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EFFECT OF THE *ORTHOSIPHON STAMINEUS*, BENTH ON AMINOPYRINE METABOLISM IN RAT HEPATOCYTES

CHIN JIN HAN[1*] AND ABAS HJ. HUSSIN[2]
[1]School of Pharmacy, University College Sedaya International,
56000 Kuala Lumpur, Malaysia
[2]School of Pharmaceutical Sciences, Universiti Sains Malaysia,
11800 USM Pulau Pinang, Malaysia

One of the commonly used herbal medicines in Malaysia is Orthosiphon stamineus, Benth (family: Lamiaceae) or locally known as Misai Kucing. This experiment was undertaken to evaluate possible interaction of methanol extract of O. stamineus with aminopyrine, a model drug, in different age groups (young, adult and old) of Sprague-Dawley (SD) female rat hepatocytes. Hepatocytes were prepared by collagenase perfusion technique. Determination of aminopyrine N-demethylase activity was done by measuring formaldehyde formed. From these findings, only normal young female rat hepatocytes in the presence of 0.001 mg/ml of methanol extract of O. stamineus showed significant increase in aminopyrine N-demethylase activity. However, aminopyrine N-demethylase activity was not affected in hepatocytes of normal adult and old female SD rats. In conclusion, exposure of methanol extract of O. stamineus could affect phase I aminopyrine metabolism in normal young female SD rat hepatocytes but this effect was age-dependent.

Keywords: *Orthosiphon stamineus, Aminopyrine, Hepatocytes, Protein kinases*

INTRODUCTION

Currently, herbal medicine gets a great attention and the demand for herbal products has been increasing from year to year. *Orthosiphon stamineus Benth*, or locally known as *Misai Kucing* (family: Lamiaceae) has been used for many centuries in South-East Asia (Indubala and Ng 2000). It is utilised for treating ailments of the bladder and kidney, diabetes mellitus and gout (Wiart 2002). Other pharmacological evaluation revealed that consumption of hydroalcohol extracts of *O. stamineus* led to increase in urine flow and urinary sodium excretion in rats were increased with *O. stamineus* (Beaux, Fleurentin and Mortier 1999). Knowledge about herb-drug interaction is very important for the design of drug therapy. Herb-drug interactions are able to cause an enhancement or attenuation in efficacy of co-administered drugs. Several mechanisms in the gastrointestinal tract (GI) have been identified to influence the absorption of drugs or herb. Some herbs have been reported to exert inhibition effect of CYP 3A4 in the intestine and increase the bioavailability of certain drugs in the systemic system. For example, grapefruit juice inhibits intestinal CYP 3A4 and increases bioavailability of some drugs in the systemic

* Corresponding author: Chin Jin Han, e-mail: jinhanchin@hotmail.com

circulation. These drugs include cyclosporine, statins (e.g. lovastatin, simvastatin), benzodiazepine (e.g. diazepam, midozolam), calcium antagonists (e.g. dihydropyridines such as felodipine) and psychiatric drugs (e.g. buspirone, sertraline) (Rotblatt and Ziment 2002). There are numerous herbs that could induce the hepatic cytochrome P450 system and affect the pharmacological and therapeutic effect of drugs. For example, the induction effect of St. John's wort and the inhibitory effect of silybin (main component of milk thistle) on human hepatic CYP 3A4 and CYP 2C9 have been previously demonstrated (Rotblatt and Ziment 2002). To prevent long term occupation of the body by foreign chemicals, the liver has developed an ability to convert non polar drugs into polar metabolites for excretion (Timbrell 2000). Drugs or herbs after being metabolised by liver will be eliminated from the body through urine or bile. Drug toxicity will be greater if the liver has some degree of hepatic diseases because ingestion of drug depends on the liver for its removal (Gibson and Skett 1994).

Hence, this study aims to investigate the possible effect of *O. stamineus* on aminopyrine metabolism in normal young, adult and old female SD rat hepatocytes. Aminopyrine, a model drug, is an antipyretic and analgesic drug, and is now rarely used because of its dangerous side effects. Cytochrome P450 is a very important protein involved in the metabolism of drugs, and xenobiotics, and they are prevalent in liver especially in the membrane of the smooth endoplasmic reticulum (Murray 1998). Aminopyrine N-demethylase enzyme is categorised as N-dealkylation reaction which belongs to one of the oxidation processes in the phase I hepatic drug metabolism reaction (Hodgson and Goldstein 2001). In this reaction, the methyl atom attached to the nitrogen atom of aminopyrine is hydroxylated, followed by the elimination of methyl from the atom nitrogen to yield formaldehyde. Hepatocytes are the major site of metabolic activity. Metabolic activity varies according to the location of the hepatocytes. There are three circulatory zones in the acinus, with zone 1 receiving blood from the afferent venules and arterioles first, followed by zones 2 and 3 (Timbrell 2000). Zone 1 hepatocytes (approximate to periportal region) are more aerobic and contain glutathione, glutathione peroxidase, alcohol dehydrogenase and are equipped for β-oxidation of fats pathway. However, zone 3 hepatocytes (centrilobular region) contain higher levels of cytochrome P450 and NADPH cytochrome P-450 reductase (Timbrell 2000). Hepatocytes in rat liver are approximately 100 times fewer in absolute number compared to humans. Hepatocytes accounted for about 60% of the total cells in the liver. The human liver contains approximately 27×10^9 hepatocytes. The life cycle for hepatocytes renewal is approximately 200 days in rat (de la Iglesia, Rebertson and Haskins 1999).

MATERIALS AND METHODS

Chemicals

All chemicals used were of standard analytical purity grade. Aminopyrine was supplied by Sigma Co., St Louis, MO, USA.

O. stamineus Methanol Extract Preparation

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

The leaves of the plant were collected in the late afternoon, from 30 to 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).

Animals

SD rats bred by the Animal House, School of Pharmaceutical Sciences, USM were used. All rats were kept under normal condition and free to access tap water. Normal young female rats (7 weeks old ± 1 week old; 100 ± 10 g body weight; n = 6), normal adult female rats (14 weeks old ± 1 week old; 170 ± 10 g body weight; n = 6) normal old female rats (53 weeks old ± 1 week old; 250 ± 50 g body weight; n = 6) rats were used throughout this study.

Hepatocytes Preparation

Isolated hepatocytes were prepared by using the collagenase perfusion technique (Hussin and Skett 1988). The cell suspension was filtered through gauze and centrifuged at 200g for five minutes. 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.

Aminopyrine N-demethylase Assay

Equal volume (1.0 ml) of serial dilutions of the methanol extract of *O. stamineus* (in distilled water) were added (10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml, 1 mg/ml) into petri dishes containing aminopyrine (25 mM final concentration), freshly isolated hepatocytes (6000 cells) and incubation medium. Control petri dishes essentially contained all of the above with the exception of the herbal preparation, being replaced with distilled water. The petri dishes were then incubated on a table top shaker (Belly dancer®) for 18 min at 37°C. Aminopyrine-N-demethylase activity was determined by measuring the quantity of formaldehyde formed according to the colorimetric method of Nash (1953).

Statistic and Results Analysis

Aminopyrine N-demethylase activity is expressed as µmol formaldehyde formed/min/million cells. The results were compared with the control, and means and standard deviation were calculated. Analysis was analysed using Dunnett test. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

In vitro herb-drug interaction study showed that aminopyrine N-demethylase activity in normal young female SD rat hepatocytes was increased significantly ($p < 0.05$) in the presence of 0.001 mg/ml of methanol extract of *O. stamineus*. Aminopyrine N-demethylase activities in the normal adult and old female rats were not affected by methanol extract of *O. stamineus* ranging from 10 ng/ml to 1 mg/ml (Table 1).

Table 1: The influence of methanol extract of *O. stamineus* on aminopyrine N-demethylase activity in normal female SD rat hepatocytes of different age groups.

Aminopyrine N-demethylase activity (µmol formaldehyde formed/min/million cells)

***O. stamineus* (mg/ml)**	**Young female**	**Adult female**	**Old female**
Control	2.28 ± 0.20	2.41 ± 0.28	2.17 ± 0.15
0.00001	2.64 ± 0.23	2.36 ± 0.25	2.25 ± 0.17
0.0001	2.56 ± 0.18	2.40 ± 0.24	2.27 ± 0.15
0.001	2.67 ± 0.13*	2.22 ± 0.19	2.19 ± 0.11
0.01	2.33 ± 0.20	2.51 ± 0.38	2.28 ± 0.24
0.1	2.43 ± 0.15	2.58 ± 0.44	2.24 ± 0.25
1	2.37 ± 0.16	2.42 ± 0.11	2.12 ± 0.19

Each value is expressed as mean ± S.D. (n = 6)
Analysed by Dunnett test; * $p < 0.05$ as compared to their respective control group

Aminopyrine is mainly N-demethylated by CYP P450 3A and 2B for rat and CYP 2C for human, although many other isoforms of cytochrome P450 are also involved (Kamataki 1993). Drugs which undergo phase I N-demethylation include cocaine, salicylamide, diazepam, erythromycin, imipramine and morphine (Abas, Saringat and Rozana 1998). This study indicates the possibility of herb-drug interaction in patients concomitantly treated with these drugs and natural products containing *O. stamineus* that would result in a decrease in the metabolism of these drugs. An increased in the phase I liver metabolising enzymes by methanol extract of *O. stamineus* may increase the metabolism of the drug in the body after chronic intake and could reduce the therapeutic effects of drugs.

Among the chemical ingredients that had been described in the hydroalcohol extracts of *O. stamineus* are caffeic, cirrchoric and rosmarinic acids, and the main flavonoids are sinensetin (SEN) and eupatorin(EUP) (Olah *et al*. 2003). Recently, researchers from USM 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), EUP and 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 herb-drug interaction effect seen is probably due to the flavonoids as they had been implicated as liver enzyme inducers (Canivenc-Lavier *et al*. 1996).

A wide variety of physiological processes including metabolism, muscle contraction, endocrine and exocrine secretion, sensory of pain, light and taste involve the G protein-coupled receptors (GPCR). GPCR-mediated signal transduction produces many second messengers such as cAMP, diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) and leads to the activation of the second messenger-activated kinases including PK_A by cAMP, PK_C by diacylglycerol and calcium/calmodulin-activated kinases by IP_3-induced calcium mobilization (Kahout and Lefkowitz 2003). Phosphorylation and dephosphorylation are important mechanisms that regulate a variety of cellular response in eukaryotic organisms (Dombradi 2002). A post-translational modification by phosphorylation has to be considered as a regulatory device. Upon phosphorylation, the typical P-450 absorption peak at 450 nm decreased while the shoulder at 420 nm developed into a peak. Phosphorylation of a single serine residue therefore converts P-450 into its P-420 form, which is known to be enzymatically inactive. In addition, the kinase recognition sequence –Arg-Arg-X-Ser- has been found exclusively in family II of the P-450 gene superfamily, which is involved in the metabolism of foreign compounds such as drugs (Pyerin and Taniguchi 1989). Therefore, further examination by using peptide mapping and immunoprecipitation with

monospecific antibodies need to be carried out to identify the involvement of phosphorylation on aminopyrine N-demethylase activity by *O. stamineus* extract in normal young female rat hepatocytes.

Many biological factors like age, gender and disease may affect drug metabolism (Gibson and Skett 1994). Sensitivity to drugs and other compounds is often different in young and geriatric animals. Many drug metabolising enzyme systems being generally reduced in neonate and decline towards old age (Timbrell 2000). The susceptibility of old animals may be due to its small active liver mass, decreased blood flow to liver and altered plasma protein binding (Timbrell 2000). However, aminopyrine has a low-extraction ratio in the normal liver, making its hepatic metabolism relatively independent of liver blood flow (Branch 1982). Therefore, the effect seen in young female rat hepatocytes in this study but not in the other groups may be caused by the direct effect on the existing hepatic enzymes for drug metabolism since some enzymes are not well-developed until adulthood. Yet, the simplicity of *in vitro* investigations using hepatocytes as compared with whole perfused organs or *in vivo* animal systems, allows for the elimination of confounding factors such as blood flow and blood protein binding (Komoroski *et al*. 2005).

CONCLUSION

Methanol extract of *O. stamineus* increased aminopyrine metabolism in the normal young female SD rat hepatocytes at 0.001 mg/ml concentration but did not affect the aminopyrine N-demethylase activity in the normal adult and old female rat hepatocytes. Further studies are necessary, such as acute and chronic *in vivo* experiments, to confirm this evidence.

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