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Hepatocellular toxicity of kava leaf and root extracts

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Abstract

Kava extracts are used widely for different purposes and were thought to be safe. Recently, several cases of hepatotoxicity have been published. To explore possible mechanisms of kava hepatotoxicity, we prepared and analyzed three different kava extracts (a methanolic and an acetonic root and a methanolic leaf extract), and investigated their toxicity on HepG2 cells and isolated rat liver mitochondria. All three extracts showed cytotoxicity starting at a concentration of 50 μg/ml (lactate dehydrogenase leakage) or 1 μg/ml (MTT test). The mitochondrial membrane potential was decreased (root extracts starting at 50 μg/ml) and the respiratory chain inhibited and uncoupled (root extracts) or only uncoupled (leaf extract) at 150 μg/ml, and mitochondrial β-oxidation was inhibited by all extracts starting at 100 μg/ml. The ratio oxidized to reduced glutathione was increased in HepG2 cells, whereas the cellular ATP content was maintained. Induction of apoptosis was demonstrated by all extracts at a concentration of 150 μg/ml. These results indicate that the kava extracts are toxic to mitochondria, leading to inhibition of the respiratory chain, increased ROS production, a decrease in the mitochondrial membrane potential and eventually to apoptosis of exposed cells. In predisposed patients, mitochondrial toxicity of kava extract may explain hepatic adverse reactions of this drug.

Keywords: Kava kava extract; *Piper methysticum*; Hepatocellular toxicity; Apoptosis; Necrosis; HepG2 cells

Introduction

Kava kava (*Piper methysticum* Forster) is a plant originating from Oceania (Polynesia, Melanesia and Micronesia). In these regions, the consumption of kava beverages has a long-standing tradition at social or ceremonial occasions and also as medicines. Medical indications for kava include induction of relaxation and/or sleep, but also the opposite, counteraction of fatigue, chronic cystitis, asthma, rheumatism, weight reduction, headache, and treatment of infections such as syphilis and gonorrhea (Singh, 1992). Traditionally, kava extracts are prepared from kava roots macerated with water or coconut milk (Johnson, 1999; Lebot et al., 1997).

Some years ago, kava products became popular also in Western countries, mainly as a herbal alternative to drugs used in patients with anxiety disorders (Schulze et al., 2003). Commonly used drugs for this indication, e.g. benzodiazepines, selective serotonin-reuptake inhibitors or buspirone, are associated with adverse drug reactions such as withdrawal syndromes, memory changes, sexual dysfunction and others (Fricchione, 2004), which have not been described for kava products.

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Until recently, apart from the so called kava dermatopathy, kava was not associated with severe adverse reactions (Bilia et al., 2002; Norton and Ruze, 1994). Kava dermatopathy includes yellowing of the skin, and the development of red eyes and a variety of allergic skin reactions, which are spontaneously reversible on stopping of the drug (Singh, 1992).

During the last few years, several patients with hepatic injury associated with the ingestion of kava have been described (Escher et al., 2001; Gow et al., 2003; Humberston et al., 2003; Russmann et al., 2001; Stickel et al., 2003). In a case series, 82 cases of liver toxicity associated with the use of kava have been reported from several countries (Schmidt, 2003). For 20 of these cases, however, there was no clear connection with the ingestion of kava. Twenty-one of these patients were treated concomitantly with potentially hepatotoxic drugs. In seven of these patients, the causality that kava was the cause for the hepatic injury could be doubted considerably for several reasons, whereas in 31 other cases, the available data were too fragmentary for a clear assessment. This left three cases, in whom hepatic injury associated with the ingestion of kava could be established as probable (Schmidt, 2003). Based on these findings, kava was banned from the market in the European countries, Canada and Australia.

Based on the clinical findings, it was hypothesized that liver injury associated with kava could have an immunological origin (Russmann et al., 2001; Schmidt, 2003; Schulze et al., 2003; Teschke et al., 2003). Several other mechanisms and/or risk factors for hepatic toxicity of kava have been proposed, including the formation of electrophilic metabolites from kavalactones (Johnson et al., 2003), possibly associated with glutathione depletion (Whitton et al., 2003), and also genetic polymorphisms of CYP2D6 (Russmann et al., 2001).

Kavalactones, also called kavapyrones, are the major lipophilic compounds in the kava root and are claimed to be responsible for the anxiolytic effect of kava (Schulz et al., 1998). Accordingly, kava containing a high amount of kavalactones is generally considered to be of high quality (Dragull et al., 2003). The amount of kavalactones varies with the different parts, the age and the cultivar of the plant (Smith, 1983). Duve (1976) found that the total kavalactone content is typically highest in the lateral roots and decreases continuously towards the aerial parts of the plants.

Since it is not clear whether kavalactones are responsible for the hepatic toxicity of kava, it was speculated that other components of kava extracts and/or the extraction method could be the reason. Nerurkar et al. (2004) and Dragull et al. (2003) have investigated the alkaloid pipermethystine, a component of kava extracts, and found a stronger toxicity on HepG2 cells than for kavalactones. Cote et al. (2004) compared the effect of an acetonic, an ethanolic and methanolic root extract with a (traditional) aqueous extract on cytochrome P450 isozymes (CYP). All extracts inhibited the CYPs investigated, but the aqueous extract was the least potent inhibitor. Whether inhibition of CYPs is somehow connected with the hepatic toxicity of kava is currently not known.

The aim of the current study was to gain further insight into the cellular mechanisms of the hepatic toxicity of kava. In addition, we wanted to find out whether extracts from different parts of the plant and extracted with different solvents are associated with a different toxicity profile.

Materials and methods

Chemicals

JC-1 and propidium iodide were from Molecular Probes (Eugene, OR, USA); Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Z-Phe-Ala-fluormethylketone (zFA-fmk) were from Enzyme Systems Products (Livermore, CA, USA); Alexa Fluor 633 labelled annexin V was a kind gift of Dr. Felix Bachmann, Aponetics Ltd. (Witterswil, Switzerland). [1-^{14}C]palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). The scintillation cocktail was from Perkin Elmer (Boston, MA, USA). All other chemicals were from Sigma (Buchs, Switzerland) and of highest quality available when not otherwise stated.

Preparation and analysis of the kava extracts

A methanolic and an acetonic extract were obtained from the root, and a methanolic extract from the leaves of the same cultivar, consisting of 3 years old greenhouse kava plants. Dried leaves and lateral roots were pulverized in a laboratory mill and extracted twice, either with methanol or acetone, in an ultrasonic bath for 15 min. The solvents were evaporated to dryness and the residue was diluted in methanol and filtered (regenerated cellulose, 0.45 μm) for HPLC analysis.

A reversed phase HPLC analysis was carried out on a Spherisorb-5 ODS column (5 μm, 250 × 4.6 mm; Waters, Milford, MA, USA) using a Jasco HPLC system (Easton, MD, USA) equipped with an autosampler and a diode array detector. The samples were chromatographed with (v:v) 22% acetonitril, 18% methanol and 60% H_3PO_4 (85%) as a solvent, at a flow rate of 0.8 ml/min and at 60 °C. The identification and quantification of the six kavalactones was based on comparing the retention times, UV spectra and peak areas with external standards (PhytoLab, Hamburg, Germany).

Methysticin, dihydromethysticin, kavain and dihydrokavain were detected at 240 nm, demethoxyyangonin and yangonin at 360 nm.

The alkaloid pipermethystine was quantified in the extracts by GC–MS as described by Dragull et al. (2003), using an HP 5890 Gas Chromatograph interfaced with an HP 5989A Mass Spectrometer in the EI mode at 70 eV.

For the toxicological studies, solutions of the dried extracts and of kavain were prepared in DMSO.

Cell culture

The human hepatocarcinoma cell line HepG2 was kindly provided by Dr. Dietrich von Schweinitz (Department of Pediatric Surgery, Children's Hospital, University of Basel). The cell line was grown in RPMI 1640 medium (supplemented with GlutaMAX^{TM}-I, 25 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin) (all from Gibco, Paisley, UK). Culture conditions were 5% CO_2 and 95% air atmosphere at 37 °C. Experiments were performed when the cells had reached a confluence of about 80%.

Cytotoxicity tests

The sulforhodamine B (SRB) test was performed according to the protocol of Skehan (Skehan et al., 1990). For the lactate dehydrogenase (LDH) assay, cells were incubated with the extracts in a 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) for the indicated time period. Two hundred microliters of the supernatant were used for the detection of the LDH activity according to the method of Vassault (1983).

To examine cell viability and activity of the mitochondrial electron transport chain, the dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used (Bruggisser et al., 2002). This assay is based on the ability of living cells to (reductively) convert the dissolved MTT (yellow) into the insoluble formazan (blue). The latter can be measured colorimetrically and is proportional to the metabolic activity of the cells investigated. For this assay, 50,000 cells/well were incubated in a 96-well plate in the presence of kava extracts for 24 h. After washing, 0.5% MTT was added for 4 h. The reaction was stopped with 100 µl sodium dodecyl sulfate 20% and absorption was measured at 550 nm (Spektra Max 250, Molecular Devices, Sunnyvale, CA, USA).

Mitochondrial membrane potential in HepG2 cells

To assay the mitochondrial membrane potential, the JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) assay was performed according to the protocol of Molecular Probes. After detachment of the cells with 10 mmol/l EDTA, they were filtered through a 40 µm mesh. Cells (100,000) were incubated in the presence of the kava extracts and JC-1 (7.5 µM). Subsequently, cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Isolation of rat liver mitochondria

Rats were anesthetized with carbon dioxide and killed by decapitation. The liver was extirpated, rinsed, minced and washed with ice-cold MSM buffer (220 mmol/l mannitol, 70 mmol/l sucrose, 5 mmol/l 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.4). Mitochondria were isolated by differential centrifugation according to the method of Hoppel et al. (1979). The mitochondrial protein content was determined using the biuret method with bovine serum albumin as standard (Gornall et al., 1949).

Oxidative metabolism of isolated mitochondria

Polarographic monitoring of oxygen consumption was carried out in a 1 ml chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30 °C as described previously (Krahenbuhl et al., 1991). The final concentration of the substrate L-glutamate was 20 mmol/l. The respiratory control ratio (RCR) was calculated according to Estabrook (1967) as the ratio between the rate of oxygen consumption in the presence of a substrate and added ADP (state 3) and the rate obtained after complete conversion of ADP to ATP (state 4).

After depletion of endogenous substrates by the addition of ADP, the substrate to be tested was added to the incubation. Subsequently, state 3 respiration was initiated by adding ADP (final concentration 100 µmol/l). The test compounds were added to the mitochondrial incubations before the addition of the substrate L-glutamate. Subsequent experiments with the F_1F_0-ATPase inhibitor oligomycin (5 µg/ml) were performed to check whether a possible drop in the RCR derives from uncoupling or increased intramitochondrial metabolism of ATP. An uncoupler is able to dissipate the proton gradient over the inner mitochondrial membrane and thereby to reinitiate the electron and oxygen flow even when the mitochondrial ATPase is inhibited.

Mitochondrial β-oxidation

β-Oxidation with freshly isolated liver mitochondria was assessed as the formation of ^{14}C-acid-soluble β-oxidation products from [1-^{14}C]palmitic acid in the presence of the kava extracts. Experiments were performed as described previously (Freneaux et al.,

1988) with the modifications described by Spaniol et al. (2001). After 5 min of preincubation with the kava extracts, the reaction was started by adding 100 µl 400 µmol/l [1-^{14}C]palmitic acid (0.925 Bq/100 µl) and the incubations were carried out at 30 °C for 15 min with slow shaking. We have shown previously that the reaction is in the linear phase at this time point (Spaniol et al., 2001). The reaction was stopped with 200 µl 20% perchloric acid and by placing the tubes on ice. After centrifugation for 5 min at 12,000*g*, the ^{14}C-acid-soluble products in the supernatant were counted. It has been verified under the conditions used that the formation of ketone bodies accounts for at least 80% of the [1-^{14}C]palmitate oxidized (Fromenty et al., 1989).

Glutathione content in HepG2 cells

In order to assess the redox status of the treated cells and possible formation of reactive metabolites, determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) was performed as follows. HepG2 cells were co incubated with kava for 2 h, scrapped from the dish, suspended in 1 ml 1 mmol/l bathophenanthrolinedisulfonic acid in 10% perchloric acid and sonicated for 30 s (sonicator from Heat Systems Ultrasonics Inc., Farmingdale, NY, USA, setting 4.5). After centrifugation, the pellet was used for protein determination (BCA protein assay kit, Pierce, Rockford, IL, USA) and the supernatant for the determination of glutathione using the enzymatic recycling assay of Tietze (1969) with the modifications of Griffith (1980). For the determination of total glutathione, the pH of the supernatant was adjusted to pH 6–7 by the addition of triethanolamine. For the determination of GSSG, the derivatization with 2-vinylpyridine was performed prior to the pH adjustment. For the enzymatic reaction, NADPH (0.21 mmol/l), DTNB (0.6 mmol/l), glutathione reductase (1 U/ml) and sample or standard, respectively, were mixed. The formation of TNB was monitored at 30 °C using a plate reader at 412 nm during 2 min. Glutathione was quantified by comparing the slope of the samples with that one of the corresponding standard curve.

Apoptosis and necrosis of HepG2 cells

Discrimination between apoptosis and necrosis was done with an annexinV/propidium iodide stain and analysis by flow cytometry (FACS Calibur, Becton Dickinson) as described previously (Kaufmann et al., 2005). HepG2 cells were incubated for 24 h with the different extracts. After trypsinization and centrifugation, cells were resuspended in RPMI 1640 medium (adjusted to 2.5 mmol/l calcium), stained with Alexa Fluor 633 labelled annexin V and propidium iodide (final concentration 1 µg/ml) and analysed by FACS. Annexin V-positive cells are in early apoptosis, whereas annexin V and propidium iodide double positive cells can be in both, late apoptosis or necrosis (Kaufmann et al., 2005).

ATP determination

The ATP content of HepG2 cells treated with kava extract was determined with the luciferin/luciferase method using an ATP bioluminescence assay kit (Sigma, Buchs, Switzerland) as described previously (Kaufmann et al., 2005). After treating the cells with the three kava extracts (each 10 or 150 µg/ml) for 24 h, the cells were trypsinized, resuspended in 600 µl water and snap-frozen in liquid nitrogen. To extract the ATP from the cells, the samples were incubated in boiling water for 10 min and centrifuged (20,000*g*, 5 min, 4 °C) (Yang et al., 2002). One hundred microliters of the supernatant were used for the determination of ATP according to the user manual of Sigma. The ATP content was calculated by comparison to a standard curve.

Statistical methods

Data represent mean ± standard error of the mean (SEM) of at least $n = 3$ replicates. Statistical analysis of differences between control incubations and incubations with kava extract was performed using analysis of variance (ANOVA) and Dunnett's multiple comparison test as posthoc test to localize differences obtained by ANOVA. A p-value <0.05 was considered to be statistically significant.

Results

Analysis of the three kava extracts

The chromatograms of the extracts showed several peaks which could be identified as kavalactones. No clear difference in the total amount of kavalactones was observed in the methanolic and the acetonic root extract, respectively. Each of them contained about 80% (w:w) kavalactones (Table 1). The leaf extract was clearly distinguishable from the root extracts, containing only 24% kavalactones (Table 1). In the root extracts, the contents of the individual kavalactones identified were more or less equal, with quantities in the range of 10–17%. In contrast, the leaf extract contained only about 0.5–1% of each kavalactone, with the exception of dihydromethysticin and dihydrokavain, which were present at about 10%. Furthermore, the leaf extract showed an additional peak which could not be identified and was not detectable in the root extract.

The pipermethystine content was in the range of 0.01% on the root extract and approximately 100 times higher in the leaf extract, reaching 1.34% (w:w). The high content of pipermethystine in leaves is consistent with previous reports (Dragull et al., 2003; Smith, 1983).

Cytotoxicity

The cytotoxicity of the three kava extracts was investigated using the hepatocarcinoma cell line HepG2. The sulforhodamine B assay (SRB) showed a concentration-dependent toxicity for all three kava extracts from a concentration of 50 µg/ml on, when incubated for 24 h (Fig. 1). These results were verified with the lactate dehydrogenase (LDH) test, which revealed toxicity already after incubations for 1, 3.5 or 5.5 h at similar concentrations (results not shown).

After having observed a cytotoxicity of the three kava extracts using the SRB and the LDH test, further steps were undertaken to clarify the mechanisms of toxicity. As mitochondria are often a target of toxicity, an MTT test was performed, which mainly reflects the mitochondrial reductive activity (Bruggisser et al., 2002). As shown in Fig. 2, reductive activity of HepG2 cells was clearly decreased by the two root extracts in a concentration-dependent fashion from 1 µg/ml (acetonic root extract) or 25 µg/ml (methanolic root extract). In comparison, the leaf extract had only a moderate effect on the reductive capacity of HepG2 cells, showing a significant toxicity from 10 µg/ml on.

Kavain, a kavalactone and thus a main component of kava extracts, was tested at concentrations according to its content in the kava plant and also to its content in the kava extracts. At concentrations between 0.77 and 7.7 µg/ml, kavain did not show any toxicity on HepG2 cells, neither in the LDH, nor in the MTT test (data not shown).

Table 1. Kavalactone and pipermethystine content of the three kava extracts

Component	Methanolic root	Acetonic root	Methanolic leaves
Methysticin	16.6	17.3	0.6
Dihydromethysticin	12.0	12.4	11.3
Kavain	16.2	14.4	0.4
Dihydrokavain	13.4	13.7	10.2
Yangonin	13.6	13.1	1.1
Demethoxyyangonin	9.8	10.6	0.5
Total kavalactones	81.6	81.4	24.1
Pipermethystine	0.011	0.011	1.34

Kavalactones were analyzed with reversed phase HPLC and pipermethystine by GC–MS as described in Materials and methods. Units are % of dried extract (w:w).

Mitochondrial toxicity of kava extracts

Since the MTT test suggested a toxic effect of kava extracts on mitochondria, the mitochondrial membrane potential was determined in HepG2 cells. Using JC-1 as a marker (Cossarizza et al., 1993), both root extracts dissipated the mitochondrial membrane potential (the methanolic root extract at 100 µg/ml and the acetonic root extract starting from 50 µg/ml, see Fig. 3). In contrast, the leaf extract did not affect the mitochondrial membrane potential up to 100 µg/ml.

Since the function of the respiratory chain is important for the maintenance of the mitochondrial membrane potential, oxidative metabolism was tested in the presence of kava extracts using isolated rat liver mitochondria. In the presence of 150 µg/ml root extract,

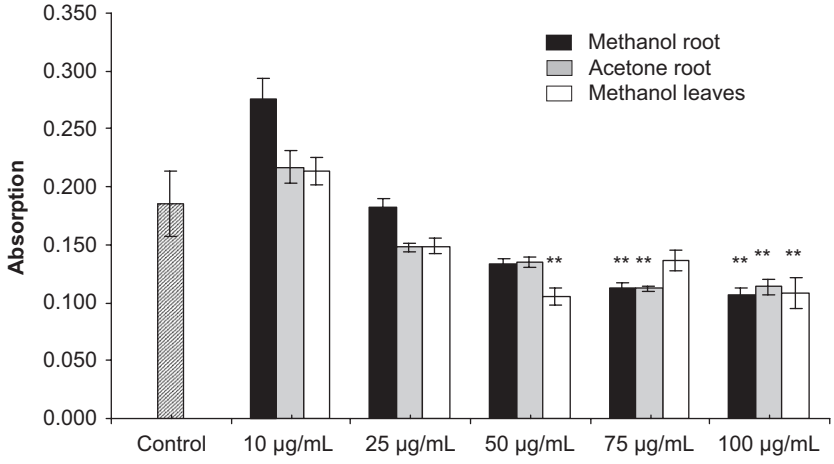

Fig. 1. Cytotoxicity of kava extracts using sulforhodamine B. Ten thousand cells were incubated for 24 h with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. Proteins of the living cells were stained with sulforhodamine B (0.4%) and the absorption was measured at 540 nm (the lower the absorption, the higher is cytotoxicity). Results are expressed as mean ± SEM of five determinations. $^{**}p < 0.01$ vs. control incubations.

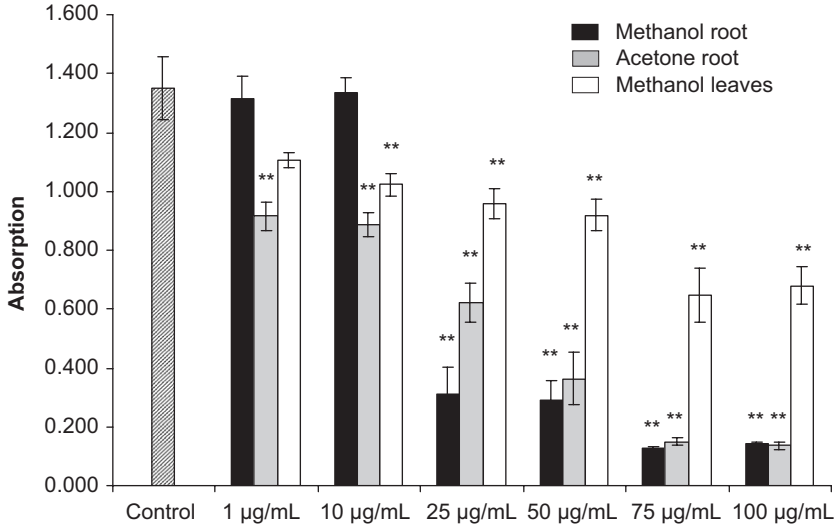

Fig. 2. Cytotoxicity using MTT, a marker for reductive activity of the cells. Cells were incubated for 24 h with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. MTT (0.5%) was added and color intensity due to the formation of formazan was measured at 550 nm. Results are expressed as mean ± SEM of 10 determinations. $^{**}p < 0.01$ vs. control incubations.

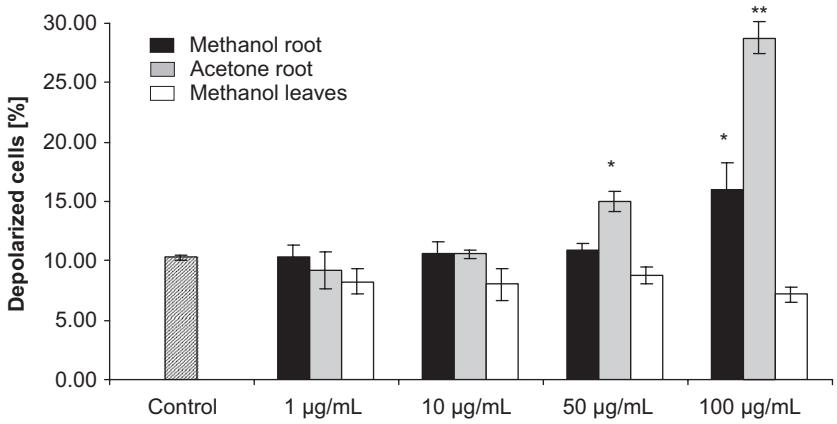

Fig. 3. Determination of the mitochondrial membrane potential using the dye JC-1. Cells were incubated for 10 min with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. The percentage of cells with a depolarized mitochondria is indicated. Results are expressed as mean ± SEM of three determinations. $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control incubations.

state 3 oxidation rates were significantly inhibited, but not in the presence of the leaf extract at the same concentrations or in the presence of 7.7 µg/ml kavain (Fig. 4A). In contrast, none of the root extracts affected state 4 oxidation rates, whereas state 4 showed a significant increase in the presence of 150 µg/ml leaf extract (Fig. 4B).

In order to investigate uncoupling of oxidative phosphorylation directly, state 4u was induced by the addition of oligomycin, an inhibitor of F_1F_0-ATPase. Under such conditions, uncouplers are able to increase oxygen consumption. As shown in Fig. 4C, both root extracts and also the leaf extract were associated with a significant increase in state 4u oxygen consumption at 150 µg/ml. In contrast, such an increase could not be shown in the presence of 7.7 µg/ml kavain.

Besides the respiratory chain, β-oxidation of fatty acids represents another target of mitochondrial toxicity which can affect the membrane potential. Using freshly isolated rat liver mitochondria, all three extracts showed a concentration-dependent inhibition of the activity of mitochondrial β-oxidation as compared to control incubations (Fig. 5). For the two root extracts, inhibition could be demonstrated starting from a concentration of 50 µg/ml, whereas the leaf extract showed toxicity starting at 200 µg/ml. At 200 µg/ml,

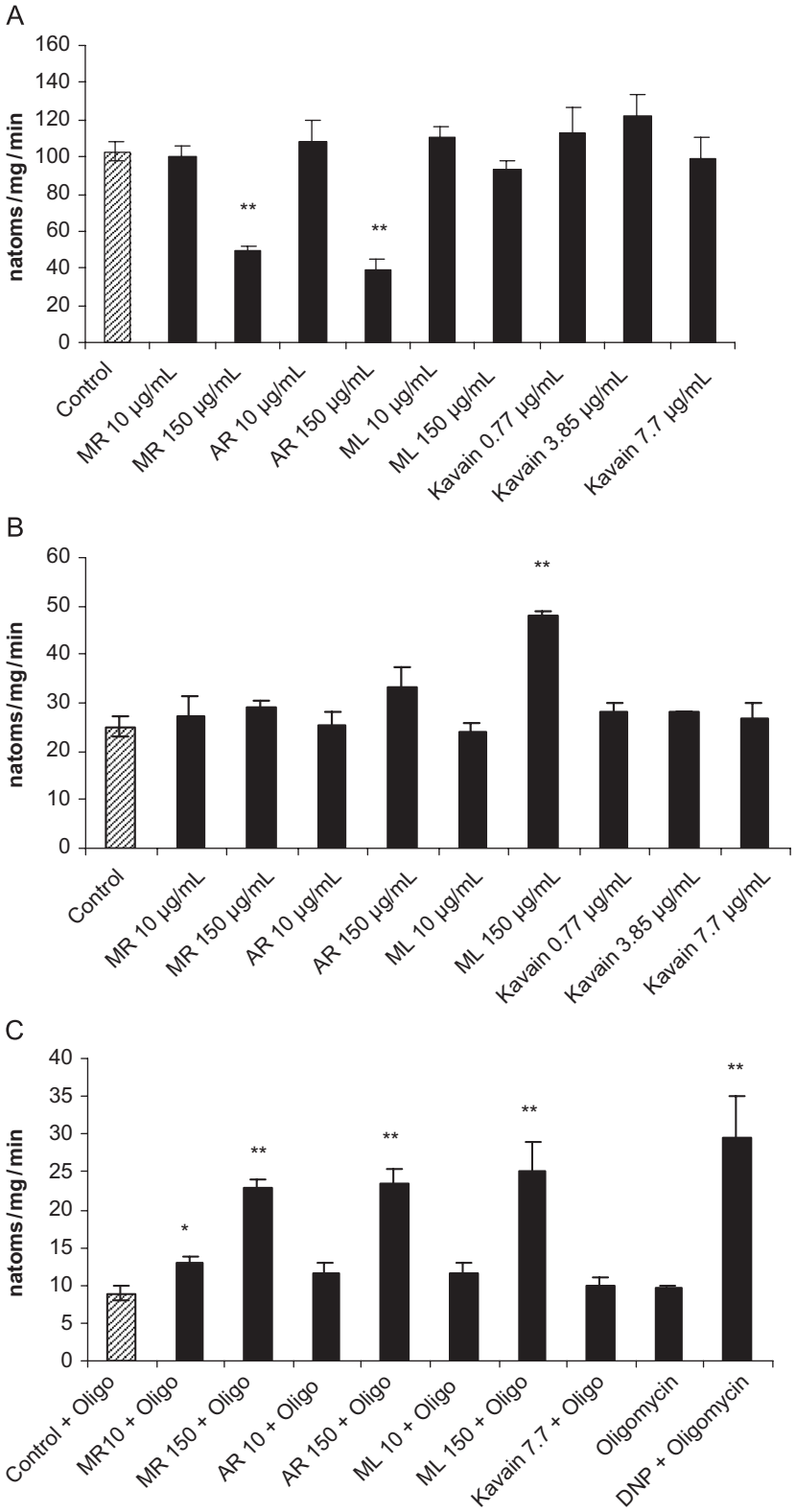

Fig. 4. Oxidative metabolism by freshly isolated rat liver mitochondria. L-Glutamate (20 mmol/l) was used as the substrate. Oxygen consumption was determined in the presence of ADP (state 3, A) and after complete conversion of ADP to ATP (state 4, B). In a second experiment, the mitochondria were pre incubated with the F_1F_0-ATPase inhibitor oligomycin (5 µg/ml) in order to investigate uncoupling of oxidative phosphorylation (C). An increase in oxygen consumption in the presence of oligomycin indicates uncoupling of the respiratory chain. MR = methanolic root, AR = acetonic root, ML = methanolic leaf extract. Oligo = oligomycin. DNP = dinitrophenol (0.1 mmol/L). Results are expressed as mean ± SEM of three determinations. Units are natoms O/min/mg mitochondrial protein. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control incubations.

the extent of inhibition was similar for all extracts, with the residual activity ranging from 14.7% to 35.9% of the activity in control incubations.

Glutathione pool of HepG2 cells

The experiments described so far have shown that kava extracts can inhibit and/or uncouple the respiratory chain and mitochondrial β-oxidation, leading to a decrease in the mitochondrial membrane potential. Since inhibition of the respiratory chain can be associated with the generation of ROS (Kaufmann et al., 2005), the redox status of HepG2 cells was assessed by measuring the glutathione pool. Treatment with kava extracts was associated with a decrease in cellular GSH and an increase in GSSG, but the differences to the control values did not reach significance (Table 2). Accordingly, the GSH to GSSG ratio of the cells treated with kava were in the range of 1.5, whereas the control ratio was 2.85. This difference reached statistical significance for the leaf extract, but not for the root extracts.

Mode of cell death

Mitochondria play a key role in the mechanisms associated with apoptotic, but also necrotic, cell death (Green and Reed, 1998; Kroemer et al., 1998; Leist et al., 1997). In order to discriminate between apoptosis and necrosis, staining with fluorescence labelled annexin V in combination with propidium iodide was performed using HepG2 cells exposed to the kava extracts. Both root extracts (Figs. 6A and B) and the leaf extract (Fig. 6C) induced a concentration-dependent increase in the apoptotic and/or necrotic/late apoptotic cell population at 150 µg/ml. The specificity of this mechanism was

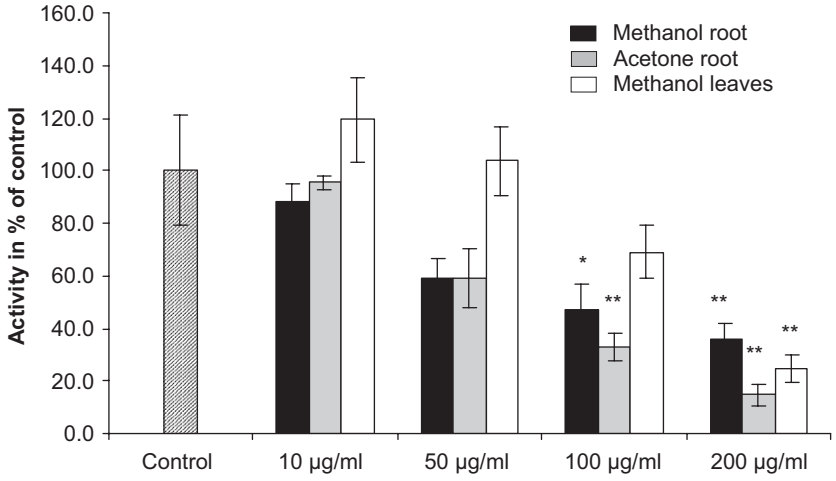

Fig. 5. Activity of mitochondrial β-oxidation. The formation of acid soluble products from ^{14}C-palmitic acid was determined in the presence of methanolic root (solid bars), acetonic root (grey bars) or methanolic leaf (open bars) extract. The rate of the control incubations (100%) was 0.61 nmoles/min/mg mitochondrial protein. Results are expressed as mean ± SEM of three determinations. $^{**}p < 0.01$ vs. control incubations.

Table 2. Glutathione pool of HepG2 cells treated with kava extracts

	GSH (µg/mg protein)	GSSG (µg/mg protein)	GSH/GSSG ratio
Control	12.6 ± 0.7	4.48 ± 0.42	2.90 ± 0.41
Antimycin A	8.88 ± 1.69	7.62 ± 2.24	1.23 ± 0.14*
MR 10 µg/ml	9.82 ± 1.24	5.82 ± 0.70	1.80 ± 0.45
MR 150 µg/ml	7.98 ± 1.29	5.30 ± 1.00	1.73 ± 0.63
AR 10 µg/ml	10.2 ± 1.2	7.49 ± 0.97	1.45 ± 0.32
AR 150 µg/ml	10.6 ± 1.6	6.89 ± 1.17	1.75 ± 0.63
ML 10 µg/ml	11.5 ± 0.8	8.47 ± 1.10*	1.43 ± 0.29*
ML 150 µg/ml	8.65 ± 2.42	9.47 ± 0.93*	0.99 ± 0.39*

Antimycin A (final concentration 10 µg/ml) was used as a positive control. GSH: reduced glutathione; GSSG: oxidized glutathione; MR = methanolic root; AR = acetonic root; ML = methanolic leaf extract; $^{*}p < 0.05$ vs. control.

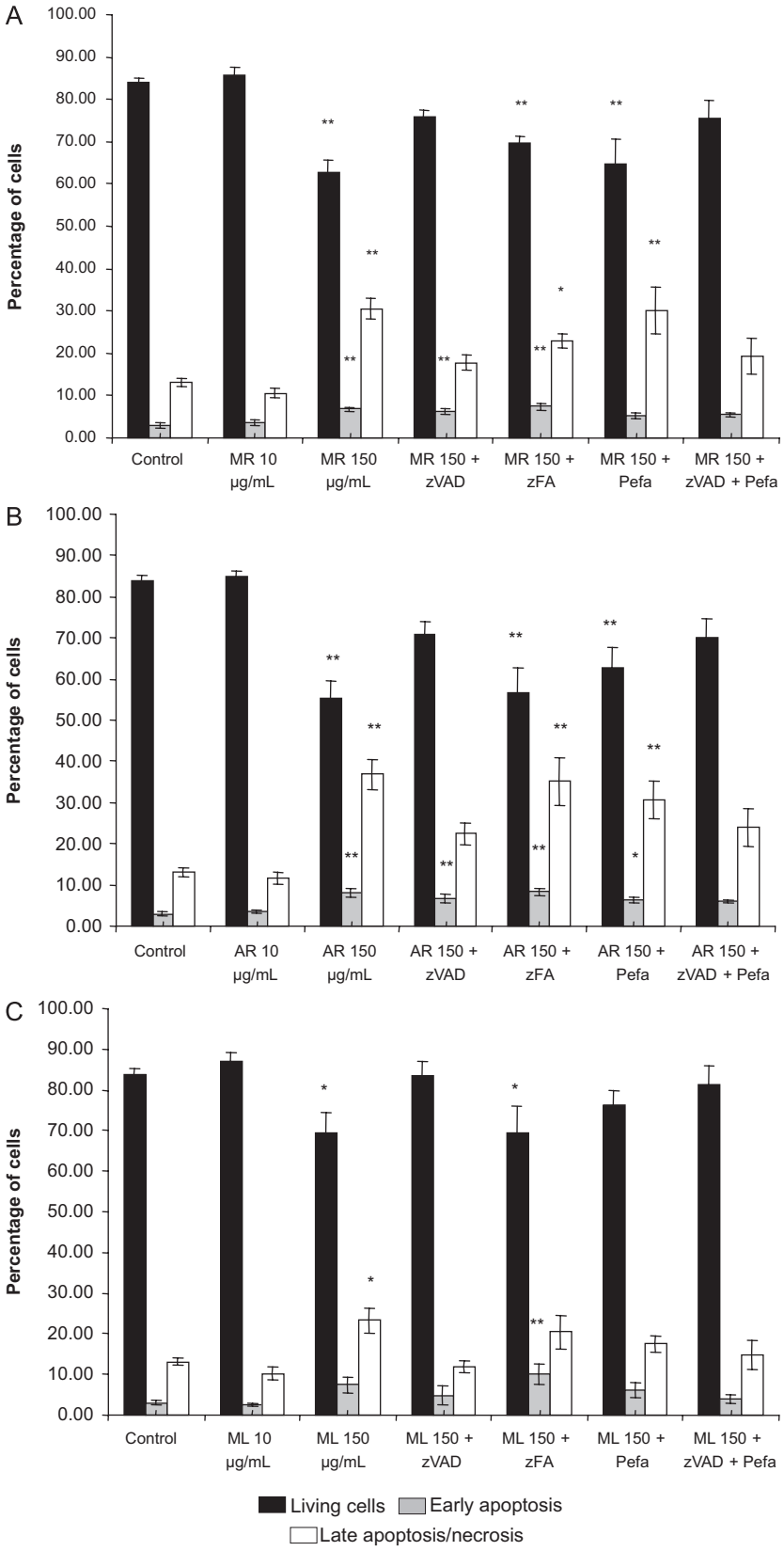

Fig. 6. Type of cell death associated with kava extracts. HepG2 cells were separated into the three categories, living cells, early apoptotic cells and late apoptotic/necrotic cells. Distinction between these three groups was performed using a combined annexin V/PI stain, analyzed with FACS as described in Materials and methods. Cells had been incubated for 24 h with a methanolic root (MR, A), an acetonic root (AR, B) or a methanolic leaf (ML, C) extract at the concentrations indicated. In addition, 3 h preincubations with the pancaspase-inhibitor zVAD, the corresponding negative control zFA, and/or the serine protease inhibitor Pefablock had been performed in selected incubations as indicated in the figure. Results are expressed as mean ± SEM of at least three determinations. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control incubations.

shown by adding the pancaspase-inhibitor zVAD-fmk to the incubations, which was able to reduce early and/or late apoptosis; this was not the case with the negative control zFA-fmk. Similarly, the serine protease inhibitor Pefablock did not affect apoptosis, since it reduced cytotoxicity only in combination with zVAD-fmk.

Cellular ATP content

When stained with annexin V/propidium iodide necrosis or (late) apoptosis could be detected. To make a further discrimination between these two modes of cell death, the ATP content of treated cells was determined. For the occurrence of apoptosis, normal levels of ATP are necessary, whereas low cellular ATP levels are indicative for necrosis (Eguchi et al., 1997; Leist et al., 1997). In our experiments, the ATP levels of the cells treated with the three kava extracts were not decreased compared to the control (data not shown), indicating that the cell death in the presence of the kava extracts is due to apoptosis and not necrosis.

Discussion

During the last few years, cases of hepatotoxicity associated with the ingestion of kava have been published in several countries, including Switzerland, Germany, Australia and Canada (Escher et al., 2001; Gow et al., 2003; Humberston et al., 2003; Russmann et al., 2001; Schmidt, 2003; Stickel et al., 2003). In many cases, the trigger for liver toxicity was not clearly identifiable as other medications were ingested concomitantly. In some cases, however, the relationship between ingestion of kava and hepatotoxicity was judged as probable or definite (Russmann et al., 2001; Schmidt, 2003; Stickel et al., 2003). To find out more about possible mechanisms involved in kava hepatotoxicity, we examined the toxic effects of different kava extracts on hepatocytes cell lines and isolated liver mitochondria.

After having demonstrated toxic effects resulting in cell death using HepG2 cells, additional tests were performed with the aim to find out the mechanisms of toxicity. One of these assays was the MTT test, a colorimetric method used to assess cytotoxicity and therefore viability of cells (Bruggisser et al., 2002). Since reduction of MTT is dependent on the activity of the mitochondrial electron transport chain, impaired formazan production from MTT is usually interpreted as indicative for mitochondrial damage. To determine whether the reduced production of formazan was indeed due to the impairment of mitochondrial function by the kava extracts, the mitochondrial membrane potential was measured using the fluorescent dye JC-1 (Cossarizza et al., 1993). Depolarization of mitochondria could be demonstrated for both root extracts, but not for the leaf extract, a result agreeing well with the MTT test, which showed also only a marginal toxicity for the leaf extract.

The experiments with isolated liver mitochondria definitively demonstrated that kava extracts are toxic for mitochondria. The root extracts were shown to act as inhibitors and also uncouplers of the respiratory chain and inhibitors of β-oxidation, and the leaf extract as an inhibitor of β-oxidation and uncoupler of oxidative phosphorylation. Considering that mitochondria are important factors in cell death induction (Green and Reed, 1998; Kaufmann et al., 2005; Kroemer et al., 1998), apoptosis and necrosis were examined directly. Annexin V FACS stains revealed that all kava extracts induced early apoptosis and also late apoptotic/necrotic cell death. Cell death could be reduced by the pancaspase inhibitor zVAD-fmk, demonstrating the involvement of caspases, which are key players of apoptosis. It can therefore be speculated that the interaction of the kava extracts with the electron transport chain and β-oxidation, in combination with uncoupling of oxidative phosphorylation, result in a decrease of the mitochondrial membrane potential, possibly via opening of the permeability transition pore (Kim et al., 2003; Lemasters, 1999). This may lead to the rupture of the outer mitochondrial membrane with the release of cytochrome *c* and other factors into the cytoplasm, leading to apoptosis and/or necrosis.

In order to find out whether ROS and/or other active metabolites are essential in cell death associated with kava extracts, we determined the glutathione pool of the cells. An increased cellular GSSG content and/or a decreased GSH/GSSG ratio would be compatible with the presence of reactive metabolites. At least a tendency pointing in this direction could be observed for all three kava extracts, most probably indicating increased production of ROS.

Our findings reveal that the leaf extract exerts a lower toxicity as compared to the root extracts, and that there is no difference between acetonic and methanolic root extracts. Considering the composition of the extracts, showing a higher kavalactone and a lower pipermethystine content in the root extracts than in the leaf extract, the toxicity appears to be associated with the kavalactones. On the other hand, kavain itself was not toxic, suggesting that methysticin, yangonin and/or one of their metabolites are associated with hepatotoxicity. These findings are in apparent contradiction to other studies, where alkaloids such as pipermethystine were considered to be associated with hepatotoxicity (Prakash et al., 1999). Furthermore, in HepG2 cells, pipermethystine was associated with a more accentuated toxicity as compared to kavalactones (Nerurkar et al., 2004). It has to be taken into account, however, that we used entire extracts, where pipermethystine is only a minor component in comparison to

the kavalactones. Although our results favor kavalactones as the hepatotoxic component of kava extracts, they do not allow proposing the preferential use of the aerial parts of the plant instead of the roots. Since the concentration of the active components, the kavalactones, is low in the aerial parts compared to the roots, ingestion of a therapeutic amount of kavalactones is associated with the high ingestion amount of other, potentially toxic components.

Finally, it can be estimated whether the investigated *in vitro* concentrations of the kava extracts correspond to relevant tissue levels in humans. According to the recommendation of the German Commission E, the daily consumption of kava should not exceed 60–120 mg kavalactones. Based on the kavalactone content of our root extracts (approximately 80%) and leaf extract (approximately 24%), 120 mg kavalactones correspond to 150 mg kava root or 500 mg leaf extract, respectively. Since kinetic data of kava extracts in humans have not been published, our assumptions are based on kinetic data of kavain and its first metabolite OH-kavain (Tarbah et al., 2003). After oral ingestion of 0.8 g kavain, plasma concentrations of kavain were in the range of 40 ng/ml after 1 h and 10 ng/ml after 4 h. Extrapolation to the *y*-axis at time 0 h using first order kinetics results in a plasma concentration of approximately 65 ng/ml. Assuming a good bioavailability, this initial plasma concentration corresponds to a very high volume of distribution (in the range of 10^4 l), suggesting an almost exclusive accumulation in tissues (Tarbah et al., 2003). After ingestion of 120 mg kavalactones (the highest recommend dose) and assuming a homogeneous tissue distribution, tissue concentrations in the range of 1–2 μg/g (corresponding to 2–10 μg extract per g tissue) could be expected, which is the lower range where we started to observe mitochondrial toxicity. Since only a minority of persons developed hepatotoxicity after ingestion of kava extracts, an individual predisposition appears also to be important, as described for other mitochondrial toxins such as valproate (Krahenbuhl et al., 2000).

Conclusion

Our data show that kava extracts exert toxic effects in *in vitro* models of liver toxicity, i.e. HepG2 cells and isolated rat liver mitochondria. How exactly these findings can be related to the *in vivo* situation in humans cannot be answered with certainty, as the pharmacokinetic properties of the kava extracts are not known exactly. Taking into account the *in vitro* effects on liver mitochondria and HepG2 cells, it can be speculated that under certain conditions, e.g. the presence of mitochondrial diseases, individual patients could develop hepatotoxicity due to ingestion of kava extracts.

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