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# In Vitro Cytotoxicity of Nonpolar Constituents from Different Parts of Kava Plant (*Piper methysticum*)

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Kava (*Piper methysticum*), a perennial shrub native to the South Pacific islands, has been used to relieve anxiety. Recently, several cases of severe hepatotoxicity have been reported from the consumption of dietary supplements containing kava. It is unclear whether the kava constituents, kavalactones, are responsible for the associated hepatotoxicity. To investigate the key components responsible for the liver toxicity, bioassay-guided fractionation was carried out in this study. Kava roots, leaves, and stem peelings were extracted with methanol, and the resulting residues were subjected to partition with a different polarity of solvents (hexane, ethyl acetate, *n*-butanol, and water) for evaluation of their cytotoxicity on HepG2 cells based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and lactate dehydrogenase and aspartate aminotransferase enzyme leakage assays. Organic solvent fractions displayed a much stronger cytotoxicity than water fractions for all parts of kava. The hexane fraction of the root exhibited stronger cytotoxic effects than fractions of root extracted with other solvents or extracts from the other parts of kava. Further investigations using bioassay-directed isolation and analysis of the hexane fraction indicated that the compound responsible for the cytotoxicity was flavokavain B. The identity of the compound was confirmed by $^{1}$H and $^{13}$C NMR and MS techniques.

**KEYWORDS: Kava; *Piper methysticum*; cytotoxicity; Kava toxicity; flavokavain**

## INTRODUCTION

Kava (*Piper methysticum*), a perennial shrub native to the South Pacific islands, has been used for the preparation of an important beverage for social ceremonies of the islanders (*1*). Since the introduction of kava to Western countries, commercial kava root extracts have become widely used for the treatment of nervous anxiety, tension, and restlessness and as an alternative treatment for various mental disorders (*2*, *3*). Kava has been regarded as effective, nonaddictive, and safe (*3*, *4*). Six major kavalactones (kavain, 5,6-dihydrokavin, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin) have been identified from kava root and demonstrated to be the active constituents responsible for the pharmacological effects on central nervous system receptors and neurotransmitters (*2*, *3*).

Recently, a number of cases of severe hepatotoxicity have been reported to be associated with the consumption of herbal products containing kava, which has resulted in a warning to the public regarding the use of these dietary supplements (*3*, *5*). Several European countries removed kava-containing products from the market. The U.S. Food and Drug Administration issued a consumer advisory statement concerning the potential risk of liver injury associated with kava dietary supplements (*6*). Following the reports of hepatotoxicity, several hypotheses of the cause of kava-linked liver injuries have been proposed. To date, most of the reported liver failures linked to kava consumption have occurred in Caucasian populations who consumed commercial herbal products containing kava extract (*5*). Approximately 5−10% of the Caucasian population are deficient in the drug-metabolizing enzyme cytochrome P450 2D6 (CYP2D6) (*5*). Very few cases of hepatotoxicity have been reported for Pacific Islanders. Because Polynesian descent found a 0% incidence of CYP2D6 deficiency (*5*), a genetic polymorphism of CYP2D6 enzyme could be a factor in the noted hepatotoxicity.

Whitton et al. reported that the traditional aqueous kava infusions contained hepatoprotective glutathione, whereas extracts obtained from organic solvents did not (*7*). An in vivo

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experiment indicated that aqueous kava extract did not produce liver damage in rats (*8*). Unger et al. reported an inhibitory activity of kavalactones to cytochrome P450 3A4 (CYP3A4) (*9*). A recent publication demonstrated that pipermethystine, a kava alkaloid, extracted from kava leaves and stem peelings, exhibited cytotoxicity in an in vitro cell culture experiment (*10*). It remains unclear whether kavalactones are responsible for the liver toxicity. The hepatotoxic constituents of kava have not been identified, and the mechanism for the hepatotoxicity of kava remains unknown. The objectives of the present study were to investigate potential hepatotoxic kava constituents by bioassay-directed isolation experiments. The cytotoxicity of different kava fractions extracted with a range of solvents from roots, leaves, and stem peelings was evaluated on HepG2 cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) enzyme leakage assay techniques. Compounds exhibiting strong cytotoxicity were isolated and identified.

## MATERIALS AND METHODS

**Chemicals and Materials.** All solvents were of high-performance liquid chromatography (HPLC) grade from J. T. Baker (Phillipsburg, NJ). Silica gel (70−230 mesh) and RP-18 silica gel for column chromatography, thin-layer chromatography (TLC) plates (250 $\mu$m thickness, 2−25 $\mu$m particle size), and MTT were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin−ethylenediaminetetraacetic acid, and antibiotics were from Invitrogen (Grand Island, NY). Diagnostic assay kits for LDH and AST activity were purchased from ThermoDMA (Melbourne, Australia).

**Chemical Analysis and Instrumentation.** TLC was performed on silica gel TLC plates (250 $\mu$m thickness, 2−25 $\mu$m particle size). The spots were detected by ultraviolet (UV) illumination and spraying with 5% (v/v) $H_2SO_4$ in an ethanol solution.

One-dimensional $^1H$ and $^{13}C$, distortionless enhancement by polarization transfer (DEPT), heteronuclear multi-bond connectivity (HMBC), and heteronuclear multiple quantum coherence (HMQC) nuclear magnetic resonance (NMR) spectra were obtained on Bruker Avance 600 MHz spectrometer (Billerica, MA). Chemical shifts are reported as a $\delta$ (ppm) value using $CDCl_3$ as the solvent. Spectra were acquired on a Bruker Avance NMR spectrometer operating at 600.133 MHz for proton and 150.903 MHz for carbon using a 5 mm triple resonance inverse probe. A DEPT135 spectrum was acquired using the standard Bruker DEPT135 pulse sequence.

The sample was further analyzed by direct exposure probe/electron ionization-mass spectrometry (DEP/EI-MS) on a ThermoFinnigan TSQ 700 mass spectrometer (San Jose, CA). The ion source temperature was 150 °C, and the electron energy was 70 eV. The quadrupole analyzer was scanned from *m*/*z* 50 to 550 in 0.5 s. The rhenium wire of the DEP was heated from 0 to 800 mA with a linear ramp of 5 mA/s.

Liquid chromatography/electrospray ionization−mass spectrometry (LC/ESI-MS) analyses were performed on the ThermoFinnigan TSQ 7000 mass spectrometer equipped with an Agilent 1100 Series HPLC. Separation of chemical components was performed with a Phenomenex HPLC column (Prodigy ODS3 2.0 mm × 250 mm × 5 $\mu$m, 100 A, Torrance, CA). The mobile phase (0.2 mL/min) was a 20 min linear gradient from 20 to 95% acetonitrile with a constant 0.1% concentration of formic acid and a 10 min hold at the end. The mass spectrometer was operated in the positive ion ESI mode with an in-source collision-induced dissociation offset of 12 V to reduce clustering with acetonitrile. Full scans were acquired from *m*/*z* 100 to 950 in 1.5 s. Other ESI conditions were as follows: spray voltage of 4.5 kV, capillary temperature set to 320 °C, sheath gas pressure of 70 psi, and auxiliary gas at 5 units. A tandem mass spectrometry (MS/MS) method was set up for product ions of *m*/*z* 285. For the MS/MS, an argon collision gas at 1.0 mTorr was used with 25 eV of collision energy while Q3 was scanned from *m*/*z* 20 to 300.

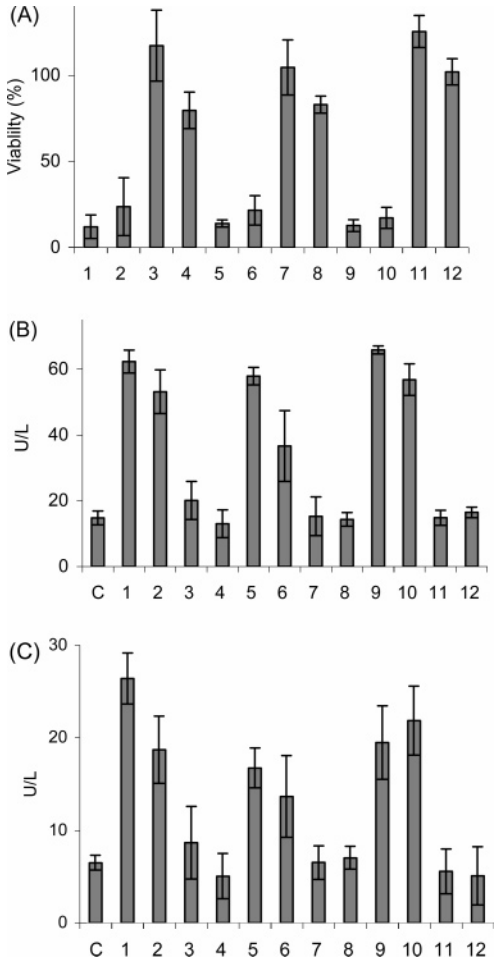


**Figure 1.** Cytotoxicity of different solvent fractions from different kava parts by (**A**) MTT assay, (**B**) LDH assay, and (**C**) AST assay on HepG2 cells. C, control; 1, hexane; 2, ethyl acetate; 3, *n*-butanol; 4, aqueous fraction of kava roots; 5, hexane; 6, ethyl acetate; 7, *n*-butanol; 8, aqueous fraction of kava leaves; 9, hexane; 10, ethyl acetate; 11, *n*-butanol; and 12, aqueous fraction of kava stem peelings; $n = 3$, 125 $\mu$g/mL treatment.

**Plant Materials and Sample Preparation.** Fresh aerial parts (leaves and stem peelings) and roots from selected healthy, mature, 2 year old kava plants (*P. methysticum* cv. Isa) were collected at the Magoon Greenhouse Facility (University of Hawaii, Manoa, Hawaii). Fresh stem peelings were obtained by using a sharp knife with minimum inclusion of the inner woody tissue, and unopened and partially opened young leaves were collected from the same cultivar. The stem peelings and leaves were air-dried at 22 °C in the dark for 1 day, followed by oven-drying overnight at 40 °C. The voucher samples were deposited at the National Center for Toxicological Research (Jefferson, AR). Kava roots, leaves, and stem peelings were ground with a coffee grinder (<80 mesh), and the ground sample (5 g) was extracted with methanol (100 mL) three times with a sonication period of 30 min. The extracts were filtered through Whatman #1 filter paper, and the organic solvent portion was dried under reduced pressure. The resulting residue was dissolved in 100 mL of distilled water and partitioned sequentially with 100 mL of each hexane, ethyl acetate, and *n*-butanol (three times each).

**HepG2 Cultures.** HepG2 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in MEM supplemented with 10% FBS, 100 $\mu$g/mL streptomycine, and 100 IU/mL penicillin. The cells were incubated at 37 °C in a 5% $CO_2$ incubator.

**Cytotoxicity Assay.** The prepared kava fractions and compound **1** (described in following sections) were dissolved in dimethyl sulfoxide (DMSO) and tested for their effects on the viability of HepG2 cells using the MTT assay. One day before treatment with the test chemicals, HepG2 cells were trypsinized and seeded at a density of $5 \times 10^4$ cells/well in a 96 well plate. The media were discarded after 24 h, and fresh

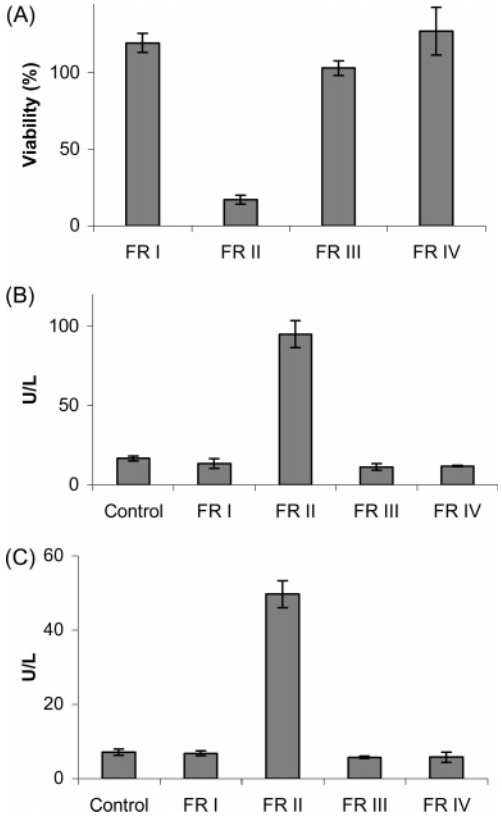


**Figure 2.** Cytotoxicity of kava root hexane fractions (FR I–IV) by (**A**) MTT assay, (**B**) LDH assay, and (**C**) AST assay on HepG2 cells; $n = 3$, 125 $\mu$g/mL treatment.

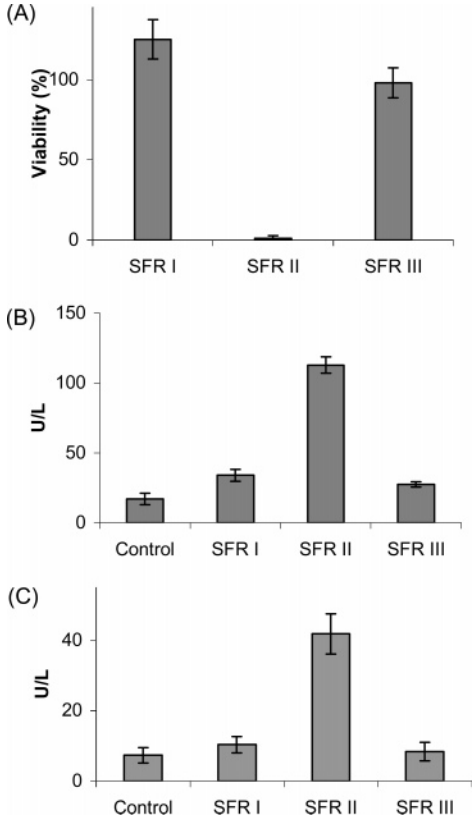


**Figure 3.** Cytotoxicity of kava root hexane subfractions (SFR I–III) by (**A**) MTT assay, (**B**) LDH assay, and (**C**) AST assay on HepG2 cells; $n = 3$, 62.5 $\mu$g/mL treatment.

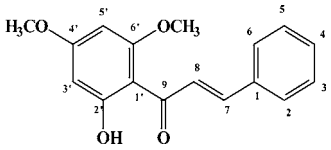


**Figure 4.** Structure of compound **1**.

FBS-free MEM media were added along with test chemicals. After 24 h of exposure with the test chemicals, the culture media were aspirated and 200 $\mu$L of MTT solution (5 mg/mL in FBS-free MEM) was added. The plate was further incubated at 37 °C for 4 h. The media were then aspirated from each well, and the purple formazan crystals were dissolved by the addition of 200 $\mu$L of DMSO to each well, followed by agitation. The absorbencies of each well were read at 550 nm. Cell viability was determined by measuring the reduction of the MTT to formazan blue. The percentage of viability was calculated by dividing by the average absorbance for the corresponding negative control.

Additionally, cell viability was assayed by measuring the release of LDH from cells with a damaged membrane and by AST activity to assess cytotoxicity. Briefly, HepG2 cells were plated in 96 well culture plates as described above. After 24 h, the media were discarded and 200 $\mu$L of fresh FBS-free MEM media was added along with the test fractions and compound **1**. After a 24 h exposure to test chemicals, the culture media were collected and utilized for LDH and AST assays. Diagnostic kits from ThermoDMA were used. Protocols supplied with the diagnostic kits were used for the application of the assay procedures and calculation of enzyme activity. The LDH activity was kinetically determined by measuring the rate of NADH production at 340 nm, while the AST activity was kinetically determined by measuring the decrease in NADH production. All assays were read using a Biotek multiplate reader (Winooski, VT).

**Isolation of Kava Constituents.** Ground kava root (180 g, <60 mesh) was extracted with methanol (500 mL) three times. The resulting solvent solution was filtered through #1 filter paper, and the solvent was evaporated in vacuo to dryness. The resulting residue (27 g) was extracted sequentially with hexane, ethyl acetate, and *n*-butanol. For further fractionation, the hexane fraction (2.9 g) was loaded onto an open column packed with silica gel (110 g) and eluted with 250 mL each of hexane, hexane/ethyl acetate (75:25, v/v), hexane/ethyl acetate (50:50), and hexane/ethyl acetate (25:75, v/v) successively. The collected fractions (FR I−IV) were dried under reduced pressure. Among the four fractions collected, FR II (578 mg) was subjected to further purification with silica gel column chromatography by eluting with 300 mL each of hexane/ethyl acetate mixtures at volume ratios of 90:10, 80:20, and 70:30. Three subfractions (SFR I−III) were collected. SFR II was further fractionated using RP-18 silica gel column chromatography and elution with 50−100% methanol, which afforded compound **1** (60 mg, purity >98% by HPLC-photodiode array).

**Compound 1.** Yellow amorphous powder. EI-MS spectral data *m*/*z* (relative intensity, %): 284 ($M^+$, 90), 283 (69), 256 (9), 267 (9), 207 (100), 181 (25), 128(6), 103 (8), 77 (6). $^1$H NMR ($CDCl_3$, 600 MHz): 3.84 (s, 3H, 4′-$OCH_3$), 3.93 (s, 3H, 6′-$OCH_3$), 5.98 (d, 1H, $J = 2.4$, H5′), 6.12 (d, 1H, $J = 2.4$, H3′), 7.41 (m, 3H, H3, H4, H5), 7.62 (dd, 2H, $J = 1.8$, 7.5 Hz, H2, H6), 7.78 (d, 1H, $J = 15.5$ Hz, H7), 7.90 (d, 1H, $J = 15.5$ Hz, H8), 14.33 (s, 1H, 2′-OH). $^{13}$C NMR ($CDCl_3$): $\delta$ 55.8 (4′-$OCH_3$), 56.1 (6′-$OCH_3$) 91.5 (C-5′), 94.0 (C-3′), 106.5 (C-1′), 127.7 (C-7), 128.6 (C-2, 6), 129.1 (C-3,5), 130.3 (C-4), 135.7 (C-1), 142.5 (C-8), 162.7 (C-6′), 166.4 (C-4′), 168.6 (C-2′), 192.8 (C-9).

## RESULTS AND DISCUSSION

In this study, different fractions from kava roots, leaves, and stem peelings obtained by solvent extraction were evaluated for their cytotoxicity on HepG2 cells based on LDH and AST enzyme leakage and MTT assay techniques. **Figure 1A** shows the results of the MTT assay experiment for individual kava fractions at a treatment concentration of 125 $\mu$g/mL. Viable cells convert MTT to purple formazan dye that can be measured spectrophotometrically after solubilization in DMSO, whereas this phenomenon does not occur in dead cells. Kava fractions

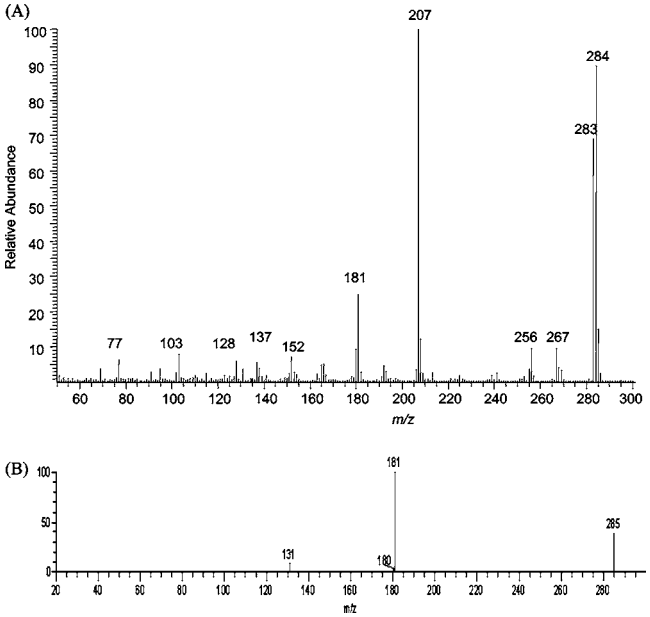


**Figure 5.** Mass spectra of compound **1**: (**A**) DEP/EI and (**B**) ESI LC-MS/MS product ions of *m/z* 285.

containing nonpolar chemical components displayed a higher cytotoxicity than polar fractions. For example, hexane fractions of kava roots, leaves, and stem peelings displayed a strong cytotoxicity showing only 12.0−13.9% of cell viability, while water fractions displayed 79.6−102% of cell viability under the same treatment conditions.

Cell viability on HepG2 cells was assayed by measuring the release of LDH from cells. The measurement of LDH leakage has been used as a sensitive marker to assess the cytotoxicity of test chemicals. When the cell membrane integrity is lost, intracellular components, including LDH, are released into the culture media. The assay results for different kava fractions are presented in **Figure 1B**, and the results paralleled with those of the MTT assay results. The cytotoxic effects of the aqueous fractions prepared from kava root, leaves, and stem peelings on HepG2 cells were negligible, up to 500 $\mu$g/mL treatment (data not shown); however, a significant increase in LDH activity in the cell culture medium was observed with the hexane and ethyl acetate fractions.

Additionally, the cytotoxicity on HepG2 was assayed by measurement of AST enzyme activity in culture media that resulted from leakage (**Figure 1C**). The results paralleled with those of the MTT and LDH assays. The hexane and ethyl acetate fractions of kava significantly increased AST enzyme leakage, while no significant increase of AST enzyme activity was observed with the aqueous kava fractions. At the present time, most of the cases of kava hepatotoxicity have been linked to the consumption of herbal products containing kava extracts that are generally prepared by solvent extraction methods. Little information is available on the hepatotoxicity in Pacific Islanders who consume water extracts/suspensions of kava roots. One report suggested that glutathione in aqueous kava infusion may have a hepatoprotective action (*7*). Singh and Devkota (*8*) reported that aqueous kava extract did not cause liver damage in rats, and results of the current study support the conclusion that aqueous extracts do not exhibit in vitro cytotoxicity.

For the isolation and identification of potential hepatotoxic components in kava, the hexane fraction of kava root, which displayed stronger cytotoxic effects on HepG2 cells than other fractions, was selected for further bioassay-directed isolation and analysis. This hexane extract was fractionated on a silica gel column to give four fractions (FR I−IV). Among the collected fractions, FR II displayed the strongest cytotoxicity on HepG2 cells (**Figure 2**). Further fractionation of FR II gave three subfractions (SFR I−III). Among these three fractions, SFR II displayed the strongest cytotoxicity as tested with MTT, LDH, and AST assays (**Figure 3**). The components of SFR II

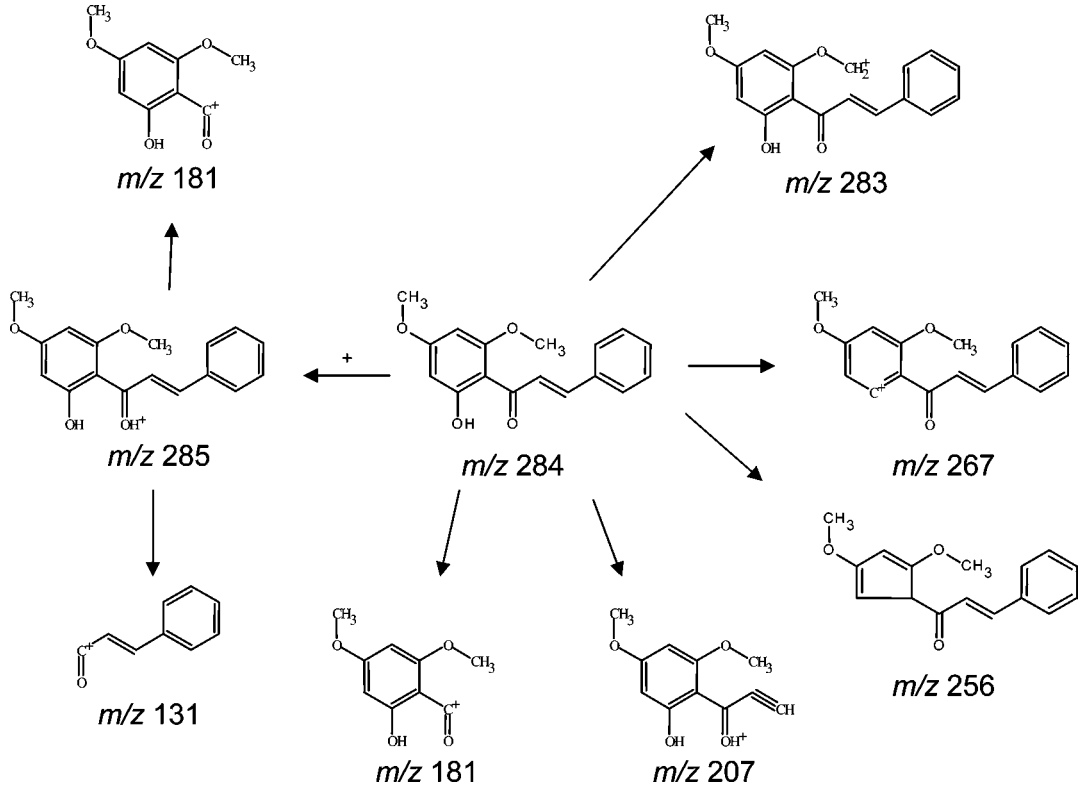


**Figure 6.** Possible structures of ions of compound **1**.

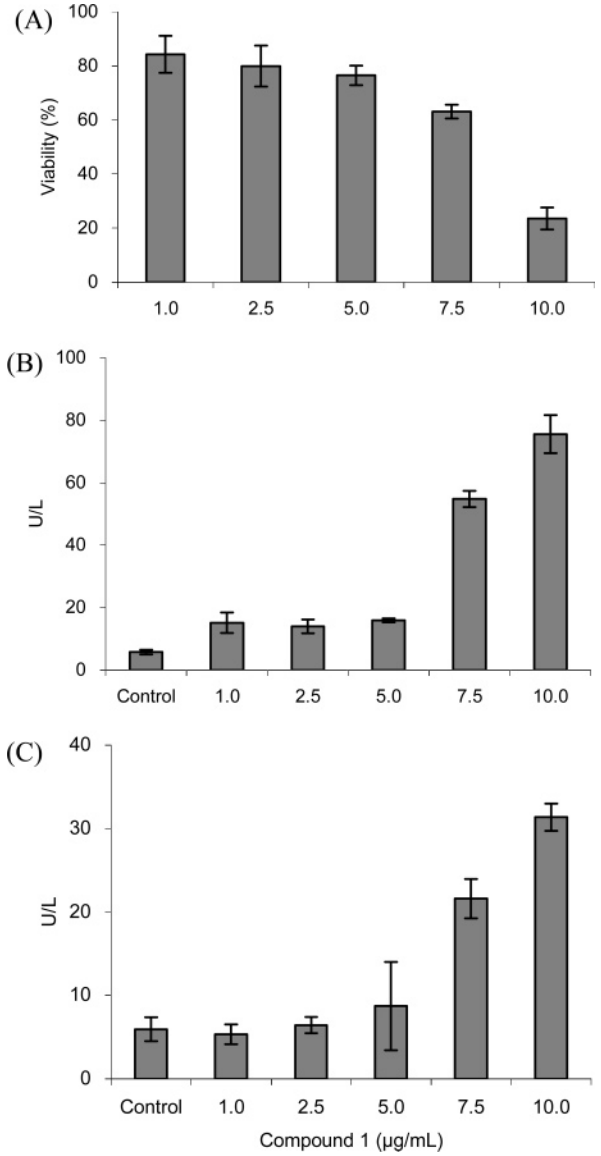


**Figure 7.** Cytotoxicity of compound **1** by (**A**) MTT assay, (**B**) LDH assay, and (**C**) AST assay on HepG2 cells; $n = 3$.

were separated on a RP-18 column as described in the Materials and Methods section and resulted in the isolation of compound **1**.

Compound **1** was isolated as an amorphous yellow solid with a molecular formula of $C_{17}H_{16}O_4$ as determined by MS, $^{13}C$ NMR, and DEPT spectra. The $^1H$ NMR spectrum of **1** (**Figure 4**) showed two trans-configured olefinic protons at $\delta_H$ 7.78 (1H, d, $J = 15.5$ Hz) and $\delta_H$ 7.90 (1H, d, $J = 15.5$ Hz) (details are described in the section of Isolation of Kava Constituents). The signals at $\delta_H$ 5.98 (1H, d, $J = 2.4$ Hz) and $\delta_H$ 6.12 (1H, d, $J = 2.4$ Hz) indicated the presence of meta-coupled protons in the aromatic ring, and the carbon signals $\delta_C$ 91.5 and $\delta_C$ 94.0 were attributed to C-5′ and C-3′, respectively. A singlet at $\delta_H$ 14.33 indicated that the compound has a hydroxyl group hydrogen bonded to a carbonyl oxygen. In addition, the presence of two methoxyl groups was indicated by the signals at $\delta_C$ 55.8 coupled to $\delta_H$ 3.84 (3H, s) and $\delta_C$ 56.1 coupled to $\delta_H$ 3.93 (3H, s) ppm. Aromatic proton resonances at $\delta_H$ 7.62 (2H, dd) and $\delta_H$ 7.41 (3H, m) and carbon resonances at $\delta_C$ 135.74 (C-1), 129.07 (C-2, C-6), 128.56 (C-3, C-5), and 130.26 (C-4) were characteristic of monosubstituted aromatic ring. The ketone carbonyl carbon showed at $\delta$ 192.8 in the $^{13}C$ NMR spectrum.

The DEP-EI/MS analysis of **1** gave a strong $M^+$ ion at *m*/*z* 284 (**Figure 5**). The EI-MS fragments at *m*/*z* 207, 181, 103, and 77 (the proposed fragmentation is shown in **Figure 6**) support the proposed structure for compound **1** (**Figure 4**). LC/ESI-MS analysis showed that the compound **1** (rentention time, 25.1 min) had a molecular weight of 284 with a protonated molecule at *m*/*z* 285. A product ion MS/MS analysis of *m*/*z* 285 was conducted and produced product ions at *m*/*z* 131 and 181 (**Figure 5**). Possible structures for the observed ions are shown in **Figure 6**.

DEPT, HMQC, and HMBC spectra further confirmed the proposed structure of compound **1**. On the basis of all of the structural analysis, compound **1** was deduced to be flavokavain B. $^1H$ and $^{13}C$ NMR spectral information were in agreement with published values (*11*, *12*). Flavokavain B has been isolated from kava with other chalcones, flavokavain A and C (*1*, *3*). The cytotoxicity of flavokavain B was evaluated on HepG2 based on MTT, LDH, and AST assay techniques. Flavokavain B displayed strong cytotoxicity toward HepG2 cells ($IC_{50} = 8.3\ \mu g$/mL). The MTT assay results showed that at the treatment level of 7.5 and 10 $\mu$g/mL of flavokavain B, cell viability was 63.1 and 23.6%, respectively (**Figure 7A**). The LDH and AST assay results were in good agreement with the MTT assay in that they showed significantly increased enzyme activity in the cell culture media at the 7.5 and 10 $\mu$g/mL levels of treatment (**Figure 7B**,**C**).

In the current study, the cytotoxicities of different kava fractions from roots, leaves, and stem peelings on HepG2 cells were evaluated to determine their potential roles in causing liver toxicity based on MTT, LDH, and AST enzyme leakage assay techniques. Bioassay-directed isolation was carried out in order to investigate the key components responsible for liver toxicity. This experiment allowed us to identify the compound responsible for cytotoxicity as flavokavain B. Chalcones are flavonoids present in a variety of plant species. Plants synthesize flavonoids from the ubiquitous amino acid phenylalanine. Specifically, the B-ring and carbon atoms 2, 3, and 4 of the C-ring are derived from L-phenylalanine, while the A-ring is derived from acetate metabolism (*13*). Various flavonoids are synthesized through a key metabolite known as chalcone The chalcones contain two aromatic rings connected through an $\alpha,\beta$-unsaturated carbonyl system. A range of biological effects have been ascribed to chalcones. An in vitro study demonstrated that hydroxychalcones were cytotoxic toward isolated rat hepatocytes, which results from the depletion of hepatic GSH (*14*). Chalcones deplete hepatic GSH by oxidation to GSSG or conjugate formation with GSH. Furthermore, it has been reported that hydroxychalcones disrupt the mitochondrial membrane potential of hepatocytes (*14*). Results of the current study suggest that flavokavain B is cytotoxic to human hepatoma HepG2 cells in vitro, which might be related to the observed hepatotoxicity. Studies are in progress to address the cytotoxicity of flavokavain analogues, such as flavokavain A and C. The cytotoxic mechanisms of flavokavain B including its in vivo hepatotoxicity effects are yet to be elucidated.

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