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In vitro modulation of cytochrome P450 isozymes and pharmacokinetics of caffeine by extracts of *Hibiscus sabdariffa* Linn calyx

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Abstract:
Background: *Hibiscus sabdariffa* beverage (HSB) is widely consumed as a medicinal herb and sometimes used concomitantly with drugs. This study evaluated the *in vitro* inhibitory potential of the aqueous extract of *H. sabdariffa* calyces (AEHS) on selected cytochrome P450 (CYP) isozymes and the effect of HSB on the pharmacokinetics of caffeine *in vivo*.
Methods: *In vitro* inhibitions of eight major CYP isozymes by AEHS were estimated by monitoring CYP-specific model reactions of 10 CYP probe substrates using *N*-in-one assay method. Subsequently, an open, randomized, two-period crossover design was used to evaluate the effect of HSB on the pharmacokinetics of single-dose 200 mg caffeine in six healthy human volunteers. Blood samples were obtained at specific times over a 24 h period. Probe drugs and metabolites were analyzed in their respective matrices with ultra-performance liquid chromatography/mass spectrometer/mass spectrometer and reversed-phase high-performance liquid chromatography/ultraviolet detection.
Results: The *H. sabdariffa* aqueous extract weakly inhibited the selected CYP isozymes *in vitro*, with IC_{50} of >100 μgmL^{-1} in the order of CYP1A2 > CYP2C8 > CYP2B6 » CYP2D6 > CYP2C19 > CYP3A4 > CYP2A6 > CYP2C9. HSB decreased terminal $t_{1/2}$ and T_{max} of caffeine by 13.6% and 13.0%, respectively, and increased C_{max} by 10.3%. Point estimates of primary pharmacokinetic endpoints, C_{max} = 1.142 (90% confidence interval (CI) = 0.882, 1.480) and $AUC_{0-\infty}$ = 0.992 (90% CI = 0.745, 1.320), were outside the 90% CI of 0.8–1.25 bioequivalence limits.
Conclusion: The aqueous extract of *H. sabdariffa* weakly inhibited eight CYP isozymes *in vitro*, but HSB modified the exposure to caffeine in human. Caution should be exercised in administering HSB with caffeine or similar substrates of CYP1A2 until more clinical data are available.

Keywords: bioequivalence, caffeine, herb-drug interaction, *Hibiscus sabdariffa* , human, pharmacokinetics
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Introduction

Several herbs and herbal preparations of different formulations have been reported to cause herb-drug interactions, some of which are potentially dangerous to patients' health [1]. Herbs are taken as powdered plant parts or whole plant, tinctures, decoctions, teas, vegetables, and herbal drinks. Examples of some of the popular herbal drinks and beverages are ginseng herbal drink and tea, licorice tea, and Hibiscus tea and beverage. Hibiscus tea or beverage has a global recognition and is known as "Rosselle" in Mexico, "Sorrel" in Latin America, "Karkade" in Germany, "Luo Shen Hua" in China, "Isapa pupa," "Zobo," or "Soborodo" in Nigeria. In folk medicine, it is used for liver disease, hypertension, diabetes, kidney diseases, fever, and as a laxative [2].

The calyx of *Hibiscus sabdariffa* has been reported to possess strong antioxidant properties both *in vitro* and *in vivo* [3], antihypercholesterolemic effect in animal models and humans [4], [5], anxiolytic and immunomodulatory [6], [7], antinociceptive and antipyretic [8], and antihypertensive activities [9], [10]. These pharmacological

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properties of *H. sabdariffa* make it a potential herb for self-medication in the treatment of diverse ailments. This raises the possibility of co-administration of *H. sabdariffa* beverage (HSB) or tea with medications for acute and chronic diseases.

Up to 40%–57% of different categories of patients use herbs along with their medications [11], [12], and it has been documented that most hospitalized patients who supplement their medications with herbs do not disclose the herbs used to their physicians [13], [14]. This practice predisposes such patients to herb-drug interactions that may modify the outcome of therapy or cause untoward effects and possibly toxic reactions.

Herb-drug interactions have been shown to occur when the pharmacokinetic profile of a drug is significantly altered by the co-administered herb. Many of these interactions involve inhibition of metabolizing enzymes and efflux transporters, resulting in increased systemic exposure and subsequent adverse drug reactions and vice versa [15], [16]. Most herb-drug interactions are metabolism-mediated through CYP isozymes responsible for the metabolism of over 70% of prescription drugs [15].

We earlier reported that the ethanolic extract of *H. sabdariffa* had a weak inhibitory activity on CYP isozymes [17], with CYP1A2 having the lowest IC_{50} value (IC_{50} = 306 μg/mL). Cytochrome P450 1A2 is an important enzyme involved in the bioactivation of precarcinogens, elimination of environmental toxins, metabolism of endogenous substances, and important chemicals and drugs such as caffeine, phenacetin, theophylline, clozapine, melatonin, olanzapine, and tizanidine [18], [19], [20]. Although a previous study used ethanolic extract of *H. sabdariffa* [17], the calyces of the plant are usually consumed as aqueous tea or beverage. Hence, this study first evaluated the *in vitro* inhibitory activity of the aqueous extract of *H. sabdariffa* calyces (AEHS) on eight major human CYP isozymes. The outcome of the *in vitro* study suggested that it may be necessary to evaluate the *in vivo* influence of HSB on the disposition of CYP1A2 substrate in healthy human volunteers, more so as *in vitro* and *in vivo* findings may not always correlate [21], [22]. As caffeine is an established probe substrate for assessing CYP1A2 activity and is incidentally the most consumed psychoactive drug globally [23], it was used in the *in vivo* herb-drug interaction study in healthy human volunteers.

Materials and methods

The study was approved by the University of Ibadan/University College Hospital Ethics Review Committee with approval number UI/EC/11/0073. Written informed consents were obtained from human volunteers.

Chemicals

Caffeine, hydroxydiclofenac, desmethylomeprazole, 3-hydroxyomeprazole, and hydroxycoumarin were purchased from Sigma-Aldrich, St Louis, MO, USA. Methanol was obtained from Fisher Scientific, Loughborough, UK; etofylline from Toronto Research Chemicals Incorporation, Toronto, Canada; and high-performance liquid chromatography (HPLC) grade acetonitrile and acetic acid (96%) from Merck KGaA, Darmstadt, Germany. Other chemicals purchased were as follows: 6-hydroxytestosterone, dextrorphan, and desethylamodiaquine (BD Bio-sciences Discovery Labware, Bedford, MA, USA); 5-hydroxyomeprazole and omeprazole sulfone (Astra Zeneca, Mölndal, Sweden); 1-hydroxymidazolam (F. Hoffmann-La Roche, Basel, Switzerland); and phenacetin (ICN Biomedicals, Costa Mesa, CA, USA). Hydroxybupropion was a free gift from Glaxo SmithKline (Research Triangle, NC, USA). Water was purified by Simplicity 185 water purifier (Millipore, Molsheim, France). Caffeine 200 mg tablets (Stay Awake®) were purchased from Walgreens Co., Deerfield, IL, USA. Other reagents and chemicals used were of analytical grade.

Preparation of plant extracts

Fresh calyces of *H. sabdariffa* obtained from Gbagi market in Ibadan, Nigeria were identified and authenticated at the Forestry Research Institute of Nigeria with voucher no. FHI 106934. For the preparation of AEHS, 500 g of powdered dried calyces of *H. sabdariffa* was extracted with 1.5 L of boiled distilled water for 4 h and filtered; the residue was further extracted with 1 L of boiled distilled water for another 4 h and filtered. The filtrates were pooled and concentrated with rotary evaporator at 40 °C and freeze dried. HSB used by healthy human volunteers was also prepared with 300 g of dried powdered calyces of the plant using a previously described method [24].

Evaluation of *in vitro* inhibitory potential of aqueous extract of *H. sabdariffa* – CYP-inhibition experiments

Using a previously reported CYP-specific model reactions and validated *N*-in-one methods with slight modification, the modulatory effect of aqueous extract of *H. sabdariffa* calyces on some CYP activity *in vitro* was investigated [25], [26]. Summarily, a cocktail of the probe substrates for each CYP isozymes at determined specific concentration (Table 1) was added to an incubation mixture. This mixture that contained 0.3 mg microsomal protein per mL, 0.1 M phosphate buffer (pH 7.4), and 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) was added to different concentrations of AEHS (0.001, 0.01, 0.1, 1, 10, 100, and 1000 μgmL^{-1}). The negative control (solvent) had no extract in the incubation mixture. Positive controls used for the *N*-in-one methods were as previously reported [17], [25], [26] and included as Supplemental Data Table 1. The reaction of the resultant mixture (200 µL) that had been preincubated in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) for 2 min at 37 °C was initiated by the addition of NADPH. Each reaction was stopped at 20 min with the addition of 200 µL of ice-cold acetonitrile while protein precipitation was achieved by cooling in an ice bath. Each determination was done in duplicates. The supernatants were collected and stored at −20 °C until analyzed. The probe metabolites from CYP-specific marker reactions (Table 1) were analyzed by adapting previously reported ultra-performance liquid chromatography/mass spectrometer/mass spectrometer (UPLC/MS/MS) methods [17], [25], [26]. The conditions for these analyses are adequately described in Table 2. The instruments used for analysis and data acquisition were the Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) and the Thermo TSQ Endura Triple Quadrupole MS (Thermo Electron North America LLC, Madison, Wisconsin, USA), respectively. The instruments were controlled using Thermo Xcalibur 3.0.63 software (Thermo Electron North America LLC, Madison, Wisconsin, USA). The validated parameters for the methods were adequate for quantification ranges of <1%–300% of the forming metabolite concentrations with limit of detection (LOD) of 0.2–10 nM, accuracies of 85%–115%, and precisions of <15% at all concentrations. The enzyme activities in the presence of the herb extract were compared with the vehicle incubations.

Table 1: Probe substrates and metabolites monitored for each CYP isozyme in the evaluation of *in vitro* inhibitory potential of aqueous extract of *H. sabdariffa* calyces.

CYP isozymes	Substrates	Concentration of substrate, µM	Metabolite monitored
1A2	Phenacetin	10	Acetaminophen
2A6	Coumarin	2	7-Hydroxycoumarin
2B6	Bupropion	2	Hydroxybupropion
2C8	Repaglinide	5	Hydroxyrepaglinide
2C9	Diclofenac	5	4-Hydroxydiclofenac
2C19	Omeprazole	5	5-Hydroxyomeprazole
2C19	Omeprazole	5	Desmethylomeprazole
2D6	Dextromethorphan	1	Dextrorphan
3A4	Midazolam	5	1-Hydroxymidazolam
3A4	Testosterone	5	6β-Hydroxytestosterone
3A4	Omeprazole	5	Omeprazole sulfone
3A4	Omeprazole	1	3-Hydroxyomeprazole

Table 2: Summary of conditions for detecting each CYP probe substrate metabolite(s) with UPLC/MS/MS.

Conditions	ACET (1A2)	7-OH-COU (2A6)	OH-BUP (2B6)	OH-REPA (2C8)	OH-DICL (2C9)	5-OH-OME (2C19)	deM-OME (2C19)	*O*-deM-DEX (2D6)	3-OH-OME (3A4)	SO_2-OME (3A4)	6β-OH-TEST (3A4)	1-OH-MDZ (3A4)
Enzyme source and supplier	Human liver microsomes (consisted of liver samples from 25 donors of both genders)/BD Bioscience (Lot # 99268)											
Column	Waters HSS C18 column (2.1 mm × 50 mm column with 1.8 µm particle size)											
UPLC eluent	A = 0.1% acetic acid (pH 3.2); B = acetonitrile											
Gradient elution	2%–65%–95% B; 0–2.5–3.5 min.											
Injection volume, µL	4											
Eluent flow rate, $\mu Lmin^{-1}$	0.5											
Run time, min.	4.5											
Retention time, min.	0.76	1.30	1.18	2.18	2.17	1.29	1.17	1.12	1.40	1.80	1.58	1.71
Incubation mixture	0.3 mg microsomal protein per mL, 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, plant extract, and probe substrates											
Extract concentration	0.001, 0.01, 0.1, 1, 10, 100, 1000, and 10,000 μgmL^{-1}											
Spray voltage	4500 V											
Vaporizer temperature	400 °C											
Transfer tube temperature	350 °C											
CID argon pressure	2.0 mTorr											
Dissolvation gas	Nitrogen 750, Lh^{-1}											
Cone gas	Nitrogen 50, Lh^{-1}											
Polarity	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+

ESI, electrospray ionization positive (+) or negative (−), ACET, acetaminophen; 7-OH-COU, 7-OH-coumarin; OH-BUP, OH-bupropion; OH-REPA, OH-repaglinide; OH-DICL, 4′-OH-diclofenac; 5-OH-OME, 5-OH-omeprazole; deM-OME, desmethylomeprazole; dextrorphan *O*-deM-DEX, 3-OH-OME, 3-OH-omeprazole; SO_2-OME, omeprazole sulfone; 6b-OH-TES, 6β-OH-testosterone; 1-OH-MDZ, 1′-OH-midazolam.

Effect of HSB on the pharmacokinetic of caffeine *in vivo*

Experimental protocol

On the basis of the outcome of the *in vitro* study, caffeine, a probe substrate for the CYP1A2 isozyme, was selected for *in vivo* study. An open-labeled, single-dose, randomized, two-period, two-treatment, two-sequence, crossover design was used for the evaluation of the effect of HSB on the pharmacokinetics of caffeine in 10 healthy human volunteers. A sample size of six healthy volunteers will be sufficient to reach a reasonable clinical conclusion according to the Food and Drug Administration [27], [28], similar pharmacokinetic studies involving other drugs [29], [30], [31] and HSB [32], [33]. The health status of the volunteers was ascertained using a health screening self-administered questionnaire, physical examination, and biochemical and hematological screening. Participants gave written informed consents, and the study was conducted in accordance with the declaration of Helsinki [34]. All volunteers were nonsmokers, and female volunteers were not pregnant. Two weeks prior to the commencement of the study and during the study, the volunteers were asked to abstain from consuming alcohol, caffeinated beverages, charbroiled foods, grapefruit juice, and any prescription or over-the-counter medications, vitamins, and herbal products.

Participants were randomly divided into two arms without bias for gender. Participants in one arm of the study took caffeine tablet 200 mg with 300 mL of water while those in the other arm took the drug with 300 mL of HSB. One week washout period was observed before the treatments were switched between the two groups in the second phase of the study. Participants were questioned about any overt adverse effects experienced with caffeine or HSB during and after each phase of the study period.

In each phase of the study, blood samples (5 mL) were collected at zero hours prior to drug administration and thereafter at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h post-dose of caffeine. They were centrifuged immediately at 3500 *g* for 10 min, and plasma obtained was stored at −20 °C until analyzed.

Analysis of caffeine

Caffeine was analyzed using the method described by Uney et al. [35] with slight modification. Briefly, to each tube containing 250 µL of plasma was added 10 µL of 125 µgmL^{-1} etofylline (IS) and 10 µL of working standard solutions of caffeine (1.25, 2.5, 10, 20, 25, 125, 250, and 375 µgmL^{-1}), respectively. Ice-cold acetonitrile (750 µL) was added to the mixture to precipitate protein. The mixture was vortexed for 30 s, centrifuged at 5000 *g* for 10 min, and the organic phase was removed and dried with nitrogen gas over a water bath at 45 °C. The residue was reconstituted with 250 µL of the mobile phase. Analysis was achieved with Agilent HPLC 1200 series equipped with isocratic pump (G1310A), online degasser, Rheodyne manual injector fitted with a 20 µL loop, and a UV detector (G1314B) set at was 280 nm. Separation of analytes was accomplished with Agilent Eclipse XDB column C18 (150 mm × 4.6 mm; 5 µm), and data were acquired with Chemstation software version Rev B.03.02 (341). Reconstituted analytes (20 µL) were injected into the HPLC column and were eluted with methanol:water (20:80 v/v) at a flow rate of 1.0 mLmin^{-1}. The retention times for etofylline and caffeine were 4.9 and 7.2 min, respectively, with total run time of 10 min.

The calibration curve for caffeine was linear over 0.05–15.0 µg/mL (R^2 = 0.9997), and lower limit of quantification (LLOQ) was 0.05 µgmL^{-1}. Within run and between run precision were 0.5%–4.9% RSD and 0.9%–7.1% RSD, respectively, while within run and between run accuracy were 80.2%–100.6% and 83.5%–98.2%, respectively. Percentage recovery for caffeine was between 83.4%–99.4%.

Data analysis

Determination of IC_{50} values

Fifty percent inhibitory concentration (IC_{50}) values were determined graphically from the logarithmic plot of aqueous extract of *H. sabdariffa* concentration vs. percentage of enzyme activity remaining after inhibition using GraphPad Prism® 7.00 software (GraphPad Software Inc., San Diego, CA, USA). The enzyme activities in the presence of AEHS were compared with the vehicle incubations control. The risk of *in vivo* herb-drug interactions from *in vitro* data was determined by estimating the IC_{50} values in Ldose^{-1} as described by Strandell et al. [36].

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters of caffeine were estimated with PKsolver® compartmental analysis software (version 2.0). The area under the plasma concentration-time curves (AUC_{0-24} and $AUC_{0-\infty}$), the peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}), the apparent total body clearance (CL/F), apparent volume of distribution (V/F), absorption rate constant (k_a), and elimination rate constant (k) were determined. The corresponding elimination half-life ($t_{½}$) and absorption half-life ($t_{½}k_a$) were also estimated.

Significant changes in the pharmacokinetic parameters following the co-administration of caffeine with HSB were estimated with Wilcoxon signed rank test at $p < 0.05$. Also, mean percentage changes in the pharmacokinetic parameters were calculated along with the 95% CIs. To demonstrate the presence of herb-drug interaction, geometric means of C_{max} and $AUC_{0-\infty}$ were determined with 90% CI when caffeine was used with HSB (T) and with water (R). If the 90% CI of the geometric mean ratio (T/R) of these pharmacokinetic parameters were outside the 80%–125% (or 0.8–1.25) bioequivalence range, the presence of a clinically significant herb-drug interaction was confirmed. Statistical analyses were performed with the Statistical Package for Social Sciences, Windows version 23 (IBM Corp, New York, NY, USA) and Minitab 17 (Minitab LLC, Pennsylvania, USA)

Results

In vitro inhibitory potential of AEHS on selected CYP isozymes

AEHS inhibited the formation of the metabolites of the CYP isozyme probe substrates investigated. The IC_{50} values ranged from 310.46 to 5249.61 μgmL^{-1} (Table 3), and the order of inhibition was 1A2 > 2C8 > 2B6 » 2D6 > 2C19 > 2A6 > 2C9 > 3A4. The herb-extract had IC_{50} values > 100 μgmL^{-1} and inhibited CYP2D6, CYP2B6, CYP1A2, and CYP2C8 by 47%, 63%, 76%, and 80%, respectively, at 1000 μgmL^{-1}. The IC_{50} values in $literdose^{-1}$ unit ranged from 0.53 $Ldose^{-1}$ (CYP3A4) to 8.85 $Ldose^{-1}$ (CYP1A2), as shown in Table 3.

Table 3: Prediction of *in vivo* CYP inhibition from *in vitro* IC_{50} data for *H. sabdariffa*.

Variables	Cytochrome P450 isozymes							
	CYP1A2	**CYP2A6**	**CYP2B6**	**CYP2C8**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4**
Human single dose[a]	8180 mg (based on quantity of powdered plant part required to make 300 mL of aqueous beverage)							
Percentage yield of extract,[b] %w/w	33.6							
Estimated extract per single human dose,[c] mg	2748.48							
In vitro IC_{50} value, μgmL^{-1}	310.46	1755.24	667.20	333.72	2134.03	1376.66–1645.49	1089.24	1554.85–5249.61
IC_{50} value ($Ldose^{-1}$ unit) = (extract dose)/IC_{50}	**8.85**	**1.57**	**4.12**	**8.24**	**1.29**	**1.67–1.20**	**2.52**	**0.53–1.77**

[a]Human single dose in the form of beverage; [b]Percentage yield of aqueous extract; [c]Assumes the same yield from aqueous beverage of *H. sabdariffa*.

Effect of HSB on the pharmacokinetics of caffeine

Six male participants with mean age of 23.1 ± 4.0 years and body mass index of 24.1 ± 4.7 kgm^{-2} completed the study out of the 10 volunteers enrolled. Similar number of participants had been used in pharmacokinetic studies [32], [37]. Three female participants dropped out for undisclosed personal reasons, while a male participant was dropped because of noncompliance to study the protocol. The mean values of laboratory tests for liver function, kidney function, and other relevant laboratory indices for the participants are shown in Table 4.

Table 4: Volunteers health indices for the study of the effect of *Hibiscus sabdariffa* beverage on the pharmacokinetics of caffeine.

Description	Mean ± SD
Age, years	23.14 ± 3.98
Clinical indices	
Body Mass Index, kgm^{-2}	24.07 ± 4.69
Fasting blood glucose, $mgdL^{-1}$	86.14 ± 12.20
Visceral fat, %	5.14 ± 2.61
Total fat, %	21.30 ± 11.45
Muscle, %	39.36 ± 6.75
Systolic blood pressure, mmHg	125.21 ± 16.73
Diastolic blood pressure, mmHg	71.86 ± 9.36
Pulse, min^{-1}	72.36 ± 10.05
Kidney function indices	
Serum creatinine, gdL^{-1}	0.67 ± 0.12
Blood urea nitrogen, gdL^{-1}	15.17 ± 5.27
Liver function indices	
Aspartate transaminase (AST), UL^{-1}	9.67 ± 1.75
Alanine transaminase (ALT), UL^{-1}	7.17 ± 2.14
AST/ALT ratio	1.43 ± 0.43
Hematological indices	
Hematocrit, %	44.33 ± 2.07
Hemoglobin, gdL^{-1}	14.72 ± 0.48
Red blood cell, μL^{-1}	7.58×10^{6} ± 0.35
White blood cell, μL^{-1}	8.63×10^{3} ± 1.44
Platelets, μL^{-1}	5.40×10^{5} ± 2.56
Lymphocytes, %	40.67 ± 12.26
Neutrophils, %	51.00 ± 9.94
Monocytes, %	4.67 ± 2.42
Eosinophil, %	2.33 ± 1.37

HSB decreased $t_{1/2}$ and T_{max} of caffeine by 13.62% (95% CI −22.91%, 0.87%) and 13.04% (95% CI −32.95%, 39.46%), respectively, while the $AUC_{0-\infty}$, elimination rate constant (k), and C_{max} increased by 2.46% (95% CI −20.08%, 26.67%), 13.33% (95% CI 2.18%, 34.06%), and 10.27% (95% CI −8.26%, 48.90%), respectively. Changes in other pharmacokinetic parameters are shown in Table 5. The plasma concentration-time profile following the co-administration of caffeine with water and HSB is shown in Figure 1. Point estimates for the primary pharmacokinetic endpoints, C_{max} and $AUC_{0-\infty}$, following co-administration of caffeine with and without HSB (T/R) were not bioequivalent as the 90% CI for these parameters were outside the 0.8–1.25 bioequivalence limits as shown in Figure 2.

Table 5: Pharmacokinetic parameters of caffeine when co-administered with water and *H. sabdariffa* beverage.

Pharmacokinetic parameter	Caffeine + water (R)	Caffeine + *Hibiscus sabdariffa* beverage (T)	% change (95% CI)	p-value[a]
$K_{a,}$, h^{-1}	2.59 ± 2.08	2.64 ± 0.98	1.89% (−12.90, 113.14)	0.463
$K_{e,}h^{-1}$	0.15 ± 0.04	0.17 ± 0.03	13.33% (2.18, 34.06)	0.102
$t_{1/2}K_a$, h	0.46 ± 0.37	0.29 ± 0.11	−36.96% (−50.32, 62.01)	0.340
$t_{1/2}K_e$, h	4.92 ± 1.54	4.25 ± 0.75	−13.62% (−22.91, −0.87)	0.116
V/F, L	42.30 ± 8.47	38.30 ± 12.11	−9.46% (−26.20, 15.51)	0.463
CL/F, Lh^{-1}	6.14 ± 0.87	6.43 ± 2.36	4.72% (−18.57, 35.49)	0.917
T_{max}, h	1.38 ± 0.54	1.20 ± 0.35	−13.04% (−32.95, 39.46)	0.600
C_{max}, μgmL^{-1}	4.48 ± 1.39	4.94 ± 0.59	10.27% (−8.26, 48.90)	0.600
AUC_{0-24}, $\mu gmL^{-1}h$	32.03 ± 3.83	33.25 ± 9.58	3.81% (−19.33, 26.78)	0.753
$AUC_{0-\infty}$, $\mu gmL^{-1}h$	33.30 ± 5.58	34.12 ± 10.07	2.46% (−20.08, 26.67)	0.753
AUMC, $\mu gmL^{-1}h^2$	170.93 ± 51.81	228.43 ± 95.01	33.64% (−9.74, 117.99)	0.249
MRT, h	5.57 ± 1.85	6.49 ± 1.19	16.52% (−10.36, 80.49)	0.917

MRT, mean residence time; AUMC, area under the first moment curve. (−) represents percentage decrease. [a]p-value for Wilcoxon signed rank test.

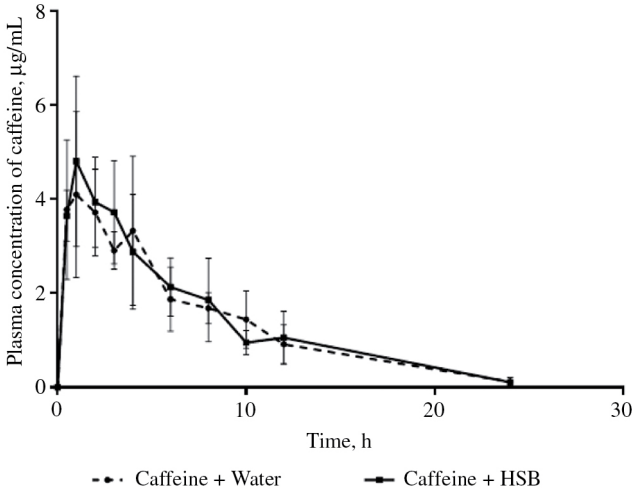

Figure 1: Plasma concentration-time curve of caffeine following co-administration with water and *Hibiscus sabdariffa* beverage (HBS).

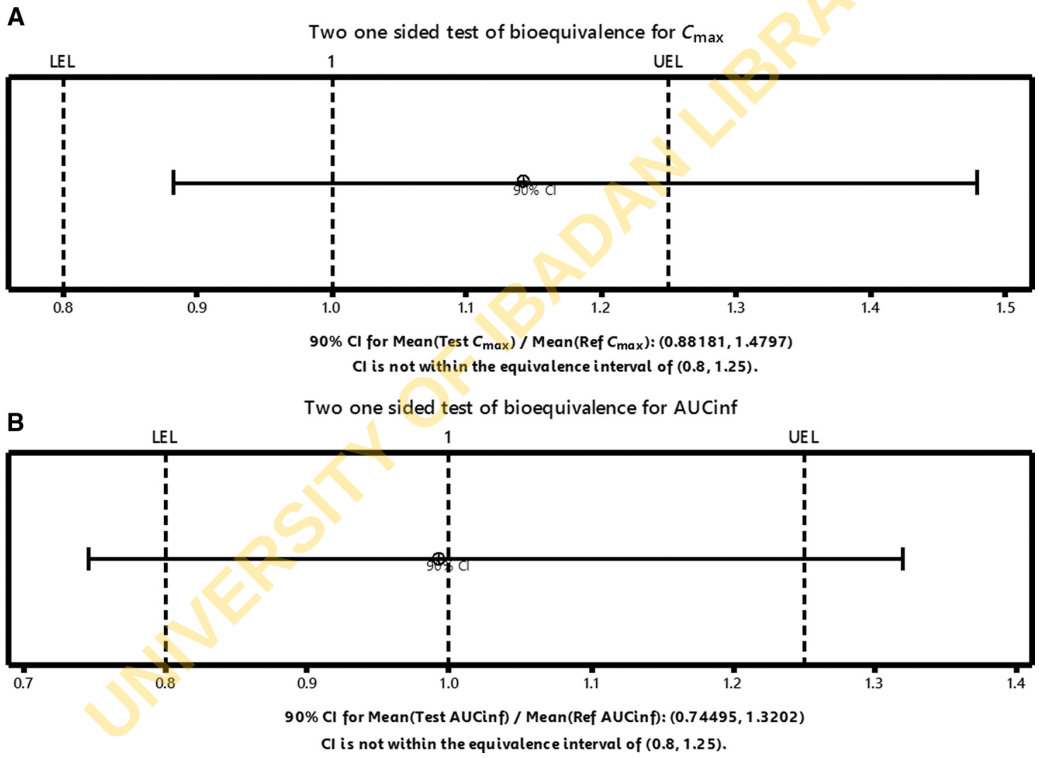

Figure 2: Two one-sided test of bioequivalence for primary pharmacokinetic endpoints, C_{max} (A) and $AUC_{0-\infty}$, (B) following the concurrent use of caffeine with water (Reference) and *Hibiscus sabdariffa* beverage (Test).
Bioequivalence is established when 90% confidence interval (CI) of the geometric mean is within 0.8 – 1.25 (80% – 125%) bioequivalence limits. LEL, lower equivalence limit; UEL, upper equivalence limit.

Discussion

The weak *in vitro* inhibitory activity observed with AEHS in this study is similar to a previous study on the ethanol extract of *H. sabdariffa* on nine CYP isozymes [17]. The ethanol and aqueous extracts of *H. sabdariffa* inhibited the CYP isozymes in similar orders: CYP1A2 > CYP2C8 > CYP2D6 > CYP2B6 > CYP2C19 > CYP3A4 » CYP2C9 » CYP2A6 and CYP1A2 > CYP2C8 > CYP2B6 » CYP2D6 > CYP2C19 > CYP3A4 > CYP2A6 > CYP2C9, respectively, showing that the extracts had effect on the magnitude of inhibition of the specific CYP isozymes. In both studies, however, CYP1A2 was the most inhibited. Prommetta et al. [38] reported that the aqueous extract of *H. sabdariffa* had no effect on the activities of CYP isozymes 1A1, 1A2, 2B1, 2B2, 2E1, and 3A using rat liver microsomes, which is different from the results obtained with human liver microsomes in our study. This is

not surprising as reports exist that CYP inhibitors do not exhibit the same selectivity in human and rat liver microsomes and there are differences in the CYP isoforms responsible for metabolism in the species [39], [40].

Studies have shown that the inhibition of CYP-mediated metabolism of drugs may lead to therapeutic failure, enhanced therapeutic effect, increased side effect, or toxicity [41], [42]. Secondary metabolites such as alkaloids, cardenolides, saponins, tannins, anthocyanins, and flavonoids present in calyces of *H. sabdariffa* are responsible for most pharmacological activities observed with its extracts [2], [43], [44]. Some of these compounds namely coumarins, saponins, anthocyanins, and flavonoids have been shown to exhibit inhibitory activities on CYP isozymes [45], [46], [47], [48]. Thus, these secondary metabolites may be responsible for the observed *in vitro* inhibitory activities of the extract on the selected cytochrome P450 isozymes. It is also expected that though weak, the *in vitro* inhibitory activities of the *H. sabdariffa* extracts on CYP1A2 may become clinically significant if co-administered with drugs with narrow therapeutic index or those that undergo capacity-limited metabolism.

According to Strandell et al. [36], herbs with *in vitro* $IC_{50} \geq 5$ $Ldose^{-1}$ likely produce *in vivo* inhibition similar to the observed *in vitro* data. Estimation of *in vivo* herb-drug interaction from the *in vitro* data obtained from the present study showed that for CYP1A2, the IC_{50} was more than 5 $Ldose^{-1}$. It would have been expected based on the Strandell et al. [36] assessment that there would be *in vivo* inhibition of CYP1A2 by HSB in healthy human volunteers. However, pharmacokinetic study in these volunteers showed that the parameters that should change if there were inhibition of caffeine by HSB were essentially not affected. There was no statistically significant change with the clearance of caffeine, which was marginally increased, and the elimination half-life, which was moderately increased. This led to the deduction that although *in vitro* findings suggested that the ethanol and AEHS are weak inhibitors of CYP1A2, actual *in vivo* study in human revealed that HSB had no obvious inhibitory activity on CYP1A2 using caffeine as a probe substrate.

Other pharmacokinetic parameters of caffeine such as the absorption rate constant, elimination rate constant, time to reach maximum plasma concentration, maximum plasma concentration, and apparent clearance in healthy human volunteers that received single-dose caffeine with water were comparable with the values obtained when co-administered with HSB. Nevertheless, going by the point estimates for the primary pharmacokinetic endpoints, C_{max} and $AUC_{0-\infty}$, co-administration of caffeine with HSB compared with caffeine taken with water were not bioequivalent, showing that the overall exposure of the drug was affected and suggests the presence of caffeine-herb interaction. The increase in C_{max} of caffeine following concomitant administration of HSB may lead to increased toxicity and adverse drug reactions especially if the beverage is co-administered with drugs with narrow therapeutic index.

In a recent study using simvastatin single dose in six healthy male volunteers, the point estimates of C_{max} and $AUC_{0-\infty}$ were also not bioequivalent following co-administration of HSB with simvastatin, a CYP3A4 substrate [33]. This is similar to our current findings; caffeine and simvastatin are substrates of different CYP isozymes. The non-bioequivalence of C_{max} and $AUC_{0-\infty}$ following co-administration of these drugs with HSB may suggest a similar mechanism of herb-drug interaction.

The result of the pharmacokinetic study should, however, be interpreted in the light of inter- and intra-individual variabilities that exist in the pharmacokinetics of caffeine and the small sample size of the study that may limit the generalizability of the findings.

Conclusion

AEHS weakly inhibited eight selected cytochrome P450 isozymes *in vitro* while the beverage of *H. sabdariffa* slightly increased the overall exposure of caffeine in healthy human volunteers. Further clinical studies with larger sample size are required to substantiate these *in vivo* findings. Nevertheless, care should be exercised by healthcare practitioners, caregivers, and patients alike in co-administering HSB with caffeine and similar substrates of CYP1A2 until more clinical data are available for informed decision.

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