UNIVERSITI PUTRA MALAYSIA

EFFECT OF METHANOL EXTRACT OF Melastoma malabathricum (MEMM) LEAVES ON CARBON TETRACHLORIDE- AND PARACETAMOL-INDUCED LIVER TOXICITY IN RATS

BY

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FPSK2 2011 10

EFFECT OF METHANOL EXTRACT OF *Melastoma malabathricum* (MEMM) LEAVES ON CARBON TETRACHLORIDE (CCL_4) – AND PARACETAMOL (PCM) - INDUCED LIVER TOXICITY MODELS IN RATS

FARAH HIDAYAH BT KAMISAN

ABSTRACT

In the modern system of medicine, there is no specific cure for a wide spectrum of liver toxicity. Thus, attempts are continuously being made to find out alternative agents from natural products sources for the treatment of hepatotoxicity. One of the plants that are being studied in our laboratory is *Melastoma malabathricum*, locally known as "senduduk". Therefore, the present study aimed to evaluate hepatoprotective effect of MEMM against CCl_4 – and PCM- induced liver toxicity in rats. For this study, 6 groups (n=6) of rats (Sprague Dawley; male; 180-200g) were used and received orally test solutions; 10% dimethyl sulfoxide (DMSO), 200 mg/kg silymarin or MEMM (50, 250 and 500 mg/kg) once daily for 7 consecutive days. On the last day, 3 hours after the test solutions administration, hepatotoxicity was induced with CCl_4 (1.5 ml/kg: intraperitoneally) and PCM (3 g/kg: orally). 48 hours after inducer administration, blood collection was carried out to obtain serum for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tests then immediately the animals were sacrificed to obtain liver for histopathological examination. As the results, the groups pretreated with MEMM at 250 and 500 mg/kg significantly ($P<0.05$) reduced the elevated serum levels of AST and ALT in CCl_4-treated group (negative control group). Based on histological examination, the negative control group has extensive interface hepatitis, multiple foci necrosis and haemorrhage of the hepatocytes. Interestingly, the groups pretreated with MEMM showed less severity of necrosis and inflammation of the hepatocytes compared to the negative control group. The pre-treated groups with MEMM showed insignificantly reduced in AST and ALT levels for PCM-treated group at dose 50 mg/kg, 250 mg/kg and 500 mg/kg. The mean score obtain for PCM-induced group showed significantly reduced at 50 mg/kg, 250 mg/kg and 500 mg/kg. As a conclusion, MEMM may possess hepatoprotective activity and further studies should be carried out to determine the exact dose that most effective for its hepatoprotective activity.

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APPROVAL

It is hereby certified that I have read this project paper entitled **Effect of Methanol Extract of Melastoma *malabathricum* (MEMM) Leaves On Carbon Tetrachloride- and Paracetamol-induced Liver Toxicity in Rats** by **Farah Hidayah binti Kamisan**, and in my opinion it is satisfactory in terms of scope, quality and presentation as a fulfillment of the requirements for the course SBP 3999.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged.

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LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CCl_4	Carbon tetrachloride
CMC	Sodium carboxymethylcellulose
Ca^{2+}	Calcium ion
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DMN	dimethylnitrosamine
GaIN	D-galactosamine
GSH	Glutathione
H&E	Haematoxylin & Eosin staining
IBS	Institute of Bioscience
in.	Inch
i.p.	Intraperitoneally
MEMM	Methanol extract of *Malastoma Malabathricum*
MOH	Ministry of Health
NAC	N-acetyl cysteine
NAPQI	N-acetyl-p-benzoquinoneimine
PCM	Paracetamol
p.o	Orally
RNA	Ribonucleic Acid
S.E.M.	Standard error mean
SOD	Superoxide dismutase
UPM	Universiti Putra Malaysia
USA	United State of America
$\cdot CCl_3$	Trichloromethyl free radical
$\cdot OOCCl_3$	Trichloromethylperoxyl radical

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CHAPTER 1

INTRODUCTION

Background of study

Liver is one of the most vital organs involved with almost all the biochemical pathways to growth, nutrient supply, fight against disease , energy provision and reproduction Besides, liver has role in transforming and clearing chemicals thus it is susceptible to the toxicity from these agents. It is always and variedly exposed to environmental toxins, and abused by poor drug habits and alcohol and prescribed over-the –counter drug which can cause various liver diseases (Md Rajib Ahsan *et al*., 2009).

In Malaysia, data on chemical poisoning and pesticide show wide variability in the total number of cases reported to the Ministry of Health (MOH) each year. During year of 1997 to 2000, the number of cases reported ranged from 552 to 945. However, the data may not reflect the actual number of poisoning cases in the country because include only those who required treatment at the MOH government health care facilities (Fathelrahman *et al*., 2005).

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The examples of hepatotoxicants are paracetamol (PCM) and carbon tetrachloride (CCl_4). PCM is one of the causes of drug-induced liver toxicity in worldwide. It is responsible for nearly 50% of liver failure, while drugs excluding paracetamol are responsible for 12% of cases based on American review (Eileen *et al*., 2007). CCl_4 is example of environmental toxin that may cause hepatotoxity through ingestion or inhalation. CCl_4 induced hepatotoxicity is broadly used for the study of hepatoprotective effects of drugs and plant extracts (Md Rajib Ahsan *et al*., 2009).

There are some drug and also active component from plant that can treat and also protect from acute liver toxicity. Drug such as N-acetyl cysteine (NAC) has been clinically approved for treatment of PCM toxicity by acting as precursor of reduced glutathione (Ehsan et al., 2010). Beside, broad range of compounds derived from natural plants such as silymarin, green tea , emodin and others have been tested ([Roomi *et al*., 2008], [El-Beshbishy, 2005], [Montilla *et al*., 2005] and [Mansour *et al*., 2006]) as an alternative to NAC treatment therapy. As referred to Ehsan *et al.* (2010), curcumin also can protect against liver damage in animals by hepatotoxic substances such as CCl_4, galactosamine, pentobarbital, 1-chloro-2,4-dinitrobenzene, and 4-hydroxynonenal.

In lack of reliable liver protective drug in the modern system of medicine, therefore attempts are perpetually being made to investigate some alternative therapy by using

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natural products. Natural products as referred to Holt and Chandra (2002) are herbs, herbal concoctions, dietary supplements, traditional Chinese medicines or alternative medicines. Natural remedies from the medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity. Some medicine traditional practices such as in Ayurveda are recommended for the treatment of liver disorders (Chatterjee, 2000).

Regarding the interest of medicinal sources from natural products, there is plant traditionally used in the Malay medicine for the treatment of diarrhoea as an astringent, post partum treatment and haemorrhoids (Susanti *et al*., 2007), besides can be used to relieve toothache and used externally against inflamed wounds (Sulaiman *et al*., 2004) which are scientifically known as *Melastoma malabathricum* .This plant belongs to the family of Melastomataceae and is commonly known among Malaysians as 'senduduk'. *M. malabathricum* is common weeds that grow abundantly in waste grounds and open fields in Malaysia, where it grows as small trees 12-13 ft. High, occasionally even up to 20 ft. They are evergreen and flower throughout the year (Susanti *et al*., 2007).

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1.2 Hypothesis

Methanol extract of *Melastoma malabathricum* leaves have hepatoprotective effect against carbon tetrachloride (CCl_4) - and paracetamol (PCM)-induce liver toxicity in rats.

Objective of the study

General

To determine hepatoprotective activity of methanol extract of *Malastoma Malabathricum* (MEMM) *leaves* using rats models

1.3.2 Specific

a) To determine hepatoprotective effect of MEMM against carbon tetrachloride (CCl_4) - and paracetamol (PCM)-induce liver toxicity in rats.

b) To determine the most effective concentration of MEMM towards different liver toxicity inducers.

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CHAPTER 2

LITERATURE REVIEW

2.1 Liver

2.1.1 Gross morphology of liver

Liver is the largest solid organ of the body (Geller and Petrovic, 2004). In an adult, liver weighs an average of 1.4 kg with a wedge-like shape that occupies most of right hypochondriac and epigastric areas, extending further to the right of body midline than to the left (Marieb, 2001). The liver is regarded as mostly consisting of a dominant right and lesser left lobe, separated by the falciform ligament, with a smaller caudate and quadrate lobes (Geller and Petrovic, 2004).

Blood enters the liver via the portal vein, which branches to become incorporated in the distinctive structural entities of the portal tract. Blood traverse the parenchyma via the sinusoids, where all of the hepatic functions take place, to reach the terminal hepatic venule, and then to hepatic veins, the inferior vena cava, and, ultimately, the heart.

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The largest vessels in the portal tracts are the portal veins and empty into periportal sinusoids through venules. The hepatic artery is a relatively minor contributor to hepatic blood flow (Geller and Petrovic, 2004).

2.1.2 Microanatomy

The basic architecture includes the portal tracts, sinusoids, and outflow vessels, which are evenly spaced throughout all lobes of the liver. The portal tracts are composed of interlobular bile ducts, hepatic arterioles, portal venules, fibroconnective tissue (collagen) and inflammatory cells. The parenchyma in the adult comprises over three-quarters of the total hepatic volume, and is composed predominantly of liver cell cords and sinusoids lined by endothelial, Kupffer, and stellate cells.

In addition, the hepatocytes comprise approximately two-thirds of the total number of cells within the, liver and are arranged in cords that are one cell thick and have three distinct cell membrane boundaries; sinusoidal, lateral and canalicular. The hepatocyte is composed of nucleus and cytoplasm. Its nucleus is centrally located that contains nucleoli and clumped chromatin while its cytoplasm comprises approximately 90% of the volume of liver cells (Kanel and Korula, 2005).

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2.1.3 Function of liver

All the nutrients resulting from the digestion of the food are taken up by the intestine and then by the liver. Besides, liver is responsible for synthesis of most of serum proteins (Dufour and Clavien, 2010) and hepatocytes also deaminate amino acids to use for ATP production or converted to carbohydrates or fats (Tortora and Derrickson, 2006). Liver also act as storage for nutrients and the energy derived from the oxidation of the nutrients. Nevertheless, liver is not only a power plant but also a cleaning device (Dufour and Clavien, 2010). It can detoxify substances such as alcohol and excrete drugs such as PCM, penicillin, erythromycin and sulphonamides into bile (Tortora and Derrickson, 2006).

2.1.4 Morphological categories of hepatic injury

2.1.4.1 Steatosis

Steatosis also known as fatty change is the presence of fat droplets (vacuoles) in hepatocytes causing displacement of the liver cell nuclei to the cell peripherally. Drugs and toxins produce variable degrees of macrovesicular or microvesicular steatosis, or both. This fatty change mostly involves parenchymal cells. Besides,

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perisinusoidal stellate (Ito) cells can also contain significant numbers of fat droplets, mostly with hypervitaminosis A. Steatosis and necrosis occur concomitantly with carbon tetrachloride-, tannic acid-, and aminitin- induced injury, but in latter steatosis predominates (Geller and Petrovic, 2004).

2.1.4.2 Necrosis

Classically, necrosis has been described as a disordered mode of cell death, that that occur in cases of severe and acute injuries or extreme physicochemical injuries. The most prominent features of necrosis are cytoplasmic swelling, rapid plasma membrane rupture, and organelle breakdown, involving remarkably few nuclear changes (Denecker *et al*., 2001). Perivenular (zone 3) liver cell necrosis is typical of toxicity induced by the paracetamol, halothane, poison mushroom Amanita phalloides, and experimentally, carbon tetrachloride (Geller and Petrovic, 2004).

2.1.4.3 Inflammation

Inflammation is a response for protection to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult. Although inflammation functions to destroy and eliminate microbes and dead tissue, the

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reaction can cause injury to normal tissues. There are two types of inflammation which is acute and chronic inflammation. Acute inflammation is rapid in onset and is characterized by fluid and plasma protein exudation and predominantly neutrophilic leukocyte accumulation.

However for chronic inflammation, it may be more insidious, longer duration (days to years), and is typified by influx of lymphocytes and macrophages. Inflammation that occurs in liver may be limited to portal tract or may be spread out into the parenchyma (Kumar *et al*., 2007).

2.1.5 Assessment of liver function

Liver enzymes such as ALT and AST are often used as markers to assess hepatic function (Ehsan *et al*., 2010). ALT is enzyme local to the liver while AST is an enzyme found in liver and the muscles (Lane *et al*., 2002). These serum enzyme tests will usually indicate the type of liver injury whether hepatocellular or cholestatic, but cannot be expected to differentiate one form of hepatitis from another or to determine whether cholestatis is intra- or extra-hepatic (Sheila and James, 2002). When levels of serum enzymes, AST and ALT are elevated that indicate cellular leakage and loss functional integrity of cell membrane in liver. In contrast, ALP is an indicator of pathological alteration in the biliary flow (L. Ranawat *et al*., 2010).

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2.2 Hepatotoxicant

The broad availability and accessibility of chemicals and their widespread use in medicine, agriculture, industry and in normal daily life lead to the increase risk of poisoning. There are varieties in the general patterns of poisoning among the developing countries.

Drugs represented the most frequently encountered group of agent which resemble the general pattern of poisoning in the USA and some countries. In the USA, the most common category of exposures is pharmacological substances followed by cleaning substances and personal care products. The majority of poisoning cases associated with drugs was responsible by paracetamol overdose, which was similar to those reported in the some Asian and western countries (Fathelrahman *et al*., 2005). Poisoning due to CCl_4 are rare, nevertheless, it hepatotoxicity effect widely studied as a model hepatotoxin for hepatoprotective effects of drugs and plants (Ahsan *et al*., 2009).

2.2.1 Carbon tetrachloride

Experimental damage that is produced by CCl_4 is histological resembles viral hepatitis (L.Ranawat *et al*., 2010). There are two hypotheses for mechanisms of cell injury with CCl_4. One is the lipid peroxidation theory whereas the other is covalent

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binding theory. The predominant mechanism of hepatotoxicity as reported is cellular damage by the free radicals. The critical process of CCl_4 hepotoxicity is underlying by the combining effect of both lipid peroxidation and the covalent binding of CCl_4 reactive metabolites to lipids and proteins (D.Dahiru *et al*., 2003).

2.2.2 Paracetamol

Accidental or intentional overdose with paracetamol (PCM) mainly cause for the acute liver failure case (Ostapowicz *et al*., 2002). Overdose of PCM resulting dose-related hepatocellular necrosis (Raymond and George, 2008).

In therapeutic doses, paracetamol is mainly metabolized via glucuronidation and sulfation and in conjugated forms is excreted from the body. In addition, Paracetamol partly is metabolized by cytochrome p450, to some metabolites mainly NAPQI, which are significantly increased in high paracetamol concentrations. Those metabolites are detoxified by glutathione and excreted out from the body. Large doses of paracetamol lead to glutathione depletion followed by an increase of NAPQI level. Result in increase of lipid peroxidation, disruption of cellular homeostasis and induced of cellular apoptosis and necrosis (Ehsan *et al*., 2010).

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Histological investigation of tissue damage in liver that have been induced by paracetamol confirmed previous findings that it causes significant focal hepatitis in the portal spaces and mild focal hepatitis in the lobules (Roomi *et al*., 2008).

2.3 Silymarin

Silymarin may be accepted as a safe herbal product, since no side effects are known in conjunction with the appropriate administration of designed therapeutic dosages. However recently reported in Australia, there are some side effects such as episodes of severe sweating, abdominal cramping, nausea, vomiting, diarrhea and weakness but the reaction was found to be due to a substance in the milk thistle product other than silibinin (Hale *et al*., 2007).

Silymarin is a standardized extract from dried fruits of milk thistle *Silybum marianum* (L.) Gaertner (Asteraceae) that is composed of a mixture of four isomeric flavonolignans, including silibinin (the major component), isosilybin, silychristin and silidianin (Martina *et al*., 2005; Jen-Chih *et al*., 2009). Seeds of the Milk Thistle have been used for more than 2,000 years to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis and icterus. It is one of the oldest and most comprehensively researched plants in the treatment of the liver diseases, also has been used from ancient times – Theoprastus (4th century BC) was probably the first to describe it under the name "Pternix" (Lidia and Wojciech, 2007).

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More recently, Silymarin has been pronounced and clinically used for its valuable effects on various liver diseases such as mushroom poisoning, drug alcohol intoxication, and viral hepatitis, whose pathogenesis involves an inflammatory response (Kren and Walterova, 2005) and (Saller *et al*., 2001). Multiple properties seem to be linked with its hepatoprotective effects: free radical scavenging activity by act against lipid peroxidation and ability to increase the cellular content of GSH (Lidia and Wojciech, 2007), membrane stabilizes effect, inhibition of arachidonic acid metabolism and increased protein synthesis by activation of RNA polymerase I (Marjan *et al*., 2010).

Besides, Silymarin is probably able to antagonize the depletion of 2 main detoxifying mechanisms which is GSH and superoxide dismutase (SOD), by reducing the free radical load and enhancing SOD activity. It can enter into the nucleus and act on RNA polymerase I enzymes and the transcription of rRNA, resulting in increased ribosomal formation. As a result this will accelerates protein and DNA synthesis, which stimulates the biosynthetic apparatus in the cytoplasm, thus leading to an increase in the synthesis rate of both structural and functional proteins (Marjan *et al*., 2010).

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2.4 *Melastoma malabathricum*

Melastoma malabathricum L.(Melastomataceae) locally known as “senduduk” may be found in the forest at the edge of a stream, on landslips or in old clearings, and these plants are evergreen and flower throughout the year. Characteristics of these plants are leaves 0.25–2 in. wide, with stalks 0.25–0.5 in. long, flowers 1–3 in. wide, calyx closely set with short chaffy and silky or silvery scale. *Melastoma malabathricum* consists of three varieties, having flowers with dark purple-magenta petals,light pink-magenta petals, and (the rare variety) white petals.

Based on Abdul malek and Asari (2001), the phytochemical analysis of M.malabathricum has demonstrated the presence of h-sitosterol, a-amyrin, sitosterol 3-O-h-d-glucopyranoside, quercetin, quercitrin and rutin. Several tannins also have been isolated from their dry leaves, hydrolysable tannin oligomers nobotanin B, malabathrins B, malabathrins C, malabathrins D, the hydrolysable tannin monomers, 1,4,6-tri-O-gal- loyl-b-D-glucose, 1,2,4,6-tetra-O-galloyl-b-D-glucose, strictinin, casuarictin, pedunculagin, nobotanin D, pterocarinin and the hydrolysable tannin oligomers, nobotanin G, nobotanin H and nobotanin J which were newly found to show potent in vitro antiviral activity against human immunodeficiency virus (Susanti *et al*., 2007).

Recent studies have also been report metabolites isolated from the Melastoma plant to have antiviral and cytotoxic activity (Lohezic-Le *et al*., 2002) ; anti-inflammatory and antipyretic properties (Zakaria *et al*., 2006), anti-helmethic and anti-spasmodic

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action (Osman *et al*., 2002),anti-nociceptive, anti cancer and anti-oxidant activity (Susanti *et al*., 2007) and anti-hypertensive activity (Fouad *et al*., 2008)

Figure 2.1 *Melastoma malabathricum* flower

Figure 2.2 *Melastoma malabathricum* leaves

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CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Collection

The leaves of *Melastoma Malabathricum*, were collected from their natural habitat around the Universiti Putra Malaysia (UPM), Serdang, Selangor campus. The time to collect the leaves is around 9.00 am to 11.00 am in the morning. A voucher specimen (ACP 0017) has been identified by a botanist from the Institute of Bioscience (IBS), UPM, Serdang, Selangor, Malaysia.

3.2 Extraction Preparation

For this planned study, methanol solvents will be used for the extraction process. The leaves of *M. Malabathricum* were dried for a week at room temperature (27 ± 2°C). Those leaves dried were pulverized in electric grinder.

Methanol extraction process (MEMM)

40 gram of dried, grounded leaves of *M. Malabathricum* weighed and soaked with 800 ml of absolute methanol, ratio of 1:20 (w/v) for 72h at room temperature.

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The mixture solutions were collected and filtered using cotton wool followed by Whatman No. 1 filter paper. Then the supernatant collected was evaporated using the rotary vacuum evaporator at 40°C under reduced pressure. This extraction process will be repeated three times using the same residue. The extract obtained were weighed and stored in 4°C before use. This method will be done according to Ozturk and Ercisli, (2006) with modification.

Induction of hepatic injury

In this study, healthy adult male Sprague-Dawley rats were used and weight in 180-220 grams. On the day of experiment, the rats were randomly and evenly distributed into 11 groups of 6 animals each . The rats will be fast 2 days from food uptakes prior to the experiment and maintained under standard laboratory condition. The treatment started with highest dose to lowest dose.

For liver toxicity screening, 2 common inducers will be used.

Carbon tetrachloride (CCl_4) -induced liver toxicity

The experimental protocol was based on previously reported studies (Naveen *et.al*, 2005) with slight modifications. MEMM (50, 250 and 500 mg/kg) dissolved in

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dimethyl sulfoxide (DMSO) and standard hepatoprotective drug silymarin will be prepared in 1% sodium carboxymethylcellulose (CMC). The drug was supplied by Sigma, USA. Group I served as normal control and received only 10ml/kg/day of DMSO by orally (p.o.).

Group II: 10% DMSO p.o. for 7 days and CCl_4 1.5 ml/kg (1:1 of CCl_4 in olive oil) intraperitoneally (i.p.) on 7th day.

Group III: Silymarin 200mg/kg (p.o.) for 7 days and CCl_4 1.5ml/kg (1:1 of CCl_4 in olive oil) i.p. on 7th day.

Group IV, V and VI: MEMM 250,500 and 1000 mg/kg p.o. for 7 days and dose of 1.5ml/kg i.p. of CCl_4 (1:1 of CCl_4 in olive oil) on the 7th day.

Paracetamol (PCM) -induced liver toxicity

For liver toxicity induce by PCM (3g/kg, p.o.) diluted with glycerol, the procedure will be follow the same procedure as mentioned above. PCM will be administering on day 7 and animals will be sacrifice by exposure to diethyl ether 48 hour after administration of PCM. This protocol modified protocol of Porchezhian and Ansari, (2005).

Group I: As normal control for both CCl_4- and PCM- induced liver toxicity

Group VII: 10% DMSO for 7 days and PCM 3g /kg on 7th day orally.

Group VIII: Silymarin 200mg/kg (p.o.) for 7 days and PCM 3g /kg on 7th day.

Group IX, X and XI: MEMM 250,500 and 1000 mg/kg p.o. for 7 days and dose of 3g/kg p.o. of PCM on the 7th day.

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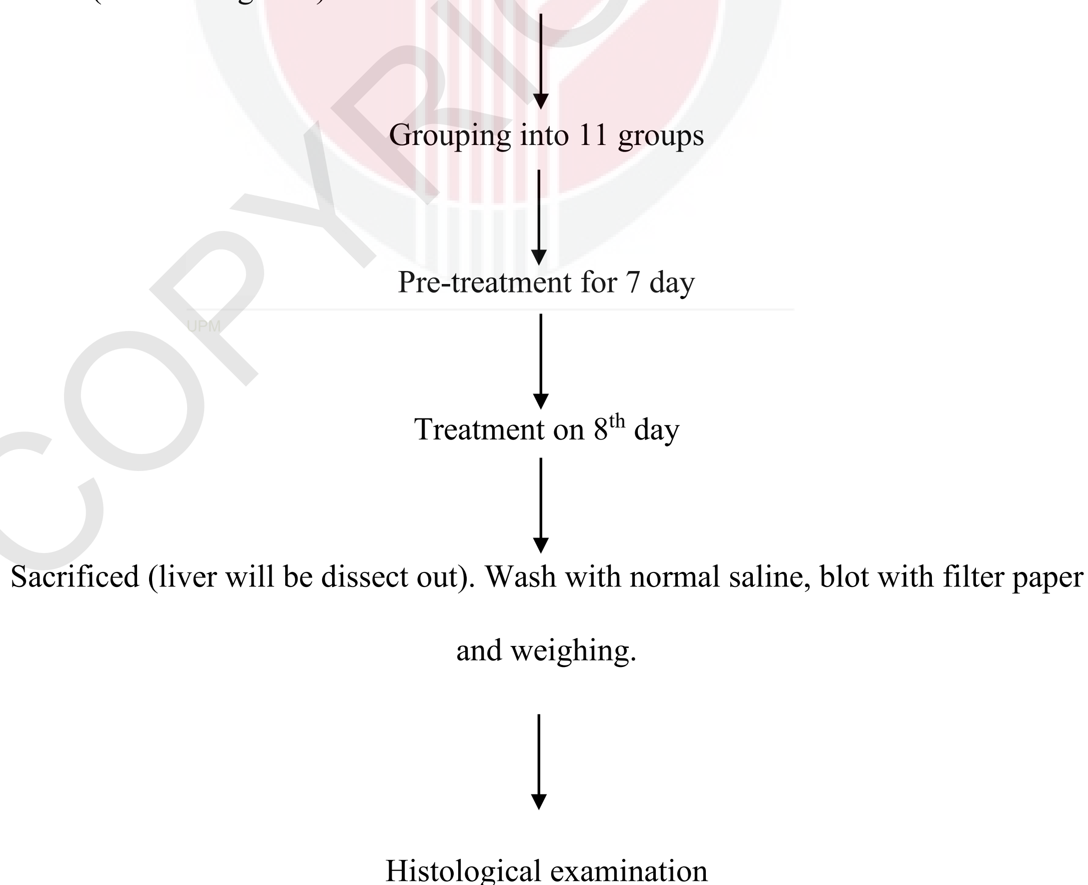
48 hours after last treatment the animal will be faint with diethyl ether and blood (3.0 ml) collected by cardiac puncture using sterile disposable syringe. The blood will be collected into the plain tube without the anti-coagulant and the serum was obtained by centrifugation (5000 rpm for 10min). Biochemical studies carried out by analyzing Alanine aminotransferase [ALT] (Mohur and Cock, 1957) and aspartate aminotansferase [AST] (Mohur and Cook, 1957). After that, the rats were sacrificed by exposure to diethyl ether and the liver was dissected out and fixed in 10% formalin for histopathology studies.



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Histological study

After the liver tissue fixed in the 10% formalin, those specimens were embedded in paraffin and sectioned (3-5µm) and stained with haematoxylin and eosin (H&E) dye. The histochemical sections evaluated under electron microscope and evaluated by the pathologist. This method applied for both inducers. Liver sections were scored on a scale of 0-3 for each histological feature as given below (Chiou *et al*., 1997).

Table 3.1 Histological scoring

Histological changes observed	Score
No necrosis/ inflammation/ hydropic degeneration or steatosis.	0
Mild hydropic degeneration / fatty change	1
Moderate hydropic degeneration or fatty change seen	2
Severe hydropic degeneration or fatty change seen	3
Mild infiltration by inflammatory cell	4
Moderate infiltration by inflammatory cell	5
Severe infiltration by inflammatory cell	6
Mild necrosis	7
Moderate necrosis	8
Severe necrosis	9

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3.5 Statistical analysis

Data obtained were analysed using the one-way analysis of variance (ANOVA) and the differences between the groups were determined using Dunnet post hoc test with $P<0.05$ as the limit of significance.

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CHAPTER 4

RESULTS

4.1 Effects of extracts on AST and ALT

4.1.1 CCl_4-induced hepatotoxicity

Based on the analysis, a significant difference in biochemical markers was observed between normal and CCl_4 control group. Both AST and ALT levels significantly increased in CCl_4 control group compared to the normal control group. This proved hepatotoxicity by the CCl_4. Then, the elevated levels of AST and ALT were significantly reduced in the animals pre-treated with MEMM as depicted in Table 4.1. Groups that pre-treated with MEMM (500 mg/kg and 250 mg/kg) showed significant hepatoprotective activity that comparable to silymarin (200 mg/kg). For MEMM at 50 mg/kg dose, it is insignificant reduced for both AST and ALT levels.

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CHAPTER 5

DISCUSSION

The aim of this study is to determine hepatoprotective effects of methanol extract of MEMM leaves using rat models on damage of liver caused by PCM and CCl_4. Liver is a vital organ that responsible for the metabolism of drugs and toxic chemicals, and therefore is the primary target organ for nearly all the toxic chemicals. There are some pharmacological or chemical substances that are known to cause hepatic injuries, such as dimethylnitrosamine (DMN), CCl_4, paracetamol and D-galactosamine (GaIN). Excessive dose exposure to the hepatotoxins may induce acute liver injury characterized by degeneration, necrosis or apoptosis of hepatocytes and abnormality of hepatic function (Wu *et al*., 2007).

It was widely believed that occurrence of drug or chemicals-induced liver injury was especially associated with oxidative stress, a cellular imbalance between the production and elimination of free radicals ((Amin and Hamza, 2005, Castro and Freeman, 2001 and Jaeschke, 2000) ; oxidative stress produced overproduction of free radicals could directly cause hepatocellular membrane injury by lipid peroxidation or other means, followed by series of cascades of cellular events such as

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massive release of inflammatory mediators or cytokines, which eventually lead to liver injuries (Dizdaroglu *et al*., 2002, and Higuchi and Gores, 2003). It is valuable to identify natural compounds that can antagonize the mechanism that cause liver injuries. In the present study, rat models of PCM- and CCl_4-induced hepatotoxicity are adopted to investigate the possible hepatoprotective effects of MEMM.

Paracetamol and CCl_4 get converted into reactive toxic metabolites by hepatic microsomal cytochrome P450 and cause hepatotoxicity (Mayuren *et al*., 2010). From the results showed that PCM and CCl_4 administration caused severe liver damage in rats, which are demonstrated by significant elevation of AST and ALT levels and classic histopathological changes. Sign of hepatic injury is leakage of cellular enzyme into plasma. When there is damaged in liver cell plasma membrane, a variety of enzymes normally located in the cytosol are released into blood stream (Kumar *et al*., 2004).

CCl_4 is biotransformed by cytochrome P450 system in the endoplasmic reticulum to produce trichloromethyl free radical ($^{\cdot}CCl_3$). Trichloromethyl free radical when combined with cellular lipids and proteins in the presence of oxygen form trichloromethylperoxyl radical ($^{\cdot}OOCCl_3$), which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus,

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trichloromethylperoxyl free radical leads to elicit lipid peroxidation. The destruction of Ca^{2+} homeostasis, finally results in cell death (Mayuren *et al*., 2010).

Pre-treatment of the rats with MEMM extract at 250 mg/kg and 500 mg/kg for 7 days before CCl_4 administration resulted in a significant protection of CCl_4- induced elevation of serum marker enzymes. MEMM appears to be effective in reducing the injurious effect of CCl_4, observed in the study. For 50 mg/kg dose MEMM, insignificant reduced of serum marker enzymes levels were observed. The mean histopathological score for CCl_4-induced model that was pre-treated with MEMM is insignificantly reduced with dose 50 mg/kg, 250 mg/kg and 500 mg/kg at $p < 0.05$. Significance of the result from histopathological scoring is not concurrent with the analysis of AST and ALT level. The limitation of this study is may due to improper handling and technique while grossing liver specimens and preparing the slides. The liver sectioning on the slides may not represent all the histological changes in the liver.

PCM is a commonly and widely used analgesic and antipyretic agent. The hepatic cytochrome P450 enzyme system metabolizes paracetamol, forming a minor yet significant alkylating metabolite known as NAPQI. NAPQI is then irreversibly conjugated with the sulfhydryl groups of glutathione. The conjugation depletes gluthathione, a natural antioxidant. Excess production of PCM metabolite causes the

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initial hepatic damage and subsequent activation of inflammatory mediator TNF-α which in turn contribute to tissue necrosis (Mayuren *et al*., 2010).

In the present investigation it was observed that the pre-treatment of MEMM reduced the mean value for AST and ALT level, but insignificantly at $p < 0.05$ compared to the negative control group. The hepatoprotective effect of MEMM was further confirmed by histopathological examination of the liver. The histological observation basically supported the results from the serum assays as MEMM administration reversed to a large extent, hepatic lesions produced by paracetamol. The histopathological mean score for PCM-induced model pre-treated with MEMM is significantly reduced at 50 mg/kg, 250 mg/kg, and 500 mg/kg ($p < 0.05$).

According to the analysis, MEMM has potential hepatoprotetive property. The ability of MEMM to exhibit protection against PCM- and CCl_4-induced hepatotoxicity may be due to the active constituents present in the extract such as flavonoids, tannins, sterols and others. It has been reported by Susanti *et al*., (2008), that the methanol extract of leaves of *Melastoma malabathricum* have yield quercitrin and kaempferol-3-*O*-(2”,6”-di-*O*-*p*-*trans*-coumaroyl)glucoside. Some metabolites isolated from this plant have also been reported to have anti-oxidant and anti-cancer properties (Susanti *et al*., 2007).

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As hepatoprotective agent, the extract may either inhibit the formation of the toxic PCM metabolite or stimulate the hepatic regeneration. This type of stimulation is known to cause the liver to become more resistant to damage by toxins (Omar *et al*., 2002).

Silymarin is also a type of flavonoids that proved in treating and preventing liver damage. Its hepatoprotective effects: free radical scavenging activity by act against lipid peroxidation and ability to increase the cellular content of GSH (Lidia and Wojciech, 2007).

From the data analysis, MEMM possess hepatoprotective effects comparable with Silymarin. Thus, the possible mechanism exerts by MEMM as hepatoprotective agent also may be due to its free radical scavenging activity. The antioxidant activity and the inhibition of free radical generation are effective in terms of protecting liver from PCM- and CCl_4- induced damage. Antioxidant properties of MEMM may be important in producing hepatoprotective effect in order to protect from liver injuries cause by free radicals. Antioxidant enzymes such as catalase,SOD and ascorbic acid are easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activities of these enzymes especially in CCl_4 toxicity (Dhanasekaran *et al*., 2009). In conclusion, the present study provides evidence on the potential hepatoprotective ability of MEMM against PCM- and CCl_4-induced hepato

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CHAPTER 6

CONCLUSION

The present study concluded that MEMM demonstrated heptoprotective activity against CCl4- and PCM-induced liver toxicity in rats, which could be attributed to its flavonoids content. From the results, pre-treated with MEMM at 250 and 500 mg/kg significantly ($P<0.05$) reduced the elevated serum levels of AST and ALT in CCl_4-treated group (negative control group). However, the mean score are insignificantly reduced at 50 mg/kg, 250 mg/kg and 500 mg/kg in CCl_4-induced group. The pre-treated groups with MEMM showed insignificantly reduced in AST and ALT levels for PCM-treated group at dose 50 mg/kg, 250 mg/kg and 500 mg/kg. Based on the histological examination, the mean score obtain for PCM-induced group showed significantly reduced at 50 mg/kg, 250 mg/kg and 500 mg/kg.

For the future directions, more parameters for biochemical analysis in assess hepatoprotective properties of the MEMM need to be done such as assess the level of natural antioxidants in the liver which is glutathione and superoxide dismutase. Further studies also need to be done in order to purify bioactive compounds in the leaf crude extracts.

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APPENDICES

APPENDIX A

TISSUE PROCESSING

Definition: any treatment of tissue necessary to impregnated them with a solid medium to facilitate production of section for microscopy.

4 major steps: 1. Dehydration

2. Clearing

3. Infiltration and impregnation

4. Embedding

Steps	Time taken
50% alcohol 70% alcohol 80% alcohol 95% alcohol 100% alcohol I,II and III Equal part of alcohol + toluene	1 and half hours each
Toluene	Overnight
Paraffin wax I, II, and III	2 hours each

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APPENDIX D

Data for level of AST and ALT level in CCl_4-induced model

GROUP	RATS	AST	ALT
Normal	1	53.7	116.8
	2	136.6	141.3
	3	36.5	86.0
	4	29.2	111.8
	5	44.8	93.7
	6	49.6	95.2
Negative control	1	2989.00	1619.30
	2	1116.00	788.80
	3	2351.00	929.70
	4	465.00	509.00
	5	1755.45	867.80
	6	1753.65	112.00
Positive control	1	858.30	551.05
	2	789.90	595.83
	3	885.70	480.60
	4	545.20	325.32
	5	345.11	154.90
	6	897.70	698.60
50 mg/kg MEMM	1	919.90	1010.56
	2	1316.10	798.99
	3	982.60	876.52
	4	1211.94	775.00
	5	965.23	648.20
	6	1388.10	386.20
250 mg/kg MEMM	1	633.17	391.69
	2	977.00	496.94
	3	921.60	310.20
	4	604.01	250.40
	5	855.87	200.00
	6	751.20	498.23
500 mg/kg MEMM	1	1183.00	201.40
	2	606.90	97.70
	3	959.00	155.60
	4	763.86	203.20
	5	976.00	175.30
	6	865.20	224.70

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APPENDIX E

Data for level of AST and ALT level in PCM-induced model

GROUP	RATS	AST	ALT
Normal	1	116.8	53.7
	2	141.3	136.6
	3	86.0	36.5
	4	111.8	29.2
	5	93.7	44.8
	6	95.2	49.6
Negative control	1	1719.95	633.17
	2	973.50	523.00
	3	1646.70	921.60
	4	599.10	604.01
	5	1719.95	605.87
	6	1714.50	251.20
Positive control	1	609.90	483.80
	2	1316.10	606.90
	3	382.60	159.00
	4	711.90	463.10
	5	364.20	276.90
	6	288.10	165.10
50 mg/kg MEMM	1	997.56	443.60
	2	976.44	315.38
	3	1145.00	661.75
	4	879.66	697.80
	5	1086.75	459.35
	6	1257.39	606.55
250 mg/kg MEMM	1	1304.70	433.37
	2	839.25	523.00
	3	874.60	521.42
	4	803.90	604.01
	5	1768.60	605.87
	6	1187.95	151.24
500 mg/kg MEMM	1	982.00	496.53
	2	983.60	385.66
	3	738.70	280.90
	4	1094.83	494.50
	5	791.67	138.17
	6	1123.84	582.10

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APPENDIX F

One Way ANOVA for CCl_4-induced model

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
AST	Between Groups	1.218E7	5	2435096.316	8.678	.000
	Within Groups	8418137.672	30	280604.589		
	Total	2.059E7	35			
ALT	Between Groups	2495817.474	5	499163.495	8.477	.000
	Within Groups	1766585.045	30	58886.168		
	Total	4262402.519	35			

Post Hoc Test for CCl_4-induced model

Multiple Comparisons

Dunnett t (2-sided)[a]

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
AST	2.00	1.00	-1679.95000*	305.83470	.000	-2492.5128	-867.3872
	3.00	1.00	.00000	305.83470	1.000	-812.5628	812.5628
	4.00	1.00	-607.70500	305.83470	.197	-1420.2678	204.8578
	5.00	1.00	-947.87500*	305.83470	.018	-1760.4378	-135.3122

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	6.00	1.00	-846.02333*	305.83470	.039	-1658.5862	-33.4605
ALT	2.00	1.00	-696.96667*	140.10254	.000	-1069.2008	-324.7325
	3.00	1.00	-336.71667	140.10254	.087	-708.9508	35.5175
	4.00	1.00	-55.18833	140.10254	.994	-427.4225	317.0458
	5.00	1.00	-446.52333*	140.10254	.014	-818.7575	-74.2892
	6.00	1.00	-628.11667*	140.10254	.000	-1000.3508	-255.8825

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

APPENDIX G

One Way ANOVA for PCM-induced model

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
AST	Between Groups	6200123.915	5	1240024.783	13.166	.000
	Within Groups	2825548.871	30	94184.962		
	Total	9025672.786	35			
ALT	Between Groups	1515938.934	5	303187.787	8.026	.000
	Within Groups	1133214.318	30	37773.811		
	Total	2649153.252	35			

Post Hoc Test for PCM-induced model

Multiple Comparisons

Dunnett t (2-sided)[a]

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound

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AST	2.00	1.00	-1288.15000*	177.18631	.000	-1758.9109	-817.3891
	3.00	1.00	-783.48333*	177.18631	.001	-1254.2442	-312.7225
	4.00	1.00	-443.17667	177.18631	.070	-913.9375	27.5842
	5.00	1.00	-265.78333	177.18631	.437	-736.5442	204.9775
	6.00	1.00	-338.48333	177.18631	.226	-809.2442	132.2775
ALT	2.00	1.00	-531.40833*	112.21083	.000	-829.5378	-233.2788
	3.00	1.00	-230.67500	112.21083	.173	-528.8045	67.4545
	4.00	1.00	-193.49833	112.21083	.310	-491.6278	104.6312
	5.00	1.00	-49.96000	112.21083	.989	-348.0895	248.1695
	6.00	1.00	107.44167	112.21083	.801	-190.6878	405.5712

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

APPENDIX H

Histology score (scale 0-9)

GROUP	RATS	CCl_4	PCM
Normal	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0
Negative control	1	3	9
	2	8	9
	3	8	7
	4	8	9
	5	8	9
	6	8	7

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Positive control	1	4	4
	2	4	4
	3	4	4
	4	4	7
	5	4	4
	6	4	4
50 mg/kg MEMM	1	8	4
	2	8	7
	3	7	7
	4	7	7
	5	7	4
	6	5	4
250 mg/kg MEMM	1	7	4
	2	7	4
	3	7	3
	4	7	3
	5	7	7
	6	7	1
500 mg/kg MEMM	1	8	4
	2	5	4
	3	7	7
	4	7	5
	5	7	8
	6	5	3

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