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Immunohistochemical analysis of expressions of hepatic cytochrome P450 in F344 rats following oral treatment with kava extract

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Abstract

Kava (*Piper methysticum*), used for relaxation and pain relief, has been one of the leading dietary supplements and several reports linking hepatic functional disturbances and liver failure to kava have resulted in a ban on sales in Europe and Canada and the issuance of warnings by the US FDA.

The National Toxicology Program conducted 14-week rat studies to characterize the toxicology of kava exposure in Fischer 344 rats [National Toxicity Program. 90 day gavage toxicity studies of KAVA KAVA EXTRACT in Fischer rats and B6C3F1 mice. Research Triangle Park, NC; 2005a; National Toxicity Program. Testing status of agents at NTP (KAVA KAVA EXTRACT M990058). Research Triangle Park, NC; 2005b. (http://ntp.niehs.nih.gov/index.cfm?objectid=071516E-C6E1-7AAA-C90C751E23D14C1B)]. Groups of 10 male and 10 female rats were administered kava extract by gavage at 0, 0.125, 0.25, 0.5, 1.0, and 2.0 g/kg/day. Increased γ-glutamyl-transpeptidase (GGT) activities were observed in the 2.0 g/kg males and 1.0 and 2.0 g/kg females, as well as increased serum cholesterol levels in males and females at 0.5 g/kg and higher. Increases in incidence and severity of hepatocellular hypertrophy (HP) were noted in males at 1.0 g/kg and females at 0.5 g/kg and higher, as well as increased liver weights. Immunohistochemical analyses of the expression of cytochrome-P450 (CYP) enzymes in liver of the control and 1.0- and 2.0-g/kg-treated groups indicated decreased expression of CYP2D1 (human CYP2D6 homolog) in 2.0 g/kg females and increased expression of CYP1A2, 2B1, and 3A1 in 1.0 and 2.0 g/kg groups of both sexes.

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The no observed adverse effect levels were decided as 0.25 g/kg in both genders, based on neurotoxic effects, increases in GGT, cholesterol, liver weight, and HP and decreases in body weight. Kava-induced hepatic functional changes in the F344 rat might be relevant to human clinical cases of hepatotoxicity following exposure.

Keywords: Kava; *Piper methysticum*; Herb; Hepatocellular hypertrophy; Cytochrome P450; F344 rat

Introduction

Kava (*Piper methysticum*) has traditionally been consumed as an aqueous extract of the root by many Pacific Island societies in a ritualistic beverage at ceremonies celebrating events such as weddings, funerals, and births; religious occasions; the welcoming of honored visitors; and the exchanging of gifts (Gruenwald, 2003; Singh, 1992). Modern uses focus on its anxiolytic and sedative effects; it induces relaxation, restful sleep, relief of headache and back pain, promotion of sociability, and relief of fatigue (Gruenwald, 2003; Norton and Ruze, 1994). Kava products are commonly standardized to contain 30% kavalactones. The recommended oral dose for usage as an anxiolytic is 50–70 mg kavalactones 2–4 times a day and, as a hypnotic, 150–210 mg in a single oral dose before bedtime (Bilia et al., 2002). Kava is being marketed on the Internet as a legal alternative to certain illicit drugs (Dennehy et al., 2005). Some commercially available beverages such as chocolate, tea, and drink mixes include kavalactones ranging between 4.7 and 135 mg per serving (De Jager et al., 2004).

Kava has reportedly been associated with hepatotoxicities in humans, including functional disturbances, hepatitis, cirrhosis, and liver failure (Campo et al., 2002; Gruenwald, 2003; Hefner, 2002; Humberston, 2003; Teschke et al., 2003). The sale of kava has been suspended in France, Germany, Switzerland, Canada, and Britain (Gruenwald, 2003; Teschke et al, 2003; Ulbricht et al., 2005). The US FDA has not regulated consumption, though the potential risk of liver injury raises concern (Food and Drug Administration (FDA), 2001, 2002; Hefner, 2002; Ulbricht et al., 2005). The Natural Standard Research Collaboration is working with Advisory Committee on Safety of Medicinal Products of the World Health Organization (WHO) on a new official report on kava and hepatotoxicity (Ulbricht et al., 2005).

The cytochrome P450 (CYP)s are Phase I enzymes that play critical roles in the bioactivation and detoxification of a wide variety of xenobiotic substances (Guengerich, 2001; Omiecinski et al., 1999; Zuber et al., 2002). Kava toxicity has been partially attributed to CYP2D6 deficiency found in 7–9% of Caucasian, 5.5% of Western European, almost 1% of Asian, and less than 1% of Polynesian populations (Ingelman-Sundberg, 2005; Poolsup et al., 2002; Wanwimolruk et al., 1998). Genetic differences may constitute significant contributory factors for increased hepatotoxicity in Caucasians (Singh, 2005). In vitro studies with human hepatocytes have shown that kava inhibits CYP1A2, 2C9, 2C19, 3A4, 2D6, 4A9/11, while CYP2A6, 2C8, and 2E1 activities are unaffected (Anke and Ramzan, 2004a, b; Mathews et al., 2002; Unger et al., 2002). Inhibition of these CYPs or a deficiency in CYP2D6 indicates that exposure to kava and other drugs and chemical agents at the same time has a high potential for causing drug interactions (Anke and Ramzan, 2004a, b; Bressler, 2005; Hu et al., 2005; Jamieson and Duffield, 1990; Mathews et al., 2002, 2005; Singh, 2005; Teschke et al., 2003; Whitton et al., 2003).

Recently, the National Toxicology Program (NTP) completed 14-week gavage toxicity studies of kava extract in F344 rats and B6C3F1 mice (National Toxicity Program, 2005a, b). The present report summarizes toxicological findings, particularly hepatic expressions of CYPs, in the F344 rats.

Materials and methods

Study design

Groups of 10 male and 10 female F344 rats, designated as core study rats, were administered kava extract in corn oil by gavage at 0 (vehicle control), 0.125, 0.25, 0.5, 1.0, 2.0 g/kg/d, 5 days per week for 14 weeks. At terminal sacrifice at 14 weeks blood samples were collected from these animals for clinical pathology (hematology and clinical chemistry) determinations. Additional groups of 10 male and 10 female F344 rats, designated as special study rats, were administered kava extract in corn oil at the same dose levels and used for clinical-pathology determinations on study days 4 and 23. They were sacrificed after blood was collected on day 23.

Chemicals

Kava extract contains 18 lactones termed kavalactones (Clouatre, 2004; He et al., 1997). Six kavalactones constituted approximately 96% of the extract: yagonin, 7,8-dehydrokawain, kawain, 7,8-dihydromethysticin,

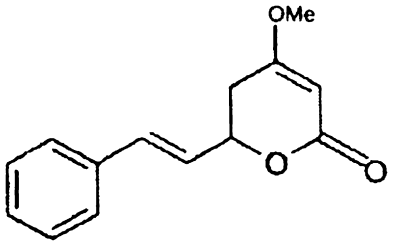
methysticin, and 5,6-dehydrokawain (Table 1). Kava extract (CAS no. 9000-38-8, Lot no. 9077SDK/Cosmo) was received in three amber glass vials as a powered extract from Midwest Research Institute (MRI, Kansas City, MO) at $<-20\,^{\circ}C$. All material in the three vials was combined into a lot (Lot no. 082203), and the purity determined by high-performance liquid chromatography with ultraviolet detection (HPLC/UV) was 100.7%. Liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry identified and quantified the six largest peaks as yangonin (42.76%), 7,8-dihydrokawain (34.69%), kawain (8.87%), 7,8-dihydromethysticin (4.03%), methysticin (3.23%), and 5,6-dehydrokawain (2.42%).

The USP-grade corn oil (Lot no. SS0711) used to prepare the vehicle for gavage formulations was obtained from Spectrum (Gardena, CA). Kava extract was formulated in the corn oil at 0, 25, 50, 100, 200, and 400 mg/ml and stored in sealed glass containers. Homogeneity of formulations was determined as indicated prior to the start of administration of dosages. Formulation was stable for 42 days at room temperature and approximately 5 °C.

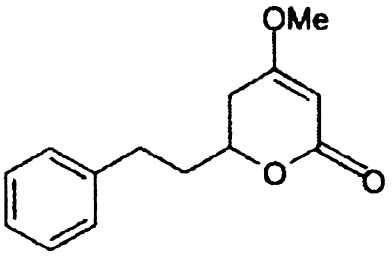
Animals

Animal studies were conducted at an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility of Battelle-Columbus Laboratories (Columbus, OH). Animal handling and husbandry were conducted in

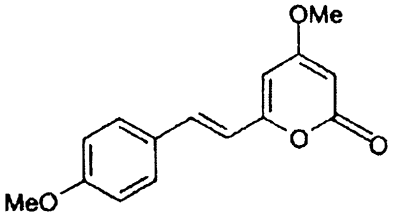
Table 1. Phytochemical compounds of kava-extract kavalactones



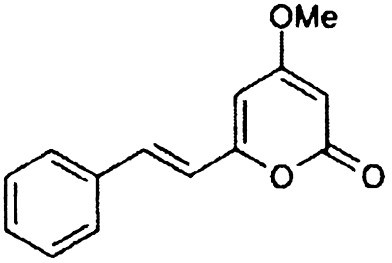
Kawain: 8.87%[a]
(LD_{50}: 1130 mg/kg)[b]



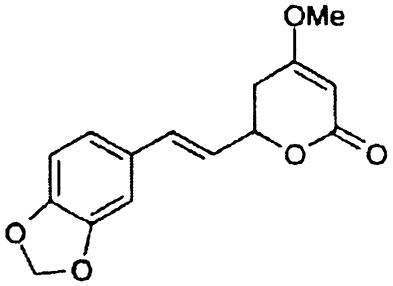
7,8-Dihydrokawain: 34.69%[a]
(LD_{50}: 980 mg/kg)[b]



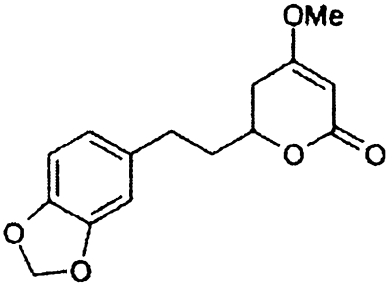
Yangonin: 42.76%[a]
(LD_{50}: >1500 mg/kg)[b,c]



5,6-Dehydrokawain (desmethoxyyangonin): 2.42%[a]
(LD_{50}: >800 mg/kg)[c,b]



Methysticin: 3.23%[a]
(LD_{50}: >800 mg/kg)[b,c]
Other compounds: 4%



7,8-Dihydromethysticin: 4.03%[a]
(LD_{50}: 1050 mg/kg)[b]

[a]Content in kava extract that produced from kava (CAS no. 9000-38-8, Lot no.9077SDK/Cosmo) by Midwest Research Institute (Kansas City, MO).

[b]Acute oral toxicity of kavalactones in mice (Gruenwald, 2003; Kretzschmar and Meyer, 1969); LD_{50}: lipid kava-extract: 360 mg/kg in mice, aqueous extract: 1.5 g/kg in mice (Gruenwald, 2003; Kretzschmar and Meyer, 1969), and that of 70% kavalactones: >1.5 g/kg in rats and mice (Teschke et al., 2003).

[c]Not determined due to low solubility.

accordance with guidelines of the National Institutes of Health (NIH) (Grossblatt, 1996). F344 rats were obtained from Taconic Laboratory Animals Service (Germantown, NY) at approximately 4 weeks of age and held under quarantine for 11 days before placed on study. Rats were housed 5/cage (polycarbonate cages) (Lab Products, Inc., Seaford, DE). Filtered room air underwent at least 10 changes per hour. The animal room was maintained at 69–75 °F with 35–65% relative humidity and 12 h each of light and darkness. Irradiated NTP-2000 pelleted feed (Zeigler Bros., Inc., Gardner, PA) and water were available ad libitum. All rats were checked twice daily for moribundity, mortality, clinical signs of ataxia and prostration, and toxicological effects including CNS depression, neurobehavioral abnormalities, and motor activity. Weekly, food and water consumptions were measured and all animals weighed.

Hematology and blood chemistry

For these parameters, blood was collected via the retroorbital sinus into prelabeled EDTA-treated tubes and/or untreated clot tubes under 70% CO_2/30% O_2 at days 4 and 23 from special study rats. The core study rats were bled at terminal sacrifice. Hematological determinations in all rats were conducted for erythrocyte count, mean corpuscular volume, hemoglobin, packed cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythrocyte morphologic assessment, leukocyte count, leukocyte differential, reticulocyte count, and platelet count and morphologic assessment. Blood chemistry was conducted for sorbitol dehydrogenase (SDH), alkaline phosphatase (ALP), creatine kinase (CK), creatinine, total protein, albumin, urea nitrogen (BUN), total bile acids, alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), glutamate pyruvate transaminase (SGPT), triglycerides, cholesterol, and glucose.

Pathological evaluation

At sacrifice, complete necropsies were performed on all animals using standardized methodology. Organ weights of liver, thymus, kidney, testis, heart, and lung were determined from all animals. All tissues, including macroscopic abnormalities, were removed and fixed in 10% neutral buffered formalin for microscopic evaluation. After fixation, the tissues were trimmed, dehydrated, cleared, and paraffin-embedded. Five-micrometer-thick sections were mounted onto glass slides, stained with hematoxylin and eosin (H&E), and examined microscopically. The severity of lesions in all of the organs was graded on a four-point scale of 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. In order to allow comparisons of severity of hepatocellular hypertrophy (HP), the histopathological changes were graded subjectively based on relative width of hepatic cords as follows: 1 = lower limit for detection of increased width beyond range of controls; 2 = approximately 25% increase in hepatic cord width; 3 = approximately 50% increase; and 4 = approximately 100% increase (Amacher et al., 1998). The pathological data underwent extensive review (Boorman et al., 2002).

Immunohistochemistry: hepatic CYP1A2, 2B1, 2D1, 2E1, and 3A1

Previous reports indicate that kava inhibits human CYP1A2, 2D6, 2E1 and/or 3A4, in vitro. (Anke and Ramzan, 2004a, b; Gurley et al., 2005; Mathews et al., 2002, 2005). Kava extract induced CYP1A2, 2B1, 2E1, and 3A1/2 and inhibited CYP2D1 in rats (Mathews et al., 2002, 2005). Although the composition and relative proportions of specific isoforms of CYPs are different in humans and rats, strong catalytic and regulatory conservation of the CYP1A1, 1A2, and 2E1 subfamilies exists among the rat isoforms and their human orthologs (Mugford and Kedderis, 1998). The above-mentioned CYPs, located mainly in liver, are involved in the metabolism of a variety of chemicals and pharmaceutical compounds (Ingelman-Sundberg, 2004; Omiecinski et al., 1999; Zuber et al., 2002). The expressions of CYP1A2, 2B1, 2D1, 2E1, and 3A1 were analyzed in this study.

Detailed staining protocols for all antibodies are listed in Table 2 and at the website of the NIEHS Laboratory of Experimental Pathology (http://dir.niehs.gov/dirlep/immuno/protocols.htm). Briefly, sections were deparaffinized, hydrated, and blocked for endogenous peroxidase. Heat-induced epitope retrieval was performed for all antibodies. These primary antibodies are reported to have the specific reactivities to each rat liver CYPs and to be used for immunohistochemical examinations in the data sheets (provided by each companies). The immunohistochemical analysis of CYPs have been reported as one of the useful method that coincides with Western-blot analysis and have been conducted in several mechanistic studies (Ejiri et al., 2005; Liu et al., 2005; Tani et al., 2001).

The intensity grade of immune staining for CYP2D1 in hepatocytes was scored from 0 to 3, corresponding to the presence of negative, weak, moderate, or strong brown staining. In order to assess the grade of expression of CYP1A2, 2B1, 2E1, and 3A1, the relative area of the hepatic lobules showing staining was graded by two pathologists as follows: (0) = no expression, (1) = <25% of hepatocytes around centrilobular area, (2) = up to ~50%, (3) = up to ~75%, and (4) = up to ~100%.

Table 2. Immunohistochemical methods for analysis of kava-induced liver lesions

Antibody protocol	Cytochrome P450				
	1A2	2B1	2D1	2E1	3A1
Heat-induced epitope retrieval	Microwave oven with citrate buffer[a]	Microwave oven with citrate buffer[a]	Microwave oven with citrate buffer[a]	Decloaker™ with citrate buffer[a]	Microwave oven with citrate buffer[a]
Blocking	Normal rabbit serum[b]	Serum-free protein block[c]	Normal goat serum[b]	Serum-free protein block[c]	Serum-free protein block[c]
Primary antibody (dilution)	Sheep anti-rat CYP1A2[d] (1:400)	Rabbit anti-rat CYP2B1[d] (1:500)	Rabbit anti-rat CYP2D1[d] (1:5000)	Rabbit anti-CYP2E1[e] (1:1250)	Rabbit anti-rat CYP3A1[d] (1:1500)
Negative control	Normal sheep serum[b]	Normal rabbit serum[b]	Normal rabbit serum[b]	Normal rabbit serum[b]	Normal rabbit serum[b]
Secondary antibody	Rabbit anti-sheep[b]	LSAB + system-HRP[c]	Goat anti-rabbit[f]	LSAB + system-HRP[c]	LSAB + system-HRP[c]
Label	Supersensitive predilute label[g]	LSAB + system-HRP[c]	Vector Elite Standard Kit[f]	LSAB + system-HRP[c]	LSAB + system-HRP[c]

[a]BioCare Medical, Walnut Creek, CA.
[b]Jackson Immunoresearch, West Grove, PA.
[c]DakoCytomation, Carpinteria, CA.
[d]Chemicon International, Temecula, CA.
[e]Stressgen Bioreagents, Victoria, BC Canada
[f]Vector Laboratories, Burlingame, CA.
[g]Biogenex Laboratories, San Ramon, CA.

Statistical analysis

The incidences of lesions were analyzed by Fisher's exact test (Gart et al., 1979) to determine significance. Data for organ and body weights, which had approximately normal distributions, were analyzed with parametric multiple comparison procedures (Dunnett,1955; Williams, 1971, 1972). Data for hematology and blood chemistry were analyzed using nonparametric multiple comparison methods of Shirley (1977) (modified by Williams, 1986) and Dunn (1964). Since CYP expression values were not normally distributed, the nonparametric median test with exact *p*-values was used (Conover, 1971). The results presented below compare each dose group with the control group and asterisks indicate significance of two-sided *p*-values.

Results

Mortality and clinical observations

Gavage administration of kava for 14 weeks resulted in treatment-related unscheduled deaths of one female in the 1.0 g/kg group and three males and four females in the 2.0 g/kg groups. The cause of death was attributable to kava-induced central nervous system (CNS) and/or respiratory depression.

From week one ataxia and lethargy occurred in 10% of males and 20% of females in the 1.0 g/kg dose groups and intermittently in 20% males and 50% females in the 2.0 g/kg dose group; these persisted for the duration of the study. Decreases in terminal body weights were observed in the 2.0 g/kg rats (males, 82.8% of control; females, 90.1%) and the 1.0 g/kg males (91.3% of control). However, food and water consumption rates between the kava-treated and control groups were similar (data not shown).

Hematology and blood chemistry

Sporadic decreases in red blood cell count, hemoglobin, and hematocrit in the 2.0 g/kg males and females were considered a result of dehydration due to ataxia and lethargy following kava administration. Levels of γ-glutamyl transpeptidase (GGT) were increased significantly in the 1.0 g/kg females at day 93 and the 2.0 g/kg males and females at days 4, 23, and 93 compared to controls (Table 3). Serum cholesterol levels in both sexes were also significantly increased at days 4, 23, and 93 in groups dosed with 0.5 g/kg and higher (Table 3). In addition, increases were noted at day 93 in mean serum total protein and albumin in males and females administered 0.5 g/kg and higher (data not shown).

Significant decrease in serum glucose levels were noted in the 2.0 g/kg males at days 4 and 93 and in the females at days 4 and 23; decrease was also observed in the 1.0 g/kg males at days 4 and 23 (data not shown). The changes were considered related to hepatic alteration resulting from kava administration.

Table 3. Clinical-chemistry data

Dose (g/kg)	Day	0	0.125	0.25	0.5	1.0	2.0
Males							
γ-Glutamyl transpeptidase (U/l)	4	0	0	1±1[a]	0	0	3±2**
	23	0	0	0	0	0	1±1**
	93	0	0	0	0	0	2±0**
Cholesterol (mg/dl)	4	95±4	100±3**	99±6	114±5**	124±9**	132±16**
	23	87±4	87±4	91±6	97±8**	97±9**	94±8
	93	77±4	75±3	79±4	83±6*	86±7**	92±10**
Females							
γ-Glutamyl transpeptidase (U/l)	4	1±1	0	1±1	0	1±1	4±2**
	23	1±1	0	1±1	1±1	1±1	4±1**
	93	1±1	2±1	1±1	2±1	3±1**	15±3**
Cholesterol (mg/dl)	4	85±8	92±10**	89±6	109±8**	112±7**	128±16**
	23	73±5	78±7	83±4**	90±4**	100±7**	107±10**
	93	69±7	83±5**	75±6	92±7**	99±6**	105±9**

*Significantly different ($p<0.05$) from vehicle control group. **$p<0.01$.
[a]Mean±SE ($n=10$).

Table 4. Mean organ weights

Dose (g/kg)	0	0.125	0.25	0.5	1.0	2.0
Males						
Liver						
Absolute (g)	11.1±0.9[a]	11.1±0.6	12.1±0.7	12.4±0.4**	13.2±0.7**	15.1±0.4**
Relative (%)	3.2±0.2	3.3±0.1	3.6±0.2**	3.7±0.1**	4.2±0.2**	5.2±0.2**
Kidney						
Absolute (g)	0.93±0.05	0.93±0.09	0.94±0.28	1.0±0.04	1.01±0.06*	1.04±0.03**
Relative (%)	0.27±0.01	0.28±0.01	0.28±0.01	0.29±0.01**	0.32±0.02**	0.36±0.01**
Females						
Liver						
Absolute (g)	6.4±0.3	6.8±0.5	6.8±0.6	8.1±0.8**	9.0±0.8**	10.3±1.1**
Relative (%)	3.3±0.1	3.4±0.2**	3.5±0.2**	4.1±0.2**	4.7±0.2**	5.8±0.5**
Kidney						
Absolute (g)	0.67±0.05	0.70±0.06	0.70±0.03	0.73±0.08	0.75±0.05**	0.78±0.04**
Relative (%)	0.34±0.02	0.35±0.03	0.36±0.01	0.37±0.02**	0.39±0.01**	0.44±0.02**

*Significantly different ($p<0.05$) from vehicle control group. **$p<0.01$.
[a]Mean±SE ($n=10$).

Pathology

Dose-related increases that occurred in mean absolute and relative liver and kidney weights in both male and female groups (Table 4) were attributable to HP resulting from administration of kava. Significant increases in absolute and relative kidney weights were not considered significant toxicologically, as no corresponding histopathological changes were noted.

Dose-related increased incidence and/or severity of histopathology were noted in liver, spleen, and thymus (Table 5). HP occurred in female rats at 0.5 mg/kg and higher and only in a single male rat treated with the 1.0 g/kg of kava extract. The effect, ranging from minimal to moderate, consisted of a diffuse increase in cell size associated with glycogen depletion and amphophilic cytoplasm of the hepatocytes (Figs. 1 and 2). Atrophy of periarteriolar lymphoid sheaths in the spleen and/or thymic necrosis was detected in the dead animals of the 2.0 g/kg dosed group, probably related to stress.

Immunohistochemical expression of CYP proteins

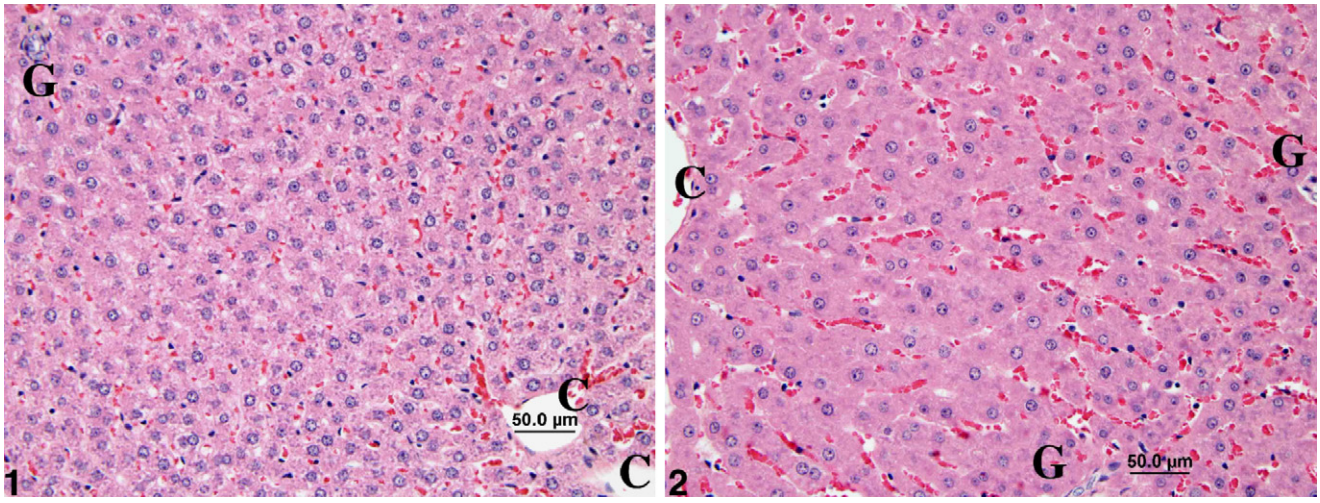
The results of immunohistochemical analyses revealing protein expressions are shown in Table 6 (Figs. 3–6: CYP2D1, Figs. 7–10: CYP2B1, Figs. 11–14: CYP3A1, Figs. 15–18: CYP1A2).

Strong immunostaining of CYP2D1 occurred diffusely in the hepatocytic cytoplasm of the control male and female rats (Figs. 3 and 4) in which the mean grades of expression were, respectively, 2.8 and 3.0 (Table 6). In the kava-treated animals, the mean grades of staining intensity were mildly decreased compared to control groups (mean grades, 2.4 and 2.6, respectively, in

Table 5. Incidences and average severity of treatment-related microscopic findings for rats in the 14-week gavage study of kava extract

Dose (g/kg)	0	0.125	0.25	0.5	1.0	2.0
Number examined	10	10	10	10	10	10
Males						
Hepatocellular hypertrophy	0[a] (0.0)[b]	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)
Spleen atrophy	0 (0.0)$^{+++}$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.7)
Thymus necrosis	0 (0.0) $^{+}$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)
Females						
Hepatocellular hypertrophy	0 (0.0) $^{+++}$	0 (0.0)	0 (0.0)	3 (1.0)	3(1.3)	10 (1.7)***
Spleen atrophy	1(2.0) $^{++}$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.0)
Thymus necrosis	1(2.0) $^{++}$	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.0)

$^{+}$Dose-related trend in incidence is significant at $p<0.05$.
$^{++}$Dose-related trend in incidence is significant at $p<0.01$.
$^{+++}$Dose-related trend in incidence is significant at $p<0.001$.
*Incidence differs from controls at $p<0.05$.
**Incidence differs from controls at $p<0.01$.
***Incidence differs from controls at $p<0.001$.
[a]Incidence.
[b]Average severity in parentheses.



Figs. 1 and 2. 1. Centrilobular area, control female rat. Note relatively smaller size of hepatocytes with cytoplasmic basophilic stippling by comparing with liver from 14-week-, 2.0 g/kg-treated rat. C: central vein. G: Glison's sheath. H&E. Bar = 50 µm. **2.** Mild (grade 2) hepatocytic hypertrophy in female rat treated with 2.0 g/kg kava extract by gavage for 14 weeks. Centrilobular hepatocytes contain more homogeneous eosinophic cytoplasm. C: central vein. G: Glison's sheath. H&E. Bar = 50 µm.

males and females treated with 1.0 g/kg, and 2.5 and 2.2, respectively in the 2.0 g/kg groups (Table 6)). The decrease in the grade achieved statistical significance in females at 2.0 g/kg ($p<0.05$). Hypertrophic change was revealed by H&E staining in female rats treated with 2.0 g/kg dose of kava extract; however, adjacent sections stained for CYP2D1 demonstrated decreased immunohistochemical staining, as shown in Figs. 5 and 6.

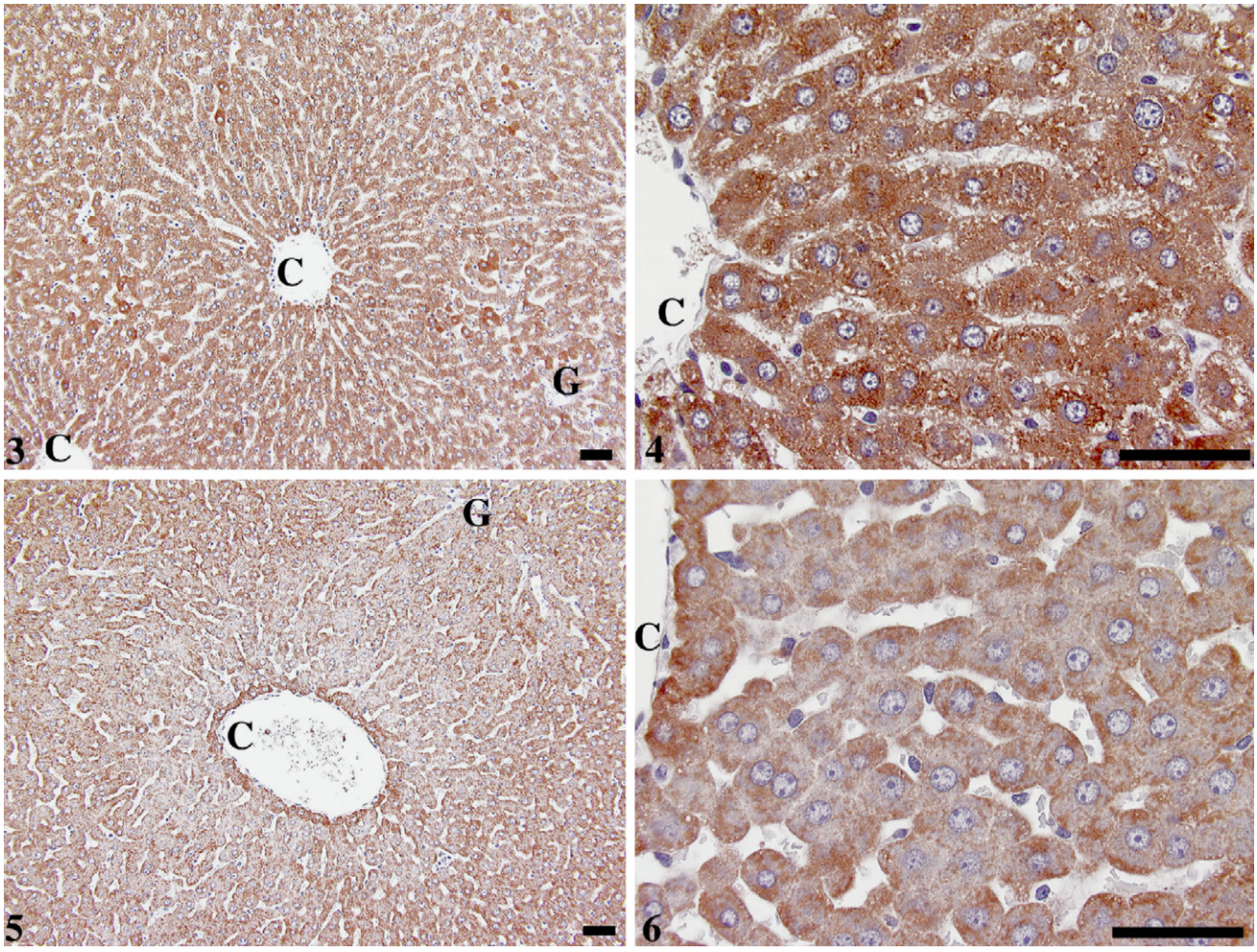
Expression of CYP2B1 was seen only in the cytoplasm of the centrilobular hepatocytes in the control male and female rats; the intensity of the staining was strong (Figs. 7 and 8). The mean grades of the expression were 0.6 and 1.0 in male and female control groups, respectively (Table 6). In the kava-treated animals, the mean grade was severely increased compared to control groups (mean grades of 4.0 and 3.8 in male and females, respectively, treated with 1.0 g/kg and grades of 4.0 and 4.0 in male and females, respectively, treated with 2.0 g/kg of kava extract) (Table 6, Figs. 9 and 10).

CYP3A1 was located only in the cytoplasm of centrilobular hepatocytes in the control male and female rats, with strong staining intensity (Figs. 11 and 12). The mean grade of expression was 1.0 in each sex of the control groups. In the kava-treated female animals, the mean grade was strongly increased compared to controls (3.6 and 4.0 in the 1.0 and 2.0 g/kg groups,

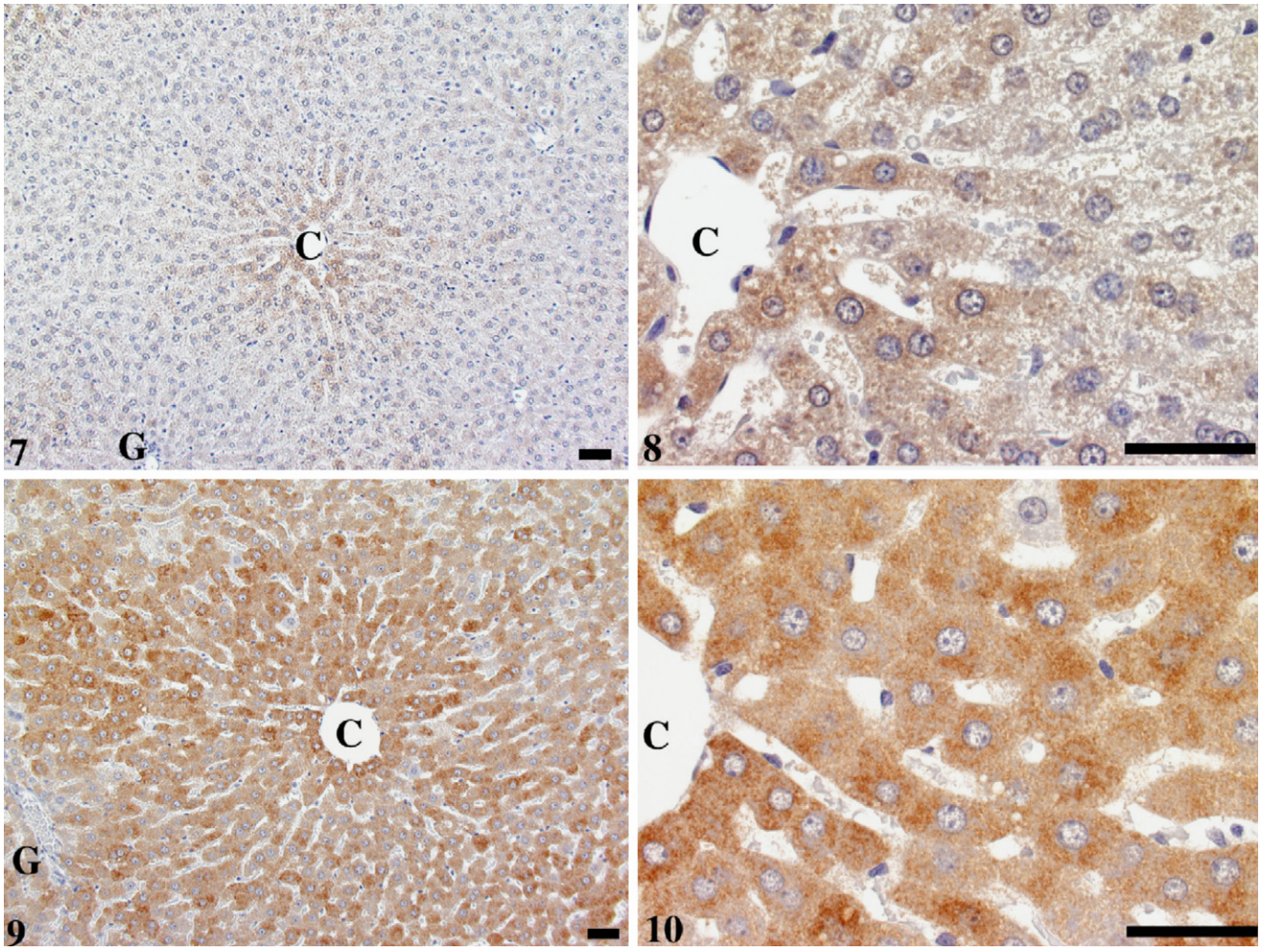
Table 6. Immunohistochemical analysis of CYPs expression in the liver treated with kava-extract for 14 weeks in rats

Dose (g/kg)	CYP1A2	CYP2B1	CYP2D1	CYP2E1	CYP3A1
Males					
0	0.0±0.0	0.6±0.2	2.8±0.3[a]	2.6±0.2	1.0±0.0
1.0	0.8±0.2*	4.0±0.0**	2.4±0.2	2.6±0.2	2.0±0.0**
2.0	2.0±0.0**	4.0±0.0**	2.5±0.3[a]	2.8±0.2	2.8±0.2**
Females					
0	0.4±0.2	1.0±0.0	3.0±0.0	2.8±0.2	1.0±0.0
1.0	1.8±0.2*	3.8±0.2**	2.6±0.2	2.8±0.4	3.6±0.2**
2.0	2.6±0.2**	4.0±0.0**	2.2±0.2*	2.6±0.2	4.0±0.0**

Mean±SE ($n = 5$).
*Differs from controls at $p < 0.05$, **differs from controls at $p < 0.01$.
Relative area of hepatic lobules showing staining for CYP 1A2, 2B1, 2E1, and 3A1 was graded by two pathologists as follows: (0) = no expression, (1) = <25% of hepatocytes around centrilobular area, (2) = up to ~50%, (3) = up to ~75%, and (4) = up to ~100%.
The intensity of staining for CYP2D1 was scored from 0 to 3 corresponding to the presence of negative, weak, moderate, or strong staining.
[a] $n = 4$.



Figs. 3–6. 3. Strong CYP2D1 expression (intensity: grade 3) in centrilobular area, control female rat; CYP2D1 detected diffusely in cytoplasm of hepatocytes of controls. C: central vein. G: Glison's sheath. Bar = 50 μm. **4.** Higher magnification of Fig. 3. C: central vein. Bar = 50 μm. **5.** Moderate expression (intensity: grade 2) of CYP2D1 in centrilobular area of hepatocytes showing mild hypertrophic changes by H&E staining, female rat treated with 2.0 g/kg kava extract by gavage for 14 weeks. C: central vein. G: Glison's sheath. Bar = 50 μm. **6.** Higher magnification of Fig. 5. C: central vein. Bar = 50 μm.



Figs. 7–10. 7. Weak expression (relative area: grade 1) of CYP2B1, centrilobular area only, control female rat. CYP2B1 protein was detected only in cytoplasm of centrilobular hepatocytes in control rats. C: central vein. G: Glison's sheath. Bar = 50 μm. **8.** Higher magnification of Fig. 7. C: central vein. Bar = 50 μm. **9.** Strong expansive expression (relative area: grade 4) of CYP2B1 in almost all of lobular area, detected in hepatocytes showing hypertrophic changes by H&E staining, female rat treated with 2.0 g/kg of kava extract by gavage for 14 weeks. C: central vein. G: Glison's sheath. Bar = 50 μm. **10.** Higher magnification of Fig. 9. C: central vein. Bar = 50 μm.

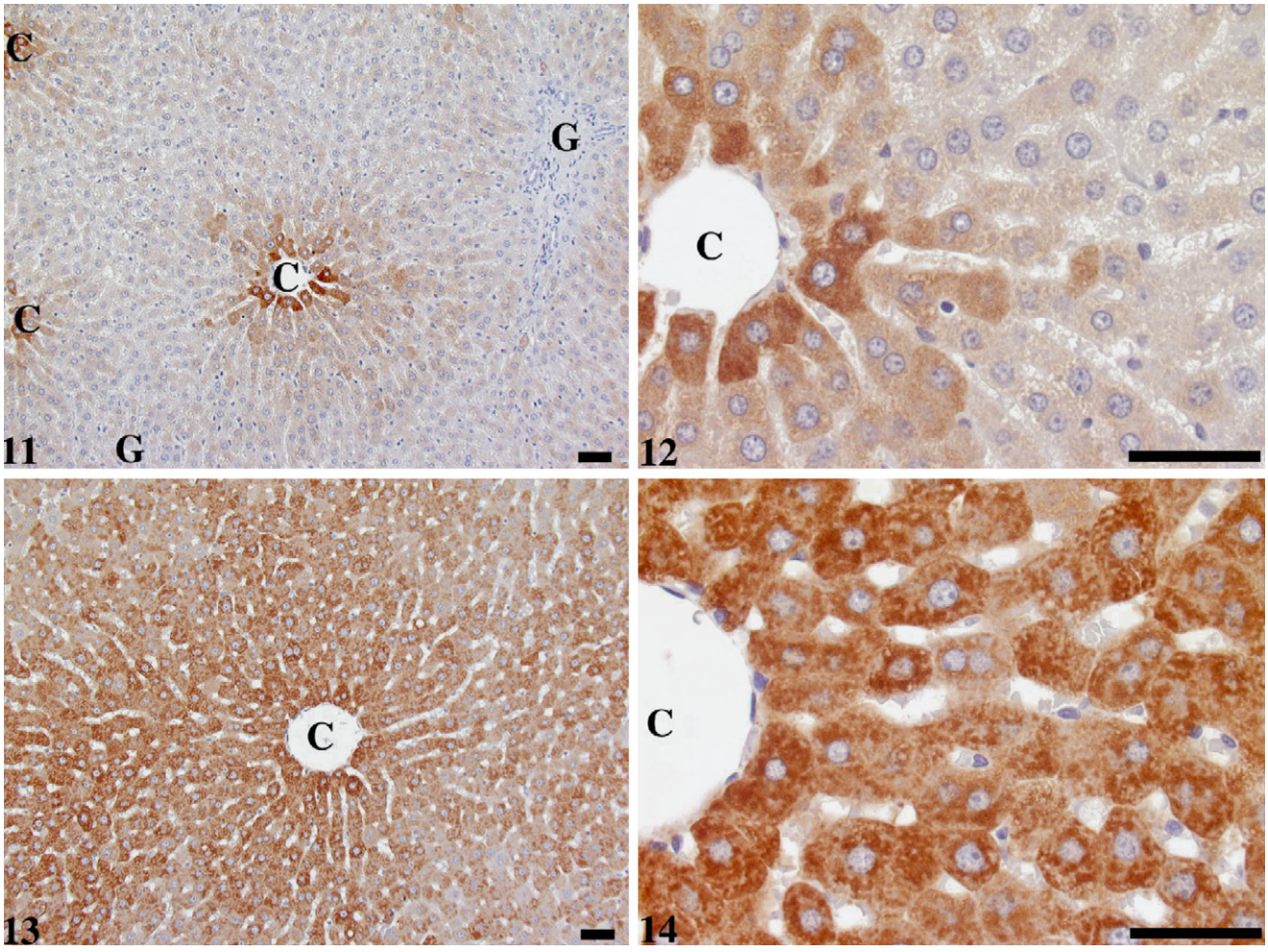
respectively) (Table 6, Figs. 13 and 14). In contrast, the tendency toward increased grades in male rats treated with kava extract was lower; intensity grades were 2.0 and 2.8 in the 1.0 and 2.0 g/kg groups, respectively.

CYP1A2 was located only in the hepatocellular cytoplasm of 2–3 layers of the centrilobular area in the control female rats; the intensity of staining was weaker than that of the other CYPs (Figs. 15 and 16). The mean grades of expression were 0.0 and 0.4 in control male and female groups, respectively. In the kava-treated animals of both sexes, statistically significant, dose-related increases of the grade were observed: 0.8 and 2.0 in the 1.0 and 2.0 g/kg male groups and 1.8 and 2.6 in the 1.0 and 2.0 g/kg female groups, respectively. (Table 6, Figs. 17 and 18). The tendency toward increased grades in female rats treated with kava extract appeared to be slightly higher.

CYP2E1 was expressed in the cytoplasm of centrilobular hepatocytes in the control male and female rats. The mean grades of the staining intensity were 2.6 and 2.8 in male and female control groups, respectively (Table 6). In the kava-treated animals, the mean grades were similar to control values: 2.6–2.8 in both sexes of rats treated with 1.0 and 2.0 g/kg.

Discussion

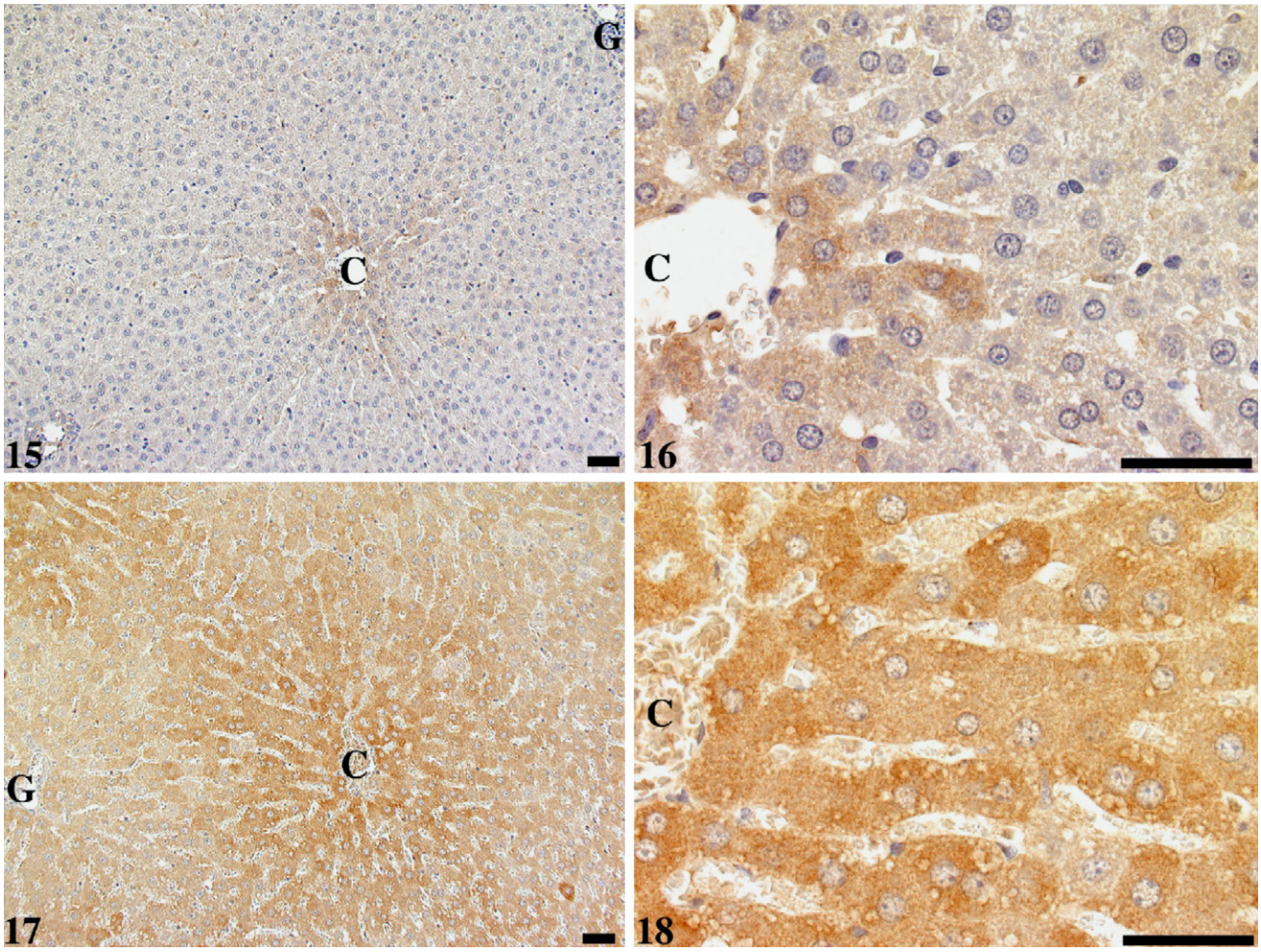
In this study, we showed that kava extract induces hepatic hypertrophy in F344 rats evidenced by dose-related liver weight increases in males and females and increased incidence and/or severity of hepatocellular hypertrophy (HP) in females treated with the 0.5, 1.0



Figs. 11–14. 11. Weak expression (relative area: grade 1) of CYP3A1 only in centrilobular area, detected locally in cytoplasm of hepatocytes around central vein, control female rat. C: central vein. G: Glison's sheath. Bar = 50 μm. **12.** Higher magnification of Fig. 11. C: central vein. Bar = 50 μm. **13.** Strong expression (relative area: grade 4) of CYP3A1 in almost all of centrilobular area detected by H&E in cytoplasm of hepatocytes showing hypertrophic changes, female rat treated with 2.0 g/kg of kava extract by gavage for 14 weeks. C: central vein. Bar = 50 μm. **14.** Higher magnification of Fig. 13. C: central vein. Bar = 50 μm.

and 2.0 g/kg and a single male treated with 1.0 mg/kg. The induction of drug-metabolizing enzymes has generally been linked with hepatomegaly and other effects in rodent liver (Amacher et al., 1998; Cattley and Popp, 2002; Vandenberghe, 1996). Enlargement of the liver, typically 10–50%, has been associated with the stimulation of drug metabolism for numerous pharmaceuticals from several pharmacological classes. Both HP and induction of microsomal enzymes constitute benign changes without significant toxicity is considered as adaptive in response to certain chemicals that stimulate the hepatic drug metabolizing enzyme system (Amacher et al., 1998; Cattley and Popp, 2002; Vandenberghe, 1996). We suggest that the dose-related increases in liver weights and the occurrence of HP in the F344 rats in this 14-week gavage study of kava extract are adaptive in nature.

Analysis of our data suggests lack of direct correlation between liver weight, incidence of HP, and degree of expression of CYPs. Absolute liver weights were 12%, 19%, and 36% higher in 0.5, 1.0, and 2.0 g/kg males than in controls, and 26%, 40%, and 60% higher in 0.5, 1.0, and 2.0 g/kg females than in controls. In contrast, the incidences of HP were 0, 1, and 0 males, and 3, 3, and 10 females in 0.5, 1.0, and 2.0 g/kg rats, respectively. Liver weight increases of 20% or greater have usually been associated with histopathological evidence of hypertrophy, but neither the severity of hypertrophy nor the magnitude of liver-weight increase have been correlated with the magnitude of elevation in drug-metabolizing enzymes (Amacher et al., 1998). The increases in liver weight in our study could not be correlated with histopathological evidence of hypertrophy. The expressions of CYP1A2, 2B1, and 3A1 which



Figs. 15–18. 15. Weak expression (relative area: grade 1) of CYP1A2 only in centrilobular area, control female rat. CYP1A2 protein was detected locally in cytoplasm of hepatocytes around the central vein. C: central vein. G: Glison's sheath. Bar = 50 μm. **16.** Higher magnification of Fig. 15. C: central vein. Bar = 50 μm. **17.** Strong expression (relative area: grade 3) of CYP1A2, centrilobular area, female rat treated with 2.0 g/kg of kava extract by gavage for 14 weeks. CYP1A2 detected in the cytoplasm of hepatocytes showing hypertrophic changes by H&E staining. C: central vein. G: Glison's sheath. Bar = 50 μm. **18.** Higher magnification of Fig. 17. C: central vein. Bar = 50 μm.

were strongly positive in centrilobular hepatocytes were increased in area in both sexes of the 1.0- and 2.0-g/kg-treated rat liver. In a previous investigation (Mathews et al., 2002), in male Fischer rats administered 256 mg/kg of kava extract (100 mg/kg kavalactones) orally for 7 days, the total P450 was not significantly different than that of vehicle-treated controls. Following daily administration of 1 g/kg kava (391 mg/kg kavalactones) to male Fischer rats for 7 days, however, the total hepatic P450 content increased 35%, and the activities of P450 enzymes increased significantly: CYP1A2 [>200%], 2B1 [>200%], and 3A1/2 [51%]; CYP2E1 was marginally increased, although the degree was much lower than that for CYP1A2, 2B1, and 3A1/2, and activities of CYP2D1 were modestly decreased by approximately 25% (Mathews et al., 2005). In our study HP might have been related to the induction of these CYPs following exposure to kava extract, although no changes in the expression of hepatic CYP2E1 were detected. Additionally, we confirmed immunohistochemically decreased levels of expression of CYP2D1 protein in the liver after treatment with 2.0 g/kg kava for 14 weeks. Hepatocellular hypertrophic change seen in our study was, therefore, not related to the induction of CYP2D1 and 2E1. Previous investigations indicate inhibition of CYP1A2, 2D6, 2E1, and/or 3A4 by kava in humans (Anke and Ramzan, 2004a, b; Gurley et al., 2005; Mathews et al., 2002, 2005). Male Sprague-Dawley rats exposed to up to 500 mg/kg for 4 weeks proved not to be a good model for studying the toxicity of kava because of their lack of mimicry of human inhibition of CYPs (Singh and Devkota, 2003). The CYP isoforms found in

male and female rats exhibit different spectral binding properties and substrate preferences; CYP2A2, 2C11, 2C13, 2C18, and 3A2 are predominantly male isoforms, and CYP2A1, 2C7, 2C12, 2C19 are prevailing female isoforms (Mugford and Kedderis, 1998). Expressed in a sex-specific manner, CYP2C, a major subfamily in rats, is not found in humans. Our immunohistochemical analysis showed that the increased expression of CYP3A1 in females was greater than that in males, although it is not classified as a sex-predominant isoform. We did not conduct immunohistochemical analysis of the hepatic expression of CYP4A. Hyperplasia and induction of microsomal CYP4A family members invariably accompany peroxisome proliferation (Cattley and Popp, 2002; Xu et al., 2005). Kava might not have induced CYP4A in the liver in our study, as it is not known to exert potential effects upon peroxisome proliferation.

In mice exposed orally to kava extract standardized to 70% kavalactones, the LD50 was greater than 1.5 g/kg; clinical signs of ataxia and lethargy occurred, and respiratory paralysis was reported as the cause of death (Teschke et al., 2003). In the current study, treatment-related unscheduled deaths of one female in the 1.0 g/kg group and three male and four female rats in the 2.0 g/kg groups were likely attributable to depression of the CNS and/or respiratory system resulting from administration of kava extract. Our findings are in agreement with those reported previously (Jamieson and Duffield, 1990; Teschke et al., 2003.

Clinical chemistry evaluations showed that GGT activities were increased in the 1.0 g/kg females and 2.0 g/kg males and females. A membrane-bound enzyme, GGT plays a role in the metabolism of glutathione and facilitates transport of amino acids. In general, serum GGT is inducible in hepatobiliary diseases, such as alcoholic hepatitis and cholestasis by microsomal enzymes of the liver (Ohta and Toda, 2001). Increased levels of GGT correlated with ALP indicate an hepatobiliary origin when cholestasis occurs in the liver (Giannini et al., 2005). In contrast, the levels are often mildly increased in microsomal enzyme induction by alcohol and anti-epileptic drugs (carbamazepine, zarontin, phenobarbital, phenytoin) in humans (Knight, 2005; Ohta and Toda, 2001; Rosalki et al., 1971; Vandenberghe, 1996), due to increased induction of GGT in the hepatocytes and its transition to blood (Ohta and Toda, 2001). In previous rat toxicity studies, hepatic induction of CYPs was generally not accompanied by substantial morphological changes or elevated serum enzyme levels considered indicative of liver injury (Amacher et al., 1998); since no data for serum GGT were included, the relationship between serum GGT change and HP was not clarified. Additionally, some microsome enzyme inducers, such as phenobarbital and other drugs, caused significantly increased hepatic cholesterol in rats (Amacher et al., 1998). Demethoxyyangonin lowered cholesterol in rats in the first 2 months but increased cholesterol after 3 months (Hsu et al., 1994). In our study, the mechanisms involving GGT and cholesterol changes were unclear; however, we suggest that increased levels of GGT and cholesterol may be related to HP caused by induction of microsomal enzymes, similar to the action of anti-epileptic drugs.

Kava-induced functional liver damages, such as temporally and reversibly elevated levels of GGT, have been reported in many human cases, including an aboriginal community (Clouatre, 2004; Clough et al., 2003; Gruenwald, 2003; Mathews et al., 1988). In Australian aboriginals, heavy use of kava (>600 g/week) may have been related to malnutrition, weight loss, hepatic abnormalities (elevated levels of serum GGT and cholesterol), renal dysfunction, rash, incoordination, pulmonary hypertension, macrocytosis of red cells, lymphocytopenia, and decreasing volumes of platelets (Mathews et al., 1988). Histopathologically, some human cases with acute hepatitis have manifested hepatocellular necrosis with hemorrhage and infiltration of lymphocytes and histiocytes (Campo et al., 2002; Humberston, 2003). Nonetheless, the incidence ratio for kava-related hepatotoxicity in humans has been very low and calculated to be 0.008 cases per 1,000,000 daily doses. By comparison, the incidence rates for benzodiazepine (bromazepam), oxazepam, and diazepam are, respectively, 0.90, 1.23, and 2.12 cases of hepatic side effects per million daily doses. A restriction of the marketing of kava products would, therefore, mean that patients would have to revert to an alternative medication with a 112- to 265-fold increase in risk of hepatic involvements (Schmidt, 2001). Moreover, in the present 14-week kava studies in rats, HP and CYP overexpression may suggest the continuation of enzyme induction over a long time period and a rapid metabolic process, followed by decreased C_{max} and AUC levels of native kava components in blood. Due to the decreased exposure levels and times of native kava components, primary severe toxicities, induced by longer exposure to native kava components, similar to those in human cases requiring transplantation, did not occur in our higher-dosed group.

In summary, exposure of F344 rats to kava extract induced decreased body weight, mild anemia considered a result of dehydration, increased serum levels of GGT and cholesterol, increased liver weight, occurrence of HP, sporadic decrease in serum glucose levels and changes in expressions of CYP enzymes. The NOAEL for these effects was 0.25 g/kg in both sex rats. These manifestations were likely related to primary and secondary toxicities induced by exposure to the kava extract. The F344 rat is suggested as a potential relevant model of human clinical side effects, such as transient

and mild abnormality of liver function, in particular change in GGT and inhibition of CYP2D. Our ongoing carcinogenicity studies will eventually elucidate the potential long-term significance of the relatively mild changes noted in the liver following the 14-week exposure and may enhance our understanding of the pathogenesis of kava-induced lethal liver failure in humans. Additional research is needed to analyze the mechanism(s) of liver failure in humans and provide understanding of the potential extrapolations from rodents to humans of kava-induced liver injuries.

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