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Interactions of *Orthosiphon stamineus* and *Morinda citrifolia* with hepatic aminopyrine metabolism by CYP3A in rats

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ABSTRACT

Herb-drug interaction studies have getting attention recently due to the increasingly use of herbal products worldwide. The porpose of the present study was to examine the *in vitro* effect of methanol leaf extract of *Orthosiphon stamineus* and *Morinda citrofolia* fruit juice extract on hepatic aminopyrine metabolism by CYP 3A in different age of normal and STZ-induced diabetic Sprague Dawley (SD) rats. Isolated rat hepatocytes were prepared using the collagenase perfusion technique. Aminopyrine was used as a probe substrate to determine the hepatic levels of CYP 3A by measuring the activity of N-demethylation of aminopyrine in rat hepatocytes according to the method described by Nash. Results obtained showed that aminopyrine N-demethylase activity measured from all diabetic rat hepatocytes was not affected by *O. stamineus* and *M. citrifolia* extract. A significant decrease in the aminopyrine N-demethylase activity was observed in the normal old male SD rat hepatocytes preincubated with 0.1 mg/kg ($P<0.01$) of methanol extract of *O. stamineus* when compared to the respective control group. *M. citrifolia* juice extract at 0.1 mg/ml ($P<0.01$) significantly increased aminopyrine N-demethylase activity in normal adult male SD rat hepatocytes as compared to the control group. For conclusion, both *M. citrifolia* and *O. stamineus* extract could affect the *in vitro* metabolism of aminopyrine by CYP3A in normal rat hepatocytes. No significant change in the hepatic aminopyrine metabolism was observed in the diabetic rats after incubating with different concentrations of *M. citrifolia* and *O. stamineus* extracts. The observed herb-drug interactions in the present study was age- and disease-dependent.

KEY WORDS: Aminopyrine, isolated hepatocytes, herb-drug interaction, *Morinda citrifolia*, *Orthosiphon stamineus*.

INTRODUCTION

Morinda citrifolia and *Orthosiphon stamineus* have getting popularity among Malaysian as a supplement to promote health. *Morinda citrifolia* (Family: Rubiaceae) or commonly known as Noni, is a small evergreen tree and is identifiable by its straight trunk, large, bright green and elliptical leaves with tubular flowers, and its distinctive, ovoid "grenade-like" yellow fruit. The antioxidant, anti-inflammatory and hepatoprotective properties of *M. citrifolia* fruit juice extract in experimental animals have been previously reported (1-3). A number of major components have been identified in the *M. citrifolia* such as anthraquinones, flavone, glycosides, and volatile oils (4-5). Based on the previous toxicological evaluation conducted in experimental animals, the fruits of *M. citrifolia* are generally considered as safe to be consumed (6). However, in 2005, two clinical cases of the acute jaundice in relation to the consumption of *M. citrifolia* have been reported in Austria (7). Thus, this toxic effect could be atrributed by other mechanisms such as herb-drug interaction and the re-examination of the safe use of *M. citrifoilia* are essentially needed.

Orthosiphon stamineus Benth. (Family: Lamiaceae) is a beautiful flowering plant, flower conspicuous, white and arranged in a terminal raceme (8). Stamens of *O. stamineus* are long, expanding and shaped like cat's whiskers and its leaves are simple, without stipules and secussate, diamond-shaped, dark green above and paler below and secondary nerves 5-6. *O. stamineus* Benth. is easily found in the 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), Misai Kucing (Malaysia), Kumis Kucing or Remujung (Indonesia), moustaches de chat (French) and Java Tea or Kidney Tea (European). This plant is believed to have several pharmacological effects such as kidney stones elimination effect, diuretic effect, anti-inflammatory effect, antioxidant effect and nitric oxide inhibition effect (8). We have recently reported that the oral administration of methanol extract of *O. stamineus* up to 5 g/kg to rats revealed no any adverse effect to liver and kidney functions (9). The most abundant phytochemical to contain in the methanol extract of *O. stamineus* is rosmarinic acid which has been identified as inducer of many antioxidative enzymes (10).

Although evidence from humans and animals experiments support the hypothesis that many natural products promote health, however, it is possible that interactions with other modern drugs may override any subtle positive effects of herbal products for clinical porposes. Due to the limited scientific studies are available for the herb-drug interactions of *O. stamineus* and *M. citrifolia* extracts, the present study was aimed to evaluate the *in vitro* effects of *O. stamineus* and *M. citrifolia* extract on rat hepatic phase I aminopyrine metabolism of CYP 3A activity and to see the impact of age and disease factors on this herb-drug interaction.

MATERIALS AND METHODS

Chemicals

Aminopyrine and trypan blue were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were obtained from local suppliers.

Preparation of methanol leaf extract of O. stamineus

The methanol extract of *O. stamineus* (spray dried powder form) was obtained from Professor Zhari Ismail, Pharmaceutical Chemistry Discipline, 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.

Preparation of M. citrifolia fruit juice extract

Semi-ripe fruits of *M. citrifolia* were collected and stored in the refrigerator at 4°C for one day. After that, the fruits were carefully washed and left to dry at room temperature. Then the fruits were weighed (average weight approximately 100 g/fruit) separately and cut into small pieces. Then the *M. citrifolia* fruits juice was extracted using a juice extractor (Braun MP80 Germany). The extract was then filtered. Finally, the Noni juice extract was placed in a dark amber bottle and was kept in the refrigerator at 4° C throughout the experiment period. The equivalent volume of juice extract to the weight of the fruit was approximately 0.4 ml/g. The equivalent dry weight of the juice extract to the volume of juice extract of the fruit was approximately to 0.01 g/ml.

Experimental animals

The animal studies were approved by the animal ethic's committee of Universiti Sains Malaysia. Normal adult male rats (14 weeks old ± 1 week old; 200 g ± 20 g body weight; n = 6) and normal old male rats (54 weeks old ± 1 week old; 250 g ± 50 g body weight; n = 6) were used throughout the experiment. All rats were provided water and food *ad libitium*. All rats were acclimatised for one week before used.

Induction of diabetic by streptozotocin (STZ)

Streptozotocin (STZ) was freshly prepared by dissolving in 0.9 % NaCl before administration. A single dose of 50 mg/kg STZ was injected intravenously to the SD rats through tail's vein. The blood glucose concentration was measured in blood from a tail vein in rats after 72 hours of STZ injection. Only the rats with blood glucose concentration higher than 15.6 mmol/L (at fasting state) were used (11).

Preparation of isolated hepatocytes

Isolated hepatocytes from normal and STZ-induced diabetic SD rats were prepared by the collagenase perfusion technique (11). The animals were anesthetised with diethyl ether and the liver was perfused *in-situ* through the hepatic portal vein with calcium-free Hanks balanced salt solution (HBSS) for about 15 minutes followed by the collagenase buffer (HBSS supplemented with 4mM calcium chloride ($CaCl_2$) and 0.5 mg collagenase/ml) until the liver appeared to have broken up. The liver was removed and homogenised. The cell suspension was filtered through gauze and centrifuged at 200 X g for 5 minutes. The supernatant was removed and the sediments which contain hepatocytes were 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 hepatocytess were then counted using a haemocytometer and assayed for viability using trypan blue. Only the hepatocytes viability greater than 85 % were used in the experiment.

Aminopyrine N-demethylase assay

Each petri dish contains 1 ml of aminopyrine (25 mM), 6000 hepatocytes, 1 ml of plant extracts ranging from 10 ng/ml to 100 µg/ml and incubation medium in the final volume of 10 ml. The petri dishes were then incubated in an incubator for 18 minutes at 37°C. The reaction was terminated by adding NaOH followed by $Ba(OH)_2$. Aminopyrine N-demethylase activity was measured at 415 nm using spectrophotometer to determine the quantity of formaldehyde formed according to the colorimetric method of Nash (12).

Data analysis

All values in the control groups were expressed as means and standard deviation. Results obtained from treatment groups were compared with the respective control. Analysis was analysed using Dunnett Test. The level of significant were set at $P<0.05$ and $P<0.01$.

RESULTS

Impact of age on the activity of aminopyrine N-demethylase activity

As compared among the control groups, the normal and diabetic old male SD rats ($P<0.05$) showed significant higher in aminopyrine N-demethylase activity than those normal and diabetic adult male rats (Figure 1).

Impact of STZ-induced diabetic on aminopyrine N-demethylase activity

No significant change in the activity of aminopyrine N-demethylase was observed between the diabetic and normal old male rat hepatocytes. However, the aminopyrine N-demethylase activity in diabetic adult male rat hepatocytes was significantly lower ($P<0.05$) than those normal adult male rats (Figure 2).

Effect of O. stamineus and M. citrifolia extract on rat hepatic aminopyrine N-demethylase activities

The activities of aminopyrine N-demethylase in the rat hepatocytes either treated with *O. stamineus* or *M. citrifolia* were compared to the respective control group. Aminopyrine N-demethylase activity in the hepatocytes obtained from all diabetic rats were not affected by *O. stamineus* and *M. citrifolia* extract as compared to the respective control group (Figure 3 & 4). Preincubated of normal adult male rat hepatocytes with 0.1 mg/ml ($P<0.01$) of *M. citrifolio* significantly increased the aminopyrine N-demethylase activity as compared to the control group (Figure 3). On the other hand, a significant decrease in the activity of aminopyrine N-demethylase was observed in the normal old male rat hepatocytes preincubated with 0.1 mg/kg ($P<0.05$) of *O. stamineus* (Figure 4).

DISCUSSION

Aminopyrine ($C_{13}H_{17}N_3O$) is common substrate used to examine liver functions. Aminopyrine undergoes N-demethylation to form monomethyl-4-antipyrine and formaldehyde (13). In rat's hepatic system, CYP3A is the major cytochrome P450 isoform responsibles for the metabolism of aminopyrine via phase I reaction although other CYP isofamilies such as CYP2D6 may also be involved (14). CYP3A plays very important roles in the metabolism a wide range of almost half of the commonly prescribed drugs in humans such as diazepam, morphine and imipramine. Among the herb-drug interaction studies, the examinations of the rate of drug metabolism based on the hepatic drug metabolising enzymes are always received greatest attention. The prediction of metabolic clearance and metabolic stability studies using isolated hepatocytes are greatly increasing in the recent years (15-16).

Biological factors such as age, gender and diseases are known to influence the activity of drug metabolising enzymes in the liver. It is generally accepted that hormonal and physiological changes could be greatly affected the expression of hepatic phase I and phase II drug metabolising enzymes in humans and experimental animals (14). Based on our results, the age and diabetes factor was shown to have influence on the activity of aminopyrine N-demethylase in rat hepatocytes. Age-dependent effect on aminopyrine N-demethylase activity was evident in the control groups of normal and diabetic old male rats by having higher enzyme activity in hepatocytes than those control normal and diabetic adult male rats (Figure 1). Although the actual reason remains unknown, however, as reported by Fu and Peng, the effect of ageing on the activity of cytochrome P450 dependent aminopyrine N-demethylase was be some relationships to the cholesterols/phospholipids ratio and membrane fluidity changes (17). The STZ-induced diabetic adult rats had lower enzyme activity compared to the normal groups (Figure 2). This observation is in parellel to the literature published by Past & Cook in which the rate of aminopyrine metabolism by diabetic rat liver microsomes was only 77% of the control rate exhibited by normal rat liver microsomes (18). In addition, phase I metabolism of drugs in the liver as exemplified by diazepam, lignocaine and imipramine shows a marked decrease in activity in diabetic rats (19).

Generally, the herb-drug interaction results indicated that only high concentration of *O. stamineus* and *M. citrifolia* juice preparations, i.e. 0.1 mg/ml had

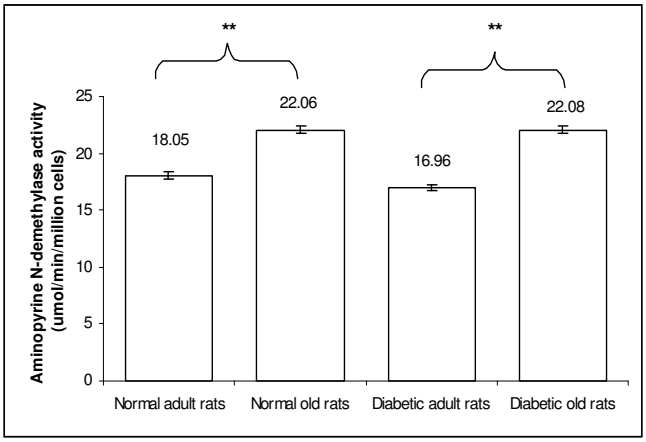

Figure 1. The impact of age factor on the activity of aminopyrine N-demethylase in the control groups of normal and STZ-induced diabetic rat hepatocytes.
Results are expressed as mean ± standard deviation; n=6 ; Results are analysed using Dunett's multiple test; ** P<0.01 between two comparison groups.

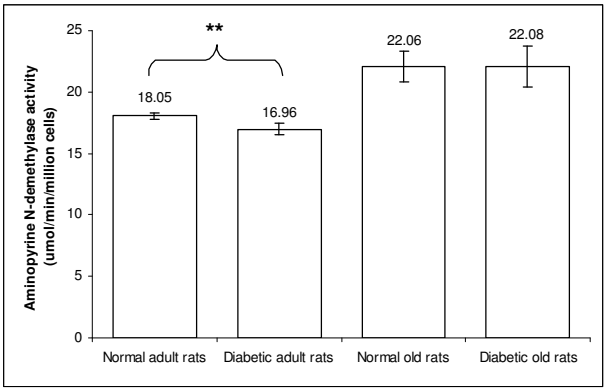

Figure 2. The impact of disease factor on the activity of aminopyrine N-demethylase in the control groups of normal and STZ-induced diabetic SD male rat hepatocytes.
Results are expressed as mean ± standard deviation; n=6; Results are analysed using Dunett's multiple test; ** P<0.01 between two comparison groups.

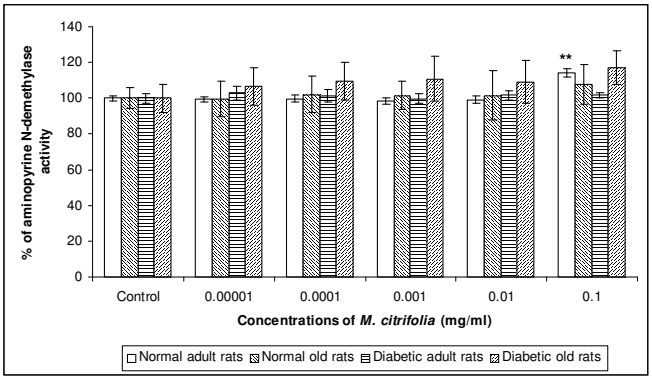

Figure 3. In vitro effect of Morinda citrifolia fruit juice extract of aminopyrine N-demethylase activity in isolated rat hepatocytes of normal and STZ-induced diabetic male SD rats.
Results are expressed as percentage of the respective control group.
N=6; Results were analysed using Dunnett's multiple test; ** P<0.01 as compared to respective control group.

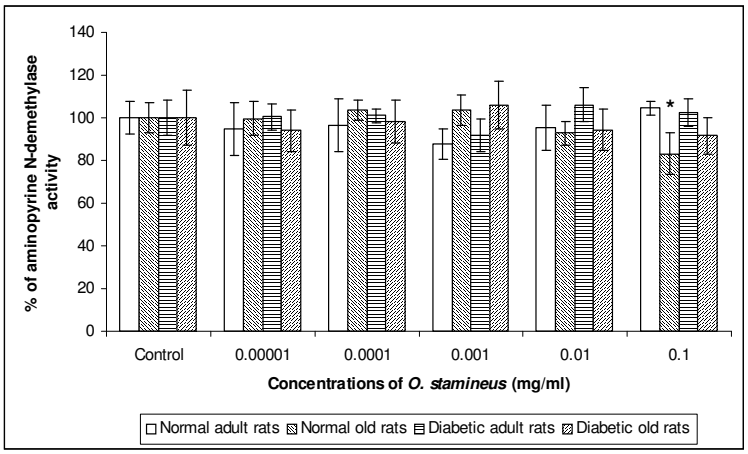

Figure 4. In vitro effect of Orthosiphon stamineus methanol leaf extract on aminopyrine Phase I metabolism in isolated hepatocytes of normal and STZ-induced diabetic male SD rats.
Results are expressed as percentage of respective control; n=6. ; Results are analysed using Dunnett's Test; * P<0.05 as compared to the respective control.

significant influence on the activity of aminopyrine N-demethylase activity and these significant effects, however, were only seen in certain groups, i.e. normal adult and old male rats. Lower concentrations of these herbal preparations did not cause any significant change in the aminopyrine metabolism in all tested normal and STZ-induced diabetic groups. This is probably due to the amounts of active phytochemicals in that particular concentrations are not sufficient to affect the enzyme activity in rat hepatocytes and it needs higher concentrations to give the responses. As seen in figure 4, 0.1 mg/kg of *O. stamineus* extract increased the rate of metabolism of aminopyrine in normal old male rat hepatocytes, indicating an increase in the activity of CYP3A in rat hepatocytes. From this observation, we suggested that *O. stamineus* extract could have the same possibility to interact with others drugs mediated by CYP3A in the liver which will be eventually reduced the therapeutic effects of these drugs due to the shorter half-life in the circulation caused by the higher metabolism rate of hepatic enzymes. On the other hand, the inhibition of the aminopyrine N-demethylase activity caused by 0.1 mg/kg of *M. citrofolia* in normal adult male rat hepatocytes could delay the drug clearance from the livers and cause the accumulate of the drugs in the hepatic system which eventually toxic to the liver. These preliminary findings provide important information about the safe use and therapeutic applications of *O. stamineus* and *M. citrifolio* extract due to the limited scientific research or clinical reports on herb-drug interactions are available. However, this *in vitro* only examines the direct action of plant extracts on the existing CYP3A enzymes in rat hepatocytes. *In vivo* studies may be varied from the *in vitro* findings due to the absorption and disposition of phytochemicals in the biological system. *In vivo* herb-drug interaction study needs to be carried out to further explain the mechanism based of *O. stamineus* and *M. citrifolia* extract in affecting aminopyrine phase I metabolism by CYP3A in rat hepatocytes and develop the correlation between both *in vitro* and *in vivo* research on herb-drug interaction study.

The projection of animal data directly to man should not be made on the assumption that the same dose of drug (in mg/kg) will attain the same concentration at the drug receptors in man as in animals (20). In general, small animals such as mice metabolise foreign compounds at a faster rate than larger animals such as humans, consistent with differences in overall metabolic rates (21). Rats are six times more efficient than man in handling xenobiotics based on its liver size/body weight (kg) which is twice that of man. Furthermore, concentrations of cytochrome P 450 in rats is three times higher than in man. Besides that, ratio of dose relative to body weight (mg) to dose relative to body surface area (mg) showed that despite exhibiting similar drug effects on rats and man, dosage given to man is actually 10-times lower than that administered in rats (22).

CONCLUSION - As a summary, *O. stamineus* methanol leaf extract and *M. citrofolia* fruit juice extract could

influence the metabolism rate of aminopyrine by CYP 3A in rat hepatocytes but these herb-drug interaction effects were age- and disease-dependent.

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