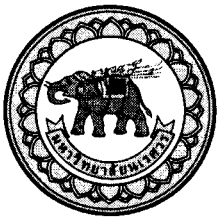

รายงานวิจัยฉบับสมบูรณ์

โครงการ การใช้โปรตีโอมิคส์ในการวิเคราะห์การเปลี่ยนแปลงของโปรตีนในตับหนูจากผลของสมุนไพรตรีกฎุก

The Proteomic analyses of rat livers affected by Trikatu

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สรุปรายงานการวิจัย

โครงการวิจัยเรื่อง การใช้โปรตีโอมิคส์ในการวิเคราะห์การเปลี่ยนแปลงของโปรตีนในตับหนู จากผลของสมุนไพรตรีกฎุก
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ABSTRACT

Trikatu is a Thai traditional herbal formulation consisting of three herbs in equal amount, *Piper nigrum*, *Piper longum* and *Zinggiber officinale*. It is suggested to be beneficial in control blood lipid. However, the scientific evidence supporting such claim has not yet been fully elucidated. The aim of this study was to identify mechanisms of Trikatu effects on lipid metabolism in rat liver using a physical parameter, biochemical parameter and proteomics approach. The 200-250 g male Wistar rats were randomly divided into six groups (n=6). The acute groups were treated with 500 and 1,000 mg/kg B.W. of Trikatu for 7 days and the subacute groups were treated with 50 and 150 mg/kg B.W. of Trikatu for 30 days. The control group was fed with 10% (w/v) propylene glycol solution. Daily changes of body weight, vital organs weight, serum lipid profile and AST/ALT enzymes were monitored. Rat livers were collected and used for proteome profile analysis. Histopathological examination of rat livers was also performed. Trikatu showed no effects on the body weight and vital organs weight (kidney, heart, lung and spleen). Significant increase of liver weight after feeding with 150, 500 and 1,000 mg/kg B.W. of Trikatu was observed. The serum AST/ALT and HDL-c level were not altered but a significant decrease in the serum triglyceride and cholesterol level was reported. In addition, the liver proteome of Trikatu fed rat was altered. These differential expressed proteins are involved in insulin signaling, glucose and lipid metabolism. Significant up-regulated proteins associated with glucose uptake and energy production were found. However, the proteins related to fatty acid oxidation and fatty acid synthesis decreased. Further

functional study of these proteins may be helpful in the elucidation of the pharmacological mechanism and identification of new drug targets for lowering lipid by Trikatu.

CHAPTER I

INTRODUCTION

Rationale for the study

Trikatu is an Ayurvedic preparation consisting of dried fruits of black pepper (*Piper nigrum* Linn; Piperaceae), dried fruits of long pepper (*Piper longum* Linn; Piperaceae) and dried rhizomes of ginger (*Zingiber Officinale Rosce*; Zingiberaceae) in the ratio of 1:1:1; w/w (Atal, et al., 1981). Trikatu is an herbal compound that is considered as potent carminative as it provides a natural and safe support system for gastric function associated with gaseous distension (Johri, *et al.*, 1992). Trikatu is known in Thailand as the "rainy season medicine" that strongly thermoregulation and removes cold, congestion, and revives weak organic functions. Trikatu is reported to be not only an anti-mucus and digestive powder used to tone up gastric and respiratory function, but also to be useful in cases of obesity, high cholesterol, high triglycerides, hypothyroid, slow metabolism and various inflammatory conditions (Sivakumar, 2004). Nevertheless, little is known about the pathways underlying the effect.

The pharmacological actions in herbal ingredient of Trikatu, in particular, on lipid control have been reported. Ginger had a significant decrease in blood glucose, total serum triglyceride and cholesterol, but increased in high-density lipoprotein cholesterol (HDL-c) (Ahmed and Sharma, 1997). However, it failed to decrease the body weight (Mascolo, et al., 1989). Sharma, et al. (1996) studied hypolipidemic and anti-atherosclerotic effects of ginger in cholesterol-fed rabbits. The administration of ginger extract increased fecal excretion of cholesterol, thus suggesting a modulation of absorption. In addition, lower total serum cholesterol and low density lipoprotein cholesterol (LDL-c) level were observed after ginger extract treatment. However, a few reports showed the lipid control action of black pepper and long pepper. The ethanol extract of the long pepper fruit yields piperlonguminine, piperine, and pipernonaline as the main antihyperlipidemic constituents. Piperlonguminine has the effect of regulating lipid metabolism by reducing the serum total cholesterol, triglyceride, LDL-c. The mechanism is likely related to increasing LDL receptor mRNA expression and decreasing apolipoprotein B (ApoB) mRNA expression (Ma, et

al., 2008). Methyl piperine has been reported to significantly inhibit the elevation of total serum cholesterol in rats fed with a high cholesterol diet (Wang, et al., 1993).

But to our knowledge, the process of lipid control of Trikatu remains unclear. Liver is a major site of metabolism, which it is keys characteristic of pharmacologic and metabolic respond of drug administration. The processes of lipid control may be regulated by many proteins in liver. The proteome-wide approach is then used to study the dynamic change of proteins in liver as a result of Trikatu. The results may provide an insight in many biochemical pathways associated with lipid control.

Purpose of the study

1. To investigate the morphological changes in Trikatu fed rat including body weight, vital organs and histopathology.
2. To monitor rat liver function and lipid profile during exposure to Trikatu.
3. To study the proteome profile of rat liver proteins after treated with Trikatu.

The importance of the study

This is the first proteomics study regarding the effect of Trikatu in rats. This can be used as a model to study the effect of herbal treatment in animals. The differential expressed proteins in rat liver after feeding with Trikatu may be used as potential biomarkers and/or therapeutic targets.

Scope of the study

The *in vivo* effects of Trikatu extracts on rat liver were investigated. The experiments were divided into 2 parts, the acute effect and the subacute effect. Male Wistar rats (200-250 g) were treated with each ethanolic extract of Trikatu by gavage for 7 days for acute effect and 30 days for sub-acute effect. Changes in daily body weight and vital organ weights (liver, kidney, lung, spleen and heart), serum lipid profile and AST/ALT enzyme, and liver proteome after exposure to Trikatu was monitored and compared to control.

Hypothesis

Trikatu was reported to reduce serum lipid in rat but had no effect on the body weight. Then, proteomic approach was used to seek for proteins respond to Trikatu treatment and associate with the regulation of lipid metabolism in the liver of Trikatu fed rat. These proteins may serve as potential therapeutic targets and or may be used to treat patients clinically.

Anticipated outcomes

1. This study may help understanding the effect of Trikatu on metabolic pathways, enzymes or transcription factors which are essential to control lipid metabolism in liver.

2. This study can support herbal drug information that is beneficial for traditional doctors and users.

CHAPTER II

LITERATURE REVIEW

Relevance of Herbal Medicine to Thailand

The World Health Organization estimates that a large proportion of the world's population relies heavily on traditional practitioners and medicinal plants in order to meet primary health care needs (WHO, 1999). Since safety and efficacy data are often not available for these drugs, the field of herbal medicine and medicinal plants requires additional research and further scholarship in the future.

The art of traditional Thai medicine was passed from father to son and many herbs were used to treat a single disease or symptom. The roots of traditional medical practices in Thailand include a mixture of Indian, Ayurvedic and Thai beliefs. During the Sukhothai period, over one hundred hospitals where traditional medicine was practiced, called Arogaya Sala, were built. This period of history began a long tradition of royal support for traditional medicine methods and techniques that would not be challenged until the nineteenth century. Later, during the Ayutthaya period, the first official textbook of Thai drug recipes was written titled "King Narai's Medicine". This textbook was the precursor for the books used today in programs of traditional medicine instruction. Traditional drug formulations were also recorded during the reigns of King Rama I, II and III when instructions were inscribed on stone tablets at the temples Wat Po and Wat Raj Oros.

King Rama V supported the production of the first medical textbook called "Tumra Paetsart Sonkau" and the national formulary of drugs called "Tumra Chabub Luang". These are two of the official books currently being used by the Thai Food and Drug Administration in their attempts to register traditional medicines.

Principle of Thai traditional medicine

The human body is composed of four elements such as earth, water, wind and fire. When the four elements of the body are in equilibrium, it will be healthy. In contrast, if an imbalance in these elements occurs or disability in any of the four elements, a person will become ill. Moreover, the imbalance in the four internal

elements and illness can also be due to an imbalance in the four external elements as well (Subcharoen, 2001; Sittitanyakit and Termwiset, 2004).

Traditional practitioners believed that herbal healing is based on the healer's belief in the power of nature and earth, and the ability to harness the power of plants and minerals for energy. Herbal treatment emphasizes adjusting the balance of the body elements using the health promotion approach (Vichai, et al., 2005). Moreover, Traditional medicine in Thailand is approached from a holistic perspective, with the idea that many factors contribute to a person's overall health and that a multitude of factors must be targeted to improve health as opposed to focusing exclusively on the narrow perspective of pathological disease.

Trikatu

Trikatu is given along with many other ancient medicines. Trikatu is basically a Sanskrit word that means "three spices". It is containing fruits of black pepper (*Piper nigrum*), long pepper (*Piper longum*) and the rhizomes of ginger (*Zingiber officinalis*) in equal proportions (Johri, et al., 1992). It is a common combination used in stimulating the digestive systems by helping in the production of right amount of gastric juices. It also promotes the secretion of hydrochloric acid from the gastric mucosa and relieves gaseous distention. Trikatu also works well on the respiratory system as it is mucolytic such as anti-mucus and expectorant due to its three pungent ingredients. It removes congestion from the lungs and works as rejuvenator for the respiratory system. It relieves cough, cold, edema, bronchitis, asthma and other breathing problems.

Trikatu is reported to be not only an anti-mucus and digestive power used to tone up gastric and respiratory function, but also to be useful in cases of obesity, high cholesterol, high triglycerides, hypothyroid, and various inflammatory conditions. Trikatu is considered to assist weight loss by maximizing metabolism along with balancing blood-glucose to decrease food cravings. Trikatu acts as an appetite suppressant while simultaneously increasing metabolism. It stimulates the production of many digestive enzymes, thereby decreasing gas, nausea, and constipation, excessive belching, bloating, and indigestion. By helping individuals to achieve and maintain an ideal weight, this treatment also helps to decrease blood pressure and

cholesterol levels. Sivakumar (2004) found that Trikatu reduces low density lipoprotein (LDL) and triglycerides level in the body. It increases high density lipoprotein (HDL) in the body thus improves dyslipidemia and prevents the risk of atherosclerosis and heart attacks. Hence 'Trikatu' can be used as a potent hypolipidemic agent and it can protect cardiac and aortic tissue as well.

The main purpose of Trikatu incorporation into numerous Ayurvedic formulations was most probably to enhance the efficacy of pharmacologically active ingredients. Several groups of investigators now attribute this bioavailability enhancing property of pepper to its main alkaloid, piperine (Kolen and Hussong, 1995). Piperine is an alkaloid with the molecular formula $C_{17}H_{19}O_3N$, which on hydrolysis with alkali gives piperic acid and piperidine (Atal, et al., 1975). The piperine content of pepper is directly proportional to its pungency.

The proposed mechanism for the increased bioavailability of drugs coadministered with Trikatu is attributed to the interaction of piperine with enzymes that participate in drug metabolism, such as mixed function oxidases found in the liver and intestinal cells (Bano, et al., 1987; 1991). Interaction with the synthesis of drug chelating molecules in the body such as glucuronic acid has also been proposed. Piperine may also interact with the process of oxidative phosphorylation, or the process of activation/deactivation of certain metabolic pathways, slowing down the metabolism and biodegradation of drugs. This action of piperine results in higher plasma levels of drugs, rendering them more available for pharmacological action (Atal, et al., 1981). These experiments revealed that Trikatu coadministered to rats orally with the drugs isoniazid increased the blood levels of isoniazid as compared to control animals who did not receive Trikatu (Karan, et al., 1998). In subsequent experiments, piperine has been proven to enhance the bioavailability of a number of drugs including rifampicin, diclofenac sodium and pefloxacin (Karan, et al., 1999; Lala, et al., 2004; Madhukar, et al., 2008)

Table 1 Use of three constituents of Trikatu from Ayurvedic literature

General name *Botanical name*	Family	Uses
Ginger *(Zingiber officinale)*	Zingiberaceae	Analgesic, Blood purifier, Carminative, Expectorant, Appetizer, Digestive, Stimulant, Sciatica, Lumbago, Rheumatism, Slip disc, Gout, Chronic arthritis, Muscular trouble, Asthma, Cough, Chronic bronchitis.
Black pepper *(Piper nigrum)*	Piperaceae	Stimulant, Carminative, Antacidic, Anti periodic, Stomachache, Digestive, Throat problem, Liver pain, Muscle pain, Piles, Spleen disorder, Leucoderma, Lumbago, Paralysis, Chronic fever, Vertigo, Arthritis, Urinary disorder, Flatulence, Indigestion.
Long pepper *(Piper longum)*	Piperaceae	Tonic, Alterative, Rejuvenator, Digestive, Carminative Cough, Chronic bronchitis, Sedative, Antidote to snakebite, Throat disorder, Anti-inflammatory, Anti-malarial, Dyspepsia, Lumbago, Splenomegaly.

Sources: Choudhury, et al., 2006

Black pepper

Black pepper is known by the botanical name of *Piper nigrum* and *Piperaceae* family. It might reach South East Asia many centuries earlier. In the seed of black pepper, it mainly contains piperine which is the major alkaloid constituent. It contains 5-9% of the alkaloids piperine and piperidine in volatile oil. Other than this, it also contains terpenes as pinene, limonene, caryophyllene and etc. (Tewtrakul, et al., 2000).

Pharmacology and clinical applications of black pepper

In the Ayurvedic descriptions, black pepper is described as a drug which increases digestive power, improves appetite, cures cold, cough, dyspnoea, diseases of the throat, intermittent fever, colic, dysentery, worms and piles (Atal, et al., 1975) also useful in tooth ache, pain in liver and muscle, inflammation, leucoderma and epileptic fits (Ayier and Kolammal, 1966; Kirtikar and Basu, 1975).

Several studies were reported both under *in vitro* and *in vivo* studies in experimental systems. Neither black pepper nor piperine produces any toxicity. In fact, it exerts liver protective action as evidenced by the studies of several workers. By enzyme modulation, piperine functions as a chemopreventive substance. Dalvi (1991) studied the hepatotoxic effect of piperine on rats by estimating the hepatic mixed function oxidases and serum enzymes as specific markers of hepatotoxicity. An intragastric dose of 100 mg/kg body weight caused an increase in hepatic microsomal enzymes after treatment (cytochrome p-450, cytochrome-b5, NADPH-cytochrome C reductase, benzphetamine N-demethylase, aminopyrine N-demethylase and aniline hydroxylase). On the other hand, an intraperitonial dose of 10 mg/kg did not produce any effect on the activities of the drug metabolizing enzymes. These treatments did not affect those serum enzymes which are specific markers of liver toxic conditions. Thus, piperine exerts significant protection against chemically induced hepatotoxicity.

Koul and Kapil (1993) reported that black pepper reduces *in vitro* and *in vivo* lipid peroxidation and prevents depletion of GSH and total thiols. Lipid peroxidation causes free radical production, which in turn produces tissue damage. GSH conjugates xenobiotics which are excreted out by subsequent glucuronidation. The hepatoprotective action of black pepper was compared with a reference compound, silymarin, a known hepatoprotective drug, and found that piperine has slightly lower activity. A dose dependent increase in the level of the hepatic biotransformation enzymes (glutathione-s-transferase, cytochrome p-450, cytochrome b-5, acid soluble sulfhydryl-SH) was obtained in a feeding experiment study using Swiss albino mice fed with a diet containing 1%, 2 % and 5 % (w/w) black pepper for 10 and 20 days (Singh and Rao, 1993). A lower level of glucuronidation due to the inhibition of the enzyme UDP-glucose dehydrogenase was observed by Reen (1993) in an *in vitro* study. While studying the hypoglycemic action of several plants, Tripathi (1979) reported that pepper fruits are devoid of any significant hypoglycemic action in rabbits. The aqueous extract of pepper leaves in a dose of 10–20 mg/kg led to a moderate increase in the blood pressure of dogs (Sridharan, et al., 1978). Piperine as well as AE (antiepilepsirine) is reported to have detoxifying qualities that may increase the bioavailability of other drugs, hence altering the pharmacokinetic parameter of the epileptic (Bano, et al., 1991).

Long pepper

Piper longum L. (*Piperaceae*), commonly known as "long pepper", is widely distributed in the tropical and subtropical regions of the world, throughout the Indian subcontinent and South East Asia countries (Kirtikar and Basu, 1980). Long pepper has been used in traditional remedies as well as in the Ayurvedic system of medicine against various disorders (Tripathi, et al., 1999). Dried unripe fruits are used as an alternative to tonic and a root is used in chronic bronchitis, cough and cold (Maitreyi, et al., 2010).

The fruit of long pepper contains a large number of alkaloids and related compounds. Piperine is the major and active constituent of long pepper. It contains 3-5% in long pepper. the most abundant of which is piperine, together with methyl piperine, piperonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, refractomide A, pregumidiene, brachystamide, brachystamide-A, brachystine, pipercide, piperderidine, longamide and tetrahydropiperine, terahydropiperlongumine, dehydropipernonaline piperidine. Piperine, terahydropiperlongumine, trimethoxy cinnamoyl-piperidine and piperlongumine have been found in the roots of long pepper (Kirtikar and Basu, 1980; Rastogi and Malhotra, 1993).

Pharmacology and clinical applications of long pepper

In view of commercial and medical importance of long pepper, several works have investigated the species chemically and also pharmacologically. An amide namely dehydropipernonaline having coronary vasorelaxant activity was isolated from the fruit of long pepper (Umeyama, et al., 2006). Methanolic extract from dried fruits, roots and nutgalls of *Piper longum, Piper sarmentosum, and Quercus infectoria* respectively, were examined for their spasmolytic activities using isolated rat or guinea pig ileum and compared with a reference anti-diarrheal drug such as loperamide and an L-type calcium channel blocker such as verapamil. The effect of methanol extract of long pepper fruits was evaluated on adriamycin-induced cardiotoxicity (i.e., biochemical changes, tissue peroxidation damage, and abnormal antioxidant levels) in Wistar rats. Histopathological studies of the heart revealed degenerative changes and cellular infiltration in rats treated with Adriamycin; however, pretreatment with long pepper reduced the intensity of these lesions. The

results indicated that long pepper offered significant protection against Adriamycin-induced oxidative stress and reduced cardiotoxicity by virtue of its antioxidant activity (Wakade, et al., 2008).

The antihyperglycemic and antilipidperoxidative effects of ethanolic extract of long pepper dried fruits in alloxan-induced diabetic rats were studied (Dhar, et al., 1968). The blood glucose level, carbohydrate metabolizing enzymes and the status of lipid peroxidation and antioxidants were assayed using specific colorimetric methods. Oral administration of dried fruits has shown significant anti-hyperglycemic, antilipidperoxidative and antioxidant effects in diabetic rats comparable to that of the standard reference drug glibenclamide (Manoharan, et al., 2007). Methyl piperine significantly inhibited the elevation of total serum cholesterol, and the total cholesterol to HDL-c ratio, in rats fed with a high cholesterol diet (Wang, et al., 1993). The unsaponificable fraction of the oil of long pepper also significantly decreased total serum cholesterol and hepatic cholesterol in hypercholesterolemia mice (Wu and Bao, 1992). The ethanol extract of the long pepper fruit yields piperlonguminine, piperine, and pipernonaline as the main antihyperlipidemic constituents. They exhibited appreciable antihyperlipidemic activity *in vivo*, which was comparable to that of the commercial antihyperlipidemic drug simvastatin (Jin, et al., 2009). Bioassay-guided isolation of chloroform extract of the fruits of long pepper showed an *in vitro* diacylglycerol acyltransferase (DGAT) inhibitory activity, led to isolation of a new alkamide together with four known alkamides. Pharmacological inhibition of acyl CoA: diacylglycerol acyltransferase by alkamides emerged as a potential therapy for the treatment of obesity and type 2 diabetes (Lee, et al., 2006). Guineensine, isolated from chloroform extract inhibited acyl-coenzyme A: cholesterol acyltransferase (ACAT) activity in a dose dependent manner (Lee, et al., 2004). Piperlonguminine has the effect of regulating lipid metabolism by reduce the serum total cholesterol, triglyceride and LDL-c levels. The mechanism is likely related to increasing LDL receptor mRNA expression and decreasing apolipoprotein B mRNA expression (Ma, et al., 2008).

Long pepper is generally assumed to be safe in moderate doses. A single oral dose in experimental animals (3 g/kg body weight) and chronic toxicity studies for 90 days revealed no adverse effects. Studies of isolated constituents in mice reported

LD_{50} values of piperine, piperlongumine and piperlonguminine as 56.2 ± 3.0, 110.1 ± 7.8, and 115.3 ± 9.5 mg/kg body weight, respectively. Thus, acute toxicity studies did not show any mortality or morbidity when administered to animals during pharmacological study (Chanda, et al., 2009). The ethanolic extract of long pepper fruits reduced the elevated levels of glutathione pyruvate transaminase (GPT), alkaline phosphatase (ALP), and lipid peroxidation (LPO) in liver and serum of radiation treated mice. But, long pepper fruits extract also increased the reduced glutathione (GSH) production to offer the radioprotection (Sunila and Kuttan, 2004). The fruit extract improved the regeneration process by restricting fibrosis, but offered no protection against acute damage or against cirrhotic changes in rodents (Rage, et al., 1984). Treatment with the ethanol extract of long pepper inhibits liver fibrosis induced by carbon tetrachloride (CCl_4) (Christina, et al., 2006). Piperine exerted a significant protection against tertbutyl hydroperoxide and carbon tetrachloride hepatotoxicity by reducing both *in vitro* and *in vivo* lipid peroxidation, enzymatic leakage of GPT and ALP, and by preventing the depletion of GSH and total thiols in the intoxicated mice. Piperine showed lower hepatoprotective potency than silymarin (Indu, et al., 1993).

Ginger

Ginger (*Zingiber officinale Roscoe*, Family *Zingiberaceae*) has a long history for its health benefits and is used as a traditional medicine in the Asian countries. The dried of rhizome extract is one of the main ingredients in Trikatu. The main active compounds are the 6-gingerols and 6-shogaols as well as some phenolic derivatives (Govindarajan, 1982).

Pharmacology and clinical applications of Ginger

Ginger is one of the herbal spices, it is commonly safe to use and proved to be effective against various human ailments. Ginger extract showed antioxidant (Stoilova, et al., 2007; Ahmed, et al., 2008), anti-cancer (Shukla and Singh, 2007), anti-inflammatory (Young, et al. 2005; Habib, et al., 2008) and antithrombotic properties (Thomson, et al., 2002).

Many studies have focused on the effect of ginger on lipids lowering in animals and humans. The results of those studies (Table 2) showed that ginger significantly reduces serum triglyceride and cholesterol level in rats (ElRokh, et al.,

2010; Giri, et al. 1984; Sanjay, et al., 2004; Fuhrman, et al., 2000; Thomson, et al., 2002). Similar effect was also found in cholesterol fed rabbits (Sharma, et al., 1996; Ahmed, et al., 2000; Bhandari, et al., 1998). The lipid lowering of ginger may be a result of elevated the activity of hepatic cholesterol-7a-hydroxylase in the liver, the rate-limiting enzyme in bile acids biosynthesis, or down-regulated HMG-CoA reductase expression. Thereby, ginger could stimulate cholesterol conversion to bile acids, resulting in elimination of cholesterol from the body (Srinivasan and Sambaiah, 1991; Tanabe, et al., 1993; Srinivas, et al., 2009).

In some cases, the hypoglycemic and antidiabetic potential of ginger were investigated and reported to be variable (Table 2). The ethanolic extract has also been shown to lower blood glucose in normal rabbits (Mascolo, et al., 1989) and diabetic rats (Ahmed and Sharma, 1997; Ojewole, 2006; Al-Amin, et al., 2006). However, the ethanolic extract of ginger was reported to elicit no effect on blood glucose in normal rats (Weidner, et al., 2000). Combination therapy often takes advantage of complementary effects of different agents. The combination of garlic and ginger is more effective in reducing serum lipids and blood glucose (Ahmed and Sharma, 1997). Similarly, the combined effect of ginger extract and atorvastatin can reduce cholesterol in hypercholesterolemic rats which are susceptible to liver function abnormalities (Gehan, et al., 2010).

Ginger is usually regarded as safe in small amounts, or approximately 2-4 grams per day (Ernst and Pittler, 2000; Chandra et al., 2002; Bryer, 2005), although certain precautions should be borne in mind. In animal experiments, ginger has not shown any teratogenic effect when applied during pregnancy (Weidner and Sigwart, 1998). Interestingly, Wilkinson (2001) found that ginger tea applied orally to rats was not materno-toxic, but increased fetal loss, although augmenting growth in the surviving fetuses.). In a human study, ginger ingested in various forms during pregnancy did not appear to increase the rates of major fetal malformations (Portnoi et al., 2003), and showed neither teratogenic effects (Fischer-Rasmussen, et al., 1990) nor mutagenic activity (Sivaswamy, et al., 1991).

Biochemical profiles for assessing Liver Function

The biochemical profile is the most common forms of the database for most diagnostic investigations. Many biochemical parameters tend to have specificity for an organ and/or a limited range of pathological processes. Interpretation of diagnostic biochemical patterns requires an understanding of the pathological implications of each abnormal result. Together with the normal results, these form a pattern which reflects one or more underlying disease process. Investigative biochemical profiles are designed to provide all the data necessary for a broad investigation of internal disease. Profiles with limited data are best used for monitoring an established diagnosis for which the results of a more wide ranging profile have already been obtained.

Transaminase

Aspartate aminotransferase (AST) is presented in many tissues and is useful in evaluating muscle and liver damage in body. It is an absolute prerequisite to eliminate extra-hepatic tissue damage as a possible source of serum AST when evaluating the enzyme related to the liver function. In combinations with the physical examination and history, the evaluation of other serum enzymes should aid in differentiating the source of increased AST levels. AST is presented in both the cytoplasm and mitochondria of hepatocytes and will elevate in states of altered membrane permeability. In such cases, levels are expected to be less than in states of frank necrosis, when both cytoplasmic and mitochondrial enzymes are released. Alanine aminotransferase (ALT) is considered to be liver specific. This enzyme is presented in high concentrations in the cytoplasm of hepatocytes. Plasma concentrations increase with hepatocellular, damage/necrosis, hepatocyte proliferation, or hepatocellular degeneration. ALT is a cytoplasmic enzyme, and is considered to be liver specific in body.

Elevation of serum levels of both AST and ALT can occur with states of altered hepatocellular membrane permeability. Because ALT is located only in the cytoplasm, serum levels tend to be relatively higher than AST, as a result of membrane leakage from the hepatocyte. Mitochondrial enzymes are less likely to be released with most of the conditions which result in increased membrane permeability. The magnitude of both AST and ALT elevations in serum is generally related to the

number of hepatocytes affected. However, the level cannot be used to predict either the type of lesion, or whether cell damage is reversible (leakage) or irreversible (frank necrosis). In fact, focal necrosis may yield a lower concentration of both AST and ALT than severe, transient hypoxia in which all cells may be affected resulting in a potentially reversible alteration in membrane permeability and diffuse enzyme leakage. Equally increases in ALT and AST may be relatively mild in cases of severe cirrhosis/fibrosis of the liver since there is no ongoing hepatocellular damage.

Another factor to be considered when interpreting AST and ALT levels is the rate of clearance from plasma. Both enzymes are molecularly too large to permit glomerular filtration and are primarily stereo chemically denatured. The half-life of these enzymes is approximately 2-4 days and some prognostic information may be gleaned with this knowledge. Thus, if an elevated serum level falls by 50% after 2-4 days, the prognosis is generally more favorable than if the enzymes remain persistently elevated or are only slightly decreased after this time period. Finally, it must be remembered that ALT is liver specific and AST is presented in many tissues, but because of organ size and relative enzyme content, it may be used with care to evaluate liver disease.

Lipid Profile

The lipid profile is a group of tests that are often ordered together to determine risk of coronary heart disease. A lipid profile measures total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. They have been shown to be good indicators of whether someone is likely to have a heart attack or stroke caused by blockage of blood vessels or hardening of the arteries (atherosclerosis).

Normally, lipids are insoluble in water but are soluble in alcohol and other solvents. When dietary fats are digested and absorbed into the small intestine, they eventually reform into triglycerides, which are then packaged into lipoproteins. Dietary fats, including cholesterol, are absorbed from the small intestines and transported into the liver by lipoproteins called chylomicrons. Chylomicrons are large droplets of lipids with a thin shell of phospholipids, cholesterol, and protein. Once chylomicrons enter the bloodstream, an enzyme called lipoprotein lipase breaks down the triglycerides into fatty acid and glycerol. After a 12 to 14 hour-fast, chylomicrons

are absent from the bloodstream. Thus, individuals who are having a lipid profile done should fast overnight to ensure that chylomicrons have been cleared.

The liver removes the chylomicron fragments, and the cholesterol is repackaged for transport in the blood in VLDL, which eventually turns into LDL. LDL consists mainly of cholesterol. Most LDL particles are absorbed from the bloodstream by receptor cells in the liver. Cholesterol is then transported throughout the cells. Diets high in saturated fats and cholesterol decrease the uptake of LDL particles by the liver. LDL particles are also removed from the bloodstream by scavenger cells, or macrophages, which are white blood cells that bury themselves in blood vessels such as arteries. Scavenger cells prevent cholesterol from reentering the bloodstream, but they deposit the cholesterol in the inner walls of blood vessels, eventually leading to the development of plaque. HDL is a separate group of lipoproteins that contain more protein and less cholesterol than LDL. HDL is produced primarily in the liver and intestine, and it travels in the bloodstream, picks up cholesterol, and gives the cholesterol to other lipoproteins for transport back to the liver (Figure 1).

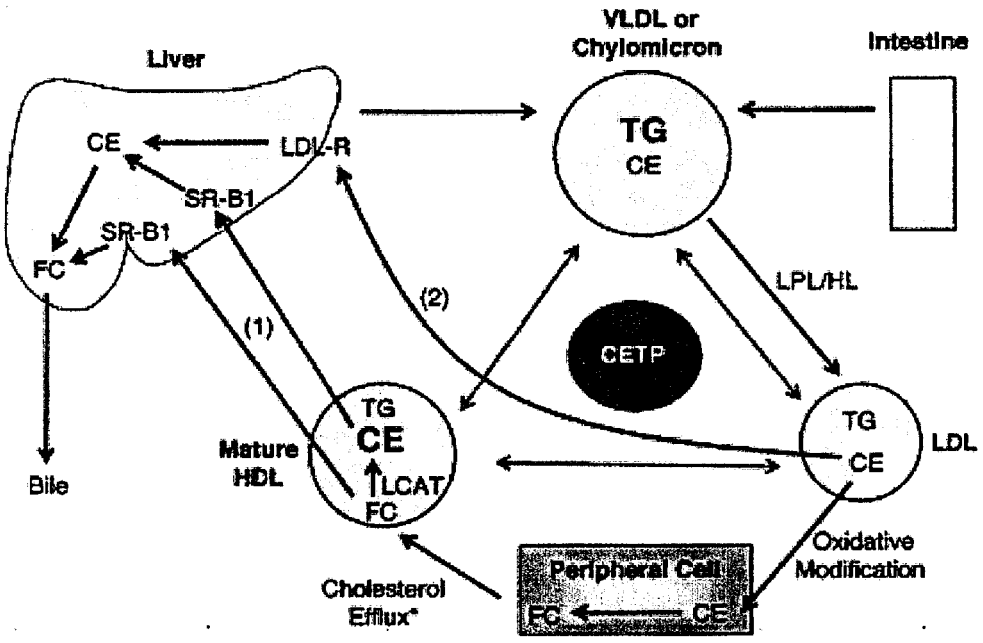

Figure 1 Lipid transport.

Sources: Philip and John, 2006

A lipid profile should be done after a nine to twelve hour-fast without food, liquids or medication. If fasting is not possible, the values for total cholesterol and HDL may still be useful. If total cholesterol is 200 mg/dl or higher or HDL is less than 40 mg/dl, the individual will need to have a follow-up lipoprotein profile done to determine LDL and triglyceride levels. Depending on the physician's request, the lipid profile may include the ratio of cholesterol to HDL. This ratio is sometimes used in place of total blood cholesterol. The ratio is obtained by dividing the HDL level by the total cholesterol.

The National Cholesterol Education Program, the American College of Cardiology, and the American Heart Association recommend diet and lifestyle modification as the first line of defense against abnormal blood lipids. These recommendations include a diet low in total fat, saturated fat, and cholesterol; a diet high in fiber; weight loss or weight management; increased physical activity; smoking cessation; increased intake of plant sterols (e.g., margarines and salad dressings made with soybean sterols) and daily use of a low-dose aspirin. Cholesterol-lowering drugs works to lower LDL by reducing cholesterol synthesis and by binding bile acids in the small intestines. However, there are possible side effects to these drugs that patients should be aware.

Table 3 Optimal, borderline, and high levels for each lipid component

Element	Optimal	Borderline	High risk
LDL Cholesterol	<100 mg/dl	130–159 mg/dl	160+ mg/dl
HDL Cholesterol	>60 mg/dl	35–45 mg/dl	<35 mg/dl
Triglycerides	<150 mg/dl	150–199 mg/dl	>200 mg/dl
Total Cholesterol	<200 mg/dl	200–239 mg/dl	>240 mg/dl
Cholesterol to HDL Ratio	<4	5	>6

Source: The National Cholesterol Education Program, the American College of Cardiology, and the American Heart Association

Proteomics

Proteomics is the study of all proteins synthesized in a cell or an organism. It is the newly developed science for the study of proteins. Genomics was used as a means to improve our understanding of disease with the hope that a comprehensive knowledge of an organism genetic makeup would lead to more efficient drug discovery. Although useful, DNA sequence analysis alone does not lead efficiently to new target identification, since one cannot easily infer the functions of gene products and protein pathways from DNA sequence. Most large pharmaceutical companies now have a proteomics oriented biotech or academic partner or have started their own proteomics division. Common applications of proteomics in the drug industry include target identification and validation, identification of efficacy and toxicity biomarkers from readily accessible biological fluids, and investigations into mechanisms of drug action or toxicity. These proteins may serve as potential therapeutic targets and or may be used to treat patients for clinical (Seyed, 2009).

Protein Expression Profiling by gel electrophoresis is a primary analysis tool used to characterize the expression of proteins in the pharmaceutical industry. Large numbers of proteins, mostly protein variants, are identified with these methods, and highly expressed proteins are easily located. The resulting differences in protein expression due to treatment with various stimulating factors are the basis for comparative gel electrophoresis maps. Proteins or peptides after separation by electrophoresis are identified by determining the sequence of amino acids comprising them. Traditionally, this was done by Edman degradation, which determined one amino acid at a time from the N-terminus of the proteins or peptides. However, the process of the identification of proteins was revolutionized by the development and application of the mass spectrometer in conjunction with the advances in genomics and bioinformatics, which made the gene and protein data available for the assignment of a particular peptide sequence to a protein and to the encoding gene.

Mass spectrometry

Mass spectrometry-based formats and industry preferences are still evolving. Proteomics applications that involve LC/MS are at similar stages of growth as drug metabolism applications during the late 1980s and early 1990s. To date, the

predominant application involves the qualitative analysis of proteins via automated database searching (i.e., protein expression profiling). Sensitive and accurate mass spectrometry approaches for quantitation of proteins appear to be destined for major advances. Mass spectrometry is unsurpassed capacity for accurate protein identification and quantitation. The principles of the mass spectrometer originated that molecules can be ionized, and the ionized molecules can be separated based on their mass-to-charge ratio by applying a magnetic force. The positively charged particles are the ionized molecules, whereas the negatively charged particles are the electrons. The results yield information about their molecular weights and chemical structure.

Components of the Instrument

A spectrometer consists of the following five major components: a port or device for the introduction of sample into the machine, a device for ionization of molecules, an analyzer for the separation of ionized molecules on the basis of their mass to charge (m/z) ratio, a detector that monitors the presence of the separated ions and records them, and a high vacuum system to allow free movement of ions within the spectrometer

Ion sources

In a mass spectrometer the role of the ion source is to create gas phase ions. Analyte atoms, molecules, or clusters are transferred into gas phase and ionized either concurrently (as in electrospray ionization) or through separate processes (as in the glow discharge). The choice of ion source depends heavily on the application. So called soft ion sources can produce intact ions of large fragile molecules.

Electrospray ionization (ESI) was first introduced by Dole and coworkers (1968) and coupled to MS by Yamashita and Fenn (1984). In ESI, a sample is vaporized by high voltage and then ions are generated as the solution of proteins or peptides is forced through a fine syringe. The sample is dissolved in a polar and transported through a needle placed at high positive or negative potential (Yamashita, et al., 1984; Aleksandrov, et al., 1984; Fenn, et al., 1989). The high electric potential (1 to 4 kV) between the needle and nozzle causes the fluid to form a Taylor cone, which is enriched with positive or negative ions at the tip. A spray of charged droplets is ejected from the Taylor cone by the electric field. The droplets shrink through evaporation, assisted by a warm flow of nitrogen gas passing across the front of the

ionization source (Figure 2). Ions are formed at atmospheric pressure and pass through a cone shaped orifice, into an intermediate vacuum region, and from there through a small aperture into the high vacuum of the mass analyzer. ESI has been used in conjunction with all common mass analyzers. The exact mechanism of ion formation from charged droplets has still not been fully elucidated and there are different theories proposed (Mora, et al., 2000; Iribarne, et al., 1976).

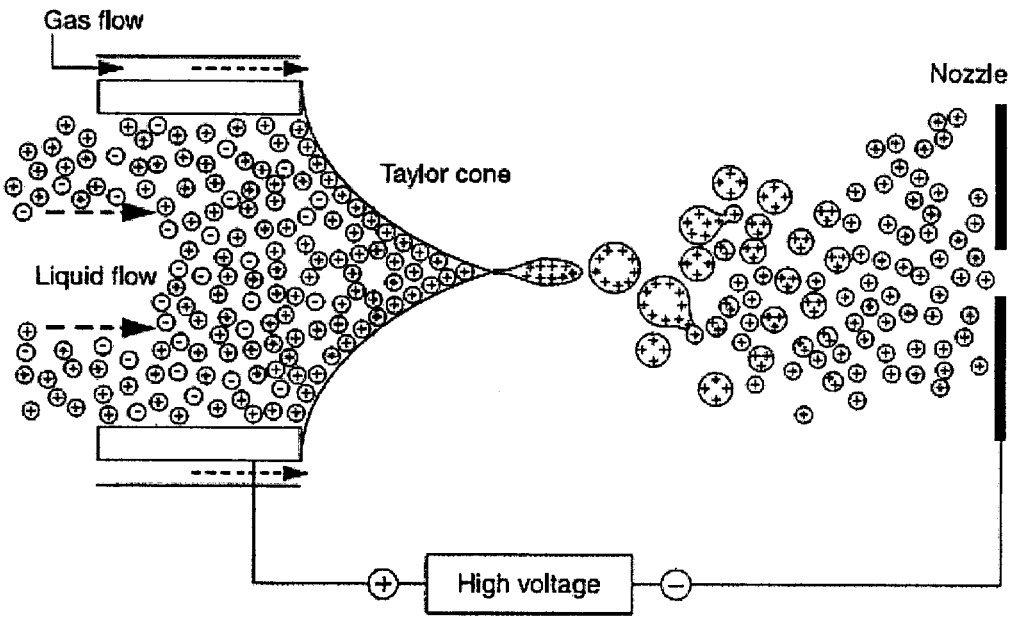

Figure 2 Schematic of electrospray ionization (ESI)

Source: Westman and Brinkmalm, 2002

Sample preparation requires only dissolution of the sample to a suitable concentration in a mixture of water and organic solvent, commonly methanol, isopropanol, or acetonitrile. A trace of formic acid or acetic acid is often added to aid protonation of the analyte molecules in the positive ionization mode. In negative ionization mode, ammonia solution or a volatile amine is added to aid deprotonation of the analyte molecules. The sensitivity of ESI-MS is good, with low femtomole or attomole detection levels for many peptides. However, the sensitivity of ESI is a function of the concentration of the injected sample. High flow rates, that is, 1 to 1000

mL/min in conventional ESI-MS, result in high sample consumption. It is therefore advantageous to use the lowest possible flow rate. A recent version of electrospray ionization called "nanospray ionization" has become more popular. In nanospray ionization, a much smaller volume of liquid as little as 1 nL/min is passed through the charged capillary needle. This results in generation of a finer spray with much reduced size of the ionized droplets and considerably higher sensitivity (Wilm, et al., 1996).

Mass analyzers

After the process of ionization, the ionized molecules of proteins or peptides enter the section of the mass spectrometer called the mass analyzer, where they are separated based on their mass-to-charge ratio by electric and/or magnetic fields or by measuring the time taken by an ion to reach a fixed distance from the point of ionization to the detector. Different kinds of analyzers are available for the separation of ionized molecules. Among the different kinds of analyzers, two particular kinds, called the quadrupole and the time-of-flight (TOF) analyzers, are the most important from the point of proteomics for their use in mass spectrometers. A particular spectrometer may use one or the other or at times a combination of both quadrupole and TOF analyzers. The separation should also be independent of the chemical conformation of the species. All mass analyzers presently in use are based on electromagnetism so ions are required to obtain separation. Therefore, an ion source has to be coupled to the analyzer. The analyzer will then separate ions coming from the source according to their m/z. There are several types of mass analyzers used in mass spectrometric research and they can be divided into different categories, such as magnetic or pure electric, scanning or non-scanning (pulse based), and trapping or non-trapping analyzers (Blaum, et al., 2006).

Ion trap analysers use a similar principle to quadrupole mass analysers but employ a system of entrance, exit and end-cap electrodes together with a ring electrode that surrounds the trap cavity (Figure 3). As with quadrupole so with ion trap, for each ion type with a given value of m/z there is a corresponding value of $\varphi 0$ when interactions between ion type and external quadrupole field are such as to enable the trapping of ion within the analyzer prior to release for detection. Ion traps are relatively inexpensive, quite sensitive and robust, so are fairly widespread, despite being less accurate than TOF and quadrupole mass analysers.

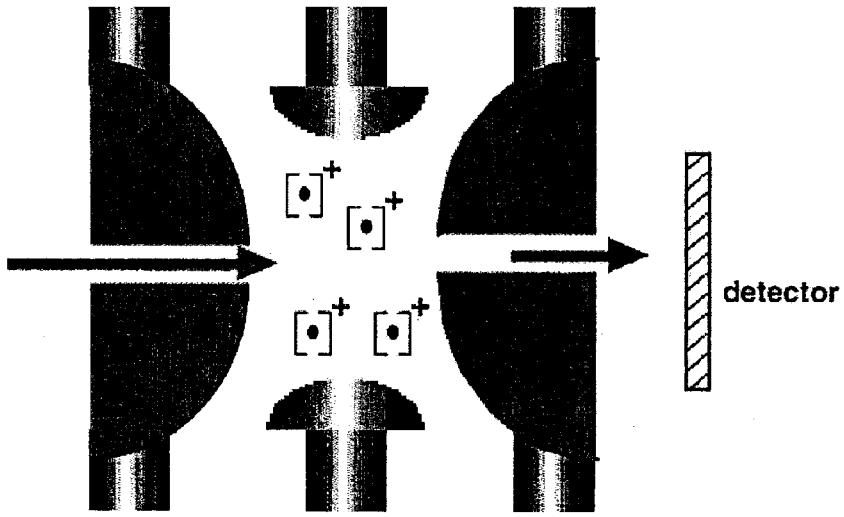

Figure 3 Schematic of an Ion Trap Mass Analyser.

Detectors

This is the final component of a mass spectrometer. Its purpose is to detect and record the presence of ions coming out of the mass analyzer hitting the detector. An electron is emitted when an ion hits the recorder and creates a small current. The low level of signal from a small number of ions coming out of the mass analyzer is amplified from 1,000 to 1 million times to become delectable and then recorded. A detector may use an electron multiplier or a photomultiplier. Photomultipliers first convert an electron produced by the ion hitting the detector plate into photon, which is detected by a phosphorescent plate in a sealed tube. Photomultipliers are preferred in a detector because they are located in a sealed tube, which reduces the noise-to-signal ratio by not allowing any outside interference to come out from the mass analyzer. All mass spectrometer are equipped with photomultipliers. These signals are then recorded on a graph by plotting the amount of signal versus m/z ratio. Mass spectrometer graphs usually show the presence of proteins/peptides of different molecular size and their abundance.

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 fed with 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 [redacted] 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 added with 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 15 min at 12,000 xg and air-dried before resuspending the protein pellet in 0.15% DOC solution.

3. Protein determination

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 (VERSA max™, Cape Cod, Inc, UK).

4. Denaturing Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 12% polyacrylamide gels mixed according to method 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 0.375 M 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, then increase the current to 70 V 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 auto sampler 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 1μl/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 identification and Gene ontology categories

DeCyder MS Differential Analaysis 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 and all peptides were detected with the PepDetect. The PepDetect module of the software was 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 DeCyder MS 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 DeCyder MS 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.

CHAPTER IV

RESULTS

Relative organ body weight ratio in rats after feeding with various concentrations of Trikatu

Analysis of organ weight in this study is an important endpoint for identification of potentially harmful effects of chemicals. Differences in organ weight among treatment groups are often accompanied with differences in body weight among these groups, making interpretation of organ weight differences more difficult. The relationship between organ weight and body weight was evaluated to determine which endpoint (organ weight and organ-to-body weight ratio) is likely to detect accurately drug target organ.

Tables 6 and 7 show the average daily body weights of rats in both experimental groups. In the acute group, 500 mg/kg Trikatu had no effect on the body weight but a significant decrease of body weight ($p<0.001$) was found in rats treated with 1,000 mg/kg Trikatu. In the sub-acute group, no change in body weight was observed in 50 and 150 mg/kg Trikatu treated male rats compared to controls.

The effect of Trikatu on weight of rat vital organs was shown in Tables 6 and 7. Liver weight was markedly increased in rats fed with high dose of Trikatu. The rats fed with 500 and 1,000 mg/kg Trikatu showed a significant increase in liver weight (14.75 and 35.1%, respectively) when compared to the control. A significant decrease of spleen weight was also found in 1,000 mg/kg Trikatu fed rats. In the sub-acute group, rats treated with 150 mg/kg Trikatu showed a significant increase in liver weight (24.22%), but rats treated with 50 mg/kg showed no change. Other organs weight in sub-acute group did not show any significant difference compared to the control.

Histopathological studies of rat liver after feeding with various concentrations of Trikatu

Hepatocytes compose about 60% of the liver arranged in plates or cords that radiate from the central vein to the portal areas. In two-dimensional sections, they are typically one-layer thick and formed anastomoses (Miyai, 1991). On one surface they are separated from the sinusoidal wall by a peri-sinusoidal space, the space of Disse, where they are exposed to tissue fluids. Kupffer cells are a self-renewing fixed macrophage composing about 10% of all liver cells (Eustis, et al., 1990). Kupffer cells are phagocytic, secrete mediators of inflammation, and catabolize lipids and proteins (Figures 4 and 5).

In this experiment, daily exposure of high doses of (500 and 1,000 mg/kg B.W.) Trikatu for 7 days caused a significant pathomorphological changes in rat liver. Hepatocyte showed hydropic degeneration. The hydropic change in hepatocytes is a type of cytoplasmic clear areas alteration manifested on H&E-stained paraffin sections as clear spaces in the cytoplasm and a centrally located nucleus when compared to control. Because of disturbance of the cell membrane integrity, accumulation of intracytoplasmic fluid may occur. This change can be caused by xenobiotics with differing lobular localization and may be a precursor to hepatocyte necrosis (Gkretsi, et al., 2007; Wang, et al., 2007; Peichoto, et al., 2006; Matsumoto, et al., 2006; Chengelis, 1988). Pathomorphological changes in liver of male rats exposed to lower doses (150 mg/kg B.W.) of Trikatu for 30 days are similar to high dose (Figures 5C and F). On the other hand, liver tissue from rats treated with 50 mg/kg Trikatu did not exhibit any significant pathological changes (Figures 4A and D).

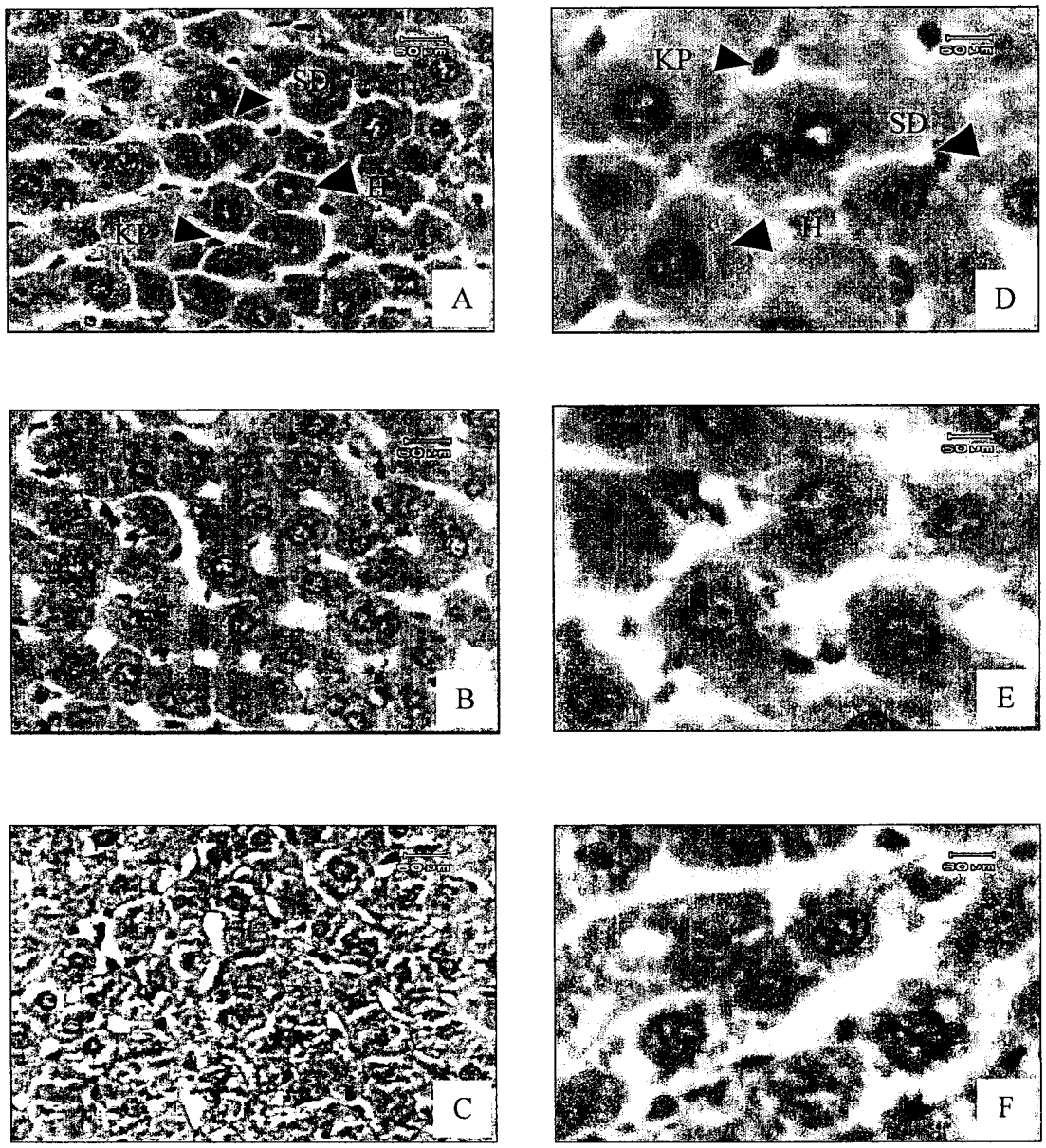

Figure 4 Acute effect of Liver histopathology

Representative liver tissue sections stained with hematoxylin and eosin, from male rats treated with Trikatu 500 mg/kg B.W. (B, E) and 1,000 mg/kg B.W.(C, F) for seven days as compared to controls (A, D). The arrows show Kupffer cells (KP) in the sinusoid (SD) that is slight space of the hepatocyte (H). The right column shows magnifications of the showed areas in the left column. Magnifications: left column, ×40; right column, ×100.

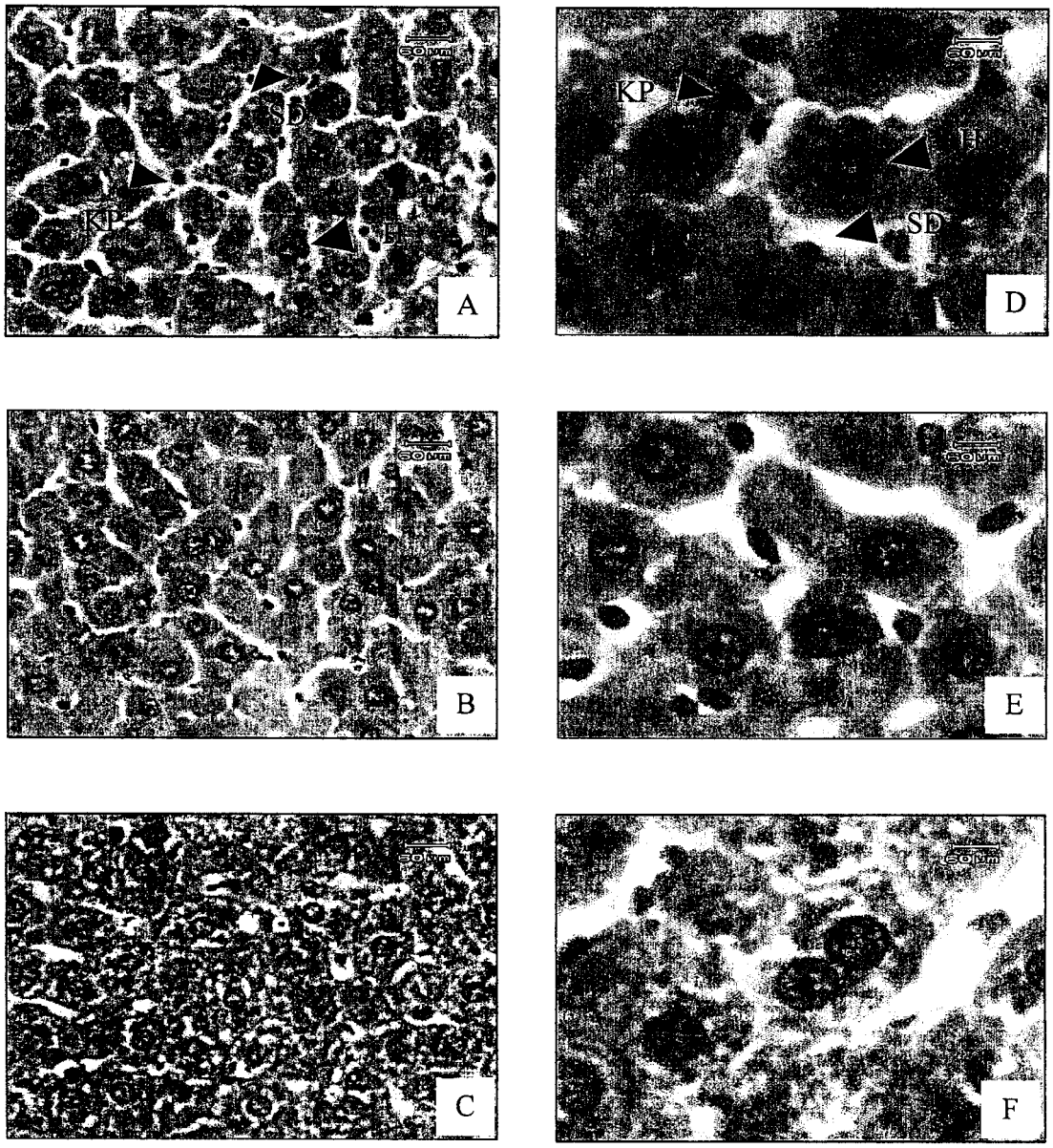

Figure 5 Sub-acute effect of Liver histopathology

Representative liver tissue sections stained with hematoxylin and eosin, from male rats treated with Trikatu 50 mg/kg B.W. (B, E) and 150 mg/kg B.W. (C, F) for 30 days as compared to controls (A, D). The arrows show Kupffer cells (KP) in the sinusoid (SD) that is slight space of the hepatocyte (H). The right column shows magnifications of the showed areas in the left column. Magnifications: left column, ×40; right column, ×100.

Liver functional test in rats after feeding with various concentrations of Trikatu

The total proteins and activities of ALT, AST in rat serum are shown in Figures 6 and 7. The acute studies of rats treated with Trikatu at the concentrations of 500 and 1,000 mg/kg did not show any significant changes in activities of AST and ALT compared to the control. Similarly, activities of both enzymes were not altered in the sub-acute rats treated with 50 and 150 mg/kg Trikatu. The ratio of AST to ALT is often used to diagnose toxicity upon liver. However, all rat treated groups showed no change in serum AST or ALT.

Serum lipid profile in rats after feeding with various concentrations of Trikatu

The effects of Trikatu on serum triglyceride, cholesterol and HDL-c are shown in Figures 8 and 9. Rats treated 500 and 1,000 mg/kg Trikatu showed a significant decrease ($P>0.05$) of triglyceride levels. The triglyceride level of the acute Trikatu treated rats was about 27.5% as compared with the level of control rats. Similarly, sub-acute oral administration of 50 and 150 mg/kg revealed a significant decrease ($P>0.01$) of cholesterol. This effect was determined to be about 37% as compared with the control group. The change in level of cholesterol was found in sub-acute treatment only. The 50 mg/kg Trikatu reduced cholesterol level in rat serum about 8.6% and stronger effect was detected in rat treated with 150 mg/kg Trikatu. The serum cholesterol can be lowered to 26.5% when compared with the control. The levels of HDL-c were not changed after treatment with the acute or sub-acute with Trikatu.

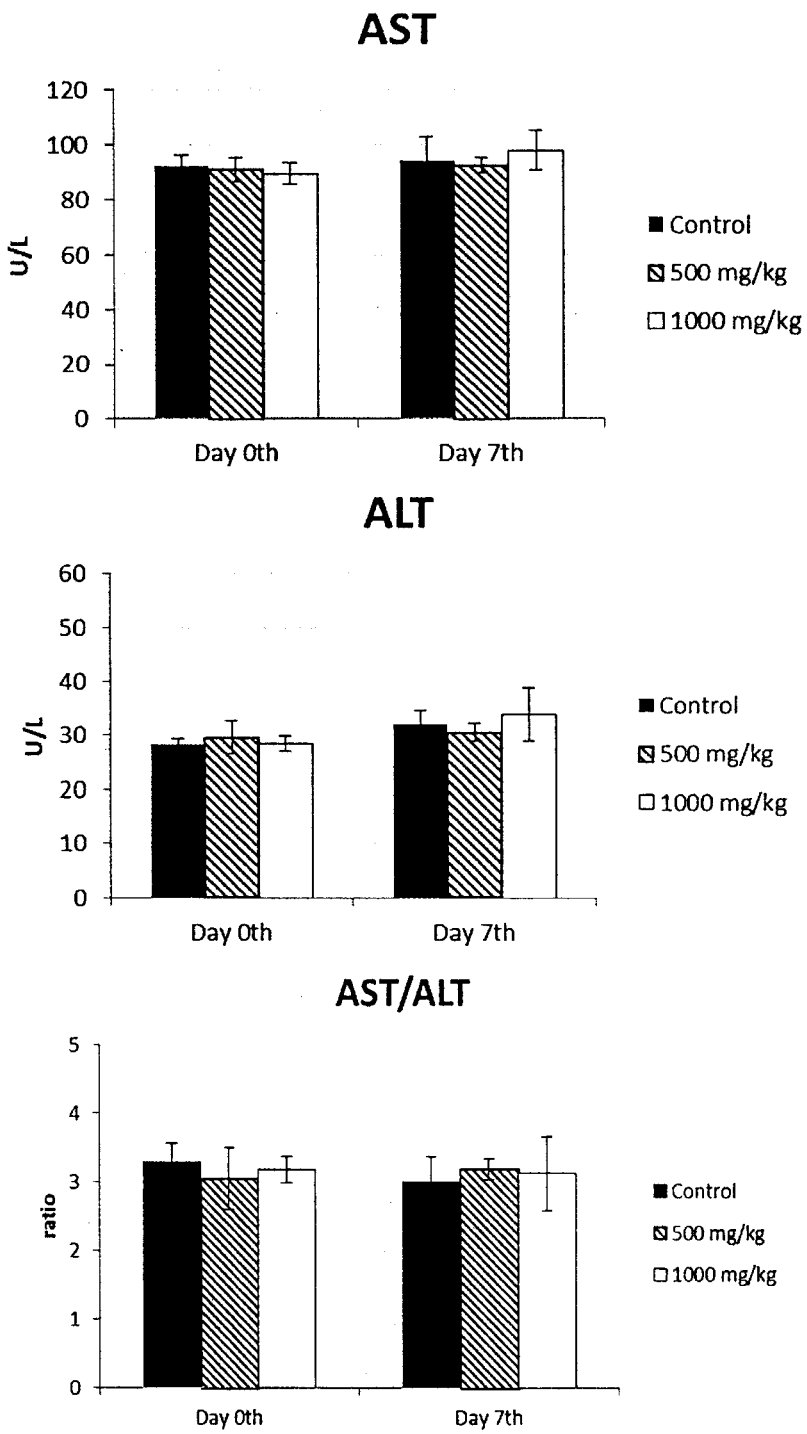

Figure 6 Serum transaminase activity in male Wistar rats after treatment with 500 mg/kg and 100 mg/kg Trikatu for 7 days.

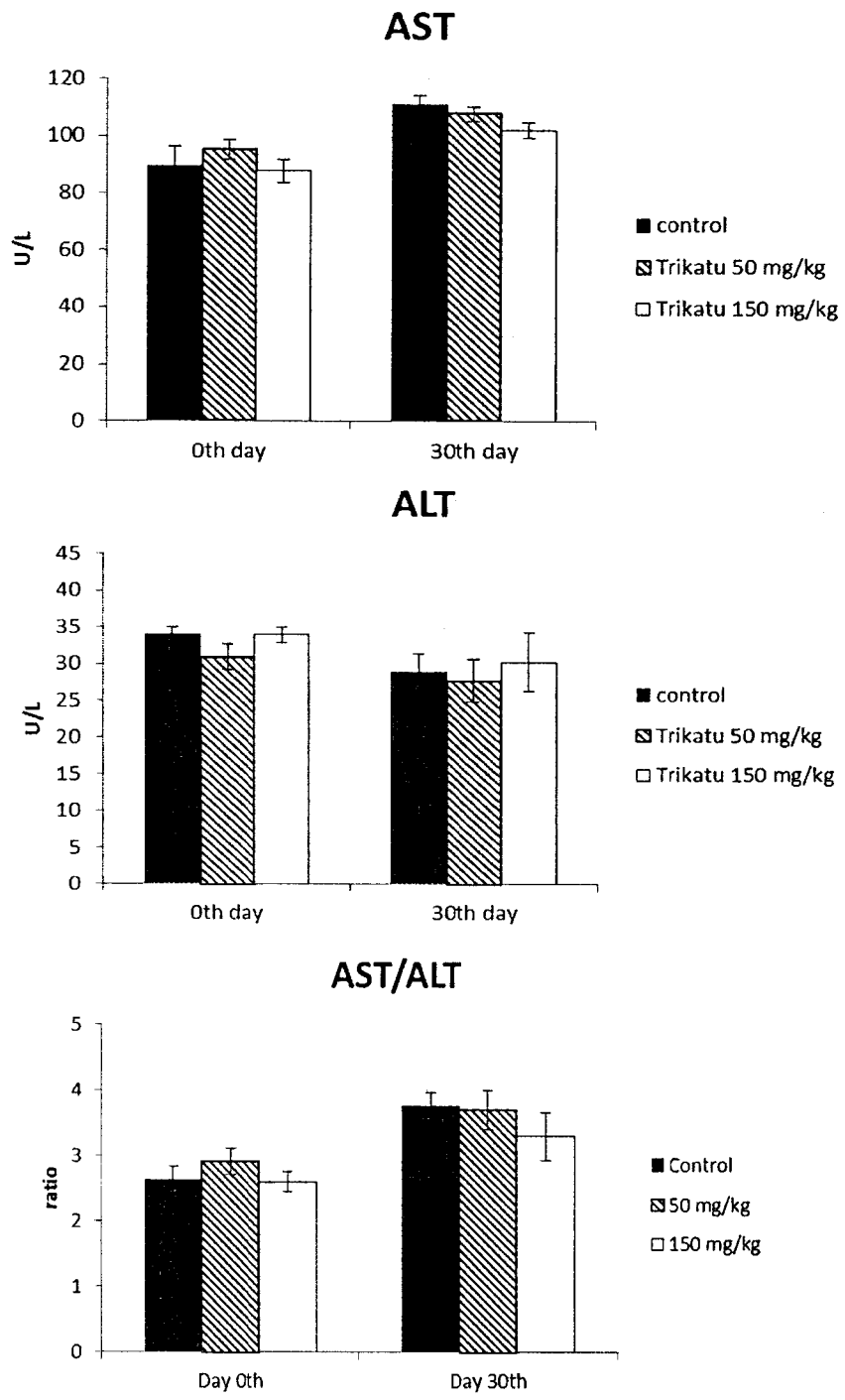

Figure 7 Serum transaminase activity in male Wistar rats after treatment with 50 mg/kg and 150 mg/kg Trikatu for 30 days.

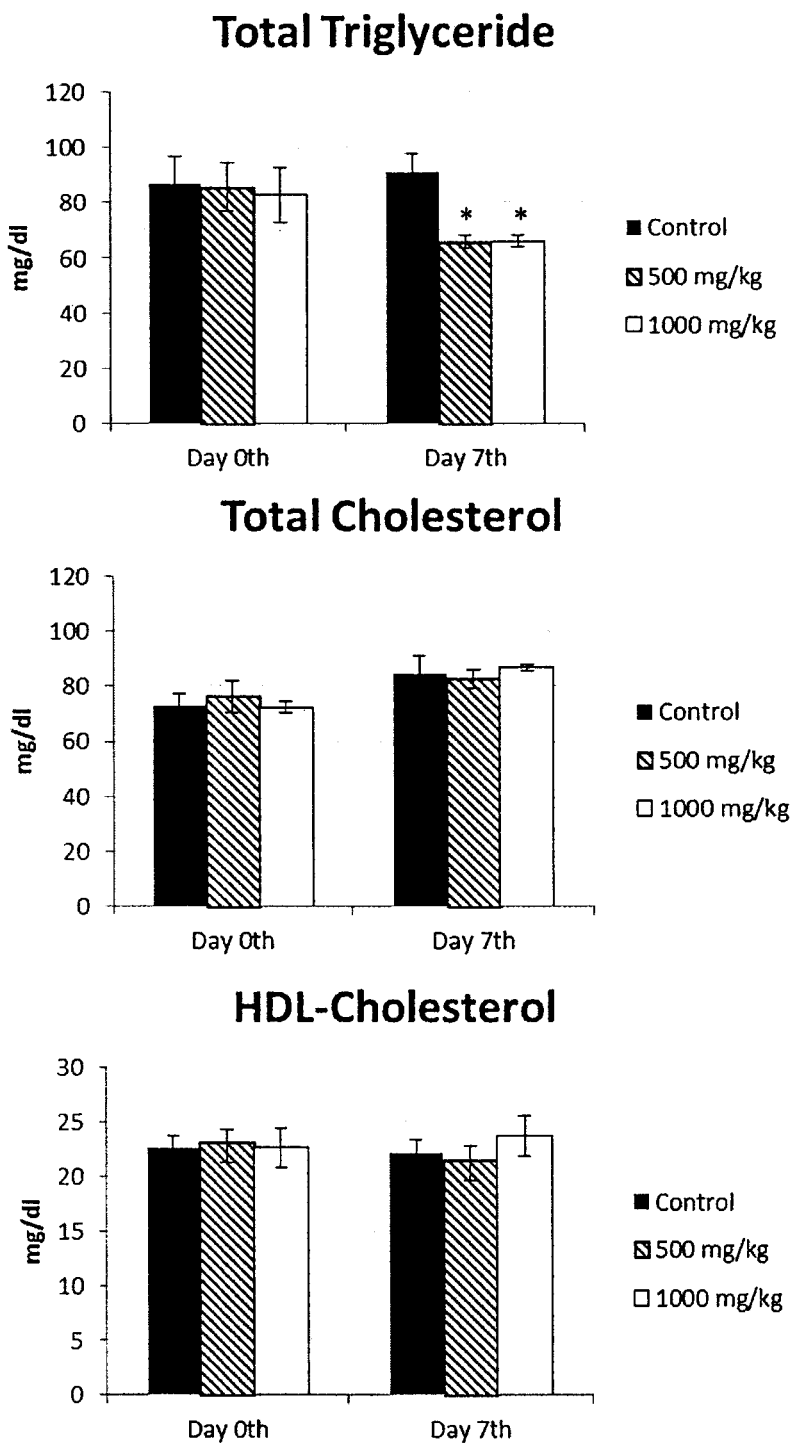

Figure 8 Serum lipid profiles in male Wistar rats after treatment with 500 mg/kg and 100 mg/kg Trikatu for 7 days.

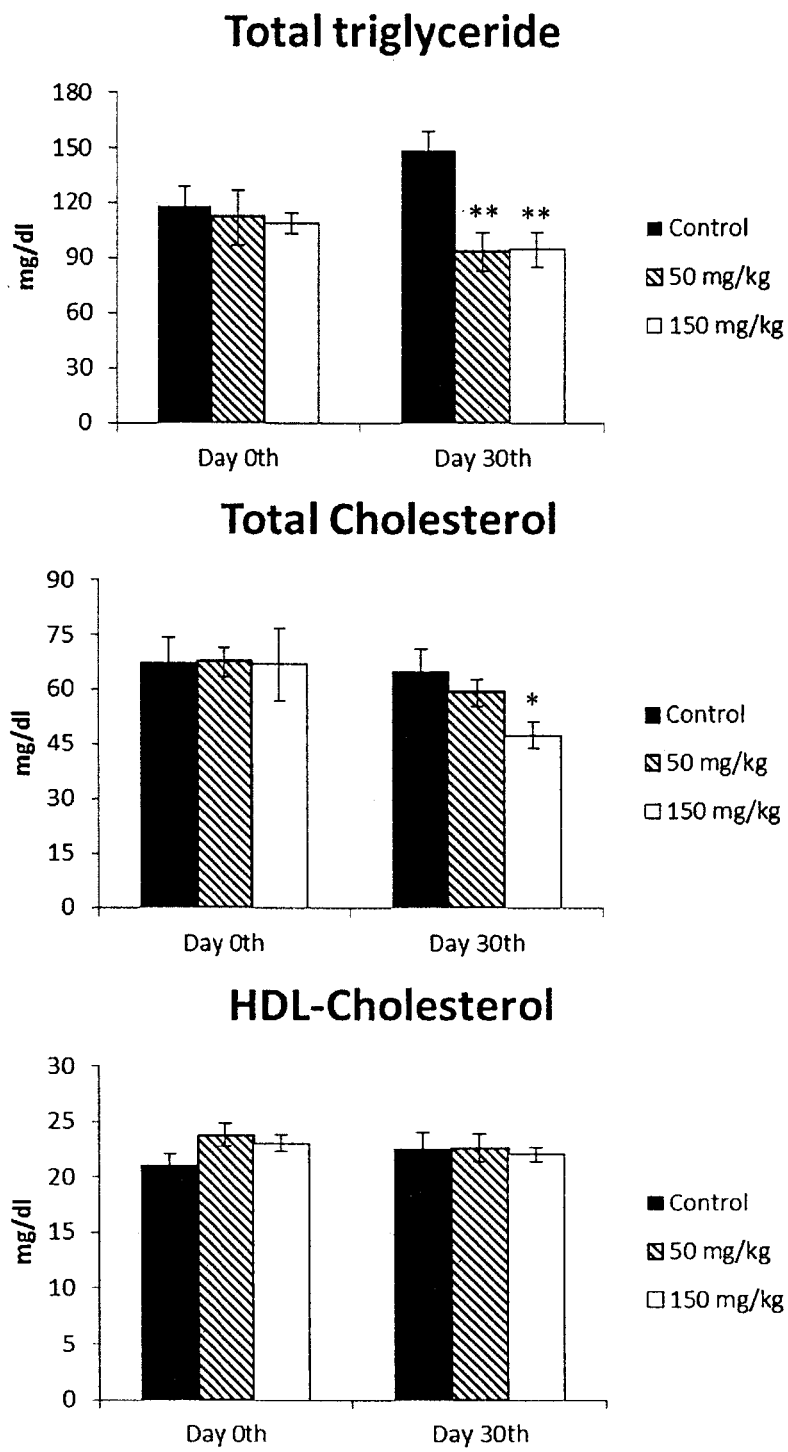

Figure 9 Serum lipid profiles in male Wistar rats after treatment with 50 mg/kg and 150 mg/kg Trikatu for 30 days.

GeLC-MS analysis of rat liver treated Trikatu

SDS-PAGE analysis of total proteins extracted from rat liver is shown in Figure 10. Few differences in protein pattern were observed between sub-acute and acute group. Metabolite or phenolic compound produced in sub-acute group may cause higher intensity of protein bands. However, the protein samples on SDS-PAGE were cut into twelve fractions (A to L) before in-gel digestion. The obtained peptides were analyzed by nano-LC-MS/MS. The mass spectrum was normalized with an internal tryptic digested BSA and evaluated by DeCyder^{TM} MS. Protein abundance data were analyzed to filter out proteins with statistically significant ($p<0.05$). The result showed that more than 1,423 proteins were differentially expressed. The biological processes of these proteins were classified by STRAP software.

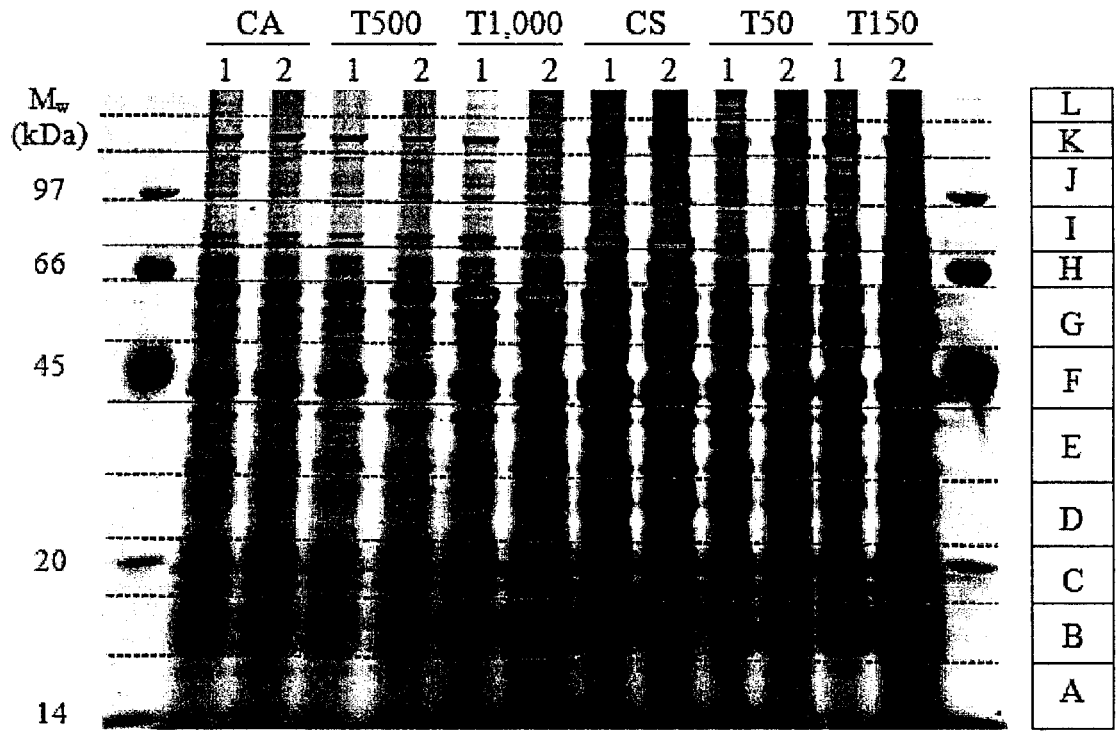

Figure 10 Fifty micrograms of total protein were separated by 1D-PAGE followed by slicing gel lanes into 12 fractions and analyzed the level of tryptic peptides in each gel plugs by LC-MS/MS.

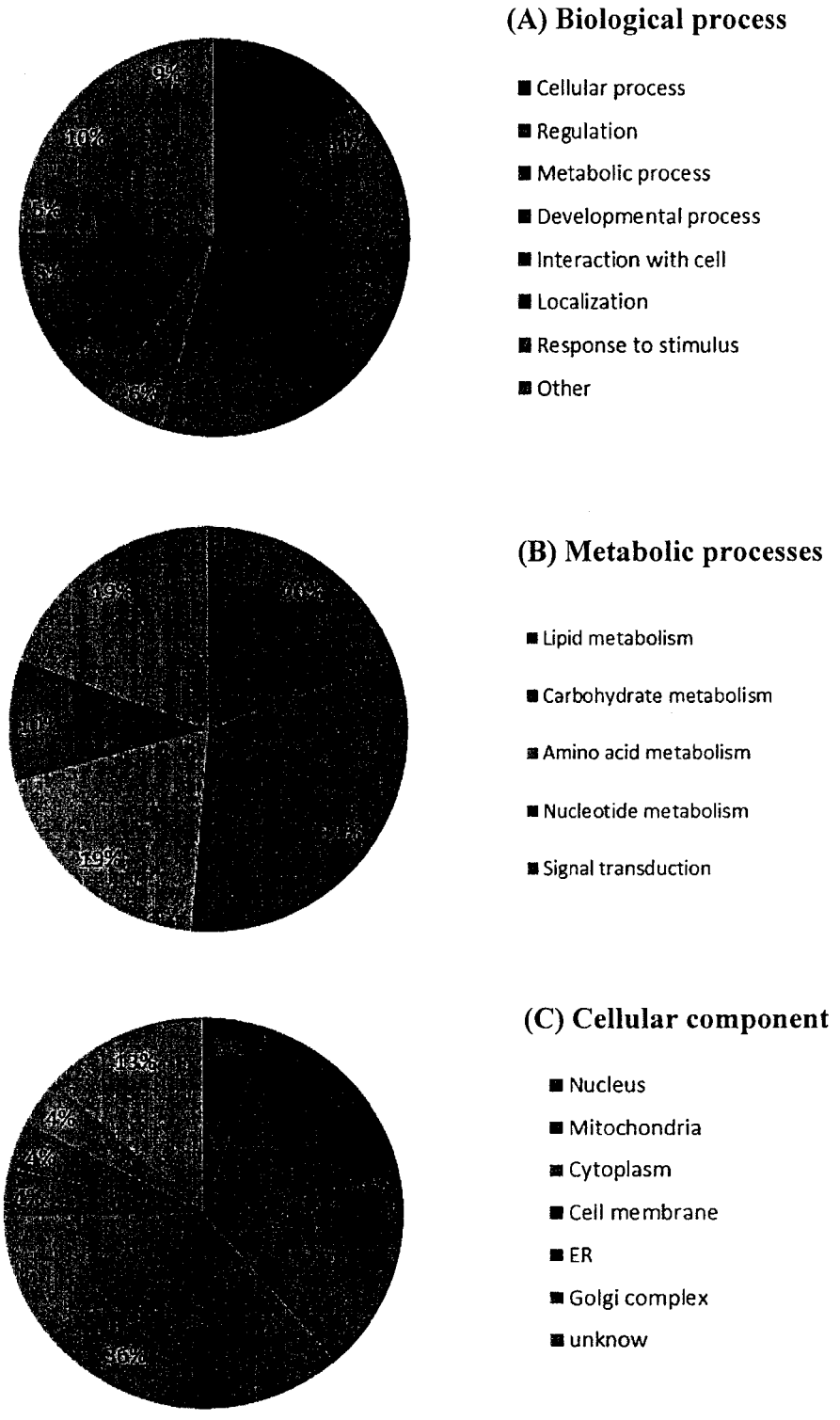

Figure 11 Protein classifications by STRAP software

The identified proteins were grouped according to their biological functions, derived from the annotated hit in the UniProtKB database and presented in Figure 11a. The proteins in metabolic process (76 proteins) were involved in lipid metabolism (14 proteins), carbohydrate metabolism (23 proteins), amino acid metabolism (14 proteins), nucleotide metabolism (7 proteins) and signal transduction (18 proteins) (Figures 11b and Table 8).

The metabolic proteins were subjected to a network interaction analysis by STRING database, which integrates protein-protein interaction data from various sources, including experimental repositories, structural computational analysis, previous knowledge and differentially expression proteins in many organelles. Interestingly, many proteins are localized in mitochondria and cytoplasm (Figure 11c). They are involved in many metabolic processes such as glycolysis, gluconeogenesis, TCA cycle, oxidative phosphorylation, lipolysis, and protein degradation. Moreover, insulin receptor and protein in insulin regulation process are linked to this network (Figure 12). These data can be used to predict the effect of Trikatu on lipid and glucose metabolism, which is believed to involve in lowering triglyceride level in rat serum.

The candidate proteins altered after Trikatu treatments were statistically analyzed by Mev software. Significance proteins were determined by one-way ANOVA and p-value cut-off of 0.05 were adjusted-alpha mode testing correction. Dihydrolipoamide acetyltransferase (Dlat) and methylcrotonoyl-CoA carboxylase alpha (Mccc1) were significantly up-regulated ($p<0.05$). Dlat is an enzyme component of the pyruvate dehydrogenase complex. The increase of this protein may cause an increase in the transformation of pyruvate from glycolysis into acetyl-CoA which is then used in the citric acid cycle to carry out cellular respiration. Mccc1 is a biotin-requiring enzyme located in the mitochondria. The increase of this protein may accelerate the breakdown of leucine to eventually yield acetyl CoA. Whereas liver glycogen phosphorylase (Pygl) and carnitine palmitoyl transferase 1 (Cpt1a) significantly down-regulated ($p<0.01$). Pygl catalyzes the rate-limiting step in the degradation of glycogen into glucose. The inhibition of glycogen phosphorylase has been proposed the release of glucose as one method for treating type 2 diabetes. Cpt1a is associated with the outer mitochondrial membrane and mediates the transport of

long-chain fatty acids across the membrane. This inhibition is a good target for future attempts to regulate Cpt1a for the treatment of metabolic disorders such as diabetes. Our candidate proteins are important to describe the toxicity and pharmacological action of Trikatu *in vivo*. However, the expression level of these proteins remained to be confirmed.

CHAPTER V

DISCUSSION AND CONCLUSION

Effect of acute Trikatu treatment in rat

Relationships between short-term treatment (7 days) and long-term treatment (30 days) of trikatu were evaluated both physically and biochemically. The body weight and relative weight of vital organs were monitored. Analysis of body weight and organ weight in pharmacological study is an important endpoint for identification of potentially harmful effects of chemicals (Steven, et al., 2004). Changes in either body weight or vital organs weight are assess to the pharmacological effects of Trikatu in rat. The results showed that body weight of rats treated with 500 mg/kg Trikatu were not changed, but 1,000 mg/kg Trikatu caused a decrease in body weight. A significant increase in liver weight was also found in both acute treatment and control. Interestingly, spleen weight decreased in 1,000 mg/kg treated rats. The administration of Trikatu at 1,000 mg/kg body weight may be not only toxic to liver and spleen but also induced the immunological response in rat.

In this case, histopathological changes in liver of rats fed with various concentrations of Trikatu were studied. Administration of Trikatu at 500 mg/kg showed minimal effect of hydropic degeneration on the liver. No apparent disruptions of the normal liver structure were detected. However, administration at 1,000 mg/kg Trikatu showed a mild hepatic injury. The administration of the Trikatu at 1,000 mg/kg may induce the hepatotoxicity rapidly with unknown mechanisms and allows accumulation of water in cytoplasm and between hepatocytes. Serum ALT and AST activity were used to state the hepatocellular membrane permeability (Giboney, 2005). ALT is a cytoplasmic enzyme, and is considered to be liver specific. AST is presented in both the cytoplasm and mitochondria of hepatocytes. No change in activities of both enzymes was detected after treatment with Trikatu. It is indicated that membrane permeability of hepatocytes did not affected by Trikatu. However, necrosis and cirrhosis/fibrosis of the liver cannot be excluded.

Effect of subacute Trikatu treatment in rat

Oral administration of 50 and 150 mg/kg Trikatu for 30 days was called the sub-acute studies. The body weights of rats after treatment with low concentration of Trikatu for a month were similar to control. Absolute and relative liver weights of rats treated with 150 mg/kg Trikatu increased. A hydropic swelling in liver section was detected. However, changes in liver weight and histopathology of hepatocytes were not found in rats treated with 50 mg/kg Trikatu. Serum ALT and AST did not elevated in both groups. Non-significant effect of 50 mg/kg Trikatu on rat livers was similar to Chanda and colleague (2009). Long-term administration of low doses of Trikatu may be considered as relatively safe, as it did not produce severe toxicological effects on body and organs of rats.

Effect of Trikatu on lipid profile in rat

Trikatu reduced serum triglyceride in acute and sub-acute groups, but HDL-cholesterol levels did not changed as compared to control. The effectiveness of reduced triglyceride was found in sub-acute group. In sub-acute group, Trikatu decreased triglyceride and cholesterol in rat serum. The cholesterol-lowering abilities of Trikatu appear to be dose-dependent. The obtained results were in agreement with the first report of Valsala and Sivakumar (2004).

Piperine is a major of active compound of long pepper and black pepper. Treatment with piperine significantly reduced not only the serum triglycerides and total cholesterol levels, but also significantly increased the HDL level, which proved its beneficial effect in reducing dyslipidemia (Shreya, et al., 2011). Srinivas (2009) investigated effects of the ethanolic extract of ginger for lipid regulating activities in high-fat diet-fed rat. The result showed that total cholesterol and triglycerides in rat serum were significantly reduced by ginger treatment. However, no significant change in serum HDL-cholesterol. Prakash and Srinivasan (2007) studied effect of dietary test spices on serum lipid profile in normal rats. They showed that piperine and ginger prominently decreased the level of serum triglycerides. The decrease in serum triglyceride was 28.2% and 27% in normal rats, respectively. Taken together, it is suggested that the decreased triglycerides and cholesterol and increased HDL-

cholesterol effect induced by Trikatu may improve dyslipidemia and prevents the risk of atherosclerosis and heart attacks.

Effect of Trikatu on protein expression in liver

Low concentration of Trikatu decreases levels of triglyceride and cholesterol in rat serum. The mechanism underlying this novel finding remained unclear. A proteomic approach is therefore selected to study the proteome change after treatment with Trikatu.

Proteins related to lipid metabolisms

Liver was targeted because it is a primary site of lipid metabolism (Nguyen, et al., 2008). The proteomics data also showed that the level of several proteins changed in response to Trikatu treatment. Of which 5 proteins were related to fatty acid oxidation, and 3 proteins were related to triglyceride synthesis and transport. The carnitine palmitoyltransferase I (Cpt-1a), short-chain-acyl-CoA dehydrogenase (Acads), long-chain-acyl-CoA dehydrogenase (Acadl), enoyl-CoA hydratase (Echdc) and acetyl-Coenzyme A acyltransferase 2 (Acaa2) were involved in the fatty acid oxidation within mitochondrial. These proteins were significantly decreased by Trikatu treatment. The Cpt-1a is an essential step in the beta-oxidation of long chain fatty acids (Kerner and Hoppel, 2000), while fatty acids are activated on the outer mitochondrial membrane and mediates the transport of long-chain fatty acids across the membrane by binding them to carnitine, the activated fatty acids are oxidized within the mitochondrial matrix in β-oxidation process (van, et al., 2000; Bonnefont, et al., 2004; Berg, et al., 2007). Cpt-1a is inhibited by malonyl-CoA, although the exact mechanism of inhibition remains to be known (Bonnefont, et al., 2004; Akkaoui, et al., 2009; Song, et al., 2010). The inhibition of Cpt-1a can be a way to prevent simultaneous oxidation of fatty acids within the liver cells. Consequently, a decrease of Cpt-1a after Trikatu treatment may be a good target for future attempts to regulation of fatty acids oxidation in metabolic disorders patients.

The inhibition of lipid transport may affect to a reduces fatty acid oxidation processes in mitochondria. Generally, mitochondrial β-oxidation of fatty acid is a cyclic process by fatty acyl-CoA dehydrogenase such as long-chain (Acadl), medium-chain (Acadm), and short-chain acyl-CoA dehydrogenase (Acads) that work on

catalyzed fatty acids which become progressively shorter, respectively (Thorpe and Kim, 1995). Enoyl-CoA hydratase (Echdc) catalyzes the second step in the breakdown of β-oxidation. The final step of β-oxidation is the cleavage of 3-ketoacyl CoA by the thiol group of another molecule of CoA. This reaction is catalyzed by Acetyl-CoA acyltransferase (Acaa). The complete breakdown of a fatty acid not only generates these acetyl-CoA molecules, but also generates a great deal of energy in the form of NADH and $FADH_2$ then feeding electrons into the electron transport system for oxidative phosphorylation to ATP (Nelson, et al.,2005). The result of proteomics showed a decrease of enzyme in β-oxidation such as Acadl, Acads, Echdc and Acaa2. This result indicated a decrease in fatty acid oxidation due to Trikatu treatment. Thus, inhibition of fatty acid uptake into mitochondria and reduction of enzymes in fatty acid oxidation simultaneously could occur simultaneously. The expression level of these enzymes may cause a decrease of acetyl-CoA produced from fatty acids within the liver (Figure 13).

Lipogenesis is the process by which acetyl-CoA is converted to fatty acid molecule (Kersten, 2001). The former is an intermediate stage in metabolism of simple sugars, such as glucose, a source of energy of living organisms. Lower expression of acetyl-CoA carboxylase 2 (Acc2) may affect the conversion rate of fatty acyl-CoA to malonyl-CoA, a molecule that is committed to fatty acid synthesis (Abu, et al., 2003). Lower expression of 1-acylglycerol-3-phosphate O-acyltransferase 1 (Agpat1) which is involved in glycerol synthesis from glycolysis was also reported. The construction of fatty acids from fatty acyl-CoA is combined with glycerol 3-phosphate, to form triacylglycerol. Thus, the decreased levels of these two enzymes in Trikatu treatment may lower concentrations of triglycerides. On the other hand, the triglyceride products may be secreted from the liver into the bloodstream in form of very-low-density lipoproteins (VLDL). The proteomics results showed a decrease in microsomal triglyceride transfer protein (Mttp) and apolipoprotein E (ApoE), which are involved in the regulation of synthesis and secretion of VLDL in the liver (Figure 13). The decrease serum triglyceride levels by Trikatu may be consistent with decrease triglyceride synthesis and/or secretion of VLDL from liver.

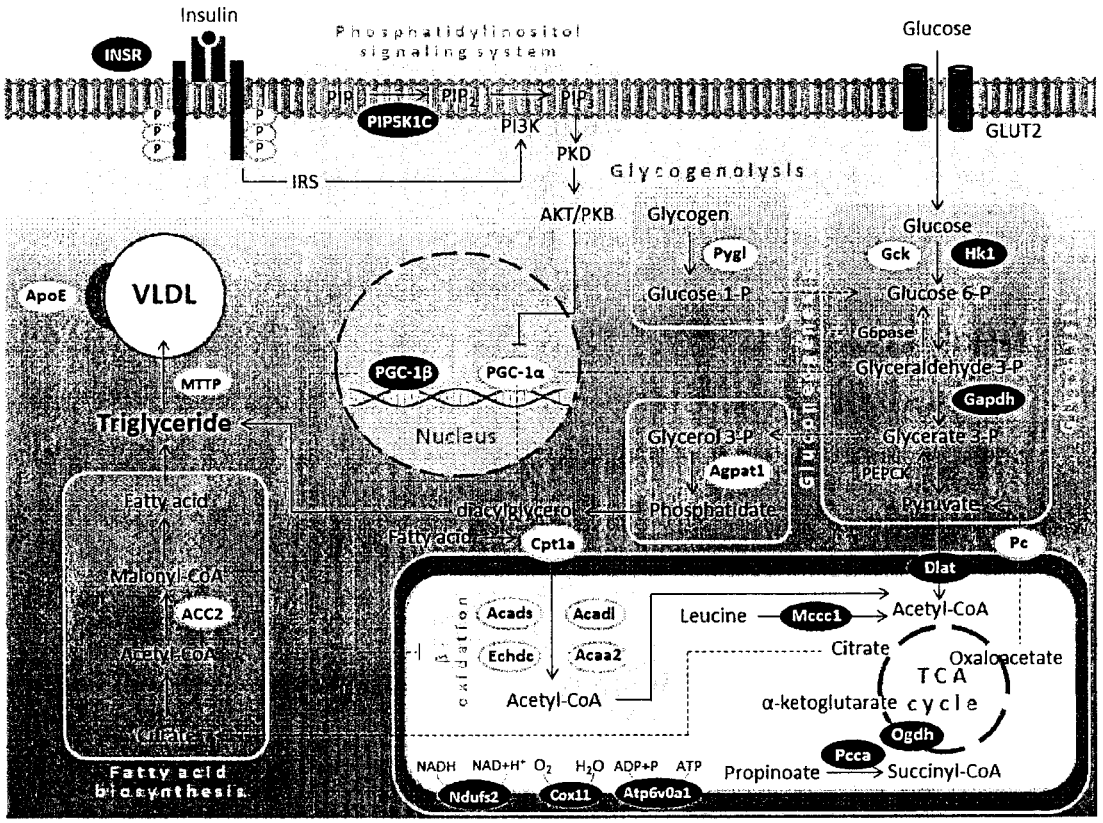

Figure 13 Schematic illustration of proposed mechanism of Trikatu treatment in rat liver. Black circles represent up-regulation and white circles represent down-regulation of protein expression.

Proteins related to carbohydrate metabolism

The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in bloodstream (Postic, et al., 2004). In this study, many upregulated proteins as a result of Trikatu were associated with glycolysis, gluconeogenesis, tricarboxylic acid cycle and electron transport chain. Glycolysis is the metabolic pathway that converts glucose into pyruvate. A molecule of glucose is metabolized through ten-stepwise chemical transformations in cytosol of the liver. Trikatu can induce various enzymes during this process. Hexokinase (Hk) and Glucokinase (Gck) are enzymes catalyzed phosphorylation of glucose to yield glucose 6-phosphate (Robey and Hey, 2006). The increased of Hk1 in Trikatu treatment indicated utilizing glucose as an energy source.

Glyceraldehyde phosphate dehydrogenase (Gapdh) is dehydrogenated to glyceraldehyde-3-phosphate in the first step in the second phase of the breakdown of glucose. Trikatu caused an increase of Gapdh enzyme that affects higher level of cellular 1,3-bisphosphoglycerate. These involved to net gain of the energy-rich molecules ATP and NADH. Then, the complete step of a glycolysis generates more pyruvate molecules. The pyruvate is metabolized by pyruvate dehydrogenase complex (PDHc) enzymes. The PDHc is responsible for the pyruvate decarboxylation step that links glycolysis into acetyl-CoA which is then used in the citric acid cycle. Trikatu could increase the level of Dihydrolipoyl transacetylase (Dlat), which is an enzyme component of the multienzyme PDHc. Dlat transferred the acetyl group from acyl-lipoamide to coenzyme A (CoASH). This results in the production of acetyl CoA, which is the end goal of pyruvate decarboxylation. High Dlat activity may also influence with high production of acetyl CoA. Most of acetyl-CoA molecules enter the TCA cycle in mitochondria and generate energy by electron transport chain.

Alpha-ketoglutarate dehydrogenase (Ogdh), one subunit of the 2-oxoglutarate dehydrogenase complex increased after Trikatu treatment. This complex catalyzes the overall conversion of alpha-ketoglutarate to succinyl-CoA and CO_2 during the citric acid cycle. Interestingly, Trikatu can increase several enzyme complexes in electron transport chain such as NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (Ndufs2), cytochrome c oxidase assembly protein 11 (COX11), cytochrome c oxidase 7A2 (COX7A2), cytochrome c oxidase subunit 4 (COX4) and ATPase, H+ transporting, lysosomal V0 subunit A1 (Atp6v0a1). Higher electrochemical proton gradient may generate more energy in the form of ATP which powers most cellular reactions (Figure13).

Regulation of hepatic lipid and glucose metabolism

The liver plays a key role in glucose homeostasis, lipid and energy metabolism. The result of proteomics showed that Trikatu increased insulin receptor (Insr), it responses to many pathways including the stimulation of lipid synthesis and storage, glycolysis and glucose storage, and the inhibition of ketogenesis and gluconeogenesis (Shaham, et al., 2008). The activation of insulin receptor can activate the intermediated proteins in PI3K-PDK-Akt/PKB cascade (Fritsche, et al., 2008). Trikatu activated phosphatidylinositol-4-phosphate 5-kinase (PIP5K1C) that is an enzyme

catalyzes phosphatidylinositol-4-phosphate (PIP) to phosphatidylinositol-4,5-bisphosphate (PIP2). PIP2 act as substrates for enzymes PI3K, which produced phosphatidylinositol-3,4,5-trisphosphate (PIP3) in plasma membrane. PIP3 regulates main classes of signalling molecules as phosphoinositide-dependent kinase 1 (PDK1), one of the serine kinases that phosphorylates and activates the serine/threonine kinase Akt/PKB (Alessi, et al., 1997). This influenced the increased glucose transports into the liver cells (Cheng, et al., 2010). Moreover, Trikatu may modulate gene transcription by activating various transcription factor such as peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) and beta (PGC-1β) in the liver.

Trikatu repressed PGC-1α by AKT cascade that controls the transcription of genes involved in gluconeogenesis, fatty acid β-oxidation and ketogenesis (Yoon, et al., 2001; Lin, et al., 2005; Finck, et al., 2006; Liang, et al., 2006). PGC-1α expression is relatively low in liver that rely in fed conditions (Daitoku, et al., 2003). The essential role of PGC-1α is induced of hepatic gluconeogenesis enzymes expression such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (Leone, et al., 2005). Thus, the reduction of PGC-1α may inhibit gluconeogenic metabolism. PGC-1β is a recently identified homologue of PGC-1α. It appears that the expression levels of PGC-1β correspond to the mitochondrial content (Meirhaeghe, et al., 2003). When overexpressed in mice, PGC-1β activity may increase in mitochondria and intracellular organelles that turn sugars and fats into heat or the cellular fuel ATP (Lelliott, et al., 2006; Junichiro, et al., 2007). PGC-1β coactivated with FOX2 that regulates hepatic lipid homeostasis by affecting the clearance rate of fatty acids through oxidation and/or secretion of lipids in response to insulin (Christian and Markus, 2006; Wolfrum, et al., 2006). Therefore, the activation of PGC-1β in Trikatu treatment may increase the energy in form of ATP and reduce serum lipid by stored its precursor in hepatocytes.

Conclusion

Trikatu has an antihyperlipidemic effect. It also has capability in reducing triglycerides and cholesterol levels. The best model of this observed study is the oral administration of Trikatu at 50 mg/kg for 30 day, which showed no obvious toxic in the liver.

This is the first proteomics study of rat liver after oral administration of Trikatu. Several proteins related to carbohydrate and lipid metabolism were altered during treatment. Up-regulations of proteins related to glycolysis and oxidative phosphorylation were reported. Conversely, down-regulations of proteins involved in lipid oxidation and triglyceride synthesis were illustrated. These Trikatu responsive pathways could be regulated through insulin receptor system. Higher level of insulin receptor accelerates glucose transport and simultaneously increase the activity of the regulatory enzymes in glycolysis. In addition, Trikatu may regulate transcription factor that leads to a decrease of fatty acid oxidation and triglyceride synthesis.

Several interesting proteins may be of benefit to further study the effect and toxicity of drugs or natural products on lipid controlling. The expression level of these proteins is necessary to be validated and the knowledge obtained will allow a better understanding of the biological pathways underlying the pharmacological of Trikatu.

Proteomic analyses of rat liver proteins affected by "Trikatu" a Thai herbal formulation

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Abstract

Trikatu is a traditional herbal formulation consisting of three herbs in equal amount, *Piper nigrum*, *Piper longum* and *Zinggiber officinale*. It is suggested to be beneficial in control lipids in the body. However, the scientific evidence supporting such claim has not yet been fully elucidated. The 200-250 g male Wistar rats were randomly divided into two groups (8 rats per group). The tested group was treated with 100 mg/kg of Trikatu and the control group was treated with the extract solution. Liver samples were collected after 4 weeks of treatment. Rat fed with Trikatu showed a significant decrease in the serum triglyceride level. Using SDS-PAGE coupled LC-MS/MS analysis, the comparative proteomics profiling of rat liver treated with Trikatu and control was reported. A down-regulation of 10 proteins in liver were observed after Trikatu treatment such as carbonic anhydrase 12, low-density lipoprotein receptor-related protein 3, TRAF3-interacting JNK-activating modulator, Selenoprotein O, Rho GTPase-activating protein 17, HEAT repeat-containing protein 1, T-box transcription factor TBX3, metal response element binding transcription factor 2, Importin subunit alpha-6, and Lysosomal-trafficking regulator. The level of triglyceride in serum may be regulated by lowering the expression of several proteins associated with carbohydrate metabolism, lipid metabolism, signaling, transcription, stress response, transcription and transport within the liver.

Keywords: Trikatu, Triglyceride, Proteomics, GeLC-MS/MS

Introduction

Trikatu is an essential ingredient of numerous formulations and prescriptions of the traditional Thai and Indian system of medicine. Trikatu is a combined herbal preparation containing dry powder of Black pepper, Long pepper and rhizome of Ginger in equal proportion [1]. It was reported that Trikatu increased bioavailability of other drug either by promoting rapid absorption from the gastrointestinal tract, or by protecting the drug from being metabolized/oxidized in its first passage through the liver after being absorbed [2]. Moreover, it is suggested to be beneficial in control of lipids in the body. However, there is no experimental evidence available to show the mechanisms of Trikatu in term of lipid metabolism.

Proteome, the entire protein complement of the genome, determines cell phenotype and functions. With rapidly developed technologies, proteomics provides a systematic approach for the quantitative and qualitative mapping of the whole proteome under drug treatment of disease conditions [3]. The altered proteins identified by proteomic approach can be further characterized as potential drug targets and the global analysis of the protein alterations can result in valuable insights to understand the drug action mechanisms. To

further understand the mechanism of Trikatu effects, we employed a proteomic approach by utilizing GeLC-MS/MS to profile the proteins treated by Trikatu in rat liver.

Materials & Methods

Preparation of the Trikatu extract

Trikatu (the mixture of the dry fruits of long pepper, dry fruits of black pepper and dry rhizome of ginger in the ratio 1:1:1 w/w) was extracted with 95% ethanol. The ethanolic extracts of Trikatu were subjected to qualitative analysis for various phytoconstituents such as gingeral and piperine. The crude was dissolved in propylene glycol at the required concentration before using.

Laboratory animals

Sixteen healthy adult male Wistar rats (weighing 200-250 g) were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornprathom, Thailand. The temperature (25 ± 5°C), humidity (50 ± 5 %) and 12 hr. light and dark cycle of animal rooms were controlled constantly. Water was supplied *ad libitum*. The rat were randomly assigned into three groups (n=8 per group) of control (Propylene glycol) and Trikatu-treated group, which received 100 mg/kg body weight for 30 days. Blood was drawn for serum lipid profile analysis. Triglyceride, total cholesterol and HDL-cholesterol parameters were monitored in all animals before the commencement of experiment and throughout the experimental period. All data are mean ± standard error of mean (SEM). Student's t-test was performed and sequential differences among the means were calculated at the level of $p < 0.05$ using Tukey-Kramer post-test analysis.

Preparation of Protein Samples

Liver samples were collected. Grind 1 g of fresh tissue to a powder with liquid nitrogen in a mortar and pestle and extracted total protein with 1 ml extraction media (8 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X-100, 50 mM DTT). The soluble protein was isolated by centrifugation at 12,000 × g for 10 minutes at 4°C. Protein quantitation is precipitated protein sample with TCA-DOC and was measured using the Lowry assay [4]. Soluble proteins (10 ug) were separated on 12% SDS-PAGE and visualized by silver staining [5]. The entire lane was excised from the gel and cut into six fractions based on molecular mass.

Mass Spectrometry and Data Analysis

Excised gel fractions were digested with trypsin and then analyzed by Ultimate 3000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonics). The samples were separated on a nano column at a flow rate of 300 nL/min. A solvent gradient (solvent A: H_2O, 0.1% formic acid; solvent B: 20%H_2O, 80% ACN, 0.1% formic acid) was performed in 40 min. For protein quantitation, Decyder™ MS Differential Analysis software (DeCyderMS) was used [6, 7]. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software. Parameters for protein searches were enzyme trypsin; miscleavages 2; charge of ions +1, +2 and +3; NCBI;species Rattus. Carbamidomethylation of cysteines and oxidation of methionine was considered a fixed modification. [8]

Results & Discussions

Effect of Trikatu on serum lipid profile

After the treatment of the rats with 100 mg/kg B.M. of Trikatu for 30 days, serum lipid profile was evaluated using commercial kit (HUMAN, Germany). We measured the Triglyceride, total cholesterol and HDL-cholesterol in the both groups of rats. The results showed that the triglyceride decreased significantly ($p<0.01$) by Trikatu treatment (Fig. 1(A)). A literature survey showed that Trikatu reduced triglycerides and low density lipoprotein (LDL) cholesterol and increased high density lipoprotein (HDL) cholesterol that could reduce the risk of hyperlipidemia and atherosclerosis [9]. Trikatu was used to benefit in control of lipids in the body.

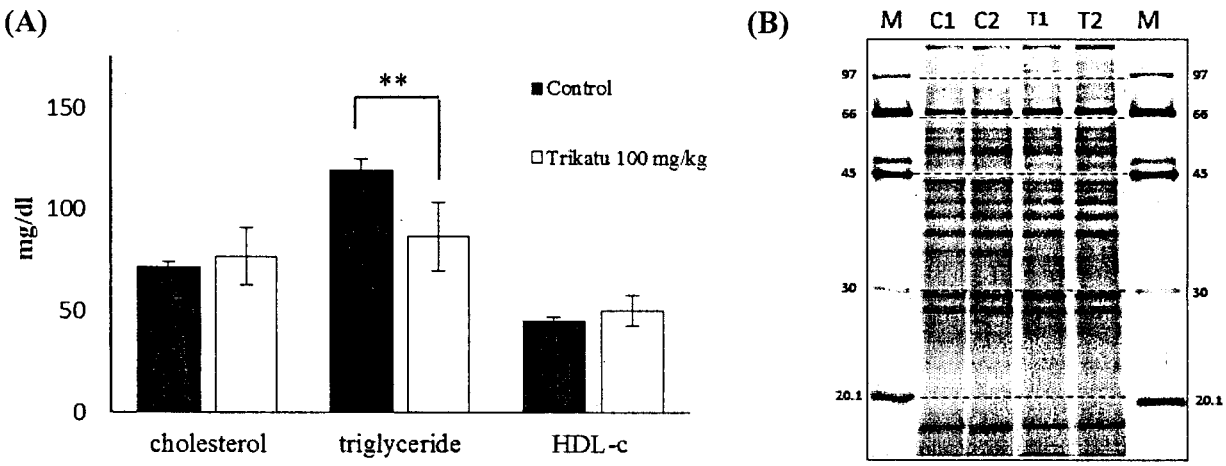

Figure 1 (A) Effect of Trikatu at 100 mg/kg body weight once orally for 30 days on serum lipid profile in male Wistar rats and (B) total protein pattern on SDS-PAGE (M= protein markers, C1-2= Control group and T1-2=Trikatu treated group)

Proteomic analysis to identify changes in protein expression stimulated by Trikatu treatment in liver

To analyze the underlying mechanisms and identify downstream mediators that were unique to the effects of Trikatu in liver, we initiated a proteomic analysis to identify target specific proteins important for pharmacological action. Liver proteins were processed simultaneously and analyzed using GeLC-MS/MS is a powerful approach for shotgun proteomic analyses. Samples are separated using 1-D SDS-PAGE and gel slices sequentially excised all the way down relevant gel lanes (Fig. 1(B)). Following in gel digestion peptides from each slice can then be analysed by MS. Discrepancies in protein were found and the significance of protein changes was evaluated using Student's t-tests.

Result of SDS-PAGE was show that no obvious difference of pattern profile. For lower 45kDa of gel piece, we also successfully identified proteins that were found 10 proteins to be lower expressed between Trikatu-treated groups and control groups (table 1). We found some proteins related to carbohydrate metabolism, signal transduction, transcription, stress response and transport , such as carbonic anhydrase 12, TRAF3-interacting JNK-activating modulator, Rho GTPase-activating protein 17, Selenoprotein O, HEAT repeat-containing protein 1, T-box transcription factor TBX3, metal response element binding transcription factor 2, Importin subunit alpha-6, and Lysosomal-trafficking regulator, which varied greatly after Trikatu treatment.

Interestingly, we found protein related to lipid metabolism that is low-density lipoprotein receptor-related protein 3. LRP3, a member of the low density lipoprotein (LDL) family, It is expressed at intermediate level in heart, brain, liver, pancreas, prostate and small intestine [10]. Functional expression failed to detect any binding of LRP3 to very low density lipoprotein (VLDL) or to the LDL receptor-associated protein (LRPAP1), suggesting that it may have a role other than lipoprotein metabolism.

Table 1 Proteins identification of differentially expressed in Trikatu-treated in rat liver

Accession no.	Protein names	Peptide	Mascot Score	Intensity ratio [a]	Functional
gi|124249068	Carbonic anhydrase 12	EAEVHA	5.42	-0.85	glucose biosynthetic
gi|31745146	T-box transcription factor TBX3	ADPEMPK	11.89	-0.83	transcriptional control
gi|149028655	metal response element binding transcription factor 2	SWPASIPHLR	6.18	-0.83	transcriptional control
gi|148283739	selenoprotein O	IMHANNPK	10.85	-0.82	response to stress
gi|157822545	HEAT repeat-containing protein 1	RNVSLPR	24.52	-0.79	response to stress
gi|68341941	importin subunit alpha-6	KVVESGGPK	11.64	-0.79	protein transport
gi|81880317	Rho GTPase-activating protein 17	RGGTLNR	9.85	-0.77	signal transduction
gi|16758280	lysosomal-trafficking regulator	VIQHIRGMYK	16.13	-0.76	lysosomal transport
gi|62078999	TRAF3-interacting JNK-activating modulator	TLGDQHR	13.80	-0.76	response to stress
gi|16758310	LDL receptor-related protein 3 precursor	SCPDGADEK	5.74	-0.75	lipid transporter activity

[a] Intensity average ratio of differential expression between Trikatu-treated and control group.

Conclusions

Trikatu is a potent hypolipidemic agent which has the capability in reducing triglyceride level. In addition, this study has been shown to identify distinct proteomic profiles associated with specific to herbal drug administration. The level of triglyceride in serum may be regulated by lowering the expression of several proteins associated with carbohydrate metabolism, lipid metabolism, signaling, transcription, stress response, transcription and transport within the liver. For those proteins associated with carbohydrate metabolism, lipid metabolism, signaling, transcription, stress response, transcription and transport within the liver. Further study of these proteins may be helpful in the further elucidation of the pharmacological effect mechanism and the identification of new targets of drugs for Trikatu treatment.

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