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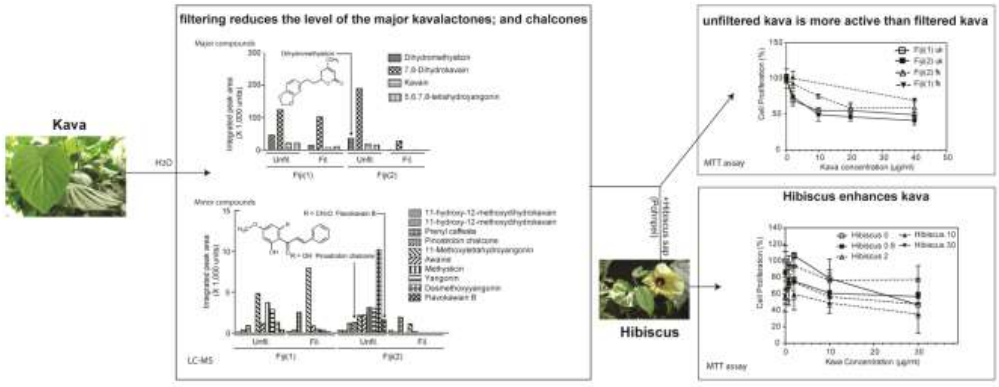
Graphical abstract



Traditional preparations of kava (*Piper methysticum*) inhibit the growth of human colon cancer cells *in vitro*

LS Einbond[a,b,d*], A Negrin[b,c], DM Kulakowski[b,c1], H-A Wu[d1,] V Antonetti[b], F Jalees[b], W Law[a], M Roller[e], S Redenti[b,c], EJ Kennelly[b,c], MJ Balick[a,c]

[a]The Institute of Economic Botany, The New York Botanical Garden, Bronx, New York 10458

[b]Lehman College, The City University of New York, Bronx, NY USA

[c]The CUNY Graduate Center, Biology, Biochemistry and Chemistry Ph.D. Programs, The City University of New York, New York, NY USA

[d]Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

[e]Naturex, Avignon, BP 81218 - 84911 Avignon cedex 9 - France;

[1]Both authors contributed equally to this manuscript.

Person to who reprint requests should be sent:

Linda Saxe Einbond, The New York Botanical Garden, Bronx, NY 10458, USA, lseinbond@gmail.com

*Corresponding author.

Linda Saxe Einbond, The New York Botanical Garden, Bronx, NY 10458, USA

Tel.: +19172091109

E-mail address: lss284@gmail.com;lseinbond@gmail.com

ABSTRACT

Background: Epidemiological studies indicate there is low incidence of colon cancer in the South Pacific islands, including Fiji, West Samoa, and Vanuatu. Cancer incidence has been shown to be inversely associated with kava (*Piper methysticum* G. Forst.) ingestion.

Hypothesis/Purpose: Kava prepared traditionally will inhibit the growth of human cancer cells. This investigation entails preparation and analysis of kava extracts and study of the growth inhibitory activity of the extracts, alone and combined with hibiscus.

Study Design: We will prepare kava as in Micronesia -- as a water extract, high in particulate content, alone or combined with sea hibiscus (*Hibiscus tiliaceus* L.) -- and examine the components and growth inhibitory activity.

Methods: We obtained ground kava prepared in the traditional way from lateral roots and sea hibiscus mucilage and sap from different sources in Micronesia, and prepared water extracts (unfiltered, as well as filtered, since in traditional use the kava beverage contains a high particulate content) and partitions. We used the MTT assay to determine the growth inhibitory activity of the preparations on colon and breast cancer cells and nonmalignant intestinal epithelial cells.. LC-MS analysis was used to examine the components of the kava and sea hibiscus extracts and partitions.

Results: Traditional preparations of kava inhibit the growth of breast and colon cancer cells. Among the kava preparations, the order of decreasing activity was Fiji(2), Fiji(1), Hawaii; the unfiltered preparations from Fiji were more active than the filtered. Phytochemical analysis indicated that

filtering reduced most kavalactone and chalcone content. For example, for Fiji(2), the ratio of dihydromethysticin in filtered/unfiltered kava was 0.01. Thus, for the extracts from Fiji, growth inhibitory activity correlates with the content of these compounds. Unfiltered and filtered kava from Fiji(1) were more active on malignant than nonmalignant intestinal epithelial cells. Since kava is prepared in Micronesia by squeezing the extract through sea hibiscus bark, we assayed the growth inhibitory activity of combinations of kava and sea hibiscus sap and found that sea hibiscus enhanced the growth inhibitory effect of kava.

Conclusion: Our results show that traditional kava, alone or combined with sea hibiscus, displays activity against human cancer cells and indicate it will be worthwhile to develop and further analyze these preparations to prevent and treat colon and other cancers. Our findings suggest it is important to examine the activity of plants in the form that people consume them.

Keywords: Piper methysticum, Piperaceae; Chalcone; *Hibiscus tiliaceus*; Malvaceae; Kavalactone

Abbreviations:

CI, Combination Index; DHK, dihydrokavain; DHM, dihydromethysticin; DMSO, dimethylsulfoxide; EtOH, ethanol; fk, filtered kava; FKA, flavokawain A; FKB, flavokawain B; F, Fiji; H, hibiscus; hk, Hibiscus Kosrae; hp, Hibiscus Pohnpei; k, kava; K, kavain; Nat-k, Naturex kava; PTFE, polytetrafluoroethylene; uk, unfiltered kava.

Introduction

There is a low incidence of colon cancer in the South Pacific Islands, including Fiji, West Samoa, and Vanuatu (Steiner, 2000: Foliaki. et al., 2011). The incidence of cancer (2000-2005) is lower for people in the Pacific (Tonga, Fiji, Cook Islands, Niue) compared to Pacific people living in New Zealand (Age-standardised cancer incidence rates:,315 per 100,00 person-years in females, 379 in males), which is similar to the rates for New Zealand overall. For Fiji, the rates were 231 and 126 (per 100,00 person-years for females and males), respectively.

Traditional aqueous preparations of kava (*Piper methysticum* Forst; family: Piperaceae; Fig. 1A) are used in Pacific Island nations as a sacred ceremonial, as well as a medicinal herb, for conditions that include inflammation (Balick and Lee, 2002; Martin et al. 2014). One study (Steiner, 2000) suggests that cancer incidence (including colon cancer) is inversely associated with kava ingestion (Steiner, 2000). This suggests that traditional preparations of kava may contain chemopreventive components. To probe this relationship, we prepared kava using traditional methods.

About 200 cultivars of kava have been identified (Martin et al., 2014). The kava act passed in Vanuatu in 2002 placed kava in 4 groups: 1) noble: traditional use as a safe drink; 2) medicinal: used by traditional herbalists; 3) Tuday ('Tu dei): have a strong effect lasting two days; 4) Wichmannii: wild varieties.

The active components are present mostly in the roots (or rhizomes). More than 30 secondary metabolites have been identified in the roots of kava, including 19 kavalactones, 3 dihydrochalcones (flavokavins, flavokawains) and 8 minor components (Fig. 1C). Traditional aqueous preparations contain about 0.3-20% kavalactones; commercial preparations (ethanol (EtOH) or acetone) contain up to 70% kavalactones.

There is disagreement about the safety of kava preparations. Studies attribute toxic effects to organic solvents used for extraction or to contamination, impurities, handling and storage, age of plant, quality of cultivars (Triolet et al., 2012; Martin et al., 2014) or using the wrong plant part, such as the leaf or stem. A kava ban instituted by Germany in 2002 was lifted in June, 2014 (Kuchta et al., 2015).

Various studies attribute negative effects to kava. The IARC report (2016) found only one epidemiological study of cancer and kava consumption (Steiner, 2000). The report regarded this study as uninformative because measures of exposure and outcome were not adequate and there was lack of tests of statistical significance. Further, Steiner (2000) believes that kava consumption results in less tobacco consumed by smokers; thus showing less cancer for those who consume kava.

The IARC report (2016) comes to the following conclusions: (1) there is not adequate evidence that kava extract is carcinogenic in humans; (2) there is sufficient evidence that kava is carcinogenic in animals. Kava induced cancers of the liver in male and female mice; (3) The overall conclusion of the study is that kava may possibly cause cancer in humans. In addition, the study reports that the NTP study (2012) found kava to induce toxic effects in the liver in mice and rats; in particular, kava caused an increase in the incidence of lesions in the liver of male and female rats and mice and inflammation in the forestomach of female mice.

On the positive side, studies indicate that extracts (organic) and components of kava possess chemopreventive activity and inhibit the growth of cancer cells (Triolet et al., 2012; Martin et al., 2014). Martin et al (2014) analyzed the: 1) chemical composition of 25 available kava products using metabolic fingerprinting and 2) the cytotoxicity on human lung adenocarcinoma cell. They found a high level of variability; the primary source of variability was solvent (100% water or 95% EtOH) and second, kava product.

Regarding *in vivo* activity, Johnson et al. (2008; 2011) reported that kava reduced tumor multiplicity in a mouse model of lung cancer and did not induce liver toxicity. Triolet et al (2012) showed that an ethanolic extract and polar (to mimic the composition of the traditional water preparation) and nonpolar fractions reduced morphological markers of colon cancer in rats treated with a carcinogen (Triolet et al., 2012). The

study also showed, in agreement with Behl et al. (2011), that kava increased relative liver weight, but did not result in liver damage, assessed by histology.

To examine the growth inhibitory activity of traditional kava, we prepared water extracts in a manner similar to the way it is prepared as a traditional ceremonial or medicinal beverage, alone or combined with hibiscus (*Hibiscus tiliaceus* L.; family: Malvaceae; Fig. 1B), and examined the components, as well as the growth inhibitory activity.

Materials and methods

Materials

All solvents and reagents were reagent grade; H_2O was distilled and deionized. We obtained an extract of kava containing 30% kavalactones from Naturex (Item #: 332679). Naturex kava (Nat-k) was dissolved in dimethylsulfoxide (DMSO) (Sigma). The kavalactone, kavain, was obtained from Sigma-Aldrich for use as a standard in the LC-MS analysis.

Plant collection

Kava: Three kava samples derived primarily from lateral roots were obtained for use in this research. One kava was obtained from a market in Fiji (1), a second was packaged in Fiji (2) ("Melanesian Fusion; variety: Madang Short; KL: 13% (HPLC*);* high in kavain and

yangonin; Kava Korporesen, Suva, Fiji Islands") and a third was grown in Hawaii ("Tuday; variety originally from Papua New Guinea; effects reported to last 2 days with high levels of DHK and DHM").

Hibiscus: Two sets of *Hibiscus tiliaceus* samples were collected during fieldwork for use in this analysis. Collections made on the islands states of Pohnpei and Kosrae, Federated States of Micronesia, consisted of *H. tiliaceus* mucilage and sap obtained from fresh stem barkwood, which was harvested using traditional methods of preparation. An additional collection of stem bark was obtained from Pohnpei for comparison of phytochemistry and activity with the mucilage/sap samples. The samples were frozen prior to being returned to the laboratory. All samples were frozen at -80 °C and then lyophilized to eliminate water content and standardize the crude extracts prior to LC-MS analysis.

Preparation of extracts

Kava (Piper methysticum)

Aqueous kava samples were prepared in the laboratory using traditional kava preparation procedures. Ground kava root samples were placed in a semi-porous cloth bag, which was soaked in HPLC grade water at a ratio of 50 g starting kava material extracted in 1.75 l water (It was one kava preparation for filtered/unfiltered. Then one aliquot was filtered, the other was left unfiltered before drying.) and slowly strained through the cloth for 10 min.. The resulting cloudy suspension of the traditional kava aqueous extract was transferred to borosilicate glass jars for storage. An aliquot of the Fiji (1) aqueous extract was passed through Whatman #4 filter paper under vacuum. Aliquots of the Fijian (2) and

Hawaiian kava water extracts were filtered using 0.45 μm polytetrafluoroethylene (PTFE) membrane syringe filters to remove any insoluble materials. The resulting Hawaii filtrate (fk) was further filtered using PTFE in order to obtain a clear filtrate (2xfk). All resulting kava aqueous extracts and filtrates were then frozen at -80 °C and lyophilized to produce a dry powder which was used for phytochemical analysis by LC-MS as well as for bioassay experiments

The starting material used for the extracts and the extract yields were as follows: For kava (Fiji 2): 10.0566 g kava (Fiji 2) starting material was extracted in 350 ml water for 10 min and freeze-dried, yielding 4.470 g kava (Fiji 2) unfiltered crude water extract (0.444 g crude kava water extract per 1.000 gram starting material). For kava (Hawaii): 10.0540 g kava (Hawaii "Tuday") starting material was extracted in 350 ml water for 10 min and freeze-dried, yielding 4.130 g Kava (Hawaii) unfiltered crude extract (0.411 g crude kava water extract per 1.000 gram starting material).

Hibiscus tiliaceus

The freeze-dried samples were ground to homogeneity and extracted in HPLC grade water as well as in 70% methanol/30% water. All extracts were then evaporated to dryness under nitrogen gas. The crude methanolic extracts were further processed using solid phase extraction (SPE). Extracts were resuspended in 0.1% formic acid (aqueous), vortexed, sonicated, and loaded onto 1 ml Strata-X 33u Polymeric Reversed Phase SPE columns (Phenomenex, Torrance, CA) before being washed with 0.1% formic acid and eluted with 100% methanol. The resulting extracts and SPE extracts were stored at -20° C prior to LC-MS analysis and bioassay.

For hibiscus (Pohnpei) the starting material used for the methanol extracts and the extract yields were as follows: 3.8018g *H. tiliaceus* sap/mucilage (Pohnpei) was frozen at -80◦ Celsius and lyophilized yielding 0.1734 g dry weight. We extracted 0.1375 g of freeze-dried *H. tiliaceus* (Pohnpei) sap/mucilage in 35 ml of 70% methanol for 60 min yielding 0.0778 g extract (0.566 grams crude hibiscus methanol extract per 1.000 gram starting material). For hibiscus (Kosrae): 3.1200g *H. tiliaceus* sap/mucilage (Kosrae) was frozen at -80◦ Celsius and lyophilized yielding 0.3249 g dry weight. We extracted 0.2925 g of freeze-dried *H. tiliaceus* (Kosrae) sap/mucilage in 35 ml of 70% methanol for 60 min yielding 0.1426 g extract (0.488 grams crude hibiscus methanol extract per 1.000 gram starting material).

Solvent-solvent partitioning of Hibiscus tiliaceus mucilage/sap extract

The crude methanolic extracts of the *Hibiscus tiliaceus* sap/mucilage from Pohnpei and Kosrae were additionally subjected to a solvent-solvent partitioning scheme based on an increasing polarity gradient in order to identify active partitions. The crude extracts were suspended in HPLC grade water and sequentially partitioned with hexanes, chloroform, and butanol before being evaporated to dryness under nitrogen gas. The resulting hexanes, chloroform, butanol, and water residues were then submitted for LC-MS analysis and bioassay.

Phytochemical analysis of extracts

Sample preparation

Traditional kava aqueous extracts and filtrates were resuspended in LC-MS grade water, vortexed, and sonicated for 5 min prior to analysis by LC-HR-ESI-MS. The *Hibiscus tiliaceus* sap aqueous extracts were redissolved in LC-MS grade water and prepared as above prior to analysis. *Hibiscus tiliaceus* sap crude methanolic extracts, SPE extracts, and partitions were redissolved in LC-MS grade 70% methanol/30% water and prepared as above. All extracts, filtrates, and partitions were then filtered with 0.45 μm PTFE membrane syringe filters prior to analysis using high resolution LC-ToF-MS and LC-qToF-MS.

LC-MS Analysis

Phytochemical analysis of kava and hibiscus samples was performed using LC-ToF-MS and LC-qToF-MS analyses. LC-ToF-MS was performed on a Waters LCT Premier XE ToF mass spectrometer (Waters, Milford, MA, USA). High resolution LC-qToF-MS was performed on a Waters Acquity UPLC tandem to a Waters Xevo G2 qToF mass spectrometer equipped with an ESI probe. A Waters Acquity BEH C18 UPLC column (50 mm x 2.1 mm i.d., 1.7 μm particle size) was employed coupled to a Waters Acquity UPLC BEH C18 VanGuard pre-column (5 mm x 2.1 mm). Gradient elution was performed with a mobile phase consisting of MS grade 0.1% formic acid (aqueous) as Solvent A and MS grade acetonitrile as Solvent B. The flow rate was held at 0.5 ml/min and the column was maintained at 40 °C during analysis. Samples were analyzed both in positive and negative polarity. The MS parameters were as follows: the capillary voltage was maintained at 3.0 kV, source temperature at 150 °C, desolvation temperature at 450 °C, desolvation gas flow at 800 l/h, cone gas flow at 50 l/h, and argon was used as collision gas at a flow

rate of 0.15 ml/min. A solution of leucine enkephalin standard (Sigma-Aldrich, St. Louis, MO) was used as lockmass during analysis. All extracts, filtrates, and partitions were filtered with 0.45 µm PTFE membrane syringe filters prior to analysis.

PCA analysis on the qToF data

Peaks were detected using MarkerLynx XS software between 100-1000 daltons for mass spectra with marker intensity thresholds above 1000 counts. Mass spectral data were aligned using relative retention time, a 0.10 min retention time window, a 0.01 dalton XIC window, and a 0.05 mass window to determine potential marker compounds.

Cell cultures

HT29 (p53 positive) colon and IEC-6 rat intestinal epithelial cells were obtained from ATCC (Manassas, VA). MCF7 human breast cancer cells were the kind gift of Dr. Moira Sauane (Lehman, CUNY, Bronx, NY). MCF7 and IEC-6 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco BRL Life Technologies, Inc, Rockville, MD) containing 10% (v/v) fetal bovine serum (FBS) (Gibco) (for IEC-6, plus insulin), while HT29 were maintained in McCoy's media plus 10% FBS; at 37 °C, 5% $C0_2$, plus Pen Strep (Gibco).

Proliferation Assay

The MTT cell proliferation assay system (Roche Diagnostic, Indianapolis, IN) was used to determine the sensitivity of the various cell lines to agents, as previously described (Einbond et al., 2007). The kava and hibiscus solutions were sonicated for 10 min before treating the cells.

Statistical analysis

For cell growth assays, the data are expressed as mean +/- standard deviation. Control and treated cells were compared using the student's t-test ($p < 0.05$).

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Calculating the Combination Index

To determine the Combination Index (CI), we treated cells with all combinations of 5 concentrations of each of the agents tested and a solvent control (Einbond et al., 2006). The results of the MTT assay were analyzed for possible synergistic effects using the median effect principle. We employed variable ratios of drugs and assumed mutually exclusive equations (Einbond et al., 2006).

Results

Phytochemical analysis of kava extracts

We prepared traditional kava water extracts, unfiltered, as well as filtered, since in traditional use the kava beverage contains a high particulate content. To reveal the components that are responsible for the growth inhibitory activity, we compared the profiles of the unfiltered

kava (uk) and filtered kava (fk) extracts using LC-MS analysis. To obtain fk, Fiji(1) uk was passed through Whatman #4 filter paper, while Fiji(2) and Hawaii uk were passed through 0.45 μm PTFE membrane syringe filters. Prior to LC-MS analysis, all extracts were passed through PTFE filters. Targeted relative quantitation (using integrated peak area) on the levels of 12 compounds, including kavalactones, chalcones and the piperidine alkaloid awaine was performed (Fig. 1C). Table 1 presents the r.t., molecular formula, exact mass and fragment ions of the tentatively identified compounds.

The most abundant compound among all samples is 7,8-dihydrokavain (DHK) (Fig. 2A, Table 3A). Other major constituents are dihydromethysticin, (DHM) 5,6,7,8-tetrahydroyangoin (THY) and kavain (K). The unfiltered extract from Fiji(2) appears to contain a low level of the chalcone flavokawain B (FLKB); the level is greater than that in the unfiltered extracts from Fiji(1) or Hawaii (FIg. 2B).

It is notable that Fiji(1) and Fiji(2) and Hawaii unfiltered extracts contain higher levels of kavalactones (major and most minor) and chalcones than the filtered; the Hawaii 2xfk contains about the same levels as the fk. For Fiji(1), the ratio fk/uk for DHM was 0.31 and for DHK was 0.83; while for Fiji(2) the ratio fk/uk for DHM was 0.01 and for DHK was 0.15. Thus filtering selectively reduced the more lipophilic kavalactone DHM versus DHK content. Every filter has different properties, including polarity (Avdeef et al., 2001; Zhao and Long, 2013). Some filters irreversibly bind certain compounds based on such properties as polarity and structural features. It may be that kavalactones bind to the filter membrane that was used.

For an unsupervised comparison of the global chemical profile between filtered and unfiltered kava extracts we performed a PCA analysis (Fig. 3A) on the qToF data, Extracts clustered according to filtered/unfiltered status revealing that filtration changes the overall composition of the extract more than the cultivar and origin of the kava. Next, the extracts were characterized into two groups; unfiltered and filtered, and OPLS-discriminatory analysis was performed to identify the variables (metabolites) most responsible for separating these two groups (Fig. 3B). The

compounds with the highest difference between filtered and unfiltered groups are K, DHM and DHK (Fig. 3C). These compounds are present in both filtered and unfiltered groups, but in unfiltered extracts they are much more abundant. The supervised OPLS-DA results (Fig. 3C) support the targeted relative quantitation described above, in that major kavalactone contents are decreased by filtration.

Traditional preparations of kava inhibit the growth of human colon cancer cells

Kava from Fiji

The growth inhibitory activity of the kava preparations was assayed on HT29 colon cancer cells. Unfiltered kava extract (uk) from Fiji was dissolved in 3 solvents: water, EtOH and DMSO; we dissolved filtered kava extract (fk) in water (S.1.1). The IC_{50} values, the concentration that caused 50% inhibition of cell growth, were as follows: uk: (water) 20 µg/ml; (EtOH): >20 µg/ml; (DMSO): >50 µg/ml; f-k (water): ~50 µg/ml; (Fig. 4A; S.1.2; Fig. S.1). Thus for the uk solutions, the order of decreasing activity was: water, EtOH, DMSO. Uk (water) was more active than fk (water) on HT29 cells.

Kava from Fiji and Hawaii

To gain insight into the activity of kava we obtained kava from two additional sources, a second sample from Fiji and from Hawaii a "Tuday" extract that claimed to have potent psychoactive (soporific and narcotic) activity that would continue for two days. For the Hawaii powder we prepared a filtered and two times filtered extract (S.1.1)

The IC_{50} values of all the extracts, assayed on human colon cancer cells, were: Fiji(2) uk: 10 µg/ml; Fiji(1) uk: 40 µg/ml; Fiji(2) fk: >40 µg/ml; Fiji(1) fk:>50 µg/ml; Hawaii-2xfk > 100 µg/ml (Hawaii-fk~Hawaii-uk>Hawaii-2xfk); Nat-k: >40 µg/ml (in a second assay: 22 µg/ml; data not shown). Thus the order of decreasing activity for the 3 extracts was: Fiji(2)>Fiji(1)>Hawaii. For Fiji kava, uk was more active than fk on HT29 cells (Fig. 4B).

Relative quantitative data for seven alkaloids in the Hibiscus tiliaceus saps, extracts and partitions

Since kava is prepared in Pohnpei and Fiji by squeezing the pounded roots through the inner bark of hibiscus (see video of this process at http://www.nybg.org/images/video/western_health.html), we next examined the components present in sea hibiscus.

We obtained sea hibiscus sap from two regions of Micronesia, Pohnpei (hp) and Kosrae (hk). To identify active partitions, the hibiscus saps were sequentially partitioned with water, methanol, chloroform (to enrich for nonpolar components) and butanol (to enrich for polar components). LC-MS analysis indicates that the crude saps contained low levels of alkaloids; and the Pohnpei sap contained more alkaloids than the Kosrae sap (S.2.2, Table 2, Fig. 2C, Table 3B). Among the hibiscus partitions, the Pohnpei-butanol fraction contained the highest level of alkaloids. The compounds were identified as alkaloids due to the high resolution LC-MS analysis of the samples (Table 2).

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Growth inhibitory activity of hibiscus preparations

The growth inhibitory activity of the sea hibiscus saps and fractions was tested on human colon cancer cells. For the crude saps (S.2.1, S.2.3), the IC_{50} values on colon cancer cells were: hp:~30 µg/ml ; hk: ~50 µg/ml (Fig. 4A). For the Pohnpei fractions, the order of activity was: butanol

(4)>chloroform (3)>methanol (2)>water (1)> aqueous residue (5) (S.2.3; Fig S.4A; Fig. 5A). The growth inhibitory activity of hibiscus appears to relate to the level of alkaloids (S.2.3; S.2.4).

Growth inhibitory activity on breast cancer cells

We compared the growth inhibitory activity of the kava and sea hibiscus extracts (uk and hp) on HT29 colon and MCF7 breast cancer cells. The preparations (Fiji(2) and hibiscus) were more active on colon than breast cancer cells; for kava: the IC_{50} for Fiji(2) uk was 14 μg/ml on HT29 and 26 μg/ml on MCF7 cells (however, Fiji(1) uk was more active on breast than colon cancer cells) (Fig. 5 A,B); for hibiscus, hp and fractions 2 (methanol) and 4 (butanol) were slightly more active on HT29 than MCF7 cells (Fig 5 A,B).

Growth inhibitory activity on malignant vs. nonmalignant cells

To assess the selectivity of the extracts, we assayed the effect on malignant (HT29) compared to nonmalignant rat intestinal epithelial cells (IEC6) and found that Fiji(1) fk preferentially inhibited the growth of malignant vs. nonmalignant cells (Fig. 4A and Fig. S.2). The IC_{50} for Fiji(1) f-k was ~50 μg/ml on HT29 and >100 μg/ml on rat IEC6 cells. Fiji(1) uk was also selective for MCF7 versus IEC6 cells (data not shown).

Hibiscus potentiates the effects of kava. on colon cancer cells

Kava is prepared in Pohnpei by squeezing the extract through the inner bark of hibiscus. Since we are interested in studying preparations that are comparable to what people consume, we combined increasing concentrations of kava (Fiji(1) uk) and sea hibiscus sap (Pohnpei) and

assessed the growth inhibitory activity on human colon cancer cells (Fig. 6A,B). The IC_{50} values for kava and hibiscus alone were 27 μg/ml and about 100 μg/ml, respectively. When increasing concentrations of kava were combined with increasing concentrations of hibiscus, the percent viable cells decreased from 92.47 % after treatment with kava (0.8 μg/ml); to 73.1 % after treatment with kava plus hibiscus 0.8 μg/ml; to 75.4 % after treatment with kava plus hibiscus 2.0 μg/ml; to 60.8 % after treatment with kava plus hibiscus 10 μg/ml: and to 56.7 % after treatment with kava plus hibiscus 30 μg/ml; $p < 0.01$. Thus, hibiscus enhances the growth inhibitory effect of kava on the human colon cancer cell line HT29.

The CI for the combination of kava (2 μg/ml) and hibiscus (10 μg/ml) was approximately 0.64, indicating moderate synergy and for the combination of kava (10 μg/ml) and hibiscus (10 μg/ml) was about 0.56 indicating strong synergy. In preliminary experiments, hibiscus Pohnpei also potentiated the effects of Fiji(2) filtered kava, although the effect was less than that for hibiscus Pohnpei and Fiji(1) unfiltered kava (Fig. S.3).

Phytochemical analysis of combinations of kava and hibiscus.

To explore the effect of kava combined with sea hibiscus, we combined the two extracts in synergistic combinations (1/1 and 1/5) and examined the components using LC-MS. The combination of kava and hibiscus had little effect on solubility of the compounds, and did not show any large changes by LC-MS detection, as shown by the data summarized in Fig. 7. Kavain standard was used for compound confirmation and relative retention time confirmations of compounds identified (Fig. S.5).

Discussion

We prepared kava in the traditional manner and examined the components, as well as the growth inhibitory activity. We have shown for the first time that: 1) unfiltered preparations of kava from Fiji were more active than filtered preparations on human colon and breast cancer cells, and (2) hibiscus potentiates the growth inhibitory activity of kava.

LC-MS analysis indicated that Fiji(1) and Fiji(2) unfiltered extracts contain higher levels of kavalactones (major and most minor) and chalcones than the filtered; therefore, the growth inhibitory activity showed a relationship to levels of kavalactones, in particular, to DHM, THY and K, as well as to the levels of chalcones FKB and pinostrobin chalcone (PC)). Thus, these compounds may be the main active constituents. Also the S-plot (Fig. 3C) shows that the major difference between filtered and unfiltered extracts are the kavalactones. However, since the decrease in activity (fk/uk: Fiji(1)~0.3; Fig. 4A) was less than the decrease in kavalactone and chalcone content, these may not be the only active compounds.

It is surprising that the Hawaii extracts are less active than the extracts from Fiji since they appear to have the same level of kavalactones. One possible explanation is that the kavalactones may not be solely responsible for activity. Minor components of aqueous preparations of kava (Xuan et al., 2008) could contribute to activity; activity may relate to the level of FKB. Lebot et al. (2014) report that the ratio of FKB/(yangonin plus desmethoxyyangonin) is higher in 2-day (0.39) and Wichmanni (0.32) versus noble (0.09) and medicinal (0.10) cultivars. From the LC-MS results, the ratio for the Hawaii extract was 0.04, which is lower than these levels; whereas the ratio for Fiji(1) uk was 0.09 and for Fiji(2) uk, 0.12. The order of ratios corresponds to the order of activity. The Hawaii powder may not be as fresh as the Fiji powders and since FKB may be less stable than the kavalactones; the level of FKB may be lower.

Our findings agree with those of Narayanapillai et al. (2014) that a commercial preparation of kava or a fraction containing mostly kavalactones opposed NNK-induced lung cancer in A/J mice. They identify DHM, not DHK, as the active compound. In addition, our results suggest that the growth inhibitory activity may relate to the relative level of the chalcones FKB and PC. This finding is consistent with studies showing that FKB and PC have anticancer and anti-inflammatory activity (Lin et al., 2009; Martin et al., 2015).

There is disagreement concerning hepatotoxic effects of flavokawain A (FKA) and FKB (Abu et al., 2015). This raises the question of the metabolic products of these kava constituents. Zenger et al (2015) found that FKC is a phase I metabolite of FKB and FKA and monoglucuronides are the main phase II metabolites; while glutathione conjugates (*in vitro*) and mercapturates (*in vivo*) have been identified as reactive metabolites of kavalactones (Olsen et al., 2011).

Our findings agree with those of Martin et al. (2014) that kava products vary widely in chemical composition and resulting cytotoxicity. However, our findings disagree in that: 1) they found no cytotoxic effect for aqueous extracts on human lung adenocarcinoma A549 cancer cells (up to 500 μg/ml); whereas we found strong activity on human colon and breast cancer cells; 2) they did not observe a correlation between concentration of compounds K, DHK, methysticin or DHM and relative cell viability, though they did find an association with the total level of 6 kavalactones; whereas we observed a relation to the level of DHM, THY and K, as well as FKB and PC. The discrepancies between our findings and Martin's (2014) may be due to differences in the cell lines, the kava product, method of preparation and/or analysis.

Traditional preparations of hibiscus weakly inhibit the growth of human colon and breast cancer cells; the activity does not increase when we partition the hibiscus saps or prepare SPE preparations. It is possible that we lose critical components when we partition and filter the preparations. Shimoda et al. (2012) suggest that *Hibiscus tiliaceus* contains mucilage (a polar glycoprotein and a water-soluble

exopolysaccharide) that functions as an excipient. Other components in the bark and stem, among these amides, may contribute to activity (Lim et al., 2014).

Hibiscus enhanced the growth inhibitory effect of both unfiltered and filtered kava. Our studies agree with those of Shimoda et al (2012) who found that the mucilage (premixed with kava) potentiated the effect of a traditional preparation of kava on calcium release in mast cells. The mucilage may function as an excipient. It may bind to, emulsify and stabilize the kava components. Interestingly, Shimoda et al (2012) showed that three purified kava components, alone or in combination, did not induce calcium release in mast cells. Thus, as is the case for kava, the crude preparations have the same or more activity as the purified preparations.

Conclusion

Our results show that kava as prepared in Micronesia, alone or combined with sea hibiscus, displays strong activity against human cancer cells and indicate it will be worthwhile to develop and further analyze these preparations to prevent and treat colon and other cancers. Our findings suggest it is important to examine the effect of traditional preparations of medicinal herbs.

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Conflict of interest

Dr. Marc Roller is the Chief Science Officer of Naturex, which produces extracts of kava, but these extracts were not the focus of this study.

Supplementary material

Supplementary material can be found at

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Table legends

Table 1. Compounds tentatively identified in *Piper methysticum* (kava) water extracts using LC-qToF-MS

Table 2. Compounds tentatively identified in *Hibiscus tiliacsue* water extracts using LC-qToF-MS

Table 3. A) Kava relative concentrations; B) Hibiscus relative concentrations

Figure legends

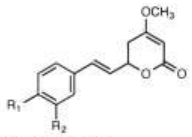
Linda S. Einbond et al. (2016) Figure 1

A

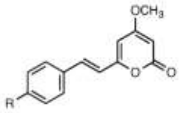
B

C

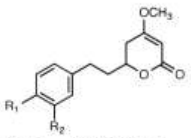
Piper methysticum **compounds**



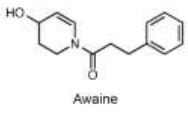
R1=H R2=H Kavain
R1-OCH2O-R2 Methysticin



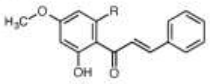
R=H Desmethoxyyangonin
R=CH3O Yangonin



R1=H R2=H Dihydrokavain
R1=CH3O R2=H 5,6,7,8-Tetrahydroyangonin
R1=CH3O R2=OH 11-Hydroxy-12-methoxydihydrokavain
R1=CH3O R2=CH3O 11-Methoxytetrahydroyangonin
R1-OCH2O-R2 Dihydromethysticin



Awaine



R=OH Pinostrobin chalcone
R=CH3O Flavokavain B

Fig. 1. Photos and chemical structures of compounds identified in *Piper methysticum* (kava).

A) *Piper methysticum* (The New York Botanical Garden, Bronx, NY);

B) *Hibiscus tiliaceus* (USDA-ARS, Tropical Agriculture Research Station, Mayaguez, Puerto Rico);

C) Chemical structures of compounds identified in kava water extracts.

Linda S. Einbond et al. (2016) Figure 2

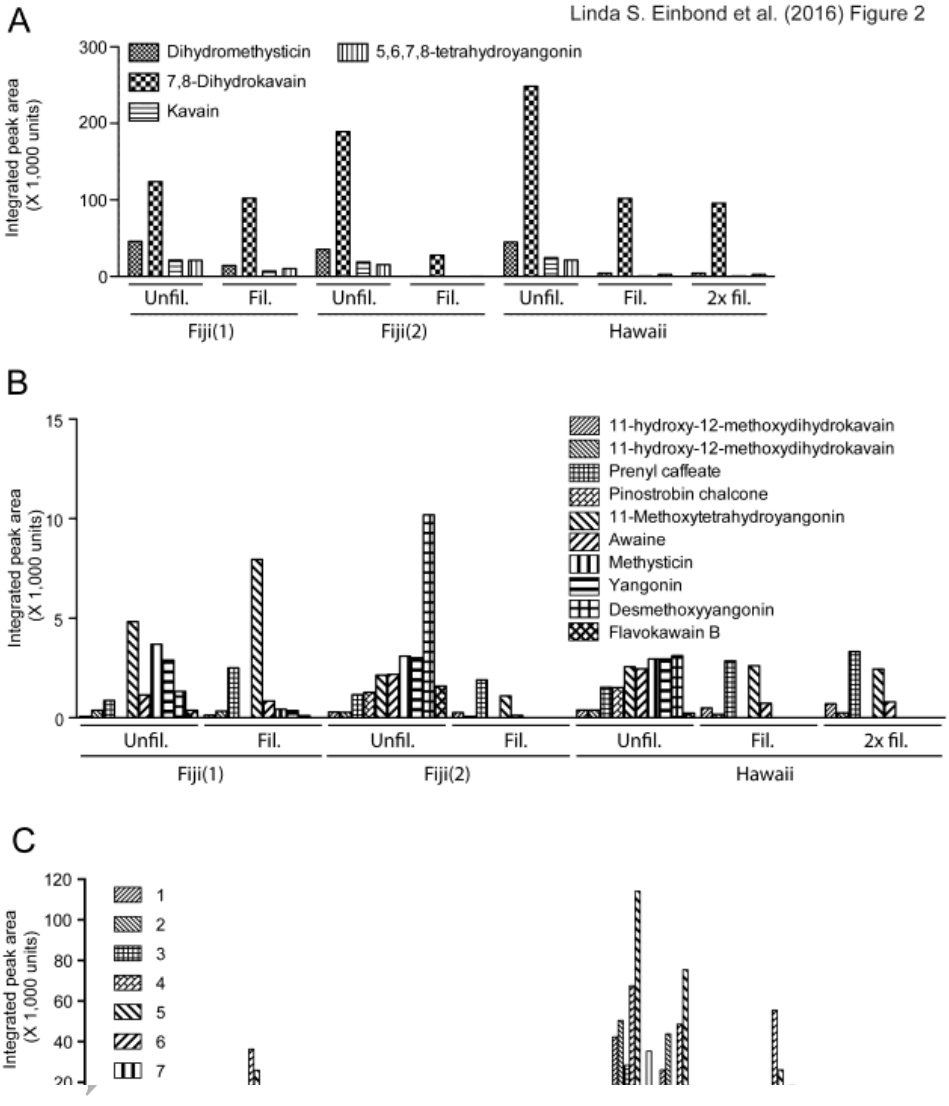


Fig. 2. Relative concentration of compounds identified in kava and hibiscus water extracts using LC-MS: A, B): Kava: A) major compounds; B) minor compounds; C) Hibiscus.

The units for the Y-axis are a measure of integrated peak area for the respective compounds.

Linda S. Einbond et al. (2016) Figure 3

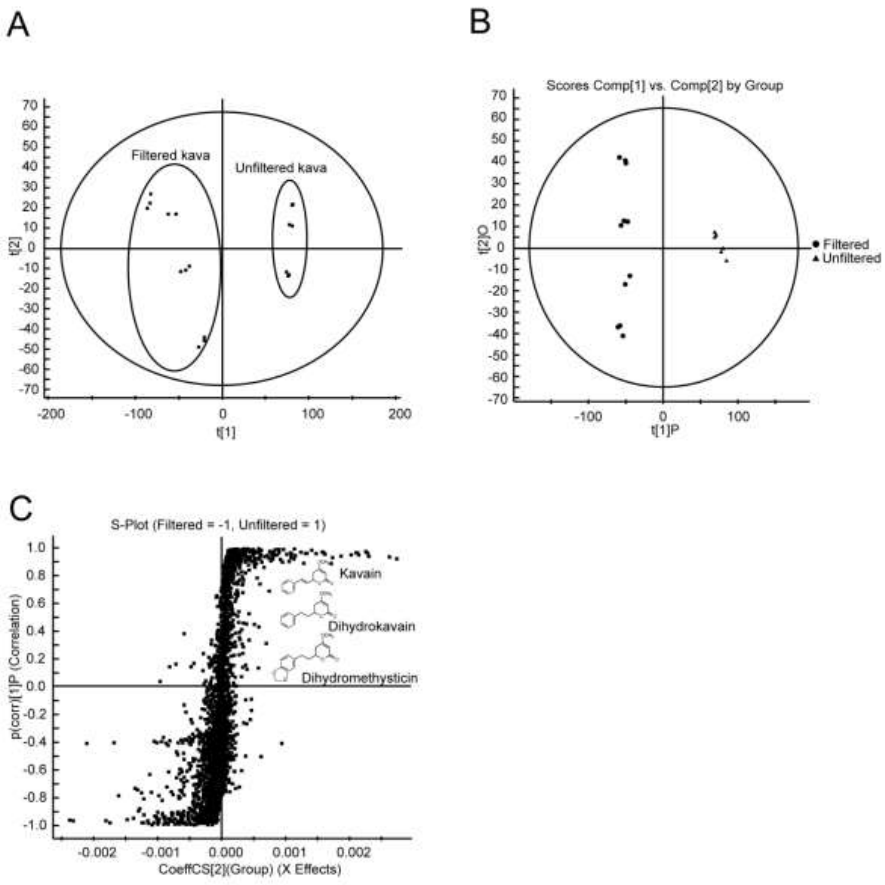


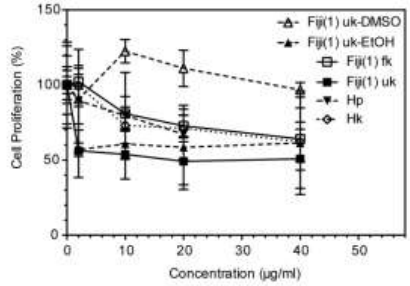
Fig. 3. Statistical Modeling (PCA) and Identification of Marker Compounds (OPLS-DA, S-Plot (Fig. S.3))

in Kava (*Piper methysticum*) water extract preparations (unfiltered/filtered).

Fig. 3 is for all 3 kava samples, both filtered and unfiltered (thus 6 samples total x replicate injections).

Linda S. Einbond et al. (2016) Figure 4

A



B

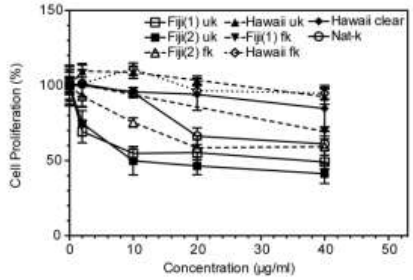


Fig. 4. A) Growth inhibitory activity of extracts of kava and hibiscus on HT29 cells.

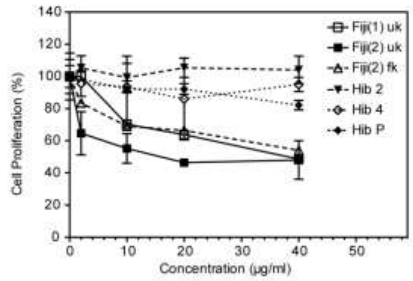
A) Fiji(1); hibiscus Pohnpei and hibiscus Kosrae; B) Fiji(1), Fiji(2) and Hawaii;

Cells were exposed to increasing concentrations of agents for 96 h and the number of viable cells determined by the MTT assay;

A,B) fk = filtered kava; uk = unfiltered kava; D = DMSO; E = ethanol; hp = hibiscus Pohnpei; hk = hibiscus Kosrae.

Linda S. Einbond et al. (2016) Figure 5

A



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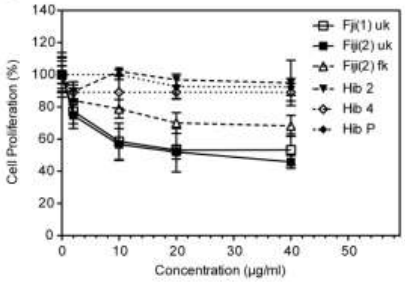


Fig. 5.

Growth inhibitory activity of extracts of kava (Fiji(1) and Fiji(2)) and hibiscus Pohnpei;

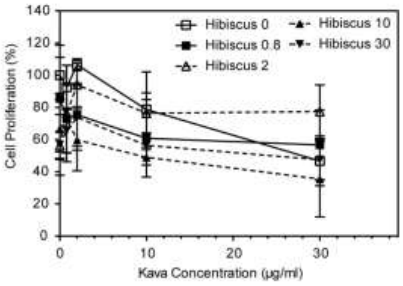
A) HT29 cells; B) MCF7 cells;

Cells were exposed to increasing concentrations of agents for 96 h and the number of viable cells determined by the MTT assay.

fk = filtered kava; uk = unfiltered kava; hibiscus Pohnpei: 2) methanol; 4) butanol; P) Pohnpei sap;

Linda S. Einbond et al. (2016) Figure 6

A



B

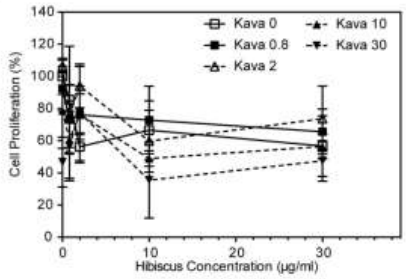


Fig. 6.

Synergistic combinations of kava (Fiji (1)) and hibiscus Pohnpei on HT29 colon cancer cells.

A: x-axis: kava; B: x-axis: hibiscus.

We treated cells with all combinations of 5 concentrations of each of the agents tested and a solvent control (Einbond et al. 2006). Cells were exposed to increasing concentrations of agents for 72 h and the number of viable cells determined by the MTT assay; the effect would be stronger if the time were 96 h.

Figure 7

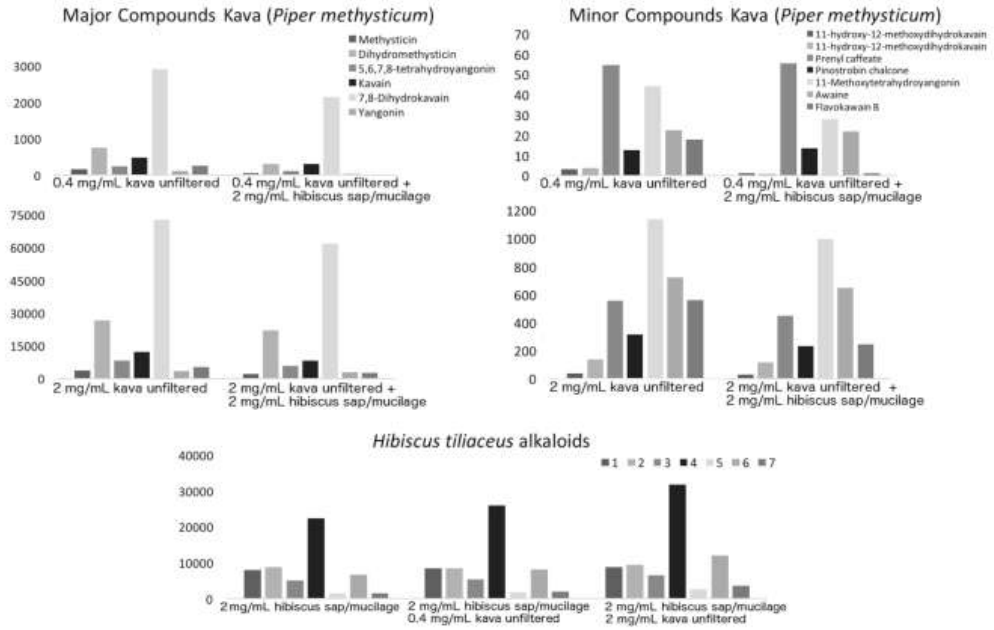


Fig. 7. Relative concentration of compounds identified in kava and hibiscus water extracts, alone or in combination, using LC-MS:

We combined kava and hibiscus in synergistic ratios (1/1 or 1/5). The units for the Y-axis are a measure of integrated peak area for the respective compounds.

Table 1. Compounds identified in *Piper methysticum* (kava) water extracts using LC-qToF-MS

Retention Time	Ion	Molecular Formula	*m/z* observed	ppm	Adduct and fragmental in exact masses $[M+X]^+$ or $[M-X]^+$"	Molecular Formula & ppm	Compound	Reference*
1.466	$[M+H]^+$	$C_{15}H_{18}O_5$	279.1230	-0.7	579.2205 $[2M+Na]^+$ 301.1054 $[M+Na]^+$ 261.1129 $[M+H-H_2O]^+$ 163.0758 $[M+H-C_9H_8O_3]^+$ 137.0604 $[M+H-C_7H_{10}O_3]^+$	($C_{30}H_{36}O_{10}Na$, −0.2) ($C_{15}H_{18}O_5Na$, −0.7) ($C_{15}H_{17}O_4$, −0.8) ($C_{10}H_{11}O_2$, −0.6) ($C_9H_9O_2$, 0.7)	11-hydroxy-12-methoxydihydrokavain	DOI: 10.1007/s11418-007-0203-2
1.533	$[M+H]^+$	$C_{15}H_{18}O_5$	279.1232	0.0	579.2214 $[2M+Na]^+$ 301.1046 $[M+Na]^+$ 261.1125 $[M+H-H_2O]^+$ 163.0760 $[M+H]^+$ 137.0602 $[M+H-C_7H_{10}O_3]^+$	($C_{30}H_{36}O_{10}Na$, 1.4) ($C_{15}H_{18}O_5Na$, −2.0) ($C_{15}H_{17}O_4$, −0.8) ($C_{10}H_{11}O_2$, 0.6) ($C_8H_9O_2$, −0.7)	11-hydroxy-12-methoxydihydrokavain	DOI: 10.1007/s11418-007-0203-2
1.604	$[M+H]^+$	$C_{14}H_{16}O_4$	249.1130	2.0	519.1993 $[2M+Na]^+$ 271.0951 $[M+Na]^+$ 231.1022 $[M+H-H_2O]^+$ 213.0917 $[M+H-H_2O-H_2O]^+$ 185.0966 $[M+H-H_2O-H_2O-CO]^+$	($C_{28}H_{32}O_8Na$, −0.4) ($C_{14}H_{16}O_4Na$, 1.8) ($C_{14}H_{15}O_3$, 0.4) ($C_{14}H_{13}O_2$, 0.5) ($C_{13}H_{13}O_1$, 0.0)	Prenyl caffeate	DOI: 10.1007/s11418-007-0203-2
1.827	$[M+H]^+$	$C_{16}H_{14}O_4$	271.0973	1.1	293.0793 $[M+Na]^+$ 269.0818 $[M-H]^-$ 167.0346 $[M+H-C_8H_8]^+$	($C_{16}H_{14}O_4Na$, 1.0) ($C_{16}H_{13}O_4$, 1.5) ($C_8H_7O_4$, 1.2)	Pinostrobin chalcone	DOI: 10.1007/s11418-007-0203-2
1.914	$[M+H]^+$	$C_{16}H_{20}O_5$	293.1386	-1.0	315.1208 $[M+Na]^+$ 275.1282 $[M+H-H_2O]^+$ 177.0916 $[M+HC_8H_8O_3]^+$ 151.0762 $[M+H-C_7H_{10}O_3]^+$ 131.0497 $[M+H-C_7H_{14}O_4]^+$	($C_{16}H_{20}O_5Na$, 0.0) ($C_{16}H_{18}O_4$, −0.4) ($C_{11}H_{13}O_2$, 0.0) ($C_9H_{11}O_2$, 2.0) ($C_9H_7O_1$, 0.0)	11-Methoxytetrahydroyangonin	DOI: 10.1007/s11418-007-0203-2
2.032	$[M+H]^+$	$C_{14}H_{17}N_1O_2$	232.1340	0.9	161.0606 $[M+H-C_4H_9N]^+$	($C_{10}H_9O_2$, 1.9)	Awaine	DOI:10.1016/S0031-9422(03)00111-0
2.413	$[M+H]^+$	$C_{15}H_{14}O_5$	275.0928	3.3	571.1580 $[2M+Na]^+$ 297.0748 $[M+Na]^+$ 257.0818 $[M+H-H_2O]^+$ 243.0663 $[M+H-CH_3OH]^+$ 233.0818 $[M+H-C_2H_2O]^+$ 225.0549 $[M+H-H_2O-CH_3OH]^+$ 159.0448 $[M+H-C_9H_8O_3]^+$	($C_{30}H_{28}O_{10}Na$, 0.0) ($C_{15}H_{14}O_5Na$, 3.0) ($C_{15}H_{13}O_4$, 1.6) ($C_{14}H_{11}O_4$, 2.5) ($C_{13}H_{13}O_4$, 1.7) ($C_{14}H_9O_3$, −1.3) ($C_{10}H_7O_2$, 1.3)	Methysticin	DOI:10.1007/s11418-011-0613-z
2.430	$[M+H]^+$	$C_{15}H_{16}O_5$	277.1078	0.7	575.1897 $[2M+Na]^+$ 299.0896 $[M+Na]^+$ 259.0972 $[M+H-H_2O]^+$ 245.0817 $[M+H-CH_3OH]^+$ 231.1024 $[M+H-C_2H_2O]^+$ 227.0710 $[M+H-H_2O-CH_3OH]^+$ 161.0604 $[M+H-C_9H_8O_3]^+$ 135.0447 $[M+H-C_7H_{10}O_3]^+$	($C_{30}H_{32}O_{10}Na$, 0.7) ($C_{15}H_{16}O_5Na$, 0.3) ($C_{15}H_{15}O_4$, 0.8) ($C_{14}H_{13}O_4$, 1.2) ($C_{14}H_{15}O_3$, 1.3) ($C_{14}H_{13}O_3$, 0.2) ($C_{10}H_9O_2$, 0.6) ($C_8H_7O_2$, 0.7)	Dihydromethysticin	DOI:10.1055/s-0034-1382949
2.585	$[M+H]^+$	$C_{15}H_{18}O_4$	263.1283	0.0	547.2311 $[2M+Na]^+$ 285.1102 $[M+Na]^+$ 245.1180 $[M+H-H_2O]^+$ 217.1229 $[M+H-CH_2O_2]^+$ 147.0810 $[M+H-C_9H_8O_3]^+$ 121.0653 $[M+H-C_7H_{12}O_3]^+$	($C_{30}H_{36}O_8Na$, 0.5) ($C_{15}H_{18}O_4Na$, −0.4) ($C_{15}H_{17}O_3$, 0.8) ($C_{14}H_{17}O_2$, 0.0) ($C_{10}H_{13}O$, 0.0) (C_8H_9O, 0.0)	5,6,7,8-Tetrahydroyangonin	DOI:10.2174/1874065000903010022
2.620	$[M+H]^+$	$C_{14}H_{14}O_3$	231.1024	1.3	483.1786 $[2M+Na]^+$ 253.0843 $[M+Na]^+$ 213.1024 $[M+H-H_2O]^+$ 199.0763 $[M+H-CH_3OH]^+$ 185.0966 $[M+HHCOOH]^+$ 181.0655 $[M+HCH_3O_3]^+$ 155.0862 $[M+H-C_2H_2O_3]^+$ 153.0705 $[M+H-C_2H_4O_3]$ 129.0705 $[M+H-C_4H_8O_3]^+$ 115.0546 $[M+H-C_5H_8O_3]^+$	($C_{28}H_{28}O_6Na$, 0.4) ($C_{14}H_{14}O_3Na$, 0.8) ($C_{14}H_{13}O_2$, 1.4) ($C_{13}H_{11}O_2$, 2.0) ($C_{13}H_{13}O$, 0.0) ($C_{13}H_9O$, 1.1) ($C_{12}H_{11}$, 0.6) ($C_{12}H_9$, 0.7) ($C_{10}H_9$, 0.8) (C_9H_7, −1.7)	Kavain	DOI:10.1007/s11418-011-0613-z

2.740	$[M+H]^+$	$C_{14}H_{16}O_3$	233.1181	1.3	487.2103 $[2M+Na]^+$ 255.0999 $[M+Na]^+$ 215.1075 $[M+H-H_2O]^+$ 187.1127 $[M+H-HCOOH]^+$ 155.0859 $[M+H-C_3H_6O_3]^+$ 117.0704 $[M+H-C_5H_8O_3]^+$	($C_{28}H_{32}O_6Na$, 1.2) ($C_{14}H_{16}O_3Na$, 0.8) ($C_{14}H_{15}O_2$, 1.4) ($C_{13}H_{15}O$, 2.1) ($C_{12}H_{11}$, −1.3) (C_9H_9, 0.0)	7,8-Dihydrokavain	DOI:10.2174/1874065000903010022
2.861	$[M+H]^+$	$C_{15}H_{14}O_4$	259.0973	-0.8	281.0792 $[M+Na]^+$ 231.1026 $[M+H-CO]^+$	($C_{15}H_{14}O_4Na$, 0.7) ($C_{14}H_{15}O_3$, 2.2)	Yangonin	DOI: 10.1007/s11418-011-0613-z
3.154	$[M+H]^+$	$C_{14}H_{12}O_3$	229.0868	1.3	479.1472 $[2M+Na]^+$ 251.0687 $[M+Na]^+$(201.0916 $[M+H-CO]^+$	($C_{28}H_{24}O_6Na$, 0.2) $C_{14}H_{12}O_3Na$, 1.2) ($C_{13}H_{13}O_2$, 0.0)	Desmethoxyyangonin	DOI: 10.1007/s10068-013-0170-1
3.326	$[M+H]^+$	$C_{17}H_{16}O_4$	285.1129	0.7	307.0949 $[M+Na]^+$ 259.0974 $[M+H-C_2H_2]^+$ 181.0503 $[M+H-C_8H_8]^+$	($C_{17}H_{16}O_4Na$, 1.0) ($C_{15}H_{15}O_4$, 1.5) ($C_9H_9O_4$, 1.1)	Flavokawain B	DOI:10.1007/s11418-007-0203-2

*Note: Please view Supplementary Materials

Table 2. Compounds tentatively identified in *Hibiscus tiliaceus* extracts and partitions using LC-qToF-MS

Compound	Ion	Molecular Formula	*m/z* Observed	ppm	Adduct and fragmental ion exact masses $[M+X]^+$ or $\{M-X\}^-$	Molecular Formula & ppm	Reference*
1	$M+H^+$	$C_{19}H_{31}NO_6$	370.2224	−1.6	352.2119 $[M+H-H_2O]^+$ 368.2068 $[M-H]^-$ 414.2128 $[M-H+HCOOH]^-$	($C_{19}H_{30}NO_5$, −1.4) ($C_{19}H_{30}NO_6$, −1.4) ($C_{20}H_{32}NO_8$, −0.5)	
2	$M+H^+$	$C_{19}H_{31}NO_6$	370.2227	−0.8	368.2070 $[M-H]^-$ 414.2128 $[M-H+HCOOH]^-$	($C_{19}H_{30}NO_6$, −0.8) ($C_{20}H_{32}NO_8$, −1.0)	
3	$M+H^+$	$C_{19}H_{29}NO_6$	368.2077	1.1	350.1969 $[M+H-H_2O]^+$ 366.1938 $[M-H]^-$	($C_{19}H_{28}NO_5$, 0.6) ($C_{19}H_{28}NO_6$, 5.7)	
4	$M+H^+$	$C_{19}H_{31}NO_5$	354.2282	0.6	336.2180 $[M+H-H_2O]^+$ 398.2176 $[M-H+HCOOH]^-$ 352.2114 $[M-H]^-$	($C_{19}H_{30}NO_4$, 1.5) ($C_{20}H_{32}NO_7$,−0.8) ($C_{19}H_{30}NO_5$, −2.8)	

5	$M+H^+$	$C_{19}H_{29}NO_5$	352.2121	-0.9	374.1947 $[M+Na]^+$ 334.2018 $[M+H-H_2O]^+$ 316.1906 $[M+H-H_2O-H_2O]^+$ 396.2020 $[M-H+HCOOH]^-$ 350.1962 $[M-H]^-$	($C_{19}H_{29}NO_5Na$, 1.1) ($C_{19}H_{28}NO_4$, 0.0) ($C_{19}H_{26}NO_3$, −2.2) ($C_{20}H_{30}NO_7$, −0.5) ($C_{19}H_{28}NO_5$, −1.4)	CAS: 1638875-82-7 DOI:10.5012/bkcs.2014.35.3.919
6	$M+H^+$	$C_{19}H_{31}NO_5$	354.2276	0.3	352.2116 $[M-H]^-$ 398.2175 $[M-H+HCOOH]^-$	($C_{19}H_{30}NO_5$, −2.3) ($C_{20}H_{32}NO_7$,−1.0)	
7	$M+H^+$	$C_{19}H_{29}NO_5$	352.2117	−2.0	350.1964 $[M-H]^-$ 396.2019 $[M-H+HCOOH]^-$	($C_{19}H_{28}NO_5$, −0.9) ($C_{20}H_{30}NO_7$,−0.8)	

*Note: Please view Supplementary Materials

Table 3A. Kava relative concentrations

Compound Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Fiji 1 Unfiltered	46018	123936	21769	21103	69	361	868	0	4823	1152	3695	2889	1328	373
Fiji 1 Filtered	14426	102385	7398	10367	130	322	2511	0	7957	840	426	368	110	0
Fiji 2 Unfiltered	35477	189300	19164	15410	284	261	1157	1275	2143	2179	3097	3012	10192	1592
Fiji 2 Filtered	440	27805	0	370	266	58	1904	0	1103	121	0	0	0	0
Hawaii Unfiltered	44948	248621	25056	21535	393	374	1531	1517	2559	2459	2953	2951	3124	241
Hawaii Filtered	4430	102030	966	2788	492	158	2856	0	2606	731	0	0	0	0
Hawaii 2x	4335	95801	884	2861	701	229	3329	0	2452	794	0	0	0	0

Filtered														

Values shown are averages of multiple injections (n=3) of integrated peak areas as determined by LC-MS analysis and are intended for comparison of relative abundances between samples and sample preparations (unfiltered/filtered).

Key of the compounds:

1	Dihydromethysticin	8	Pinostrobin chalcone
2	7,8-Dihydrokavain	9	11-Methoxytetrahydroyangonin
3	Kavain	10	Awaine
4	5,6,7,8-tetrahydroyangonin	11	Methysticin
5	11-hydroxy-12-methoxydihydrokavain	12	Yangonin
6	11-hydroxy-12-methoxydihydrokavain	13	Desmethoxyyangonin
7	Prenyl caffeate	14	Flavokawain B

Table 3B. Hibiscus relative concentrations

Compound Number	1	2	3	4	5	6	7
3966 Pohnpei sap	1439	1831	1057	2585	4925	444	1269
3966 Pohnpei Water extract	759	1060	652	645	2684	400	638
3966 Pohnpei MeOH extract	3099	4242	2378	13080	11021	2825	3384
3966 Pohnpei Chloroform partition	2407	3408	1270	5025	4294	1056	2175
3966 Pohnpei Butanol partition	11983	16628	10985	55531	26214	8888	18088
3966 Pohnpei Water residue	733	654	276	566	645	66	173
3966 Pohnpei SPE	42330	50510	28436	67596	114042	11502	35371
3964 Pohnpei SPE	26281	27954	19748	62993	67091	14788	22707
3965 Pohnpei stem bark SPE	26168	43919	12607	48632	75380	9525	8801
3967 Kosrae sap	27	225	25	671	469	16	0
3967 Kosrae Water extract	14	108	10	528	194	14	0
3967 Kosrae MeOH extract	34	288	31	1661	600	0	0
3967 Kosrae Hexane partition	0	0	0	111	44	0	0
3967 Kosrae Chloroform partition	29	246	9	1140	275	23	0
3967 Kosrae Butanol partition	62	696	0	3619	0	0	0
3967 Kosrae Water partition	31	325	0	861	0	37	0
3967 Kosrae Water residue	0	0	0	0	0	0	0
3967 Kosrae SPE	2032	13992	2599	36405	25945	2635	391

Values shown are averages of multiple injections (n=3) of integrated peak areas as determined by LC-MS analysis and are intended for comparison of relative abundances between preparations.