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Composition and biological activity of traditional and commercial kava extracts

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Abstract

For centuries the South Pacific islanders have consumed kava (*Piper methysticum*) as a ceremonial intoxicating beverage. More recently, caplets of kava extracts have been commercialized for their anxiolytic and antidepressant activities. Several cases of hepatotoxicity have been reported following consumption of the commercial preparation whereas no serious health effects had been documented for the traditional beverage. A detailed comparison of commercial kava extracts (prepared in acetone, ethanol or methanol) and traditional kava (aqueous) reveals significant differences in the ratio of the major kavalactones. To show that these variations could lead to differences in biological activity, the extracts were compared for their inhibition of the major drug metabolizing P450 enzymes. In all cases (CYP3A4, CYP1A2, CYP2C9, and CYP2C19), the inhibition was more pronounced for the commercial preparation. Our results suggest that the variations in health effects reported for the kava extracts may result from the different preparation protocols used.

Keywords: Kava; *Piper methysticum*; Kavalactone; Methysticin; Kavain; Yangonin; P450; CYP; Inhibition; Inhibit

Extracts of *Piper methysticum*, or kava, have been consumed in the South Pacific Islands for centuries without any reported serious side effects [1,2]. The traditional extract is an infusion of ground kava roots (1.0–1.5 g) in cold water (100–150 mL) used for its psychotropic and hypnotic properties [3]. The consumption of kava has now spread to the Western world where the traditional kava root infusion has been replaced by commercially available caplets. Sixteen different kavalactones have been identified as the active principles of this extract. Methysticin, dihydromethysticin, kavain, dihydrokavain, demethoxyyangonin, and yangonin are the major ones and account for 96% of the organic extract [4].

Reported beneficial effects of kava at low concentrations include relaxation, euphoria, anticonvulsant properties, neuroprotection, analgesia, and attenuation of menopausal symptoms [1,3–7]. After being approved for the treatment of anxiety and nervous disorders in 1990 [3], kava was banned from Germany in June 2002 based on the reevaluation of its risk-to-benefit ratio by the German Health authorities [8–10]. Kava has since been removed from the market in all of Europe (including UK), Australia, and Canada, and a warning has been issued in USA [2,8,9]. Many clinical studies suggest that the kava extract offers an effective alternative for the treatment of anxiety [11–14] and conclude that it is relatively safe when used alone at recommended dosage for a short period of time (1–24 weeks) [15–17]. On the other hand, 78 cases of hepatotoxicity have been reported following ingestion of commercial kava caplets [8,9,18–21] but none have been recorded for the traditional extract. In several of these cases, the hepatic failure has required liver transplantation or has been fatal [2,3,8,9,18–21]. Two cases have been attributed to depletion of human CYP2D6, the enzyme responsible for

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kavalactones metabolism [2,5,21]. Most other cases involved concomitant ingestion of drugs known for their potential hepatotoxicity [9] or of other pharmaceuticals, which suggests that herb–drug interactions may be implicated.

P450 inhibition or induction is an important factor leading to drug interactions. The inhibition of human P450 isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP4A9/11 (from either microsomes or human hepatocytes) by individual kavalactones, by commercial kava caplets, and by an ethanolic kava root extract has been reported [22–24]. This family of enzymes is important for the degradation of toxins and pharmaceuticals, and for the biosynthesis of steroids and prostaglandins. P450s are most abundant in the liver where a few isoforms, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, account for 80% of the total hepatic metabolism [25]. One of the most promiscuous human isoforms, CYP3A4, is involved in the phase I metabolism of more than 50% of the current pharmaceuticals. It is also the major P450 in the small intestine, whereas CYP2D6, the enzyme responsible for kavalactones metabolism [2,5,21], is the main P450 in the brain [25].

Although the hepatoxicity of kava is still debated [21], multiple hypotheses have been proposed to explain the difference in toxicity between the commercial caplets and the traditional kava extracts. Commercially available kava caplets are usually prepared by ethanolic or acetonic extraction of the full plant [2,26]. Since kava has traditionally been consumed as a water infusion of the root, it has been suggested that the use of organic solvents may extract more toxic components from the kava plant [2]. The use of the aerial part of the plant by the producers of kava caplets is also suspected to lead to toxicity of the extract. Indeed, the cytotoxic alkaloid pipermethysticin has recently been isolated from this part of the plant [27]. Finally, it has been suggested that the addition of a racemic mixture of synthetic kavain, sometimes added to boost the activity of the commercial extract, may increase the toxicity [9].

Our aim was to compare kava caplets and kava root extracts prepared using water, methanol, ethanol or acetone by analytical methods. The ratio of the major kavalactones of the aqueous extract was found to be different from that of all other extracts and it was envisaged that these disparities could lead to variations in biological activities. This hypothesis was confirmed by comparing the inhibition of the major drug metabolizing human P450s by the aqueous and organic extracts.

Materials and methods

Chemicals and enzymes. Human liver CYP1A2 supersomes containing P450 reductase were purchased from BD Gentest (Woburn, MA). Human liver CYP3A4, CYP2C19, and CYP2C9 microsomes, which included P450 reductase and cytochrome b_5, were also obtained from BD Gentest. The substrates 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluorocoumarin (MFC), and 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), as well as the fluorescent products 7-hydroxy-4-trifluoromethylcoumarin (HFC) and 3-[2-(*N*,*N*-diethyl-*N*-methylamino) ethyl]-7-hydroxy-4-methylcoumarin (AHMC) were also purchased from BD Gentest.

The kava roots (Moi variety) were generously provided by Dr. Will McClatchey from Hawaii University (occasionally via Jonathan Yee, a kava farmer in Hawaii). The kavalactone standards methysticin (M), dihydromethysticin (DHM), kavain (K), dihydrokavain (DHK), demethoxyyangonin (DMY), and yangonin (Y) were purchased from ChromaDex (Santa Ana, CA). Kava caplets (150 mg per caplet each containing 45 mg of kavalactones) were from Nature's Resource (Mission Hills, CA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO). Solvents used were HPLC grade, except acetone which was ACS grade. All the measurements described below were done in duplicate.

Instruments. Kinetic assays were performed in black Costar 96-well microplates. Fluorescence of the products was monitored using a GEMINI XS model microplate reader (Molecular Devices, Sunnyvale, CA). NADPH consumption UV assays were carried out using a Spectra Max 190 model microplate reader (Molecular Devices). Analytical HPLC analyses were performed on an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a fluorescence detector, a thermostatted column compartment, and a ChemStation (for LC 3D A.09.03) data system.

Extracts preparation. The kava roots (repeated with two different batches of kava Moi varieties, one of the most popular variety) were dried at 37 °C for 48 h and ground in a coffee grinder. For the preparation of organic kava root extracts, the organic solvent (75 mL of either methanol, acetone or ethanol) was added to ground kava root (1 g) and the heterogeneous mixture was shaken at 250 rpm and 40 °C for 90 min. The mixture was then filtered to remove the root, the solvent was evaporated in vacuo, and the residues were each reconstituted in methanol (10 mL). The aqueous kava root extract was generated from ground kava root (1 g) stirred in water (125 mL) at room temperature for 10 min. The mixture was subsequently filtered and lyophilized. The residue was reconstituted in methanol (20 mL). The commercial caplet extract was prepared using the powder from 4 Nature's Resource kava caplets mixed in methanol (75 mL) and shaken at 250 rpm and 40 °C for 90 min. The insoluble material was filtered off and the solvent was evaporated in vacuo. The residue was reconstituted in methanol (10 mL).

HPLC and LC-MS separation. Separation and quantification of the kavalactones were achieved by HPLC using a modification of the procedure reported by Shao et al. [28]. The column used was a 250 × 4.6 mm SYNERGI 4μ Hydro-RP 80Å (Phenomenex, Torrance, CA, USA). Isocratic elution with water:acetonitrile:methanol (52:23:25) was used at a flow rate of 1 mL/min. The absorption spectra were recorded from 200 to 400 nm. Quantification was accomplished at 240 nm for methysticin, dihydromethysticin, kavain, and dihydrokavain and at 350 nm for demethoxyyangonin and yangonin. The column was thermostatted at 40 °C. Confirmation of the identification of the different kavalactones was achieved by LC-MS and with the use of authentic samples. LC-MS analysis was performed on a Spectra System P4000 (Spectro Separation Products) equipped with a UV 2000 detector and a Finnigan LCQ DUO (ion trap) mass detector setup in Atmospheric Pressure Chemical Ionization (APCI) negative mode, controlled by the X-calibur software version 1.2. The elution method was the same as described above.

Stock solutions used for enzyme activity and inhibition studies by fluorescence-based assays. The P450 liver microsomes were diluted in 100 mM (CYP3A4, CYP1A2, and CYP2D6) or 25 mM (CYP2C19 and

CYP2C9) potassium phosphate buffer at pH 7.4 to a final concentration of 0.1 μM. The stock solution of BFC (5.5 μM, substrate for CYP3A4 and CYP2C19) was prepared from an aqueous dilution of a 300 μM solution in acetonitrile. The CYP2D6 substrate AMMC was first dissolved in acetonitrile (1 mM) and then diluted in water to 15 μM. MFC, the substrate used with CYP2C9, was similarly prepared by aqueous dilution to 165 μM of a 1 mM solution in acetonitrile. Finally, the stock solution of the CYP1A2 substrate ethoxyresorufin (ER) was prepared by aqueous dilution to 5.5 μM from a 1 mM stock solution in acetonitrile. Their respective products were dissolved in the same solvent mixtures. The coenzyme NADPH was dissolved to give a final concentration of 25 mM in the same buffer used to dilute the enzyme tested. All aqueous solutions were prepared using water from a Milli-Q purification system (Millipore, Bedford, Mass).

Stock solutions used for NADPH consumption assays. The stock solution of quinidine (16.7 mM), the substrate used with CYP3A4, was prepared in aqueous solution. The CYP1A2 substrate, ER, was dissolved (4.2 mM) in chloroform:acetonitrile (15:85). The stock solution for the CYP2C9 substrate MFC (2.5 mM) was prepared in potassium phosphate buffer (pH 7.4):acetonitrile (50:50). The stock solution of tolbutamide, a CYP2C19 substrate, was prepared in acetonitrile (195 mM).

Enzyme activity assays. The activities of CYP3A4 and CYP2C19 were assayed by monitoring the debenzylation of BFC to HFC. A solution of CYP3A4 (3.3 nM) and BFC (7.5 μM) in 100 mM potassium phosphate at pH 7.4 (total volume 300 μL) was incubated for 5 min at 37 °C. CYP2C19 (15 nM) and BFC (2.3 μM) were diluted in 25 mM potassium phosphate at pH 7.4 (total volume 200 μL) and incubated for 5 min at 37 °C. The reactions were initiated by the addition of NADPH (830 or 125 μM for CYP3A4 or CYP2C19, respectively) and they were monitored at 37 °C for 20 min. The production of HFC was followed at an excitation wavelength (λ_{ex}) of 410 nm and an emission wavelength (λ_{em}) of 530 nm.

The activity of CYP1A2 was measured by following the production of resorufin from ER. Each incubation mixture (300 μL) contained EDTA (1.5 mM), CYP1A2 (5 nM), and 7-ethoxyresorufin (1 μM) in 100 mM potassium phosphate at pH 7.4. It was incubated for 5 min at 37 °C, and following the addition of NADPH (250 μM), the reaction was monitored at λ_{ex} = 530 nm and λ_{em} = 582 nm for 20 min.

The assay used to study the activity of CYP2C9 involved the demethylation of MFC to HFC at 37 °C over 30 min. During these experiments λ_{ex} = 410 nm and λ_{em} = 530 nm were used. The reaction mixture (150 μL) contained CYP2C9 (10 nM), MFC (40 μM), and NADPH (750 μM) in 25 mM potassium phosphate buffer at pH 7.4.

Enzyme inhibition assays. Each experiment was carried out in duplicate and included a blank (without enzyme), a control of activity as described above, and appropriate control reactions to ensure the absence of inhibition by the trace of solvents used. The concentrations of aqueous kava extract used for inhibition studies were 0.3, 1.4, 2.9, 4.3, 5.8, 8.7, 10.1, 11.6, 14.5, 17.4, 20.3, and 40.6 μg extract/mL for CYP3A4, 0.1, 1.0, 5.0, 10.0, 20.0, 30.0, and 50.0 μg extract/mL for CYP1A2, 0.1, 1.0, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 120.0, 150.0, 170.0, and 200.0 μg extract/mL for CYP2C9, and 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 30.0, and 60.0 μg extract/mL for CYP2C19. The concentrations of acetonic kava extract used were 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, 10.0, and 20.0 μg extract/mL for CYP3A4, 0.01, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 100.0 μg extract/mL for CYP1A2, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 50.0, 70.0, and 100.0 μg extract/mL for CYP2C9, and 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, 3.0, 5.0, 10.0, and 20.0 μg extract/mL for CYP2C19. Finally, the concentrations of commercial kava caplet extract used were 0.3, 0.7, 1.3, 2.6, 5.2, and 10.4 μg extract/mL for CYP3A4, 0.01, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 100.0 μg extract/mL for CYP1A2, 0.1, 1.0, 3.0, 5.0, 7.0, 10.0, 25.0, and 50.0 μg extract/mL for CYP2C9, and 0.1, 0.5, 1.0, 1.5, 3.0, 5.0, 10.0, 25.0, and 50.0 μg extract/mL for CYP2C19. IC_{50} values were calculated from dose–response curves using GraphPad Prism 4.0. Possible interference with our assays (native fluorescence and/or quenching) from the different components of the kava extracts was verified by HPLC and UV and found not to be significant. No corrections were required on the calibration curves of the fluorescent products.

NADPH consumption assays. To each enzyme (50 nM CYP3A4, CYP2C9, CYP1A2 or CYP2C19) in potassium phosphate buffer at pH 7.4, the desired kava extract (2.5 or 25 μg/mL for the acetone extract and 5 or 50 μg/mL for the other ones), the corresponding substrate (275 μM quinidine for CYP3A4, 85 μM MFC for CYP2C9, 1.3 mM tolbutamide for CYP2C19 or 140 μM ER for CYP1A2) and NADPH (830 μM) were added. Control experiments were carried out without inhibitor and without NADPH, separately. The consumption of NADPH was monitored at 37 °C for 20 min by fluorescence for CYP2C9 (λ_{ex} = 380 nm, λ_{em} = 420 nm), CYP2C19 (λ_{ex} = 340 nm, λ_{em} = 445 nm), and CYP1A2 (λ_{ex} = 360 nm, λ_{em} = 455 nm) and by absorbance for CYP 3A4 (360 nm).

Results and discussion

Two different batches of kava root (Moi variety, one of the most popular) were ground and extracted separately in water to reproduce the preparation of traditional kava, and in acetone, in ethanol, and in methanol to mimic commercial extracts. Commercial kava caplets were also studied to validate our results with the kava root. Identification and quantification of the constituents of each extract was achieved by LC-MS and HPLC (Fig. 1). The chromatograms show that the same kavalactones are found in all of the extracts whereas highly polar constituents are present in negligible amounts in the organic extracts and in the caplets (data not shown). The ratio of the six major kavalactones however, is significantly different for the aqueous extract (note in particular the very low concentration of yangonin) compared to the organic extracts (acetone, ethanol, and methanol) which are almost identical to one another (Fig. 2). The commercial kava caplets also displayed a comparable pattern of kavalactones except for kavain and dihydrokavain. The higher proportion of kavain and dihydrokavain detected in the caplets may be explained by a different variety of kava used by the manufacturer, by the standardization of the extract or by the intentional addition of racemic material to increase the biological activity [9]. Calculation of the total amount of kavalactones per milligram of extract reveals that the aqueous kava root extract contains the lowest proportion of kavalactones of all the root extracts, as expected from the reported low water solubility of kavalactones (Table 1).

Differences in the ratio of kavalactones imply possible variations in biological activity. Inhibition of human P450 enzymes is an important pharmacological criterion since it is a primary cause of drug interactions and has been suggested as one possible source of kava hepatotoxicity. Fluorescence-based assays are commonly used to monitor the in vitro activity of P450 enzymes however they only allow the detection of the fluorescent product.

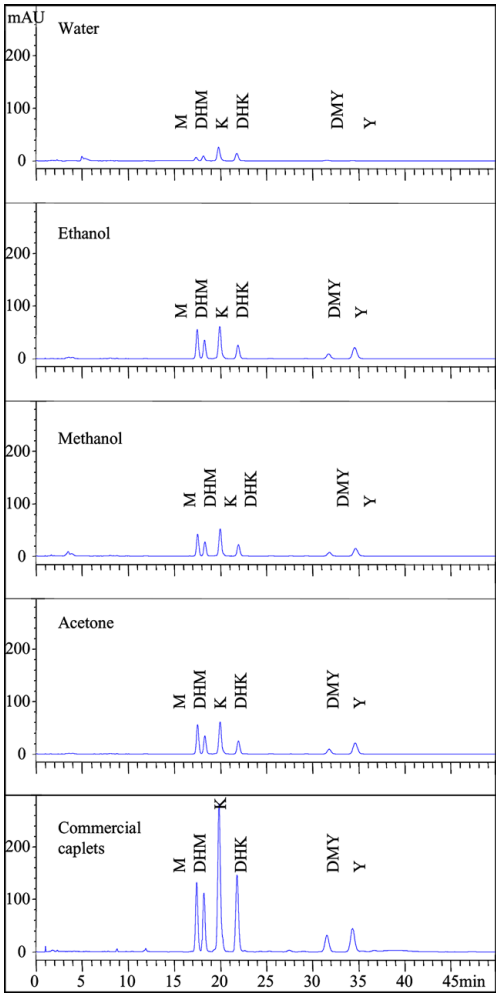

Fig. 1. HPLC chromatograms of the kava root extracts and the commercial kava caplets showing the separation of the six major kavalactones: methysticin (M), dihydromethysticin (DHM), kavain (K), dihydrokavain (DHK), demethoxyyangonin (DMY), and yangonin (Y).

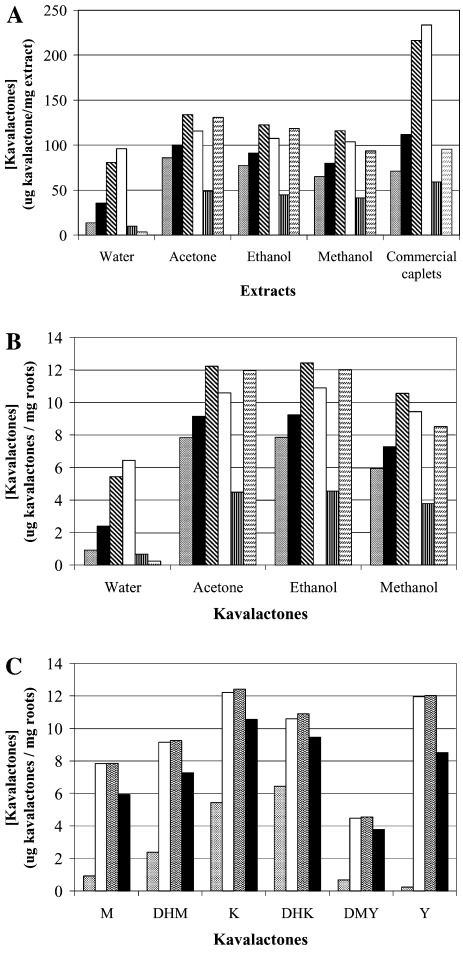

Fig. 2. (A) Comparison of the amount of methysticin (▨), dihydromethysticin (■), kavain (▩), dihydrokavain (□), demethoxyyangonin (▥), and yangonin (▨) quantified in kava root extracts and in commercial kava caplets. (B) Comparison of the amount of methysticin (▨), dihydromethysticin (■), kavain (▩), dihydrokavain (□), demethoxyyangonin (▥), and yangonin (▨) present in the kava root extract adjusted for the mass of kava root used. (C) Same data as B but the histograms are ordered to facilitate comparison of the amount of each kavalactone in the extracts prepared using water (▨), acetone (□), ethanol (▨), or methanol (■). The abbreviations M, DHM, K, DHK, DMY, and Y are used for methysticin, dihydromethysticin, kavain, dihydrokavain, demethoxyyangonin, and yangonin, respectively.

When another molecule is added, a decrease in formation of the fluorescent product can result from fluorescence quenching, from its competing transformation by the enzyme, or from inhibition of the enzyme by this molecule. NADPH consumption assays were performed to eliminate the possibility that kava extracts be substrates of P450 enzymes as opposed to inhibitors. This assay strongly suggested that the extracts tested all inhibited CYP3A4, CYP2C9, and CYP2C19. In the absence of substrate and in the presence of kava extract, a modest NADPH consumption was detected for CYP1A2, revealing that the extract is transformed by this enzyme but considerably more slowly than 7-ethoxyresorufin. CYP1A2 inhibition was confirmed by a dramatic decrease in NADPH consumption in the presence of both the substrate and the kava extracts. Because of their higher sensitivity, fluorescence-based assays were used for detailed kinetic studies. Control experiments were carried out to validate the methodology (see Materials and methods). Since the acetonic, ethanolic, and methanolic kava root extracts presented similar concentrations and ratio of kavalactones (Fig. 2), P450 inhibition was only tested with the acetonic extract, the aqueous extracts and the commercial kava caplets. The results (Table 2) revealed significant inhibition in all cases (here μg/mL ~ μM) yet consistently less inhibition was observed with the aqueous extract. This was expected since kavalactones are known to inhibit P450s [23] and the aqueous extract contains less kavalactones per milligram of extract (Table 1). Moreover, these results suggest that "non-kavalactones" components present in the aqueous extract do not inhibit P450s

Table 1
Total amount of kavalactones (KL) in kava root extracts prepared from different solvents and in commercial caplets

Water (μg KL/mg extract)	Acetone (μg KL/mg extract)	Ethanol (μg KL/mg extract)	Methanol (μg KL/mg extract)	Caplets (μg KL/mg extract)
240	616	562	500	788

Table 2
Inhibition of human P450 by different kava root extracts and by commercial caplets expressed in microgram of extract per milliliter

P450 isoform	IC_{50} waters extract (μg extract/mL)	IC_{50} acetone extract (μg extract/mL)	IC_{50} caplet (μg extract/mL)
3A4	7.8 ± 1.3	2.2 ± 0.3	5.9 ± 0.9
1A2	18.4 ± 8.9	4.0 ± 0.6	2.7 ± 0.9
2C9	40.5 ± 13.1	24.8 ± 2.9	5.1 ± 1.8
2C19	3.8 ± 0.3	2.0 ± 0.2	1.8 ± 0.2

Table 3
Inhibition of human P450 by different kava root extracts and by commercial caplets expressed in microgram of kavalactones per milliliter

P450 isoform	Water extract (μg KL/mL)	Acetone extract (μg KL/mL)	Caplets (μg KL/mL)
3A4	1.9 ± 0.3	1.4 ± 0.2	4.6 ± 0.7
1A2	4.4 ± 2.1	2.5 ± 0.4	2.1 ± 0.7
2C9	9.7 ± 3.1	15.3 ± 1.8	4.0 ± 1.4
2C19	0.9 ± 0.1	1.2 ± 0.1	1.4 ± 0.2

significantly. When calculated from the amount of extract, lower inhibition is observed for the aqueous extract with all the enzymes tested. On the other hand, when the IC_{50} is expressed per microgram of kavalactone present in each extract (Table 3), no significant differences are apparent except for CYP2C9. Again, this was expected from the known P450 inhibition by kavalactones [23]. The IC_{50} for the inhibition of CYP2C9 by the acetonic extract (15.3 μg/mL) is higher than those for the caplet (4.0 μg/mL) and the aqueous extract (9.7 μg/mL). Those results may be explained by the increasing amount of yangonin in the different extracts (aqueous < caplet < acetone). Yangonin has been reported by Zou et al. [24] to have no effect on the activity of CYP2C9 and this may explain the lower inhibition by the acetone extract which contained the higher proportion of this kavalactone.

In summary, comparison of traditional aqueous kava extracts with organic kava extracts and commercial kava caplets revealed differences in the ratio of kavalactones which suggest deviations in biological activity. Indeed, although all of the extracts inhibited human CYP3A4, CYP1A2, CYP2C9, and 2C19 in the low micromolar range, the aqueous extract was the least potent for all these P450s. Kava is often consumed in combination with other pharmaceuticals including antidepressants such as benzodiazepines which are known to be metabolized by CYP3A4 [29,30]. Not surprisingly, harmful interactions have been reported between kava and alprazolam [31]. Our results suggest that other potential drug interactions are possible with kava extracts and warrant more in vivo studies. Finally, the data presented here indicate that if the hepatotoxicity reported for the commercial caplet is the result of P450 inhibition, the traditional extract should also be hepatotoxic at higher doses.

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