TOXICOLOGICAL AND ANTI-INFLAMMATORY EFFECTS OF LIGNOSUS RHINOCEROTIS COOKE RYVARDEN (TIGER MILK MUSHROOM)

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ABSTRACT

Lignosus rhinocerotis (Cooke) Ryvarden (Tiger Milk mushroom) has been traditionally used to treat a variety of diseases, including asthma, fever, food poisoning, joint pain, cancers, kidney disorders, body swelling, chronic cough, chronic hepatitis, gastric ulcer and as a general tonic. The sclerotium of the mushroom is the part with medicinal value. This rare mushroom has recently been successfully cultivated, making it possible to be fully exploited for its medicinal and functional benefits. Sub-acute toxicity of the sclerotial powder of *L. rhinocerotis* from the wild type and two cultivars (termed TM02 and TM03) as well as chronic toxicity of the sclerotial powder of cultivar TM02 were evaluated. There was no treatment-related sub-acute toxicity in rats following 28-days oral administration of 250, 500 and 1000 mg/kg TM02, 1000 mg/kg TM03 as well as 1000 mg/kg wild type *L. rhinocerotis* sclerotial powder, as measured by haematological, clinical biochemistry, weight, general observations and histological examinations of heart, kidney, spleen, lung and liver. There was also no treatment-related chronic toxicity in rats following the long term (180-days) oral administration of 250, 500 and 1000 mg/kg of *L. rhinocerotis* (TM02) sclerotial powder, as shown by the clinical observations, body weight gain, haematological analysis, clinical biochemistry, urinalysis, absolute organ weight, relative organ weight and histological examinations of the organs. Thus, the no-observed-adverse-effect level (NOAEL) doses for both sub-acute and chronic toxicity of the respective sclerotial powders were more than 1000 mg/kg. The sclerotial powder of *L. rhinocerotis* (TM02) at 100 mg/kg did not cause adverse effect on fertility nor teratogenic effect on the offspring of the treated rats. The bacterial reverse mutation assay also showed that the sclerotial powder (TM02) was not mutagenic. Cold water extract (CWE), hot water extract (HWE) and methanol extract (ME) of the sclerotial powder of *L. rhinocerotis* cultivar TM02 possessed anti-acute inflammatory activity as was

measured by carrageenan-induced paw oedema test using rats, with CWE having the most potent activity. The acute anti-inflammatory activity of CWE was mainly contributed by its high molecular weight (HMW) fraction isolated by Sephadex G-50 gel filtration chromatography. CWE at 200 mg/kg did not inhibit transudative and proliferative phase of chronic inflammation, as shown by using the cotton pellet induced granuloma in rats. The anti-inflammatory activity of CWE of TM02 which was measured by inhibition of lipopolysaccharide induced TNF-alpha production in RAW 264.7 macrophage cells was mainly contributed by the protein component (also containing carbohydrate) of the HMW fraction as it exhibited strong inhibitory effect on TNF-alpha production with an IC_{50} of 9.35 ± 0.48 μg/ml based on total carbohydrate and protein content. The protein component was subjected to fractionation by anionic exchange chromatography (Resource™ Q) and two active fractions (F5 and F6) with the strongest inhibitory effect on TNF-alpha production were separated by SDS-PAGE. LC-MS/MS (QTOF) analysis of SDS-PAGE gel section and literature research suggested possible anti-inflammatory candidate(s) of F5 and F6 to be serine proteases (the most potential candidate), lectins and/or immunomodulatory proteins.

ABSTRAK

Lignosus rhinocerotis (Cooke) Ryvarden (Cendawan Susu Harimau) lazimnya digunakan untuk mengubati penyakit asma, deman, keracunan makanan, sakit sendi, kanser, penyakit buah pinggang, bengkak-bengkak badan, batuk kronik, hepatitis kronik, ulser gastrik dan sebagai tonik. Ubi (atau lebih dikenali sebagai sklerotium) cendawan tersebut merupakan bahagian yang mempunyai nilai perubatan. Kini, cendawan yang jarang ini telah berjaya dikultur. Melalui kaedah pengkulturan ini, bekalan cendawan ini makin stabil dan peranan cendawan ini dalam bidang perubatan dapat dikaji. Kajian ketoksikan sub-akut ubi *L. rhinocerotis* jenis liar dan dua kultivar (digelar TM02 dan TM03) serta kajian ketoksikan kronik ubi daripada kultivar TM02 telah dijalankan. Pemerhatian ujikaji menunjukkan bahawa ubi *L. rhinocerotis* tidak toksik pada dos 250, 500 dan 1000 mg/kg TM02, 1000 mg/kg TM03 serta 1000 mg/kg (jenis liar) berikutan rawatan secara oral selama 28 hari. Ujian hematologi dan biokimia serta berat badan, pemerhatian umum dan histologi jantung, buah pinggang, limpa, paru-paru dan hati telah dijalankan dan tiada sebarang tanda kemudaratan ditemui. Kajian ketoksikan kronik (dos 250, 500 dan 1000 mg/kg *L. rhinocerotis* (TM02) ubi *L. rhinocerotis*) berikutan rawatan secara oral selama 180 hari juga tidak menunjukkan sebarang tanda ketoksikan (melalui pemerhatian klinikal, perbezaan berat badan, analisis hematologi, biokimia, ujian kencing, berat organ mutlak, berat organ relative dan pemeriksaan histologi organ). Oleh yang demikian, dos no-observed-adverse-effect level (NOAEL) untuk kajian ketoksikan sub-akut dan kronik daripada ubi *L. rhinocerotis* masing-masing telah ditentukan melampaui 1000 mg/kg. Ubi *L. rhinocerotis* (TM02) pada 100 mg/kg juga didapati tidak membawa kemudaratan kepada kesuburan tikus dan tiada kesan teratogenik diperhatikan pada anak tikus. Pencerakinan mutasi berbalik bakteria telah menunjukkan bahawa ubi tersebut tidak mempunyai sebarang sifat mutagenik. Ekstrak air sejuk (CWE), ekstrak air

panas (HWE) dan ekstrak metanol (ME) bagi serbuk ubi *L. rhinocerotis* (TM02) didapati mempunyai aktiviti anti-radang akut. Ini terbukti melalui uji kaji dengan menggunakan karrageenan yang berupaya menyebabkan bengkak pada tapak kaki tikus. CWE didapati paling berpotensi dalam aktiviti anti-radang ini. Kajian lanjut telah menunjukkan bahawa aktiviti anti-radang akut oleh CWE disumbangkan terutamanya oleh bahagian yang mempunyai berat molekul tinggi (HMW) dalam serbuk ubi tersebut. Bahagian ini telah berjaya diasingkan dengan menggunakan kaedah penurasan gel dengan Sephadex G-50 sebagai media penurasan. CWE pada 200 mg/kg didapati tidak menghalang fasa transudatif dan proliferatif dalam radang kronik seperti yang ditunjukkan dalam ujikaji pelet kapas yang berupaya menyebabkan granuloma dalam tikus. Model penghasilan TNF-alpha oleh sel RAW 264.7 akibat dorongan lipopolisakarida (LPS) telah digunakan dalam penganalisaan aktiviti anti-randang yang selanjutnya. Didapati bahawa aktiviti anti-radang CWE TM02 disumbangkan terutamanya oleh komponen protein dalam bahagian HMW (P-HMW) yang juga mengandungi karbohidrat. P-HMW berupaya merencat TNF-alpha dengan IC_{50}: 9.35 ± 0.48 μg/ml dan seterusnya diasingkan melalui kaedah kromatografi penukaran ion (Resource™ Q). Dua pecahan (F5 dan F6) dengan kesan perencatan yang paling kuat ke atas penghasilan TNF-alpha oleh sel RAW 264.7 yang didorong LPS telah diasingkan melalui kaedah SDS-PAGE. Juzuk yang menyumbangkan aktiviti anti-randang bagi kedua-dua pecahan tersebut berkemungkinan besar merupakan sebagai serine protease (paling berpotensi), lectins dan/atau protein immunomodulasi melalui analisis LC-MS/MS (QTOF).

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LIST OF SYMBOLS AND ABBREVIATIONS

184B5:	Human breast epithelial cell line
A-549 :	Human lung carcinoma cell line
AA:	Arachidonic acid
ABTS•+:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical
APTT :	Activated partial thromboplastin time
ALT:	Alanine transaminase
AST:	Aspartate aminotransferase
BALB/3T3:	Mouse embryonic fibroblast cell line
BSA:	Bovine serum albumin
CCD-18CO:	Human colonic myofibroblasts
COX:	Cyclooxygenase
COX-2:	Cyclooxygenase-2
CSF:	Colony-stimulating factors
CWE:	Cold water extract
DMARDs:	Disease-modifying anti-rheumatic drugs
DMSO:	Dimethyl sulfoxide
DPPH•:	1,1-diphenyl-2-picrylhydrazyl radical
ERK:	Extracellular signal-regulated protein kinase 1/2
FIPs:	Fungal immunomodulatory proteins
FRAP:	Ferric reducing antioxidant power
GOPOD:	Glucose oxidase/peroxidase mixture
GRO:	Growth-regulated oncogene
GGT:	Gamma-glutamyl transpeptidase

LIST OF SYMBOLS AND ABBREVIATIONS

H_2O_2:	Hydrogen peroxide
H&E:	Haematoxylin and eosin
HCT-116:	Human colorectal carcinoma cell line
HeLa:	Human epithelial carcinoma cell line
HepG2:	Human hepatocellular carcinoma cell line
HK-1:	Human nasopharyngeal carcinoma cell line
HL-60:	Human acute promyelocytic leukaemia cell line
HMW:	High molecular weight fraction
HSC-2:	Human squamous carcinoma cell line
HWE:	Hot water extract
ICAM-1:	Intercellular adhesion molecule 1
IC_{50}:	Half-maximal inhibitory concentration
IC_{70}:	70% inhibitory concentration
ID_{50}:	Median inhibitory dose
IDF:	Insoluble dietary fiber
IL:	Interleukin
IFN-γ:	Interferon gamma
iNOS:	Inducible nitric oxide synthase
IP-10:	IFN-γ-inducible protein 10
ITS:	Internal transcribed spacer
JNK:	c-Jun amino-terminal kinase
K-562:	Human chronic myelogenous leukemia cell line
LDH:	Lactate dehydrogenase
LMW:	Low molecular weight fraction

LIST OF SYMBOLS AND ABBREVIATIONS

LPS:	Lipopolysaccharide
MAPKs:	Mitogen-activated protein kinases
MCF-7 and MDA-MB-231:	Human breast adenocarcinoma cell line
MCH:	Mean corpuscular haemoglobin
MCHC:	Mean corpuscular haemoglobin concentration
MCP:	Monocyte chemotactic protein
MCV:	Mean corpuscular volume
MD:	Human spleen monocyte/macrophage cell line
ME:	Methanol extract
MIP:	Macrophage inflammatory protein
MMW:	Medium molecular weight fraction
MNCs:	Mononuclear cells
MRC-5:	Human lung fibroblast cell line
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88:	Myeloid differentiation factor 88
N2a:	Neuroblastoma-2a cell line
NAP-2:	Neutrophil-activating protein-2
NDCs:	Non-digestible carbohydrates
NF-κB:	Nuclear transcription factor kappa-B
NGF:	Nerve growth
NK cells:	Natural killer cells
NL-20:	Human lung epithelial cell line
nNOS:	Neuronal nitric oxide synthase

LIST OF SYMBOLS AND ABBREVIATIONS

NO:	Nitric oxide
NOAEL:	No-Observed Adverse Effect Level
NP-HMW:	Non-protein component of HMW fraction
NP-MMW:	Non-protein component of MMW fraction
NRU:	Neutral red uptake
NSAIDS:	Non-steroidal anti-inflammatory drugs
O_2^-:	Superoxide anion radical
PC-3:	Human prostate adenocarcinoma cell line
PC-12:	Pheochromocytoma cell line
PCV:	Packed cell volume
PECs:	Peritoneal exudate cells
PG:	Prostaglandin
P-HMW:	Protein component of HMW fraction
PMA:	Phorbol myristate acetate
P-MMW:	Protein component of MMW fraction
PMSF:	Phenylmethylsulfonyl fluoride
RAW 264.7:	Murine macrophage cell line
RBC:	Red blood cell
S.D.:	Standard deviation
SD rats:	*Sprague Dawley* rats
SDF:	Soluble dietary fiber
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

LIST OF SYMBOLS AND ABBREVIATIONS

S.E.M.:	Standard error of the mean
SGOT:	Serum glutamic oxaloacetic transaminase
SGPT:	Serum glutamic pyruvic transaminase
SOD:	Superoxide dismutase
sTNF-α:	Soluble tumour necrosis factor alpha
TEMED:	N,N,N',N'-tetramethylethylenediamine
THP-1:	Human acute monocytic leukemia cell line
tmTNF-α:	Transmembrane tumour necrosis factor alpha
TNF-α:	Tumour necrosis factor alpha
TNFR1:	TNF-α receptor 1
TNFR2:	TNF-α receptor 2
VCAM-1:	Vascular cell adhesion molecule 1
VEGF:	Vascular endothelial growth factor
WBC:	White blood cell
WRL-68:	Human embryonic liver cell line

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Mushrooms have long been appreciated as an ingredient for gourmet cuisines across the globe due to their unique flavour and texture. Mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Trametes versicolor*, *Inonotus obliquus* (Chaga) and many others have been used as traditional medicine as a remedy for different diseases in Japan, China and Korea and eastern Russia (Wasser, 2002; Lee et al., 2012a). Modern scientific researches show that medicinal mushrooms contain bioactive substances with anti-microbial, anti-viral, anti-tumour, anti-allergic, anti-inflammatory, anti-atherogenic, immunomodulating, hepatoprotective, hypoglycaemic and central activities (Lindequist et al., 2005). For these reasons, mushrooms have been receiving increasing attention as a valuable source of pharmaceuticals, as functional food and nutraceuticals (Xu et al., 2011; Giavasis, 2014). Furthermore, mushrooms are low in cholesterol, fat, sodium and calories, but rich in carbohydrate, protein, vitamins, minerals and fiber (Aida et al., 2009). These medicinal and nutritional benefits of mushrooms make them potential candidates in the formulation of novel nutraceuticals and functional foods.

Lignosus rhinocerotis (Cooke) Ryvarden (Tiger Milk mushroom) is an important medicinal mushroom in Southeast Asia and China. It has been traditionally used in the treatment for a variety of diseases, including asthma, fever, food poisoning, joint pain, cancers, kidney disorders, body swelling, chronic cough, chronic hepatitis, gastric ulcer and as a general tonic (Chang & Lee, 2004; Nasir, 2006; Wong & Cheung, 2008; Lee et al., 2009; Tan, 2009; Ligno ™, 2012). The sclerotium of *L. rhinocerotis* is the part with medicinal value. It can only be found when the cap and stipe of the mushroom sprout from the ground under favourable conditions. This mushroom is rarely solitary in the jungle and thus the collection of the mushroom's sclerotia in large quantity is a difficult

task. Encroachment of deforestation, modern development and pollution have led to the scarcity of this precious mushroom (Vikineswary & Chang, 2013). Hence, the sclerotium is expensive (US$15-25 per sporophore including the sclerotium) and supply is limited (Abdullah et al., 2013). In 2012, Tan et al. reported a successful cultivation of *L. rhinocerotis* (sclerotia) in specially formulated culture medium and biotechnological approach with good yield. Subsequently, Abdullah et al. (2013) reported a pilot cultivation of *L. rhinocerotis* (mycelia) using an optimised formulation comprising paddy straw and sawdust. These successful cultivation methods have produced large quantity of the mushroom, enabling scientific research and exploitation of its medicinal benefits. In this study, sclerotia of *L. rhinocerotis* (from cultivars TM02 and TM03) were provided by Ligno Biotech Sdn. Bhd (Selangor, Malaysia).

The sclerotial extracts of *L. rhinocerotis* have been demonstrated to exhibit anti-proliferative, anti-oxidant, anti-microbial and immunomodulatory effect, and neurite outgrowth stimulation (Lai et al., 2008; Wong et al., 2009; Wong et al., 2011; Eik et al., 2012; Lee et al., 2012b; Mohanarji et al., 2012; Lau et al., 2013; Phan et al., 2013; Yap et al., 2013; Zaila et al., 2013). Non-digestible carbohydrates (NDCs) from the sclerotia may function as novel prebiotics (Gao et al., 2009). In view of the traditional claims as well as the scientifically validated therapeutic effects of the sclerotium, the mushroom sclerotia have the potential to be used as health supplement (nutraceutical) and hence warrants an in depth evaluation of the safety of sclerotium before it is to be marketed. Therefore, in the present study, sub-acute toxicity studies (28-days) of the sclerotium of wild type *L. rhinocerotis* and the cultivars (termed TM02 and TM03) were carried out. In addition, assessment of 180-day chronic toxicity, reproductive toxicity (anti-fertility and teratogenic effects) and genotoxicity of the sclerotium of *L. rhinocerotis* cultivar TM02 were also carried out.

The traditional uses of *L. rhinocerotis* sclerotium for treatment of asthma, cough, joint pain, chronic hepatitis and gastric ulcer are presumably related to its anti-inflammatory effect. There is, however, a lack of information in this aspect. Hence, in this study, we also investigate the anti-inflammatory properties of the sclerotial extracts and fractions (cultivar TM02), as well as the bioactive component(s) contributing to the anti-inflammatory activity.

1.1 Objectives

The objectives of the present study are:

1. To assess the sub-acute toxicity (28-days) of the sclerotium of wild type *L. rhinocerotis* and its cultivars (TM02 and TM03), using a rat model.
2. To evaluate the chronic toxicity of TM02 (180-days), using a rat model.
3. To evaluate the reproductive toxicity (anti-fertility and teratogenic effects) of TM02 using a rat model as well as genotoxicity of TM02 using bacterial reverse mutation assay.
4. To investigate the anti-inflammatory effects of TM02, using rat model and cell line.
5. To investigate the bioactive compound(s) in TM02 that may contribute to its anti-inflammatory effects.

CHAPTER 2: LITERATURE REVIEW

2.1 *Lignosus rhinocerotis* (Cooke) Ryvarden, Tiger Milk Mushroom

Lignosus rhinocerotis (Cooke) Ryvarden (Tiger Milk mushroom, synonym: *Lignosus rhinocerus; Polyporus rhinocerus; Fomes rhinocerus*), known locally as 'cendawan susu rimau' (literally means 'tiger milk mushroom') is one of the most popular medicinal mushrooms used by the indigenous communities of Peninsular Malaysia, local Malay and Chinese to treat a variety of ailments (Mycobank, n.d.; Chang & Lee, 2004; Lee et al., 2009; Tan, 2009). The name "Tiger Milk" mushroom was derived from folklore whereby the mushroom grows at the spot where the tigress would have dripped its milk while feeding the cubs (Ligno ™, 2012). The mushroom is distributed in the tropical rainforest in the regions of South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea (Tan et al., 2012).

The first record of the mushroom was by John Evelyn (1664) who described "*Lac Tygridis*" (Tiger's milk) as a fungi-liked, weighty and a concretion or coagulation of some other matter (Bray, 1901). This mushroom was initially given the scientific name *Fomes rhinocerotis* using a specimen from Penang (Cooke, 1879). This mushroom was later reported by Sir Henry Nicholas Ridley as a valuable medicine of the Malay community for treatment of asthma and other chest complaints (Ridley, 1890). It was recorded by Burkill, Director of Garden, Straits Settlements as one of the economic products with medicinal values in Malay Peninsula (Burkill, 2002).

Sporophore (fruiting body) of *L. rhinocerotis* is unusual when compared to most of the polypores as it consists of a cap on a central stem and grows from an underground sclerotium rather than from wood (Figure 2.1) (Abdullah et al., 2013). It is a polypore mushroom that releases its spore through pores under its cap instead of gills (Bessette et al., 1997). The mushroom is classified according to taxonomy as follows (Mycobank, n.d.):

Kingdom: Fungi

Phylum: Basidiomycota

Subdivision: Agaricomycotina

Class: Agaricomycetes

Order: Polyporales

Family: Polyporaceae

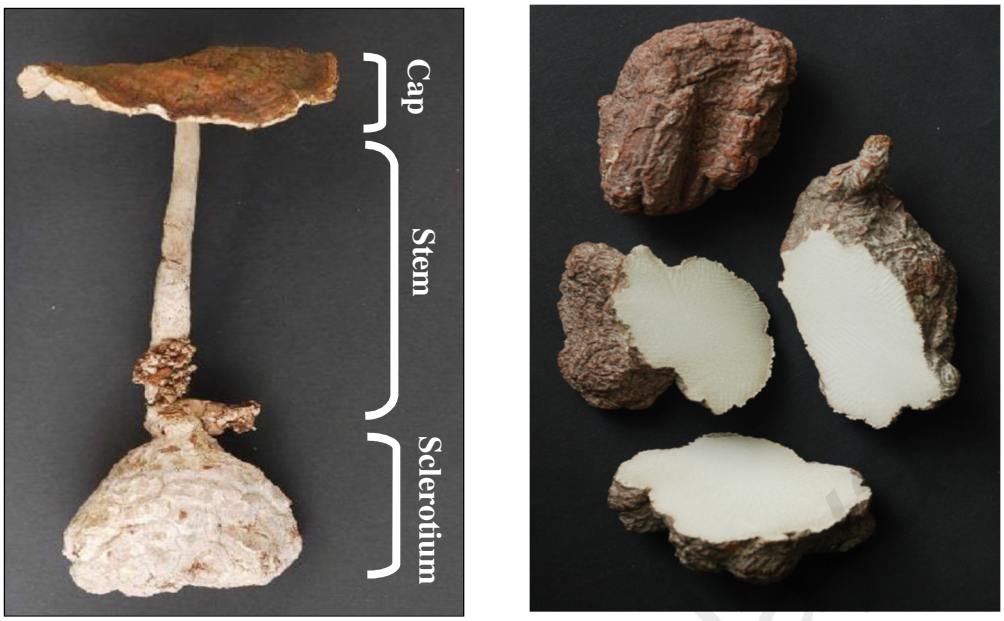

Figure 2.1: ***Lignosus rhinocerotis***

(A) Morphology of *L. rhinocerotis* consists of fruiting body (cap and stem) and sclerotium; (B) Cross-section of *L. rhinocerotis* sclerotium.

(Pictures courtesy of Ligno Biotech Sdn. Bhd.)

2.2 The Sclerotium of *Lignosus rhinocerotis*

"Sclerotia are loosely described as morphologically variable, nutrient-rich and multihyphal structures which can remain dormant or quiescent under adverse environment" (Willetts & Bullock, 1992, p.801). When conditions are favourable, they germinate to reproduce the fungus (Willetts & Bullock, 1992). During sclerotial development, considerable amount of the nutrients are utilised to provide energy and building blocks nutrients for the developing sclerotium (Wong & Cheung, 2008).

The sclerotium of *L. rhinocerotis* is the part of the mushroom with medicinal value. The sclerotium of the mushroom is subterranean with a spherical, oval, or even irregular shape, and its size is approximately 4-5 cm in diameter (Wong & Cheung, 2008). The rind of sclerotia (rough and wrinkly surface) is white to pale brown in colour while the internal structure is white and powdery (Wong & Cheung, 2008).

2.3 Morphological and Genetic Identification of *Lignosus rhinocerotis*

According to Tan et al. (2013), the pore and basidiospore sizes are the two sufficiently reliable characteristics used to identify *Lignosus* species. Table 2.1 shows that *L. rhinocerotis* has 7-8 pores per mm and has basidiospores size 3-3.5 x 2.5-3 µm (Tan et al., 2013). However, specimens obtained from field collections are usually without intact cap and stem, thus making it hard to distinguish *L. rhinocerotis* from other *Lignosus* species. Species identification of the sclerotium, however, can be confirmed by genetic marker (using specific primer) through their internal transcribed spacer (ITS) regions of the ribosomal DNA (Tan et al., 2010). In this approach, the ITS region (ITS-1, 5.8S rRNA and ITS-2) was PCR amplified using primers which were designed according to the conserved region for most fungi. The amplified PCR product was then purified from agarose gel using glass-milk matrix, followed by DNA sequencing and analysis. DNA sequences in ITS1 region of sclerotia from five isolates which were collected from different locations of Malaysia (Cameron Highland (4.4721^{o}N; 101.3801^{o}E), Hulu Langat (3.1131^{o}N; 101.8157^{o}E) and Gerik (5.4285^{o}N; 101.1297^{o}E)) were almost identical and highly conserved and hence it was chosen to design specific primer for the development of PCR-based genetic marker for *L. rhinocerotis* identification. Recently, Yap et al. (2014a) developed a genetic marker based on ITS region for the authentication of the other two *Lignosus* species, *L. tigris* and *L. cameronensis*.

Table 2.1: Pore and basidiospore sizes in *Lignosus* species

(Source: Tan et al., 2013)

Species	Pores (per mm)	Basidiospores (µm)
L. goetzii	0.5-2	6-9 x 5-8
L. tigris	1-2	2.5-5.5 x 1.8-3.6
L. sacer	1-3	5-7 x 3-4.5
L. cameronensis	2-4	2.4-4.8 x 1.9-3.2
L. ekombitii	2-4	8.1-9.3 x 2.5-3.8
L. hainanensis	3-4	4.9-6 x 2.2-2.9
L. dimiticus	6-8	3-4.5 x 2.5-3
L. rhinocerotis	7-8	3-3.5 x 2.5-3

2.4 Cultivation of *Lignosus rhinocerotis*

The sclerotia of *L. rhinocerotis* were successfully cultivated by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia) and two cultivars (termed TM02 and TM03) were provided for entire studies throughout the thesis. According to Tan et al. (2012), tissues and/ or spores from stem, pileus and sclerotium of *L. rhinocerotis* were cultured in a specially formulated media. In order to obtain a clean and pure culture, mycelium growth was subjected to many sub-culturing cycles. The cultivation of sclerotia took approximately 6 months. Cultivation of *L. rhinocerotis* in different developmental stages are demonstrated in Figure 2.2.

Figure 2.2: Different developmental stages during cultivation of *Lignosus rhinocerotis*

(A) Culture of *L. rhinocerotis* mycelium on nitrified agar (2 weeks culture);

(B) Mycelial cultures of *L. rhinocerotis* on solid medium (1 to 2 months cultures);

(C) Newly formed sclerotia on the surface of culture medium (4 to 6 months culture).

(Source: Yap et al., 2014b).

2.5 Nutritional Composition of *Lignosus rhinocerotis*

According to Wong et al. (2003) and Yap et al. (2013), dry matter of both wild type and cultivated (TM02) sclerotia of *L. rhinocerotis* contained substantial amount of carbohydrates, moderate amount of protein and very low lipid content. Protein content of cultivated sclerotia (TM02) was at least 3.6 times higher than the wild type (Yap et al., 2013). Low sugar content was found in both wild type and cultivated sclerotia (Yap et al., 2013).

Wong et al. (2003) reported that almost 90% of the carbohydrate content of wild type sclerotia of *L. rhinocerotis* was in the form of dietary fibers. On the other hand, only around 42% of the carbohydrate content of cultivated sclerotia of the mushroom (named TM02) composed of dietary fiber (unpublished data by Yeannie Yap Hui Yeng).

Dietary fiber can be classified into soluble (SDF) and insoluble fractions (IDF) according to its solubility in an aqueous medium (Wong et al., 2003). Increased intake of SDF enhances glycaemic control and insulin sensitivity in non-diabetic and diabetic individuals (Anderson et al., 2009). IDF are especially effective in increasing fecal mass and promoting regularity (Anderson et al., 2009). The wild type sclerotia contained a notably high level of IDF (98%) with remarkably high levels of non-starch polysaccharides (sum of amino, neutral and uronic acids polysaccharides residues) in which the predominant sugar residue was glucose, followed by glucosamine and uronic acids (Wong et al., 2003). It, however, contained only low amount of SDF (2%). Almost 97% of the dietary fiber of cultivated sclerotia of the mushroom (TM02) was found to be IDF (unpublished data by Yeannie Yap Hui Yeng).

According to Yap et al. (2013), both wild type and cultivated sclerotia of *L. rhinocerotis* showed substantial amount of potassium and magnesium with modest amounts of calcium and sodium. The higher K/Na ratio in TM02 as compared to wild type sclerotia means that TM02 has a good electrolytic balance as a diet (Yap et al., 2013). Total essential amino acid content of TM02 was found to be much higher than wild type sclerotia (Yap et al., 2013).

2.6 Traditional Claims and Consumption of *Lignosus rhinocerotis*

L. rhinocerotis is one of the most popular medicinal mushrooms used by the indigenous communities of Peninsular Malaysia to treat fever, cough, asthma, cancer, food poisoning, joint pain and as a general tonic (Chang et al., 2004; Nasir, 2006; Lee et al., 2009). It is used by Malay traditional practitioners to treat leukemia, cervical cancer, stomach cancer, breast cancer, kidney disorders and body swelling (Tan, 2009). Local Chinese uses it to treat asthma, chronic cough, fever and to strengthen weak constitution (Ligno ™, 2012). In China, the sclerotium of the mushroom is an expensive traditional medicine used for the treatment of liver cancer, chronic hepatitis and gastric ulcers (Wong & Cheung, 2008).

A common way of consumption of *L. rhinocerotis* sclerotium by the Malaysian local community is decoction (Azlina et al., 2012). The sclerorium is sliced and boiled with other herbs such as "tongkat ali" (*Eurycoma longifolia*) root, and the resulting decoction is drunk (Chang & Lee, 2004). It is also found to be administered in a betel-quid in the interior of Pahang (Burkill, 2002). In Kelantan, this sclerotium is given after childbirth where the vegetative parts are pounded with raw rice, infused and drunk (Burkill, 2002).

2.7 Scientifically Validated Therapeutic Effects of the Sclerotia of *Lignosus rhinocerotis*.

2.7.1 Anti-proliferative Activity

Anti-proliferative activities of cultivated and wild type sclerotium of *L. rhinocerotis* against human leukemia, liver cancer, lung cancer, colon cancer, nasopharyngeal cancer, skin cancer, breast cancer and prostate cancer cell line(s) are summarised in Table 2.2. Among all the tested cancer cell lines, human breast adenocarcinoma cell line (MCF-7) was found to be the most susceptible cytotoxic target of cold water extract of the cultivated sclerotium of *L. rhinocerotis* (Lee et al., 2012b; Lau et al., 2013; Yap et al., 2013) in which no significant cytotoxicity was found against the corresponding non-tumourigenic cell line (184B5) (Lee et al., 2012b). These findings provide scientific evidence for traditional use of the sclerotium in breast cancer treatment.

Table 2.2: Anti-proliferative activity of the sclerotia of *Lignosus rhinocerotis*

Wild type (WT)/ cultivated (C)	Type of extract	Cell line (s)	IC_{50} (µg/ml)	Reference
WT	Hot water	HL-60	100	Lai et al. (2008)
		K-562	388	
		THP-1	> 400	
WT	Cold alkali	HL-60, K-562, THP-1	No inhibition	
C (TM02)	Cold water	MCF-7	97	Lee et al. (2012b)
		A-549	467	
WT	Cold water	MCF-7	206	Yap et al. (2013)
	Hot water and methanol		>1000	
C(TM02)	Cold water	MCF-7	90	
	Hot water and methanol		>1000	
C	Cold water	A-549	41	Lau et al. (2013)
		HepG2	120	
		HCT-116	37	
		HK-1	88	
		HSC-2	57	
		MCF-7	37	
		MDA-MB-231	79	
		PC-3	43	
		HL-60	355	
	Hot water	Same as cold water extract	>500	
WT	Methanol pressurised liquid	HCT-116	600	Zaila et al. (2013)
	Hot aqueous pressurised liquid	HCT-116	1200	

Abbreviations: HL-60: human acute promyelocytic leukemia cells; K-562: human chronic myelogenous leukemia cells; THP-1: human acute monocytic leukemia cells; A-549: human lung carcinoma cells; HepG2: human hepatocellular carcinoma cells; HCT-116: human colorectal carcinoma; HK-1: human nasopharyngeal carcinoma cells; HSC-2: human squamous carcinoma cells; MCF-7 and MDA-MB-231: human breast adenocarcinoma cells; PC-3: human prostate adenocarcinoma cells.

Lai et al. (2008) demonstrated that sclerotial glucomannan-protein complexes from hot water extract of wild type *L. rhinocerotis* exhibited anti-proliferative effects on human acute promyelocytic leukemia cells (HL-60), human chronic myelogenous leukemia cells (K-562) and human acute monocytic leukemia cells (THP-1) whereas no inhibition activity was seen in glucans derived from cold alkali extract. Anti-proliferative effect of the hot water extract on the most susceptible cell line, HL-60 was mediated by apoptosis as a result of cell cycle arrest at G_1 phase. However, no specific cell cycle arrest was seen in K-562 cells treated with the same extract.

Zaila et al. (2013) reported cytotoxic effect of alkaloid rich methanol and hot aqueous pressurised extracts of wild type sclerotium of *L. rhinocerotis* against human colorectal carcinoma cells (HCT-116) with no toxic effect to the corresponding non-tumourigenic cells (CCD-18CO).

Cold water extract of the sclerotium of *L. rhinocerotis* which was cultivated by Lau et al. (2013) (IC_{50}: 37-355 µg/ml) exerted stronger cytotoxicity than hot water extract (IC_{50} > 500 µg/ml) against several cancer cell lines, including human breast adenocarcinoma (MCF-7), human colorectal carcinoma (HCT-116), human lung carcinoma (A-549) and human prostate adenocarcinoma cells (PC-3), amongst the most susceptible cell lines. The cold water extract, despite of its potent cytotoxicity against several cancer cells, was also cytotoxic to two non-tumourigenic cell lines: human embryonic liver cells (WRL-68) and human lung fibroblast (MRC-5). This implied that cellular toxicity of the cold water extract towards the cancer cells was not selective. Hot water extract, on the other hand, did not affect the viability of both non-tumourigenic cell lines.

Cold water extract of *L. rhinocerotis* cultivar TM02 showed anti-proliferative effect against human lung carcinoma (A-549) and human breast carcinoma (MCF-7) cell lines in which the cytotoxic action was suggested to be mediated by apoptosis (Lee et al., 2012b). In comparison, no significant cytotoxicity was found against two corresponding non-tumourigenic human cell lines, NL-20 (human lung cell) and 184B5 (human breast cell). Cytotoxic action of the extract was contributed by high molecular weight fraction (protein or protein-carbohydrate complex) which could be isolated by Sephadex G-50 gel column (Lee et al., 2012b). Yap et al. (2013) reported the comparison of anti-proliferative activity of wild type and cultivated sclerotia of *L. rhinocerotis* (cultivar TM02). Cold water extract (IC_{50}: 90 µg/ml) of the cultivar had a higher cytotoxic effect against human breast carcinoma (MCF-7) cells than the wild type (IC_{50}: 206 µg/ml).

2.7.2 Immunomodulatory Effect

Stimulation effects of cold alkali extract consisting of β-glucan and hot water extract consisting of polysaccharide-protein complex on human innate immune cells have been reported by Wong et al. (2009). Cold alkali extract of wild type sclerotium of *L. rhinocerotis* significantly stimulated the proliferation of natural killer cell line (NK-92MI) with a corresponding increase in the expression of cytokines IL-2 and I-309, which belong to the chemokine subfamily and are known to be chemotactic for monocytes. Incubation of both hot water and cold alkali extract for 72 hours stimulated proliferation of human primary natural killer cells (CD 56^+ cells) and human normal spleen monocytes/macrophages (MD) cells.

Wong et al. (2011) studied immuno-stimulatory effect of wild type sclerotium of *L. rhinocerotis* on innate and adaptive immune system by normal BALB/c mice. Intraperitoneal injection of hot water extract of the sclerotium exerted significant ($p < 0.05$) increase in spleen weight (49.0%) that indicates an enhancement of mitogenic activity results in the maturation of lymphocytes of normal BALB/c mice while the cold alkali extract had less significant effect (9.80%).

Immunophenotyping of spleen mononuclear cells (MNCs) and peritoneal exudate cells (PECs) isolated from normal BALB/c mice treated with hot water or cold alkali extract were analysed through their cell surface antigens (Wong et al., 2011). Percentage of T-helper cell population ($CD3^+/CD4^+$) in MNCs of normal BALB/c mice treated with hot water or cold alkali extract were found to be at least 30% more than control group. Within MNCs of normal BALB/c mice, expression of NK 1.1^+ natural killer cells were significantly increased ($p < 0.05$) by 9.6% and 18.2% for mice injected with hot water and cold alkali extract, respectively. Both extracts showed an increase in the percentage of Mac-3^+ macrophage population in the peritoneal exudate cells (PECs), with cold alkali extract having greater increment than hot water extract, as compared to control group. Therefore, both hot water and cold alkali extracts were suggested to activate adaptive and innate immunities to different extent (Wong et al., 2011).

A spontaneous mutation of the *Foxn1* gene (forkhead box N1 gene) causes T-cell deficiency and a partial defect in B-cell production in athymic nude mice (Costigan et al., 2009). Due to its immunodeficiency, this animal model has been used extensively to investigate therapeutic effect of novel molecules prior to human clinical trials in cancer research as it does not reject allografts and often does not reject xenografts (Reid et al., 1979; Ng et al., 2007).

Wong et al. (2011) evaluated immunomodulatory effect of wild type sclerotium of *L. rhinocerotis* on athymic nude mice by analysing their sera cytokine profile following treatment. Cold alkali extract was mainly responsible for recruitment of neutrophils in which mice injected with the extract had the sera cytokine profile showing a higher expression of IL-17 and two colony-stimulating factors (G-CSF and GM-CSF), as compared to the control group. Hot water extract might have promising anti-angiogenic activity due to its high expression of IL-12 and the downregulation of vascular endothelial growth factor (VEGF) in the serum of mice. According to Colombo & Trinchieri (2002), IL-12 may exert anti-angiogenic activity by acting on NK cells and T-cells, and results in increasing production and activity of cytotoxic lymphocytes. It is also responsible for the differentiation of T helper 1 cell, a potent producer of IFN-γ (Colombo & Trinchieri, 2002). IL-12 inhibits angiogenesis through its downstream mediators such as IFN-γ-inducible protein 10 (IP-10) (Strieter et al., 1995) and monokine induced by IFN-γ (Mig) (Suyama et al., 2005). VEGF can induce angiogenesis (required for the growth of most tumours) and lymphangiogenesis (promotion of metastatic spread) (Eklund et al., 2013). These findings provide insight into potential cancer treatment through immunomodulation by cold alkali and hot water extract.

2.7.3 Anti-oxidant Activity

According to Yap et al. (2013), ferric reducing antioxidant power (FRAP) values of hot water, cold water and methanol extracts of *L. rhinocerotis* sclerotium (both wild type and cultivar TM02) ranged from 0.006 to 0.016 mmol min^{-1} g^{-1} extract while the phenolic content ranged from 19.32 to 29.42 mg gallic acid equivalents g^{-1} extract. The DPPH•, $ABTS^{\bullet+}$ and superoxide anion radical scavenging activities of the extracts ranged from 0.52 to 1.12, 0.05 to 0.20 and -0.98 to 11.23 mmol Trolox equivalents g-1 extract, respectively. In general, the three mushroom extracts showed relatively low FRAP value,

DPPH• and ABTS•$^{+}$ radical scavenging activity, as compared to the positive controls (rutin and quercetin). However, hot and cold water extract of wild type (11.23 and 9.09 mmol Trolox equivalents g^{-1} extract, respectively) and cultivated (8.00 and 9.90 mmol Trolox equivalents g^{-1} extract, respectively) sclerotia of *L. rhinocerotis* exhibited very strong superoxide anion radical scavenging activity with potency comparable to that of rutin (9.62 mmol Trolox equivalents g $^{-1}$ extract) and quercetin (11.43 mmol Trolox equivalents g-1 extract), which are both strong free radical scavengers. The finding showed that cultivar TM02 of *L. rhinocerotis* may be beneficial in preventing oxidative stress induced by superoxide anion radicals, consequences of aerobic metabolism which can lead to oxidative damage to DNA, proteins, lipids and other cellular components in which an excess of them can cause extensive cell damage and cell death (Čáp et al., 2012).

2.7.4 Anti-microbial Activity

Petroleum ether, chloroform, methanol, and water (30^{o}C) extracts of wild type *L. rhinocerotis* sclerotium were screened for its anti-bacterial and anti-fungal activities against several Gram-positive and negative bacteria as well as fungi by measuring diameter of zone of inhibition through disc diffusion method (Mohanarji et al., 2012) (Table 2.3). Aqueous and methanol extract (30 mg/ml) of the sclerotium showed higher anti-bacterial and anti-fungal activity against the tested pathogens in comparison to petroleum ether and chloroform extract. The presence of various chemical constituents in both aqueous and methanol extract including alkaloids, gums, mucilage, protein and flavonoids might be responsible for the anti-microbial activity.

Table 2.3: Type of bacteria and fungi used for anti-microbial study of several extracts of wild type *Lignosus rhinocerotis* sclerotium

(Source: Mohanarji et al., 2012).

Type of pathogen		Type of strain
Bacteria	Gram-positive	*Staphylococcus aureus* *Corynebacterium diphtheriae* *Bacillus cereus* *Stapyhlococcus epidermidis* *Streptococcus pyogenes* *Strepococcus viridians* *Micrococcus luteus*
	Gram-negative	*Klebsiella pneumoniae* *Salmonella typhi* *Entorobacter aerogenes* *Vibro cholera* *Escherichia coli* *Pseudomonas aeruginosa* *Serratia marcescens* *Proteus hauseri*
Fungi		*Candida albicans* *Candida tropicalis* *Candida krusei* *Mucor racemosus*

2.7.5 Neurite Outgrowth Stimulation and *in vitro* Neurotoxicity Study

Eik et al. (2012) demonstrated an enhanced stimulation of neurite outgrowth at 17.7% when a combination of 20 µg/ml (w/v) aqueous extract of cultivated sclerotia of *L. rhinocerotis* (TM02) and 30 ng/ml (w/v) of nerve growth factor (NGF) were added to rat pheochromocytoma (PC-12), as compared to the aqueous extract alone. In addition, aqueous extract was also found to significantly promote neurite outgrowth in neuroblastoma-2a (N2a) cells by 38.1% (Phan et al., 2013).

In vitro neuro-toxic and embryo-toxic effects of aqueous extract of cultivated sclerotia of *L. rhinocerotis* (TM02) were evaluated by Phan et al. (2013) with the use of neuroblastoma-2a (N2a) cells and mouse embryonic fibroblast (BALB/3T3), respectively. After 24 h exposure of N2a and 3T3 cells to the extract, no cytotoxicity was found by using tetrazolium (MTT), lactate dehydrogenase (LDH) and neutral red uptake (NRU) release assays. Therefore, cultivar TM02 may be developed as a safe dietary supplement for brain and cognitive health (Phan et al., 2013).

2.7.6 Prebiotics

A prebiotic is known as a non-digestible food ingredient that benefits the host health by selectively stimulating growth and/or activity of bacteria in the colon (Gibson & Roberfroid, 1995). Galactooligosaccharides, fructooligosaccharides, maltooligosaccharides, inulin and its hydrolysates as well as resistant starch are prebiotics usually used in human diet (Al-Sheraji et al., 2013).

According to Gao et al. (2009), non-digestible carbohydrates (NDCs) from wild type sclerotia of *L. rhinocerotis* significantly stimulated the growth of *Bifidobacterium longum* (14.3%), as compared to positive control, lactulose (9.16%). The percentage of increase of *Lactobacillus brevis* between NDCs from the sclerotia and lactulose were comparable. NDCs of the sclerotium showed significantly stronger inhibition against *Clostridium celatum*, an important pathogen in human colon ($p < 0.05$) than lactulose. Owing to the stimulation of the growth of beneficial bacteria (*Bifidobacterium longum* and *Lactobacillus brevis*) and inhibition of the pathogenic ones (*Clostridium celatum*), the sclerotium might be developed into a novel prebiotic for gastrointestinal health (Gao et al., 2009).

2.8 The Genome of *Lignosus rhinocerotis*

The *de novo* draft genome sequence of cultivated sclerotia of *L. rhinocerotis* (named TM02) was recently reported by Yap et al. (2014b). *L. rhinocerotis* genome of 34.4 Mb was found to encode 10,742 putative genes with 84.30% of them being homologous to known proteins deposited data in public databases (InterPro, NCBI nr, SwissProt and TrEMBL databases). A total of 1,686 genes in *L. rhinocerotis* genome were predicted to encode for hypothetical proteins.

According to Yap et al. (2014b), phylogenetic analysis from a concatenated alignment of 144 shared proteins in 18 genomes of fungal species revealed a closer evolutionary relationship of *L. rhinocerotis* to other white rot members: *Ganoderma lucidum*, *Dichomitus squalens* and *Trametes versicolor* in the core polyporoid clade. However, *L. rhinocerotis* shows distinct growth habit and morphological features from these three fungi (Yap et al., 2014b). Its terrestrial growth habit with the development of an underground sclerotium is however similar to brown-rot *Wolfiporia cocos* while the three

white rot-fungi grow on wood. In terms of morphological features, the sclerotium of *L. rhinocerotis* has an oblong or irregular shape, centrally stipitate fruiting body and an isodiametric cap. The caps of *G. lucidum* and *T. versicolor* are offset and sometimes indistinct with either a base stipe for the former or lacking one for both fungi. *D. squalens*, on the other hand, has a basidiocarp with poroid hymenophore and lacks a stipe. *W. cocos* with similar growth habit to *L. rhinocerotis* has resupinate fruiting body and spherical sclerotium. These indicate the uniqueness of *L. rhinocerotis* when compared to the sequenced Basidiomycota (Yap et al., 2014b).

The genome of *L. rhinocerotis* encodes cytochrome P450s, lectins, fungal immunomodulatory proteins, laccases as well as a repertoire of enzymes responsible for carbohydrate and glycoconjugate metabolism (Yap et al., 2014b). *L. rhinocerotis* fungal immunomodulatory proteins (FIPs) were found to be homologous to LZ-8 (a member of the FIP family from *G. lucidum* with immunomodulatory and anti-cancer activity) with 64% identities (Yap et al., 2014b). Lectins and laccases isolated from mushrooms exhibited anti-proliferative activity against cancer cells and human immunodeficiency virus (HIV)-1 reverse transcriptase inhibitory activity (Li et al., 2010; Zhao et al., 2010; Hu et al., 2011; Zhao et al., 2014). Lectin isolated from *Ganoderma lucidum* demonstrated anti-fungal activity (Girjal et al., 2011).

Genes of enzymes that may be engaged in the biosynthesis of 1,6-β-glucans and uridine diphosphate glucose (UDP-glucose, precursor of glucans) were found in genome of *L. rhinocerotis* and this suggested the capabilities of the mushroom to produce 1, 3-β- and 1,6-β-glucans (Yap et al., 2014b). This is an important finding as beta glucan in mushroom is known to exhibit wide range of bioactivities such as immunomodulatory and hypocholesterolemic effects (Cheung, 1996; Ji et al., 2007) as well as anti-tumour and anti-inflammatory activities (Zhang et al., 2014; Smiderle et al., 2008).

Genes encoding key enzymes involved in secondary metabolite biosynthesis from nonribosomal peptide, polyketide and triterpenoid pathways were also identified in the genome of *L. rhinocerotis* (Yap et al., 2014b). In particular, the genome is enriched with sesquiterpenoid biosynthesis genes in which up to 12 sesquiterpene cyclase genes were found. Sesquiterpene cyclase is an enzyme that catalyse biosynthesis of sesquiterpenoid through cyclisation mechanism (Davis & Croteau, 2000). Sesquiterpenoid hydroquinones produced by *Ganoderma pfeifferi* Bres. which have been named ganomycins exhibited moderate growth inhibition against several bacteria strains, especially Gram-positive strains such as *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* (Mothana et al., 2000). A new sesquiterpenoid which was isolated from *Pleurotus cornucopiae* showed anti-proliferative effect against cancer cell lines: HeLa (IC_{50} values of 70.6 μM) and HepG2 cells (IC_{50} values of 76.8 μM) (Wang et al., 2013).

2.9 Genome-based Proteomic Analysis of *Lignosus rhinocerotis*

Yap et al. (2015) reported the identification of a total of 16 non-redundant, major proteins in sclerotium of *L. rhinocerotis* cultivar TM02 by using two-dimensional gel electrophoresis coupled with mass spectrometry analysis. The *L. rhinocerotis* genome was used as custom mapping database. Putative lectins (39.13%), immunomodulatory proteins (2.52%), superoxide dismutase (0.91%) and aegerolysin (0.37%) in the sclerotium may have pharmaceutical potential (Yap et al., 2015). Serine proteases, the other major constituents of the sclerotium (11.08 %) may be used as detergent enzyme for industrial application (Kumar et al., 2008; Yap et al., 2015). Other proteins are involved in nutrient mobilisation and the protective antioxidant mechanism in the sclerotium (Yap et al., 2015).

The bioactivities of lectins and immunomodulatory proteins have been described in section 2.8. Superoxide dismutase (SOD) is an anti-oxidant enzyme that catalyses the dismutation of superoxide anion (O_2^-) into H_2O_2 and molecular oxygen (Turkseven et al., 2005). Aegerolysins possessed anti-tumoural, anti-proliferative and anti-bacterial activities (Berne et al., 2009). These proteins may have the potential to be used for atherosclerosis prevention, as vaccines, for improvement in the cultivation of some commercially important edible mushrooms and as specific markers in cell and molecular biology (Berne et al., 2009).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Sprague Dawley (SD) rats of both male and female were supplied by Chenur Supplier (Selangor, Malaysia). The animals were kept under standard conditions (temperature at 22 ± 2^{o}C, 12 h light, 12 h dark), and given food and water *ad libitum*. Animals were acclimatised before use.

3.1.2 Animal Ethical Clearance

Experimental protocols reported in this thesis (involving animal use) were approved by Institutional Animal Care and Use Committee, University of Malaya (UM IACUC-Ethics reference no. PM/16/11/2010/0812/FSY (R)) (Appendix L)

3.1.3 Anaesthesia

Anaesthetic drugs ketamine (45 mg/kg) and xylazine (4.5 mg/kg) were injected intramuscularly into rats.

3.1.4 Chemicals and Consumables

37% formaldehyde (analytical grade), methanol (analytical grade), 96% sulphuric acid, acetic acid glacial (analytical grade), acetonitrile (HPLC grade), 85% formic acid (analytical grade) and dimethyl sulfoxide were purchased from Friedemann Schmidt, Washington, U.S.A.

Ammonium acetate, absolute ethanol (HPLC grade), 85% ortho-phosphoric acid (analytical grade), 37% hydrochloric acid, potassium hydroxide, isopropanol (analytical grade), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anhydrous disodium phosphate, sodium dihydrogen phosphate and Millipore® Ziptips C18 were bought from Merck KGaA, Darmstadt, Germany.

Bovine serum albumin, λ-carrageenan, indomethacin, N-N'-methylene bisacrylamide, ammonium persulphate, b-mercaptoethanol, ammonium bicarbonate, dithiothreitol, iodoacetamide, lipopolysaccharides from *Escherichia coli* 0127:B8, N,N,N',N'-tetramethylethylenediamine (TEMED), phenol, ammonium sulphate, acrylamide, Coomassie Brilliant blue G-250, Sephadex® G-50, Sephadex® G-25 gel medium, trypsin from bovine pancreas, phenylmethanesulfonyl fluoride (PMSF), Pefabloc® SC and Whatman filter paper were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Sodium bicarbonate and riboflavin were purchased from BDH Chemical Ltd., Poole, England.

Blue dextran 2000 was bought from Pharmacia Fine Chemicals, Uppsala, Swedan.

Bromophenol blue, Tris base, phosphate buffered saline, sodium dodecyl sulphate (SDS) and CHAPS were purchased from MP Biomedicals, LLC, California, USA.

Sodium chloride and absolute ethanol (analytical grade) were purchased from J. Kollin Corporation, Germany while glycine was purchased from GMbiolab Co, Ltd., Taiwan.

Pierce™ trypsin protease, MS grade and Spectra™ multicolor broad range protein ladder (10 to 260 kDa) were purchased from Thermo Scientific, Massachusetts, USA.

Normal saline was purchased from Klean and Kare, Bangkok, Thailand.

D-glucose was purchased from HmbG Chemicals, Hamburg, Germany.

Urea, thiourea, 2-D quant kit and 2-D clean-up kit were purchased from GE healthcare Life Science, Amersham, UK.

RAW 264.7 (ATCC® TIB-71™) cell line and Dulbecco's Modified Eagle's medium (ATCC® 30-2002™) were purchased from ATCC, Manassas, Virginia, United States.

Heat inactivated fetal bovine serum was purchased from Gibco, Grand Island, New York, USA and Biowest, Nuaillé, France, respectively.

BD Falcon disposable petri dishes (100 x15 mm) was purchased from BD Biosciences, New Jersey, United States.

Flat Bottom MicroWell™ 96-Well Microplates, T25 and T75 cell culture flasks with filter caps were purchased from Nunc, Roskilde, Denmark.

Vivaspin 15R hydrosart (5000 MWCO and 2000 MWCO) was purchased from Sartorius Stedim Biotech GmbH, Göttingen, Germany.

Kontes™ Chromaflex™ Column was purchased from Kimble-Chase, Vineland, New Jersey, United States.

TNF-α mouse ELISA kit was purchased from Life Technologies, Carlsbad, USA.

Accutase was purchased form ebioscience, Santa Clara, California, USA.

Ilium Xylazil-100 and ketamil at 100mg/ml were purchased from Troy laboratories, Glendenning, New South Wales, Australia.

Ethilon monofilament polyamide 6 suture was bought from Ethicon, Cincinnati, Ohio, U.S.A.

Needle at 23 G x 1" (0.60 x 25 mm), 26 G x 1/2" (0.45 x 13 mm) and 25 G x 5/8" (0.5 x 16 mm) as well as U-100 insulin needle with syringe at 27 G x 1/2" (0.40 x13 mm, 1 ml) and syringe without needle were purchased from Terumo, Shibuya-ku, Tokyo, Japan.

3.2 General Methods

3.2.1 Preparation of the Sclerotial Powder of *Lignosus rhinocerotis*

The sclerotia of *L. rhinocerotis* cultivar TM02 and TM03 as well as the wild type were provided by Ligno Biotech Sdn. Bhd. (Selangor, Malaysia) and were identified by their internal transcribed spacer (ITS) regions of the ribosomal DNA (Tan et al., 2010). Sclerotia were freeze-dried and milled into powder using 0.2 mm sieve. The powder for wild type and cultivars are white and light brown, respectively. They are dry, fluffy with milk-like taste.

3.2.2 Preparation of 10% Buffered Formalin

10% buffered formalin was prepared with 100 ml of 37% formaldehyde, 4 g of sodium dihydrogen phosphate, 6.5 g of anhydrous disodium phosphate and 900 ml of distilled water.

3.2.3 Determination of Total Carbohydrate and Protein Contents of the Sclerotial Extracts or Fractions of *Lignosus rhinocerotis* Cultivar TM02

3.2.3.1 Protein Determination

<u>Bradford Method (Bradford, 1976)</u>

a) Preparation of Bradford Reagent

Coomassie Brilliant blue G-250 (100 mg) was dissolved in 50 ml of 95% ethanol. To this solution, 100 ml of 85% phosphoric acid was added. The resulting solution was diluted to a final volume of 1 L.

b) Standard Curve Preparation

Bovine serum albumin (BSA) at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg in a volume of 0.1 ml was used for standard curve preparation. Standard curve used for the determination of protein amount in unknown samples were generated by plotting the absorbance of standards against corresponding amount of protein.

c) Bradford Assay

The Bradford reagent (5 ml) was added to 0.1 ml of sample or standard, and the mixture was vortexed. The absorbance at 595 nm was measured after 2 min and before 1 h in a cuvette by using spectrophotometer.

2-D Quant Kit (GE Healthcare Life Sciences)

Standard curve was prepared using bovine serum albumin standard solution at 0, 10, 20, 30, 40 and 50 µg. Standard or protein sample was mixed with 500 µl of precipitant by vortexing. After incubation 2-3 min at room temperature, 500 µl of co-precipitant was added to each tube (containing sample or standard), followed by a brief vortex and centrifugation at a minimum of 10,000 x g for 5 min to sediment protein. The resulting supernatant from each tube was decanted. A brief pulse was done and remaining supernatant was removed. Copper solution (100 µl) and milliQ water (400 µl) were added to each tube, followed by a brief vortex to dissolve precipitated protein. Subsequently, 1ml of working colour reagent was added to each tube, followed by mixing and incubation at room temperature for 15-20 min. Absorbance of each sample and standard was read at 480 nm within 40 min of the addition of working colour reagent. Protein concentration of a sample was determined from a standard curve which was generated by plotting the absorbance of the standards against the amount of protein.

3.2.3.2 Determination of Carbohydrate Content

Carbohydrate content was determined by phenol sulphuric acid method according to Dubois et al. (1956). D-glucose ranging from 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 µg in 0.2 ml were used to generate standard curve for the determination of carbohydrate content of an unknown sample. An aliquot of 0.2 ml phenol solution (5%) was added to each sample or standard. One milliliter of sulphuric acid (96%) was rapidly added to each tube. After 10 min, solution in each tube was shaken and placed in water bath at 25-30ºC for 10-20 min. Absorbance of mixture in each tube was measured at 490 nm. Carbohydrate concentration of a sample was determined from a standard curve which was generated by plotting the absorbance of the standards against the amount of D-glucose.

3.2.4 Determination of the Glucan Content of the Sclerotial Extracts, Fractions and Protein and Non-protein Components Derived from HMW Fraction of *Lignosus rhinocerotis* Cultivar TM02

Glucan content was determined by Mushroom and Yeast Glucan Assay Kit (Megazyme International Ireland Ltd, Bray Co, Wicklow, Ireland) according to manufacturer's instructions. To determine percentage of total glucan plus oligomers etc., sample was solubilised in concentrated hydrochloric acid (37%) and hydrolysed by1.3 M HCl at 100°C for 2 h. KOH (2 M) was then added to neutralise the hydrolysates. Hydrolysis to D-glucose was completed by incubation with a mixture of highly purified exo-1,3-β-glucanase (20 U/ml) and β-glucosidase (4U/ml). The hydrolysates were then incubated with glucose oxidase/peroxidase mixture (GOPOD) reagent. Absorbance was measured at 510 nm against the GOPOD reagent blank and compared to D-glucose standard to calculate percentage of total glucan plus oligomers etc.

To determine percentage of alpha glucan plus oligomers etc., sample was solubilised and hydrolysed in 2 M KOH and amyloglucosidase (1630 U/ml) plus invertase (500 U/ml). The hydrolysates were incubated with GOPOD reagent. Absorbance was measured at 510 nm against reagent blank and compared to D-glucose standard to calculate percentage of alpha glucan plus oligomers etc. Percentage of beta glucan content was obtained by subtracting percentage of total glucan plus oligomers etc. and alpha glucan plus oligomers.

3.2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970) and Studier (1973) in an electrophoresis slab gel system by using Mini PROTEAN® Tetra cell (Bio-Rad, USA).

3.2.5.1 Preparation of Solutions and Buffers

Solution A: 29.2% (w/v) acrylamide, 0.8% (w/v) N-N'-methylene bisacrylamide in milliQ water, stored at 4^{o}C in dark.

Solution B: 1.5 M Tris-HCl buffer containing 0.4% (w/v) SDS, pH 8.8.

Solution C: 10% (w/v) ammonium persulphate, freshly prepared.

Solution D: 0.5 M Tris-HCl containing 0.4% (w/v) SDS, pH 6.8.

Electrophoresis buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS

Sample incubation buffer: 62 mM Tris-HCl (pH 6.8), 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) b-mercaptoethanol.

Fixing solution: 40% (v/v) methanol, 10% (v/v) acetic acid in milliQ water

Staining solution: 0.2% (w/v) Coomassie Blue R-250 in the fixing solution.

Destaining solution: 5% (v/v) methanol, 7% (v/v) acetic acid in water.

3.2.5.2 Preparation of Resolving and Stacking gels

12.5% Resolving Gel

A mixture of 3.75 ml Solution A, 3 ml of solution B, 2.25 ml of milliQ water and 50 µl of solution C were prepared. TEMED (5 µl) was added to the mixture, and the solution was rapidly poured between two glass plates, up to about 2.5 cm below the notch. Isopropanol was layered on top of the gel to achieve even surface. Gel will be polymerised in 20 min. Isopropanol layer was removed and the space between top of two glass plate was washed a few times with milliQ water.

4% Stacking Gel

A mixture of 0.7 ml of solution A, 1.25 ml of solution D, 3.05 ml of milliQ water, 0.1 ml of solution C and 5 µl TEMED were prepared and poured on top of resolving gel. A comb was rapidly inserted. Gel will be polymerised in 20 min.

3.2.5.3 Preparation of Protein Sample and Running Condition

Protein samples were filtered with 0.2 µm syringe filter and were cleaned up by 2-D clean-up kit. Briefly, 300 µl precipitant was mixed with protein containing sample in each tube. Co-precipitant (300 µl) was added to the mixture, and the tube was vortexed. The mixture was centrifuged at maximum speed (at least 12,000 × g) for 5 min. Supernatant was decanted without disturbing the pellet. A brief pulse was done and the remaining supernatant was removed. Co-precipitant (40 µl) was layered on top of the pellet and the tube was sit on ice for 5 min. Each tube was centrifuged for 5 min and the wash was removed by pipette tip. MilliQ water (25 µl) was added on top of pellet, followed by vortexing for 5-10 s in order to disperse the pellet. Following addition of 1ml of wash buffer and 5 µl of wash additive, the pellet was fully dispersed by vortexing. The protein samples were incubated at -20°C for at least 30 min and vortexed for 20-30s once every 10 min. The samples were again centrifuged at the same maximum speed for 5 min. Supernatant was removed and white pellets were air dried briefly. Pellets which were solubilised in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS) were mixed with sample incubation buffer and boiled in water bath for 3-5 min. Protein sample in sample incubation buffer was loaded to the gel. Spectra™ multicolor broad range protein ladder (10 to 260 kDa) from Thermo Scientific was added as size reference. Electrophoresis was carried out at constant voltage of 90 V for 3 h.

3.2.5.4 Fixing, Staining and Destaining

After electrophoresis, SDS-PAGE gel was fixed in fixing solution for 5 min and then stained with staining solution for 15 min. The gel was destained overnight until clear.

3.2.6 Cell Culture

The murine macrophage cell line RAW 264.7 (ATCC® TIB-71TM) was grown in DMEM (ATCC) supplemented with 10% heat inactivated FBS at 37°C in a humidified 5% CO_2 incubator. The medium was routinely changed every two to three days. For all studies which were carried out in chapter 7, cells were cultured in non-tissue culture grade petri dish and detached with accutase when they attained confluence.

CHAPTER 4: SUB-ACUTE TOXICITY STUDY OF THE SCLEROTIUM OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

4.1 Introduction

In view of the wide ethno-botanical usages as well as scientifically validated therapeutic effects of the *L. rhinocerotis* which were elaborated in Chapter 1 and 2, it is believed that the cultivated *L. rhinocerotis* will rapidly become a popular health supplement. It is therefore necessary to examine the toxicity of the sclerotium of the mushroom. Present studies reported investigation on the sub-acute toxicity of the sclerotia of the wild-type and two cultivars of *L. rhinocerotis* (termed TM02 and TM03). Five treatment groups (5 rats/group/sex) were orally administered with various concentrations of wild type sclerotial powder of *L. rhinocerotis* as well as two cultivars (TM02 and TM03) once daily, while control group received distilled water. After 28 days, blood and organs of the animals were collected for analysis. The sub-acute toxicity study was carried out in compliance with the guidelines from the Organization of Economic Cooperation and Development (OECD, 2008). The highest dose used at 1000 mg/kg was chosen based on a preliminary 7-day acute toxicity study of the sclerotial powder of cultivar TM02 (unpublished data by Ligno Biotech Sdn. Bhd.). The study did not reveal any treatment-related toxicity in rats following oral administration of 2000 mg/kg of the sclerotial powder ($n = 5$).

4.2 Literature Review

4.2.1 Sub-acute Toxicity Study

A 28-day sub-acute toxicity study provides information on the effects of repeated oral exposure and selection of concentrations for long term studies (OECD, 2008). Animals are observed closely each day during the period of administration for signs of toxicity (OECD, 2008). At the end of the study, haematological examinations, clinical biochemistry determinations and histopathological examinations will be carried out. Results obtained from the study can be used to characterise toxicity of a test substance, to indicate any dose response relationship and to determine the no-observed-adverse-effect level (NOAEL), which is defined as the highest dose level where no adverse treatment-related findings are observed (OECD, 2008).

4.2.2 Haematological Examination

Haematopoietic system is evaluated to reflect physiological and pathological status in human and animal as it is one of the most sensitive targets for toxic compounds (Gautam & Goel, 2014). Red blood cell indices including red blood cell count, haemoglobin concentration, packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) are used to evaluate possibility of haemolytic anemia and polycythemia (Alasil et al., 2014). White blood cell counts (basophil, eosinophil, lymphocyte, monocyte as well as atypical lymphocyte) are used to monitor the effect of target components on immune system (Sireeratawong et al., 2012). Abnormality of haemostasis can be evaluated by platelet count (Mitchell & Bussel, 2013).

4.2.3 Clinical Biochemistry

Clinical biochemistry refers to analysis of major toxic effects found in tissues and specifically effects on liver (total protein, albumin, serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase) and kidney (urea and creatinine levels) (OECD, 2008). Besides, serum electrolytes (calcium, potassium and sodium) as well as total cholesterol and glucose levels are monitored.

4.2.4 Histopathological Analysis

Histopathology is the study of the structural manifestations of disease at the light microscopic level, enabling toxicologic and risk assessment of foods, chemicals, drugs, medical devices and biologics (Crissman et al., 2004). Tissue specimens are first fixed for preservation (Histology sample preparation, n.d.). Processing step is followed to dehydrate, clear and infiltrate the tissue with paraffin wax (Histology sample preparation, n.d.). After that, embedding allows orientation of the specimen in a "block" that can be sectioned for the ease of storage and handling (Histology sample preparation, n.d.). Finally, tissue block is sectioned with microtome to produce thin sections to be placed on a microscope slide for staining and analysis (Histology sample preparation, n.d.).

4.3 Methods

4.3.1 Animals and Oral Feeding of Sclerotial Powder of *Lignosus rhinocerotis*

Five male (7 weeks old) and five female (7-8 weeks old) SD rats were acclimatised for 14 days before treatment. The animals were divided into five treatment groups and one control group (5 rats/group/sex). Treatment groups received 1000 mg/kg wild type sclerotial powder of *L. rhinocerotis*, 1000 mg/kg TM02, 500 mg/kg TM02, 250 mg/kg TM02 or 1000 mg/kg TM03. The animals were orally administered with the sclerotial powder in the form of suspension in distilled water, once daily for 28 consecutive days. Control group received distilled water by oral gavage for 28 consecutive days. Body weight of each animal was measured and any gross behavioral changes and toxic symptoms were observed daily. The doses were selected according to OECD guidelines. For TM02, two descending doses at 500 mg/kg and 250 mg/kg were selected to demonstrate if there is any dose related response.

4.3.2 Blood Analysis

Rats were fasted for 18 hours at the end of day 28. At day 29, blood samples from rats were collected (by cardiac puncture) following anaesthesia with ketamine (45 mg/kg) and xylazine (4.5 mg/kg). Haematological parameters and clinical biochemistry were analysed using Advia 2120 Haematology System (Siemen, Germany) and Advia 2400 Chemistry System (Siemen, Germany), respectively. The parameters for haematological examination included red blood cell count, haemoglobin concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, white blood cell count, neutrophil count, lymphocyte count, monocyte count, eosinophil count,

basophil count and atypical lymphocyte count. Biochemical tests included glucose, urea, creatinine, calcium, sodium, potassium, total cholesterol, total protein, albumin, serum glutamic oxaloacetic transaminase or aspartate aminotransferase (SGOT or AST) and serum glutamic pyruvic transaminase or alanine transaminase (SGPT or ALT).

4.3.3 Histopathological Analysis

After blood collection, vital organs including liver, spleen, heart, lung and kidney were removed and preserved in 10% buffered formalin. The fixed tissues were dehydrated by serial ethanol solution, cleared with xylene, paraffin embedded, sectioned and stained with haematoxylin and eosin (H&E). Light microscopic examinations of multiple tissue sections from each organ were performed.

4.3.4 Statistical Analysis

All data were expressed as mean ± standard deviation (S.D.) and analysed using One-way Analysis of Variance (ANOVA). Statistical differences between the means of control group and treatment groups were determined using Dunnett's t (two-sided) test. In case of variance heterogeneity, Dunnett T3 test was used. The homogeneity of variances was calculated using Levene statistics. Results were considered significant at $p < 0.05$.

4.4 Results

4.4.1 General Observation

Neither behavioral changes, toxic symptoms (abnormality in fur, eyes color, piloerection, locomotor activity or diarrhea) nor death was observed in all the treated rats (including control group). Figures 4.1 and 4.2 show the growth rate for male and female rats, respectively which were treated with 1000 mg/kg of TM02, and that of the control group. The net body weight gain of all the treated groups were not significantly different from the control animals ($p > 0.05$) (Table 4.1).

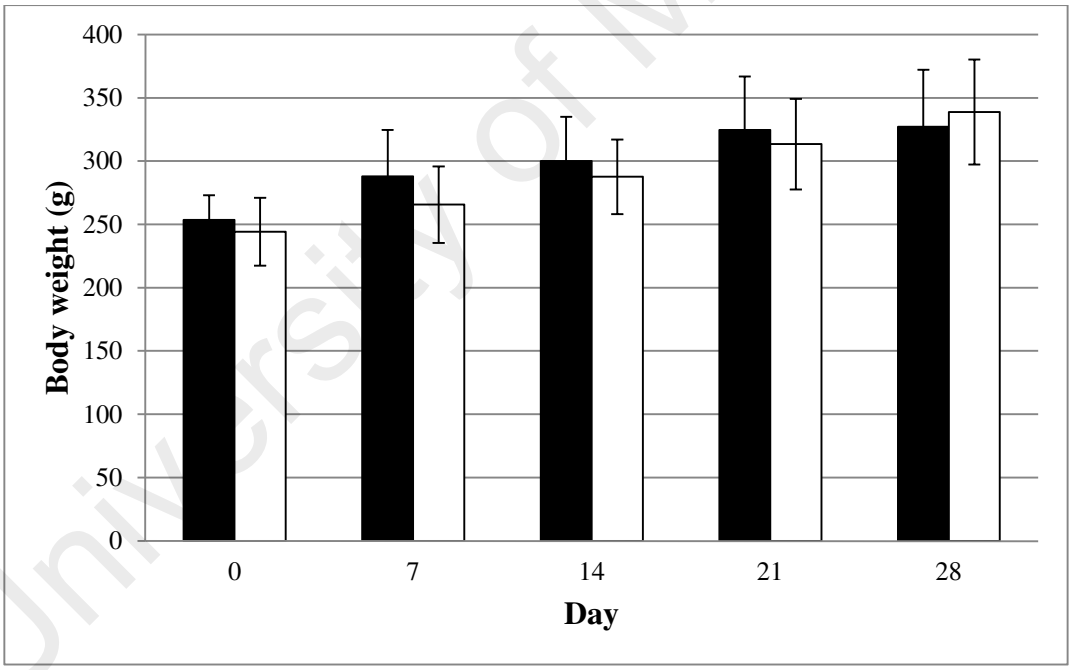

Figure 4.1: Body weight of male rats treated with 1000 mg/kg sclerotial powder of *Lignosus rhinocerotis* cultivar TM02 and the control group

Body weight is shown as mean ± S.D. ($n = 5$). Black column ■ is control group, white column □ is the 1000 mg/kg TM02 treated group.

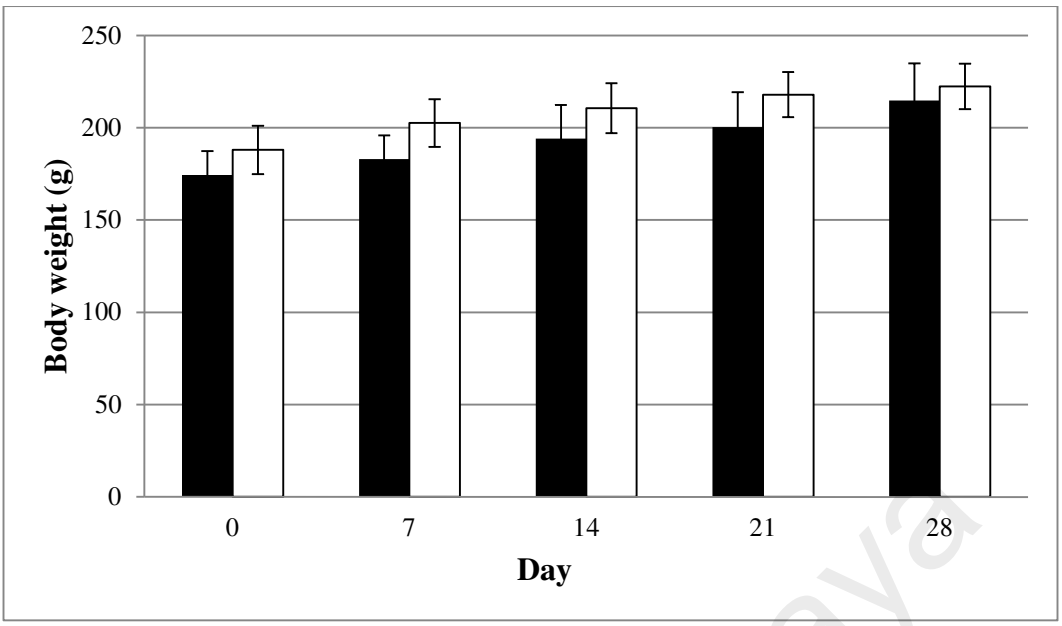

Figure 4.2: Body weight of female rats treated with 1000 mg/kg sclerotial powder of *Lignosus rhinocerotis* cultivar TM02 and the control group

Body weight is shown as mean ± S.D. (n = 5). Black column ■ is control group, white column □ is the 1000 mg/kg TM02 treated group.

Table 4.1: Body weight gain of rats treated with various sclerotial powder of *Lignosus rhinocerotis* for 28 days

Treatment	Body weight gain (g)			
	Day 7	Day 14	Day 21	Day 28
Male rats				
Control	34.40 ± 24.17	46.40 ± 23.46	71.00 ± 34.15	73.60 ± 37.46
1000 mg/kg Wild type	21.40 ± 10.71	43.60 ± 12.52	60.40 ± 18.46	76.80 ± 17.50
1000 mg/kg TM02	21.40 ± 10.64	43.40 ± 15.06	69.20 ± 19.64	94.60 ± 26.91
500 mg/kg TM02	32.20 ± 14.43	59.40 ± 14.40	74.60 ± 13.37	103.60 ± 7.54
250 mg/kg TM02	24.80 ± 10.40	29.00 ± 36.37	65.40 ± 14.01	96.40 ± 20.26
1000 mg/kg TM03	21.60 ± 12.58	33.20 ± 18.28	44.00 ± 20.14	65.00 ± 15.70
Female rats				
Control	9.00 ± 6.82	20.20 ± 12.07	26.40 ± 13.09	40.80 ± 14.08
1000 mg/kg Wild type	14.20 ± 9.83	25.00 ± 10.65	29.20 ± 11.39	33.20 ± 10.83
1000 mg/kg TM02	14.60 ± 7.06	22.60 ± 7.83	30.00 ± 9.51	34.40 ± 9.94
500 mg/kg TM02	15.60 ± 6.11	30.00 ± 8.37	41.20 ± 15.53	44.60 ± 13.07
250 mg/kg TM02	8.20 ± 11.21	14.00 ± 15.56	21.20 ± 13.03	22.00 ± 10.84
1000 mg/kg TM03	5.80 ± 7.09	18.80 ± 4.76	21.00 ± 6.08	28.20 ± 8.35

The male and female rats were orally fed with various types and doses of sclerotial powder of *L. rhinocerotis* for 28 consecutive days. The values for body weight gain are expressed as mean ± S.D. (n = 5/group/sex). The weight gain between the various treatment groups were not significantly different from the control group ($p > 0.05$).

4.4.2 Blood Analysis

4.4.2.1 Haematological Examinations

The results of the haematological examinations of the blood samples of the treated and control groups, after the 28 days treatment are shown in Tables 4.2 and 4.3, respectively for male and female rats. Generally, the values of red blood cell count, haemoglobin, PCV, MCV, MCH, MCHC, platelet count, WBC and differential leucocyte counts of rats from all the treated groups were not significantly different from the control group. The only exception was for female rats of the 250 mg/kg TM02 treated group, in which the mean corpuscular volume (MCH) of the treatment group (20.60 ± 0.55 pg) was slightly higher than that of the control group (18.20 ± 1.30 pg, $p < 0.05$) (Table 4.3).

Table 4.2: Haematological parameters of male rats treated with various types and doses of sclerotial powder of *Lignosus rhinocerotis* for 28 days

Treatment (mg/kg)	Control (n = 5)	1000 mg/kg wild type (n = 5)	1000 mg/kg TM02 (n = 5)	500 mg/kg TM02 (n = 5)	250 mg/kg TM02 (n = 5)	1000 mg/kg TM03 (n = 5)
RBC ($x10^{12}$/l)	8.30 ± 0.39	8.22 ± 0.69	7.96 ± 0.87	8.10 ± 0.37	8.50 ± 0.21	8.44 ± 0.26
Haemoglobin (g/dl)	13.66 ± 0.73	14.52 ± 0.60	13.46 ± 1.09	13.16 ± 0.84	14.36 ± 0.39	14.42 ± 0.43
PCV (%)	45.80 ± 1.92	45.80 ± 4.15	45.40 ± 3.36	44.20 ± 2.77	47.00 ± 1.58	45.80 ± 1.79
MCV (fl)	55.20 ± 1.30	55.20 ± 2.39	57.20 ± 2.77	54.60 ± 2.61	55.80 ± 2.68	54.40 ± 0.55
MCH (pg)	16.60 ± 0.55	17.60 ± 1.67	17.00 ±1.00	16.20 ± 0.45	16.80 ± 0.84	17.20 ± 0.45
MCHC(g/dl)	29.80 ± 0.84	31.60 ± 2.51	29.80 ± 0.45	29.80 ± 1.30	30.60 ± 0.55	31.60 ± 0.55
Platelet count ($x10^9$/l)	878.60 ± 239.30	1060.00 ± 189.83	763.00 ± 103.62	1052.40 ± 204.99	912.40 ± 147.58	877.80 ± 198.93
WBC ($x10^9$/l)	11.46 ± 5.53	10.10 ± 2.81	7.22 ± 2.20	11.06 ± 2.52	10.34 ± 3.76	8.38 ± 2.12
Lymphocyte (%)	69.00 ± 11.90	68.40 ± 7.57	70.60 ± 6.90	67.00 ± 3.74	56.40 ± 14.71	60.80 ± 13.57
Monocyte (%)	2.00 ± 1.58	4.40 ± 1.14	3.80 ± 0.84	4.20 ± 1.64	5 .00 ± 1.87	2.00 ± 1.22
Eosinophil (%)	0.40 ± 0.55	1.80 ± 2.17	0.60 ± 0.89	1.20 ± 1.30	0.00 ± 0.00	0.40 ± 0.89
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.45
Atypical lymphocyte (%)	1.40 ± 1.14	0.00 ± 0.00	2.20 ± 2.17	1.00 ± 1.41	0.60 ± 0.89	0.00 ± 0.00

Values are mean ± S.D. (n = 5/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 4.3: Haematological parameters of female rats treated with various types and doses of sclerotial powder of *Lignosus rhinocerotis* for 28 days

Treatment (mg/kg)	Control (n = 5)	1000 mg/kg wild type (n = 5)	1000 mg/kg TM02 (n = 5)	500 mg/kg TM02 (n = 5)	250 mg/kg TM02 (n = 5)	1000 mg/kg TM03 (n = 5)
RBC (x10^{12}/l)	7.60 ± 0.39	6.72 ± 0.30	7.24 ± 0.44	7.22 ± 0.56	6.98 ± 0.08	7.46 ± 0.42
Haemoglobin (g/dl)	13.86 ± 0.69	12.86 ± 0.77	13.66 ± 0.23	13.10 ± 1.10	14.34 ± 0.49	14.08 ± 1.10
PCV (%)	43.20 ± 2.95	38.00 ± 2.24	40.80 ± 3.11	39.80 ± 3.56	40.00 ± 1.22	40.40 ± 1.95
MCV (fl)	57.00 ± 2.00	56.40 ± 1.14	56.40 ± 2.07	55.20 ± 0.84	58.00 ± 1.22	54.40 ± 2.07
MCH (pg)	18.20 ± 1.30	19.20 ± 0.45	19.00 ± 1.22	18.20 ± 1.64	20.60 ± 0.55*	18.80 ± 1.48
MCHC (g/dl)	32.20 ± 3.11	33.80 ± 0.45	33.60 ± 2.61	32.80 ± 3.03	35.40 ± 0.89	34.60 ± 2.41
Platelet count (x10^{9}/l)	800.20 ± 152.53	753.00 ± 274.14	773.20 ± 119.70	828.60 ± 464.65	825.60 ± 158.59	983.20 ± 119.58
WBC (x10^{9}/l)	6.70 ± 2.38	5.72 ± 1.75	7.66 ± 1.52	7.14 ± 3.79	6.82 ± 3.27	6.82 ± 2.20
Lymphocyte (%)	58.40 ± 6.77	66.00 ± 7.31	59.80 ± 6.94	69.40 ± 12.42	68.40 ± 8.41	68.80 ± 7.92
Monocyte (%)	4.00 ± 2.00	2.20 ± 1.92	4.00 ± 1.73	2.80 ± 2.05	3.20 ± 2.17	3.60 ± 2.07
Eosinophil (%)	1.20 ± 0.84	0.20 ± 0.45	2.60 ± 4.72	2.40 ± 1.95	0.00 ± 0.00	1.20 ± 1.64
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Atypical lymphocyte (%)	0.00 ± 0.00	0.60 ± 0.55	0.60 ± 0.89	0.40 ± 0.55	0.80 ± 0.84	0.40 ± 0.89

Values are mean ± S.D. (n = 5/group). *Significant difference with control (ANOVA, Dunnett's t (two-sided), $p < 0.05$).

4.4.2.2 Clinical Biochemistry

The results of clinical biochemistry of male and female rats are shown in Tables 4.4 and 4.5, respectively. Generally, levels of serum glucose, urea, creatinine, calcium, sodium, potassium, total cholesterol, total protein, albumin, SGOT and SPGT of the treated groups were not significantly different ($p > 0.05$) from the control group, except for the following:

- Glucose levels in male group treated with 1000 mg/kg TM03 (5.94 ± 0.47 mmol/l) were significantly lower than that of the control group (8.02 ± 0.94 mmol/l, $p < 0.05$);
- Calcium levels in the male group treated with 1000 mg/kg wild type *L. rhinocerotis* (2.36 ± 0.03 mmol/l) were slightly lower than the control group (2.49 ± 0.03 mmol/l, $p < 0.05$);
- Total protein level in the male group treated with 1000 mg/kg wild type *L. rhinocerotis* (69.60 ± 1.67 g/l) was slightly higher than the level in control group (64.00 ± 1.58 g/l, $p < 0.05$).

Table 4.4: Clinical biochemistry parameters of male rats treated with various types and doses of sclerotial powder of *Lignosus rhinocerotis* for 28 days

Treatment (mg/kg)	Control (n = 5)	1000 mg/kg wild type (n = 5)	1000 mg/kg TM02 (n = 5)	500 mg/kg TM02 (n = 5)	250 mg/kg TM02 (n = 5)	1000 mg/kg TM03 (n = 5)
Glucose (mmol/l)	8.02 ± 0.94	6.28 ± 0.78	7.58 ± 1.21	6.78 ± 1.69	6.50 ± 1.18	5.94 ± 0.47*
Urea (mmol/l)	7.52 ± 1.40	7.40 ± 0.78	6.16 ± 0.96	7.46 ± 1.95	6.5 ± 0.86	6.36 ± 1.08
Creatinine (µmol/l)	54.80 ± 6.50	47.80 ± 11.37	49.80 ± 3.49	48.00 ± 3.32	46.40 ± 16.26	48.60 ± 4.04
Calcium (mmol/l)	2.49 ± 0.03	2.36 ± 0.03*	2.43 ± 0.10	2.16 ± 0.28	2.40 ± 0.12	2.39 ± 0.06
Sodium (mmol/l)	143.60 ± 1.14	146.00 ± 1.41	144.60 ± 0.55	144.00 ± 0.74	144.60 ± 2.79	144.60 ± 0.55
Potassium (mmol/l)	4.80 ± 0.29	4.34 ± 0.30	4.70 ± 0.34	4.98 ± 0.74	4.52 ± 0.57	4.50 ± 0.84
Total cholesterol (mmol /l)	1.58 ± 0.33	1.44 ± 0.32	1.38 ± 0.34	1.34 ± 0.34	1.40 ± 0.23	1.40 ± 0.22
Total protein (g/l)	64.00 ± 1.58	69.60 ± 1.67*	66.60 ± 2.61	61.00 ± 4.30	67.60 ± 4.28	66.20 ± 3.11
Albumin (g/l)	33.00 ± 0.71	35.60 ± 2.07	35.00 ± 1.58	31.80 ± 2.17	34.60 ± 2.41	34.60 ± 1.52
SGOT(AST) (IU/l)	214.60 ± 19.77	250.60 ± 49.97	221.20 ± 33.00	246.80 ± 33.25	271.00 ± 42.47	267.60 ± 36.56
SGPT(ALT) (IU/l)	62.40 ± 6.35	57.80 ± 11.37	57.00 ± 12.98	58.60 ± 7.89	64.00 ±8.69	60.00 ± 4.85

Values are mean ± S.D. (n = 5/group). *Significant difference from control (ANOVA, Dunnett's t (two-sided) test, $p < 0.05$).

Table 4.5: Clinical biochemistry parameters of female rats treated with various types and doses of sclerotial powder of *Lignosus rhinocerotis* sample

Treatment (mg/kg)	Control (n = 5)	1000 mg/kg wild type (n = 5)	1000 mg/kg TM02 (n = 5)	500 mg/kg TM02 (n = 5)	250 mg/kg TM02 (n = 5)	1000 mg/kg TM03 (n = 5)
Glucose (mmol/l)	6.78 ± 0.67	6.20 ± 0.73	7.54 ± 2.68	6.74 ± 0.82	6.54 ± 0.89	7.16 ± 0.89
Urea (mmol /l)	7.26 ± 0.85	6.90 ± 0.69	9.66 ± 2.04	8.24 ± 0.68	8.36 ± 0.72	8.80 ± 1.10
Creatinine (µmol/l)	47.20 ± 4.15	42.00 ± 2.45	46.80 ± 9.76	47.40 ± 12.22	33.80 ± 6.76	43.00 ± 14.75
Calcium (mmol/l)	2.55 ± 0.08	2.58 ± 0.12	2.42 ± 0.08	2.47 ± 0.07	2.54 ± 0.11	2.48 ± 0.08
Sodium (mmol/l)	140.60 ± 1.52	143.40 ± 1.14	140.40 ± 1.34	142.40 ± 1.34	142.40 ± 1.52	141.20 ± 1.48
Potassium (mmol/l)	4.64 ± 0.71	4.08 ± 0.26	4.78 ± 0.54	4.38 ± 0.49	4.20 ± 0.41	4.18 ± 0.25
Total cholesterol (mmol/l)	1.88 ± 0.52	1.78 ± 0.13	1.64 ± 0.30	1.52 ± 0.31	2.12 ± 0.33	1.72 ± 0.38
Total protein (g/l)	73.80 ± 5.59	70.00 ± 3.74	71.20 ± 1.92	68.60 ± 5.03	74.40 ± 3.65	72.4 ± 2.51
Albumin (g/l)	38.40 ± 4.16	37.80 ± 1.79	37.00 ± 1.58	36.40 ± 4.28	41.80 ± 3.35	37.4 ± 3.97
SGOT(AST) (IU/l)	126.20 ± 28.45	144.80 ± 25.12	160.00 ± 36.03	179.60 ± 45.91	164.60 ± 34.85	180.2 ± 38.49
SGPT(ALT) (IU/l)	44.60 ± 7.89	55.60 ± 8.02	46.80 ± 3.56	43.00 ± 5.79	63.60 ± 23.11	47.8 ± 7.79

Values are mean ± S.D. (n = 5/dose). There was no significant difference between control and treatment groups ($p > 0.05$).

4.4.3 Histopathological Analysis

Microscopic examinations of the vital organs (heart, kidney, liver, spleen and lung) of rats in all treated and control groups showed no histopathological changes following 28 days of oral feeding. Figures 4.3 to 4.12 show the histological sections of heart (Figures 4.3 and 4.4), kidney (Figures 4.5 and 4.6), liver (Figures 4.7 and 4.8), spleen (Figures 4.9 and 4.10) and lung (Figures 4.11 and 4.12) of male and female rats subjected to the different treatments. For all the treated rats, the heart showed normal cardiac muscle fibres; the kidney showed normal glomeruli, tubules and interstitium; and the liver showed normal architecture with normal hepatocytes and portal tracts, while the spleen also showed normal histology. Peribronchial and interstitial inflammatory cell infiltration of mainly lymphocytes, at similar degree were found in the lungs of all treated and control groups, and the findings in TM02 treated groups were not dose-related (Figures 4.11 and 4.12). The lungs of male or female rats without any treatment displayed the same histological observations (Figure 4.13). Histological findings of the organs of the male rats were similar to those of females.

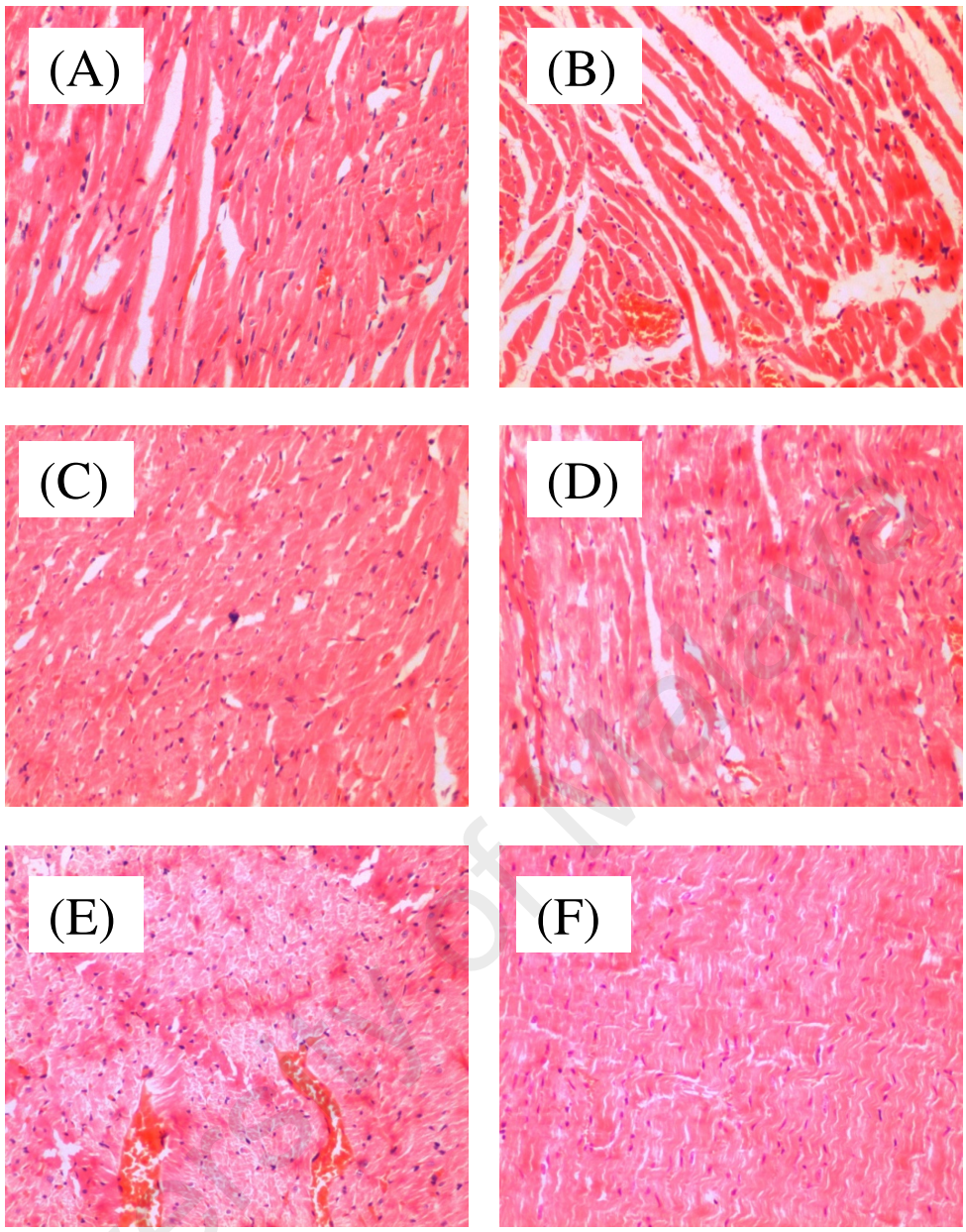

Figure 4.3: Heart of male rats subjected to various treatments showing normal cardiac muscle fibers (H&E stain x 100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

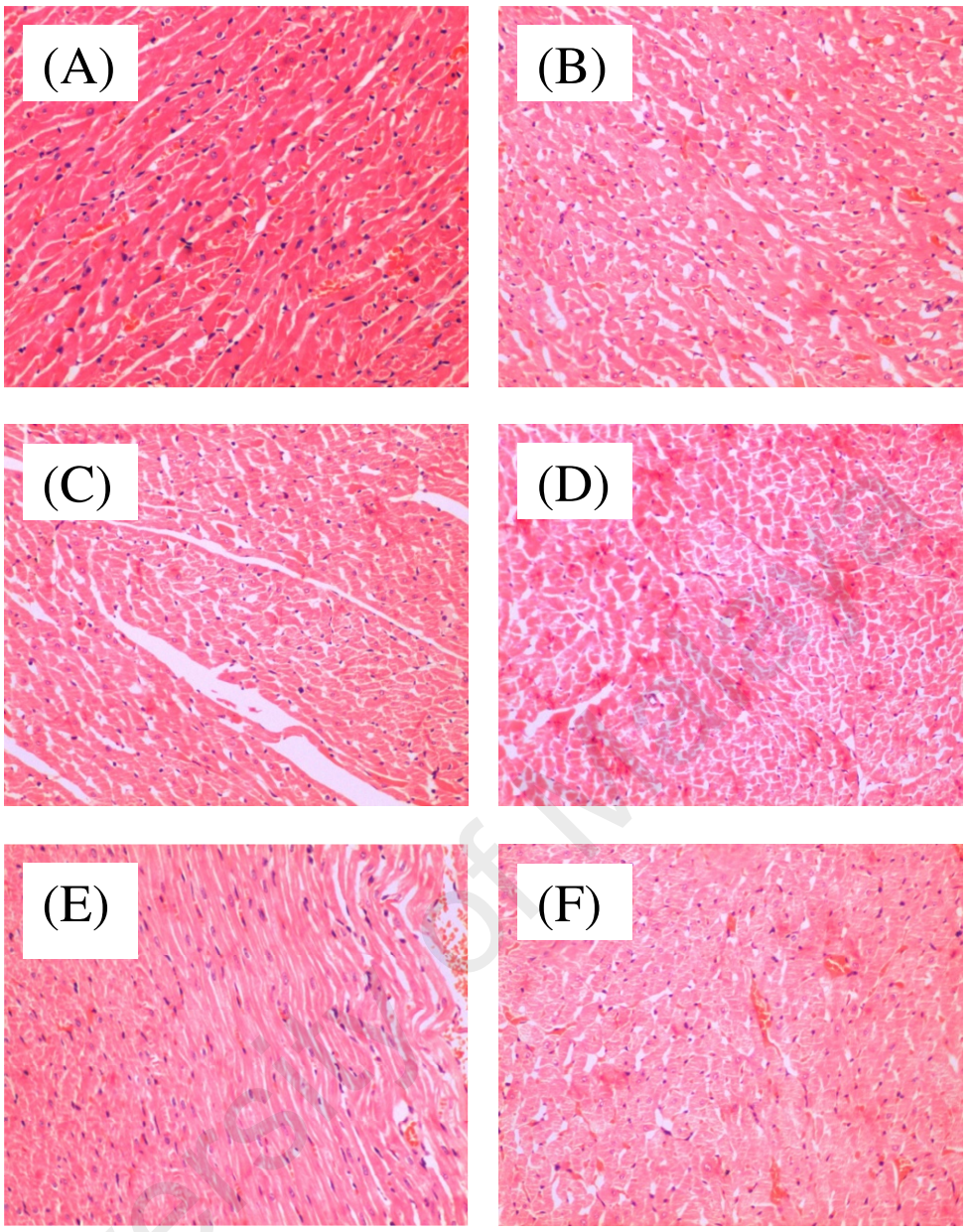

Figure 4.4: Heart of female rats subjected to various treatments showing normal cardiac muscle fibers (H&E stain x 100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

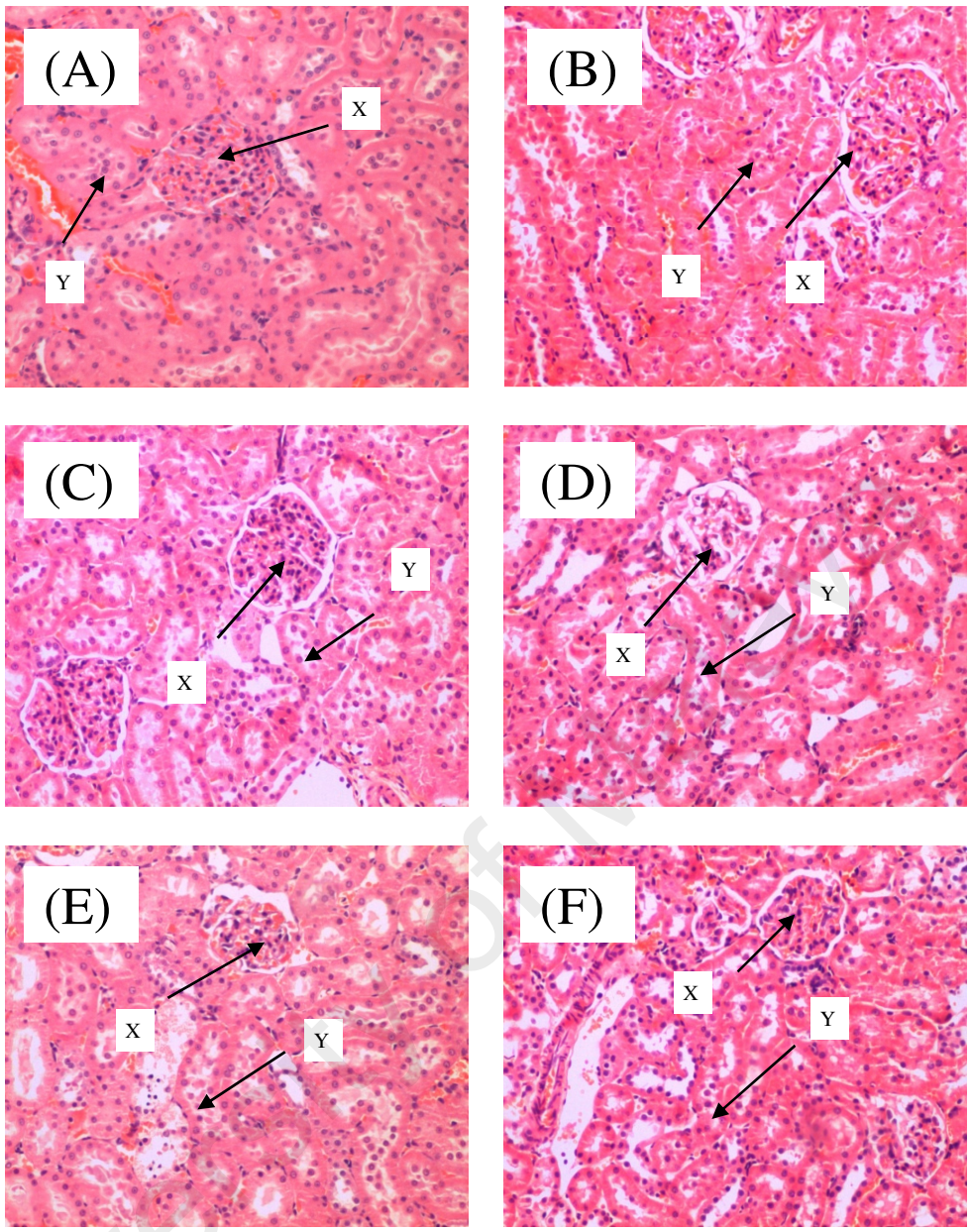

Figure 4.5: Kidney of male rats subjected to various treatments showing normal glomerulus, tubules and interstitium (H&E stain x 100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

'X' and 'Y' show the position of glomerulus and tubule, respectively.

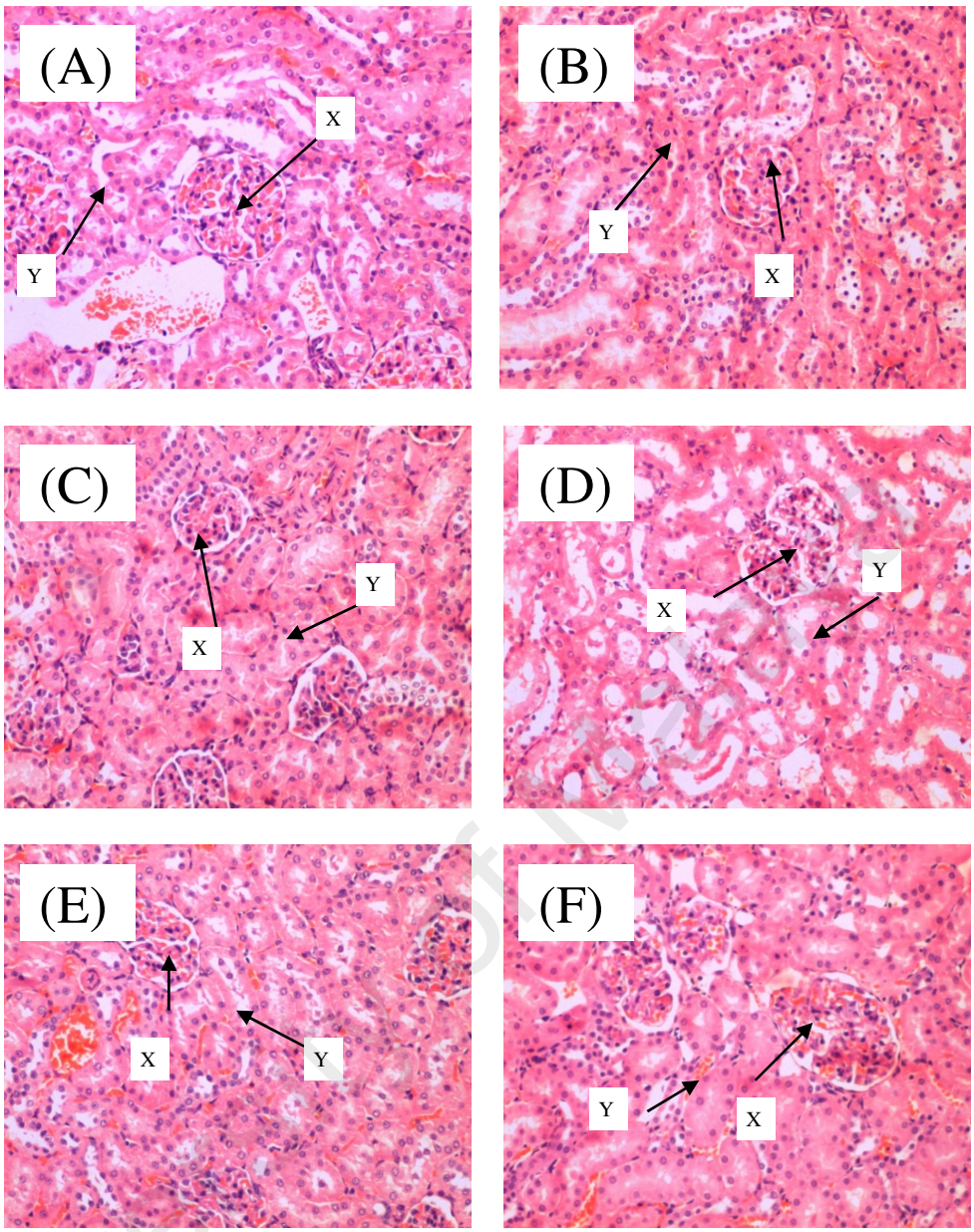

Figure 4.6: Kidney of female rats subjected to various treatments showing normal glomerulus, tubules and interstitium (H&E stain x 100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

'X' and 'Y' show the position of glomerulus and tubule, respectively.

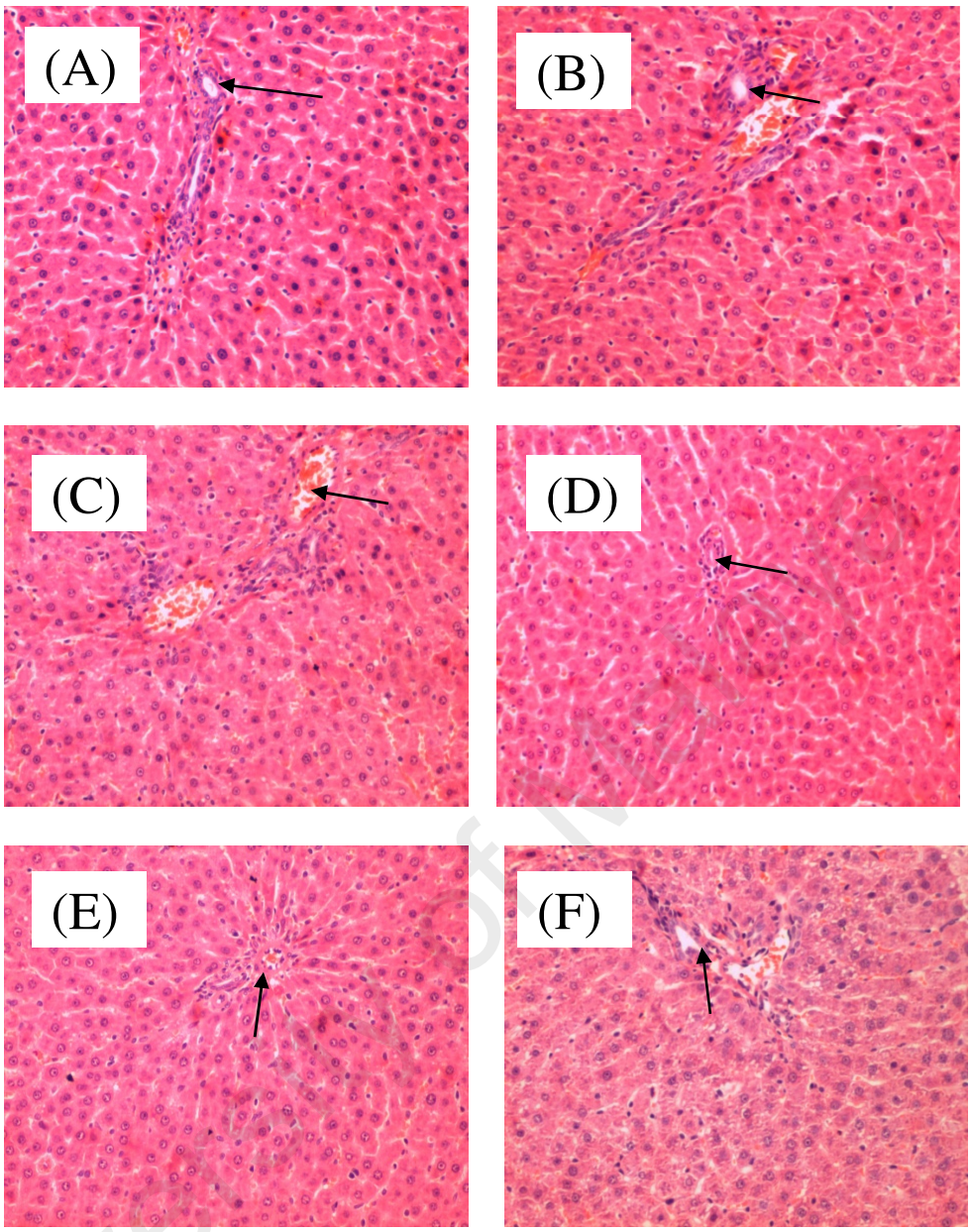

Figure 4.7: Liver of male rats subjected to various *Lignosus rhinocerotis* treatments showing normal architecture with normal hepatocytes and portal tract (H&E stain x100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

Arrows showed the position of portal tracts.

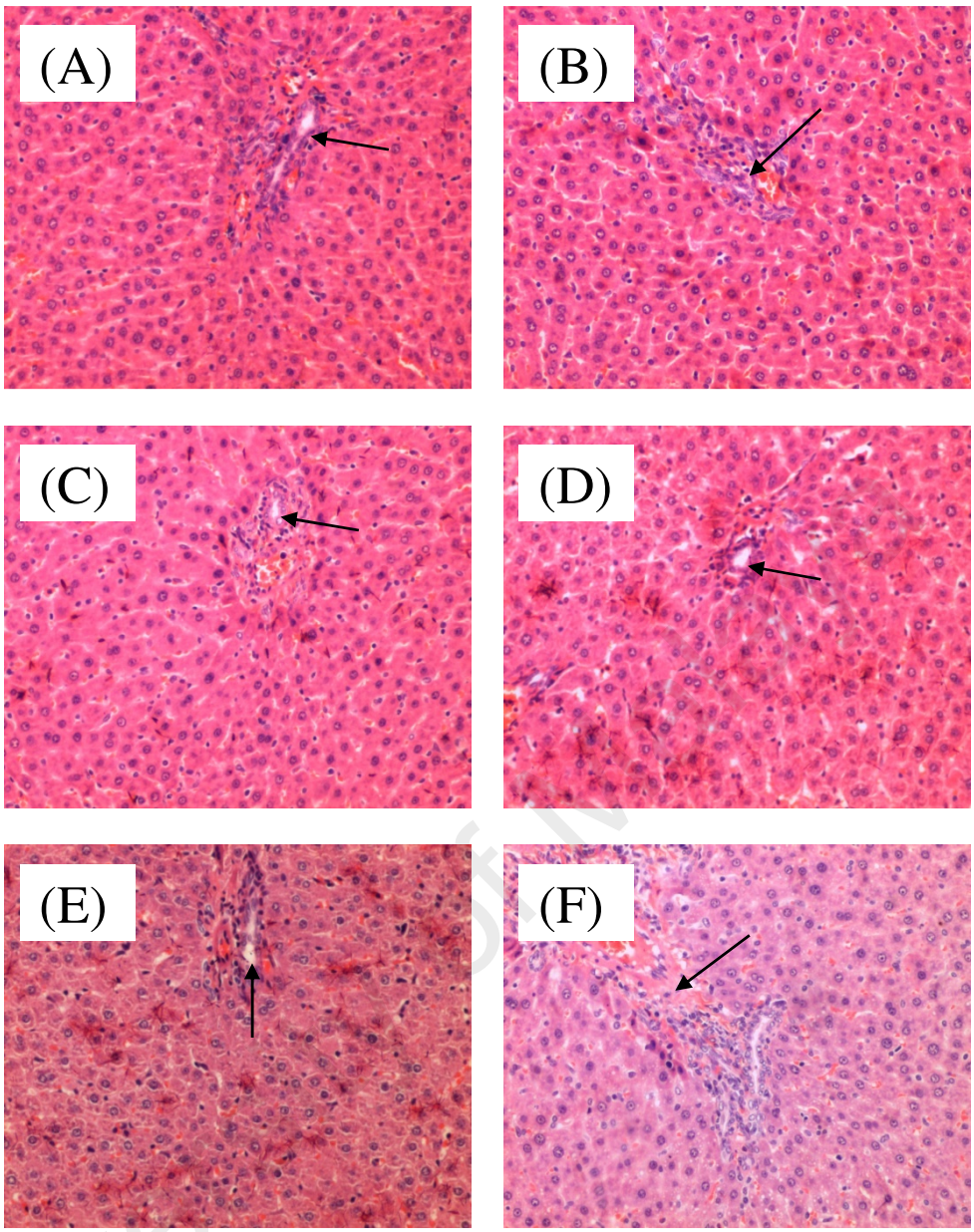

Figure 4.8: Liver of female rats subjected to various *Lignosus rhinocerotis* treatments showing normal architecture with normal hepatocytes and portal tracts (H&E stain x100)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500mg/kg; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

Arrows showed the position of portal tracts.

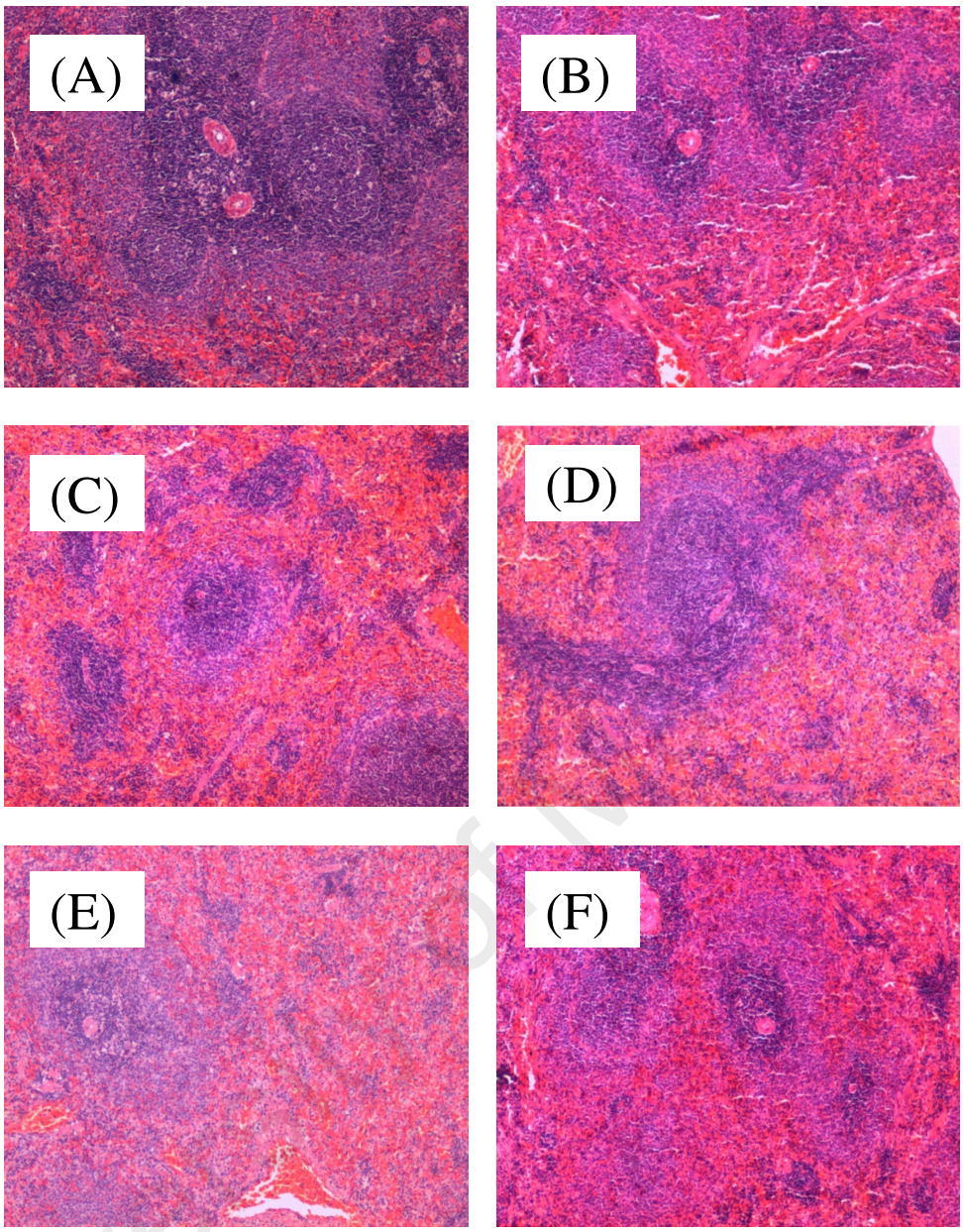

Figure 4.9: Spleen of male rats subjected to various treatments showing normal histology (H&E stain x 40)

(A) control; (B) wild type *L. rhinocerotis,* 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500 mg/kg ; (E) TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

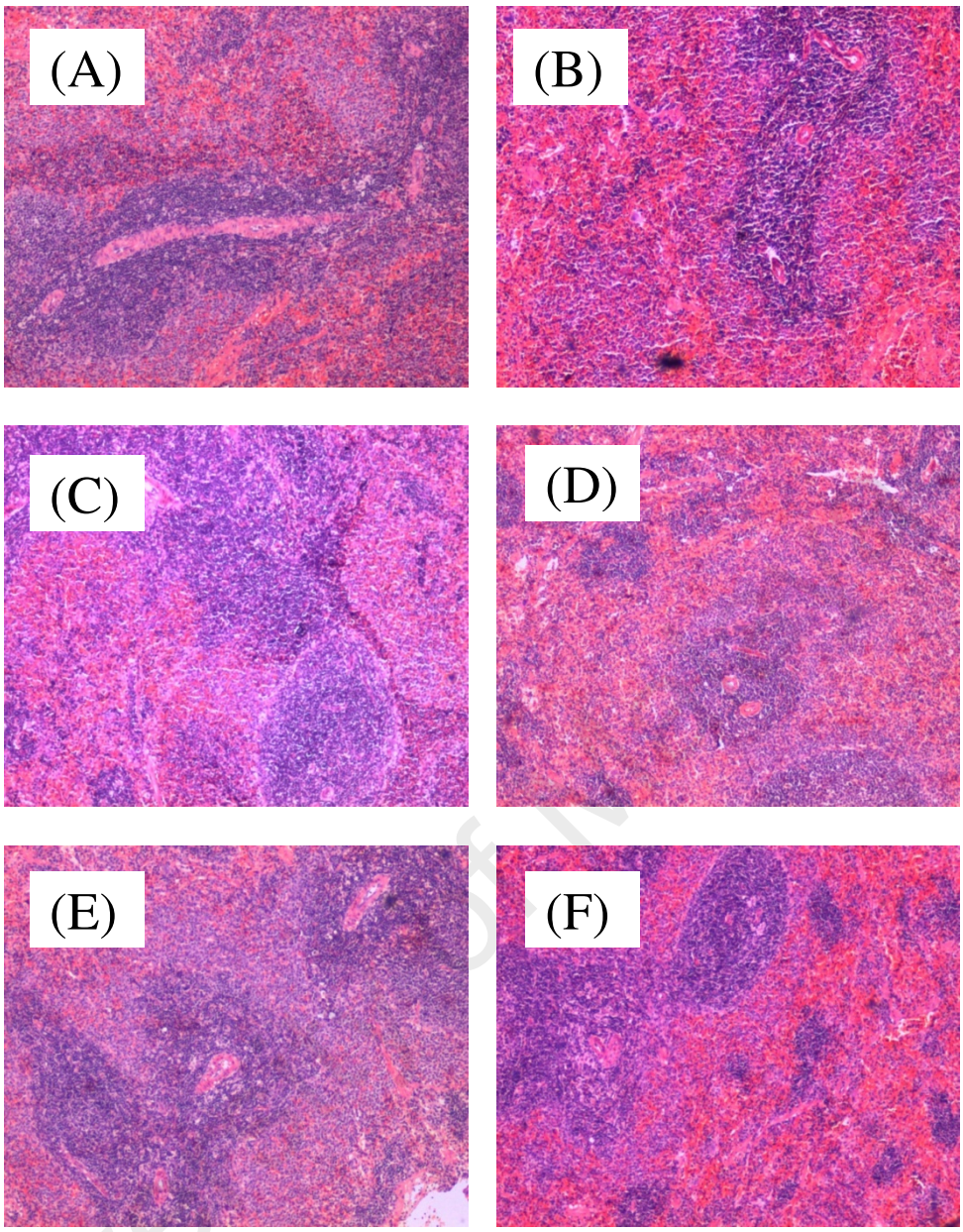

Figure 4.10: Spleen of female rats subjected to various treatments showing normal histology (H&E stain x 40)

(A) control; (B) wild type *L. rhinocerotis,* 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500 mg/kg ; (E)TM02, 250 mg/kg; (F) TM03, 1000 mg/kg.

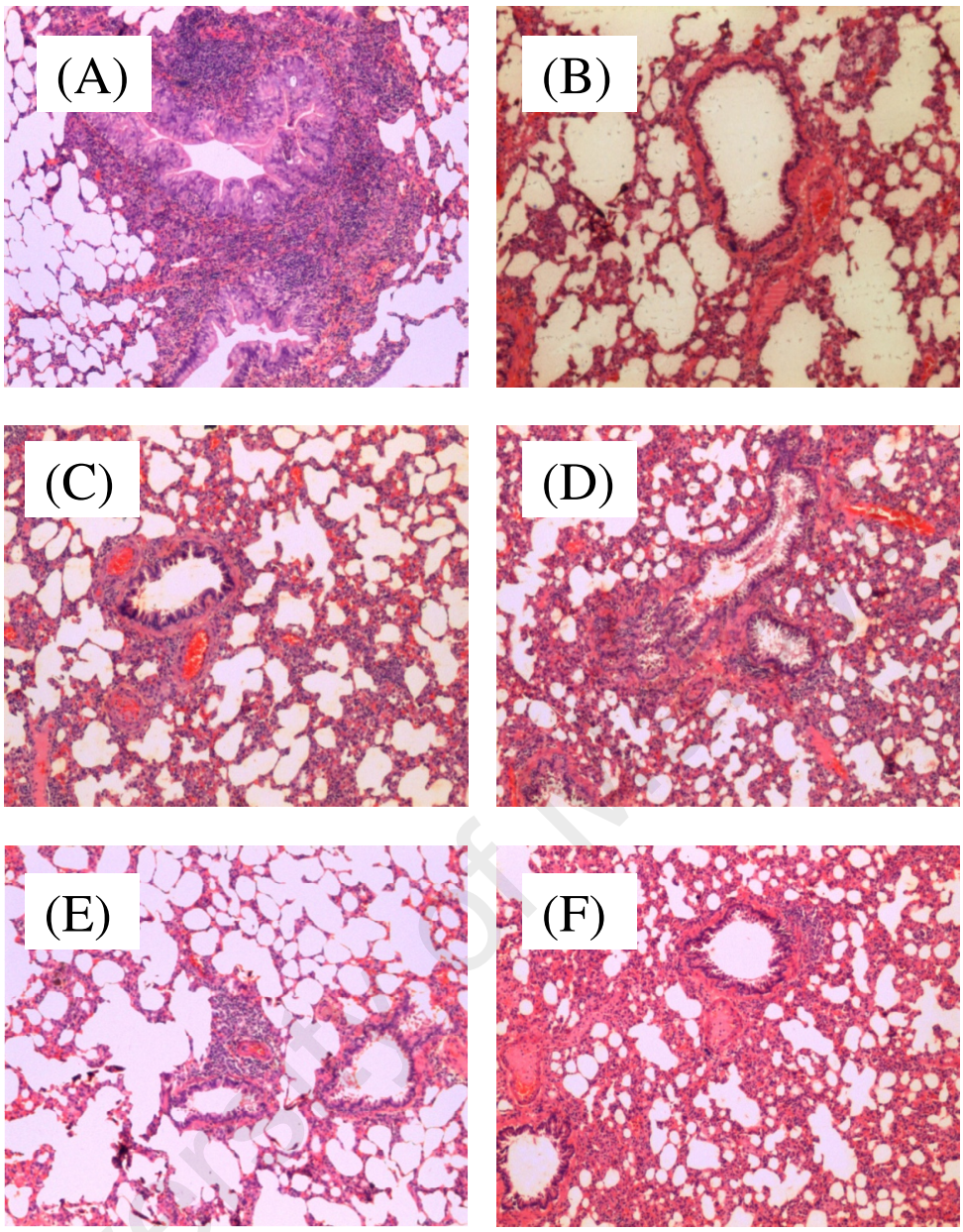

Figure 4.11: Lung of male rats subjected to various treatments showing interstitial inflammatory cell infiltrate of mainly lymphocytes (H&E stain x 40)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500 mg/kg; (E) TM02, 250 mg/kg (F) TM03, 1000 mg/kg.

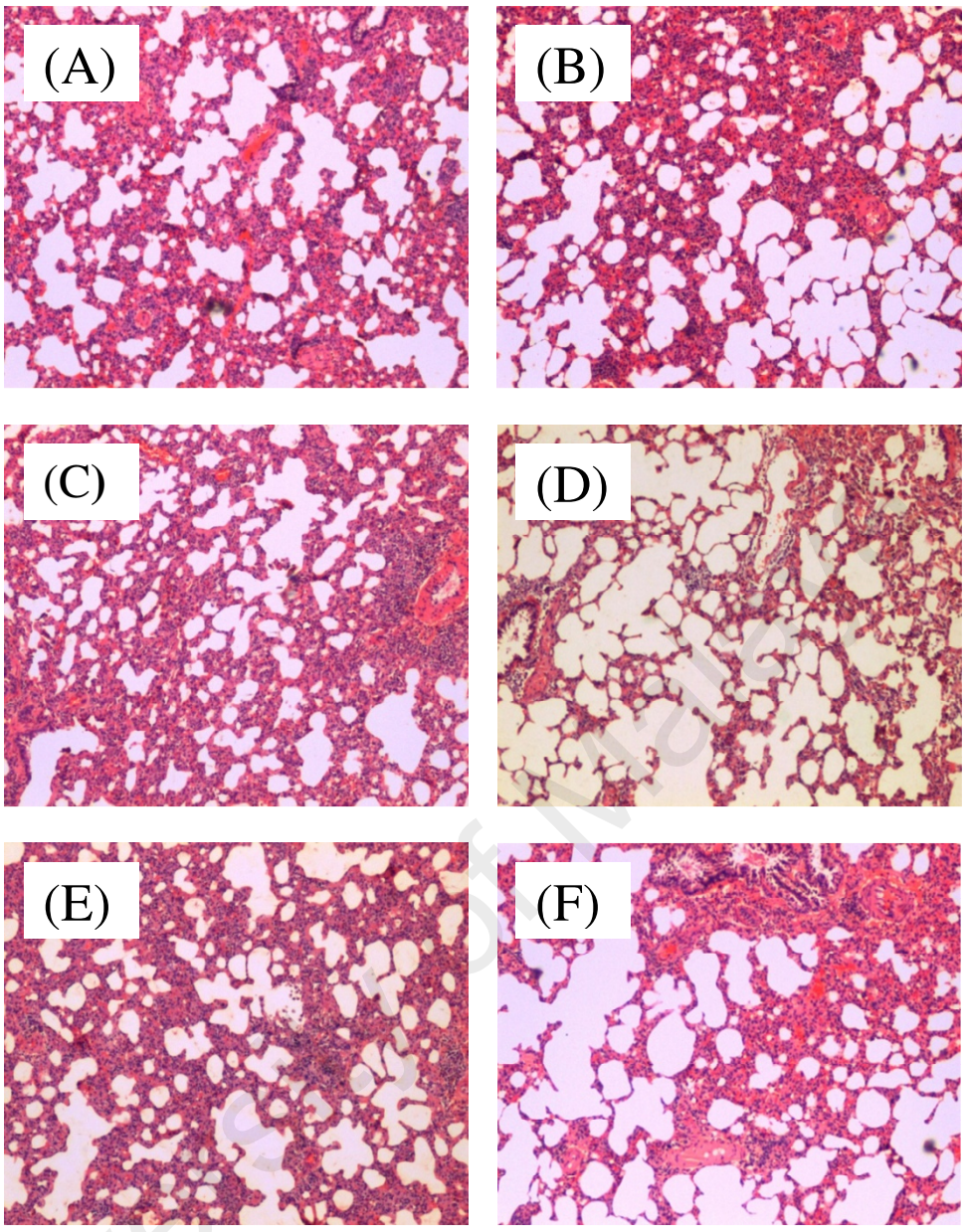

Figure 4.12: Lung of female rats subjected to various treatments showing interstitial inflammatory cell infiltrate of mainly lymphocytes (H&E stain x 40)

(A) control; (B) wild type *L. rhinocerotis*, 1000 mg/kg; (C) TM02, 1000 mg/kg; (D) TM02, 500 mg/kg; (E) TM02, 250 mg/kg (F) TM03, 1000 mg/kg.

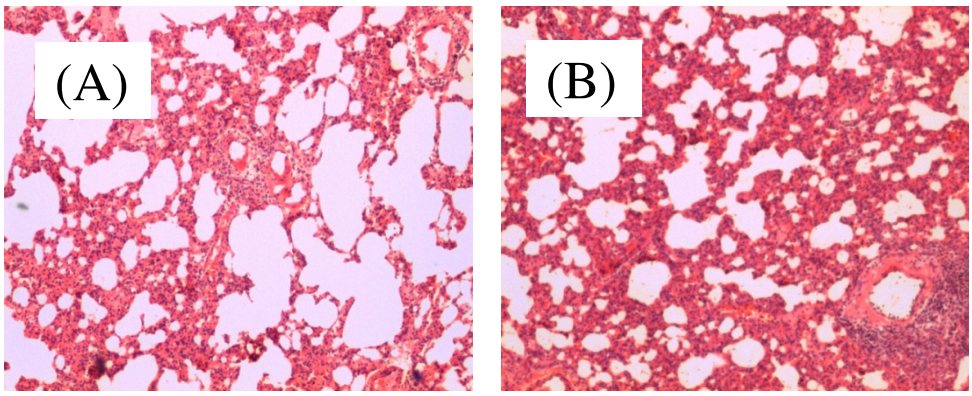

Figure 4.13: Lung of (A) male and (B) female rats which were not subjected to any treatments showing interstitial inflammatory cell infiltrate of mainly lymphocytes (H&E stain x 40)

4.5 Discussion

Adverse effects of drugs and chemicals can be indicated by changes in body weight (Hilaly et al., 2004; Mukinda & Eagles, 2010). The similar growth pattern as shown by body weight gain (Table 4.1; Figures 4.1 and 4.2) indicated that oral administration of the three sclerotial powder of *L. rhinocerotis* at a daily dose of up to 1000 mg/kg had no adverse effect on the growth of the SD rats.

It has been established that the highest overall concordance of toxicity in animals with humans is haematological parameters (Olson et al., 2000). Haematological parameters of rats fed with wild type, TM02 and TM03 *L. rhinocerotis* sclerotia for 28 days were not significantly different from the control group, except for 250 mg/kg TM02 treatment group, where the MCH of the female rats was slightly higher than that of the control group (Tables 4.2 and 4.3). The value, however, was within the maximum normal range established for SD rats at 18-19 week old (Petterino & Argentino-Storino, 2006). Other related haematological parameters such as red blood cell count, haemoglobin, PCV, MCV and MCHC of the rats in the same group were all normal. Therefore, it can be concluded that *L. rhinocerotis* sclerotia had no adverse effect on the haematological parameters of the rats.

Generally, clinical biochemistry studies showed that various types and doses of *L. rhinocerotis* treatments did not affect the renal functions (urea and creatinine levels), hepatic functions (albumin, total protein, SGOT and SGPT), serum electrolytes (calcium, potassium and sodium) as well as total cholesterol and glucose levels (Tables 4.4 and 4.5) of the rats. Although blood glucose levels of the male rats treated with 1000 mg/kg TM03 were reduced to 5.94 ± 0.47 mmol/l ($p < 0.05$), it does not necessarily reflect a hypoglycaemic effect by TM03, as the value was still within the reference range

established by Petterino & Argentino-Storino (2006). On the other hand, the blood glucose levels of TM02 (all 3 doses) and 1000 mg/kg wild type *L. rhinocerotis* treatment groups were both within normal range. In the 1000 mg/kg wild type *L. rhinocerotis* treatment group, though the calcium and total protein levels in the male group were slightly different from the control group ($p < 0.05$), the levels also fell within the reference range (Petterino & Argentino-Storino, 2006) and thus these minor differences are unlikely to be of any clinical significance.

Histological examinations supported the conclusions from clinical biochemistry studies that oral feeding of up to 1000 mg/kg of TM02, TM03 and wild type *L. rhinocerotis* sclerotial powder did not induce any pathological changes or damage in heart, kidney, liver and spleen of the rats (Figures 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9 and 4.10).

The peribronchial and interstitial inflammatory cell infiltration observed in all treatment groups were not induced by the *L. rhinocerotis* sclerotia, as the same infiltration was also found in both control groups (male and female rats) as well as rats without any treatment (Figures 4.11, 4.12 and 4.13). Therefore, there were no dose-related inflammatory responses in TM02, as observed by the use of three different doses. The inflammatory response may be caused by the housing environment of the animal supplier who used wood chips bedding for the animals (during the experimental period, however, the animals were housed on paper crumbs). The lung inflammation might be caused by particles generated from wood chips bedding, as *in vivo* mouse model showed that repeated airway exposure to wood dust can elicit lung inflammation (Määttä et al., 2006).

CHAPTER 5: CHRONIC TOXICITY OF THE SCLEROTIUM OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

5.1 Introduction

The no-observed-adverse-effect level (NOAEL) of the sclerotial powder of *L. rhinocerotis* cultivar (termed TM02) was demonstrated to be higher than 1000 mg/kg in a 28-day sub-acute toxicity study using rats (refer to chapter 4). To firmly establish the safety of the consumption of this cultivar, a 180-day chronic toxicity study was carried out. The study estimates the no-observed-adverse effect level which can be used to establish safety criteria for human exposure (OECD, 2009).

In this present studies, SD rats (10 rats/group/sex) received 250, 500 and 1000 mg/kg of sclerotial powder of TM02 by oral gavage. Rats were orally administered with the sclerotial powder once daily for 180 consecutive days. Haematological and clinical biochemical parameters, urine profiles, organ weight as well as histopathology were examined at the end of the experiment. The chronic toxicity study was carried out according to the guidelines of the Organization of Economic Cooperation and Development (OECD, 2009).

5.2 Literature Review

5.2.1 Chronic Toxicity Study

The chronic toxicity study is used to evaluate possible health hazards associated with repeated exposure of a test substance covering a considerable part of the lifespan of the species used (OECD, 2009). The study provides information on the toxic effects of the substance, indicates target organs toxicity and the possibility of toxicity accumulation (OECD, 2009). Chronic toxicity study should only be conducted after obtaining initial information from repeated dose 28-day and/or 90-day toxicity studies (OECD, 2009). The test substance is orally administered once daily and consecutively for 180 days to several groups of experimental animals. General observation, morbidity or mortality of all animals should be checked at least once a day (OECD, 2009). At the end of the oral feeding period, analysis of haematological and clinical biochemical parameters, urine profiles, organ weight as well as histopathological analysis are carried out.

5.2.2 Haematological Examination

Most of the haematological examinations were explained in chapter 4. Neutrophil count which is included in this chapter is to monitor the effect of target components on immune system (Sireeratawong et al., 2012). The prothrombin time (PT) and activated partial thromboplastin time (APTT) are commonly used to screen for coagulation abnormality in blood (Adaeze et al., 2014).

5.2.3 Clinical Biochemistry

Most of the clinical biochemistry assessments were explained in chapter 4. Altered serum uric acid concentrations (out of normal levels) have been associated with a number of disease states (Kutzing & Firestein, 2008). An abnormally high uric acid level has been correlated with cardiovascular disease, gout, renal disease and hypertention whereas a reduced uric acid concentration has been linked to Parkinson's disease, multiple sclerosis, optic neuritis and Alzheimer's disease (Kutzing & Firestein, 2008). Serum total protein, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase and gamma-glutamyl transferase (GGT) are used to assess hepatic function. Serum electrolytes such as inorganic chloride and inorganic phosphatase are evaluated as well.

5.2.4 Urinalysis

Urinalysis is an examination of urine by physical appearance, chemical composition and microscopic analysis (Echeverry et al., 2010). Physical appearance of urine is examined by its appearance, volume, colour and specific gravity. Examination of chemical composition of urine includes the identification of glucose, protein, ketone, occult blood, urobilinogen, bilirubin and pH. Microscopic examination includes the detection of epithelial cells, red blood cells and white blood cells in urine.

5.2.5 Organ Weight

In toxicology studies, analysis of organ weight is an important endpoint for identification of potentially harmful effects of chemicals (Bailey et al., 2004). Organ weight can be one of the most sensitive indicators of an effect of an experimental substance as significant differences in organ weight between treated and untreated animals may be seen in the absence of any morphological changes (Bailey et al., 2004). Differences in organ weight between treatment groups are always accompanied by body weight differences between these groups, making interpretation of organ weight differences more difficult (Bailey et al., 2004). Therefore, an analysis of the ratio of organ weight to body weight is commonly used (Bailey et al., 2004).

5.3 Methods

5.3.1 Animals and Oral Feeding of Sclerotial Powder of *Lignosus rhinocerotis*

SD rats (5 weeks old, male and female) were acclimatised for 14 days before use. The animals (7 weeks old) were divided into three treatment groups and one control group (10 rats/group/sex). Treatment groups received 250, 500 or 1000 mg/kg sclerotial powder of *L. rhinocerotis* (cultivar TM02) by oral gavage. The animals were orally administered with the sclerotial powder in the form of suspension in distilled water, once daily for 180 consecutive days. Control group received distilled water by oral gavage throughout the experiment. The highest dose used, 1000 mg/kg was chosen based on the results of the 28-day sub-acute toxicity study (Chapter 4). Two descending doses at 500 and 250 mg/kg were chosen to demonstrate if there is any presence of dose related response.

5.3.2 Blood Analysis

Rats were fasted for 18 hours at the end of experiment (day 180). At day 181, blood samples from rats were collected (by cardiac puncture) following anaesthesia with ketamine (45 mg/kg) and xylazine (4.5 mg/kg). Haematological parameters and clinical biochemistry were analysed using Advia 2120 Haematology System (Siemen, Germany) and Advia 2400 Chemistry System (Siemen, Germany), respectively. The parameters for haematological examination included red blood cell (RBC) count, haemoglobin concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, white blood cell (WBC) count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, atypical lymphocyte count, prothrombin time and activated partial thromboplastin time (APTT). Biochemical tests

included testing for glucose, urea, creatinine, calcium, inorganic phosphate, uric acid, sodium, potassium, chloride, total cholesterol, total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase, serum glutamic oxaloacetic transaminase or aspartate aminotransferase (SGOT or AST), serum glutamic pyruvic transaminase or alanine transaminase (SGPT or ALT) and gamma-glutamyl transpeptidase (GGT).

5.3.3 Urinalysis

At day 180, rat urine was collected over 18 hours using metabolic cages. Appearance, colour and volume of urine were recorded. Specific gravity, pH, total protein, glucose, ketone, occult blood, urobilinogen and bilirubin were analysed using SD UroColor^{TM} and Urometer 720^{TM} (Standard Diagnostics, INC, Korea). White blood cells, red blood cells and epithelial cells were observed using microscope (Olympus CH, America).

5.3.4 Harvesting of the Organs and Histopathological Analysis

After blood collection, the following organs were collected and weighed: the adrenals, brain, epididymis, heart, kidneys, liver, lungs, ovaries, spleen, testes and uterus. Organ weight relative to final body weight was calculated as follows:

$$\text{Organ weight relative to body weight (\%)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100\ \%$$

In addition to these organs, the aorta, caecum, cervix, coagulating gland, colon, duodenum, eyes, Harderian gland, ileum, jejunum, lacrimal gland (exorbital), lymph nodes, oesophagus, pancreas, prostate, rectum, salivary gland (parotid, submaxillary and sublingual), seminal vesicle, skeletal muscle, skin, stomach, thymus, trachea, urinary

bladder and vagina were removed and preserved in 10% buffered formalin. The tissues were dehydrated by serial ethanol solution, cleared with xylene, paraffin embedded, sectioned and stained with haematoxylin and eosin. Light microscopic examinations of multiple tissue sections from each organ were performed.

5.3.5 Statistical Evaluation

All data were expressed as mean ± standard deviation (S.D.). Data for chronic toxicity study was analysed using One-way Analysis of Variance (ANOVA). Statistical differences between the means of control and treatment groups were determined using Dunnett's t (two-sided) test. In case of variance heterogeneity, Dunnett T3 test were used. The homogeneity of variances was calculated using Levene statistics. Results were considered significant at $p < 0.05$.

5.4 Results

5.4.1 Body Weight and General Clinical Observations

Neither treatment related abnormality in the rats nor death in animals was observed following oral administration of the sclerotial powder of *L. rhinocerotis* (cultivar TM02) at all the doses tested. Body weight of rats treated with various doses of the sclerotial powder, and that of the control group for 180 days (26 weeks) were shown in Figures 5.1 and 5.2, respectively for male and female rats. There were no significant changes in the net body weight gain between the treated groups and the control group (Tables 5.1 and 5.2, $p > 0.05$).

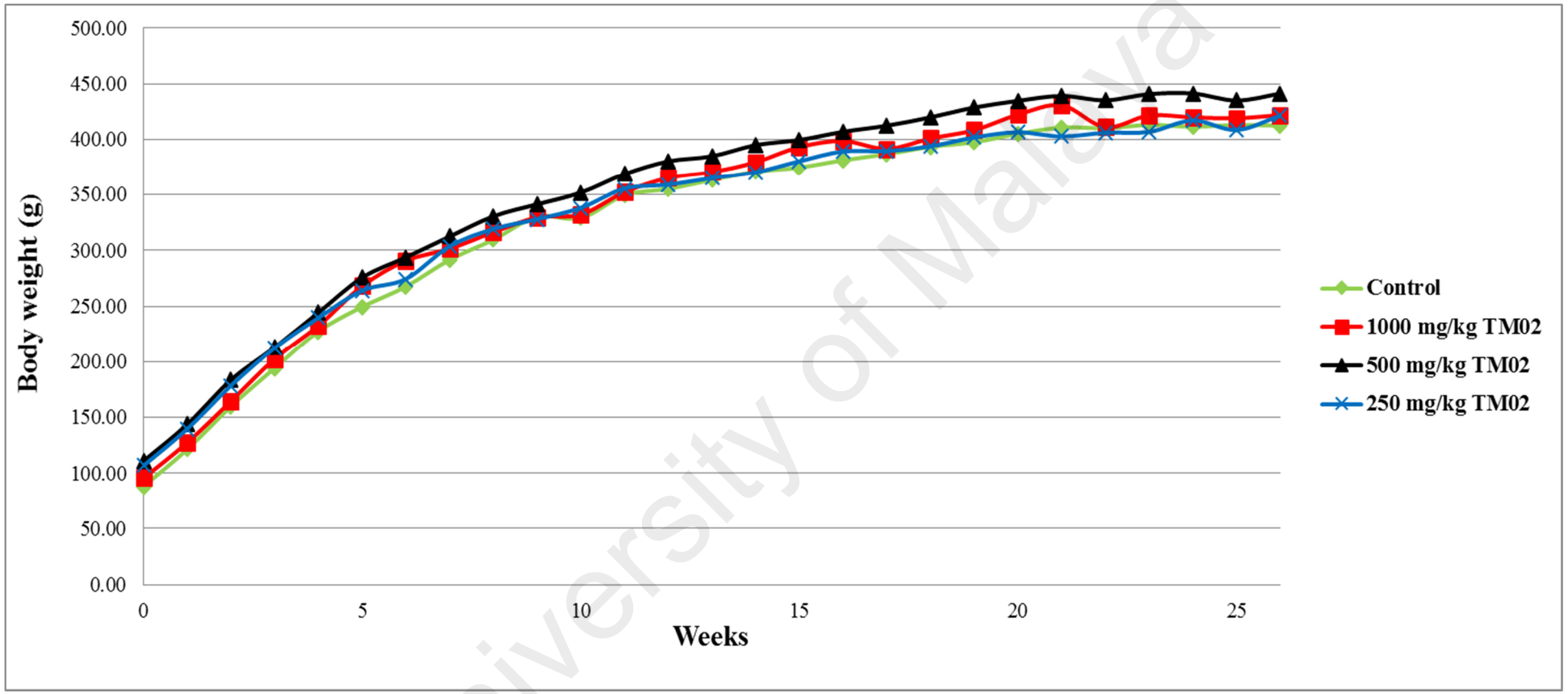

Figure 5.1: Body weight of male rats treated with 1000, 500 and 250 mg/kg sclerotial powder of *Lignosus rhinocerotis* (TM02) and the control group for 26 weeks (180 days)

Body weight in the figure is shown as mean of 10 rats per group.

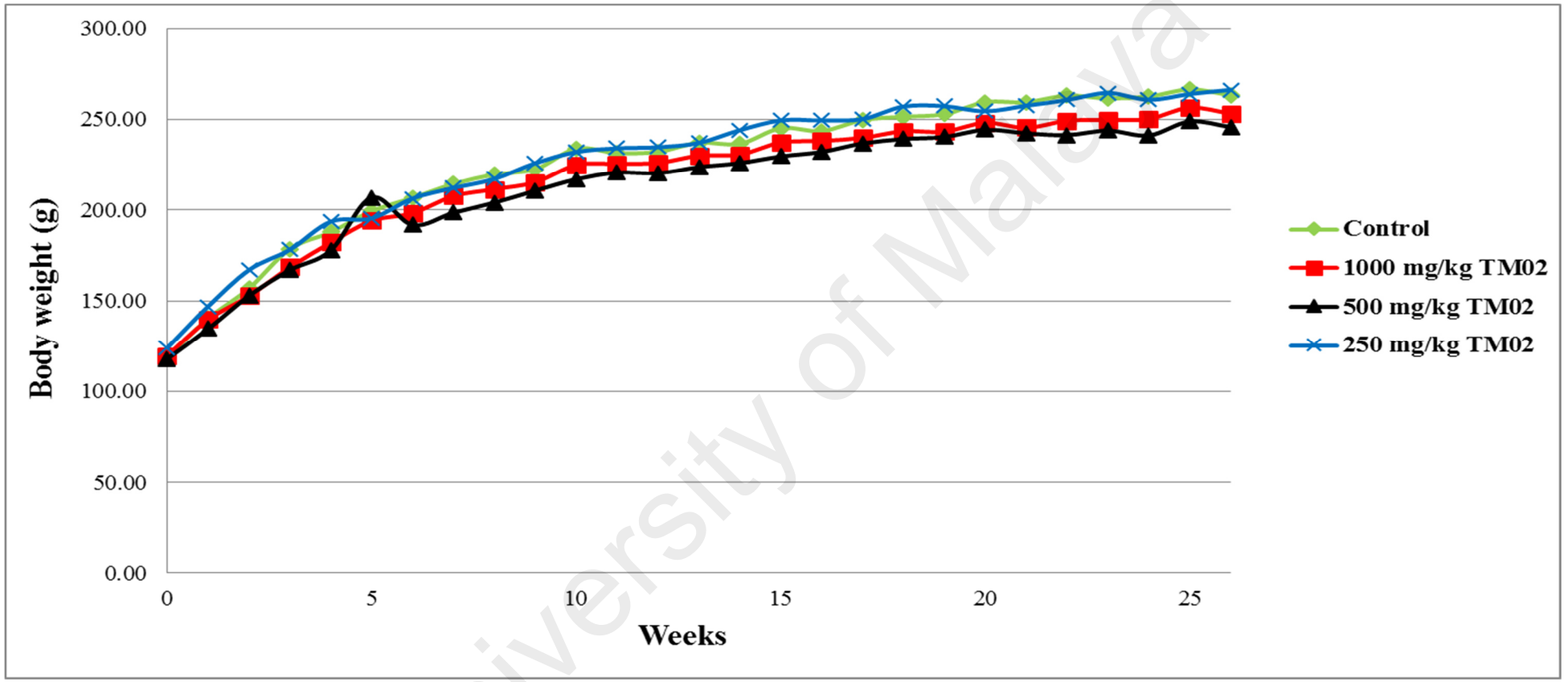

Figure 5.2: Body weight of female rats treated with 1000, 500 and 250 mg/kg sclerotial powder of *Lignosus rhinocerotis* (TM02) and the control group for 26 weeks (180 days)

Body weight in the figure is shown as mean of 10 rats per group.

Table 5.1: Body weight gain of male rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 26 weeks (180 days)

Treatment	Body weight gain (g)								
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Control	33.30 ± 8.50	71.70 ± 13.95	106.00 ± 19.71	139.20 ± 24.62	161.20 ± 32.24	179.40 ± 34.89	203.30 ± 38.94	221.30 ± 43.19	241.80 ± 33.23
1000 mg/kg TM02	31.80 ± 6.83	68.90 ± 20.54	106.80 ± 25.98	136.80 ± 23.67	178.40 ± 32.70	195.00 ± 32.43	205.70 ± 33.24	220.50 ± 36.25	233.60 ± 43.59
500 mg/kg TM02	32.80 ± 9.75	72.60 ± 11.89	101.90 ± 35.50	133.30 ± 39.65	164.60 ± 34.96	182.70 ± 33.41	201.30 ± 30.35	219.40 ± 32.10	230.20 ± 32.84
250 mg/kg TM02	32.90 ± 7.69	71.70 ± 11.09	105.60 ± 15.27	132.80 ± 18.29	157.20 ± 23.14	167.60 ± 32.69	196.80 ± 29.60	212.20 ± 31.78	221.00 ± 33.09
Treatment	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18
Control	240.70 ± 44.70	261.10 ± 48.07	266.80 ± 47.09	275.00 ± 48.84	282.60 ± 48.98	286.30 ± 50.00	293.00 ± 51.17	298.50 ± 50.61	305.10 ± 51.45
1000 mg/kg TM02	236.30 ± 46.61	256.40 ± 43.16	269.30 ± 42.60	274.60 ± 41.29	283.60 ± 43.76	289.80 ± 45.51	295.10 ± 49.16	295.70 ± 44.19	305.10 ± 43.77
500 mg/kg TM02	240.80 ± 31.69	257.50 ± 34.76	269.10 ± 32.14	272.70 ± 31.07	283.80 ± 34.48	288.40 ± 33.68	295.80 ± 31.67	301.30 ± 36.66	308.80 ± 36.49
250 mg/kg TM02	231.20 ± 31.76	248.60 ± 32.85	252.10 ± 34.77	258.10 ± 35.27	263.40 ± 33.74	273.40 ± 35.01	282.30 ± 37.83	282.60 ± 37.42	287.00 ± 37.16
Treatment	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Week 25	Week 26	
Control	309.30 ± 51.63	316.80 ± 52.50	322.40 ± 54.54	321.90 ± 52.48	324.80 ± 56.78	323.20 ± 58.20	324.80 ± 58.23	324.10 ± 59.91	
1000 mg/kg TM02	312.60 ± 47.17	318.80 ± 47.21	326.80 ± 48.32	308.10 ± 62.60	317.40 ± 48.31	315.90 ± 52.35	314.40 ± 52.39	316.70 ± 58.36	
500 mg/kg TM02	317.60 ± 35.05	323.60 ± 36.19	328.00 ± 33.67	324.10 ± 38.03	322.40 ± 36.48	322.80 ± 41.09	323.90 ± 43.16	330.10 ± 42.75	
250 mg/kg TM02	294.90 ± 39.53	299.60 ± 41.88	295.80 ± 40.20	299.20 ± 41.77	299.90 ± 40.55	310.20 ± 39.08	301.70 ± 37.91	314.50 ± 38.99	

The values for body weight gain are expressed as mean ± S.D. (n = 10/group/sex). The weight gain between the control and treatment groups were not significantly different ($p > 0.05$).

Table 5.2: Body weight gain of female rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 26 weeks (180 days)

Treatment	Body weight gain (g)								
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Control	21.30 ± 7.73	38.00 ± 11.47	59.70 ± 10.73	68.90 ± 10.48	80.50 ± 11.43	87.80 ± 10.62	95.50 ± 12.80	100.80 ± 13.45	103.70 ± 16.84
1000 mg/kg TM02	19.90 ± 6.14	31.82 ± 110.68	49.45 ± 10.39	64.09 ± 13.38	76.45 ± 14.24	82.36 ± 19.03	92.36 ± 21.66	95.82 ± 20.29	100.27 ± 20.81
500 mg/kg TM02	19.50 ± 5.13	34.90 ± 7.85	49.10 ± 13.34	59.90 ± 12.11	89.00 ± 18.07	73.90 ± 11.91	80.60 ± 13.39	86.20 ± 16.84	92.80 ± 17.43
250 mg/kg TM02	23.00 ± 8.65	43.10 ± 11.65	54.30 ± 14.61	69.70 ± 15.41	71.50 ± 13.20	82.50 ± 14.85	88.30 ± 16.24	93.30 ± 16.82	102.10 ± 17.14
Treatment	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18
Control	115.10 ± 16.29	112.70 ± 17.73	113.40 ± 17.15	118.40 ± 17.79	117.80 ± 17.91	126.40 ± 15.84	124.80 ± 17.62	130.90 ± 19.33	132.70 ± 21.23
1000 mg/kg TM02	109.91 ± 21.55	111.09 ± 22.32	111.09 ± 23.02	115.73 ± 23.69	117.09 ± 25.14	124.73 ± 28.92	124.55 ± 26.33	126.27 ± 25.99	129.91 ± 26.42
500 mg/kg TM02	99.10 ± 15.23	102.80 ± 14.45	102.50 ± 14.84	105.90 ± 14.27	108.10 ± 13.50	111.80 ± 15.61	114.30 ± 16.51	119.00 ± 15.19	121.40 ± 13.70
250 mg/kg TM02	108.20 ± 18.61	110.30 ± 18.57	110.90 ± 21.73	113.30 ± 22.61	120.30 ± 22.46	125.70 ± 23.40	125.70 ± 23.81	126.40 ± 23.34	133.30 ± 22.95
Treatment	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Week 25	Week 26	
Control	134.10 ± 20.10	140.70 ± 19.22	140.70 ± 17.91	144.30 ± 18.73	142.80 ± 18.61	143.80 ± 21.10	147.70 ± 19.17	144.60 ± 20.61	
1000 mg/kg TM02	129.82 ± 26.69	134.27 ± 24.95	132.00 ± 26.80	135.27 ± 26.97	135.91 ± 26.52	136.64 ± 26.59	142.91 ± 28.62	140.27 ± 29.01	
500 mg/kg TM02	122.60 ± 14.76	126.50 ± 14.14	124.60 ± 19.30	123.60 ± 16.22	126.10 ± 16.66	123.40 ± 18.12	131.10 ± 14.34	128.00 ± 13.90	
250 mg/kg TM02	133.50 ± 28.30	130.70 ± 26.42	133.80 ± 26.07	136.90 ± 25.77	140.80 ± 27.29	137.00 ± 26.95	140.20 ± 29.39	142.50 ± 28.86	

The values for body weight gain are expressed as mean ± S.D. (n = 10/group/sex). The weight gain between the control and treatment groups were not significantly different ($p > 0.05$).

5.4.2 Blood Analysis

5.4.2.1 Haematological Examinations

The results of the haematological examinations of the blood samples from the treated and control groups following 180 days treatment were shown in Tables 5.3 and 5.4, respectively for male and female rats. The values of RBC, haemoglobin, PCV, MCV, MCH, MCHC, platelet count, WBC, neutrophil, lymphocyte, monocyte, eosinophil, basophil and atypical lymphocyte of the treated group were not significantly different from the control group ($p > 0.05$).

5.4.2.2 Clinical Biochemistry

Tables 5.5 and 5.6 show the results of clinical biochemistry for male and female rats, respectively. Generally, the levels of serum glucose, urea, creatinine, calcium, inorganic phosphate, uric acid, sodium, potassium, chloride, total cholesterol, total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase, SGOT, SPGT and GGT of the treated groups were not significantly different from the control group ($p > 0.05$), with following exceptions:

- Sodium levels in the female group treated with 500 mg/kg (147.30 ± 1.89 mmol/l) and 250 mg/kg (146.30 ± 1.70 mmol/l) sclerotial powder of TM02 were slightly higher than the control group (144.00 ± 1.94 mmol/l, $p < 0.05$);
- Chloride level in the female group treated with 500 mg/kg sclerotial powder of TM02 (107.50 ± 2.01 mmol/l) was slightly higher than the control group (105.00 ± 1.94 mmol/l, $p < 0.05$).

Table 5.3: Haematological parameters of male rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days

Treatment (mg/kg)	Control (n = 10)	1000 mg/kg sclerotial powder (n = 10)	500 mg/kg sclerotial powder (n = 10)	250 mg/kg sclerotial powder (n = 10)
RBC (x10^{12}/l)	9.45 ± 0.56	9.00 ± 0.45	9.02 ± 0.34	9.07 ± 0.43
Haemoglobin (g/dl)	15.53 ± 0.71	15.13 ± 1.00	14.95 ± 0.45	15.01 ± 0.77
PCV (%)	46.10 ± 2.60	45.90 ± 2.42	44.60 ± 1.07	44.70 ± 2.00
MCV (fl)	48.90 ± 2.47	51.00 ± 1.89	49.40 ± 1.71	49.40 ± 2.37
MCH (pg)	16.50 ± 0.71	16.80 ± 0.42	16.70 ± 0.67	16.60 ± 0.70
MCHC (g/dl)	33.80 ± 0.63	33.00 ± 1.05	33.40 ± 0.70	33.60 ± 0.84
Platelet count (x10^{9}/l)	1047.30 ± 140.63	985.70 ± 205.09	1188.50 ± 170.68	1129.20 ± 206.65
WBC (x10^{9}/l)	8.42 ± 2.39	11.01 ± 3.46	9.21 ± 1.92	11.16 ± 2.74
Neutrophil (%)	39.20 ± 8.55	35.50 ± 8.02	31.30 ± 4.76	32.60 ± 7.04
Lymphocyte (%)	52.70 ± 7.76	55.20 ± 9.16	55.70 ± 3.53	55.70 ± 6.52
Monocyte (%)	7.80 ± 3.33	8.90 ± 2.42	12.60 ± 4.50	11.40 ± 2.07
Eosinophil (%)	0.30 ± 0.67	0.40 ± 0.84	0.40 ± 0.97	0.30 ± 0.95
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Atypical lymphocyte (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Prothrombin time (s)	9.39 ± 0.47	8.95 ± 0.51	9.18 ± 0.74	9.40 ± 0.49
APTT (s)	19.80 ± 1.69	18.70 ± 0.95	19.50 ± 1.35	18.40 ± 1.43

Values are mean ± S.D. (n = 10/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 5.4: Haematological parameters of female rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days

Treatment (mg/kg)	Control (n = 10)	1000 mg/kg sclerotial powder (n = 10)	500 mg/kg sclerotial powder (n = 10)	250 mg/kg sclerotial powder (n = 10)
RBC ($x10^{12}$/l)	8.27 ± 0.76	8.05 ± 0.48	8.16 ± 0.41	8.12 ± 0.37
Haemoglobin (g/dl)	14.64 ± 0.94	14.60 ± 0.62	14.69 ± 0.50	14.80 ± 0.86
PCV (%)	45.30 ± 3.06	44.60 ± 1.26	44.40 ± 1.51	44.40 ± 2.01
MCV (fl)	55.30 ± 4.81	55.60 ± 3.24	54.70 ± 3.23	55.10 ± 2.56
MCH (pg)	17.80 ± 1.03	18.30 ± 0.48	18.10 ± 0.74	18.30 ± 1.06
MCHC (g/dl)	32.20 ± 1.23	32.90 ± 1.10	33.10 ± 0.99	33.10 ± 0.57
Platelet count ($x10^9$/l)	953.80 ± 143.67	1029.70 ± 188.36	1035.50 ± 144.66	992.40 ± 190.33
WBC ($x10^9$/l)	7.45 ± 2.90	7.95 ± 1.94	6.98 ± 1.47	7.77 ± 2.86
Neutrophil (%)	29.40 ± 5.76	24.40 ± 7.59	23.50 ± 4.74	27.30 ± 10.32
Lymphocyte (%)	65.70 ± 7.47	70.20 ± 5.71	71.70 ± 6.57	67.80 ± 9.59
Monocyte (%)	4.10 ± 2.73	4.70 ± 3.20	4.80 ± 2.94	4.40 ± 2.01
Eosinophil (%)	0.60 ± 0.84	0.70 ± 1.06	0.80 ± 1.03	0.50 ± 0.85
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Atypical lymphocyte (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Prothrombin time (s)	N.D.	N.D.	N.D.	N.D.
APTT (s)	N.D.	N.D.	N.D.	N.D.

Blood samples collected from female rats was insufficient for the determination of prothrombin time and APTT-not determined (N.D.) Values are mean ± S.D. (n = 10/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 5.5: Clinical biochemistry parameters of male rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days

Treatment (mg/kg)	Control (n = 10)	1000 mg/kg sclerotial powder (n = 10)	500 mg/kg sclerotial powder (n = 10)	250 mg/kg sclerotial powder (n = 10)
Glucose (mmol/l)	8.29 ± 0.58	8.23 ± 0.90	8.42 ± 1.16	8.10 ± 1.13
Urea (mmol/l)	6.77 ± 0.90	6.92 ± 1.21	6.25 ± 0.94	7.10 ± 1.05
Creatinine (µmol/l)	40.60 ± 10.28	44.00 ± 9.68	36.30 ± 4.35	38.40 ± 6.65
Calcium (mmol/l)	2.47 ± 0.04	2.45 ± 0.09	2.45 ± 0.04	2.54 ± 0.08
Inorganic pohosphate (mmol/l)	2.26 ± 0.36	2.44 ± 0.31	2.24 ± 0.25	2.33 ± 0.16
Uric acid (mmol/l)	0.08 ± 0.05	0.09 ± 0.04	0.06 ± 0.01	0.07 ± 0.01
Sodium (mmol/l)	144.30 ± 3.53	144.90 ± 1.37	143.20 ± 0.79	143.00 ± 2.05
Potassium (mmol/l)	4.56 ± 0.69	4.57 ± 0.40	4.50 ± 0.20	4.53 ± 0.25
Chloride (mmol/l)	104.10 ± 1.52	103.60 ± 2.22	103.10 ± 1.45	105.70 ± 3.20
Total cholesterol (mmol /l)	1.47 ± 0.12	1.45 ± 0.14	1.52 ± 0.27	1.30 ± 0.23
Total protein (g/l)	77.20 ± 4.49	73.80 ± 4.94	74.60 ± 3.98	78.10 ± 4.56
Albumin (g/l)	35.80 ± 1.99	33.50 ± 3.21	35.90 ± 1.29	36.10 ± 2.38
Globulin (g/l)	41.00 ± 3.27	40.30 ± 3.77	38.70 ± 3.43	42.00 ± 4.00
A/G ratio	0.88 ± 0.06	0.82 ± 0.11	0.93 ± 0.08	0.87 ± 0.11
Total bilirubin (µmol/l)	3.10 ± 0.32	3.30 ± 0.48	3.10 ± 0.32	3.20 ± 0.42
Alkaline phosphatase (IU/l)	105.40 ± 20.51	79.00 ± 34.79	107.30 ± 28.56	113.40 ± 28.41
SGOT(AST) (IU/l)	162.70 ± 27.11	187.80 ± 32.55	172.10 ± 38.21	191.30 ± 31.88
SGPT(ALT) (IU/l)	59.70 ± 11.51	57.10 ± 9.73	52.10 ± 7.45	60.10 ± 13.36
GGT (IU/l)	1.90 ± 2.28	0.00 ± 0.00	0.20 ± 0.63	0.40 ± 0.97

Values are mean ± S.D. (n = 10/group). There was no significant difference between control and treatment groups ($p > 0.05$).

Table 5.6: Clinical biochemistry parameters of female rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days

Treatment (mg/kg)	Control (n = 10)	1000 mg/kg sclerotial powder (n = 10)	500 mg/kg sclerotial powder (n = 10)	250 mg/kg sclerotial powder (n = 10)
Glucose (mmol/l)	8.29 ± 1.18	8.59 ± 0.84	8.58 ± 1.51	8.65 ± 1.00
Urea (mmol/l)	7.90 ± 1.61	7.53 ± 1.06	7.71 ± 0.68	7.65 ± 1.45
Creatinine (µmol/l)	47.80 ± 5.59	48.10 ± 610	42.40 ± 6.35	46.70 ± 8.06
Calcium (mmol/l)	2.67 ± 0.10	2.58 ± 0.11	2.63 ± 0.12	2.69 ± 0.09
Inorganic pohosphate (mmol/l)	1.79 ± 0.33	1.91 ± 0.64	2.02 ± 0.41	1.95 ± 0.24
Uric acid (mmol/l)	0.08 ± 0.02	0.09 ± 0.05	0.06 ± 0.01	0.06 ± 0.02
Sodium (mmol/l)	144.00 ± 1.94	144.70 ± 2.54	147.30 ± 1.89*	146.30 ± 1.70*
Potassium (mmol/l)	4.10 ± 0.31	4.36 ± 0.66	4.11 ± 0.25	4.18 ± 0.36
Chloride (mmol/l)	105.00 ± 1.94	102.70 ± 2.26	107.50 ± 2.01*	106.50 ± 2.27
Total cholesterol (mmol /l)	2.09 ± 0.46	2.12 ± 0.36	1.85 ± 0.32	2.44 ± 0.53
Total protein (g/l)	84.50 ± 5.28	80.50 ± 6.92	81.10 ± 4.98	84.30 ± 5.87
Albumin (g/l)	44.40 ± 4.25	41.10 ± 4.77	41.20 ± 3.46	43.60 ± 4.03
Globulin (g/l)	40.10 ± 3.73	39.40 ± 3.44	39.90 ± 3.51	40.70 ± 3.62
A/G ratio	1.13 ± 0.14	1.06 ± 0.13	1.03 ± 0.13	1.08 ± 0.11
Total bilirubin (µmol/l)	4.00 ± 0.00	3.50 ± 0.53	4.00 ± 0.00	3.70 ± 0.48
Alkaline phosphatase (IU/l)	56.10 ± 28.75	68.70 ± 21.96	58.40 ± 19.24	71.80 ± 20.47
SGOT(AST) (IU/l)	156.20 ± 33.03	152.90 ± 30.53	148.70 ± 39.06	150.90 ± 18.39
SGPT(ALT) (IU/l)	52.10 ± 25.02	43.90 ± 12.12	53.20 ± 20.78	48.30 ± 14.59
GGT (IU/l)	0.10 ± 0.32	0.00 ± 0.00	0.20 ± 0.42	0.00 ± 0.00

Values are mean ± S.D. (n = 10/dose). *Significant difference from control (ANOVA, Dunnett's t (two-sided) test, $p < 0.05$).

5.4.3 Urinalysis

There were no significant changes in the physical appearance (appearance, volume, colour and specific gravity), chemical composition (glucose, total protein, ketone, occult blood, urobilinogen, bilirubin and pH) and microscopic examination (the presence or absence of white blood cell number, red blood cell and epithelial cells) of the urine samples of all treated groups, as compared to the control group of both sexes.

5.4.4 Absolute and Relative Organ Weight

No significant changes were found in the absolute organ weights (Table 5.7) and relative organ weights (Table 5.8) in all male and female rats which were orally fed with the sclerotial powder at various doses, as compared to the control group.

Table 5.7: Absolute organ weight of male and female rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days

Treatment (mg/kg)	Control (n = 10)		1000 mg/kg sclerotial powder (n = 10)		500 mg/kg sclerotial powder (n = 10)		250 mg/kg sclerotial powder (n = 10)	
	male	Female	male	female	male	female	Male	female
Heart	1.29 ± 0.31	0.85 ± 0.05	1.29 ± 0.17	0.87 ± 0.12	1.29 ± 0.27	0.84 ± 0.09	1.29 ± 0.14	0.86 ± 0.10
Left lung	0.67 ± 0.07	0.56 ± 0.11	0.72 ± 0.15	0.56 ± 0.13	0.73 ± 0.07	0.49 ± 0.06	0.68 ± 0.08	0.51 ± 0.08
Right lung	1.39 ± 0.24	1.15 ± 0.36	1.43 ±0.18	1.09 ± 0.21	1.38 ± 0.13	0.98 ± 0.12	1.27 ± 0.16	1.03 ± 0.23
Liver	10.22 ± 1.75	6.64 ± 0.87	10.26 ± 1.59	6.29 ± 0.66	11.19 ± 1.61	6.14 ± 0.82	9.74 ± 1.76	6.84 ± 1.11
Left kidney	1.26 ± 0.16	0.78 ± 0.08	1.34 ± 0.15	0.76 ± 0.08	1.28 ± 0.14	0.72 ± 0.08	1.24 ± 0.15	0.81 ± 0.12
Right kidney	1.26 ± 0.16	0.79 ± 0.07	1.36 ± 0.15	0.78 ± 0.09	1.31 ± 0.12	0.76 ± 0.08	1.27 ± 0.14	0.82 ± 0.12
Left adrenal	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Right adrenal	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00
Spleen	0.60 ± 0.16	0.44 ± 0.06	0.62 ±0.11	0.47 ± 0.10	0.65 ± 0.09	0.46 ± 0.08	0.63 ± 0.09	0.48 ± 0.05
Brain	1.85 ± 0.06	1.76 ± 0.10	1.94 ± 0.04	1.79 ± 0.12	1.91 ± 0.18	1.72 ± 0.07	1.85 ± 0.08	1.80 ± 0.10
Left testes/ovary	1.40 ± 0.12	0.05 ± 0.01	1.52 ± 0.21	0.04 ± 0.01	1.27 ± 0.60	0.05 ± 0.01	1.33 ±0.18	0.05 ± 0.01
Right testes/ovary	1.43 ± 0.11	0.05 ± 0.01	1.53 ± 0.20	0.04 ± 0.01	1.35 ± 0.42	0.05 ± 0.01	1.32 ± 0.17	0.05 ± 0.01
Left epididymis/uterus	0.66 ± 0.05	0.58 ± 0.16	0.66 ± 0.12	0.59 ± 0.15	0.64 ± 0.09	0.58 ± 0.20	0.61 ± 0.05	0.56 ± 0.11
Right epididymis	0.69 ± 0.08	-	0.64 ± 0.09	-	0.66 ± 0.12	-	0.61 ± 0.06	-

Values are mean ± S.D. (n = 10/group). There was no significant difference between control and treatment groups of rats at each sex ($p > 0.05$).

Table 5.8: Organ weight relative to body weight of male and female rats treated with various doses of sclerotial powder of *Lignosus rhinocerotis* (TM02) for 180 days.

Treatment (mg/kg)	Control (n = 10)		1000 mg/kg sclerotial powder (n = 10)		500 mg/kg sclerotial powder (n = 10)		250 mg/kg sclerotial powder (n = 10)	
	male	Female	male	female	male	female	Male	female
Heart	0.32 ± 0.06	0.35 ± 0.03	0.32 ± 0.04	0.36 ± 0.04	0.31 ± 0.05	0.35 ± 0.01	0.34 ± 0.04	0.35 ± 0.02
Left lung	0.17 ± 0.03	0.24 ± 0.06	0.18 ± 0.04	0.23 ± 0.05	0.18 ± 0.03	0.22 ± 0.03	0.18 ± 0.03	0.21 ± 0.02
Right lung	0.36 ± 0.09	0.48 ± 0.17	0.35 ± 0.05	0.45 ± 0.08	0.34 ± 0.05	0.44 ± 0.06	0.34 ± 0.05	0.41 ± 0.07
Liver	2.57 ± 0.31	2.73 ± 0.25	2.52 ± 0.19	2.61 ± 0.20	2.70 ± 0.23	2.62 ± 0.21	2.55 ± 0.33	2.78 ± 0.40
Left kidney	0.32 ± 0.02	0.32 ± 0.03	0.33 ± 0.03	0.32 ± 0.04	0.31 ± 0.03	0.32 ± 0.03	0.33 ± 0.04	0.33 ± 0.04
Right kidney	0.32 ± 0.02	0.33 ± 0.03	0.34 ± 0.04	0.32 ± 0.04	0.32 ± 0.02	0.34 ± 0.03	0.33 ± 0.04	0.34 ± 0.04
Left adrenal	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Right adrenal	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ±0.00	0.01 ± 0.00
Spleen	0.15 ± 0.04	0.18 ± 0.03	0.16 ± 0.03	0.20 ± 0.04	0.16 ± 0.02	0.20 ± 0.03	0.17 ± 0.02	0.20 ± 0.02
Brain	0.47 ± 0.06	0.73 ± 0.08	0.48 ± 0.06	0.75 ± 0.07	0.46 ± 0.06	0.78 ± 0.07	0.48 ± 0.03	0.74 ± 0.08
Left testes/ovary	0.35 ± 0.06	0.02 ± 0.00	0.38 ± 0.07	0.02 ± 0.00	0.30 ± 0.14	0.02 ± 0.00	0.35 ± 0.07	0.02 ± 0.01
Right testes/ovary	0.35 ± 0.06	0.02 ± 0.01	0.38 ± 0.07	0.02 ± 0.00	0.31 ± 0.10	0.02 ± 0.00	0.35 ± 0.06	0.02 ± 0.01
Left epididymis/uterus	0.17 ± 0.03	0.24 ± 0.06	0.17 ± 0.04	0.25 ± 0.07	0.15 ± 0.02	0.26 ± 0.09	0.16 ± 0.02	0.23 ± 0.04
Right epididymis	0.18 ± 0.04	-	0.16 ± 0.03	-	0.16 ± 0.03	-	0.16 ± 0.02	-

Values are percentage mean ± S.D. (n = 10/dose). There was no significant difference between control and treatment groups of rats at each sex ($p > 0.05$).

5.4.5 Histopathological Examinations

Histopathological examinations were only performed on organs from animals in the control and highest-dose treated group of both sexes (1000 mg/kg sclerotial powder of TM02) as no macroscopic abnormality was observed in the organs of the control and all treated rats. After 180 days treatment, there were no alterations worthy of note in the microscopic examinations of the organs in all treated and control rats. Figures 5.3 to 5.7 show the histological sections of vital organs including heart, spleen, liver, kidney and lung of male and female rats in control group and subjected to the treatment of sclerotial powder of TM02 at 1000 mg/kg. Normal histology of heart and spleen were found in both control group and group treated with 1000 mg/kg sclerotial powder of TM02 (Figures 5.3 and 5.4). In both male and female rats, focal portal lymphocytic infiltration of liver was observed in rats treated with 1000 mg/kg sclerotial powder of TM02 and control group (Figure 5.5). However, no evidence of cholangitis, venulitis or arteriolitis was found. Besides, congestion of glomeruli were found in male and female rats of both 1000 mg/kg sclerotial powder of TM02 treated group and control group (Figure 5.6). However, no evidence of glomerular nephritis was found. Tubules were found normal and there was no evidence of necrosis or tubulitis. Chronic inflammatory cell infiltration in the interstitium was found in the lungs of the treated group (1000 mg/kg sclerotial powder of TM02) and control group, at similar degree (Figure 5.7). The inflammatory response may be caused by the housing environment of the animal supplier who used wood chips bedding for the animals which was explained in chapter 4.

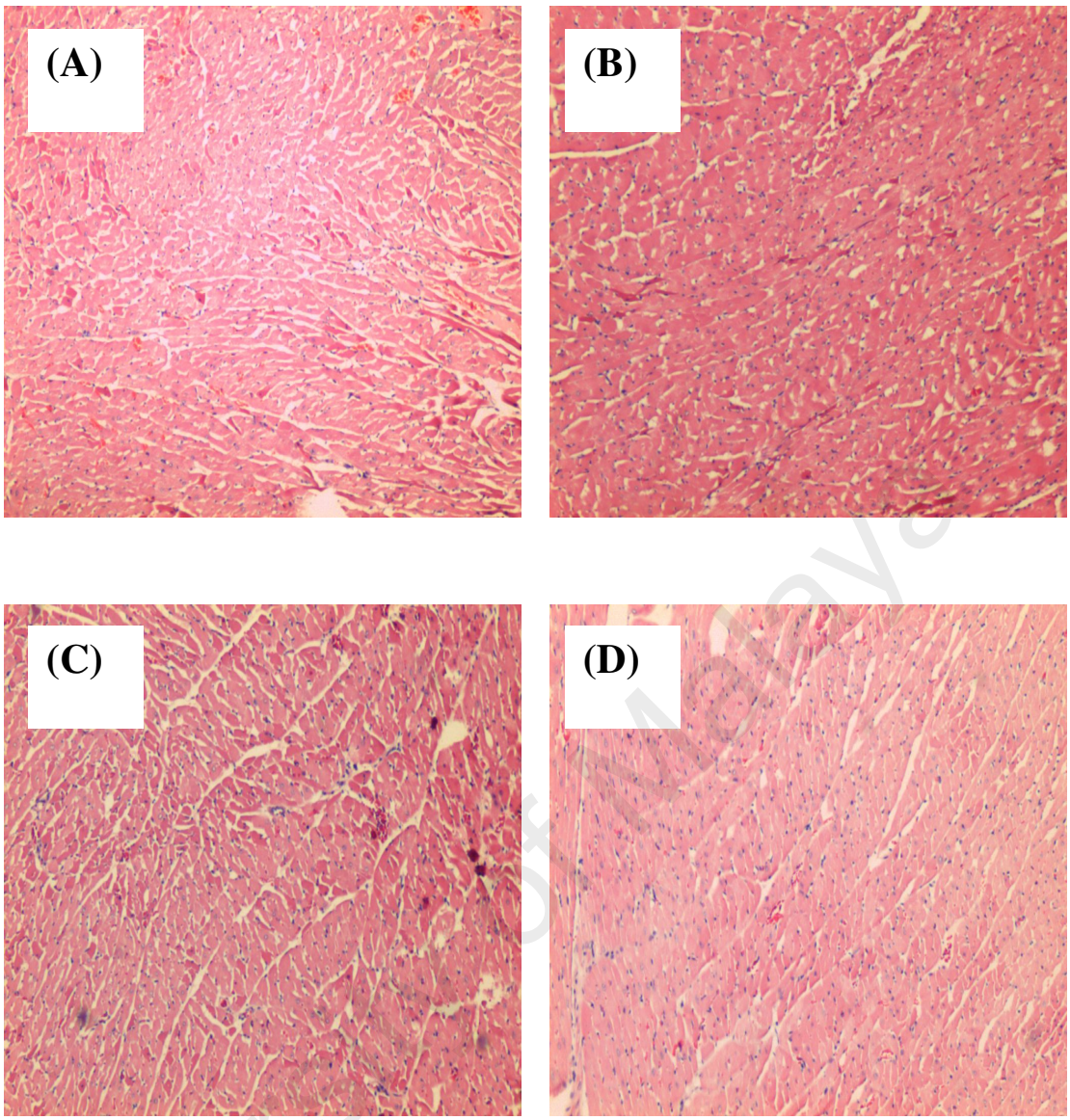

Figure 5.3: Heart of male and female rats subjected to respective treatments showing normal cardiac muscle fibers (H&E stain x 40)

(A) control, male; (B) TM02, 1000 mg/kg, male; (C) control, female; (D) TM02, 1000 mg/kg, female.

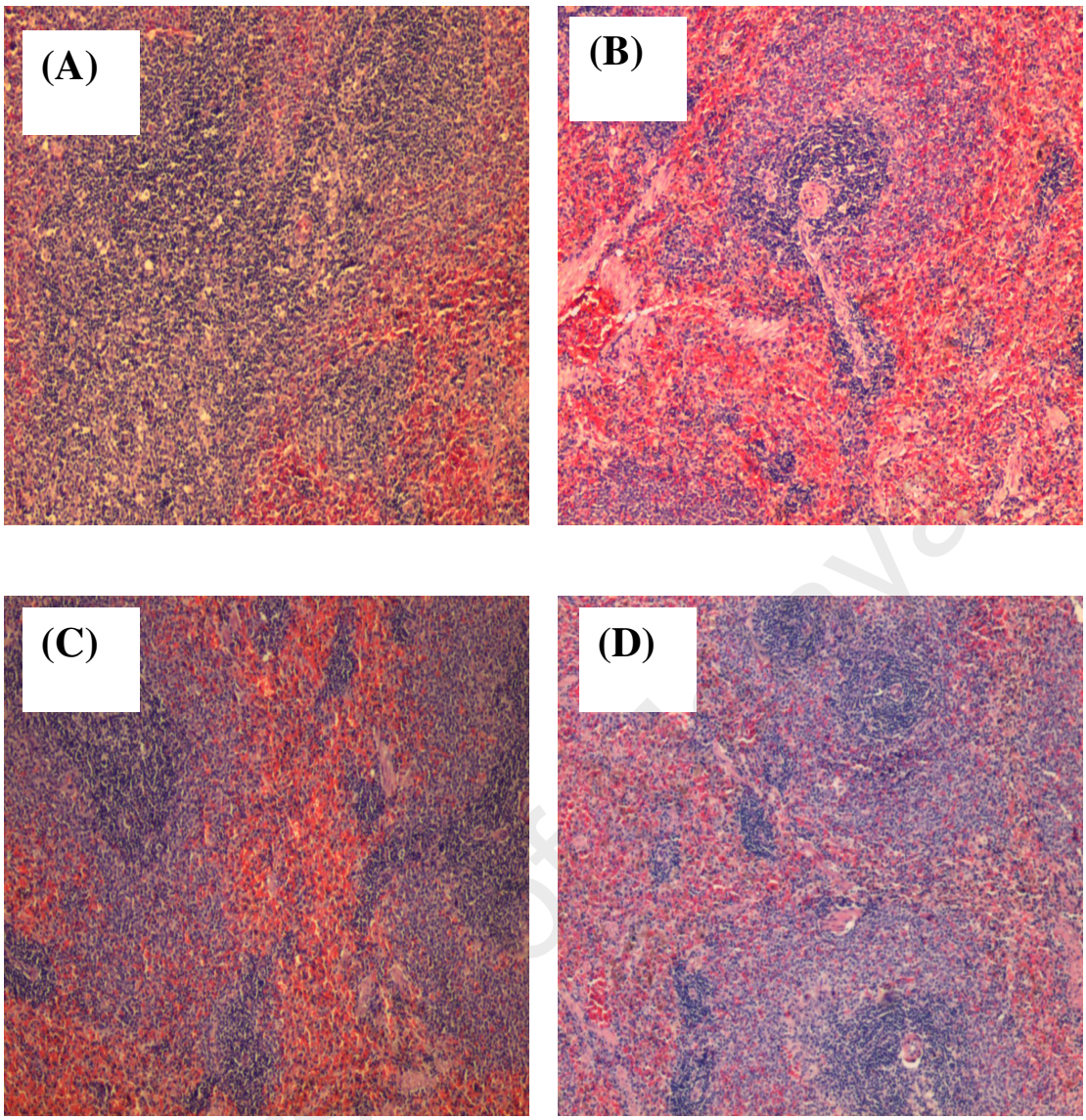

Figure 5.4: Spleen of male and female rats subjected to respective treatments showing normal histology (H&E stain x 40)

(A) control, male; (B) TM02, 1000 mg/kg, male; (C) control, female;

(D) TM02, 1000 mg/kg, female.

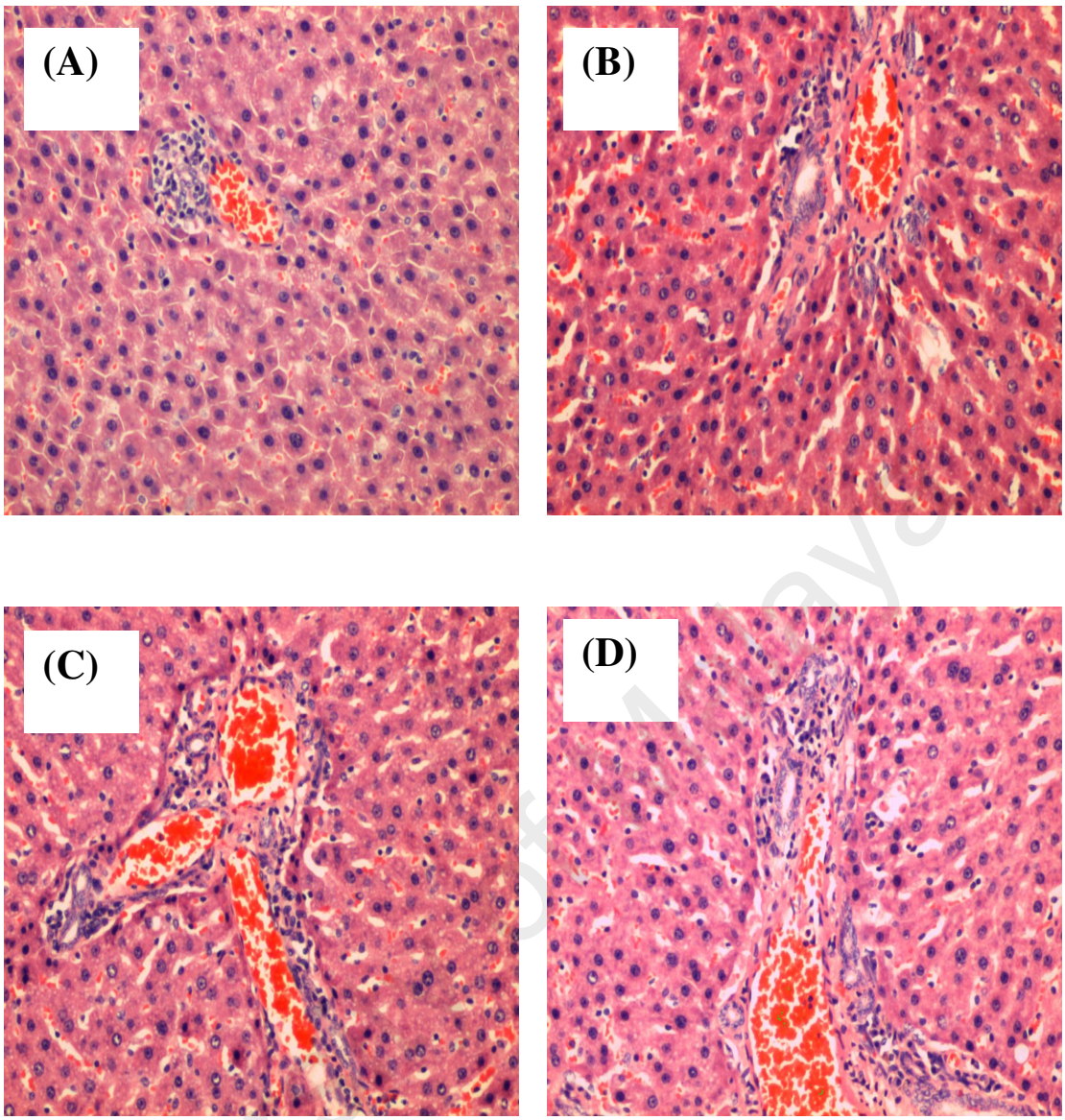

Figure 5.5: Liver of male and female rats subjected to respective treatments showing focal portal lymphocytic infiltration of liver with no evidence of cholangitis, venulitis or arteriolitis (H&E stain x 40)

(A) control, male; (B) TM02, 1000 mg/kg, male; (C) control, female; (D) TM02, 1000 mg/kg, female.

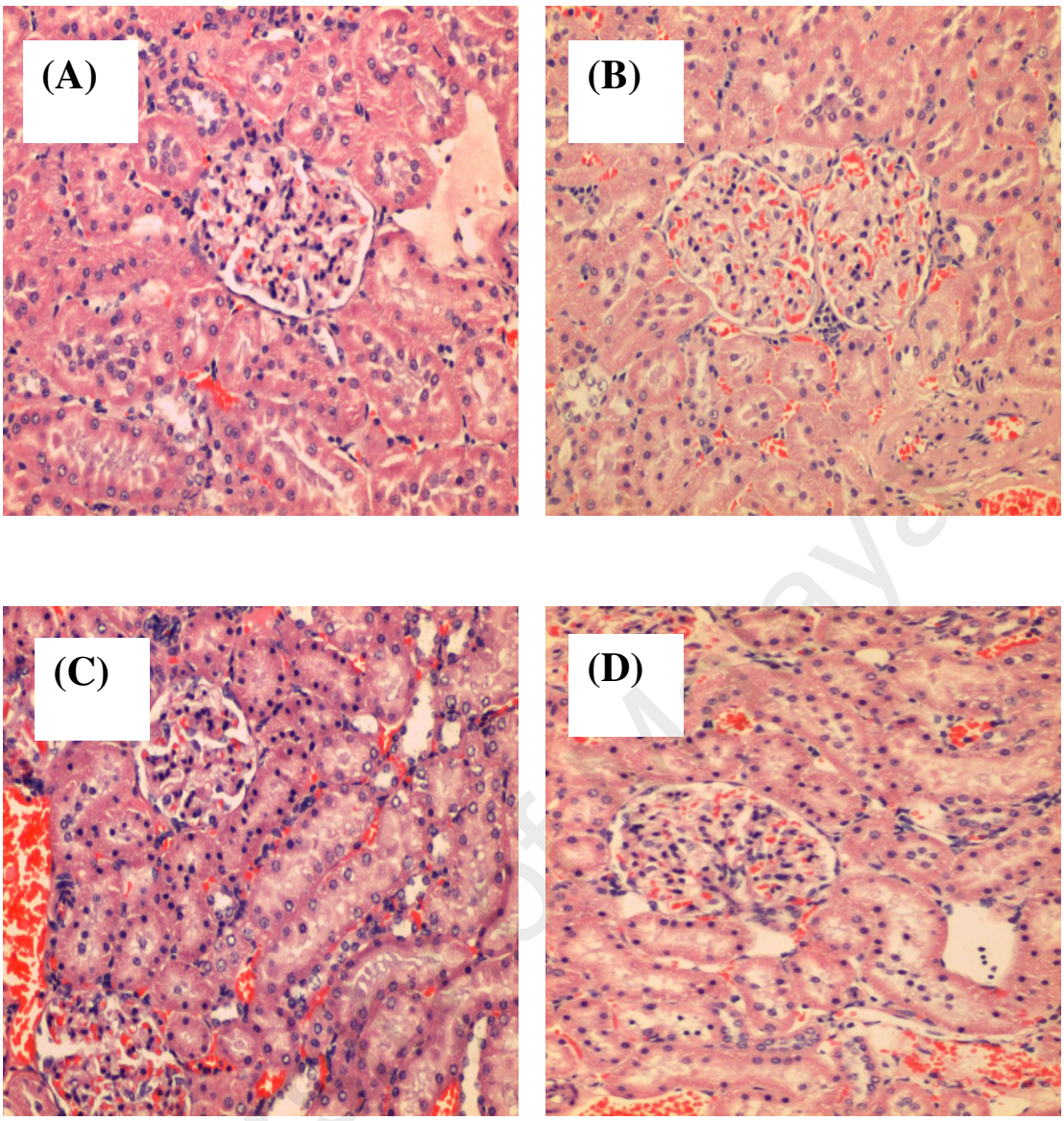

Figure 5.6: Kidney of male and female rats subjected to respective treatments showing normal tubules and interstitium

Congestion of glomeruli was found in both male and female groups with no evidence of glomerular nephritis (H&E stain x 100). (A) control, male; (B) TM02, 1000 mg/kg, male; (C) control, female; (D) TM02, 1000 mg/kg, female.

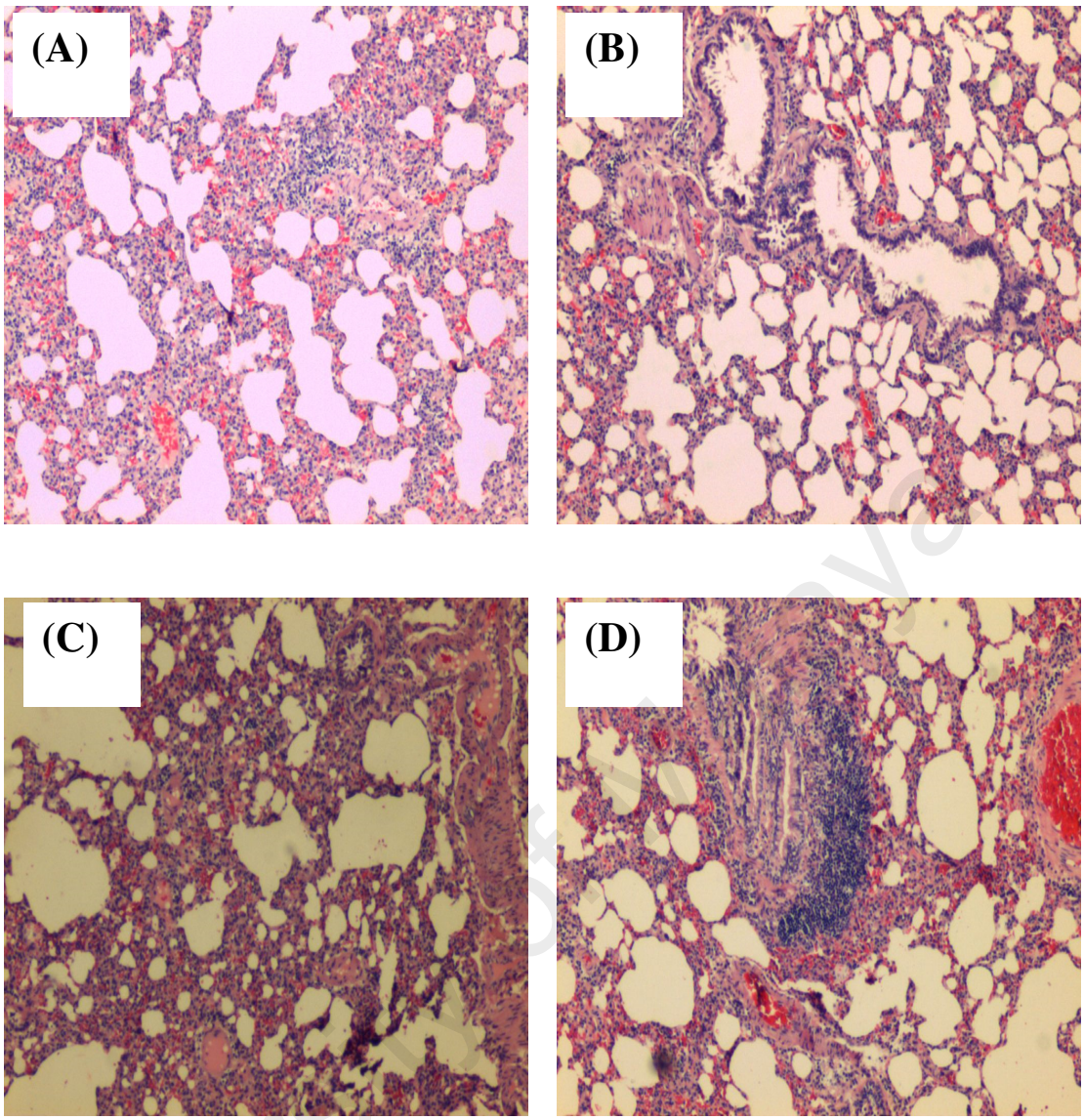

Figure 5.7: Lung of male and female rats subjected to respective treatments showing chronic inflammatory cell infiltrate in the interstitium (H&E stain x 40)

(A) control, male; (B) TM02, 1000 mg/kg, male; (C) control, female;

(D) TM02, 1000 mg/kg, female.

5.5 Discussion

In the present studies, the similar growth pattern as shown by body weight (Figures 5.1 and 5.2) and body weight gain (Tables 5.1 and 5.2) for the treated and control groups indicated that oral administration of sclerotial powder of *L. rhinocerotis* (TM02) at a daily dose of up to 1000 mg/kg for 180 days had no adverse effect on the growth of the rats.

Haematological parameters of rats treated with sclerotial powder of *L. rhinocerotis* for 180 days were not significantly different from the control group (Tables 5.3 and 5.4). This indicates that the sclerotia of the mushroom had no adverse toxic effect on haematopoietic system of rats, as assessed by haematological examinations. Clinical biochemistry studies showed that the 180 days' treatment with the sclerotial powder up to 1000 mg/kg did not affect the renal functions (urea, creatinine and uric acid levels), hepatic functions (total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase, SGOT, SGPT and GGT), serum electrolytes (calcium, inorganic phosphate, sodium, potassium and chloride) as well as total cholesterol and glucose levels of the rats (Tables 5.5 and 5.6). Even though the sodium levels of the 250 and 500 mg/kg female treated groups and the chloride levels in the 500 mg/kg female treated group were slightly higher ($p < 0.05$) than the control group (144.00 ± 1.94 and 105.00 ± 1.94 mmol/l, respectively), the values were within the reference range established by Sharp and La Regina (1998). Besides, the differences observed were not in dose dependent manner. Therefore, these very minor variations are unlikely to be of any toxicological significance.

Organ weight changes are regarded as a sensitive indicator of chemically induced changes to organs (Michael et al., 2007). In present study, no significant alterations in the absolute and relative organ weights were observed in rats following treatment with the sclerotial powder of *L. rhinocerotis* (TM02) ($p > 0.05$) (Tables 5.7 and 5.8), indicating that consumption of the sclerotial powder for an extended period (180 days) did not induce organ changes. Histological examinations of the organs revealed that oral feeding of up to 1000 mg/kg of sclerotial powder of *L. rhinocerotis* (TM02) did not induce histopathological changes to all the internal organs examined (Figures 5.3, 5.4, 5.5, 5.6 and 5.7). Thus, the no-observed-adverse-effect level (NOAEL) dose of the sclerotial powder of *L. rhinocerotis* (TM02) in 180-day chronic toxicity study was more than 1000 mg/kg.

CHAPTER 6: EVALUATION OF REPRODUCTIVE TOXICITY (ANTI-FERTILITY AND TERATOGENIC EFFECTS) AS WELL AS GENOTOXICITY OF THE SCLEROTIUM OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

6.1 Introduction

The no-observed-adverse-effect level (NOAEL) dose of the sclerotial powder of *L. rhinocerotis* (TM02) was more than 1000 mg/kg, as demonstrated in 28-day sub-acute and 180-day chronic toxicity studies. There is, however, still a need to conduct reproductive toxicity study to obtain information on the effects of repeated oral exposure of the sclerotial powder of *L. rhinocerotis* (TM02) on the fertility of rats and teratogenic effects to their offspring. It is also necessary to conduct genotoxicity test to rule out any potential genotoxic effect of the sclerotial powder. This was conducted using bacterial reverse mutation test. The results of reproductive toxicity as well as genotoxicity study will complement findings of sub-acute and chronic toxicity studies for establishment of safety criteria for human consumption of the sclerotial powder of *L. rhinocerotis*.

6.2 Literature Review

6.2.1 Reproductive Toxicity

Reproductive toxicity is broadly described as "adverse effects on sexual function and fertility in adults, and developmental toxicity in their offspring" (OECD, 2004, p.12). Therefore, reproductive toxicity of the sclerotium of *L. rhinocerotis* cultivar TM02 was measured by its anti-fertility and teratogenic effects.

An agent is considered to have teratogenic potential if it has the capacity to cause abnormal development in an embryo or a foetus under certain exposure conditions (FDA, 2012a). Physical and chemical nature of an agent influences teratogenic exposure as some agents are inherently more risky than others (Friedman & Hanson, 2013). Another critical factor of teratogenicity is developmental stage of the embryo at time of teratogen exposure (Friedman & Hanson, 2013). Human embryo is most susceptible to teratogens during organogenesis when the tissues and organs are forming (3 to 8 weeks post conception) (FDA, 2005). However, exposures in late pregnancy can also lead to important abnormalities such as the cartilage defects, foetal alcohol syndrome and effects on renal function seen with the use of warfarin, alcohol abuse and ACE inhibitors, respectively (FDA, 2005).

Dose is also an important feature of any teratogenic exposure by which adverse effect of exposure below a threshold does not occur (FDA, 2005; Friedman & Hanson, 2013). For instance, alcohol exposure at lower repetitive doses during pregnancy is associated with slight intellectual impairment, growth disturbances and behavioral changes, whereas at higher repetitive doses, foetal alcohol syndrome can be developed with growth deficiency, microcephaly, dysfunction in neurobehavioral development, facial

abnormalities and skeletal defects (Jones, 2003; Ornoy & Ergaz, 2010).

The route of exposure of teratogens is also associated with the development of teratogenicity (Friedman & Hanson, 2013). Exposure of an agent without systemic absorption is unlikely to be risk associated (Friedman & Hanson, 2013). In general, topical application corresponds to very low circulating drug levels and is unlikely to exert any risk to the foetus (Ferreira et al., 2013). A few agents such as ionising radiation has direct access to embryo while others require extensive metabolism by the mother (Friedman & Hanson, 2013). Maternal and foetal genotypes may also influence the teratogenicity of an exposure (Friedman & Hanson, 2013).

6.2.2 Genotoxicity

The purpose of genotoxicity test is to establish potential harmful effects of substances on genetic material (Van Leeuwen & Vermeire, 2007). Genotoxicity test is also very useful in pre-screening potential genotoxic carcinogen (Van Leeuwen & Vermeire, 2007).

Genotoxicity can be assessed with bacterial reverse mutation test which has been demonstrated to detect large numbers of mutagenic carcinogens in rodent and is used as a clue for the prediction of human carcinogenicity (McCann & Ames, 1976; Zeiger, 1987). Genotoxicity should also be evaluated *in vitro* and/or *in vivo* by using mammalian cells (FDA, 2012b). *In vitro* mammalian cell systems which are sufficiently validated and widely used include *in vitro* micronucleus assay, mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay (MLA) and *in vitro* metaphase chromosome aberration assay (FDA, 2012b). *In vivo* genotoxicity assays such as mammalian bone marrow chromosome aberration test or mammalian erythrocytes micronucleus test are necessary to be carried out as some agents appear to be mutagenic *in vivo* but not *in vitro*,

and factors such as absorption, distribution, metabolism and excretion are accounted in these assays (FDA, 2012b).

In present study, genotoxicity of the sclerotium of *L. rhinocerotis* cultivar TM02 was evaluated by bacterial reverse mutation assay. In this assay, amino acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* were used to detect point mutations that involves addition, deletion or substitution of one or a few DNA base pairs (OECD, 1997).

Many human genetic diseases are resulted from point mutations, and these mutations in proto-oncogenes and tumour suppressor genes have been proved to involve in tumourigenesis and cancer progression (OECD, 1997; Adjei, et al., 2001; Rivlin et al., 2011).

Bacterial reverse mutation test is based on the principle to detect mutations which revert mutations present in the test strains, and restore functional capability of bacteria to synthesise an essential amino acid (OECD, 1997). The revertant bacteria are able to grow in the absence of the amino acid required by the parent strain (OECD, 1997).

S. typhimurium strains with a prefix "TA" detect reversion from $his^- \rightarrow his^+$ (Young et al., 2014) while *E.coli* strains with a prefix "WP2" measure reversion from $trp^- \rightarrow trp^+$ (Young et al., 2014). The *S. typhimurium* strains are constructed to detect base pair (TA 100, TA 1535) and frame-shift mutations (TA 98 and TA1537) (Maron & Ames, 1983). The *E.coli* strain WP2 uvrA detects base substitution mutagens (McMahon et al., 1979). It is known that the four *S. typhimurium* strains may not detect certain crosslinking agents, oxidising mutagens and hydrazines (OECD, 1997). These substances, however may be detected by *E. coli* WP2 strains and thus the bacteria strain is included in the test (OECD, 1997).

Bacteria system which is used in the reverse mutation assay does not duplicate mammalian metabolism in activating pro-mutagens (Ames et al., 1973). Therefore, an exogenous activation system such as S9-fraction derived from liver of rodents is added to overcome the drawback (Ames et al., 1973; OECD, 1997).

6.3 Methods

6.3.1 Animals

SD rats (5 weeks old, male and female) were acclimatised for 14 days before use.

6.3.2 Reproductive Toxicity Studies: Anti-fertility and Teratogenicity Effects

Reproductive toxicity studies (anti-fertility and teratogenicity effects) were carried out according to Oliveira et al. (1991) and Tabach et al. (2009), with modification. The rats were divided into two groups (5 female rats and 2 male rats/group). First group (control group) was orally fed with 10 ml/kg distilled water daily while second group (test group) was orally administered with 100 mg/kg sclerotial powder of *L. rhinocerotis* cultivar TM02 daily. After 28 days (without interruption of treatment), the 5 female rats were left to mate with the 2 male rats from the same treatment group for 10 days. Following mating period, male rats were separated from the female rats, and the female rats were continually fed with the sclerotial powder until delivery. The female rats gave birth within 7 to 8 weeks of the treatment.

The main parameters used to assess anti-fertility effect and possible teratogenic effect on the offspring were: number of female rats with delivery, number of offspring/delivery, any external signs of malformation of the litter, weight gain/loss, day of eyes opened, righting reflex and ambulation of the litter (Oliveira et al., 1991; Tabach et al., 2009). Seven pups per litter were randomly selected for analysis of righting reflex and ambulation. Body weight per litter was recorded on day 1, 7, 14 and 21. The righting reflex was evaluated on days 1, 3 and 7 by placing the animals on their backs and recording the time needed to return to the right posture. The ambulation was recorded on

days 8 and 13 by placing each animal on a surface divided in 9 squares of 10 cm each and recording the number of squares crossed in 2 min.

6.3.3 Assessment of Genotoxicity of the Sclerotial Powder of *Lignosus rhinocerotis* (Bacterial Reverse Mutation Assay)

The genotoxicity of the sclerotial powder of the *L. rhinocerotis* cultivar TM02 or its potential to induce gene mutations was assessed using the plate incorporation test and pre-incubation test. The bacterial reverse mutation assay was carried out by BSL Bioservice (City, Germany) according to internationally accepted guidelines: OECD (1997), EC (2008) and EPA (1998).

The bacterial strains tested included: *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and *E. coli* WP2 uvrA. Each assay was conducted with and without metabolic activation of a mammalian microsomal fraction S9 mix in triplicate (including the controls). The bacterial strains were exposed to the sclerotial powder at 3.16, 10.0, 31.6, 100, 1000, 2500 or 5000 µg/plate. The mutation factor was calculated by dividing the mean value of the revertant counts through the mean values of the solvent control.

According to BSL Bioservice, a test item is considered as mutagenic if:

a) A clear and dose-related increase in the number of revertants occurs and/or
b) A biologically relevant positive response for at least one of the dose groups occurs in at least one tester strain with or without metabolic activation.

A biologically relevant increase is described as follows:

(a) If in tester strains TA 98, TA 100 and *E.coli* WP2 uvrA, the number of reversions are at least twice as high

(b) If in tester strains TA 1535 and TA 1537, the number of reversions are at least three times higher, as compared to the reversion rate of the solvent control.

According to the OECD (1997), the biological relevance of the results is the criterion for results interpretation whereas a statistical analysis of the results is not regarded as necessary. A test item is considered to be non-mutagenic in the system when neither a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose group is produced.

6.3.4 Statistical Evaluations

All data were expressed as mean ± standard deviation (S.D.). Results for anti-fertility effect and teratogenic effect of the sclerotial powder treatment on the offspring were analysed using Independent Samples T-test (SPSS v14). Results were considered significant at $p < 0.05$.

6.4 Results

6.4.1 Assessment of the Reproductive Toxicity (Anti-fertility and Teratogenic Effects) of the Sclerotial Powder of *Lignosus rhinocerotis*

Oral administration of 100 mg/kg sclerotial powder of *L. rhinocerotis* for 7-8 weeks did not significantly ($p > 0.05$) alter the number of pregnant female rats, number of pups per litter, day of eyes opened of the litter, weight of pups, righting reflex as well as the ambulation of the litter (Tables 6.1 and 6.2). Besides, there were no external signs of malformation of the litter.

Table 6.1: Assessments of the anti-fertility and teratogenic effects of consumption of *Lignosus rhinocerotis* sclerotial powder in rats

Treatment	Number of pregnant female rats	Number of pups per litter (mean ± S.D.)	Day of eyes opened of the litter (mean ± S.D.)	External signs of malformation of the litter
Control	4/5	8.33 ± 1.15	16.38 ± 0.50	No
100 mg/kg TM02	5/5	9.00 ± 1.87	16.64 ± 0.74	No

The values for each parameter are expressed as mean ± S.D. (except number of pregnant female rats). All parameters of the treated group (100 mg/kg sclerotial powder) were not significantly different from the control group ($p > 0.05$).

Table 6.2: Assessments of the possible teratogenic effects of consumption of *Lignosus rhinocerotis* sclerotial powder in rats

Treatment	Weight of pups (g)				Righting reflex (s)			Ambulation	
	Day 1	Day 7	Day 14	Day 21	Day 1	Day 3	Day 7	Day 8	Day13
Control	6.46 ± 0.74	13.59 ± 1.45	24.52 ± 2.62	37.11 ± 5.00	12.00 ± 7.71	8.62 ± 7.97	1.90 ± 2.61	2.19 ± 1.63	3.43 ±2.04
100 mg/kg TM02	6.14 ± 0.67	12.30 ± 3.29	22.90 ± 4.22	34.63 ± 7.27	12.17 ± 7.05	10.24 ± 9.28	1.60 ± 1.01	1.40 ± 1.63	3.40 ± 2.25

Weight of pups was recorded on day 1, 7, 14 and 21. Righting reflex was evaluated on day 1, 3 and 7. Ambulation was assessed on day 8 and 13. The values for each parameter are expressed as mean ± S.D. All parameters for the offspring of the treated group were not significantly different from the control group ($p > 0.05$).

6.4.2 Assessment of the Genotoxicity of the Sclerotial Powder of *Lignosus rhinocerotis* (Bacterial Reverse Mutation Assay)

Plate incorporation test and pre-incubation test were used to evaluate potential of the sclerotial powder of *L. rhinocerotis* in the induction of gene mutations. The results showed that there were no toxic effects in any of the five tester strains used, and up to the highest dose group (5000 μg/plate), evaluated with and without metabolic activation. Treatment with the sclerotial powder at any concentration level (either in the presence or absence of metabolic activation) did not induce biologically relevant increases in revertant colony numbers of any of the five tester strains. The validity of the experiments were indicated by a distinct increase of revertant colonies by the reference mutagens.

6.5 Discussion

The recommended daily consumption of the sclerotial powder of *L. rhinocerotis* as neutraceutical is approximately 5-10 mg/kg, whereas for cancer patients, up to 100 mg/kg of sclerotial powder is recommended per day (above figures provided by supplier of the sclerotial powder, Ligno Biotech Sdn. Bhd., Selangor, Malaysia). As such, a dose 100 mg/kg was used in the anti-fertility and teratogenicity studies. Results showed that oral administration of 100 mg/kg sclerotial powder of *L. rhinocerotis* (TM02) for 7-8 weeks did not cause any adverse effect to the fertility of the rats, nor did the treatment induce teratogenic effect on their offspring. Genotoxicity studies using bacterial reverse mutation assay also demonstrated that the sclerotial powder of *L. rhinocerotis* did not cause gene mutations by base pair changes or frame-shifts in the genome of the tester strains used.

Result of bacterial reverse mutation assay of sclerotial powder of *L. rhinocerotis* cultivar TM02 in present study was in accordance with Chen et al. (2013) in which mycelium of the mushroom did not provoke mutagenicity in bacterial reverse mutation assay which was tested up to the highest dose of 5000 μg/plate (Chen et al., 2013). According to Sumiya et al. (2008), aqueous extract of *Agaricus blazei* Murrill did not induce genotoxicity, as determined by bacterial mutation test (up to 5000 μg/plate), either in the presence or absence of S9 mix.

CHAPTER 7: ANTI-INFLAMMATORY EFFECT OF THE SCLEROTIUM OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

7.1 Introduction

Inflammation is a defence mechanism in response to tissue injury as a result of physical trauma (burn, cut or bruise), exposure to microbiologic agents or noxious chemicals, or even autoimmune disease (Mothana, 2011). Severe inflammation can cause physiological decompensation, organ failure and death cascade (Sherwood & Toliver-Kinsky, 2004). Conventional non-steroidal anti-inflammatory drugs (NSAIDS) are widely used in the world for effective treatment of inflammation (Laine, 2001). However, they are associated with several side effects, especially gastrointestinal toxicity including ulcerations, erosions, life-threatening perforations or severe hemorrhage (Raskin, 1999). Other anti-inflammatory drugs such as selective cyclooxygenase-2 (COX-2) inhibitor, disease-modifying anti-rheumatic drugs (DMARDs) and anti-TNF biologic agents are also not devoid of adverse effects. Therefore, there is a need to search for alternative anti-inflammatory agent with reduced side effects.

Mushrooms can be a potential bio-source for the production of natural anti-inflammatory metabolites as they contain highly diversified bioactive compounds such as polysaccharides, proteoglucans, terpenoids, phenolic compounds, steroids and lectins (Elsayed et al., 2014).

The sclerotium of *L. rhinocerotis* (Cooke) Ryvarden (Tiger Milk mushroom) is used as a traditional medicine to relieve cough, asthma and chronic hepatitis. The traditional uses of the sclerotium are presumably related to its anti-inflammatory effect. The present study was carried out to investigate the anti-inflammatory potential of the sclerotium of *L. rhinocerotis* cultivar TM02.

7.2 Literature Review

7.2.1 Inflammation

Inflammation is "a protective attempt by an organism to remove injurious stimuli, initiate healing process and to restore both structure and function of tissue" (Das, 2011, p.15). It is characterised by five cardinal signs, namely redness, swelling, heat, pain and loss of function (Punchard et al., 2004). The heat sensation in inflammation is caused by increased blood flow through dilated vessels into cooled extremities and the increase number of red blood cells passing through the area results in redness. The swelling (oedema) is caused by fluid accumulation from dilated and permeable blood vessels into surrounding tissues, cell infiltration into the injured area and deposition of connective tissue as a result of prolonged inflammatory responses. Pain is chemical mediated and a result of the stretching of sensory nerves due to oedema formation. The loss of function can be loss of mobility in a joint as a result of pain and oedema formation, or the replacement of functional cells with scar tissue (Punchard et al., 2004).

7.2.1.1 Acute Inflammation

Acute inflammation is an early and nonspecific response to injury (Sommer & Porth, 2004). It is usually of short duration and typically occurs before the establishment of immune response and plays a role in removing injurious agent and limiting the extent of tissue damage (Sommer & Porth, 2004). Acute inflammatory response involves vascular and cellular events with chemical mediation.

Vascular Events

Vascular events can be elaborated by haemodynamic changes as well as changes in vascular permeability (Mohan, 2010). After a transient vasoconstriction of arterioles that lasts for a few seconds, vasodilation occurs (Mohan, 2010). This results in increased blood volume in microvascular bed of the affected area, followed by redness and warm sensation at the site of acute inflammation (Mohan, 2010). Progressive vasodilation results in transudation of fluid into extracellular space. Increased vascular permeability leads to the escape of a protein rich fluid (exudate) into the insterstitium and causes oedema formation (Cotran et al., 1999). Exudate contains essential nutrients for epithelial cells, facilitates the ingress of white cells and provides the moist environment which is important for healing (Cutting, 2003). It also contains electrolytes and a number of inflammatory components, such as fibrin, fibrinogen and leucocytes (Cutting, 2003). Stasis develops when blood circulation in the local vessels slows down as a result of increased permeability with fluid extravasation into extravascular spaces, followed by blood cells concentration in the small vessels and increased blood viscosity (Elgazzar & Elmonayeri, 2006). This can facilitate emigration of leucocytes into extravascular space (Mohan, 2010).

Cellular Events

After stasis develops, leukocytic margination along the vascular endothelium via adhesion molecules occurs (Elgazzar & Elmonayeri, 2006). After that, leucocytes transmigrate across the endothelium (a process called as diapedesis) and migrate to the site of injury under the influence of chemotactic agents (Cotran et al., 1999). During the final stage of cellular response, neutrophils, monocytes and tissue macrophages are activated to engulf and degrade offending agents in a process called phagocytosis (Porth,

2011). During chemotaxis and phagocytosis, activated leucocytes may release toxic metabolites and proteases extra-cellularly, potentially causing endothelial and tissue damage (Gozhenko et al., 2009).

Chemical Mediators of Acute Inflammation

Chemical mediators of acute inflammation originate either from the plasma or cells (Waite, 2013). The plasma derived mediators include product of complement activation, kinin system and clotting system. Cell derived mediators include vasoactive amines, lipid mediators, cytokines, chemokines and nitric oxide.

a) Plasma Derived Mediators

The complement fragments C3a and C5a (anaphylatoxins) are released as protein cleavage by-products during activation of the complement system (William, 1983). These substances cause contraction of smooth muscle, increase in vascular permeability and histamine release (Björk et al., 1985; El-Lati et al., 1994; Drouin et al., 2001). Besides, C5a stimulates neutrophil adhesion to endothelial cells and induces chemotaxis in neutrophil and mast cell (Foreman et al., 1996; Hartmann et al., 1997; Haynes et al., 2000).

Proteolysis by thrombin, plasmin or Hageman factor generates fibrinopeptide B, fibrin degradation products or kinins, respectively that cause vasodilation and increased vascular permeability (either directly or by inducing the release of histamine from mast cells) (Medzhitov, 2008). Neutrophil chemotactic potency of fibrinopeptide B and fibrin degradation products were reported by Skogen et al., (1988) and Leavell et al. (1996). Bradykinin, the main product of kallikrein-kinin cascade has potent pro-algesic effect

(Medzhitov, 2008).

b) Cell Derived Mediators

Vasoactive amines (histamine and serotonin) are released when mast cells degranulate (Wernersson & Pejler, 2014). They cause increased vascular permeability, vasodilation, or vasoconstriction (Majno & Palade, 1961; Owen et al., 1980; Vanhoutte, 1987; Jin et al., 2006).

Arachidonic acid (AA) is a precursor for the generation of lipid mediators known as eicosanoids that include prostaglandins (Harizi et al., 2008). Monocytes and macrophages are important source of eicosanoids because their membranes typically contain large amount of AA (Calder & Yaqoob, 2003). AA is metabolised by cyclooxygenases (COX) metabolic pathways which produce prostaglandins (Cabral, 2005). The prostaglandins PGE_2 and PGI_2 are important in mediating vasodilatation in inflammation (Williams, 1979). PGE_2 also contributes to hyperalgesia and fever (Syriatowicz et al., 1999; Wilhelms et al., 2014).

Several cytokines are important in mediating acute inflammatory reactions such as TNF-α, interleukins (IL-1, IL-6, IL-8 and IL-11) and other chemokines (Feghali & Wright, 1997). Their primary sources in acute inflammation are monocytes/macrophages, neutrophils and mast cells (Feghali & Wright, 1997).

Cytokines play several roles in inflammatory response, including activation of the endothelial cells and macrophage (Medzhitov, 2008). Activation of endothelial cells by cytokines such as TNF-α and IL-1, increase surface adhesivity for polymorphonuclear leucocytes, monocytes and lymphocytes to endothelium and this can facilitate their

emigration (Cotran & Pober, 1989). Macrophages are subjected to classical (Th1) or alternative (Th2) activation into M1 or M2 macrophages, respectively based on types of cytokines that they are exposed to (Sindrilaru et al., 2011; Hao et al., 2012; Boorsma et al., 2013). The M1 macrophage is often associated with tissue injury and inflammation, whereas the M2 macrophage involves in tissue repair and fibrosis (Ploeger et al., 2013).

Based on the position of cysteine residues, chemokines are classified into two major sub-families, CXC and CC (Graves & Jiang, 1995). Generally, CXC chemokines (such as IL-8, NAP-2, gro-α, gro-β and gro-γ) are chemotactic for neutrophils, while CC chemokines (such as MCP-1, MCP-2, MCP-3, MIP-1α and MIP-1β) are chemotactic for monocytes and a small sub-set of lymphocytes (Graves & Jiang, 1995). Chemokines play a pivotal role in leucocyte extravasation by promoting firm adhesion of leucocytes to vascular endothelium and directing subsequent trans-endothelial migration to the site of inflammation (Johnston & Butcher, 2002).

Nitric oxide (NO) generated primarily by inducible nitric oxide synthase (iNOS) is a mediator of tissue injury and inflammation (Fakhrzadeh et al., 2002; Sharma et al., 2007). The direct toxicity of nitric oxide to tissue is modest but is markedly enhanced after reaction with superoxide to form peroxynitrite (Beckman & Koppenol, 1996). Peroxynitrite, an oxidative stress inducing mediator can be further decomposed into other cytotoxic reactive species (Ischiropoulos & al-Mehdi, 1995; Chen et al., 2014). Furthermore, it was found to enhance neutrophil adhesion to endothelial cells through an increased expression of adhesion molecules and to display pro-inflammatory activity (Zouki et al., 2001a; Zouki et al., 2001b; Sohn et al., 2003).

Outcomes of Acute Inflammation

Acute inflammatory reactions typically have one of three outcomes: complete resolution, healing by connective tissue replacement (scarring or fibrosis) or progression of the response to chronic inflammation (Kumar et al., 2015). Complete resolution of inflammation involves the elimination of granulocytes and ultimate return of tissue mononuclear cell (macrophage and lymphocyte) numbers to basal levels as well as cessation of pro-inflammatory signaling (Maskrey et al., 2011). These result in restoration of tissue homeostasis. Healing by connective tissue replacement occurs when non-regenerable tissues or severely damaged tissues are repaired by scarring process (the laying down of connective tissue) (Krafts, 2010). It involves "the influx of debris removing inflammatory cells, formation of granulation tissue (tissue consisting of fibroblasts and delicate capillaries in a loose extracellular matrix) and conversion of the granulation tissue into fibrous tissue that is remodeled over time to form a scar" (Krafts, 2010, p. 5). Transition from acute to chronic inflammation occurs when acute inflammatory response cannot be resolved. (Kumar et al., 2015).

7.2.1.2 Chronic Inflammation

Chronic inflammation may develop as a continuation of acute inflammation or low-grade smoldering response without acute inflammatory action (Sommer & Porth, 2004). Sustained chronic inflammation can lead to many pathological conditions, such as type 2 diabetes (Xu et al., 2003), rheumatoid arthritis (Adlan et al., 2014), chronic obstructive pulmonary disease, atherosclerosis (Bäck, 2008), Alzheimer's disease (Liu & Chan, 2014) and cancer (Kundu & Surh, 2012).

Granulomatous Inflammation

Granulomatous inflammation is a special variety of chronic inflammation in which the predominant cell types are of mononuclear phagocyte system, including macrophages, multinucleated giant cells and/or epithelioid cells as well as an usual admixture of other cells, especially lymphocytes, fibroblasts and plasma cells (Williams & Williams, 1983). Granulomas are mostly formed by aggregation of these cells into well demarcated focal lesions, although a looser and more diffuse arrangement may be found (Williams & Williams, 1983). The causative agents of granulomatous inflammation appear to be non-degradable by non-active macrophages and neutrophils (Pawale et al., 2011). Thus, macrophages are transformed into multinucleated giant cells and/or epithelioid cells to wall off, sequester and contain causative agent of granuloma, if not destroy altogether (Williams & Williams, 1983).

Epithelioid cells have little or no phagocytic potential (Hirsh & Johnson, 1984). They secrete a variety of pro-inflammatory and fibrogenic cytokines (Corrin et al., 2012). Multinucleated giant cells are formed by macrophage fusion, making them large enough to resorb or sequester extracellular material (Quinn & Schepetkin, 2009). These cells exhibit features of phagocytosis which are mediated by endoplasmic reticulum (McNally & Anderson, 2005), and were found to regulate granulomatous inflammation by generating inflammatory and anti-inflammatory cytokines (Hernandez-Pando et al., 2000)

Granuloma can be classified into high and low turn-over granuloma according to turnover of participating macrophages (Ramakrishnan, 2012). In low turn-over granuloma (or foreign-body granulomas), inert substances (such as talc and silica) are surrounded by long-lived macrophages that are not replaced. In high turn-over granuloma (or epithelioid

granulomas), the provocative antigenic agent is toxic to constituent macrophages and thus leading to their death and continual replenishment by newly arrived cells (Ramakrishnan, 2012).

Foreign body granuloma is often phagocytosed by macrophages and multinucleated giant cells, whereas the participation of epithelioid cells is generally scarce or absent (Brito & Franco, 1994). Immune mechanisms are of minor importance in the pathogenesis of foreign body granuloma (Brito & Franco, 1994).

Granuloma deposition in response to indigestible antigenic material is mediated by complex interaction between antigen-presenting mononuclear phagocytes and T lymphocytes (predominantly T-helper cells) as well as a variety of cytokines (Mihailovic-Vucinic, 2012). The maturation of recruited macrophages, their localisation at sites of antigen deposition in the form of epithelioid cells and multinucleated giant-cells, leads to the formation of mature granulomas (Mihailovic-Vucinic, 2012).

Chronic granulomatous inflammation can cause tissue damage and fibrosis (Co et al., 2004). Tissue damage in granulomatous inflammation is due to local secretory products of macrophages and neutrophils (Brito & Franco, 1994). Fibrosis is attributed to excessive deposition of extracellular matrix components such as collagen (Wyne, 2008). The resolution of granuloma may proceed without alteration of the tissue in which granulomas are embedded (Mornex et al., 1994). Erosion or replacement by scar tissue as a result of fibrosis may be involved in the resolution process (Mornex et al., 1994). The resolution can also cause permanent tissue damage even after the provocative agent has been eliminated (Williams & Williams, 1983). For example, pulmonary and hepatic fibrosis are important long-term complications of sarcoidosis and schistosomiasis, respectively (Williams & Williams, 1983).

7.2.2 Anti-inflammatory Drugs

7.2.2.1 Cyclooxygenase (COX) Inhibitor

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat a variety of clinical conditions characterised by pain, inflammation and fever (Knights et al., 2006). Cyclooxygenase (COX), the main target of NSAIDs, is the key enzyme to commit arachidonic acid to prostaglandin (PG) biosynthesis (Claria & Romano, 2005). There are two isoforms of COX, namely COX-1 and COX-2. Broadly speaking, COX-1 is constitutively expressed in various tissues for the production of PGs involved in gastric mucosal integrity, platelet homeostasis and regulation of renal blood flow (Nagi et al., 2015). In contrast, COX-2 is inducible upon inflammatory stimuli such as IL-1β, TNF-α, phorbol myristate acetate (PMA) and LPS (Park & Kwon, .2011).

Inhibitors of COX activity associated with anti-inflammatory properties include: (1) conventional non-selective non-steroidal anti-inflammatory drugs (ns-NSAIDs) that inhibit both COX-1 and COX-2, such as Fenoprofen, Ibuprofen and Indomethacin; (2) selective COX-2 inhibitors (also known as COXIBs), such as celecoxib, etoricoxib and parecoxib (Radi & Khan, 2006; Boursinos et al., 2009). Patients receiving non-selective NSAIDs often encounter abdominal discomfort and some of them experience serious gastrointestinal complications, such as ulceration, bleeding, perforation or obstruction (Akarca, 2005). Therefore, selective COX-2 inhibitors were introduced with reduced gastrointestinal complications, as compared to conventional NSAIDs (Laine et al., 1999; Hawkey et al., 2000; Goldstein et al., 2001).

However, the use of selective COX-2 inhibitors is still controversial due to the increased cardiovascular risks including myocardial infarction, stroke, heart failure, and hypertension (Mathew et al., 2011). COXIBs such as rofecoxib (Vioxx®) and valdecoxib (Bextra®) were withdrawn from the market in 2004 and 2005, respectively as long term use of the drugs are associated with increased risk of heart attacks and strokes (Krishna et al., 2013).

7.2.2.2 TNF alpha Inhibitor

A cytokine, tumour necrosis factor α (TNF-α) is produced by monocytes, macrophages, lymphocytes, fibroblasts and keratinocytes in response to injury, infection, inflammation and other environmental challenges (Baud & Karin, 2001). Excessive production of TNF-α is associated with pathogenesis of inflammatory disorders, such as rheumatoid arthritis (Matsuno et al., 2002; Moelants et al., 2013), Crohn's disease (Gibson, 2004), ulcerative colitis (Sands & Kaplan, 2007), psoriasis (Sato et al., 2014), type-2 diabetes mellitus (Swaroop et al., 2012), sepsis (Qiu et al., 2011) and refractory asthma (Berry et al., 2007).

TNF-α is first synthesised as a transmembrane protein (tmTNF-α) by activated macrophages, lymphocytes as well as other cell types (Olmos & Lladó, 2014). The cleavage of the extracellular domain of tmTNF-α by the matrix metalloprotease TNF-α-converting enzyme (TACE) releases a soluble TNF-α (sTNF-α) homotrimer (Olmos & Lladó, 2014). The sTNF-α binds the two distinct cell surface receptors, TNF-α receptor 1 (TNFR1 or p55TNFR) and TNF-α receptor 2 (TNFR2 or p75TNFR), followed by recruitment of a number of adaptor proteins to the cytoplasmic domains of the TNFR1 and TNFR2, which in turn initiate complex intracellular events including activation of nuclear factor-κB (Diamantopoulos et al., 2013). This cascade may result in the

production of other pro-inflammatory cytokines, such as IL1β, IL6, IL8, and TNF-α itself (Brightling et al., 2008).

Multiple leucocyte adhesion molecules, such as E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were expressed by endothelial cells in response to TNF-α (Chandrasekharan et al., 2007). Adhesion molecules interact with specific leucocyte receptors and this results in rolling, firm adhesion and transmigration of leucocytes from the blood to site of inflammation (Chandrasekharan et al., 2007).

TNF-α facilitates the transition from innate to adaptive immunity by enhancing antigen presentation and T cell activation (Chen et al., 1998; Aspalter et al., 2003). It was reported to be able to enhance number and function of human dendritic cell in presenting antigens to $CD4^+$ T cells *in vitro* (Chen et al., 1998). Aspalter et al. (2003) demonstrated that TNF-α interaction with TNFR2 can costimulate T cell receptor-mediated T cell activation, thereby enhancing T cell proliferation, expression of T cell activation markers (human leucocyte antigen-DR, CD25 and TNFR2) and secretion of cytokines such as TNF-α and interferon-γ.

In view of the critical roles of TNF-α in controlling cytokine cascade, recruitment of leucocyte into site of inflammation as well as transition from innate to adaptive immunity, TNF-α can be a promising therapeutic target for inflammatory diseases.

Inhibition of TNF-α can be achieved with the use of fully human anti-TNF-α mAbs, anti-TNF-α chimeric, recombinant soluble p75 TNF receptors and small anti-TNF-α molecules (Song et al., 2008). The fully human anti-TNF-α mAb, adalimumab (Humira®); the chimeric anti-TNF-α mAb, infliximab (Remicade®); and the

recombinant dimeric soluble p75 TNF receptor, etanercept (Enbrel®) have been used clinically for the treatment of rheumatoid arthritis (Chen et al., 2006), psoriatic arthritis (Rodgers et al., 2001), ankylosing spondylitis (McLeod et al., 2007) and Crohn's disease (Dretzke et al., 2011). Anti-TNF therapy was found to produce a modest but significant decrease in the risk of dying in sepsis patients (Qiu et al., 2013). According to Brightling et al. (2008), preliminary studies on small numbers of asthma patients showed improved lung function, airway hyperresponsiveness, asthma quality of life and exacerbation rate after receiving anti-TNF therapy. Small-molecules agents that inhibit synthesis of TNF-α in various stages of preclinical and clinical development include thalidomide and p38 MAP kinase inhibitors (Palladino et al., 2003).

Anti-TNF therapy is however associated with an increased risk of bacterial and fungal infection, particularly of reactivating latent tuberculosis (Miller and Ernst, 2009). The evidence for the risk association between malignancies and anti-TNF therapy (abatacept, adalimumab, anakinra, certolizumab, etanercept, golimumab, infliximab, rituximab, and/or tocilizumab) is still conflicting (Bongartz et al., 2006; Wolfe & Michaud, 2007; Askling et al., 2009; Lopez-Olivo et al., 2012). Hence, anti-TNF therapy should be used with caution in patients with active or prior cancer or those with high risk for developing cancer (Raval & Mehta, 2010).

7.2.3 Medicinal Mushrooms with Anti-inflammatory Properties

The roles of natural products as remedies have long been recognised. Natural products play a pivotal role in human health by prevention and treatment of a wide variety of disorders, including inflammatory conditions (Yuan et al., 2006). Medicinal mushroom can be an excellent source of anti-inflammatory agents because it produces a vast diversity of bioactive compounds such as polysaccharides, polysaccharide-protein

complexes, lectins, fungal immunomodulatory protein (FIPs), lipid components, terpenoids and phenolic compounds (Garcia-Lafuentea et al., 2010; Elsayed et al., 2014). Table 7.1 summarised *in vitro* and *in vivo* anti-inflammatory activity of some medicinal mushrooms.

Table 7.1: Medicinal mushroom that exhibits anti-inflammatory activity

Mushroom species	Bioactive compound(s)/ extract	Assay model(s)	Result/mechanism of anti-inflammatory activity	References
Ganoderma lucidum	Polysaccharides	Carrageenan and formalin induced paw oedema in mice	• 100 mg/kg of polysaccharides showed 57.6% and 58.2% of significant paw oedema inhibition, respectively, as compared to control ($p < 0.01$).	Joseph et al. (2011)
	Triterpene extract	(i)LPS-stimulated RAW264.7 macrophage cells (ii) LPS-induced endotoxemic mice.	• Triterpene extract exerted anti-inflammatory effects by inhibiting NF-κB and AP-1 signaling in LPS-stimulated macrophages, finally resulting in the attenuation of the production of TNF-α, IL-6, NO and PGE_2. • The extract at 12 mg/ml significantly suppressed the production of IL-6 and TNF-α in LPS-induced endotoxemic mice ($p < 0.001$).	Dudhgaonkar et al. (2009)
	Fungal immunomodulatory protein (LZ-8)	LPS-stimulated RAW264.7 macrophage cells	• LZ-8 lowered the production of NO and IL-6, but had no effect on the level of TNF-α.	Huang et al. (2014)
Poria cocos	Ethanol extract	LPS-stimulated RAW264.7 macrophage cells	• Ethanol extract suppressed the inflammatory response of macrophages via inhibition of COX-2, iNOS, TNF-α and IL-1β through down regulation of the NF-κB signaling pathway.	Jeong et al. (2014)
Inonotus obliquus	80% ethanol extract, hot water-soluble polysaccharide fraction, polyphenolic fraction and ethyl acetate /water extracted fraction	LPS-stimulated RAW264.7 macrophage cells	• All fractions or extract significantly inhibited the levels of IL-1β, IL-6, TNF-α and NO ($p < 0.05$).	Van et al. (2009)
	Steroids compounds (ergosterol and ergosterol peroxide) and triterpenes compound (trametenolic acid)	LPS-stimulated RAW264.7 macrophage cells	• Three compounds exerted significant inhibition effects on NO production and NF-κB luciferase activity ($p < 0.05$).	Ma et al. (2013)

Table 7.1, continued

Mushroom species	Bioactive compound(s)/ extract	Assay model(s)	Result/mechanism of anti-inflammatory activity	References
Polyporus dermoporus	Extract rich in β-glucan-protein complex	(i) Carrageenan-induced pleurisy in mice	• Extract at 30 mg/kg significantly reduced number of leucocytes and concentration of NO in pleural infiltrate at 92.5% and 68.7%, respectively, as compared to the control ($p < 0.001$).	Guerra Dore et al. (2014)
Pleurotus florida	NO production: methanol, acetone and hot water extracts iNOS expression and carrageenan induced paw oedema: acetone extract	(i) LPS-stimulated RAW264.7 macrophage cells (ii) Carrageenan induced paw oedema in rat	• NO production and iNOS expression were inhibited significantly by the mushroom extract(s) from 0.5 to 2 mg/ml in a dose dependent manner ($p \leq 0.01$). • Acetone extract at 5, 15 and 50 mg/kg significantly reduced paw oedema by 45.81%, 53.01% and 55.97%, respectively ($p \leq 0.01$).	Im et al. (2014)
Antrodia camphorate	Polysaccharide fraction containing glycoprotein	LPS-stimulated RAW264.7 macrophage cells	• NO production and the protein expression of iNOS were inhibited in a dose-dependent manner (50-200 μg/ml).	Chen et al. (2007)
	Lanostanoids and lactone derivatives	LPS-stimulated RAW264.7 macrophage cells	• Significant suppression of the NO concentration with IC_{50} values ≤10 μM.	Liaw et al. (2013)

7.2.4 Experimental Models to Investigate Anti-inflammatory Activity

7.2.4.1 Acute Inflammation: Carrageenan Induced Paw Oedema Rat Model

Carrageenan is a family of gel-forming and viscosifying polysaccharides which are extracted from certain species of red seaweeds (Necas & Bartosikova, 2013). There are three main types of carrageenan: lambda, kappa and iota (Morris, 2003). The lambda form is injectable to induce an inflammatory response as it does not gel strongly at room temperature (Morris, 2003).

Carrageenan induced paw oedema model has been commonly employed to evaluate activity of anti-inflammatory agents because of its high degree of reproducibility, reliability and its responsiveness to doses well below the toxic level, as compared to other phlogistic agent such as formalin, mustard and kaolin (Winter et al., 1962). Acute inflammatory response in carrageenan induced paw oedema model occurs in three distinct phases. (Di Rosa & Willoughby, 1971). An initial phase (first hour) is attributed by the liberation of histamine and serotonin. Subsequently, the increased vascular permeability is maintained by kinin release during secondary phase (Di Rosa & Willoughby, 1971). In the final phase, the release of prostaglandin from 2.5-6.0 h is closely linked to the migration of leucocytes into the site of inflammation (Di Rosa &Willoughby, 1971).

Nitric oxide (NO) was found to be another important mediator in acute inflammatory response in carrageenan induced paw oedema model. It contributes to oedema formation and development of hyperalgesia (Handy & Moore, 1998; Osborne & Coderre, 1999). Carrageenan induces the release of NO, the production and its effect on hyperalgesia were found to be mediated by two isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS) in the early phase (up to 3 h) and by both nNOS and inducible (iNOS) in the late

phase (2.5-8 h) of carrageenan-induced inflammation (Handy & Moore, 1998; Omote et al., 2001; Tao et al., 2003).

Histological examination on carrageenan injected feet at approximately 2 hours revealed a predominant polymorphonuclear cells emigration into the lesion (Di Rosa et al., 1971). At 4 and 6 hours, numbers of both polymorphonuclear and mononuclear cells in the lesion were increased with the former contains the most. Non-steroidal anti-inflammatory drugs (such as indomethacin, butazolidin, aspirin and mefenamic acid) were reported to suppress mainly prostaglandin phase of the carrageenan induced paw oedema model (Di Rosa et al., 1971). Their abilities to suppress this phase were found to correlate directly with their ability to inhibit mononuclear leucocytes (important in chronic inflammatory processes) migration into the inflamed tissues. Therefore, this model of acute inflammation can be used in finding new non-steroidal anti-inflammatory as well as possible anti-chronic inflammatory agents (Di Rosa et al., 1971).

7.2.4.2 Chronic Inflammation: Cotton Pellet Induced Granuloma Rat Model

Cotton pellet induced granuloma (a typical foreign body granuloma) test is widely used to assess transudative, exudative and proliferative phase of chronic inflammation (Swingle & Shideman, 1972). In transudative phase of non-inflammatory origin, cotton pellet absorbs its maximum capacity of fluid that is low in protein (Swingle & Shideman, 1972). This can be measured as in the increased of wet weight of the pellet. Exudative phase represents a vascular permeability change in inflammation, is defined as leakage of Evan blue from the bloodstream around the granuloma. Proliferative phase corresponds to the appearance of collagen (reflects new connective tissue synthesis) in the granuloma can be measured as the increased in dry weight of the granuloma (Swingle & Shideman, 1972). Mucopolysaccharides and fibroblasts formation are the other two natural

proliferative agents involved in the formation of granuloma tissue in proliferative phase (Swingle, 1974). Histopathological finding by Mali et al. (2013) showed the presence of giant cells in granulomatous tissue induced by cotton pellet.

7.2.4.3 *In vitro* Anti-inflammatory Model: Lipopolysaccharides (LPS) Induced Inflammatory Response by RAW 264.7

In recent years, lipopolysaccharide (LPS), derived from the cell wall of Gram-negative bacteria activated RAW264.7 cells (macrophage cell line) are widely used as an *in vitro* model to screen for effective anti-inflammatory agents and to investigate underlying anti-inflammatory mechanisms. Macrophages play a central role in the inflammatory response and serve as an essential interface between innate and adaptive immunity (Lu et al., 2011).

LPS is recognised by a complex of CD14 and the TLR4/MD-2 on the cell surface of macrophages (Wang et al., 2009). After complex recognition, several downstream signaling pathways were activated, including mitogen activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) through adaptor proteins of the myeloid differentiation factor 88 (MyD88) family, finally resulting in the up-regulation of pro-inflammatory mediator gene expression (Wang et al., 2009).

Nuclear transcription factor kappa-B (NF-κB) is an ubiquitous transcription factor that regulate genes involved in inflammatory responses, cellular proliferation and cell adhesion (Won et al., 2006). In most cell types, NF-κB dimers bind to its inhibitory protein IκBs and maintain inactive in the cytoplasm (Lee et al., 2006). After activation by a variety of cellular stimuli, IκBα become phosphorylated, ubiquitylated and degraded by the proteasome complex, followed by NF-κB translocation to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes. Transcription of pro-

inflammatory mediators and cytokines such as inducible isoforms of NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumour necrosis factor alpha (TNF-α) and interleukins (IL-1β, IL-6, and IL-8) were then induced (Lappas et al., 2002; Kang et al., 2004; Rajapakse et al., 2008). Prostaglandin E2 (PGE_2) and nitric oxide (NO) are produced by COX-2 and NO synthase (iNOS), respectively upon LPS induction (Won et al., 2006).

Three major groups of MAPKs are known to differentially regulate many cellular functions including inflammation: the extracellular signal-regulated protein kinase 1/2 (ERK), the c-Jun amino-terminal kinase (JNK) and the p38 MAP kinases (p38) (Peroval et al., 2013). In macrophage, ERK, JNK and p38 become activated in response to LPS and this leads to the production of IL-1β, IL-6, IL-12, TNF-α, NO and/or PGE_2 (Swantek et al., 1997; Carter et al., 1999; Feng et al., 1999; Chen et al., 2001; Kim et al., 2004; Tseng et al., 2014).

7.3 Methods

7.3.1 Preparation of Extracts from the Sclerotial Powder of *Lignosus rhinocerotis* Cultivar TM02

For cold water extraction, the sclerotial powder was extracted by continual stirring with distilled water for 24 h at 4°C. The resultant extract was termed CWE TM02. For hot water extraction, the sclerotial powder was extracted with the use of an oil bath for 2 h at 95°C to 100°C. The resultant extract was termed HWE TM02. Methanol extraction was performed by continual stirring of the sclerotial powder in methanol at 25°C for 24 h. The resultant extract was termed ME TM02. All extractions were carried out with sample to solvent ratio of 1 g: 20 ml. After each extraction, the mixture was centrifuged at 8000 x g for 30 min, followed by filtration of the supernatant with Whatman paper to remove insoluble materials. The aqueous extracts were freeze-dried while the methanol extract of the sclerotial powder was evaporated to dryness using rotary evaporator at 37°C. Protein and carbohydrate contents of each fraction were determined by Bradford and phenol sulphuric acid method, respectively, as described in chapter 3.

7.3.2 Fractionation of CWE of the Sclerotial Powder of *Lignosus rhinocerotis* by Sephadex G-50 Gel Filtration

CWE TM02 was fractionated using Sephadex G-50 (Sigma-Aldrich, USA) superfine column (v = 112 ml) and eluted with 0.05 M ammonium acetate buffer at 0.5 ml/min. High molecular weight (HMW) fraction appeared at void volume (mol. wt. >10 kDa dextran or 30 kDa protein) while low molecular weight (LMW) fraction emerged at bed volume. Medium molecular weight (MMW) fraction appeared between void and bed volume. Protein and carbohydrate contents of each fraction were determined by Bradford

and phenol sulphuric acid method, respectively, as described in chapter 3.

7.3.3 Animals

Six male SD rats per group were used in carrageenan induced paw oedema study and cotton pellet induced granuloma study. Rats of 9 weeks old (240-270g) and 8 weeks old (170-200g) were used in the former and latter study, respectively.

7.3.4 Carrageenan Induced Paw Oedema Study

Carrageenan induced paw oedema method was carried out according to Arawwawala et al. (2010), with modifications. SD rats were intraperitoneally injected (2 ml/kg for each rat) with CWE TM02 (25, 50, 100 or 200 mg/kg), HMW, MMW and LMW fractions of the CWE (53, 35 and 112 mg/kg, respectively), HWE TM02 (200 mg/kg), ME TM02 (200 mg/kg), reference drug (indomethacin at 10 mg/kg) or control group (6% sodium bicarbonate in saline). The dosages used for HMW, MMW and LMW fractions were calculated based on amount isolated from 200 mg/kg of CWE TM02 using Sephadex G-50 gel filtration chromatography. All extracts and the reference drug were prepared in 6% sodium bicarbonate in saline. Intraperitoneal injection was carried out one hour before the carrageenan injection. Two hundred microliters of 1.5% carrageenan in saline was injected into the sub-plantar of the right hind paw of all animals. Following paw oedema induction, the right hind paw volumes of these rats were measured using a plethysmometer (IITC INC., USA) at hourly intervals up to 5 h. Paw oedema volume was calculated by measuring paw volume increment: (V_t - V_0). Vt is the average volume of the right hind paw at each time interval after carrageenan injection while V_0 is the average volume of the right hind paw before carrageenan injection. The percentage inhibition of oedema volume at each hour compared to the control group was calculated using the

following formula:

Percentage inhibition = $[(A - B)/A] \times 100$

Where $A = (V_t - V_0)_{control}$; $B = (V_t - V_0)_{treatment}$

The median inhibitory dose (ID_{50}) for the inhibition of inflammation at 3rd and 4th h (peak oedema) after inflammation induction was calculated.

7.3.5 Cotton Pellet Induced Granuloma Study

The inhibition effect of CWE on granuloma formation in rats was examined as described by Barua et al. (2011). The rats (*n* = 6/group) were anaesthetised with ketamine (45 mg/kg) and xylazine (4.5 mg/kg) and shaved on their backs. Sterilised cotton pellets weighing 20 ± 1 mg were subcutaneously implanted on the back of animals. The animals were orally fed with 6% sodium bicarbonate in saline at 10 ml/kg in control group or CWE at 200 mg/kg in treatment group, from day 1 to 7. Indomethacin group (1 mg/kg) received treatment by oral gavage for every 2 days. On the 8th day, the animals were euthanised using an overdose of carbon dioxide. The cotton pellets with granulomatous tissue were removed and the wet pellets were weighed. Subsequently, the pellets were dried at 60^{o}C overnight to a constant weight. The mean dry weight of granulomatous tissue (after subtracting the weight of cotton pellet) was determined. The percentage inhibition was calculated by comparing the mean dry or wet weight of the treated group with the control group.

7.3.6 Isolation of the Protein and Non-protein Components (P-HMW, P-MMW, NP-HMW and NP-MMW) of the HMW and MMW Fractions

The proteins of HMW and MMW fraction (P-HMW and P-MMW, respectively) were precipitated with ammonium sulphate, which was added gradually to the fraction at 4ºC until 100% saturation was reached. After stirring for 1 h, the solution was centrifuged at 8000 x g for 20 min to collect the precipitated proteins. The supernatants resulted from protein precipitation of HMW and MMW fractions were desalted by Sephadex G-50 (column size v = 228.3 ml) and Sephadex-G-25 (column size v = 200 ml) (Sigma-Aldrich, USA) fine chromatography, respectively. Both columns were eluted with 0.05 M ammonium acetate buffer at 2 ml/min. The fractions collected were then freeze-dried. The sample was termed non-protein component of the HMW or MMW fraction (NP-HMW or NP-MMW, respectively). The protein pellets derived from HMW and MMW fraction were dissolved in milliQ water and desalted with Vivaspin 15R at 5000 MWCO and 2000 MWCO, respectively and were termed protein component of the HMW and MMW fraction (P-HMW and P-MMW, respectively). The protein and carbohydrate contents of P-HMW, P-MMW, NP-HMW and NP-MMW were determined by 2-D quant kit and phenol sulphuric acid method, respectively, as described in chapter 3.

7.3.7 Anionic Exchange Chromatography

Desalted P-HMW which was filtered with 0.2 µm syringe filter was fractionated with anionic exchange chromatography with Resource™ Q column, 1 ml (GE Healthcare Life Sciences). Eluents used were 20 mM Tris-HCl, pH 8.0 (buffer A) and 0.5 M NaCl in buffer A, pH 8.0 (buffer B). Flow rate was set at 1 ml/min. Linear gradient from 0% B to 100% B were performed in 45 min. 100% B was maintained for 25 min before the end of each run. Each fraction from several runs were pooled and desalted with Vivaspin 15R

(5000 MWCO). Carbohydrate and protein contents of each fraction were determined by phenol sulphuric acid method and 2-D quant kit, respectively, as described in chapter 3.

7.3.8 In-gel Tryptic Digestion and Desalting of Protein Sections from SDS-PAGE Gel

In-gel tryptic digestion of protein sections from SDS-PAGE was performed using Pierce™ Trypsin Protease, MS grade (Thermo Scientific, Massachusetts, USA) according to manufacturer's instructions with modification. Bands or regions in SDS-PAGE gel were excised with surgical blade according to assigned sections. Each gel section was excised into gel plugs of 1 mm x1 mm. Gel plug was destained with 50% acetonitrile in 50 mM ammonium bicarbonate at 37ºC. Dithiothreitol (10 mM) in 100 mM ammonium bicarbonate was used to reduce disulphide bonds of proteins at 60ºC for 30 min. Alkylation was done by incubating gel plug with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 min in the dark at 37ºC. Gel plugs were washed with 50% acetonitrile in 100 mM ammonium bicarbonate at 37ºC. Gel plugs were then dehydrated with 100% acetonitrile for 15 min at room temperature and dried in speed vacuum at ambient temperature. Trypsin in 50 mM ammonium bicarbonate was added into gel plug in each tube at 500 ng. Trypsin digestion was carried by incubating gel plug overnight at 37 ºC. Digested solution was transferred to a new tube. Gel plug was shaken in 50% acetonitrile for 15 min. Solution from gel plug was transferred into the previous tube containing digested solution. Gel plug was shaken with 100% acetonitrile for 15 min. Solution from gel plug was transferred into the same tube containing digested solution. Subsequently, digested solution was dried in speed vacuum and stored at -20ºC until further use. Digested proteins were desalted with ZipTip® pipette tips (Merck Milipore) before LC-MS/MS analysis. Briefly, the pipette tip was wetted and equilibrated with 50% acetonitrile and 0.1% formic acid, respectively. The pipette tip was then aspirated and

dispensed with digested proteins which were dissolved in 0.1% formic acid. Washing step for the pipette tip was carried out in 0.1% formic acid. Following sample elution into 20 µl of 0.1 % formic acid in 50% acetonitrile, desalted samples were dried in speed vacuum and stored at -20ºC until LC-MS/MS analysis.

7.3.9 LC-MS/MS Analysis

An Agilent 1200 HPLC-Chip/Ms Interface coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS was used to analyse protein composition of P-HMW. Desalted protein sample was reconstituted with 5 µl of solution A (0.1% formic acid in water). One µl of the reconstituted sample was injected into a large capacity chip containing a 160 nl enrichment column packed with C18 (300 Å) at 4 µl/min for sample pre-concentration. Subsequent analytical separation was accomplished over a 75 µm x 150 mm analytical column at 0.3 µl/min in an initial gradient of 3% solvent B, 50% solvent B at 30 min, 95% solvent B at 32 min and 95% solvent B at 39 min. Solvent A consists of 0.1% formic acid in water while solvent B contains 90% acetonitrile in solvent A. Total run time was 47 min including post-run of 8 min. For subsequent MS (rate: 8 spectra/s, time: 125 ms/spectrum) and MS/MS (rate: 4 spectra/s, time: 250 ms/spectrum) analyses, spectra were acquired in a MSMS mode with scan range from 110 to 3000 m/z and 50 to 3000 m/z, respectively. Capillary and fragmentor voltage were 1980 V and 175 V, respectively with drying gas flow rate of 5.0 l/min at 325°C. Generated raw data were searched against *L. rhinocerotis* genome database using Agilent Spectrum Mill MS Proteomics Workbench software packages. The following parameters and filters were implemented for protein and peptide identification: MH+ scan range from 600 to 4000 Da, carbamidomethylation of cysteines was set as a fixed modification, protein score > 11, peptide score > 6, and % scored peak intensity > 60.

Protein composition of two P-HMW derived fractions (F5 and F6) which were separated by anionic exchange chromatography were also determined by LC-MS/MS analysis but with different instrument, parameters and settings. An Agilent 1260 HPLC-Chip/MS Interface coupled with Agilent 6550 Accurate-Mass Q-TOF LC/MS was used to analyse protein composition of F5 and F6. Desalted protein sample was reconstituted with 7 µl of solution A (0.1% formic acid in water). Sample injection volume was 2 µl. After sample pre-concentration, analytical separation was accomplished over a 75 µm x 150 mm analytical column at 0.4 µl/min in an initial gradient of 5% solvent B, 50% solvent B at 11 min, 70% solvent B at 15 min, 70% solvent B at 18 min and 5% solvent B at 19 min. Solvent A consists of 0.1% formic acid in water while solvent B contains 100% acetonitrile in solvent A. Total run time was 25 min including post-run of 6 min. For subsequent MS (rate: 8 spectra/s, time: 50 ms/spectrum) and MS/MS (rate: 4 spectra/s, time: 125 ms/spectrum) analyses, spectra were acquired in a MSMS mode with scan range from 200 to 3000 m/z and 50 to 3200 m/z, respectively. Capillary and fragmentor voltage were 1800 V and 175 V, respectively with drying gas flow rate of 5.0 l/min at 290°C. Generated raw data were searched against *L. rhinocerotis* genome database using Agilent Spectrum Mill MS Proteomics Workbench software packages. The following parameters and filters were implemented for protein and peptide identification: MH+ scan range from 100 to 3200 Da, carbamidomethylation of cysteines was set as a fixed modification, protein score > 20, fast discovery rate < 1%.

Proteins with less than 2 distinct peptides were considered not significant and thus filtered out. Relative intensity of each gel section to entire selected gel lane was calculated with MYImageAnalysis software (Thermo Scientific). Protein percentage in each gel section was calculated with the following formula:

Protein percentage in each gel section = $(A/\sum A) \times B$

A represents mean peptide spectra intensity of a protein. $\sum A$ represents the sum of mean peptide spectra intensity of all proteins in a gel section. B represents relative intensity of each gel section to the entire selected gel lane.

7.3.10 Cell Viability Assessment for the Measurement of Inhibition of TNF-alpha Production

To examine cell viability, MTT assay was carried out according to Chou et al. (2013), with modification. RAW 264.7 cell line (seeded at 2×10^5 cells/well in 96-well plate) was pre-treated with the respective extracts/fractions for 1 h followed by LPS stimulation (12.50 ng/ml) for 24 hours. Medium was then aspirated and 100 µl of 0.50 mg/ml MTT was added to each well and incubated for 1 h at 37°C in the dark. After removing the solution in each well, 100 µl DMSO was added to the cells to dissolve the formazan crystals. The absorbance of each well was measured by a microplate reader (Bio-Rad, USA) at 570 nm. Absorbance of pre-treatment groups was normalised to the LPS-treated control, and expressed as percentage viability.

7.3.11 Measurement of Inhibition of TNF-alpha Production

RAW 264.7 macrophages were cultured in 96-well plate with CWE and HMW at 6.25, 12.50, 25.00, 50.00, 75.00 and 100.00 µg/ml (based on dry weight); HWE, MMW and LMW at 6.25, 12.50, 25.00, 50.00, 75.00, 100.00 and 150.00 µg/ml (based on dry weight); P-HMW and P-MMW at 5.00, 10.00, 15.00, 25.00, 35.00, 45.00 µg/ml (additional 7.50 and 3.75 µg/ml for P-HMW and P-MMW, respectively; based on total carbohydrate and protein concentration); NP-HMW and NP-MMW at 150.00 and 100.00 µg/ml (based on dry weight); and anionic exchange chromatography separated fractions derived from P-HMW up to 2.50 µg/ml (based on total carbohydrate and protein concentration) for 1 h, and then incubated with 12.50 ng/ml LPS for 24 h. Supernatants were obtained and frozen at -80°C until analysis. TNF-alpha in the culture medium was determined by TNF-α mouse ELISA kit (Novex®) according to the manufacturer's recommended protocols. Briefly, 100 µl of samples or standards were pipetted into a 96-well ELISA stripped plate. Biotinylated Ms TNF-α biotin conjugate solution (50 µl) was added into each well except for chromogen blank well. Plate was covered and incubated at room temperature for 90 min. Solution from each well was decanted and the plate was washed 4 times with wash buffer. Streptavidin-HRP working solution (100 µl) was added to each well except for chromogen blank. Plate was covered and incubated for 30 min at room temperature. Solution from each well was decanted and the plate was washed 4 times. Stabilised chromogen (100 µl) was added to each well and the plate was incubated at room temperature for 20 min in the dark. Subsequently, 100 µl of stop solution was added to each well. Absorbance of each well was read at 450 nm having blanked against a chromogen blank. Standard curve was generated with four parameter logistic fit curve by MYassay online software (http://www.myassays.com/). The result was expressed as percentage of inhibition of TNF-alpha production by various treatment groups as

compared to LPS stimulated control without pre-treatment, and IC_{50} and IC_{70} values were then calculated.

7.3.12 Protease Assay

Protease activities of P-HMW derived fraction 5 and 6 which were separated by anionic exchange chromatography were measured according to method described by Kunitz (1947) with modification. Test sample (20 µl) was mixed with 140 µl of 1 % (w/v) casein in phosphate buffer (pH 7.2), followed by incubation at 37°C for 15 min. An aliquot of of 5 % trichloroacetic acid (600 µl) was added to the mixture, followed by centrifugation at 16000 xg for 5 min. Absorbance of the supernatant was read at 280 nm and water was used as blank. Protease activity is expressed in absorbance units/min at 280 nm. Trypsin was used as the positive control.

7.3.13 Statistical Analysis

All data were expressed as mean ± S.E.M., except for results obtained from LC-MS/MS analysis as well as protein and carbohydrate percentage of fractions separated by anionic exchange chromatography from P-HMW (Table 7.6). In all assays, three independent experiments were carried out in triplicate, except for animal studies, LC-MS/MS analysis as well as protein and carbohydrate percentage of fractions separated by anionic exchange chromatography from P-HMW (Table 7.6). In carrageenan induced paw oedema and cotton pellet induced granuloma model, all data were analysed using One-way Analysis of Variance (ANOVA). Statistical differences between the means of control and treatment groups were determined using Dunnett's t (two-sided) test. In case of variance heterogeneity, Dunnett's T3 test was used. The homogeneity of variances was calculated using Levene statistics. Statistical differences between results on 200 mg/kg CWE TM02

and ME TM02 as well as HWE TM02 in carrageenan induced paw oedema study were analysed using Independent Samples t-test. Data collected from the comparison of maximal inhibitory concentration of TNF-α production by sclerotial extracts, fractions separated by sephadex G-50 gel filtration chromatography as well as P-HMW derived fractions separated by anionic exchange chromatography were subjected to One-way ANOVA and the statistical significance between pre-treatment means were determined by Tukey's Honestly Significant Differences (HSD) test. Statistical differences between maximal inhibitory concentration of TNF-α production of P-HMW and P-MMW were analysed using Independent Samples t-test. All results were considered significant at $p < 0.05$.

7.4 Results

7.4.1 Extraction Yield of CWE TM02, HWE TM02 and ME TM02

The extraction yield of CWE, HWE and ME were 20.3 ± 0.5%, 37.3 ± 0.6% and 4.8 ± 0.2% by dry weight (w/w), respectively.

7.4.2 Determination of Total Carbohydrate and Protein Content of the Various Sclerotial Extracts

The CWE, HWE and ME consisted of 68.4 ± 1.7%, 92.2 ± 2.7% and 12.8 ± 0.4% carbohydrate by dry weight (w/w), respectively, and 2.0 ± 0.1%, 0.2 ± 0.0% and 0.2 ± 0.0% protein by dry weight (w/w), respectively (Table 7.2).

Table 7.2: Carbohydrate and protein composition of extracts and fractions of sclerotia

Extract/fraction/protein or non-protein component of fraction	Carbohydrate percentage by dry weight (w/w)	Protein percentage by dry weight (w/w)
CWE	68.4 ± 1.7	2.0 ± 0.1
HWE	92.2 ± 2.7	0.2 ± 0.0
ME	12.8 ± 0.4	0.2 ± 0.0
HMW	78.6 ± 1.5	4.4 ± 0.1
MMW	54.0 ± 0.8	1.3 ± 0.1
LMW	23.9 ± 0.9	0.3 ± 0.0
P-HMW	91.2 ± 0.3	8.8 ± 0.3
NP-HMW	92.9 ± 2.1	0.2 ± 0.1
P-MMW	75.3 ± 0.9	24.7 ± 0.9
NP-MMW	49.5 ± 3.5	ND

Each value is mean ± S.E.M (n = 3). CWE-cold water extract; HWE-hot water extract; ME-methanol extract; HMW-high molecular weight fraction; MMW-medium molecular weight fraction; LMW-low molecular weight fraction; P-HMW-protein component of HMW fraction; P-MMW-protein component of MMW fraction; NP-HMW-non-protein component of HMW fraction; NP-MMW-non-protein component of MMW fraction; ND-not detectable.

7.4.3 Fractionation of CWE

HMW, MMW and LMW fractions were obtained from Sephadex G-50 gel filtration chromatographic separation of CWE (Figure 7.1). The carbohydrate and protein content of HMW fraction were determined to be 78.6 ± 1.5% and 4.4 ± 0.1% by dry weight (w/w), respectively. The MMW fraction consisted of 54.0 ± 0.8% carbohydrate and 1.3 ± 0.1% protein by dry weight (w/w), while the LMW fraction contained 23.9 ± 0.9% carbohydrate and 0.3 ± 0.0% protein by dry weight (w/w) (Table 7.2).

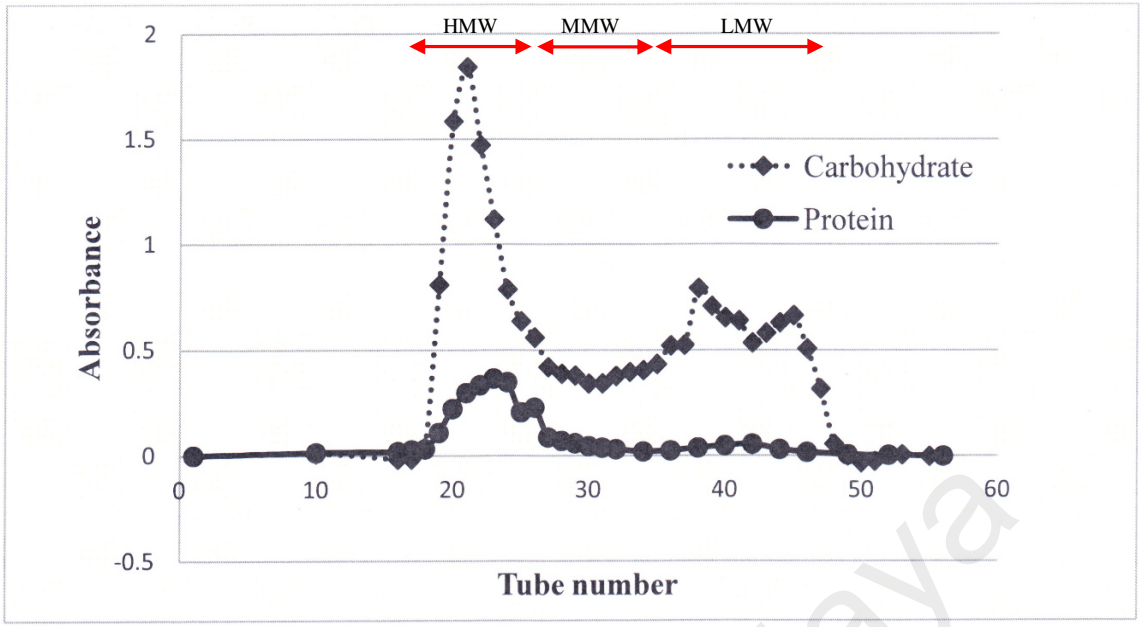

Figure 7.1: Sephadex G-50 superfine gel filtration chromatography of the cold water extract (CWE)

The cold water extract (CWE, 500 mg dissolved in 5.6 ml milliQ water) was loaded to the column (v = 112 ml) and 3 ml per fraction was collected into each test tube.

7.4.4 Carrageenan Induced Paw Oedema Study

CWE exhibited inhibitory activity against carrageenan induced paw oedema formation in SD rats at all doses employed (25, 50, 100 and 200 mg/kg) after 1-5 h of carrageenan injection (Table 7.3). Rats treated with CWE at 50, 100 and 200 mg/kg showed significant inhibition of paw oedema formation from 2nd to 5th hour post carrageenan injection, as compared to the control group ($p < 0.05$). At first hour post carrageenan injection, a significant inhibition of paw oedema formation was observed in the rats treated with CWE at 200 mg/kg. Paw oedema suppression in dose dependent manner was observed in rat treated with CWE at all doses employed after 2-5 hour of carrageenan injection.

Paw oedema size of SD rat treated with CWE was maximal at 3rd to 4th hour post carrageenan injection. The median inhibitory doses (ID_{50}) of the CWE for the suppression of paw oedema at the 3rd and 4th hour were both 88 mg/kg. Inhibitory effect on paw oedema formation by 200 mg/kg CWE was found to be higher than 10 mg/kg indomethacin (standard drug), after 1-5 hour of carrageenan injection. HMW fraction of CWE (53 mg/kg) displayed inhibitory effect on paw oedema formation which was higher than MMW (35 mg/kg) and LMW (112 mg/kg) fractions of the same extract.

Paw oedema inhibitory activity by HWE and ME at 200 mg/kg were significantly lower (Independent Samples t-test, $p < 0.05$) than CWE at the same dosage (after 1-5 hour of carrageenan injection), with ME TM02 having the least potent activity.

Table 7.3: Effects of sclerotial extracts of *Lignosus rhinocerotis* cultivar TM02 on carrageenan induced paw oedema in rats

Group	Dose (mg/kg body weight)	Paw oedema volume (ml)				
		1st hour	2nd hour	3rd hour	4th hour	5th hour
Control	**-**	0.28 ± 0.02	0.76 ± 0.04	0.85 ± 0.03	0.90 ± 0.04	0.86 ± 0.04
CWE TM02	**25**	0.25 ± 0.02 (12)	0.52 ± 0.04* (32)	0.70 ± 0.03 (18)	0.70 ± 0.03* (22)	0.65 ± 0.04* (25)
	50	0.18 ± 0.02 (36)	0.40 ± 0.07* (48)	0.50 ± 0.06* (42)	0.51 ± 0.05* (44)	0.46 ± 0.05* (47)
	100	0.18 ± 0.01 (35)	0.22 ± 0.04* (71)	0.33 ± 0.04* (62)	0.41 ± 0.03* (55)	0.35 ± 0.02* (60)
	200	0.11 ± 0.03* (61)	0.13 ± 0.03* (83)	0.10 ± 0.03* (88)	0.11 ± 0.03* (88)	0.10 ± 0.03* (89)
HMW	**53****	0.11 ± 0.02* (63)	0.12 ± 0.02* (84)	0.12 ± 0.02* (86)	0.09 ± 0.02* (90)	0.08 ± 0.02* (91)
MMW	**35****	0.16 ± 0.03* (44)	0.30 ± 0.04* (61)	0.35 ± 0.05* (65)	0.51 ± 0.05* (43)	0.44 ± 0.03* (49)
LMW	**112****	0.23 ± 0.03 (20)	0.46 ± 0.05* (39)	0.51 ± 0.05* (41)	0.51 ± 0.04* (43)	0.47 ± 0.04* (46)
ME TM02	**200**	0.24 ± 0.05 (15)	0.68 ± 0.04 (11)	0.78± 0.04 (9.0)	0.73 ± 0.04* (19)	0.69 ±0.03* (20)
HWE TM02	**200**	0.20 ± 0.03 (31)	0.41 ± 0.06* (46)	0.50 ± 0.05* (42)	0.49 ± 0.04* (46)	0.47 ± 0.03* (45)
Indomethacin	**10**	0.18 ± 0.03 (35)	0.23 ± 0.03* (69)	0.26 ± 0.03* (69)	0.37 ± 0.03* (59)	0.34 ± 0.03* (57)

Each value is mean ± S.E.M of six rats. *$p < 0.05$, compared with control using Dunnett's t (two-sided) test. Values given in parentheses represent the percentage inhibition of oedema volume at each hour as compared to the control group. **Amount isolated from 200 mg/kg of CWE TM02 using Sephadex G-50 gel filtration chromatography. CWE-cold water extract; HWE-hot water extract; ME-methanol extract; HMW-high molecular weight fraction; MMW-medium molecular weight fraction; LMW-low molecular weight fraction.

7.4.5 Cotton Pellet Induced Granuloma Study

The wet and dry weight of granuloma from rats treated with CWE at 200 mg/kg were not significantly suppressed, as compared to the control group (Table 7.4). However, treatment with 1 mg/kg indomethacin for every 2 days significantly reduced the wet and dry weight of granuloma in rats by 20.8% and 20.3%, respectively.

Table 7.4: Effect of the cold water extract (CWE) and indomethacin on cotton pellet induced granuloma in rats

Treatment	Dose (mg/kg)	Granulomatous tissue	
		Wet weight (mg)	Dry weight (mg)
6% sodium bicarbonate (control)	-	281.0 ± 13.8	39.6 ± 1.4
CWE TM02	200	281.2± 13.9	40.2 ± 2.4
Indomethacin	1	222.7 ± 14.3* (20.8%)	31.5 ± 2.6* (20.3%)

Values are mean ± S.E.M. Values given in parentheses represent percentage of inhibition.* Significant difference with control (ANOVA, Dunnett's t (two-sided) test, $p < 0.05$).

7.4.6 Determination of Total Carbohydrate and Protein Content of Protein and Non-protein Components of the HMW and MMW Fraction

The HMW and MMW fractions were subjected to 100% ammonium sulphate precipitation to yield the protein (P-HMW and P-HMW) and non-protein components (NP-HMW and NP-MMW). Carbohydrate to protein ratio of P-HMW and P-MMW were 10 and 3, respectively (Table 7.2). Carbohydrate contents of NP-HMW and NP-MMW were 92.9 ± 2.1% and 49.5 ± 3.5%, respectively. Very small amount of protein was found

in NP-HMW (0.2 ± 0.1%) while no protein was detected in NP-MMW.

7.4.7 Determination of Glucan Content of Various Sclerotial Extract, Fraction as well as Protein and Non-protein Component of HMW Fraction of *Lignosus rhinocerotis* Cultivar TM02

Alpha glucan and oligomers etc. were found to be the major glucan component in almost all extracts or fractions obtained (CWE, HWE, HMW, MMW, LMW, P-HMW and NP-HMW) (Table 7.5), except for ME, where beta glucan content was 3.8 times more than alpha glucan and oligomers etc. Total glucan and alpha glucan content (including oligomers etc.) of fractions separated by Sephadex G-50 gel filtration chromatography were in the order of: HMW (45.3 ± 0.7%; 43.6 ± 0.9%) > MMW (39.7 ± 1.0%; 33.0 ± 0.7%) > LMW (10.3 ± 0.3%; 7.7 ± 0.2%). MMW contained the highest percentage of beta glucan (6.7 ± 0.3%), followed by LMW (2.7 ± 0.1%) and HMW (1.7 ± 0.3%). In P-HMW, about 77.3 ± 0.9% of the carbohydrate was alpha glucan and oligomers etc. Only 1.2 ± 0.2% of the carbohydrate was beta glucan in the P-HMW. Carbohydrate of NP-HMW was 61.4 ± 1.1% alpha glucan and oligomers etc., and 3.4 ± 0.5% of beta glucan.

Table 7.5: Glucan percentage of sclerotial powder of *Lignosus rhinocerotis* cultivar TM02

Extracts/fractions	Percentage by dry weight; % w/w			Percentage by dry weight of carbohydrate content; % w/w		
	Total glucan and oligomers etc.	Alpha glucan and oligomers etc.	Beta glucan	Total glucan and oligomers etc.	Alpha glucan and oligomers etc.	Beta glucan
CWE TM02	39.7 ± 1.1	37.4 ± 1.1 (94.1)	2.3 ± 0.1 (5.9)	58.0 ± 1.6	54.5 ± 1.6	3.4 ± 0.1
HWE TM02	61.0 ± 1.0	57.7 ± 0.8 (94.5)	3.3 ± 0.3 (5.5)	66.1 ± 1.1	62.5 ± 0.9	3.6 ± 0.3
ME TM02	6.2 ± 0.2	1.3 ± 0.1 (21.1)	4.9 ± 0.2 (78.9)	48.4 ± 1.3	10.4 ± 0.6	38.0 ± 1.8
HMW fraction	45.3 ± 0.7	43.6 ± 0.9 (96.2)	1.7 ± 0.3 (3.8)	53.2 ± 0.8	51.2 ± 1.0	2.0 ± 0.4
MMW fraction	39.7 ± 1.0	33.0 ± 0.7 (83.1)	6.7 ± 0.3 (16.9)	62.1 ± 1.5	51.6 ± 1.0	10.5 ± 0.5
LMW fraction	10.3 ± 0.3	7.7 ± 0.2 (74.3)	2.7 ± 0.1 (25.7)	43.1 ± 1.3	32.0 ± 0.8	11.1 ± 0.6
P-HMW fraction	71.6 ± 1.0	70.5 ± 0.8 (98.5)	1.1 ± 0.2 (1.5)	78.5 ± 1.1	77.3 ± 0.9	1.2 ± 0.2
NP-HMW fraction	60.3 ± 1.1	57.1 ± 1.1 (94.7)	3.2 ± 0.4 (5.3)	64.9 ± 1.1	61.4 ± 1.1	3.4 ± 0.5

Each value is mean ± S.E.M (n = 3). Values given in parentheses under percentage by dry weight represent the percentage of alpha glucan and oligomers etc. or beta glucan by total glucan and oligomers etc.

7.4.8 Carbohydrate and Protein Determination of P-HMW Derived Fractions Separated by Anionic Exchange Chromatography

A total of 8 fractions were obtained from high performance Resource Q ion exchange chromatographic separation of P-HMW. The fractions were named as F1 to F8 (Figure 7.2). Carbohydrate contents of F1, F2, F3, F4 and F8 were higher than protein contents while F5, F6 and F7 contained larger amount of protein in relative to carbohydrate (Table 7.6).

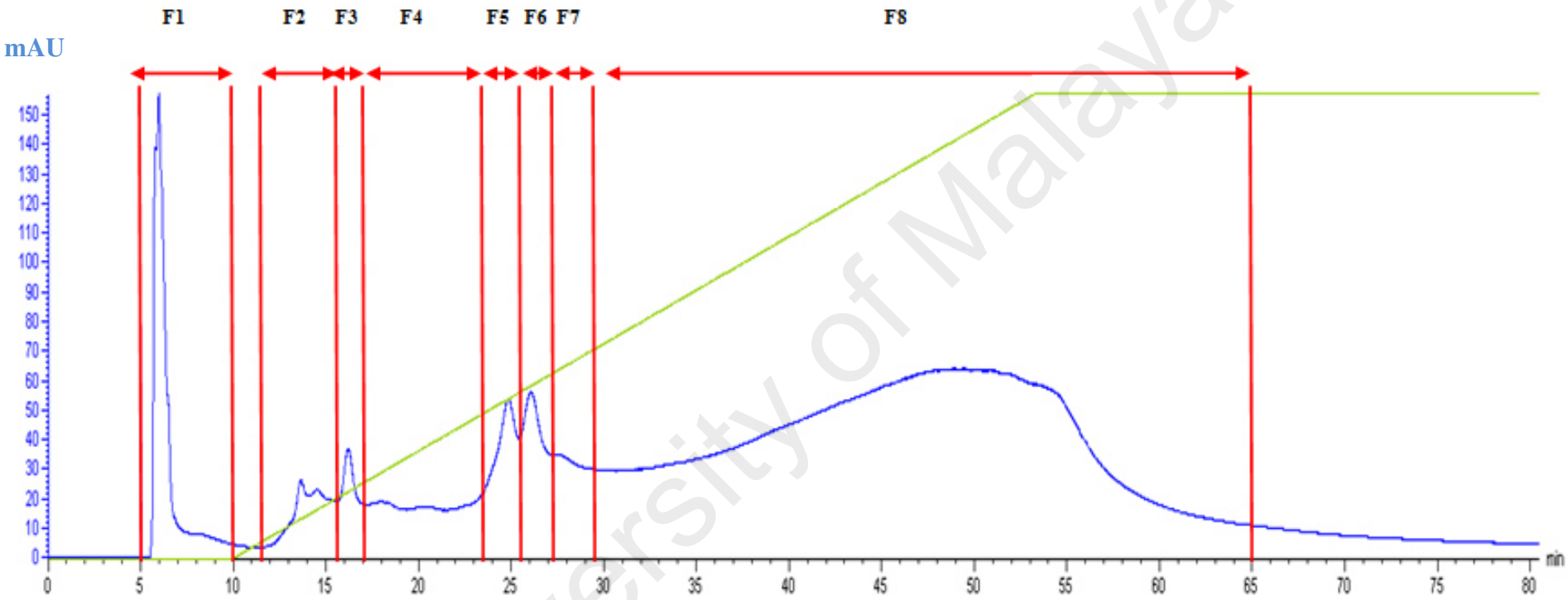

Figure 7.2: Anionic exchange chromatography by Resource™ Q column (1ml) of P-HMW

Green line represents gradient of buffer B (%) while blue line represents absorbance at 280nm. F1 to F8 are fractions collected from the chromatography.

Table 7.6: Protein and carbohydrate percentage of fractions separated by anionic exchange chromatography from P-HMW

Fraction	Protein percentage by total carbohydrate and protein content (w/w)	Carbohydrate percentage by total carbohydrate and protein content (w/w)
1	0.2	99.8
2	10.6	89.4
3	32.0	68.0
4	20.0	80.0
5	65.9	34.1
6	75.0	25.0
7	60.2	39.8
8	7.8	92.2

Results shown are the mean values of a single experiment performed in triplicate.

7.4.9 Measurement of Inhibition of TNF-alpha Production

In all experiments, cell viability of RAW 264.7 after LPS-stimulation alone was at least 83% while the cell viability of pre-treatment groups normalised to LPS-stimulated control was at least 77%. CWE exhibited TNF-α inhibition activity with IC_{50} of 30.00 ± 1.33 µg/ml while the inhibitory effect by HWE was not detectable up to 150.00 µg/ml (Table 7.7). HMW (IC_{50}: 25.79 ±1.77 µg/ml) and MMW (IC_{50}: 52.83 ± 2.74 µg/ml) fraction of CWE exhibited inhibitory activity on TNF-α production while the LMW fraction did not show any activity up to 150.00 µg/ml. Protein components of HMW and MMW fraction (P-HMW and P-MMW) exhibited very potent TNF-α inhibition activity with IC_{50} of 9.35 ± 0.48 and 5.10 ± 0.36 µg/ml, respectively when based on total protein and carbohydrate content; and IC_{50} of 0.82 ± 0.04 and 1.31 ± 0.09 µg/ml, respectively when based on total protein content alone (Table 7.8). No inhibitory effect on TNF-α production was detected on fraction 1 to 3 (F1-F3) derived from P-HMW at tested concentration up to 2.50 µg/ml (Table 7.9). F4 and F8 displayed weak inhibitory effect on TNF-α production with percentage inhibition of 6.13 ± 1.51% and 10.63 ± 0.24 %, respectively corresponding to tested concentration at 2.5 µg/ml. F5 and F6 showed similar potency on TNF-α inhibition activity with IC_{50} of 0.45 ± 0.02 and 0.45 ± 0.03 µg/ml, respectively. F7 exhibited weaker inhibitory activity (IC_{50} of 1.11 ± 0.03 µg/ml), as compared to F5 and F6.

Table 7.7: The inhibitory effect of sclerotial extracts and fractions separated by sephadex G-50 gel filtration chromatography on LPS-induced TNF-α production in RAW 264.7 cells

Extract/fraction	Maximal inhibitory concentration of TNF-α production (µg/ml)	
	IC_{50}	IC_{70}
CWE	30.00 ± 1.33^{a}	56.17 ± 1.59^{a}
HWE	ND	ND
HMW	25.79 ± 1.77^{a}	40.37 ± 1.88^{b}
MMW	52.83 ± 2.74^{b}	97.17 ± 5.64^{c}
LMW	ND	ND

Each value is mean ± S.E.M (n = 3) in which the value is based on total dry weight. ND-not detectable. Means within a column with the identical letters are not significantly different at the 95% level of confidence using Tukey's HSD test.

Table 7.8: The inhibitory effect of protein and non-protein component isolated from HMW and MMW fraction which are separated by sephadex G-50 gel filtration chromatography on LPS-induced TNF-α production in RAW 264.7 cells

Pre-treatment group	Maximal inhibitory concentration of TNF-α production (µg/ml)	
	IC_{50}	IC_{70}
P-HMW	9.35 ± 0.48 (0.82 ± 0.04)	17.52 ± 1.48 (1.53 ± 0.13)
NP-HMW	ND	ND
P-MMW	5.10 ± 0.36 (1.31 ± 0.09)	8.27 ± 0.55 (2.13 ± 0.14)
NP-MMW	ND	ND

Each value is mean ± S.E.M (n = 3) in which the values of P-HMW and P-MMW are based on total carbohydrate and protein concentration. ND-not detectable. Values given in parentheses represent IC_{50} and IC_{70} based on total protein concentration alone. Statistical comparisons of IC_{50} and IC_{70} within a column were made using Independent-Samples T Test. IC_{50} of P-HMW and P-MMW pre-treated group (both total carbohydrate and protein concentration as well as protein concentration alone) were significantly different ($p < 0.05$). Same result was obtained in IC_{70} of the same groups.

Table 7.9: The inhibitory effect of P-HMW derived fractions separated by anionic exchange chromatography on LPS-induced TNF-α production in RAW 264.7 cells

Fraction	Maximal inhibitory concentration of TNF-α production (µg/ml)			
	IC_{50}		IC_{70}	
	Total carbohydrate + protein	Total protein	Total carbohydrate + protein	Total protein
F1	ND	ND	ND	ND
F2	ND	ND	ND	ND
F3	ND	ND	ND	ND
F4	ND	ND	ND	ND
F5	0.45 ± 0.02^{a}	0.30 ± 0.01^{a}	0.92 ± 0.03^{a}	0.61 ± 0.02^{a}
F6	0.45 ± 0.03^{a}	0.33 ± 0.02^{a}	0.91 ± 0.02^{a}	0.68 ± 0.02^{a}
F7	1.11 ± 0.03^{b}	0.67 ± 0.02^{b}	1.72 ± 0.09^{b}	1.04 ± 0.06^{b}
F8	ND	ND	ND	ND

Each value is mean ± S.E.M (n = 3) in which the values are based on total carbohydrate and protein concentration or total protein concentration alone. ND-not detectable. F1 to F8 represent anionic exchange chromatography separated fractions. Means within a column with the identical letters are not significantly different at the 95% level of confidence using Tukey's HSD test.

7.4.10 Analysis of Protein Composition of P-HMW, P-MMW and P-HMW Derived Fractions Separated by Anionic Exchange Chromatography

Protein composition of P-HMW, F5 and F6 were analysed using SDS-PAGE gel–LC-MS/MS (Figures 7.3 and 7.4). Percentage of each protein in each gel section of P-HMW, F5 and F6, as well as complete protein profile of F5 and F6 were provided in Appendix D, E and F, respectively. LC-MS/MS report of F5 and F6 in details were inserted in Appendix G and H, respectively.

The most abundant proteins in P-HMW, F5 and F6 were found to be serine proteases, with content of 36.3%, 50.0% and 54.1%, respectively (Figures 7.5, 7.6 and 7.7). Serine proteases were found in all gel sections of P-HMW, F5 and F6 (Figures 7.8, 7.9 and 7.10). Most of the serine proteases were found in the gel section(s) with molecular mass between 25 and 35 kDa. Four serine proteases were detected in P-HMW with genome database accession number of 4347, 8711, 7804 and 5911 (Table 7.10). Serine protease 4347, 8711, 7804 and 5901 in P-HMW accounted for 31.8%, 3.8%, 0.5% and 0.2% of total proteins, respectively. Only serine protease 4347 and 8711 were found in both F5 and F6. It is interesting that the total serine protease composition of F5 and F6 were similar, and that serine proteases of F5 at 25-35 kDa were mainly serine protease 4347 (serine protease 4347 at 21.3% and 8711 at 3.3%) while in F6, the percentage of serine protease 4347 (16.1%) and 8711 (14.2%) were comparable.

Multiple sequence alignment of serine protease 4347 and 8711 with their most homologous fungal serine proteases were shown in Appendix J. Catalytic triad of serine protease 4347 and 8711 were predicted to be Asp176 His207 Ser381 and Asp160 His191 Ser346, respectively by using NCBI's conserved domain database (CDD). Percentage sequence identity that measures the extent to which two amino acid sequences have the

same residues at the same positions in an alignment (Fassler & Cooper, 2011) between serine protease 4347 and 8711 with their best-matching sequences (serine protease from *Dichomitus squalens* LYAD-421 SS1 with sequence ID: XP_007359794.1) were found to be 54% and 55%, respectively (Appendix E). Percentage of sequence similarity refers to the percentage of aligned residues that have at least similar physicohemical characteristics and can be more readily substituted for each other (Xiong, 2006). Percentage of sequence similarity between serine protease 4347 and 8711 with serine protease XP_007359794.1 were found to be 65% and 67%, respectively (Appendix E). Pairwise sequence alignment of serine protease 4347 and 8711 by using Bl2seq revealed that they were 61% identical and 69% similar (Appendix K).

To confirm protease activity of serine proteases in F5 and F6, protease assay in which casein was used as substrate was carried out (Figure 7.11), and the results showed that F5 and F6 exhibited comparable protease activity ranging from 0 to 0.023/ min at tested concentration from 0 to 60.8 µg/ml.

To examine the effect of serine protease inhibitors on the protease activity of the serine proteases 4347 and 8711 in F5 and F6, two irreversible serine protease inhibitors: phenylmethylsulfonyl fluoride (PMSF) and Pefabloc® SC (Powers et al., 2002) were used. Prior to protease activity assay, fractions or trypsin were mixed with the respective serine protease inhibitor and incubated in room temperature for 15 min. In the presence of 1 mM PMSF, protease activity of trypsin (at 60.8 µg/ml) was reduced by 27.4 ± 1.3% (compared to control). In the presence of Pefabloc® SC at 1, 2 and 4 mM, the protease activity of the same trypsin solution was reduced by 24.9 ± 0.6%, 44.8 ± 1.3% and 61.6 ± 0.8%, respectively. However, surprisingly, protease activity of F5 and F6, at 60.8 µg/ml, was not inhibited by 1 mM of PMSF or 4 mM of Pefabloc® SC.

Glycoside hydrolase family proteins were found to be the second most abundant proteins in F5 that accounted for 24.1% of total protein (Figure 7.6). The most abundant glycoside hydrolase family proteins in F5 and F6 were found to be glycoside hydrolase family 27 proteins with database accession number of 9375 and 9376. The rest of the glycoside hydrolase family proteins constituted less than 0.8% of total protein. Glycoside hydrolase 9375 and 9376 contributed 10.8% and 12.5%, respectively of the total protein of F5 and 4.2% and 5.1%, respectively of the total protein of F6. Phosphatidylserine decarboxylase-like proteins (database accession number 4019) which were the second abundant protein in F6 accounted for 13.8% of total protein.

Lectins, the third most abundant proteins in P-HMW and F6 accounted for 11.0% and 10.7% of total protein, respectively (Figure 7.5 and 7.7). Total lectin content in F5 was only 2.6% (Figure 7.6). Figure 7.8 (d) showed that the major proteins in the SDS-PAGE gel section 4 of P-HMW were lectins and 69.1% of total lectins were found in this section.

Immunomodulatory proteins (immunomodulatory protein 8 and immunomodulatory protein FIP-Fve) which were found to be the most abundant proteins in gel section 5 of P-HMW (10 to 15 kDa) accounted for 57.1% of the total protein of section 5 (Figure 7.8 (e)). It was the second most abundant protein of P-HMW, and constituted 18.1% of total protein (Figure 7.5). However, this protein (only immunomodulatory protein 8) was only found in moderate amount in F5 (3.5% in total) and small amount in F6 (0.9% in total) (Figures 7.6 and 7.7).

Hypothetical proteins in P-HMW, F5 and F6 accounted for 10.4%, 9.5% and 5.4% of total protein, respectively (Figures 7.5, 7.6 and 7.7). A total of 8 hypothetical proteins with genome database accession number of 4537, 7308, 6918, 3033, 5536, 6244, 1186 and 9837 were found in P-HMW. Hypothetical proteins 9837, 6918, 1261, 1186 and 4537

were found in F5 while only two hypothetical proteins: 9837 and 6918 were found in F6.

In addition, a number of minor proteins were also found in P-HMW, F5 and F6, including putative chitin synthase, phosphoglycerate mutase-like protein and aldo/keto reductase. Generally, they accounted for <5% of the total proteins.

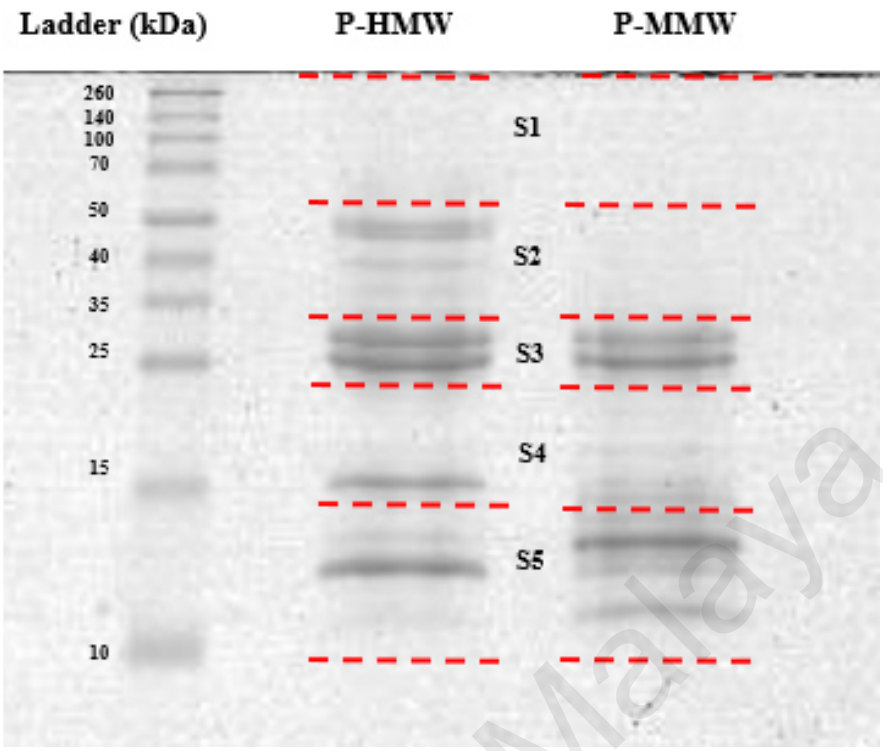

Figure 7.3: Separation of 35 µg P-HMW and P-MMW (based on protein amount) by 12.5% reducing SDS-PAGE

Gel sections 1 to 5 were labelled as S1 to S5.

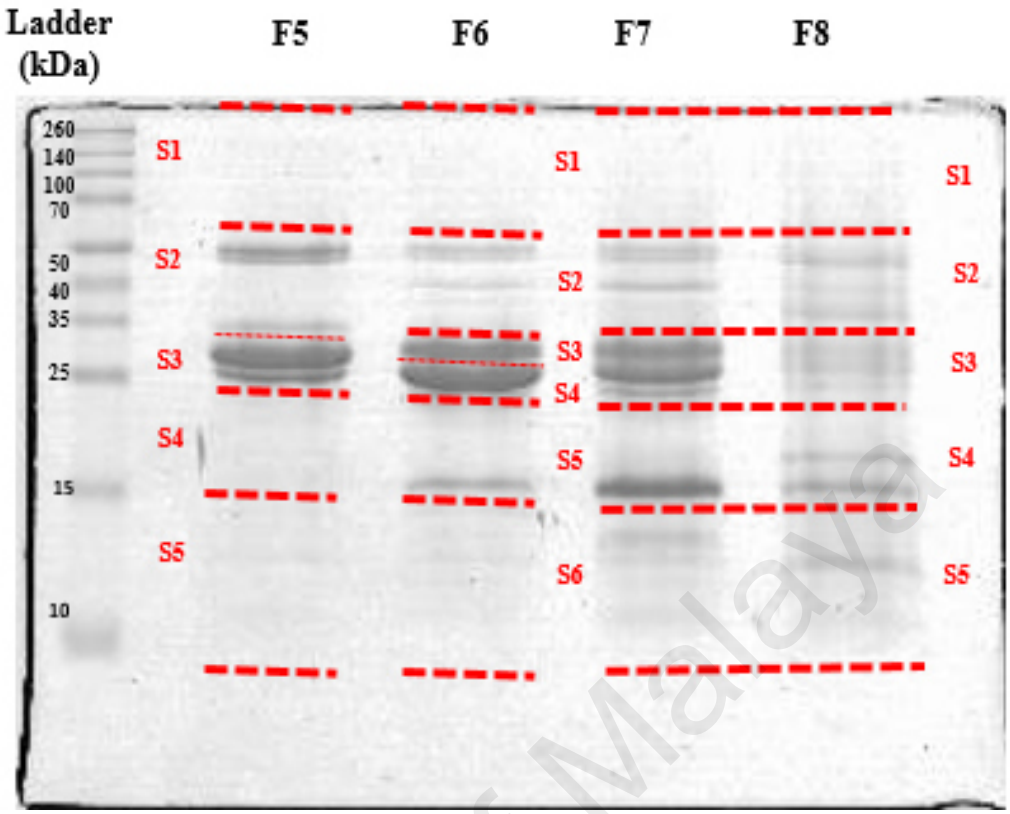

Figure 7.4: Separation of F5, F6, F7 and F8 (based on protein amount) by 12.5% reducing SDS-PAGE

F5, F6 and F7 were loaded at 35µg while F10 was loaded at 18.5 µg. Gel sections 1 to 6 were labelled as S1 to S6.

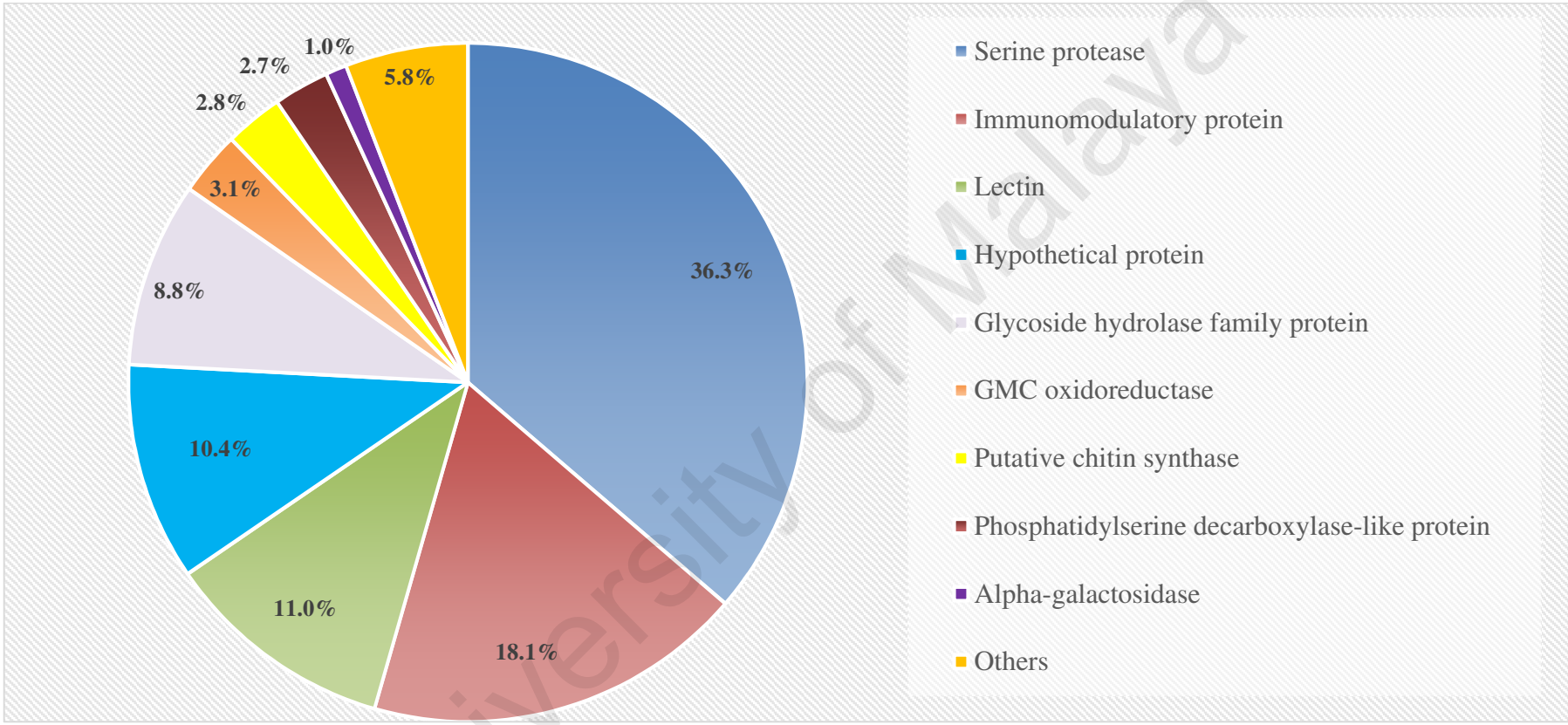

Figure 7.5: Total protein composition of P-HMW

SDS-PAGE gel of P-HMW as shown in Figure 7.3 was cut into 5 sections and total protein composition was determined by LC-MS/MS (QTOF) analysis searching through *Lignosus rhinocerotis* genome database.

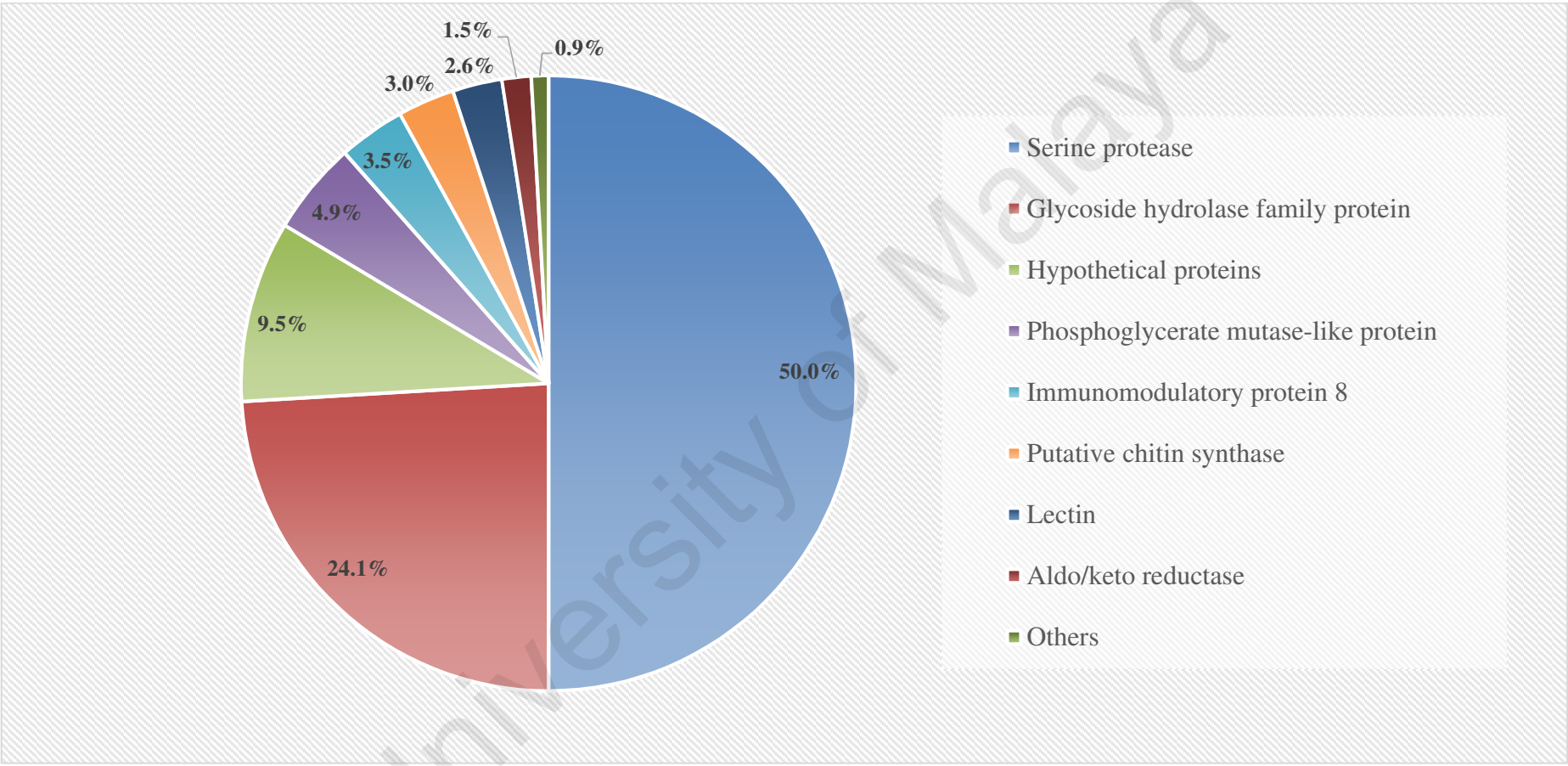

Figure 7.6: Total protein composition of F5

SDS-PAGE of F5 as shown in Figure 7.4 was cut into 5 sections and total protein composition was determined by LC-MS/MS (QTOF) analysis by searching through *Lignosus rhinocerotis* genome database.

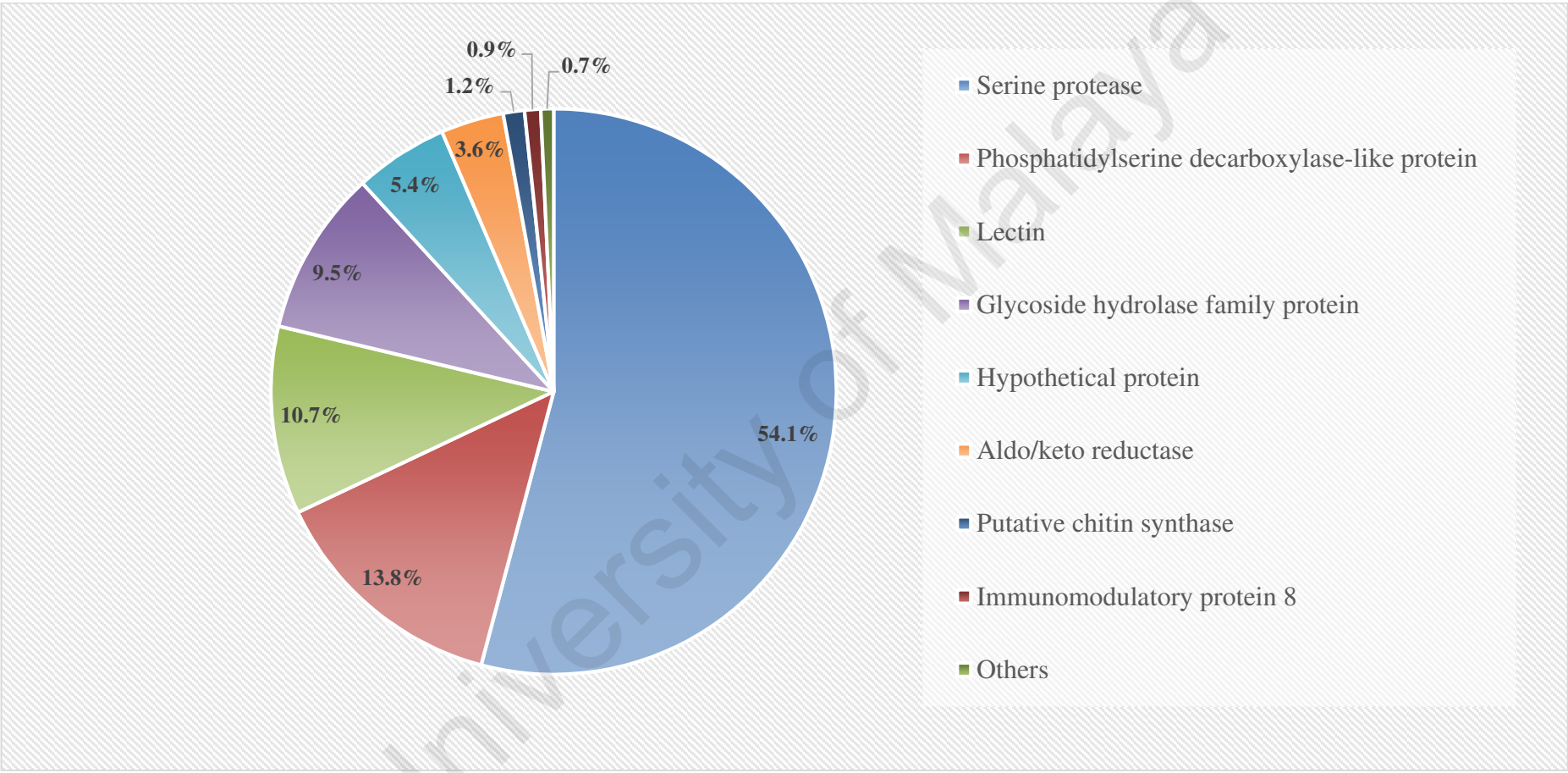

Figure 7.7: Total protein composition of F6

SDS-PAGE gel of F6 as shown in Figure 7.4 was cut into 6 sections and total protein composition was determined by LC-MS/MS (QTOF) analysis by searching through *Lignosus rhinocerotis* genome database.

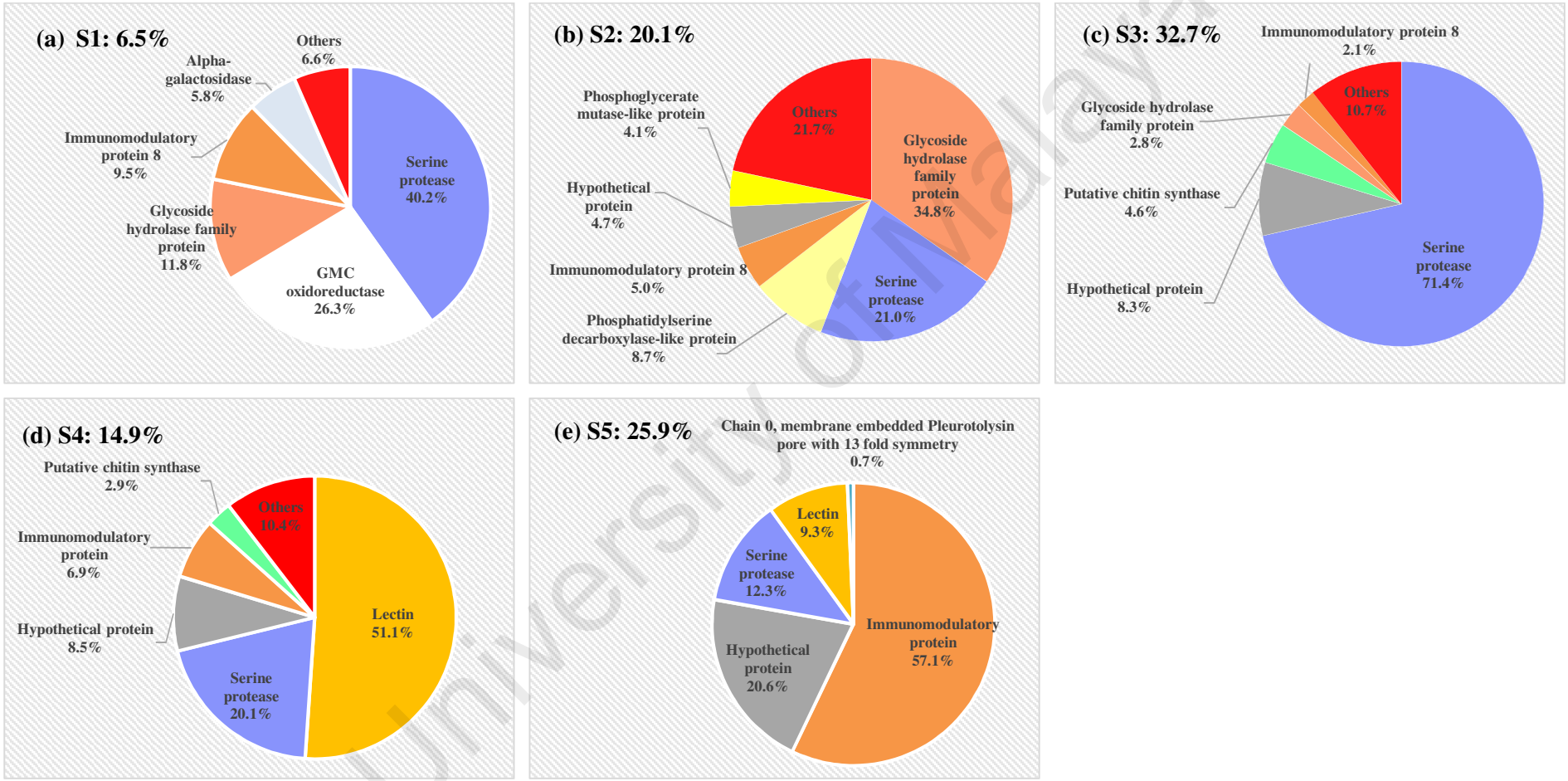

Figure 7.8: Percentage of proteins derived from P-HMW in each SDS-PAGE gel sections as demonstrated in Figure 7.3

Relative protein percentage of each gel section was displayed at the top left of each sub-figures.

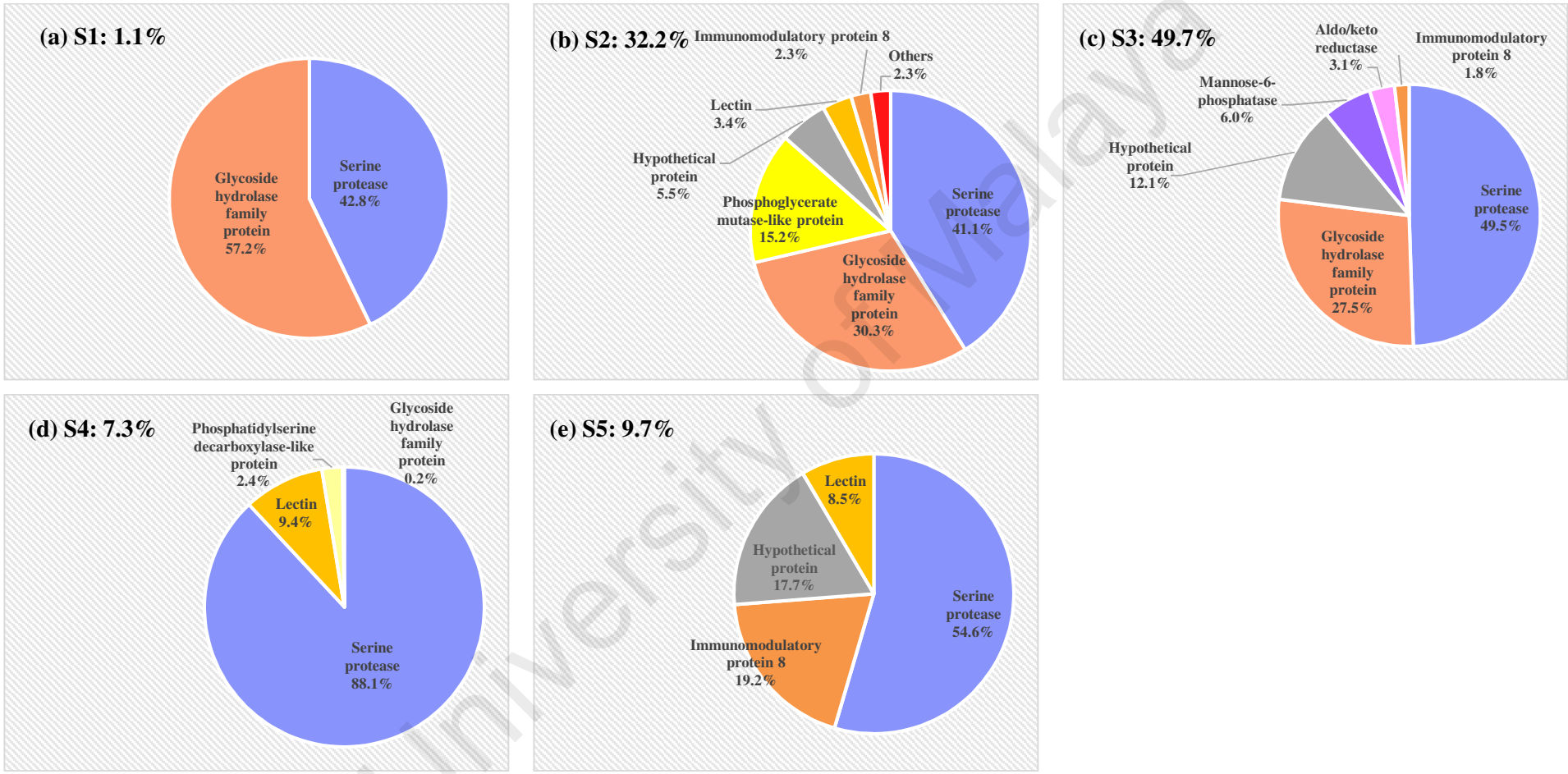

Figure 7.9: Percentage of proteins derived from F5 in each SDS-PAGE gel sections as demonstrated in Figure 7.4

Relative protein percentage of each gel section was displayed at the top left of each sub-figures.

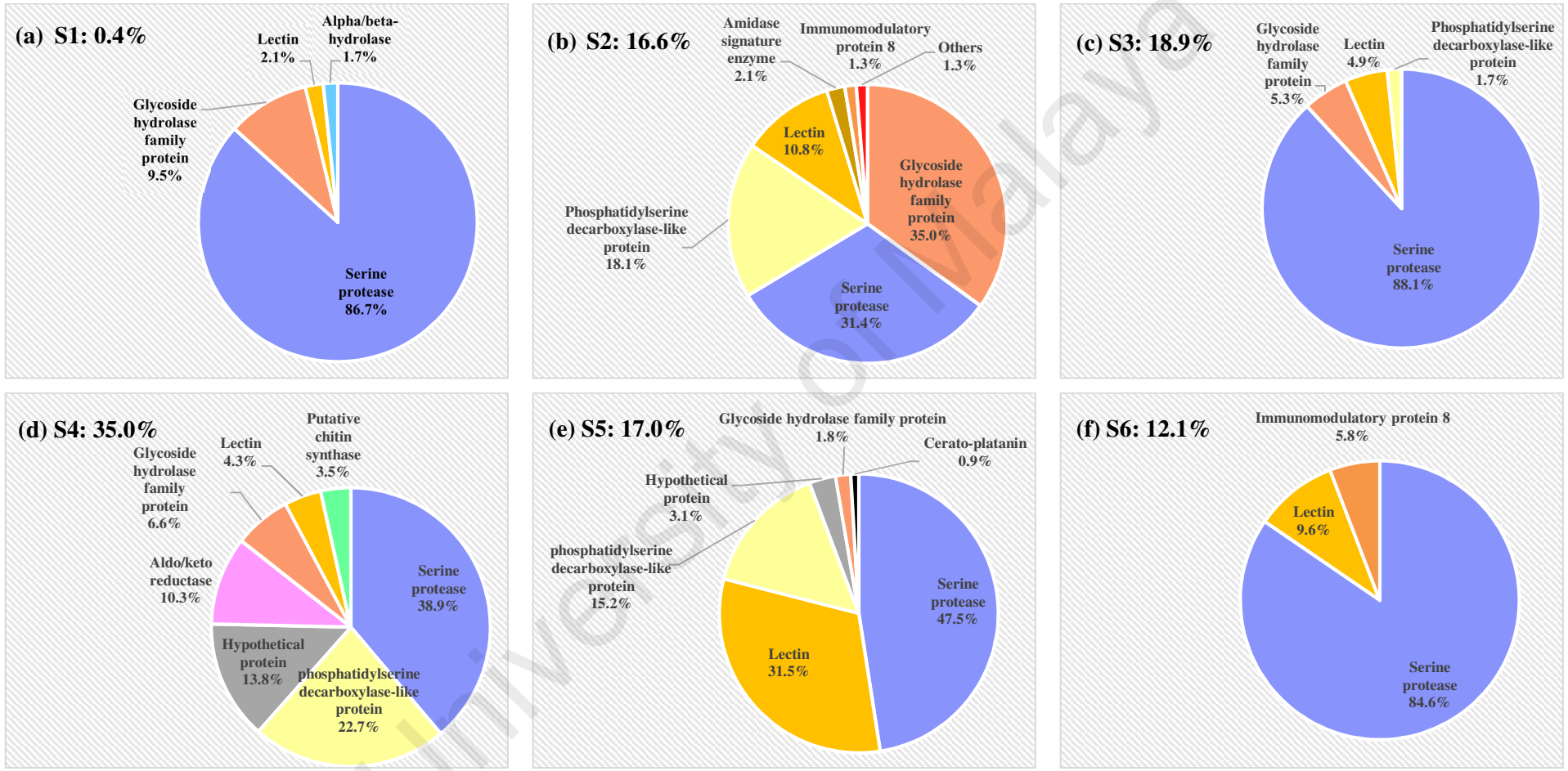

Figure 7.10: Percentage of proteins derived from F6 in each SDS-PAGE gel sections as demonstrated in Figure 7.4

Relative protein percentage of each gel section was displayed at the top left of each sub-figures.

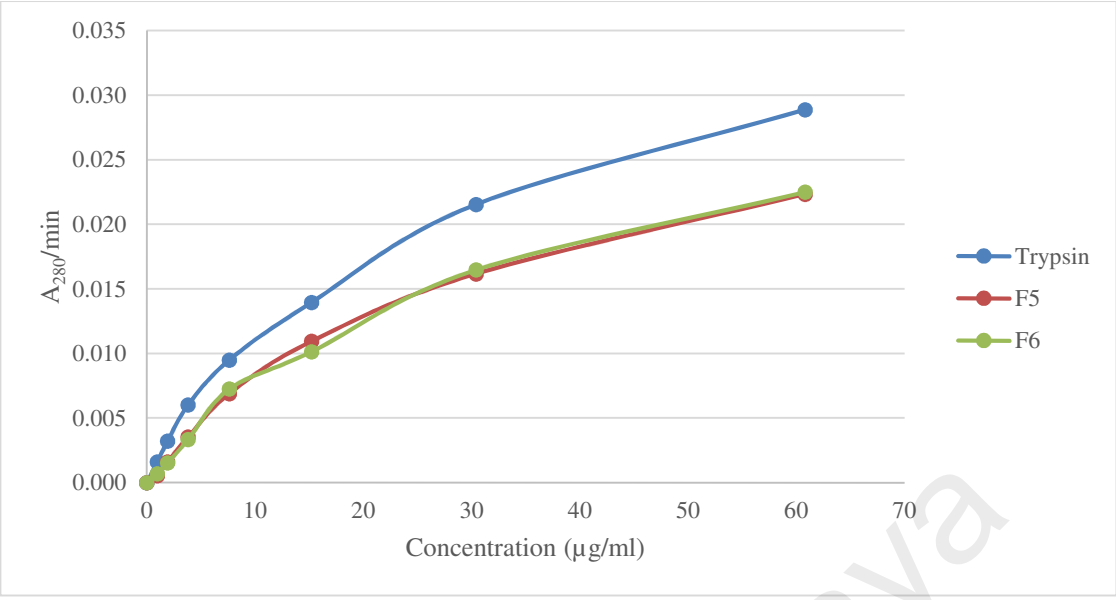

Figure 7.11: Protease activity of F5 and F6

Protease activities of F5 and F6 were tested from 0 to 60.8 µg/ml in which casein was used as substrate. Trypsin was used as positive control at same concentrations. Protease activity is expressed in absorbance units/min at 280 nm.

Table 7.10: Distribution of four different serine proteases in each gel section of P-HMW, F5 and F6

Accession number	Sample	Protein percentage (%)					
		S1	**S2**	**S3**	**S4**	**S5**	**S6**
4347	P-HMW	2.6	3.0	20.0	3.0	3.2	-
	F5	0.5	3.3	21.3	6.3	5.3	-
	F6	0.2	1.5	5.1	11.0	4.5	10.2
8711	P-HMW	-	1.2	2.6	-	-	-
	F5	-	9.9	3.3	0.1	0.1	-
	F6	0.1	3.7	11.6	2.6	3.6	0.0
7804	P-HMW	-	-	0.5	-	-	-
	F5	-	-	-	-	-	-
	F6	-	-	-	-	-	0.0
5911	P-HMW	-	-	0.2	-	-	-
	F5	-	-	-	-	-	-
	F6	-	-	-	-	-	0.0

7.5 Discussion

7.5.1 The Anti-inflammatory Activity of the Sclerotium of *Lignosus rhinocerotis*

The medicinal mushroom *L. rhinocerotis* has been used extensively by the indigenous people from Peninsular Malaysia. The sclerotium of *L. rhinocerotis* is usually grated on a hard surface with some water, and the resulting aqueous mixture is further diluted with water and consumed, with or without boiling (Chan, 1953; Chang & Lee, 2004). Therefore, cold and hot water extractions mimicking the preparation methods were used in the investigation of anti-inflammatory activity of the mushroom. The methanol extraction was used to investigate anti-inflammatory activity of the non-polar and water immiscible substances of the mushroom.

Carrageenan induced rat hind paw oedema model has been widely used for the evaluation of anti-inflammatory drugs as it is well researched and highly reproducible (Morris, 2003). The triphasic events occur in carrageenan induced paw oedema model were outlined in section 7.2.4.1.

The cold water extract (CWE) exhibited anti-acute inflammatory activity by reducing paw oedema induced by carrageenan at all the doses tested (25, 50, 100 and 200 mg/kg), during all three phases of oedema development (Table 7.3). Paw oedema was significantly inhibited in a dose dependent manner by treatment with CWE from 25-200 mg/kg ($p < 0.05$), at 2nd-5th hours post carrageenan injection. CWE showed similar anti-inflammatory pattern with 10 mg/kg of indomethacin (an NSAID) by inhibiting all three phases of oedema development induced by carrageenan. At 200 mg/kg, the CWE displayed paw oedema inhibition to a greater extent than 10 mg/kg indomethacin during all three phases of oedema development.

Ethanol extract of *Ganoderma lucidum* mycelium at 1000 mg/kg inhibited paw oedema formation by 65% after 3 hours of carrageenan injection while CWE at lower dosage (200 mg/kg) showed higher (88%) paw oedema inhibitory activty (Lakshmi et al., 2003). Im et al. (2014) reported that acetone extract from fruiting bodies of *Pleurotus florida* at 50 mg/kg displayed 62.1% of paw oedema inhibition after 2 hours of carrageenan injection whereas CWE at the same dosage gave 48% paw oedema inhibition. However, it is not possible to make accurate quantitative comparison as the experimental design of the above mentioned findings (including ours) vary from one another.

The amount of the high, medium and low molecular weight fractions (HMW, MMW and LMW) corresponding to 200 mg/kg of CWE were 53, 35 and 112 mg/kg, respectively. HMW fraction at 53 mg/kg showed comparable paw oedema inhibition activity with 200 mg/kg CWE, whereas both 35 mg/kg of MMW and 112 mg/kg of LMW fraction exhibited substantially lower anti-inflammatory activity. This showed that the anti-acute inflammatory activity of CWE was mainly contributed by HMW fraction but the possibility of synergistic effect between HMW, MMW and LMW fractions cannot be excluded.

In comparison to CWE at 200 mg/kg, the hot water extract (HWE) and methanol extract (ME) at the same dosage were far less effective in reducing paw oedema induced by carrageenan in all three phases of oedema development. The extraction yield of CWE, HWE and ME were 20.3 ± 0.5% (w/w), 37.3 ± 0.6% (w/w) and 4.8 ± 0.2% (w/w), respectively. The major anti-acute inflammatory principle(s) of the sclerotial powder of *L. rhinocerotis* cultivar TM02 were in the CWE, in particular, its high molecular weight fraction (HMW). Though the HWE exhibited weaker anti-acute inflammatory activity, it also contained substantial amount of anti-inflammatory principle due to its high extraction yield. The yield of ME was so low that it contained only negligible amount of

the bioactive substance.

Granulomatous inflammation is "a focal chronic inflammatory response to tissue injury evoked by a poorly soluble substance characterised by the accumulation and proliferation of leucocytes, principally of the mononuclear type" (Warren, 1976, p. 7; Hirsh & Johnson, 1984, p. 90). Cotton pellet induced granuloma test is widely used to assess transudative, exudative and proliferative phase of inflammation (Swingle & Shideman, 1972). In this study, CWE at 200 mg/kg did not show reduction of wet and dry weight of implanted cotton pellet (Table 7.4), indicating that it might not inhibit the transudative and proliferative phase of chronic inflammation.

The anti-inflammatory activity of the sclerotia of *L. rhinocerotis* was also measured by inhibition of lipopolysaccharide (LPS) induced TNF-α production in RAW 264.7 macrophage cell line. CWE exhibited TNF-α inhibition activity with IC_{50} of 30.00 ± 1.33 µg/ml while the inhibitory effect by HWE was not detectable up to 150.00 µg/ml (Table 7.7). Among three fractions of CWE (separated with Sephadex G-50 gel filtration chromatography), HMW fraction (25.79 ±1.77 µg/ml) exhibited the strongest inhibitory activity on TNF-α production, followed by MMW fraction (IC_{50}: 52.83 ± 2.74 µg/ml). LMW fraction did not show any inhibitory activity up to 150.00 µg/ml. Protein components of HMW and MMW fraction (P-HMW and P-MMW) exhibited very potent TNF-α inhibitory activity with IC_{50} of 9.35 ± 0.48 and 5.10 ± 0.36 µg/ml, respectively when based on total protein and carbohydrate content; and IC_{50} of 0.82 ± 0.04 and 1.31 ± 0.09 µg/ml, respectively when based on total protein content alone (Table 7.8). Non-protein components of HMW and MMW fraction (NP-HMW and NP-MMW) did not exhibit any inhibitory activity up to 150 µg/ml.

The extraction yield of P-HMW and P-MMW (based on total carbohydrate and protein content) were 14.4% and 2.7% (w/w by dry weight of CWE), respectively. In view of higher extraction yield of P-HMW (5.3x higher for total carbohydrate and protein content and 6x higher for protein content only) as compared to P-MMW, majority of the anti-inflammatory principle of CWE apparently is in P-HMW, which was then chosen for further investigation.

P-HMW was subjected to Resource Q anionic exchange chromatography and a total of 8 fractions were collected, termed fraction 1 to fraction 8 (F1-F8). No inhibitory effect on TNF-α production was detected in F1 to F3 at tested concentration up to 2.5 µg/ml. The rest of the fractions displayed strong to weak inhibitory effect on TNF-α production in the following order: F5 = F6 > F7 > F8 > F4 (Table 7.9).

7.5.2 Bioactive Components That May Contribute to the Anti-inflammatory Activity of *Lignosus rhinocerotis*

Since both F5 and F6, which exhibited potent anti-inflammatory activity, contained several proteins (refer to Results section), the possibility that one of these proteins might be responsible for the anti-inflammatory activity is considered as below.

Serine Proteases

LC-MS/MS analysis of SDS-PAGE gel section revealed that the most abundant proteins in P-HMW, F5 and F6 were serine proteases (Figures 7.5, 7.6 and 7.7). Four serine proteases were detected in P-HMW with genomic database accession number of 4347, 8711, 7804 and 5911 while only the first two serine proteases (4347 and 8711) were found in F5 and F6 (Table 7.10). The distribution of two serine proteases (4347 and 8711) in gel section 3 and 4 of F6 suggested the possibility of post-translational modification, particularly of glycosylation due to the presence of relatively high amount of carbohydrate in the fraction (25.0%, w/w by total carbohydrate and protein content) (Table 7.6). Protein glycosylation is an enzymatic process to attach glycosidic linkages of saccharides to protein (Marth & Grewal, 2008). N-linked and O-linked glycosylation (two major types of glycosylation) are both involved in protein intracellular trafficking and secretion, in protein protection from proteolytic degradation and in the maintenance of protein conformation and activity (Varki, 1993; Roth et al., 2012).

Prediction of N-glycosylation and O-glycosylation sites on the serine proteases were performed using NetGlyc 1.0 and NetOGlyc 4.0 server, respectively (http://www.cbs.dtu.dk/services/NetNGlyc/). Serine protease 4347 has two putative N-linked glycosylation consensus sites at positions Asn66 (N-V-T) and Asn159 (N-Y-T)

while serine protease 8711 has one at position Asn143 (N-Y-T). Nine O-glycosylation sites were predicted for serine protease 4347 and six for serine protease 8711.

There are only several reports on the anti-inflammatory effects of serine protease, though none are from medicinal mushroom. Ito et al., (1979) reported that oral administration of a mixture of proteases, bromelain and trypsin to rabbits synergistically inhibited paw oedema induced by carrageenan, histamine, dextran or egg albumin or skin oedema induced by thermal stimulation and anti-rabbit serum. Inhibitory action of the mixture against oedema formation might be contributed by inhibitory action of increased vascular permeability and the anti-inflammatory action may be specific for acute exudative inflammation. Jutila et al. (1991) reported that low-dose chymotrypsin treatment inhibited neutrophil extravasation to sites of inflammation *in vivo*. According to Viswanatha Swamy and Patil (2008), three serine proteases: chymotrypsin, trypsin and serratiopeptidase displayed dose dependent anti-inflammatory activity in carrageenan induced paw oedema model and cotton induced granuloma model. The combination of low doses of these enzymes with aspirin resulted in synergistic anti-inflammatory activity with reduced ulcerogenic effect (adverse effect of aspirin). Ghaffarinia et al. (2014) demonstrated that intra-CSF injection of 0.2 mg/ml chymotrypsin into rats ameliorated clinical signs of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. Administration of chymotrypsin at 0.2 mg/ml decreased IL-17 production along with increased IL-4 and FoxP3 in the brain and spinal cord of animals. These finding suggested anti-inflammatory effect of chymotrypsin on multiple sclerosis. Gleeson et al. (2014) reported that exposure to factor Xa (a serine protease crucial for blood coagulation) significantly impaired pro-inflammatory cytokine production from THP-1 monocytic cells, lipopolysaccharide-treated peripheral blood mononuclear cells and murine macrophages. Factor Xa also attenuated nuclear factor-kappa B activation in

THP-1 reporter cells, requiring phosphatidylinositide 3-kinase activity for its anti-inflammatory effect. Thus, serine proteases are the potential candidate as bioactive substance with the anti-inflammatory activity.

In this respect, it is interesting to note that F5 and F6, the two most potent fractions that inhibited TNF-α production with comparable IC_{50} and IC_{70} values, also contained comparable amount of serine proteases. This further supported the hypothesis that the serine proteases might be the main anti-inflammatory substance of the fractions.

Percentage of sequence identity between serine protease 4347 and 8711 with their best-matching sequences (serine protease from *Dichomitus squalens* LYAD-421 SS1 with sequence ID: XP_007359794.1) were found to be 54% and 55%, respectively (Appendix E). It is believed that when two proteins share 50% or higher sequence identity, their backbones will differ by less than 1 A˚ RMS deviation (Chung & Subbiah, 1996), suggesting that the two serine proteases may be structurally very similar to XP_00735974.1. Protein sequences of serine protease 4347 and 8711 are quite similar to each other as they share 69% of sequence similarity.

An attempt to inhibit protease activity of the serine proteases in F5 and F6 was carried out using two irreversible serine protease inhibitors: phenylmethylsulfonyl fluoride (PMSF) and Pefabloc® SC. It was hoped that inhibition of the protease activity will bring concomitant inhibition of anti-inflammatory activity, thus establishing the correlation between serine protease activity and anti-inflammatory activity. Protease activity of trypsin at 60.8 µg/ml was indeed inhibited by PMSF and Pefabloc® SC. However, protease activities of F5 and F6 at the same concentration were not inhibited by either inhibitors. PMSF and Pefabloc® SC are known to inhibit serine proteases of families S1 (Trypsin- and Chymotrypsin-like) and S8 (subtilisin-like) (Basak, et al., 2004; Phrommao

et al., 2011; Horn et al., 2014). Both serine protease 4347 and 8711 carry the conserved domains of peptidase_S8 (Subtilase family) with predicted catalytic triad of Asp-His-Ser, and were expected to be inhibited by either of the two serine protease inhibitors. The unexpected resistance of the two proteases against inhibition by PMSF and Pefabloc® SC indicated that these proteases may have unusual structure/active site, and it also means that we are unable to use inhibitory studies to demonstrate whether the serine proteases contribute to the anti-inflammatory activity.

Glycoside Hydrolase and Phosphatidylserine Decarboxylase-like Proteins

Glycoside hydrolase family proteins were the second most abundant proteins in F5 that accounted for 24.1% of total protein (Figure 7.6). The most abundant glycoside hydrolase family proteins in F5 and F6 were glycoside hydrolase family 27 proteins with database accession number of 9375 and 9376. Phosphatidylserine decarboxylase-like proteins (database accession number 4019) were the second most abundant protein in F6 (13.8% of total protein) (Figure 7.7). To date, no anti-inflammatory properties was reported on glycoside hydrolase family 27 proteins or phosphatidylserine decarboxylase. Therefore, glycoside hydrolase family 27 proteins or phosphatidylserine decarboxylase-like proteins are unlikely to be the anti-inflammatory candidates in F5 and F6.

Lectins

Lectins, the third most abundant proteins in P-HMW and F6 accounted for 11.0% and 10.7% of total protein, respectively (Figure 7.5 and 7.7). Its content in F5 was however, very low (2.6%) (Figure 7.6). None of the lectins from mushroom exhibit anti-inflammatory activity, though lectins in plant and marine algae were reported to be a potential source of anti-inflammatory agent (Silva et al., 2010; Vanderlei et al., 2010;

Araújo et al., 2013). Silva et al. (2010) found that lectin from the marine alga *Pterocladiella capillacea* (PcL), when administered to Swiss mice via *i.v* injection significantly reduced the number of writhes. Intravenous injection of Swiss mice with purified lectin from green marine alga *Caulerpa cupressoides* (CcL) at 3, 9 or 27 mg/kg significantly inhibited number of writhes induced by acetic acid by 37.2%, 53.5% or 86.0%, respectively (Vanderlei et al., 2010). The same lectin also (at 27 mg/kg by *i.v* injection) reduced the second phase of the formalin test. Besides, CcL lectin (9 mg/kg) when administered *i.v*. into Wistar rats 30 min before carrageenan injection (intraperitoneally) caused a reduction of neutrophil counts by 65.9%. Araújo et al. (2013) reported that two lectins from *Moringa oleifera* seeds, termed cmol and WSMoL (6.25 µg/ml) showed inhibitory activity against lipopolyssaccharide-stimulated inflammation in murine macrophages by reducing the production of TNF-α and nitric oxide.

Thus, there is a possibility that lectins may also contribute to the anti-inflammatory activity of the P-HMW. Nevertheless, the fact that F5 and F6, which are almost equally potent in anti-inflammatory activity, contain vastly different amount of lectins (2.6% and 10.7%, respectively) suggests that it is a less likely candidate to be the anti-inflammatory substance in the fractions.

Immunomodulatory Proteins

Most of the immunomodulatory proteins (immunomodulatory protein 8 and immunomodulatory protein FIP-Fve) of P-HMW were located in gel section 5 (10 to 15 kDa). It was the second most abundant protein found in P-HMW, and it constituted 18.1% of the total protein (Figure 7.5). This protein (only immunomodulatory protein 8) was found in moderate amount in F5 (3.5% in total) and small amount in F6 (0.9% in total) (Figures 7.6 and 7.7). According to Huang et al. (2014), the

immunomodulatory protein (LZ-8) lowered the production of nitric oxide and interleukin-6 in lipopolysaccharide-stimulated RAW 264.7 macrophages, but had no effect on the level of tumour necrosis factor-α. Hsu et al. (2013) reported that LZ-8 induced Treg expansion (important in the regulation of intestinal homeostasis) was capable of alleviating acute colitis in mice. Chang et al. (2014) reported that oral administration of fungal immunomodulatory proteins (FIPs) from *Flammulina velutipes* alleviated respiratory syncytial virus (RSV)-induced airway hyperresponsiveness (AHR) and suppressed IL-6 expression in bronchoalveolar lavage fluid (BALF) of RSV-infected BALB/c mice. Based on the above reports, the immunomodulory protein in F5 and F6 may also contribute to the anti-inflammatory activity of the fraction. Again, the fact that F5 and F6, which are almost equally potent in anti-inflammatory activity, contain vastly different amount of the immunomodulatory proteins (3.5% and 0.9% respectively) indicates that the protein is a less likely candidate to be the anti-inflammatory substance in the fractions.

Hypothetical Proteins

Proteins annotated as hypothetical proteins in P-HMW, F5 and F6 accounted for 10.4%, 9.5% and 5.4% of total protein, respectively (Figures 7.5, 7.6, 7.7). Expression of hypothetical proteins are predicted from an open reading frame but no experimental evidence of their translation is known (Ijaq et al., 2015). Thus, anti-inflammatory potential of the proteins which are identified as hypothetical proteins are unknown.

Putative Chitin Synthase, Phosphoglycerate Mutase-like Protein and Aldo/keto Reductase

Putative chitin synthases were found in moderate amount in F5 (3.0%) and F6 (1.2%) (Figures 7.6 and 7.7). Phosphoglycerate mutase-like protein constituted 4.9% total proteins of F5 but was not detected in F6. Aldo/keto reductases were found to be 1.5% and 3.6% in F5 and F6, respectively. However, these proteins have not been reported to exhibit anti-inflammatory activity.

Thus, comparison of the relative protein composition of F5 and F6, and literature search on the anti-inflammatory potential of the various proteins suggest that the serine proteases to be the most likely candidate as the bioactive substances that contribute to the anti-inflammatory activity of the fractions, even though the involvement of lectins and immunomodulatory protein cannot be ruled out.

CHAPTER 8: CONCLUSION

8.1 Preclinical Toxicological Evaluation of the *Lignosus rhinocerotis* Sclerotium

There was no treatment-related sub-acute toxicity effect in rats following 28-days oral administration of 250, 500 and 1000 mg/kg TM02, 1000 mg/kg TM03 as well as 1000 mg/kg wild type *L.rhinocerotis* sclerotial powder, as observed by haematological analysis, clinical biochemistry tests, body weight and other general gross observations. Histological examinations of heart, kidney, spleen, lung and liver of animals treated with up to 1000 mg/kg of the three samples of *L. rhinocerotis* sclerotial powder for 28 days did not reveal any significant pathology concerns. Thus, the no-observed-adverse-effect level (NOAEL) doses of the sclerotial powders in 28-day sub-acute toxicity study were more than 1000 mg/kg.

There was no treatment-related chronic toxicity in rats for both sexes following the long term (180-days) oral administration of 250, 500 and 1000 mg/kg of *L. rhinocerotis* (TM02) sclerotial powder, as shown by the clinical observations, body weight gain, haematological analysis, clinical biochemistry tests, urinalysis, absolute organ weight, relative organ weight and histological examinations of the organs. Thus, the NOAEL dose of the sclerotial powder of *L. rhinocerotis* (TM02) in 180-day chronic toxicity study was more than 1000 mg/kg.

Oral administration of 100 mg/kg sclerotial powder of *L. rhinocerotis* (TM02) for 7-8 weeks did not cause any adverse effect to the fertility of the rats, nor did the treatment induce any teratogenic effect on their offspring.

Data on safety evaluation of the sclerotial powder of *L. rhinocerotis* (TM02) had been submitted to the National Pharmaceutical Control Bureau (NPCB) prior to the registration of the sclerotial powder as a commercial product in terms of traditional medicine. The recommended daily consumption of the sclerotial powder of *L. rhinocerotis* (TM02) as neutraceutical is approximately 5-10 mg/kg per day (assuming the average body weight is 50 kg). The dose is 100-200 times less than the highest dosage used in sub-acute and chronic toxicity study and 10-20 times less than dosage used in reproductive toxicity study. Adults with serious health concerns may take 10 mg/kg, twice daily. For cancer patients, up to 100 mg/kg of sclerotial powder is recommended per day. To date, there has not been report of any adverse effects from sclerotial powder of *L. rhinocerotis* (TM02) consumption according to dosage guide provided by Ligno Biotech Sdn. Bhd.

Bacterial reverse mutation assay also showed that the sclerotial powder was not mutagenic. However, it is known that no single test can detect every genotoxin. Other *in vitro* and/or *in vivo* genotoxicity tests in mammalian system: *in vitro* micronucleus assay, the mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay (MLA), the *in vitro* metaphase chromosome aberration assay, mammalian erythrocytes micronucleus test and/or mammalian bone marrow chromosome aberration test may be conducted in future to complement results obtained from bacterial reverse mutation assay.

8.2 Anti-inflammatory Effect of the Sclerotium of *Lignosus rhinocerotis*

Cold water extract (CWE), hot water extract (HWE) and methanol extract (ME) of the sclerotial powder of *Lignosus rhinocerotis* cultivar TM02 possessed anti acute-inflammatory effect but most of the bioactive substance(s) appeared to be extractable by cold water. The anti-inflammatory activity of the cold water extract (CWE) was mainly contributed by its high molecular weight (HMW) fraction, in particular its protein component, which was very potent in the inhibition of lipopolysaccharides (LPS) induced TNF-α production in RAW 264.7 with an IC_{50} of 9.35 ± 0.48 μg/ml based on total carbohydrate and protein content and IC_{50} of 0.82 ± 0.04 μg/ml based on total protein only. However, the possibility of synergistic effect between HMW, MMW and LMW fractions cannot be excluded. Cold water extract at 200 mg/kg did not inhibit transudative and proliferative phase of chronic inflammation, as shown by cotton pellet induced granuloma model.

Protein component of HMW fraction (P-HMW) was subjected to Resource Q anionic exchange chromatography and a total of 8 fractions were collected, termed fraction 1 to fraction 8 (F1-F8). No inhibitory effect on LPS induced TNF-α production in RAW 264.7 was detected in F1 to F3 at tested concentration up to 2.5 μg/ml. The rest of the fractions displayed strong to weak inhibitory effect on TNF-α production in the following order: F5 = F6 > F7 > F8 > F4.

LC-MS/MS analysis of SDS-PAGE gel sections and literature research suggested possible anti-inflammatory substances of F5 and F6 to be: serine proteases, lectins and immunomodulatory proteins. The possibility of synergistic effect between components in F5 and F6 cannot be excluded.

LC-MS/MS analysis of SDS-PAGE gel sections revealed that the most abundant proteins in F5 (50.0%) and F6 (54.1%) were serine proteases (database accession number of 4347 and 8711). IC_{50} and IC_{70} (based on total carbohydrate and protein as well as total protein content alone) for the inhibition of TNF-α production by F5 and F6 were found to be comparable, which were possibly due to the presence of similar content of serine proteases. Therefore, serine proteases appear to be the most likely components of F5 and F6 that contribute to their anti-inflammatory activity. Attempts to verify this hypothesis by protease inhibitor studies, however, were unsuccessful due to the unusual resistance of the serine proteases against the serine protease inhibitors used (PMSF and Pefabloc® SC).

Future Studies on the Bioactive Substance(s) Contributing to the Anti-inflammatory Activity of *L. rhinocerotis*

Further purification of F5 and F6 is required to obtain insight on the bioactive component(s) contributing to its anti-inflammatory effect. A preliminary attempt using Superdex gel filtration chromatography to separate the two protein bands (25 to 35 kDa) in either F5 or F6 was unsuccessful (data not shown). Further purification may have to involve more steps of ion exchange chromatography or reverse-phase HPLC. One foreseeable challenge is the possible 'unusual' molecular nature of the two serine proteases (4347 and 8711), as both F5 and F6 appear to contain comparable amount of serine proteases, even though the two fractions can be separated apart by the Resource Q ion exchange chromatography.

The other possible approach is by molecular cloning and expression of the serine proteases 4347 and 8711, followed by examination of its protease and anti-inflammatory activity, as the serine proteases are the most likely anti-inflammatory substances in the fractions F5 and F6.

Once the anti-inflammatory substance can be obtained in pure form, then further investigation of pathway of anti-inflammatory action can be carried out.

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LIST OF PUBLICATIONS

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