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Chemical and in vitro toxicity analysis of a supercritical fluid extract of Kava kava (*Piper methysticum*)

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A B S T R A C T

Ethnopharmacological relevance: Kava and kava extracts have shown great potential as a way to minimize anxiety-associated symptoms and to help alleviate pain. Hepatoxicity has been associated with the consumption of kava products. The chemical compounds, kavalactones (KL) and flavokavains (FK) have been implicated in kava's psychotropic and possible hepatotoxic properties.

Aim of the study: To investigate the kavalactone and flavokavain content and in vitro toxicity of KAVOA™, a supercritical carbon dioxide extraction (SFE) of kava.

Materials and methods: Kavalactone and flavokavain content of SFE kava and noble kava root were determined following extraction in acetone, cell culture media, and water using ultra high-performance liquid chromatography (UHPLC). Using water extractions of the kava products, the cell viability and toxicity on the human hepatocellular carcinoma cell line (HepG2) were determined using luminescent and fluorescent assays, respectively. The half maximal inhibitory concentration (IC_{50}) of the SFE kava and noble kava root, extracted in cell culture media, were determined utilizing a luminescent cell viability assay.

Results: Quantification of the KAVOA™, a SFE extract of kava and kava root showed similar profiles of kavalactone and flavokavain content. Water extracted SFE and root kava did not show a negative impact on cell viability and toxicity when compared to the vehicle control treated cells. IC_{50} values were determined for the SFE kava and kava root extracted in cell culture media in respect to cell viability, 78.63 and 47.65 μg/mL, respectively.

Conclusions: KAVOA™, a supercritical carbon dioxide extract of kava displays a similar kavalactone profile to a noble variety of kava. In relation to total kavalactone content, KAVOA™ also has a lower content of the cytotoxic compound FKB. Aqueous extractions of KAVOA™ and noble kava root had no significant negative impact on cell viability and toxicity on HepG2 cells when compared to vehicle controlled treated cells. Results indicate KAVOA™ demonstrates a similar in vitro safety profile to that of noble kava root when experiments are normalized to kavalactone content.

1. Introduction

Kava kava, *Piper methysticum* G.Forst., is a perennial shrub and member of the pepper family. Indigenous to the South Pacific, aqueous extracts have been used for over a thousand years as a ceremonial and medicinal drink (Singh, 1992). Traditional preparations of kava are made from the root or rhizome of the plant, where the ground root material is mixed with water and/or coconut water, filtered and then consumed. This tonic has been used to treat a variety of ailments ranging from asthma to warts. In particular, kava has been shown to be effective at minimizing anxiety-related symptoms and recently shown an increase in use among athletes to alleviate pain (Ooi et al., 2018). Extracts of kava prepared using organic solvents or supercritical fluid extraction have been produced to concentrate the active components that have been associated with the beneficial effects. These active components include a unique class of lactones known as kavalactones. As many as eighteen kavalactones have been identified in the rhizome and root of the kava plant with six of the kavalactones; methysticin (M), dihydromethysticin (DHM), kavain (K), dihydrokavain (DHK), yangonin (Y), and desmethoxyyangonin (DMY), constituting 96% of the total kavalactone content. Also, a group of chalcones known as; flavokavain A (FKA), flavokavain B (FKB), and flavokavain C (FKC), are commonly found in kava, in significantly lower quantities (Côté et al., 2004; Lebot et al., 2014).

Despite its potential benefits related to stress relief, relaxation, and sleep support, kava usage has been called into question with approximately 100 cases of liver damage having been associated with the consumption of kava or kava extracts (Organisation mondiale de la santé,

Abbreviations: KL, kavalactones; FK, flavokavains; SFE, supercritical fluid extraction; UHPLC, ultra high-performance liquid chromatography; HepG2, human hepatocellular carcinoma cell line; IC_{50}, half maximal inhibitory concentration; FKB, flavokavain B; M, methysticin; DHM, dihydromethysticin; K, kavain; DHK, dihydrokavain; Y, yangonin; DMY, desmethoxyyangonin; FKA, flavokavain A; FKC, flavokavain C; PM, pipermethystine; UHPLC-UV, ultra high-performance liquid chromatography- ultraviolet; HPLC, high-performance liquid chromatography; ACN, acetonitrile; MeOH, methanol; MΩ, megaohm; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; PES, polyethersulfone; MCE, mixed cellulose ester; FBS, fetal bovine serum; ANOVA, analysis of variance; RxU, relative unit; RLU, relative luminescent unit; RFU, relative fluorescent unit; γ-GT, γ-glutamyl-transferase;; NTP, National Toxicology Program; APAP, acetaminophen

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2007). Beginning in 2002, kava products were being pulled from the market in Australia, Europe, and the US, due to their potential hepatotoxic effects (Centers for Disease Control and Prevention (CDC), 2002). The reports also elicited an advisory from the FDA in the United States (Nutrition, n.d.). Initially thought to be related to organic solvent extractions of kava, it has since been suggested that markers used to assess liver damage can also be credited to traditional kava preparations (Teschke et al., 2009). Detailed analysis of these cases, found the evidence lacking to suggest kava as the casual factor (Teschke et al., 2003). In addition, investigations of hepatotoxicity related to kava are complicated by various factors that are common to herbal products. These include; preexisting liver conditions, concurrent use with drugs and/or other herbal products, product adulteration, improper use, and possible contamination. Other confounding factors specifically related to kava include lack of quality control at the producer level (inappropriate cultivar, plant quality, plant parts used, and mold contamination), and production level (kavalactone content and profile, solvent use, metal contaminants and toxin testing)(Teschke et al., 2011). The German ban on kava was finally overturned in 2014 due to lack of conclusive evidence associating kava with liver toxicity (Kuchta et al., 2015).

Since the reports linking kava to liver toxicity, research into the possible mechanism of action for liver toxicity has been undertaken. The impact of kava extracts and individual kavalactones and flavokavains on factors related to liver function has been investigated, both in vitro and in vivo. These include possible perturbations of the phase I and II detoxification pathways through the inhibition of cytochrome P450 enzyme isoforms and glutathione depletion, respectively, suggesting the potential for interactions with drugs (Mathews et al., 2002; Schmidt, 2003). Additionally, investigation into the impact of kavalactones on cyclooxygenase (COX) activity demonstrated the ability of all six of the major kavalactones and flavokavain A to inhibit both COX-I and COX-II activity, further suggesting the possibility of the interaction of kava with other drugs (Wu et al., 2002).

A variety of in vitro studies using hepatic and non-hepatic derived cells have been performed to investigate the impact of kava on cell viability and health. Interestingly, results varied based on not only the cell-type being used, but also the assay being used to evaluate cytotoxicity. In summary: A) the chalcones, FKA, FKB, and FKC, were reported to have the greatest negative impact on the cell viability of hepatic-derived cells (Jhoo et al., 2006).; B) all six of the major kavalactones have been shown to negatively impact cell viability, individually, at high concentrations.; C) the alkaloid pipermethystine (PM), a possible contaminant found in kava leaves, significantly decreased the cell viability of HepG2 cells (Lim et al., 2007); D) extracts made from kava negatively impact cell viability, with extracts made using organic solvents showing the greatest impact and aqueous extracts showing significantly less impact (Côté et al., 2004; Lüde et al., 2008; Martin et al., 2014; Teschke et al., 2009). Interpretation of cell viability studies using extracts of kava are made difficult due to the lack of information relating to the kava quality and lack of quantitative data relating to kavalactone and flavokavain content of the resulting extract. This lack of data makes it difficult to correlate the results of the individual components to the whole extract.

In this report, we compare the impact of a unique extract of kava on cell proliferation and viability with that of its root source, a noble variety of kava. We demonstrate that the extract impacts cell proliferation and viability in a similar manner as the root source, when samples are normalized to kavalactone content, providing initial evidence that the safety of the extract at the in vitro level is similar to the safety of a traditional source of kava.

2. Materials and methods

2.1. Chemicals

2-propanol (HPLC grade), *o*-phosphoric acid (ACS (85%), acetonitrile (ACN, HPLC grade), methanol (MeOH, LC-MS grade), and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (USA). acetic Acid (ACS grade) was purchased from Research Products International (USA). The chromatography standards, methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain, and flavokavain B were purchased from ChromaDex (USA). The chromatography standard for flavokavain C was purchased from ExtraSynthese (France). The cell toxicity positive control, staurosporine, was purchased from Sigma-Aldrich (USA). Ultrapure (18 MΩ) water was produced using a Barnstead™ GenPure™ Pro Water Purification System from Thermo Scientific (Germany).

2.2. Sample quantification

Chromatographic determination of kavalactones was performed using a Shimadzu Nexera X2 UHPLC system equipped with the gradient pump (model LC-30 CE), autosampler (model SIL-30AC), column oven (model CTO-20AC), degasser (model DGU-20A), controller (model CBM-20A), and photodiode array detector (model SPD-M30A). Chromatograms were monitored at a wavelength of 239 and 350 nm. The full UV spectra were monitored from 190 nm to 800 nm by Nexera X2 UHPLC. System control, data collection and data evaluation were performed using Lab Solution from Shimadzu.

The preferred method for the chemical analysis of kava is liquid chromatography analysis due to its low detection levels and high reproducibility (Bilia et al., 2004). The method used for the analysis of kava samples is that of Meissner and Häberlein with modifications (Meissner and Häberlein, 2005). Samples (10 µL) were separated on an Agilent Zorbax SB-C8 (100 × 2.1 mm, 1.8 µm) reversed phase column under gradient conditions. The mobile phase consisted of solvent A (0.1% formic acid in ultrapure water) and solvent B (50% 2-propanol: 35% ACN: 15% MeOH) at a flow rate of 0.44 mL/min. The following gradient condition was applied: 15% B, 0–0.3 min; 15–31% B, 0.3 – 0.8 min; 31% B, 0.8–11.8 min; 31–50% B, 11.8–12.0 min; 50% B, 12.0–18.5 min; 50–100% B, 18.5–19.0 min; 100% B, 19.0–21.5 min; 100–15% B, 21.5–22.0 min; 15%B, 22.0–25.0 min.

Identification of kavalactones and flavokavains were based on the comparison of retention time and UV spectra to that of kavalactone and flavokavain standards. Quantification was performed using peak area of samples compared to calibration curves of kavalactone and flavokavain standards. The limit of detection (LOD) and limit of quantification (LOQ) of the Kava standard assay were determined by calibration curve method, based on ICH Q2 guidance. Level of detection (LOD) and Level of quantification (LOQ), shown in Table 1, were calculated using the following equations:

$$\mathrm{LOD} = \frac{3.3 \times \textit{Stdev of y – intercept of calibration curve}}{\textit{Slope of calibration curve}} \mathrm{LOQ}$$

$$= \frac{10 \times \textit{Stdev of y – intercept of calibration curve}}{\textit{Slope of calibration curve}}$$

2.3. Sample preparation

Samples used for quantification and cell viability studies are KAVOA™, referred to as SFE kava, and kava root, the starting material used for the production of the KAVOA™ product. KAVOA™ is a supercritical carbon dioxide extract of a noble variety of *Piper methysticum* G. Forst. root from Fiji. Kava root with a minimum age of two years and no aerial components were used for the production of KAVOA™. The following criteria has been suggested as a means to help determine whether kava is of noble or non-noble origin; FKB/(DMY + Y) ≤ 0.09; FK/KL ≤ 0.29; FKB percentage of < 0.15%, FKB/K ratio of < 8.5, and K/(KL-K) ratio of > 25 (Lebot et al., 2014; Fratini Vergano Law Firm, 2015). The source root in this study meets those criteria with 0.03 FKB/(DMY + Y) ratio, 0.01 FK/KL, 0.05% FKB, 3.39 FKB/K ratio, and 25.42 K/(KL-K) ratio. Furthermore, the root has the chemotype 246351 which

Table 1
Kavalactone and flavokavain content of acetonic extractions.

	LOD		LOQ		Acetone					
	µg/mL	µg/mL	mg/g		mg/g		% of Sample		% of Total Kavalactone content	
			SFE	Source	SFE	Source	SFE	Source	SFE	Source
M	0.23	0.77	0.62	0.15	37.38 ± 1.66	14.99 ± 5.2	3.74 ± 0.17	1.50 ± 0.52	15.91 ± 0.67	18.62 ± 3.07
DHM	0.13	0.71	0.57	0.14	25.67 ± 2.99	8.05 ± 3.12	2.57 ± 0.30	0.80 ± 0.31	10.92 ± 1.24	9.88 ± 1.20
K	0.90	0.39	0.31	0.08	48.56 ± 0.95	17.15 ± 9.02	4.86 ± 0.10	1.72 ± 0.90	20.67 ± 0.37	20.27 ± 0.65
DHK	0.24	2.74	2.19	0.55	75.48 ± 4.28	25.47 ± 15.91	7.55 ± 0.43	2.55 ± 1.59	32.13 ± 1.88	29.24 ± 4.39
DMY	0.72	0.73	0.58	0.15	11.32 ± 1.72	4.36 ± 2.25	1.13 ± 0.17	0.44 ± 0.22	4.82 ± 0.75	5.17 ± 0.11
Y	0.16	2.19	1.75	0.44	36.51 ± 2.17	13.95 ± 6.29	3.65 ± 0.22	1.39 ± 0.63	15.54 ± 0.93	16.83 ± 0.89
FKC	0.20	0.49	0.39	0.10	0.54 ± 0.10	0.20 ± 0.02	0.05 ± 0.01	0.02 ± 0.002	0.23 ± 0.04	0.28 ± 0.17
FKA	0.27	0.60	0.48	0.12	0.75 ± 0.04	0.34 ± 0.04	0.08 ± 0.003	0.03 ± 0.004	0.32 ± 0.02	0.45 ± 0.17
FKB	0.26	0.83	0.66	0.17	0.84 ± 0.04	0.52 ± 0.08	0.08 ± 0.004	0.05 ± 0.008	0.36 ± 0.02	0.68 ± 0.24

is indicative of a noble cultivar of kava due to the higher amount of K (4) and minimal amount of DHM (5) (Siméoni and Lebot, 2002)

Acetonic extractions for kavalactone/chalcone quantification were prepared at a ratio of 1.5 g/L or 5 g/L of SFE kava or kava root to acetone, respectively. Samples were sonicated for 30 min at 40 °C in a Fisher sonication bath (Fisher Scientific, USA). Following 10-min centrifugation at 12,000 × g, samples were diluted 2.5x with 18 MΩ water for the determination of kavalactone content, while undiluted samples were used for the determination of flavokavain content. Prior to UHPLC-PDA analysis, samples were centrifuged at 12,000 × g for 10 min.

Water extraction of kava samples for cell treatment were performed by adding approximately 2 g of SFE kava or 4 g of kava root to 25 mL of 18 MΩ water in a 50 mL conical tube. Samples were sonicated for 30 min at 40 °C. Following sonication, samples were centrifuged at 12,000 × g for 15 min. The supernatant was retained and the pellet was further extracted with 25 mL of 18 MΩ water using the above procedure. Again, following sonication, each sample was centrifuged at 12,000 × g for 15 min and the supernatant was retained. The retained supernatant underwent rotary evaporation to remove the solvent. The residue was dissolved in methanol using sonication. Samples were stored in 15 mL conical tubes at 4 °C until use. Samples stored in methanol were diluted 100-fold in methanol and quantitated for kavalactone content using UHPLC-PDA. For cell culture assays, 25 mg of kavalactones of each sample was dried under vacuum and resuspended at a concentration of 50 mg/mL in DMSO and stored at 4 °C.

Preparations for IC_{50} determination were performed in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) complete cell culture media as follows: 0.5 g of SFE or root were mixed with 5 mL DMEM complete media. Samples were sonicated in a water bath at 40 °C for 30 min. Following centrifugation at 4200 rpm using a swing bucket rotor, samples were filtered using a 0.2 µm PES syringe filter under sterile conditions into a sterile 15 mL conical tube and stored at 4 °C until use. Please note, initially a 0.2 µm mixed cellulose ester (MCE) syringe filter was used, but was found to retain the kavalactones. Prior to use, 200 µL of sample was extracted with 700 µL of acetonitrile and analyzed for kavalactone content using UHPLC-PDA.

2.4. Cell culture

HepG2 cells (HB-8065) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/mL Penicillin-Streptomycin at 37 °C in a 5% CO_2 incubator under saturating humidity. Cell passages were maintained in T75 flasks and passages 4 – 15 were used for experiments. Prior to treatments, cells were dissociated with TrypLE Express (Gibco, USA), seeded at the appropriate density for assay, and allowed to adhere overnight. Cell count and viability were determined using an automated cell counter (TC20, Bio-Rad, USA) after Trypan Blue staining (Gibco, USA). Only cells with a viability greater than 95% were used for subsequent assays. Luminescent assays were performed using white, cell culture-treated 96-well microplates (Cat# 136101, Thermo Scientific Nunc, USA) and black-walled, cell culture-treated 96-well microplates (Ref#655090, Greiner Bio-one, Germany) were used for fluorescent assays.

2.5. Cell viability assays

Cell viability was determined using the RealTime-Glo™ MT assay kit (Promega). Assays were performed following the manufacturer's protocol with the following modifications; cells were plated at 1250 cells/well in 50 µL total volume and allowed to adhere overnight; 4 times (4x) concentrated solutions of reagents and cell treatments were made, with 25 µL of each being added to the 50 µL cell volume for a total volume of 100 µL to begin the treatments. The 4x treatments were also performed using water for kavalactone quantification. Viability experiments were performed using extracts resuspended in DMSO. The DMSO concentrations did not exceed 0.1% and each plate contained a DMSO vehicle control for comparison.

Luminescence was measured using a BioTek Cytation 5 multimode reader kept at 37 °C and set for discontinuous kinetic reading. Luminescence was set to autogain.

The RealTime-Glo™ MT assay was used for the IC_{50} and cell viability assay. The IC_{50} experiments were performed twice in triplicate, and the cell viability assay was performed twice with six replicates of each treatment.

2.6. Cell toxicity assays

Cell toxicity was determined using the CellTox™ Green assay kit (Promega). Assays were performed following the manufacturer's protocol with the following modifications; cells were plated at 5000 cells/well in 50 µL total volume and allowed to adhere overnight; 4x solutions of reagents and cell treatments were made, with 25 µL of each being added to begin the treatments. The 4x treatments were also performed in water for kavalactone quantification. Toxicity assays were performed using extracts resuspended in DMSO. The DMSO concentrations did not exceed 0.1% and each plate contained a DMSO vehicle control for comparison.

Fluorescence was measured using a BioTek Cytation 5 multimode reader kept at 37 °C and set for discontinuous kinetic reading. Fluorescence was set to auto.

2.7. Data and statistical analysis

All statistical data are expressed as the mean ± SD and were analyzed using GraphPad Prism version 7.04 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Quantification of

acetonic kavalactone and flavokavain content and average IC_{50} data was analyzed using an unpaired student's *t*-test with statistical significance set at $P < 0.05$. Percentage activity data was analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test and was performed with statistical significance set at $P < 0.05$. The percentage of total kavalactones was calculated as follows: $\frac{x}{M + DHM + K + DHK + Y + DMY} * 100$.

3. Results

3.1. Sample extraction and quantification

Samples were extracted with acetone, water, and cell culture medium to assess their kavalactone and flavokavain profile from UHPLC-PDA quantitation analysis (Tables 1, 2). Acetone was used to give the overall profile due to its ability to extract a majority of the kavalactones (Wang et al., 2015). Data for the acetonic extraction is represented as mg/g of starting material, percent of sample, and percent of total kavalactone content (Table 1). Concentrations in cell culture medium and water extracts are in μg/mL, normalized to a total kavalactone content of 50 μg to represent concentrations determined during cell treatments (Table 2). Data for the water extraction does not have a standard deviation associated with it as only one SFE lot and one source root were used for the extraction. The LOD and LOQ are shown in Table 1.

Acetone extraction of the SFE kava and the source root yielded no significant differences in the kavalactone and flavokavain profile when normalized to percentage of total kavalactone content (Table 1 and Fig. 1). Overall kavalactone percentage of the SFE and source root kava were 23.5% (± 0.06) and 8.4% (± 4.2), respectively.

Samples were also extracted in water and cell culture medium to perform cell viability and IC_{50} assays, respectively. Quantification by UHPLC-PDA was performed to ensure equal kavalactone content was used for the cell viability assays. Following extraction in water, samples were dried using rotary evaporation and resuspended in methanol. The methanol suspension was quantified using the UHPLC-PDA method. Based on quantification, samples in methanol were dried under vacuum and resuspended in DMSO to yield a kavalactone concentration of 50 mg/mL. Following extraction in cell culture medium, samples were filter sterilized using a 0.2 μm PES syringe filter. A sample of the extract was treated with 79% acetonitrile to precipitate the cell culture medium matrix and the supernatant was subsequently analyzed to determine the kavalactone/flavokavain content using UHPLC-PDA. The kavalactone/flavokavain concentration was used to determine the IC_{50} value. Quantification data is shown in Table 2, the three flavokavains were below LOQ.

3.2. IC_{50} determination of SFE and root source kava

IC_{50} values were determined using Promega's RealTime Glo MT assay. The average IC_{50} values of 78.63(± 7.6) and 47.65(± 11.8) μg/mL were determined for three SFE lots and source roots, respectively. Statistically, using an unpaired *t*-test, a P = value of 0.0002 was determined between the SFE kava and source root (Fig. 2C). Experiments were performed in triplicate, with each SFE lot and source root lot being tested in duplicate. A representative IC_{50} curve and individual lot data are shown in Fig. 2A and B, respectively.

Table 2
Kavalactone profile of IC_{50}, cell viability, and cell toxicity treatments.

	Cell Culture Media		Water	
	μg/mL (50 μg total)		μg/mL (50 μg total)	
	SFE	Source	SFE	Source
M	2.84 ± 0.39	1.78 ± 0.03	2.83	1.00
DHM	3.57 ± 0.48	3.23 ± 0.47	3.52	3.69
K	13.29 ± 1.45	12.79 ± 3.02	14.59	7.24
DHK	28.81 ± 2.03	32.1 ± 2.6	26.55	36.69
DMY	1.34 ± 0.42	0.81 ± 0.07	1.48	0.90
Y	1.11 ± 0.42	0.37 ± 0.18[a]	0.82	0.35

[a] – below LOQ.

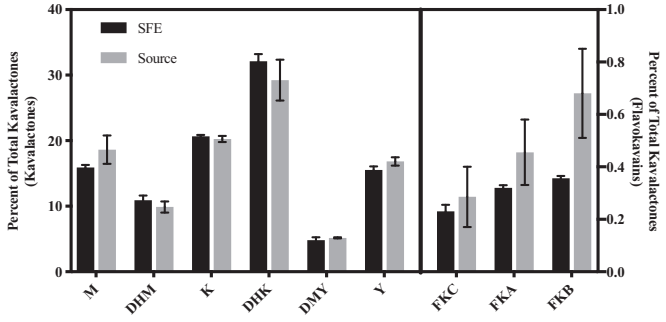

Fig. 1. Kavalactone and Flavokavain content of acetone extract of SFE and source root kava. Data is represented as percent of total kavalactone content ± SEM.

3.3. Cell viability and toxicity of SFE and Kava root source extracted with water

Further analysis was performed on HepG2 cells to investigate cell viability and toxicity. Cell viability/proliferation was determined using the RealTime™ Glo MT assay from Promega. The RealTime™ Glo assay allows for minimal media changes during the assay and also allows for kinetic readings of cell growth. As a measure of cell death, experiments were performed in parallel using Promega's CellTox™ Green assay kit. The data allows us to determine if the impact of kava on the cells is due to a lower proliferation rate as measured by RealTime™ Glo, or an increase in cell death as measured by CellTox™ Green.

Experiments were performed twice using six replicates per trial. The percentage activity of samples compared to the DMSO vehicle control was calculated over a 24-h period as follows:

$$\%Activity = [(RxU_{treatment\ \ 24hr} - RxU_{DMSO\ \ 24hr})/RxU_{DMSO\ \ 24hr}] \times 100$$

RxU represents the Relative Luminescent Unit (RLU) or Relative Fluorescent Unit (RFU) value used for the cell viability or cell toxicity assay, respectively. The percent activity was rescaled to get a baseline of zero percent activity (Hsieh et al., 2017).

The percent activity was compared to cells treated with the DMSO vehicle and 1 μM staurosporine as a positive control. All cells were treated with a total kavalactone concentration of 50 μg/mL, as precipitation occurred at concentrations above 50 μg/mL.

Cells treated with 50 μg/mL SFE kava extracted with water displayed a significant ($p < 0.0001$) increase in luminescent activity of 19% when compared to the DMSO-treated control cells. Whereas, the source root treated cells only exhibited an increase of 1.5% ($p < 0.5$). Positive control treated cells showed a decrease of 27% activity ($p < 0.0001$) over the same 24-h period (Fig. 3A).

In parallel, cells were evaluated for cell toxicity using a fluorescent nuclear dye only permeable to compromised cell membranes. Both SFE kava and the source root showed insignificant increases of fluorescent activity of 8.5% ($p < 0.5$) and 1.8% ($p < 0.5$) when compared to the vehicle control treated cells, respectively. Staurosporine showed an increase of 130% ($p < 0.0001$) (Fig. 3B).

4. Discussion

The current study shows that a supercritical fluid extract of kava (*Piper methysticum*) displays a similar kavalactone profile to the source root from which it was derived. Additionally, the class of compounds

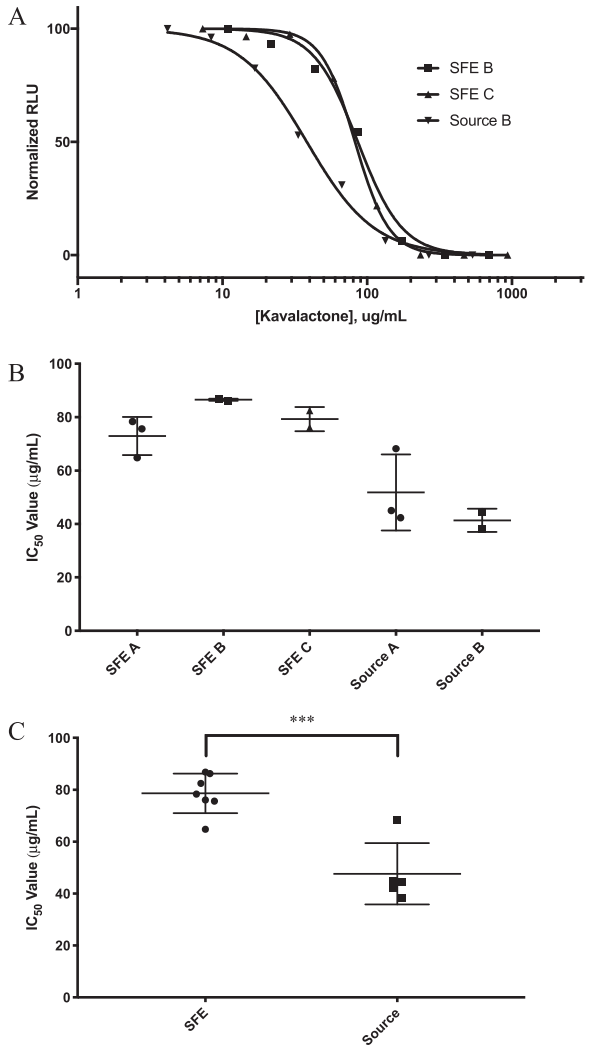

Fig. 2. Effect of SFE and source root kava extracted in cell culture media on cell viability/proliferation. HepG2 cells were treated for 24 h with different concentrations of either SFE or source root kava extracted in cell culture media. Treatments were quantified for kavalactone content (µg/mL). A) Representative IC_{50} plot. B) Individual IC_{50} data of three SFE kava lots and two source root kavas. C) Average IC_{50} data from SFE and source root kava. Scatter plots display the mean (µg/mL) ± SD. (*** represents student's *t*-test of $p < 0.0005$).

known as flavokavains, which have been shown to display in vitro cytotoxicity, are reduced, relative to total kavalactone content, through the supercritical fluid extraction process. Furthermore, when normalized to kavalactone content, the effect of the SFE kava on in vitro viability and toxicity assays were consistent with the SFE kava having a reduced impact on cell viability when compared to the source root.

A major component in this study is not only the complete quantification of the six major kavalactones and three flavokavains in the SFE kava and the source root, but the subsequent use of the quantification to normalize samples prior to viability and toxicity assays. Many of the prior studies investigating the effects of kava preparations on cell viability took place before the presence of the cytotoxic flavokavains were identified, making the interpretation of their results difficult in light of this missing information. Additionally, comparison of kava preparations on cell viability are difficult to interpret due to the lack of standardization in respect to cell treatments. It is well known that organic solvents like ethanol, methanol, and acetone are efficient at extracting the individual kavalactones and the flavokavains when compared to using water as is done in the traditional preparation of kava (Wang et al., 2015; Xuan et al., 2007). However, instead of comparing the effects of different kava preparations on cell viability using the kavalactone content as the normalizing factor, results are typically communicated with respect to residue content following kava preparation protocols. Exceptions to this are when individual kavalactone or flavokavains are being studied.

A major finding in the current study is the reduced impact the SFE kava on cell viability in relation to the source root when treatments are normalized to kavalactone content. Impact of kava treatments on the hepatoma cell line, HepG2 were evaluated using Promega's cell viability assay RealTime-Glo™ MT and their cytotoxicity assay, CellTox™ Green. Following the extraction of the SFE and source kava using water to; mimic the traditional preparation of kava, simulate the aqueous environment of the body when used as supplements, and simulate the use of kava in ready to drink preparations, samples were quantitated for their kavalactone and flavokavain content. Following quantification, treatment for cellular assays were standardized to a 50 µg/mL final kavalactone content in cell culture medium. The percent activity compared to DMSO-treated cells was determined for the cell viability and cell toxicity after a 24-h treatment. Cell toxicity of both the SFE kava and the root showed no significant increase compared to the DMSO control cells, whereas the positive control treated cells (staurosporine) showed a significant increase in activity when compared to the DMSO treated cells. Interestingly, the results of the cell viability assay indicated a significant increase in activity with the SFE kava treated cells, while the root showed no significant change over the DMSO treated cells. As expected, the staurosporine treated cells showed a significant decrease in activity compared to control. Combined, the data shows that treatment with 50 µg/mL kavalactone content from either the SFE or root kava has no negative impact on cell viability or increase in cell toxicity compared to the vehicle control treated cells. The surprising increase in cell viability activity with the SFE kava will be investigated in future studies.

Using a different approach to address the impact of the kava samples on cell viability, kava samples were extracted in cell media to create a "saturated" solution with respect to kavalactone content. Samples were subsequently quantified for kavalactone content and diluted, allowing for the calculation of an IC_{50} value. Similar to the previous viability results, the cells treated with the SFE kava had a higher IC_{50} compared to the root kava. An important factor is the lack of the flavokavains found in the culture medium extractions, suggesting any impact on cell viability has little to do with the flavokavain content itself, but may be related to total kavalactone/flavokavain content or a yet undetermined compound(s).

Numerous studies have addressed the effect of crude extract and individual kavalactones/flavokavains on a variety of assays relating to cell health and viability. These assays include; LDH release, ATP levels, mitochondrial membrane potential, release of caspase-3, MTT, and AST assays (Abu et al., 2014; Einbond et al., 2017; Jhoo et al., 2006; Lüde et al., 2008; Martin et al., 2014; Mathews et al., 2005; Nerurkar et al., 2004; Sakai et al., 2012; Tang et al., 2011; Teschke et al., 2009). Additionally, studies have been performed to investigate the impact of kava on cytochrome P450 activity and p-glycoprotein, important factors related to liver metabolism and possible drug-drug interactions (Li et al., 2011; Lim et al., 2007; Mathews et al., 2002; Weiss et al., 2005; Yamazaki et al., 2008; Zou et al., 2004).

Of particular interest to the current study is the effect of crude extracts on these measures. Typically, crude extracts are evaluated to determine the effect of extraction solvent on cell viability, specifically, aqueous versus organic. Overwhelmingly, studies have shown kava extracted with aqueous solvents, display minimal impact on cell viability compared to extraction with organic solvents like acetone, ethanol, or methanol. Due to the lack of standardization of protocols, it is difficult to determine if the difference between solvents is related to a preferential concentration of cytotoxic compounds, or the difference in kavalactone content. Coté et al. looking at the alteration of cytochrome P450 activity between kava extracted with water and acetone showed a

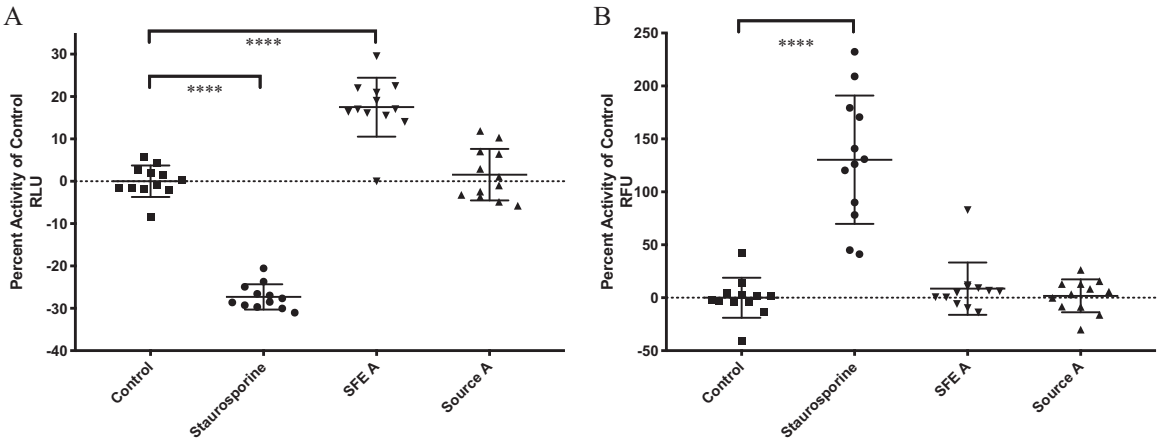

Fig. 3. Effect of 50 μg/mL SFE and source root kava extracted in water on 24-h cell viability and toxicity on HepG2 cells. HepG2 cells were treated for 24 h with SFE and source root kava at 50μg/mL total kavalactone content, DMSO vehicle control and 1 μM staurosporine. A) Cell viability assay represented as percent activity (relative luminescent units) of DMSO control. Data is represented in a scatterplot with mean ± SD (percent activity). * * * * represents an adjusted p-value of < 0.0001. B) Cell toxicity assay represented as percent activity (relative fluorescent units) of DMSO control. Data is represented in a scatterplot with mean ± SD (percent activity). * * * * represents an adjusted p-value of < 0.0001.

greater impact of the acetone extract on the inhibition of the cytochrome P450 isoforms; 3A4, 1A2, 2C9, and 2C19, as indicated by lower IC_{50} values (Côté et al., 2004). Interestingly, when the data was normalized to the kavalactone content, as opposed to the amount of extraction residue, the impact was not only negated, but the IC_{50} values for isoforms 2C9 and 2C19 were lower in the water extractions compared to the acetone extraction. It is likely a similar effect is occurring in the current study.

The unexpected result of increased activity and higher IC_{50} value associated with the SFE derived kava over kava root may suggest the possibility of a non-kavalactone component not found or minimized in the SFE kava that has a negative impact on cell viability/proliferation. Shimoda et al. demonstrated proinflammatory characteristics associated with aqueous extracts of kava that were not replicated using purified individual kavalactones or combinations of individual kavalactones (Shimoda et al., 2012). The SFE kava was standardized to a content of 20% kavalactone and due to the method of extraction may lack these probable components found in the extract of whole root.

Beyond in vitro toxicology studies on kava, numerous animal and human studies have been performed due to the continued interest in kava's psychotropic effects. Studies investigating the effect of self-reported kava usage on measures of liver health have identified increases in the liver enzymes γ-glutamyl-transferase (γ-GT) and alkaline phosphatase (Clough et al., 2003). Interestingly, clinical trials investigating the efficacy of kava, using both well-defined kava extracts and daily kavalactone dosages of less than 250 mg, have yet to identify any hepatoxicity issues, even when used daily for over a month (Gastpar and Klimm, 2003; Lehrl, 2004; Sarris et al., 2013, 2012, 2011, 2009; Volz and Kieser, 1997). Similarly, in rats, long-term administration of kava, up to 380 mg/kg/day show lack of adverse liver effects (DiSilvestro et al., 2007; Narayanapillai et al., 2014; Singh and Devkota, 2003; Sorrentino et al., 2006; Yamazaki et al., 2008). Higher dosages up to 2 g/kg of kavalactones, as demonstrated in a study done by the National Toxicology Program (NTP), lead to an increase in γ-GT, serum cholesterol, liver weight, and increased frequency of hepatocellular hypertrophy (National Toxicology Program, 2012). Interestingly, both the Clough et al and NTP study failed to show an increase in other enzymes (i.e. alanine transaminase (ALT) and aspartate aminotransferase (AST)) that are normally associated with liver toxicity (Clough et al., 2003; National Toxicology Program, 2012). However, co-administration of kava with acetaminophen (APAP) in mice, suggest the possibility of kava potentiating APAP-induced hepatoxicity (Narayanapillai et al., 2014). Furthermore, the authors identified synergistic interaction of the chalcones, FKA and FKB, with APAP in hepatotoxicity. Counter to the APAP results in mice, DiSilvestro et al. failed to show any potentiating effects of kava treatment on rats treated with the hepatotoxin galacatosamine (DiSilvestro et al., 2007). Numerous in vitro studies support the negative effects of the flavokavains on cell health, further suggesting the importance of quantifying and minimizing the amount of the flavokavains found in kava products (Abu et al., 2014, 2016; Chang et al., 2017; Ji et al., 2013; Sakai et al., 2012).

5. Conclusion

In this study we demonstrated that using supercritical carbon dioxide fluid extraction for the extraction of kava produces a product that displays a kavalactone profile similar to the noble kava root it was produced from. In addition, levels of the cytotoxic chalcones, FKA, FKB, and FKC were reduced as a percentage of total kavalactone content. Subsequent assays addressing the effect of the SFE kava and the root source on cell viability and toxicity, demonstrated similar effects when treatments were normalized to kavalactone content. Unexpectedly, the SFE extraction positively impacted the measure of cell viability as indicated by an increase in activity compared to the vehicle control treated cells. This will warrant further investigation in the future.

Author contribution

Greg E. Petersen – Study concept and design, acquisition of data, analysis and interpretation of data, and drafting of manuscript.

Yijin Tang - Acquisition of data, analysis and interpretation of data, and critical review of manuscript.

Christine Fields - Study concept and design, analysis and interpretation of data, and critical review of manuscript.

Author declaration

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G.E.P, Y.T., and C.F. are employees of Applied Food Sciences, Inc.

CRediT authorship contribution statement

Greg E. Petersen: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Yijin Tang:** Investigation, Writing - review & editing. **Christine Fields:** Conceptualization, Formal analysis, Project administration, Writing - review & editing.

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