Biosci. Biotechnol. Biochem., **72** (12), 3075–3083, 2008

Safety Evaluation of the Aqueous Extract Kothala Himbutu (*Salacia reticulata*) Stem in the Hepatic Gene Expression Profile of Normal Mice Using DNA Microarrays

Ryanghyok IM, Hiroshi MANO, Sachie NAKATANI, Jun SHIMIZU, and Masahiro WADA†

Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

Received November 15, 2007; Accepted August 19, 2008; Online Publication, December 7, 2008
[doi:10.1271/bbb.70745]

Kothala himbutu is a traditional Ayurvedic medicinal plant used to treat diabetes. We aimed to evaluate the safety of an aqueous extract of Kothala himbutu stem (KTE) in normal mice. The mice were divided into two groups: one was administered KTE and the other distilled water for 3 weeks. During the test period, the groups showed no significant differences in body weight gain or plasma parameters, such as fasting blood glucose level, oral glucose tolerance test, or aspartate transaminase (AST) or alanine transaminase (ALT) activity. DNA microarray analysis revealed that expression of genes of known function, such as those for the stress response, ribosomal proteins, transcription, cell function, the inflammatory/immune response, and metabolism (xenobiotic, glutathione, *etc.*) remained largely unaffected by KTE. However some genes such as catechol-*o*-methyltransferase and succinyl-CoA synthetase were regulated by KTE, indicating that KTE is not toxic to normal mice and might be effective as a functional food.

Key words: *Salacia reticulata*; DNA microarray; food safety; C57BL/6J mice

Salacia reticulata (Hippocrateaceae), referred to as Kothala himbutu (KT) in Singhalese, is a woody climber native to Sri Lanka, where it is a well-known traditional medicinal plant. The aqueous extract of the roots and stems of this plant is extensively used in Ayurvedic medicine in India and Sri Lanka in treatment in the initial stages of diabetes.[1,2] It reduces fasting blood glucose levels in rats and mild type-2 diabetic patients.[3,4] Controlling the fasting blood glucose level is a critical step in the treatment of type-2 diabetes, but little is known about the mechanism behind this effect.

Previous studies in our laboratory using a diabetic model of KK-Ay mice have revealed the mechanisms by which the aqueous extract of the Kothala himbutu stem (KTE) decreases fasting blood glucose. An active compound in KTE, mangiferin, acts directly on liver cells and downregulates the gluconeogenic pathway by regulating fructose-1,6-bisphosphatase expression, resulting in lowered fasting blood glucose levels in the diabetic patient.[5]

The recent concept of functional food, intermediates between food and medicine, has gained considerable attention as a key concept in the global food industry.[6,7] As a result, many people in the United States, Japan, and other industrialized countries now use plants as functional foods in the form of herbal teas and dietary supplements to prevent disease and maintain good health. KT is available commercially as a dietary supplement for the prevention of diabetes and obesity in countries such as the United States and Japan.

An assessment of the safety of KTE, including tests for oral toxicity and chromosomal aberrations in Sprague-Dawley rats, has been performed.[8] Only limited information is available on the safety of KTE, while no gene expression profile of the effects of KTE in normal mice has been reported yet.

There are various methods, including *in vitro* and *in vivo* studies, of evaluating food safety.[9] Nutrigenomics, which utilizes high through-put analysis tools, has emerged as an immensely valuable strategy in nutrition research.[10] DNA microarray technology is one such tool useful in the evaluation of the safety of foods and natural products.[11,12] Hence, we investigated the effects of KTE on gene expression in the liver of normal mice using DNA microarrays carrying approximately 10,000 known mouse genes.

The purpose of this study was to evaluate the safety of KTE consumption by non-diabetic mice with a view to validating its presumed benefits in healthy and prediabetic people for its supposed preventive effect. The safety of KTE consumption in normal C57BL/6J mice was evaluated by examining plasma parameters, includ-

† To whom correspondence should be addressed. Fax: +81-49-271-7240; E-mail: mwada@josai.ac.jp

Abbreviations: CYP, cytochrome P450; GST, glutathione S-transferase; KT, Kothala himbutu; KTE, aqueous extract of the stem of Kothala himbutu; KTED, freeze-dried aqueous extract of the stem of Kothala himbutu

ing postprandial and fasting blood glucose levels, AST and ALT activities, and the hepatic gene expression profile.

Materials and Methods

Preparation of aqueous extract of KT. Whole stems (excluding leaves and roots) of KT, harvested in Sri Lanka, were purchased from the Ayurveda Kothalahimbutu Association (Tokyo). The plant was identified by Dr. G. A. S. Premakumara, Herbal Technology Section, Industrial Technology Institute (Colombo, Sri Lanka). A voucher specimen of the stems was deposited in our laboratory (Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Sciences, Josai University, Japan). The stems were dried and ground to a fine powder. One hundred g of the powder was boiled in 12.5 liters of distilled water for 2 h. The solvent was filtered and centrifuged for 10 min at $3{,}000 \times g$ to remove particulate matter. The supernatant was designated KT aqueous extract (KTE). KTE was freeze dried (Freeze Dryer System, Labconco, Kansas City, MO) yielding the KTED (9 g). The yield (w/w) of KTED was about 3.8%. The amount of KTED obtained from KTE was about 0.45 mg of dry matter/ml.

Animals and diet. We purchased 12 male 4-week-old C57BL/6J mice from Clea Japan (Tokyo). The mice were housed individually in an environmentally controlled room under constant temperature ($25 \pm 3\,^{\circ}C$) and a 12-h light/dark cycle. Experiments were performed on the mice after an acclimatization period of 1 week.

Under standardized conditions, the mice (5 weeks old at that time) were divided into two groups. One group was treated with KTE, and the other (the control) was administered distilled water. The treatment and control groups were given with free access to KTE and distilled water respectively. On an average, the mice in the treatment group consumed approximately 2.3 mg of dry matter/5 ml of KTE per d, whereas those in the control group drank approximately 5 ml of distilled water per d. Both groups were given free access to a standard diet (MF diet, Oriental Yeast, Tokyo) for 3 weeks.

The mice were euthanized by cervical dislocation at the age of 8 weeks after overnight fasting. They were then dissected, and the liver tissue was harvested for total RNA isolation. Animal care was in conformance with "Standards Relating to the Care and Management of Experimental Animals" laid down by the Ministry of Education of Japan and the Institutional Animal Care and Use Committee of Josai University.

Fasting blood glucose levels and oral glucose tolerance test. The fasting blood glucose levels of the mice the were examined after fasting them for 10 h. The blood samples were collected from the tail vein. The blood glucose concentration was measured using a Glucocard meter (Arkray, Kyoto).

The oral glucose tolerance test (OGTT) was performed on overnight-fasted mice. The control and KTE-treated mice were administered 200 µl of distilled water and 200 µl of KTE respectively, followed by 2 g/kg of glucose. Blood was collected from the tail vein immediately before and 30, 60, and 120 min after administration of glucose to determine blood glucose levels.

Biochemical analysis of plasma. The plasma concentration of glucose was measured with a Glucose C II test Wako kit (Wako Pure Chemicals, Tokyo). Alanine aminotransferase (ALT, EC1.1.1.27) activity and aspartate aminotransferase (AST, EC2.6.1.1) activity were measured using a Transamilase C-II test Wako kit (Wako Pure Chemicals, Tokyo).

Preparation of RNA. Total RNA was isolated using Trizol reagent (Invitrogen, Tokyo) according to the manufacturer's instructions. It was dissolved in diethyl pyrocarbonate-treated distilled water. The concentration of total RNA was estimated from the absorbance at 260 nm. RNAs from the liver tissue were used for DNA microarray analysis.

DNA microarray analysis. All experiments and analyses were performed according to the protocol in the GeneChip Expression Analysis Technical Manual (Affymetrix). Equal amounts of total RNA from six mice in each group were pooled. The mRNAs were reverse-transcribed with T7-$(dT)_{24}$ primer and copied into double-strand cDNAs (Superscript Choice System, Invitrogen). Biotin-labeled cRNAs were synthesized (High Yield RNA Transcript Labeling Kit, Affymetrix) and fragmented by heating at $94\,^{\circ}C$ for 35 min. Fragmented cRNAs were hybridized with a GeneChip Murine Genome U74A Array (Affymetrix) according to the Affymetrix protocol. After hybridization and subsequent washing and staining using Affymetrix Fluidics Station 400, the arrays were scanned using an Affymetrix array scanner, and the fluorescence intensity was measured using Microarray Suite 5.0 (Affymetrix).

Statistical analysis. Data were expressed as means ± standard deviation (SD). Treatment effects were analyzed by the Student's *t*-test. Differences with $P < 0.05$ were considered statistically significant. Expression analysis systemic explorer (EASE) analysis[13] was carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID).[14]

Results

Body weight gain, liver weight, fasting blood glucose levels, and plasma parameters

Food intake during the 3-week experimental period did not differ significantly between the two groups. The body weights of the mice in both groups increased

Table 1. Body Weight Gain, Liver Weight, Fasting Blood Glucose, and Plasma Parameters at the End of the Experimental Period

	Control	KTE
Body weight gain (3 weeks)	5.1 ± 1.1	4.3 ± 1.4
Liver weight (g/100 g body weight)	5.64 ± 0.53	5.29 ± 0.39
Fasting blood glucose (mg/dl)	96.7 ± 4.2	91.7 ± 1.2
Plasma		
AST (U/I)	32.2 ± 4.2	34.4 ± 9.0
ALT (U/l)	14.1 ± 2.1	14.0 ± 5.0

Data are as mean ± SD, $n = 6$–8.
AST, asparate aminotransferase; ALT, alanine aminotransferase

steadily during the experimental period. At the end of study, there was no significant difference between the body weight gains of the KTE-treated mice and the control mice. The liver weight (g/100 g of body weight) did not significantly differ between the two groups. As compared to the control mice, the KTE-treated mice tended to have decreased fasting blood glucose levels and plasma glucose concentrations, but the differences between the groups were not significant. Moreover, there were no significant differences in aspartate aminotransferase (AST) activity or alanine aminotransferase (ALT) activity between the KTE-treated and the control mice (Table 1).

Oral glucose tolerance test

The influence of KTE on postprandial blood glucose levels was examined in glucose-loaded mice. There was no significant difference in the postprandial blood glucose levels between the KTE-treated and the control mice (Fig. 1).

DNA microarray analysis of liver tissue of KTE-treated mice

Changes in gene expression profiles in the liver cells of the treatment and control groups were investigated by DNA microarrays (Table 2). A total of 5,808 genes were identified as present in both groups by Microarray Suite 5.0 (Affymetrix) on the basis of expression profiles. These genes were then classified into 10 groups on the basis of their known biological functions: transcription (818), cell function (705), inflammatory/immune response (110), metabolism (1403), angiogenesis (59), transport (814), stress response (39), ribosomal proteins (34), and other (522). Genes to which a function could not be ascribed were classified as unknown (1,363). We found that 207 genes were upregulated (n = 194) or downregulated (n = 13) by 2-fold or more in the KTE-treated mice as compared with the control mice. The EASE score (a Fisher Exact Statistic) was used to indicate the probability that a set of genes was present by sampling of annotated genes on the GeneChip Murine Genome U74A Array. Analysis of EASE score was carried out using web-accessible tools available in DAVID on the National Institute of Allergy and Infectious Disease (NIAID) website, as described previously.[13,14] The EASE scores of all categories were determined to be greater than 0.05 (Table 2).

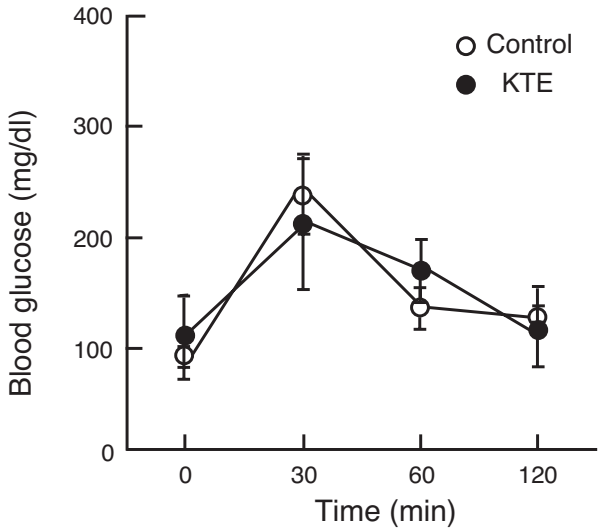

Fig. 1. Influence of KTE on Blood Glucose Levels during the Oral Glucose Tolerance Test (OGTT).

OGTT was performed on C57BL/6J mice administered KTE or distilled water for drinking for 4 weeks. After 16 h of fasting, glucose (2 g/kg) was administered orally. Before the administration of glucose, the control mice were administered 200 µl of distilled water, and the KTE treated mice were administered 200 µl of KTE. Blood samples were collected before and at 30, 60, and 120 min after glucose administration. Each point represents the mean ± SD ($n = 6$–8).

CYP51, CYP2C70, and CYP2A5 were upregulated and CYP 7B1 was downregulated by KTE. Additionally, glutachione S-transferases (GSTA2, GSTA4, GSTT1, GSTM2, GSTM5, and GSTM6) were also upregulated by KTE (Table 2). Moreover, the genes for some enzymes involved in the energy metabolic process exhibited changes in expression: catechol-*o*-methyltransferase and monoamine oxidase A (the enzyme that degrades catecholamines), and succinyl-CoA synthetase and 3-hydroxy-3-methylglutaryl-CoA reductase were upregulated, and acyl-CoA synthetase medium-chain family member 3 was downregulated by KTE (Table 2).

Discussion

The metabolic syndrome, comprising obesity, insulin resistance, and dyslipidemia, has become a worldwide epidemic of type-2 insulin-resistant diabetes mellitus.[15] KTE is used as an Ayurvedic dietary supplement in United States and Japan for the prevention of metabolic syndrome, especially diabetes. In this study, we focused on evaluating the safety of KTE consumption in non-diabetic mice, with a view to validating its presumed benefits to healthy people consuming KTE as a functional food.

There was no significant difference in food intake or body weight gain between the KTE-treated and the control mice during the experimental period. The values

Table 2. Gene Expression in C57BL/6J Mouse Livers Treated with KTE

Affymetrix ID	Gene	KTE/Control ratio	EASE score
Transcription (818)			0.760
Upregulated			
92554_at	C-terminal binding protein 2	2.31	
93802_at	metadherin	2.22	
94292_at	serine/threonine kinase receptor associated protein	2.03	
98951_at	transcription factor 25	2.14	
97506_at	ring finger protein 2	2.06	
101529_g_at	transcription elongation factor A (SII) 1	2.11	
102062_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin	2.16	
102344_s_at	transcription elongation factor A (SII) 3	2.56	
103073_i_at	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated fact	2.20	
103668_at	suppressor of Ty 6 homolog	2.29	
97974_at	zinc finger protein, multitype 1	2.05	
99076_at	nuclear receptor subfamily 1, group D, member 2	2.85	
101465_at	signal transducer and activator of transcription 1	3.26	
104010_at	zinc finger with KRAB and SCAN domains 14	2.06	
93006_at	nuclear factor I/C	2.02	
93164_at	ring finger protein 2	2.25	
97185_at	aryl hydrocarbon receptor nuclear translocator	2.25	
102144_f_at	splicing factor proline/glutamine rich	2.33	
160117_at	thyrotroph embryonic factor	2.53	
160377_at	TAR DNA binding protein	2.24	
160495_at	aryl-hydrocarbon receptor	2.02	
160681_at	poly (A) polymerase alpha	2.36	
160941_at	phosphodiesterase 8A	2.23	
160979_at	C-terminal binding protein 2	2.56	
161562_f_at	zinc finger protein 787	2.43	
93829_at	ROD1 regulator of differentiation 1	2.19	
160663_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	2.04	
160722_at	RNA methyltransferase like 1	2.06	
Downregulated			
94480_at	DNA segment, Chr 1, ERATO Doi 161	0.28	
Cell function (705)			0.83
Upregulated			
98948_at	guanine nucleotide binding protein-like 3	2.28	
97506_at	ring finger protein 2	2.06	
94338_g_at	growth arrest specific 2	2.40	
93164_at	ring finger protein 2	2.25	
160843_at	spindlin 1	2.72	
93764_at	NADH dehydrogenase (ubiquinone) 1	2.06	
94524_at	death associated protein 3	3.19	
97519_at	secreted phosphoprotein 1	2.25	
94338_g_at	growth arrest specific 2	2.40	
95030_at	prolactin receptor	2.05	
96753_at	B-cell CLL/lymphoma 7C	2.07	
102921_s_at	fas (TNF receptor superfamily member)	2.06	
103446_at	interferon induced with helicase C domain 1	2.27	
104157_at	FAST kinase domains 5	3.21	
100033_at	mutS homolog 2	2.44	
92596_at	calcyclin binding protein	2.17	
96104_at	Ring finger protein 145	2.47	
98635_at	ubiquitin-conjugating enzyme E2Z	2.14	
104037_at	RWD domain containing 4A	2.10	
98972_at	ubiquitin specific peptidase 8	2.02	
99990_at	retinoblastoma binding protein 6	2.02	
99669_at	lectin, galactose binding, soluble 1	2.14	
99062_at	poliovirus receptor-related 3	2.52	
103506_f_at	desmocollin 2	2.76	
97288_at	PDZ domain containing 1	2.06	
98946_at	WD repeat and SOCS box-containing 1	2.41	
Downregulated			
98067_at	cyclin-dependent kinase inhibitor 1A	0.28	
102016_at	cell death-inducing DFFA-like effector	0.26	

Continued on next page

Table 2. *Continued*

Affymetrix ID	Gene	KTE/Control ratio	EASE score
Inflammatory/Immune response (110)			0.188
Upregulated			
103617_at	CD55 antigen	2.02	
92222_f_at	histocompatibility 2, Q region locus 1	2.16	
Metabolism (Xenobiotic, Glutathion, Carbohydrate, Lipid, Catecholamin, *etc.*) (1403)			0.51
Upregulated			
94916_at	cytochrome P450, family 51	2.43	
95043_at	cytochrome P450, family 2, subfamily c, polypeptide 70	3.01	
102847_s_at	cytochrome P450, family 2, subfamily a, polypeptide 5	3.66	
93009 at	glutathione S-transferase, mu 2	2.78	
96085_at	glutathione S-transferase, alpha 4	5.09	
96258_at	microsomal glutathione S-transferase 3	5.30	
100042_at	hydroxyacyl glutathione hydrolase	2.17	
100629_at	glutathione S-transferase, mu 5	2.89	
95019_at	glutathione S-transferase, theta 1	2.13	
101872_at	glutathione S-transferase, alpha 2	4.20	
104637_at	glutathione S-transferase, mu 6	2.38	
96789_i_at	galactose mutarotase	2.49	
96803_at	glucan (1,4-alpha-), branching enzyme 1	2.57	
161594_f_at	galactose mutarotase	2.29	
162031_f_at	galactose mutarotase	2.13	
160428_at	succinyl-CoA synthetase	2.00	
96627_at	phenylalkylamine Ca2+ antagonist (emopamil) binding protein	2.41	
102769_f_at	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog	2.63	
103665_at	ELOVL family member 6, elongation of long chain fatty acids	3.42	
104285_at	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	2.28	
101945_g_at	lysophospholipase 1	2.27	
160388_at	sterol-C4-methyl oxidase-like	3.66	
160737_at	lanosterol synthase	2.05	
93542_at	phosphotriesterase related	2.17	
93749_at	monoamine oxidase A	2.09	
98535_at	catechol-O-methyltransferase	2.02	
94380_at	insulin degrading enzyme	2.02	
102938_at	leukocyte cell-derived chemotaxin 2	2.03	
160934_s_at	SH3-domain GRB2-like (endophilin) interacting protein 1	2.07	
102017_at	PRP4 pre-mRNA processing factor 4 homolog B	2.19	
99960_at	mitogen activated protein kinase kinase 4	2.10	
92192_s_at	proprotein convertase subtilisin/kexin type 5	2.01	
92767_at	bone morphogenetic protein receptor, type 1A	2.07	
92937_at	fibroblast growth factor receptor 4	2.03	
160632_at	protein kinase C	2.50	
99521_at	adenylate kinase 3 alpha-like 1	3.16	
99959_at	adenylate kinase 3 alpha-like 1	2.06	
92492_at	adenylate kinase 3	2.48	
101489_at	S-adenosylmethionine decarboxylase 1	2.14	
100323_at	S-adenosylmethionine decarboxylase 1	2.19	
102773_at	carbonic anhydrase 8	2.38	
160375_at	carbonic anhydrase 3	3.80	
103391_at	cysteine conjugate-beta lyase 2	3.11	
160628_at	glycine C-acetyltransferase	2.28	
Downregulated			
92898_at	cytochrome P450, family 7, subfamily b, polypeptide 1	0.45	
161345_f_at	cytochrome P450, family 7, subfamily b, polypeptide 1	0.49	
102192_r_at	acyl-CoA synthetase medium-chain family member 3	0.35	
Angiogenesis (59)			1.00
Upregulated			
96038_at	ribonuclease, RNase A family 4	2.00	
102373_at	glutamyl aminopeptidase	2.20	
102698_at	endothelial PAS domain protein 1	2.31	
Transport (814)			0.510
Upregulated			
101913_at	chloride channel 5	2.32	
102662_at	asialoglycoprotein receptor 2	2.70	
103271_at	low density lipoprotein receptor-related protein 6	2.84	

Continued on next page

Table 2. *Continued*

Affymetrix ID	Gene	KTE/Control ratio	EASE score
103958_g_at	transferrin receptor	2.33	
94792_at	macrophage scavenger receptor 1	2.37	
96078_g_at	solute carrier family 17 (sodium phosphate), member 1	2.02	
94433_at	solute carrier family 38, member 2	2.00	
100491_at	solute carrier family 16 (monocarboxylic acid transporters), member 2	2.01	
94797_at	solute carrier family 26 (sulfate transporter), member 1	2.26	
96331_at	sorting nexin 2	2.08	
97505_at	ADP-ribosylation factor-like 1	2.07	
103703_f_at	similar to integral membrane transport protein UST1R	2.91	
92294_at	ADP-ribosylation factor-like 5A	2.28	
93711_at	secretory protein SEC23 related gene	2.22	
94322_at	squalene epoxidase	3.12	
92840_at	nucleoporin 54	2.07	
103619_at	cytochrome b5 type B	2.38	
96276_r_at	transmembrane 9 superfamily member 3	2.25	
103702_i_at	similar to integral membrane transport protein UST1R	2.36	
96227_at	serine (or cysteine) peptidase inhibitor, clade A, member 6	3.94	
98969_at	ATP-binding cassette, sub-family D (ALD), member 1	3.73	
100491_at	solute carrier family 16 (monocarboxylic acid transporters), member 2	2.01	
100951_at	polycystic kidney disease 2	2.15	
103702_i_at	similar to integral membrane transport protein UST1R	2.36	
104421_at	flavin containing monooxygenase 3	4.67	
92763_at	ATP-binding cassette, sub-family B (MDR/TAP), member 7	3.01	
Downregulated			
94642_at	guanosine diphosphate (GDP) dissociation inhibitor 2	0.30	
100078_at	mouse apolipoprotein A-IV	0.38	
Stress (39)			0.403
Upregulated		—	
Downregulated		—	
Ribosomal (34)			0.633
Upregulated		—	
Downregulated		—	
Others (522)			0.0825
Upregulated			
103076_at	chromatin modifying protein 5	2.30	
102240_at	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	2.52	
94379_at	kinesin family member 1B	2.04	
94325_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	2.34	
101837_g_at	protein phosphatase 1B, magnesium dependent, beta isoform	2.10	
97778_at	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	3.94	
94818_at	O-linked N-acetylglucosamine (GlcNAc) transferase	2.05	
96623_at	UDP-glucose ceramide glucosyltransferase	2.86	
93575_at	gamma-glutamyl hydrolase	2.46	
98934_at	lethal, Chr 7, Rinchik 6	2.20	
99669_at	lectin, galactose binding, soluble 1	2.14	
93183_at	CDC91 cell division cycle 91-like 1	2.07	
96775_at	chromobox homolog 1	2.23	
99535_at	CCR4 carbon catabolite repression 4-like	3.79	
96207_at	RNA binding motif, single stranded interacting protein 1	2.12	
103944_at	RAD51-like 1	2.07	
102225_at	RAB GTPase activating protein 1-like	2.12	
102696_s_at	phosphatidylinositol transfer protein, beta	2.23	
104202_at	transmembrane protein 1	2.50	
93212_at	protein tyrosine phosphatase-like A domain containing 1	2.02	
96572_at	5-azacytidine induced gene 2	2.25	
160135_at	nitrilase family, member 2	2.28	
160365_at	eukaryotic translation initiation factor 2, subunit 2	2.15	
98349_at	interleukin 6 signal transducer	2.02	
Downregulated			
97487_at	serine (or cysteine) peptidase inhibitor, clade E, member 2	0.46	
102016_at	cell death-inducing DFFA-like effector c	0.26	
100431_at	leptin receptor	0.47	

Continued on next page

Table 2. *Continued*

Affymetrix ID	Gene	KTE/Control ratio	EASE score
Unknown (1363)			0.0797
Upregulated			
95050_at	cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protei	2.32	
96258_at	microsomal glutathione S-transferase 3	5.30	
96351_at	basic transcription factor 3-like 4	2.06	
98530_at	growth arrest specific 5	3.74	
99649_at	glutamate cysteine ligase	2.01	
100042_at	hydroxyacyl glutathione hydrolase	2.17	
101966_s_at	ring finger protein 13	2.11	
103871_at	sec23 interacting protein	2.09	
96215_f_at	uc53a11.r1 Mus musculus cDNA, 5 end	3.00	
97402_at	indolethylamine N-methyltransferase	3.74	
98428_at	spastin	2.80	
99988_at	dymeclin	2.24	
100949_at	cDNA sequence BC004044	2.25	
101380_at	nudix (nucleoside diphosphate linked moiety X)-type motif 14	2.17	
101393_at	annexin A3	2.32	
101404_at	coiled-coil domain containing 115	2.16	
103497_at	cDNA sequence BC025546	2.15	
103519_at	carboxylesterase 1	2.54	
103744_at	SH3 domain binding glutamic acid-rich protein like 2	2.32	
103982_s_at	alcohol dehydrogenase 4 (class II), pi polypeptide	2.57	
103983_at	alcohol dehydrogenase 4 (class II), pi polypeptide	3.32	
103986_at	inhibin beta-C	2.88	
104398_at	tetraspanin 33	2.09	
104640_f_at	josephin domain containing 3	2.08	
104643_at	WW, C2 and coiled-coil domain containing 1	2.26	
93646_at	twinfilin, actin-binding protein, homolog 1	2.22	
94192_at	ganglioside-induced differentiation-associated-protein 10	3.30	
96494_at	kelch-like 24	2.49	
96518_at	WW, C2 and coiled-coil domain containing 1	2.19	
101179_at	aspartate-glutamate-alanine-aspartate box polypeptide 6	2.44	
160678_at	tetraspanin 12	2.35	
160697_at	expressed sequence C77080	2.39	
162058_f_at	AV271456 Mus musculus cDNA, 3 end	3.11	
160217_at	RIKEN cDNA 2310001A20 gene	2.26	
103403_at	RIKEN cDNA 2310007H09 gene	3.18	
98594_at	RIKEN cDNA 1190002N15 gene	2.58	
102920_at	RIKEN cDNA 1810054D07 gene	2.01	
Downregulated			
X00686_M_at	mouse gene for 18S rRNA.	0.30	
103460_at	DNA-damage-inducible transcript 4	0.43	

Categories are gene ontology biological processes. EASE score is the Fisher Exact Statistic for the likelihood that the number of genes found in the filtered list occurred by chance sampling of the MG-U74A probe set population.

for liver weight and plasma parameters such as fasting and postprandial blood glucose levels and AST and ALT activities did not differ significantly between the two groups. Although KTE reduced the fasting blood glucose level in the type-2 diabetic model,[5] it did not reduce the fasting or the postprandial blood glucose level in normal mice.

DNA microarray analysis revealed that the expression of genes of known function, such as those for stress response, ribosomal proteins, transcription, cell function, the inflammatory/immune response, angiogenesis, transport, and metabolism (xenobiotic, glutathione, carbohydrate, lipid, catecholamine, *etc.*) remained largely unaffected by KTE. EASE analysis indicated that KTE did not have any selective effects on the expression of the genes associated with the above categories. Furthermore, the expression of oncogenes (alk, cyclin D1, erbA/ear, erB, fos, jun, myc, ras, and src) and antioncogenes (p16, p53, p107/p130, rb, and wt1) also was not modulated by KTE (data not shown).

The drug metabolism system comprises phase I and II enzymes. Phase I enzymes, mainly CYPs, detoxify a variety of endogenous and exogenous chemicals and activate many carcinogens.[16] The major CYPs involved in drug metabolism in the human body are hCYP1A2, hCYP2C9, hCYP2C19, hCYP2D6, hCYP2E1, and hCYP3A4.[17] Although there are more than 50 CYPs, 90% of drugs are metabolized by just six of these, the two most significant enzymes being hCYP3A4 and hCYP2D6,[18] but KTE upregulates the expression of mCYP2C70, whose human ortholog is hCYP2C9. In

humans, hCYP2C9 is the rate-limiting enzyme in the metabolic clearance of clinically used drugs such as the hypoglycemic agents tolbutamide and glipizine, the anticonvulsant phenytoin, and the *S*-enantiomer of the anticoagulant warfarin.[19,20] KTE can influence the effects of these drugs, but it remains to be seen whether the changes in CYP2C70 mRNA expression induced by KTE are reflected as alterations in drug metabolism, since the induction level of the mRNA was not very high (2- to 5-fold). Further studies are necessary to determine the exact function of mCYP2C70 in mice.

Phase II enzyme systems catalyze the conjugation of phase I metabolites to various water-soluble molecules such as glutathione or glucronic acid, thereby accelerating the metabolic excretion rate. Glutathione S-transferase (GST) is one of the most important phase II enzymes. In the present study, KTE slightly induced GSTs, suggesting that it modulated the drug-metabolizing capacity of the mice.

It has been reported that some functional foods, such as comfrey (*Symphytum officinale*), noni (*Morinda citrifolia*), and black cohosh (*Cimicifuga racemosa*), are hepatotoxic in experimental animals and humans.[21–24] Using DNA microarray analysis, Mei *et al.* founds that comfrey drastically regulates many CYP genes (*e.g.*, CYP2A12, CYP4A12, CYP7A1, CYP2C12, and CYP26), GST genes (Gsta3, Gstm3, and Gstp1), ATP-binding cassette transporters (*e.g.*, Abcb9 and Abcc3), and other metabolism-associated genes in the rat liver.[21] Although KTE altered the mRNA levels of drug-metabolizing enzymes by 2- to 5-fold in our study, comfrey was found to change them by as much as 20-to 100-fold.[21,22]

In this study, there were no significant differences in AST or ALT activities between the KTE-treated and the control mice. Furthermore, hepatoprotective and antioxidative effects have been reported for hot-water extracts of the roots and stems of KT in liver injury model mice.[25] Although KTE induced gene expression of some drug-metabolizing enzymes, it is probably not hepatotoxic in normal mice.

An anti-obesity effect has been reported for the aqueous extract of the roots of KT in Zucker fatty.[26] There are, however, only a few reports on the effect of KT consumption in normal mice. Kishino *et al.* have reported an antiobesity effect of a mixture of *Salacia reticulata* extract and cyclodextrin in normal C57BL/6J mice fed a high-fat diet.[27] The mechanisms of the antiobesity effect of KTE in normal mice has not yet been reported.

In this study, we used DNA microarray analysis and found that KTE regulated the mRNA level of genes related to energy metabolism, such as catechol-*o*-methyltransferase, monoamine oxidase A, succinyl-CoA synthetase, 3-hydroxy-3-methylglutaryl-CoA reductase, and acyl-CoA synthetase medium-chain family member 3. This data suggests that KTE influences energy metabolism through gene regulation. Further research to clarify the mechanism of gene regulation by KTE is planned.

This study indicates that KTE has no significant acute hepatotoxicity. In conclusion, normal mice can safely consume KTE, and it might be effective as a functional food to maintain good health.

Acknowledgments

The authors wish to express their gratitude to Professor Keiko Abe and Associate Professor Ichiro Matsumoto, Graduate School of Agricultural and Life Sciences, The University of Tokyo, for their help in performing DNA microarray analyses.

References

1) Warrir, P. K., Nambir, V. P. K., and Ramankutty, C., *Salacia reticulata* wight. In "Indian Medical Plants," Orient Longman, Chennai, pp. 47–48 (1993).
2) Jayawardena, M. H., de Alwis, N. M., Hettigoda, V., and Fernando, D. J., A double blind randomized placebo controlled cross over study of a herbal preparation containing *Salacia reticulata* in the treatment of type 2 diabetes. *J. Ethnopharmacol.*, **97**, 215–218 (2005).
3) Karunanayake, E. H., Welihinda, J., Sirimanne, S. R., and Sinnadorai, G., Oral hypoglycaemic activity of some medicinal plants of Sri Lanka. *J. Ethnopharmacol.*, **11**, 223–231 (1984).
4) Kajimoto, O., Kawamori, S., Shimoda, H., Kawahara, Y., Hirata, H., and Takahashi, T., Effects of a diet containing *Salacia reticulata* on mild type 2 diabetes in humans. *J. Jpn. Soc. Nutr. Food Sci.*, **53**, 199–205 (2000).
5) Im, R., Mano, H., Matsuura, T., Nakatani, S., Shimizu, J., and Wada, M., Mechanisms of blood glucose-lowing effect of aqueous extract from stems of Kothala himburu (*Salacia reticulata*) in the mouse. *J. Ethnopharmacol.*, in press.
6) Swinbanks, D., and O'Brien, J., Japan explores the boundary between food and medicine. *Nature*, **364**, 180 (1993).
7) Arai, S., Morinaga, Y., Yoshikawa, T., Ichiishi, E., Kiso, Y., Yamazaki, M., Morotomi, M., Shimizu, M., Kuwata, T., and Kaminogawa, S., Recent trends in functional food science and the industry in Japan. *Biosci. Biotechnol. Biochem.*, **66**, 2017–2029 (2002).
8) Shimoda, H., Furuhashi, T., Naitou, K., Nagase, T., and Okada, M., Thirteen-week repeat dose oral toxicity study of *Salacia reticulata* extract in rats. *Jpn. J. Med. Pharm. Sci.*, **46**, 527–541 (2001).
9) Arai, S., Studies on functional foods in Japan: state of the art. *Biosci. Biotechnol. Biochem.*, **60**, 9–15 (1996).
10) Muller, M., and Kersten, S., Nutrigenomics: goals and strategies. *Nat. Rev. Genet.*, **4**, 315–322 (2003).
11) Narasaka, S., Endo, Y., Fu, Z., Moriyama, M., Arai, S., Abe, K., and Kato, H., Safety evaluation of hypoallergenic wheat flour by using a DNA microarray. *Biosci. Biotechnol. Biochem.*, **70**, 1464–1470 (2006).
12) Kamakura, M., Maebuchi, M., Ozasa, S., Komori, M., Ogawa, T., Sakaki, T., and Moriyama, T., Influence of royal jelly on mouse hepatic gene expression and safety

assessment with a DNA microarray. *J. Nutr. Sci. Vitaminol.*, **51**, 148–155 (2005).
13) Hosake, D. A., Dennis, G., Jr., Sherman, B. T., Lane, H. C., and Lempicki, R. A., Identifying biological themes within lists of genes with EASE. *Genome Biol.*, **4**, R70 (2003).
14) Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., and Lempicki, R. A., DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.*, **4**, P3 (2003).
15) Moller, D. E., New drug targets for type 2 diabetes and the metabolic syndrome. *Nature*, **414**, 821–827 (2001).
16) Guengerich, F. P., and Shimada, T., Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem. Res. Toxicol.*, **4**, 391–407 (1991).
17) Lynch, T., and Price, A., The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects. *Am. Fam. Physician.*, **76**, 391–396 (2007).
18) Faber, M. S., Jetter, A., and Fuhr, U., Assessment of CYP1A2 activity in clinical practice: why, how, and when? *Basic Clin. Pharmacol. Toxicol.*, **97**, 125–134 (2005).
19) Miners, J. O., and Birkett, D. J., Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br. J. Clin. Pharmacol.*, **45**, 525–538 (1998).
20) Goldstein, J. A., Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br. J. Clin. Pharmacol.*, **52**, 349–355 (2001).
21) Mei, N., Guo, L., Zhang, L., Shi, L., Sun, Y. A., Fung, C., Moland, C. L., Dail, S. L., Fuscoe, J. C., and Chen, T., Analysis of gene expression changes in relation to toxicity and tumorigrnrsis in the livers of Big Blue transgenic rats fed comfrey (*Symphytum officinale*). *BMC Bioinformatics*, **7**, S16 (2006).
22) Gue, L., Mei, N., Dail, S., Fuscoe, J., and Chen, T., Comparison of gene expression profiles altered by comfrey and riddelliine in rat liver. *BMC Bioinformatics*, **8**, S22 (2007).
23) Stadlbauer, V., Fickert, P., Lackner, C., Schmerlaib, J., Krisper, P., Trauner, M., and Stauber, R. E., Hepatotoxicity of NONI juice: report of two cases. *World J. Gastroenterol.*, **14**, 4758–4760 (2005).
24) Vitetta, L., Thomsen, M., and Sali, A., Black cohosh and other herbal remedies associated with acute hepatitis. *Med. J. Australia*, **180**, 598–600 (2004).
25) Yoshikawa, M., Ninomiya, K., Shimoda, H., Nishida, N., and Matsuda, H., Hepatoprotective and antioxidative properties of *Salacia reticulata*: preventive effects of phenolic constituents on CCl_4-induced liver injury in mice. *Biol. Pharm. Bull.*, **25**, 72–76 (2002).
26) Yoshikawa, M., Shimoda, H., Nishida, N., Takada, M., and Matsuda, H., *Salacia reticulata* and its polyphenolic constituents with lipase inhibitory and lipolytic activities have mild antiobesity effects in rats. *J. Nutr.*, **132**, 1819–1824 (2002).
27) Kishino, E., Ito, T., Fujita, K., and Kiuchi, Y., A mixture of the *Salacia reticulata* (Kotala himbutu) aqueous extract and cyclodextrin reduces the accumulation of visceral fat mass in mice and rats with high-fat diet-induced obesity. *J. Nutr.*, **19**, 433–439 (2007).