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Research paper

In vitro inhibition of carboxylesterase 1 by Kava (*Piper methysticum*) Kavalactones

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ABSTRACT

Kava refers to the extracts from the rhizome of the plant *Piper methysticum* which is of particular significance to various indigenous cultures in the South Pacific region. Kavalactones are the active constituents of kava products and are associated with sedative and anxiolytic effects. Kavalactones have been evaluated in vitro for their potential to alter the activity of various CYP450 enzymes but have undergone little systematic investigation as to their potential influence on esterases. This study investigated the inhibition effects of kava and its kavalactones on carboxylesterase 1 (CES1) in an in vitro system and established associated kinetic parameters. Kava and its kavalactones were found to produce reversible inhibition of CES1 to varying degrees. Kavain, dihydrokavain, and desmethoxyyangonin displayed competitive type inhibition, while methysticin, dihydromethysticin, and yangonin displayed a mixed competitive-noncompetitive type inhibition. The inhibition constants (K_i) values for each of the kavalactones were as follows: methysticin (35.2 μM), dihydromethysticin (68.2 μM), kavain (81.6 μM), dihydrokavain (105.3 μM), yangonin (24.9 μM), and desmethoxyyangonin (25.2 μM). With consideration to the in vitro K_i for each evaluated kavalactone as well as available clinical kavalactone concentrations in blood circulation, co-administration of CES1 substrate medications and kava products at the recommended daily dose is generally free of drug interaction concerns. However, uncertainty around kavalactone exposure in humans has been noted and a clinically relevant CES1 inhibition by kavain, dihydrokavain, and dihydromethysticin is indeed possible if the kavalactone consumption is higher than 1000 mg in the context of over-the-counter usage. Further clinical studies would be required to assess the possibility of clinically significant kava drug-drug interactions with CES1 substrate medications.

1. Introduction

According to the Center for Disease Control and Prevention (CDC), from 2017 to 2018 in the US over 40% of adults aged 20 and older were estimated to have used a dietary supplement [1]. Despite the popularity of dietary supplement usage in adults, the US Food and Drug Administration (FDA) does not require the same scrutiny and rigor of a full approval process that are required for conventional medications drugs, creating the possibility of unrecognized drug-drug interactions [2]. In the US, kava (*Piper methysticum*) is classified as a dietary supplement and is promoted to improve sleep and enhance relaxation. While it is difficult to quantify the popularity and prevalence of kava in the United States, based on a report from the American Botanical Council, in 2020 kava supplements were among the top-selling herbal supplements purchased

Abbreviations: α, indicator of type of inhibition; AB-free kava extract, flavokavain A and flavokavain B-free kava extract; b, shaping exponent; CES1, carboxylesterase 1; C_{max}, maximum plasma concentration; DDI, drug-drug interaction; DME, drug-metabolizing enzymes; HED, human equivalent dose; IC, half-maximal inhibitory concentration; IC_{50}, inhibitor concentration that achieves 50% of enzyme activity; I_{max}, maximal percentage of inhibition; K_i, inhibition constant; K_m, Michaelis-Menten constant; OST, oseltamivir phosphate; OC, oseltamivir carboxylate; PBS, Phosphate buffered saline; R_v, the ratio of metabolite formation with inhibitor to control without inhibitor; R_1, the ratio of intrinsic clearance values of CES1 substrate in the absence and presence of inhibitor; V_{max}, maximum velocity of a reaction.

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through natural, healthy food, specialty retail outlets, and kava bars, in the US [3].

Kava is primarily cultivated in the South Pacific islands where it has been in use for thousands of years as a medicine and for ritual purposes [4]. The major active constituents responsible for the pharmacological activity of kava are referred to as kavalactones, of which there are six major ones; kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin [4]. The proportion of kavalactones present in a given kava extract and the dosage of kavalactones received from consuming kava can vary based on a variety of factors including country of origin, types of cultivars, processing and extraction methods and pharmaceutical formulation [4]. Two randomized placebo-controlled clinical trials support the utility of kava in treating symptoms of anxiety over a period of three weeks or more [5,6]. These potential benefits could lead patients to use kava as an alternative treatment for anxiety symptoms and to potentially use kava concomitantly with conventional medications.

The liver is the primary site responsible for drug metabolism due to the presence of multiple drug-metabolizing enzymes (DMEs) in high abundance. These include the cytochrome P450s, UDP-glucuronosyltransferases, esterases and others. Among the hepatic DMEs, it has been established that in human liver microsomes and human liver S9 fractions, the esterase CES1 is by far the most predominant [7]. CES1 is responsible for the metabolism of a wide array of structurally dissimilar drugs (and prodrugs) with representatives from essentially every major drug class [8].

Pharmacokinetic DDIs are those based on the general principle that the plasma concentrations of a "victim" drug are altered by another administered "perpetrator" drug or substance leading to inhibition and/or induction of the metabolism (or drug transporter-mediated influences) of the victim drug. Unrecognized or mismanaged DDIs are a significant cause of therapeutic failure, preventable adverse events, and hospitalization [9,10]. Although there is a lack of clinical DDI studies investigating the influence of CES1 inhibition on CES1 substrate drugs, carriers of the G143 *CES1* loss of function genetic mutation provide strong evidence of altered drug metabolism and disposition as a consequence of compromised CES1 function. Indeed, the metabolism of CES1 substrate drugs including methylphenidate, clopidogrel and oseltamivir are significantly impaired in individuals carrying this *CES1* variant [11–14]. Accordingly, sufficient exposures to metabolic inhibitors of CES1 would be anticipated to produce similar effects. While CES1 inhibition by natural products has not been thoroughly evaluated clinically, there are numerous in vitro reports of CES1 inhibition by botanical extracts [15].

Previously published in vitro studies have assessed the potential for kava extracts and/or their individual kavalactones to inhibit various DMEs. Available reports suggest that kava and its kavalactones were able to inhibit the activity of major cytochrome P450 (P450) enzymes [16–18]. However, only recently a kava extract (200 μg/mL) was shown to inhibit CES1 activity by around 50%; however, the kinetics of the inhibition by kava and its individual constituents on CES1 was not evaluated [19]. The aim of the present investigation was to evaluate the influence of kava and its constituents more fully on CES1 via in vitro assay and to determine if inhibition was significant.

2. Materials and methods

Materials. Oseltamivir phosphate (OST) was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Oseltamivir carboxylate (OC) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Additional OST and ritalinic acid were purchased from Cayman Chemical (Ann Arbor, MI). PBS was purchased from Corning (Manassas, VA). Flavokavain A and flavokavain B-free (AB-free) kava extract was obtained by a previously described method [20]. Kavain, dihydrokavain, methysticin, dihydromethysticin, and desmethoxyyangonin were obtained using a previously described method, while yangonin was obtained using the same method but slightly modified (Fig. 1.) [21]. All other chemicals and reagents were of the highest analytical grade and were commercially available.

Preparation of CES1 Wildtype Cell S9 fractions. Human embryonic kidney cells (Flp-In-293; Invitrogen, Carlsbad, CA) expressing wild type CES1 were cultured using Dulbecco's modified Eagle's Medium with 10% FBS, 2 mM L-glutamine, 1% Pen-Strep, and 100 μg/mL hygromycin as previously described [11]. The cells were harvested when visual inspection determined they achieved around 80%–90% confluence and were suspended in PBS. The cells were then sonicated to disrupt the membrane and release the enzyme which was then centrifuged at 9000 g for 30 min at 4 °C. The supernatant containing the CES1 S9 fractions were then transferred to 1.5 mL Protein Lobind tubes (Eppendorf Tubes®) and stored in a −70 °C freezer. The total protein concentration was determined by a Pierce BCA protein assay kit.

CES1 Substrate Metabolism. The influenza neuraminidase inhibitor, oseltamivir (OST), served as the probe CES1 substrate in the conducted experiments. OST is a prodrug requiring CES1-mediated hydrolysis to form the active moiety, oseltamivir carboxylate (OC; Fig. S1 Supplementary Data). It has been employed in CES1 inhibition studies previously by our group and others [22,23]. Samples containing OST and S9 fractions with a final reaction volume of 100 μL in 2 mL microcentrifuge tubes were incubated at 37 °C to form OC. All OST concentrations and S9 fractions were prepared in 50 mM phosphate buffer. To minimize spontaneous hydrolysis of OST to OC, the 2 mL tubes were kept on ice during preparation of pre-mixture solutions, but the reaction was initiated by incubating the samples in a 37 °C water bath system. Our preliminary studies suggested that the reactions were linear over an *enzyme* range of 0–80 μg/mL and incubation time range of 10–20 min. The final CES1 S9 fraction concentration and incubation time were determined as 20 μg/mL and 15 min for subsequent inhibition studies. To terminate the reaction, samples were taken out of the incubator, put on ice and 400 μL acetonitrile with 50 nM of ritalinic acid as the internal standard was added. Each sample was then centrifuged at 16,100 g for 10 min at 4 °C and 50 μL of supernatant was further diluted with 150 μL of 50% water and 50% acetonitrile and 1% formic acid. The final solution of 200 μL was then transferred to glass vials with inserts for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Screening of kavalactones for inhibition of CES1. Kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin (all pre-dissolved in DMSO) were all screened initially at a concentration of 10 μg/mL to investigate their inhibitory potential of CES1. A final OST substrate concentration of 100 μM was utilized for the screen. A final volume of 100 μL with 1% DMSO was incubated for 15 min. The negative controls in these experiments contained 1% DMSO but no kavalactones. After the incubation period, all reactions were terminated with the addition of ritalinic acid in acetonitrile and underwent LC-MS/MS analysis.

Assessment of Time-Dependent Inhibition of CES1 by the Individual Kavalactones. This assessment was utilized for each individual kavalactone to evaluate for the presence of time-dependent inhibition of CES1. Each of the 6 kavalactones were preincubated at varying concentrations (0–50 μg/mL) for 30-min with CES1 S9 fractions. Individual kavalactone pre-dissolved in DMSO (0.5% in final concentration) was added to of the pre-incubation mixture containing CES1 S9 while another group that served as the no preincubation group was simultaneously preincubated with 0.5% DMSO in place of the individual kavalactones. After a 30-min pre-incubation, individual kavalactone pre-dissolved in DMSO (0.5% final concentration) was added to the no preincubation samples while 0.5% DMSO was added to the pre-incubation samples to achieve the same final DMSO concentration (1%) between both groups. 90 μL of the solution for each individual kavalactone concentration was mixed with 10 μL of OST for a final volume of 100 μL with final CES1 S9 fraction concentrations of 20 μg/mL and final OST concentrations of 200 μM for each of the kavalactones. This

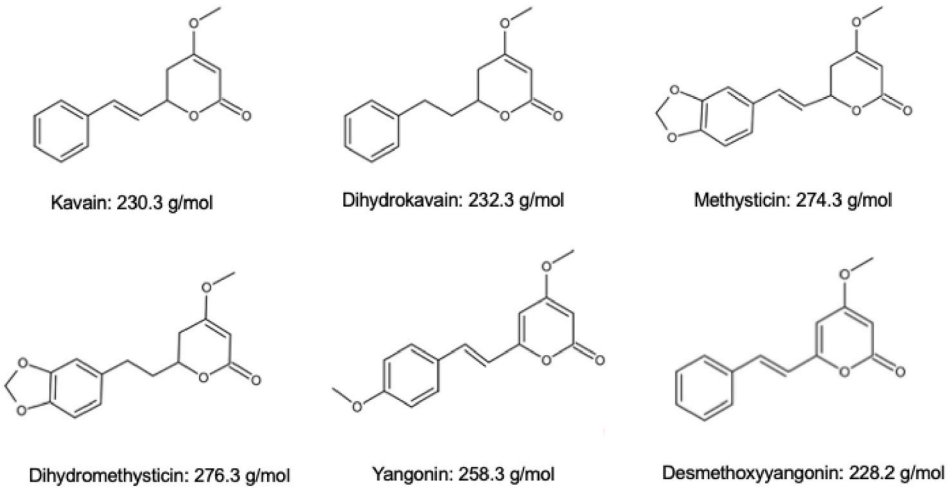

Fig. 1. Kavalactone chemical structures. Kavalactones and their respective molecular weights selected for analysis.

evaluation was performed similarly on the AB-free kava extract containing all 6 kavalactones. AB-free kava extracts have had flavokavain A and flavokavain B removed due to their being linked to kava associated hepatotoxicity [24]. The samples were then incubated for a period of 15 min at 37 °C. After this incubation period, all samples were prepared for LC-MS/MS evaluation as described below.

In Vitro Inhibition Study by Individual Kavalactones. These experiments permitted the determination of the inhibition constant (K_i) and type of inhibition. Substrate concentrations of 0, 200, 400, 800, 1600, 2500, and 5000 μM were used along with kavalactone concentrations of 0–48 μg/mL (0–208.46 μM). The total reaction volume was 100 μL with 1% DMSO. Each reaction was initiated with the final addition of substrate to all samples on ice that were then transferred to a water bath for a 15-min incubation period at 37 °C. Substrate was added to the samples on ice to minimize premature OC formation prior to the incubation period. After the incubation period, all samples were prepared for LC-MS/MS evaluation as described below.

LC-MS/MS Analysis. OST's active metabolite OC was determined using a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) coupled to an AB Sciex API 3000 Triple-Quadrupole Mass Spectrometer (Applied Biosystems, Foster City, CA). Chromatographic separation was achieved on a C18 reverse-phase analytic column (Aqua, 50 × 2.0 mm, 5 μm; Phenomenex Inc., Torrance, CA). A gradient mobile phase was used with 0.1% formic acid in water as the aqueous phase and methanol as the organic phase and was delivered at a flow rate of 0.25 mL/min as previously described [23]. A gradient method was used starting with 90% aqueous phase and 10% organic phase. At 2 min there was a switch to 10% aqueous phase and 90% organic phase, and at 6 min the ratio returned to 90% aqueous phase and 10% organic phase with a total run time of 11 min. The mass spectrometric analysis was performed via electrospray ionization in positive mode, and the mass transitions of the mass/charge ratios were 285.3 → 138.3 for OC and 220.2 → 84.4 for ritalinic acid (Fig. S2 Supplementary Data.).

Data Analysis. OST was prepared without CES1 S9 fractions at each selected concentration of OST for each assay due to its propensity for spontaneous hydrolysis to OC as reported previously [23]. This spontaneous hydrolysis of OST was accounted for and subtracted from the final quantity of OC formation from the samples containing S9 fractions of CES1 (Table S1 Supplementary Data). A one-way ANOVA with a Dunnett's multiple comparisons test was used to assess statistical significance for the individual kavalactones inhibition screen in comparison to the control ($\alpha = 0.05$).

To quantify the results from the time-dependent inhibition assays, a nonlinear regression analysis with the modified Hill equation (Eq. (1)) below was performed [23]:

$$R_v = 100 \cdot \left(1 - \frac{I_{max} \cdot [I]^b}{[I]^b + IC^b}\right) \quad (1)$$

The metabolite formation was expressed as a ratio of remaining enzyme activity (R_v) in comparison to each group's respective controls without any individual kavalactone and plotted as CES1 activity, with ([I]) as the inhibitor concentration already determined, the maximal inhibitory percentage (I_{max}), the half-maximal inhibitory concentration (IC) and (b) which is a shaping exponent. For the methysticin preincubation group, 50% inhibition of CES1 was not achieved. This resulted in some instability in our model for the methysticin preincubation group not allowing for an accurate prediction of IC_{50} for the methysticin preincubation group; however, this instability was corrected by fixing the preincubation I_{max} to the I_{max} generated from the no preincubation group which did achieve greater than 50% inhibition. Regardless of the inhibition mechanism, the inhibitor and substrate's interactions should be the same between identical assay systems containing the same enzyme. Furthermore, the preincubation group did not see a higher I_{max}, which is further indicative of a lack of time-dependent inhibition. This allowed us to compare the IC_{50} predictions for both methysticin groups. These generated parameters were then used to calculate the inhibitor concentration that inhibits 50% of enzyme activity (IC_{50}) in the equation below (Eq (2)):

$$IC_{50} = \frac{IC}{(2 \cdot I_{max} - 1)^{1/b}}. \quad (2)$$

A nonlinear regression analysis was performed using a modified Michaelis-Menten model for mixed competitive-noncompetitive inhibition model for the evaluation of the in vitro potential of the kavalactones which is shown in the equation below (Eq. (3)):

$$V = \frac{V_{max} \cdot [S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{\alpha \cdot K_i}\right)} \quad (3)$$

The predetermined variables by the assay were the substrate concentration of OST ([S]) and the concentration of kavalactone ([I]) which were both expressed in μM. (V) is the OC formation velocity, which was used as the marker for CES1 activity, was determined through LCMS analysis of the samples and was expressed as nmol/min/mg protein. The remaining parameters were estimated using equation (3) that were the reaction's Michaelis-Menten constant (K_m), the maximum velocity of the reaction (V_{max}), the inhibitor constant (K_i) and the type of inhibition indicator (α), where α values equal to 1 are indicative of a noncompetitive inhibition type, α values approaching infinity are indicative of a

competitive inhibition type, and values ranging between 1 and infinity are indicative of a mixed-type inhibition. The definitions and interpretations of our enzyme kinetics have largely been adapted from Segel [25]. Lineweaver-Burke plots were inspected and zoomed in to visualize the type of inhibition for each of the kavalactones. Lineweaver-Burke plots were only used as a qualitative visual aid to demonstrate the type of inhibition and were not used diagnostically. A relative weighted linear regression ($1/Y^2$) was used for the Lineweaver-Burke plots. The highest kavalactone concentrations for methysticin, dihydromethysticin, dihydrokavain, and yangonin and the lower substrate concentrations of OST for dihydromethysticin and dihydrokavain were excluded because too few amounts of metabolite OC formed.

Software. Excel version 16.51 for Mac (Microsoft, Redmond, WA) was used to quantify and store gathered data. Nonlinear regression data analysis and graph visualization were performed using GraphPad Prism version 9.2.0 for macOS (GraphPad Software, La Jolla, CA).

3. Results

Screening of Kavalactones. The metabolite formation of OC in the presence of each kavalactone were quantified relative to a control containing no kavalactone and were expressed as a ratio. In the first screen (Fig. S3 Supplementary Data.) at a concentration of 10 μg/mL for each kavalactones, all six kavalactones produced a reduction in metabolite formation. Yangonin inhibited CES1 activity by 45.9%, being the most of all six kavalactones, while dihydrokavain inhibited CES1 activity by 22.3%, being the least, but each kavalactone showed statistical significance in comparison to control ($p < 0.05$). After this screen, it was determined that further analysis was warranted for categorizing and quantifying the in vitro inhibition exhibited by the AB-free kava extract and its kavalactones.

Kavalactones Demonstrate Reversible Inhibition of CES1 and OST hydrolysis. There was a more marked decrease of OC formation at higher concentrations of each of the individual kavalactones (Fig. 2. A, B, C, D, E, F). Using both a preincubation group and a non-preincubation group allowed for a comparison of IC_{50} values and thereby determination of inhibition mechanism. All IC_{50} ratios for each kavalactone of preincubation to no preincubation were greater than 1, indicating that with a 30-min preincubation phase there was no increase in the potency of inhibition (Table 1). Since the preincubation group for each of the kavalactones did not show an increase in inhibition potency, it was concluded that neither irreversible inhibition nor time-dependent inhibition occurred. The AB-free kava extract containing each kavalactones displayed a similar behavior as the individual kavalactones (Fig. S4 Supplementary Data).

In-Vitro Categorization and Quantification of Inhibition Potential of the Six Kavalactones. Using nonlinear regression analysis, the extent and type of inhibition the kavalactones exhibit with CES1 was determined (Fig. 3. A, B, C, D, E, F). The analytical findings were then further visualized with the usage of Lineweaver Burke plots (Fig. 4. A, B, C, D, E, F). The Ki values for methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin and desmethoxyyangonin were 35.2, 68.2, 81.6, 105.3, 24.9, and 25.2 μM respectively (Table 2). The order of the inhibition potency in vitro for each kavalactone is as follows: yangonin

Table 1
Preincubation effect with each kavalactone on CES1 activity.

Kavalactones	No preincubation	30 min preincubation	Preincubation/No Preincubation
	IC_{50} (μM)	IC_{50} (μM)	
Methysticin	23.58 ± 12.81	67.53 ± 15.07	2.86
Dihydromethysticin	56.86 ± 14.00	63.87 ± 18.64	1.12
Kavain	117.2 ± 70.55	137.4 ± 95.61	1.17
Dihydrokavain	90.26 ± 42.64	105.22 ± 22.22	1.17
Yangonin	68.79 ± 35.97	85.36 ± 10.49	1.24
Desmethoxyyangonin	48.24 ± 16.41	63.16 ± 28.03	1.31

The IC_{50} estimates (±S.E.) were obtained from nonlinear regression analysis using the model for IC determination from [Eq. (1)] and [Eq. (2)].

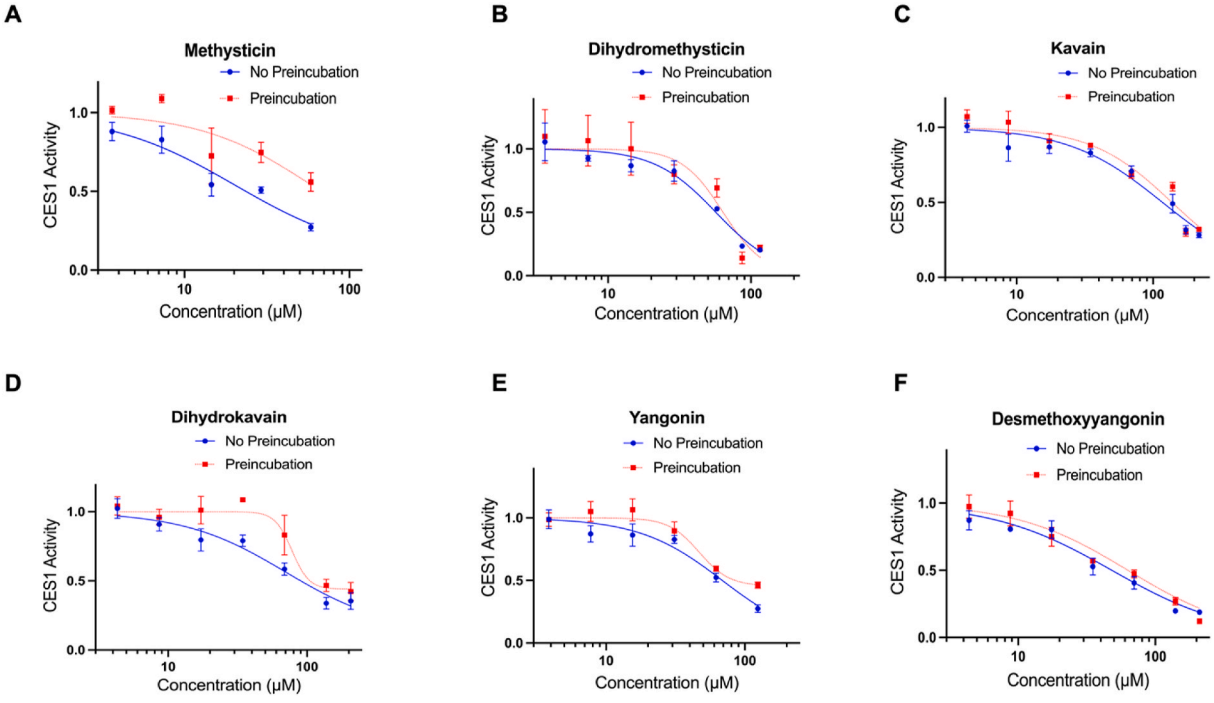

Fig. 2. Time-dependent inhibition curve for each kavalactone: (A) methysticin, (B) dihydromethysticin, (C) kavain, (D) dihydrokavain, (E) yangonin, (F) desmethoxyyangonin. Each kavalactone was added with the CES1 S9 fractions before a 30-min preincubation in the preincubation group and after in the no preincubation group. The OST (substrate) concentration was 200 μM and the CES1 S9 fraction (enzyme) concentration was 20 μg/mL. CES1 activity was a ratio that was relative to control with no inhibitor. Individual points represent the mean (±S.D.) done in triplicate. Plots were generated using [Eq. (1)].

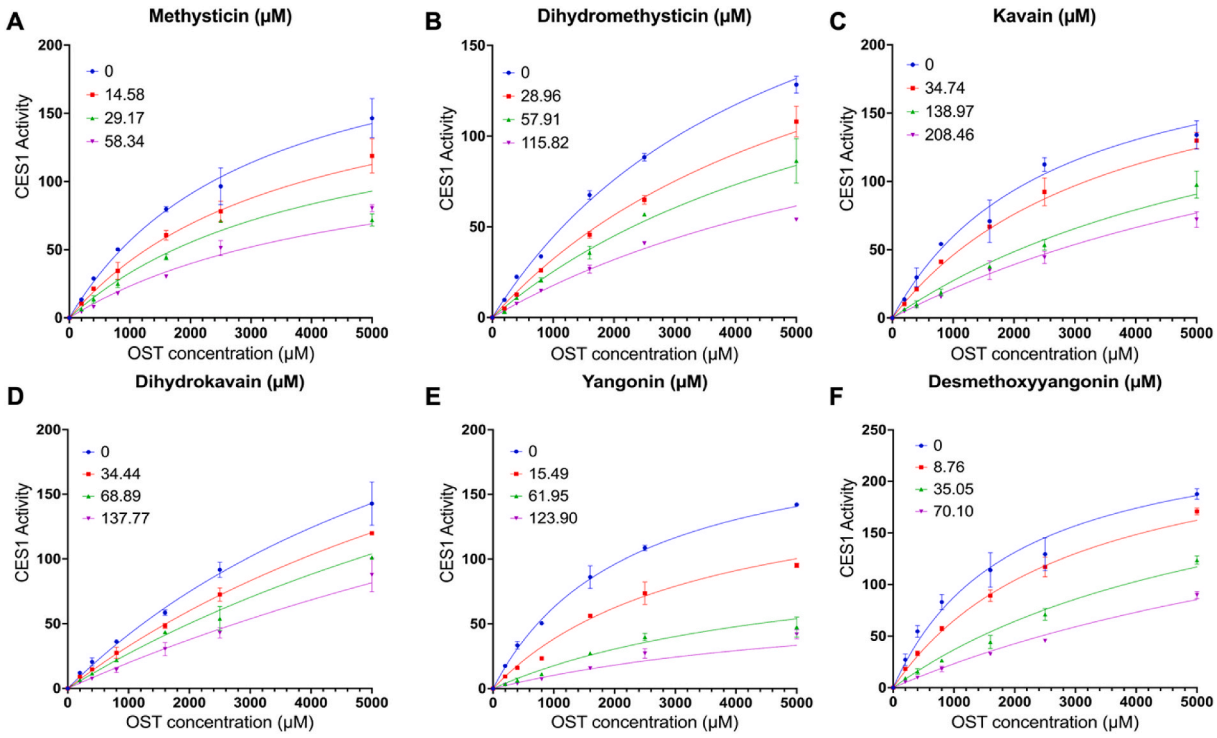

Fig. 3. The kinetic analysis for each kavalactone (A) methysticin, (B) dihydromethysticin, (C) kavain, (D) dihydrokavain, (E) yangonin, (F) desmethoxyyangonin in an in vitro system with CES1. CES1 S9 fractions were incubated with the absence and presence of each kavalactone. Individual points represent the mean (±S.D.) of duplicate samples. Each kavalactone was replicated in triplicate with 1 of the 3 curves being represented. CES1 activity represents the velocity of the reaction (nmol/min/mg protein). Plots were generated by utilizing [Eq. (3)].

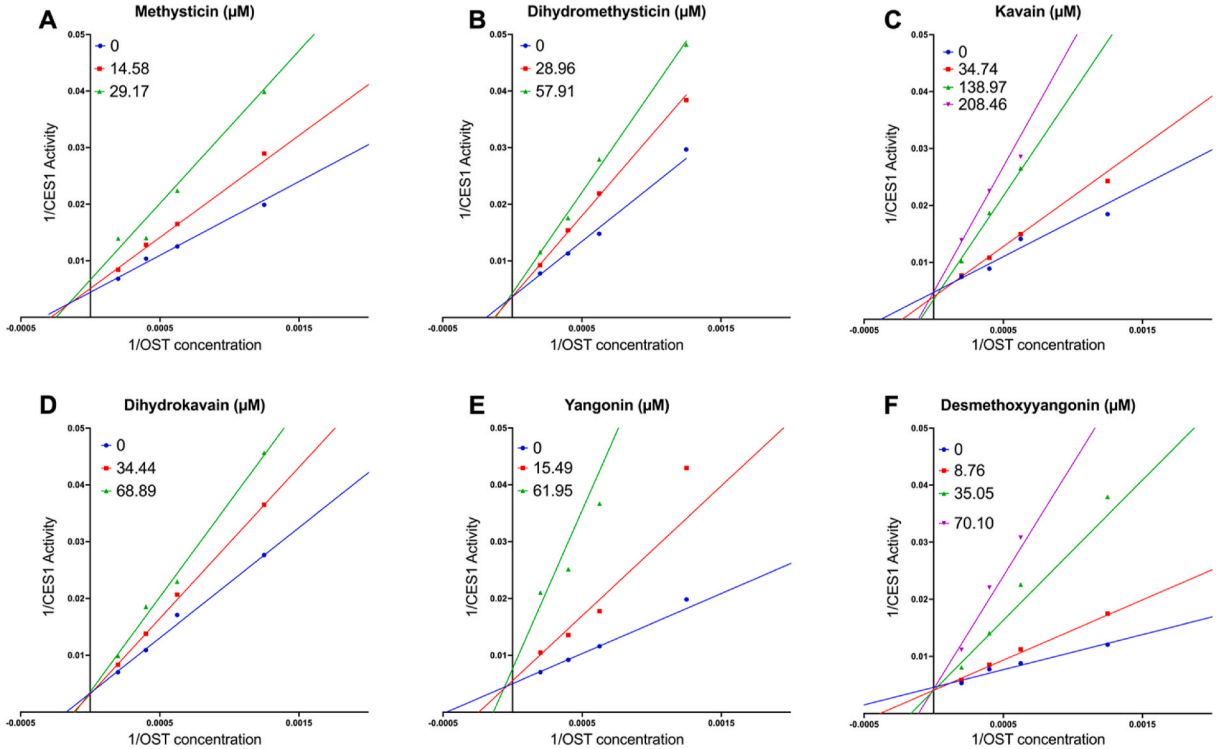

Fig. 4. The corresponding Lineweaver-Burk plots for each kavalactone (A) methysticin, (B) dihydromethysticin, (C) kavain, (D) dihydrokavain, (E) yangonin, (F) desmethoxyyangonin in an in vitro system with CES1. CES1 S9 fractions were incubated with the absence and presence of each kavalactone. Individual points represent the mean (±S.D.) of duplicate samples. Each kavalactone was replicated in triplicate with 1 of the 3 curves being represented. Plots were generated using linear regression.

Table 2
Parameter estimates of in vitro kavalactone inhibition studies on CES1 activity.

Kavalactones	K_m (μM)	K_i (μM)	α	V_{max} (nmol/min/mg protein)
Methysticin	4014 ± 841	35.19 ± 10.74	1.8 ± 0.7	261 ± 29.6
Dihydromethysticin	4581 ± 1194	68.19 ± 7.38	1.9 ± 0.6	249 ± 18
Kavain	2751 ± 452	81.59 ± 11.22	∞	203 ± 25
Dihydrokavain	6831 ± 912	105.29 ± 16.97	∞	332 ± 71
Yangonin	3509 ± 1667	24.86 ± 5.41	3.1 ± 1.2	244 ± 67
Desmethoxyyangonin	2413 ± 441	25.22 ± 12.60	∞	214 ± 46

All parameter estimates were obtained from nonlinear regression analysis using the modified Michaelis-Menten equation in [Eq. (3)]. The values are the mean estimated parameters and S.D. in triplicated runs of duplicate samples.

> desmethoxyyangonin > methysticin > dihydromethysticin > kavain > dihydrokavain. Methysticin, dihydromethysticin, and yangonin demonstrated a mixed competitive-noncompetitive inhibition. Kavain, dihydrokavain, and desmethoxyyangonin demonstrated competitive inhibition.

4. Discussion

We determined that the AB-free kava extract and its kavalactones have the ability, to varying degrees, to inhibit OST hydrolysis by CES1 in vitro. After initially screening each kavalactone at a concentration of 10 μg/mL, OST hydrolysis was inhibited by ≥ 20% when compared to a negative control. Each kavalactone exhibited a reversible mechanism of inhibition, as a 30-min preincubation period with the kavalactones and CES1 S9 fractions did not result in an increase in potency of inhibition. Finally, using the mixed type of inhibition Michaelis-Menten model, we found that the rank order of CES1 inhibition for the kavalactones were as follows: yangonin, desmethoxyyangonin, methysticin, dihydromethysticin, kavain and dihydrokavain. This model also enabled us to use generated α parameters to determine the type of inhibition of each of the kavalactones. We found that methysticin, dihydromethysticin and yangonin demonstrate a mixed competitive-noncompetitive inhibition model while kavain, and dihydrokavain, desmethoxyyangonin demonstrate a competitive model.

These results add to what has been previously reported relative to inhibition of DMEs by kava and its kavalactones. A number of in vitro studies has demonstrated that various extracts of kava and the six major kavalactones have the ability to inhibit CYP1A2, 2C9, 2C19, 2D6, and 3A4 [16–18]. However, these findings have generally not been confirmed in formal human clinical studies [26–29]. Despite the extensive in vitro data and available clinical data on kava and kavalactones and the CYP 450 system, only recently has kava itself been shown to inhibit a CES1 mediated metabolism of 4-nitrophenyl acetate in an in vitro system by 50% at a concentration of 200 μg/mL [19]. CES1 can catalyze the activation of prodrugs such as oseltamivir or deactivate therapeutic compounds like methylphenidate. CES1 substrates encompass a wide variety of therapeutic areas including antihypertensives, anti-hyperlipidemic agents, antiplatelet agents, anticoagulants, antiviral agents, various CNS agents, and others [30].

One of the first major clinical trials involving kava for the treatment of generalized anxiety disorder established the safety of using kava doses standardized up to a total of 240 mg of kavalactones that was achieved by taking two 3 g kava tablets (containing 60 mg of kavalactones) twice a day [6]. These investigators determined that of their standardized 60 mg per tablet of kavalactones contained kavain (21%), dihydrokavain (26%), methysticin (14%), dihydromethysticin (18%), yangonin (13%), and desmethoxyyangonin (8%) meaning that the patients received up to 50.4, 62.4, 33.6, 43.2, 31.2, 19.2 mg each of the respective kavalactones a day [6]. A commercially available ethanolic extract containing a total of 150 mg/mL kavalactones and 75 mg total kavalactone capsule were previously profiled for their kavalactone content [31]. It was determined that the components for the ethanolic extract were kavain (27.2%), dihydrokavain (39.1%), methysticin (3.4%), dihydromethysticin (14.1%), and desmethoxyyangonin (16.2%) and the components for the capsule were kavain (33.7%), dihydrokavain (18.2%), methysticin (10.4%), dihydromethysticin (27.3%), and desmethoxyyangonin (10.4%) [31]. This highlights both the variability of each individual kavalactone based on the supplier and the formulation of product. Recently, the kavalactone content of 28 differing commercially available kava products was quantified demonstrating a difference in kavalactone content and composition [32]. Depending on the cultivar, manufacturer, and supplier patients could be exposed to a wide range of dosing for the individual kavalactones when consuming kava products.

There is a paucity of reported pharmacokinetic characterization of kavalactones in humans. One of the first studies to quantify the amount of individual kavalactones present in human plasma includes two subjects who received one kava soft gel capsule (containing 75 mg of kavalactones) three times daily for a total daily dose of 225 mg kavalactones for one week before blood samples were drawn [31]. The quantified plasma concentrations of kavain (0.01–0.02 μg/mL), dihydrokavain (0.03–0.05 μg/mL), methysticin (0.007–0.012 μg/mL), dihydromethysticin (0.05–0.08 μg/mL), and desmethoxyyangonin (0.0003–0.0008 μg/mL) are generally in the low ng/mL range with the consideration that these are not the reported C_{max} and are most likely not the highest achieved plasma concentrations of the individual kavalactones within the two subjects [31]. Another important consideration is that as these individual kavalactones are being considered for their individual benefits, there may be formulations that are designed to have more of a certain kavalactone or only contain one kavalactone, such as the study that gave a single dose of 800 mg of kavain in an unreported number of human subjects in order to determine its pharmacokinetics [33]. They found that subjects attained serum concentrations ranging from 0.01 to 0.04 μg/mL within 1–4 h of administration, again these were not reported as a C_{max} and may not be the highest attainable concentration of kavain within a human subject [33].

Potential of clinical drug-drug interactions caused by kavalactone-mediated CES1 inhibition is evaluated utilizing a basic reversible inhibition model recommended in the FDA guidance for in vitro drug interactions. The model is as follows (Eq. (4)):

$$R_1 = 1 + \left(\frac{I_{max,u}}{K_{i,u}}\right) \quad (4)$$

$I_{max,u}$ represents the maximal unbound plasma concentrations of the inhibitor at steady state, $K_{i,u}$ represents the experimentally determined unbound inhibition constant that was determined in the experiments, and R_1 is the ratio of intrinsic clearance values of a CES1 substrate in the absence and presence of inhibitor. An R_1 value of 1.02 or greater is considered to have the potential of observing clinical interactions and needs further investigation by either a more mechanistic model or clinical study. In this study, the reported concentrations of kavalactones were evaluated for their likelihood to mediate drug interactions with CES1 substrates. Since there is no known or reported plasma protein binding associated with the individual kavalactones, a similar unbound fraction was assumed between in vivo and our in vitro assay.

Although limited in scope, the available physiological concentrations of several kavalactones (namely kavain, dihydrokavain, methysticin, dihydromethysticin, and desmethoxyyangonin) were utilized in our assessment of DDI potential. Using the limited clinical data, the highest R_1 value of 1.004 was calculated among all the kavalactones after multiple doses of a 75 mg thrice daily total kavalactones regimen [31, 33]. This dosing regimen provides similar daily intake of total

kavalactones to the recommended 120 mg twice daily dose [5,6]. Since the R_1 value extrapolated from available human physiological concentrations of kavalactones does not meet the significance cut off defined by the FDA, clinically relevant inhibition of CES1 is not expected with multiple doses of 120 mg twice daily kavalactones. However, it needs to be noted that the reported physiological concentrations of the kavalactones were based on a limited sample size in the referenced studies and a C_{max} was not clearly documented.

Notably, kava extracts are unregulated and widely available as over-the-counter supplements and beverages in kava bars [6]. It is a reasonable assumption that many consumers of kava routinely use these products daily in amounts far exceeding the recommended ceiling dose of 240 mg/day (120 mg twice daily). In the scenario of consumption of 1000 mg or higher daily kavalactones, this would be anticipated to become sufficient to produce measurable inhibition of CES1 and potentially influence the metabolism of co-administered CES1 substrate medications. Recently, a clinical study involving human patients sought to assess the impact of consuming more than 2000 mg of kavalactones in a 6 h drinking period, which is considered an average time for a Fijian or Polynesian kava session [34]. At a kavalactone concentration of 145 mg/100 mL, the average kava consumption of the participants was 3059 mL (7/20 subjects in the kava drinker group were able to drink a total 3600 mL) during the 6-h period, resulting in over 4000 mg kavalactones being consumed in less than a day [34]. Based on the previous predictions, it is certainly plausible in this type of setting to expect drug interactions in patients who are also taking medications that are CES1 substrates. In addition, as indicated by the mouse pharmacokinetic study, there appeared to be an accumulation of kavalactones in the liver as compared to serum [31]. The liver/serum concentration ratios ranged from 5 to 49-fold for the studied kavalactones. Since the liver is the major site of metabolism as well as expression of CES1, the hepatic concentration may provide a more accurate prediction and a higher magnitude of drug interactions may be possible in this case.

Finally, the typical limitations of extrapolating in vitro assessments of DDI potential with botanical constituents to the clinical situation applies here. That is, there is little available data on the absorption or bioavailability of kavalactones in humans, uncertainty regarding clinically relevant concentrations of free compound versus conjugates or other metabolites formed in vivo, and in general, metabolites of botanical extracts are poorly characterized yet may potentially contribute to the net inhibitory effects observed [35].

In conclusion, we have shown that kava and its kavalactones can potently inhibit CES1 catalytic activity in vitro. Using these findings together with limited available clinical data we predict that clinically relevant drug interactions involving inhibition of CES1 by kavalactones at the daily recommended dose of kava are unlikely. Meanwhile, several uncertain factors have been discussed and a drug interaction potential cannot be fully excluded, particularly in the scenario of over-the-counter use of more than recommended dose. To rule out clinically relevant drug interactions and ensure the safe usage of kava-related products, further clinical DDI studies with a standardized kava extract is warranted.

Authorship contributions

Participated in research design: Melchert, Qian, Zhang, Xing, Markowitz. Conducted experiments: Melchert, Qian. Performed data analysis: Melchert, Qian, Zhang, Xing, Markowitz. Wrote and contributed to the writing of the manuscript: Melchert, Zhang, Xing, Klee, Markowitz.

Declaration of competing interest

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

Dr. Chengguo Xing has disclosed that he maintains a proprietary interest in the AB-free kava extract from which the kavalactones are extracted. All other authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2022.109883.

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