Electron Spin Resonance and Cell Biology Studies Of Potentially Hepatotoxic Kava Root Extracts

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This study was performed to analyze the hepatotoxic activity of kava kava root extracts The structures of these compounds are similar to structures of compounds known to produce free radicals upon oxidation. This free radical activity in the liver caused by the reactive oxygen species resulting from this oxidation could lead to oxidative stress. Such stress might activate a pathway leading to the activation of an enzyme, caspase-3, which is responsible for apoptosis (programed cell death). This study attempted to compare the free radical activity of kavalactones and chalcones using ESR spectroscopy to the activation of caspase-3 enzymes using a caspase-3 colorimetric assay. Due to the difficulty of obtaining pure kavalactones/chalcones, ESR studies could not be performed on these compounds. However, one compound extracted from the kava root extract, borynl cinnamate, was analyzed through ESR spectroscopy but yielded no free radical activity under oxidation conditions. One kavalactone, yangonin, was obtained commercially in milligram quantity to perform a caspase-3 assay. The results of the assay revealed a possibility that concentrations of yangonin could induce cell death through apoptosis.

INTRODUCTION

Kava Kava

Piper methysticum (commonly referred to as Kava Kava) is a root harvested from the South Pacific Islands . The organic-solvent extracts of the Kava root are promoted for relaxation to relieve stress, anxiety, and tension along with relief of sleeplessness and symptoms of menopause. In 2002 the United States Food and Drug Administration (FDA) advised consumers of possible liver injury associated with the use of Kava-containing supplements. The plant is usually harvested for its rootstock which is knotty and thick. After drying the harvested rootstock, the contents are approximately 43% starch, 20% fibers, 12% water, 3.2% sugars, 3.6% proteins, 3.2% minerals, and 15% kavalactones. The rootstock color varies from white to dark yellow depending on the amount of kavalactones that are contained in the resin. The kavalactones are the psychoactive components of the rootstock. Once the dry rootstock is harvested, it can be sold as is or extracted with supercritical CO_2 and placed into pill or paste form containing a higher kavalactone content.[1] Contrasting the many uses of this root, several cases of liver damage have been associated with kava kava exposure such as hepatitis, cirrhosis, liver failure, and death. No mechanism of toxicity has been reported in the relevant literature.

Many substances that are reported to cause liver damage in the same manner as kava kava, have associated free radical chemistry leading to oxidative stress and ultimately apoptosis. The chemical structures of kavalactones include aromatic methoxy groups that have been reported in previous studies of similar compounds to display unique free radical chemistry (e.g. methyl engenol). These radicals can be formed through oxidation by cerium (IV) sulfate.

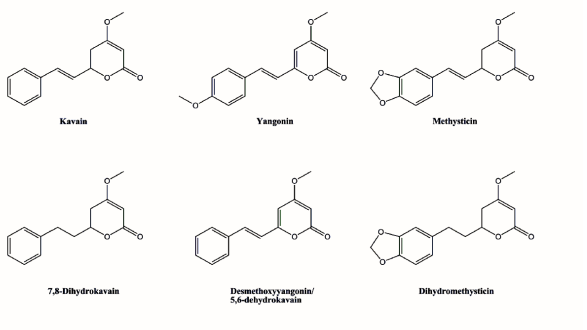


Fig1. Major Kavalactones in the Kava Kava Extract all with aromatic methoxy groups.[3]

Free Radical Chemistry

A free radical is any species capable of independent existence that contains one or more unpaired electrons. The presence of unpaired electrons causes free radical species to possess a magnetic moment, and causes them to be highly reactive. For the kavalactones and chalcones focused on in this study, oxygen radicals may be formed through oxidation with a compound such as cerium (IV) sulfate. A subset of these radicals formed are referred to “reactive oxygen species” and can have negative effects on the body’s tissues.[4]

In healthy aerobic organisms, the production of reactive species is appropriately balanced with antioxidant defense systems, which control the level of reactive species in the body to a point where the levels of most reactive species are minimized (except for the levels that remain to perform essential roles). If this balance is upset, perhaps by the presence of oxygen free radicals on kavalactones and chalcones, oxidative stress may occur. Oxidative stress is described as having too many reactive oxygen

species in relation to the available antioxidants. Oxidative stress can lead to oxidative damage.[4]

The type of oxidative damage that this study will focus on is apoptosis (programmed cell death). Apoptosis plays a role in cell loss during the body's reaction to certain diseases. Neurodegenerative diseases and some viral infections are often combated by the body's apoptosis mechanisms. The molecular pathway for this process is laid out in figure 2. Ligands that bind to these receptors include members of the TNF family (TNF-α), Fas (fibroblast associated cell surface ligand L or CD95L), and TRAIL (TNF-related apoptosis-inducing ligand). One or a combination of these ligands binds to death receptors and cause them to aggregate into trimers. The cytoplasmic 'tails' of these receptors recruit other proteins such at TRADD (TNF receptor-associated death domain) which carries a death-effector domain (DED). The DED binds procaspase-8 and incorporates it into the death-inducing signaling complex (DISC) The activation of procaspase-8 leads to the activation of caspases-3, -6, and -7.[4]

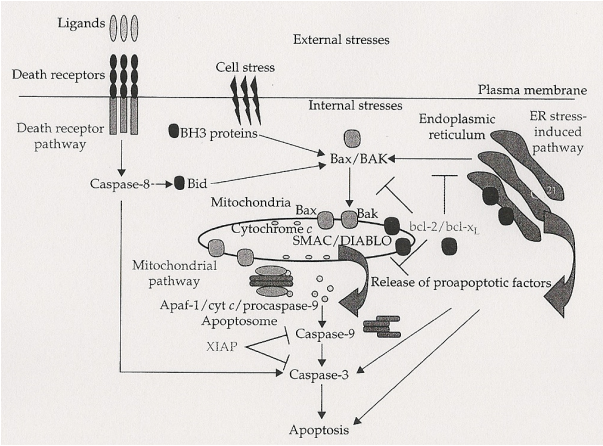


Fig 2. Molecular Pathway of Apoptosis showing the initiation of cell stress to the activation of the cytochrome c pathway leading to the activation of caspase-3 for apoptosis.[5]

This study focuses on the apoptosis of hepatocytes caused by an oxidative stress mechanism. In human HepG2 hepatoma cells exist antioxidants such as ascorbic acid, glutathione, uric acid, carotenes, α-tocopherol, catalase, and ubiquinol. If these antioxidants are overwhelmed by oxidative stress mechanisms (free radicals) cell death will be induced by the apoptosis mechanism presented above.[4]

Antioxidant	Fraction of Liver	Concentration (μmol/kg tissue)
Ascorbate	Homogenate	260 ± 20
	Cytosol	340 ± 40
Urate	Homogenate	1600 ± 150
	Cytosol	1700 ± 200
GSH	Homogenate	6400 ± 400
	Cytosol	3800 ± 400
α-Tocopherol	Homogenate	50 ± 20
	Cytosol	nd

Table 1. Concentrations of Main Antioxidants in Hepatocytes

These antioxidants are used within the body to absorb damaging substrates such as superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ can reduce the activity of antioxidant defense systems (such as catalase). Since superoxide is highly reactive in organic solvents, any $O_2^{\bullet-}$ produced in the lipophilic membrane interior of a cell could be very damaging. Superoxide derived species (such as hydrogen peroxide, hydroxyl radicals, and phenoxyl radicals) are considered cytotoxic because of their ability to react with DNA.

METHODS

All materials unless otherwise specified were obtained from the chemistry stocks at Hampden-Sydney College or from Sigma-Aldrich. Instruments used include Varian CP-3800/2200 GC/MS (gas chromatograph/mass spectrometer), JEOL 400 MHz NMR (nuclear magnetic resonance spectrometer) and an EXCALIBUR HE Series FTS 3100 FTIR (fourier transform infrared spectrometer). ESR (electron spin resonance) spectra were obtained on a JEOL RE-1X (Peabody, MA) spectrometer equipped with a TE-011 cylindrical cavity

A. Extraction

Kava paste, 5 grams, (containing 84% kavalactones) supplied by Kona Kava Farm (Kailua-Kona, HI) was dissolved in dichloromethane and placed on a silica gel column (100g). Flash chromatography was performed with dichloromethane solvent to elute 6, 250 mL fractions. One final 1000 mL methanol fraction was collected.[17] The fractions were analyzed by GC/MS using a polar column. The fractions containing desired product were combined and concentrated by rotary evaporation. ^{13}C NMR, ^{1}H NMR, and HMQC spectroscopic measurements were utilized along with the mass spectrum to determine the identity of the isolated extract.

B. ESR Sampling Techniques[9]

Two major sampling techniques are used for aqueous samples in ESR spectroscopy: aspiration (static) and fast-flow (FF) ESR spectroscopy.

Aspiration sampling ESR is used for observing relatively stable free radical species. These radicals last for a longer period of time (minutes to hours). To begin this method, a sample is prepared (in a test tube) and aspirated into the flat cell through a stainless steel cannula via an external aspiration pump.

Fast-flow ESR is used for unstable, short-lived radicals (lifetime on the order of seconds or less). In this method, two solutions separate from each other are pumped by a peristaltic pump into the flat cell where they are mixed. The two solutions are mixed at the moment they enter the flat cell so that the radicals produced can immediately be observed by the spectrometer. The flow rates of these two solutions can be varied from 15 mL/min to greater than100 mL/min. Since the flat cell volume is about 0.1 mL, the contents of the flat cell are renewed every 200 to 30 milliseconds at these flow rates. The resulting "slow-flow" ESR occurs at a much slower flow rate through the spectrometer.

C. Cell Culture[22]

1. Medium Preparation

A 1:1 mixture of Dulbecco's modified Eagle's medium and Hams F12 medium (obtained from Dr. Ed Devlin, Hampden-Sydney College) is supplemented with 0.005mg/mL insulin (obtained from NIEHS: Research Triangle Park, NC), 0.005mg/mL transferrin, 5ng/mL selenium, 40ng/mL dexamethasone, and 10% (by volume) fetal bovine serum. Penicillin/streptomycin antibiotic solution (5 mL) is added to the medium The medium solution is stored at 4°C.

2. Thawing of Cell Line

The vial of frozen mouse hepatocyte cell line AML12 (obtained from ATCC) was. Growth media is added to the liquid cell line in increments: 50µL, 100µL, 200µL, 400µL, 800µL, and 8.5mL (1 minute between each addition, 5 minutes before the last). The cells and media are centrifuged and the supernatant is removed The pellet is resuspended and added to a cell-culturing flask with 15mL of fresh growth medium.

Subculturing

When proper confluency is observed (2-3 days with AML12), the flask is removed and culture medium discarded. The supernatant is poured off and the pellet is resuspended in 15mL fresh medium in a new culture flask. The flask is placed in a 37°C CO_2 incubator and observed routinely for proper confluency (percent coverage of the cell culture flask).

3. Counting and Freezing

When proper confluency (80-90%) is observed (2-3 days with AML12), the flask is removed and culture medium is discarded. The supernatant is poured off and the pellet is resuspended in 4mL of culture medium. 20µL of cell suspension and 20µL of Trypan Blue are added to a single well of a 96-well plate. A hemocytomer is loaded with 15mL of the cell-Trypan Blue solution, and the cells are counted according to the hemocytometer protocol. The number of cells per mL is calculated according to Formula:

#cells/ml = (#cells counted)(hemocytometer factor)(dilution factor)

The cells are resuspended and centrifuged. The pellet is resuspended in 3mL growth medium supplemented with DMSO (cryoprotectant). The cells are placed in an -80°C freezer until they are ready to use.

D. Caspase-3 Colorimetric Assay[15]

Caspase-3/CPP32 Colorimetric Assay Kit provided by BioVision (Catalog #K106-25) Apoptosis is induced by plating 1mL aliquots of $1e^6$ AML12 cells with differing concentrations of 1mM stock solution of yangonin (obtained from LKT Labratories) in methanol. An untreated control and methanol-only controls (vehicles) are also plated.

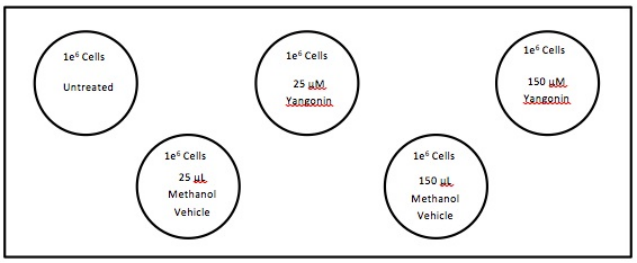


Fig 3 Colorimetric Assay Experimental Setup showing various treatments. Hepatocytes were exposed for a 24-hour incubation.

Previous studies[23] have listed N^{ω}-hydroxy-nor-Arginine (NOHA, Cayman Product #: 10006861 as a positive control in caspase-3 activation studies. A second plate was setup with an untreated condition, 1mM NOHA (in PBS) condition, 1mM yangonin condition, and a 1 mL methanol vehicle condition.

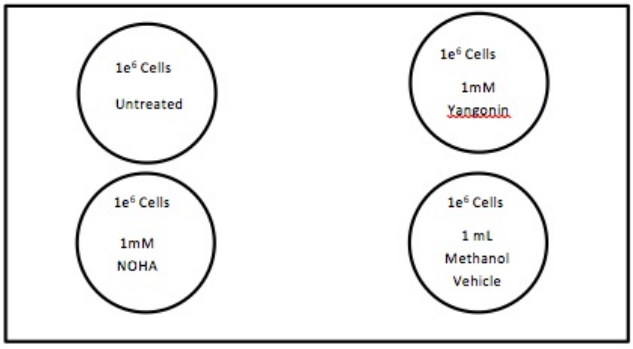


Fig 4 Colorimetric Assay Experimental Setup showing various treatments. Hepatocytes were exposed for a 48-hour incubation.

The plated cells are incubated for 24 hours at 37° in a CO_2 incubator. The cells are counted, pelleted, and resuspended in 50µL of chilled Cell Lysis Buffer and incubated on ice for 10 minutes. The solution is centrifuged for 1 minute in a microcentrifuge (10,000 × g). The supernatant (cytosolic extract) is transferred to a fresh tube and put on ice for immediate assay or aliquoted and stored at -80°C for future use. The protein concentration is assayed. 50-200µg protein to 50µL cell lysis buffer is diluted for each assay. 50Ml of 2X Reaction Buffer (containing 10mM DTT) is added to each sample. 5µL of the 4mM DEVD-pNA substrate (200µM final concentration) is added and the tubes are incubated for 1-2 hours. The samples are read at 400-405nm in a microtiter plate reader. These results are compared with the level of uninduced control in order to determine fold-increase in CPP32 activity.

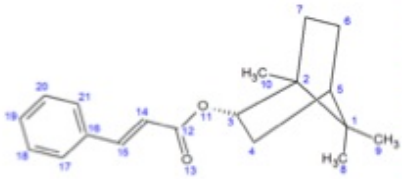
The ENDOR spectrum was obtained using the same apparatus but in addition an ENI (Rochester, NY) A-300 RF power amplifier, a Philips (Eindhoven, Netherlands) PM5193 programmable synthesizer / function generator, and a JEOL ENDOR ES-LHC3X cavity was used. The parameters for the acquisition of the ENDOR spectrum were as follows. B_0 was 3474.4G, the phase was set at 100.05°, the sweep range was twenty MHz, the RF amplitude was 0.6, the ENDOR frequency range was 14.79MHz from 4.79 to 24.79 MHz, each frequency step was 19.53kHz and 5 scans were conducted with 1024 points recorded.

The acquisition of higher quality ENDOR spectra has continued after preparation of this report.

RESULTS AND DISCUSSION

Extraction

Bornyl cinnamate (2.0 grams) was obtained after extracting commercial kava paste with dichloromethane by flash chromatography



Carbon No.	CHn	ACD Predicted Chemical Shift (ppm)	Actual Chemical Shift (ppm)
1	C	19.53	22.7206
2	C	49.18	62.0180
3	CH	78.67	68.9384
4	CH2	37.70	31.9581
5	CH	44.72	33.9386
6	CH2	28.36	27.1864
7	CH2	28.36	27.1864
8	CH3	19.93	24.8465
9	CH3	19.93	24.8465
10	CH_3	12.93	14.0567
12	C	168.48	172.8289
14	CH	118.40	129.6087
15	CH	144.30	172.4618
16	C	134.30	131.0265
17	CH	127.90	129.8840
18	CH	128.70	128.5607
19	CH	130.00	130.0063
20	CH	128.70	130.0063
21	CH	127.90	129.8840

Figure 5. ^{13}C NMR of flash chromatography extract suggesting that the extract is bornyl cinnamate

These spectral results provide reasonable evidence that the extracted compound is borynl cinnamate.

Electron Spin Resonance Studies

Fast flow ESR studies were performed on 3 compounds: 4-methoxycinnamic acid, methyl-*trans*-cinnamate, and the extracted bornyl cinnamate. 4-methoxycinnamic acid and methyl-trans-cinnamate were tested as known compounds with similar structures to the known kava root extracts.

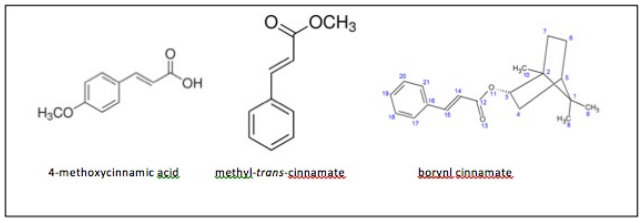


Fig 6. Compounds Studied with ESR

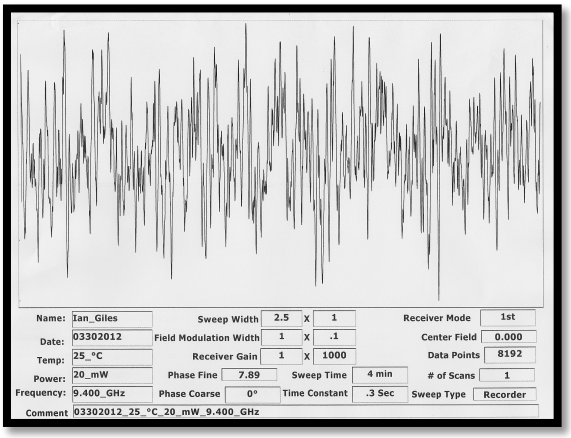


Fig 7. Background ESR Signal (empty cavity)

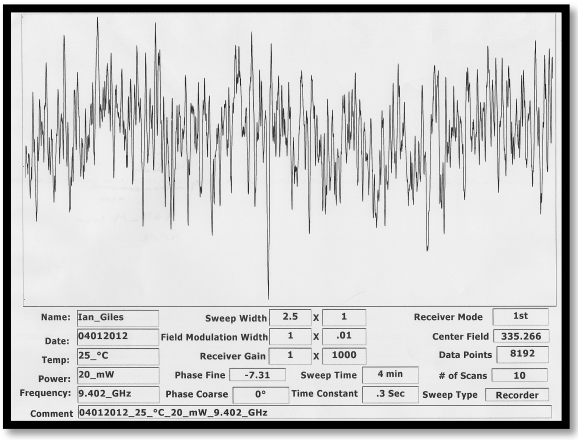


Fig 8. ESR signal of 4- methoxycinnamic acid oxidized by Ce(IV)SO4

ESR signal of methyl-trans-cinnamate and of borynl cinnamate appear similar to both the background and the 4-methoxycinnamic acid spectra, revealing no free radical activity induced by cerium (IV) sulfate in any of the three compounds.

Caspase-3 Colorimetric Assay

Caspase-3 Colorimetric Assay Results		
Treatment	**Absorbance**	**Absorbance - Blank**
Untreated	0.119	0.054
25 µM Yangonin	0.142	0.077
25 µL Methanol	0.118	0.053
150 µM Yangonin	0.14	0.075
150 µL Methanol	0.17	0.105
Blank	0.065	0

Table 2. Hepatocyte Caspase-3 Colorimetric Assay Results

The activation of caspase-3 increases with yangonin treatments over the untreated condition. The most caspase-3 activation occurs when hepatocytes are treated with a high volume (150µL) of methanol. This indicates that the vehicle used to dissolve yangonin (methanol) may induce caspase-3 activation as well.

Caspase-3 Colorimetric Assay Results		
Treatment	**Absorbance**	**Absorbance - Blank**
Untreated	0.065	0.002
1 mM NOHA	0.071	0.008
1 mM Yangonin	0.483	0.42
1 mL MeOH	0.387	0.324
Blank	0.063	0

Table 3. Hepatocyte Caspase-3 Colorimetric Assay Results

The activation of caspase-3 increases with the increasing dose of yangonin. The methanol vehicle also induced caspase-3 activation. The NOHA positive control shows no activation.

Though no free radical activity was observed on the three compounds studied, this result does not rule out the absence of free radical activity stemming from the kava root extract. The only pure compound that was able to be extracted from the kava root was

borynl cinnamate. No kavalactones or chalcones were able to be extracted with the facilities available at Hampden-Sydney College. Most reports of isolating kavalactones from the kava root extract include preparatory HPLC extraction. One kavalactone, yangonin (which was purchased in nominal amounts), was able to undergo caspases-3 colorimetric testing which gave preliminary evidence that caspase-3 was the mechanism of hepatoxicicity. The caspase-3 activity resulting from yangonin treatments was also found to be dose dependent, as the higher treatments of yangonin (1 mM) gave a 4× higher activation than lower treatment concentrations (25 μM, 150 μM).

Continuing work on this project would consist of finding a way to obtain kavalactones in quantities large enough to perform fast flow ESR experiments. These compounds can be obtained by HPLC extraction from the kava root extract, synthesis, or purchase.

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