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Hepatoprotective effect of *Orthosiphon stamineus* Benth against acetaminophen intoxication in rats

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Abstract

Orthosiphon stamineus Benth (Lamiceae) is commonly used by the Malaysian for the treatment of many diseases such as diabetes mellitus and hypertension. In the present study, the hepatoprotective effect of methanol extract of *O. stamineus* against acetaminophen (APAP)-induced hepatotoxicity in Sprague Dawley (SD) rats was studied. Fourty-two young male SD rats were randomly assigned to seven groups: normal control group, APAP control group, silymarin treated group and four groups receiving methanol extract of *O. stamineus* in varying dosages. The hepatoprotective effect of methanol extract of *O. stamineus* extract on SD rats was determined by using serum enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) levels and the percentage of hepatocytes viability. 2 g/kg of APAP caused a significant liver damage in APAP control group compared to normal control group. However, pretreatment with 500 mg/kg of methanol extract of *O. stamineus* to rats was significantly reverted all the APAP-induced changes in serum enzymes and the percentage of hepatocytes viability compared to the APAP control group. The results suggest that the use of methanol extract of *O. stamineus* appeared to be beneficial to rats in preventing the liver damage induced by APAP and may be of therapeutic value in the treatment of APAP-induced hepatotoxicity.

Keywords: *Orthosiphon stamineus*; Acetaminophen; Serum enzymes; Hepatocytes

1. Introduction

Orthosiphon stamineus Benth (Lamiceae) or locally known as Misai Kucing became a popular herbal tea among the community of Southeast Asia and European countries [1]. *O. stamineus* is also found in other countries such as Thailand, Indonesia and Europe. In these countries, *O. stamineus* is also known as yaa Nuat Maeo, Rau Meo or Cay Bac (Thailand),

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Kumis Kucing or Remujung (Indonesia), Moustaches de chat (French) and Java Tea and Kidney Tea (European). In Malaysia, the *O. stamineus* products are appearing in the forms of tea sachets, capsules and dried leaves. The leaves of *O. stamineus* are arranged in opposite pairs. The petiole is relatively short, about 0.3 cm in length and reddish purple in colour.

The flowers are borne on verticals about 16 cm length, white to bluish in colour with long far-exerted filaments, making it look like cat's whispers [2]. There are many different species of this plant which can be easily distinguished by its flower, these including *Orthosiphon aristatus* with white flower and *Orthosiphon grandifolis* with red colour flower.

This plant is believed to have several pharmacological and therapeutic effects. Traditionally, *O. stamineus* has been widely used by Malaysian community as a diuretic and for treating catarrh of the bladder. It has also been used to eliminate stones in the bladder [1]. In the recent years, many scientific studies have been undertaken with *O. stamineus*, in Malaysia in attempt to develop new remedies for treatment of many diseases such as diabetes mellitus, inflammation and urinary tract problem. The methanol extracts of this plant have shown the inhibitory activity on nitric oxide production in macrophage like cells [3].

The aqueous extract of *O. stamineus* has shown its anti-hyperglycemic effect in both normal and diabetic rats [4]. Protection of methanol extract of *O. stamineus* against acetaminophen (APAP)-induced liver damage in rats has not been previously reported. Hence, the present study aims to examine the hepatoprotective effect of methanol extract of *O. stamineus* on APAP-induced hepatotoxicity SD rats.

2. Methods

2.1 Chemicals

Potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), disodium hydrogen phosphate (Na_2HPO_4) were purchased from R & M Chemicals, UK. Trypan blue, acetaminophen and collagenase IV were purchased from Sigma-Aldrich chemicals Co. St Louis, MO, USA Glucose, magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$) and calcium chloride ($CaCl_2$) were purchased from BDH Laboratory Supplies, UK.

2.2 Plant material and preparation of the extract

Plants were grown from cuttings using standard agronomic practices at Kepala Batas, Penang, Malaysia. The leaves were collected from 30-45-day-old white-flowered plants. The specimen was labeled, numbered and annotated with the date of collection and locality. Voucher specimen of the plant material was deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Malaysia.

Plant leaves were ground to a homogeneous powder in a Wiley mill (no. 20 mesh) after drying in an oven (35°C). The dried powdered leaves were extracted with methanol by using soxhlet apparatus. After the solvent was removed under reduced pressure, portion of the concentrated extract was spray-dried [5].

2.3 Experimental Animals

A total of 42 male Sprague Dawley (SD) rats weighting 100 g ± 10 g were used throughout the present experiment. They were bred in the animal house unit, School of Pharmaceutical Sciences, Universiti Sains Malaysia. They were housed in a room temperature maintained at 25 ± 1°C with 12 hour light: 12 hour dark cycle. Food and water were provided *ad libitum* [6].

2.4 Determination of hepatoprotective effect of O. stamineus

After one week of acclimatisation, animals were divided into seven groups. Each group is consisting of six animals (n=6). Group I treated with vehicle (distilled water) for 14 days was kept as normal control group. Group II treated with vehicle (distilled water) was kept as APAP control group. Group III was pretreated with standard drug, 100 mg/kg of silymarin, for 14 days and followed by group IV, V, VI and VII were pretreated with a single dose daily of 5 mg/kg, 31.25 mg/kg, 125 mg/kg and 500 mg/kg of the methanol extract of *O. stamineus* up to 14 days. All rats (except normal control group) were orally fed with 2 g/kg of acetaminophen suspended into gum tragacath 2 h after the last dose administration at day-14 [7].

2.5 Behavioural observation

Four groups of rats treated with methanol extract of *O. stamineus* were observed continuously first hour after feeding for any gross behavioural changes, symptoms of toxicity and mortality if any and intermittently for the next 6 h and then again, 24 h after dosing with *O. stamineus* extract.

2.6 Biochemical parameters examination

Blood samples were collected using cardiac puncture post 48 hours of 2 g/kg of acetaminophen administration. After overnight fasting (at least 16 hours), all rats were briefly anesthetised by diethyl ether inhalation and blood was taken via cardiac puncture by using a needle (size 0.50 x 16 mm, Terumo) to collect about 2.0 ml of blood [8]. Blood samples obtained from SD rats were left for clotting at room temperature for 60 minutes. Then, blood samples were centrifuged at 3,000 RPM, at room temperature for 15 minutes to obtain blood serum. A total of three serum biochemical tests were tested. They were AST, ALT and ALP. All serum biochemical testing was conducted at Lam Wah Ee hospital, Penang, Malaysia using Roche (Intergra 700®) machine.

2.7 Determination of hepatocytes viability

All rats were subjected to liver perfusion. Hepatocytes obtained were examined for percentage of viability. Hepatocytes were isolated by collagenase perfusion technique [9]. Hepatocytes which excluded trypan blue were considered viable cells while hepatocytes with trypan blue stain were considered damaged cell [10].

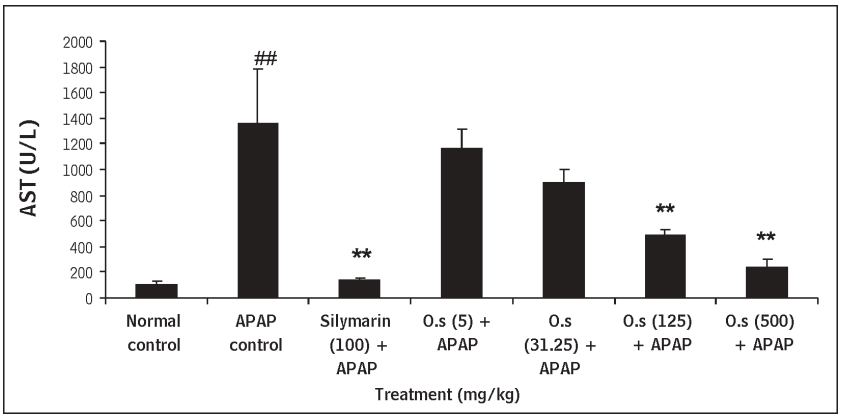

Fig. 1. Effect of methanol extract of *O. stamineus* on serum AST level in APAP-induced hepatotoxicity SD rats. Values are mean ± S.D, n=6; analysed using Dunnett's test. ** P<0.01 significantly different from APAP control; ## P<0.01 significantly different between normal control group and APAP control group.

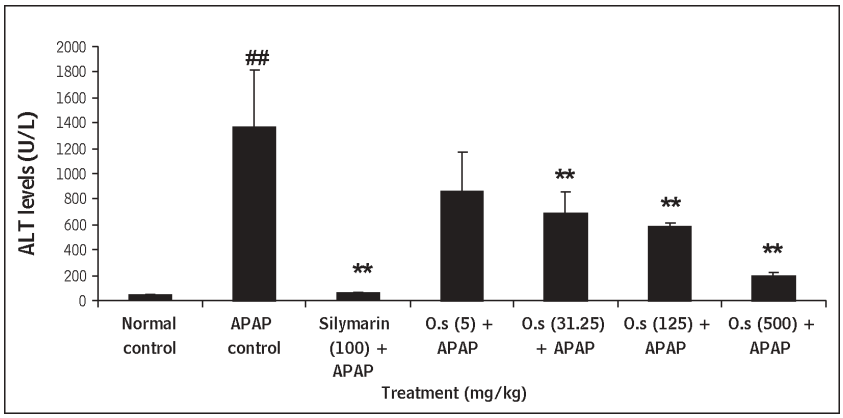

Fig. 2. Effect of methanol extract of *O. stamineus* on serum ALT level in APAP-induced hepatotoxicity SD rats. Values are mean ± S.D, n=6; Analysed using Dunnett's test. ** P<0.01 significantly different from APAP control; ## P<0.01 significantly different between normal control group and APAP control group.

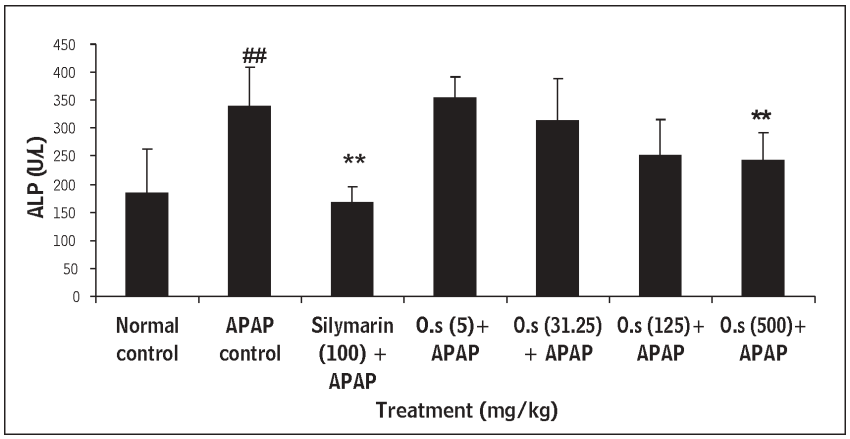

Fig. 3. Effect of methanol extract of *O. stamineus* on serum ALP level in APAP-induced hepatotoxicity SD rats. Values are mean ± S.D, n=6; Analysed using Dunnett's test. ** P<0.01 significantly different from APAP control; ## P<0.01 significantly different between normal control group and APAP control group.

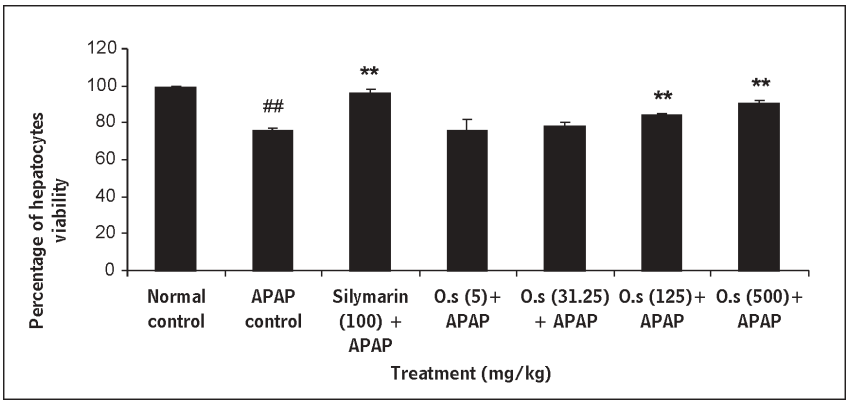

Fig. 4. Effect of methanol extract of *O. stamineus* on the percentage of hepatocytes viability in APAP-induced hepatotoxicity SD rats. Values are mean ± S.D, n=6; Analysed using Dunnett's test. ** P<0.01 significantly different from APAP control; ## P<0.01 significantly different between normal control group and APAP control group.

Percentage of hepatocyte viability obtained from rats fed with *O. stamineus* extract was compared to APAP control rats in order to see any protection effect on hepatocytes against acetaminophen-induced liver damage.

2.8 Data analysis

Data were expressed as mean ± standard deviation of mean (S.D). Statistical comparisons were performed using Dunnett's test [11]. P values less than 0.05 and 0.01 were considered as significant.

3. Results

No mortality or any toxic symptom occurred within 24 h with the four tested doses of the methanol extract of *O. stamineus* in rats. The LD_{50} of the methanol extract of *O. stamineus* through oral administration in rats was believed greater than 500 mg/kg (data not shown). APAP significantly produced acute liver intoxication in APAP control group. The serum ALT, AST and ALP values in the APAP control group were significantly increased ($P<0.01$) associated with the significant decrease in hepatocytes viability ($P<0.01$) compared to that of normal control group (Fig. 1-4). Pretreatment with 5 mg/kg of methanol extract of *O. stamineus* did not produce any hepatoprotective effect against APAP-induced hepatotoxicity in rats. The other doses such as 31.25 mg/kg and 125 mg/kg used in the study did not produce a conclusive hepatoprotective effects although some enzyme levels showed a gradual decrease for these treated groups. However, the difference from the normal control group was still significantly higher. No significant elevation on serum ALP values were also observed in those rats pretreated with 31.25 mg/kg and 125 mg/kg of methanol extract of *O. stamineus* as when compared to APAP control group (Fig. 3). Serum AST level was found not to be elevated in the rats pretreated with 31.25 mg/kg of *O. stamineus* extract compared to that of APAP control group (Fig. 1). The hepatocytes viability basically supported the results seen from serum AST and ALT levels in rat treated with 125 mg/kg and 500 mg/kg of methanol extract of *O. stamineus*. The percentage of hepatocytes viability was increased after pretreated with methanol extract of *O. stamineus* at 125 mg/kg and 500 mg/kg compared to APAP control group (Fig. 4). Among these tested doses, 500 mg/kg of methanol extract of *O. stamineus* was found to be most effective against APAP-induced liver intoxication as evident from decreased levels in all serum biochemical parameters compared to APAP control group, indicating the improvement of the functional status of liver, which was also supported by the increment of percentage of hepatocytes viability finding.

4. Discussion

Liver plays a central role in metabolising drugs and xenobiotics and it is easily exposed to xenobiotics and toxic metabolites. A large number of drugs and xenobiotics are reported to be potentially hepatotoxic. Acetaminophen (APAP) is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses [12]. APAP is absorbed from the gastrointestinal tract and it is metabolised by microsomal enzyme in the liver. The chemical substances, drug, xenobiotic and endogenous compound undergo several types of phase I (functioning) and phase II (conjugation) reactions before changing into hydrophilic products, more suitable for excretion. Cytochrome P_{450} enzymes are the major phase I enzymes involved the metabolism of drugs or xenobiotics. Glutathione conjugation, catalyzed by glutathione-S-transferase (GST) represents a major phase II detoxification reaction involved in the biotransformation of a wide variety of drugs, environmental chemicals and numerous

endogenous compounds. APAP is primarily metabolised via sulfate by the transferase enzymes and conjugation of glucoronide acid and about 10-15 % of APAP is oxidized to the reactive oxygen species through cytochrome P_{450} [13]. However, only a very small percentage of a non-toxic dose of APAP is converted to N-acetyl-p-benzoquinoneimine (NAPQI). Later, the products of the conjugation of NAPQI with the glutathione (GSH) can be readily excreted in the bile or urine. However, when APAP is taken in overdose may lead to the saturation of the glucuronidation and sulfation pathways, allowing more APAP to be activated by cytochrome P_{450}. Thus, it leads to the increase formation of toxic metabolites which then covalently bind to the macromolecules of cells which later are causing damage to membranes and other tissues [14].

Leakage of large quantities of cytosolic enzymes into the blood stream is often associated with massive necrosis of the liver due to the APAP intoxication. Thus, an obvious sign of liver damage is leakage of cellular enzymes into blood [15]. The measurement of AST, ALT and ALP levels in serum is a very useful indicator for the hepatocellular and hepatobiliary damage. AST is found in the liver, cardiac muscles, skeletal muscle, pancrease, lungs, kidney, brain whereas ALT concentration is highest in the liver than the other organs. Therefore, ALT appears to be more sensitive test to hepatocellular damage than AST [16]. In the present study, the serum level of AST, ALT and ALP were increased and reflected the hepatocellular damage in the APAP-induced hepatotoxicity in SD rats.

Many laboratory models have been developed for the study of APAP-induced hepatotoxicity using experiment animals such as rats, mice and hamster. Among these species, acute hepatotoxicity-induced by APAP varies with strain, age and sex [17]. As mentioned by Raheja *et al*. (1983), Sprague Dawley (SD) male rats are more sensitive than females towards APAP toxicity [18]. Neonates are more resistant than young rats in APAP intoxication, and their sensitivity to APAP is diminished in 2-year-old rats [19, 20]. These are the few important factors which were considered when designing the present study.

Many phytochemical analyses suggested that *O. stamineus* may be a natural antioxidant. Recently, researchers from the University Sains Malaysia had reported that rosmarinic acid is the main component in this 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 [5]. *O. stamineus* contains polyphenols that have been shown to selectively induce antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-P_x) and phase II metabolic enzymes which increase the formation and excretion of detoxified metabolites resulting from xenobiotic metabolism [21]. This is in parallel with our previous findings which have shown that the treatment of methanol extract of *O. stamineus* up to 14 days to SD rats demonstrated antioxidant properties as evident from the significant increased of the hepatic SOD, CAT and GSH-P_x levels [22]. This likely to be the one of the possible protection mechanisms of methanol extract of *O. stamineus* against APAP-induced liver intoxication. It is generally accepted that the administration of overdose of APAP would deplete the hepatic GSH concentration and GSH-P_x activity [23]. The increment of antioxidant enzymes could prevent the process of APAP toxicity. The threshold for toxicity is passed when the amount

of NAPQI generated greatly exceeds the ability that can be conjugated by GSH and thus NAPQI is free to react with proteins and macromolecules [17]. Thus, maintaining the balance between the reactive oxidative species and antioxidant activity has been suggested to play a crucial role in drug toxicity such as from APAP and could serve as major mechanism in preventing damage by oxidative stress [24].

Standard drug, Silymarin used in the present study is a well known hepatoprotective compound. The hepatoprotective properties of silymarin have also been confirmed in clinical studies [25]. Silymarin is a mixture of flavanolignans, primarily consisting of silybin, silydianin and silychristin [26]. It is reported to have marked protective effect on the plasma membrane of hepatocytes [10]. Other reported mechanisms underlying the hepatoprotective properties of silymarin include the prevention of GSH depletion [27], destruction of free radicals [28], maintenance of hepatic protein synthesis via RNA activation [29] and preservation of mitochondrial transport function [30]. Based on our findings, the recovery towards normalization of serum enzymes and percentage of hepatocytes viability which contributed by methanol extract of *O. stamineus* was found to be less effective to that caused by silymarin in APAP-induced liver damage. However, the present study was not designed to determine the role of individual compounds present in the *O. stamineus* extract. Rather, it was designed as a pilot study to determine whether the crude methanol extract of *O. stamineus* has any protective role in general in APAP-intoxicated rats.

5. Conclusion

Taken as whole, although the effect of 500 mg/kg methanol extract of *O. stamineus* in preventing APAP-induced hepatotoxicity as reflected in the serum biochemical parameters and percentage of hepatocytes viability does not seem to be drastic and as effective as standard drug, silymarin, it points to a favorable disposition towards recovery among all the parameters. In summary, administration of 500 mg/kg of methanol extract of *O. stamineus* exerts the most significant protective role against the APAP-intoxicated rats. Further studies are worthy to be carried out to isolate the individual bioactive compounds with regard to the protective mechanism of the *O. stamineus* against APAP-induced liver injury.

6. Acknowledgement

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