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Liver Toxicity and Carcinogenicity in F344/N Rats and B6C3F1 Mice Exposed to Kava Kava

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Abstract

Kava Kava is an herbal supplement used as an alternative to antianxiety drugs. Although some reports suggest an association of Kava Kava with hepatotoxicity, it continues to be used in the United States due to lack of toxicity characterization. In these studies F344/N rats and B6C3F1 mice were administered Kava Kava extract orally by gavage in corn oil for two weeks, thirteen weeks or two years. Results from prechronic studies administered Kava Kava at 0.125- to 2g/kg body weight revealed dose-related increases in liver weights and incidences of hepatocellular hypertrophy. In the chronic studies, there were dose-related increases in the incidences of hepatocellular hypertrophy in rats and mice administered Kava Kava for up to 1 g/kg body weight. This was accompanied by significant increases in incidences of centrilobular fatty change. There was no treatment-related increase in carcinogenic activity in the livers of male or female rats in the chronic studies. Male mice showed a significant dose-related increase in the incidence of hepatoblastomas. In female mice, there was a significant increase in the combined incidence of hepatocellular adenoma and carcinoma in the low and mid dose groups but not in the high dose group. These findings were accompanied by several nonneoplastic hepatic lesions.

Keywords

Kava; gavage; liver; toxicity; rats; mice

INTRODUCTION

The assessment of the efficacy and safety of herbal plants and herbal dietary supplements is important for human health protection (FDA, 2001, 2002; Fong, 2002; Fu *et al.*, 2008a,b; Parkman, 2002). Although the herbal market has been rapidly growing, data on the identification and toxicological characterization of biologically active constituents in many herbs are lacking.

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Kava Kava, an extensively used herbal product, is derived from the tropical shrub known as *Piper methysticum*. Traditionally, it has been widely cultivated for its rootstock in three geographic regions of the Pacific: Polynesia, Melanesia, and Micronesia (Norton and Ruze, 1994), where it is used as a ritual beverage to promote relaxation and a sense of well-being. The active principles of Kava Kava rootstock are mostly contained in the lipid-soluble resin. The isolates of Kava Kava resin fall into three general categories: arylethylene-apyrone, chalcones and other flavanones, and conjugated diene ketones. The compounds of greatest pharmacological interest are the substituted α -pyrones or Kava pyrones, commonly known as kavalactones. Fifteen lactones have been isolated from Kava Kava rootstock of which six are present in the highest concentrations and account for approximately 96% of the lipid resin. These are yangonin, methysticin, dihydromethysticin, kavain, dihydrokavain, and desmethoxyyangonin (Shulgin, 1973; Lebot *et al.*, 1992; Dentali, 1997).

Kava Kava extract is one of the most widely used herbals in the United States with estimated sales of \$106 million and is readily available at health stores, pharmacies, and super-stores (ABC News, 1998; Mirasol 1998). It is used by approximately 2.2 million people in the United States as a natural alternative to anti-anxiety drugs such as Xanax® and Valium® (Gardiner *et al.*, 2007). It has also been claimed to have diuretic and antiseptic properties, (Norton and Ruze, 1994; JNC Corp., 1998), reported to help children with hyperactivity (Symmetry, 1998), and it is often used as a skin-conditioning agent in cosmetics (Robinson *et al.*, 2009). The recommended oral dose for usage of Kava Kava as an anxiolytic is 50 to 70 mg kavalactones two to four times a day and, as a hypnotic, 150 to 210 mg in a single oral dose before bedtime (Bilia *et al.*, 2002).

The main concern with the use of Kava Kava is hepatotoxicity in humans (Russmann *et al.*, 2001, 2003; Campo *et al.*, 2002; De Smet, 2002; Parkman, 2002; Brauer *et al.*, 2003; Clough *et al.*, 2003; Humberston *et al.*, 2003; Ernst, 2006). However, evidence on the hepatotoxic effects of Kava Kava currently remain conflicting with some reports indicating an association of Kava Kava administration with hepatotoxicity including hepatitis, cirrhosis, and liver failure (Escher *et al.*, 2001; Campo *et al.*, 2002; Hefner, 2002; Gruenwald and Skrabal, 2003; Humberston *et al.*, 2003; Teschke *et al.*, 2003), while others demonstrating that it is safe in most individuals at recommended doses (Denham *et al.*, 2002; Kopp, 2003).

The sale of Kava Kava has been suspended in some countries due to potential hepatotoxic effects but it continues to be on the US market although the Food and Drug Administration (FDA) has issued several warnings to consumers about the association between Kava Kava use and serious liver damage (FDA, 2001, 2002). However, whether the dose or duration of use may be correlated with the risk of liver damage remains unknown. It is also unclear if the safety profile of Kava Kava is comparable to other agents used in the management of anxiety. In addition, there are difficulties inherent in causality assessment when dealing with herbal hepatotoxicity such as daily overdose, prolonged therapy, coingestion with up to five other herbals, dietary supplements, and synthetic drugs (Teschke *et al.*, 2008).

Mechanistically, the toxicity of Kava Kava in humans has been partially attributed to the CYP2D6 deficiency seen in 7% to 9% of Caucasian, 5.5% of Western European, almost 1% of Asian, and less than 1% of Polynesian populations (Wanwimolruk *et al.*, 1998; Poolsup *et al.*, 2000; Ingelman-Sundberg, 2005). Reports suggest that genetic differences may constitute significant contributory factors for increased hepatotoxicity in Caucasians (Singh, 2005). Inhibition of these CYPs or a deficiency in CYP2D6 indicates that exposure to Kava Kava and other drugs and chemical agents at the same time has a high potential for causing drug interactions (Jamieson and Duffield, 1990a,b; Mathews *et al.*, 2002, 2005; Unger *et al.*,

2002; Zou *et al.*, 2002, 2004; Raucy, 2003; Teschke *et al.*, 2003; Whitton *et al.*, 2003; Anke and Ramzan, 2004a,b; Bressler, 2005; Hu *et al.*, 2005; Singh, 2005).

In the literature, there are a limited number of animal studies on the toxicity of Kava Kava extract and its constituents. Most of these studies focus on the potential for clinical signs for hepatotoxicity. For example, a study by Singh and Devkota (2003) showed that Kava Kava administered to Sprague Dawley rats by gavage at 200–500 mg/kg/day for 2 and 4 weeks, exhibited no increases in serum markers of hepatotoxicity or malondialdehyde production. In another study, administration of Kava Kava root extract (100 mg/kg) by gavage in male F344/N rats for 2 weeks failed to elicit any significant changes in serum markers of hepatotoxicity (alanine aminotransferase and aspartate aminotransferase) or markers of hepatic lipid peroxidation and apoptosis (Lim *et al.*, 2007). Subchronic studies following oral exposure to Kavain (10 to 400 mg/kg) in dogs for 91 days revealed the presence of mild toxicity with multicentric liver necrosis in one high-dose dog (Hapke *et al.*, 1971). There are no long-term studies to identify and characterize the potential for chronic toxicity or carcinogenicity following Kava Kava exposure.

Kava Kava continues to be used widely in the United States, although the correlation of dose and duration of use with the risk of liver damage is unclear. Therefore, Kava Kava was nominated for toxicological assessment by the National Cancer Institute (NCI) due to its increasing use as a dietary supplement in the mainstream United States market, reports of liver toxicity among humans, and lack of sufficient toxicity and carcinogenicity data.

The NTP conducted 2-week, 3-month, and 2-year toxicity and carcinogenicity studies in F344/N rats and B6C3F1 mice to address these issues. The current results focus on the toxic effects on the liver, the main target organ of concern following Kava Kava administration. Details of the complete study can be found in the NTP Technical Report (NTP 2011).

MATERIALS AND METHODS

Chemicals and Reagents

Kava Kava extract (CAS 9000-38-8), a medium yellow powder was obtained from Cosmopolitan Trading Co. (Seattle, WA). A combination of chromatographic and spectrometric techniques was used to characterize the test article; an authentic standard of kavain was used for quantitation. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with ultraviolet (UV) light detection was used to determine the composition of the bulk chemical.

The batches of Kava Kava extract used in these studies were consistent with the composition of commercially available Kava Kava extract. The identified kavalactones in the extract included methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, and desmethoxyyangonin. The dose formulations were prepared by mixing Kava Kava extract with corn oil to give the required concentrations. The dose formulations were stored at room temperature in clear glass bottles sealed with Teflon®-lined lids enclosed in amber plastic bags for up to 37 days (2-week studies) or 42 days (3-month and 2-year studies) and were stable during this period.

Animals and Exposures

The studies were conducted at Battelle Memorial Institute (Columbus, OH). Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Laboratory (Germantown, NY). Rats and mice were quarantined for 14 days and were five to six weeks of age at the beginning of the studies. The animals were distributed randomly into groups of approximately equal initial mean body weights and identified by tail tattoo. Male rats were

housed five per cage in the subchronic and three per cage in chronic studies, female rats were housed five per cage, male mice were housed individually, and female mice were housed three to five per cage. Tap water and NTP-2000 diet (Zeigler Brothers, Inc. Gardners, PA) were made available *ad libitum*. The care of animals on this study was according to NIH procedures as described in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 1996). These studies were conducted in compliance with the Food and Drug Administration Good Laboratory Practice Regulations (Food and Drug Administration 1987).

Study Design

In the prechronic studies, groups of male and female F344/N rats and B6C3F1 mice (n = 5/dose/sex in 2-week studies and 10/sex/dose in 3-month studies) were administered Kava Kava extract by gavage in corn oil at concentrations of 0, 0.125, 0.25, 0.5, 1 and 2 g/kg. For the subchronic studies, groups of 10 male and 10 female rats and mice were dosed at concentrations of 0, 0.125, 0.25, 0.5, 1 and 2 g/kg. For the chronic studies, groups of 50 male and 50 female rats and mice were dosed at concentrations of 0, 0.3, 1.0 g/kg (rats) and 0, 0.25, 0.5, 1.0 g/kg (mice) respectively.

Body weights were recorded initially, weekly, and at study termination for the subchronic studies. For the chronic studies, body weights were recorded initially, weekly for the first 13 weeks, monthly thereafter, and upon study termination. Clinical findings were recorded once a week beginning on day 1 and at the end of the studies for the subchronic and at 4-week intervals beginning at week 5 until study termination for the chronic studies. Additional groups of 10 male and 10 female rats designated for clinical chemistry evaluations were administered the same doses for up to 23 days in the 3-month studies and up to 18 months in the chronic studies. Blood was collected via the retroorbital plexus (rats) or sinus (mice) of the clinical pathology study rats on days 4 and 23 and from the main study rats and mice at the end of the studies for hematology (only mice at day 4) and clinical chemistry (rats only). For the chronic studies, blood was collected from the retroorbital plexus of clinical chemistry study rats at 6, 12, and 18 months.

Necropsies were performed on all study animals. In the prechronic studies, the heart, right kidney, liver, lung, right testis, and thymus of rats and mice were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes were first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μ m, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all vehicle control and 2.0 g/kg rats and mice and on study animals that died early; tissues were examined to a no-effect level in the remaining core study groups. For the chronic studies, at necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved as described above. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. A complete microscopic examination was performed in all chronic study animals. Additional details regarding the pathology data generation, quality assurance review, and NTP pathology working group are available in the NTP technical report (NTP 2011).

Statistical Analysis

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided. The poly-3 test was used to assess neoplasm and nonneoplastic lesion incidence (Bailer and

Portier 1988; Piegorsch and Bailer 1997; Portier and Bailer 1989). To reduce bias of tumor incidence based on decrease in body weight, a statistical model was applied to adjust for body weight, average survival time, housing, and route of administration (Haseman et al., 1997).

Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971). The hematology and clinical chemistry data, which typically have skewed distributions was analyzed using the modified (Dunn 1964; Williams 1986) nonparametric multiple comparison methods of Shirley (1977).

RESULTS

2-Week Studies in Rats and Mice

In the two-week studies, rats and mice were administered 0, 0.125, 0.25, 0.5, 1 and 2 g/kg Kava Kava extract by gavage. There were no treatment- related effects on survival or body weight in rats or mice. Clinical findings included abnormal breathing, ataxia, and lethargy in the high dose- groups of male and female rats and mice. No gross lesions related to Kava Kava extract administration were observed. The liver was found to be the major target organ of toxicity in both rats and mice. This was reflected by dose-related increases in absolute and relative liver weights in 1.0 and 2.0 g/kg male and 0.5 g/kg or greater female rats accompanied by significantly increased incidences of minimal hepatocellular hypertrophy in the 2.0 g/kg male and in 0.25 g/kg or greater female rats. In mice, liver weights were significantly increased in 2.0 g/kg males and females with accompanying increases in the incidence of hepatocellular hypertrophy in the 2.0 g/kg female mice (Data not shown). Since there were no treatment-related effects on survival or body weights, and histopathologic changes were minimal; the same doses were selected for the 3-month studies.

3-Month Studies in Rats

There were treatment- related decreases in survival and body weights in the highest dose group in male and female rats administered Kava Kava for 3- months (Table 1). Clinical findings included ataxia and lethargy in both sexes in the 1.0 g/kg groups during week 1 and persisted in the 2.0 g/kg groups throughout the study. No chemical-related gross lesions were seen in early death or terminal sacrifice animals. There were dose-related increases in the absolute and relative liver weights of 0.25 g/kg or greater males and 0.5 g/kg or greater females compared to the vehicle controls (Table 1). While there was no statistically significant increased incidence of hepatocellular hypertrophy in the males, the females showed significant dose-related increases in incidence and severity. Microscopically, the hypertrophy was minimal to mild in severity and consisted of a diffuse increase in cell size associated with glycogen depletion and amphophilic cytoplasm of the hepatocytes.

Several alterations in clinical chemistry consistent with the histopathological lesions were observed (Table 2). The most prominent was a multiple-fold increase in serum gamma-glutamyltransferase (GGT) activity in the 2.0 g/kg males and females at all time-points. In addition, the 1.0 g/kg females were affected at week 14. In general, increases in serum GGT activity are used as a marker of cholestasis. Other markers of cholestasis, however, were either decreased (ALP activity) or unaffected (bile salts concentration) and did not support the increased GGT activities (data not shown). Dose-related increases in cholesterol concentrations occurred in the top three male and female dose groups (≥ 0.5 , g/kg) at all time points. Lower-dose groups (0.125 and 0.25 g/kg) also demonstrated this alteration, but less consistently. Triglyceride concentration, another marker of lipid metabolism, was unaffected (data not shown). Total protein and albumin concentrations were minimally increased

(<15%) in the top four female dose groups (0.25, 0.5, 1.0, and 2.0 g/kg) on day 23. By week 14, all female and the top three male dose groups (0.5, 1.0, and 2.0 g/kg) demonstrated these changes in serum proteins. The increases protein fractions were proportional. There were no changes in the hematology results considered attributable to Kava Kava extract administration (data not shown).

The highest dose for the 2-year gavage study in rats was selected to be 1.0 g/kg per day. This was based on findings in the 3-month study including decreases in the survival and body weights of 2.0 g/kg males and females. The increases in liver weights and incidences of hepatocellular hypertrophy in 1.0 g/kg males and females were considered to be minimal and alterations in GGT activity at 1.0 g/kg were not considered dose-limiting.

3-Month Studies in Mice

There were treatment- related decreases in survival in the top- dose groups in mice administered Kava Kava for 3- months (Table 3). There was no effect on body weight between the control and treated groups. Clinical signs included transient ataxia and lethargy in males and females in the 1.0 and 2.0 g/kg groups. No chemical-related gross lesions were seen in early death or terminal sacrifice mice. There were no changes in the hematology data of mice that were considered attributable to Kava Kava extract administration. There were treatment –related increases in the absolute and relative liver weights in both sexes (Table 3). Non-neoplastic lesions included a dose-related increase in the incidence of centrilobular hypertrophy in both males and females with minimal to moderate severities. Microscopically, the lesion consisted of enlarged hepatocytes, primarily located in the centrilobular regions, and was characterized by increased hepatocellular size and ground-glass cytoplasmic eosinophilia and decreased cytoplasmic glycogen content. The no-observed-effect level for this lesion was 0.25 g/kg in males and females. The doses of 0.25, 0.5, and 1.0 g/kg were selected for the 2-year gavage study in mice based on decreased survival in 2.0 g/kg males and females.

2-Year Studies in Rats

Chronic administration of Kava Kava did not significantly affect survival or body weight in either males or females (Table 4). Clinical findings included ataxia and lethargy that occurred in the 1.0 g/kg groups and persisted randomly and intermittently throughout the study. Similar to the 3-month studies, there were increases in GGT activity which were statistically significant at 18- months in males and at 6, 12 and 18- months in females (Table 5). In addition, there was an increase in bile salt concentrations in both sexes.

There were increases in several non-neoplastic lesions in the livers of the rats following Kava Kava exposure (Table 4). Hepatocellular hypertrophy was noted in both males and females and was microscopically characterized by irregular increase in the size of hepatocytes, usually in a centrilobular distribution (Figures 1, 2). There was also a significant increase in the incidence of centrilobular fatty change in 0.1 and 1.0 g/kg males, and in 1.0 g/kg females. This lesion consisted of poorly demarcated areas of hepatocytes with large, clear cytoplasmic vacuoles, usually in the centrilobular and midzonal regions. Cystic degeneration was observed in all dosed groups of males, and the incidence in 1.0 g/kg males was significantly increased. Microscopically, cystic degeneration consisted of multilocular cystic areas containing a finely granular or flocculent eosinophilic material, apparently resulting from the distention and occasional rupture of adjacent hepatocytes (Figure 3). In addition, a unique lesion was noted in the pancreas in the 1.0 g/kg males and females which included incidences of metaplasia of pancreatic acinar cells to a hepatocytic morphology. The increase was significant in the male rats (incidence: 0/49, 0/50, 0/50, 6/50). Microscopically, this lesion was characterized by the presence of small clusters of

apparently normal hepatocytes adjacent to islets of Langerhans (Figure 4). The etiology of this lesion in the current studies remains unknown.

2-Year Studies in Mice

Chronic administration to Kava Kava in male and female B6C3F1 mice did not affect survival (Table 6). There was a slight exposure-related reduction in body weight gain in the females in the highest exposure group. Clinical findings included ataxia and lethargy that occurred in both sexes in the 1.0 g/kg groups during the first week but persisted only in the females.

There were several neoplastic lesions noted in the livers of mice in both sexes. The males revealed dose-related increases in hepatoblastomas. The incidences of hepatoblastomas in 0.5 and 1.0 g/kg males exceeded the concurrent and historical controls while the 0.25 g/kg males exceeded only historical controls. There were also statistically significant increases in hepatocellular adenomas (multiple) in the top-dose group males and in the 0.5 g/kg group in females. There was also an increase in hepatocellular carcinomas in all dosed group females which was statistically significant at the low dose (0.25 g/kg). Importantly, there was a statistically significant increase in the combined incidences of hepatocellular adenomas or carcinomas in the 0.25 and 0.5 g/kg groups. These values exceeded the historical range for corn oil gavage studies, but not for all routes combined. Histologically, the hepatocellular carcinomas were variably sized, nodular lesions composed of well-differentiated, neoplastic hepatocytes that typically compressed the adjacent hepatic parenchyma. Portal areas and central veins were typically absent, and mild cellular atypia was often present. Hepatocellular carcinomas were variably well demarcated from the surrounding hepatic parenchyma and were composed of neoplastic hepatocytes that displayed mild to marked cellular and nuclear pleomorphism and mitoses. The predominant pattern displayed by most neoplasms in this study was trabecular, although focal areas had glandular or solid patterns of growth (Figures 5A, 5B). Necrosis was occasionally quite extensive, and metastasis to the lung was frequently observed. Hepatoblastomas tended to arise within hepatocellular adenomas or carcinomas and were composed of small, basophilic fusiform cells with a high nucleus to cytoplasm ratio (Figures 6A, 6B). Mitoses, large, irregularly shaped cystic areas filled with blood, and areas of necrosis were common.

The neoplastic lesions were accompanied by several non-neoplastic effects. There were dose-related increases in the incidences and severities of centrilobular hypertrophy in both sexes.

Microscopically, the hypertrophy was characterized by enlargement of centrilobular hepatocytes with increased amounts of eosinophilic cytoplasm and enlarged nuclei. This lesion was often variable in its presence and severity between lobes and within regions of the same lobe. There were significant increases in eosinophilic foci in the 0.5 g/kg males and in 1.0 g/kg males and females. The eosinophilic foci consisted of well-differentiated hepatocytes containing increased amounts of eosinophilic cytoplasm (Figure 7). Portal areas and central veins were often present, and minimal compression of the adjacent parenchyma occurred occasionally. The incidences of angiectasis increased in a dose-related manner in males, and the increase in the 1.0 g/kg group was significant. This lesion was characterized by variably sized dilatations of the hepatic sinusoids, typically occurring in small clusters randomly arranged throughout the hepatic parenchyma and without a sub-anatomic orientation (Figure 8). The sinusoids were lined by an attenuated to unapparent endothelium. The incidences of hepatocellular necrosis were significantly increased in 0.25 and 1.0 g/kg males. Microscopically, the hepatocellular necrosis was characterized by focal, widely scattered, randomly distributed areas of necrosis of hepatocytes often infiltrated by small

numbers of mixed inflammatory cells. Necrosis was not diagnosed when it was deemed to be secondary to neoplasia.

DISCUSSION

Kava Kava has been reported to treat anxiety in humans, with effects observed after as few as one to two doses with progressive improvements over 1 to 4 weeks (Pittler and Ernst, 2000, 2002, 2003; Basch *et al.*, 2002). Although commercial preparations of Kava Kava were used world-wide as anxiolytics, they have been withdrawn in several European and Canadian markets due to safety concerns (Stafford, 2001; Ernst, 2002a,b; Boon and Wong, 2003; Mills *et al.*, 2003; Schulze *et al.*, 2003). Several cases of liver damage have been associated with Kava Kava exposure in Europe including hepatitis (Bujanda *et al.*, 2002; Humberston *et al.*, 2003; Stickel *et al.*, 2003), cirrhosis and liver failure (Escher *et al.*, 2001; Kraft *et al.*, 2001), and death (Gow *et al.*, 2003; Thomsen *et al.*, 2004). However, since the correlation of dose and duration of Kava Kava use with the risk of liver damage is unclear, and the assessment of its safety profile compared with other agents used in the management of anxiety remains an area of controversy, it continues to be used widely in the United States. Hence, Kava Kava was nominated for study to the NTP due to the lack of sufficient information in the literature on its toxicity characterization.

The liver was consistently seen as the major target organ of toxicity in both rats and mice in the 3-month studies and 2-years as indicated by liver enlargement and persistent hepatocellular hypertrophy. To identify a potential mechanism for liver toxicity in the 3-month studies, microarray, real-time polymerase chain reaction (RT PCR) and immunohistochemistry studies were conducted on livers of rats and mice administered Kava for 3- months; results are published elsewhere (Clayton *et al.*, 2007; Guo *et al.*, 2009, 2010). Findings from the microarray studies revealed a dose-dependent induction in several drug metabolizing enzymes belonging to the cytochrome P450 family, notably CYP1A1. These findings were confirmed using RT PCR (Guo *et al.*, 2009, 2010) and immunohistochemical analysis (Clayton *et al.*, 2007). In addition, dose-related alterations were noted in other Phase I and Phase II drug metabolizing enzymes as well as transporters (Guo *et al.*, 2009, 2010). Clinical chemistry parameters in the 3- month and 2-year studies showed several-fold increases in serum GGT activity, an important marker of cholestasis. The females appeared to be more affected compared to the males although the reason for the sex difference remains unknown. Studies in literature suggest that GGT synthesis can be induced by steroids and other xenobiotics (Sulake *et al.*, 1990; Hall, 2007). It is possible that the increase in GGT activity may be related to the observed hepatocellular hypertrophy through an intrahepatic cholestatic mechanism such as enzyme induction as seen in our previous studies (Guo *et al.*, 2009, 2010). In addition, there were dose-related increases in total protein, albumin and cholesterol concentration at all time points in the 3- month studies. Although other serum markers of cholestasis (ALP activity and bile salt concentration) were unaffected in our 3- month studies, there was an increase in bile salt concentration in the 2-year findings. This is in concordance with findings in literature which suggest that the liver plays an important role in cholesterol metabolism and albumin synthesis (Wagner *et al.*, 1999; Nguyen, 1999). Hence, there may be potential changes in hepatic metabolism following Kava Kava exposure thereby impacting clinical markers. This is supported by our microarray findings which showed liver enzyme induction in the 3- month studies (Guo *et al.*, 2009, 2010).

In the 2-year studies, survival of the dosed groups of rats and mice was comparable to that of the vehicle controls. Mean body weights were generally decreased (10% or more) in 1.0 g/kg male and female rats and female mice at the end of the study. The liver was the major site of carcinogenic activity in mice with increased incidences of eosinophilic foci, which

are often viewed as histopathologic precursors to hepatocellular neoplasms, in the 0.5 and 1.0 g/kg males and females.

In male mice, there were dose-related increases in the incidences and multiplicities of hepatoblastomas and hepatocellular adenomas as well as an increase in the combined incidence of hepatocellular carcinomas or hepatoblastomas. Hepatoblastomas are believed to arise *de novo* or from within hepatocellular adenomas or carcinomas, and approximately 25% are believed to metastasize (Allen *et al.*, 2004). Since hepatoblastomas are malignant, have a low spontaneous background incidence in mice, and the incidences in the current study exceed the historical controls, it was concluded that there was clear evidence of carcinogenic activity in male mice.

The incidences of hepatocellular carcinomas and the combined incidences of hepatocellular adenomas or carcinomas in female mice treated with 0.25 and 0.5 g/kg Kava Kava were significantly increased compared to vehicle controls. The incidences exceeded the historical control range for corn oil gavage studies. However, there were no significant increases in the incidences of liver neoplasms in the 1.0 g/kg group. A lack of effect in the high-dose group of 1.0 g/kg could be attributed to decreases in body weight. The body weight adjusted tumor incidence (Haseman *et al.*, 1997) was similar to the observed tumor incidence suggesting little evidence for a chemical-related increase in the high dose group. Hence, although no neoplastic lesions were seen in the high dose-group, the significant increases in the combined incidences and multiplicities of hepatocellular adenomas or carcinomas in the low and mid dose- groups suggest that Kava Kava induces carcinogenicity in female mice. In conclusion, even though liver toxicity was observed in rats and mice in the 3-month and 2-year studies, Kava Kava extract did not induce liver neoplasms in rats suggesting species differences in the sensitivity of liver neoplasm induction. This pattern is corroborated with findings in the literature that demonstrate that mice appear to be more sensitive to the induction of liver neoplasms than rats (NTP, 1993; Bucher *et al.*, 1998). The results of the NTP bacterial mutagenicity and *in vivo* micronucleus studies as well as the available published information (Jhoo *et al.*, 2007; Whittaker *et al.*, 2008) suggest that Kava Kava extract is not mutagenic, therefore, it appears that the carcinogenic activity in mice is most likely mediated through nongenotoxic mechanisms involving enzyme activation and/or free radical generation (Guo *et al.*, 2009; 2010). Although these studies were not designed to assess herb-drug interactions in humans, the findings raise the possibility that Kava Kava consumption as a dietary supplement may result in hepatic toxicity due to its enzyme modulating effects thereby producing profound effects on the pharmacokinetics of many coadministered drugs or other food supplements, potentially exacerbating hepatotoxicity.

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Highlights

- Rats and mice were administered Kava Kava by gavage for two weeks, thirteen weeks or two years
- Prechronic studies revealed dose-related increases in liver weights and hepatocellular hypertrophy
- Dose-related increases in non-neoplastic lesions seen in rats and mice in chronic studies
- Additionally, hepatocarcinogenicity seen in male and female mice with chronic exposure

Figure 1.
Photomicrograph of a liver section from a control male rat from the 2 year Kava Kava study.
Note (arrows), the normal aspect of the centrilobular hepatocytes. Compare with Figure 1B.
X 32. H&E

Figure 2.
Photomicrograph of a liver section from a female rat treated with 1 g/kg Kava Kava for 2 years. Note (arrows), mild (grade 2) centrilobular hypertrophy. Compare with Figure 1A. X 32. H&E

Figure 3. Photomicrograph of a liver section from a male rat treated with 1 g/kg Kava Kava for 2 years. Note (arrows), mild (grade 2) cystic degenerationX 16. H&E

Figure 4. Photomicrograph of minimal (grade 1) metaplasia of pancreatic acinar cells to a hepatocytic morphology (arrows) from a female rat treated with 1 g/kg Kava Kava for 2 years. X₁₆. H&E

Figures 5.

Photomicrographs of hepatocellular carcinoma from a male mouse treated with 0.25 mg/kg Kava Kava for 2 years. Figure 5A: Note (arrows), margins of the carcinoma compressing the adjacent normal tissue. X 2. Figure 5B: Higher magnification of Figure 5A. Note (arrows) the trabecular architecture displayed by the neoplastic cells. X 10. H&E

Figures 6.

Photomicrograph of hepatoblastoma from a male mouse treated with 1 mg/kg Kava Kava for 2 years (arrows). Note the hyperbasophilic appearance of the mass due to presence of basophilic fusiform cells with a high nucleus to cytoplasmic (N:C) ratio. Fig. 6A: X 2. Figure 6B: x 10. H&E

Figure 7.
Photomicrograph of eosinophilic focus (arrows) from a male mouse treated with 0.25 mg/kg Kava Kava for 2 years X 5. H&E

Figure 8.
Photomicrograph of angiectasis (arrows) from a male mouse treated with 1 g/kg of Kava Kava for 2 years. X 10. H&E.

Table 1
Survival, Body weight and Liver Pathology for Rats Administered Kava Kava for 3-months

Male Rats	Control	0.125 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg	2.0 g/kg
Survival	10/10	10/10	9/10	10/10	10/10	7/10
Body Weight (g)	346	334 ^b (97) ^c	334 (97)	339 (98)	316 ^{**} (91)	286 ^{**} (83)
Absolute Liver weight (g)	11.05 ± 0.47	11.05 ± 0.29	12.09 ± 0.35 ^{ad}	12.42 ± 0.20 ^{**c}	13.20 ± 0.33 ^{**c}	15.06 ± 0.21 ^{**d}
Relative liver weight	32.09 ± 0.59 ^b	33.36 ± 0.26 ^b	35.98 ± 0.82 ^{**c}	37.44 ± 0.64 ^{**c}	41.56 ± 0.86 ^{**c}	51.92 ± 1.13 ^{**d}
Hepatocyte, Hypertrophy	0	0	0	0	1 ^e (1.0) ^f	0
Female Rats						
Survival	9/10	10/10	10/10	10/10	9/10	6/10
Body Weight (g)	197	196 ^d (100) ^b	193 (98)	196 (99)	190 (96)	178 ^{**} (90)
Absolute Liver weight (g)	6.42 ± 0.11	6.75 ± 0.16	6.82 ± 0.17	8.06 ± 0.25 ^{**}	8.97 ± 0.27 ^{**}	10.29 ± 0.46 ^{**}
Relative liver weight	32.52 ± 0.34	34.34 ± 0.64	35.26 ± 0.63 [*]	41.11 ± 0.77 ^{**}	47.22 ± 0.81 ^{**}	57.84 ± 2.06 ^{**}
Hepatocyte, Hypertrophy	0	0	0	3 (1.0)	3 (1.3)	10 ^{**} (1.7)

* P ≤ 0.05;

** p ≤ 0.01

^a body weight at the end of 3- months;

^b % body weight compared to control;

^c n=4;

^d n=3;

^e lesion incidence;

^f average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked;

^g Data are presented as mean ± standard error.

Table 2

Selected Clinical Chemistry for Rats Administered Kava Kava for 3-months

Male Rats	Control	0.125 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg	2.0 g/kg
Γ- Glutamyltransferase (IU/L)						
Day 4	0.2 ± 0.1 ^a	0.2 ± 0.1	0.6 ± 0.3	0.3 ± 0.2	0.1 ± 0.1	2.8 ± 0.6 ^{**}
Day 23	0.0 ± 0.0	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.9 ± 0.2 ^{**}
Week 14	0.2 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 0.1 ^{**}
Cholesterol (mg/dL)						
Day 4	95 ± 1	100 ± 1 ^{**}	99 ± 2 [*]	114 ± 2 ^{**}	124 ± 3 ^{**}	132 ± 6 ^{**}
Day 23	87 ± 1	87 ± 2	91 ± 2	97 ± 2 ^{**}	97 ± 3 ^{**}	94 ± 3 ^{**}
Week 14	77 ± 1	75 ± 1	79 ± 1	83 ± 2 [*]	86 ± 2 ^{**}	92 ± 4 ^{**}
Total Protein						
Day 4	6.1 ± 0.1 ⁸	6.1 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.1 ± 0.1	5.9 ± 0.1
Day 23	6.9 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	7.0 ± 0.1	7.1 ± 0.1	6.9 ± 0.1
Week 14	6.8 ± 0.1	6.7 ± 0.0	6.8 ± 0.1	7.1 ± 0.1 ^{**}	7.4 ± 0.1 ^{**}	7.9 ± 0.3 ^{**}
Albumin						
Day 4	4.3 ± 0.0	4.3 ± 0.0	4.4 ± 0.0	4.4 ± 0.1	4.3 ± 0.1	4.2 ± 0.1
Day 23	4.8 ± 0.1	4.7 ± 0.1	4.8 ± 0.0	4.8 ± 0.0	4.9 ± 0.1	4.8 ± 0.0
Week 14	4.6 ± 0.0	4.6 ± 0.0	4.6 ± 0.0	4.8 ± 0.0 ^{**}	5.0 ± 0.1 ^{**}	5.3 ± 0.1 ^{**}
Female Rats						
Γ- Glutamyltransferase (IU/L)						
Day 4	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.9 ± 0.2	4.3 ± 0.7 ^{**}
Day 23	0.5 ± 0.2	0.2 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	1.0 ± 0.1	3.9 ± 0.4 ^{**}
Week 14	1.3 ± 0.3	1.5 ± 0.3	1.4 ± 0.2	1.7 ± 0.3	3.1 ± 0.3 ^{**}	15.3 ± 1.0 ^{**}
Cholesterol (mg/dL)						
Day 4	85 ± 3	92 ± 3	89 ± 2		109 ± 2 ^{**}	128 ± 5 ^{**}
Day 23	73 ± 1	78 ± 2	83 ± 1 ^{**}	90 ± 1 ^{**}	100 ± 2 ^{**}	107 ± 3 ^{**}
Week 14	69 ± 2	83 ± 2 ^{**}	75 ± 2 [*]	92 ± 2 ^{**}	99 ± 2 ^{**}	105 ± 4 ^{**}

Female Rats									
Total Protein	6.0 ± 0.1 ^e	6.1 ± 0.1	6.1 ± 0.0	6.2 ± 0.0 [*]	6.1 ± 0.1	5.9 ± 0.1			
Day 23	5.8 ± 0.1 [^]	6.0 ± 0.1	6.1 ± 0.1 [*]	6.2 ± 0.0 ^{**}	6.2 ± 0.1 ^{**}	6.3 ± 0.1 ^{**}			
Week 14	6.4 ± 0.1	6.7 ± 0.1 ^{**}	6.5 ± 0.1 [*]	6.8 ± 0.1 ^{**}	6.8 ± 0.1 ^{**}	7.2 ± 0.1 ^{**}			
Albumin Day 4	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.5 ± 0.0	4.4 ± 0.0	4.3 ± 0.0			
Day 23	4.4 ± 0.0	4.5 ± 0.1	4.5 ± 0.0 [*]	4.6 ± 0.0	4.6 ± 0.0 ^{**}	4.6 ± 0.1 ^{**}			
Week 14	4.6 ± 0.0	4.8 ± 0.0 ^{**}	4.8 ± 0.0 [*]	4.9 ± 0.1 ^{**}	4.9 ± 0.0 [*]	5.1 ± 0.0 ^{**}			

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test

** P ≤0.01;

^a Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.

Table 3

Survival, Body weight and Liver Pathology in Male and Female Mice Administered Kava Kava for 3-months

Male Mice	Contr	0.125 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg	2.0 g/kg
Survival	10/10	10/10	10/10	10/10	10/10	6/10
Body Weight (g)	34.4	35.5 ^a (103)	35.4 (105)	36 (105)	35.3 (103)	32.4 (94)
Absolute Liver weight (g)	1.41 ±	1.41±0.09 ^c	1.46 ± 0.08 ^c	1.61 ±0.06 ^c	1.60 ±0.05 ^c	1.90 ±0.05 ^{***d}
Relative liver weight	42.10	39.24 ± 1.79 ^c	41.84 ± 2.17 ^c	44.62 ± 1.53 ^c	47.513 ± 0.63 ^{*c}	60.83 ± 1.53 ^{***d}
Centrilobular Hypertrophy	0	0	0	9 ^{***e} (1.0) ^f	10 ^{**} (2.0)	7 ^{**} (2.6)
Female Mice						
Survival	10/10	10/10	10/10	10/10	10/10	6/10
Body Weight (g)	28.1	28.5 (101)	27.7 (99)	29.1 (104)	27.1 (96)	27 (96)
Absolute Liver weight (g)	1.12 ±	1.199 ± 0.02	1.16 ±0.03	1.22 ±0.03 [*]	1.25 ±0.05 ^{**}	1.65 ± 0.03 ^{***}
Relative liver weight	40.19	42.21 ± 0.93	41.87 ±0.94	41.95 ±0.83	45.96 ± 0.72 ^{**}	61.21 ±1.31 ^{**}
Centrilobular Hypertrophy	0	0	0	2 (1.0)	5 [*] (1.0)	4 [*] (1.5)

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test

*** P≤0.01

^a body weight at the end of 3-months;

^b % body weight compared to control;

^c n=4;

^d n=2;

^e lesion incidence

^f average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Table 4

Survival, Body Weight and Non-neoplastic Lesions of the Liver in Male and Female Rats Administered Kava Kava for 2-years

Male Rats	Control	0.1 g/kg	0.3 g/kg	1.0 g/kg
Survival	34/49	35/50	34/50	31/50
Body Weight (g)	501	493 ^a (98) ^b	508 (101)	449 (90)
Hepatocyte, Hypertrophy	0	2 ^c (1.0) ^d	2 (1.0)	22 ^{**} (1.4)
Centrilobular fatty change	1 (2.0)	7 [*] (1.3)	4 (1.3)	21 ^{**} (1.7)
Degeneration, Cystic	0	4 (1.0)	3 (1.3)	5 [*] (1.2)

Female Rats				
Survival	34/50	35/50	24/50	34/50
Body Weight (g)	327	327 (100)	323 (99)	282 (86)
Hepatocyte, Hypertrophy	5 (1.6)	2 (1.0)	3 (1.3)	33 ^{**} (1.8)
Centrilobular fatty change	0	0	0	2 (2.0)
Degeneration, Cystic	0	0	0	1 (1.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test

** $P \leq 0.01$

^a two-year body weights (weeks 53–101)

^b % body weight compared to control

^c lesion incidence

^d average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Table 5

Selected Clinical Chemistry for Rats Administered Kava Kava for 2-years

Male Rats	Control	0.1 g/kg	0.3 g/kg	1.0 g/kg
Γ- Glutamyltransferase (IU/L)				
Month 6	0.5 ± 0.2	0.0 ± 0.0*	0.1 ± 0.1	0.6 ± 0.2
Month 12	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.2
Month 18	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	1.1 ± 0.2**
Bile Salts (μmol/L)				
Month 6	6.2 ± 1.2	7.4 ± 1.5	5.6 ± 1.2	5.6 ± 0.7
Month 12	3.8 ± 0.5	8.0 ± 1.6	4.5 ± 0.7	9.9 ± 1.6**
Month 18	9.4 ± 1.9	10.1 ± 2.4	9.5 ± 1.5	16.4 ± 3.5

Female Rats	Control	0.1 g/kg	0.3 g/kg	1.0 g/kg
Γ- Glutamyltransferase (IU/L)				
Month 6	1.7 ± 0.2	2.2 ± 0.3	1.6 ± 0.3	4.3 ± 0.3**
Month 12	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.2	3.5 ± 0.6**
Month 18	0.5 ± 0.2	0.4 ± 0.3	0.5 ± 0.2	4.4 ± 0.7**
Bile Salts (μmol/L)				
Month 6	7.1 ± 1.3	5.2 ± 0.9	4.1 ± 0.4	11.3 ± 1.8
Month 12	4.9 ± 0.5	6.6 ± 0.9	7.8 ± 0.7*	7.3 ± 0.9
Month 18	5.5 ± 0.8	10.9 ± 2.5*	11.6 ± 1.3**	15.4 ± 2.0**

Table 6

Survival, Body Weight, Neoplastic and Non-neoplastic Lesions of the Liver in Mice Administered Kava Kava for 2-years

Male Mice	Control	0.25 g/kg	0.5 g/kg	1.0 g/kg
Survival	34/50	33/50	35/50	36/50
Final Body Weight (g)	55.1	55.2 ^a (100) ^b	55.2 (100)	53.6 (97)
Centrilobular Hypertrophy	0	34 ^{**c} (1.0) ^d	30 ^{**} (2.0)	39 ^{**} (2.0)
Eosinophilic Foci	28	32	42 ^{**}	43 ^{**}
Angiectasis	3 (1.0)	6 (1.0)	7 (1.1)	10 [*] (1.7)
Necrosis	3 (1.7)	10 [*] (2.0)	7 (2.0)	13 ^{**} (2.0)
Hepatocellular Adenoma, Multiple	13	19	19	23 [*]
Hepatocellular Carcinoma ^e	20	18	26	20
Hepatoblastoma ^f	0/50 ^{**}	4/50 (9.4) ^g	9/50 [*] (20.1)	12/50 ^{**} (26.4)

Female Mice	Control	0.25 g/kg	0.5 g/kg	1.0 g/kg
Survival	38/50	34/50	45/50	37/50
Final Body Weight (g)	63.1	61.6 (98)	60.8 (96)	51.3 (81)
Centrilobular Hypertrophy	0	20 ^{**} (1.0)	48 ^{**} (1.9)	49 ^{**} (2.0)
Eosinophilic Foci	9	7	16	26 ^{**}
Hepatocellular Adenoma, Multiple	0	4	6 [*]	1
Hepatocellular Carcinoma ^h	3/50 (6)	13/50 [*] (26)	8/50 (16)	8/50 (16)
Hepatocellular adenoma or carcinoma ⁱ	10/50 (22.1)	21/50 [*] (45.1)	20/50 ^{**} (41.2)	13/50 (28)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test

** $P \leq 0.01$

^a two-year body weights (weeks 53–101)

^b % body weight compared to control

^c lesion incidence

^d average severity grade of lesion in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^e Historical incidence for corn oil gavage studies: 72/250 (28.8% $\pm$ 9.0%), range 16%–40%; all routes: 389/1,248 (31.2% $\pm$ 10.3%), range 16%–56%

^f Historical incidence for corn oil gavage studies: 10/250 (4.0% $\pm$ 3.2%), range 0%–8%; all routes: 50/1,248 (4.0% $\pm$ 6.7%), range 0%–34%

^g Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^h Historical incidence for corn oil gavage studies: 14/247 (5.7% $\pm$ 3.0%), range 2%–10%; all routes: 136/1,296 (10.5% $\pm$ 9.7%), range 0%–46%

ⁱ Historical incidence for corn oil gavage studies: 51/247 (20.7% $\pm$ 10.4%), range 8%–35%; all routes: 428/1,296 (33.0% $\pm$ 18.8%), range 6%–76%