Published in final edited form as:
Food Chem Toxicol. 2010 February ; 48(2): 686–696. doi:10.1016/j.fct.2009.11.050.

Gene expression profiling in male B6C3F1 mouse livers exposed to kava identifies – Changes in drug metabolizing genes and potential mechanisms linked to kava toxicity

Lei Guo[a,*], **Qiang Shi**[a], **Stacey Dial**[a], **Qingsu Xia**[b], **Nan Mei**[c], **Quanzhen Li**[d], **Po-Chuen Chan**[e], and **Peter Fu**[b,*]

[a]Divisions of Systems Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, USA

[b]Biochemical Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, USA

[c]Genetic and Reproductive Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, USA

[d]Microarray Core Facility, UTSW Medical Center, Dallas, TX 75390, USA

[e]National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

Abstract

The association of kava products with liver-related health risks has prompted regulatory action in many countries. We used a genome-wide gene expression approach to generate global gene expression profiles from the livers of male B6C3F1 mice administered kava extract by gavage for 14 weeks, and identified the differentially expressed drug metabolizing genes in response to kava treatments. Analyses of gene functions and pathways reveal that the levels of significant numbers of genes involving drug metabolism were changed and that the pathways involving xenobiotics metabolism, Nrf2-mediated oxidative stress response, mitochondrial functions and others, were altered. Our results indicate that kava extract can significantly modulate drug metabolizing enzymes, potentially leading to herb–drug interactions and hepatotoxicity.

Keywords

Drug metabolizing enzyme; Drug metabolizing gene; Gene expression; Kava extract; Microarray; TaqMan assay

1. Introduction

Herbal plants, including the traditional Chinese herbs, have been used for thousands of years as medicines, functional foods, and teas. Since the United States Congress passed the Dietary Supplement Health and Education Act (DSHEA) in 1994, herbal products, including

*Corresponding authors. Tel.: +1 870 543 7048 (L. Guo), tel.: +1 870 543 7027 (P. Fu)., lei.guo@fda.hhs.gov (L. Guo), peter.fu@fda.hhs.gov (P. Fu).

Conflict of interest statement The authors declare that there is no conflict of interest.

Disclaimer This article is not an official guidance or policy statement of the National Toxicology Program (NTP) or the US Food and Drug Administration (FDA). No official support or endorsement by the US FDA and NTP is intended or should be inferred.

herbal dietary supplements, have been the fastest growing segment of the vitamin, mineral supplements, and herbal products industry in the United States. The American Herbal Products Association estimates that there are about 3000 species of plants, in as many as 50,000 different products, sold as herbal supplements in the United States (Zurer and Hanson, 2004). St. John's wort, *Ginkgo biloba* (ginkgo), golden seal, *panax* ginseng, kava, *Aloe vera*, and mild thistle extract are among the most widely-used of these products (Chan and Fu, 2007a; Chan et al., 2007; Fu, 2007a; Guo et al., 2009). In the United States kava-containing products remain popular and their consumption is dramatically increasing. These products continue to be sold in health food stores and ethnic markets regardless of the fact that kava has been banned in several Western countries following reports of alleged hepatotoxicity (CDC, 2002).

While it has been reported that a number of herbal dietary supplements cause adverse health effects (Chan and Fu, 2007b; Chan et al., 2007; Fu, 2007b; Fu et al., 2007, 2008b; Gurley et al., 2005a, 2007; Hu et al., 2005; Singh, 2005), to date, safety issues concerning potential side effects and toxic contamination of herbal products have not been addressed adequately. Consequently, assessment of the safety of herbal plants and herbal dietary supplements is timely and important (FDA, 2001, 2004a,b; Fong, 2002; Fu et al., 2002). Recently, a number of herbal dietary supplements and active ingredients have been nominated by the US Food and Drug Administration (FDA) and the US National Institutes of Health for the US National Toxicology Program (NTP) for determination of their toxicity and tumorigenicity. *Ginkgo biloba* (ginkgo), *panax* ginseng, kava, *Aloe vera*, and green tea are among the herbal dietary supplements currently being investigated in NTP toxicity and tumorigenicity bioassays.

When a test substance is found tumorigenic in NTP chronic tumorigenicity bioassays, the mechanism of action should be determined so that the relevance to humans can be established. A substance that is determined to be a tumorigen with a known mechanism and that possesses a significant human exposure potential will be listed as either "*known to be a human carcinogen*" or "*reasonably anticipated to be a human carcinogen*" in "Report on Carcinogens", a US congressionally mandated document prepared by the NTP (http://ntp.niehs.nih.gov/go/roc).

In general, approaches for determining the mechanism by which a pure chemical induces toxicities or tumors have been well established. Nevertheless, to date, it is still a challenge to determine the mechanisms of toxicities or tumor induction elicited by a mixture of many chemical components, such as herbal plants and herbal dietary supplements. For chemical mixtures, there is a need for new approaches for elucidating mechanisms.

Toward this goal, we previously studied the alterations in gene expression of drug metabolizing enzymes in the livers of Fischer 344 male rats administered kava extract by gavage for 14 weeks. Our results indicate that kava extract can significantly modulate drug metabolizing enzymes, particularly the CYP isozymes, which can potentially cause herb–drug interactions and may lead to hepatotoxicity (Guo et al., 2009). The gene expression profile correlated well with immunohistochemical data determined by Clayton et al. (2007). In addition, we observed that kava altered the expression of Cyp1a1 and many other Cyp genes that can metabolize various xenobiotics and drugs. These findings illustrate that, without obtaining the whole spectrum of gene expression change, some important information may be missed (Guo et al., 2009). Our study also suggested that analysis of the gene expression profiles using microarrays in the livers of rodents treated with herbal dietary supplement is potentially a practical approach for understanding the mechanism of action (Guo et al., 2009).

As a continuation of our mechanistic work on herbal dietary supplements, we have analyzed changes in gene expression of drug metabolizing enzymes in the livers of male B6C3F1 mice following oral treatment with kava extract by gavage at 0, 0.125, 0.25, 0.5, 1.0, or 2.0 g/kg/day for 90 days. The altered genes were also examined for their possible involvement in toxicity related pathways.

2. Material and methods

2.1. Study design

Groups of 10 male and female B6C3F1 mice 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, a complete necropsy was performed and blood and tissues were saved for hematology and histopathology evaluations according to the NTP procedure.

About 130–150 mg of tissue from the left liver lobe was collected from each mouse and placed in RNAlater (Qiagen, Valencia, CA) per the manufacturer's instructions and stored at −20 °C until processed for microarray analysis.

2.2. Chemicals

The powdered kava extract was received in three amber glass vials from Midwest Research Institute (MRI, Kansas City, MO). The extract was prepared from the rhizome of the kava tropical shrub plant, *Piper methysticum* Forst F. by extraction with methanol. All material in the three vials was combined into a lot (Lot No. 082203). High-performance liquid chromatography with ultraviolet detection (HPLC/UV) analysis showed the extract contained 30% kavalactones. Of the 30% kavalactone, 96% were a mixture of yangonin, demethoxyyangonin, methysticin, dihydromethysticin, kavain, and dihydrokavain. 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 same kava extract was used for the NTP 14-week toxicity bioassays and for the NTP 2-year chronic tumorigenicity bioassays, and the chemical analysis of its kavalactones constituents have been previously published (Clayton et al. 2007).

The USP-grade corn oil (Lot No. SS0711), used as 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 prior to the start of administration of dosages. During the 14-week experimental period, stock dose formulations of kava extract in corn oil were prepared every 3 weeks. Aliquots of approximately 30 ml of the dose formulation were sealed in amber glass jars with Teflon-lined lids, with minimum air space and refrigerated (5 °C). The dose formulations were homogenized for 30 s with Polytron before use. In a 42-day stability study at 7, 14, 21, 28, and 42 days the stock dose formulations were analyzed by HPLC along with kavain standards. A 3-h simulated dosing study in which the dose formulations were analyzed after exposure to light and air for 3 h. The relative stability of methysticin, dihydromethysticin, dihydrokavain, yangonin, and desmethoxyyangonin in the dose formulations were also monitored. The analysis data showed that the dose formulations were stable for at least 42 days (within 10% of target) except yagonin showed 17.6% degradation. The decrease in yongonin peak area coincided with an increase in the peak area of peak 6, an unidentified kava component. The 3-h simulated dosing study indicated that the dose formulations were stable for up to 3 h in air and light. Yangonin degradation was also

observed in neat kava, which indicated that the yongonin loss is inherent in the kava and not caused by the presence of the corn oil matrix.

2.3. 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 accordance with guidelines of the National Institutes of Health (NIH). B6C3F1 mice 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. Mice were individually housed in polycarbonate cages (Lab. Products, Inc., Seaford, DE). Filtered room air underwent at least 10 changes per hour. The animal room was maintained at 21–24 °C with 35–65% relative humidity and 12 h each of light and darkness. Irradiated NTP-2000 pelleted feed (Zeigler Bros., IncGardner, PA) and water were available *ad libitum*. All animals were checked twice daily for moribundity, mortality, clinical signs of ataxia and prostration, and toxicological effects including CNS depression, neurobehavioral abnormalities, and motor activity. Body weights were measured weekly.

2.4. RNA isolation

Total RNA was isolated using an RNeasy Midi Kit (Qiagen). The yield of the extracted RNA was determined spectrophotometrically by measuring the optical density at 260 nm. The purity and quality of extracted RNA were evaluated using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). High quality RNA with RNA integrity numbers (RINs) greater than 8.5 was used for microarray experiments and TaqMan gene expression assays.

2.5. Microarray analysis

Gene expression profiling was performed using Illumina's multi-sample format MouseWG-6 BeadChip that contains 45,281 transcripts and profiles six samples simultaneously on a single chip (Illumina Inc., San Diego, CA).

2.5.1. Sample labeling and quality control of labeled aRNA—For each sample, 200 ng total RNA was labeled using a MessageAmp II-biotin enhanced kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, double stranded cDNA was synthesized using T7-oligo (dT) primers and followed by an *in vitro* transcription reaction to amplify aRNA while biotin was incorporated into the synthesized aRNA probe. The aRNA probe was then purified and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The size distributions of aRNA were made by running 200 ng of each of sample on an Agilent Bioanalyzer (Wilmington, DE) using the RNA 6000 Nano Chip kit and the Eukaryotic mRNA Assay with smear analysis.

2.5.2. Sample hybridization—Biotinylated cRNA probe was hybridized to the MouseWG-6 BeadChip Array (Illumina). Labeled aRNA (1.5 μg) was used for hybridization to each array. The hybridization, washing, and scanning were performed according to the manufacturer's instructions with the addition of a 10-min wash in High-temp wash buffer (Illumina) at 55 °C for 10 min in a Scigene Hybex Microarray Incubation System (SciGene Corporation, Sunnyvale, CA) with a water bath insert following the overnight hybridization.

2.5.3. Scanning and data outputs—The arrays were scanned using a BeadScan 2.3.0.10 (Illumina) at a multiplier setting of "2." The microarray images were registered and extracted automatically during the scan according to the manufacturer's default settings.

2.5.4. Normalization—Raw microarray intensity data had background subtracted and were normalized using the cubic spline normalization method according to the manufacturer's recommendation.

2.5.5. Microarray data analysis—The differentially expressed genes were identified based on *t*-tests and fold-change cutoffs. Hierarchical Cluster Analysis (HCA) was conducted within ArrayTrack (http://www.fda.gov/nctr/science/centers/toxicoinformatics/Array-Track/). Additional calculations were performed within JMP 7.0 (SAS Institute, Cary, NC). The pathways, networks, and functional analyses were generated through the use of Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com).

2.6. TaqMan gene expression assays

Microarray-based mRNA expression was validated using real-time PCR with TaqMan assays (Applied Biosystems) as previously described (Guo et al., 2009). The gene expression of the following four drug metabolizing genes were confirmed by TaqMan assays: Cyp1a1 (Mm00487218_m1); Cyp3a11 (Mm00731567_m1); Gata2 (Mm00833353_mH); Nqo1 (Mm00500821_m1). The following two genes were used for endogenous controls: Polr2a (Mm00839493_m1) and Actb (Mm00607939_s1). The fold induction calculation was as described previously (Guo et al., 2009).

3. Results

3.1. Animal results

Survival of this NTP 14-week mouse gavage study of kava at 0, 0.125, 0.25, 0.5, 1.0, or 2.0 g/kg showed that unscheduled early deaths occurred with four 2.0 g/kg mice. The deaths occurred on Day 2 (1 death), Day 3 (2 deaths), and Day 6 (1 death). All mice in the other dose groups survived. Pathological examination of the early deaths revealed no gross or microscopic lesions that could be assigned as a cause of death. The early deaths were possibly due to central nervous system and/or respiratory depression. In week 1, ataxia was observed in 5 and lethargy in seven mice in the 1.0 g/kg group; and ataxia was observed in 2 and lethargy in 10 mice in the 2.0 g/kg group. The surviving animals soon developed tolerance and the ataxia and lethargy observed in week 1 were no longer observed in week 2. In this study, except deaths at early time points, animals tolerated up to the maximum given dose. No LD_{50} of kava was established. Kava extract caused no statistically significant reduction in terminal body weight in any of the dose groups of mice. Mean body weights (32.4 ± 1.0 g) of the 2.0 g/kg group of mice at sacrifice were about 6% lower compared to controls (34.4 ± 2.9 g), the decrease was not significant. Absolute (1.90 ± 0.10 g) and relative (6.08 ± 0.21) liver weights of the 2.0 g/ kg mice were significantly increased compared to controls (1.4 ± 0.1 g and 4.2 ± 0.1, respectively, $P < 0.01$). The Fischer's least significant difference method was used in the statistic analysis. Histopathology results included minimal to moderate hepatocellular centrilobular hypertrophy in the males administered 0.5 g/kg and higher dosages of kava extract and severity increased with increasing dose (data not shown). Blood was collected at terminal sacrifice. Hematology measurements were recorded; no clinical chemistry study was evaluated due to insufficient serum.

3.2. DNA microarray data quality

In this study, the gene expression profiles from the livers of B6C3F1 mice treated with kava extract for 14 weeks were determined by genome-wide gene expression microarray analysis. In order to avoid possible confounding effects on liver gene expression by cyclical female hormonal changes, RNA isolated from male mice were used for the study. There were one control group and five treatment groups, each group containing 4–6 biological replicates, with a total of 33 RNA samples processed. For microarray quality control purposes, two randomly selected samples (E48 and E50) were analyzed twice and served as technical replicates. Thus, a total of 35 microarrays were performed in this study.

To explore the treatment effects and to determine the relationship of the samples based on expression profiles, unsupervised two-way Hierarchical Cluster Analysis (HCA) was employed for the control and the kava-treated groups. The $\log_2$ intensity of the entire gene set was scaled by Z-score transformation; then these values were hierarchically clustered using the distance metric of Ward's. The resulting cluster analysis is displayed in Fig. 1 with the heat map of gene expression depicting the distance between samples. HCA showed there were two large clusters and samples were grouped according to the treatment, namely, high-dose groups (0.5, 1.0, and 2.0 g/kg kava treatment, first cluster in red) vs. control and low-dose groups (0, 0.125, and 0.25 g/kg kava treatment, second cluster in black). In the first cluster, the highest dose treatments (1.0 and 2.0 mg/kg) did not separate well. In the second cluster, the three groups were separated and clear separation was observed for the treated vs. controls. These results indicated treatment effects were detectable between the treatment groups. Furthermore, the effects were different between the low dose and high-dose groups with the low dose (0.125 and 0.25 g/kg) treatment groups showing a certain degree of similarity to the controls.

Interestingly, our findings from the clustering data demonstrate concordance with histopathological data in which hepatocellular hypertrophy was seen in the male mice administered high doses of kava (0.5, 1.0, and 2.0 g/kg), but not in the mice belonging to the low-dose groups (data not shown).

To assess the overall quality and reproducibility of the DNA microarray data, Pearson's correlation coefficient of pair-wise $\log_2$ intensity correlation was calculated. Pearson's correlation matrix of 35 arrays was calculated based on all data points with no filtering applied. As shown in Table 1, the median rank of correlation was 0.990 across 35 arrays with the range of 0.970–0.996.

The reproducibility was also assessed for the technical replicates. For visualization purposes, the raw $\log_2$ intensity data of all 45,281 probes from the technical replicates were plotted against each other. As shown in the scatter plot (Fig. 2), for most spots the intensity values from the replicate microarrays were accumulated along the diagonal axis with a correlation >0.995, indicating that the data from the technical microarrays were highly reproducible.

The $\log_2$ transformed intensity of any two gene expression profiles was plotted and compared as shown in Fig. 3. When the intensity of the entire probe set obtained from control sample E2 was plotted against the control sample E3, most of data points were gathered along the diagonal axis of the scatter plot with a correlation coefficient value of 0.992, demonstrating good repeatability of the two biological replicates (Fig. 3A). In contrast, the comparison between the control sample E2 and 2.0 g/kg kava-treated sample E54, exhibited many data points were scattered, indicating a large number of genes were altered in response to the treatment with the high dose of kava (Fig. 3B).

3.3. Analysis of differentially expressed genes

3.3.1. Total differentially expressed genes—A differentially expressed gene was identified based on the criteria of a fold-change greater than 1.5 (up or down) and a *P*-value less than 0.05 in comparison to the control group. Based on these two criteria, we identified 349, 353, 880, 1339, and 1674 significantly up or down-regulated genes in the livers exposed to 0.125, 0.25, 0.5, 1.0, and 2.0 g/kg kava, respectively. These results demonstrate a dose–response relationship for the number of genes affected. The commonly regulated genes within various treatments were identified as displayed in the Venn diagrams (Fig. 4) and the majority of the genes altered in the low-dose treatment group were also found in the high-dose treatment groups. For instance, out of 349 genes altered in the 0.125 g/kg kava treatment, 280 genes (80%) overlapped with either the 0.25 or 0.5 g/kg kava-treatment group. About 90% (796 out of 880) of the altered genes identified in the 0.5 g/kg-treatment group were found either in the 1.0 or 2.0 g/kg-groups. Since the largest numbers of differentially expressed genes were detected in the 2.0 g/kg treatment, we focused further analyses, including gene functions and pathways, primarily on these 1674 genes.

Among the 1674 genes altered in response to the 2.0 g/kg kava treatment, 1162 were up-regulated and 512 genes were down-regulated, as displayed in a “Volcano” plot (Fig. 5). Upon further examination, we found that the up-regulated genes with the most prominent fold changes were drug metabolizing genes. It is worth mentioning that gene Gsta1, which was repeatedly identified by multiple probes on the microarray, exhibited the same trend of change in terms of fold induction. This could further server as microarray quality control purpose.

3.3.2. Total differentially expressed genes of drug metabolizing enzymes—Since many therapeutic drugs and herbal products, such as kava, under enzymatic metabolism, it is expected that simultaneous use of kava and therapeutic drugs could potentially raise herb–drug interactions, causing hepatotoxicity (Fu et al., 2008a). We investigated the gene expression changes of drug metabolizing enzymes for 2.0 g/kg kava treatment in detail. We identified changes in 115 drug metabolizing genes; of these genes 20 were duplicated on the microarray chip leaving a total of 95 effected genes. Table 2 shows the 95 drug metabolizing genes with unique Gene Bank IDs whose expression was significantly changed by the 2.0 g/kg kava treatment. As tabulated in Table 2, among the 95 drug metabolizing enzyme associated genes, 28 genes were associated with Phase I metabolizing enzymes; 29 genes with Phase II metabolizing enzymes; and 38 genes with transporters (Phase III).

As shown in Table 2, a dramatic change in terms of fold induction was detected in two Gata (glutathione S-transferase) genes (Gata1 and Gata2), as well as Cyp2a5, 2b20 and 2c55. We investigated the dose dependency of these genes. As demonstrated in Fig. 6, the expression of these 5 genes (Gsta1, Gsta2, Cyp2b20, Cyp2c55, and Cyp2a5) increased in response to increasing dose of Kava. For instance, 1.8-, 3.0-, 11-, 29- and 50-fold induction for gene Gsta1 was seen for the 0.125, 0.25, 0.5, 1.0, and 2.0 g/kg treatment, respectively.

3.4. Real-time PCR validation

Quantitative, real-time PCR has been developed to specifically measure template numbers and it is considered the “gold standard” for the measurement of gene expression. In this study, we used TaqMan assays to confirm the results of the gene expression changes measured with microarrays. As shown in Fig. 7, based on triplicate measurements for each RNA sample, the Cyp1a1, Cyp3a11, Gata2 and Nqo1 genes were significantly up-regulated when compared to control ($P > 0.05$). It should be noted that the real-time PCR data correlated well with the microarray results except for the gene Cyp1a1. The expression of

Cyp1a1 exhibited a 12.6-fold increase as measured with TaqMan while there was no change as measured with Illumina's microarray platform. We further explored the expression of Cyp1a1 by obtaining data using a different microarray platform, Phalanx Mouse OneArray (Phalanx Biotech Group, Inc., HsinChu, Taiwan), and found that Cyp1a1 was 5.1-fold increased with $P = 0.0001$ (data not shown). The reason for the discrepancy between the platforms was not clear, but one possible explanation could be that the probes are designed for different regions and measure different transcript variants. Nevertheless, the observed pattern of changes was constant with the two different techniques, although the real-time PCR seemed more sensitive than microarray.

3.5. Pathway analysis of the differentially expressed genes

Ingenuity Pathway Analysis (IPA) software was used to determine the most relevant biological pathways of the affected genes. Out of the 1674 differentially expressed genes that were identified from the 2.0 g/kg kava treatments, 1470 were mapped to the IPA database and were used for canonical pathways analysis. This analysis identified the pathways from the IPA library of canonical pathways that were most significantly altered in the data set. Differentially expressed genes that were associated with a canonical pathway in the IPA database were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: (i) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the pathway and (ii) Fischer's exact test was used to calculate a *P*-value determining the probability that the association between the genes in the data-set and the canonical pathway is explained by chance alone.

Forty-two pathways were found to be significantly altered with a *P*-value <0.05. The top 20 significant pathways are listed in Table 3. The entire pathway list and associated genes can be found in the Supplemental Table 1. Expectedly, as listed in Table 3, the top two pathways are xenobiotic metabolism related, namely "Metabolism of Xenobiotics by Cytochrome P450" and "Xenobiotic Metabolism Signaling", with *P*-values of 6.31E–15 and 1.58E–13, respectively. Very interestingly, "NRF2 (nuclear factor erythroid-related factor 2)-mediated Oxidative Stress Response" was identified with a significance of $P = 2.75E–7$ and a large number of genes (28 genes) were found to be linked to this pathway (Supplemental Table 1). In the pathway regulated by NRF2, most of the genes were up-regulated. The up-regulated genes include genes coding for detoxifying enzymes such as; Nqo1, Gstms, Gstas and Gstp1 (Table 2). Nqo1 exhibited a 4-fold induction as confirmed by real-time PCR (Fig. 7).

4. Discussion

The potential hepatotoxicity associated with kava-containing dietary supplement products in humans has long been reported (CFSAN, 2002). The US NTP has started determining the possible toxicity and tumorigenicity of kava extract, the subject of our study. Although the mechanisms by which kava induces hepatotoxicity are not clearly understood, it has been proposed that the kava-induced hepatotoxicity is via by the induction of herb–drug interactions through modulation of metabolizing enzymes which could effect drug metabolism (Fu, 2007a).

In this study, we analyzed the whole gene expression changes in the livers of male B6C3F1 mice orally-treated daily with five different doses of kava extract for 14 weeks. The high quality and reproducibility of the microarray data were obtained. The results of the gene expression changes measured by microarrays were also validated by real-time PCR (Fig. 7).

In the high dose (2.0 g/kg) treatment group, there were 95 drug metabolizing enzyme associated genes significantly altered including 28 Phase I metabolizing enzymes genes; 29

Phase II genes; and 38 transporters (Table 2). Based on the results of microarray, Taq-Man assay, and Phalanx Mouse OneArray, the significant detected gene expression changes of note included Gstal, Gsta2, Cyp1a1, Cyp1a2, Cyp2a5, Cyp2b20, Cyp2c55, and Cyp3a11. The increased expression of Cyp1a1 upon the exposure of kava is in concordance with our previous finding (Guo et al., 2009) and the report by Yamazaki et al. (2008) with rat liver tissue.

It is well recognized that the Phase I enzymes (CYP superfamily) are the most important metabolizing enzymes in the metabolism of drugs and the metabolic activation of toxic and carcinogenic xenobiotics. The CYP1 superfamily is important in metabolism of xenobiotics while CYP2 and CYP3 families are important in the metabolism of drugs and other substances (Gonzalez and Gelboin, 1994; Gonzalez and Yu, 2006). The Phase II enzymes can eliminate drugs and carcinogenic metabolites of xenobiotics by forming water soluble conjugated metabolites. The balance between the Phase I and Phase II enzyme components determines the metabolic fate of endogenous and exogenous chemicals. Consequently, co-administration of herbal dietary supplements, such as kava extract, and therapeutic drugs very likely raise the potential for herb–drug interactions, which may lead to serious clinical as well as toxicological consequences (Bressler, 2005; Gurley et al,. 2005a, 2007; Hu et al., 2005; Mathews et al., 2002; Schulze et al., 2003; Singh, 2005). Indeed, our results demonstrated many drug metabolizing genes were altered in response to kava treatment; this is consistent with the report that kava-induced herb–drug interaction through modulation of metabolizing enzymes is a highly possible cause of hepatotoxicity (Fu, 2007a).

In vivo and *in vitro* studies have been conducted to study inductive/inhibitory effects on drug metabolizing enzymes by kava in humans and rodents (Clayton et al., 2007; Guo et al., 2009; Gurley et al., 2005b; Mathews et al., 1988, 2002; Raucy, 2003; Russmann et al., 2005; Strahl et al., 1998). Unfortunately, the results obtained with *in vitro* or *in vivo* studies investigating the effects on drug metabolizing enzymes are inconsistent or contradictory. It is necessary to investigate systematically the modulation of drug metabolizing enzymes in order to better understand the underlying mechanism of food–drug interactions. Microarray technology is a useful tool to rapidly detect the induction/inhibition of drug metabolizing enzymes after toxicant treatment and it has greatly contributed to the understanding of drug metabolizing enzyme function and expression (Blomme et al., 2009; Rezen et al., 2007). Although levels of gene expression do not fully represent the levels of enzyme activities, investigation at the gene expression level reveals that there is a high degree of correlation for Phase 1 enzymes between the fold inductions of the enzymatic activity and mRNA expression in liver samples (Iyer and Sinz, 1999; Roymans et al., 2004). We took the advantages of microarray that enable simultaneous measurement of thousands of genes and systematically investigated the modulations of drug metabolizing genes. Our study provided substantial evidence that kava causes remarkable changes in drug metabolism enzymes; raising the possibility that kava might profoundly affect the pharmacokinetics of many co-administrated drugs or other food supplements, thus leading to hepatotoxicity.

Histopathology results from the NTP 14-week mouse studies showed minimal to moderate hepatocellular centrilobular hypertrophy (increased severity with increasing dose) in the male mice administered 0.5 g/kg and higher dosages of kava extract. At the present time it is not known which gene expression changes are responsible for these pathological effects. It is plausible that part of these drug metabolizing enzyme gene expression changes results in hepatocellular centrilobular hypertrophy. Other than hypertrophy, no severe liver toxicities were observed. As discussed previously (Guo et al., 2009), there are three possible explanations to interpret this absence of hepatotoxicity: (i) kava extract does not induce hepatotoxicity in mice, i.e. the observed metabolizing gene expression does not contribute to kava-induced hepatotoxicity; (ii) kava-induced hepatotoxic effects requires a latency period

longer than 14 weeks for histopathological changes to be observed; and (iii) kava-induced hepatotoxicity is through an idiosyncratic mechanism.

Even though there was no clear clinical sign of kava-induced liver toxicity, the signatures of gene expression would be a powerful tool for predictive toxicity. In this study, kava exposure resulted in the significant stimulation of the Nrf2-mediated oxidative pathway (Supplemental Table 1). Keap1-Nrf2-ARE signaling plays a critical role in protecting cells from endogenous and exogenous stresses, and is involved in antioxidative response, detoxification of xenobiotics, and proteome maintenance. Under normal physiological conditions, the transcription factor Nrf2 localizes in the cytoplasm and interacts with Keap1. Upon oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus and subsequently activates its various downstream target genes (Kensler et al., 2007). The target genes show a wide spectrum of functions including inactivating oxidants, increasing the levels of glutathione, and enhancing toxin export via transporters to enhance cell survival. Nrf2-knock out mice showed higher sensitivity to chemical toxicity. Studies using Nrf2-knock out mice showed that they are more susceptible to acetaminophen-induced hepatocellular injury (Chan et al., 2001) and benzo[a]pyrene-induced tumor formation exhibiting higher levels of DNA adducts (Ramos-Gomez et al., 2001). This susceptibility is partly due to a reduced level in the expression of detoxification enzymes (Aleksunes and Manautou, 2007; Kensler et al., 2007). Activation of detoxification enzymes plays a pivotal role in protecting cells from oxidative insult when cells encounter toxin challenge.

In our study, the expressions of a group of genes involved in detoxification process (Nqo1, Gstms, Gstas and Gstps) were elevated. The most prominent changes were observed in the 2.0 g/kg treatment group, with Gsta1, Gsta2 and Nqo1 increased by about 50-, 24- and 4-fold, respectively. These altered genes are the well-known target genes controlled by the transcription factor Nrf2. We therefore speculate that the enhanced expressions of these genes were caused by Nrf2 activation. Indeed, our IPA analysis identified Nrf2 as a significantly changed pathway. However, the mechanisms for Nrf2 activation after Kava treatment remain elusive. It has been demonstrated that multiple mechanisms are involved in Nrf2 activation, among which, Keap1-dependent pathway and kinase (MAPKs, PKC and PI3K) signal pathway are the best characterized (Shen et al., 2004). While the former pathway is usually oxidative stress (OS)-driven, the latter could be either OS-dependent or OS-independent. As to Kava effects, a recent study showed that Kava ingredients activated Nrf2 by mechanisms of OS-independent MAPKs, specifically ERK1/2 kinase signal pathway in the neural cells (Wruck et al., 2008). Whether this observation could be extended to liver or hepatocytes is worth further investigation. Of note, our study appear supportive of this mechanism, as we indeed observed perturbations in the ERK/MAPK signaling pathway by IPA analysis, though it was not statically significant ($P > 0.05$, Supplementary Table).

Most recent studies (Reisman et al. 2009a,b) using Nrf2-knock out and/or Keap1-knock down mice showed that activation of Nrf2 increased biliary excretion of sulfobromophthalein (BSP) and enhanced elimination of the metabolite of acetaminophen (AA-glucuronide). These indicate that activation of Nrf2 pathway can potentially alter the pharmacokinetics of different xenobiotics, causing enhanced elimination or altered biotransformation of drugs. Further study on kava–drug interaction should be focused on activation of Nrf2 pathway.

To date, it is still a challenge to determine the mechanisms of toxicity induced by a mixture of many chemical components such as kava and other herbal plant extracts. Under this circumstance, microarray followed by real-time PCR should be a highly practical initial approach for revealing the whole spectrum of modifying gene expression by a chemical mixture (e.g., kava). After obtaining this information at the gene level, several conventional

methodologies, including determining protein expression by immunoblotting and enzyme activity determination by quantitative metabolism, can be followed. Without obtaining the whole spectrum of gene expression change, some important pathways for study will most likely be missed. Consequently, our gene expression microarray study in kava-treated mice, the present study, and rats, the previous study, represents a good example of this advantage, e.g., measuring the effect of kava on the whole spectrum of liver genes, including all the genes of drug metabolizing enzymes at the same time.

Acknowledgments

We thank Drs. Frederick A. Beland, Donna Mendrick, James C. Fuscoe, Tucker Patterson, Rick Irwin and Scott Auerbach for their critical review of this manuscript. Dr. Qiang Shi is supported by the Research Participation Program at the NCTR administrated by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

References

Aleksunes LM, Manautou JE. Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease. Toxicol Pathol 2007;35(4):459–473. [PubMed: 17562481]

Blomme EA, Yang Y, Waring JF. Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. Toxicol Lett 2009;186(1):22–31. [PubMed: 18996174]

Bressler R. Herb–drug interactions: interactions between kava and prescription medications. Geriatrics 2005;60(9):24–25. [PubMed: 16153141]

CDC. Hepatic Toxicity Possibly Associated with Kava-Containing Products –United States, Germany, and Switzerland, 1999–2002. Morbidity and Mortality Weekly Reports (MMWR) 2002;(51):1065–1067.

CFSAN. US Department of Health and Human Services, Food and Drug Administration; Rockville, Maryland: 2002 Mar 25. 2002 Center for Food Safety and Applied Nutrition (CFSAN): Kava-containing Dietary Supplements may be Associated with Severe Liver Injury. <http://www.cfsan.fda.gov/~dms/ds-warn.html>

Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci USA 2001;98(8):4611–4616. [PubMed: 11287661]

Chan PC, Fu PP. Toxicity of Panax ginseng – an herbal medicine and dietary supplements. J Food Drug Anal 2007a;15:416–427.

Chan PC, Fu PP. Toxicity of Panax ginseng – an herbal medicine and dietary supplements. J Food Drug Anal 2007b;(15):416–427.

Chan PC, Xia Q, Fu PP. *Ginkgo biloba* leave extract: biological, medicinal, and toxicological effects. J Environ Sci Health C Environ Carcinogen Ecotoxicol Rev 2007;25(3):211–244.

Clayton NP, Yoshizawa K, Kissling GE, Burka LT, Chan PC, Nyska A. Immunohistochemical analysis of expressions of hepatic cytochrome P450 in F344 rats following oral treatment with kava extract. Exp Toxicol Pathol 2007;58(4):223–236. [PubMed: 17059882]

FDA. FDA Advises Dietary Supplement Manufacturers to Remove Comfrey Products from the Market. 2001 [July 6, 2001]. <http://www.cfsan.fda.gov/~dms/supplmnt.html>

FDA. FDA Public Health News, FDA Announced Major Initiatives for Dietary Supplements. 2004a November 5;

FDA. Final Rule Declaring Dietary Supplements Containing Ephedrine Alkaloids Adulterated because they Present an Unreasonable Risk Federal Register. 2004b February 11;

Fong HH. Integration of herbal medicine into modern medical practices: issues and prospects. Integr Cancer Ther 2002;1(3):287–293. discussion 293. [PubMed: 14667286]

Fu PP. Quality assurance and safety of herbal dietary supplements. J Food Drug Anal 2007a;15:400–415.

Fu PP. Quality assurance and safety of herbal dietary supplements. J Food Drug Anal 2007b;(15):333–334.

Fu PP, Xia Q, Chou MW, Lin G. Detection, hepatotoxicity, and tumorigenicity of pyrrolizidine alkaloids in Chinese herbal plants and herbal dietary supplements. J Food Drug Anal 2007;(15): 400–415.

Fu PP, Xia Q, Guo L, Yu H, Chan PC. Toxicity of kava kava. J Environ Sci Health C Environ Carcinogen Ecotoxicol Rev 2008a;26(1):89–112.

Fu PP, Xia Q, Guo L, Yu H, Chan PC. Toxicity of kava kava. Environ Carcinogen Ecotocicol Rev 2008b;(26):1–24.

Fu PP, Yang YC, Xia Q, Chou MW, Cui YY, Lin G. Pyrrolizidine alkaloids – tumorigenic components in Chinese herbal medicines and dietary supplements. J Food Drug Anal 2002;(10): 198–211.

Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994;26(1–2):165–183. [PubMed: 8082563]

Gonzalez FJ, Yu AM. Cytochrome P450 and xenobiotic receptor humanized mice. Annu Rev Pharmacol Toxicol 2006;46:41–64. [PubMed: 16402898]

Guo L, Li Q, Xia Q, Dial S, Chan PC, Fu P. Analysis of gene expression changes of drug metabolizing enzymes in the livers of F344 rats following oral treatment with kava extract. Food Chem Toxicol 2009;47(2):433–442. [PubMed: 19100306]

Gurley BJ, Gardner SF, Hubbard MA, Williams DK, Gentry WB, Cui Y, Ang CY. Clinical assessment of effects of botanical supplementation on cytochrome P450 phenotypes in the elderly: St. John's wort, garlic oil, *Panax ginseng* and *Ginkgo biloba*. Drugs Aging 2005a;22(6):525–539. [PubMed: 15974642]

Gurley BJ, Gardner SF, Hubbard MA, Williams DK, Gentry WB, Khan IA, Shah A. In vivo effects of goldenseal, kava kava, black cohosh, and valerian on human cytochrome P450 1A2, 2D6, 2E1, and 3A4/5 phenotypes. Clin Pharmacol Ther 2005b;77(5):415–426. [PubMed: 15900287]

Gurley BJ, Swain A, Barone GW, Williams DK, Breen P, Yates CR, Stuart LB, Hubbard MA, Tong Y, Cheboyina S. Effect of goldenseal (*Hydrastis canadensis*) and kava kava (*Piper methysticum*) supplementation on digoxin pharmacokinetics in humans. Drug Metab Dispos 2007;35(2):240–245. [PubMed: 17079360]

Hu Z, Yang X, Ho PC, Chan SY, Heng PW, Chan E, Duan W, Koh HL, Zhou S. Herb–drug interactions: a literature review. Drugs 2005;65(9):1239–1282. [PubMed: 15916450]

Iyer KR, Sinz MW. Characterization of Phase I and Phase II hepatic drug metabolism activities in a panel of human liver preparations. Chem Biol Interact 1999;118(2):151–169. [PubMed: 10359459]

Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007;47:89–116. [PubMed: 16968214]

Mathews JD, Riley MD, Fejo L, Munoz E, Milns NR, Gardner ID, Powers JR, Ganygulpa E, Gununuwawuy BJ. Effects of the heavy usage of kava on physical health: summary of a pilot survey in an aboriginal community. Med J Aust 1988;148(11):548–555. [PubMed: 3374423]

Mathews JM, Etheridge AS, Black SR. Inhibition of human cytochrome P450 activities by kava extract and kavalactones. Drug Metab Dispos 2002;30(11):1153–1157. [PubMed: 12386118]

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in Nrf2 transcription factor-deficient mice. Proc Natl Acad Sci USA 2001;98(6):3410–3415. [PubMed: 11248092]

Raucy JL. Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. Drug Metab Dispos 2003;31(5):533–539. [PubMed: 12695340]

Reisman SA, Csanaky IL, Aleksunes LM, Klaassen CD. Altered disposition of acetaminophen in Nrf2-null and Keap1-knockdown mice. Toxicol Sci 2009a;109(1):31–40. [PubMed: 19246624]

Reisman SA, Csanaky IL, Yeager RL, Klaassen CD. Nrf2 activation enhances biliary excretion of sulfobromophthalein by inducing glutathione-S-transferase activity. Toxicol Sci 2009b;109(1):24–30. [PubMed: 19246623]

Rezen T, Contreras JA, Rozman D. Functional genomics approaches to studies of the cytochrome p450 superfamily. Drug Metab Rev 2007;39(2–3):389–399. [PubMed: 17786628]

Roymans D, Van Looveren C, Leone A, Parker JB, McMillian M, Johnson MD, Koganti A, Gilissen R, Silber P, Mannens G, et al. Determination of cytochrome P450 1A2 and cytochrome P450 3A4 induction in cryopreserved human hepatocytes. Biochem Pharmacol 2004;67(3):427–437. [PubMed: 15037195]

Russmann S, Lauterburg BH, Barguil Y, Choblet E, Cabalion P, Rentsch K, Wenk M. Traditional aqueous kava extracts inhibit cytochrome P450 1A2 in humans: protective effect against environmental carcinogens? Clin Pharmacol Ther 2005;77(5):453–454. [PubMed: 15900292]

Schulze J, Raasch W, Siegers CP. Toxicity of kava pyrones, drug safety and precautions – a case study. Phytomedicine 2003;10(Suppl. l4):68–73. [PubMed: 12807347]

Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, Keum YS, Han J, Gallo MA, Kong AN. Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J Biol Chem 2004;279(22):23052–23060. [PubMed: 15020583]

Singh YN. Potential for interaction of kava and St. John's Wort with drugs. J Ethnopharmacol 2005;100(1–2):108–113. [PubMed: 16005588]

Strahl S, Ehret V, Dahm HH, Maier KP. Necrotizing hepatitis after taking herbal remedies. Dtsch Med Wochenschr 1998;123(47):1410–1414. [PubMed: 9856112]

Wruck CJ, Gotz ME, Herdegen T, Varoga D, Brandenburg LO, Pufe T. Kavalactones protect neural cells against amyloid beta peptide-induced neurotoxicity via extracellular signal-regulated kinase 1/2-dependent nuclear factor erythroid 2-related factor 2 activation. Mol Pharmacol 2008;73(6): 1785–1795. [PubMed: 18334601]

Yamazaki Y, Hashida H, Arita A, Hamaguchi K, Shimura F. High dose of commercial products of kava (*Piper methysticum*) markedly enhanced hepatic cytochrome P450 1A1 mRNA expression with liver enlargement in rats. Food Chem Toxicol 2008;46(12):3732–3738. [PubMed: 18930106]

Zurer P, Hanson D. Chemistry puts herbal supplements to the test. Chem Eng News 2004;82(14):16.

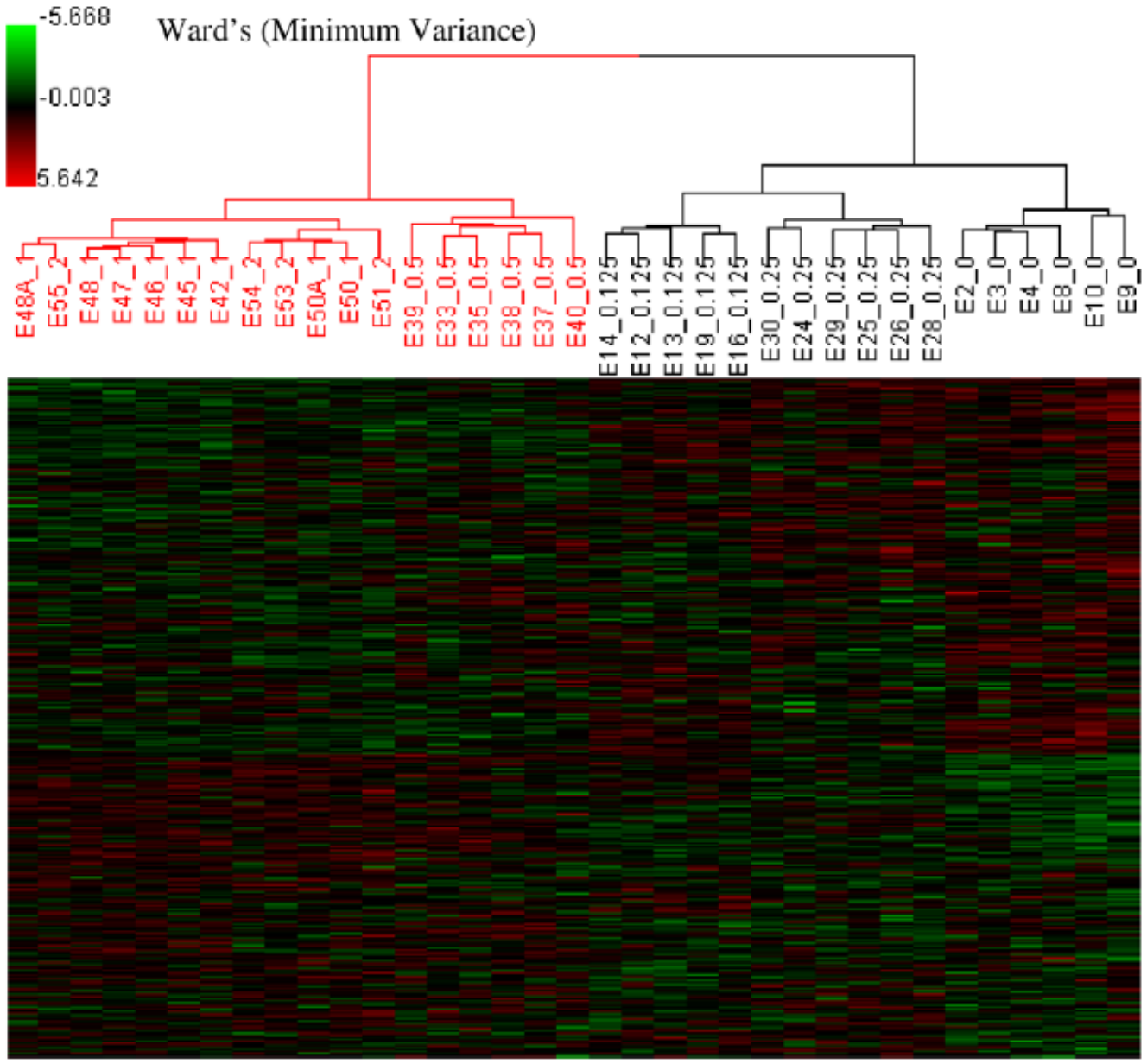


Fig. 1.

Each column represents the results from an individual hybridization. Each row represents the $\log_2$ intensity values of the 35 samples for one particular gene. Samples are labeled according to the convention of Animal ID_Dose (g/kg/day). E48A and E50A are the technique replicates for E48 and E50.

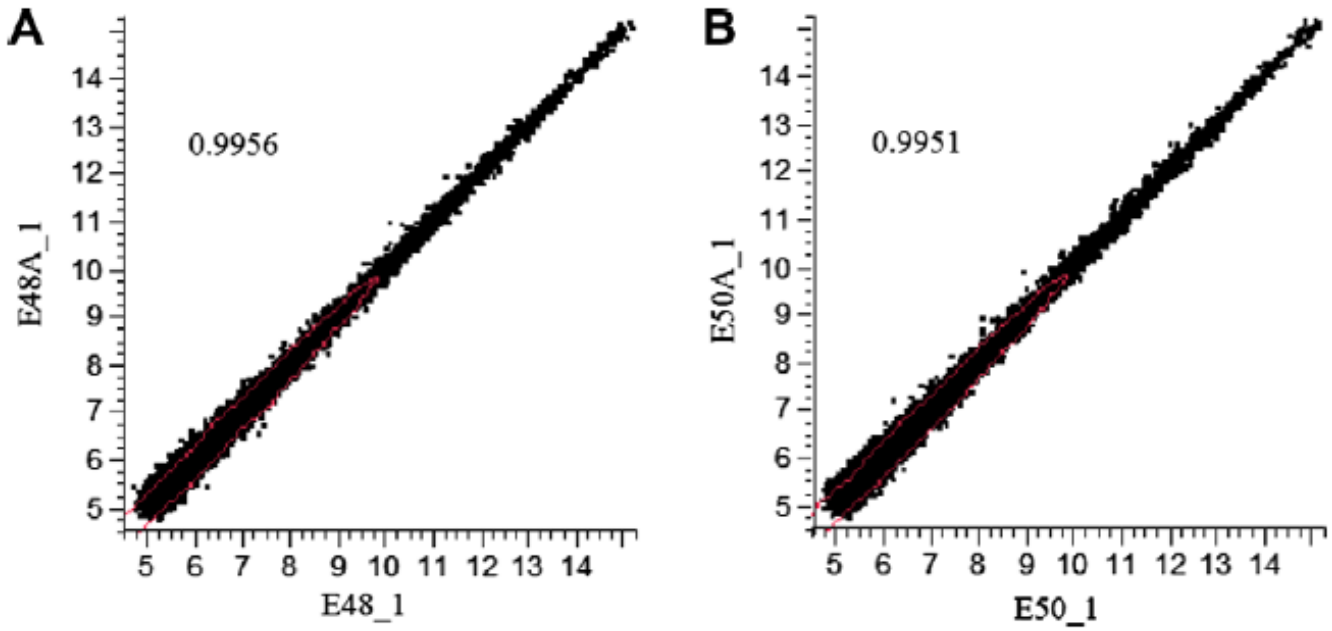


Fig. 2.
The $\log_2$ raw intensity data of the two technical replicates (E48/E48A and E50/E50A) were plotted against each other.

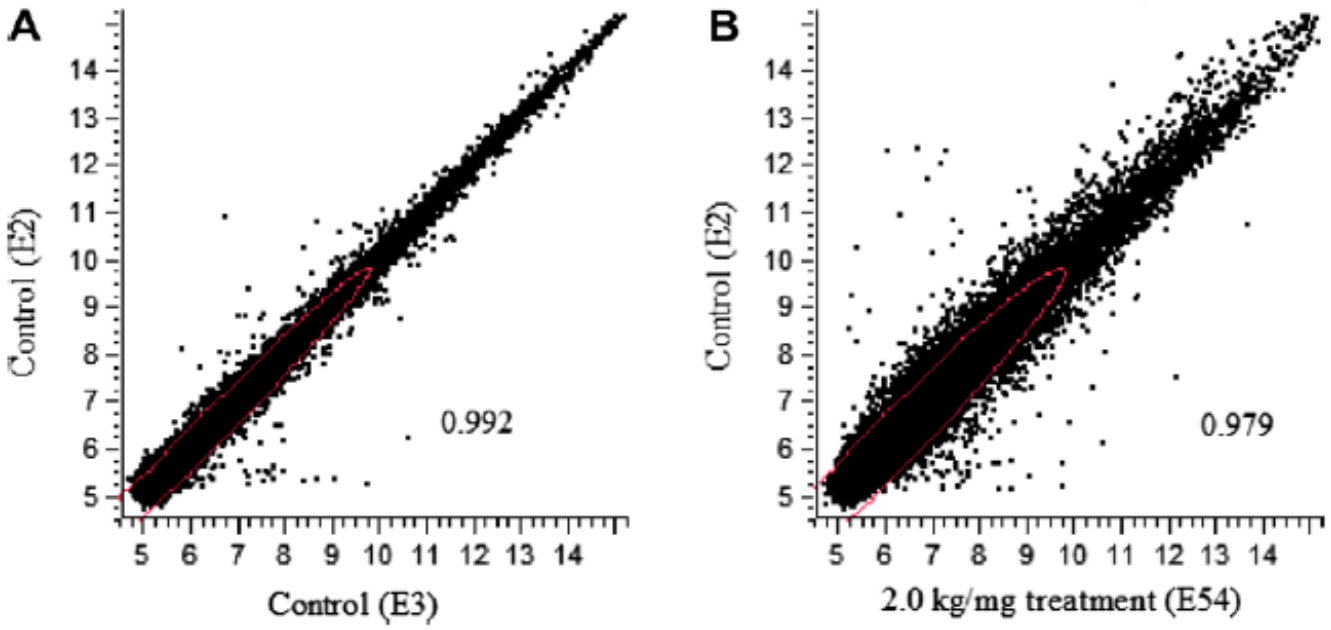


Fig. 3.

Overall gene expression profiles. The $\log_2$ transformed intensity was used for scatter plotting. (A) two control samples (E2 & E3) and (B) control (E2) and 2.0 g/kg kava-treated samples (E54) were plotted against each other.

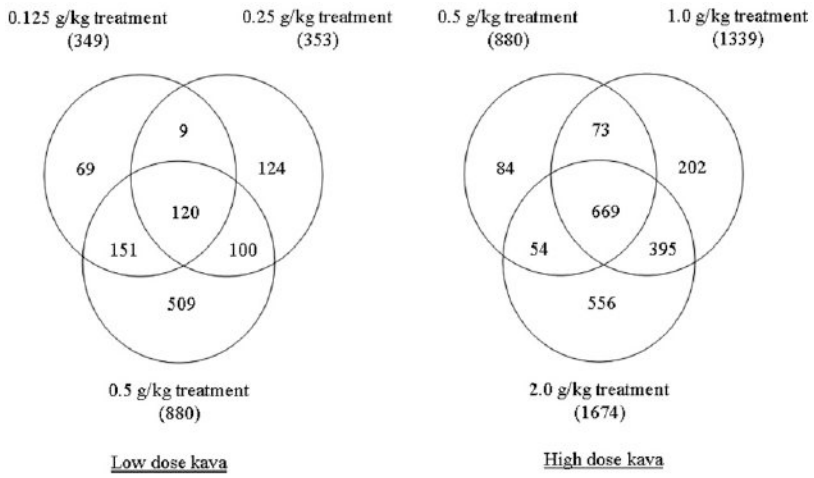
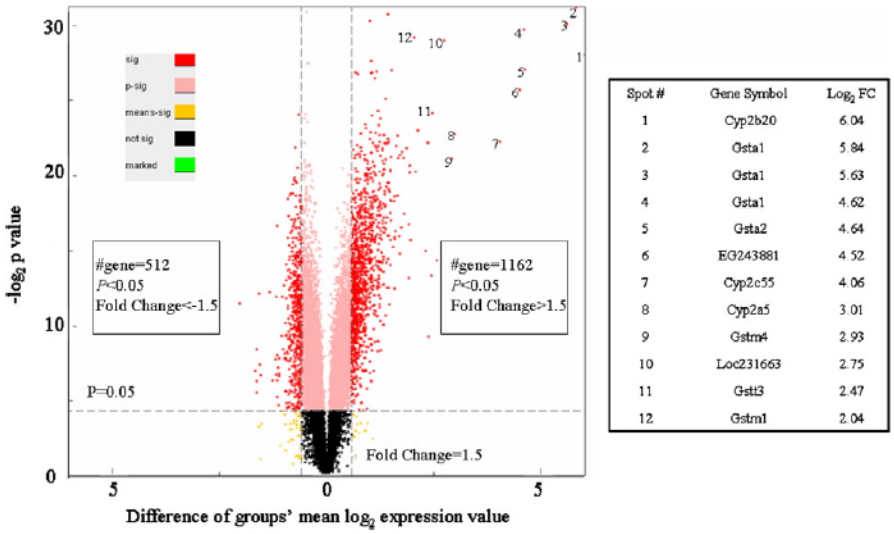


Fig. 4.

Venn diagrams show the numbers of differentially expressed genes regulated by 0.125, 0.25, 0.5, 1.0 and 2.0 g/kg kava treatments and the overlaps of these genes. A gene was identified as differentially expressed if the fold-change was greater than 1.5 (up- or down-regulated) and the *P*-value was less than 0.05 in comparison to the control group.



Spot #	Gene Symbol	Log_2 FC
1	Cyp2b20	6.04
2	Gsta1	5.84
3	Gsta1	5.63
4	Gsta1	4.62
5	Gsta2	4.64
6	EG243881	4.52
7	Cyp2c55	4.06
8	Cyp2a5	3.01
9	Gstm4	2.93
10	Loc231663	2.75
11	Gstt3	2.47
12	Gstm1	2.04

Fig. 5.

Significant gene expression changes in the mice livers exposed to 2.0 g/kg kava treatment. The relationship between *P*-value (*Y* axis, $-\log_2$ *P*-value) and mean expression ration of the changes (*X* axis, $\log_2$ expression ration) in the treatment group compared to control group is displayed in "Volcano" plot. 1162 genes are unregulated and 512 genes are downregualted. 12 genes with most prominent fold changes are labeled with numbers and their names and fold changes are listed in the enclosed box.

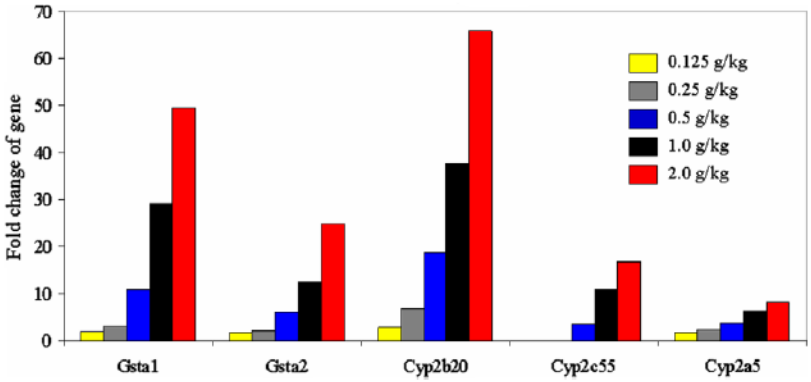


Fig. 6.

Dose-dependent induction of gene expression by kava treatments: microarray study showed the increased gene expression of Gsta1, Gsta2, Cyp2b20, Cyp2c55 and Cyp2a5 were dose-dependent.

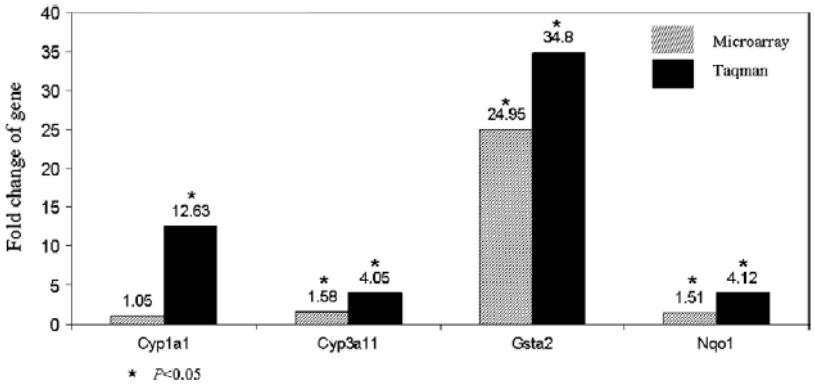


Fig. 7.

Real-time PCR validation for selected genes: TaqMan assays were used to verify the results of gene expression changes measured using microarray. RNAs from three control animals and three 2.0 g/kg kava-treated animals were employed for TaqMan assay and the assay was run in triplicate for each RNA sample

Table 1

Assessment of microarray data quality the microarray data reproducibility was assessed by the Pearson's correlation coefficient of pair-wise $\log_2$ intensity correlation. The median rank of correlation is 0.990 across all 35 arrays with the range of 0.970–0.996. Pearson's correlation matrix of 35 arrays was calculated based on all data points (45,281 probes).

	E2	E3	E4	E8	E9	E10	E12	E13	E14	E16	E19	E24	E25	E26	E28	E29	E30	E33	E35	E37	E38	E39	E40	E42	E45	E46	E47	E48	E48A	E50	E50A	E51	E53	E54	E55
E2_0	1.000	0.992	0.990	0.988	0.986	0.987	0.988	0.986	0.988	0.987	0.987	0.988	0.987	0.986	0.986	0.986	0.988	0.983	0.985	0.987	0.985	0.984	0.986	0.984	0.984	0.985	0.984	0.985	0.985	0.982	0.982	0.979	0.980	0.979	0.984
E3_0		1.000	0.991	0.991	0.988	0.988	0.989	0.987	0.990	0.989	0.988	0.990	0.989	0.988	0.988	0.987	0.989	0.982	0.984	0.987	0.985	0.984	0.987	0.984	0.984	0.985	0.985	0.985	0.985	0.982	0.982	0.978	0.981	0.979	0.984
E4_0			1.000	0.992	0.989	0.988	0.988	0.988	0.989	0.987	0.986	0.987	0.986	0.987	0.987	0.985	0.988	0.980	0.983	0.986	0.984	0.984	0.986	0.981	0.981	0.983	0.982	0.982	0.982	0.981	0.981	0.976	0.978	0.980	0.982
E8_0				1.000	0.986	0.988	0.988	0.989	0.989	0.986	0.985	0.986	0.985	0.986	0.985	0.984	0.987	0.979	0.981	0.985	0.982	0.984	0.985	0.981	0.981	0.982	0.982	0.981	0.982	0.980	0.980	0.975	0.978	0.979	0.982
E9_0					1.000	0.988	0.986	0.982	0.985	0.986	0.985	0.984	0.986	0.986	0.988	0.984	0.985	0.977	0.980	0.981	0.979	0.978	0.982	0.976	0.977	0.977	0.977	0.977	0.977	0.978	0.978	0.974	0.977	0.978	0.978
E10_0						1.000	0.985	0.982	0.985	0.983	0.982	0.983	0.983	0.982	0.984	0.980	0.984	0.973	0.975	0.980	0.978	0.977	0.981	0.974	0.975	0.975	0.976	0.976	0.976	0.975	0.975	0.970	0.972	0.972	0.976
E12_0.125							1.000	0.989	0.992	0.991	0.991	0.989	0.988	0.988	0.989	0.988	0.991	0.985	0.986	0.989	0.988	0.987	0.989	0.986	0.988	0.987	0.988	0.988	0.988	0.986	0.987	0.982	0.985	0.986	0.988
E13_0.125								1.000	0.991	0.990	0.990	0.987	0.986	0.987	0.985	0.988	0.987	0.985	0.986	0.986	0.984	0.988	0.984	0.986	0.986	0.986	0.986	0.986	0.987	0.986	0.986	0.981	0.983	0.984	0.986
E14_0.125									1.000	0.992	0.991	0.990	0.989	0.990	0.989	0.990	0.991	0.987	0.988	0.990	0.989	0.988	0.990	0.988	0.989	0.990	0.990	0.989	0.989	0.988	0.989	0.985	0.987	0.987	0.989
E16_0.125										1.000	0.994	0.989	0.991	0.990	0.990	0.991	0.990	0.989	0.990	0.989	0.988	0.987	0.988	0.987	0.989	0.988	0.989	0.989	0.989	0.989	0.990	0.985	0.988	0.988	0.988
E19_0.125											1.000	0.989	0.990	0.990	0.990	0.991	0.989	0.989	0.990	0.989	0.988	0.987	0.987	0.988	0.989	0.988	0.989	0.989	0.989	0.989	0.990	0.986	0.988	0.988	0.988
E24_0.25												1.000	0.991	0.989	0.990	0.990	0.992	0.988	0.988	0.989	0.988	0.988	0.989	0.988	0.989	0.988	0.989	0.989	0.989	0.988	0.988	0.984	0.986	0.984	0.988
E25_0.25													1.000	0.991	0.991	0.992	0.990	0.988	0.989	0.988	0.988	0.986	0.987	0.986	0.988	0.988	0.988	0.988	0.988	0.989	0.989	0.985	0.987	0.986	0.987
E26_0.25														1.000	0.991	0.991	0.990	0.986	0.988	0.987	0.986	0.987	0.987	0.984	0.986	0.986	0.987	0.986	0.986	0.987	0.987	0.984	0.987	0.986	0.986
E28_0.25															1.000	0.990	0.991	0.986	0.987	0.988	0.987	0.985	0.988	0.985	0.987	0.986	0.987	0.986	0.986	0.987	0.987	0.983	0.986	0.987	0.986
E29_0.25																1.000	0.990	0.990	0.991	0.988	0.987	0.987	0.986	0.987	0.989	0.988	0.989	0.988	0.988	0.989	0.989	0.986	0.989	0.987	0.988
E30_0.25																	1.000	0.986	0.987	0.990	0.988	0.988	0.989	0.987	0.989	0.988	0.989	0.989	0.989	0.987	0.987	0.984	0.986	0.985	0.988
E33_0.5																		1.000	0.994	0.990	0.990	0.989	0.987	0.991	0.991	0.992	0.992	0.992	0.992	0.992	0.992	0.990	0.991	0.990	0.991
E35_0.5																			1.000	0.991	0.991	0.989	0.988	0.991	0.991	0.992	0.992	0.992	0.991	0.992	0.992	0.990	0.990	0.990	0.990
E37_0.5																				1.000	0.993	0.990	0.991	0.990	0.991	0.992	0.992	0.992	0.991	0.990	0.990	0.987	0.989	0.988	0.991
E38_0.5																					1.000	0.989	0.991	0.990	0.990	0.992	0.992	0.991	0.991	0.990	0.990	0.987	0.989	0.988	0.991
E39_0.5																						1.000	0.987	0.989	0.990	0.990	0.990	0.991	0.990	0.990	0.989	0.986	0.987	0.987	0.989
E40_0.5																							1.000	0.989	0.989	0.990	0.990	0.989	0.990	0.988	0.988	0.985	0.987	0.986	0.990
E42_1																								1.000	0.993	0.994	0.994	0.994	0.993	0.992	0.992	0.990	0.991	0.989	0.993
E45_1																									1.000	0.994	0.995	0.995	0.994	0.993	0.992	0.991	0.992	0.990	0.993
E46_1																										1.000	0.995	0.995	0.994	0.992	0.992	0.991	0.992	0.990	0.994
E47_1																											1.000	0.995	0.994	0.993	0.993	0.991	0.993	0.991	0.994
E48_1																												1.000	0.996	0.993	0.992	0.991	0.992	0.990	0.994

	E2	E3	E4	E8	E9	E10	E12	E13	E14	E16	E19	E24	E25	E26	E28	E29	E30	E33	E35	E37	E38	E39	E40	E42	E45	E46	E47	E48	E48A	E50	E50A	E51	E53	E54	E55
E48A_1																													1.000	0.992	0.993	0.991	0.992	0.990	0.994
E50_1																														1.000	0.995	0.991	0.993	0.992	0.992
E50A_1																															1.000	0.991	0.993	0.993	0.993
E51_2																																1.000	0.992	0.990	0.991
E53_2																																	1.000	0.993	0.993
E54_2																																		1.000	0.992
E55_2																																			1.000

Table 2

Genes involved in three phases of drug metabolism altered by 2.0 g/kg kava treatment in mouse liver.

Gene symbol	Gene bank ID	Fold-change	*P*-value	Gene description
Phase I metabolism				
Aldh1a1	NM_013467	2.25	<0.0001	Aldehyde dehydrogenase family 1, sub-family A1
Aldh1a7	NM_011921	2.54	<0.0001	Aldehyde dehydrogenase family 1, sub-family A7
Aldh3b1	NM_026316	1.63	0.0002	Aldehyde dehydrogenase 3 family, member B1
Aldh6a1	NM_134042	1.60	0.0018	Aldehyde dehydrogenase family 6, sub-family A1
Aldh8a1	NM_178713	1.60	0.0014	Aldehyde dehydrogenase 8 family, member A1
Cyp1a2	NM_009993	1.77	<0.0001	Cytochrome P450, family 1, sub-family a, polypeptide 2
Cyp26a1	NM_007811	1.91	0.0187	Cytochrome P450, family 26, sub-family a, polypeptide 1
Cyp2a4	NM_009997	2.75	<0.0001	Cytochrome P450, family 2, sub-family a, polypeptide 4
Cyp2a5	NM_007812	8.05	<0.0001	Cytochrome P450, family 2, sub-family a, polypeptide 5
Cyp2b10	NM_009998	2.75	<0.0001	Cytochrome P450, family 2, sub-family b, polypeptide 10
Cyp2b20	NM_009999	65.73	<0.0001	Cytochrome P450, family 2, sub-family b, polypeptide 20
Cyp2c29	NM_007815	3.83	<0.0001	Cytochrome P450, family 2, sub-family c, polypeptide 29
Cyp2c37	NM_010001	2.84	<0.0001	Cytochrome P450, family 2. sub-family c, polypeptide 37
Cyp2c50	NM_134144	2.59	<0.0001	Cytochrome P450, family 2, sub-family c, polypeptide 50
Cyp2c54	NM_206537	3.87	<0.0001	Cytochrome P450, family 2, sub-family c, polypeptide 54
Cyp2c55	NM_028089	16.63	<0.0001	Cytochrome P450, family 2, sub-family c, polypeptide 55
Cyp2d26	NM_029562	−1.83[a]	0.0002	Cytochrome P450, family 2, sub-family d, polypeptide 26
Cyp2d9	NM_010006	1.60	<0.0001	Cytochrome P450, family 2, sub-family d, polypeptide 9
Cyp2u1	NM_027816	−1.57	0.0001	Cytochrome P450, family 2, sub-family u, polypeptide 1
Cyp3a11	NM_007818	1.58	0.0006	Cytochrome P450, family 3, sub-family a, polypeptide 11
Cyp3a25	NM_019792	1.71	<0.0001	Cytochrome P450, family 3, sub-family a, polypeptide 25
Cyp4a10	NM_010011	−2.92	0.01	Cytochrome P450, family 4, sub-family a, polypeptide 10
Cyp4a12	NM_172306	1.57	0.0037	Cytochrome P450, family 4, sub-family a, polypeptide 12B
Cyp4b1	NM_007823	−1.51	0.0008	Cytochrome P450, family 4, sub-family b, polypeptide 1
Cyp4v3	NM_133969	−1.60	<0.0001	Cytochrome P450, family 4, sub-family v, polypeptide 3
Dpyd	NM_170778	1.72	<0.0001	Dihydropyrimidine dehydrogenase
Esd	NM_016903	1.63	0.0003	Esterase D/formylglutathione hydrolase
Fmo5	NM_010232	2.14	0.0001	Flavin containing monooxygenase 5
Phase II metabolism				
Acsm3	NM_212442	−1.72	<0.0001	Acyl-CoA synthetase medium-chain family member 3
Ces1	NM_021456	2.36	<0.0001	Carboxylesterase 1
Ces3	NM_053200	1.50	0.0006	Carboxylesterase 3
Ephx1	NM_010145	1.66	<0.0001	Epoxide hydrolase 1, microsomal
Ephx2	NM_007940	1.81	0.0002	Epoxide hydrolase 2, cytoplasmic
Gnmt	AK082459	−3.08	0.0131	Glycine N-methyltransferase
Gsta1	NM_008181	49.47	<0.0001	Glutathione S-transferase, alpha 1 (Ya)
Gsta2	NM_008182	24.92	<0.0001	Glutathione S-transferase, alpha 2 (Yc2)
Gsta3	NM_010356	1.94	<0.0001	Glutathione S-transferase, alpha 3

Gene symbol	Gene bank ID	Fold-change	*P*-value	Gene description
Gsta4	NM_010357	1.90	0.0003	Glutathione S-transferase, alpha 4
Gstk1	NM_029555	1.97	<0.0001	Glutathione S-transferase kappa 1
Gstm1	NM_010358	4.12	<0.0001	Glutathione S-transferase, mu 1
Gstm2	NM_008183	3.65	<0.0001	Glutathione S-transferase, mu 2
Gstm3	NM_010359	2.86	<0.0001	Glutathione S-transferase, mu 3
Gstm4	NM_026764	7.64	<0.0001	Glutathione S-transferase, mu 4
Gstm6	NM_008184	1.98	0.0002	Glutathione S-transferase, mu 6
Gstp1	NM_013541	1.92	0.0001	Glutathione S-transferase, pi 1
Gstp2	NM_181796	5.23	0.0017	Glutathione S-transferase, pi 2
Gstt1	NM_008185	1.60	0.0002	Glutathione S-transferase, theta 1
Gstt2	NM_010361	2.37	0.0002	Glutathione S-transferase, theta 2
Gstt	NM_133994	5.15	<0.0001	Glutathione S-transferase, theta 3
Mgst1	NM_019946	1.70	<0.0001	Microsomal glutathione S-transferase 1
Mgst3	NM_025569	2.28	0.0002	Microsomal glutathione S-transferase 3
Nqo1	NM_008706	1.51	0.0009	NAD(P)H dehydrogenase, quinone 1
Sult1d1	NM_016771	1.57	0.0019	Sulfotransferase family 1D, member 1
Sult5a1	NM_020564	1.82	0.0083	Sulfotransferase family 5A, member 1
Tpmt	NM_016785	2.01	<0.0001	Thiopurine methyltransferase
Ugt1a12	NM_201644	3.07	<0.0001	UDP glucuronosyltransferase 1 family, polypeptide A9
Ugt1a9	NM_201410	−1.53	0.0033	UDP glucuronosyltransferase 1 family, polypeptide A6B
Phase III metabolism				
Abca6	NM_147218	1.88	<0.0001	ATP-binding cassette, sub-family A (ABC1), member 6
Abca7	NM_013850	−1.62	<0.0001	ATP-binding cassette, sub-family A (ABC1), member 7
Abcc2	NM_013806	1.97	0.0001	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
Abcc3	XM_358306	1.52	0.0008	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
Abcc4	XM_139262	2.27	<0.0001	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
Abcd1	NM_007435	1.94	<0.0001	ATP-binding cassette, sub-family D (ALD), member 1
Abcd3	NM_008991	2.40	<0.0001	ATP-binding cassette, sub-family D (ALD), member 3
Atp11b	XM_358349	1.51	0.0081	ATPase, class VI, type 11B
Atp11c	NM_001037863	−1.70	<0.0001	ATPase, class VI, type 11C
Atp13a1	NM_133224	2.02	<0.0001	ATPase type 13A1
Atp1b1	NM_009721	2.15	0.0012	ATPase, Na^+/K^+ transporting, beta 1 polypeptide
Atp2a2	NM_009722	−1.76	0.0031	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2
Atp5a1	NM_007505	1.70	0.0001	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1
Atp6ap2	NM_027439	−1.52	0.0001	ATPase, H^+ transporting, lysosomal accessory protein 2
Mvp	NM_080638	1.53	0.0001	Major vault protein
Slc12a6	NM_133649	−1.50	0.0004	Solute carrier family 12, member 6
Slc17a2	NM_144836	−1.72	<0.0001	Solute carrier family 17 (sodium phosphate), member 2
Slc1a2	NM_001077515	−1.66	<0.0001	Solute carrier family 1 (glial high affinity glutamate transporter), member 2
Slc20a2	NM_011394	1.72	0.0001	Solute carrier family 20, member 2
Slc25a11	NM_024211	1.75	<0.0001	Solute carrier family 25 (mitochondrial carrier oxoglutarate carrier), member 11

Gene symbol	Gene bank ID	Fold-change	*P*-value	Gene description
Slc25a17	NM_011399	1.73	<0.0001	Solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17
Slc25a38	NM_144793	−1.61	0.0041	Solute carrier family 25, member 38
Slc25a39	NM_026542	1.67	0.0004	Solute carrier family 25, member 39
Slc29a1	BC004828	−1.55	0.0208	Solute carrier family 29 (nucleoside transporters), member 1
Slc2a2	NM_031197	1.56	0.0086	Solute carrier family 2 (facilitated glucose transporter), member 2
Slc2a9	NM_145559	1.53	0.0005	Solute carrier family 2 (facilitated glucose transporter), member 9
Slc35b1	XM_128634	1.62	<0.0001	Solute carrier family 35, member B1
Slc35d1	NM_177732	1.87	<0.0001	Solute carrier family 35, member D1
Slc37a4	NM_008063	2.95	<0.0001	Solute carrier family 37 (glucose-6-phosphate transporter), member 4
Slc38a2	NM_175121	−1.95	0.0205	Solute carrier family 38, member 2
Slc38a4	NM_027052	2.28	<0.0001	Solute carrier family 38, member 4
Slc39a1	NM_013901	1.68	0.0001	Solute carrier family 39 (zinc transporter), member 1
Slc39a7	NM_008202	1.88	0.0001	Solute carrier family 39 (zinc transporter), member 7
Slc43a1	XM_130259	−1.87	0.0019	Solute carrier family 43, member 1
Slc6a9	NM_008135	1.90	<0.0001	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9
Slc7a2	NM_007514	−1.65	0.0001	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 2
Slco2a1	NM_033314	−1.66	0.0001	Solute carrier organic anion transporter family, member 2a1
Vdac2	NM_011695	1.7231	0.0001	Voltage-dependent anion channel 2

[a]The symbol of minus (−) indicates down-regulation.

Table 3

Top 20 canonical pathways altered by kava treatment.

	Canonical pathway	*P*-values
1	Metabolism of xenobiotics by cytochrome P450	6.31E–15
2	Xenobiotic metabolism signaling	1.58E–13
3	LPS/IL-1 mediated inhibition of RXR function	3.39E–09
4	Glutathione metabolism	3.63E–09
5	NRF2-mediated oxidative stress response	2.75E–07
6	Aryl hydrocarbon receptor signaling	5.13E–07
7	Tryptophan metabolism	7.41E–07
8	Fatty acid metabolism	1.41E–06
9	Mitochondrial dysfunction	1.00E–05
10	Acute phase response signaling	1.62E–05
11	PXR/RXR Activation	7.76E–05
12	Retinol metabolism	9.33E–05
13	Valine, leucine and isoleucine degradation	1.51E–04
14	Citrate cycle	1.70E–04
15	Pentose phosphate pathway	1.78E–04
16	Arachidonic acid metabolism	1.95E–04
17	Protein ubiquitination pathway	3.98E–04
18	β-Alanine metabolism	4.90E–04
19	Pyruvate metabolism	6.17E–04
20	Lysine degradation	8.13E–04