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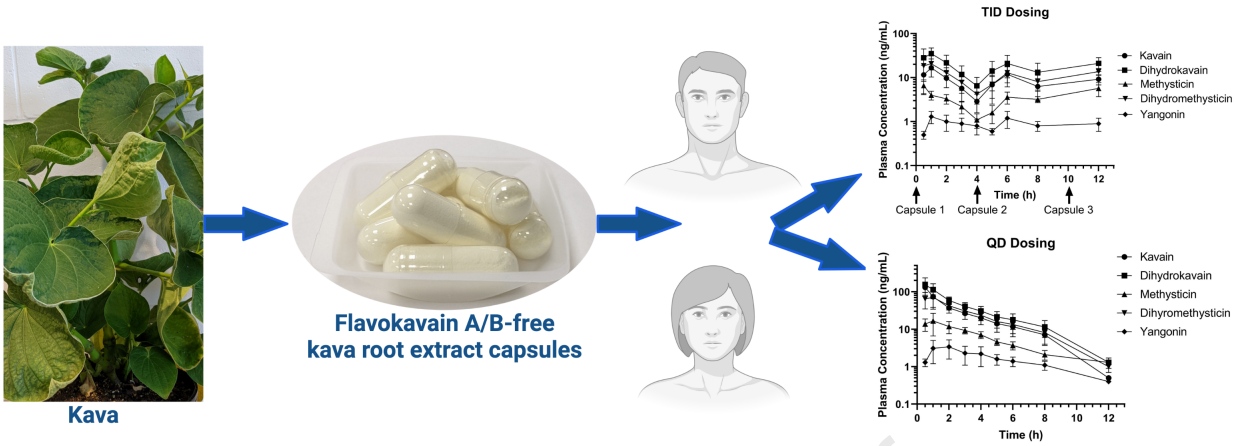
Siva Rama Raju Kanumuri: Methodology, Investigation, Data curation, Writing – original draft, preparation. **Jessica Mamallapalli:** Methodology, Investigation, Writing – review & editing. **Robyn Nelson:** Investigation, Writing – review & editing. **Christopher R McCurdy:** Conceptualization, Supervision, Writing – review & editing. **Carol A. Mathews:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition. **Chengguo Xing:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition. **Abhisheak Sharma:** Methodology, Investigation, Supervision, Data curation, Writing – original draft, preparation.



Clinical Pharmacokinetics of Kavalactones after Oral Dosing of Standardized Kava Extract in Healthy Volunteers

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Abstract

Ethnopharmacological Relevance: Piper methysticum G. Forst. (Piperaceae), commonly known as kava, has been used as a traditional beverage for centuries for its relaxing properties. Kavalactones are considered to be the major constituents responsible for kava's beneficial effects. Despite the extensive use of kava, clinical pharmacokinetic data is not available in the literature; therefore, the findings of this study will be critical for the dosage calculations for future clinical evaluation of kava.

Aim of the study: The aim of the current study is to examine the clinical pharmacokinetics of six major kavalactones following dosage of flavokavain A/B-free standardized kava extract capsules in healthy volunteers using two dosage regimens.

Materials and Methods: A sensitive, reliable, and specific ultra-high pressure liquid chromatography-mass spectrometry (UPLC-MS/MS) method was developed and validated for the simultaneous quantification of six major kavalactones (kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin) and two flavokavains (A and B) in human plasma. Pharmacokinetic profiles were assessed in ten healthy volunteers after oral doses of standardized kava product, and plasma samples were analyzed for six kavalactones and two flavokavains using the validated UPLC-MS/MS method. Concentration-time data was subjected to pharmacokinetic analysis.

Results: The systemic exposure of the kavalactones was found to be in the following order: dihydrokavain > dihydromethysticin > kavain > methysticin > yangonin. Desmethoxyyangonin was observed only at a couple of time points, while flavokavain A and flavokavain B were not present in any of the plasma samples. Fast absorption of five kavalactones was observed with time to reach the maximum plasma concentration of 1-3 h. A dose proportionality in pharmacokinetics was observed from 75-225 mg of kavalactone doses. In the multiple-dose study, a significant reduction in the extent of absorption of kavalactones with food was observed.

Conclusion: Single and multiple-dose clinical pharmacokinetic studies for kava were performed in healthy volunteers, and higher exposure to the kavalactones was observed after single-dosing (225 mg), while a longer duration of exposure was observed after three times a day (3 x 75 mg) dosing.

Keywords

Kava, kavalactones, flavokavain, UPLC-MS/MS, clinical pharmacokinetics

1. Introduction

Kava is a perennial shrub belonging to the pepper family and scientifically known as *Piper methysticum* G. Forst. (Piperaceae) which is native to the South Pacific islands. Kava has been used as a celebratory and recreational beverage for centuries by indigenous peoples of the pacific islands for its relaxing properties (FAO, 2016). Traditionally the fresh roots and rhizomes of the plant were chewed or ground to powder and used for preparing water-based suspensions for consumption (Swift et al., 2020). Kava has been reported to possess several pharmacologic effects, including anxiolytic (Kinzler et al., 1991), sedative (Gleitz et al., 1996), spasmolytic (Singh, 1997), antithrombotic (Gleitz et al., 1997), stress relieving(Kinzler et al., 1991), muscle relaxant (Singh, 1983), local anesthetic (Singh, 1992; Singh, 1997), neuroprotective (Gleitz et al., 1996), hypnotic (Wheatley, 2001), anti-inflammatory (Pollastri et al., 2009), memory enhancement (Munte et al., 1993), anticonvulsant (Gleitz et al., 1996), analgesic (Jamieson and Duffield, 1990) and anti-cancer (Einbond et al., 2017; Johnson et al., 2011; Johnson et al., 2008; Leitzman et al., 2014; Li et al., 2012; Narayanapillai et al., 2014a; Tang et al., 2016; Triolet et al., 2012) activities. Several preclinical studies on the therapeutic effects of kava indicate that most of the neurobiological effects are imparted by the kavalactones, which include kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin (Figure 1). These kavalactones are present primarily in the root and rhizomes of the plant and consist of around 4-8% of the dried products (Singh, 1992). Kavalactones may act as anxiolytics through the mechanisms of blockade of voltage-gated sodium ion channels and calcium ion channels, enhanced ligand binding to gamma-aminobutyric acid (GABA) type A receptors, reversible inhibition of monoamine oxidase B, and reduced neuronal reuptake of serotonin and dopamine (Davies et al.,

1992; Gleitz et al., 1996; Jussofie et al., 1994). There are also chalcone-based minor ingredients in kava, known as flavokavain A and flavokavain B, which are reported to be associated with the hepatotoxic effects of certain kava preparations (Li et al., 2011; Narayanapillai et al., 2014b; Olsen et al., 2011; Zhou et al., 2010). All of these kavalactones and flavokavains are present in varying proportions in different cultivars of kava. The kava preparations primarily used by the western world (United States, Canada, Australia, and Europe) are processed products containing either aqueous or organic (acetone or ethanolic) extracts in the form of tablets or capsules to promote relaxation. These extracts have concentrated kavalactone content of between 30-70% (FAO, 2016). The ethanolic extracts contain even higher amounts of kavalactones and flavokavains, potentially leading to some adverse effects when consumed at higher doses (FAO, 2016; Kuchta et al., 2015). Some kava preparations may induce hepatotoxicity in rare cases (thought to be caused by the presence of flavokavains A and B), leading the Food and Drug Administration (FDA) to issue a public health advisory and suspended clinical studies of kava in 2002 following a few cases of liver toxicity with a few resulting in death, reported in the US, Germany, and Switzerland (Centers for Disease Control and Prevention, 2002). A subsequent safety evaluation by the World Health Organization (WHO) published in 2007 reported that kava-associated hepatotoxicity was rare at lower doses (120-240 mg/day), particularly with proper preparation and reduction or elimination of the non-kavalactone content, following which most of the restrictions for clinical studies were lifted in several parts of the world (WHO, 2007).

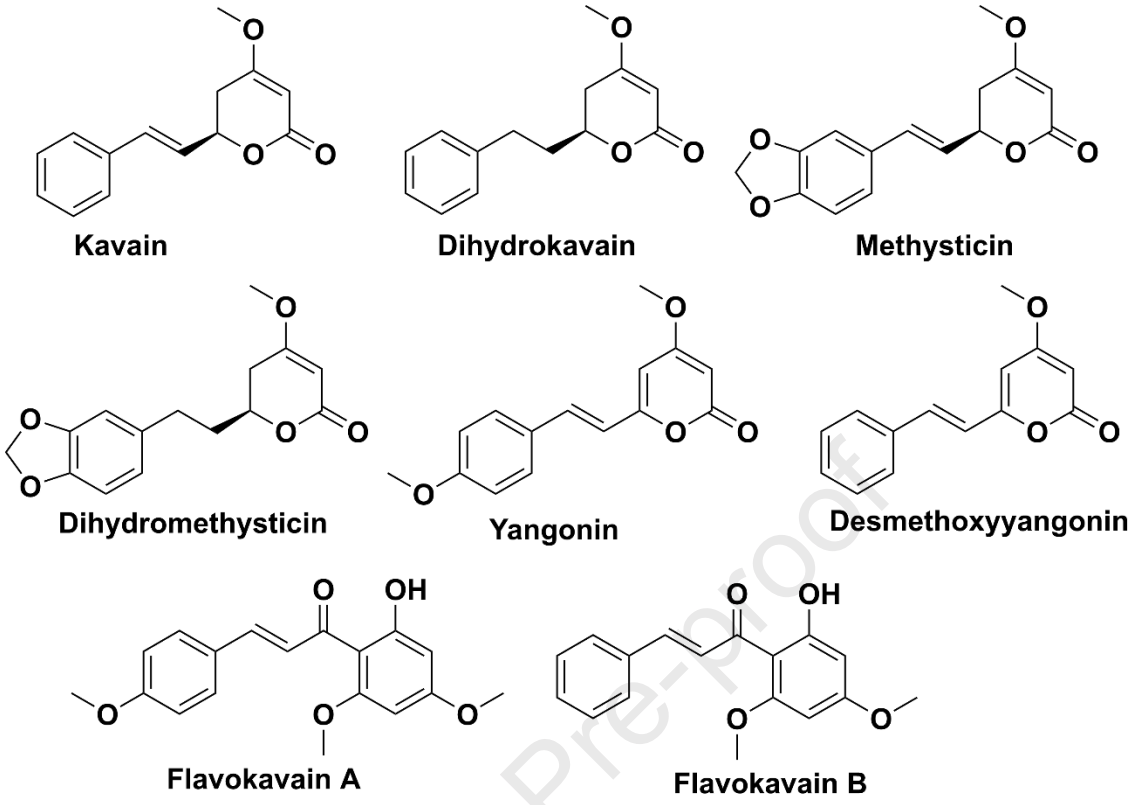

Fig. 1. Chemical structures of kavalactones and flavokavains.

Some of the clinical trials conducted from 2001 to 2020 in the US, Germany, and Australia to evaluate the pharmacodynamic effects of kava products in placebo-controlled studies suggested that kava is more effective than placebo for mild to moderate anxiety. The clinical studies in Germany were conducted using acetonic extracts containing 70% kavalactones, while the studies in Australia were conducted using aqueous extracts (Connor and Davidson, 2002; Gastpar and Klimm, 2003; Geier and Konstantinowicz, 2004; Lehrl, 2004; Malsch and Kieser, 2001; Sarris et al., 2020; Sarris et al., 2009a; Sarris et al., 2009b; Sarris et al., 2013). The most recent multicentered clinical trial in Australia was inconclusive in terms of effectiveness compared to placebo treatment (Sarris et al., 2020). These clinical studies were performed using dose ranges from 120 to 280 mg

kavalactones per day for multiple days or weeks without any significant adverse effects other than those that were also observed in the placebo arm at similar rates (Connor and Davidson, 2002; Gastpar and Klimm, 2003; Geier and Konstantinowicz, 2004; Lehrl, 2004; Malsch and Kieser, 2001; Sarris et al., 2020; Sarris et al., 2009a; Sarris et al., 2009b; Sarris et al., 2013). While the mechanisms behind kava's anxiolytic properties are not well-established, its clinical use showed no signs of addiction or withdrawal (Connor and Davidson, 2002; Gastpar and Klimm, 2003; Geier and Konstantinowicz, 2004; Lehrl, 2004; Malsch and Kieser, 2001; Sarris et al., 2020; Sarris et al., 2009a; Sarris et al., 2009b; Sarris et al., 2013).

Despite these clinical trials and kava's common use as a dietary supplement in the US market, human pharmacokinetic information is lacking; such information is important for its future effective usage either as a dietary supplement or as an herbal therapeutic. This clinical study was designed and performed to collect pharmacokinetic data on six major kavalactones after oral dosing of flavokavain A/B-free standardized kava extract in healthy subjects, and to determine whether flavokavains A and B were successfully removed, with the rationale to determine an optimal dosing regimen to achieve the desired pharmacodynamic effects.

2. Material and Methods

2.1. Chemicals and reagents

Standardized flavokavain A/B-free kava extract capsules (75 mg total kavalactones/capsule) with the same lot number were obtained from Thorne Research Inc (USA). US FDA Investigation of new drug (IND) (FDA IND Number 142838) and UF-Institutional Review Board (IRB number 201900074) approval were obtained for all

aspects of this study. Reference standards for kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin, flavokavain A, and flavokavain B were isolated and purified in-house (Mamallapalli et al., 2021). The deuterated internal standards kavain-d3, dihydrokavain-d3, methysticin-d3, dihydromethysticin-d2, desmethoxyyangonin-d3, and flavokavain B-d6 were synthesized following reported procedures with the corresponding deuterium-labeled starting materials (Mamallapalli et al., 2021). LC-MS grade methanol, acetonitrile, isopropanol, and formic acid were procured from Fisher Scientific. HPLC grade sodium lauryl sulfate, acetic acid, and ammonium acetate were procured from Sigma Aldrich. Triple distilled water was obtained using the MilliQ water purification system.

2.2. UPLC-MS/MS instrumentation and analytical conditions

Quantitative analysis of the six kavalactones and two flavokavains was carried out using a Waters Acquity Class I Plus UPLC coupled with a Waters Xevo TQ-S Micro triple quadrupole mass spectrometer (MS/MS) (Waters Corp, USA). The analytical method reported by Mamallapalli et al., 2021 (Mamallapalli et al., 2021) was modified and validated for bioanalytical purposes as per US FDA bioanalytical method validation guidance (Food and Drug Administration, 2018). The chromatographic separation was carried out on an Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm) using the mobile phase consisting of 0.1 % formic acid (A) – acetonitrile (B) with a gradient program consisting of 80 % A held for the first 1 min, then decreased to 50 % in 2 min followed by decreasing further to 45 % until 3 min and held there until 4.7 min, then sharply increased back to the initial conditions by 4.8 min and maintained until 5.5 min. The mobile phase was delivered at a flow rate of 0.35 mL/min. The mass spectrometer was operated in

positive ionization and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring transitions of kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin, flavokavain A, flavokavain B, and internal standards as shown in Table 1. The column oven temperature was kept at 40 °C, and the autosampler temperature was at 4 °C. The injection volume was 4 µL for each sample analysis. MassLnyx software version 4.2 was used for instrument control and TargetLynx for data analysis. Each compound was monitored using two precursor-to-daughter ion transition (*m/z*) pairs, one as a quantifier and another as a qualifier to achieve better selectivity for each compound. Ion spray voltage was set at 1000 V, desolvation temperature was 400 °C, desolvation gas flow was 1000 L/h, and the cone gas flow was 40 L/h.

Table 1 Ion Transitions Monitored for Analytes and Internal Standards

Compound	**Type**	**Parent (*m/z*)**	**Daughter (*m/z*)**	**Cone (V)**	**Collision (V)**
Kavain	Quantifier	231.1	114.9	24	12
Kavain	Qualifier	231.1	185.1	24	10
Dihydrokavain	Quantifier	233.1	91.0	22	24
Dihydrokavain	Qualifier	233.1	116.9	22	20
Methysticin	Quantifier	275.1	102.9	14	36
Methysticin	Qualifier	275.1	159.0	14	10
Dihydromethysticin	Quantifier	277.1	130.9	18	26
Dihydromethysticin	Qualifier	277.1	160.9	18	10
Yangonin	Quantifier	259.0	160.9	32	22
Yangonin	Qualifier	259.0	231.0	32	14
Desmethoxyyangonin	Quantifier	229.1	141.0	54	24
Desmethoxyyangonin	Qualifier	229.1	130.9	54	20
Flavokavain A	Quantifier	315.0	181.0	8	20
Flavokavain B	Quantifier	284.9	180.9	28	18
Kavain-d3	IS	234.1	114.9	26	12
Dihydrokavain-d3	IS	236.1	91.0	24	26
Dihydromethysticin-d2	IS	279.1	136.9	26	20
Methysticin-d3	IS	278.1	159.0	12	12
Desmethoxyyangonin-d3	IS	232.1	102.9	50	34
Flavokavain B-d6	IS	291.1	187.0	18	20

2.3. Preparation of calibration and quality control standards

Primary stock solutions of the kavalactones and flavokavains were prepared in acetonitrile (1 mg/mL) separately and stored at -20 °C. The mixed working standard solutions were prepared by combining the aliquot of each primary stock solution and diluting with acetonitrile to obtain the concentrations of 3.125, 6.250, 12.500, 25.00, 62.50, 125.00, 250.00, 625.00, 1125.00, and 1250.00 ng/mL. Another set of mixed working stock solutions of 3.125, 9.375, 500.00, and 1000.00 ng/mL were prepared similarly for separately weighted primary stocks for quality control standards. Primary stocks of internal standards (IS), kavain-d3, dihydrokavain-d3, methysticin-d3, dihydromethysticin-d2, desmethoxyyangonin-d3, and flavokavain B-d6, were prepared at a concentration of 1 mg/mL separately and then combined to get a mixed IS working stock at a concentration of 0.20 mg/mL each in acetonitrile. A quenching solution for protein precipitation was prepared by adding IS mixture to acetonitrile at a concentration of 10 ng/mL. All the stock solutions were stored at -20 °C until use. Calibration standards of kavalactones and flavokavains (0.25, 0.50, 1, 2, 5, 10, 20, 50, 90, and 100 ng/mL) were prepared by spiking 23 µL of pooled drug-free human plasma with the appropriate working standard solutions (2 µL). Quality control (QC) samples were prepared by individually spiking pooled blank human plasma at four concentration levels 0.25, 0.75, 40, and 80 ng/mL as lower limit of quantification (LLOQ), QC low (LQC), QC medium (MQC), and QC high (HQC), respectively.

2.4. Sample preparation

A simple and high throughput protein precipitation method was implemented for the extraction of analytes and removal of endogenous substances from human plasma. Plasma samples (25 µL) were aliquoted in microfuge tubes and 75 µL of acetonitrile

containing IS was added. Samples were vortex mixed for 2 min and filtered through a Solvinert (0.45 µm) 96 well filter plate (Millipore, Burlington, MA, USA) by centrifugation at 2000 rpm for 10 min at 4 °C. The filtrate was injected to UPLC-MS/MS for quantitative analysis.

2.5. Method validation

The bioanalytical method for the simultaneous quantification of kavalactones and flavokavains in human plasma was validated according to the United States Food and Drug Administration (US-FDA) Bioanalytical Method Validation guidance (US Food and Drug Administration, 2018). The method was validated for different parameters: specificity, selectivity, sensitivity, calibration curve, precision and accuracy, extraction efficiency, matrix effect, dilution integrity, and stability.

2.5.1. Specificity and selectivity

The interferences from the endogenous components of plasma and mobile phase at the peaks of the analytes and internal standards were assessed by comparing the chromatograms of blank plasma samples of ten different individuals with the spiked sample at LLOQ (0.25 ng/mL, each) using the optimized extraction method and chromatographic condition.

2.5.2. Sensitivity and calibration curve

Calibration curves for each analyte were constructed by plotting the peak area ratios of the analyte to that of the IS against the nominal concentrations. For each analyte, the corresponding deuterated compound was used as IS except for yangonin and flavokavain A, where desmethoxyyangonin-d3 and flavokavain B-d6 respectively were used as IS. The final concentrations used for plotting the calibration curve were 0.25, 0.50, 1, 2, 5,

10, 20, 50, 90 and 100 ng/mL. The linearity was fitted using linear regression with $1/X^2$ as a weighing factor. The acceptance criterion for each back-calculated standard concentration was a 15 % deviation from the nominal value except at LLOQ, which was set at 20 %. The LLOQ was considered the lowest concentration of the calibration curve which gave at least fifteen times the response (signal to noise ratio $\geq$ 15) compared to noise. The limit of detection (LOD) was calculated using the signal-to-noise ratio of greater than 3.

2.5.3. Precision and accuracy

The assay was validated for its intra-day accuracy and precision by analyzing five replicates of each sample at four different concentrations LLOQ (0.25 ng/mL), LQC (0.75 ng/mL), MQC (40 ng/mL), and HQC (80 ng/mL). The inter-day accuracy and precision of the method were determined by running four QC's in five replicates for 3 different days (n=15). The accuracy was calculated as percent of the nominal concentration while precision was calculated as the percent relative standard deviation (% RSD). The accuracy and precision should be within 15% except at LLOQ where it can be up to 20%.

2.5.4. Extraction efficiency, matrix effect, and dilution integrity

The extraction efficiency of each analyte through the protein precipitation using acetonitrile was determined at LLOQ (0.25 ng/mL), LQC (0.75 ng/mL), MQC (40 ng/mL), and HQC (80 ng/mL) by comparing the peak areas of analytes in the pre-extraction spiked sample with post-extraction spiked samples. The matrix effect was calculated by comparing the areas of the post-extraction spiked samples with that of the neat samples (no matrix) at respective concentrations. The matrix effect on the internal standards was

determined at a concentration of 10 ng/mL. The dilution integrity was tested at 10-folds of HQC concentration (800 ng/mL) by diluting with blank human plasma.

2.5.5. Stability experiments

A battery of stability studies was conducted to evaluate the autosampler, bench-top, freeze-thaw, and long-term stability of kavalactones in human plasma at two QC levels (LQC and HQC) in five replicates each. The benchtop stability was assessed by keeping plasma samples on the bench for 2 h. The autosampler stability was assessed at 4 °C for 24 h in the autosampler. The freeze-thaw stability was assessed for 3 cycles of freezing and completely thawing to room temperature (FT-3) while long-term stability was assessed by analyzing the QC samples stored at -80 ± 10 °C for 6 weeks. Stability samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ± 15% deviation) and precision (i.e., 15 % RSD) as compared to freshly prepared QC samples. The stock solution stability was accessed for six months at -20 °C.

2.6. Capsule content and dissolution testing

Kavalactones and flavokavains content of the standardized kava extract capsules (N = 10) was determined as described by Mamallapalli et al. (Mamallapalli et al., 2021) using UPLC-MS/MS method. Each capsule was emptied in a separate 100 mL volumetric flask and 50 mL of acetonitrile was added and vortex mixed for 5 min at room temperature. The volume was made up to 100 mL and mixed upside down for 1 min. A small aliquot of extraction solvent was taken and filtered through a 0.22 µm syringe filter. The extraction solvent was diluted with acetonitrile-water (1:1 v/v) to get the concentration within the linearity range. The samples were analyzed after adding 3 volumes of acetonitrile with internal standard to one volume of the sample using UPLC-MS/MS.

In vitro dissolution testing of kava capsules was performed as per the United States Pharmacopeia (USP) guidelines for disintegration and dissolution of dietary supplements (32, 2011). The testing was performed at pH 4.5 (acetate buffer, USP) with the addition of 0.2% sodium lauryl sulfate to increase the aqueous solubility of the kavalactones. Dissolution apparatus with Paddle (USP II, Distek 2100A apparatus) was used with sinkers for the capsule. Each capsule was loaded to a sinker and added per vessel in 900 mL of buffer at 37 ^{o}C (± 0.5 ^{o}C) and the rotation speed was 75 rpm. The dissolution was assessed in six replicates. Samples were collected at 0, 10, 20, 30, 45, and 60 min for analysis of kavalactones and flavokavains concentrations and analyzed using a validated UPLC-MS/MS method.

2.7. Clinical pharmacokinetic study

The clinical pharmacokinetics study was approved by the University of Florida Institutional Review Board (IRB201900074). A total of ten healthy volunteers were enrolled in the pharmacokinetic study. The inclusion criteria for the participants were: healthy adults ages 18-50 years, non-smokers, not pregnant or with plans to become pregnant in the next 12 weeks, and not experiencing liver disease or other chronic health conditions. Liver function tests were assessed at the time of screening visit and were required to be within the clinical laboratory derived standard ranges. Liver function tests were also performed during the 12 week follow up visit. There were no restrictions on ethnicity, social background, or gender. Volunteers (5 men and 5 women) enrolled in this study had a median age of 21 years (range, 18-40 years), a median weight of 70.4 kg (range, 49-107.8 kg), and median body mass index (BMI) of 23.1 kg/m^2 (range, 16.4-37.1 kg/m^2). Among the ten volunteers, seven were white, one was African American and two were Asian. With respect to CYP 2D6 phenotyping, one was a poor metabolizer, four were

intermediate metabolizers and five were normal metabolizers. Each participant stayed in the University of Florida (UF) outpatient Clinical Research Center (CRC) during the 12 hours of the study period. Participants were asked to refrain from food, liquids other than water, and medications for 12 h before entering the CRC of the UF. An indwelling venous catheter was placed in the lower arm to facilitate multiple blood draws during the study. The participants were randomized into 2 groups of five participants each. One group received flavokavain A/B-free standardized kava extract capsules equivalent to 225 mg kavalactones according to the label (3 capsules, each 75 mg total kavalactones) orally as a single dose (QD- once-daily dosing). Another group was administered one kava capsule (75 mg, total kavalactones) three times a day (TID; morning, noon, and evening) with a total dose of 225 mg kavalactones/day. The blood samples were collected in 2 mL EDTA vacutainers at pre-dose, and 0.5, 1, 2, 3, 4, 5, 6, 8, and 12 h post-dose after the first dose of kava. The blood samples were centrifuged at 2000 rpm at 4 ^{o}C for 10 min and plasma was separated and stored at -80 ^{o}C till analysis. A standard diet was provided to all participants 5 hr post-dose for a single dose (group 1) and along with the second capsule dosing for TID dosing (group 2).

2.8. Pharmacokinetic data analysis

The plasma concentration - time data of two oral dosage regimen groups was subjected to non-compartmental analysis using Phoenix WinNonlin (version 8.3, Certara USA Inc., USA). Observed maximum plasma concentration (C_{max}) and time to reach the maximum plasma concentration (T_{max}) were obtained by visual inspection of the experimental data. Systemic exposure measured as the area under the curve ($AUC_{0\text{-}4\ h}$, $AUC_{4\text{-}8\ h}$, and $AUC_{0\text{-}last}$) was calculated using the linear trapezoidal rule. Data were expressed as the mean ±

SEM (standard error of the mean). Pharmacokinetic parameters were subjected to the student's t-test. A P-value <0.05 was taken to indicate a significant difference.

3. Results and Discussion

3.1. UPLC-MS/MS method development and validation

Chromatographic conditions, source, and compound-related parameters were adopted from the analytical UPLC-MS/MS method previously published by our group (Mamallapalli et al., 2021) with modifications for the bioanalysis of the kavalactones and flavokavains in the human plasma and validated as per the US FDA bioanalytical guidance (US Food and Drug Administration, 2018).

Sample preparation techniques were optimized to develop a simple, selective, sensitive, precise, and high throughput assay method for the quantitation of six kavalactones and two flavokavains. A protein precipitation method was preferred as the sample preparation method as it is cost-effective and efficient. Different solvents (e.g., acetonitrile and methanol) with and without modifiers were tried as the quenching solvents. Acetonitrile was selected as the extraction solvent as it gave a better, more reproducible, and concentration-independent recovery, along with a clean and interference-free extract with good peak shapes and sensitivity (0.25 ng/mL) for all the analytes and IS.

As shown in Figure 2, the absence of interfering peaks in the blank sample at the retention times for kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin, flavokavain A, flavokavain B, and deuterated internal standards indicated that the method is selective and specific for these analytes.

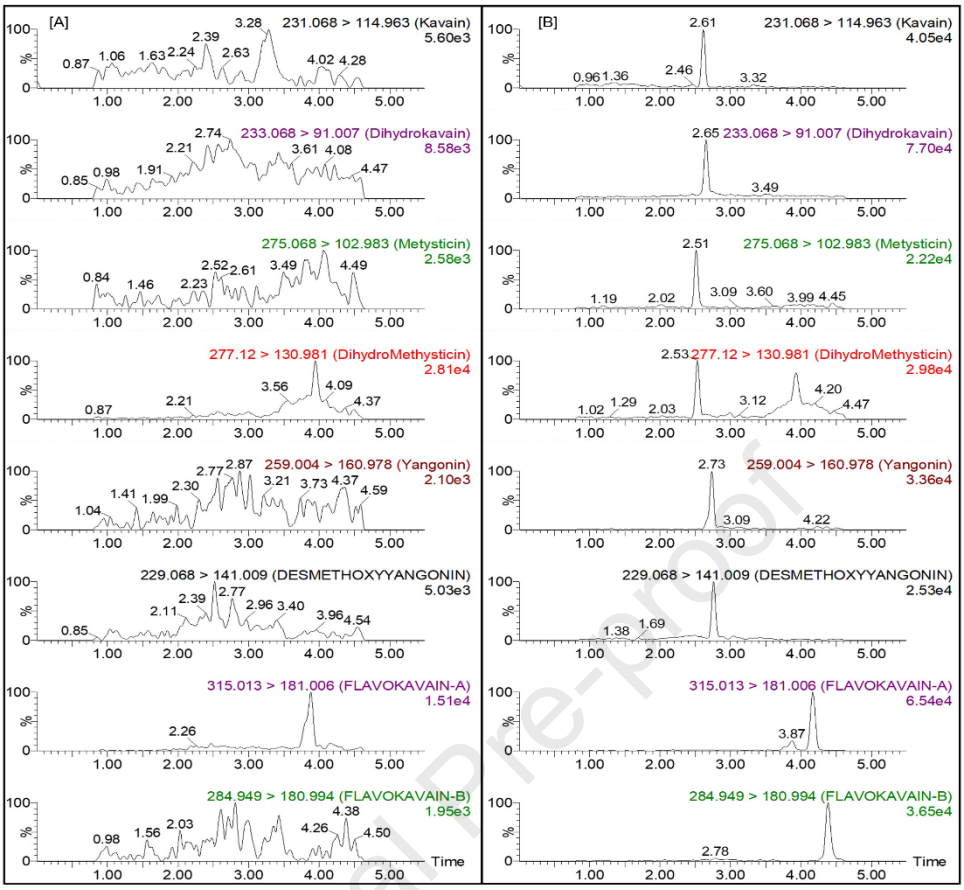

Fig. 2. UPLC-MS/MS chromatograms for kavalactones and flavokavain in A) blank plasma, B) plasma spiked with kavalactones and flavokavains at LLOQ (0.25 ng/mL).

A total of ten calibration points ranging from 0.25-100 ng/mL were utilized to determine the linearity by plotting the area ratios of analytes to IS against nominal concentrations. The method was found to be linear over the concentration range of 0.25-100 ng/mL for all the eight analytes with the correlation coefficients of greater than 0.99 using the linear regression with a $1/x^2$ weighing factor. The lower limit of quantification was found to be 0.25 ng/mL while the limit of detection was found to be 0.05 ng/mL for all analytes. The extraction recovery was found to be greater than 90% for all the analytes at LLOQ, LQC, MQC, and HQC, while the matrix effect was found to be in the range of 95.7 to 108.8%

for all the analytes. Dilution integrity was tested for 10-fold dilution to a final concentration of 80 ng/mL, in five replicates. The accuracy was within the 15% deviation and precision below 15% which are within the FDA's acceptable limits.

The intra- and inter-day accuracy of all the analytes were found to be between 95-110 % while the intra- and inter-day precision calculated as % RSD was less than 10% (Table 2). The precision and accuracy values on both occasions (intra- and inter-day) were found to be within the acceptable limits.

Table 2 Intra- and inter-day accuracy and precision of kavalactones and flavokavains of the bioanalytical method.

Analyte	Concentration (ng/mL)	Intra-day (n=15)			Inter-day (n=15)		
		Mean ± SD	Accuracy (%)	Precision (%RSD)	Mean ± SD	Accuracy (%)	Precision (%RSD)
Kavain	0.25	0.25 ± 0.01	99.23	4.28	0.26 ± 0.01	103.79	5.72
	0.75	0.73 ± 0.04	97.19	5.14	0.76 ± 0.04	101.60	5.28
	40	39.01 ± 0.46	97.52	1.18	41.79 ± 0.55	104.47	1.31
	80	79.85 ± 3.05	99.82	3.82	82.8 ± 1.14	103.50	1.38
Dihydrokavain	0.25	0.24 ± 0.02	95.55	7.18	0.26 ± 0.02	104.29	7.78
	0.75	0.73 ± 0.04	97.60	5.27	0.76 ± 0.05	101.42	6.80
	40	41.68 ± 1.88	104.21	4.52	41.71 ± 0.45	104.28	1.09
	80	82.15 ± 3.36	102.69	4.09	83 ± 0.75	103.74	0.90
Methysticin	0.25	0.24 ± 0.02	96.75	8.21	0.26 ± 0.03	103.55	9.68
	0.75	0.73 ± 0.03	97.69	4.11	0.77 ± 0.04	102.04	5.08
	40	39.2 ± 0.56	98.00	1.43	40.97 ± 1.37	102.42	3.34
	80	82.86 ± 2.68	103.57	3.23	83.13 ± 1.63	103.92	1.96
Dihydromethysticin	0.25	0.25 ± 0.02	101.09	7.19	0.24 ± 0.02	95.79	7.91
	0.75	0.73 ± 0.04	97.33	4.99	0.75 ± 0.05	99.52	7.20
	40	39.31 ± 0.39	98.28	0.99	40.93 ± 1.18	102.32	2.89
	80	80.7 ± 2.85	100.87	3.53	81.83 ± 2.23	102.29	2.73
Yangonin	0.25	0.25 ± 0.02	99.65	6.63	0.25 ± 0.01	100.19	5.96
	0.75	0.76 ± 0.03	101.02	4.09	0.75 ± 0.04	100.09	5.00
	40	43.06 ± 1.22	107.66	2.83	42.06 ± 1.11	105.15	2.63
	80	84.7 ± 2.94	105.87	3.47	83.15 ± 2.54	103.93	3.05
Desmethoxyyangonin	0.25	0.25 ± 0.02	101.23	8.89	0.25 ± 0.02	99.55	7.45
	0.75	0.72 ± 0.04	96.62	5.11	0.74 ± 0.05	98.67	6.74

	40	43.15 ± 1.39	107.87	3.23	42.22 ± 1.25	105.56	2.97
	80	84.64 ± 2.55	105.80	3.02	83.65 ± 1.8	104.57	2.16
Flavokavain A	0.25	0.25 ± 0.02	99.89	6.81	0.26 ± 0.02	102.80	8.34
	0.75	0.75 ± 0.04	99.33	5.61	0.76 ± 0.04	101.42	5.57
	40	43.6 ± 0.58	109.01	1.32	42.02 ± 1.12	105.06	2.66
	80	84.42 ± 2.43	105.52	2.88	83.19 ± 2.49	103.99	2.99
Flavokavain B	0.25	0.25 ± 0.02	101.92	8.47	0.25 ± 0.02	101.47	7.40
	0.75	0.77 ± 0.03	102.98	3.36	0.77 ± 0.05	102.13	6.22
	40	42.45 ± 0.83	106.14	1.95	42.17 ± 1	105.43	2.38
	80	83.45 ± 1.46	104.31	1.75	83.69 ± 2.24	104.61	2.67

Stability studies were performed under different conditions that can be encountered during the bioanalysis of the samples and were performed at two QC levels, LQC (0.75 ng/mL) and HQC (80 ng/mL). Benchtop stability (BT, 2 h), autosampler stability (AS, 24 h at 4 °C), three freeze-thaw cycles (FT-3 at -80 ± 10 °C) and long-term stability (LTS, 6 weeks at -80 ± 10 °C) are as shown in Table 3. All the analytes were found to be stable with the accuracy (%) and precision (%RSD) ranging between 91.47–107.54 and 0.96–10.15, respectively indicating that the plasma samples and processed samples were stable enough under the conditions of storage, processing, and analysis. The stock solutions were found to be stable for up to 6 months with the accuracy of the QC samples prepared ranging that 92.47-103.29 %.

3.2. Capsule content and dissolution testing

For the quality control of the capsules used for the clinical study, the total amount of kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin, flavokavain A, and flavokavain B was determined using the UPLC-MS/MS method. The average content of kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin was found to be 44.2 ± 0.4, 21.5 ± 0.3, 8.1 ± 0.1, 8.2 ± 0.1, 10.5 ± 0.2 and 7.4 ± 0.1 % of total kavalactones, respectively. Flavokavain A and flavokavain B

Table 3 Stability of kavalactones and flavokavains under different storage and processing conditions.

Analyte	Conditions	Autosampler (4 ºC, 24 h)		Bench Top (RT, 2 h)		3 Freeze-Thaw Cycles (FT-3 at -80 °C)		Long term (6 weeks, -80 ºC)	
	Nominal Concentration (ng/mL)	0.75	80	0.75	80	0.75	80	0.75	80
Kavain	Mean ± SD	0.75 ± 0.03	77.82 ± 1.86	0.76 ± 0.03	83.22 ± 0.78	0.76 ± 0.02	77.04 ± 1.16	0.78 ± 0.03	76.78 ± 0.98
	Accuracy (%)	102.61	98.12	103.43	104.93	102.89	97.14	106.15	96.81
	Precision (%RSD)	3.93	2.39	4.26	0.94	2.21	1.51	4.25	1.28
Dihydrokavain	Mean ± SD	0.75 ± 0.03	79.54 ± 2.08	0.75 ± 0.01	83.99 ± 0.81	0.74 ± 0.03	79.06 ± 1.04	0.74 ± 0.02	79.21 ± 1.23
	Accuracy (%)	101.91	95.91	102.34	105.89	101.36	95.34	100.54	95.52
	Precision (%RSD)	3.71	2.62	1.46	0.96	4.10	1.32	3.37	1.55
Methysticin	Mean ± SD	0.77 ± 0.05	83.01 ± 0.65	0.75 ± 0.03	78.86 ± 0.85	0.72 ± 0.02	79.24 ± 1.18	0.76 ± 0.03	79.03 ± 1.2
	Accuracy (%)	97.72	99.98	95.44	94.98	91.65	95.45	95.95	95.19
	Precision (%RSD)	6.94	0.78	3.33	1.07	3.33	1.49	3.54	1.52
Dihydromethysticin	Mean ± SD	0.75 ± 0.03	82.39 ± 1.02	0.76 ± 0.01	77.05 ± 0.93	0.74 ± 0.03	77.6 ± 0.94	0.78 ± 0.05	77.08 ± 1.52
	Accuracy (%)	101.19	101.57	102.81	94.09	100.38	94.75	104.98	94.12
	Precision (%RSD)	3.71	1.23	1.61	1.20	4.20	1.21	6.79	1.97
Yangonin	Mean ± SD	0.74 ± 0.03	80.19 ± 0.69	0.74 ± 0.07	81.73 ± 1.38	0.75 ± 0.02	81.9 ± 0.81	0.73 ± 0.03	79.75 ± 1.17
	Accuracy (%)	96.85	98.26	96.59	100.14	97.90	100.36	96.06	97.72
	Precision (%RSD)	4.22	0.86	10.15	1.69	2.78	0.98	3.67	1.47
Desmethoxyyangonin	Mean ± SD	0.74 ± 0.02	80.7 ± 1.02	0.7 ± 0.04	81.55 ± 1.19	0.73 ± 0.06	80.63 ± 1.23	0.74 ± 0.03	85.2 ± 2.25
	Accuracy (%)	98.15	97.13	92.06	98.15	96.56	97.04	97.62	102.55
	Precision (%RSD)	3.22	1.26	5.24	1.46	7.99	1.53	4.22	2.64
Flavokavain A	Mean ± SD	0.74 ± 0.02	79.18 ± 1.69	0.74 ± 0.02	78.67 ± 1.89	0.75 ± 0.02	80.18 ± 1.37	0.74 ± 0.03	80.68 ± 1.74
	Accuracy (%)	96.74	95.05	97.03	94.44	97.48	96.25	96.41	96.85
	Precision (%RSD)	2.39	2.13	2.50	2.40	2.18	1.70	3.95	2.15
Flavokavain B	Mean ± SD	0.74 ± 0.04	78.94 ± 2.17	0.74 ± 0.02	78.93 ± 2.09	0.75 ± 0.02	78.46 ± 1.65	0.74 ± 0.02	78.53 ± 1.88
	Accuracy (%)	96.87	94.86	97.20	94.85	98.32	94.28	96.97	94.36
	Precision (%RSD)	4.88	2.75	2.37	2.65	2.12	2.10	2.44	2.39

were found to be less than 0.02% w/w of the capsule content indicating they are in negligible quantities. The dissolution testing of the capsules was performed to check the release profile of the kavalactones from the capsules. During the capsule dissolution studies performed at pH 4.5, percent of each kavalactone released in 60 min was greater than 85% for kavain, dihydrokavain, dihydromethysticin, and desmethoxyyangonin, while total release of methysticin and yangonin was 75% of total capsule content (Figure 3). Slow release of kavalactones indicates the solubility limited dissolution.

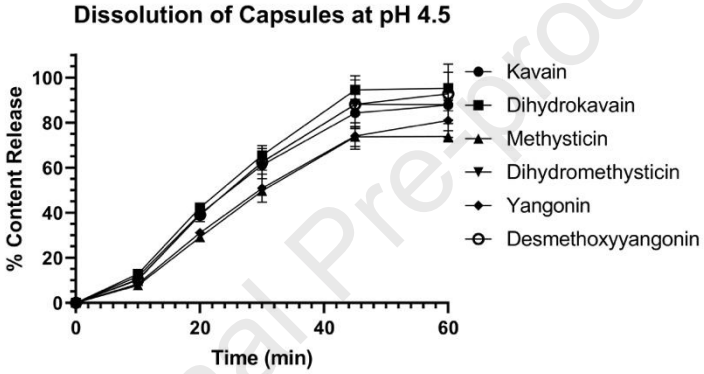

Fig. 3. Release profile of kavalactones from standardized kava extract capsule to dissolution media at pH 4.5.

3.3. Clinical Pharmacokinetics

To our knowledge, this study is the first clinical pharmacokinetic study to identify the exposure of kavalactones following an oral dose of flavokavain A/B free standardized kava extract given either as a single dose (once a day, QD) of 225 mg kavalactones or as three times a day (TID) of 75 mg kavalactones (total 225 mg/day). The only previously reported kavalactone plasma concentration data available in the literature to date was conducted by Wang et al (Wang et al., 2018), and they reported kavalactone plasma and urine concentrations at a single time-point in a single individual following the consumption of kava for 7 days.

Given the reports that associated flavokavain content to hepatotoxic effects of kava products (Narayanapillai et al., 2014b), we conducted this clinical study using a standardized kava extract that was devoid of flavokavains (A & B) and subsequently aimed to confirm, using standard approaches, that this was indeed the case. No major clinically relevant adverse events were observed in any of the volunteers, and no liver function abnormalities were noted in any volunteer over the 12 weeks of follow-up. We found that following oral dosing of kava capsules, only 5 of the 6 kavalactones (kavain, dihydrokavain, methysticin, dihydromethysticin, and yangonin) were consistently quantifiable in the plasma samples while desmethoxyyangonin was observed only at a couple of time points. As expected, flavokavain A and flavokavain B were not quantifiable in any of the plasma samples. Data fitting approaches using different compartmental models were unsuccessful; thus, the pharmacokinetic parameters of these kavalactones were estimated using non-compartmental analysis. Plasma concentration-time profiles of the kavalactones are as shown in Figure 4 and pharmacokinetic parameters are given in Table 4. Although kavain (~44%) was the major kavalactone present in the capsules, the maximum plasma concentration (C_{max}) achieved following single dosing for dihydrokavain (173.5 ± 173.1 ng/mL) was higher than that of kavain (112.0 ± 117.2 ng/mL), while the C_{max} of dihydromethysticin (92.9 ± 73.4 ng/mL) was comparable to kavain. The C_{max} of methysticin and yangonin were found to be 19.8 ± 20.5 and 3.9 ± 1.7 ng/mL, respectively. The order of the plasma concentrations of the kavalactones was similar to those reported by Wang et al. (Wang et al., 2018). All five kavalactones showed fast absorption with the time to reach maximum concentration (T_{max}) following single dosing ranging between 1-3 hr post-dose. Both dihydrokavain and dihydromethysticin

were absorbed faster than the others. Similar profiles were observed with TID dosing during the first 4 hours (prior to dosing the second capsule). The C_{max} of kavain, dihydrokavain, methysticin, dihydromethysticin, and yangonin were 26.0 ± 16.9, 55.1 ± 32.4, 6.7 ± 2.8, 33.2 ± 15.7, and 1.2 ± 0.5 ng/mL, respectively. The T_{max} for TID dosing was observed earlier than QD dosing indicating that lower doses have faster absorption compared to the higher doses. The C_{max} of kavalactones normalized to 75 mg kavalactone dose (per capsule) following administration of QD and TID capsule dosing are comparable except for kavain, which showed higher C_{max} with QD dosing than with TID dosing (Table 4), indicating that dose linearity in pharmacokinetics can be expected from 75 mg to 225 mg total kavalactones dosing. For TID dosing, the second dose of kava was given after food and the absorption of kavalactones was reduced significantly with food intake, resulting in a lesser second C_{max} compared to the first C_{max} (~half of C_{max1}) for all the kavalactones (Figure 4 and Table 5). The total systemic exposure of kavalactones measured as the area under the curve ($AUC_{0\text{-last}}$) was higher for QD dosing compared to TID (Table 4). This may be due to the decreased absorption from the second and third capsules, which were dosed with food following the initial dosing, which was done in a fasted state, and lack of elimination phase time points beyond 12 h following last capsule dosing at 10 h post first capsule dosing. Although the absorption of the kavalactones was reduced significantly with food, the $AUC_{0\text{-}4\ h}$ following first and second capsule doses were comparable. This may be due to the lasting exposure of kavalactones from the first capsule dose and continued absorption from the second capsule giving cumulative exposure. Significant variability in the exposure between the volunteers was observed. Covariate analysis was conducted to assess potential effects of race, gender, weight,

Table 4 Pharmacokinetic parameters of kavalactones following oral dosing of standardized kava extract capsules.

Parameter	Kavain		Dihydrokavain		Methysticin		Dihydromethysticin		Yangonin	
	QD	TID	QD	TID	QD	TID	QD	TID	QD	TID
C_{max} (ng/mL)	112.0 ± 117.2	26.0 ± 16.9	173.5 ± 173.1	55.1 ± 32.4	19.8 ± 20.5	6.7 ± 2.8	92.9 ± 73.4	33.2 ± 15.7	3.9 ± 1.7	1.2 ± 0.5
C_{max}/Capsule (ng/mL)	37.3 ± 39.1	26.0 ± 16.9	57.8 ± 57.7	55.1 ± 32.4	6.6 ± 6.8	6.7 ± 2.8	31.0 ± 24.57	33.2 ± 15.7	1.3 ± 0.6	1.2 ± 0.5
T_{max} (h)	1.9 ± 1.1	1.2 ± 0.8	1.3 ± 0.9	1.2 ± 0.8	1.9 ± 1.1	1.2 ± 0.8	1.5 ± 1	1.20 ± 0.8	1.9 ± 0.9	1.8 ± 0.8
$AUC_{0\text{-}last}$ (h*ng/mL)	212.9 ± 156.7	100.4 ± 84.2	333.4 ± 205.5	210.2 ± 203.3	58.3 ± 37.3	39.9 ± 13.6	221.6 ± 110.1	131.2 ± 79.1	15.6 ± 7.0	7.1 ± 3.2

QD- Once a day; TID- Three times a day

Table 5 Pharmacokinetic parameters of kavalactones following TID dosing of kava capsules

Compound	Parameter	First Dose	Second Dose
Kavain	C_{max} (ng/mL)	26.0 ± 17.0	11.9 ± 12.8
	$AUC_{0\text{-}4h}$ (h*ng/mL)	35.2 ± 22.5	32.5 ± 16.5
Dihydrokavain	C_{max} (ng/mL)	55.1 ± 32.4	21.0 ± 24.9
	$AUC_{0\text{-}4h}$ (h*ng/mL)	77.3 ± 56.0	62.3 ± 35.5
Methysticin	C_{max} (ng/mL)	7.2 ± 2.6	3.6 ± 2.4
	$AUC_{0\text{-}4h}$ (h*ng/mL)	11.4 ± 3.7	10.7 ± 2.4
Dihydromethysticin	C_{max} (ng/mL)	33.2 ± 15.7	12.9 ± 11.9
	$AUC_{0\text{-}4h}$ (h*ng/mL)	47.8 ± 22.2	36.7 ± 15.3
Yangonin	C_{max} (ug/L)	1.2 ± 0.5	1.2 ± 1.0
	$AUC_{0\text{-}4h}$ (h*ng/mL)	2.4 ± 1.1	3.0 ± 1.0

body mass index (BMI), and CYP2D6 phenotype on the pharmacokinetic parameters, but none of these accounted for the observed interindividual variability for each kavalactone. It is possible that low solubility and other variabilities associated with absorption account for the inter-individual variabilities, although these potential explanations cannot be assessed within the current study.

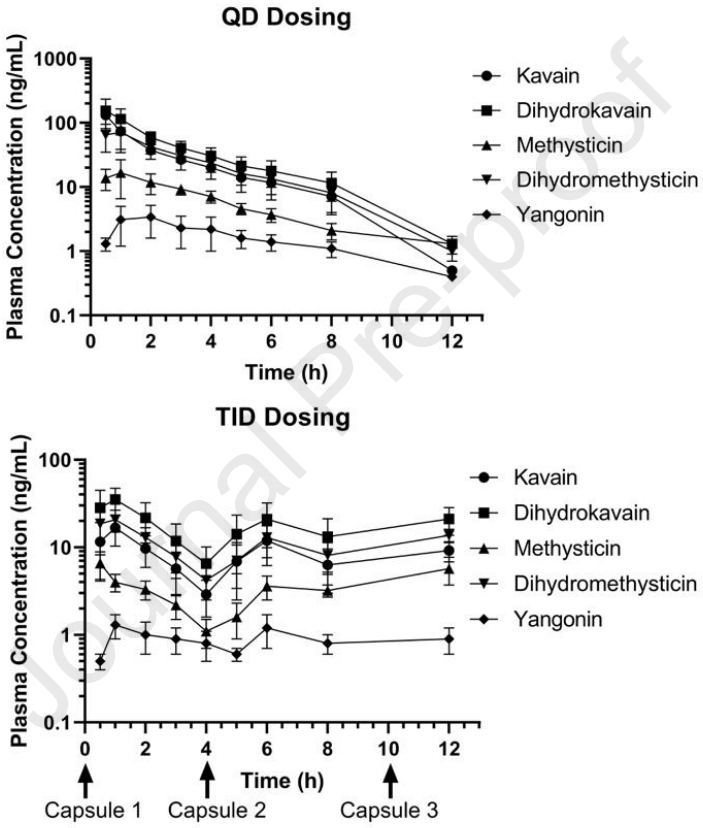

Fig. 4. Plasma concentration-time profiles of kavalactones after oral dosing as QD (upper) and TID (below) dosing regimens.

4. Conclusion

A sensitive and selective UPLC-MS/MS-based bioanalytical method for the simultaneous quantification of six major kavalactones along with two flavokavains in human plasma has

been developed and validated. The clinical pharmacokinetic study was performed in healthy volunteers to access the systemic exposure of kavalactones following ingestion of kava capsules as single and three times a day dosing. We found that overall higher exposure to the kavalactones as a group was obtained with a single-dose, while a longer duration of exposure was observed after three times a day dosing. Substantial inter-subject variability was observed, and we were unable to identify the source of this variability with the covariates available. Additional studies with larger sample sizes will thus be required to better understand the population pharmacokinetics of major kavalactones.

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CRediT authorship contribution statement

Siva Rama Raju Kanumuri: Methodology, Investigation, Data curation, Writing – original draft, preparation. **Jessica Mamallapalli:** Methodology, Investigation, Writing – review & editing. **Robyn Nelson:** Investigation, Writing – review & editing. **Christopher R McCurdy:** Conceptualization, Supervision, Writing – review & editing. **Carol A. Mathews:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding

acquisition. **Chengguo Xing:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition. **Abhisheak Sharma:** Methodology, Investigation, Supervision, Data curation, Writing – original draft, preparation.

Declaration of competing interest

The authors declare no competing financial interest.

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Highlights:

- A selective and sensitive UPLC-MS/MS method has been developed and validated for the simultaneous quantification of six major kavalactones and two flavokavains in human plasma over a range of 0.25-100 ng/mL.
- A clinical pharmacokinetic study of kavalactones was performed in healthy volunteers for the first time.
- Food intake altered the absorption of kavalactones and lowered their systemic exposure.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: