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Original Article

In vitro effects of Air Jamu Pak Tani, an herbal product, on aminopyrine hepatic phase I n-demethylase activity in spontaneous hypertensive rats: the mechanism involved

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ABSTRACT:

Objective: Pharmacokinetic interactions of co-administered herbal isolates with pharmaceuticals and the mechanisms of interactions remain to be elucidated. Herein, we evaluated the *in vitro* effects of Air Jamu Pak Tani (AJPT), a mixture of plant isolates, on phase I aminopyrine metabolism and the molecular mechanism involved in spontaneous hypertensive rat (SHR) livers. **Material and Methods**: Hepatocytes were isolated by the collagenase perfusion technique. Aminopyrine n-demethylase activity was determined using the colorimetric method of Nash. **Results:** Compared to control AJPT dose independently increased significantly the aminopyrine n-demethylase activity by 29.6% ($P<0.05$). Molecular mechanism elucidation, using protein stimulants (or inhibitors), showed that the effect of AJPT was significantly abrogated by the pre-incubation of hepatocytes with 3-isobutyl-methylxanthine and okadaic acid. Trifluoperazine and genistein did not change the effect of AJPT, while no significant observation was verified in the presence of guanylyl-5'-imidodiphosphate and furafylline. In conclusion, administration of AJPT induced dephosphorylation of cytochrome P450 in young male SHR hepatocytes. The effect of AJPT was mediated through different molecular mechanisms, most probably, including inhibition of tyrosine kinases, calmodulin, and cAMP/PK_A pathways, and also due to activation of protein phosphatases. Therefore, caution should be considered when AJPT is used with n-demethylase substrate 'drugs' as it may reduce their bioavailability.

Keywords: Drug metabolism; Hepatocytes; Aminopyrine; Herbal product; Cytochrome 450.

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INTRODUCTION:

Plants have always been a source of drugs, and herbal medicines are one of the ancient therapies that have stood the test of time. Extracts of medicinal plants have been used nowadays and for thousands of years to preserve public health. These extracts are commonly used either alone or in combination with chemotherapy. Most of populations use medicinal plants following experiences accepted through their ancestors. The majority of people think that natural things are good and healthy, whereas synthetic source is bad and serious. Accordingly, WHO reported that the use of herbal medicines should be incorporated into primary care to give the best of getting the goal of health-care for the whole population [1].

Most extracts, either obtained from one plant or mixture of plants, need to be evaluated scientifically to understand the skills obtained during its long-established uses. Indeed, herbal preparations, used by humans, contain a combination of plant isolates. Hence, the active constituents are uncertain. Consequently, the therapeutic effects and the adverse effects are obtained from the whole of those elements present within the herbal extract. So, herbal drugs prepared from medicinal plants have to fulfill the global requirements on the efficacy and safety [2]. Therefore, evaluation of the herbal extracts is of the most important step with respect to the health benefit and risk to the users.

Drug-herb interactions can induce either increase or decrease in the efficacy of co-administered medications. Although these interactions can be beneficial, the harm they pose is just as great. Pharmacokinetic interaction studies, *in vivo* and *in vitro*, have showed that changes in drug metabolites after co-administered herbs are attributed to phosphorylation and de- phosphorylation of hepatic drug-metabolizing enzymes, in particular cytochrome P450 (CYP450) [3]. So, as the uses of herbal medicines from natural sources are increased, the necessities for pharmacokinetic studies are also increases for safe pharmacodynamics effects.

A folklore uses of plant remedies, while taking prescribed medications, remains popular in developing as well as developed countries. So, there is a possibility of interactions and one substance may alter the bioavailability of another by inducing phase I hepatic enzymes that can affect the therapeutic outcomes or even generate toxicity. Indeed, activities of CYP450 enzymes are influenced by various endogenous and exogenous factors [3]. In line, plant extracts are significant elements that can influence CYP450 behaviors. Inhibition and induction of CYP450 enzymes are well known, and phosphorylation was stated as a crucial element in the adaptation of drug-metabolizing CYP450 [4]. Several herb extracts and their natural constituents were classified as inducers or inhibitors of the CYP450 [5, 6]. It has been shown that induction and inhibition of enzymes occurs through several molecular pathways inside the cell. So, when any drug 'a substrate' occupied a given receptor on a target cell, different pathways are triggered as a secondary messenger such as cyclic adenosine monophosphate (cAMP), protein kinase A (PG_A), protein kinase G (PK_G), protein kinase C (PK_C), phosphatidyl inositol triphosphate and cyclic guanosine monophosphate (cGMP) pathways. Along these lines, research has primarily been conducted *in vitro*, and the influence of co-administered herbal remedies further complicates these molecular mechanisms.

Aminopyrine, a modern drug, is a painkiller; presently is infrequently used due to its critical side effects. Studies, in rats, have showed that aminopyrine is metabolized to monomethyl-4-aminoantipyrine and formaldehyde by hepatic CYP450 3A and 2B, is mainly n-demethylase [7]. Pharmacokinetic aminopyrine-herb interaction, as example of phase I metabolism, using isolated liver hepatocytes were reported in a number of studies [4, 8, 9]. It was shown that herbal products considerably modified phase I drug metabolism [9, 10]. With this background, the present study was aimed to evaluate the influence of Air Jamu Pak Tani® (AJPT), a mixture of 12 different water extract of several Malay plants, sold as dietary supplements and widely, traditionally/over-the-counter, used for health cares (fig. 1), on the metabolism of aminopyrine in spontaneously hypertensive rat (SHR) hepatocytes. Also, it was designed to investigate the possible molecular mechanisms involved in the AJPT-aminopyrine phase I interaction in liver hepatocytes.

MATERIALS AND METHODS:

Experimental animals

All rats were bred in the Animal Research and Service Centre of the Universiti Sains Malaysia, Penang, Malaysia. Spontaneously hypertensive rats (SHR) aged 8-10 weeks; weighting 85-103 g (young and male; n=6 each group) were used in all experiments. Rats were housed on normal husbandry condition of temperature and humidity with 12 hrs. light/dark cycle. Animals, 2-4 animals per cage, had free access to animal pellets (Gold

Coin®) and tap water was provided as *ad libitum*. The study protocol was approved by the Animal Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia. Laboratory animal Care and Use was in accordance with the guidelines of animal welfare.

Blood pressure measurement:

Animals' blood pressures were measured, at room temperature, by an indirect tail cuff method using blood pressure analyzer (IITC Life Science). Rats with systolic blood pressure over 160 mmHg were selected [11].

Chemicals

All chemicals and reagents used were of analytical grade purity. 3-isobutyl-1-methyl-xanthine (IBMX), 5'guanylylmidodiphosphate tetralithium salt (Gpp), okadaic acid potassium salt (OKA) and genistein were purchased from Calbiochem, Germany. Trifluoperazine dihydrochloride (TPA), furafylline, aminopyrine, collagenase (type IV), formaldehyde solution 37% and trypan blue were purchased from Sigma Chemicals Co, (St. Louis, MO, USA).

Air Jamu Pak Tani

AJPT (Perusahaan Jamu Arjuna, Sdn. Bhd. Johor, Malaysia; batch no. 59902) was bought from local market, Penang, Malaysia. It is a water extract of certain Malay plants used traditionally as a supplement for blood building and to enhance stamina and vitality. Also, it is used to relieve backache and lumbago, stiff limbs, muscular pain and to enhance appetite. Furthermore, it is used to improve bowels movement and blood circulation. It also, provide instant fitness relief, build up natural resistance against bacteria, increase immunity against seasonal ailments such as cough, influenza and fatigue and relief menstrual pain. It is recommended to people who require instant regain of energy such as sportsmen, fishermen, carpenter and to women after birth. The compositions of 100 ml of AJPT, as written on the bottle container, were as follows: Radix Polygoni Multiflori (4%); Radix Codonopsitis (7%); Astragali (4%); Cortex Eurycommae (4%); Rhizoma Liqustici wallichii (4%); Menthol (B.P China) (7%); Rhizoma Rehmanniac (4%); Radix Angelicae Sinensis (4%); Fructus Lycii (4%); Caulis Cistanchis (4%); Peppermint (BP China) (7%); Honey sugar and mineral water to 10g (47%), (Figure 1). Dosage: *children*-5 ml two times per day after meal; *adults*-10 ml two times per day after meal.

Preparation of hepatocytes

Hepatic cells comprise a variety of metabolizing enzymes that are normally localized in a particular organelle within the cells. The present study used freshly isolated hepatocytes to evaluate, *in vitro*, the aminopyrine n-demethylase enzyme activity (n=6 each group). The method used for isolation of liver cells was executed by the use of modified collagenase perfusion technique as previously described by Taher and Hussin [9]. Briefly, SHR young male rats were anaesthetized with ether, and the abdomen was cut open and the portal vein was freed of any fats. Each rats' liver was then perfused, *in situ*, with a peristaltic pump (Watson Marlow, UK), through the hepatic portal vein, just before it branches into the liver, for 15 minutes at speed of 15 $mL.min^{-1}$, with calcium-free Hank's Balanced Salt Solution (HBSS) until the size of the liver reached twice its normal mass. The vena cava was then cut and the perfusion rate reduced to 10 $ml.min^{-1}$. Hereafter, the perfusion was continued with a collagenase buffer (HBSS supplemented with 4 mM $CaCl_2$ and 500 µg/ml of collagenase; at 37°C) for another 15 minutes until the liver seemed to have choppy. The liver was taken out from the animal into a petri dish containing ice cooled incubation medium and was homogenized using a steel forceps. The cell suspension was filtered immediately *via* gauze and then centrifuged at 300 RPM for 5 minutes (Hettich Universal Centrifuge) to remove the parenchymal cells of debris. The supernatant was emptied out, and the remained cells were then re-suspended in incubation medium (HBSS supplemented with 1 $g.L^{-1}$ glucose, 100 mg. L^{-1} MgSo4, 100 mg. L^{-1} $MgCl_2$ and 185 mg. L^{-1} $CaCl_2$; pH 7.4). After, the cells were counted by the use of hemocytometer. The viability of cells was assayed using trypan blue (1%) under the microscope (Olympus R, Optical works, ERMA, Tokyo). For all isolated livers, the average yield was $28x10^7$ cells g^{-1} liver, and the viability was more than 85%.

Aminopyrine n-demethylase assay (study I)

The enzyme activity was measured as previously described [9]. Briefly, $6.0x10^3$ hepatocytes were plated in a petri dish (Medical Product, Malaysia) with; 1ml aminopyrine (final concentration, 25 mM), 1ml of AJPT (ranges from one 1000^{th} to one 5^{th} dilutions) and the final volume of reaction mixture was adjusted to 10 ml by the addition of incubation medium. Control petri dishes were treated with similar way but had the AJPT replaced with distilled water. Each run of experiment was done in quadruplicates. All petri dishes were then shaken using a table top shaker (Belly Dancer, Stoval, Life Science, INC, USA) at room temperatures (30± 1°C) for 18 minutes pre-incubation period. After, the reaction was stopped

by adding 0.5ml $ZnSO_4$ (25% w/v) and followed by 0.5 ml saturated Ba $(OH)_2$ after 5 minutes. The mixture was centrifuged using table-top-centrifuge machine (Eppendorf) at 1000 RPM for 10 minutes. Next, 1ml of supernatant was taken out and added to 2ml of Nash reagent. Nash reagent is a mixture of 30% (w/v) ammonium acetate and 0.4% (v/v) acetyl acetone [4]. The combination was incubated at 60°C for 30 minutes in a water-bath with shaking (Belly Dancer, Stoval, Life Science, INC, USA). After, tubes were let to cool down at room temperature. Lastly, to detect the amount of formaldehyde formed according to the colorimetric method of Nash [12], 200 µl were taken from the mixture and measured, spectrophotometry, at 415 nm using 96 Well plate (Costar, USA), by micro-plate reader (Anthos, labtec, USA). The aminopyrine n-demethylase activity was determined indirectly, by converting the absorbance to concentration using formaldehyde calibration curve. The formula used for the calculation of enzyme activity was as follows:

Enzyme activity = V_E x [(*formaldehyde conc.*) / (*incubation time* x *no. of cells used*)]

Where;

V_E = supernatant volume of centrifuged solution in which enzyme activity have ceased

Determination the mechanism of action of AJPT on phase I aminopyrine metabolism (study II)

In this study, six cellular protein stimulants (or inhibitors) were used to investigate the possible mechanism through it AJPT influence the activity of aminopyrine n-demethylase enzyme. The concentrations used of these protein stimulants (or inhibitors) were based on the IC_{50} according to previous studies. The experiment was executed according to procedure previously described by Purwantiningsih and his colloquies [4]. Briefly, each petri dish contains isolated hepatocytes in the incubation medium were firstly pre-incubated with specific IC_{50} of genistein (IC_{50}=10 µM), IBMX (IC_{50}=50 µM), Gpp (IC_{50}=1 µM), OKA (IC_{50}=15 nM), TPA (IC_{50}=100 µM) and furafylline (IC_{50}=70 nM) for 15 minutes at room temperature. Control petri dishes were pre-incubated similarly but had the protein stimulants (or inhibitors) replaced with DMSO. After, 1ml of AJPT serial dilution (ranges from one 20^{th} to one 5^{th} dilutions) in distilled water was added. Subsequently, 1ml of aminopyrine, dissolved in distilled water, was added to each petri dish at a final concentration of 25 mM. Each run of experiment was done in quadruplicates. The experiments were divided into six main groups according to protein stimulants (or inhibitors), and each group had five subgroups. The 1^{st} group was negative control [$C^{-/-}$] "in the absence of AJPT and protein stimulants (or inhibitors)". The 2^{nd} group was positive control [$C^{+/-}$] "in the presence of protein stimulants (or inhibitors) but in the absence of AJPT", whereas, the 3^{rd}, 4^{th} and 5^{th} groups [E1-E3] were test groups "in the presence of protein stimulants (or inhibitors) and AJPT". Aminopyrine n-demethylase activity was determined as described above, measured at 415 nm to determine the quantity of formaldehyde formed according to the colorimetric method of Nash.

Statistical analysis

Data are expressed as mean enzyme activity ± SEM. Formaldehyde concentration was calculated using linear regression equation depending on the formaldehyde standard curve for each experiment. Overall, the coefficient of variation was 'r = 0.98'. Aminopyrine n-demethylase activity, the total amount of formaldehyde formed (in absolute value) as a result of aminopyrine metabolism is expressed as nmol min^{-1} $million^{-1}$ cells (shown in table 1) and as a percentage of activity compared to the control group (shown in fig. 3). Results were analyzed using one way analysis of variance (ANOVA) followed by Tukey's *Post hoc* test. The probability value was set at $P<0.05$ for all the statistical tests. Analyses were performed using GraphPad Prism (GraphPad Software Inc., version 3.0, San Diego, USA).

RESULTS:

The standard curve was obtained with a good coefficient of variation (r = 0.98) for formaldehyde with six concentrations points measured, in triplicate, for concentrations from 0 to 10^{-10} mmol/L (Figure 2, n=6). The absorbance (y-axis) versus concentration (x-axis) was plotted and the slope, $\Delta y/\Delta x$, was evaluated. The unknown formaldehyde concentration, formed as a metabolite of aminopyrine, was calculated using a linear regression equation "y = ax + b" from the strait line of standard curve.

In study (I), our data demonstrated that co-administration of AJPT, an herbal product of multiple ingredients, a plant extract in origin, and traditionally used for treatment of several diseases, modified the hepatic phase I metabolism of aminopyrine in rat hepatocytes. Figure 3 shows that, in young male SHR hepatocytes, *in vitro* incubation of AJPT with aminopyrine did significantly ($P<0.05$) increased the metabolism of aminopyrine by 29.6 % mainly at higher concentrations used, (at 1:5, 1:10 and 1:20 dilutions) whereas at one 50^{th} and higher dilutions had no effect on the enzyme

activity, compared with control group. The observed increased acceleration in n-demethylase activity was not dose dependent since increased AJPT concentrations did not further increase the hepatic phase I metabolism of aminopyrine.

Molecular mechanism studies of AJPK in affecting aminopyrine metabolism. Table 1 illustrates that aminopyrine metabolism was significantly increased (P<0.05) in the presence of TPA and was significantly decreased when incubated with protein inhibitors OKA, IBMX, genistein, Gpp and furafulline compared with negative control.

Table 1. *In vitro* effects of AJPT on aminopyrine n-demethylase activity (study I) and on hepatic phase I aminopyrine metabolism in the absence and presence of protein stimulant (or inhibitor; study II) in young male SHR rats' hepatocytes. Data are expressed as mean enzyme activity ± SEM, n=6. Each dose runs in quadruplicate. C1 had AJPT sample dilutions replaced with distilled water. $C^{-/-}$ = negative control; "in the absence of AJPT and protein stimulant or inhibitor". $C^{+/-}$ = positive control; "in the presence of protein stimulant (or inhibitor) but in the absence of AJPT", E1, E2, E3 = test groups; "in the presence of protein stimulant (or inhibitor) and AJPT". *P<0.05 vs. C_1 (study I). $^{€}$P<0.05 vs. $C^{-/-}$. $^{\#}$P<0.05 vs. $^{(a)}$, the respective control in study I. DS = distil water, OKA= Okadaic acid, Fura= Furafylline, Genis= Genistein.

Dilutions	Enzyme activity (nmol formaldehyde formed/min/million cells) Mean (SEM)							
	study (I)	*study (II)*						
			IBMX	Fura	OKA	TPA	Genis	GPP
Aminopyrine + DS (C_1)	583.70 (21.64)	$C^{-/-}$	583.70 (21.64)	583.70 (21.64)	583.70 (21.64)	583.70 (21.64)	583.70 (21.64)	583.70 (21.64)
		$C^{+/-}$	380.33 (7.44) $^{€}$	496.99 (15.92) $^{€}$	437.96 (7.53) $^{€}$	728.24 (12.78) $^{€}$	703.47 (18.01) $^{€}$	421.76 (19.48) $^{€}$
Aminopyrine $^{(a)}$ + AJPT (1:5)	756.48 (33.56) *	E1	440.51 (35.32) $^{\#}$	795.37 (37.19)	766.90 (18.17)	709.96 (13.82)	792.36 (9.32)	789.58 (14.41)
Aminopyrine $^{(a)}$ + AJPT (1:10)	678.47 (26.64) *	E2	448.15 (12.90) $^{\#}$	704.86 (25.58)	442.13 (35.59) $^{\#}$	687.96 (21.42)	644.21 (14.91)	626.39 (13.85)
Aminopyrine $^{(a)}$ + AJPT (1:20)	677.55 (20.99) *	E3	418.29 (32.3) $^{\#}$	682.18 (39.42)	415.05 (11.16) $^{\#}$	631.95 (45.24)	629.39 (14.41)	698.15 (17.17)

Our data showed that incubation of rat hepatocytes with IBMX, the positive control, decreased aminopyrine metabolism significantly by 34.8 % (P<0.05) compared with negative control Table 1. Addition of AJPT to rat hepatocytes after pretreatment with IBMX did not increase aminopyrine metabolism at the dilutions used, 1:5, 1:10, and 1:20, (P> 0.05), compared with findings in study (I), in the absence of phosphodiesterase inhibitor, IBMX. Pre-incubation of rat hepatocytes with Gpp, the positive control, significantly decreased the metabolism of aminopyrine by 27.7 % (P<0.05) compared with negative control. Yet, pre-incubation of rat hepatocytes with Gpp did not affect AJPT accelerated metabolism of aminopyrine. The augmentation in metabolism of aminopyrine induced by AJPT is comparable (P>0.05) to their respective control, in the absence of GPP (study I).

In the present study inhibition of calmodulin activation by TPA, in the positive control group, significantly increased the formation of formaldehyde by 24.8 % (P<0.05), as a result of aminopyrine metabolism, compared with negative control Table 1. Also, after pre-incubation with TPA, AJPT significantly increased the metabolism of aminopyrine in rat hepatocytes at all AJPT dilutions used. This increase in aminopyrine metabolism was not significantly different (P>0.05) from that in the absence of TPA (study I). The protein inhibitor OKA, in the positive control group, induced significant decrease in aminopyrine metabolism and formation of formaldehyde by 24.9 % (P<0.05) compared with negative control Table 1. Treatment of rat hepatocytes with AJPT after preincubation with OKA did not increase aminopyrine metabolism at dilutions 1:10 and 1:20, (P<0.05) as it does in study (I), in the absence of phosphatase inhibitor, OKA. In contrast, pre-incubation of rat hepatocytes with OKA did not abrogate the effect of AJPT at higher concentration (1:5 dilutions, Table 1).

Incubation of rat hepatocytes with genistein produced increased formation of formaldehyde due to enhanced metabolism of aminopyrine. The metabolism of aminopyrine was increased by 20.5

% (P<0.05) compared with negative control group Table 1. Treatment of rat hepatocytes with AJPT significantly increased the metabolism of aminopyrine (P<0.05; study I). Equally, as shown in Table 1, after pre-incubation of rat hepatocytes with genistein, AJPT significantly increased the metabolism of aminopyrine at all dilutions used. This increase in aminopyrine metabolism was comparable, non-significant (P>0.05), to that in the absence of tyrosine kinase inhibitor, genistein (study I). Lastly, our data showed that incubation of rat hepatocytes with furafylline significantly decreased the metabolism of aminopyrine by 14.9 % (P<0.05) compared with negative control Table 1. The significant increase in metabolism of aminopyrine induced by AJPT was not changed by pre-incubation of rat hepatocytes with furafylline.

Figure 1. The herbal product Air Jamu Pak Tani

Figure 2. Standard curve of absorbance versus known formaldehyde concentrations.

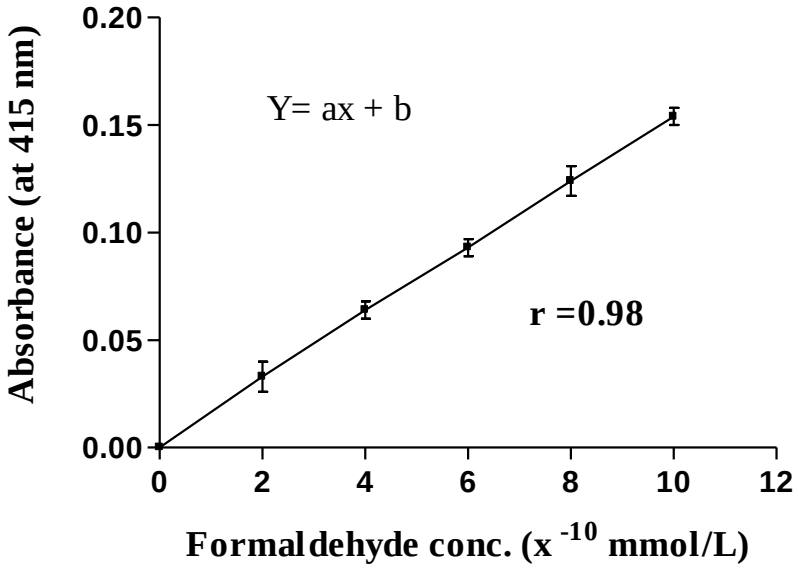


Figure 3. Dose-response effects of AJPT on aminopyrine metabolism in young male SHR rat hepatocytes. Data are expressed as mean percentage of enzyme activity compared with control group ± SEM. Each dose runs in quadruplicate, n=6 each. C_1 (control) had AJPT sample dilutions replaced with distilled water. *P<0.05 vs. C_1.

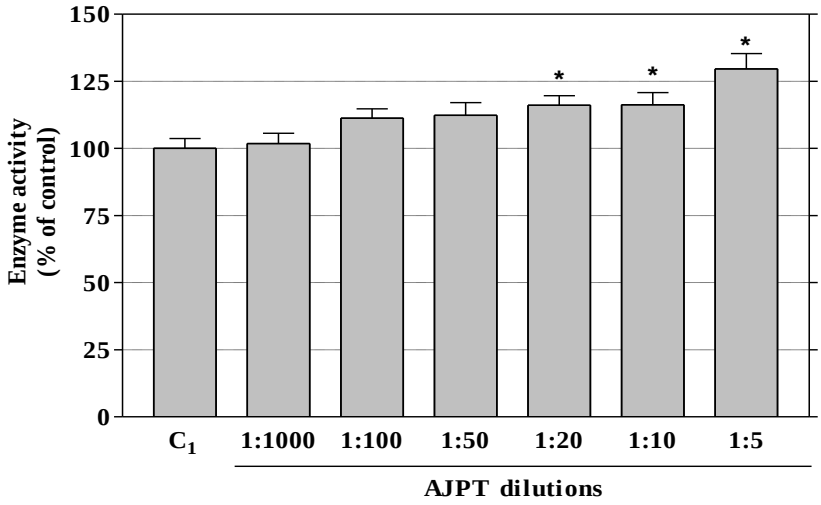
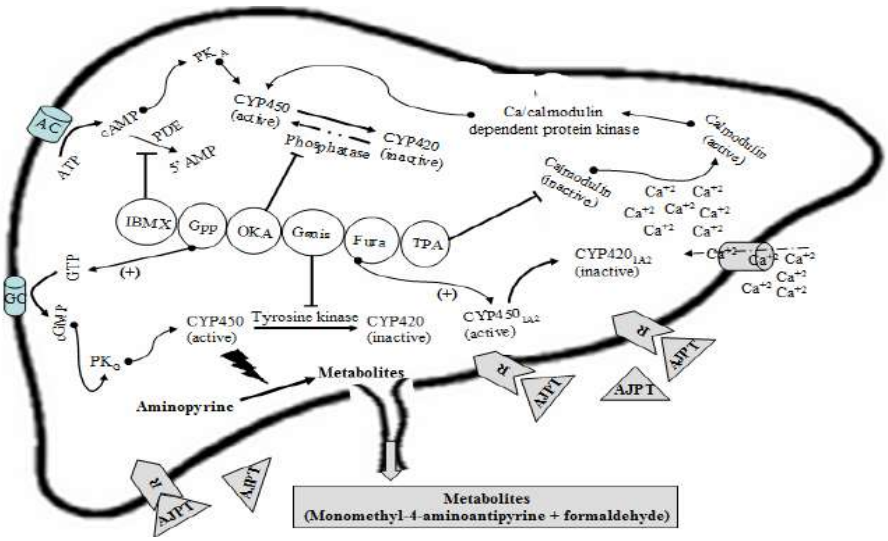


Figure 4. Schematic illustration of the molecular mechanisms involved in the effect of AJPT in modifying metabolism of aminopyrine in young male SHR hepatocytes in the presence of protein stimulants (or inhibitors). *Key*: IBMX = 3-isobutyl-1-methyl-xanthine; Gpp = 5'guanylylmidodiphosphate tetralithium salt; OKA = okadaic acid; genis = genistein; TPA = Trifluoperazine; Fura = furafylline; PDE = phosphodiesterase; PK_A = protein kinase A; PK_G = protein kinase G; R: receptor; AC: adenylyl cyclase; GC: guanylyl cyclase. Sold arrows represent CYP450 phosphorylation; dotted arrows represent CYP420 dephosphorylation; Sold arrows with black circle represent molecular mechanism pathway; blocked lines represent inhibition.



DISCUSSION:

The present study demonstrated that hepatic enzymes were stimulated after treatment with AJPT. The influence of AJPT on aminopyrine metabolism was due to its direct effect on liver n-demethylase enzyme activity. Our findings are in agreement with others [8], who demonstrate that the metabolism of aminopyrine is influenced by herbal extracts in SHR hepatocytes. However, the plant extract/s, present in AJPT, responsible for the alteration in the metabolism of aminopyrine are not known at present, since AJPT is a concoction of 12 natural plant extracts. It appears likely that AJPT did change the activity of cytochrome P450. AJPT did de-phosphorylate cytochrome P450, as they had been implicated as liver enzyme inducers, in young male SHR rats. Our finding is comparable with several studies [13, 14] that reported herbs and their

natural isolates are identified as inhibitors/ or inducers for cytochrome P450 system. The molecular mechanisms through which AJPT did modify the activity of cytochrome P450 and metabolism of aminopyrine were through various secondary messenger pathways are discussed below.

The molecular mechanisms through which AJPT affecting metabolism of aminopyrine

Pharmacokinetic interactions and hepatic drug biotransformation are well documented in rodent models [5, 8, 9]. It has been shown that cytochrome P450s enzymes are accountable for phase I drug metabolism. In phase I drug metabolism, various reactions are initiated such as reduction, de-alkylation and hydroxylation. De-alkylation reactions arise with medications that have alkyl substituted thiol group or alkoxy as a functional group and are presented in a form of secondary and tertiary amine. These drugs, during reaction, produce formaldehyde as end metabolite products [15]. The reaction is either N-, O- or S- de-alkylation according to the type of atom that connects the alkyl group [15, 16]. Several drugs such as aminopyrine, diazepam, erythromycin, morphine and the antidiabetic drug, rosiglitazone, are metabolized through n-demethylation. Herein the biotransformation of aminopyrine follows two steps; the first is binding of the methyl group, next to the nitrogen, with the hydroxyl group. The second step is the breakdown of this intermediate metabolite and the release of formaldehyde.

Interaction of herbal products with pharmaceutical drugs was the subject of different studies [5, 9, 10, 17, 18]. Previous studies showed that metabolism of aminopyrine was influenced in the presence of various plant isolates. These extracts changed the pharmacokinetic, in particular the metabolism, of aminopyrine through their effects on CYP450 activities in rat hepatocytes [4, 8]. A number of studies have showed that CYP450 activity was changed after its phosphorylation [19]. In fact, phosphorylation and dephosphorylation are important mechanisms that regulate a variety of cellular responses [20]. CYP450 enzymes activities are influenced by either cellular protein stimulants (or inhibitors). In the present study we used six protein stimulants (or inhibitors) that are well known to targeted G-protein kinases, either cAMP, cGMP, phosphodiesterase; phosphatase; tyrosine kinase; calmodulin pathway and $CYP450_{1A2}$ isoenzymes.

IBMX is a specific phosphodiesterase inhibitor. It modifies the metabolism of drugs through the inhibition of the alteration of active cAMP into inactive 5'-AMP [21, 22]. Inhibition of phosphodiesterase enzyme increases CYP450 phosphorylation due to activation of protein kinase A (PK_A) by increased intracellular cAMP. So, PK_A facilitate the conversion of CYP450, the active form, to the enzymatically inactive form CYP420 [10], and this leading to a decrease in drug metabolism. Our findings that IBMX did abrogates AJPT increases aminopyrine metabolism at the dilutions used in study (I), indicates a role for cAMP pathway in the effect of AJPK on metabolism of aminopyrine.

Our findings in study (I) suggests that AJPT activated the enzyme phosphodiesterase and so increased the metabolism of aminopyrine. Similar to IBMX, Gpp induced CYP450 phosphorylation by activation of PK_G and so reduced aminopyrine metabolism. Gpp is a non-hydrolysable analogue of guanosine-5′- triphosphate (GTP) and is a potent activator of guanylate cyclase enzyme. Conversion of inactive GTP to active cGMP is mediated by guanylate cyclase enzyme. So, Gpp after it occupied the receptor will stimulates PK_G through the cGMP pathway. PK_G initiates the phosphorylation of CYP450 to the inactive CYP420 form and results in a decrease in aminopyrine metabolism. The observation that uses of Gpp did not change AJPT accelerated metabolism of aminopyrine indicates that cGMP pathway was not involved in the effect of AJPT increased aminopyrine metabolism.

Studies have reported that TPA influenced drug metabolism by inhibition of calmodulin pathway [23]. Release of Ca^{2+} from the store and increase its entrance to the cell provokes conversion of inactive calmodulin to the active form. Active calmodulin will activate Ca/calmodulin dependent protein kinase and thus stimulates CYP450 phosphorylation into inactive CYP420. So, this led to a decrease in drug metabolism. The observation that uses of TPA with AJPK did significantly increase the metabolism of aminopyrine indicated that the mechanism through which AJPK enhanced metabolism of aminopyrine was most probably mediated, in part, by inhibition of the calmodulin path way.

OKA is a phosphatase enzyme inhibitor. Phosphatase enzyme has the responsibility to maintain CYP450 in the active form. So, inhibition of phosphatase enzyme by OKA will phosphorylate CYP450 to the inactive form CYP420 [24]. Our present findings demonstrated that phosphatase enzyme pathway was shared only in the effect of AJPK on metabolism of aminopyrine when using lower concentrations of AJPT, while, at higher

concentration of AJPT other mechanisms were involved.

Studies have should that activation of tyrosine kinase decreases the metabolism of drugs since it induces the phosphorylation of CYP450 and, so it converts the active form CYP450 to CYP420, the enzymatic inactive form [25]. Genistein is an isoflavone, modified the metabolism of drugs by inhibition of tyrosine kinases. Hence, the present finding suggests that increased metabolism of aminopyrine by AJPT was seemly mediated, in part, by inhibition of tyrosine kinase enzyme. Furafylline is a methylxanthine derivative. It is a highly selective inhibitor of $CYP450_{1A2}$. It accelerates the phosphorylation of $CYP450_{1A2}$ to the inactive isoenzyme $CYP420_{1A2}$ [26]. Therefore, our observation that influence of AJPT on metabolism of aminopyrine was not changed by the use of furafylline indicated that $CYP450_{1A2}$ pathway was not participated.

In summary, the present study showed that the effect of AJPT on hepatic phase I aminopyrine metabolism in young male SHR was mediated through different molecular mechanisms; activation of phosphodiesterase enzyme and so inhibition of cAMP pathway; inhibition of calmodulin pathway; inhibition of tyrosine kinase and activation of protein phosphatase enzyme. Our findings are consistent with others who showed that natural plant isolates have induced (or inhibited) these molecular pathways and thereby influenced the activity of CYP450 phase I drug metabolism [4, 8, 10].

CONCLUION:

The current study demonstrated that AJPT, *in vitro*, modifies the pharmacokinetic profile, in particular metabolism, of aminopyrine. AJPT, at higher concentrations used, considerably inhibited phosphorylation of cytochrome P450. AJPT, dose independently, increased the metabolism of aminopyrine in young male SHR hepatocytes. The effect of AJPT on hepatic phase I aminopyrine metabolism was mediated by different molecular mechanisms; most probably due to inhibition of $cAMP/PK_A$ pathway; inhibition of calmodulin pathway and activation of protein phosphatase and tyrosine kinase enzymes. While, activation of G-protein and a role for $CYP450_{1A2}$ isoenzyme were excluded Figure 4. So, it is most likely that the increased metabolism of aminopyrine induced by AJPT is attributed to the summation effects elicited by all those ingredients present inside the AJPT dosage form and conceivably *via* different molecular mechanisms of action. Therefore, caution should be considered when AJPT are used with n-demethylase substrate drugs, such as erythromycin, rosiglitazone, diazepam and morphine, and uncertainly reduces their bioavailability. So, more studies are needed to further elucidate whether these *in vitro* data are assigned to the *in vivo* situation. Also to characterize the effects and mechanisms of other herbal extracts on the pharmacokinetics of other clinically used modern drugs.

Conflict of interests

There are no conflicts of interest and no financial support and sponsorship

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