Results are expressed as mean ± S.D; Results are analyses using Dunnett Test; ** P<0.01 significant compared to APAP control.*

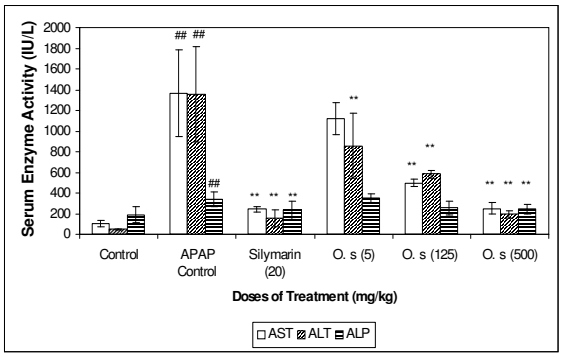

Figure 2: The effect of methanol leaf extract of Orthosiphon stamineus on serum enzyme levels in acetaminophen-induced liver injury male SD rats.

*n=6; Results are expressed as mean ± S.D; Results are analyses using Dunnett Test; ## P<0.01 significant compared to control; ** P<0.01 significant compared to APAP control.*

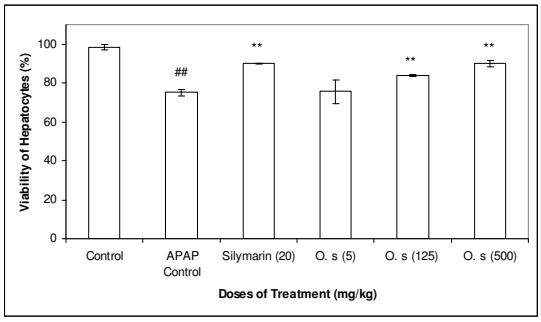

Figure 3: The effect of methanol leaf extract of Orthosiphon stamineus on viability of hepatocytes in acetaminophen-induced liver injury male SD rats.

*n=6; Results are expressed as mean ± S.D; Results are analyses using Dunnett Test ## P<0.01 significant compared to control; ** P<0.01 significant compared to APAP control.*

All serum biochemical parameters such as ALT, ALT and ALP and hepatocytes viability were significantly changed between the control and APAP control groups. Among all the doses of *O. stamineus* extract, the *O. stamineus* extract at 500 mg/kg (P<0.01) demonstrated the most potent effect in protecting rats against acetaminophen-induced liver damage, as evidenced by the decrease in all serum levels of AST, ALT and ALP and increased hepatocytes viability compared to the control.

DISCUSSION

The role of the liver in metabolising a wide range of chemicals and xenobiotics has been extensively studied (15). The increased levels of AST, ALT and ALP are conventional indicators of liver injury (16). An obvious sign of hepatic injury is the leaking of intracellular enzymes into the plasma due to the disturbance caused in the transport functions of hepatocytes. The present study revealed a significant increase in the levels of AST, ALT and ALP in blood after treating to 2 g/kg of acetaminophen indicating considerable liver injury. In the present study, we examined the effect of pretreatment with *O. stamineus* on acetaminophen induced hepatotoxicity. We found that the administration of 500 mg/kg of methanol extract of *O. stamineus* significantly reduced serum AST, ALT and ALP levels and increased hepatocytes viability in acetaminophen-treated rats. This is an indication of the stabilisation of plasma membrane, as well as repair of hepatic tissue damage due to the acetaminophen toxicity. The recovery of serum enzymes and percentage of hepatocytes viability contributed by 500 mg/kg of methanol extract of *O. stamineus* was found to be comparable to that of silymarin treatment.

Silymarin, a flavonolignan from *Silybum marianum,* is widely used to treat liver diseases. Several reported mechanisms underlying the hepatoprotective properties of silymarin include the prevention of GSH

depletion and destruction of free radicals (17-18).

According to the cage-side observation, the doses of methanol extract of *O. stamineus* at 5 mg/kg, 125 mg/kg and 500 mg/kg are safe in rats without causing any observable toxic signs or relative organ weight changes. The oral LD_{50} of the methanol extract of *O. stamineus* in adult male SD rats is suggested higher than 500 mg/kg body weight due to the no lethality incident was observed during the experimental period. For the assessment of hepatic drug metabolising enzymes, it is observed that pretreatment with *O. stamineus* for fourteen days resulted in significant induction of hepatic phase II enzyme, GST activity but no significant change was seen in the phase I hepatic aminopyrine N-demethylase mediated by CYP 3A in the liver when compared to the control group. Previously, methanol extract of *O. stamineus* has been reported to have an induction effect on another hepatic phase II enzyme, known as UDP-glucuronosyltransferase (UGT) activity in male rats (19). The induction of GST activity in animals is partly under control of the Ah gene locus or the antioxidant responsive element and it is usually correlated with the induction of UGT (20). A lower ratio of phase I enzymes (activating) to phase II (conjugating) would indicate effective conjugation resulting in an increase in the elimination of the active metabolites (21). From the result obtained, methanol extract of *O. stamineus* could have a greater influence on phase II enzymes compared to phase I enzyme. This suggests that the liver protection observed in the present study may have occurred by a detoxification mechanism rather than the prevention of bioactivation of acetaminophen catalysed by CYP P450 system. As reported by Trakshel and Maines (1988), GSH, glutathione peroxidase, GST and glutathione reductase are among the major antioxidant defense systems that eliminate lipid peroxides and reactive oxygen species (22). We have previously reported the *in vivo* antioxidant activity of this plant in rats as evidenced by the increase in hepatic antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase (7). An increase in GST activity by pretreatment of *O. stamineus* indicates its protective role, as GST is involved in the conjugation of a wide range of chemicals including the reactive intermediates, NAPQI generated from acetaminophen metabolism to non-toxic conjugates. On the other hand, *O. stamineus* might affect the therapeutic effects of certain drugs if they are concurrently consumed together. Methanol extract of *O. stamineus* could reduce the half-life and the bioavailability of co-administered drugs by enhancing the clearance in non-toxic glutathione conjugates.

CONCLUSION

The liver protective property exhibited by methanol extract of *O. stamineus* in the present study suggests they may be useful in the treatment of acute liver diseases by increasing the conjugation of hepatotoxins *via* glutathione conjugation.

ACKNOWLEDGEMENTS

This work was supported by research grant from the Ministry of Science, Technology and Innovative, Malaysia. We thank Prof. Zhari Ismail for his generous help in supplying methanol extract of *O. stamineus*.

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