CHAPTER III

MATERIALS AND METHODS

Preparation of Trikatu extracts

Trikatu were prepared by ethanolic extraction of ginger, black pepper and long pepper.It was provided by Assist. Prof. Dr. Sakchai Wittaya-areekul (Faculty of Pharmaceutical Science, Naresuan University). The dried powder of ginger, black pepper and long pepper were extracted by refluxing with ethanol (95% v/v) for 3 h with extraction machine (TCE206, GBF Co. Ltd., China). The ethanol extracts were filtered through a filter paper and concentrated under a vacuum at 40°C. The crude extracts were subjected to a qualitative analysis for various phytoconstituents using HPLC (Thermo separation product, USA). The 6-gingeral content was about 3.57% of ginger extract. Piperine content was 27.37% and 22.27% of black pepper and long pepper extract, respectively. All extracts were pooled in the ratio of 1:1:1 (w/w) and stored at 4°C.

Experimental animals and treatment

The 36 adult male Wistar rats, weighing ranged (200-250g) were obtained from National Laboratory Animal Centre, Mahidol University. The use of animals for this research was approved by the Ethical Committee of Naresuan University. The animals were normalized for 1 week and then randomly assigned into six groups of six rats each. The acute testing group rats were fed with 500 and 1,000 mg/kg B.W. of Trikatu extracts in 3 ml of 10% (w/v) propylene glycol solution for 7 days, respectively which control group was given with 3 ml of 10% (w/v) propylene glycol solution. In sub-acute study, rats were fed with 50 and 150 mg/kg B.W. of Trikatu extracts in 3 ml of 10% (w/v) propylene glycol solution for 30 days. The control group was fedwith 3 ml of 10% (w/v) propylene glycol solution. The oral administration of test compound used gavage dosing techniques. All rats were housed in stainless steel cages and exposed to a normal daylight/dark cycle under humid tropical condition

(25 ± 2°C). They were fed with a standard laboratory diet and tap water *ad libitum* freely available throughout the duration of the experiment.

Preparation of serum

The blood was collected by rat tail artery (fast 8-12 h before a procedure to obtained 1 ml of blood) in all animals before the commencement of experiment and throughout the experimental period. Then, serum was harvested by centrifugation at 3,000xg for 5 min. The serum was kept at -20°C prior to biochemical analysis.

Biochemical analysis of liver function and lipid profile

1. Determination of the serum AST and ALT activity

The serum AST and ALT were assayed by the method of Reitman and Frankel (1957). The method is based on the principle that oxaloacetate formed from the aspartate aminotransferase catalyzed reaction between alpha-ketoglutarate and L-aspartate. The ALT activity based on that pyruvate formed from the alanine aminotransferase catalyzed reaction between alpha-ketoglutarate (oxoglutarate) and L-alanine.These reactions are coupling with chromogenic solution (2, 4- dinitrotrophenyl hydrazine (DNPH)) in alkaline medium to form colored hydrazine and that the concentration of which is proportional to the AST and ALT activity as measured with a colorimeter. The procedures in Tables 4-6 were performed in 96-well plates. The spectrophotometer was set to zero using distilled water at 492-510 nm and the absorbance of TBK (Test blank), QC (quality control) and TEST was measured in order. In the measurement of both serum AST and ALT, pyruvate is used as the standard. One unit/L of AST or ALT is defined as the calibration curve by plotting the corresponding absorbance of standards against their respective AST or ALT activity (Table 6). It is noted that a value of AST 60-300 U/L and ALT 25-55 U/L in rat is a laboratory useful reference limit of AST and ALT activity, respectively (Shayne and Christopher, 1992).

Table 4 The protocol for estimation of serum AST activity

	TBK	QC	TEST
AST substrate (µl)	25	25	25
distilled water /QC/Test sample	5	5	5
Mix and incubate the plate at 37°C in a waterbath for 60min			
2,4 DNPH (µl)	25	25	25
Mix and leave the plate for 20 min at room temperature (25-35°C)			
0.4M NaOH (µl)	250	250	250
Mix and leave the plate for 5 min at room temperature (25-35°C)			

Table 5 The protocol for estimation of serum ALT activity

	TBK	QC	TEST
ALT substrate (µl)	25	25	25
distilled water /QC/Test sample	5	5	5
Mix and incubate the plate at 37°C in a waterbath for 30 min			
2,4 DNPH (µl)	25	25	25
Mix and leave the plate for 20 min at room temperature (25-35°C)			
0.4M NaOH (µl)	250	250	250
Mix and leave the plate for 5 min at room temperature (25-35°C)			

Table 6 The protocol for plotting the AST or ALT calibration curve

	Blank	Std 1	Std 2	Std 3	Std 4
Pyruvate standard (µl)	0	2.5	5	7.5	10
ALT or AST substrate (µl)	25	22.5	20	17.5	15
distilled water (µl)	5	5	5	5	5
2,4-DNPH (µl)	25	25	25	25	25
Mix and leave the plate for 20 min at room temperature (25-35°C)					
0.4 M NaOH (µl)	250	250	250	250	250
Mix and leave the plate for 20 min at room temperature (25-35°C)					
Equivalent AST in serum (U/L)	-	24	61	114	190
Equivalent ALT in serum (U/L)	-	28	57	97	150

2. Lipid profile measurement

The levels of total triglycerides, total cholesterol and HDL-c in serum were measured by enzymatic colorimetric commercial kit (Human Gesellschaft fur Biochemica und Diagnositca Human GmbH, Germany). For cholesterol or triglyceride measurement, the 10 µl of each reagent blank, sample and standard (concentration at 200 mg/dl) were added into the 96-wells plate, mixed and incubated for 5 min at 37°C. For HDL-c measurement, 75 µl of reagent were added into the wells and incubated at 37°C for 5 min. After that, 25 µl of substrate was added and then incubated again for 10 min at 37°C. The microplates were read using a microplate reader (Labsystem, Helsinki, Finland). Measurement the absorbance at 492-500 nm was used for cholesterol and triglycerides and 520 nm were used for HDL-C measurements within 60 min. The total cholesterol, triglyceride and HDL-c concentration was calculated using the followed equations:

$$\text{Total cholesterol or triglyceride} = \frac{(\text{O. D. sample} - \text{O. D. blank})}{(\text{O. D. standard} - \text{O. D. blank})} \times 200 \text{ mg/dl}$$

$$\text{HDL cholesterol} = \frac{(\text{O. D. sample} - \text{O. D. blank})}{(\text{O. D. standard} - \text{O. D. blank})} \times 56.7 \text{ mg/dl}$$

A range value of 42-90 mg/dl, 30-90 mg/dl and 20-35 mg/dl in rat is a laboratory useful reference limit of total cholesterol, total triglyceride and HDL-c value, respectively (Shayne and Christopher, 1992).

3. Statistical Analysis

The data obtained from the present study were expressed as Mean ± SEM. They were analyzed by one-way analysis of variance (ANOVA). In brief, multiple comparisons of means were made using the Least Significant Difference (LSD) test with the Statistical Package for Social Sciences (SPSS) for windows version 16.0 package. Differences were considered significant at $p<0.05$ level of significance.

Histopathological Study

The rat was individually weighed daily on a top loader electronic balance and weight recorded in grams. At the end of this experiment, rats were sacrificed by cervical dislocation. The vital organs of rat (liver, kidney, lung, spleen and heart) were isolated and washed with freshly prepared 1x PBS buffer solution. Adherent fat and surrounding tissue were cleaned off. The vital organs were rapidly weighed and calculated relative organ weight ratio and kept at -80 °C prior to use.

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (in gram)/}}{\text{Body weight on day of sacrifice (in gram)}} \times 100$$

The rat livers were investigated for influence of the tested extract on the histopathology. Livers from each sacrificed rat were fixed in 10% neutral formalin and prepared for histopathological examination according to the method of Lillie and Fullmen (1976). The liver tissues were cut into 3 mm, fixed in ethanol (dehydration) and xylene (clearing agent) and then embedded in paraffin. Paraffin blocks were cut in to rotary microtome at the 6-7 micrometers and picked up on a glass microscope slide. The glass slides were deparaffined, stained with hematoxylin and eosin dye and covered with a thin glass coverslip. Hematoxylin stains the nuclear components of cells and becomes a dark blue while eosin stains the cytoplasmic organelles resulting in varying shades of pink, red or orange. This effect can be observed under light microscopy.

Proteomics analysis

1. Total Proteins Extraction of Liver

The frozen liver piece (100-150 mg) was ground into powder with liquid nitrogen with a mortar and pestle. Then, 5 mL of extraction media (0.175 M Tris-HCl, pH 8.8, 5% SDS, 15% glycerol and 0.3 M DTT) was added directly to mortar and continue grinding for an additional 30 sec. The colloidal tissues were filtered through two layers of cheesecloth into a 50 mL Falcon tube at room temperature. Four volumes of ice cold 100% acetone was added to filtered homogenate, the mixture was mixed by vortexing, placed at -20°C for at least 1 h before centrifugation at 5,000 xg

for 15 min to collect precipitated cell. Cell pellet were washed in 15-20 mL of cold 80% acetone and total protein was extracted with 1 mL of IEF extraction solution (8 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X-100 and 50 mM DTT) by incubating sample for 1 h at room temperature. After centrifugation at 12,000 xg for 10 min, protein concentration in supernatant was determined by Lowry assay using bovine serum albumin as a standard protein (Lowry, et al., 1951).

2. Protein precipitation

To remove interference in protein sample, Deoxycholic acid (DOC) and Trichloroacetic acid (TCA) were used (Peterson, 1983). Aliquot of protein solution were addedwith 0.1 ml of 0.15% DOC solution, mixed thoroughly and incubated for 15 min at room temperature. Then, 0.1 ml of 72% TCA solution was added, mixed properly and incubated for 2 h at 4°C. After centrifugation at 12,000 xg, 4°C for 15-30 min, the supernatant was carefully decanted without disturbing the pellet. Ten volumes of ice-cold acetone were added, mixed gently and incubated at -20°C for 15 min or overnight. The supernatant was collected by centrifugation at 4°C for 15min at 12,000 xg and air-dried before resuspending the protein pellet in 0.15% DOC solution.

3. Proteindetermination

Protein concentration was estimated by the method of Lowry using bovine serum albumin as standard (2-10 mg/ml BSA). The diluted sample (1:25) was mixed with 0.2 ml freshly prepared alkaline copper solution made by mixing 0.4% $CuSO_4.7H_20$ in Tatalic acid, 5% SDS, 0.8 M NaOH and 20% sodium carbonate. The reaction was incubated for 30 min at room temperature before adding 0.05 ml 20% Folin-Ciocalteu phenol reagent. The mixture was vigorously mixed and allowed to stand at room temperature for 30 min. The absorbance at 750 nm was measured using a microplate reader (VERSAmax™, Cape Cod, Inc, UK).

4. Denaturing Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 12% polyacrylamide gels mixed according tomethod of Laemmli (1970). Prior to sample loading, the extracts were mixed with a one-fifth volume of a 5-fold concentrated sample buffer to yield a final concentration of 0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue and heated at 95°C for 5 min before applied to a gel lane. The upper and lower reservoirs of the electrophoresis apparatus were filled with electrophoresis buffer

(0.025M Tris, 0.192M glycine, 0.1% SDS). The individual proteins are separated electrophoretically at a constant voltage of 50V of constant current for a stacking gel until the bromophenol blue tracking dye enters the separating gel, and then increase the current to 70V until tracking dye has reached the bottom of the separating gel. Protein bands were visualized by silver staining.

5. Silver staining

At the end of each electrophoresis, the gel protein was fixed in the fixing solution (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde to 100 ml fixing solution) for 30 min. The gel was removed in the washing solution (35% ethanol) 2 times for 5 min each and sensitizing in 0.02% sodium thiosulfate for 2 min. After washing in water twice for 5 min each, the gel was stained with silver nitrate (2%) for 20 min. The gel was shaken in the developing solution until regarded protein bands were visualized and stopped quickly in the stopping solution for 20 min. The gel was kept in 0.1% acetic acid at room temperature.

6. In-gel trypsin digestion

Protein bands were excised into twelve sections. Each band was further cut into approximately ~1X1 mm pieces and subjected to in-gel digestion using an in-house method developed by Proteomics Research Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Gel pieces were washed with distilled water and dehydrated with 100% acetonitrile (ACN) for 5 min. The gel pieces were reduced with 10mM Dithiothreitol (DTT) in 10mM ammonium bicarbonate at room temperature for 1 h and alkylated with 100mM iodoacetamide (IAA) in 10mM ammonium bicarbonate at room temperature for 1 h in the dark. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for 3 h or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and gels were incubated at room temperature for 10 min in a shaker. Final solution in extraction process were evaporated or dried at 40°C for 3-4 h

or overnight. The peptide samples from the extracted were redissolved in 0.1% formic acid and pooled together into the insert tube for mass spectrometric analysis.

7. Nano-LC-MS/MS analysis

The protein digests from gel electrophoresis were separated in Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany)). Briefly, the sample were loaded using an autosampler and separated on a monolithic Trap Column (PS-DVB, 300 µm i.d. x 5 mm) at a flow rate of 20 µl/min following separated on a Pepswift monolithic column (PS-DVB, 100 µm i.d. x 5 cm) at a flow rate of 1 µl/min. The peptide mixtures were separated and eluted with a 0-50% gradient solution (Buffer A, 0.1% formic acid in water; Buffer B, 0.1% formic acid in water and 50% acetonitrile in formic acid and 95% ACN) within 20 min and were then online detected in ESI-Ion Trap mass spectrometer.

8. Protein identificationand Gene ontology categories

DeCyder MS Differential Analysis software (GE Healthcare) was used to quantify the peptide in all samples (Johansson, et al., 2006; Thorsell, et al., 2007). Acquired LC-MS raw data were converted to mzXML file by CompassXport software andall peptides were detected with the PepDetect. The PepDetect module of the softwarewas used for automated peptide detection, charge state assignments based on resolved isotopic peaks and consistent spacing between consecutive charges states, and quantitation based on MS signal intensities of individual LC-MS analyses. The final step consisted of matching peptides across different signal intensity maps using the PepMatch module resulting in a quantitative comparison. Acquired MS/MS data from the analysis of the DeCyderMS software were submitted to database search using the MS/MS Ions Search on Mascot software available on-line at www.matrixscience.com (Matrix Science, London, UK) (Perkins, et al., 1999).

The data was searched against the NCBI database (March 2011), for protein identification. Database interrogation was; taxonomy (Rattus); enzyme (trypsin); variable modifications carbamidomethyl and oxidation of methionine residues; mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1^+, 2^+ and 3^+). Therefore, the

Mascot DAT files were merged and evaluated on the peptide level with the built-in DeCyderMS software and exported to Microsoft Excel.

9. Gene ontology annotation and mapping of protein networks

Gene ontology annotation was performed using Software Tool for Rapid Annotation of Proteins (STRAP) version 1.1.0.0 (Bhatia, et al., 2009). STRAP allows collection and annotation of information about the proteins in a data set. First, protein was imported from protein lists text file formats. It then downloads information about each protein from several online databases, focusing on information from the UniProt Knowledgebase database and then compiles all of the protein annotation information and displays it in a Gene ontology term that includes biological process, cellular component and molecular function, respectively. The final distribution pie charts were generated using Microsoft Excel.

Moreover, the KEGG IDs of identified proteins were simultaneously analyzed by iPath2.0 program (http://pathways.embl.de) to search for the visualization and analysis of cellular pathways (Takuji, et al., 2011). The content of iPath2.0 is summarized in three separate overview maps such as central metabolism, secondary metabolite biosynthesis and regulatory pathways from their orthologous protein information defined in KEGG database. The UniProt IDs of the identified proteins were simultaneously submitted to The Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org) to search for understanding of cellular functions and annotate all functional interactions among proteins in the cell (Damian, et al., 2010).

10. Quantification of the changes in protein Analysis

Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using *MultiExperiment Viewer* (Mev) software version 4.6.1 (Howe, et al., 2010). Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. They performed statistical tests of variance of differences (ANOVA) for these data sets p-value less than 0.05 that statistically significant proteins.