Complementary Therapies in Medicine 46 (2019) 87–94

Contents lists available at ScienceDirect

Complementary Therapies in Medicine

journal homepage: www.elsevier.com/locate/ctim

Caffeine/*Angelica dahurica* and caffeine/*Salvia miltiorrhiza* metabolic inhibition in humans: In vitro and in vivo studies

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ARTICLE INFO

Keywords:
Caffeine
Cytochrome
Furanocoumarin
Pharmacokinetic interaction
Tanshinone

ABSTRACT

Background: caffeine is a major constituent in numerous foods, beverages, dietary supplements and medications.*Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav, and *Salvia miltiorrhiza* Bunge are traditional medicines commonly used in Asia.

Objectives: to compare the pharmacokinetics of caffeine in humans before and after consuming an aqueous extract of *A. dahurica* or *S. miltiorrhiza*, and to propose a mechanistic explanation for *in vivo* caffeine metabolism inhibition based on *in vitro* data obtained with human liver microsomes.

Methods: Each of the four human volunteers was given a single oral dose of caffeine before and after consuming an *A. dahurica* or *S. miltiorrhiza* extract. Saliva samples were collected from the volunteers at pre-determined time points after receiving caffeine. The saliva samples were analyzed for unchanged caffeine using liquid chromatography.

Results: *A. dahurica* and *S. miltiorrhiza* extracts were capable of inhibiting caffeine metabolism in the human volunteers. In a separate study, cytochrome (CYP) 1A2-mediated caffeine demethylase activity was studied in incubation containing human liver microsomes, β-nicotinamide adenine dinucleotide phosphate, and an herbal extract (or a pure bioactive chemical from the herbs). In all cases, CYP1A2 activity was decreased with an increasing inhibitor concentration, confirming the inhibition of caffeine metabolism *in vivo*. Caffeine metabolism inhibition most likely involved the competitive and/or non-competitive mechanism.

Conclusion: Because a high level of caffeine in the plasma may result in adverse health effects in humans, care must be exercised when caffeine is consumed together with *A. dahurica* or *S. miltiorrhiza.*

1. Introduction

Caffeine is found naturally in tea, coffee, cocoa, and chocolate or fortified-beverages such as cola soft drinks and energy drinks.[1] Caffeine also is a major component in many over-the-counter medications designed for the treatment of headaches[2] and sleep apnea in infants.[3] The adverse effects of caffeine in humans include nausea, vomiting, irritability, dehydration, and sleep deprivation.[4,5] Caffeine overdose can lead to acute and chronic health effects in the cardiovascular, behavioral, and reproductive systems. Indeed, acute cardiovascular effects may cause deaths in sensitive individuals.[6]

The pharmacokinetics of caffeine in humans has been reviewed thoroughly by Benowitz.[7]. After oral administration, caffeine is rapidly absorbed into the blood and metabolized by the hepatic cytochrome (CYP) P450 enzymes with < 3% unchanged caffeine being excreted into the urine.[8] Approximately 70–80% of the caffeine absorbed is metabolized by the CYP1A2 isozyme to paraxanthine which may be further metabolized to 1-methylxanthine, 1-methyluric acid, 5-acetylamino-6-formylamino-3-methyluracil, and 1,7-dimethyluric acid.[9,10] As such, caffeine is often used as a probe drug to monitor the activity of

Abbreviations: AUC, area under the concentration-time curve; C+B, caffeine + *A. dahurica* treatment; C+D, caffeine + *S. miltiorrhiza* treatment; CL, clearance; C_{max}, maximum concentration of drug in plasma; CO, caffeine only; CTS, cryptotanshinone; CYP, cytochrome; DMSO, dimethyl sulphoxide; FC, furanocoumarin; HLMs, human liver microsomes; HPLC, high-performance liquid chromatography; M, imperatorin; ISOIM, isoimperatorin; NADPH, β-nicotinamide adenine dinucleotide phosphate; PT, Phelloptarin; PK, pharmacokinetic; $t_{1/2}$, half-life of drug in the body; TN, Tanshinone; TNI, Tanshinone I; TNIIA, Tanshinone II A; UV, Ultra-Violet; WT, weight

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https://doi.org/10.1016/j.ctim.2019.07.024
Received 10 June 2019; Received in revised form 28 July 2019; Accepted 30 July 2019
Available online 31 July 2019

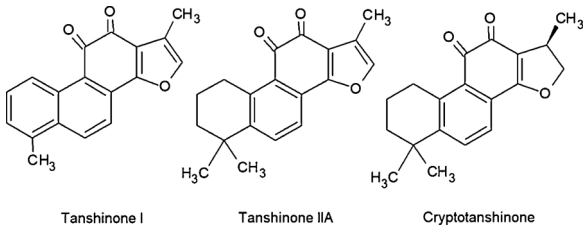

Fig. 1. Structures of tanshinones found in *S. miltiorrhiza*.

hepatic CYP1A2 in humans[11] although CYP2C8/9, CYP2E1, and CYP3A4 are also involved in the metabolism of caffeine.[8–10]

Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav (*a.k.a.* baizhi; Chinese name: 白芷) and *Salvia miltiorrhiza* Bunge (*a.k.a.* danshen; Chinese name: 丹参) are widely used traditional medicines in Asian countries.[12,13] *S. miltiorrhiza* is mostly used to treat angina pectoris, cerebral artherosclerosis, and thrombophlebitis whereas *A. dahuria* is mainly used to treat headache, toothache, and skin diseases.[12] The recommended oral doses for *S. miltiorrhiza* and *A. dahurica* are 5–30 and 3–60 g/day respectively, depending on the desired treatment.[14,15] The major bioactive components of *S. miltiorrhiza* are the tanshinones (TNs) which include tanshinone I (TSI), tanshinone IIA (TSIIA), and cryptotanshinone (CTS) (see Fig. 1).[16,17]

The TNs are potent inhibitors of hepatic CYP1A2, CYP2C9 and CYP2E1 isozymes.[18,19] The major bioactive chemicals in *A. dahurica* are the furanocoumarins (FCs) which include imperatorin (IM), isoimperatorin (ISOIM), and phellopterin (PT) (see Fig. 2).[20–22] The FCs of grapefruits are potent inhibitors of CYP3A4 isozyme[23] but IM and ISOIM mainly are inhibitors of CYP1A2, CYP2B6, CYP2C19 and CYP2D6.[24] Also, psoralen and angelicin,[25] and isopimpinellin [26] are potent inhibitors of CYP1A2 isozyme.

Human consumption of caffeinated beverages especially energy drinks has increased in recent years.[27] The use of traditional herbal remedies to prevent and treat diseases also has increased in North America.[28] Because caffeine, *A. dahurica*, and *S. miltiorrhiza* are all metabolized by the same hepatic CYP1A2 isozyme, caffeine/*A. dahurica* and caffeine/*S. miltiorrhiza* interactions may occur more frequently than our current belief. Yet relatively little is known of the pharmacokinetic (PK) interactions of caffeine/*A. dahurica* and caffeine/*S. miltiorrhiza*, and the mechanism(s) behind these interactions. The goals of this study were: to compare the pharmacokinetics of caffeine in the saliva/plasma of humans before and after treatment with an *A. dahurica* or *S. miltiorrhiza* extract, and to investigate the mechanism(s) of caffeine/*A. dahurica* and caffeine/*S. miltiorrhiza* interaction using incubations containing human liver microsomes (HLMs) (refer to Fig. 3 for an overview of experiments).

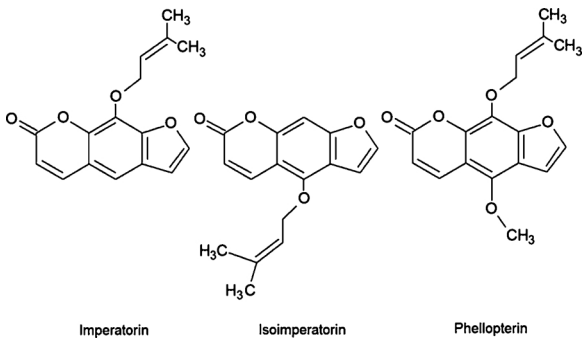

Fig. 2. Structures of furanocoumarins found in *A. dahurica*.

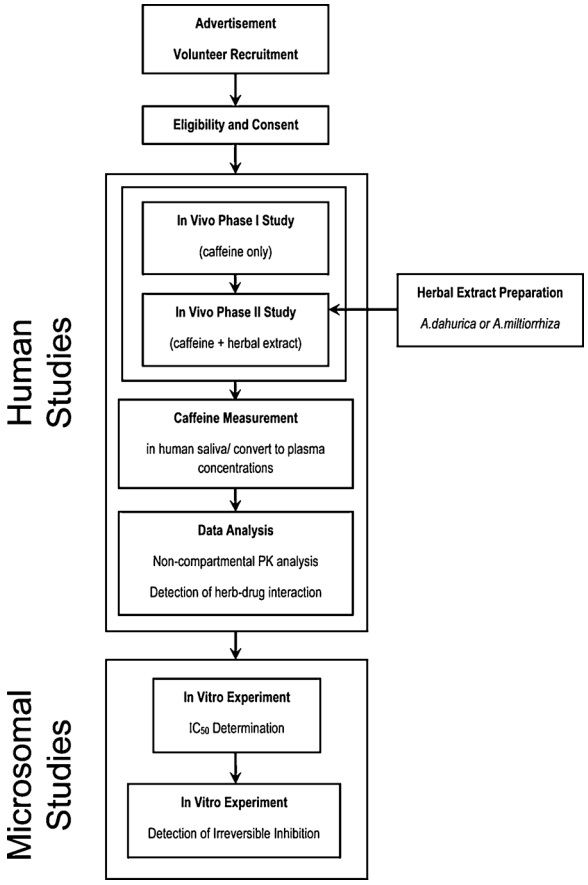

Fig. 3. A flowchart presenting the methodological steps for *in vivo*and *in vitro* experiments in this study.

2. Materials and methods

2.1. Source of herbs, chemicals, and microsomes

A. dahurica and *S. miltiorrhiza* were obtained from Spring Wind Herbs (El Cerrito, CA). The herbs were authenticated and certified to be free of insecticides and preservatives by the supplier. IM and ISOIM standards with > 97% chemical purities were obtained from Chromadex Corp. (Irvine, CA). TSI, TSIIA and CTS with > 99% chemical purities were purchased from Hangzhou Dayangchem Co., Ltd (Hangzhou, China). [3′-*N*-^{14}C-methyl]-caffeine (specific activity 55 mCi/mmol, radiochemical purity > 98%) and liquid scintillation cocktail were purchased from Perkin Elmer Life Sciences and Analytical Services, Inc. (Wellesley, MA). Dipotassium phosphate and trichloroacetic acid (TCA) were purchased from Anachemia (Norman Lachine, QC). Dimethyl sulphoxide (DMSO) and mono-potassium phosphate were obtained from Caledon (Bolton, ON). Ultrapure water was produced using a Millipore (Billerica, MA) system with a minimum resistivity of 16.0 MΩ cm at 25 °C. The remaining chemicals, solvents, and materials were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON). HLMs were obtained from Becton and Dickinson BioSciences (Woburn, MA) and stored at -80 ^{0}C prior to use.

2.2. A. Dahurica and S. Miltiorrhiza water extracts

2.2.1. Preparation of herbal extracts

Nine grams of dried *A. dahurica* and *S. miltiorrhiza* roots were accurately weighed and reduced to fine powders with a Salton Food Processor (Dollard-des-Ormeaux, QC). The powders were suspended in 600 mL filtered drinking water and sonicated at room temperature for

3 h. Aliquots of the mixture (2 mL) were filtered through a 0.2-micron Millipore IC MILLEX LG filter (Etobicoke, ON) and used in the study.

2.2.2. Bioactive chemicals in herbal extracts

The amounts of bioactive chemicals in an herbal product may vary from one supplier to another due to growing herbs in different geographic locations and the season of harvesting.[29] Therefore, it is important to analyze the *A. dahurica* and *S. miltiorrhiza* for bioactive chemicals the present study. Thus, the bioactive chemicals in *A. dahurica* and *S. miltiorrhiza* extracts were quantified using a Hewlett-Packard 1090 high performance liquid chromatography (HPLC) (Palo Alto, CA) equipped with a 250 mm pentafluorophenylpropyl (PFPP) column (4.6 mm internal diameter), a guard column from Phenomenex (Torrance, CA), and an Ultra-Violet (UV). The mobile phase of the gradient elution HPLC consisted of solvent A (ultrapure water) and solvent B (acetonitrile). The following described the percent of solvent B at different run times: (a) For *S. miltiorrhiza* extracts, the TNs were separated and quantified according to the HPLC procedure of Liu et al [30] with modification. Solvent B was increased linearly from 45 to 60% in 0–3 min and held steadily at 60% from 3 to 20 min before being increased to 80% at 21 min. Solvent B was decreased to 45% in 10 min and the column was washed with 45% solution B for 14 min. The injection volume was 100 µl. The mobile phase flow rate was fixed at 1.2 mL/min. The UV detector wavelength was set at 270 nm. (b) For *A. dahurica* extracts, the FCs was separated and quantified using the HPLC method of Frerot and Decorzant [31] with modification. Solvent B (10%) was held from 0 to 5 min. From 5–35 min, solvent B was increased linearly from 10 to 90%, and then stabilized at 90% for 10 min to wash the column. The column was re-equilibrated by reducing solvent B to 0% from 45 to 55 min. The injection volume was 100 µL. The mobile phase flow rate was fixed at 1.0 mL/min. The UV detector wavelength was set at 310 nm.

The bioactive chemicals in an herbal extract were identified initially using retention times. The identity of the bioactive chemical was confirmed by spiking the herbal extract with the pure chemical. Linearity of a calibration curves was established using the B.E.N. Software (DIN 32645) developed by the Institute of Legal Medicine and Traffic Medicine, Germany.

2.3. Caffeine pharmacokinetics in the saliva/plasma of humans

2.3.1. Human volunteers

Four healthy human volunteers between 21–30 years old were selected for the study. None of the subjects smoked, used medications or had any health issues that would affect the results of the study. We used a crossover study design in which each subject acted as his/her control. The subjects were assorted into *A. dahurica* (A1-A4) or *S. miltiorrhiza* (B1-B4) treatment groups. They were asked to avoid consuming caffeinated drinks and caffeine-containing foods for 12 h prior to and over the lifetime of the caffeine-only (CO) and herbal product pre-treatment studies. The subjects also were asked to refrain from consuming solid food 3 h prior to each study. The study protocol was approved by the Simon Fraser University Office of Research Ethics under approval number 2012s0565 and registration number ISRCTN83028296. All volunteers provided written consent and their identities were kept private.

2.3.2. Saliva sampling

Each subject took a single dose of 200 mg caffeine tablets (Adrem Inc., Scarborough, ON) orally before providing 1–2 mL of saliva samples: (a) For the CO study, saliva samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12 and 24 h post-dosing. A pre-dosing saliva sample also was collected to determine the background level of caffeine, if any. (b) For the interaction study, the same group of volunteers in the CO study was used after a 4-day washout period. In this study, each volunteer was asked to consume an herbal extract prepared from 9 g of *A. dahurica* (or *S. miltiorrhiza*) powders (see section 2.2) 3 h before taking 200 mg of caffeine. Saliva samples (1–2 mL) were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 48 h after caffeine consumption.

2.3.3. HPLC analysis of saliva samples

Caffeine concentrations in the saliva samples were quantified according to Perera et al [32] with modification. An aliquot (200 µL) of the saliva sample was extracted once using ethyl acetate (4 mL) containing 100 µL of the 50 µg/mL benzotriazole solution which served as the internal standard. The solution was vortexed and centrifuged at 5000 rpm for 0.5 min. The ethyl acetate layer was separated and evaporated to dryness under a steady stream of nitrogen gas. The residues were re-suspended in 200 µL of mobile phase. A 100 µL aliquot of the reconstituted sample was injected into an HPLC equipped with a 250 mm Agilent (Santa Clara, CA) Eclipse Plus Zorbax XDB-C18 column (4.6 mm i.d.) and a guard column with the same phase. The HPLC column was eluted at room temperature under isocratic conditions. The mobile phase which consisted of ultrapure water, acetonitrile, and acetic acid at 900:100:1 (v:v:v) with a flow rate of 1.5 mL/min. The UV detector wavelength was set at 280 nm.

2.3.4. Non-compartmental analysis of plasma concentration-time curve

Caffeine concentration in the saliva was converted to plasma concentration using a correction factor of 0.79.[33] Plasma caffeine concentrations were plotted against sampling times. The resulting concentration-time curve was analyzed with the non-compartmental approach of WinNonlin® (Pharsight Corporation, Mountain View, CA) to determine the area under the concentration-time curve (AUC), clearance (CL), and half-life ($t_{1/2}$). However, the maximum plasma caffeine concentration (C_{max}) was determined by visual inspection of the concentration-time data.

2.4. in vitroI inhibition of human liver microsomal CYP1A2 activity by an herbal extract or a pure chemical inhibitor inhibition of human liver microsomal CYP1A2 activity by an herbal extract or a pure chemical inhibitor

2.4.1. in vitro inhibition of caffeine metabolism Inhibition of caffeine metabolism

[1-^{14}C-methyl]-caffeine was used as the probe substrate in determining *in vitro* CYP1A2 activity.[34] A typical incubation consisted of 40 µL of 400 µM ^{14}C-labeled caffeine, 220 µL of 50 mM phosphate buffer (pH 7.4), 0.2 mg HLMs (40 µl), 20 µL of 0.01 mg/µL NADPH and 1 µL of herbal extract (or pure bioactive chemical) inhibitor in a final volume of 321 µL. All incubations were carried out in a Dubnoff metabolic incubator at 37 ^{0}C. Blank and control incubations which did not contain NADPH and inhibitor, respectively also were carried out concurrently.

Multiple inhibitor concentrations were used to study *in vitro* caffeine metabolism inhibition. The herbal extract stock solution was prepared as a 20 mg dried herb/mL solution. The stock solutions from herbal extracts and pure chemicals were diluted serially with DMSO.[35] Thus, the inhibitory effects of herbal extract and pure bioactive chemical were examined under similar incubation conditions although their IC_{50} units were different because pure chemical IC_{50} was based on chemical weight (WT) and herbal extract IC_{50} was based on the dry WT of an herb.

At the conclusion of a 10-min incubation, the reaction was stopped by adding 10% ice-cold TCA, placed on ice for 5 min, and centrifuged at 3500 rpm for 5 min to separate protein precipitates from the supernatant. Exactly 300 µL of the supernatant was removed and passed through a pre-conditioned Supelclean Envi-Carb® solid phase extraction tube cartridge (Supelco Canada, Oakville, ON). The eluant was collected and mixed with 14 mL of Ultima Gold scintillation cocktail (Perkin Elmer Scientific, MA) in a liquid scintillation vial which was

counted in a Beckman Coulter LSC 6500 Multipurpose Scintillation Counter (Beckman Coulter Canada, Mississauga, ON). The ^{14}C counts represented the [^{14}C]-formaldehyde and [^{14}C]-formic acid formed in the incubation; and were used to calculate the rate of caffeine demethylation.[34] CYP1A2 inhibition was expressed as the residual activity of control incubation.

2.4.2. IC_{50} determination

The IC_{50} was determined from the CYP1A2 activity inhibition *vs.* concentration plot. CYP1A2 inhibition data were first normalized from 0%–100% and then plotted against log inhibitor concentrations. The resulting concentration-inhibition curves were fitted to a four-parameter logistic function (Eq. 1) to determine the IC_{50} values. Curve fitting was performed using the GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA). The IC_{50} represented the concentration of a pure bioactive chemical (or an herbal extract), which evoked a half-maximal inhibition of the CYP1A2 activity.

$$\text{Response} = \text{min} + (\text{max} - \text{min}) / \left[1 + 10^{(\log IC_{50} - \log c)(-m)}\right] \quad (1)$$

where response represents 50% maximum inhibition; log c, the logarithm of a pure bioactive concentration (or dilution volume of herbal extract); max and min, respectively, are the 100% and 0% inhibition of the concentration-inhibition curve; m, the Hill slope.

2.5. Irreversible inhibition of caffeine metabolism

The two-step procedure described by Obach et al[36] was used to determine if the interaction between caffeine and individual bioactive chemicals might involve irreversible inhibition mechanism. In the first step, HLMs and NADPH were pre-incubated with one of the FCs or TNs for 5, 10 or 15 min. An aliquot was drawn from the pre-incubation and diluted with buffer to minimize competitive inhibition between caffeine and the inhibitor. The second incubation was initiated by the addition of ^{14}C-caffeine and fresh NADPH. If ^{14}C-caffeine metabolism was unaffected by the pre-incubation periods, the tested bioactive chemicals (*i.e.*, FCs or TNs) did not act *via* the irreversible inhibition mechanism.

2.6. Statistical analysis of data

Pairwise statistical analysis was performed with JMP 10 (SAS Institute, Cary, NC). Differences between two comparable datasets were considered significant when $p < 0.05$.

3. Results

3.1. Caffeine pharmacokinetics in humans

Fig. 4 shows a typical HPLC chromatogram of the saliva samples. Peak 1, which co-eluted with caffeine, had a RT of 10.4 min. No caffeine peak was found in the pre-dosing saliva samples except volunteers A-1 (see Table 1) and B-4 (see Table 2) of whom both showed trace levels of caffeine in the samples. Also, we were unable to find any bioactive or inactive chemicals either from *A. dahurica* or *S. miltiorrhiza* in the saliva samples.

Fig. 5 depicts the caffeine concentration-time curves in the plasma of volunteers before and after *A. dahurica* extract pre-treatment. The concentration-time curves show caffeine was rapidly absorbed after oral administration but eliminated relatively slow within 24 h. Table 1 summarizes the PK parameters in these studies. Pre-treatment with *A. dahurica* extract significantly altered the PK profiles of caffeine in the plasma: mean AUC increased from 66.9 ± 19.1 to 146.4 ± 53.7 μg-h/mL with a concomitant decline in CL. Mean $t_{1/2}$ of caffeine increased from 7.9 ± 2.7 h to 19.4 ± 9.8 h. However, the increase in mean C_{max} post *A. dahurica* treatment was insignificant statistically.

Fig. 6 shows the plasma concentration-time curves of caffeine in the volunteers before and after pre-treatment with *S. miltiorrhiza* extract. Table 2 summarizes the PK parameters of these studies. *S. miltiorrhiza* pre-treatment also significantly changed the PK profiles of caffeine in the plasma: mean AUC increased from 59.7 ± 28.1 to 116 ± 39.2 μg-h/mL with a concomitant decline in CL. Both C_{max} and $t_{1/2}$ of caffeine also were increased significantly (Fig. 7).

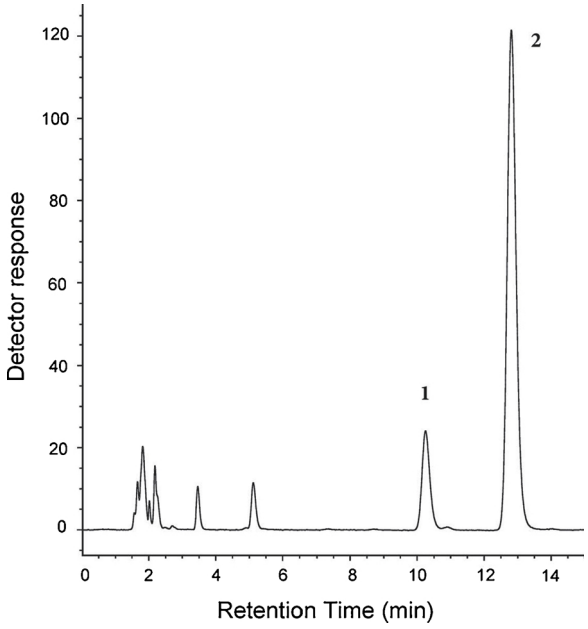

Fig. 4. A typical HPLC chromatogram of human saliva. 1) caffeine; 2) benzotriazole (internal standard).

3.2. Analyzing herbal extracts chemically using HPLC

Bioactive chemical concentrations in the herbal extracts were determined using gradient elution HPLC (see Materials and Methods). The RT of CTS, TSI and TSIIA under our gradient elution HPLC conditions were 34.05, 34.55, and 37.85 min, respectively. TSI, TSIIA, and CTS concentrations in the *S. miltiorrhiza* extract were found to be 7.32 ± 1.84, 1.77 ± 0.28, and 0.34 ± 0.07 mg/g dry herb, respectively. The RT of IM and ISOIM under our gradient elution HPLC conditions were 20.64 and 21.73 min, respectively. IM and ISOM concentrations in the *A. dahurica* extract were 0.21 ± 0.08 and 0.15 ± 0.03 mg/g dry herb, respectively. The concentration of PT in *A. dahurica* extract could not be determined because PT standard chemical was not available at the time. Nevertheless, our findings confirmed previous reports that IM and ISOIM were the bioactive components of *A. dahurica*[20–22] and TSI, TSIIA, and CTS were the bioactive components of *S. miltiorrhiza*.[15,16]

3.3. In vitro inhibition of hepatic CYP1A2 activity by herbal extracts and pure bioactive chemicals

The IC_{50} of *A. dahurica* and *S. miltiorrhiza* extracts on CYP1A2 inhibition were determined in incubations containing NADPH-fortified HLMs. Both *A. dahurica* and *S. miltiorrhiza* extracts were able to inhibit caffeine metabolism with increasing extract concentrations (see Fig. 7). The IC_{50} of *A. dahurica* and *S. miltiorrhiza* on hepatic CYP1A2 activity were 3.58 and 4.98 μg herb dry WT/mL, respectively. These results confirmed our *in vivo* inhibition results (Section 2.3) that *A. dahurica* and *S. miltiorrhiza* extracts were able of inhibit caffeine metabolism in humans.

When caffeine was incubated with individual IM, ISOIM, TSI, TSIIA, and CTS chemicals in the presence of NADPH-fortified HLMs, we observed similar inhibition straight lines as those in the whole herbal extracts (see Fig. 7). At the highest test concentrations, all pure

Table 1
Caffeine pharmacokinetic parameters in human volunteers before and after pre-treatment with *A. dahurica* extract.

Parameter	Human Volunteers †									
	A-1		A-2		A-3		A-4		Mean ± SD	
	CO	C + B	CO	C + B	CO	C + B	CO	C + B	CO	C + B
AUC (μg/mL*h)	85.3	211.0	48.5	146.0	86.8	150.0	47.1	79.0	66.9 ± 19.1	146.4 ± 53.7 §
CL (mL/kg/h)	36.9	14.9	49.0	16.3	38.7	22.3	68.0	40.5	48.1 ± 12.4	23.5 ± 11.8 §
C_{max} (μg/mL)	7.7	8.0	7.7	5.0	7.9	10.3	5.8	11.5	7.3 ± 0.9	8.7 ± 2.9
$t_{1/2}$ (h)	10.9	29.8	4.7	22.2	10.2	19.3	6.0	6.3	7.9 ± 2.7	19.4 ± 9.8 §

† PK parameters were obtained by analyzing individual concentration-time curves with the non-compartmental approach. CO represented volunteers receiving a single oral dose of caffeine; C + B represented volunteers receiving a dose of *A. dahurica* extract 3 h before consuming caffeine.
§ Significantly different to the corresponding caffeine-only parameters ($p < 0.05$).

chemicals with the exception of TSIIA were able to inhibit caffeine metabolism by > 90%. Table 3 summarizes the IC_{50} of the pure bioactive chemicals on caffeine metabolism, which may range from 0.73 to 6.63 μM. The IC_{50} of PT was not studied in this study because the standard chemical was not available at the time. Since the IC_{50} units for pure chemicals and herbal extracts are different (μM for pure chemical *vs.* g of dry WT/mL for herbs), a comparison of results was not possible. Nevertheless, these results demonstrated that the bioactive chemical mixtures were responsible for the *in vivo* (see Figs. 4 and 5) as well as *in vitro* (see Fig. 6) inhibition of caffeine metabolism by the whole herbal extracts.

Table 4 shows the rates of caffeine metabolism do not change significantly after a 5-, 10- or 15-min pre-incubation period. Therefore, it was unlikely that the irreversible inhibition mechanism was involved in caffeine metabolism inhibition by individual bioactive chemicals or the whole herbal extracts. Instead, our results (see Figs. 4–6) showed caffeine metabolism inhibition most likely involved the competitive and/or non-competitive inhibition mechanisms.

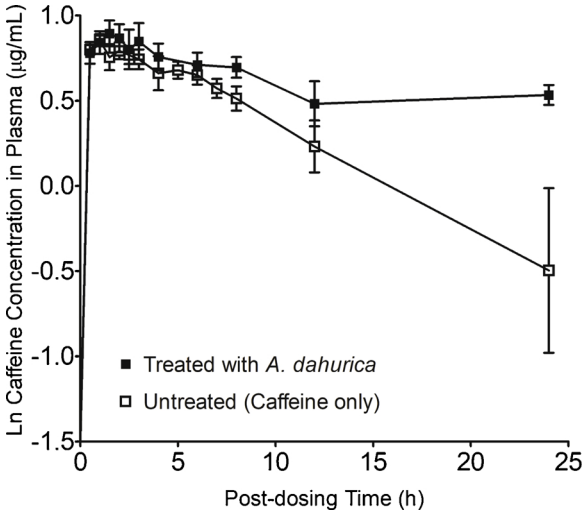

Fig. 5. Mean caffeine concentration-time curves in the plasma of healthy volunteers ($n = 4$) with and without *A. dahurica* extract pre-treatment. The time-points of 36 and 48 h have been omitted for better display and comparison.

4. Discussion

The caffeine PK study is conducted using a single-blinded, non-randomized, crossover design in which each volunteer receives an oral dose of 200 mg caffeine tablets with or without herbal extract pre-treatment. The AUC of CO or untreated volunteers (see Tables 1 and 2) are in agreement with the AUC in previously reported studies. For example, in a study conducted by Kamimori et al [37], the mean AUC in healthy human volunteers is 60.8 ± 23.7 μg-h/mL after receiving an oral dose of 5 mg/kg caffeine. This is very close to the AUC of our untreated volunteers (see Tables 1 and 2). In contrast, Fuhr et al [38] administer 167 mg of caffeine orally to 12 volunteers and report a mean AUC of 28.5 ± 14.9 μg-h/mL which is markedly lower than our results (see Tables 1 and 2). The discrepancy in results probably is due to the fact that 6 of the volunteers in their study are smokers and cigarette smoking is shown to enhance or induce caffeine biotransformation.[39]

According to the USFDA,[40] the aqueous extracts of *A. dahurica* and *S. miltiorrhiza* may be classified as 'weak' or 'moderate' inhibitors of caffeine 3′-*N*-demethylase activity (see Tables 1 and 2). Mean AUC and $t_{1/2}$ values in the plasma increase significantly after pre-treating the volunteers with *A. dahurica* (see Table 1) or *S. miltiorrhiza* (see Table 2) extract. Although the C_{max} of caffeine increases significantly after *S. miltiorrhiza* treatment (see Table 2), it remains unchanged after *A. dahurica* treatment (see Table 1). A 50% or more reduction in caffeine CL also is observed in the volunteers after pre-treatment with the extracts (see Tables 1 and 2). Because *A. dahurica* consumption does not lead to an increase in C_{max} (*i.e.*, caffeine uptake), the increases in AUC and $t_{1/2}$ most likely are due to a reduction in CL *i.e.,* caffeine metabolism. A similar conclusion may be drawn from *S. miltiorrhiza* pre-treated

Table 2
Caffeine pharmacokinetic parameters in human volunteers before and after pre-treatment with *S. miltiorrhiza* extract.

Parameter	Human Volunteers †									
	B-1		B-2		B-3		B-4		Mean ± SD	
	CO	C + B	CO	C + B	CO	C + B	CO	C + B	CO	C + B
AUC (μg/mL*h)	101.0	174.0	46.8	95.8	37.4	87.8	53.9	107.0	59.7 ± 28.1	116.0 ± 39.2 §
CL (mL/kg/h)	25.1	14.5	65.0	31.8	65.8	28.1	45.7	23.1	50.4 ± 19.3	24.3 ± 7.5 §
C_{max} (μg/mL)	7.7	13.6	5.4	7.2	5.7	6.6	7.2	6.4	6.5 ± 1.1	8.5 ± 3.4 §
$t_{1/2}$ (h)	11.4	13.1	6.8	16.0	4.6	9.2	16.7	21.5	9.9 ± 5.4	14.9 ± 5.2 §

† PK parameters were obtained by analyzing individual concentration-time curves with the non-compartmental approach. CO represented volunteers receiving a single oral dose of caffeine; C + B represented volunteers receiving a dose of S. miltiorrhiza extract 3 h before consuming caffeine.
§ Significantly different to the corresponding caffeine-only parameters ($p < 0.05$).

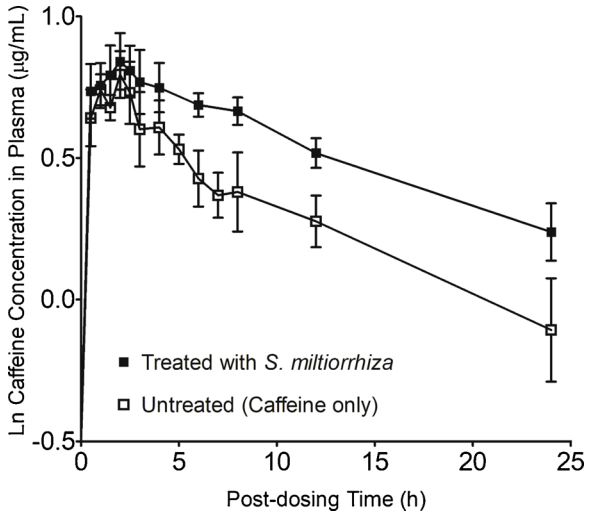

Fig. 6. Mean caffeine concentration-time curves in the plasma of healthy volunteers ($n = 4$) with and without *S. miltiorrhiza* pre-treatment. The time-points of 36 and 48 h have been omitted for better display and comparison.

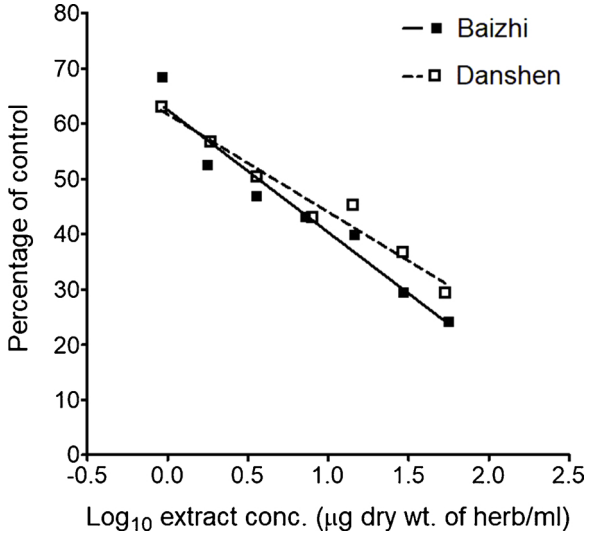

Fig. 7. *In vitro* inhibition of human liver microsomal CYP1A2 activity by *A. dahurica* (baizhi) and *S. miltiorrhiza* (danshen) extracts.

Table 3
In vitro IC_{50} values of pure *A. dahurica* and *S. miltiorrhiza* bioactive chemicals.

Individual bioactive	IC_{50} (μM) [†]
Imperatorin	1.3
Isoimperatorin	1.39
Tanshinone I	0.94
Tanshinone IIA	6.63
Crytotanshinone	0.73

[†] The IC_{50} was determined from a plot of % CYP1A2 activity inhibition *vs.* inhibitor concentrations.

volunteers despite an increase in mean C_{max} (see Table 2) because the increase in C_{max} is mainly due to a single volunteer (*i.e.*, B-1). If volunteer B-1 were excluded from the analysis, the increase in mean C_{max} would become insignificant. Lampe et al[41] have shown that *apiaceous* vegetables containing FC bioactive chemicals which are potent inhibitors of caffeine metabolism. Our results are consistent with their findings.

We are unable to find any bioactive chemicals from *A. dahurica* and *S. miltiorrhiza* in the saliva samples although these chemicals are present in the serum of laboratory animals after receiving *A. dahurica*[22] or *S. miltiorrhiza*. [17] Most likely, this is related to the type of biological fluid sampled in the study *i.e.*, saliva, blood, or urine. As with animal studies, the bioactive chemicals of *A. dahurica* and *S. miltiorrhiza* most likely are absorbed into the blood of humans after oral administration. However, the bioactive chemicals are unable to partition from the blood into the saliva due to large molecular sizes (270–300 g/mol) and high lipophilicity (3.31–5.57 log $K_{o/w}$). Another possible but unlikely explanation may be that the bioactive chemicals of *A. dahurica* and *S. miltiorrhiza* have low oral bioavailability in humans even though they are readily absorbed by laboratory animals[17,22]

The IC_{50} of pure TSI and CTS (see Table 3) are comparable to those reported previously.[18,19] In contrast, the IC_{50} of TSIIA in our study (see Table 3) is much higher than those reported in the previous studies. An explanation for the discrepancy in the IC_{50} of TSIIA is not available but may be related to the different probe substrates, TSIIA concentrations, and/or HLMs used in the studies. In contrast, the IC_{50} of IM and ISOIM in our study (see Table 3) are just slightly higher than that reported.[24] Ishihara et al. [42] have shown that *A. dahurica* is an inhibitor of CYP2C, CYP3A and CYP2D1 isoforms; yet little or no information is available on the inhibition of CYP1A2 isozyme by whole *A. dahurica* extract.

The bioactive chemicals in *A. dahurica* and *S. miltiorrhiza* probably act as competitive or non-competitive inhibitors of caffeine metabolism since CYP1A2 inhibition increases with an increasing inhibitor concentration (see Figs. 5–7). Both Qiu et al [18] and Wang et al. [19] have shown that TSI, TSIIA and CTS are competitive and/or non-competitive inhibitors of CYP1A2. Our results are in agreement with their findings. Since a 5-, 10- or 15-min pre-incubation period does not cause significant increases in CYP1A2 inhibition (see Table 4), caffeine metabolism inhibition is unlikely to involve the irreversible inhibition mechanism. A similar conclusion may be drawn for the IM and ISOIM inhibitors (see Table 3) even though they have been identified as irreversible inhibitors for other CYP isozymes.[23,24]

The CYP1A2 is the main metabolic enzyme involved in caffeine metabolism.[43] Inter-variation for CYP1A2 enzyme activity has been reported for different ethnic groups and populations.[44] It has been reported that the magnitude of induction for CYP1A2 enzyme activity depends on the gene allele (e.g. *CYP1A2*1A/*1F* or *CYP1A2*1 F/*1F* are hyperinducers).[45] However, in this study we only give a single dose of herbal extract to the volunteers, which unlikely causes CYP1A2 enzyme activity induction which would require multiple dosing. The herb-drug interaction in this study reflects the magnitude of reversible (i.e. competitive) CYP1A2 inhibition in a relatively short period of time.

This study has limitations that are worthwhile to mention. The herbal extracts oral doses are not based on volunteer's body WT which might explain the slight variability in caffeine PK parameter values. The reason is that consumers (including patients) do not consume herbal medicine and decoctions based on body WT in real life. Despite the limited number of four volunteers for each extract, the findings of all volunteers were similar and had an average AUC ratio that is statistically significant. The results in this study only reflects the magnitude of interaction in non-smoking healthy volunteers aged 21–30 years. The magnitude, and probably the adverse effects, of consuming the herbs and caffeine/CYP1A2 substrate(s) might be greater in sensitive groups such as the elderly, children, people with cardiac and hepatic illnesses.

5. Conclusion

To our knowledge, this is the first study in which the PK of caffeine in the saliva/plasma is modulated after pre-treating humans with a single oral dose of *A. dahurica* or *S. miltiorrhiza* extract. The *in vivo* PK parameters are in agreement with the *in vitro* data that both *A. dahurica* and *S. miltiorrhiza* extracts are potent inhibitors of caffeine metabolism in humans at relevant single oral doses. In view of the potential

Table 4
Effects of pre-incubation time on *in vitro* caffeine metabolism.

Pre-incubation Time	Velocity of Metabolism (pg of formic acid formed/min/mg protein) [†]														
min	IM			ISOIM			TSI			TSIIA			CTS		
5.0	69.92	±	0.73	82.54	±	0.33	131.00	±	1.83	175.70	±	1.84	125.40	±	1.49
10.0	75.29	±	0.23	81.26	±	0.57	125.40	±	1.83	178.30	±	1.41	126.30	±	1.67
15.0	68.52	±	1.51	77.25	±	1.96	124.50	±	1.95	167.60	±	0.58	127.00	±	3.30

[†] mean caffeine *N*-demethylation rate ± SD in the secondary incubations. The two-sided *t*-test showed pre-incubation time did not affect the caffeine *N*-demethylation rate significantly ($p > 0.05$).

occurrence of caffeine/*A. dahurica* and caffeine/*S. miltiorrhiza* interactions, care must be exercised when caffeine is co-administered with these herbal products.

Declaration of Competing Interest

None.

Acknowledgements

We thank Mr. Chandler Q. Zhang, Department of Biological Sciences, Simon Fraser University for his advice and technical assistance on CYP1A2 assay.

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