UNIVERSITI PUTRA MALAYSIA

HEPATOPROTECTIVE ACTIVITY OF Dicranopteris linearis (Burm. f.) Underw. LEAF METHANOL EXTRACT AND ITS ETHYL ACETATE FRACTION

FARAH HIDAYAH BINTI KAMISAN

FPSK(P) 2017 41

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By

FARAH HIDAYAH BINTI KAMISAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2017

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Zainul Amiruddin Zakaria, PhD
Faculty : Medicine and Health Sciences

Liver disease is a major global concern with extremely poor prognosis and high mortality rate due to the lack of effective preventive or treatment options. Besides, a reliable liver protective drug with fewer side effects is still lacking in modern medicines. Therefore, attempts are perpetually being made to investigate several alternative therapies that use natural plants. In the first part of the *in vivo* study, methanol extract (MEDL) of *Dicranopteris linearis* leaves was investigated for hepatoprotective activity against carbon tetrachloride (CCl_4)-induced and paracetamol (PCM)-induced liver damage. Rats (n=6) used in the study were divided into several groups and given a daily administration of 10% dimethyl sulfoxide (negative control group), 200 mg/kg Silymarin (positive control group) or MEDL (50, 250 or 500 mg/kg) for 7 days, followed by the induction of hepatotoxicity either CCl_4 or PCM. Crude methanolic extracts also were further partitioned using solvents of increasing polarity: petroleum ether <ethyl acetate <water. Semi-purified partitions were called petroleum ether (PEDL), ethyl acetate (EADL) and aqueous (AQDL) extracts. In the second part of in vivo study, these partitions were assayed on PCM-induced hepatotoxicity study. All the semi-purified partitions with intermediate doses (250 mg/kg) were tested on the PCM-induced hepatotoxicity model. The best partition proceeded with another two doses of 50 mg/kg and 500 mg/kg. Blood samples and livers were collected and subjected to biochemical and microscopic analyses. The crude and all the semi-purified partitions were also subjected to antioxidant assays, 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), superoxide dismutase scavenging activity assay (SOD), and oxygen radical absorbance capacity assay (ORAC), and anti-inflammatory assays using lipoxygenase (LOX) and xanthine oxidase (XO) assay. Besides that, total phenolic content (TPC), phytochemical screening and high-performance liquid chromatography (HPLC) analysis and gas chromatography-mass spectrometer (GCMS) were also carried out. MEDL exhibited significant ($p<0.05$) hepatoprotective activity against both inducers. Pre-

treatment of MEDL at a high dose markedly attenuated the increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and prevented a marked decrease in superoxide dismutase (SOD) and catalase (CAT) levels in CCl_4-and PCM-induced hepatotoxic rats. These observations were supported by the histologic findings and scoring, where the liver tissues in groups pre-treated with MEDL and Silymarin showed mild necrosis and inflammation of the hepatocytes compared to the DMSO pre-treated group (negative control group). For the partition extracts, EADL partition exhibited the best activity in protecting the liver against PCM-induced toxicity. Interestingly, EADL, at a low dose (50 mg/kg) significantly (p<0.05) reduced ALP, ALT and AST to 198.5 ± 19.78 U/L, 108.7 ± 29.00 U/L and 313.0 ± 65.99 U/L, respectively and the total bilirubin levels (1.133 ± 0.1687 umol/L) in PCM-induced hepatotoxic rats. EADL also increased the activity of SOD (17.98 ± 0.09 U/g tissue) and CAT (116.9 ± 2.71 U/g tissue) while significantly attenuated the malondialdehyde (MDA) levels of the liver to 2.61 ± 0.70 µM in the liver homogenates. EADL partition also ameliorated histopathological changes to liver tissues by the PCM intoxication. In the *in-vitro* antioxidant assay, MEDL showed the highest DPPH- and superoxide anion-radical scavenging activity (98.94 ± 1.14% and 93.2 ±1.18%) as well as high TPC (1757.25 ± 29.39 mg/100g GAE) and ORAC (24 272.50 ± 2056.53 µM TE/ 100g) values, indicating a high antioxidant activity. In addition, EADL with low TPC (352.18 ± 48.40 mg/100g GAE) exhibited a high scavenging activity against DPPH- and superoxide anion with 93.68 ± 3.0% and 92.6 ± 2.17%, respectively. EADL was also the highest in ORAC value with 555 000 ± 12 700 µM TE/ 100g. The phytochemical screening of MEDL and EADL showed the present of saponins, flavonoids, tannins and polyphenolic compounds. HPLC analysis of MEDL also identified the presence of flavonoids, Rutin and Quercetin in the extracts. Moreover, GCMS analysis revealed the presence of 48 volatiles compounds in the MEDL extracts with some of them reported to exhibit antioxidant and anti-inflammatory activity. MEDL and EADL also exerted hepatoprotective activity that could be partly contributed by its antioxidant activity and high phenolic content, and the presence of various bioactive compounds that might act synergistically to enhance the hepatoprotective effect.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN HEPATOPROTEKTIF OLEH EKSTRAK METANOL DAN PECAHAN ETIL ESITAT EKSTRAK DARI DAUN *Dicranopteris linearis* (Burm. f.) Underw

Oleh

FARAH HIDAYAH BINTI KAMISAN

September 2017

Pengerusi : Profesor Madya Zainul Amiruddin Zakaria, PhD
Fakulti : Perubatan dan Sains Kesihatan

Penyakit hati adalah antara perkara yang membimbangkan secara global dan penyakit ini mempunyai prognosis yang lemah dan kadar kematian yang tinggi disebabkan oleh kekurangan kaedah pencegahan yang efektif atau pilihan rawatan. Selain itu, dalam sistem perubatan moden, terdapat juga kekurangan dadah yang merawat hati dengan kurang kesan sampingan. Oleh itu, usaha dilakukan untuk mengkaji terapi alternatif dengan menggunakan tumbuhan semulajadi. Untuk bahagian pertama kajian in vivo, ekstrak metanol daun *Dicranopteris linearis* (MEDL) telah dikaji untuk aktiviti hepatoprotektif melawan kerosakan hati oleh karbon tetraklorida (CCl_4) dan parasetamol (PCM). Tikus-tikus (n=6) dibahagikan kepada beberapa kumpulan dengan diberi sekali sehari 10% dimetil sulfoksida (kumpulan kawalan negatif), 200 mg/kg Silymarin (kumpulan kawalan positif), atau MEDL (50, 250 or 500 mg/kg) masing-masing selama 7 hari diikuti dengan induksi kerosakan hati oleh CCl_4 dan PCM. Ekstrak methanol kemudian melalui kaedah partisi dengan menggunakan larutan berbeza polarity secara menaik: petroleum eter< etil asetat< air. Partisi- partisi semi tulen tersebut dipanggil petroleum eter (PEDL), Etil asetat (EADL) dan akua (AQDL). Untuk bahagian kedua kajian in- vivo, partisi-partisi tersebut diuji untuk mencegah kerosakan hati oleh PCM. Pada dos sederhana (250 mg/kg), semua partisi-partisi diuji untuk mencegah kerosakan hati oleh PCM. Partisi yang terbaik dilanjutkan untuk ujian pada dos 50 mg/kg dan 500 mg/kg. Sampel darah dan hati dikumpul untuk analisa biokimia dan mikroskopi. Ekstrak mentah MEDL dan semua partisi- partisinya juga diuji untuk antioksidan asai iaitu asai skaveng radikal 2, 2-difenil-1-picrilhidrazil (DPPH), asai skaveng radikal superoksida dismutase (SOD), dan asai kapasiti penyerapan oksigen radikal (ORAC), dan asai anti-inflamasi iaitu asai lipoksigenas (LOX) and xanthine oxidase (XO). Selain itu, jumlah kandungan fenol (TPC), saringan fitokimia, analisa kromatografi cecair berprestasi tinggi (HPLC) dan analisa kromatografi gas- spectrometer jisim (GCMS) juga dijalankan. MEDL menunjukkan aktiviti hepatoprotektif yang ketara ($p< 0.05$) mencegah

kerosakan hati. Pra-rawatan dengan MEDL pada dos tinggi menurunkan peningkatan paras alanin aminotransferas (ALT), aspartate aminotransferas (AST), dan alkalin fosfatas (ALP) dalam serum secara ketara dan juga mencegah penurunan paras enzim superoksida dismutase (SOD) dan katalas (CAT) dalam hati tikus yang telah dirangsang kerosakan hati oleh CCl_4 dan PCM. Hasil pemerhatian ini juga disokong oleh hasil dari analisa dan indeks histology, dimana hati yang telah pra-rawat oleh MEDL dan Silymarin menunjukkan kurang nekrosis dan inflamasi apabila dibandingkan dengan kumpulan kawalan negative. Untuk ektsrak partisi, partisi EADL menunjukkan aktiviti yang terbaik dalam melindungi hati dari kerosakan oleh PCM. Menariknya, EADL pada dos rendah 50 mg/kg, menurunkan paras ALP, ALT dan AST yang ketara kepada 198.5 ± 19.78 U/L, 108.7 ± 29.00 U/L, dan 313.0 ± 65.99 U/L dan juga paras jumlah bilirubin dalam serum kepada 1.133 ± 0.1687 umol/L). EADL juga telah meningkatkan aktiviti SOD (17.98 ± 0.09 U/g tisu) dan CAT (116.9 ± 2.71 U/g tisu) manakala menurunkan secara ketara paras malondialdihida (MDA) kepada 2.61 ± 0.70 μM dalam hati. Partisi EADL juga mengurangkan perubahan histopatologi kepada tisu hati dari toksik oleh PCM. Dalam asai in vitro antioksidan, MEDL menunjukkan aktiviti yang tertinggi dalam skaveng radikal DPPH dan superoksida (98.94 ± 1.14% dan 93.2 ±1.18%) dan tinggi TPC (1757.25 ± 29.39 mg/100g GAE) dan nilai ORAC (24 272.50 ± 2056.53 μM TE/ 100g) menunjukkan tinggi aktiviti antioksidan. Tambahan pula, EADL dengan jumlah TPC yang rendah (352.18 ± 48.40 mg/100g GAE) menunjukkan aktiviti yang tinggi dalam skaveng radikal DPPH dan superoksida, masing- masing dengan 93.68 ± 3.0% dan 92.6 ± 2.17%. EADL juga tinggi nilai ORAC sebanyak 555 000 ± 12 700 μM TE/ 100g. Dalam saringan fitokimia MEDL dan EADL, menunjukkan adanya sebatian saponin, flavonoid, tannin dan juga polefenolik. Analisa HPLC MEDL juga telah mengenal pasti adanya Rutin dan Quercetin di dalam ekstrak. Selain itu, analisa GCMS juga merungkai terdapat 48 sebatian yang meruap terdapat dalam ekstrak, dimana sebahagian dari sebatian tersebut mempunyai aktiviti antioksidan dan antiinflamasi. MEDL dan EADL mempunyai aktiviti heptoprotektif mungkin disumbang oleh aktiviti antioksidan dan jumlah kandungan fenol di dalamnya, serta disebabkan kehadiran beberapa sebatian bioaktif yang bertindak secara bersinergisma antara satu sama lain untuk mempertingkat efek hepatoprotektif.

ACKNOWLEDGEMENTS

Alhamdulillah, All Praise and Thanks to Allah Subhanahu wa ta`ala for helping me in completing my journey in pursuing the degree of Doctor of Philosophy.

I would like to express my greatest appreciation to my supervisor, Associate Professor Dr Zainul Amiruddin Zakaria who has never stopped encouraging and helping me throughout the course of my study. I thank him for giving me the opportunity to be a postgraduate student under his supervision.

My appreciation also goes to my co-supervisor, Associate Professor Dr Norhafizah Mohtarruddin for her compassion in guiding me in the histological study that is undeniably vital in completing this project. I would also like thank my co-supervisor, Dr Siti Farah Binti Md Tohid and Professor Dr. Mohd Zaki Salleh for the opportunity to conduct a research under their guidance and for their continuous encouragement and willingness to share their knowledge with me.

My sincere appreciation also goes to the staff members of the Pharmacology and Toxicology Laboratory, Histopathology and Chemical Pathology Laboratory and also those from the Animal House, Faculty of Medicine and Health Science, UPM for their cooperation in allowing me to perform my research in the laboratory unit and the excellent facilities that have helped the analyses to run smoothly.

I would also like to acknowledge and express my gratitude to all my fellow friends, Farhana, Roihana, Tavamani, Siti Syariah, Nur Diyana, Nur Liana, Erin, Wahida, Khusairi, Mohd Hijaz, Muhammad Hafiz, Salahhuddin and Fauzi Fahmi for their support, assistance and kindness towards me in completing this research project successfully.

Last but not least, my heartfelt gratitude goes to my beloved parents, Encik Kamisan bin Misran and Puan Nor'Aishah Zakaria, my beloved husband, Mohammad Sya'rani Najmuddin and my little daughter Nur Syifa Humaira for their love, patience, moral support and encouragement that have truly motivated me to complete my journey as a postgraduate student successfully. To my family, including members of my family in-law and everyone who has been involved in this project whether directly or indirectly, thank you so much. I can never repay your supports and love. May Allah bless all of them.

Thank you so much.

I certify that a Thesis Examination Committee has met on 7 September 2017 to conduct the final examination of Farah Hidayah binti Kamisan on her thesis entitled "Hepatoprotective Activity of *Dicranopteris linearis* (Burm.f.) Underw. Leaf Methanol Extract and its Ethyl Acetate Fraction" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Muhammad Nazrul Hakim bin Abdullah, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mohamad Taufik Hidayat bin Baharuldin, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Patimah binti Ismail, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Lyndy Joy Mcgaw, PhD
Professor
University of Pretoria
South Africa
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 30 November 2017

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Zainul Amiruddin Zakaria, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Norhafizah Mohtarrudin, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Siti Farah Md Tohid, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Mohd. Zaki Salleh, PhD
Professor
Faculty of Pharmacy
Universiti Teknologi MARA
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Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Norhafizah Mohtarrudin

Signature: _____
Name of Member of Supervisory Committee: Dr. Siti Farah Md Tohid

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Mohd. Zaki Salleh

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatise
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APAP	Acetyl-para-aminophenol
AQDL	Aqueous extract of *Dicranopteris linearis*
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CAT	Catalase
CAM	Complementary and alternative medicine
$CCl_3\cdot$	Trichloromethyl free radical
CCl_4	Carbon tetrachloride
$Cl_3COO\cdot$	Trichloromethyl peroxy
CMC	Sodium carbomethyl cellulose
COX	Cyclooxygenase
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EADL	Ethyl acetate extract of *Dicranopteris linearis*
EDTA	Ethylenediaminetetra acetic acid
FDA	Food and Drug Administration
eNOS	Endothelial nitric oxide synthase
GSH	Glutathione
Hb	Haemoglobin
HPLC	High performance liquid chromatography
i.p	Intraperitoneally
IC_{50}	Median inhibitory concentration
IBS	Institute of Bioscience
ICR	Institute of Cancer Research
iNOS	Inducible nitric oxide synthase
LD_{50}	Lethal dose of 50% population
LOX	Lipooxygenase
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MEDL	Methanol extract of *Dicranopteris linearis*
MeOH	Methanol
NAC	N-acetyl cystein
NAPQI	N-acetyl-p-benzoquinoneimine
NO	Nitric oxide
OECD	Organisation for Economic Cooperation and Development
o.p	Orally
ORAC	Oxygen radical absorbance capacity
PCM	Paracetamol
PCV	Packed cell volume

PEDL	Petroleum ether extract of *Dicranopteris linearis*
RBC	Red blood cell
ROW	Relatives organ weight
ROS	Reactive oxygen species
SEM	Standard error mean
SOD	Superoxide dismutase
TB	Total bilirubin
TPC	Total phenolic content
WBC	White blood cell
WHO	World Health Organization
w/v	Weight/ volume
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Background of the 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. Nevertheless, factors such as toxic chemicals, alcohol abuse and viral infections can lead to different extent of liver injury. Liver disease can be classified as acute or chronic hepatitis (inflammatory liver disease), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder leading to fibrosis of the liver) (Kumar et al., 2012). Nowadays, liver disease has become one of the main concerns threatening human health at high prevalence (Hou et al., 2013). For this reason, medical experts and the general population are increasingly paying attention to ways to maintain a healthy liver. Scientific research on natural plants or their compounds with potential hepatoprotective activity for functional food and nutraceutical development has also been an important topic in many fields, including nutrition and pharmaceutical.

Moreover, the World Health Organization (WHO) recorded that 80% of the world's population depends on either, wholly or partly on plant-derived pharmaceuticals (MacDonald et al., 2016). The world population have shifted from synthetic drugs to natural products because of the very expensive cost and associated intolerable side effects of most synthetic drugs used for hepatic disorders (Khan et al., 2017; MacDonald et al., 2016). Herbal medicines have gained significance and popularity in recent years due to safety, efficacy and cost effectiveness associated with them. Extracts of a wide range of natural plants have been used as raw drugs, and they possess varied medicinal properties. Besides, these plants also contain a wide range of phyto-constituents that can be used to treat diseases. The different parts that are used include the leaves, stems, flowers, fruits and twigs exudates (MacDonald et al., 2016).

One group of plants in biodiversity that has been neglected as far as its economic value is concerned is the pteridophytes. Used as folk medicine, the pteridophytes, which constitute fern and ferns allies, have been known to man for more than 2000 years (Talukdar et al., 2011). *Dicranopteris linearis* is one of the neglected plants that is widely distributed in Malaysia, and it has been used in folk medicine in some countries including Malaysia, Papua New Guinea and India (Zakaria et al., 2008). The Malays call it "Resam" and have been using its leaves to control fever as it helps in reducing body temperature. *D.linearis* leaves are squeezed in water before the water is drunk as tonic, or the wet leaves are applied to one's body as poultice (Zakaria et al., 2008). Several other biological attributes of *D. linearis* leaves have also been reported; nevertheless, none on them were on the

hepatoprotective properties. Therefore, this study is to assess the potential of *D. linearis* leaves as a hepatoprotective agent.

1.2 Problem statement

Liver disease is a health problem worldwide. The causes that may contribute to liver disease are excessive alcohol consumption, drug overdose (eg. Paracetamol), viral infection and obesity. According to the Malaysian Liver Registry, Association of Clinical Registries Malaysia (2005), digestive disorder was responsible for 3.2% of the total burden disease in Malaysia in 2000, and cirrhosis of the liver was the main and important cause of burden of disease among digestive disorders causal as it was responsible for up to 60% of the total burden in this category. In Malaysia, liver cirrhosis is mostly caused by the viral hepatitis B and C infections and also alcohol consumption (Qua and Goh, 2011). However, recently there is a rising prevalence of non-alcoholic fatty liver disease (NAFLD) due to diabetes and obesity which are common among Malaysians. The fatty accumulation in liver could result in liver inflammation, liver tissue scarring (fibrosis), cirrhosis and even liver cancer (Ho, 2013). In addition, overdose of paracetamol (PCM) is recognised to result in a range of hepatic damage, leading to acute liver failure (ALF) and death. This problem is infrequent in Asia; however, it is a common means of self-poisoning worldwide because of its wide availability and accessibility (Marzilawati et al., 2012). Acute liver failure due to PCM overdose has been extensively reported in the United Kingdom (UK), the United States (US), France, Canada and Australia. 60 to 75% of acute liver failure in the UK was due to paracetamol overdoses (Marzilawati et al., 2012).

1.3 Justification of the study

Despite remarkable advances in modern medicines, liver disease remains a health problem worldwide. Hence, the search for new treatments is still ongoing. The currently used conventional or synthetic drugs in the treatment of liver disease are insufficient and have severe side effects. Besides, some of the available treatments also have low bioavailability and poor solubility (Khan et al., 2017). Due to the serious side effects of available treatments, there is an urgent need to investigate and develop more efficient hepatoprotective agents with high bioavailability, better solubility, but at the same time, inexpensive and safe. In recent times, focus on plant research has increased all over the world and interestingly, utilisation of herbal medicines to prevent various chronic diseases has been a common clinical practice for long in Asian countries (Ghosh et al., 2011). Thus, investigation of hepatoprotective activity of methanol extract of *D. linearis* (MEDL) leaves and its partitions, may contribute to the identification of another natural plant product that may be effective as a hepatoprotective agent. To date, there is no complete study on the hepatoprotective activity of methanol extract of *D. linearis* leaves and its partitions. Recently, investigations of *D. linearis* leaves have demonstrated that they exert anti-oxidant and anti-inflammatory activities (Zakaria et al., 2011; Zakaria et al., 2008). Both activities are crucial as a mechanism of liver protection against toxic substances. Literature review shows that some plants are useful in

protecting the liver by improving the antioxidant and anti-inflammatory statuses (Makni et al., 2011; Makni et al., 2010; Makni et al., 2008). In addition, phytochemical studies on *D.linearis* leaves have reported to show the presence of various types of flavonoids, particularly of flavonol 3-o-glycosides types and triterpenes, saponins and steroids (Hussaini et al., 2012). Flavonoids possess a wide range of bioactive capacities, especially their antioxidant activity as antioxidants play a significant role in liver protection (Pareek et al., 2013). Supported by these facts, *D. linearis* leaves may also have the potential to exert hepatoprotective activities thus, justifying the investigation of hepatoprotective activity of *D. linearis*. This study may contribute in the search for other plant products that can be used in the treatment of liver disease.

1.4 Hypothesis

Methanol extract of *D. linearis* leaves (MEDL) possesses hepatoprotective activity against both carbon tetrachloride (CCl_4)- and Paracetamol (PCM)-induced liver injury, and one of its partitions either petroleum ether partition (PEDL), ethyl acetate partition (EADL) or aqueous partition (AQDL) possesses the best hepatoprotective agent against PCM-induced liver injury.

1.5 Objective of the study

1.5.1 General objective

This study aimed to investigate the *in vivo* hepatoprotective properties of the methanol extract of *Dicranopteris linearis* leaves (MEDL) and its petroleum ether (PEDL), ethyl acetate (EADL) and aqueous (AQDL) partitions using rat models.

1.5.2 Specific objectives

1. To determine the acute toxicity effects of *D. linearis* methanol extract *in vivo.*
2. To investigate the potential of the methanol extract of *D. linearis* (MEDL) as a hepatoprotective agent against CCl_4 and PCM toxicity.
3. To determine whether (PEDL, EADL or AQDL) is the best to possess the hepatoprotective activity against PCM- induced liver injury.
4. To assess the antioxidant activities of MEDL and its partitions via determination of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), superoxide dismutase scavenging activity (SOD), and oxygen radical absorbance capacity (ORAC) assays that may be involved to act as a pathway in hepatoprotective properties of *Dicranopteris linearis* against liver toxicants.
5. To assess the *in vitro* anti-inflammatory activity of MEDL and its partitions using lipoxygenase and xanthine oxidase assays.

6. To screen the bioactive compounds present in MEDL crude extract and its partitions using high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometer (GCMS).

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